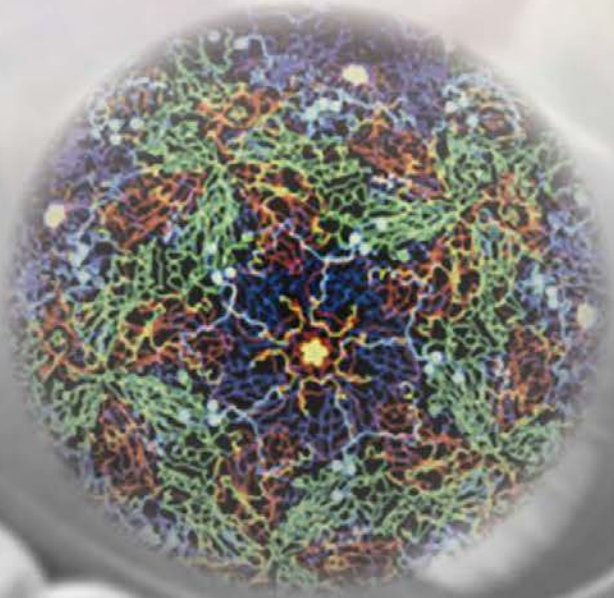
Essentials of Medical Microbiology

Rajesh Bhatia
Rattan Lal Ichhpujani

Fourth Edition



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Rajesh Bhatia
MD (Medical Microbiology)
Formerly Director National Institute of Biologicals, New Delhi
and
Department of Microbiology
Maulana Azad Medical College, New Delhi
India

Rattan Lal Ichhpujani
MD (Medical Microbiology)
Additional Director and
Chief of Division of HIV/AIDS and Related Diseases
National Institute of Communicable Diseases, Delhi
Formerly Department of Microbiology
Medical College, Rohtak
Haryana
India

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Rel: +91-11-32558559, Fax: +91-11-23276490, +91-11-23245683
e-mail: jaypee@jaypeebrothers.com Visit our website: www.jaypeebrothers.com

Branches

- 2/B, Akruti Society, Jodhpur Gam Road Satellite
  Ahmedabad 380 015, Phones: +91-79-26926233, Rel: +91-79-32988717
  Fax: +91-79-26927094, e-mail: ahmedabad@jaypeebrother.com
- 202 Batavia Chambers, 8 Kumara Krupa Road, Kumara Park East
  Bengaluru 560 001, Phones: +91-80-22285971, +91-80-22382956, +91-80-22372664
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- 282 IIIrd Floor, Khaleel Shirazi Estate, Fountain Plaza, Pantheon Road
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  Fax: +91-44-28193231, e-mail: chennai@jaypeebrothers.com
- 4-2-1067/1-3, 1st Floor, Balaji Building, Ramkote Cross Road
  Hyderabad 500 095, Phones: +91-40-66610020, +91-40-24758498
  Rel:+91-40-32940929 Fax:+91-40-24758499, e-mail: hyderabad@jaypeebrother.com
- No. 41/3098, B & B1, Kuruvi Building, St. Vincent Road
  Kochi 682 018, Kerala, Phones: +91-484-4036109, +91-484-2395739
  +91-484-2395740 e-mail: kochi@jaypeebrothers.com
- 1-A Indian Mirror Street, Wellington Square
  Kolkata 700 013, Phones: +91-33-22651926, +91-33-22276404, +91-33-22276415
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- Lekhraj Market III, B-2, Sector-4, Faizabad Road, Indira Nagar
  Lucknow 226 016, Phones: +91-522-3040553, +91-522-3040554
  e-mail: lucknow@jaypeebrothers.com
- 106 Amit Industrial Estate, 61 Dr SS Rao Road, Near MGM Hospital, Parel
  Mumbai 400 012, Phones: +91-22-24124863, +91-22-24104532, Rel: +91-22-32926896
  Fax: +91-22-24160828, e-mail: mumbai@jaypeebrothers.com
- "KAMALPUSHPA" 38, Reshimbag, Opp. Mohota Science College, Umred Road
  Nagpur 440 009 (MS), Phone: Rel: +91-712-3245220, Fax: +91-712-2704275
  e-mail: nagpur@jaypeebrothers.com

Essentials of Medical Microbiology

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To

Indu, Peromila,
Parul, Vrinda and Tushar
for being so wonderful and
understanding during the
genesis of this work

Preface to the Fourth Edition

Teaching of Medical Microbiology to the MBBS students is changing rapidly. This is primarily due to emergence of newer pathogens, newer diagnostic tools, emerging and re-emerging infections and above all shrinking time available to accommodate all this. Microbiology continues to be the backbone of understanding diagnosing and controlling the communicable diseases. Our primary focus of this Fourth Edition is to update various chapters, delete the contents that have become relatively unimportant and enhanced focus on the topics of current interest. For easy assimilation and reproducibility of the subject by the students, a lot of tables, figures and flow charts have been included. The book is for the first time being printed in colour which will definitely enhance its acceptability by our valued readers.

We are deeply indebted to Shri Jitendar P Vij, Chairman and Managing Director, Jaypee Brothers Medical Publishers and his dedicated team for their magnificent support in bringing this book to its current shape. We continue to be thankful to our family members, friends, well-wishers and above all our dear students for their continuous support.

We are optimistic that this revised edition will be liked by our readers. We look forward to their patronage.

Rajesh Bhatia
RL Ichhpujani

Preface to the First Edition

The development of the science of medical microbiology—from Leeuwenhoeck's astonishing observations of animalcules to Pasteur's first use of rabies vaccine on a human being; to Fleming's discovery of penicillin; to today's race to develop a vaccine against AIDS—has been indeed one of the most amazing stories of mankind behind which is hidden the intricate and intimate relationship between the man and the microbe. For every single cell in the human body, ten microbes are harboured by the man. At any time an adult human being harbours 100 trillion microorganisms. These organisms are most of the times beneficial to man. Pathogenicity is a trait exhibited by only a few of them. Yet, tremendous mortality and morbidity have been inflicted on the mankind by these organisms since time immemorial. Rapid advances in medical sciences may have brought down the incidence of microbial infections in some parts of the world but an overwhelming population is still afflicted by a variety of infectious diseases. Medical microbiology is the science which deals with the study of organisms that cause infectious diseases.

This book has been designed chiefly for the students pursuing an undergraduate course in medical microbiology or any of the allied fields. This has deliberately been written in a simple, straightforward, functional, easily reproducible and user-friendly style. Special attention has been given to provide the updated information. Clear, attractive and easy-to-make illustrations have been used in abundance to ensure better understanding of the subject. About three dozen coloured figures have been appended in the beginning of the book to create long-lasting impressions of the appearances of the microorganisms. Numerous boxes coupled with tables have been inserted to highlight salient features.

So much has been the information explosion in medical microbiology that no book, however, hefty it may be, can stake a claim to be complete and up-to-date in all aspects of the subject. Apropos to this reality we decided to present in this book a prudent mix of essentials of fundamentals, recent advances and applications to the practice of medicine. The book has been accordingly titled as *Essentials of Medical Microbiology*. For those desirous of going into details, a few relevant references have been appended at the end of the text of each chapter.

While we derived immense pleasure in conceiving the concept of this book and giving it the shape of the manuscript, getting it through various stages of publication was also an scintillating experience. We are deeply indebted to Shri Jitendar P Vij of Jaypee Brothers, and his dedicated team for the magnificent support extended by them.

Finally, we thank our affectionate families for their constant encouragement without which we would have never contemplated, let alone complete, this book.

Rajesh Bhatia
RL Ichhpujani

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PART I HISTORY AND SCOPE OF MEDICAL MICROBIOLOGY

1 History of Medical Microbiology

Microbiology is the study of living organisms of microscopic size. The term was introduced by the French chemist Louis Pasteur.

At a very early stage man developed concept that contagious disease was caused by invisible living things. Invention of the microscope proved it to be a reality. Antony van Leeuwenhoek (1632-1723) designed a single lens microscope and demonstrated the little agents of disease, which he designated as *animalcules*. These animalcules are now well established entities belonging to bacteria, viruses and several other pathogens. The organisms being invisible to naked eye are known as microorganisms.

For many years it was believed that the microorganisms arose from dead, especially decomposed organic matter. This was known as *theory of spontaneous generation*.

CONTRIBUTIONS OF LOUIS PASTEUR (FIG. 1.1)

Louis Pasteur (1822-1895), a French chemist generated strong evidence to show that the microorganisms did not rise *de novo* or spontaneously in the media but were introduced from without. Pasteur showed that these organisms were maximum in the dusty air of towns and minimum in air of hilly areas where human habitation did not exist.

Pasteur also showed that microorganisms were inactivated by:

- boiling
- at 120°C under pressure (autoclaved)
- at 170°C (hot air oven).

Pasteur's demonstration of airborne microbes prompted Joseph Lister's (1827-1912) work on wound sepsis. He introduced the practice of protecting the wounds from airborne microbes by applying antiseptic dressing and making medical and paramedical workers wash their hands with antiseptic solution before they touched any exposed part of a patient. He achieved strikingly successful results and brought down tremendously the mortality due to sepsis.

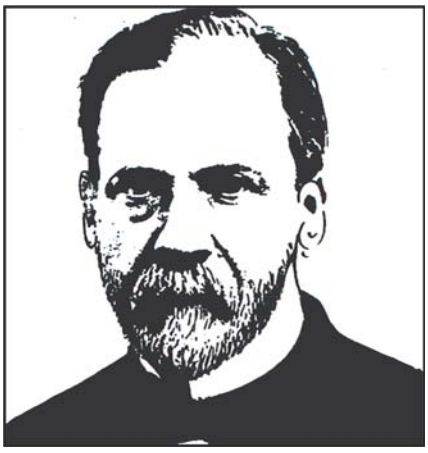


Fig. 1–1. Louis Pasteur—father of microbiology

Pasteur's achievements in the field of sterilization were closely followed by many other workers.Tyndall introduced the method of sterilization by repeated heating with appropriate intervals for germination of spores between them and their subsequent destruction. The method is known as *tyndallization* and is practiced even today.

Pasteur developed vaccines against chicken cholera and anthrax by using attenuated suspension of bacteria.

In order to show that the process was akin to Jenner's use of cowpox, he referred to this as vaccination. These attenuated organisms, on injection into animals, protected them from the effects of virulent bacteria. Soon anthrax immunization was widely practised with an enormous reduction in mortality amongst sheep.

However, his epoch making discovery was the development of rabies vaccine from the spinal cord of rabbits. It has been responsible for saving innumerable human lives consequent to bites by rabid animals.

The discoveries made by Pasteur can be summarised as follows:

CONTRIBUTIONS OF LOUIS PASTEUR

- Microbial theory of fermentation and proving that all forms of life including microorganisms arise from their like and not spontaneously
- Principles and practice of sterilisation
- Development of initial bacteriological techniques
- Control of diseases of silkworms
- Development of vaccines against:
  - — Anthrax
  - — Chicken cholera
  - — Rabies

CONTRIBUTIONS OF ROBERT KOCH (FIG. 1.2)

Robert Koch's first contribution to science was demonstration of the character and mode of growth of causative bacillus of anthrax.

In 1882, Koch discovered tubercle bacillus and in 1883 the cholera vibrio. For his manifold discoveries in bacteriology, Koch is considered as father of bacteriology.

Koch's Postulates

To confirm the claim that a microorganism isolated from a disease was indeed the cause of this, Koch postulated a set of criteria. According to these postulates, a microorganism can be accepted as a causative agent of an infectious disease only if the followings are satisfied:

A. The isolate should be found in every case of the disease and under conditions which explain the pathological changes and clinical features
B. It should be possible to isolate the causative agent in pure culture from the lesion
C. When such pure culture is inoculated into appropriate laboratory animal, the lesion of the disease should be reproduced

Fig. 1–2. Robert Koch—father of bacteriology

D. It should be possible to reisolate the causative agent in pure culture from the lesion produced in the experimental animal (Fig. 1.3).

Subsequently another criterion has been introduced which demands that specific antigens or antibodies to the bacterium should be detectable in the serum of the patient suffering from the disease.

Exceptions to Koch's Postulates

Some of the exceptions of these postulates are:

a. Inability to grow *Treponema pallidum* and *Mycobacterium leprae*-known causative agents of syphilis and leprosy respectively on artificial media
b. Inability to grow many viruses and rickettsial pathogens on artificial media.

The lifelong achievements of Robert Koch are summarised as under:

CONTRIBUTIONS OF ROBERT KOCH

- Verification of germ theory of diseases
- Introduction of staining techniques for visualisation of microorganisms
- Discovery and use of solid medium in bacteriology
- Discovery of causative agents of:
  - — Tuberculosis
  - — Cholera
  - — Anthrax
- Koch's postulates
- Use of laboratory animals for experimental infections

During the last quarter of the nineteenth century, succession of discoveries were reported which had bearing on the relation of bacteria to human and animal diseases. Table 1.1 shows some important discoveries.

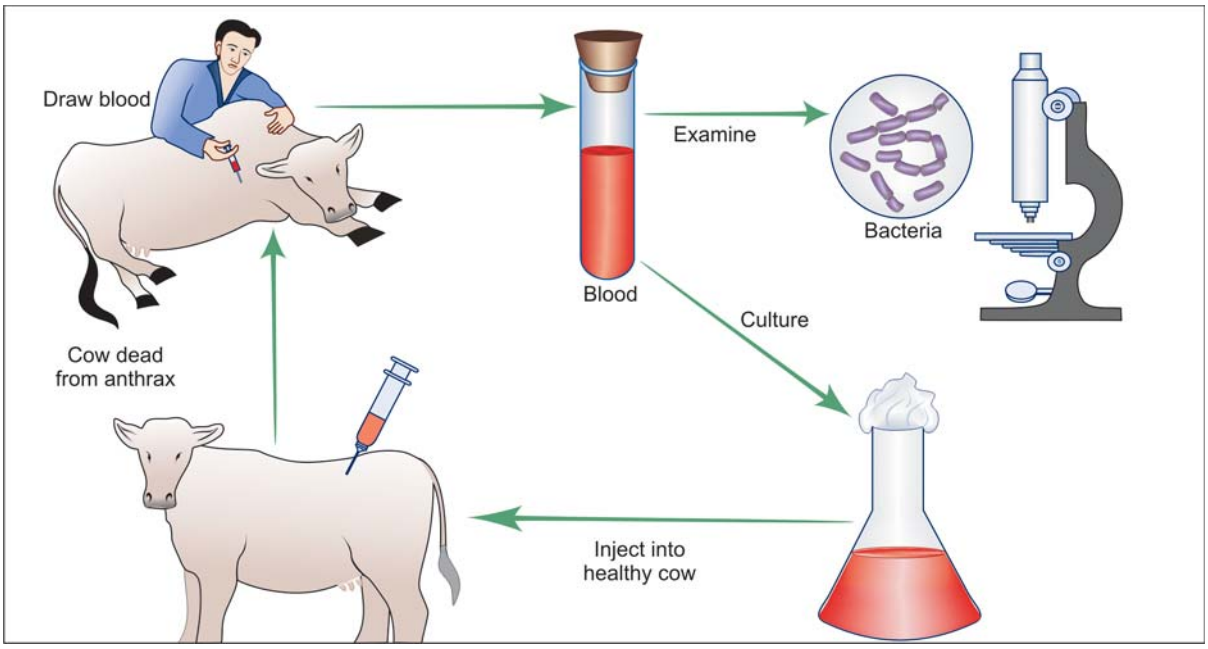


Fig. 1–3. Koch's postulates

Table 1–1. Discoveries of bacteria in 19th century

Year	Organism	Discovered by
1874	*Mycobacterium leprae*	Hansen
1879	*Neisseria gonorrhoeae*	Neisser
1880	*Salmonella typhi*	Eberth
1881	*Staphylococcus*	Ogston
1881	*Pneumococcus*	Pasteur and Sternberg
1882	*M. tuberculosis*	Robert Koch
1882	*Bacillus glanders*	Loeffler and Shutz
1883	*Vibrio cholerae*	Robert Koch
1883	*Streptococcus*	Fehleisen
1884	*C. diphtheriae*	Loeffler
1885	*Clostridium tetani*	Nicolaier
1887	*Neisseria meningitidis*	Weichselbaum
1887	*Brucella melitensis*	Bruce
1892	*Haemophilus influenzae*	Pfeiffer
1892	*Clostridium welchii*	Welch and Nuttall
1894	*Yersinia pestis*	Yersin and Kitasato
1896	*Clostridium botulinum*	Ermengem
1896	*Shigella*	Shiga

ANTIMICROBIAL AGENTS

With the identification and confirmation of bacteria as causative agents of human diseases, efforts were made to develop effective agents which could kill bacteria in the body of the man without damaging the host tissue. Pioneer work was done by Paul Ehrlich (Fig. 1.4) who is justly described as the father of chemotherapy. In the 1900s he cured one form of trypanosomiasis in rats with the dye trypan red and another form in mice with an organic arsenic compound, atoxyl.

In 1910, Ehrlich successfully treated syphilis using compound '606'' (dioxydiaminoarsenobenzol dihydrochloride) and called it Salvarsan. Fleming discovered penicillin and Waksman streptomycin. Subsequently, several fungi have been used as source of antimicrobial substances.

Fig. 1–4. Paul Ehrlich—father of chemotherapy

CONTRIBUTIONS OF PAUL EHRLICH

- Discovery of salvarsan as a chemotherapeutic agent against syphilis. This opened the new field of antimicrobial agents which has saved the lives of millions of people till date
- Identified that mycobateria have acid fastness nature
- Proposed a theory for the production of antibody called as "side chain theory"
- Refined the science of staining the organisms
- Advocated standardisation of biologicals including toxins and antitoxins to ensure uniformity

DISCOVERY OF VIRUSES

By the end of nineteenth century many infectious diseases had been proven to have a bacterial aetiology. The trend continued in twentieth century. But yet there remained many diseases of common occurrence for which no bacterium could be demonstrated. These included smallpox, chickenpox, measles and common cold. Advent of electron microscopy in 1934 by Ruska made morphological examination of viruses possible. The first human disease proven to have a virus aetiology was yellow fever.

Table 1–2. Nobel laureates in microbiology and immunology

Year	*Nobel Laureate*	*Contribution in/discovery of*
1901	Von Behring	Diphtheria antitoxin
1902	Sir Ronald Ross	Malaria
1905	Robert Koch	Tuberculosis
1908	Paul Ehrlich	Theories on immunity
1908	Metchnikoff	Phagocytosis
1913	Richet	Anaphylaxis
1919	Bordet	Immunity
1928	Nicolle	Typhus
1930	Landsteiner	Blood group
1939	Domagk	Prontosil as antibacterial
1945	Fleming, Chain and Florey	Penicillin
1951	Marx Theiler	Yellow fever
1952	Waksman	Streptomycin
1954	Enders, Weller and Robbins	Poliomyelitis
1958	Beadle, Tatum and Lederberg	Bacterial genetics
1960	Burnet and Medawar	Immunological tolerance
1962	Watson, Crick and Wilkins	Structure of DNA
1965	Jacob, Monod and Lwoff	Protein synthesis in bacteria
1968	Holley, Khorana and Nirenberg	Genetic code
1969	Delbruck, Hershey and Luria	Mechanism of viral infection of bacteria
1972	Edelman and Porter	Nature and structure of antibody
1975	Dulbecco, Temin and Baltimore	Reverse transcriptase and causation of cancer
1976	Blumberg and Gajdusek	Chronic viral infections and cancers
1977	Yalow	Radioimmunoassays
1978	Nathans, Smith and Arber	Restriction enzymes
1980	Benacerraf, Snell and Dausset	MHC genes and transplantation
1983	Barbara McClintok	Transposons
1984	Milstein,Kohler and Jerne	Monoclonal antibody
1987	Susumu Tonegawa	Genetics of antibody production
1989	Bishop and Varmus	Oncogenes
1990	Murray and Thomas	Use of immunosuppressive drugs in transplantation
1993	Sharp and Roberts	Gene splicing
1996	Doherty and Zinkernagel	Recognition of viruses by immune system
1997	Prusiner	Prions
1999	Gunter Blobel	Intrinsic signals in proteins
2001	Hartwell, Hunt and Nurse	Key regulators of cell cycle
2002	Brenner, Horvitz and Sulston	Genetic regulation of organ development and cell death

Table 1–3. Recently recognised pathogenic microbes and infectious diseases

Year	*Microbe*	*Type*	*Disease*
1973	Rotavirus	Virus	Major cause of infantile diarrhoea worldwide
1975	Parvovirus B-19	Virus	Aplastic crisis in chronic haemolytic anaemia
1976	*Cryptosporidium parvum*	Parasite	Acute and chronic diarrhoea
1977	Ebola virus	Virus	Ebola haemorrhagic fever
1977	*Legionella pneumophila*	Bacteria	Legionnaires disease
1977	Hantaan Virus	Virus	Haemorrhagic fever with renal syndrome (HRFS)
1977	*Campylobacter jejuni*	Bacteria	Enteric pathogen distributed globally
1980	Human T- lymphotropic virus I (HTLV-1)	Virus	T-cell lymphoma -leukaemia
1981	Toxin producing strains of *Staphylococcus aureus*	Bacteria	Toxic shock syndrome
1982	*Escherichia coli* O157:H7	Bacteria	Haemorrhagic colitis; haemolytic uremic syndrome
1982	HTLV-II	Virus	Hairy cell leukaemia
1982	*Borrelia burgdorferi*	Bacteria	Lyme disease
1983	HIV	Virus	AIDS
1983	*Helicobacter pylori*	Bacteria	Peptic ulcer disease
1985	*Enterocytozoon bieneusi*	Parasite	Persistent diarrhoea
1986	*Cyclospora cayatanensis*	Parasite	Persistent diarrhoea
1988	Human herpes virus-6 (HHV-6)	Virus	Roseola subitum
1988	Hepatitis E Virus	Virus	Enterically transmitted hepatitis
1989	*Ehrilichia chafeensis*	Bacteria	Human ehrlichiosis
1989	Hepatitis C Virus	Virus	Parenterally transmitted liver infection
1991	Guanarito virus	Virus	Venezuelan haemorrhagic fever
1991	*Encephalitozoon hellem*	Parasite	Conjunctivitis; disseminated disease
1991	New sps. of *Babesia*	Parasite	Atypical babesiosis
1992	*Vibrio cholerae* O139	Bacteria	New strain associated with epidemic cholera
1992	*Bartonella henselae*	Bacteria	Cat-scratch disease; bacillary angiomatosis
1993	Sin nombre virus	Virus	Adult respiratory distress syndrome
1993	*Encephalitozoon cunculi*	Parasite	Disseminated disease
1994	Sabia virus	Virus	Brazilian haemorrhagic fever
1995	HHV-8	Virus	Associated with Kaposi's sarcoma in AIDS patients
1999	Nipah	Virus	Encephalitis
2002	SARS CoVirus	Virus	SARS
2004	Influenza virus H5N1	Virus	Avian influenza

In 1930s viruses could be grown in bacteria free, living chick embryo—a technique perfected by Goodpasteur. By 1940, growth in tissue culture of susceptible mammalian cells was established. The availability of well defined cell lines have now replaced tedious methods of growing viruses in the living animals.

NOBEL LAUREATES

A number of Nobel laureates in Medicine and Physiology were awarded this prize for their work in Microbiology and Immunology (Table 1.2).

Discovery of New Organisms

The discovery of new microorganisms is a continuous phenomenon. A large number of new organisms have been discovered in recent past (Table 1.3). Some of these have acquired considerable importance because of the mortality and morbidity caused by them (HIV, hepatitis B, hepatitis C) and others have the capability to cause international scare (SARS CoVirus) or even pandemic (influenza virus H5N1).

2 Scope of Medical Microbiology

Medical microbiology is the biological study of bacteria, viruses, fungi, protozoa and algae which are collectively called as microorganisms, and unlike macroscopic organisms that are readily visible, these require magnification to be seen with the help of a microscope.

MICROORGANISM

A microorganism is one of a very diverse group of organisms that typically are microscopic and occur as independent, rapidly producing units that are comparatively less complex than plants and animals and exists almost everywhere. The easy way to remember these features is

M = Microscopic
I = Independent units
C = Complex (less)
R = Rapid growth rates
O = Omnipresent (present everywhere)

MICROBIOLOGY AND PATIENT

Medical microbiology is concerned with aetiology, pathogenesis, laboratory diagnosis and treatment of infections in an individual and with the epidemiology and control of infection in the community. The ideal bacteriological management of a patient with infection consists of following steps:

a. Establishment of a clinical diagnosis
b. Isolation of causative agent
c. Determination of antibiotic susceptibility in laboratory and administration of effective drug
d. Monitoring of therapy
e. Measurement of antimicrobial agents in body fluid
f. Confirmation of bacteriological cure.

The treating physician often identifies the disease causing organism on the basis of his clinical findings and accordingly treats the patient. There are many clinical conditions which are manifested only by fever and can be caused by a large number of organisms. The laboratory comes to the aid of physician in all such cases. Many a times even when clinical syndrome is diagnosed such as sore throat, urinary tract infection and acute diarrhoea, it becomes difficult to prescribe most appropriate chemotherapeutic agent because of large number of organisms which can cause these syndromes. Laboratory investigation provides the information regarding organisms as well as the drug to be used.

Predicting Antibiotic Sensitivity

For many organisms it is not essential to perform antibiotic sensitivity every time these are isolated from a clinical sample because bacteriology laboratory, on the basis of its experience in that area can always predict with fairly high degree of accuracy, the useful drugs for a particular type of infection.

Organisms with Predictable Sensitivity

Bacterium like *Streptococcus pyogenes* which commonly causes sore throat is almost always sensitive to penicillin and *Yersinia pestis* and many other gram-negative bacilli are invariably resistant to penicillin. Similarly organism like *Haemophilus influenzae* is almost always sensitive to cotrimoxazole, chloramphenicol, ampicillin and tetracycline. Regular patterns of sensitivity to many chemotherapeutic agents can be shown for streptococci, pneumococci, *Yersinia*, *Salmonella* and *Clostridium*. Thus, every regional microbiological laboratory should have and provide this information.

Many a times infection is of such a nature that antimicrobial agent given alone fails to eradicate the infection. A combination therapy is usually the answer to such problems and only a bacteriology laboratory can provide the best combination. It can also be first detected only in a laboratory that a combination of drugs shall result in a synergistic or an antagonistic effect.

MICROBIOLOGY AND HEALTHY INDIVIDUAL

Immunoprophylaxis against a large number of infectious diseases has now become an established practice. The development and testing of these vaccines in human beings fall within the domain of microbiology laboratory.

MICROBIOLOGY AND COMMUNITY

Microbiology is closely concerned with the epidemiology and control of infection in any community where the transmission and disease producing capacity of the microorganisms is facilitated by the environmental factors such as overcrowding, contaminated food, drink or air, malnutrition, tissue damage etc. The availability of microbiological services has strengthened the epidemiological services by providing new and better tools. Among the early benefits were recognition of carriers—convalescent or chronic and their role in spread of infection in a community. The importance of water, milk and food as vehicles of infection was also established.

The laboratory helps in establishing correct diagnosis of outbreak and the availability of epidemiological markers for various organisms assists in tracing the source of infection.

Microbiology extensively influences the following components of community infections (including infections in closed settings such as hospitals and institutions):

a. Collection of precise data
b. Laboratory investigation
c. Source and spread of infection
d. Zoonoses
e. Experimental epidemiology.

BRANCHES OF MICROBIOLOGY

Bacteriology. Study of bacteria—the smallest, simplest single celled organisms.

Mycology. The study of fungi which includes both microscopic forms (molds and yeasts) and larger forms (mushrooms).

Parasitology. Study of parasites which traditionally includes pathogenic protozoa and helminths.

Virology. Study of viruses-minute, unicellular particles that parasitize living things.

Immunology. Study of systems of body defenses that protect against infection. This science includes serology, a discipline that tests the products of immune reactions in blood serum and helps in the diagnosis of infectious diseases.

Microbial taxonomy. Study of classification, naming and identification of microorganisms.

Biotechnology. This discipline includes any process in which humans use systems or process of organisms to arrive at a desired product.

Genetic engineering and recombinant DNA technology. This is the most powerful and rapidly growing area in modern microbiology which involves techniques that deliberately alter the genetic make-up of organisms to introduce new compounds, different genetic combinations and even unique organisms.

ROLE OF MEDICAL MICROBIOLOGY

Medical microbiology plays an important role in the diagnosis, prevention, treatment and control of infectious diseases. Some of its applications are listed in Table 2.1.

Table 2–1. Applications of medical microbiology

• Confirmation of diagnosis which helps in starting specific treatment
• Detection of newer pathogens
• Determination of antimicrobial susceptibility patterns
• Development of vaccines and immunotherapeutic agents
• Development of newer antimicrobials/biocides
• To trace the source/reservoir of infection
• To trace the route of spread of pathogens using epidemiological markers such as gene sequencing
• Development of newer regimens/treatment schedules
• Monitoring of changing profile of pathogens
• Detection of asymptomatic infections or chronic carriers
• Distinction between recent and past infections
• Help in monitoring the response to treatment
• Monitoring the quality of air, water and food

PART II CELLULAR AND MOLECULAR MICROBIOLOGY

3 Bacterial Morphology

Traditionally all living objects had been divided into plant and animal kingdoms. There are some organisms that do not fulfil criteria for either of the two kingdoms. A new kingdom, Protista was created to include these organisms. Protista has been divided into viruses, prokaryota and eukaryota (Table 3.1).

The prokaryotes have primitive nucleus (pro-, primitive: karyote-, nucleus) whereas eukaryotes have a true (eu-) and well defined nucleus. The essential distinguishing features between prokaryotes and eukaryotes have been summarised in Table 3.2.

Table 3–1. Classification of protista

Subcellular organization
- Viruses

Thallus unicellular, multicellular or plasmodial
- Nuclear apparatus without membrane
  — Prokaryota
- Nuclear apparatus with membrane
  — Eukaryota

Protista includes algae, protozoa, fungi, bacteria and viruses. A few distinctive characteristics of bacteria,

Table 3–2. Differences between prokaryotes and eukaryotes

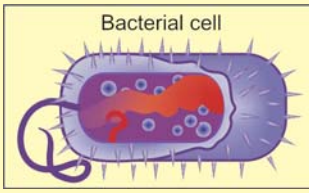
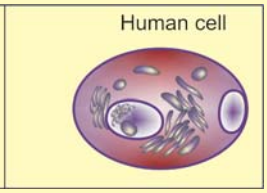
Function/structure	*Characteristic*	*Prokaryotic cell*	*Eukaryotic cell*
Genetics	DNA	Circular	Linear
	Chromosome number	One	More than one
	True nucleus	–	+
	Nuclear membrane	–	+
Reproduction	Mitosis	–	+
Biosynthesis	Golgi apparatus	–	+
	Endoplasmic reticulum	–	+
Respiration	Mitochondria	–	+
Size	µm	1	2-100
Example		Bacterial cell	Human cell
			

Table 3–3. Distinguishing features of bacteria, viruses, fungi and parasites

Feature	*Bacteria*	*Viruses*	*Fungi*	*Parasites*
Size (µm)	1-5	0.02-0.2	3-10	15-28
Nucleic acid	Both RNA and DNA	Either RNA or DNA	Both RNA and DNA	Both RNA and DNA
Ribosomal type	70S	Nil	80S	80S
Mitochondria	Absent	Absent	Present	Present
Outer surface	Rigid containing peptidoglycan	Protein capsid and lipid envelope	Rigid wall with chitin	Flexible membrane
Replication	Binary fission	No binary fission	Binary fission and budding	Binary fission
Motility	Some	None	None	Most

viruses and other closely related organisms have been shown in Table 3.3.

THE BACTERIAL CELL

Bacteria are microorganisms and because of small size these cannot be visualized by naked eye. To understand their size, one needs to revisit various measurement units. The unit of measurement used in bacteriology is the micron (µ) or also called micrometre (µm)

1 µm = One thousandth of a millimetre

1 millimicron (mµ) or nanometre (nm) = one thousandth of a micron or one millionth of millimetre.

1 angstrom unit (Å) = one tenth of a nanometre.

1 millimetre = one thousandth of a metre.

A bacterium shall characteristically have a cell envelope which includes a layered cell wall and external surface adherents. The appendages of cell wall include flagellae—the organs of locomotion and fimbriae which help in adhesion of bacteria. Internally the bacterium has loose arrangement of DNA, i.e. nuclear apparatus surrounded by an amorphous cytoplasm which contains ribosomes. Mesosomes and inclusion granules are other structures present in bacterium. An artist's impression of a bacterium is shown in Figure 3.1.

Shape and Size of Bacteria

Bacteria can have any of the following three shapes (Fig. 3.2):

- Spheroidal (cocci),
- Cylinderical (bacilli or rods) and
- Spirillar (spirochetes).

Cocci are true spheres with diameter ranging between 0.75 to 1.25 µm (and average of 1 µm).

Bacilli vary in length from 2-10 times their width.

Coccobacilli are very short bacilli

Filaments are long threads of bacilli which have not separated into single cells.

Curved bacterial rods vary from small, coma shaped or mildly helical shaped organisms with only one curve such as *Vibrio cholerae*.

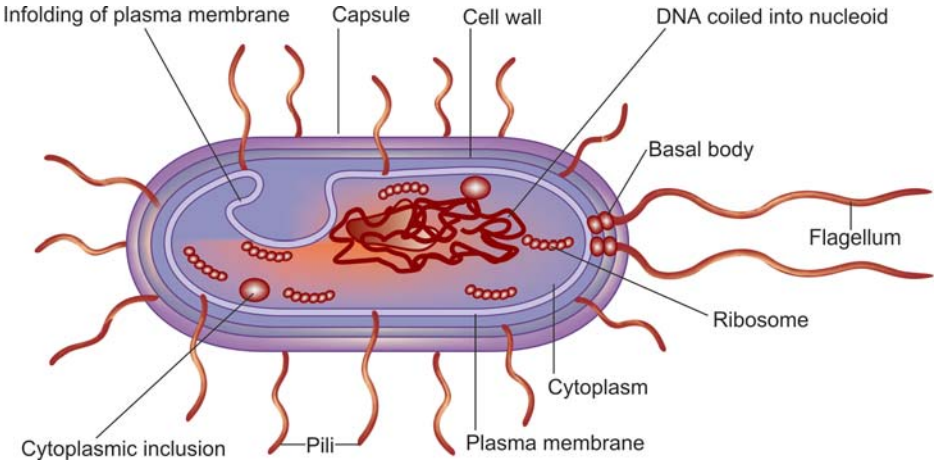


Fig. 3–1. Artist's impression of a bacterium

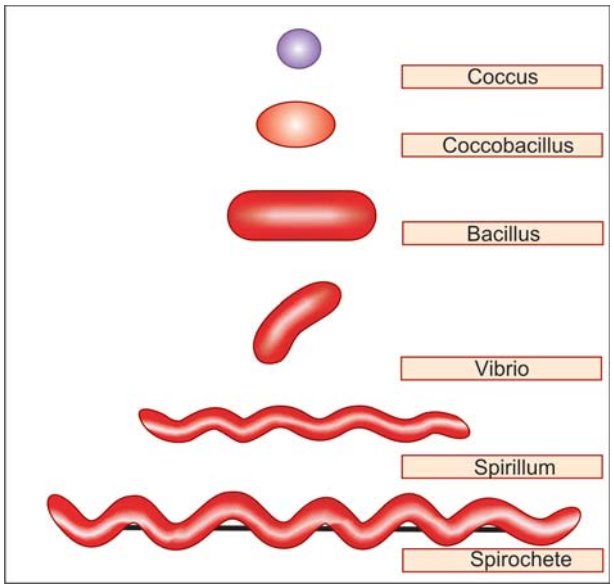


Fig. 3–2. Common bacterial shapes

Spirochetes are long and sinously curved bacteria with as many as 20 coils.

Apart from the length and width of the bacilli the shape of the ends often shows features that are of differential value. They may be:

- Rounded,
- Square cut or
- Sharply pointed.
- Gently round ends are seen in *Salmonella typhi*, squared ends in *Bacillus anthracis* and fusiform bacilli, which are present in oral cavity and gastrointestinal tract, are tapered at both the ends. Some bacteria vary widely in form even within a single culture, a phenomenon known as *pleomorphism*.

Arrangement of Bacterial Cells

Cocci may occur in pairs as in diplococci. Some of the bacilli also have the tendency to cluster together in different patterns but in the absence of any consistency in the patterns it is difficult to assign them any characteristic arrangement. Some of the commonly seen arrangements of bacteria are shown in Figure 3.3.

The layers that surround the prokaryotic cell are referred to collectively as cell envelope. The structure and organization of the cell envelope differs in gram-positive and gram-negative bacteria. The cell envelope of gram-positive cells is relatively simple consisting of 2-3 layers: the cytoplasmic membrane, a thick peptidoglycan layer and some bacteria have an outer layer, either a capsule or an S-layer (slime layer). The cell envelope in gram-negative bacteria is highly complex multilayered structure. The cytoplasmic membrane (called the inner membrane in gram-negative bacteria) is surrounded by a single sheet of peptide glycan to which is anchored a complex layer called the outer membrane. An outermost capsule or S-layer may also be present. The space between the inner and outer membrane is called the "periplasmic space".

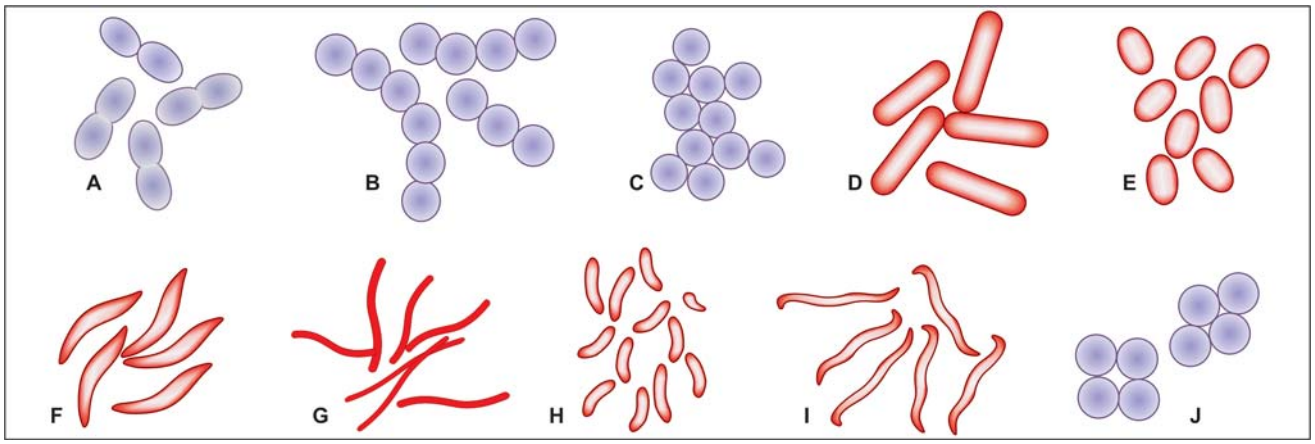
When examined from outside and as shown in Figure 3.1 bacteria have:

- Surface adherents and appendages
- Cell wall
- Cytoplasm
- Nucleus

Surface Adherents and Appendages

Capsule and Slime

These two are now collectively called as glycocalyx. All bacteria have at least a thin slime layer. For detection



Figs 3–3A to J. Bacterial forms: A, *diplococci*; B, *streptococci*; C, *staphylococci*; D, *bacilli*; E, *coccobacilli*; F, *fusiform bacilli*; G, *filamentous bacillary forms*; H, *vibrios*; I, *spirilla*; J, *sarcinae*

of capsule, negative staining by India ink is commonly employed in which capsule stands out as a halo. Capsule can also be specifically stained. Sometimes no capsule can be detected with staining but on reacting the bacterium with the specific anti-capsule serum, an antigen-antibody reaction takes place (Quellung reaction). Such capsules are known as *microcapsules*.

The virulence of bacteria is often associated with the capsule production (e.g. pneumococci). Capsule protects the bacterium from being phagocytosed. Loss of capability to produce capsule correlates with increased destruction by phagocytosis and decreased virulence. However, loss of capsule does not have any effect on the viability of the bacterium. The development of capsules is often dependent on the existence of favourable environmental conditions. Thus the size may vary with the amount of carbohydrate in the culture medium.

Presence of capsule does not let the flagella act and hence, capsulated organisms are invariably nonmotile.

Salient features of capsule are summarised in Table 3.4.

Table 3–4. Features of bacterial capsule

- Usually weakly antigenic
- Not necessary for viability of the bacterium
- Endows virulence
- Protects from phagocytosis
- Plays a role in the adherence of bacteria
- Capsulated strains are invariably nonmotile
- Visualised by:
  - — negative staining (India ink)
  - — special capsular staining
- Detected by quellung reaction

When the viscid material around the cell surface is a loose, undemarcated secretion, it is called as "slime layer". Some bacteria such as *Streptococcus salivarius* may have both—a capsule and a slime layer. Bacteria secreting large amounts of slime produce mucoid growth on agar media.

Flagella

These are the organs of locomotion. Their presence can be detected by observing the motility of organism either by a hanging drop preparation (Fig. 3.4) or by the spread and movement of bacteria over the surface of agar medium or through it. Organisms which are motile are vibrios, spirilla and spirochetes. Pathogenic cocci and capsulated organisms are nonmotile. A bacterial flagellum is made up of several thousand molecules of a protein subunit called *flagellin*. The flagellins of different bacterial species presumably differ from one another in primary structure. These are highly antigenic (H-antigens). If flagella are removed by mechanically agitating a suspension of bacteria, new flagella are rapidly formed by synthesis of flagella subunits.

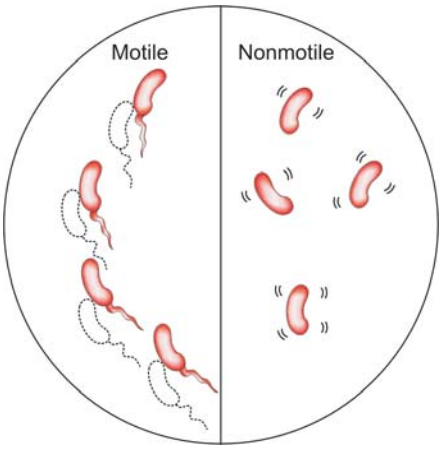


Fig. 3–4. A hanging drop slide can be used to detect motility by observing the microscopic behaviour of the cell. In true motility, the cell swims and progresses from one point to another. It is assumed that a motile cell has one or more flagella. Nonmotile cells oscillate in the same relative space due to bombardment by molecules, a physical process called Brownian movement

Dimensions. Flagella vary in their length from 3 to 20 µm and in diameter from 0.01 to 0.013 µm. The flagellar antigen is called as 'H' (Hauch) antigen.

Arrangement. The flagella may have characteristic arrangement in different bacteria (Fig. 3.5). These are:

Monotrichous

Bacteria which have one polar flagellum, e.g. *Vibrio cholerae.*

Lophotrichous

Bacteria with a tuft of several polar flagella, e.g. in *Spirilla.*

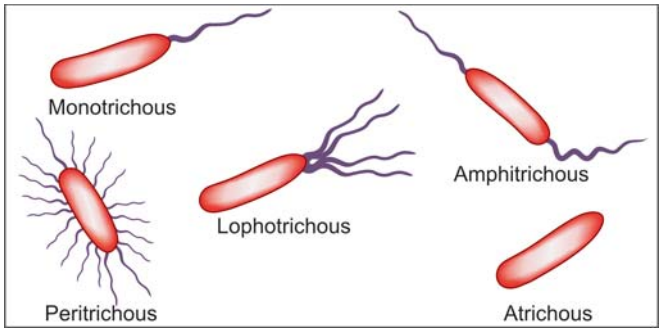


Fig. 3–5. Arrangement of flagella

Amphitrichous

Bacteria with flagella at both the ends, e.g. in some *campylobacter sps.*

Peritrichous

Bacteria with flagella distributed all over the surface of the bacterium, it is called as *peritrichous,* e.g. in *Salmonella typhi*.

Atrichous

Bacteria which do not possess any flagellum, e.g. in *Shigella sps*.

Anatomy. A flagellum consists of three parts: The filament, the hook and the basal body. The hook-basal body structure is embedded in the cell envelope whereas the filament is the only part which is external (Fig. 3.6).

Mechanism of motility. This is shown in Figure 3.7. Motility can be observed either microscopically or by noting the occurrence of spreading growth in semi-solid agar medium. Microscopic observation of a hanging drop preparation shows motile bacteria swimming in different directions across the field. True motility can be differentiated from Brownian movements because of oscillatory movements of latter. Some important features of flagella are:

- Flagellar antibodies are not protective, may help in serodiagnosis.
- Though different genera of bacteria have flagella of same chemical composition, they are antigenically different.
- Polar flagellated bacteria move the fastest, e.g. darting mobility in *Vibrio cholerae*, may be as fast as 200 µm per second.
- The flagellated bacteria move faster than the fastest animal. The speed could be equivalent to 50 body lengths/second as compared to a mere 25 body lengths per second of Cheetah (the fastest moving animal).

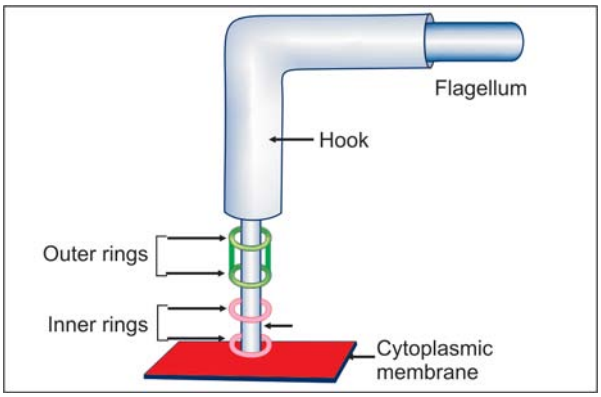
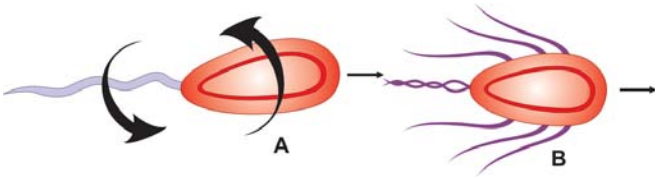


Fig. 3–6. Anatomy of a flagellum



Figs 3–7A and B. The operation of flagella and the mode of locomotion in bacteria with polar and peritrichous flagella, (A) In general, when the flagellum rotates in one direction, the cell rotates in the opposite direction. (B) In peritrichous forms, all flagella sweep towards one end of the cell and rotate as a single group

Pili (Fimbriae)

Pili (*sing*ular Pilus) or fimbriae are hair-like structures located on the surface of certain gram-negative bacteria. Pili in latin mean "hairs" while fimbriae in latin means "fringes". Fimbriae are the organs which help bacteria in adhering to the surfaces. Whereas pili specifically allow attachment to other bacteria. These are composed of protein called pilin. The only gram-positive organism which has these pili is *Corynebacterium renale.*

Eight morphologic types of pili are known which may be classified as *common* or *sex pili* on the basis of the function. Pili of different bacteria are antigenically distinct-and elicit the formation of antibodies by the host.

Differences between pili and flagella are shown in Table 3.5.

Table 3–5. Differences between flagella and pili

Character	*Flagella*	*Pili*
Size	Large	Small
Thickness	+++	+
Appearance	Straight	Never straight
Attached to cell wall	–	+
Origin	Cytoplasmic membrane	Cell wall
Organ of movement	+	–
Organ of adhesion	–	+
Required for conjugation	–	+

Functions of pili are:

i. Organs of adhesion, which helps them to anchor in nutritionally favourable environment
ii. Genetic transfer of material between bacteria
iii. For colonization of bacteria.

Cell Wall

It refers to the portion of the cell envelope that is external to the cytoplasmic membrane and internal to the capsule of glycocalyx.

The cell wall of bacteria is multilayered and constitutes about 20% of the dry weight of the bacterium. The cell wall has an average thickness of 0.15 to 0.50 µm.

The functions of cell wall are given in Table 3.6

Table 3–6. Functions of cell wall

- Provides shape to bacterium
- Gives rigidity to organism
- Protects from environment
- Contains receptor sites for phages
- Site of action of antibody
- Provides attachment to complement
- Contains components toxic to host
- Determines the Gram staining character
- Site of action of colicin

Chemical Structure

Chemically cell wall is composed of mucopeptide scaffolding formed by N-acetyl glucosamine and N acetyl muramic acid molecules alternating in chains which are cross-linked by peptide chains. Gram-positive bacteria produce a variety of specific proteins and polysaccharides which are attached to peptidoglycan. Example of this would include teichoic acids (Fig. 3.8)

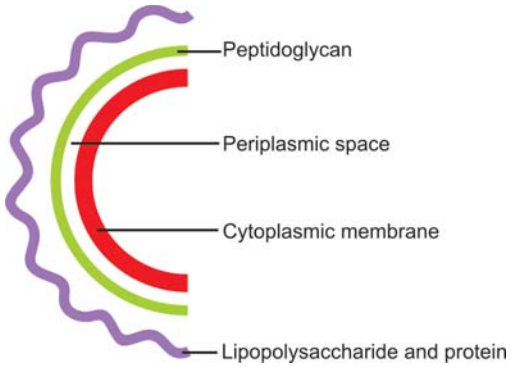
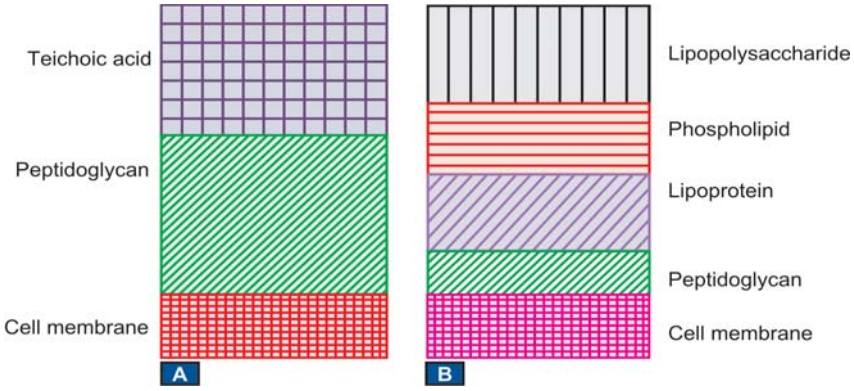


Fig. 3–9. Cell wall of gram-negative bacteria

group polysaccharide of streptococci and capsular substances of pneumococci.

The cell wall of gram-negative bacteria has three distinct and loosely arranged layers. These are outer membrane of 6 to 20 nm thickness; middle layer of 1.5-3 nm thickness and inner plasma membrane of about 8 nm thickness (Fig. 3.9).

Certain properties of cell walls result in different staining reactions which can be used to differentiate between gram-positive, gram-negative and acid-fast bacilli (Table 3.7).



Figs 3–8A and B. Cell wall of gram-positive (A) and gram-negative (B) bacteria

Table 3–7. Characteristics of cell walls of gram-positive, gram-negative and acid-fast bacteria

Character	*Gram +ve bacteria*	*Gram -ve bacteria*	*Acid-fast bacteria*
Peptidoglycan	Thick layer	Thin layer	Small amount
Lipids	Very little	Lipopolysaccharide (major)	Mycolic acid and other waxes
Outer membrane	Absent	Present	Absent
Periplasmic space	Absent	Present	Absent
Cell shape	Always rigid	Rigid or flexible	Rigid or flexible
Result of enzyme digestion	Protoplast	Spheroplast	Difficult to digest
Sensitivity to dyes and antibiotics	+++	++	+

Periplasm is the space which can be easily visualised in gram-negative bacteria but only with great difficulty in gram-positive bacteria. It refers to the area between the cell membrane and outer membrane.

Cytoplasmic Structures

Plasma or Cytoplasmic Membrane

This is a very delicate structure which is also called as cytoplasmic or cell membrane. It separates the rigid cell wall from the contents of the cytoplasm. Plasma membranes are dynamic, constantly changing entities. Materials constantly move through pores and through the lipids themselves. Functions of plasma membrane are summarised in Table 3.8. The outstanding difference between the cytoplasmic membrane of bacteria and the eukaryotes is the absence of sterols in bacterial cytoplasmic membrane.

Table 3–8. Functions of plasma membrane

- Regulates the transport of material into and out of cell
- Synthesis of cell wal component
- Assists in DNA replication
- Secretes proteins
- Carries on respiration
- Captures energy in ATP
- Is the site of action of certain antibiotics, e.g. polymyxin

Mesosomes

These are the principal sites of respiratory enzymes which are analogous to mitochondria in higher forms of life. These can easily be demonstrated in gram positive bacteria. These are essential in bringing about cell division. These are convoluted invaginations of the plasma membrane and are also called as chondroids.

Ribosomes

Ribosomes are the small cytoplasmic particles which are the sites of protein synthesis in the organisms. These are composed of RNA (70%) and proteins (30%) and constitute upto 40 per cent of total protein and 90 per cent of total RNA. Ribosomes are often grouped in long chains as polyribosomes. Have a sedimentation co-efficient of 70S and are compared of 30S and 50S subunits (Fig. 3.10).

Polyamines

Three types of polyamines are produced by bacteria. These are *putrescine*, *spermidine* and *spermine*. These are found associated with bacterial DNA, ribosomes and cytoplasmic membrane and are required for the growth of certain species of *Haemophilus*.

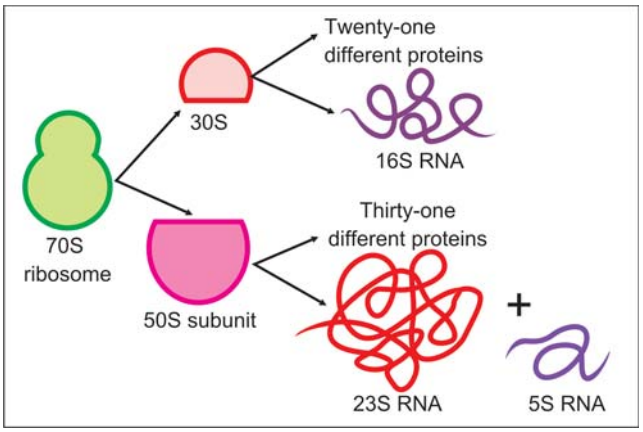


Fig. 3–10. Bacterial ribosomes

Cytoplasmic Granules

Three types of granules have been seen in bacteria (Table 3.9). These are granules of glycogen, poly-beta hydroxybutyrate and Babes-Ernst (volutin) granules. Usually the granules represent accumulated food reserves.

Table 3–9. Cytoplasmic granules in bacteria

Nature of granules	*Examples of bacteria*
Glycogen	Enteric bacteria
Poly-beta hydroxy butyrate	*Bacillus*
	Pseudomonas
Babes-Ernst	*Corynebacterium*
	Yersinia pestis

Nuclear Apparatus

The bacterial genome consists of single molecule of double stranded DNA arranged in a circular form which may open under certain conditions to form a long chain of around 1,000 µm. The bacterial chromosome replicates by single fission. Bacterial DNA represents only 2-3 percent of the cell weight but it occupies almost 10 percent of the volume. This is because of the diffuse chromatin network which allows for ready diffusion of soluble material to all parts of nuclear apparatus. It is not surrounded by a nuclear membrane nor does it contain a mitotic apparatus. Unlike the eukaryotic cells, the nuclear region in the bacteria is not organised and hence is called "nucleoid".

Plasmids: Apart from nuclear apparatus bacterium may have some extrachromosomal genetic material (Fig. 3.11) in the form of DNA which is known as plasmid.

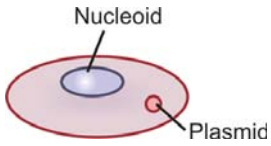


Fig. 3–11. Plasmid in bacteria

The plasmids range in size from less than 1% to about 10% of the size of the bacterial chromosome. There may be only one or many plasmids in a cell. The plasmids do not play any role in the normal physiological functioning of the bacterium, but may confer certain additional properties, (e.g. virulence, drug resistance) which may facilitate survival and propagation of the organism.

Protoplasts and Spheroplasts

The form of bacteria which are devoid of peptidoglycan component of cell envelope and which are thus sensitive to the osmolarity of the medium belong to the groups of spheroplasts or protoplasts (Fig. 3.12). When the surface of bacterium is completely free of cell wall components, it is called as *protoplast* (in gram-positive bacteria) whereas in spheroplasts outer membrane is present (in gram-negative bacteria). Protoplasts cannot revert to normal morphology by re-forming their cell-walls but spheroplasts can do so under suitable conditions.

L Forms

These have also been called as L phase variants. These were first detected in the Lister Institute, London. The designation 'L' has been after the name of the Institute.The L forms are the variants of bacteria which can replicate as pleomorphic, filterable elements with defective or absent cell walls. These can develop in synthetic media in the presence of agents such as penicillin or with hypertonic osmolarity.

BACTERIAL SPORES

Under conditions of limited supply of nutrition, certain bacteria form highly resistant, dehydrated forms called spores or endospores. These endospores are capable of surviving under adverse conditions viz. heat, drying, freezing, action of toxic chemicals and radiation. Spores are formed when the cells are unable to grow, but never by actively growing cells.

Formation and Structure

Spores are usually smooth walled and ovoid. In some species these are spherical. In bacilli, spores usually fit into the normal cell diameter except in clostridia where these may cause a bulge, which may be either terminal (*drum stick*) or more central. The spores usually do not take up ordinary stains. In bacteria spores look like areas of high refractility under light microscope. The structure of spore has been depicted in Figure 3.13 and their arrangement in Figure 3.14. The general life cycle of a spore-forming bacterium is depicted in Figure 3.15.

Germination

The overall process of converting a spore into a vegetative cell is often called as germination. It is much faster than sporulation and under ideal conditions may take less than two hours. It has three stages: Activation, germination proper and outgrowth.

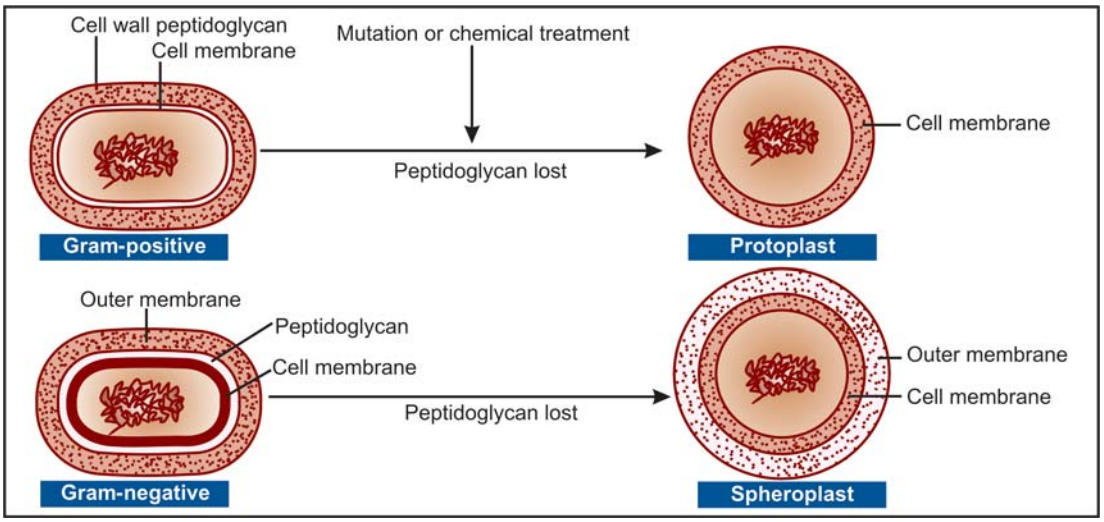


Fig. 3–12. Conversion of bacteria into protoplast and spheroplast

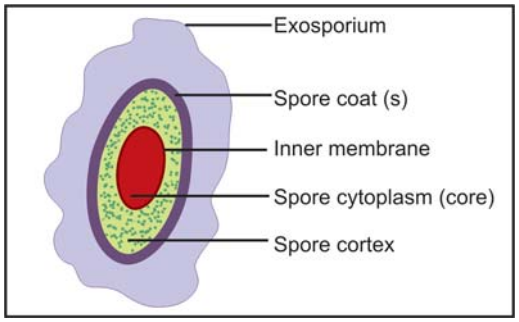


Fig. 3–13. Structure of bacterial spore

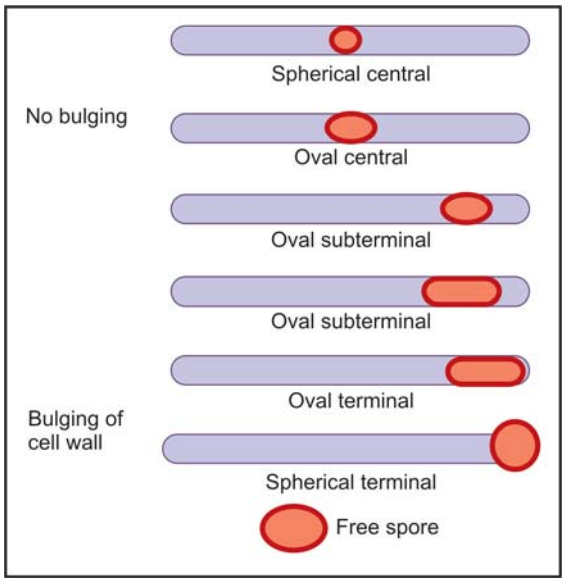


Fig. 3–14. Arrangement of spores

Activation Some spores activate spontaneously but some require the effect of an activator such as heat, low pH or an SH compound.

Germination proper This requires water and a triggering agent. Different bacterial species respond to different agents which include metabolites such as alanine, and inorganic ions like manganese which penetrate the damaged coat.

Outgrowth This occurs following germination in a nutrient medium. In a starvation medium or under adverse conditions the cell may become rehydrated but does not become a vegetative cell. After outgrowth, gradual resumption of vegetative life commences.

Sporulation involves an extensive shift in the pattern of gene transcription which is brought about by changes in the specificity of RNA polymerase.

Examples of spore-forming bacteira

i. Bacillus species
ii. Clostridium species
iii. Gram-positive coccus sporosarcina

Some important features of bacterial spores are given in Table 3.10.

Table 3–10. Features of bacterial spores

- Are formed in response to certain adverse nutritional conditions
- Are inactive bacterial cells
- More resistant to desiccation, heat and various chemicals
- Contain calcium dipicolinate which aids in heat resistance within the core
- Germinate under favourable nutritional conditions
- Helpful in identifying some species of bacteria
- Spore germinate to produce a single vegetable cell

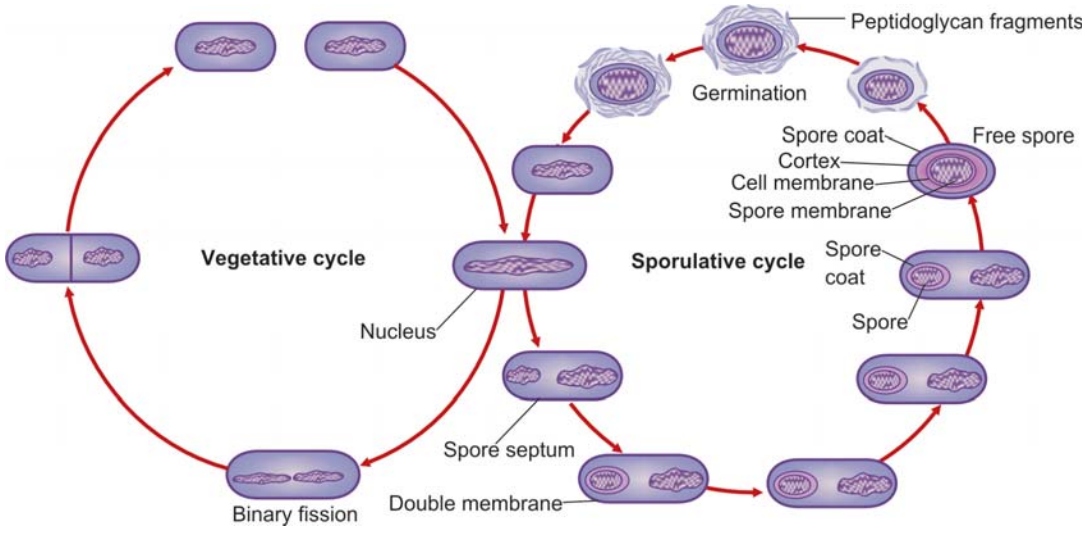


Fig. 3–15. The general life cycle of a spore forming bacterium

CLASSIFICATION OF BACTERIA

Classification of bacteria is a mixture of easily determined practical characteristics (size, shape, colour, staining properties, respiration, reproduction) and of more sophisticated analysis of immunological and biochemical criteria. Their genetic characters can be used to divide the organisms into conventional taxonomic groupings. Bacteria are then classified below the species level in several different ways.

On the basis of these characters, bacteria have been classified into various genera. Those which are of medical importance have been listed in Table 3.11.

Table 3–11. Bacteria of major pathogenic significance

Diagnostic characteristics	*Genus*	*Common species*
Gram-positive bacteria		
Cocci	*Staphylococcus*	*Staph.aureus*
	Streptococcus	*S. pyogenes*
		S.pneumoniae
Bacilli		
Aerobic	*Bacillus*	*B. anthracis*
	Mycobacterium	*M. tuberculosis*
	Nocardia	*N. asteroides*
Anaerobic	*Clostridium*	*Cl. tetani*
	Actinomyces	*A.israeli*
Facultative anaerobic	*Listeria*	*L. monocytogenes*
	Erysepelothrix	*E. rhusiopathiae*
	Corynebacterium	*C. diphtheriae*
Gram-negative bacteria		
Cocci	*Neisseria*	*N. meningitidis*
Bacilli		
Aerobic	*Pseudomonas*	*P. aeruginosa*
	Streptobacillus	*S. moniliformis*
	Brucella	*B. abortus*
	Bordetella	*B. pertussis*
Anaerobic	*Bacteroides*	*B. fragilis*
	Fusobacterium	*F. nucleatum*
Facultative anaerobic	*Escherichia*	*Esch. coli*
	Salmonella	*S. typhi*
	Shigella	*Sh. flexneri*
	Klebsiella	*K. pneumoniae*
	Yersinia	*Y. pestis*
	Campylobacter	*C. fetus*
	Haemophilus	*H. influenzae*
	Vibrio	*V. cholerae*
Spiral	*Treponema*	*T. pallidum*
	Borrelia	*B. recurrentis*
	Leptospira	*L. icterohaemorrhagiae*
Other bacteria		
Wall deficient	*Mycoplasma*	*M. pneumoniae*
Obligate intracellular parasites	*Rickettsia*	*R. prowazekii*
	Coxiella	*C. burnetti*
	Chlamydia	*C. trachomatis*

TECHNIQUES TO STUDY MORPHOLOGY OF BACTERIA

Microscopic Examination

Currently available light microscopes have a double magnifying lens system, a lamp in the base that gives off visible light to illuminate the specimen, and a special lens called the *condenser* that converges or focusses the rays of light to a single point on the object. To be most effective a microscope should provide adequate magnification, resolution and clarity of image. Magnification of the object or specimen by a compound microscope occurs in two phases. The first lens in this system (the one closer to the specimen) is the *objective lens* and the second (the one closest-to the eye) is the *ocular lens* or eye piece. The *total power of magnification* of the final image formed by the combined lenses is a product of the separate power of each lens.

Power of objective lens ×	Power of ocular lens = Total magnification
10 × (low power)	20 × = 200 ×
40 × (high dry-objective)	10 × = 400 ×
100 × (oil immersion)	10 × = 1000 ×

Microscopes are equipped with a nose piece holding 3 or more objectives that can be rotated into position as needed (Fig. 3.16). The power of the ocular lens usually remains constant for a given microscope. Depending upon the power of the ocular lens, the total magnification of standard light microscopes can vary from 40 × with the lower power objective (called the scanning objective) to 2000 × with the highest power objective (the oil immersion objective).

Resolution or the *resolving power* is the capacity of an optical system to distinguish or separate two adjacent objects or points from one another. A simple equation for determining the resolution power is given below.

$$\text{Resolving power (RP)} = \frac{\text{Wavelength of light in nm}}{2 \times \text{Numerical aperture of objective lens}}$$

Thus, the factors influencing the resolution power of the microscope are wavelength of light and numerical aperture of the objective lens.

In practical terms, this means that the oil immersion lens can resolve any cell or cell part as long as it is at least 0.2 µm in diameter and that it can resolve two adjacent objects as long as they are no closer than 0.2 µm. The factor that most limits the clarity of a microscope image is its resolving power.

Variations in Optical Microscope

Optical microscopes that use visible light may be described by the nature of their *field*, meaning the circular area viewed through the ocular lens. With special

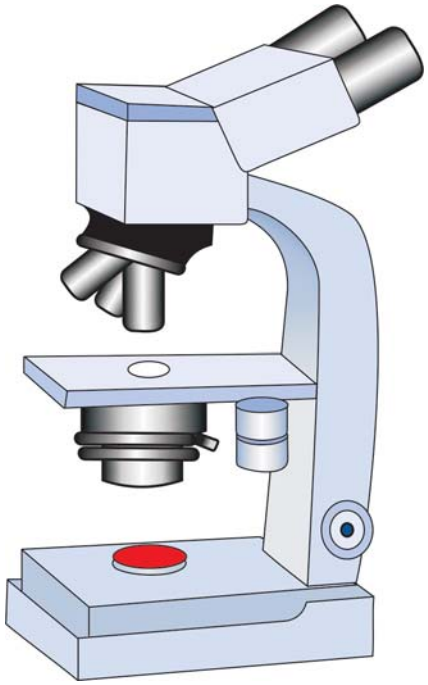


Fig. 3–16. Compound microscope

adaptations in lenses, condenser, and light sources, many special types of microscopes can be described:

i. Bright field
ii. Dark-field
iii. Phase contrast
iv. Fluorescence microscopy
v. Electron microscopy

i. *Bright field microscopy* is the most widely used type of light microscope. It forms its image when light is transmitted through the specimen. The specimen being denser and more opaque than its surroundings absorbs some of this light, and rest of the light is transmitted directly up through the ocular into the field. As a result the specimen will produce an image that is darker than the surrounding brightly illuminated field.

ii. *Dark field microscopy* A bright field microscope may be adapted as a dark field microscope by adding a special disc called a *stop* to the condenser. The *stop* blocks all light from entering the objective lens except peripheral light that is reflected off the sides of the specimen itself. The resulting image is particularly striking of brightly illuminated specimens surrounded by a dark (block) field. The most effective use of dark-field microscopy is to visualize unstained living cells that would be distorted by drying or heat.

iii. *Phase contrast microscopy* Internal components of a live unstained cell are difficult to distinguish because they lack contrast, but cell structures do differ slightly in density, enough that they may alter the light that passes through them in subtle ways. The phase contrast microscope has been constructed to take advantage of this situation. The internal details of the organisms are thus better visualised with phase contrast microscopy than bright or dark field microscopy.

iv. *Fluorescence microscopy* Fluorescent microscope is a specially modified compound microscope furnished with an ultraviolet (UV) radiation source and a filter that protects the viewer's eye from injury by these dangerous rays. The name of this type of microscopy originates from certain dyes (acridine, fluorescein) and minerals that possess the property of fluorescence. This means that they emit visible light when bombarded by shorter UV rays. This has been widely used in diagnostic microbiology to detect both the antigen and antibodies, may they be in pure form or mixed form.

vi. *Electron microscopy* One of the most impressive features of the electron microscope is the high resolution it provides. Unlike the light microscope, which is limited by wavelength and numerical aperture, the electron microscope forms an image with a beam of electrons that behave somewhat like a wave (Fig. 3.17). Because resolving power is a function of the wave length, electrons have tremendous power to resolve

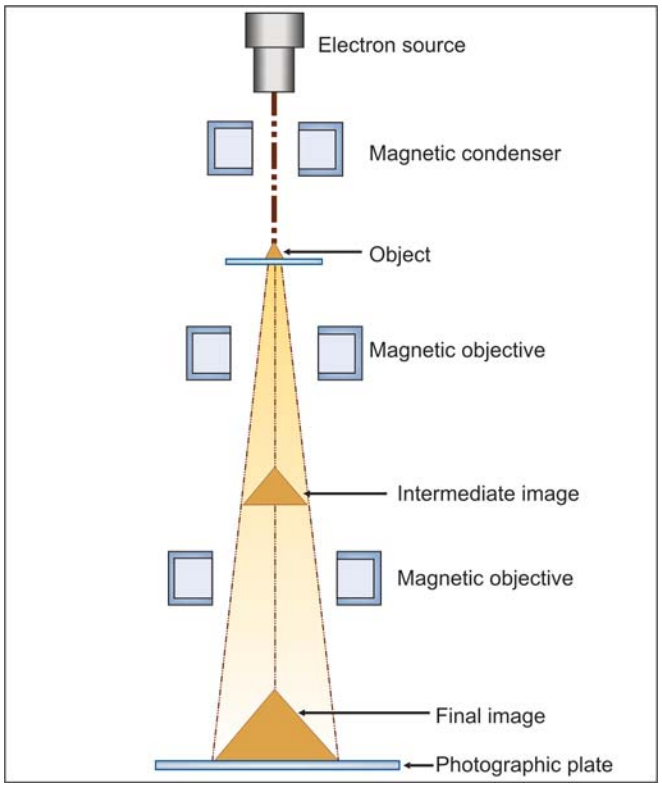


Fig. 3–17. Operational schematic of electron microscope

minute structures. Accordingly magnification can also be maximum. Thus, electron microscope helps in seeing the finest structures (called the ultrastructure). The general forms of electron microscopy are:

a. *Transmission electron microscopy (TEM)*: In which microscope produces its image by transmitting electrons through the specimen.
b. *Scanning electron microscopy (SEM)*: Provides some of the most dramatic and realistic images in existence. It provides detailed three-demensional picture.

The comparison of light and electron microscopy is given in Table 3.12.

Comparative features of different types of microscopy are presented in Table 3.13.

Fresh living preparations. Live samples of microorganisms are placed in wet or *hanging drop* mounts so that they can be observed as much as possible in their natural state. The cells are suspended in a suitable fluid (water, broth, saline) that temporarily maintain viability and provide space and medium for locomotion. A wet mount consists of a drop or two of the culture placed on a slide and overlaid with a cover glass. Although this type of mount is quick and easy to prepare, but the coverslip may damage large cells, slide is very susceptible to drying and can contaminate the slide handler. A more satisfactory alternative is the hanging drop preparation made with a special concave (depression) slide, a vaseline adhesive/sealant film (Fig. 3.18). These types of preparations, though temporary, provide very useful information. Similarly saline mount, iodine mount and KOH mounts can be used.

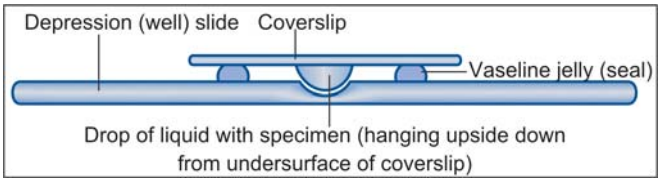


Fig. 3–18. Hanging drop preparation

Fixed stained smears. A more permanent mount for long-term study can be obtained by preparing fixed stained specimens.

a. *Smear* It is the mainstay of the modern microbiology laboratory, the smear was developed by Robert Koch more than 100 years ago. The technique consists of spreading a thin film made from a liquid suspension of cells on a slide and air drying it (Fig. 3.19).
b. *Fixation* During the step called fixation, the air dried smear is usually heated gently by passing through flame (Fig. 3.20). It inactivates and secures the specimen to the slide. It is also meant to arrest various cellular components in a natural state without

Table 3–12. Comparison of light microscopy and electron microscopy

Characteristic	*Light microscopy*	*Electron microscopy*
1. Useful magnification	2000 ×	over 100,0,000 ×
2. Maximum resolution	200 nm	0.5 nm
3. Image produced by	Visible light rays	Electron beams
4. Image focussed by	Glass objective lens	Electromagnetic objective lenses
5. Image viewed through	Glass ocular lens	Fluorescent screen
6. Specimen placed on	Glass slide	Copper mesh
7. Specimen may be alive	Yes	No
8. Special stains or treatment of specimen	Not always	Yes
9. Coloured images produced	Yes	No

Table 3–13. Comparison of types of microscopy

Microscope	*Maximum useful magnification*	*Resolution*	*Important features*
1. Bright field	2000 ×	0.2 mm	Commonly used, good for both live and stained specimens
2. Dark-field	2000 ×	0.2 mm	Best for observing unstained specimen which get destroyed by drying or heat
3. Phase contrast	2000 ×	0.2 mm	Used for live specimen. Excellent for internal details
4. Differential interference	2000 ×	0.2 mm	Provides brightly coloured, highly contrasting three dimensional images of live specimens
5. Fluorescent	2000 ×	0.2 mm	Specimen stained with flourescent dyes. Both antigen and antibodies can be detected. Excellent diagnostic tool.
6. Transmission electron microscopy (TEM)	1,000,000 ×	0.5 nm	Very high magnification finest details seen.
7. Scanning electron microscopy (SEM)	100,00 ×	10 nm	Produces striking three dimensional image

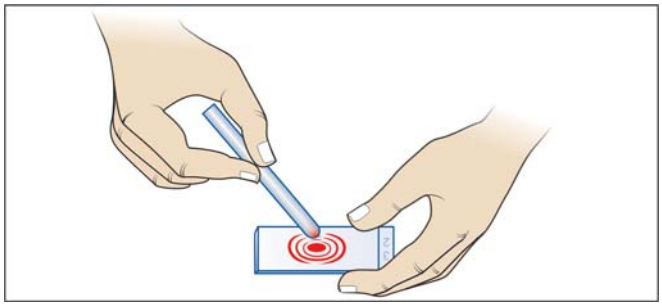


Fig. 3–19. Preparation of smear

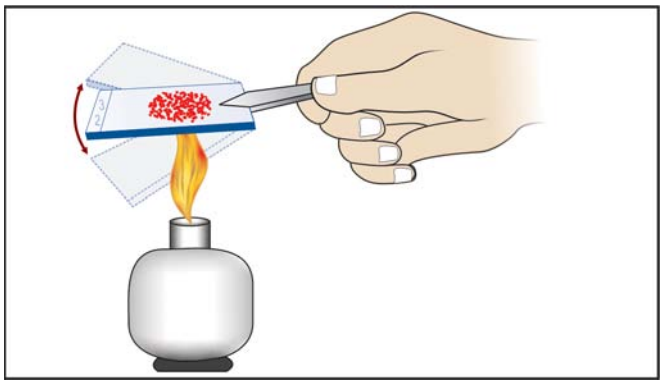


Fig. 3–20. Fixation of smear

undue distortion. The fixation can also be achieved by some chemicals such as alcohol and formalin.

c. *Staining* Staining is a procedure in which coloured chemicals called *dyes* are added to smears. Dyes impart a colour to cells or cell parts by becoming affixed to them through a chemical reaction. In general, dyes are classified as *basic dyes*, which have a positive charge or *acidic dyes* which have a negative charge. Because chemicals of opposite charge are attracted to each other, those cell parts that are negatively charged will attract basic dyes and those that are positively charged will attract acidic dyes (Table 3.14).

Table 3–14. Basic and acidic dyes

Basic dyes	*Acidic dyes*
• Crystal violet • Methylene blue • Safranin • Malachite green	• Nigrosin • India Ink

Negative versus positive staining. Two basic types of staining techniques are used, depending upon how the dye reacts with the specimen. Most procedures involve a *positive stain*, in which the dye actually sticks on the specimen and gives it a colour. A *negative stain*, on the other hand, is just the reverse (like a photographic negative) because the dye does not stick to the speciman but settles around its outer boundary. In a sense negative staining "stains" the glass slide to produce a dark field that highlights the cells in the specimen. Dye used for this purpose is usually India Ink. The value of negative staining is its relative simplicity and the reduced shrinkage or distortion of cells, as the smear is not heat fixed. Negative staining is useful in characterising the capsule that surrounds certain bacteria and yeasts.

The features of positive and negative staining are compared in Table 3.15.

Table 3–15. Comparison of positive and negative staining

Feature	*Positive staining*	*Negative staining*
Appearance of cell	Coloured by dye	Colourless
Background	Usually not stained	Stained, usually dark
Dyes used	Basic dyes	Acidic dyes
Types of stain	Several	Few types

Simple versus differential staining. Positive staining methods are classified as simple, differential or special. While the *simple stains* require only a single dye and an uncomplicated procedure, *differential stains* use two different coloured dyes, called the *primary dye* and the *counterstain*, to separate or distinguish cell types or parts. These stains tend to be more complex and may require additional chemical reagents to reveal a particular staining reaction.

Special stains are used to bring out or emphasize certain cell parts such as flagella and spores that are not revealed by conventional staining methods. Some staining techniques fall into more than one category.

Examples of the three types of stains are shown in Table 3.16.

Table 3–16. Simple, differential and special stains

Simple stain	*Differential stains*	*Special stains*
Methylene blue	Gram's stain Acid-fast stain Spore stain	Capsule stain Flagella stain Spore stain Granules stain Nucleic acid stain

The Gram's staining was introduced in 1880 by the Danish bacteriologist, Christian Gram. Bacteria can be divided into two groups on the basis of their reaction to this staining technique-gram-positive bacteria colour *violet* and gram-negative appear *pink* under the light microscope. This reaction is dependent upon thickness of the cell wall, pore size, and permeability properties

Table 3–17. Differences between gram-positive and gram-negative bacteria

Features	*Gram-positive*	*Gram-negative*
Thickness of cell wall	+++	+
Variety of amino acid in cell wall	A few	Many
Lipids in cell wall	+/-	+++
Digestion by gastric juices	–	+
Lysis by lysozyme	+	–
Inhibition by penicillin	+	–
Inhibition by sulphonamides	+	–
Inhibition by streptomycin	+	–
Inhibition by crystal violet	+	–
Isoelectic range	pH 2-3	pH 4-5
Nutritional requirements	Complex	Simple
Toxin production	Exotoxin	Endotoxin

of the intact cell envelope. Some important differences in gram-positive and negative bacteria are summed up in Table 3.17.

Acid-fast Staining

The acid-fast staining is primarily used for mycobacteria because it is extremely difficult to stain these bacteria with Gram's stain. Mycobacteria are lipophillic and difficult to stain even with other techniques. However, once a stain is taken up by the mycobacteria, it is difficult to decolourize these. Carbol Fuchsin stain is made to penetrate cell wall of mycobacteria by steaming the stain covered slide over a flame for several minutes. After washing, slide is destained with alcohol and counterstained with methylene blue. Under the light microscope, the acid-fast bacilli appear to be red in colour against a blue background.

4 Nutrition and Growth of Bacteria

Bacteria have well defined requirements of proper nutrients, oxygen, pH, and temperature. *Nutrition* is a process by which chemical substances called *nutrients* are acquired from the surrounding environment and used in cellular activities such as *metabolism* and growth.

Bacterial growth refers to an increase in bacterial cell numbers (multiplication) which results from a programmed increase in the biomass of the bacteria. Growth usually occurs asynchronously, i.e. all cells don't divide at precisely the same moment.

NUTRIENTS

Two categories of essential nutrients required are *macronutrients* and *micronutrients*. Macronutrients are required in relatively large quantities and play principal role in cell structure and metabolism. *Micronutrients* sometimes called as trace elements are needed in much smaller amounts for enzyme and pigment structure and function.

Most of the bacteria of medical importance will grow only if a source of organic material as a nutrient is available. For the growth of some bacteria a single source will suffice where some others are more exacting and require complex media where several organic matters are available. Bacteria can be categorized into various groups on nutritional requirements (Table 4.1).

An *autotroph* is a microorganism that depends on no more than CO_2 for its carbon needs.

A *photoautotroph is* an autotroph whose energy needs are met by light.

A *chemoautotroph* is an autotroph whose energy needs are met from inorganic substances.

A *heterotroph* refers to bacteria that require preformed organic compounds, e.g. sugar, amino acids for growth.

A *saprobe* satisfies its nutritional needs by feeding upon the dead.

Bacteria also require a source of nitrogen and a number of salts to have a supply of potassium, magnesium, iron, phosphate and sulphate. Minor concentrations of calcium and manganese are also required whereas growth is facilitated when trace quantities of cobalt, zinc, chlorine, copper, nickel, etc. are present in the medium.

OXYGEN AND CARBON DIOXIDE

The need of oxygen for a particular bacterium reflects its mechanism to meet the requirement of energy. On this basis bacteria have been divided into following groups (Fig. 4.1).

i. *Aerobe (aerobic organism)* grows well in the presence of normal atmospheric oxygen. An organism that

Table 4–1. A nutritional classification of microorganisms

Nutritional type	*Energy source*	*Carbon source*	*Examples*
Autotrophs			
Photoautotroph	Light	Carbon dioxide (CO_2)	Photosynthetic bacteria (green sulfur and purple sulfur bacteria), cyanobacteria, algae
Chemoautotroph	Inorganic compounds	Carbon dioxide (CO_2)	*Nitrosomonas, Nitrobacter*
Heterotrophs			
Photoheterotroph	Light	Organic compounds	Purple nonsulfur and green nonsulfur bacteria
Chemoheterotroph	Organic compounds	Organic compounds	Most bacteria; all fungi; and protozoa

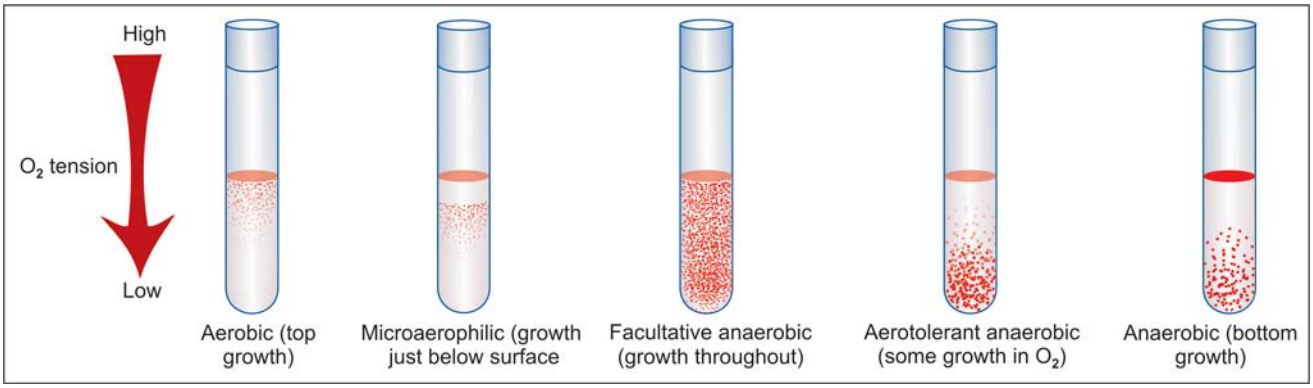


Fig. 4–1. Oxygen requirement of bacteria

cannot grow without oxygen is *obligate aerobe.* Most fungi, protozoa and many bacteria such as genus bacillus are strictly aerobic in their metabolism.

ii. *Microaerophile* does not grow at normal atmospheric tensions, but requires a small amount of oxygen in metabolism. Examples: *Actinomyces israelii* and *Treponema pallidum*.

iii. *Facultative anaerobe* is an aerobic organism capable of growth in the absence of oxygen—that is O_2 is not absolutely required for its metabolism. The examples are enteric bacilli and staphylococci.

iv. *Aerotolerant anaerobes* do not utilize oxygen but can survive in its presence. These organisms are not killed by oxygen, mainly because they possess alternative mechanisms for breaking down peroxides and superoxide. They display "Pasteur effect" in which the energy needs of the cell are met by consuming less glucose under respiratory metabolism than under a fermentative metabolism. Examples are lactobacilli and anaerobic streptococci.

v. *Anaerobe (anaerobic organism)* does not grow in normal atmospheric oxygen and it lacks the metabolic enzyme systems for using oxygen in respiration. Microbes *killed* or inhibited by oxygen are called *strict* or *obligate anaerobes*. Example are: *Clostridium tetani, Bacterioides sps Trichomonas* .

Capnophiles. Although all microbes require some CO_2 in their metabolism, the *capnophiles* grow best at a higher CO_2 tension than is normally present in the atmosphere (e.g. in Candle Jar in Figure 4.2).

The **oxidation-reduction (Redox) potential (Eh)** of the culture medium is a critical factor determining growth. The Eh of most media in contact with air is about +0.2 to 0.4 volt at pH 7. The strict anaerobes are unable to grow unless Eh is as low as -0.2 volt.

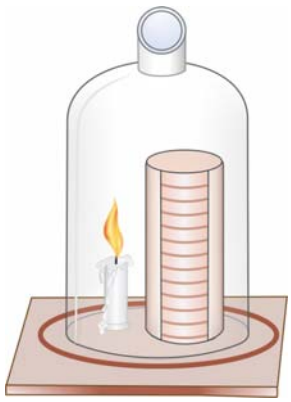


Fig. 4–2. Candle jar

TEMPERATURE

Most of the bacteria have a narrow range of temperature requirement for their optimal growth. Beyond the ideal range of temperature, the growth is either reduced drastically or bizarre and irregular morphological forms are produced. On the basis of temperature requirements, three groups of bacteria are recognised (Fig. 4.3).

i. *Psychorophile* is a microorganism that grows optimally below 15°C and is capable of growing at 0°C. It is obligate with respect to cold and generally cannot grow above 20°C. Room temperature is lethal to the organism. Storage in refrigerators incubates rather than inhibits. They are rarely, if ever, pathogenic to man.

ii. *Mesophile* are organisms that grow at moderate temperatures the optimal range being 20–40°C. Most human pathogens fall in this group. *Thermo-*

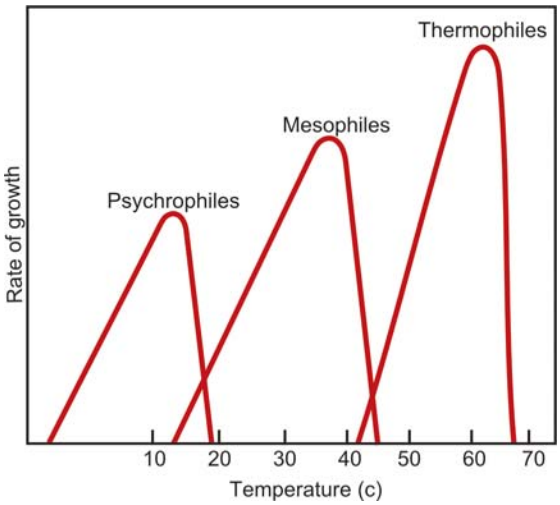


Fig. 4–3. Temperature requirement of bacteria

duric microbes can survive short exposure to high temperature, but normally mesophiles are common contaminants of heated or pasteurized foods.

iii. *Thermophile* is a microbe that grows optimally at temperatures greater than 45°C. Such heat loving microbes live in soil and water associated with volcanic activity and in habitats directly exposed to sun. Most thermophiles are spore-forming sps. of *Bacillus* and *Clostridium* and a small number are pathogens.

pH

The pH of the medium of growth of bacteria has profound effect upon the multiplication of organisms. Most pathogenic bacteria require a pH of 7.2-7.6 for their optimal growth.Some bacteria can flourish in the presence of considerable degree of acidity and are termed *acidophilic*, e.g. *Lactobacillus species*. Some others are very sensitive to acid, but are tolerant of alkali, e.g. *Vibrio cholerae*.

MOISTURE AND DESICCATION

Moisture is absolutely necessary for growth of bacteria. The capability to survive under *dry* environment varies from organism to organism. The Gonococcus and *Treponema pallidum* die quickly in dry conditions but *Staphylococcus aureus* and tubercle bacilli can survive for weeks or months under similar conditions.

Bacterial growth and viability are favoured by darkness. Ultraviolet rays quickly kill the bacteria and a similar effect is produced by ionizing radiations.

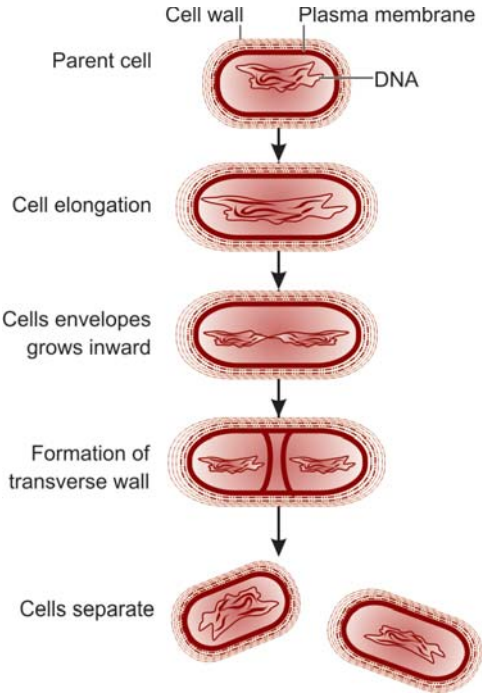


Fig. 4–4. Binary fission in bacteria

BACTERIAL METABOLISM

BACTERIAL GROWTH

Bacteria do not have an obligatory life cycle. Whenever adequate nutrition and conducive environmental factors are available a bacterium enlarges and eventually divides by binary fission to form two daughter cells (Fig. 4.4). The conversion of a parental cell into two daughter cells constitutes the bacterial life cycle and the time taken to complete one cell cycle is known as **generation time** or **doubling time** or **replication time**.

How Fast Do Bacteria Grow?

Compared to the growth rates of most other living things, bacteria are notoriously speedy. The average generation (doubling) time is 30-60 minutes under optimum conditions. Longest generation time occurs in *Mycobacterium leprae* (10-30 days). Most pathogens have relatively shorter doubling time. *Salmonella enteritidis* and *Staphylococcus aureus* both causes of food poisoning, double in 20–30 minutes.

Bacterial Growth Curve

In the presence of fresh growth medium bacteria show following four phases during their growth (Fig. 4.5).

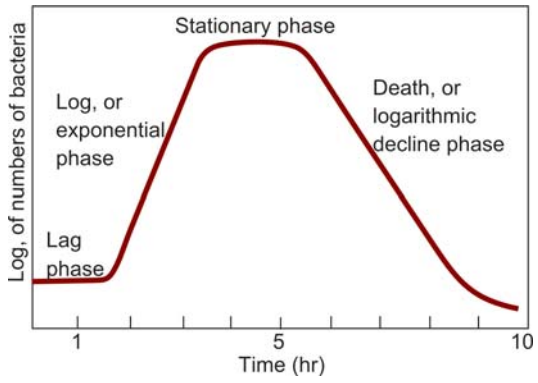


Fig. 4–5. Bacterial growth curve

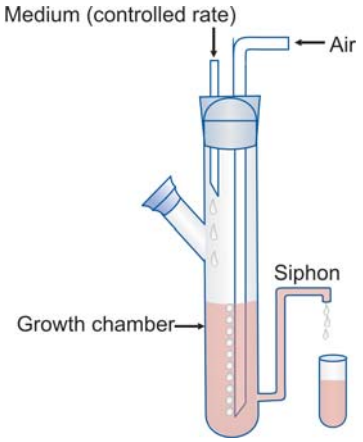


Fig. 4–6. Chemostat

- The lag phase
- The log phase
- The stationary phase
- The decline phase

The Lag Phase

This phase is of a short duration in which bacteria adapt themselves to new environment in such a way that the bacterial machinery brings itself in conformity with the nutrition available. It is 'flat' period on the graph because newly inoculated cells require a period of adjustment, enlargement and synthesis. Enzymes and intermediates are formed and accumulate until they are present in concentration that permit growth to resume.

The Log Phase (Exponential Phase)

Regular growth of bacteria occurs in this phase which is also of short duration since the nutrients present in the medium are utilised by the bacteria and daughter cells. Once exhaustion of nutrients occurs, slowing down of growth also takes place and bacterium passes onto stationary phase. The morphology of bacteria is best developed in this phase and organisms manifest typical biochemical characters.

This phase will continue as long as cells have adequate nutrients and the environment is favourable. Hence, this phase can be prolonged by continuous supply of fresh nutrients at a constant and predefined rate. **Chemostat** (Fig. 4.6) and **turbidostat** are examples of techniques by which this phase can be prolonged, since in both continuous availability of the nutrients is assured.

Chemostats or turbidostats are

- Devices that maintain a bacterial culture in a specific phase of growth or at a specific cell concentration
- Are used to keep bacterial culture in the exponential phase
- Toxic products and cells are removed at the same rate as fresh nutrients are added and new cells synthesised.

The Stationary Phase

This phase ensues when the culture conditions are so changed that further balanced growth and cell division cannot be sustained.

The Decline Phase

The phase of decline is also called as *death phase*. Due to depletion of nutrients and accumulation of toxic end products the number of bacteria dying is much more than those dividing and hence there is a gradual decline in the total number of organisms. The growth curve now dips downwards. For a microbial cell, death means the irreversible loss of the ability to reproduce (growth and divide). The empirical test of death is the culture of cells on solid media; a cell is considered dead if it fails to give rise to a colony on any medium, provided a suitable culture medium is chosen.

Synchronous Growth

Refers to a situation in which all the bacteria in a culture divide at the same moment. It can be achieved by various methods such as selective filtration of older and young cells, alternate cycles of low and optimal incubation temperature, etc.

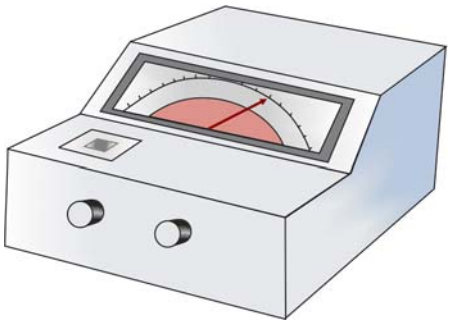


Fig. 4–7. Spectrophotometer

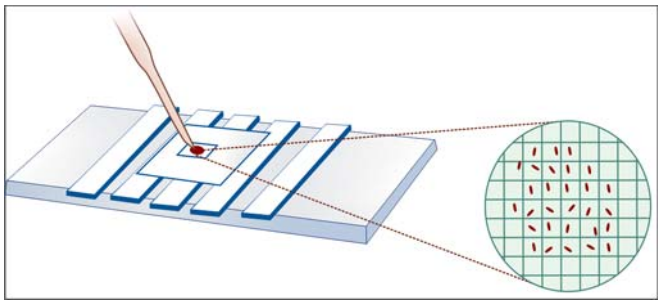


Fig. 4–8. Total cell count

Measurement of Bacterial Growth

It can either be defined in terms of *mass* of cellular material or *cell numbers* depending upon the type of study to be performed.

The cell mass can be measured in terms of dry weight, packed cell volume, or nitrogen content. A convenient method is to determine *turbidity* by photoelectric colorimeter or spectrophotometer (Fig. 4.7).

The cell number can be counted as *total cell number* (Fig. 4.8) as well as *viable count*. Viable number of bacteria can be counted by inoculating the suspension onto solid growth medium and counting the number of colonies. Since each colony is the end product of one viable bacterium, their count gives the number of viable bacteria in the suspension.Total number of bacteria can be ascertained in specially designed chambers such as Coulter counter.

5 Bacterial Genetics

Genetics is the science of heredity. It includes the study of what genes are, how they carry information, how they replicate and pass information to future generation.

In 1944, three American microbiologists, Oswald Avery, Colin MacLead and Maclyn McCarty discovered that a substance called deoxyribonucleic acid (DNA) is the material of which genes are made. In 1953, James Watson and Francis Crick determined the physical structure of DNA. DNA and another substance called ribonucleic acid (RNA) are together called as nucleic acids because these were first discovered in the nuclei of cells.

According to Watson and Crick model, a DNA molecule consists of 2 long strands wrapped around each other to form a double helix (Fig. 5.1) which looks like a twisted ladder. Amino acids are the structural units of proteins, nucleotides are the structural units of nucleic acids. Each strand of DNA double helix consists of many nucleotides. Each chain has a back bone of deoxyribose and phosphate residues arranged alternatively. Attached to each deoxyribose is one of the 4 nitrogenous bases, the purines: adenine (A) and guanine (G) and pyrimidines: thymine (T) and cytosine (C). The purines (A and G) are double-ring structures whereas pyrimidines (T and C) are single ring structures. The term nucleoside refers to the combination of a purine or pyrimidine plus a pentose sugar, it does not contain a phosphate group. Purine 'A' is always paired with pyrimidine 'T' and that purine 'G' is always paired with pyrimidine 'C'. The bases are held together by hydrogen bonds, A-T is held by two hydrogen bonds and G-C is held by three. The order in which the nitrogen-base pairs occur along with backbone is extremely specific and contains the genetic instructions for the organism. In DNA the proportion of A equals that of T and the proportion of G equals that of C. The base ratio of a given DNA is thus (A + T)/(G + C) and remains constant for a species.

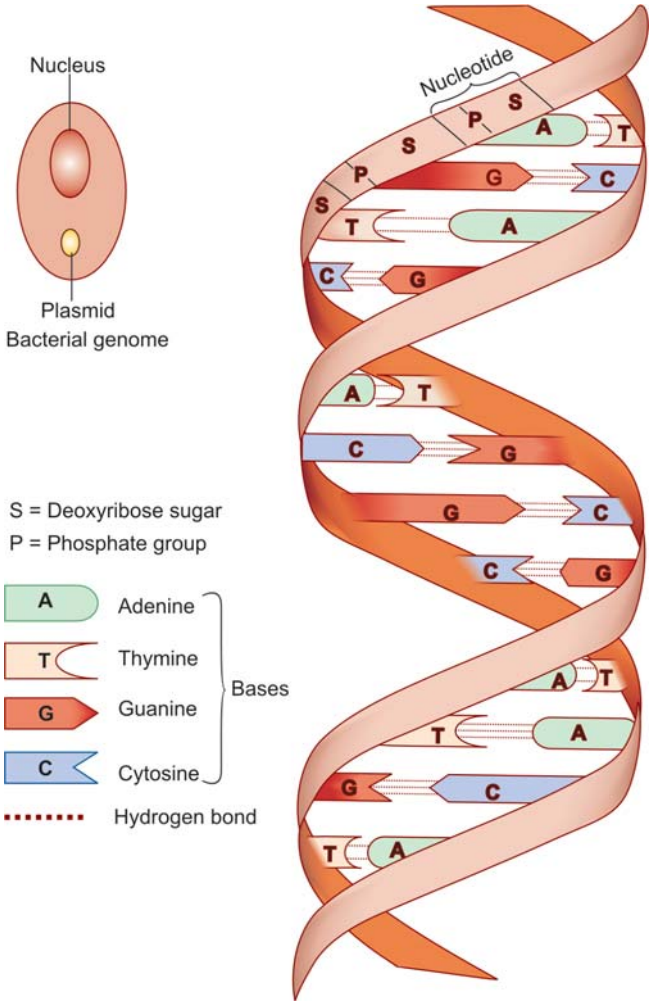


Fig. 5–1. Bacterial genome and double helix structure of DNA

Structure of RNA

Ribonucleic acid (RNA), the second principal kind of nucleic acid, differs from DNA in the following ways.

- Whereas DNA is double stranded, RNA is usually single stranded.

- The five carbon sugar in the RNA nucleotide is ribose as compared to deoxyribose in DNA.
- One of the RNA's bases is uracil (U) instead of thymine. The other 3 bases (A, G and C) are the same in as DNA.
- At least 3 kinds of RNA—ribosomal RNA, messenger RNA and transfer RNA are involved in protein synthesis.

Gene. A gene can be defined as a segment of DNA (sequence of nucleotides in DNA) that codes for a functional product. A DNA molecule consists of a large number of genes each of which contains hundreds of thousands of nucleotides. The length of DNA is usually expressed in kilobases (1 kb = 1000 base pairs) and bacterial DNA is about 4000 kb in length.

Genotype. The genotype of an organism is its genetic make up, the information that codes for all particular characteristics of the organism. The genotype represents the potential properties but not the properties themselves.

Phenotype. Refers to the actual, expressed properties. Phenotype is thus the manifestation of the genotype.

DNA Replication

A semiconservative model for the replication of DNA ensures transmission of genetic information present in the parent is faithfully transmitted to the progeny (Fig. 5.2). It also means that after one generation DNA is present in a hybrid form which contains half old and half new DNA. It was also found that replication of DNA occurs at a growing point (fork) that moves linearly from an origin to a terminus usually in both the directions.

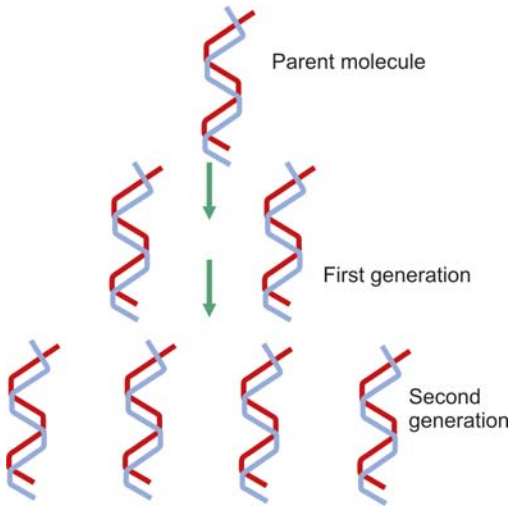


Fig. 5-2. Replication of DNA

Genetic Code

Genetic code is the information which resides in the nucleic acids of the organisms. From DNA it is passed onto mRNA through which it is translated into the primary structure of proteins.

DNA

↓ Transcription

RNA

↓ Translation

Proteins

Extrachromosomal Genetic Material (Plasmids)

The extrachromosomal genetic elements, called as *plasmids* are autonomously replicating, cyclic, double stranded DNA molecules which are distinct from the cellular chromosome (Fig. 5.1). The plasmids carry genes that are not essential for host cell growth while the chromosome carries all the necessary genes.

The general properties of plasmids are shown in Table 5.1. Most of the times plasmids express their characters in the host. Some of the small plasmids sometimes fail to do so and such plasmids are called as *cryptic* plasmids. The commonly observed phenotypic effects of plasmids on bacteria are shown in Figure 5.3.

Table 5–1. Properties of plasmids

• Autonomously replicate in host cell • Plasmid specificity is shown by host • May express phenotypically • Some may have apparatus for transfer • Can reversibly integrate into host chromosome • Can transfer even chromosomal genes • Free DNA is transferred by transfection

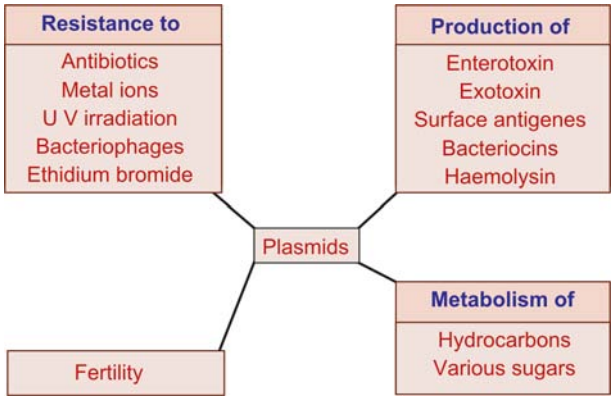


Fig. 5–3. Phenotypic effects of plasmids on bacteria

Bacterial Variations

Bacterial variations are of two types:

Phenotypic Variations

These are acquired during life of a bacterium and may not be passed down to progeny.

Genetic Variations

These variations influence the genetic composition of the bacterium and are transmitted to the next generation. The genetic variations can be due to two reasons:

Alterations in the genome structure due to mutations

Acquisition of genetic material through gene transfer.

MUTATIONS

Mutation can be defined as any change in the sequence of bases of DNA, irrespective of detectable change in the cell phenotype.

Mutation may be *spontaneous* or *induced* by mutagenic agents. Those mutations which do not express phenotypically are known as *silent mutations.*

Point mutation consists of a change in a single nucleotide. *Frameshift mutation* consist of the *insertion* or *deletion* of a single nucleotide.

Among the replacements, *missense mutation* causes one amino acid to replace another and the resultant protein may retain its function without any major change in tertiary structure or active sites.

An altered enzyme function due to mutation may result into the whole organism becoming temperature sensitive. *Nonsense mutations* create a codon that prematurely terminates the growing peptide chain and almost always destroys the function of the proteins.

Mutations may occur

- Spotaneously without any external influence or may be
- Induced by several factors

Spontaneous mutations occur in the absence of any known mutagen and appear to be due to errors in base pairing during DNA replication. Induced mutations are mutations produced by agents called *mutagens*. These are:

1. *Agents which alter the pyrimidines* or *purines* so as to cause error in base pairing. These include nitrous acid and alkylating agents.
2. *Agents which interact with DNA and its secondary structure* producing local distortions in the helix thus giving rise to errors of replication. Acridine dyes such as proflavine and acridine orange belong to this category.
3. *Base analogs which are incorporated into the DNA* and cause replication errors. To this group belongs 5-bromouracil which acts as analog of thymine and pair with adenine.

Mutation Rate

The mutation rate is the probability of a gene mutating each time a cell divides. The rate is usually stated as power of 10, and because mutations are very rare, the exponent is always a negative number. For example, if there is one chance in 10,000 that a gene will mutate when the cell divides, the mutation rate is 1:10,000 which is expressed as 10^{-4}.

Ames Test

It is used to test whether a particular substance can induce mutations or not. Special strains of *Salmonella* that have lost their ability to synthesize amino acid histidine are used in this test. These strains easily undergo another mutation that restores their ability to synthesize histidine. Ames test is based on the hypothesis that if a substance is a mutagen, it will increase the rate at which these organisms revert to histidine synthesizers (Fig. 5.4).

GENE TRANSFER

Unlike eukaryotes where the gene transfer takes place through sexual reproduction—a mechanism which does not exist in bacteria, following four methods result into transfer of genetic material in bacteria:

1. Transformation: uptake of naked DNA
2. Transduction: infection by a nonlethal bacteriophage
3. Conjugation: mating between cells in contact
4. Protoplast fusion.

Transformation

Gene transfer by soluble DNA is transformation. Transformation requires that DNA be adsorbed by the cell, gain entrance to the cytoplasm and undergo recombination with the host genome (Fig. 5.5). The size of the DNA is related to the transforming ability. DNA with less than 0.3 million dalton molecular weight usually fails to transform.

Naturally competent transformable bacteria are found in several genera and include *Bacillus subtilis*, *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Streptococcus pnuemoniae*.

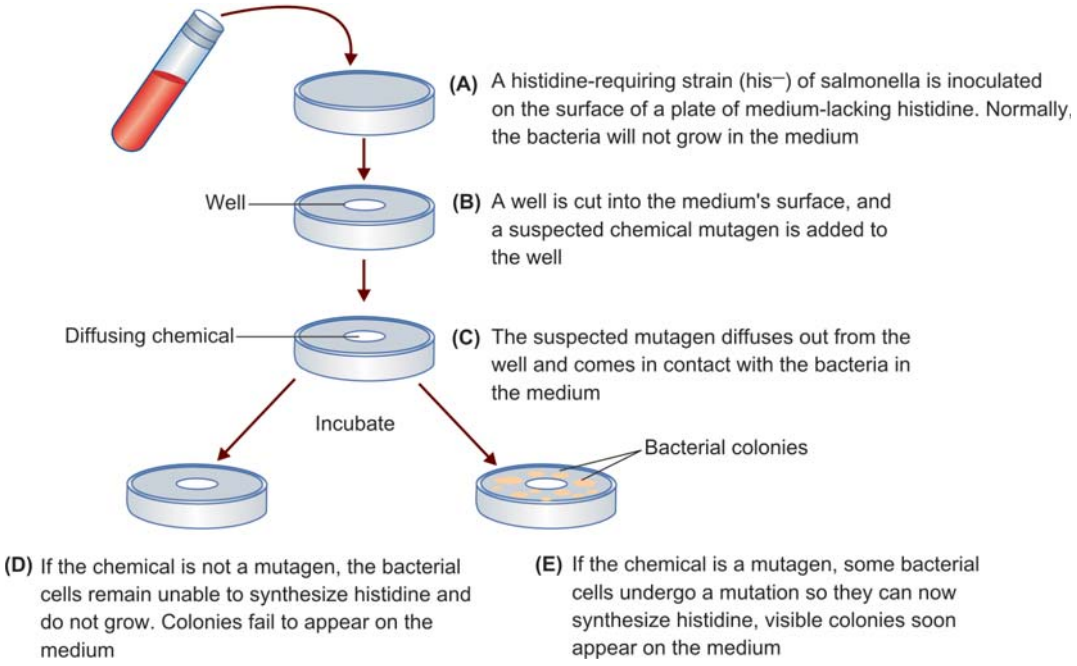


Fig. 5–4. Ames test

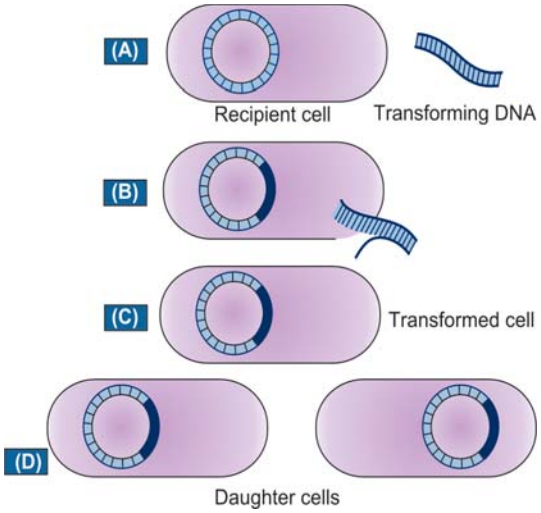


Fig. 5–5. Transformation of a bacterial cell by integration of DNA. (A) Competent recipient cell and transforming DNA. (B) Transforming DNA attacks competent cell and is degraded to single strand by cell nuclease. (C) Single strand DNA pairs with complementary bases of recipient cell chromosome. (D) Integrated transforming DNA is replicated and transformed daughter cells are formed

Griffith in 1928 found that mice died when injected with a mixture of live non-capsulated (R) pneumococci and heat killed (S) pneumococci, neither of which separately proved fatal. If in the experiment the live (R) pneumococci were derived from capsular type II and the killed (S) strain from type III, from blood cultures of mice that had died, live type III capsulated pneumococcus could be isolated, showing that some factor in the heat-killed type III pneumococcus had transferred the information for the capsule synthesis to the live rough strain.

Artificial Transformation (Transfection)

Some of the bacteria (such as *Escherichia coli*) resist transformation until they are subjected to some special treatment such as $CaCl_2$ to make the bacterium permeable to DNA.

Transduction

The type of gene transfer in which the DNA of one bacterial cell is introduced into another bacterial cell by viral infection is known as transduction. This introduces only a small fragment of DNA. Because the DNA is protected from damage by the surrounding phage coat transduction is an easier to perform and more reproducible process than transformation.

Two types of transductions are known. When a phage picks up fragments of host DNA at random and can transfer any genes, it is called as *generalized transduction* (Fig. 5.6). In *specialized transduction* phage DNA that has been integrated into the host chromosome is excised alongwith a few adjacent genes, which the phage can then transfer.

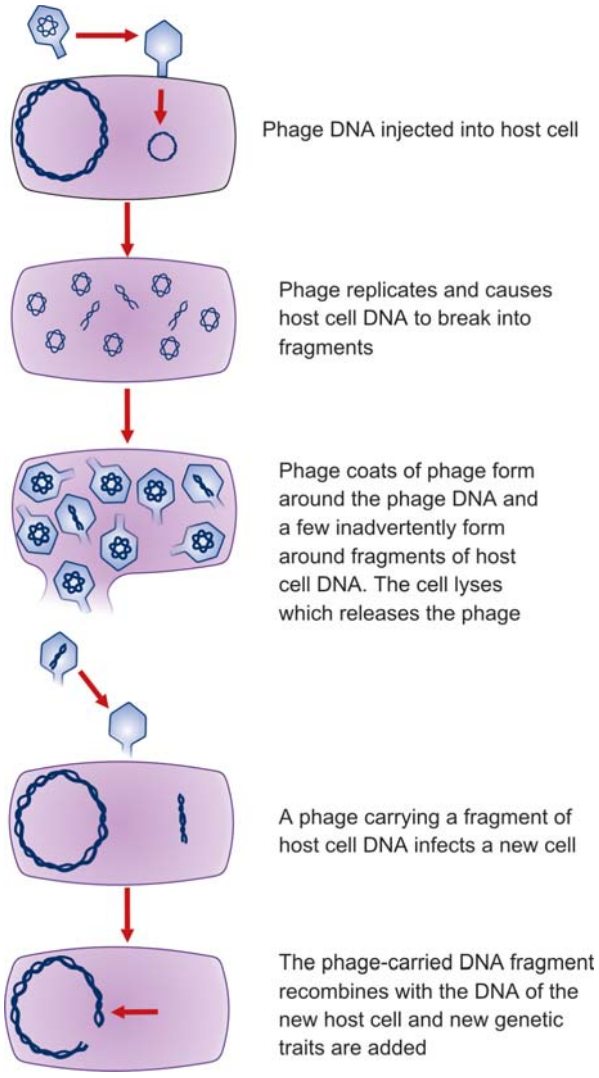


Fig. 5–6. Generalized transduction

In transduction, adsorption of the bacteriophage to receptor sites on the bacterial surface is followed by injection into the cell of the DNA contained within the phage particle. If the phage is of *virulent type,* new phage particles will be synthesized and cell lysis shall result. However, if it is a *temperate phage*, a lysogenic response shall result and phage DNA shall be incorporated into the host chromosome in such a way that the two genomes are linearly contiguous. The phage genome in this stage is known as **prophage**. The host cell acquires a significant new property as a consequence of lysogeny because it becomes immune to infection by homologous phage.

Penicillinase gene in staphylococci is usually located on a plasmid and it may be transferred into other staphylococcal strains by transduction.

Abortive Transduction

If due to any reason the phage DNA fails to integrate into the host chromosome, the process is called as abortive transduction.

Co-transduction: transfer of more than one gene at a time is limited to linked bacterial genes.

Significance of Transduction

- It transfers genetic material from one bacterial cell to another and alters the genetic characteristics of the recipient cell
- The incorporation of the prophage into a bacterial chromosome demonstrates a close evolutionary relationship
- The existence of a prophage in a cell for a long period of time suggests a mechanism for the viral origin of cancer
- Specialized transduction provides a way to study gene linkage which can help in mapping of chromosome.

Lysogenic Conversion

In this the phage DNA becomes integrated with the bacterial chromosome as the prophage which multiplies synchronously with the host DNA and is transferred to the daughter cells. This process is called lysogeny and bacteria harbouring prophages are called lysogenic bacteria. For more details please refer to chapter on Bacteriophages.

Conjugation

Conjugation is defined as the transfer of DNA directly from one bacterial cell to another by a mechanism that requires cell-to-cell contact. DNA is transferred in a nuclease resistant form which differentiates it from transformation and without the aid of bacteriophage which distinguishes it from transduction.

For conjugation to occur, the donor and recipient cells must come in contact. The pili make the initial contact between these two cells and then are retracted into the donor to draw two cells together until direct contact is made. Gradually, this combination is stabilized and significant force is required to separate them.

Significance of Conjugation

- Very important and common mode of drug resistance particularly in enteric bacteria
- Because of precise linear transfer of genes it is useful for gene mapping

- It is especially important in increasing genetic diversity.

The various established techniques of transfer of genetic material have been summarised in Table 5.2.

Transposable Genetic Material

Transposons (Tn) are DNA sequences which are incapable of autonomous existence and which transpose blocks of genetic material back and forth between the cell chromosome and smaller replicons such as plasmids. These can subsequently transfer the DNA blocks to other cells.

Insertion sequences (IS) are another similar group of nucleotides which can move from one chromosome to another genetic material. Such elements (IS and Tn) are called as transposable elements because of their common ability to transpose on different DNA molecules. These elements were also given the name of *jumping genes* because of their ability to insert at large number of sites on chromosomal as well as plasmid DNAs.

These transposable elements are now recognised to play an important role in bringing about various types of mutations in the chromosomes.

NEWER APPROACHES TO BACTERIAL GENETICS

Genetic variation is a continuous process in nature. Mutation, transformation, transduction and conjugation are the processes by which genotype of organisms undergoes change. The selection of new or altered genotype is a complex process and is also done by nature. Intricacies of this section and time period in which such changes take place cannot be defined. It has been since long an endeavour of man to bring about genetic variation or modification in the genotype in a directed and predetermined way. The technique by which it is done is known as **genetic engineering, gene cloning or recombinant DNA technology**. With this it has been possible to study the gene structure and regulation as well as to manipulate the genetic material. The technique has tremendous potential of developing microorganisms that are able to produce many useful products that are difficult or impossible to produce by other methods.

GENETIC ENGINEERING

Genetic engineering are the techniques in which DNA is manipulated artificially to identify and derive useful genes and genetic products. The most prominent of the technologies in this field involves recombinant DNA and gene cloning.

Recombinant DNA Technique

Recombinant DNA (rDNA) techniques unite DNA sequences from different organisms. The technique is essentially based upon the observation that various bacteria accept genetic material such as plasmids from

Table 5–2. Genetic transfer mechanisms

Genetic process	*Represntative organism*	*State of DNA as transfer agent*	*Direction of transfer*	*Amount of transfer*
Transformation	*Streptococcus pneumoniae* *Haemophilus influenzae* *Bacillus* species *Neisseria* spp., Oral streptococci (*Streptococcus sanguis*, *Streptococcus mitior*)	'Naked' DNA	DNA donor → DNA recipient	Few genes (1/200 of chromosomes)
Transduction	*Staph. aureus* *Escherichia coli* *Shigella*	Bacteriophage carrier	Phage donor → phage recipient	Small linkage groups genes
Lysogenic conversion	*Corynebacterium diphtheriae* and other exotoxin producing bacteria	Phage/prophage	Lysogenic → nonlysogenic	1 or 2 genes
Conjugation	*E. coli*, *Shigella*, *Salmonella*, *Proteus*, *Bacillus*, *Streptococcus mutans*, *Bacteroides* spp, *Streptococcus faecalis*	DNA via cytoplasmic	Hfr — F^- (F^+ — F^-)	Large linkage group

other organisms and allow this foreign genetic material to express phenotypically either by the production of proteins or by conferring additional qualities on the host bacterium. The process of introduction of gene and its multiplication in a host is called as **cloning**.

Clone and Cloning Host

A clone has been defined as genetically identical strain or organism that originated from same parent. In molecular biology, **clone** refers to the duplication of a gene isolated from another organism by the cells of a microbial host. For research purposes *Escherichia coli* has been traditionally considered as an ideal **cloning host**. However, because of its potential for virulence such as endotoxin production and capability to establish itself as normal flora, the common brewer's yeast *Saccharomyces cerevisiae* is widely used in this industry since it fulfils most of the following desirable features of the cloning host:

- Rapid overturn, fast growth rate
- Grows readily *in vitro*
- Nonpathogenic with simple genome
- Well characterised genetic make-up
- Capable of accepting plasmid vector
- Maintains foreign genes through multiple generation
- Secrete high yield of proteins from expressed foreign gene.

Vector

Cloning technique requires some sort of vector to carry the foreign DNA into the cloning host. Most vectors are plasmids or bacteriophages. These are of three types:

- R-factor (plasmid that carries genetic markers for resistance)
- Charon phage vector
- A hybrid vector (cosmid) combining both plasmid and phage.

Plasmids are inserted into cloning hosts by transformation and phage-based vectors are inserted by transduction.

Technique of Gene Cloning

The gene cloning requires isolating the desired gene from an animal, plant, virus, or bacterium and inserting it into a vector, cloning the vector with its foreign gene in a cloning host and isolating the end product (Fig. 5.7).

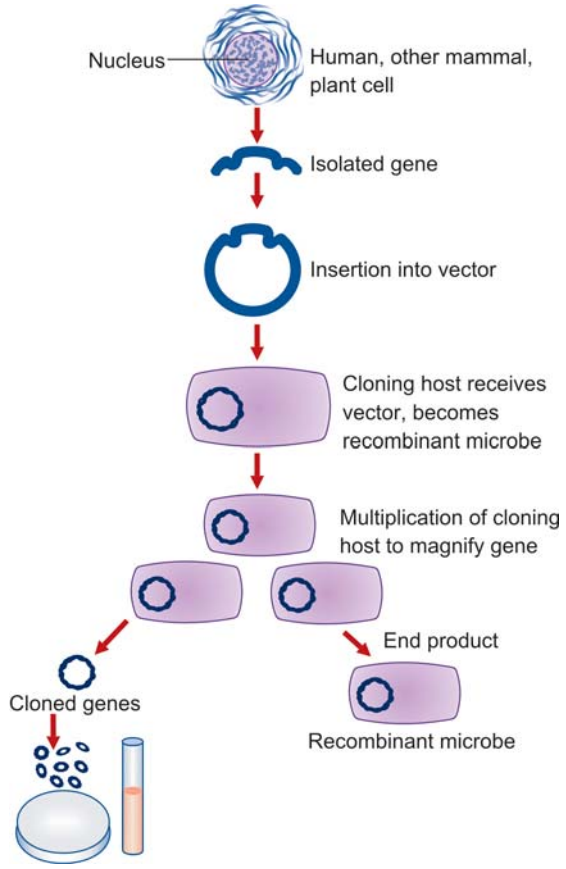


Fig. 5–7. Gene cloning and rDNA technique

Applications of rDNA Technology

Various fields have found unlimited usage for this technology (Table 5.3). Synthesis of humulin (human insulin) and protropin (hormone for children with dwarfism) are being produced by rDNA technology. Vaccine against hepatitis B produced by rDNA methodology has been widely used.

The desired uses of these techniques are three-fold:

- To mass produce protein products such as enzymes and hormones
- To increase the number of cloned genes to be used for gene probes andresearch, and
- To create cultures of genetically recombined organisms for biotechnological applications.

DNA Probes

The advances in the field of immunology and molecular biology have affected the field of diagnosis and detection of microorganisms based upon unique sequences of DNA or RNA. With the development of gene

Table 5–3. Applications of rDNA technology

Production of proteins
- Insulin
- Growth hormones
- Interferon
- Interleukin-2
- Factor VIII

Production of vaccines
- Hepatitis B
- HIV capsid protein (under trial)
- Malaria (in experimental stage)

Antimicrobial agents

Enzymes

Cloned genes
- DNA probes
- RNA probes
- Gene mapping
- Gene therapy

Recombinant microorganisms
- Oil-eating bacteria
- Virus resistant plants
- Fungicide producing bacteria
- Pollutants degrading bacteria

cloning technique and oligonucleotide synthesis almost any nucleic acid sequence can be prepared in large quantities for use as a probe. These nucleic acid probes can be used for various purposes (Table 5.4).

The technique is based upon hybridization of test DNA (to be detected in a test sample/culture) with DNA probe. A DNA probe is a sequence of DNA which is tagged with an easily detectable marker like radioactive isotope or an enzyme. If the test DNA is present in the sample it will conjugate with itself the DNA probe. The sequence of DNA which is used to prepare a probe is unique for that organism and is usually responsible for particular pathogenic property such as toxigenicity or invasion.

Table 5–4. Applications of nucleic acid probes

- Detection of organisms which are difficult to culture.
- Detection of organisms which do not have diagnostic antigens
- Differentiation of avirulent strains from pathogenic ones
- Identification of antibiotic resistance genes
- Detection of latent virus infection
- Development of epidemiological markers
- Rapid confirmation of cultured organisms

In order for a nucleic acid probe to recognize a complementary sequence in a complex mixture of DNA or RNA, the probe must be single stranded and should hybridise with the complementary strand under controlled conditions. Double stranded nucleic acid can be denatured to single strands by chemical or heat treatment.

Probes. The choice of the probe is the most critical step. As discussed earlier DNA sequence is selected which is specific to the pathogen. Once it has been identified, a probe can be prepared by various methods (Fig. 5.8).

For labelling the probes, radioactive material has been used since long. Certain enzymes can also be coupled. These include alkaline phophatase and peroxidae. Fluorescein labelled oligonucleotides have also been prepared. Biotin has also been employed.

Target nucleic acid. Nucleic acid of the test pathogen needs to be extracted and usually denatured before they can be hybridised with the probes. Target DNA can be readily extracted with the help of NaOH. Hybridization reaction can be carried out with the target nucleic acid either in solution or fixed to a solid support such as nitrocellulose or nylon filter. The latter technique is often referred to as *colony hybridization* or as **spot blots, dot blots** or **slot blots**. The probe once mixed with sample seeps out and binds to its

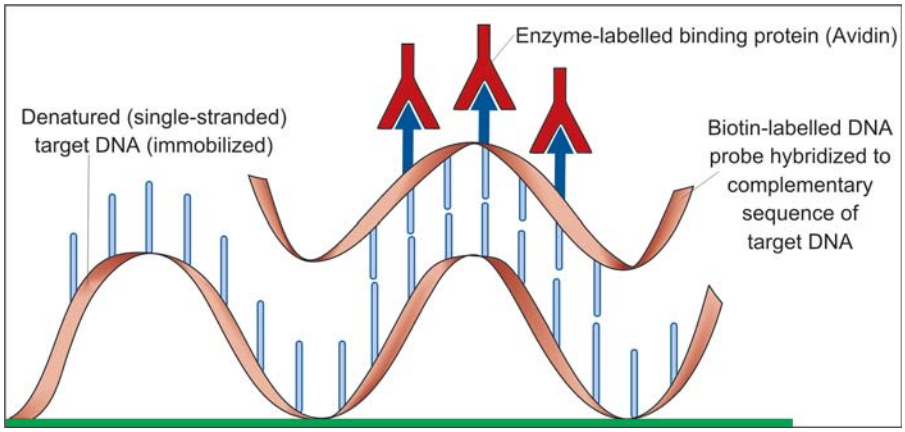


Fig. 5–8. DNA probes

complementary nucleic sequence. The double stranded nucleic acid is then separated from the rest of the labelled probe DNA and sample. The amount of probe bound to sample is recorded and the degree of binding is compared to that found in positive and negative controls to determine whether the sample contained the target sequence in question, and the infectious agent in question. Many DNA probes are currently commercially available (Table 5.5).

DNA probes provide reliable results in a short time (usually less than one day) on a large number of specimens. Though the identification and production of a nucleotide sequence is highly sophisticated and expensive, once produced, they are very cost effective.

Polymerase Chain Reaction (PCR)

The development of the polymerase chain reaction (PCR) in 1983 was major methodological breakthrough in molecular biology. In a short span of fifteen years the method has found its way into nearly every type of laboratory from forensic to ecology and from diagnosis to pure research.

PCR is an *in vitro* method for producing large amounts of specific DNA fragment of defined length and sequence from small amounts of complex template. Perhaps the most obvious application of this technique is to enhance gene probe detection of specific gene sequences. By exponentially amplifying a target sequence, PCR significantly enhances the probability of detecting rare sequences in a heterologous mixture of DNA.

PCR involves three stages which are as follows:

1. Melting of DNA (at 94°C) to convert double stranded DNA to a single stranded DNA.
2. Annealing of primers to target DNA (at 50-70°C) and
3. Synthesis of DNA by addition of nucleotides from primers by action of DNA polymerase.

The oligonucleotide primers are designed to hybridize to region of DNA flanking a desired target gene sequence. The primers are then extended across the target sequence using DNA polymerase derived from *Thermus aquaticus* (Taq) in the presence of free deoxynucleotide triphosphate resulting in duplication of starting material. Melting the product of DNA duplexes and repeating the process many times results in exponential increase in the amount of target DNA (Fig. 5.9).

If PCR is performed at 100% theoretical maximum efficiency, one could generate 100 mg of a 1kb unique human DNA fragment from 100 ng of total DNA in only 25 PCR cycles. Under controlled conditions, however, only a few mg are generally produced.

PCR technique has found applications in a large number of fields (Table 5.6).

PCR amplification permits the detection of as few as 100 cells per 100 gm sample and is useful in tracking genetically engineered microorganisms and monitoring indicator population and pathogens in water, soil and sediments. The PCR products can also be quantified, permitting estimates of organisms and specific mRNAs in the environmental samples. PCR is useful for measuring gene expression by viable microorganisms as well as detecting specific populations based upon diagnostic gene sequence. PCR is also useful for cloning genes, permitting sequence genes and thus demonstrates an extremely wide applications.

RNA PCR

A modification of PCR technique has allowed the amplification starting from RNA template. Basically a complementary copy (cDNA) of the desired RNA target is made by reverse transcriptase and this is followed by a routine PCR which amplifies the cDNA.

Table 5–5. Commercially available diagnostic DNA probes

- *Legionella pneumophila*
- *Mycoplasma pneumoniae*
- *Campylobacter jejuni*
- *Helicobacter pylori*
- *Mycobacterium tuberculosis*
- *M. avium*
- *M. intracellulare*
- *Escherichia coli* (LT and ST toxins)
- Herpes simplex type 1 and 2
- Hepatitis B virus
- *Plasmodium falciparum*
- Rotavirus type A
- Human immunodeficiency virus

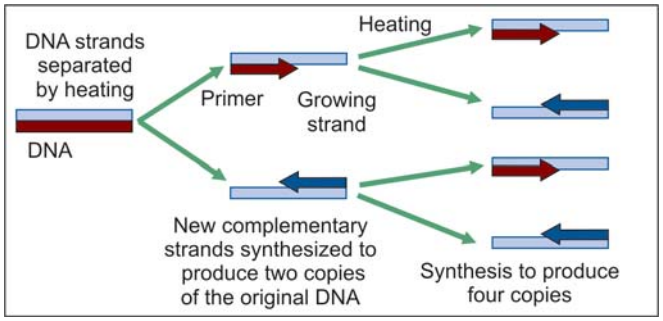


Fig. 5–9: Polymerase chain reaction

Table 5–6. Microbes for which PCR has been performed

Bacteria
Helicobacter pylori
Legionella pneumophila
Mycobacterium tuberculosis
Chlamydia trachomatis
Mycoplasma pneumoniae
Viruses
Coxsackieviruses
Cytomegalovirus
Herpes simplex virus
Hepatitis B virus
Hepatitis C virus
Human immunodeficiency virus
Measles virus
Human papillomavirus
Rotavirus
Rubella virus
Fungi
Candida albicans
Protozoa
Toxoplasma gondii
Pneumocystis carinii
Trypanosoma cruzi
Rickettsia
Rickettsia rickettsiae

Ligase Chain Reaction (LCR)

The LCR, or ligase amplification reaction, was first described in 1989 and modified in 1991. This requires two sets of oligonucleotide pairs which are allowed to anneal to their target DNA at 65°C. Enzyme ligase is allowed to join the pair at ligation junction when complementary base pairing occurs. A mismatch at the pair junction, however, prevents ligation between two oligonucleotides. The reaction mixture is then heated at 94ºC to denature the ligated product from the target and cooled to 65ºC to allow the annealing and ligation, and then the cycle is repeated (Fig. 5.10). Newly formed ligation products are used as templates for ligation of still more substrates. Once ligated products form, subsequent cycles increase the amounts of products at exponential rates. The principal advantage of LCR is its ability to detect single base-pair mismatches between target DNAs.

Uses of LCR

LCR based probe amplification has been used for the detection of *Mycobacterium tuberculosis, Borrelia burgdorferi* and *Neisseria gonorrhoeae.*

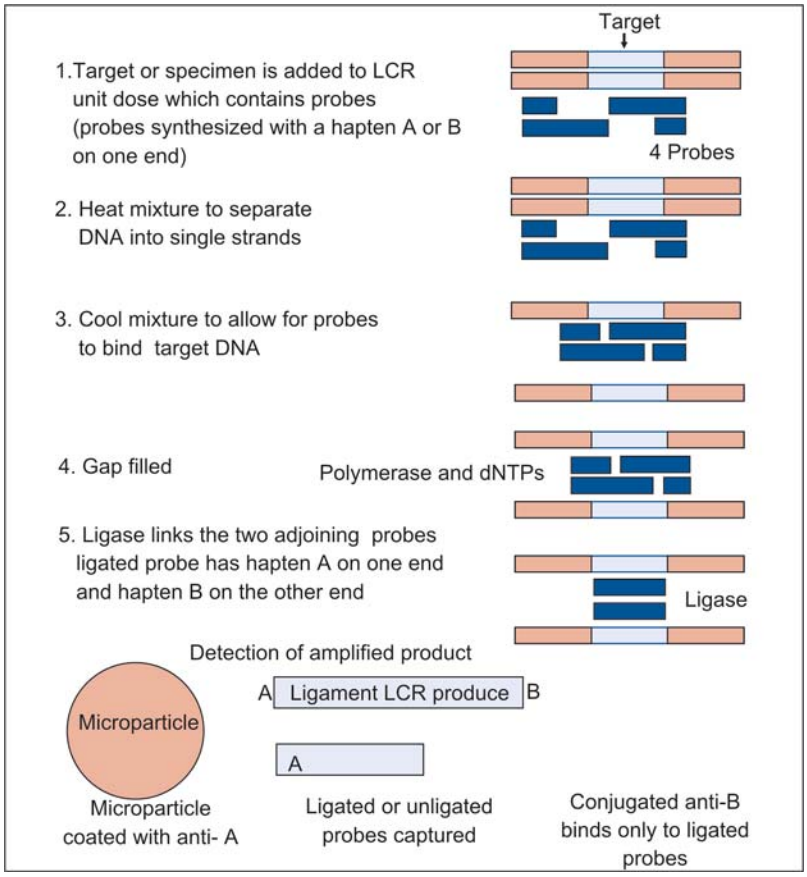


Fig. 5–10. Ligase chain reaction (LCR) amplification of DNA

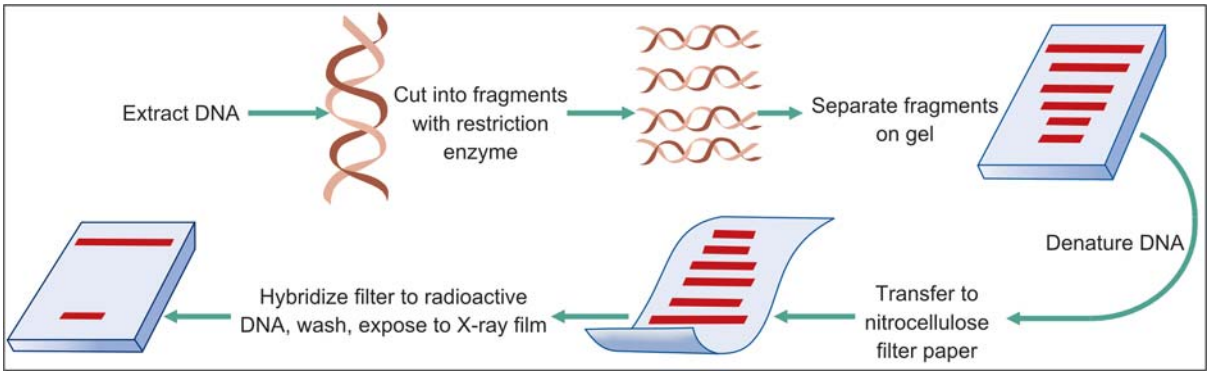


Fig. 5–11. Southern blotting

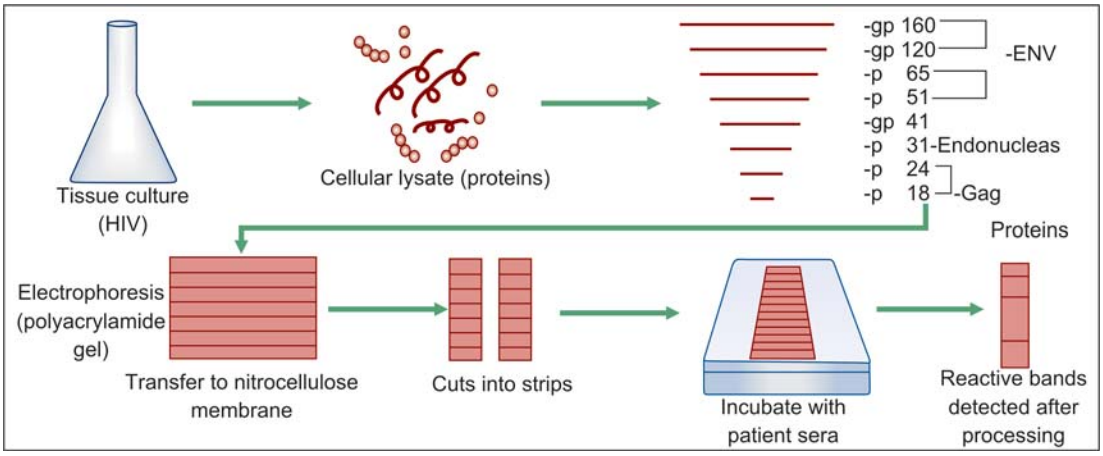


Fig. 5–12. Western blot technique

Blotting Techniques

Southern Blotting

The technique has been named after the scientist who developed it—EM Southern. In this the DNA fragments obtained by digestion with restriction enzymes and separated by gel electrophoresis are transferred by blotting on nitrocellulose or nylon membrane which can bind the DNA. This membrane bound DNA is converted into single stranded form and treated with radioactive single stranded DNA probes. This will result in radioactive double stranded segments which can be detected on X-ray films. This DNA: DNA hybridization is called as Southern blotting (Fig. 5.11).

Northern Blotting

Northern blotting is similar to southern blotting and it is used for the analysis of RNA.

Western Blotting

Western blotting (also known as immunoblotting) is the technique used for identification of proteins. Here all steps are same as in southern blotting except that probe is specific radiolabelled or enzyme labelled antibodies. This test has been used for confirmation of HIV-antibodies (Fig 5.12).

6 Bacteriophage

Definition

The bacteriophages (*phage*: to eat) are the viruses that infect bacteria. These are usually called as phages. These have been used extensively in basic molecular research and epidemiological tracings.

Morphology of Phages

The phages have a simple structure comprising of a **genome** which is surrounded by a protein covering known as **phage capsid**.

The genome usually comprises of single, linear, double stranded DNA molecule. Large phages generally consist of a head and a tail.

The Head

It is a hexagonal structure which encompasses the genome. The head consists of tightly packed core of nucleic acid surrounded by a protein coat or capsid and measure in size from 28-100 nm.

The Tail

The tail is a cylinderical structure which is used as an organ of attachment as well as the conduit through which phage DNA passes into the host cell (Fig. 6.1). It consists of a hollow core and is surrounded by a contractile sheath and a terminal base plate to which are attached pins or tail fibres or both.

The salient characteristics of phages have been summarised in Table 6.1.

Chemical Structure

Bacteriophages comprise of protein and only one type of nucleic acid which in majority of phages is DNA. However, a group of phages which specifically attack male strains of *Esch. coli* contain only RNA. The nucleic acid makes about 50% of the dry weight of phages.

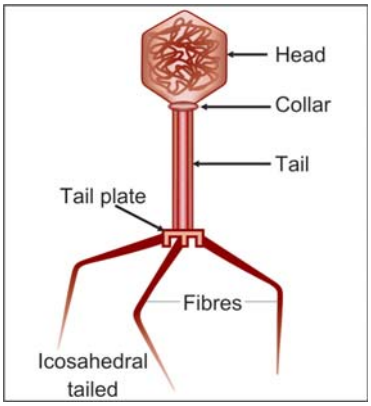


Fig. 6–1. Structure of a phage

Table 6–1. Features of bacteriophages

- Have high host specificity
- Pass through filters which hold back the bacteria
- Lytic phages lyse bacteria best during the active phase of bacterial growth
- Boiling inactivates phages
- Lytic phages can be propagated indefinitely in association with growing bacterial cultures
- Intestinal bacterial flora of man and animals is commonest habitat
- Phage lysis also seen in actinomycetes, bacteria and some yeasts

Life Cycle of Phages

Phages exhibit two different types of life cycles:

a. *Lytic cycle* in which intracellular multiplication of the phage results in the lysis of the host bacterium and release of progeny virions. This is also known as *virulent cycle*.

b. *Lysogenic cycle* in which phage DNA becomes integrated into the bacterial genome and replicates with the bacteria without causing any harm to the host cell. This is also called as *temperate cycle*.

Lytic Cycle

The lytic cycle of bacteriophages can be described in following steps:

Adsorption. Adsorption is the first step and depends upon the susceptibility of the bacterium to the specific phages and is dependent upon the presence of chemical receptors on the surface of the bacterium. It is a very rapid process and is completed within minutes.

Penetration. Penetration follows adsorption. Phages inject the phage DNA into the periplasmic membrane-space between the cell wall and the cell membrane. In most phages the empty head and tail remains outside the bacterium after the injection of phage DNA.

After entry, phage DNA converts into circular form and the process is called as *circulation of phage DNA*.

Eclipse Phase. For several minutes following infection active phage is not detectable. During this period a number of new proteins are being synthesized which include enzymes necessary for synthesis of phage DNA. These are also known as *early proteins*.

Synthesis of Phage Components. After the eclipse phase is over, the synthesis of bacterial proteins stops and that of protein subunits of phage's head and tail starts. These proteins are called as *late proteins*.

Assembly. The protein subunits of the phage head and tail aggregate spontaneously (self-assembly) to form the compact capsid.

Maturation. The assembly of the phage components into the mature infective phage particle is known as *maturation.*

Lysis and Liberation of New Phages. Phage synthesis continues until the cell disintegrates liberating infectious phages as in virulent phages. The cell bursts as a result of osmotic pressure after the cell wall has been weakened by the lysozyme. This is called *lysis from within*.

The interval between the infection of a bacterial cell and the first release of infectious phage particles is known as *latent period*. The average yield of progeny phages from the infected bacterial cells is known as *burst size*. The period during which infectious phages released rise is known as the *rise period.* The lytic cycle is depicted in Figure 6.2.

Lysogenic Cycle

Infection with every phage does not result into lysis of the host cell. Some phages on entering into the cell, do not multiply in the manner described above for virulent phages. Instead, these *integrate into the genome of the host chromosome.* The integrated genome of the phage is called as *prophage*. This phenomenon is known as *lysogeny*. Bacteria which have prophage integrated into their chromosome are designated as *lysogens* and such phages are called as *lysogenic* or *temperate* phages. Lysogenic cycle is described in Figure 6.3.

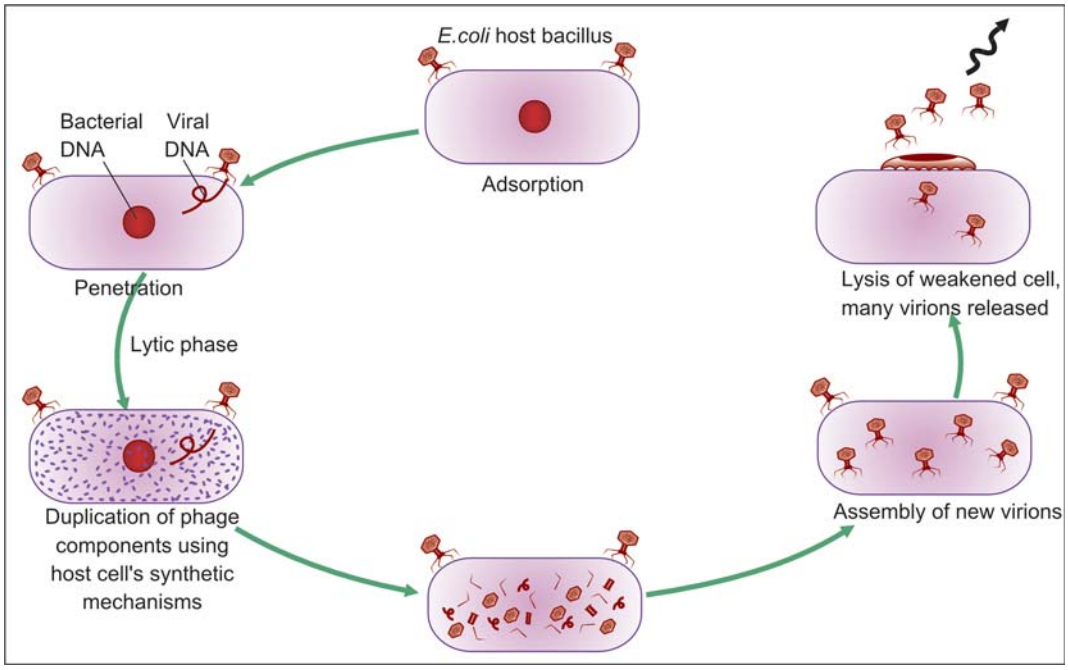


Fig. 6–2. Lytic cycle of a phage

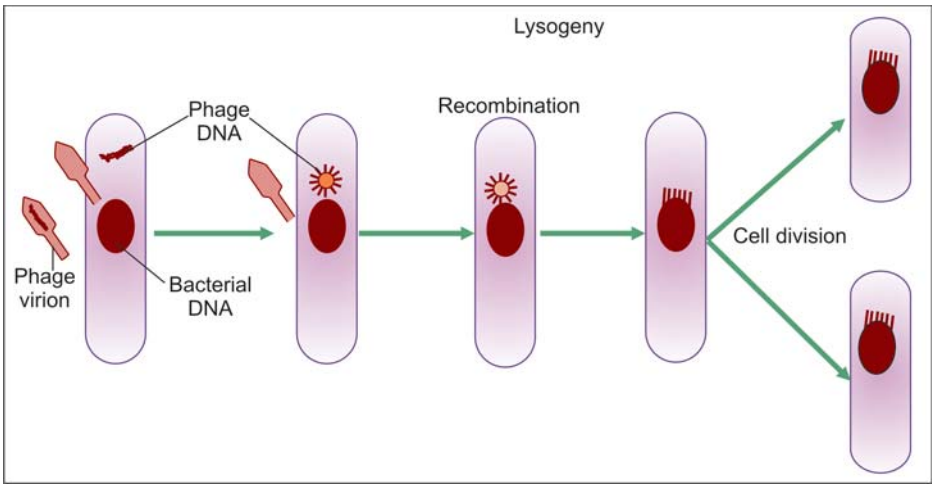


Fig. 6–3. Lysogenic cycle of bacteriophage

Lysogeny. After entering the host cell, the DNA gets integrated into the host DNA. The host cell, now called a lysogenic cell, replicates the phage DNA (prophage) every time it divides. Infection by a temperate phage can also lead directly to a lytic cycle, and on rare occasions, a prophage excises from the bacterial chromosome and initiates a lytic cycle.

Significance of Lysogeny

The prophage confers certain new properties on the lysogenic bacterium. This is known as lysogenic conversion or phage conversion. Some examples are:

a. Phage mediated conversion of somatic antigens of *Salmonella*
b. Phage mediated toxigenicity of *Corynebacterium diphtheriae*
c. Phage mediated toxicity in *Clostridium botulinum*

A lysogenic bacterium is resistant to reinfection by the same or related phages. This is known as *superinfection immunity*.

Bacteriophage Typing

Different strains of a serologically or otherwise identical species of bacteria are susceptible to one or more different strains or types of species-specific bacteriophages. Suspensions of phages are deposited onto agar plates on which a lawn culture has been made with the suspected pathogen. Susceptible bacteria are lysed by the phages, leaving clear areas known as *plaques* (Fig. 6.4). On the basis of this reaction, a bacterial species can be divided into various types. This method has been used in epidemiological tracing of infections or outbreaks due to *Staphylococcus aureus*, *Salmonella* and *Vibrio cholerae*, etc.

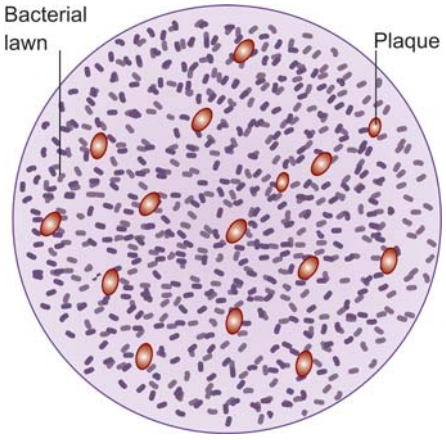


Fig. 6–4. Plaques produced by bacteriophages

Importance of Bacteriophages

1. Bacteriophages may act as carriers of genes from one bacterium to another—a process known as *transduction*. An important example of this is drug resistance seen in *Staphylococcus aureus*.
2. Phage typing is used as an important epidemiological marker which helps in establishing the path of transmission of infectious agent and identifying the reservoir of infection.
3. Subtyping of a species or genus is also possible on the basis of the reaction to phages (vibrios, staphylococci, salmonellae).
4. Bacteriophages may endow the property of toxigenicity to some organisms such as *C. diphtheriae.*
5. Bacteriophages have been extensively utilized in studying host-parasite relationships.

7 *In Vitro* Cultivation of Bacteria

Clinical materials *per se* do not exhibit organisms in a form on the basis of which diagnosis can be confirmed. A presumptive diagnosis on the basis of morphological examination of organism from the clinical sample can be made in exceptionally few instances (e.g. faucial diphtheria, gonorrhoea). It is, therefore, essential to grow bacteria *in vitro* in laboratory by providing essential nutrients and other growth requirements. This process of growing bacteria (*culture of bacteria*) helps in not only having bacteria in pure form but also plenty of growth in pure form can be utilized to undertake further tests to confirm the identity of the bacterium.

BACTERIOLOGICAL MEDIA

The substrate on which bacterial culture is done is known as *medium* which provides nutrition and pH to the organism.

Liquid and Solid Bacteriological Media

There are two broad groups of media: *liquid* and *solid*. Both are extensively used in bacteriological laboratories. To obtain a pure growth one has to use solid medium because of the following disadvantages with the liquid media:

1. Growth does not show character on the basis of which a tentative diagnosis can be established.
2. If it is a mixed culture of more than one organisms, their separation cannot be performed.

However, once a pure growth has been obtained on solid media, liquid media are used to perform further tests because of the homogenous growth in these media.

Agar Agar

To convert liquid media, which were in use till 1880s, gelatin was used by some bacteriologists to make solid media but the real advancement was made by Robert Koch when he could make stable solid media by adding agar agar to the liquid medium. Since then, agar agar (commonly called as agar) has been used as the most effective solidifying agent.

Properties of Agar

Important properties of agar are shown in Table 7.1.

Table 7–1. Properties of agar

- Derived from certain seaweeds
- Melts at 95°C and solidifies at 42°C
- Remains firm at temperatures of incubation
- Heat labile material can be added to it even at temperature of 45°C thus retaining their potency
- Bacteriologically inert
- Resistant to action of all medically important bacteria
- Easily available
- Economical

Types of Culture Media

Some bacteria can grow with minimum and ordinary available growth conditions whereas many others are very specific in their demands. These can be in liquid or solid state; with or without oxygen. Some media have simple composition whereas others may be either synthetic or complex in composition. Accordingly, different types of media are available. Media can be divided into two broad groups: defined synthetic media and routine laboratory media.

Defined Synthetic Media

These are prepared from chemicals and their exact composition is known. These are used for research purposes and are of two types:

Simple synthetic media: Simple synthetic media contain a carbon and energy source, an organic source of nitrogen and various inorganic salts in buffered aqueous solution.

Complex synthetic media: The complex synthetic media possess, in addition to components of simple media, amino acids, purines, pyramidines and many other growth factors depending upon the nature and growth requirements of the organism.

Routine Laboratory Media

These media attempt to provide nutrition and pH to the organisms which are akin to that of tissues and body fluids. Many of these nutrients are supplied by aqueous extracts of meat and peptone. There are various types of these media.

Basal Media

These include nutrient broth and peptone water which are the simplest liquid media and form basis of all other laboratory media. Addition of agar to nutrient broth results in nutrient agar medium which is a solid basal medium.

Enriched Media

Addition of substances such as blood and serum to basal media results in formation of enriched media which can support the growth of those bacteria which are comparatively exacting in their demands. Blood agar is an example of such medium.

Selective Media

These media contain substances that inhibit the growth of a large number of bacteria and permit the growth of a few selected bacteria. This property is the result of addition of some chemical to the basal media. MacConkey agar and bismuth sulphite agar belong to this category.

Enrichment Media

These are liquid media which selectively favour the growth of certain organisms or inhibit the growth of some bacteria from mixed inocula. The examples of this category of media are tetrathionate broth and selenite F medium. These cultures, however, do not indicate the proportion of the selected organism in the mixture.

Indicator Media

When some substance is added to a medium which would produce a visible change in the medium by the growth of certain bacteria, it is designated as indicator medium. MacConkey agar has a dye which in the presence of lactose fermenting organisms changes the colour of the colonies to pink, thus helping in identification of lactose fermenting bacteria. Blood tellurite agar used for isolation of *C. diphtheriae* is another example.

Transport Media

The basic purpose of such medium is to sustain the viability of the organisms when a clinical sample is to be transported from periphery to the laboratory. The medium prevents the growth of contaminants during transit. Stuart's transport medium and Amies transport medium are examples of this kind of media.

Storage Media

These media help in preservation and storage of bacteria for a considerable long period. These include Dorset's egg medium, nutrient agar stabs, blood agar slopes in screw capped bottles and Robertson's cooked meat media.

Sugar Media

The term 'sugar' denotes fermentable substance. The usual sugar consists of 1% of the chosen type of sugar (lactose, sucrose, mannose, dextrose inulin, arabinose, etc) in peptone water alongwith an appropriate indicator. A small tube (Durham's tube) is kept inverted in the sugar tube to detect gas production.

Anaerobic Media

Medium such as Robertson's cooked meat medium is used for growing anaerobic organisms.

Various types of media are summarised in Table 7.2.

Media Recommended for Routine Use in Laboratory

A large number of media are now available and that makes it difficult to select few which can be used routinely in the bacteriology laboratory. Ideally speaking such selection is done only on the basis of the type of workload in that particular laboratory. However, common experience suggests the use of following media for almost all the clinical samples in the initial phase of isolation:

a. Nutrient broth
b. Nutrient agar (or still better blood agar)
c. MacConkey agar
d. Sensitivity test agar (this can be nutrient agar for most of the commonly used antibiotics except for sulpha-or sulpha containing drugs).

Table 7–2. Types of bacteriological media

Name	*Purpose*	*Examples*
Basal media	Simple liquid media Base for other media	Nutrient broth, peptone water
Enriched media	Support the growth of nutritionally demanding bacteria	Blood agar, chocolate agar Loeffler's serum slope
Selective media	Suppress growth of unwanted bacteria and encourage desired ones	MacConkey agar Bismuth sulphite agar
Enrichment media	Liquid media and increase number of desired microbes to detectable levels	Tetrathionate broth, Selenite F Alkaline peptone water
Indicator media	Distinguish colonies of one type from another	MacConkey agar
Selective + Indicator Transport media	Both functions in same medium Sustain microbes during transportation	MacConkey agar Stuart's medium Cary Blair medium
Storage media	Preserve bacteria	Nutrient agar, Dorset egg media

Preparation of Media

The components of any medium are combined together in recommended proportion without sterile precautions. All media are distributed as liquids. Agar media are first melted and distributed when temperature is around 55°C. Clean but unsterile glassware is used and container alongwith media are subsequently sterilized. If autoclaving does not damage the medium, it should be the method of choice for sterilization.

If any of the ingredients of the medium are liable to be spoiled by autoclaving, the complete medium should not be sterilized by heat. In such cases, it is usual to autoclave the thermostable ingredients of medium and to add sterile heat sensitive ingredients with sterile precautions. Some heat labile substances such as blood can be procured sterile from natural sources whereas the remaining have to be filtered.

Blood for use in bacteriology laboratory must be collected under sterile precautions from natural source and should be preserved in sterile container. It should be rendered noncoagulable by defibrination or by the addition of anti-coagulants such as citrates or oxalates. Defibrination is better since it does not involve the addition of any substance that might have some influence upon the nutritive properties of media.

Serum can be sterilized by filteration and hence can be prepared from unsterile, defibrinated or oxalated blood. While handling blood and serum, care should be taken to avoid their contact with human body because of the possibility of transmission of HIV and hepatitis B and C virus infections.

The prepared medium is distributed in test tubes (prior to sterilization) and in petridishes (usually called as plates).

The tubes should not be filled to more than 50 per cent of its capacity and petridish should have around 20 ml of the medium.

Storage of Media

If the sterilized medium is in screw capped bottles, it can be stored at room temperature for weeks. With the passage of time, however, some deterioration in the quality of medium does manifest. Medium in petri dishes deteriorates rather quickly at room temperature. Blood, serum and media can be preserved for longer time if these are kept at low temperatures. Plates of media can be kept in a refrigerator for 7 to 10 days. Screw capped bottles having media can be stored for months in refrigerator. Blood and serum can be stored for still longer periods at –10°C to –40°C. Media should never be frozen since it is detrimental to quality of nutrition.

CULTIVATION OF BACTERIA

The indications for culture of bacteria include:

- To obtain pure culture
- To characterise and confirm the isolate
- To carry out antibiotic sensitivity tests
- To carry out other epidemiological marker studies
- To estimate viable counts
- To store the bacterial isolates for future use.

Instrument for Seeding Media

This is selected according to the nature of the medium and inoculum. Platinum or nichrome wires of different gauges are used. Nichrome is oxidising and hence in

some of the tests where this property of bacterium is to be tested (e.g. oxidase test) instead of nichrome, platinum wire should be used. This wire is sterilized by holding it vertically in the flame of the burner so that the whole length of wire becomes red hot. It is allowed to cool down before it touches any material suspected to be having bacteria to avoid heat killing of organisms. Pre-sterilized disposable loops are now available commercially. The wire can be used as:

- Straight wire (Fig. 7.1) to stab the culture, picking of single colonies as well as for inoculating the liquid media.
- Thick wire which is useful for lifting the viscid material such as sputum, and
- Wire loop which is usually of a diameter of 2 mm is most useful of all inoculating wires. These are preferred to seed a plate of medium as the straight wire usually cuts the agar.

Seeding a Culture Plate

There are three commonly employed techniques for seeding culture plates. Most common of these technique is shown in Figure 7.2.

The inoculum from the clinical material or another plate is first spread out in the form of a primary inoculum (as at A in Fig. 7.2) which is also called as 'well-inoculum' or only 'well'. The successive series of strokes B, C, D and E are made with the loop sterilized between each sequence. At each step the inoculum is derived from the most distal part of the immediately preceding strokes so as to gradually reduce the number of bacteria. This helps in obtaining isolated colonies.

In an alternative plating procedure one edge of a large loop is used to make a secondary well (see B in Fig. 7.3). The other edge is then used to make succession of strokes across the remaining unseeded area.

When the inoculum is small or the medium is selective it can be more heavily inoculated (Fig. 7.4). Several loop-fulls of the specimen are used to spread the primary inoculum.

After sterilizing the loop, it is recharged by rubbing it over area A and the plate is seeded in parallel strokes B, C and D (Fig. 7.4).

Seeding a Liquid Medium

If the tubes have got cotton plugs, the mouth of the tubes should be heated in flame before and after any handling of tube to prevent contamination from the rims of tubes getting into the medium. It is not required when metal caps and screw capped tubes are handled. Incline the liquid medium containing tube to 45° and deposit the inoculum on its wall above the surface

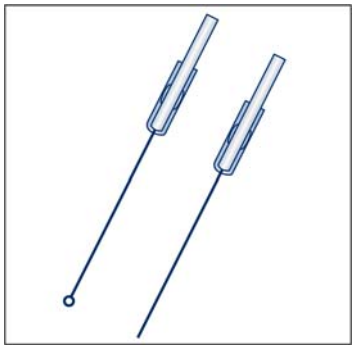


Fig. 7–1. Loop and inoculating wire

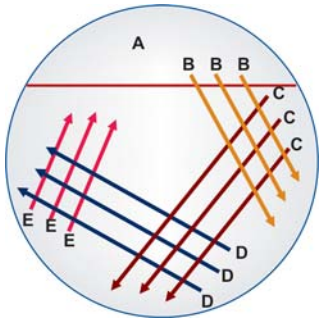


Fig. 7–2. Seeding a culture plate

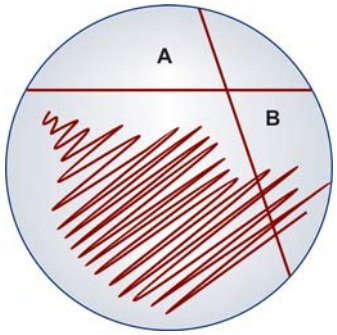


Fig. 7–3. Seeding a culture plate

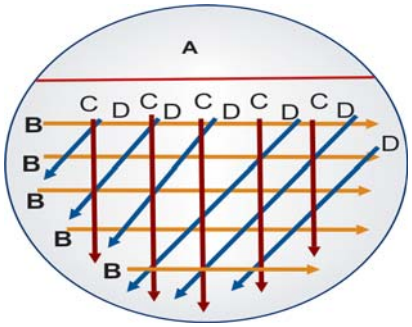


Fig. 7–4. Seeding a culture plate

of the liquid at its lower end. Return the tube to vertical position. Now the inoculum shall be below the surface of the liquid.

Seeding Solid Media in Test Tubes

Slopes of solid media are inoculated by streaking the surface of the agar with loop in a zig-zag manner. Stab cultures are inoculated by plunging the wire into the centre of the medium.

Aerobic Incubation of Cultures

For bacteria of medical importance incubation is usually done at 37°C. Some bacteria require special temperature such as Campylobacters grow better at 43°C and leptospires at 30°C. Depending upon the work load a laboratory may have a table top incubator or a walk-in incubator. For prolonged incubations as are required for the growth of *Mycobacterium tuberculosis* screw capped bottles should be used instead of petri dishes or tubes to prevent the drying of medium.

Incubation in an atmosphere with added carbon dioxide

Extra carbon dioxide is needed for optimal growth of organisms such as *Brucella abortus,* Pneumococcus and Gonococcus, etc. The concentration of additional carbon dioxide nedded is 5-10 per cent. The simplest method for having this environment is to put the plates in a container and generate CO_2 inside by lighting a candle in it just before putting on the lid. Pure CO_2 can also be introduced in a container.

Methods of isolating pure cultures

When there is a mixture of bacteria, following methods can be utilised to isolate bacteria in pure cultures.

a. Use of enrichment, selective and indicator media as described above can help in isolation of pure cultures.
b. Pretreatment of clinical sample with substances which destroy unwanted bacteria e.g. culture of tubercle bacilli. The sputum is pretreated with alkali/acids.
c. Plating on solid media to obtain isolated colonies, for selecting the desired ones.
d. Obligate aerobes and anaerobes can be separated by incubating in appropriate conditions.
e. The vegetative forms and spore forms can be separated by heating at 80°C which will kill the vegetative forms.
f. Motile and non-motile bacteria can be separated by inoculating the Craigie's tube which permits the travel of motile bacteria to appear in the outside of the tube.
g. Bacteria of different sizes can be separated by use of selective filters with different pore sizes.
h. Pathogenic organisms can be separated from non-pathogenic by animal inoculation tests.

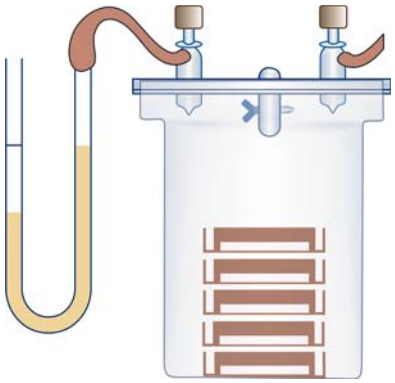


Fig. 7–5. Anaerobic jar

ANAEROBIC CULTURES

For obligatory anaerobic bacteria, oxygen acts as a lethal poison and hence for the growth of these an environment which is free of oxygen has to be created.

The MacIntosh and Fildes technique of 1916 is still widely practised with some modifications. In this the inoculated medium to be incubated anaerobically is placed in the air tight jar and with the help of vacuum pump it is evacuated to –660 mm Hg. Hydrogen (90%) and carbon dioxide (10%) are added to this through built in valves (Fig. 7.5). The oxygen is removed by its combination with hydrogen in the presence of palladium catalyst which is present in the jar. More hydrogen or gas mixture is then introduced to equiliberate the pressure and the jar is incubated at 37°C.

Commercially available kits such as Gaspak and Gaskit are used along with Tatlock anaerobic jar. Gaspak is commercially available as a disposable envelope, containing chemicals which generate H_2 and CO_2 on the addition of water. After the inoculated plates are kept in the jar, the Gaspak envelope, with water added, is placed inside and the lid screw tight. H_2 and CO_2 are released which in the presence of cold catalyst in the envelope permits the H_2 and O_2 to produce anaerobic environment. Gaspak obviates the need of drawing a vacuum and addition of hydrogen.

Reduced methylene blue which remains colourless under anaerobic conditions is used as indicator.

Glove box or anaerobic chamber is used in modern laboratories with high quantum of work.

8 Identification of Bacteria

The most important task of a bacteriology laboratory is to identify the pathogens from the clinical samples so that appropriate treatment can be instituted. The accurate identification, in an overwhelming number of cases, can be accomplished by a series of procedures which are:

- Isolation in pure form
- Morphology of bacterial colony
- Morphology and staining reaction
- Biochemical tests
- Antigenic characters
- Fluorescent microscopy
- Typing of bacteria
- Animal pathogenicity
- Antibiotic sensitivity determination
- Molecular techniques

Isolation in Pure Form

Studies on the biochemical, antigenic and other characters of bacteria can be done only if the organism is available in pure form. From a few clinical samples usually pure culture is obtained. These include blood, spinal fluid, closed abscess but many other clinical samples especially sputum, faeces, skin and body orifices shall provide a mixed culture. For the isolation of organism in pure form following techniques are available:

Plating on Solid Culture Media

Clinical sample is streaked onto a solid medium (nutrient agar, blood agar or MacConkey agar) in such a way so as to ensure isolated discrete colonies. A colony is presumed to be a pure culture since all the bacteria in it are descendents of a single bacterium. This colony can be picked up with a straight wire to obtain a pure subculture.

Use of Selective, Enrichment or Indicator Media

A selective medium such as blood tellurite agar is used to obtain pure growth of diphtheria bacilli because majority of other bacteria will not grow on this medium. Similarly, growth of *Salmonella* is favoured by enrichment medium such as selenite F and a subculture from selenite F within the recommended time shall give a pure growth of *Salmonella*. In indicator media some bacteria shall present with an altered visible appearance of colonies and the same can be picked up and sub-cultured to procure a pure culture, e.g. *Escherichia coli* and *Klebsiella* grow as pink coloured (lactose fermenting) colonies on MacConkey agar as compared to *Salmonella* and *Shigella* colonies which do not ferment lactose and hence are pale yellow in appearance.

Use of Selective Growth Conditions

Most important example of this is the growth of anaerobic bacteria which will not take place in an environment having oxygen.

Animal Inoculation

Some laboratory animals are selectively susceptible to some bacteria and use of this property is made to obtain pure growth of bacteria. If a mixture of organisms having pneumococci is injected into mouse, the animal would die of pneumococcal septicaemia in 12 to 48 hours and from the heart blood of the mouse the organisms can be obtained in pure form.

Morphology of Bacterial Colony

The following characters of the colonies are noted as these are produced by different bacteria (Fig. 8.1).

- Size (diameter in mm)

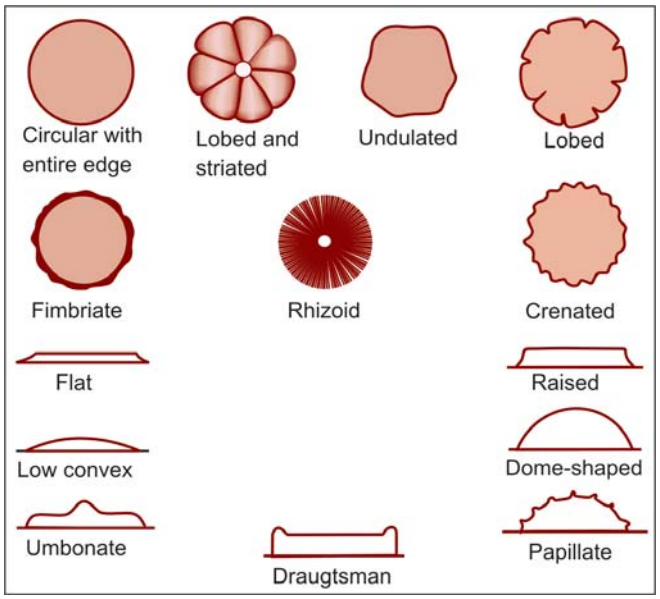


Fig. 8–1. The shape of bacterial colonies

- Outline (circular, entire, wavy, indented)
- Elevation (flat, raised, low convex, dome shaped)
- Translucency (clear and transparent, opaque, trasluscent)
- Colour (colourless, white, yellow, black, pink)
- Changes in medium (haemolysis)
- Mucoid
- Adherence to medium
- Surface : glistening or dull
- Consistency : butyrous, brittle etc.
- Odour : Some bacteria have distinctive odour

Morphology and Staining Reaction

The colonies are subjected to basic staining with Gram stain and their motility is checked by making a 'hanging drop' or wet preparation. These steps show the morphology and motility of bacteria which are of great help in identifying them.

Depending upon source and growth characters, other differential stains can be used to further confirm or classify the bacteria.

Biochemical Tests

A large number of tests are available which help in identifying the bacteria. These can be classified as:

- Tests for metabolism of carbohydrates and related compounds
  - — Tests to distinguish between aerobic and anaerobic break down of carbohydrates.
  - — Tests to show the carbohydrates that can be attacked such as glucose, sucrose, mannitol, lactose etc.
  - — Tests for specific breakdown products such as MR, VP tests.
  - — Tests to show ability to utilize substrates such as citrate, malonate etc.
- Tests for metabolism of proteins and amino acids
  - — Gelatin liquefaction
  - — Indole production
  - — Amino acids decarboxylase tests
  - — Phenylamine deaminase test
- Test for metabolism of fats
  - — Hydrolysis of tributyrin
- Tests for enzymes
  - — Catalase test
  - — Oxidase test
  - — Urease test
  - — ONPG test
  - — Nitrate reduction

Antigenic Characters

The absolute specificity of antigen—antibody reaction is made use of in confirming the identity of an isolate in the laboratory by treating it with its specific antibody (commonly called as antiserum). Such antisera may show agglutination of the isolate if they are specific for each other or are homologous. Confirmation of *Salmonella*, *Shigella* and *Vibrio* is done by the slide agglutination test employing respective homologous antisera.

Fluorescent Microscopy

Antibody molecule labelled with a fluorescent dye is used in direct fluorescent antibody test (FAT) to detect the antigen. If antigen is present, the antibody gets tagged to it and the fluorescent dye, on exposure to ultraviolet rays shall emit bright light indicating the presence of homologous organisms. Various diseases for which rapid diagnosis can be made with fluorescent microscopy include rabies, plague, influenza etc.

Typing of Bacteria

A single bacterial species may contain many strains which vary from one another in minor characters. Thus, different similar strains may constitute types within the same species. There are various methods by which these types can be ascertained. Some of these typing techniques are shown in Table 8.1.These techniques are also used for tracing the source of infection.

Table 8–1. Typing techniques for bacteria

Designation	*Character upon which based*	*Examples*
Biotyping	Biochemical tests	*Vibrio cholerae, Salmonella*
Phage typing	Bacteriophage susceptibility	*Staph. aureus, Salm. typhi*
Serotyping	Surface antigens	Pneumococcus, *H. influenzae*
Bacteriocine typing	Production of bacteriocine	*Pseudomonas, Klebsiella*
Antibiogram typing	Susceptibility to antibiotics	*Salm.typhi, Staph.aureus*
Resistogram typing	Susceptibility to chemicals such as heavy metals	*Salmonella, Klebsiella*
Plasmid profile	Plasmid	All bacteria

Animal Pathogenicity

Isolation of certain organisms is facilitated by the inoculation of clinical material into selectively susceptible animals. However, animal models are more frequently employed for ascertaining whether isolated strain is pathogenic (toxigenic) or not. Some of these are:

Disease	*Animal*
Diphtheria	Guinea pig
Botulism	Mice
Tetanus	Rats
Plague	Mice
Tuberculosis	Guinea pig
Enterotoxins	Rabbit

Antibiotic Sensitivity Determination

Bacteria can be tested in the laboratory to find the most suitable antimicrobial agent. Two methods (Fig. 8.2) are currently practised. These are Stokes' method in which the test strain is tested against standard reference strain for sensitivity to antibiotics and the results are based on the comparison between the zone of inhibition produced by the same drug for test and standard strain. The second method is modified Kirby Bauer's method (NCCLS method) where zone of inhibition produced by antimicrobial agent for a particular strain is measured and its diameter is compared with the standard figures. Based upon this the strain is pronounced as sensitive, or resistant to that particular antimicrobial agent.

Molecular Techniques

Recent molecular techniques which have been used for the identification of bacteria include: DNA probes, polymerase chain reaction (PCR), nucleic acid hybridization and flowcytometry.These tools provide rapid and sensitive means of diagnosis.

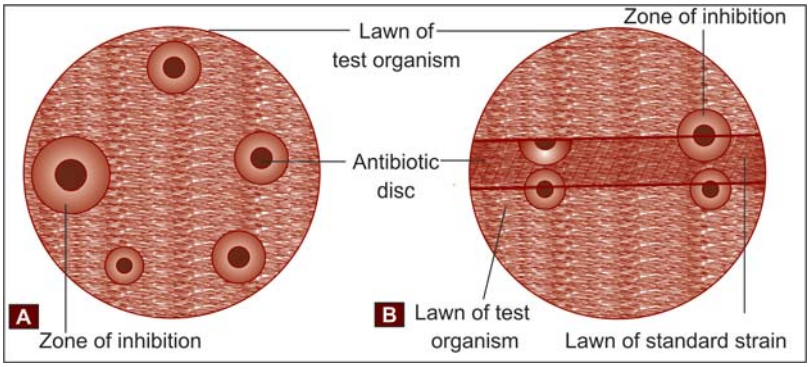


Fig. 8–2. Antibiotic susceptibility determination
A = Kirby Bauer's method, B = Stokes method

PART III MICROBIAL CONTROL AND MECHANISM OF PATHOGENICITY

9 Disinfection and Sterilization

DEFINITIONS

Antiseptic. A substance that inhibits or destroys microorganisms. The term is applied specifically for substances applied topically to living tissues.

Biocide. A substance that kills all living microorganisms including spores, both pathogenic and nonpathogenic (for example bacteriocides, virucides).

Biostat. An agent that prevents the growth of microorganisms but does not necessarily kill them (for example bacteriostatic, fungistatic).

Decontamination. The removal of microorganisms with no quantitative implication. The term is relative and the end can be achieved by sterilization or disinfection. When a highly infectious or pathogenic organism is suspected of being present, decontamination is best achieved by a process that renders the material sterile.

Disinfection. A process that reduces or completely eliminates all pathogenic microorganisms except spores.

Germicide. A substance that destroys microorganisms, especially pathogenic microorganisms. A germicide does not destroy spores.

Sanitation. The process by which microbial contamination is brought to a "safe" level. This process refers primarily to the process of "cleaning" inanimate objects.

Sterilization. The use of physical and/or chemical procedures to completely eliminate or destroy all forms of microbial life. This term is absolute and indicates complete freedom from microorganisms and their spores.

The agent which would perform the functions to fulfil above mentioned definitions can be divided into two broad groups: physical agents and chemical agents (Fig. 9.1).

PHYSICAL AGENTS

Physical agents are usually preferred over chemical agents for performing sterilization. Heat, in one form or another occupies the most important place as physical agent to sterilize. It is the method of choice wherever possible. Both moist as well as dry heat can be applied.

Moist Heat

This is heating in the presence of water and can be employed in the following ways:

- Temperature below 100°C
- Temperature around 100°C
- Temperature above 100°C

Temperature below 100°C

The best and widely used examples of this technique are holder method of Pasteurization where 60°C for 30 minutes is employed for sterilization and the 'flash' modification of the same wherein objects are subjected to a temperature of 71.1°C for 15 seconds.

Temperature around 100°C

Tyndallization is an example of this methodology in which steaming of the object is done for 30 minutes on each of the 3 consecutive days. The principle is that

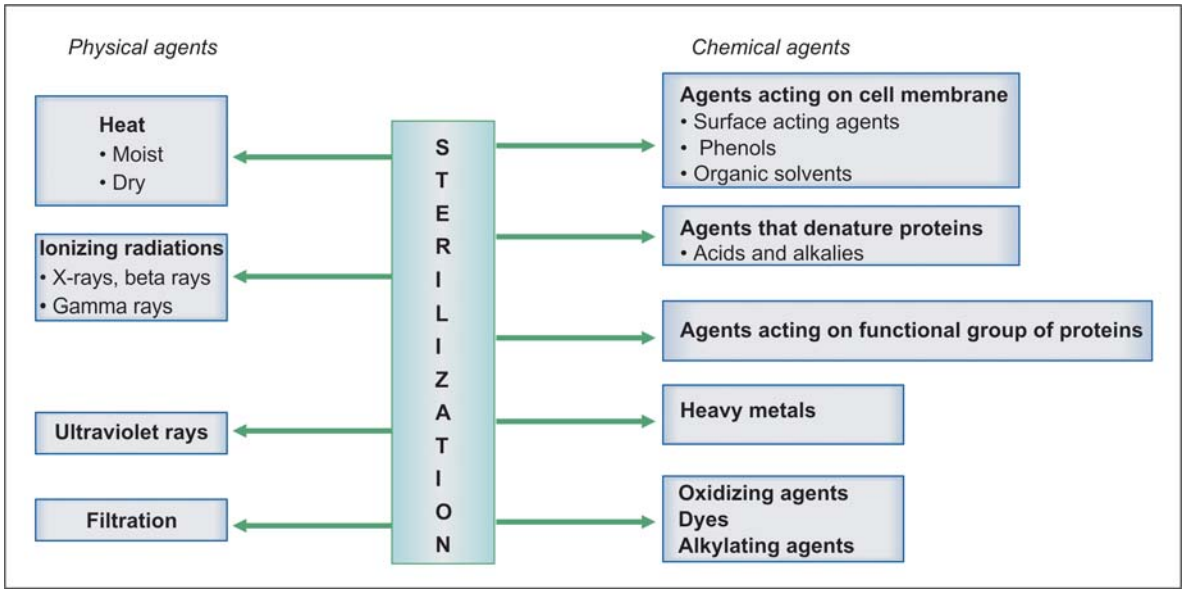


Fig. 9–1. Methods of sterilization

spores which survived the heating process would germinate before the next thermal exposure and would then be killed.

Temperature above 100°C

Dry saturated steam acts as an excellent agent for sterilization because of:

- High temperature
- Wealth of latent heat
- Ability to form water of condensation
- Instantaneous contraction in volume that occurs during condensation.

Superheated steam is not that effective because it is hotter than dry saturated steam and the process is akin to dry heat which is not as efficient as moist heat.

Autoclaves are designed upon the principles of moist heat (Fig. 9.2). The ideal time-temperature relationship in moist heat and dry sterilization processes is given in Table 9.1.

Table 9–1. Time-temperature relationship in heat sterilization

Process	*Temperature*	*Holding period*
Moist heat	121°C	15 minutes
(Autoclaving)	126°C	10 minutes
	134°C	3 minutes
Dry Heat	160°C	120 minutes
	170°C	60 minutes
	180°C	30 minutes

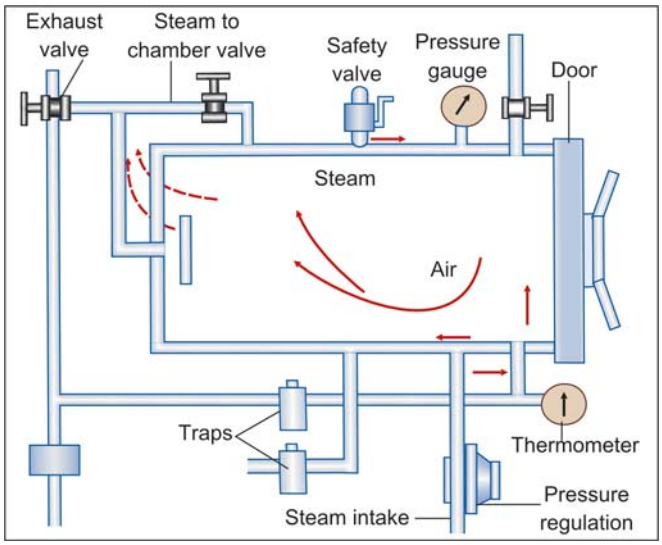


Fig. 9–2. Steam autoclave

Autoclave

Structure: A laboratory autoclave consists of vertical or horizontal cylinder of gun metal or stainless steel in a supporting iron case. The lid is fastened by screw clamps and made airtight by washer. There is a discharge tap for air and steam, a pressure gauge and a safety valve on the lid. Heating is done by electricity or can also be done by gas.

Functioning of an Autoclave

- Put sufficient quantity of water in the cylinder
- Put the materials to be sterilized on the tray
- Start the heating
- Screw tight the lid, keeping the discharge tap open
- Adjust the safety valve to the required pressure
- Allow the steam-air mixture to escape freely till all the air has been displaced. This can be tested by leading the escaping steam into a pail of water through a rubber tubing close the discharge tap when no more air bubbles come through
- Count the holding time from the time when the safety valve opens and the excess steam escapes
- Turn off the heater when the holding time is complete
- Allow it to cool, tilt the pressure gauge indicates that the pressure inside is equal to the atmospheric pressure
- Open the discharge tap slowly and let the air into autoclone.

Drawbacks of Autoclaving

- The method of air discharge is inefficient and it is difficult to assess when the process is completed
- No facility to dry the materials after the process of autoclaving is completed.

A variety of autoclaves are now coming in the market to avoid these problems.

Cautions

i. For effectiveness, completely remove the air from the autoclave
ii. Pack the articles to be autoclaved in such a way that there is easy penetration of the steam
iii. Don't open the tap when the pressure inside is high than atmospheric pressure, it can lead to explosion or spilling of liquids.

Low Temperature Steam with Formaldehyde

Low temperature steam at 80°C was found to be more effective than water at the same temperature. The addition of formaldehyde to low temperature steam achieves a sporocidal effect and it has been found to be suitable for thermolabile equipment.

Mechanism of Microbial Inactivation by Moist Heat

Various mechanisms come into play for killing non-sporulating bacteria as well as destroying the spores (Table 9.2).

Sterilization by autoclaving is invariably successful if properly done and two common sense rules are followed:

Table 9–2. Mechanisms of microbial inactivation by moist heat

Bacterial spores	*Non-sporulating bacteria*
• Denaturation of spore enzyme • Impairment of germination • Damage to membrane • Increased sensitivity to inhibitory agents • Structural damage • Damage to chromosome	• Damage to cytoplasmic membrane • Breakdown of RNA • Coagulation of proteins • Damage to bacterial chromosome

- Articles should be placed in autoclave so that steam can easily penetrate them
- Air should be evacuated from autoclave so that the chamber fills with steam.

Wrapping objects in aluminium foil is not recommended because it may interfere with steam penetration.

Dry Heat

Dry heat is less efficient process than moist heat and bacterial spores are most resistant to it. Spores may require a temperature of 140°C for three hours to get killed.

Dry heat can be used by following means:

Incineration

This is an efficient method for sterilization and disposal of contaminated materials at a high temperature.

Red Heat

Inoculating wires, loops and points of forceps are sterilized by holding them in the flame of a Bunsen burner until they are red hot.

Flaming

Direct exposure for a few seconds may be used for scalpels and the neck of flasks, but it is of uncertain efficacy.

Hot Air Sterilizers (Ovens)

Dry heat sterilization is usually carried out in hot air ovens in which a number of time-temperature combinations can be used (Table 9.1). It is essential that hot air should circulate between the objects being sterilized and these must therefore, be loosely packed and given adequate air space to ensure optimum heat transfer. A fan is also essential to prevent the wide variations in temperature that would otherwise occur. In case there is no fan the holding time has to be doubled for that particular temperature.

Mechanism of Inactivation

Microbial inactivation by dry heat is primarily an oxidation process. However, the possibility of DNA damage is also now incriminated as one of the mechanisms.

Dry heat is employed for sterilization of glassware, glass syringes, oil and oily injections and metal instruments.

D Value

Several measurements have been defined to quantify the killing power of heat. The thermal death point is the time required to kill all the bacteria in a particular culture at a specified temperature.

The decimal reduction time, also known as the DRT or D value, is the length of time needed to kill 90 per cent of the organisms in a given population at a specified temperature.

Ionizing Radiations

Ionizing radiations include X-rays, gamma rays and beta rays. These can induce single stranded and sometimes double stranded breakdown of DNA. In contrast ultraviolet rays do not possess enough energy to eject electrons to bring about chemical changes and hence are not ionizing radiations. The efficacy of ionizing radiations is influenced by following factors:

1. **Type of organisms.** Spores are more resistant than non-sporulating bacteria.
2. **Pre-irradiation treatment.** Organisms which are derived from media having serum are more resistant.
3. **Oxygen effect.** This concerns the effect on bacterial spores of gaseous environment during and after radiation.
4. **Stage of sporulation.** Resistance to irradiation develops earlier than resistance to heat.

Mechanism of Microbial Inactivation

These radiations induce defects in the microbial DNA. These are not always irreversible because the natural DNA repair mechanism tries to repair. If no repair takes place, DNA synthesis is inhibited and errors in the protein synthesis manifest resulting into cell death. Spores are more resistant to ionizing radiations than non-sporulating bacteria because of followings:

a. Possible presence of a radioprotective substance in spores.
b. Spore coat confers protection.
c. DNA is in different state in spores.

The ionizing radiations are used for the sterilization of single use disposable medical items.

Ultraviolet Radiations

The UV rays have wavelength varying between 210-328 nm and wavelength of 240-280 nm has been found to be most efficient in sterilizing. With UV rays the quantum of energy liberated is less and these rays have lesser penetration power which makes these rays less effective than other types of radiations. Bacterial spores are more resistant to UV rays than the vegetative cells. Even viruses are often more resistant than vegetative bacteria. Whereas *Sarcina lutea* is highly sensitive to UV rays, *Salmonella typhimurium* is moderately sensitive and organisms such as *Escherichia coli, Staphylococcus* and *Proteus vulgaris* are mildly sensitive to the action of these rays.

Mechanism of Inactivation

Exposure to UV rays results in the formation of purine and pyramidine dimers between adjacent molecules in the same strand of DNA resulting in noncoding lesions in DNA and bacterial death.

UV rays have practical applications in disinfection of drinking water, obtaining pyrogen free water, air disinfection (especially in safety laboratories, hospitals, operation theatres) and in places where dangerous microorganisms are being handled.

Because of the presence of UV rays, the sunlight has got bactericidal activity. Sunlight can inactivate even spores provided the exposure is of prolonged duration. The desert surfaces are found to be sterile not only because of immense dry heat but also because of prolonged exposure to UV rays present in the sunlight.

Sterilization by Filtration

Filters have been used for last many centuries in one form or another primarily to purify water and sewage by allowing them to percolate through beds of sand, gravel or cinders. Several types of filters are now available. These include:

Unglazed ceramic filters. These are manufactured in different grades of porosity and used for large scale clarification of water. Chamberland and Doulton filters are examples of this type. These filters can be cleaned with chemical agents after use.

Asbestos filters. These filters have high adsorbing capacity but suffer from disadvantages of alkalinizing the solutions and possible carcinogenesis. These include Seitz, Carlson and Sterimat filters.

Sintered glass filters. These contain finely powdered glass particles of different sizes—the size being adjusted

according to the required pore size. These are expensive and being glass, fragile.

Membrane filters. These are widely used nowadays and consist of cellulose ester. These are most suitable for preparing sterile solutions. The range of pore size in which they are available is 0.05–12 µm (required pore size for sterilization is in the range of 0.2–0.22 µm).

The membrane filters have found extensive usage in medical and industrial fields (Table 9.3).

Table 9–3. Applications of filtration

- Sterilization of thermolabile parenteral and ophthalmic solutions
- Sterility testing of pharmaceutical products
- Clarification of water supplies
- Microbiological evaluation of water purity
- Viable counting procedures
- Determination of viral particle size
- Air sterilization
- Sera sterilization

Indicators of Sterilization Process

The wide application of sterilization processes makes it mandatory to impose strict control measures in these processes to validate the results obtained. These processes are of three broad types: physical, chemical and biological (Table 9.4). In addition, sterility tests on the treated products are necessary. The commonly employed methods are chemical indicators, autoclave tapes and thermocouples.

A summary of important features of physical agents used to control microorganisms has been shown in Table 9.5.

CHEMICAL AGENTS

In contrast to chemotherapeutic agents which show a high selectivity for certain bacterial species, chemical disinfectants are highly toxic for all types of the cells. Their efficacy, however, also depends upon the conditions in which they are made to work and these include:

- Concentration of bactericidal agents
- Time of exposure
- pH of the medium where action is to take place
- Temperature of the medium
- Nature of the targetted organisms
- Presence of extraneous materials.

The chemical agents can be classified into various groups depending upon their site of action or mechanism of inactivating the organisms (Table 9.6).

Agents that Interfere with Membrane Function

The passage of nutrients and fluids into and out of the cell is regulated by the cell membrane which comprises of lipids and proteins in an orderly arrrangement. Any interference with the integrity of the structure of the membrane shall result into damage to active transport as well as energy metabolism resulting into cell death.

Surface Acting Agents

These substances bring about changes in the energy relationship at surface thus bringing down the surface tension. These are widely used as detergents, wetting agents and emulsifiers. They are of three types:

1. **Cationic Surface Acting Agents.** These include quaternary ammonium compounds which are more active at alkaline pH and denature proteins. These are bactericidal for many bacteria especially gram-positive organisms.
2. **Anionic Surface Acting Agents.** These include soaps which act better at acid pH and are effective against gram-positive organisms. Gram-negative bacteria are relatively resistant because of the presence of lipopolysaccharide in their cell wall. These should not be used alongwith cationic agents because they neutralize the action of each other. Both these types of surface acting agents are inhibited by the presence of extraneous proteins in the medium.
3. **Nonionic Surface Acting Agents.** Tween 80 and triton X-100 are examples of this type of detergents which are relatively nontoxic and a few of these even promote the growth of bacteria. Tween 80 by providing oleic acid in the medium promotes the growth of tubercle bacilli.

Table 9–4. Methods of validating sterilization processes

Process	*Physical methods*	*Chemical methods*	*Biological test organism used*
Dry heat	Temperature recording chart	Colour change indicators	*B. subtilis var niger*
Moist heat	Temperature recording chart	Colour change indicators	*B. stearothermophilus*
Ionizing radiations	Dosimeters	Radiochromic chemicals	*B. pumilus*
Filtration	Bubble point pressure test	None	*Ser. marcescens, Ps. diminuta*

Table 9–5. Summary of physical agents used to control microorganisms

Physical method	*Conditions*	*Instrument*	*Object of treatment*	*Examples of uses*	*Comment*
Direct flame	A few seconds	Flame	All microorganisms	Laboratory instruments	Object must be disposable or heat-resistant
Hot air	160°C for 2 hr	Oven	Bacterial spores	Glassware, powders oily substances	Not useful for fluid materials
Boiling water	100°C for 10 min 100°C for 2 hr	— —	Vegetative form of microorganisms Bacterial spores	Wide variety of objects	Total immersion and precleaning necessary
Pressurized steam	121°C for 15 min at 15 lb/in^2	Autoclave	Bacterial spores	Instruments Surgical materials Solutions and media	Broad application in microbiology
Fractional sterilization	30 min/day for 3 successive days	Arnold sterilizer	Bacterial spores	Materials not sterilized by other methods	Long process sterilization not assured
Pasteurization	62.9°C for 30 min 71.6°C for 15 sec	Pasteurizer	Pathogenic microorganisms	Dairy products	Sterilization not achieved
Hot oil	160°C for 1 hr	—	Bacterial spores	Instruments	Rinsing necessary
Filtration	Entrapment in pores	Berkefeld filter Membrane filter	All microorganisms	Fluids	Many adaptations
Ultraviolet light	265 nm energy	UV light	All microorganisms	Surface and air sterilization	Not useful in fluids
X-rays Gamma rays	Short-wave length energy	Generator	All microorganisms	Heat-sensitive materials	Possible toxic chemicals
Ultrasonic vibrations	High-frequency sound waves	Sonicator	All microorganisms	Fluids	Few practical applications

Table 9–6. Classification of chemical disinfectants

Agents interfering with membrane functions	
Surface acting agents	Quaternary ammonium compounds
	Tween 80
	Soaps and fatty acids
Phenols	Phenol, cresol
	Black fluid, hexylresorcinol
Organic solvents	Chloroform
	Toluene
	Alcohol
Agents that denature proteins	
Acids and alkalies	Organic acids
	Hydrochloric and sulphuric acids,
	Formic acid
Agents that destroy/modify functional groups of proteins	
Heavy metals	Copper, Silver, Mercury and their compounds
Oxidizing agents	Hydrogen peroxide, ozone, iodine, chlorine
Dyes	Acridine orange
	Acriflavine
Alkylating agents	Formaldehyde
	Ethylene oxide

Phenols

These compounds in low concentration precipitate proteins as well as orient themselves in the interfaces of the cell membrane resulting in damage to cell. The oxidases and dehydrogenases which are bound to cell membrane are inactivated by phenols. These chemicals are bactericidal in nature.

Most of the phenols have low solubility in water and hence are formulated with the addition of soaps which also increase their bactericidal activity. Cresol is one such preparation in which soap is added to phenol.

The halogenated diphenyl compounds (e.g. hexachlorophene) were shown to be highly effective against Gram-positive organisms. Hexachlorophene was used in large variety of cosmetics and soaps and daily bathing of newborn children with soap having this compound became an established practice in early 1960s to avoid colonization of umbilical stumps with *Staphylococcus aureus*. Within a decade, its use was banned because of the neurotoxicity observed with this compound after dermal absorption.

Organic Solvents

Alcohol is the most important example of this group. Alcohol disorganizes the lipid structure of the cell membrane by penetrating into the hydrocarbon region. It also denatures the proteins of the cell. Alcohols are, however, restricted by their inability to kill spores at normal temperatures and hence should not be used for sterilization of instruments.

Chloroform and toluene are used to keep the solutions sterile and to disrupt permeability barriers.

Ethanol is used to sterilize the skin prior to cutaneous injections. It is active against Gram-positive, Gram-negative and acid-fast organisms and acts best at concentration of 50–70 per cent. Isopropyl alcohol is more active than ethanol and is less volatile and can be used to sterilize thermometers.

Agents that Denature Proteins

The proteins are functional only when these are in proper characteristic conformation. Agents that alter the structure of proteins make them nonfunctional. The denaturation of proteins causes unfolding of the polypeptide chain so that chains become randomly looped or coiled.

Acids and Alkalies

These agents release their free H^+ and OH^- ions and then through undissociated molecules or by altering the pH of the medium they denature the proteins of the organisms. The whole molecule of the acid (e.g. benzoic acid) is also as effective as dissociated molecules. Benzoic acid and salts of propionic acid are effective as food preservatives.

Agents that Destroy or Modify Functional Groups of Proteins

The binding of bacterial enzymes to their respective substrates and initiation of catalytic action is done by certain specific functional groups of the enzymes. If these groups are altered or destroyed, the bacteria do not survive. Mercury and arsenic compounds combine with sulphadryl groups and formaldehyde reacts with amino and imidazole groups. Basic dyes react with the acidic groups.

Heavy Metals

Soluble salts of silver, mercury, arsenic and other heavy metals destroy enzymatic activity by combining with sulfhydryl groups of cystine residues. As compared to mercury chloride, which was earlier extensively used as a disinfectant, the organic mercurials such as merthiolate and mercurochrome are less toxic and useful antiseptic agents.

Silver compounds are widely used as antiseptics. The organic silver salts are efficient bactericidal agents. Most commonly employed compound is silver nitrate which is highly bactericidal for gonococci and is used for the prophylaxis of ophthalmia neonatorum in newborn infants.

Oxidizing Agents

This group includes halogens, hydrogen peroxide and potassium permanganate. These convert functional-SH groups into non-functional oxidized S-S form.

Chlorine and iodine are most useful disinfectants. Iodine as a skin disinfectant and chlorine as water disinfectant have given consistently magnificent results. Their activity is almost exclusively bactericidal and they are effective against sporulating organisms also. Iodine acts best at pH less than 6 and its efficacy decreases as the pH rises beyond 7.5. Mixtures of various surface acting agents with iodine are known as *iodophores* and these are used for the sterilization of dairy equipments.

Apart from chlorine, hypochlorite, inorganic chloramines and organic chloramines are good disinfectants they act by liberating chlorine. Although chlorine is one of the most potent bactericidal agent, its activity is markedly influenced by the presence of organic matter and hence it becomes necessary to ascertain the chlorine demand of the water before it is disinfected.

Hydrogen peroxide in a 3 per cent solution is a harmless but very weak disinfectant whose primary use is in the cleansing of the wounds. Potassium permanganate is another oxidizing agent which is used in the treatment of urethritis.

Dyes

Various dyes not only stain the bacteria but also inhibit their growth. These dyes include aniline dyes and the acridines. The dyes are, however, readily neutralized by serum and other proteins. Of the aniline dyes malachite green, crystal violet and brilliant green are extensively used. The acridine dyes (proflavine and acriflavin) exert a bactericidal as well as bacteriostatic action upon a number of bacteria.

Alkylating Agents

The important members of this group are formaldehyde, glutaraldehyde and ethylene oxide and all these exert lethal effect upon proteins. Damage produced by such agents is irreversible resulting in inhibition of enzymic activity.

Formaldehyde is one of the least selective agent acting on proteins. It is a gas that is usually employed as its 37 per cent solution, formalin. When used in sufficiently high concentration it destroys the bacteria and their spores. It is also used for coagulating and preserving fresh tissues for microscopic studies.

Ethylene oxide is an alkylating agent extensively employed in gaseous sterilization. It is active against all kinds of bacteria and spores. It can sterilize almost

any object but its greatest utility is in sterilizing those objects which are damaged by heat (e.g. heart lung machine).

In addition to its action on proteins, ethylene oxide also acts on and damages nucleic acids. It has been found to be mutagenic for bacteria and hence its use as a disinfectant poses dangers of mutagenicity and carcinogenicity for human beings also.

Ethylene oxide is being used to sterilize fragile, heat sensitive equipments, powders as well as components of space crafts.

Required concentration and time for chemical destruction of selected microbes is shown in Table 9.7.

Table 9–7. Required concentration and times for chemical destruction of selected microbes

Organism	*Concentration*	*Time*
Agent: Aqueous Iodine		
Staphylococcus aureus	2%	2 min
Escherichia coli	2%	1.5 min
Enteric viruses	2%	10 min
Agent: Chlorine		
Mycobacterium tuberculosis	50 ppm	50 sec
Entamoeba cysts (protozoa)	0.1 ppm	30 min
Hepatitis A virus	3 ppm	30 min
Agent: Phenol		
Staphylococcus aureus	1:85 dil	10 min
Escherichia coli	1:75 dil	10 min
Agent: Ethyl Alcohol		
Staphylococcus aureus	70%	10 min
Escherichia coli	70%	2 min
Poliovirus	70%	10 min
Agent: Hydrogen Peroxide		
Staphylococcus aureus	3%	12.5 sec
Neisseria gonorrhoeae	3%	0.3 sec
Herpes simplex virus	3%	12.8 sec
Agent: Quaternary Ammonium Compound		
Staphylococcus aureus	450 ppm	10 min
Salmonella typhi	300 ppm	10 min
Agent: Silver Ions		
Staphylococcus aureus	8 mg/ml	48 hrs
Escherichia coli	2 mg/ml	48 hrs
Candida albicans (yeasts)	14 mg/dl	48 hrs
Agent: Glutaradehyde		
Staphylococcus aureus	2%	< 1 min
Mycobacterium tuberculosis	2%	< 10 min
Herpes simplex virus	2%	< 10 min
Agent: Ethylene oxide Gas		
Streptococcus faecalis	500 mg/l	2-4 min
Influenza virus	10,00 mg/l	25 hrs
Agent: Chlorhexidine		
Staphylococcus aureus	1:10 dil	15 sec
Escherichia coli	1:10 dil	30 sec

Sterilants

This term is sometimes used for small range of chemical compounds (e.g. ethylene oxide, formaldehyde and glutaraldehyde) which under controlled conditions, can kill sporing bacteria.

A summary of important features of chemical agents is shown in Table 9.8.

IDEAL DISINFECTANT

The properties of an ideal disinfectant (Table 9.9) can be used to compare those of any other compound which is in use or is about to be introduced.

Evaluation of Disinfectants

Various methods are being employed to evaluate the efficacy of the disinfectant as well as their capacity to retain their activity when repeatedly used microbiologically. To the former category belongs the phenol coefficient test and the later category is represented by Kelsey-Sykes test.

Phenol Coefficient Test

In this phenol is taken as a standard reference material. The ratio of the concentration of the disinfectant being tested to the concentration of the reference standard required to kill in a specified time is referred to as phenol coefficient. In the United Kingdom, this activity is determined against *Salmonella typhi*. In the USA, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are also taken as specified strains for testing. Two methods are currently in practice for this test: Rideal-Walker and Chick-Martin method; in the latter test only a source of organic material (e.g. killed yeast suspension) is used to simulate the practical situation. Yet this test has many pitfalls because neither of the methods used takes into consideration all the variables which are likely to be present in real clinical situations.

A phenol coefficient of 1.0 means that the disinfectant being compared has the same effectiveness as phenol. A coefficient less than 1.0 means it is less effective; a coefficient greater than 1.0 means it is more effective than phenol.

Kelsey-Sykes Test

This test measures the capacity of a disinfectant to retain its activity when repeatedly used microbiologically. The standard organism (*Staphylococcus aureus*, *Escherichia coli, Ps. aeruginosa*) is added to disinfectant in three

Table 9–8. Summary of chemical agents used to control microorganisms

Chemical agent	*Antiseptic or disinfectant*	*Mechanism of activity*	*Applications*	*Limitations*	*Antimicrobial spectrum*
Chlorine	Chlorine gas Sodium hypochlorite Chloramines	Protein oxidation Membrane leakage	Water treatment Skin antisepsis Equipment spraying Food processing	Inactivated by organic matter Objectionable taste, odor	Broad variety of bacteria, fungi, protozoa, viruses
Iodine	Tincture of iodine iodophors	Halogenates tyrosine in proteins	Skin antisepsis Food processing Preoperative preparation	Inactivated by organic matter Objectionable taste, odor	Broad variety of bacteria, fungi protozoa, viruses
Phenol and derivative	Cresols Trichlosan Hexachlorophene Chlorhexidine	Coagulates proteins Disrupts cell membranes	General preservatives Skin antisepsis with detergent	Toxic to tissues Disagreeable odor	Gram-positive bacteria Some fungi
Mercury	Mercuric chloride Methiolate Metaphen	Combines with —SH groups in proteins	Skin antiseptics Disinfectants	Inactivated by organic matter Toxic to tissues Slow acting	Broad variety of bacteria, fungi, protozoa, viruses
Copper	Copper sulfate	Combines with proteins	Algicide in swimming pools Municipal water supplies	Inactivated by organic matter	Algae Some fungi
Silver	Silver nitrate	Binds proteins	Skin antiseptic Eyes of newborns	Skin irritation	Organisms in burned tissue Gonococci
Alcohol	70% ethyl alcohol	Denatures proteins Dissolves lipids Dehydrating agent	Instrument disinfectant Skin antiseptic	Precleaning necessary Skin irritation	Vegetative bacteiral cells, fungi, protozoa, viruses
Formaldehyde	Formaldehyde gas Formalin	Reacts with functional groups in proteins and nucleic acids	Embalming Vaccine production Gaseous sterilant	Poor penetration Allergenic Toxic to tissues Neutralized by organic matter	Broad variety of bacteria, fungi, protozoa, viruses
Glutaraldehyde	Glutaraldehyde	Reacts with functional groups in proteins and nucleic acids	Sterilization of surgical supplies	Unstable Toxic to skin	All microorganisms, including spores
Ethylene oxide	Ethylene oxide gas	Reacts with groups in proteins and nucleic acids	Sterilization of instruments, equipment, heat-sensitive objects	Explosive Toxic to skin Requires constant humidity	All microorganisms, including spores
Chlorine dioxide	Chlorine dioxide gas	Reacts with functional groups in proteins and nucleic acids	Sanitizes equipment, rooms, buildings	Explosive	All microorganisms, including spores
Hydrogen peroxide	Hydrogen peroxide	Creates aerobic environment Oxidizes protein groups	Wound treatment	Limited use	Anaerobic bacteria
Cationic detergents	Commercial detergents	Dissolve lipids in cell membranes	Industrial sanitization Skin antiseptic Disinfectant	Neutralized by soap	Broad variety of microorganisms

Table 9–9. Properties of an ideal disinfectant

1. *Broad spectrum:* Should always have the widest possible antimicrobial spectrum.
2. *Fast acting:* should have a rapidly lethal action on all vegetative forms and spores of bacteria and fungi, protozoa and viruses.
3. *Not affected by physical factors:*
   a. Active in the presence of organic matter such as blood, sputum, and faeces (i.e. cleaning capability).
   b. Should be compatible with soaps, detergents and other chemicals encountered in use.
4. Nontoxic
5. Surface compatibility:
   a. Should not corrode instruments and other metalic surfaces
   b. Should not cause the disintegration of cloth, rubber, plastics, or other materials.
6. Residual effect on treated surfaces
7. Easy to use
8. Odorless: an inoffensive odor would facilitate its routine use.
9. Economical: cost should not be prohibitively high.

successive lots at 0, 10 and 20 minutes. Each increment is in contact with disinfectant for eight minutes and samples are transferred at 8, 18 and 28 minutes to a recovery medium. The test is carried out under both clean and 'dirty' conditions. Based upon the results the dilution of the disinfectant to be most effective is recommended for use.

Filter Paper Method

This method uses small filter paper discs, each soaked with a different chemical agent. It is simpler than the determination of phenol coefficient. The discs are placed on the surface of an agar plate that has been inoculated with a test organism. A different plate is used for each organism. An agent that inhibits growth of a test organism is identified by a clear area around the disc where the bacteria have been killed.

In Use Dilution Test

It uses standard preparations of certain test bacteria. These bacteria are added to tubes containing different dilutions of a chemical agent, the tubes are incubated, and then the tubes are observed for the presence or absence of growth. Agents that prevent growth at the greatest dilutions are considered most effective.

Comparison of Different Methods

The advantages and disadvantages of various commonly used methods are shown in Table 9.10.

The methods that are currently in use for the sterilization of common articles are given in Table 9.11.

Table 9–10. Advantages and disadvantages of various sterilization methods

Method	*Advantages*	*Disadvantages*
Autoclaving	1. Short cycle time 2. Good penetration 3. Wide range of material can be processed without destruction	1. Corrosion of unprotected carbon steel instruments 2. Dulling of unprotected cutting edges 3. Package may remain wet at end of cycle 4. May destroy heat sensitive materials
Dry heat	1. Effective and safe for sterilization of material instruments and mirrors 2. Does not dull cutting edges 3. Does not rust or corrode 4. Destroy heat labile items.	1. Long cycle required for sterilization 2. Poor penetration of edges 3. May discolour and char fabric
Unsaturated chemical vapor	1. Short cycle time 2. Does not rust or corrode metal, including carbon steel 3. Does not dull cutting edges 4. Suitable for orthodontic stainless wires	1. Instruments must be completely dried before processing 2. Will destroy heat sensitive plastics 3. Chemical odour in poorly ventilated areas
Ethylene oxide	1. High penetration 2. Does not damage heat-labile materials (including rubber and handpipes) 3. Evaporates without leaving a residue 4. Suitable for materials that cannot be exposed to moisture	1. Slow- requires very long cycle times 2. Retained in liquid and rubber material for prolonged intervals. 3 Causes tissue irritation, if not well aerated. 4. Requires special 'spark-shield' – explosive in presence of flame or sparks

Table 9–11. Preferred methods of sterilization for common use articles

Autoclaving	*Hot air oven*	*Ethylene oxide*	*Glutaraldehyde*	*Filtration*
Animal cages	Glass ware	Fabric	Endoscope	Antibiotics
Sugar tubes	Beakers	Bedding	Cystoscope	Serum
Lab. coats	Flasks	Blanket		Vaccines
Cotton	Petridish	Clothing		
Filters	Pipette	Mattresses		
Instruments	Slides	Pillows		
Culture media	Syringes	Disposable instruments		
Rubber	Test tubes	Blades		
Gloves	Glycerine	Knives		
Stopper	Needles	Scalpels		
Tubing	Oils	Scissors		
Glass	Paper	Books		
Slides	Matrix band	Cups		
Syringe and needles		Plates		
Test tubes		Plastics		
Enamel metal trays		Flasks		
Wire baskets		Petri dish		
Wood		Tubes		
Tongue depressor		Tubing		
Applicator		Rubber		
Steel tumbler		Catheters		
		Drains		
		Gloves		
		Bronchoscope		
		Cystoscope		
		Heart lung machine		

COMBINED TREATMENTS

Enhanced sterilizing activity can take place if two or more processes, chemical or physical, are employed together. There are various kinds of such treatments.

Thermochemical treatment. With the increase in temperature the antimicrobial activity of various compounds increases. Use of ethylene oxide at 60°C and low temperature steam with formaldehyde are salient examples of these combined treatments.

Chemical treatment and irradiation. During radiation if certain chemicals are also present, the spores get sterilized and respond better to action of irradiation. These findings have yet to find application.

Thermoradiation. Simultaneous use of heat and ionizing radiations can also provide good results provided temperature is carefully selected to avoid paradoxical inversion of thermorestoration which may occur at certain temperatures.

Certain other combinations are being tried but these have yet to find acceptance for application.

10 Antimicrobial Chemotherapy

Antimicrobial agents are widely employed to cure bacterial diseases. Antibiotics are substances that are derived from various species of microorganisms and are capable of inhibiting the growth of other microorganisms when administered in small concentrations. Antimicrobial agents that are produced synthetically but have action similar to that of antibiotics have been defined as chemotherapeutic agents (sulphonamides, quinolones).

Bacteriostatic and Bactericidal Agents

Antimicrobial agents that reversibly inhibit growth of bacteria are called *bacteriostatic* (tetracycline, chloramphenicol) whereas those with an irreversible lethal action on bacteria are known as *bactericidal* (penicillins, isoniazid).

Ideal Antimicrobial Agent

Before an antimicrobial agent is accepted for use in human beings it must demonstrate most, if not all, of the properties listed in Table 10.1.

Mechanism of Action of Antibiotics

Various antibiotics act on different sites of bacteria and some agents have more than one primary site of action or mechanism by which bacteria are destroyed. Figure 10.1 shows various sites of bacteria on which different antimicrobial agents act. The detailed description of these agents is given in pharmacology books. Some of the important antimicrobial agents are briefly described.

The Penicillins

Penicillins and cephalosporins act by inhibiting transpeptidases, the enzyme that catalyzes the final cross-linking step in synthesis of peptidoglycan. Due to this reason, penicillin is bactericidal for the growing bacteria since new peptidoglycan is synthesized at that stage only. In non-growing cells, no new cross-linkages are required and penicillin is inactive. An intact beta-lactam is essential for the antibacterial activity of the penicillin.

Penicillins have been classified into following groups according to their action and antibacterial spectrum:

Table 10–1. Desirable properties of an antimicrobial agent

- Selective toxicity: should act on bacteria without damaging the host tissues
- Bactericidal rather than bacteriostatic
- Effective against a broad range of bacteria
- Should not be allergenic
- Should remain active in plasma, body fluids etc.
- Stable and preferably water soluble
- Desired levels should be reached rapidly and maintained for adequate period of time
- Should not give rise to resistance in bacteria
- Long shelf life
- Reasonable cost

Classification of Penicillins

Natural

- Benzylpenicillin (penicillin G)
- Phenoxymethyl penicillin (penicillin V)

Semisynthetic and penicillinase resistant

- Methicillin
- Nafcillin
- Cloxacillin
- Oxacillin
- Dicloxacillin
- Floxacillin

Extended spectrum

- Aminopenicillins
  - — Ampicillin
  - — Amoxycillin
- Carboxypenicillins
  - — Carbenicillin
  - — Ticracillin
- Ureidopenicillin
  - — Piperacillin

Resistance to penicillin is mainly due to the modification of the antibiotic by the enzyme beta lactamase or penicillin amidase, commonly called as penicillinase. This enzyme opens the beta lactam ring hydrolytically and thus converts the antibiotic to inactive penicilloic acid. The enzyme penicillinase is found both in Gram positive and Gram negative organisms.

Beta Lactamase Inhibitors. The activity of beta lactamase can be inhibited by clavulinic acid which is a product of *Strept.clavuligerus*. It acts against the activity of staphylococcal beta lactamase, most plasmid mediated beta lactamase of Gram-negative bacteria and some chromosomal enzymes such as those from *Bact. fragilis*.

Sulbactam. This is a semisynthetic sulfone derivative with weak antibacterial activity of its own. Its antibacterial spectrum is similar to that of clavulinic acid. It acts best when administered in combination with ampicillin.

Tazobactam. This is a penicillonic acid sulfone derivative. On its own it is poorly antibacterial. It gives best results when used in combination with piperacillin.

Cephalosporins

Cephalosporins are also beta-lactam drugs that act in the same manner as penicillins. The structures, however, are different. Cephalopsorins are the products of molds of genus *Cephalosporium,* except cefoxilin which is produced from *Streptomyces.*

These drugs have been found to be effective against a wide array of organisms and accordingly have been grouped (Table 10.2).

Other Beta Lactam Antibiotics

Carbapenems. These drugs have the widest antibacterial range. Structurally these differ from other beta lactam antibiotics. Imipenem is the first compound of this group that has been clinically used.

Table 10–2. Grouping of cephalosporins

Group	*Activity against*	*Antibiotics*
I	Gram-positive organisms	Cefazolin Cephalothin Cephacetrile
II	Enterobacteria	Cephamandole Cefuroxime Cephalexin
III	Pseudomonas	Ceftazidime Cefsulodin Cefoperazone
IV	Enterobacteria and Bacteriodes	Cefoxitin

Quinolones

Quinolones (or 4-quinolones) are the first wholly synthetic antimicrobials. The commonly used quinolones and the antibacterial spectrum of this group of drugs is mentioned in Table 10.3. Quinolones act on DNA gyrase (topoisomerase II) which prevents DNA polymerase from proceeding at replication fork and consequently stopping DNA synthesis.

Table 10–3. Quinolones and their antimicrobial spectrum

Quinolones	*Antimicrobial spectrum*
Nalidixic acid	*Enterobacteriaceae*
Norfloxacin	*Staphylococcus aureus*
Ofloxacin	*Pseudomonas aeruginosa**
Pefloxacin	*Vibrio cholerae*
Ciprofloxacin	*Haemophilus influenzae*
	Moraxella
Amifloxacin	*Neisseria gonorrhoeae*
	Gardenerella vaginalis
	Chlamydia species

* Variable activity

Aminoglycosides

Aminoglycosides are a group of antibiotics in which amino sugars are linked by glycoside bonds. Best known aminoglycoside is streptomycin which was discovered in 1944. This was the first effective drug against tuberculosis.

Streptomycin, other aminoglycoside and tetracyclines act at the level of ribosomes not compatible with ribosomal subunit and inhibit initiation phase of protein synthesis (Fig. 10.1). All the aminoglycoside antibiotics (gentamicin, neomycins, paromomycins, tobramycins, kanamycins, and spectinomycins) have action against a wide spectrum of organisms and are bactericidal with the exception of spectinomycin.

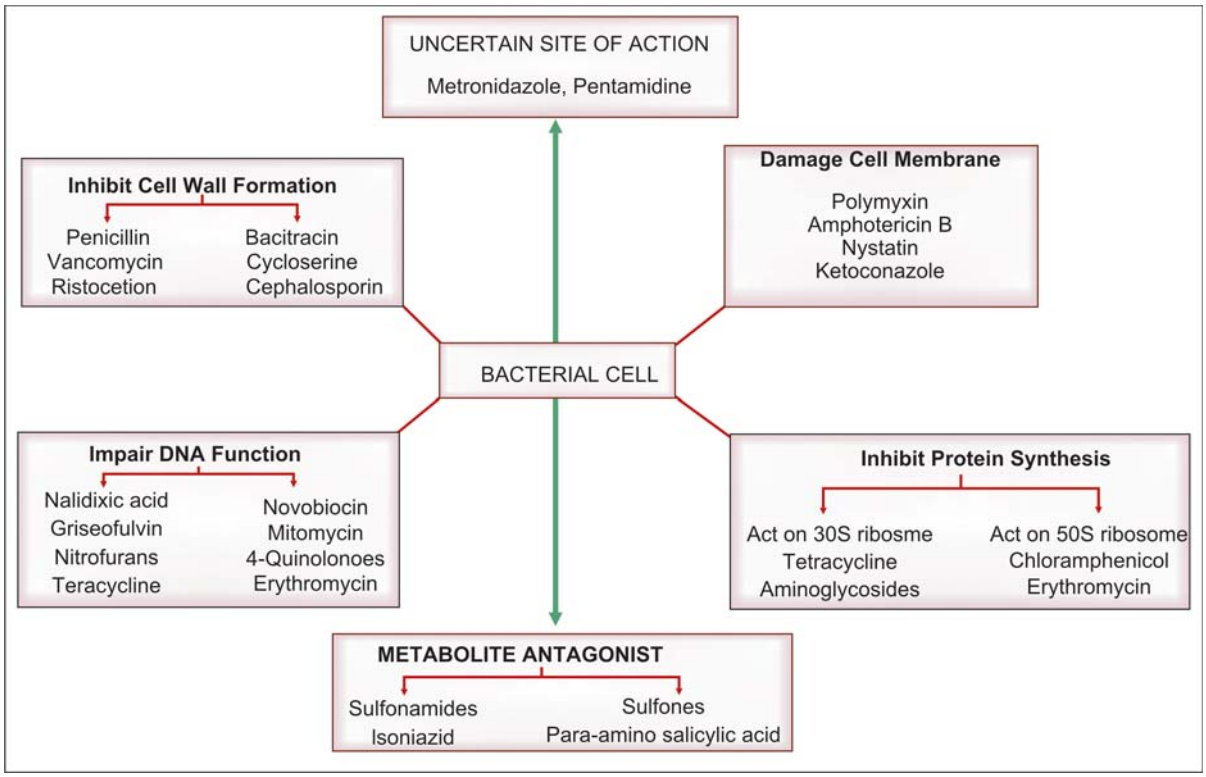


Fig 10–1. Chemotherapeutic agents and their sites of action

Tetracyclines Tetracyclines are a group of broad spectrum antibiotics produced by *Streptomyces species*.

Three of the commonly used tetracyclines are, oxytetracycline, chlortetracycline and tetracycline itself.

The tetracyclines are bacteriostatic and inhibit only rapidly multiplying organisms. Resistance to tetracyclines develops slowly and is attributed to alterations in cell membrane permeability to enzymatic inactivation of the drug.

Chloramphenicol is also a bacteriostatic drug but must be used with caution since it can induce lethal bone marrow depression as well as grey baby syndrome.Chloramphenicol interferes with the protein synthesis.

Macrolides

Macrolides are a group of antibiotics named for the presence of a macrocyclic lactone ring. The only one in clinical use is erythromycin. It acts by inhibiting the protein synthesis. Its spectrum is like penicillin and quite frequently is used as an alternative to penicillin.

Antibiotics Against Anaerobes

Most of the anaerobes (anaerobic cocci, clostridia and *Bacteroides* except *Bact. fragilis*) are susceptible to benzylpenicillin. *Bact. fragilis* as well as many other anaerobes are treatable with erythromycin, lincomycin, tetracyclines and chloramphenicol. Clindamycin is another important antibiotic which is effective against almost all the strains of *Bacteroides*.

1960s saw the emergence of metronidazole as a wonder drug against trichomonas, amoebiasis and protozoal infections of humans. In 1973, its role against anaerobes was discovered and since then it has been a leading agent in treating infections with anaerobes. Prolonged usage in high doses may cause peripheral neuritis.

ANTIVIRAL DRUGS

Several antiviral compounds are now licensed in developed countries for clinical use (Table 10.4).

With increase in HIV burden, several antiviral drugs have been developed with specific action on HIV.

ANTIFUNGAL DRUGS

Polyenes. Two commonly used antifungal drugs that belong to this group are nystatin and amphotericin B. Both are fungicidal. These drugs combine with the sterols in fungal plasma membranes, making the membranes excessively permeable and killing the cell.

Table 10–4. Antiviral compounds of clinical use

Compound	*Effective against*
Acyclic guanosine (acyclovir)	Herpes simplex type 1 and 2 Varicella-zoster virus
Azidothymidine (AZT, ziduvudine) (+several other anti-HIV drugs)	HIV
Amantadine	Influenza A
Ribavirin	Respiratory syncytial virus
Idoxuridine (IDU)	Herpes simplex type 1
Triflurothymidine (TFT)	Herpes simplex type 1
Adenine arabinoside (Vidarabine)	Herpes infection of brain

Imidazole. To this group belong miconazole, clotrimazole and ketaconazole which primarily interfere with sterol synthesis in fungi.

Griseofulvin. This antifungal agent is produced by *Penicillium* species. It has the property of binding selectively to keratin found in the skin, hair follicles and nails. Its mode of action is to interfere with mitosis and thereby inhibiting fungal reproduction.

DRUG RESISTANCE

With the introduction of antimicrobial agents, it was thought that soon mankind would get rid of the infectious diseases. But this was not to be. One of the major factor contributing to the persistence of infectious diseases has been the tremendous capacity of the microorganisms for circumventing the action of inhibitory drugs (Fig. 10.3). This ability has also offered a serious threat to the future usefulness of present day chemotherapeutic agents. The basis of drug resistant strains could be genetic or non-genetic (Fig. 10.2).

Origin of Drug Resistant Strains

The drug resistant strains arise either by mutation and selection or by genetic exchange in which a sensitive organism receives the genetic material (part of DNA) from the resistant organism and the part of DNA carries with it the information of mode of inducing resistance against one or multiple antimicrobial agents.

The drug resistance can arise by random mutation and when with it the organism becomes resistant to a drug, the application of that drug shall select out the resistant organisms and let them multiply.

Transmissible (Infectious) Drug Resistance

Apart from chromosomal resistance, extrachromosomal material which is called as *plasmid* (or episome) is also capable of conferring resistance to antibiotics.

There is adequate evidence to suggest that transfer of resistance can occur within the intestinal tract of human beings as well as the animals.

BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE

The biochemical changes which are seen in bacteria which have become resistant due to mutation are:

- Increased synthesis of drug antagonist
- Decreased permeability to drug
- Increased destruction of inhibitor
- Induction of different relative affinity of substrate and antagonist.

The mechanism of drug resistance induced by plasmids is different than what is seen in mutated organism (Table 10.5). In bacterial resistance to some drugs, e.g. sulphonamides more than one mechanism may be operative.

Table 10–5. Plasmid mediated drug resistance

Antimicrobial agent	*Mechanism of resistance*
Sulphonamides	Reduced permeability
Erythromycin	Modification of ribosomes
Lincomycin	Ribosomal modification
Tetracycline	Reduced permeability
Neomycin	Phosphorylation of drug
Chloramphenicol	Acetylation of drug
Kanamycin	Phosphorylation of antibiotic
Streptomycin	Adenylation of drug
Penicillin	Hydrolysis of lactam ring

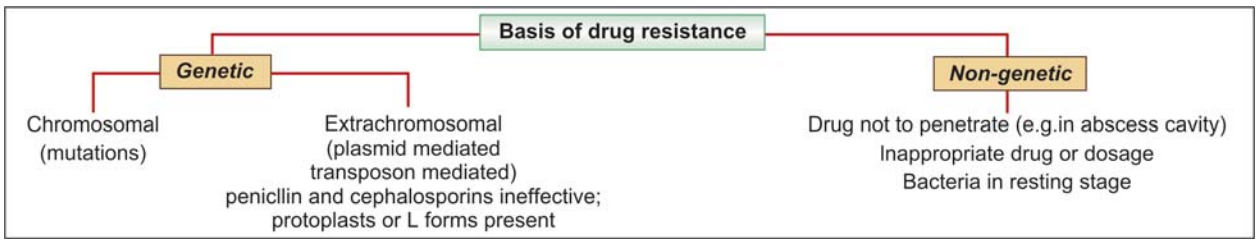


Fig. 10–2. Basis of drug resistance

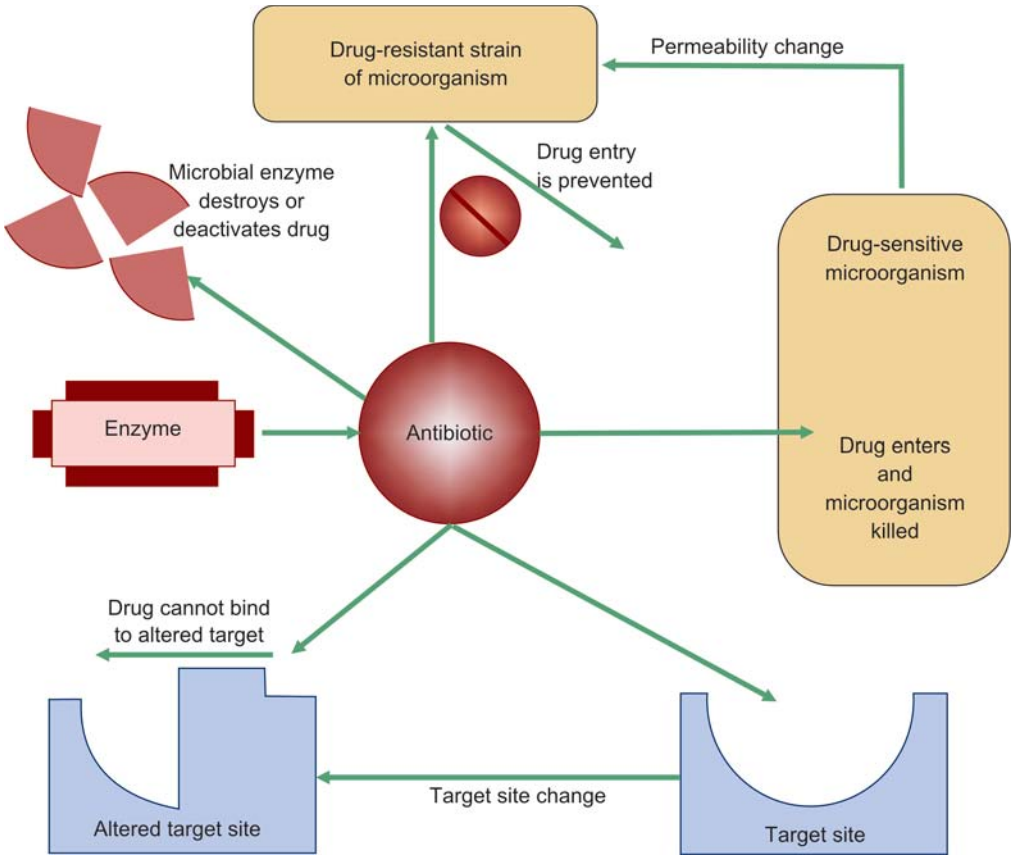


Fig. 10.3: Basis of resistance in bacterium

There are a few important differences between drug resistance induced by mutation and transferable drug resistance (Table 10.6).

Table 10–6. Distinguishing features between mutation and transferable drug resistance

Feature	*Mutation resistance*	*Transferable*
No. of drugs to which resistant	Usually one	Usually multiple
Degree of resistance	Low	High
Can be overcome by increasing dose	Yes	No
Preventable by combination of drugs	Yes	No
Infectious	No	Yes
Metabolically defective	Yes	Normal
Virulence of bacterium	Low	Not decreased

11 Infection and Disease

All organisms living in or on the bodies of other organisms and drawing their sustenance from them are considered *parasites*. In medical terminology, parasites include intestinal worms and organisms causing malaria, kala azar or amoebic dysentery whereas bacteria and viruses do not find mention as parasites.

Any relationship in which two organisms live together in an intimate association is considered to be symbiosis. The relationship can be *mutualistic* if both members of the pair benefit, *commensal* if one benefits and the host is not harmed, or *parasitic* if the organism benefits and the host is harmed to a greater or lesser degree. Hence, this relationship can be equal or unequal in various ways.

Predators

These are the organisms that hunt, catch and kill other organisms before eating them, whereas parasites do not hunt, catch and kill their prey before eating it, rather they eat it while it continues to live.

Saprophytes

Saprophytes eat dead organisms that they did not kill. The term *saprophyte* is reserved for microbes such as bacteria, protozoa and fungi that digest and thus degrade the bodies of dead plants and animals.

Infestation

When the parasite worms and arthropods that live on the surface of the body or in the lumen of the hollow organs such as intestines are usually described as *infesting* their hosts.

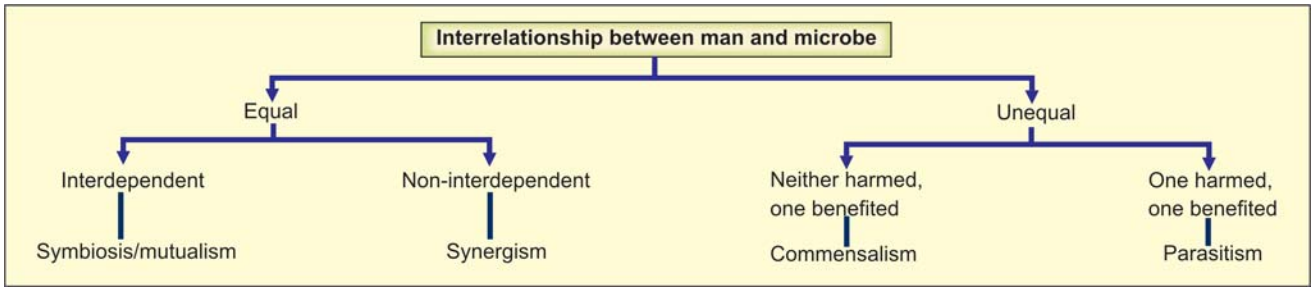
Infection

The bacteria, fungi, protozoa and viruses that are parasitic are usually described as *infecting* their hosts. The implication of the word infection is a generalised invasion of the host's tissues whereas an *infestation* is more superficial.

Infection and Disease

Infection and disease are not synonyms and must be distinguished.Infection is the invasion or colonization of the body by pathogenic microorganisms. Disease occurs when an infection results in any change from a state of health.

When microorganisms first associate with a host, the host is said to be *contaminated*. If the microbes establish themselves and grow and multiply for a period



of time, the host is said to be *infected* and if the infection causes the damage , the host is said to have an *infectious disease.*

Pathogens

Microbes capable of causing infections that result in disease are called *pathogens. T*his characteristic is called as pathogenicity of the organism.

Virulence

Degree of pathogenicity of a microbe is referred to its *virulence*. Virulence is a quantitative term. Infection of a susceptible animal with a highly virulent pathogen will almost always result in disease, whereas infection with a pathogen of low virulence will often fail to produce disease or only produce a mild disease.

DYNAMICS OF INFECTIOUS DISEASES

For an infectious disease to perpetuate there has to be a reservoir of microorganism from where the causative agent should be transmitted to a susceptible host either directly or through the agency of a vehicle or a vector (Fig. 11.1).

Source and Reservoir

The source of infection is the person, animal, object or substance from which an infectious agent passes or disseminates to the host whereas a reservoir is defined as any person, animal, arthropod, plant, soil or substance (or combination of these) in which an infectious agent lives and multiplies. These can be of three types:

- Humans
- Animals
- Non-living substances

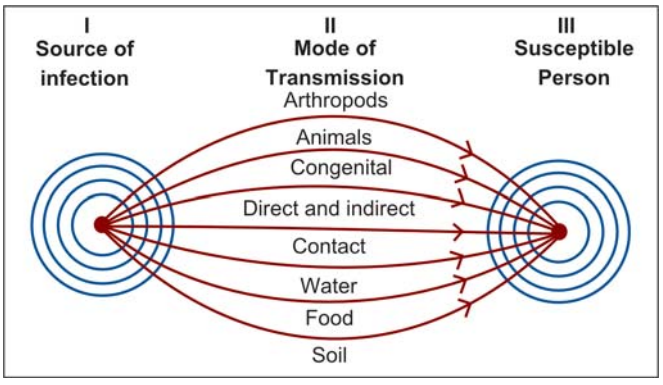


Fig. 11–1. Sources and vehicles of infections

Humans

For infectious diseases of human beings, man is the most important reservoir. He may be a *case or a carrier*.

Human Case

A case may be having a clinical disease or subclinical infection which remains unmanifested or abortive. In latter, the disease agent may multiply in the host but does not manifest itself by signs and symptoms.

Human Carriers

Some microorganisms do not get completely eliminated from the host after natural cycle of disease or after treatment. Such persons become carriers of the agents. A *carrier is defined as an infected person or animal that harbours a specific infectious agent in the absence of overt clinical signs and serves as a potential source of infection for others*. Though carriers are less infectious than cases, they carry greater epidemiological importance because of the prolonged duration for which they can silently excrete organisms.

The carriers can be:

- incubatory,
- convalescent or
- healthy.

Depending upon the duration of excretion of microorganisms they can be designated as:

- temporary (acute) or
- chronic carriers.

The incubatory and convalescent carriers are usually temporary whereas chronic carriers are otherwise healthy individuals. Chronic carrier state occurs in various diseases notably typhoid fever and hepatitis B.

Animals

Animals and birds can also pass on microorganisms to man. These may also manifest as case or exist as carriers. The diseases which are naturally transmissible between man and animals are called as *zoonoses*.These diseases are of great importance in countries where a close contact between man and animals is inevitable such as occurs in rural areas of India. Some of the important zoonotic infections are rabies; plague, brucellosis, leptospirosis, hydatidosis.

Non-living Substances

Soil and inanimate objects can also occur as reservoir of some microorganisms such as causative agents of tetanus, anthrax, hookworm disease and mycetoma.

Table 11–1. Types of bacterial infections

Types of infection	*Description*	*Example*
Subclinical	No detectable clinical symptoms of infection	Asymptomatic gonorrhoea in women and men
Latent	Carrier state	Typhoid carrier
Accidental	Zoonosis and environmental exposures	Anthrax, cryptococcal infection
Opportunistic	Infection caused by normal flora or transient bacteria when normal host defences are compromised	*Serratia* or *Candida* infection of the genito-urinary tract
Primary	Clinical apparent invasion and multiplication of microbes in body tissues causing local tissue injury	*Shigella dysenteriae*
Secondary	Microbial invasion subsequent to primary infection	Bacterial pneumonia following viral lung infection
Mixed	Two or more microbes infecting same tissue	Anaerobic abscess (*Esch coli* and *Bacteroides fragalis*)
Acute	Rapid onset (hrs or days); brief duration (days/weeks)	Diphtheria
Chronic	Prolonged duration (months, years)	Mycobacterial diseases (tuberculosis and leprosy)
Localized	Confined to a small area or to an organ	Staphylococcal boil
Generalized	Disseminated to many body regions	Gram-negative bacteremia (gonococcemia)
Pyogenic	Pus forming	Staphylococcal and streptococcal infections
Retrograde	Microbes ascending in a duct or tube against the flow of secretions or excretions	*Esch coli* urinary tract infection
Fulminant	Infections that occur suddenly and intensely	Airborne *Yersinia pestis* Pneumonic plague

Modes of Transmission

The microorganisms can be transmitted to human beings directly or indirectly.

Direct transmission occurs through:

- Contact with man, animal or inanimate objects
- Droplet infection
- Breach of skin or mucous membrane
- Transplacental and congenital

The *indirect transmission* is possible by any of the following mechanisms

- Vehicles (water, food etc.)
- Vectors (mechanical or biological)
- Air (droplet or dust)
- Fomites
- Unclean hands and fingers

Mechanism of Infection

To produce infection in man a microbe has to gain entry into the host. The most frequent portals of entry are the respiratory tract, the gastrointestinal tract and breaks in the superficial mucous membranes and skin. From the portal of entry the parasite may spread directly through the tissues or may proceed via lymphatic channels to the blood stream, which distributes it widely and permits it to reach tissues particularly suitable for its multiplication. Nevertheless,

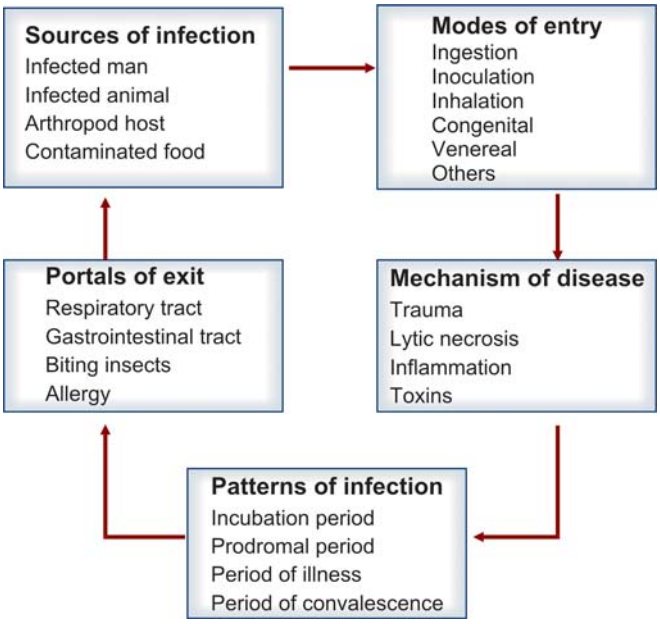


Fig. 11–2. Dynamics of infection

for the perpetuation of a parasitic species a satisfactory portal of exit of the parasite from the host and an effective mechanism for transmission to new hosts are also of paramount importance (Fig. 11.2).

Development of Disease

Once the microorganism overcomes the defences of the host, development of infectious disease follows a sequence of events.

Period of Incubation

This is the time interval between the actual infection and the appearance of first clinical feature.

Prodromal Period

This relatively short period follows incubation period in some diseases. It is characterised by mild early symptoms such as general aches and malaise.

Period of Illness

This is the acute phase of illness characterised by typical clinical features of the infectious disease.

Period of Decline

During this period the signs and symptoms subside. The fever decreases and the feeling of malaise diminishes. During this period, the person may be attacked by secondary infection.

Period of Convalescence

The person regains strength during this period and body returns to prediseased state.

Local or Generalised Infections

An infection may be restricted to the point of entry (local) or may spread throughout the body (generalized).

Generalised Infections

Bacteraemia, septicaemia and *pyaemia* are some of the generalised infections whichspread through blood:

Bacteraemia

Bacteraemia is defined as the circulation of bacteria in the blood. It may be *transient* or of *long duration* depending upon the duration for which bacteria are present in the blood.

Transient bacteraemia is a frequent event which occurs even in healthy individuals while chewing, brushing of teeth and straining while passing stools.

Bacteraemia of long duration occurs with pathogenic organisms such as *Salmonella typhi* and *Brucella* and is essential for the initiation of disease process.

Septicaemia

This is a condition in which bacteria circulate and multiply in the blood, form toxic products and cause high swinging type of fever. This can be due to both endotoxin as well as exotoxin producing organisms (Table 11.2).

Table 11–2. Organisms producing septicaemia

Exotoxic	*Endotoxic*
C. diphtheriae	Salmonella
Clostridium tetani	Shigella
Clostridium perfringens	*Escherichia coli*

Pyaemia

This is a condition in which pyogenic bacteria (e.g. *Staphylococcus aureus*) produce septicaemia with multiple abscesses in internal organs such as liver, spleen and kidney.

12 Microbial Pathogenicity

PATHOGENIC MICROORGANISMS

Any microorganism that is able to infect a host and produce disease is called a pathogen. The infection and disease do not always occur together. Bacteria can cause a multitude of different infections, ranging in severity from inapparent to fulminating (Fig. 12.1). The capacity of the organism to cause disease reflects its relative pathogenicity. On this basis, bacteria can be organised into three major groups.

Frank Pathogens

When isolated from a patient, frank pathogens are considered to be probable agents of a disease, e.g. *Salmonella typhi, Neisseria gonorrhoeae* and *Yersinia pestis*.

Opportunistic Pathogens

These are isolated from patients whose defense mechanisms have been compromised, e.g. *Staph. epidermidis* infection.

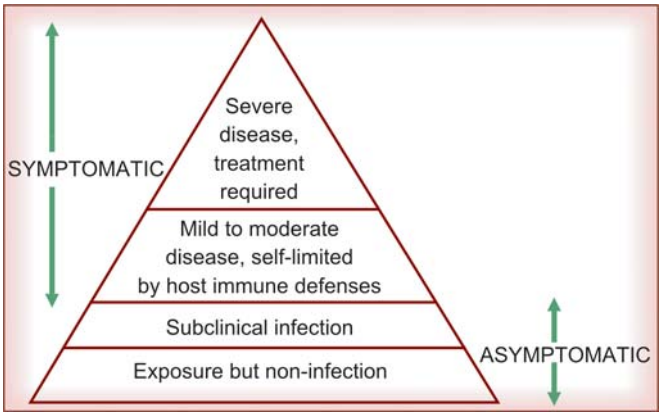


Fig. 12–1. Host response infection

Non-pathogens

These rarely or never cause human disease. *Bacillus subtilis* is an example of nonpathogenic microorganism.

Whether disease ensues as a result of a host's encounter with a pathogen is dependent both upon the condition of the host and particular characteristics of the microorganism. The characteristics that contribute to the ability of a microbe to produce disease are referred to as *virulence* factors.

VIRULENCE FACTORS OF MICROORGANISMS

The virulence factors may be broadly subdivided into:

- *invasiveness*
  (ability to enter host tissues, multiply there, and spread)
- *toxigenicity*
  (ability to produce toxic substances)
- *plasmid* mediated phenotypic expressions

These have been summarised in Table 12.1. In addition, microorganisms adopt many strategies to circumvent the defenses of the host.

Different microbes possess these attributes in varying degrees. Most pathogens have many such factors and although some have been identified, it is still not known in all cases precisely how each factor functions in the production of disease.

The nature and mode of action of some of the known virulence attributes in pathogenesis is described here.

Invasiveness

Adhesin

Most infections actually begin on the mucous membranes of the respiratory, gastrointestinal or urogenital tracts. In these environments, the potential pathogen

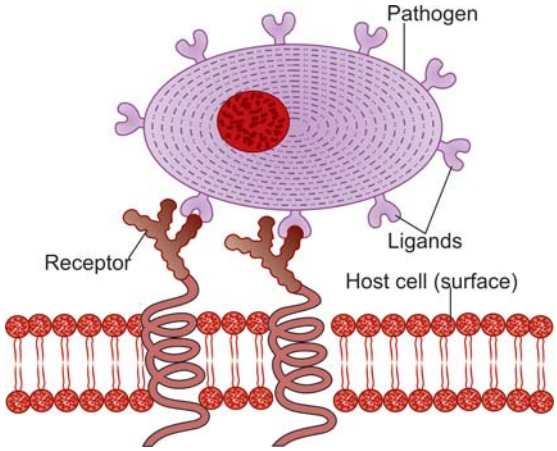


Fig. 12–2. Adhesion of pathogen to host cell

must first adhere to the host cell (Fig. 12.2). Adherence prevents the microorganism from being flushed away in mucus secretions and renders it less susceptible to the effects of enzymes and secretory IgA. If disease is to result from the effects of a toxin, close adherence of the bacterium assures that the toxin will be delivered in high concentrations directly to the host cell. Adherence must occur before penetration of host cells (invasion) is possible.

The organisms attach to the receptors present on the surface of the cells with the help of distinct surface structures called as *adhesins*. In Gram-positive organisms these are known as *fibrillae* whereas those of Gram-negative organisms are called as *fimbriae*.

Antiphagocytic Factors

Adherence to a host cell is by no means the end of the microorganism's struggle to establish itself in the host. The potential pathogen must withstand attack by the phagocytic cells of the host, the polymorphonuclear leukocytes and the macrophages whose job it is to rid the body of invading organisms by ingesting and destroying them. There are several ways by which bacteria thwart this process (Table 12.1).

Toxins and Enzymes

Endotoxin

Endotoxin has been identified as a component of Gram-negative bacterial cell wall envelope and is composed of a complex of lipopolysaccharide with outer membrane proteins (Fig. 12.3). Most of the toxic effects of endotoxins are due to the lipid A component of the lipopolysaccharide. The biologic effects of endotoxins are shown in Table 12.2 and Figure 12.4.

Table 12–1. Factors facilitating pathogenicity

Invasiveness
- Factors Mediating Adhesion
  - — Adhesins
- Antiphagocytic Factors
  - — Capsule
  - — M protein
  - — Cytotoxin and impairment of chemotaxis
- Survival within the Phagocyte
  - — Interference with oxidative burst
  - — Prevention of fusion and degranulation
  - — Resistance to lysosomal enzymes
  - — Escape from phagosomes
- Siderophores and iron acquisition

Toxins and Enzymes
- Endotoxin
- Enzymes
- Exotoxins
- **Plasmid** mediated phenotypic expressions

Table 12–2. Biologic effects of endotoxin

- Fever
- Cardiovascular shock
- Increase in nonspecific resistance
- Adjuvanticity and immune stimulation
- Protection against X-irradiation
- Greater susceptibility to stress (heat, cold)
- Lethality

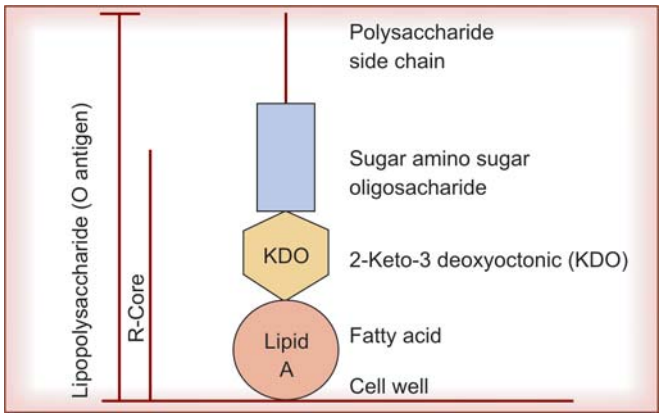


Fig. 12–3. LPS of Gram-negative bacteria

Enzymes

The presence of a microorganism in the host evokes an inflammatory response, resulting in certain amount of tissue damage. Some bacteria also have the ability to

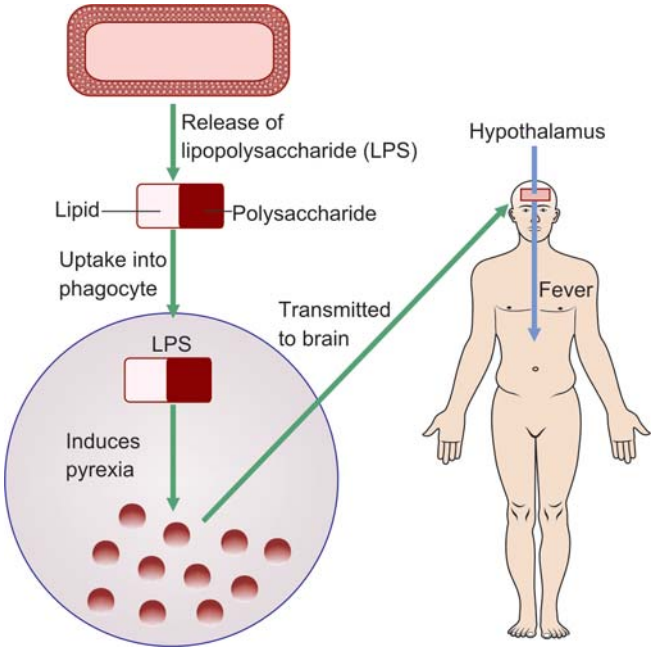


Fig. 12–4. Biological effect of LPS

produce enzymes that can directly damage host tissue. Few important enzymes produced by bacteria are:

- Proteases
- Kinases
- Hyaluronidase
- Collagenase
- Coagulase

Proteases specifically cleave IgA which is the most important immunoglobulin for protection at mucosal surface thus diminishing the protective function of the IgA molecule.

Both virulent streptococci and staphylococci produce kinase that enhance the spread of microbe by dissolving fibrin clots and inhibiting the clotting of plasma. These organisms also produce hyaluronidase that enhances dissemination of the infecting microorganism by breaking down the intercellular mucopolysaccharide hyaluronic acid that 'cements' cells together. Virulent strains of staphylococci produce coagulase which causes deposition of fibrin around the invading cocci and thus protects them from the phagocytes.

Collagenase produced by *Clostridium perfringens* produces breakdown of collagen in connective tissue. This results in destruction of the muscle tissue and enhances the spread of the bacteria.

Exotoxins

Exotoxins are proteins which are secreted by certain viable bacteria (Table 12.3). These are the most potent poisons known. Most of these are composed of two subunits:

Fragment A

Fragment is the toxic or enzymatic unit that exerts pathologic action.

Fragment B

Fragment B is the carrier, binds to the host cell receptor and enables fragment A to enter the cell.

Table 12–3. Exotoxins with known role in pathogenicity

Causative organism	*Toxin*	*Disease*
Corynebacterium diphtheriae	Diphtheria toxin	Diphtheria
Clostridium tetani	Tetanus toxin (Tetanospasmin)	Tetanus
Clostridium botulinum	Botulinum toxin	Botulism
Clostridium perfringens (type B,C,D)	beta and epsilon toxins	Enterotoxaemia in domestic animals
Vibrio cholerae	Cholera enterotoxin	Cholera
Escherichia coli (enteropathogenic strains)	Heat labile (LT) and stable (ST) enterotoxin	Diarrhoeal disease
Staphylococcus aureus	Staphylococcal enterotoxin	Food poisoning
Clostridium perfringens	Clostridial enterotoxin	Food poisoning
Staphylococcus aureus	Epidermolytic toxin	Scalded skin syndrome
Streptococcus pyogenes	Erythrogenic toxin	Scarlet fever
Pseudomonas aeruginosa	Exotoxin A	Opportunistic infection

Table 12–4. Differences between exotoxins and endotoxins

Exotoxins	*Endotoxins*
1. Protein polypeptides with mol.wt ranging between 10000-900000	1. Lipopolysaccharide complex. Lipid A portion is probably responsible for toxicity
2. Secreted by living cells into medium from cell cytoplasm. Found in high concentration in fluid medium	2. Present in cell wall as a constituent. Released only on disruption of cells
3. Heat labile. Toxicity is destroyed rapidly on exposure to temperature above 60°C	3. Relatively heat stable and can withstand heat over 60°C for hours without loosing toxicity
4. Highly antigenic	4. Poorly, if at all, antigenic
5. Stimulates immune system to produce specific antitoxin which can neutralize toxin	5. No antitoxin is formed. Only antibodies against polysaccharide
6. Can be converted into antigenic but nontoxic, toxoid by formalin, acid, heat etc.	6. Cannot be converted into toxoids
7. Do not produce fever in host	7. Usually produce fever in host
8. Specific for particular tissue (e.g. tetanus toxin for nervous system)	8. Nonspecific in action
9. Produced mainly by Gram +ve bacteria and some gram-negative	9. Produced mainly by Gram –ve bacteria
10. Frequently controlled by extra chromosomal genes	10. Synthesis directed by chromosomal genes

Exotoxins can be converted into toxoids which are often used as immunogens for prophylactic immunization.

Many organisms produce exotoxins which exert their activity in gastrointestinal tract. These are called as *enterotoxins.* Enterotoxin production may take place in contaminated food, as is the case with staphylococcal enterotoxin, or in the gastrointestinal tract, as occurs with *Salmonella* and other bacteria. Although the mechanism of action varies, the end results are usually similar.

Though both exotoxin and endotoxin are attributes of organisms which result into disease, there are lot of differences between the two (Table 12.4).

Genetic Mechanism (R-factors)

Plasmids are extrachromosomal DNA segments that carry genes for antibiotic resistance known as R-factors. These factors are readily transferrable by conjugation to bacterial cells of the same species as well as to cells of different species and even genera, and can confer resistance to many antibiotics.

Many bacteria of the normal flora carry these R-factors, but they present no problem until prolonged or improper use of antibiotics selectively enhances the survival of drug resistant clones, making further therapy difficult and thus giving edge to bacteria for its survival and propagation within the host.

Plasmids also code for pathogenic mechanisms such as colonization factors, enterotoxin production and siderophore synthesis. Some of these plasmids may get integrated with bacterial chromosome. Bacteriophages may also contribute to the virulence of bacteria. Toxin production by *C. diphtheriae* occurs as a result of lysogenic infection of the bacterium by a specific phage.

Microbes Escape Mechanisms

To survive the powerful forces of natural and adaptive host immunity against it, a parasite must have one or several survival strategies. By definition, this is true of

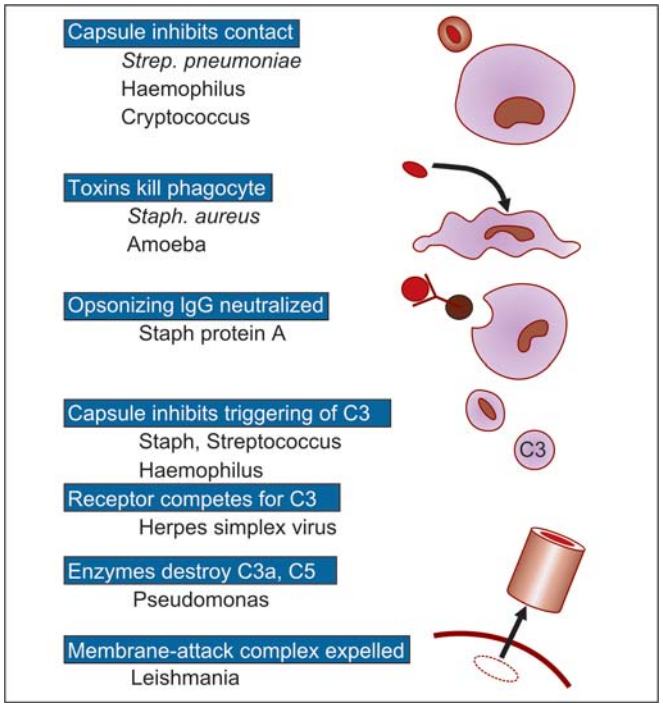


Fig. 12–5. Survival within phagocytes

all successful parasites, which include all those of medical interest. These include:

Avoiding Phagocytosis (Fig. 12.5)

- Avoiding Death in the Phagocyte
- Avoiding Complement
- Antigenic Variation

Immunosuppression. The power of HIV to suppress T cell immunity is all too familiar, but similar effects are seen with many protozoa (trypanosomes, malaria), bacteria (TB) and, more transiently, other viruses (measles). Some parasite-derived molecules go to the opposite extreme and activate practically all lymphocytes, which effectively means that specific responses are 'crowded out'.

Immunosuppression by microbes often involves actual infection of immune cells e.g.

- T cells (HIV, measles)
- B cells (Epstein-Barr Virus)
- Macrophages (HIV, Leishmania)
- Dendritic cells (HIV).

PART IV IMMUNOLOGY

13 Nonspecific Defenses of Host

HOST-PARASITE RELATIONSHIP

Among the most dangerous enemies to whom human beings act as hosts are the microorganisms. Many organisms exist in a balance with the host (Fig. 13.1). Any decrease in host resistance or increase in virulence of the microorganism can result in the development of disease because of imbalance in host parasite relationship that had previously been innocuous.

Host-parasite interaction is a dynamic equilibrium. When host resistance is impaired, organisms that are even normally present may cause disease.

RESISTANCE OF HOST TO MICROORGANISMS

There are a variety of mechanisms by which human beings resist attack by microorganisms. These can be broadly divided into:

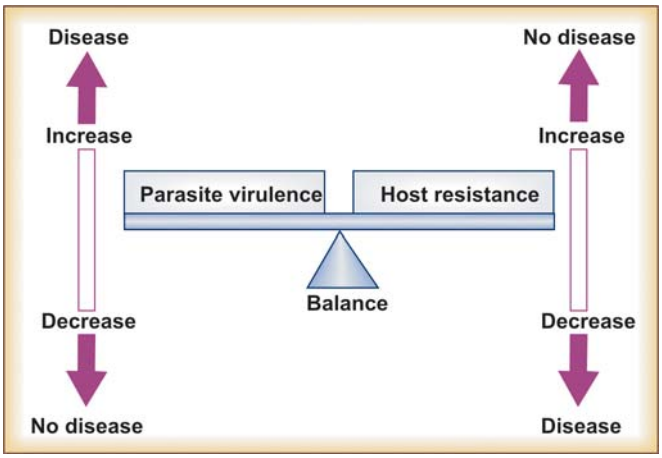


Fig. 13–1. Host-parasite relationship

- Natural resistance, (innate immunity, nonspecific resistance, or constitutive immunity).
- Acquired immunity, (specific, inducible, or adaptive immunity).

Natural (innate) resistance for host protection develops during ontogeny without contact with any parasite or its products and is not specific for any particular parasite. Inducible mechanisms are only developed following exposure to a particular parasite or its products and are specific for the inducing parasite (Table 13.1).

Table 13–1. Types of host defenses

Type of defense	*Type of system*
Nonspecific immune response	Physical barriers; skin, cilia, and flushing action,
	Physiological barriers; inflammation, fever, phagocytes, lymphokines
Specific immune response	Specific immune system; lymphoid system, immune response, antibodies, cell-mediated immunity

The term natural resistance is often used in place of constitutive immunity since the word immunity has a connotation of specificity that is usually not present in natural resistance mechanisms.

Natural (Innate) Resistance (Constitutive Immunity)

Natural resistance or innate immunity refers to that type of resistance which each individual has by virtue

of being the individual he or she is in terms of species, race, sex or other factors associated with genetically controlled resistance. Important factors influencing effectiveness of natural resistance are discussed below:

Species

A given pathogenic organism is often capable of producing disease in one animal species but not in another. Humans get mumps, but dogs and cats do not; mammals may contract anthrax, but birds do not.

Racial or Genetic Basis

Within one animal species there may be marked racial and genetic differences in susceptibility. Blacks appear to be more susceptible than whites to tuberculosis.

Nutrition

Low protein diets make a person more susceptible to infections. Vitamin deficiencies often exhibit significant effects on host defense. Vitamin A and vitamin C deficiencies are well known to increase susceptibility to bacterial infections. Similarly deficiency of zinc and folic acid predisposes to some infections.

Hormone Related Resistance

Hormone imbalance (such as in diabetes mellitus, pregnancy) has a direct effect on susceptibility to a number of infectious diseases. Staphylococcal, streptococcal and certain fungal diseases definitely occur more frequently in diabetics. Pregnancy is associated with marked hormonal alterations and an increase in urinary tract infections.

Miscellaneous

The age of an individual also has a marked effect on innate immunity. The very young and the very aged always have more infectious diseases than the middle-aged groups, possibly because of less phagocytic activity. Likewise fatigue, climate, including simple climatic variation and numerous other factors can significantly alter host resistance.

Mechanism of Natural Resistance

Natural resistance operates nonspecifically during the early phase of an immune response. It functions through activation of nonspecific cells which are phagocytic cells of the mononuclear and granulocytic systems and natural killer (NK) cells. The most significant phagocytic cells are the macrophages and large granular lymphocytes (LGL).

Natural resistance is the result of the action of a number of systems. These may be differentiated into external and internal systems which are bridged with acquired immunity by the process of inflammation (Table 13.2). The external system prevents the entry of microbes into the body and the internal system comes into play following injuries that breach the physical barriers. In addition, inflammation also aids the host in containing the infectious agent.

Table 13–2. Components of natural resistance

- *External defense systems*
  - The skin
  - The respiratory tract
  - The mouth
  - The digestive tract
  - The urogenital tract
  - The eye
- *Internal defense systems*
  - Phagocytic cells
  - Chemotaxis
  - Phagocytosis
  - Opsonization
  - Ingestion
  - Oxygen dependent killing of microbes
  - Oxygen independent killing of microbes
  - Destruction of ingested microbes
- *Inflammatory response*
  - Local
  - Generalised

Characteristics of Components of Natural Resistance

The components of natural resistance are:

- *Preformed*—the components are present before challenge
- *Standardized*—the response magnitude is consistent
- *Without memory*—the host does not realise that it has been reexposed to same antigen
- *Nonspecific*—it does not differentiate between invaders.

The External Defense Systems

The first line of defense against infection are the external systems. Major components of the external systems are the mechanical barriers, the effectiveness of which is enhanced by various antimicrobial secretions. These have been summarised in Table 13.3.

The Internal Defense Systems

Microbes that by one means or another succeed in passing the external barriers encounter the second line of defense, the internal systems. These include a variety

Table 13–3. External barriers to entry of microorganisms

Body site	*Defense mechanism*	*Conditions that foster entry*
Skin	Dryness, acidity, toxicity, constant shedding	Wounds, excess moisture, serous discharge.
Respiratory tract	Ciliated cells constantly moving mucus to throat	Reduced movement of ciliated cells as in smoking chilling, narcotics, viral infection.
Gastrointestinal tract	0.2% hydrochloric acid, enzyme pepsin	Reduced stomach acid, ingestion of antacid.
Vagina	Lactobacilli during childbearing years	Reduced numbers of lactobacilli from douching, soaps, menopause, antibiotic therapy.
Urinary tract	Flushing action of urination	Short urethra in women, incomplete or infrequent urination, sexual intercourse

Table 13–4. Natural internal defense systems

Phagocytosis	Neutrophils Monocytes Macrophages
Leukotaxis Opsonins	C5a, C(5,6,7) C3b Miscellaneous proteins
Complement activation	Alternative pathway (polysaccharides)
Macromolecules	Glycoproteins Transferrin Lysozyme Various polyamines

of antimicrobial substances in the blood and body fluids and process of phagocytosis through various cells (Table 13.4).

Some important nonspecific antimicrobial factors have been shown in Table 13.5.

The phagocytic cells are a major factor in this line of defense.

Phagocytic cells. The major phagocytic cells are the neutrophils and the monocytes of the blood and the monocytes and the macrophages in the tissues. The neutrophils also may enter tissues as part of the inflammatory response.

Chemotaxis. Phagocytic cells are attracted to an area of infection or tissue damage by microbial and host-derived chemotactic factors. The host derived chemotactic factors include components of the complement, clotting, fibrinolytic, and kinin systems that are activated either directly by contact with microbes, or indirectly by the action of proteolytic enzymes released by the invading microbe on damaged cell. The complement C5a is a chemotactic factor.

Phagocytosis. The phagocytes scavenge foreign material including microbes, that may enter the body. They do this based on recognition mechanisms in their

Table 13–5. Some important nonspecific antimicrobial factors

Antimicrobial factor	*Chemical*	*Source*	*Effects*
Lysozyme	Protein	Most body fluids	Destroys bacterial cell wall also within phagocytes
Beta-lysin	Protein	Serum leukocytes	Attacks cytoplasmic membrane; active against Gram-positive bacteria
Peroxidase (peroxidase enzymes)	Protein	Leukocytes, saliva and other sources of peroxidases	Kills a variety of microorganisms important killing mechanism in saliva and within neutrophils
Interferon	Protein	Leukocytes and tissue cells	Interferes with the multiplication of viruses by causing the formation of antiviral protein.
Complement system	Many distinct proteins	Produced by macrophages and other host cells	Proteins acting in special sequence to produce effects such as chemotaxis, opsonization, and cell lysis

membranes and with the aid of constitutive and inducible opsonins. The phagocytes also participate in the inflammatory response and initiate and regulate the inducible immune response that develops in vertebrates following the breaching by particles of the physical barriers to infection.

Opsonization. Opsonins are the substances in the serum that coat organisms and cause them to be engulfed by phagocytic cells (opsonization). After attachment of an opsonized particle to the phagocyte, the phagocyte will surround the particle by forming pseudopods (Fig. 13.2).

The killing of ingested microbes and the destruction of ingested material is carried out by a variety of enzymes which are similar to enzymes of digestive tract and are generally present in lysosome of phagocytes.

Inflammatory Response

Inflammation is a complex series of events that is a part of the response of all multicellular organisms to the introduction into their tissues of living and non-living foreign agents. The word *"inflame"* means literally 'to set on fire', its use here refers to all the changes that occur during the local and systemic responses to tissue damage. Though inflammatory response is protective in nature, it may cause some tissue damage.

The five cardinal signs of acute inflammation are *redness*, or *erythema; swelling; pain; heat* and *loss of function.* These are caused by processes related to vascular changes. Vasodilatation causes heat and erythema. Exudation by blood plasma and leukocytes causes swelling. Pressure on nerve endings in swollen tissue and the effects of some prostaglandins causes pain. Loss of function may result from buildup of extravascular fluids and cells at the inflammed site. Function may also be restricted as a result of pain.

Systemic effects of inflammation Followings are systemic effects of inflammation:

- Induction of *fever.*
- Stimulation of production of a group of substances termed *colony stimulating factor* (CSF), that stimulate the production of granulocytes and monocytes from bone marrow precursor cells.
- Induction of *acute phase reactants* which are a group of plasma proteins that serve as nonspecific host resistance factors and may also function to aid tissue repair. Some of these are C3, alpha 1-antitrypsin, alpha 1-antichymotrypsin, hepatoglobulin, transferrin, fibrinogen and C-reactive protein.

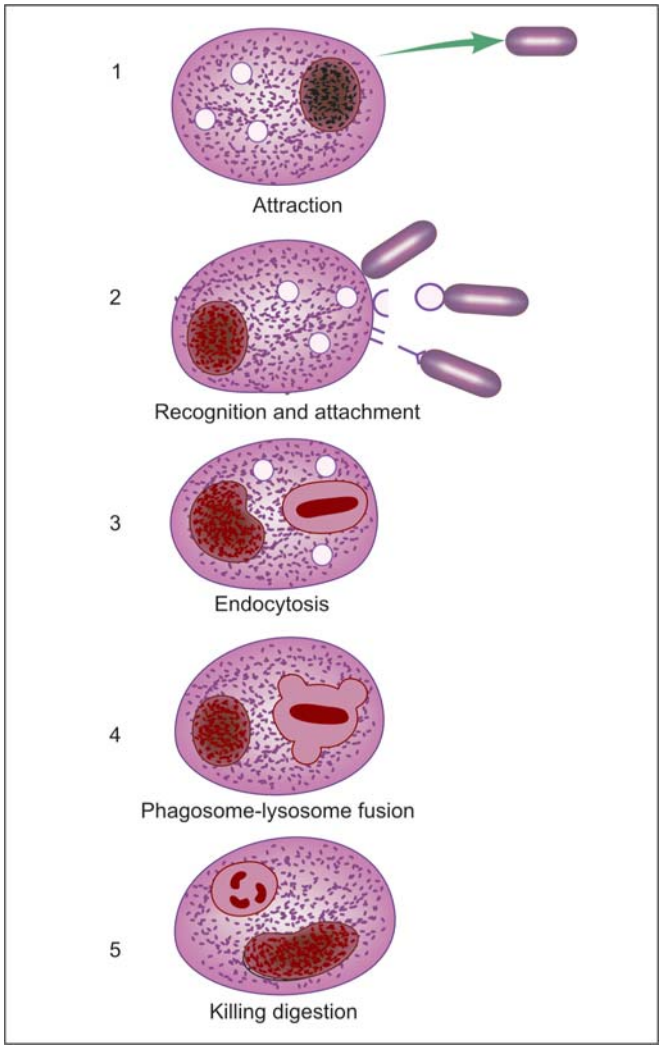


Fig. 13–2. Phagocytosis is a multi-step process. Here a typical bacterium is shown being dealt with by a phagocyte. Note that all stages except stage 2 require the expenditure of energy by the phagocyte, whereas attachment is based on simple physical forces between receptors on the cell and the bacterium or molecules bound to it (i.e. complement, antibody). The mechanisms used for stage 5 are classified as oxidative or non-oxidative, depending on whether they require a source of oxygen

Inflammation has many features which are common to natural resistance and some it shares with acquired immunity. It is hence, often referred to as *bridge between natural resistance and acquired immunity.* Inflammation differs from acquired immunity in not having memory and lacking specificity for particular microbe. It provides protection against a variety of organisms, not just the one inducing it.

PART IV

14 Overview of Acquired Immunity

Immunity and Susceptibility

The word *immune* literally means *free from burden*. Used in a general sense, *immunity* refers to the ability of an individual to recognise and defend itself against infectious diseases. *Susceptibility*, the opposite of immunity, is the vulnerability of the host to harm by infectious agents.

Immunology and Immune System

Immunology is the study of *specific* immunity and how the immune system responds to specific infectious agents. The i*mmune system* consists of various cells, especially lymphocytes, and organs such as thymus gland, which participate in providing the host with specific immunity to infectious agents.

The *immune system* protects an individual against invasion by foreign bodies specially microbial agents and their toxic products.

The integrity of the body is maintained by the multiple defense systems including immune response. The general defenses of the host (discussed in Chapter 13) try to protect the individual against invading microorganism, regardless of the type of microbe. Immunity produced by such types of defenses is called as *nonspecific immunity*. In contrast, *specific (or acquired) immunity* is the ability of a host to mount a defense against particular infectious agent by physiological responses *specific to that infectious agent*. These have been briefly described in Tables 14.1 and 14.2.

Types of Immune System

The immune system of humans consists of two parts—humoral (antibody mediated) immune system and cell-mediated immune system, abbreviated as AMI and CMI

The *humoral immune system* involves antibodies that are dissolved in the blood plasma and lymph. These antibodies are produced by lymphocytes called *B cells*.

The *cellular, or cell-mediated immune system* depends on lymphocytes called *T cells*, which are located both in the blood and in the lymphoid tissues. T cells do not secrete antibodies into body fluids (Table 14.3).

Table 14–1. Natural resistance and acquired immunity

Immunity	*Type*	*Characters and examples*
Natural resistance	Inborn as a result of the genetic constitution of the individual; independent of previous experience	Immunity in humans to distemper virus of dogs; protection provided by phagocytes and lysozymes etc.
Acquired	• Naturally acquired *Active* • Naturally acquired *Passive* • Artificially acquired *Active* • Artificially acquired *Passive*	Antibodies and specialized cells acquired after natural exposure to a foreign agent; long lasting and specific Transfer of immunity to disease from mother to foetus through placenta; temporary Acquired following immunization with vaccines Long lasting; specific Acquired by administration of readymade preformed antibodies, e.g. rabies immunoglobulins, Anti-diphtheric serum (ADS), Anti-tetanus serum (ATS) Anti-rabies serum (ARS) etc, temporary

Table 14–2. Comparison of natural and acquired immune systems

	Natural	*Acquired*
Major elements/Soluble factors	Lysozyme, complement, acute phase proteins e.g. C-reactive protein, interferon	Antibody
Cells	Phagocytes Natural killer (NK) cells	Lymphocytes
Response to microbial infection		
First contact	+	+
Second contact	+	++++
	Non-specific No memory resistance not improved	Specific Memory persists resistance improved

Table 14–3. Classification of acquired immunity

Effector	*Type of response*	*Outcome of defense response*
Cellular	Engulfment	Uptake of foreign material (phagocytosis)
	Cytotoxic	Destruction of infected cells or nonself cells by contact
Humoral	Agglutination	Clumping of organisms and phagocytosis
	Precipitation	Clumping of soluble molecules and phagocytosis
	Neutralization	Inactivation of toxins Blocking of infection by viruses and other intracellular parasites
	Complement	Lysis of parasites fixation Phagocytosis of parasites

Specific Immunity (Acquired, Adaptive or Inducible)

Acquired immunity refers to that immunity which a person develops during his lifetime. Acquired immunity is the surveillance mechanism that *specifically* recognizes foreign antigens and *selectively* eliminates them, and on re-encountering the antigen has an enhanced response.

Characteristics of acquired immunity. There are six major characteristics of acquired immunity:

- Specificity
- Inducibility
- Diversity
- Memory
- Distinguishing self from nonself
- Self-limiting

Humoral immunity. Immunity based on antibodies is called as humoral immunity. Antibodies are produced by B subset of lymphocytes. probably the most formidable type of immunity. This form of immunity is conveniently subdivided into that which is *actively acquired* and that which is *passively acquired* (Table 14.4).

The humoral immunity protects against circulating extracellular antigens such as bacteria, microbial exotoxins and viruses in their extracellular phase; that is, antibodies normally interact with circulating antigens but are unable to penetrate living cells.

In active immunity the individual synthesizes his own antibodies, whereas in passive immunity the individual receives these antibodies from some other individual, either a human or a lower animal. Both active and passive types may each be further divided into natural and artificial. The artificial types are those that result from intervention by physicians.

Actively acquired immunity. A degree of naturally acquired active immunity results from any infection from which a person recovers, whether the illness is serious or subclinical. During the illness the individual receives an antigenic stimulus which initiates antibody production against that specific pathogen. On a subsequent visitation by the same or an antigenically related pathogen, these antibodies will assist in the defense of the body. Because many microbes produce diseases with a high morbidity, this is not a very satisfactory way of developing immunity. This problem could be overcome by producing safe and potent vaccines or toxoids that are being used to induce active immunity.

Table 14–4. Differences between active and passive immunity

Character	*Active immunity*	*Passive immunity*
Production by host's immune system	+	–
Induced by contact with antigen	+	–
Protection induced lasts long	+	–
Immunity effective after lag phase	+	–
Immunological memory	+	–
Used as immunoprophylaxis	+	–
Used as immunotherapy	–	+
Type of immunity involved	CMI and humoral	Only humoral
Immunity inherited	–	+
Useful in immunodeficient patients	±	+
Duration of immunity	Long	Short
Negative phase	May occur	No negative phase

The immunity resulting from the injection of these immunogens is said to be of the artificially acquired type, since it is a man made procedure.

Passively acquired immunity. Passive immunity may also be acquired by *natural or artificial means*. Naturally acquired passive immunity usually refers to the transplacental passage of antibodies from the mother to the unborn child during the later part of pregnancy. This is caused almost entirely by IgG since other immunoglobulins do not pass the placental barrier. Colostrum contains secretory IgA and IgM which are not destroyed by the poorly developed digestive system. Even if not absorbed, these antibodies passively coat the infants digestive tract and ward off intestinal infections. Types of immunity naturally and artificially acquired are summed up in Table 14.5.

Artificial passive immunity refers to the original production of antibodies in some other individual (either human or lower mammal) and the acquisition of these antibodies through parenteral route. Injections of hyperimmune serum (against rabies, tetanus, hepatitis, etc.) represent this type of immunization.

Cell-mediated immunity (CMI). Cell-mediated immune response is a complex process which is based mainly upon thymus dependent lymphocytes called as T cells. Two sub-populations of T cells play important role in CMI: T helper (T_H) cells and T cytotoxic (T_C) cells.

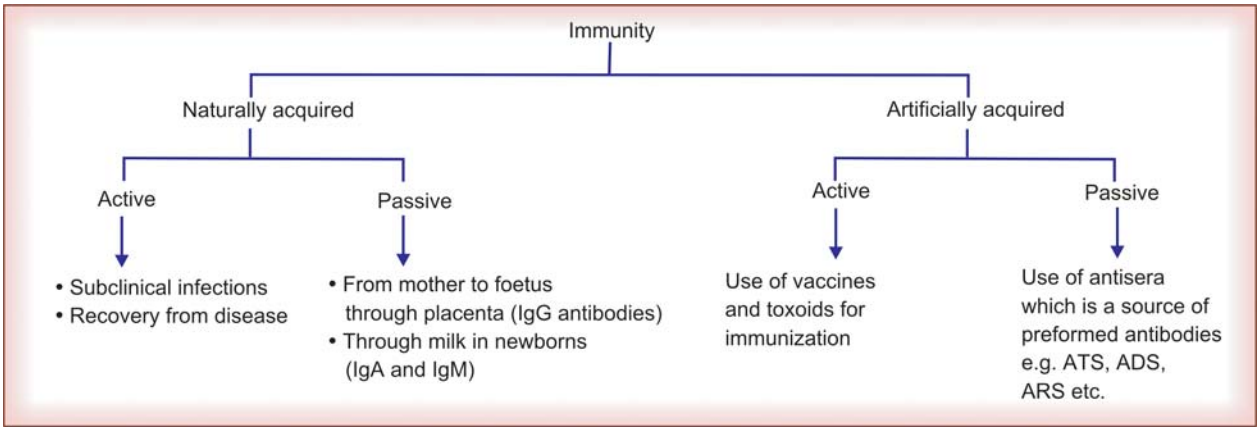
Cell-mediated immunity protects against intracellular microorganisms, such as viruses, and is important in the rejection of organ transplants and tumour cells.

Essentials of Immune Response

The essentials of immune response can be summarised in four Rs. These are:

1. **Recognise** self from nonself. Normally the body recognises its own cells as nonantigenic; therefore an immune response generally is triggered only in response to agents that the body identifies as foreign. In autoimmune disorders the ability to differentiate self from nonself is disrupted and the immune system attacks the body's own cells as if they were foreign antigens.
2. **Respond** to nonself invaders. The immune system responds in part by producing antibodies that target specific antigens for destruction. New antibodies are produced in response to new antigens. Deficits in the ability to respond can result in immunodeficiency disorders.
3. **Remember** the invader. The ability to remember antigens that have invaded the body in the past is the immune system's memory. This characteristic allows for a quicker response if subsequent invasion by the *same* antigen occurs.

Table 14–5. Summary of types of immunity



4. **Regulate** its action. Self-regulation allows the immune system to monitor itself by *turning on* when an antigen invades and *turning off* when the invasion has been eradicated. Regulation prevents the destruction of healthy or host tissue. The inability to regulate could result in chronic inflammation and damage to the host tissue.

Local Immunity

This refers to availability of immunity at the site of the entry of the pathogens. For example oral vaccines developed against various intestinal pathogens provide immunity both at the gut level and systemically in contrast to parentral vaccines which don't provide immunity at the gut level. A special class of immunoglobulins called secretory antibodies (IgA) form the major component of local immunity.

Herd Immunity

This refers to the overall level of immunity in a community and is relevant in the control of epidemic diseases. When a large proportion of community (herd) are immune to a pathogen, the herd immunity to the pathogen is satisfactory. When herd immunity is low the disease can spread as epidemics. With the stoppage of smallpox vaccination and eradication of smallpox, there is no herd immunity against this disease, and if the disease had to occur now, it would spread like wildfire.

15 Functional Anatomy and Development of Immune System

The immune system comprises of a variety of organs which are concerned with growth, development and deployment of lymphocytes. These are called as lymphoid organs. However, lymphoid tissue can contain other cell types and at the same time lymphocytes and antigen processing cells are important constituents of nonlymphoid tissues such as those in the respiratory and gastrointestinal tracts.

Functions of Lymphoid Organs

Four broad functions have been attributed to lymphoid organs:

- To provide an environment for the maturation of various lymphoid cells
- To concentrate lymphocytes into organs that drain areas of antigen *insult*
- To permit interaction between various types of lymphocytes
- To efficiently disburse products of lymphocytes (antibodies or cytokines).

Types of Lymphoid Organs

Lymphoid organs have been divided by their functions into two categories:

- Primary or central lymphoid organs
- Secondary or peripheral lymphoid organs

Primary lymphoid organs are those organs in which proliferation and differentiation of lymphocytes takes place independent of antigenic stimulation. In human beings following constitute primary lymphoid organs:

- Thymus
- Bone marrow
- Intestinal epithelium

In the tissues of these organs, the evolution of stem cell progeny into mature lymphocytes takes place. The mature lymphocytes then seed into periphery, where exposure to antigen and appropriate immune responses occur.

Those organs which receive and maintain functional lymphocytes are called *secondary lymphoid organs.* These include followings:

- Lymph nodes
- Spleen
- Gut or mucosa associated lymphoid tissue
- Appendix
- Tonsils
- Adenoids

Lymph nodes develop throughout the body and are most concentrated at areas where pathogens are more likely to enter.

The major components of immune system in humans have been shown in Figure 15.1.

Primary or Central Lymphoid Organs

Thymus. The thymus is a small organ in the anterior mediastinum. It has an outer cortex and an inner medulla. Immature cells called prothymocytes, which are derived from stem cell in bone marrow migrate through the circulation into the cortex where they proliferate and acquire membrane proteins characteristic of mature T lymphocytes. During this phase, the cells are termed thymocytes. T lymphocyte maturation is most vigorous during foetal and neonatal development and proceeds actively until puberty, after which the thymus atrophies. As the immune response becomes capable of generating long lived memory cells to react against antigen to which it has been previously exposed, the

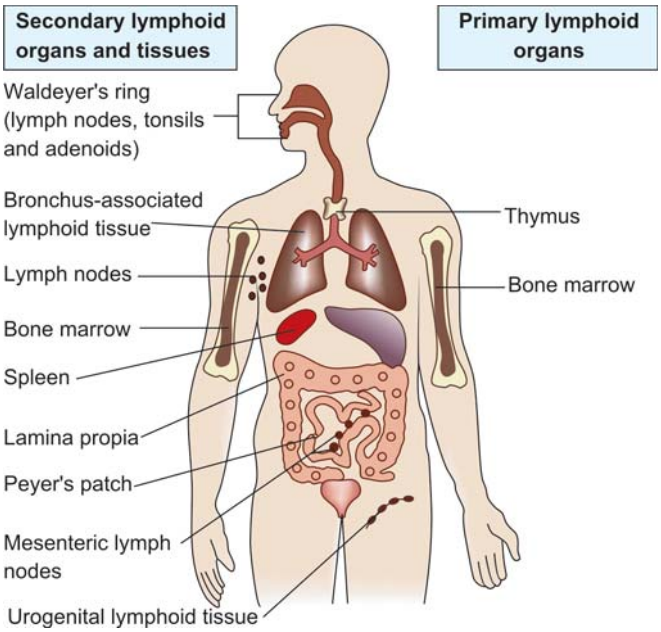


Fig. 15–1. Anatomy of immune system

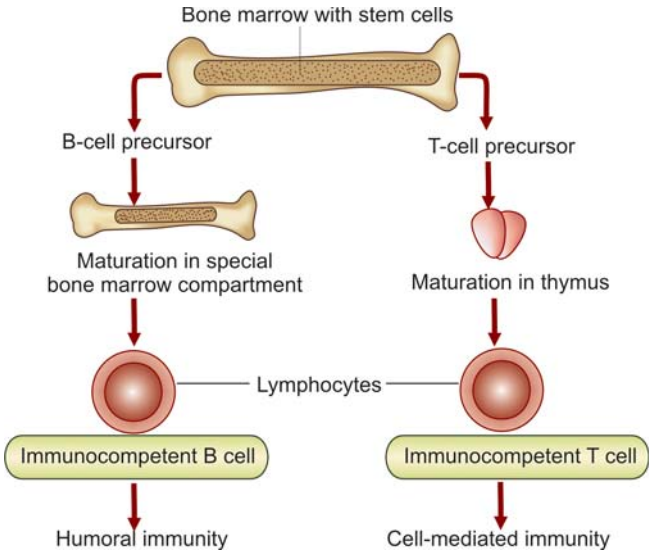


Fig. 15–2. Lymphopoiesis

need for newly derived thymocytes decreases. Thus removal of thymus in adulthood causes little immune impairment whereas its absence during neonatal life can produce a profound immunodeficiency.

The thymocytes in the cortex are small to medium in size. They become progressively smaller and functionally mature on migrating into medulla. In the medulla, the lymphocytes complete their maturation process and migrate into the blood as mature T lymphocytes capable of responding to antigenic exposure.

Thymic hormones. The differentiation and maturation of cells from bone marrow that terminate in the recognition of a mature T cell are regulated by a number of polypeptides, which are described as thymic hormones. These include thymosin, thymic humoral factor, thymulin, thymopoietin and serum factor.

Bone marrow. The maturation of pre-B cells into functionally mature B lymphocytes occurs in bone marrow after which they leave bone marrow and settle into secondary lymphoid organs.

The bone marrow is the source of erythrocytes, platelets, granulocytes, monocytes and lymphocytes. The differentiation of the pluripotent stem cells into mature B and T cells is shown in Figure 15.2.

Intestinal epithelium. Until recently, the thymus was thought to be the sole source of T cells. However, now it is established that intestinal epithelium is a major site for development of thymus-independent T-cell development. In adults it contains the largest collection of T cells in the body.

Secondary Lymphoid Organs

The stem cells arising in the bone marrow differentiate into immunocompetent T and B cells in the primary lymphoid organs. These cells then colonise the secondary lymphoid tissues where immune responses are organised.

Lymph nodes. The lymph nodes are the arena in which the encounter between an antigen and a naive immune system takes place (Fig. 15.3). Four major immunological functions are performed by the lymph nodes.

- Provide a site for phagocytosis and antibody production
- Act as junction between circulatory system and lymphatic system
- Support the development of lymphocytes
- Allow recirculation of lymphocytes.

Spleen. The architecture of spleen is similar, but not identical, to that of lymph node. Unlike lymph nodes, the spleen has no lymphatic drains and it is primarily an organ to respond to blood borne antigens. Spleen has two seggregated areas, the red pulp and the white pulp. The red pulp consists of cords of cells that divide venous sinuses. These cords are composed of a reticular connective tissue lined with the fixed macrophages. The white pulp consists of sheaths of lymphocytes organised around penetrating, or trabecular

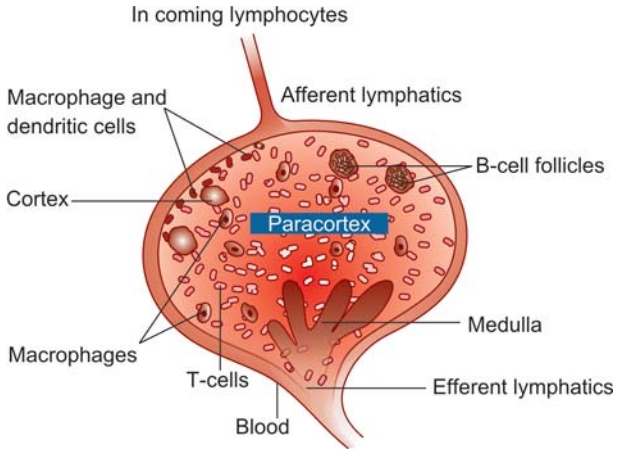


Fig. 15–3. Lymph node

arteries. At branch points of trabecular arteries, the white pulp expands into large structures known as splenic follicles. The follicles, the T-independent areas, consist of collection of the lymphocytes in varying degrees of activation.

Other Lymphoid Organs

The tonsils are lymphoid tissues that, like the thymus, are rather large in childhood and tend to diminish in size with age. Tonsillar lymphocytes are largely B cells.

Other potentially important collections of lymphoid tissue exist in appendix, lamina propria and Peyer's patches of the intestine. The lymphocyte population of Peyer's patches is a mixture of B and T lymphocytes. The lamina propria contains T cells and a large population of plasma cells. In man, the lingual, palatine and pharyngeal tonsils, the Peyer's patches and appendix have also been designated as mucosal-associated lymphoid tissue **(MALT)**. The MALT of the gut is called as gut associated lymphoid tissue **(GALT)**.

Development of Immune System

The basic pattern of the immune system found in the adult animal is developed during embryogenesis. All components of the human immune system begin formation during the first three months *in utero*. After about six weeks of development, the thymus, spleen and bone marrow begin to form.

The last portions of the immune system to form are the lymphatic channels and lymph nodes. In humans, six lymphatic sacs of the mesodermal tissue are formed. As the foetus develops, these elongate into channels. At about 3 months masses of mesodermal cells form beside the channels, connective tissue capsule form around them, and they become vascularised. The newly developed lymph nodes sink into the channels and become populated with lymphocytes from the thymus and spleen. Lymph node development is completed within a short period after parturition.

16 Biology of Immune Response

DYNAMICS OF ANTIGEN ELIMINATION

The basic objective of defense mechanisms of body is to eliminate antigen. Both immunological and non-immunological activities play important roles.

The injection of an antigen into an animal may initiate several important changes. For intravenously injected antigen, three phases of antigen removal are easily detected. These are:

- Phase of equilibration
- Phase of non-immune catabolic degradation
- Phase of immune catabolism.

Phase of Equilibration

First phase is of equilibration which lasts for 10 to 20 minutes if particulate antigens are used and a longer time if antigen is soluble.

Phase of Non-immune Catabolic Degradation

The second phase lasts for 4-7 days during which catabolic degradation and removal of antigen takes place.

Phase of Immune Catabolism

The third phase is of immune catabolism which is the result of combination of newly formed antibody molecules against the antigen, enhancing phagocytic engulfment, digestion and removal of antigen. The absolute removal of all antigen from an immunised animal can take many months or years (Fig. 16.1).

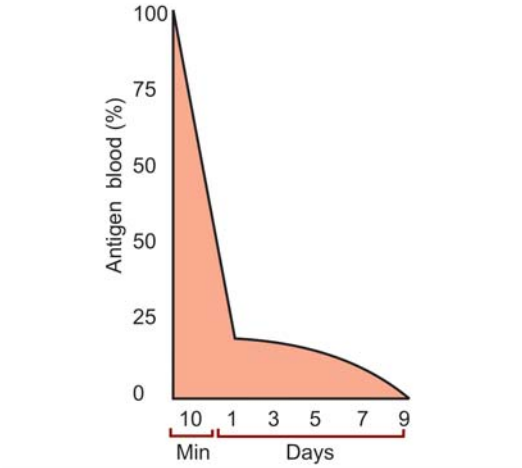


Fig. 16–1. Antigen elimination curve

IMMUNE RESPONSE

It can be described as:

- Primary immune response
- Secondary immune response

Primary Immune Response

The primary immune response is defined as the host's first exposure to a specific antigen which results in appearance of specific serum antibodies. All immunogens can induce a primary antibody response.

In the primary response, after the first injection of antigen there is a lag of several days (latent or inductive period) before antibody appears. The factors that influence primary antibody response are:

- Chemical and physical nature of antigen
- Use of adjuvants
- Dosage of antigen
- Frequency of antigen exposure
- Route of antigen exposure
- Genetic make up of host receiving the antigen.

On stimulation, a B cell may produce antibody within 20 minutes but since very few cells produce antibody in beginning, it cannot be measured. Usually measurable antibody appears between the 5th and 10th day. The general shape of primary response curve is the typical sigmoid curve, with an extended decay phase. The exact shape of the response curve is detected by many variables.

The antibody secreting cells gradually disappear from the system because of influence of regulatory cells, their cytokines and antibodies. The memory cells, however, persist in lymphoid tissues. The memory cells do not participate in the initial response, but they retain their ability to recognise a particular antigen. They can survive *without dividing* for many months to many years and play important role in inducing secondary antibody response.

Table 16–1. Salient features of secondary immune response

- It is not the result of sudden release of preformed antibody but is due to bulk of synthesis of antibody
- May be induced any time after primary response
- Repeatable within physiologic limits
- May be induced
- Fall in antibody titre is gradual
- Contains more IgG
- IgM anamnesis does not occur
- May not always be safe (e.g. anaphylaxis)
- Primary response due to antigen coupled with adjuvant mimicks secondary response.

Secondary Immune Response

In the secondary response if the same animal is subsequently exposed to same antigen, there occurs a sharp drop in circulating antibody because it complexes with the newly injected antigen. After two or three days, a marked increase in antibody level becomes evident. This increase continues for several days and ultimately results in level of antibody which is higher than what is seen in primary response. This response is also often called as **memory,** or **booster response** or **secondary immune response.**

Table 16–2. Comparison of primary and secondary antibody responses

Parameter	*Primary response*	*Secondary response*
Responding B cell	Naive B cell	Memory B cell
Latent period	5-10 days	1-3 days
Peak antibody titre	Smaller	Larger
Persistence of antibody titre	Short	Long
Predominating Ab class	IgM, IgG	IgG
Induced by	All immunogens	Only protein antigens
Dose for immunization	High	Low
Adjuvant	May be needed	Not needed

Few salient features of secondary immune response are summarised in Table 16.1. The primary and secondary responses are compared in Figure 16.2 and Table 16.2.

USES OF PRIMARY AND SECONDARY IMMUNE RESPONSES

Primary and secondary responses may occur during microbial infections. When we recover from an infection without the help of antibiotics, it is usually because of the primary response. If, at a later time, we contact the same microorganism, the secondary response can be so swift that the microorganisms are quickly destroyed and no clinical feature of the disease is exhibited.

Secondary immune response also provides basis for immunization against certain diseases. On administering initial immunization, the immune system gets *primed*. Should the antigenic stimulus be encountered

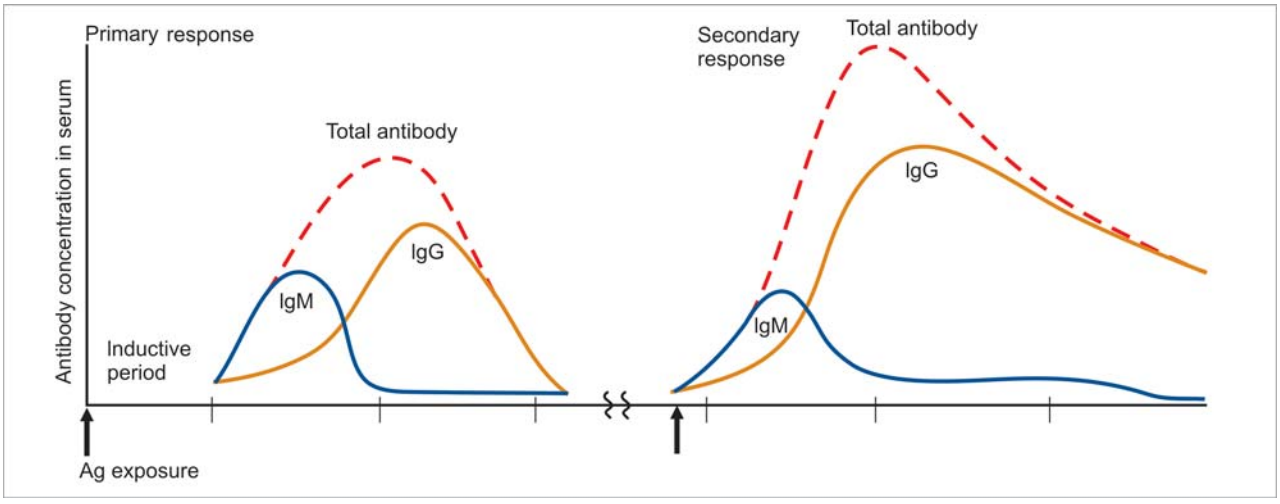


Fig. 16–2. Immune response

Table 16–3. Factors influencing antibody production

1. Genetic factors	:	There are "poor-responders" and "good-responders" in antibody production, which is genetically determined.
2. Nutrition	:	Malnutrition affects the immune response adversely. Amino acids deficiency has been shown to cause a decrease in antibody synthesis.
3. Age	:	The immune system is weak at extreme of age. Full competence is developed by about 5-7 years of age for IgG and 10-15 years for IgA.
4. Route of administration	:	Best response when antigen is given parenterally. Route also decides the type of antibodies produced. IgA produced after oral or nasal route administration. Hepatitis B and tissue culture antirabies vaccines are less effective when given in intragluteal region than in deltoid region because of the fat present in gluteal region.
5. Dose size	:	There is a "critical size" about every antigen. The quantities lesser or higher than that are not good for antibody production
6. No. of doses	:	The first dose gives lesser quantum of antibodies, which rises with booster doses.
7. Multiple antigens given together	:	The response could be same as if the antigens were given singly or may be less or increased. For optimal effect, the relative proportion of different antigens in a mixture should be carefully adjusted.
8. Adjuvants	:	When antigens are given alongwith adjuvants, the antibody response is better.
9. Simultaneous use the immunosuppressive agents	:	The response of antibody production diminishes when the person is receiving immunosuppresive agents such as corticosteroids, anti-cancerous drugs, etc.
10. Concomitant use of passive immunization	:	When active and passive immunity is given together, the antibody response is reduced.

again as the infecting microorganism or *booster dose* of vaccine, the body experiences the secondary response. A booster dose literally boosts the antibody titre to a higher level.

FACTORS INFLUENCING ANTIBODY PRODUCTION

There are many factors which affect the antibody production in a positive or negative way. These are summarized in Table 16.3.

17 Antigen

Definition

The term *antigen* was coined from the words *antibody* and *generator*. Thus, an antigen was originally considered to be a substance that induced production of antibody. However, with greater insight into mechanisms of immune response following definition is considered appropriate for antigen.

"An antigen when introduced into a host induces the formation of specific antibodies and T lymphocytes that are reactive against the antigen."

Immunogenicity and Antigenicity

Antigens have two very important characteristics: *immunogenicity*, or the ability to stimulate the specific immune response, and *antigenicity*, the ability to react specifically with antibodies. An antigen with both these characteristics is called a **complete antigen**. An antigen which has reactivity but not immunogenicity is called an **incomplete antigen** or **hapten** (*haptein*: to grasp). A hapten can be made into a complete antigen by combining it with a larger carrier molecule, such as a protein.

Determinants of Antigenicity

There are several important determinants of any antigenicity.

- Macromolecular size
- Molecular complexity
- Biodegradibility
- Foreignness
- Specificity.

Macromolecular Size

Proteins of molecular weight exceeding 10,000 daltons are good antigens. Tetanus toxoid, egg albumin, thyroglobulin and haemocyanin—all with molecular weight ranging between 14,000–6,000,000 are examples of good protein antigens. Polysaccharides are poor antigens.

Molecular Complexity

The antigenic potency of macromolecule increases with the complexity of structure and accordingly quaternary structures are antigenically most potent.

Biodegradibility

If a substance is insoluble in body fluids and cannot be converted to soluble forms by tissue enzymes, it may not act as an antigen. All cellular antigens, bacteria, viruses and red blood cells are quickly engulfed by phagocytic macrophages and digested to their soluble constituents.

Foreignness

To be antigenic, the macromolecule must be foreign to the animal being immunized. Foreignness here denotes being of different antigenicity as the host. The more foreign the antigen source better it will be.

Specificity

Antigen attaches specifically to an antibody because of the "fit" between the antigenic determinant on its surface as well as the receptor on antigen binding site on antibody. The effect of antibody is activated only after the "fit" has taken place. The specificity can be:

Species specific: Human antigens v/s animal antigens with the exception of heterophile antigens which are present in more than one species.

Organ specific: Every organ has its specific antigens. In exceptional instances there is sharing which may lead to immunological problems.

Isospecificity: These antigens differentiate members of same species viz HLA and blood group antigens.

Antigen Nomenclature

The antigens that require T cells in order to generate an immune response are called as *T cell-dependent* (*TD*) *antigens*. Antigens that stimulate B cells without the intervention of T cells are the *T cell-independent (TI) antigens.* The booster or memory response is mediated through T cells and hence can be initiated only by TD antigens.

There are certain other terms which are in common use regarding some varieties of antigens.

Autologous Antigen

An autologous antigen is one's own antigen, which under appropriate circumstances would induce auto-antibody formation. Thus, autologous antigen is synonymous with *auto* or *self-antigen*.

Heterologous Antigen

An heterologous antigen is merely an antigen different from that used in immunization; it may or may not react with the antiserum depending on its chemical similarity to homologous antigen.

Homologous Antigen

The homologous antigen is the antigen used in the production of antiserum.

Isophile Antigen

The isophile antigens or **isoantigens** are the molecules of one individual of a species that are antigenic in another member of same species. Best example of isoantigens is blood group system.

Cross Reactivity of Antigens

An antigen can be a complex mixture of many antigenic molecules, e.g. a microbe. Cross reactivity may occur if different complex antigens have similar antigenic molecules.

Cross reactivity may also result from the presence of a variety of molecules in the preparation, some of which are shared. As immunological reaction is directed against small regions of molecules *(epitopes)* and not against whole molecule, cross reactivity may occur because some epitopes on a given molecule may be similar and others distinct. Hence, immune sera raised against a preparation containing only a single type of molecule may react with preparations of different molecules if same epitopes are shared.

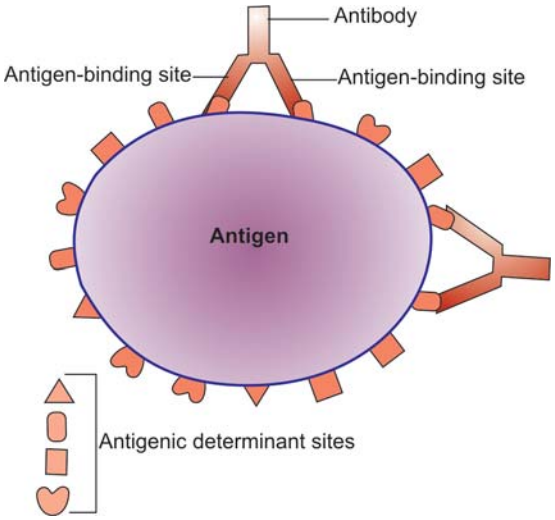


Fig. 17–1. Antigenic determinant sites

Antigenic Determinant Sites (Epitopes)

The whole antigen does not induce an immune response. Only a limited part of an antigen molecule is inducer of B and T cell responses. It is also that part of antigen with which the antibody or T cell reacts. This is called an antigenic determinant site or *epitope* (Fig. 17.1).

Haptens

Haptens are too small molecules to be antigenic in their own right. Injection of hapten into an animal does not normally induce an immune response. At the same time hapten is capable of reacting with antibody induced by injection of a hapten-carrier complex. Thus, an hapten is defined as a molecule that is not immunogenic in itself but that can react with preformed antibody of right specificity.

Haptens can be covalently coupled to existing established antigens *(carrier)* to create new antigenic determinants. These hapten-antigen (carrier) complexes, or conjugated or neoantigens, generate antibodies with specificity for haptenic groups. Haptens are of two types:

a. *Simple haptens* combine with specific antibody but do not produce any antigen-antibody product viz precipitation.
b. *Complex haptens* do combine with specific antibody to produce precipitates because of presence of multiple antibody combining sites on its surface.

Adjuvants

Adjuvants (*adjuvare*: to help) are agents used to potentiate the immune response-both humoral and cell-mediated.Adjuvants are customarily administered with the antigen. An adjuvant may not be an antigen itself.

Classification

Adjuvants are broadly classified into particulate or insoluble and nonparticulate or soluble (Table 17.1).

Table 17–1. Classification of adjuvants

Particulate	*Nonparticulate*
Aluminium	Muramyl dipeptide
Calcium	Lipid A
Oil-in-water	Saponin
Water-in-oil	Cytokines
ISCOM	Carbohydrate polymers
Liposomes	Bacterial toxins

The aluminium and calcium salts are best known examples of adjuvants. These form an insoluble complex with antigen which slows down tremendously the escape of antigen from a subcutaneous or intramuscular depot thus prolonging the original stimulus over a period of 3-4 weeks. This creates the effect of multiple, microbooster exposures. These adjuvants, by increasing the physical size of the antigen, also enhance phagocytosis.

Water-in-oil emulsifying adjuvants have had a restricted use in humans.

A classic example of this type of adjuvant is *Freund's adjuvant*. This is used in two forms-incomplete and complete. The incomplete form consists of a mixture of a light mineral oil and an emulsifier such as mannide mono-oleate.

Freund's complete adjuvant contains, in addition to components of incomplete adjuvant, a supplement of 0.5 mg/ml of killed mycobacteria. Freund's complete adjuvant produces such serious granulomas that it is not recommended for use in human beings.

Bacteria such as *Bordetella pertussis* have also been used as adjuvants. The smoother phase contains a lymphocytosis promoting factor that seems to mobilize with B and T cells.

Mechanism of Action of Adjuvants

The precise mechanism of action of adjuvants remains uncertain. However, various possible mechanisms have been shown in Table 17.2. To summarise adjuvants are believed to bring about:

a. Direct increase in number of cells involved in antibody production
b. Ensure a more efficient processing of antigen
c. Prolong the duration of antigen in the immunized animal, and
d. Increase the synthesis and release of antibody from antibody forming cells.

ISCOMS (immune stimulating complexes) are new chemical adjuvants which have shown great initial promise. These comprise of saponin, cholesterol and phospholipid. Similarly liposomes which contain cholesterol and phospholipids are emerging as important group of adjuvants.

Table 17–2. Possible modes of action of adjuvants

Principle	*Mechanism*
Immunomodulation	Modify cytokine network and better processing of antigen
Presentation of antigen	Optimal antigenic determinant is presented to effector cells
Induction of cells	CD8 cells are induced for cytotoxic response
Targetting of cells for antigen	Better delivery of antigen to immune effector cells
Depot generation	Sustained and long-term release of antigen for boosting the immune response

18 Antibody

The production of antibody is a part of the response of the immune system to antigenic stimulation. Antibodies are blood proteins, all of which are globulins (hence the synonymous immunoglobulins) part of gamma fraction of serum. In addition to those found in blood *(humoral antibodies)*, some types of antibodies are fixed to body cells or tissues or exist in body secretions *(cell bound antibodies)*.

Structure

Immunoglobulin (Ig) molecules are symmetrical structures. In solution they become 'Y'-shaped after binding to an antigen. Each molecule consists of four polypeptide chains: two identical heavy (H) chains and two identical light (L) chains. These are designated light or heavy based upon their molecular weight which is 50,000 to 70,000 daltons for H chains and 20,000 to 25,000 daltons for light chains (Fig. 18.1). The L-chain is attached to H-chain by a disulphide bond. The two H-chains are joined together by 1 to 5 S-S-bonds depending on the class of immunoglobulins. The H-chains are structurally and antigenically distinct for each class of immunoglobulin. The L-chains are similar in all classes of Ig. They occur in two types: Kappa (κ) and lambda (λ). A molecule of immunoglobulins could have either kappa or lambda but never both. L and H-chains are subdivided into *variable regions* and *constant regions.* An L-chain consists of one variable domain (V_L) and one constant domain (C_L). Most H-chains consist of one variable domain (V_H) and 3 or more constant domains (C_H). Each domain is approximately 110 amino acids long. Variable regions are responsible for antigen binding while the constant regions are responsible for biological functions. In the variable regions of both L and H-chains are three extremely

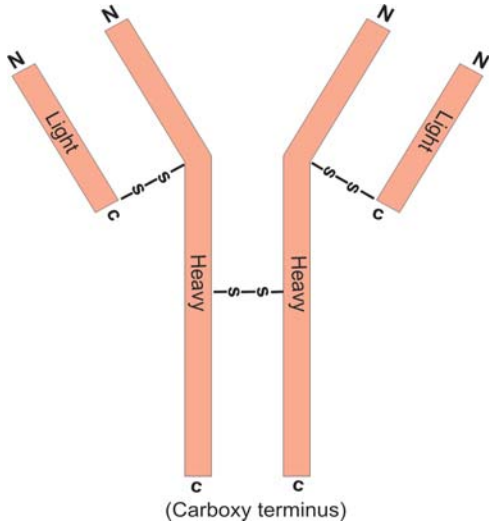


Fig. 18–1. Antibody molecule

variable (hypervariable) amino acid sequences that form the antigen-binding site.

The antibody molecule can be split by papain to yield two identical fragments, each with a single combining site for antigen. This is called as *Fab-fragment antigen binding*. The third fragment which lacks the ability to bind to antigen is termed as *Fc-fragment crystallizable* (Fig. 18.2). In an experimental setting, enzymes can be used to cleave the antibody into Fc and Fab fragments, which have several uses:

Enzyme	*Location of cleavage*	*First fragment*	*Second fragment*
Papain	At hinge region	Two Fab fragments	Fc fragment
Pepsin	Below hinge region	One F(ab')2 fragment	Fc fragment

- *Fc region*: The Fc region (fragment, crystallizable), is derived from the stem of the "Y," and is composed

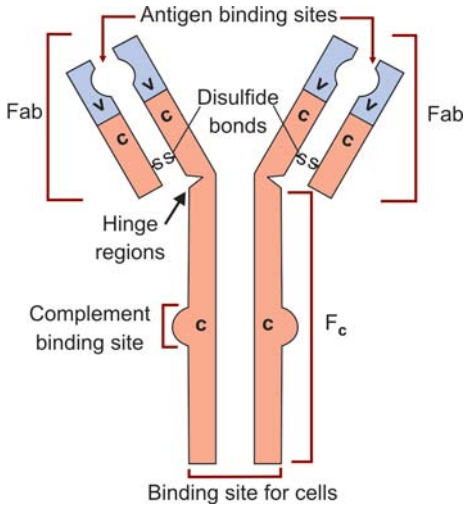


Fig. 18–2. Structure of Ab molecule

of two heavy chains that contribute two or three constant domains depending on the class of the antibody. The Fc region binds to various cell receptors and complement proteins. In this way, it mediates different physiological effects of antibodies such as opsonization, cell lysis, degranulation of mast cells, basophils and eosinophils and other processes.

- *Fab region:* Each end of the forked portion of the "Y" on the antibody is called the Fab region *(fragment, antigen binding).* It is composed of one constant and one variable domain of each of the heavy and light chain. These domain shape the paratope—the antigen binding site—at the amino terminal end of the monomer. The two variable domains bind the epitope on their specific antigens.

Functions of Fab and Fc

The Fab and Fc fragments of the immunoglobulin molecule have distinct functions. The Fab fragment binds to antigen because it contains the antibody's binding site. Fab fragment combines with antigen to form soluble complexes that do not precipitate. The Fc portion does not bind to antigen, but on it are sites that determine effector function of antibody molecules, such as complement fixation, binding to phagocytes, transplacental transfer, binding to mast cell and secretion into body fluids.

Classes (Isotype of Ig)

Based upon the structure of their heavy chain constant region, immunoglobulins are classed into major groups called *classes* and also termed as *isotypes.* In human beings there are five classes: immunoglobulin G, IgA, IgM, IgD and IgE. The heavy chains of these immunoglobulins are designated by greek letter as gamma (γ), alpha (α), mu (μ), delta (δ) and epsilon (ε) respectively. Within class IgG, based upon different distinctive heavy chains and differing functional properties, there are four subclasses: IgG1, IgG2, IgG3 and IgG4. Similarly there are two subclasses each of IgA and IgM.

Characteristics of Immunoglobulins

Physical and biological characteristics of the five major immunoglobulin classes in the humans have been summarised in Table 18.1. The following description shall supplement the information given in Table 18.1.

Immunoglobulin G

Immunoglobulin G (IgG) is the class of immunoglobulin which has maximum concentration in serum and is the major immunoglobulin to be synthesized during the secondary immune response. It is the major line of defence in a newborn because of its capability to pass through placenta as well as to be secreted in colostrum. IgG can be further subgrouped into four isotypic subclasses - IgG1, IgG2, IgG3, and IgG4.

Table 18–1. Characteristics of human immunoglobulins

Class	*IgG*	*IgM*	*IgA*	*IgD*	*IgE*
Molecular weight	150,000	900,000	160,000	150,000	200,000
Valency for antigen binding	2	10	2 or 4	2	2
Heavy chains class	Gamma	Mu	Alpha	Delta	Epsilon
Subclasses	4	1	2	–	–
J chain	–	+	+	–	–
Secretory piece	–	–	+	–	–
Present in epithelial secretions	–	–	+	–	–
Percent of total Ig	70-80	5-10	10-15	1	0.01

Immunoglobulin A

The main function of IgA is to defend the exposed external surfaces of the body against attack by micro-organisms. It appears selectively in saliva, tears, nasal fluid, sweat, colostrum and secretions of lungs, genito-urinary and gastrointestinal tracts. IgA is synthesized locally by plasma cells. It prevents the entry of microbes into body tissues. Aggregated IgA binds to polymorph, and also activates the alternative pathway of complement.

Two subclasses of IgA have also been found, IgA1 and IgA2. The human secretory IgA is associated with two proteins, termed *secretory component* and *J chain* (Fig. 18.3).

Secretory Component. Secretory IgA differs from serum IgA in having an additional protein called secretory component (SC). The functional role of SC remains enigmatic.

J-chain. This protein has been named as J-chain *(joining chain)* because it is found only in immunoglobulins composed of more than one four peptide units. The biological role of J chain is believed to initiate polymerization of IgA and IgM molecules.

Immunoglobulin M

Because of high molecular weight (900,000) and a polymer of five four peptide units, the IgM is also referred to as the *macroglobulin* antibody.

These antibodies are extremely efficient, appear early in response to infection and are largely confined to the bloodstream.

Immunoglobulin D

IgD is present on the surface of a proportion of blood lymphocytes where it seems likely that they may operate as mutually interacting antigen receptors for the control of lymphocyte activation and suppression.

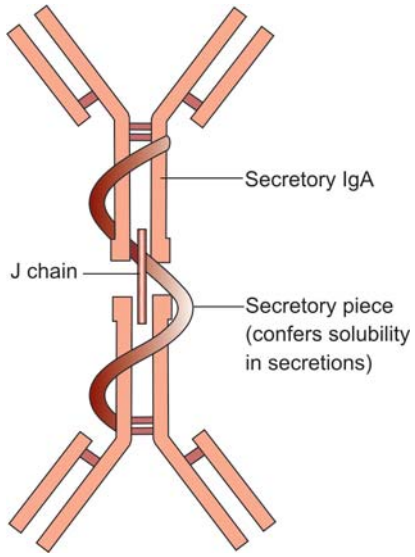


Fig. 18–3. Structure of IgA with J chain and secretory piece

Immunoglobulin E

Very few plasma cells in body synthesize this immunoglobulin and the concentration of IgE in serum is also very low. IgE antibodies remain firmly fixed for an extended period when injected into human skin where they are probably bound to mast cells. Contact with antigen leads to release of vasoactive amines.

The main physiological role of IgE seems to be protection of external mucosal surfaces of the body through triggering an acute inflammatory reaction.

Biological Properties of Immunoglobulins

These have been summarised in Table 18.2.

Theories of Immunoglobulin Formation

Two theories have been put forth to explain the synthesis of immunoglobulin.

Table 18–2. Biological properties of immunoglobulins

Property	*IgG*	*IgA*	*IgM*	*IgD*	*IgE*
Major characteristics	Most abundant internal Ig	Protects external surfaces	Efficient against bacteraemia	Mainly lymphocyte receptor	Initiates inflammation, raised in parasitosis, causes allergy
Antigen binding	++	++	++	++	++
Complement fixation (classical)	++	–	+++	–	–
Cross placenta	++	–	–	–	–
Binding to					
Macrophages	++	+/–	–	–	+/–
Polymorphs	++	+/–	–	–	+/–

PART IV

Ehrlich's Instructive Theory

This theory assumes that the antigen can inform a cell in some way to make an antibody against that antigen. As per this theory, the antigen enters a cell that is routinely engaged in normal gamma globulin synthesis. The antigen interferes with this process, possibly by complexing with mRNA in the polysome. This results into a change in gamma globulin synthesis which now takes the shape of antibody. The antibody dissociates itself from antigen and is excreted into the blood.

Clonal Selection Theory

A clone is a population of cells arising from a single parent cell. As per this theory, in a mature animal the lymphocyte is genetically endowed with the capability of synthesizing immunoglobulins. At rest, unstimulated by antigen, only small amounts of immunoglobulins are formed. On contact with the corresponding antigen, lymphocyte capping heralds a change of that lymphocyte to reproduce and differentiate into a clone of immunoglobulin secreting plasma cells. The resulting clone of cells would consist of a large enough population that the antibody produced would become measurable into the blood.

Soluble Antibodies and Membrane Bound Antibodies

Antibodies occur in two forms: A soluble form that is secreted from cells and released into the blood and tissue fluids, and a membrane-bound form that is attached to the surface of a B cell and is called the B cell receptor (BCR). The BCR on the surface of B cells allows the B cell to detect when a specific antigen is present in the body. Once the B cell binds to an antigen the B cell can be activated - interaction of the B cell with a T helper cell is necessary to produce full activation of the B cell. The activated B cell differentiates into either soluble antibody generating factories called plasma cells, or into memory cells that will survive in the body for years afterwards, allowing an organism to remember that antigen and respond faster upon future exposures.

Antimicrobial Actions of Antibodies

The major antimicrobial actions of antibodies are shown in Table 18.3.

Table 18–3. Antimicrobial actions of antibodies

- Opsonization for phagocytosis
- Complement activation, enhancing phagocytosis and inducing lysis
- Prevention of attachment of Ag to host cells
- Prevention of penetration by Ag of host cells
- Neutralization of toxins
- Inhibition of motility of parasites
- Agglutination of parasites
- Inhibition of microbial growth and metabolism

MONOCLONAL ANTIBODY (HYBRIDOMAS)

One of the most significant advance in immunology was made in 1965 with the discovery of immunoglobulin secreting hybridoma and for this a Nobel Prize was awarded in 1984 to Kohler and Milstein. The hybridomas are produced by fusion of high density antibody forming cells and nonsecreting myeloma cells. These cells have several advantages as given below:

a. Pure antibodies are produced from crude antigen preparation.
b. Antibodies to those antigens can be synthesized which are not detectable by conventional means.
c. Antibody produced is of single Ig class and specific for single epitope.
d. The produce is constant.
e. The quality is same since large quantity of uniform quality can be produced.
f. Can help in antigen mapping.
g. Dynamics of mutation in antibody forming cells can be studied.

The hybridoma technique provides a novel avenue for investigating the antigenic nature of infectious agents, tumour antigens, HLA antigens, differentiation of antigens and provides reagents of a purity never before available.

19 Cells of Immune System

MAJOR CELLS OF IMMUNE SYSTEM

The immune system is made of four major kinds of cells:

- B lymphocytes : produce antibodies
- T lymphocytes : cooperate with B cells and produce cytokines
- Macrophages
- Natural killer (NK) cells : non-specific hunter-killers

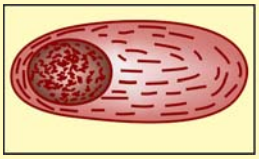
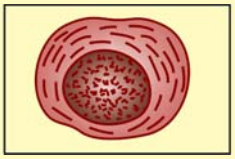
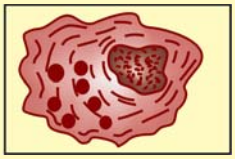
The B and T lymphocytes are two groups of *non-phagocytic, morphologically indistinguishable, but functionally different lymphocytes.* However, there are certain features that are common to B and T cells. Some of these are:

- Unlike all other blood cells, B and T lymphocytes recirculate around the body from blood to tissues and back again into circulation.
- Each lymphocyte has individual receptor in its plasma membrane to enable it to recognise a particular antigen. This confers specificity.
- On getting stimulated with specific antigen these lymphocytes undergo clonal proliferation.
- They show property of memory giving rise to a faster and bigger immune response on re-challenge with the same antigen.

B Lymphocytes

B line stem cells are present in the bone marrow and are the source of pre-B cells which on maturation become precursors of plasma cells. The antibodies are produced by stimulated plasma cells. Various other characteristics of B cells have been mentioned in Table 19.1.

Table 19–1. Characteristics of immune cells

Characteristic	*B cell*	*T cell*	*Macrophages*
Site of production	Bursal equivalent	Thymus tissue	–
Type of immunity	Humoral	Cell mediated	Humoral and cell mediated
Subpopulation	Plasma cells Memory cells	Helper Suppressor	Cytotoxic Fixed and Wandering
Presence of surface Ig	+	–	–
Presence of receptors for antigens	+	+	–
Phagocytic action	–	–	+
Life span	Short	Long and short	Long
Secretory product	Antibodies	Lymphokines	Interleukin-1
			

Distribution of B Lymphocytes

In an adult approximately 30% of the lymphocytes circulating in the blood can be identified as B cells on the basis of their surface Ig markers. Their distribution in various important organs/tissues is as under:

Peripheral blood	15-30%
Lymph nodes	20%
Bone marrow	75%
Thymus	10%
Tonsillar lymphocytes	50%
Splenic lymphocytes	50%

Activation of B-cells

Each B cell carries the genetic instruction to produce antibody of unique antigen specificity as a membrane receptor. On getting stimulated mature B cells differentiate into antibody secreting plasma cells.

T Lymphocytes

T cells, just like B cells, also originate from precursor cells of the bone marrow but mature in the thymus. Several subsets of the T cells arise during this maturation process, each becoming focussed upon a specific function.

Distribution of T Lymphocytes

Thymus, lymph nodes and peripheral blood are rich in T lymphocytes. Their distribution is as under:

- Peripheral blood 55-75%
- Lymph nodes 75%
- Bone marrow 10%
- Thymus 75%

Functions of T Cells

- T cells mediate cell-mediated immunity (CMI) through the production of cytokines.
- T cells can directly act on and destroy virus infected cells, tumour and foreign cells (cytotoxic action).
- T cells act as regulatory cells that modulate the activity of other T cells, macrophages or B cells. Regulation can be in the form of help or suppression.

Nomenclature of T Lymphocytes

With the help of monoclonal antibodies various types of T cells have been identified which indicate presence of different physicochemical structures on the surface of these cells. These were earlier designated as T1, T2, T3 and so on. However, WHO has suggested a terminology for T cells in which abbreviation CD is to be used. CD refers to *cluster of differentiation*. At present the numbering system for CD is equivalent to T so that CD4 cells are T4 whereas CD8 cells are T8 cells.

T Cell Subsets and their Functions

The purification of T lymphocytes has revealed that there are three major functional subsets of these cells (Fig. 19.1).

- Helper T cells
- Cytotoxic T cells
- Delayed type hypersensitivity T cells

Helper T Cells (CD4 Cells)

These constitute 55-70% of all T cells and are usually designated as T_H cells. As the name indicates these cells help B cells and other T cells to multiply into large clones and carry out their role in immune response.

Cytotoxic T Cells (CD8 Cells)

These T cells are responsible for killing virus-infected cells, transplant tissue, and cancer cells by secreting a series of molecules known as *lymphotoxins* (LT).

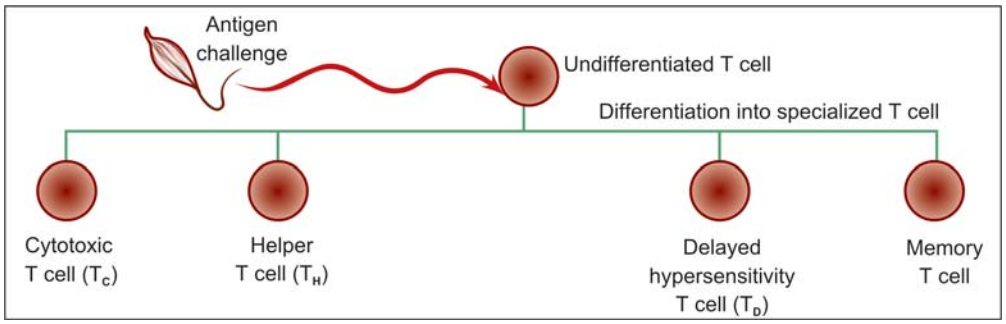


Fig. 19–1. Subsets of T cells

Delayed Type Hypersensitivity T (T_D) cells

T cells have been found to be responsible for delayed type hypersensitivity and act by releasing macrophage chemotaxin and macrophage migration inhibition factor.

Natural Killer (NK) Cells

The NK cells are large granular lymphocytes (LGL) and constitute around 3% of the peripheral lymphocytes. The salient characteristics of these cells have been shown in Table 19.2.

Table 19–2. Characteristics of LGL cells

- More granular cytoplasm than B or T cells
- Non-phagocytic
- Nonadherent to surfaces
- Destroy target cells without prior training
- IL-2 receptors present
- Activated by IL-2 and IFN

Macrophages

Macrophages circulate in blood as monocytes and get rooted to some tissues as tissue macrophages. These are not antigen specific and hence also called as *accessory cells* of immune system.

Macrophages carry out a remarkable array of functions (Fig. 19.2). They play a key role in CMI and produce various lymphokines, enzymes and factors that are involved in reorganization and repair following tissue damage.

Cytokines

Cytokines are a class of nonantibody molecules that are produced by many different cells-immune and non-immune, in a highly regulated manner. They exert different biologic effects which change the behaviour and function of many cells that are needed in immune response. Broad functions of cytokines are:

- Control of lymphocyte growth
- Activation of innate immunity mechanism including inflammation
- Control of bone marrow haematopoiesis

Cytokines is now considered as a generic term that encompasses *lymphokines* (cytokines derived from lymphocytes), *monokines* (cytokines that are derived from macrophages), interleukins and interferons.

Chemical Nature and Production

Cytokines are usually low molecular weight glycoproteins that are biochemically distinct. Cytokines can be obtained from followings:

- Lymphoid tissues
- Peripheral blood cells
- Lymphoid cell lines
- Monocyte lineage cells
- Fibroblasts
- Thymic epithelial cells
- Keratinocytes
- Endothelial cells

Target Cells for Cytokines

The antigen-independent activity of cytokines is directed mainly towards those cells which have specific receptors for these on their surfaces. These cells are:

- *Inflammatory cells:*
  - — Neutrophils
  - — Macrophages
  - — Lymphocytes

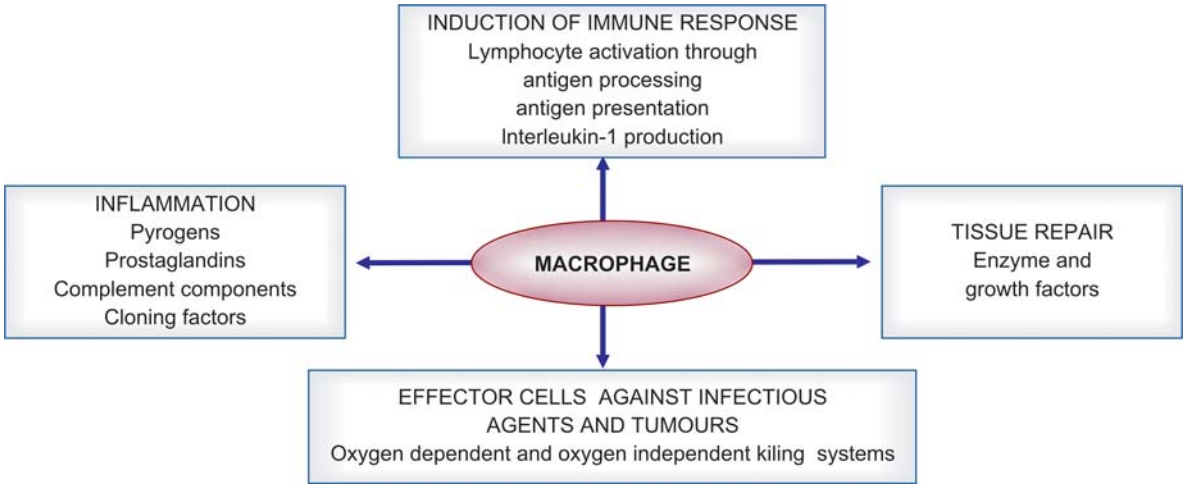


Fig. 19–2. Central role of macrophage

- *Non-inflammatory cells*
  - — Endothelial cells
  - — Osteoclasts
  - — Fibroblasts

Families of Cytokines

- Five families are recognised currently:
  - — Interleukins(IL1-IL15)
  - — Tumour necrosis factor
  - — Interferons
  - — Colony stimulating factors
  - — Others

Interferons (IFN)

Interferons are a set of proteins which are released by virus infected cells *in vivo* and which react with uninfected cells so as to render them resistant to infection with viruses. Around 20 human interferons have been characterized. Perhaps an equal number have been isolated from various animal species.

General Characteristics of Interferons

i. These are a family of glycoproteins
ii. These do not have any direct action on viruses
iii. Their activity is not virus specific. Interferon induced by one virus is effective against many other viruses
iv. These are usually species specific
v. The production of interferons is augmented at elevated temperatures such as 40°C
vi. Steroids and high oxygen tension depress the synthesis of interferons
vii. Synthesis of interferons begins within one hour of induction and takes around 12 hours to reach its maximum activity
viii. These are sensitive to the action of proteolytic enzymes but resist destruction by nucleases and lipases
ix. Interferons are resistant to heat at 56°C for upto 60 minutes
x. These are nondialysable
xi. Interferons do not get sedimented even if these are centrifuged at 100,000 g for several hours
xii. Antisera against three types of interferons are now available and with their help it has been shown that the three types are antigenically distinct.

Types of Interferons

These belong to three antigenically and chemically distinct types, known as alpha (α), beta (β) and gamma (γ) which have certain characteristic features (Table 19.3).

The gamma interferon is not induced by viral infection but is continuously produced by lymphocytes following stimulation with mitogens and hence is also one of the *lymphokines*. None of the interferons can be produced in the virus infected cell cultures.

Interferon Inducers

Most RNA viruses are good interferon inducers and with the exception of pox viruses all DNA viruses are poor interferon inducers. Other than infectious viruses followings are the interferon inducers.

1. Viruses such as influenza, Newcastle disease, reo and bovine enterovirus which have been inactivated by heat or UV rays.
2. Double stranded RNA, the replicative form of single stranded viral RNAs and the RNA present in certain mycophages, i.e. viruses of fungi.
3. Certain synthetic polycarboxylic acids and pyran copolymers as well as bacterial endotoxins. Commonly used among them is poly I:C.

Table 19–3. Characteristics of IFNs

IFN	*Alpha*	*Beta*	*Gamma*
Type	I	I	II
Cell source	T cell	Fibroblasts	T cell
Inducers	Virus ds RNA	Virus ds RNA	Antigen Mitogens
Molecular weight	17,000	17,000	15,000
Number of amino acids	188	187	166
Number of variants	13	5	Unknown
Chromosome of origin	9	9	12
Antiviral immunity	++	++	±
Immune regulation	±	±	++
Anticancer agent	+(?)	–	–

Unfortunately, none of the interferon inducers mentioned above fulfil the essential requirements for use in man, i.e. safety and effectiveness.

Mechanism of Action of Interferons

It is now believed that interferons belong to the family of 'nonclassical hormones' which cannot only inhibit the viral replication but also modulate the immune system and may act as antitumour agent.

Interferon binds to specific receptors on the plasma membrane thus inducing the production of three enzymes:

1. 2-5 A synthetase
2. RNase L (endoribonuclease)
3. Protein kinase.

By various mechanisms these three enzymes inhibit the protein synthesis in interferon treated virus infected cells.

Interferons also activate T lymphocytes, macrophages and NK cells in the immediate vicinity of virus infected cell to develop their cytotoxic potential. These effector cells not only destroy the target but on coming in contact with viral antigen on the surface of cells, get stimulated to produce more interferons. This cascade effect greatly amplifies the lytic arm of the immune response.

Biological Effects of Interferons

1. Induction of resistance to viral infections
2. Induction of resistance to some of other intracellular microbial infections such as malaria, toxoplasmosis
3. Inhibition of growth of tumour cells
4. Increased expression of MHC antigens on surfaces
5. Enhanced cytotoxic effects of NK, K and T cells.

Human interferon is now available in unlimited quantities because of its production by cloning it in bacteria. The recombinant interferon is now extensively used in therapy of chronic hepatitis due to hepatitis C virus. Local application in high doses has shown some promise in infections with herpesvirus (keratitis), and respiratory viruses as well as genital warts.

Interferons have been extensively tried in pre- and postexposure prophylaxis against rhinoviruses causing common cold. Till date spray of interferon in nose in established cases with common cold has been found to be of no use. Pre-exposure administration of interferon has been of great use but is associated with certain side effects especially coryza.

20 The Complement System

Complement (written as c′) is one of the protective cascading systems in blood.

A cascading system comprises of a large number of components or subsystems which activate each other in a sequential manner to produce a specified action. Four cascade systems of molecules are present in the plasma, waiting to respond to tissue damage by various agents including microorganisms as well as to induce inflammation. These are clotting, kinin, fibrinolytic and complement systems. Of these complement system is most important in relation to infections. It is also the most complex comprising of about 25 proteins. It can be activated in two different ways which are called as *classical pathway* and *alternate pathway of* complement activation. The activated complement cascade culminates in three useful results—phagocytosis, lysis and inflammation.

The name complement was given almost a century back on account of its property of complementing the action of antibody in disposing off bacteria.

General Properties

Some of the general properties of the complement distinguish it from immunoglobulins and other serum proteins are depicted in Table 20.1.

Complement binding or complement fixation refers to the union of complement or one of its fractions with a substance, usually an Ig or antigen. This results into a new biologic activity and change in complement is called as *complement activation.*

Classical Activation Pathway (Fig. 20.1)

As mentioned in the general properties, there are 9 components of protein complex in complement activation by classical pathway-C1 to C9. The C1 molecule is activated by certain antigen-antibody reactions and then a cascading effect results into activation of other components of complement.

Table 20–1. General properties of complement

- Present in all normal sera
- Does not increase on immunization
- Non-specific serologic reagent
- Destroyed at 56°C in 30 mts.
- Not a single substance —complex
- In classical pathway; complex is of 9 proteins
- In alternate pathway; complex is of 13 proteins
- IgM, IgG1, IgG2, IgG3 react with complement
- Activation by antigen-antibody complex
- Alternate pathway activated by polysaccharide/enzymes
- Produces cytolytic destruction by specific antibody
- Inactivators and inhibitors present in serum.

The nine components (units) of complement can be grouped into three:

- Recognition Unit: C1 (C1q, C1r, C1s)
- Activation Complex: C4, C2, C3
- Membrane Attack Complex: C5,C6, C7, C8, C9

Recognition Unit: C1

The first component of the complement cascade is a true macromolecule with a molecular weight of approximately 750,000. C1 dissociates into three subunits—C1q, C1r, C1s.For C1q to initiate the complement cascade it must attach to two immunoglobulin molecules. Since these are adjacent in IgM, IgM is described as a better complement binding antibody than IgG. IgA, IgD and IgE do not bind C1q and hence cannot catalyze the complement cascade.

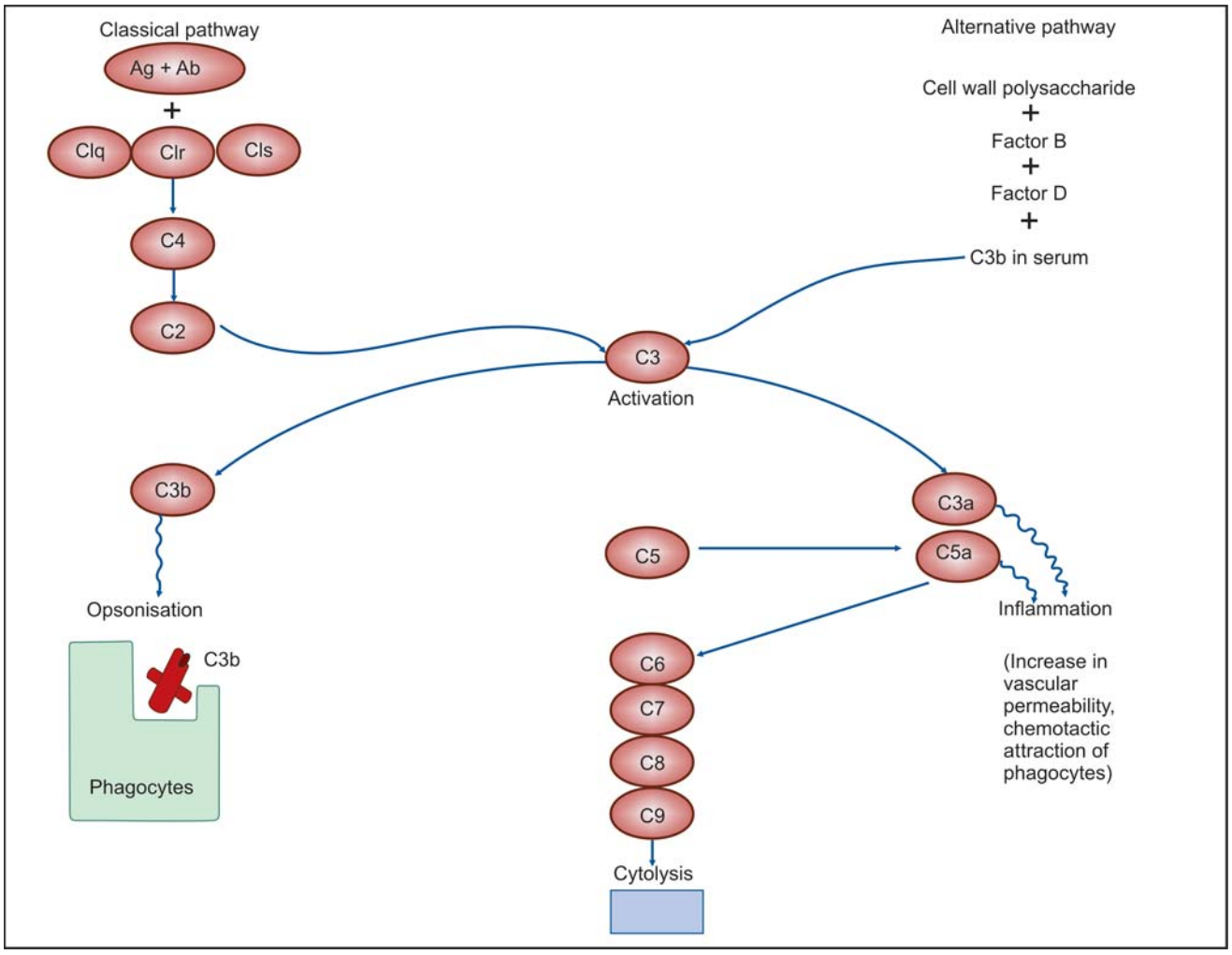


Fig. 20–1. Classical and alternate pathways of complement activation

Activation Unit: C4

After the activation of C1q, C1r and C1s get activated and C1s activates C4 which gets split into C4a and C4b. The latter attaches to erythrocyte surfaces, bacterial cell membranes, and other antigens. It does not normally attach to C1.

C4a molecule has now been recognised as an anaphylatoxin. Anaphylatoxins bind to mast cells and basophils and cause them to discharge their cytoplasmic granules. When freed from these cells, the contents of these granules contract smooth muscles, causing oedema and shortness of breath.

Activation Unit: C2

The alteration of C2 during the complement sequence shows that it binds to C4b and is cleaved by C1s into C2a and C2b. C2a functions with C4b to activate C3. This is also called as C3 convertase.

Activation Unit: C3

C3 is also known as 3rd component and is the heart of the complement cascade. It is a major serum protein whose enzymatic splitting or *activation* has dramatic effects. The substrate for C4b2a is C3 which on getting split produces C3a and C3b. If it occurs on the surface of the bacterial cell, most of the C3b is left there. Since phagocytes have receptors for C3b, bacterium cells with C3b attached to it get marked for phagocytosis. In such a case, the bacterium is said to be opsonized. The new complex C4b2a3b is also known as C5 convertase, which, activates C5 to ultimately bring about lysis of bacterium.

Table 20–2. Activators of the human blood complement system

Activator	*Classical pathway*	*Alternative pathway*
Immunologic activators	IgM > IgG1, IgG3 > IgG2	IgA, IgE and some IgG subclasses
Non-immunologic activators	Trypsin like proteases (plasmin,streptokinase, and lysosomal proteases)	Trypsin like proteases (Plasmin, streptokinase, etc)
	Deoxyribonucleic acid polyinosinic acid	Lipopolysaccharides, damaged mammalian cell walls
	Staphylococcal protein A	Plant or bacterial polysaccharides
	C-reactive protein, polyanion/polycation complexes	Cobra venom factor

Membrane Attack Complex : C5

C5 is derived from a precursor molecule called pro C5 from macrophages. The C5 convertase (C4b 2a 3b) splits C5 into C5a and C5b. C5b serves as the receptor for C6 and C7 and is the first element of membrane attack complex.

Membrane Attack Complex : C6 and C7 to C9

C5b attaches to membrane of target cells alongwith C6, C7, C8 and C9 to bring about lysis of cells.

Activators of Complement

The activators of classical as well as alternate pathway of complement have been shown in Table 20.2.

Alternate Pathway for C Activation (Fig. 20.1)

The initiating factor for alternate pathway has not yet been clearly identified but is probably the combination of one of the activators with C3b which is obtained from endogenous production from classic pathway. The actual amount of C3b needed for alternate pathway is trivial.

Proteins in Alternate Pathway

The proteins which play important role in alternate pathway of complement include factor B, factor D and properdin (P) as well as C3 convertase.

Factor B. Factor B, the C3 proactivator, is a normal serum protein. The enzyme nature of factor B is not expressed until it is bound to C3b and acted on by a protease. This protease is factor D.

Factor D. It is also called as C3 proactivator convertase since it enzymatically converts C3 proactivator (factor B) and generates two fragments—Ba and Bb. Ba is released but Bb remains bound to C3b. The complex of C3b, Bb now displays the typical features of a serine easterase and is able to hydrolyze C3. It hydrolyses C3 at the same peptide bond which is cleaved by C4b, 2a enzyme of the classic pathway. However, the C3 convertase in alternate pathway is labile and decays rapidly. It is stabilized by the addition of properdin.

Properdin. Properdin is composed of four peptide subunits, each with a molecular weight of 46000 held together by hydrogen and ionic bonds.

With the generation of stable C3 and C5 convertases, the remainder of the pathway from C6 to C9 is ensured. Since the alternate pathway usually occurs in solution rather than on cell surface, cell lysis is not thought of as a part of this system. Only when the neighbouring cell density is high will the C5-C9 complex associate with cells in so called bystander lysis.

Immunologic and Biologic Activities of C'

The vast number of proteins involved in the complement system and the complexities of their interaction, plus the fact that two separate mechanisms have evolved for the activation of the complement system, almost dictate that several important biological activities would emerge from this system. Table 20.3 shows some of these activities.

Although most of these activities may be known yet a rather large number of these remain unknown. The activities arising from C1, C4 and C2 are expressed only when complement is activated through the classic pathway; those arising from C3 through C9 are expressed after either the classic or alternate pathway activation.

Table 20–3. Biological activities of complement components

Complement		*Activity*
C2b	(Fluid phase)	Kinin like, increased vasodilatation
C4a		Anaphylatoxin-histamine release
C3a		Anaphylatoxin
C5a		Anaphylatoxin-strong chemotactic factor
C4b	(membrane)	Immune adherence, opsonization
C3b	(membrane)	Immune adherence, opsonization
C567		Weak chemotactic factor
C5b-C9	(polymer)	Membrane disruption
C4,C2		Virus neutralisation

Immunoconglutinins

Immunoconglutinins are the antibodies that display a specificity towards antigenic determinants that are exposed by fixed complement but which are unavailable in free complement. These are autoantibodies since these are produced by an animal against its own complement.

These antibodies are produced during activation of complement, i.e. in infections and after immunization. In the course of complement fixation, new antigenic determinants are exposed. These new sites appear to be in C3b, although some experiments have suggested that they appear in C4b.

Conglutinin is a beta globulin with a molecular weight of 750000. It is activated by conglutinin activating factor (KAF) and combines with haemolytically inactive C3b to cause haemagglutination of erythrocytes that have combined with non-agglutinating quantities of antibody. This conglutinin-complement fixation test is a sensitive indicator of a serologic reaction.

Regulators of Complement Mediated Functions

Because of large number of biological activities associated with complement, some regulatory mechanisms have to be in existence to modulate these functions which would otherwise continue until exhaustion of complement occurs. These can be grouped into two.

1. **Inactivators** which are mostly enzymes that destroy the primary structure of complement protein. These include factor I, factor H, Ana INH, C4 binding protein and C6 INAC.
2. **Inhibitors** which combine with complement molecule to halt its further function. These include C1 sINH and S protein.

Complement Receptors

Four types of complement receptors have been identified so far. These are:

- Complement receptor type 1 (CR1)
- CR2
- CR3
- Miscellaneous receptors for C proteins.

CR1

These are found on a variety of cells including erythrocytes, neutrophils, monocytes, macrophages, B cells and T cells. Important roles assigned to the receptors are phagocytosis, clearance of immune complexes, converting B cells into antibody secretors and may serve in soluble form as a cofactor for Factor 1.

CR2

It binds to C3b, C3d and C3d-g. Apart from acting as receptor for E-B virus, no function of CR2 has been ascertained.

CR3

Like CR1, it has a wide cell distribution that includes macrophages, LGL, neutrophils and erythrocytes.

Physiological roles recognized for complement receptors are given in Table 20.4.

Table 20–4. Physiological roles of C' receptors

- Facilitates phagocytosis
- Moderation of immune response
- Receptors for viruses
- Surface markers

Other physiological roles may be recognized in the future.

Complement Fixation Test (CFT)

The complement fixation test (CFT) is an important immunological test which is based upon two properties of complement viz.

- C′ combines with all Ag-Ab complexes whether or not it is required for that reaction
- C′ is needed in immunolytic reactions.

The details of this test have been provided in Chapter on Antigen—Antibody Reactions.

Source of Complement

The main source for the complement proteins are the hepatocytes, epithelial cells of the gut, blood monocytes and tissue macrophages. More than 90% of plasma C3, C6, C8 and C9 is synthesised in liver. Table 20.5 describes the sources of various components.

Complement deficiencies have been described for most of the components and regulatory proteins.

Deficiency of Complement

Complement activity can be impaired by the absence of one or more of its protein components. Impaired complement activity causes various diseases (Table 20.6).

The complement deficiencies can be *congenital* or *acquired.* Acquired diseases result from temporary depletion of a complement protein; which subside when cells again become able to synthesise that protein. Congenital complement deficiencies are due to perma-

Table 20–5. Sources of complement components

Component	*Source*
C1q	Macrophages
C1r	Macrophages
C1s	Blood monocytes, tissue macrophages and epithelial cells
C2	Macrophages
C3	Hepatocytes
C4	Macrophages
C5	Macrophages
C6	Hepatocytes
C7	Hepatocytes
C8	Cells in spleen
C9	Hepatocytes
Factor B	Macrophages, lymphocytes and hepatocytes
Factor D	?

nent genetic defects that prevent synthesis of one or more complement components.

The most significant effect of complement deficiencies is lack of resistance to infection. Although deficiencies in several complement components have been observed, the greatest degree of impaired complement function occurs with a deficiency of C3. This situation is not surprising because C3 is the key component in the system. In patients with C3 deficiencies, chemotaxis, opsonization and cell lysis are impaired.

Table 20–6. Disease states related to complement deficiency

Disease state	*Complement deficiency*
Severe recurrent infections	C3
Recurrent infections of lesser severity	C1,C2, C5
Systemic lupus erythematosus	C1,C2,C4,C5,C8
Glomerulonephritis	C1,C8
Gonococcal infections	C6,C8
Meningococcal infections	C6

21 Antigen-Antibody Reactions

If molecules of antigen and antibody (which is specific for the antigen) are brought together in solution, linkage is formed between Fab portion of immunoglobulin molecule and antigenic determinants of antigen molecule. This interaction forms the basis of the humoral response of the immune system to antigenic stimulation. These reactions can be visualised by allowing the antigen and antibody to combine under controlled *in vitro* conditions. These conditions shall indicate qualitative or quantitative assay of constituents but may not reflect the precise *in vivo* response to same antigen. Nevertheless, some of these reactions, e.g. neutralisation of toxin or virus do express the biological role of antibody.

Antigen-Antibody Binding

Once a linkage is established between antigen and antibody molecule, many other forces come into play which provide strength to the antigen antibody complex. The better the fit, the closer the contact and stronger the antigen antibody bond. Some of these intermolecular forces are:

Van der Waal's Bonds

These arise from close juxtaposition of two molecules or atoms, the force rising with close proximity. The complementary spatial relation between antigen and antibody molecules contributes to the exertion of such forces and increases the tendency of the two molecules to bind together.

Hydrostatic Bonds (Columbic Bonds)

These occur between two positively charged groups, e.g. amino and carboxyl groups when these are in ionised forms. In this case the force varies with the square of the distance.

Hydrogen Bonding

Whenever a covalently bond hydrogen atom, which has a positive charge, comes adjacent to a negatively charged atom, a hydrogen bond develops between them. The force is more when hydrogen atom is nearer to negatively charged atom.

Hydrophobic Properties

Antibody molecules have surface groups which attract water (hydrophilic). When antigen combines with antibody, these surface groups become lesser accessible to water and hydrophobic bonding increases as two protein molecules become closely opposed.

Effect of Electrolytes

Formation of visible aggregates of antigen-antibody (agglutination) is dependent upon the presence of electrolytes in the solution. In the absence of electrolytes antigen-antibody binding may occur but formation of visible aggregates shall require adequate concentration of electrolytes in solution.

ANTIGEN-ANTIBODY REACTIONS

The principal types of antigen-antibody reactions and their various modifications have been shown in Table 21.1. Characteristics which are common to various antigen-antibody reactions are shown in Table 21.2.

Few important definitions which shall help in better understanding of antigen-antibody reactions are given here:

Table 21–1. Antigen-antibody reactions

Reaction/test	*Modified test*
Precipitation	Immunoelectrophoresis
	Immunoprecipitation
Agglutination	Latex agglutination
	Indirect haemagglutination
	Coagglutination, Coombs test
Complement fixation	Conglutination
Neutralization	Plaque assays
	Measurement of LD
Immunofluorescence	Indirect immunofluorescence
	Immunofluorimetric assay
Enzyme immunoassay	Enzyme linked immunosorbent assay
Radioimmunoassay	Immunoradiometric assay

Table 21–2. Characteristics of antigen-antibody reactions

- Specific cross reaction may also occur
- Antigen-antibody combination is firm but reversible
- No denaturation of antigen or antibody during reaction
- Binding takes place at surface
- Entire molecules react.

Affinity. Affinity of the antigen-antibody reaction refers to the intensity of the attraction between antigen and antibody molecules.

Avidity. Avidity is the strength of the bond after the formation of antigen-antibody complex.

Sensitivity. Sensitivity refers to the ability of the test to detect even very minute quantities of antigen or antibody. A test shall be called as highly sensitive if false negative results are absent or minimal.

Specificity. Specificity refers to the ability of the test to detect reaction between homologous antigen and antibodies only, and with no other. In a highly specific test, false positive reactions will be minimal or absent.

Measurement of Antigen and Antibody

Various methods that are currently available for the detection and measurement of antigen and antibody have been depicted in Table 21.1. Measurement is usually in terms of *units* or *titre*. The antibody titre of a serum is the highest dilution of the serum which gives an observable reaction with the antigen in a particular test. Similarly value of an antigen in terms of its titre can be detected against a known serum.

PRECIPITATION

This reaction takes place only when *antigen is in soluble form.* Such an antigen when comes in contact with specific antibody in a suitable medium results in formation of an insoluble complex which precipitates. This precipitate usually settles down at the bottom of the container. If it fails to sediment and remain suspended as floccules, the reaction is known as flocculation. Precipitation can take place not only in liquid media but also in system with gels. By electrically moving the antigen and the antibody, the process of precipitation can be hastened. Precipitation also depends upon the optimal concentration of NaCl, suitable temperature and appropriate pH.

Zone Phenomenon

Precipitation occurs most rapidly and abundantly when antigen and antibody are in optimal proportions or equivalent ratio. These proportions are constant for all dilutions of the same reagent. The aforesaid principle can be illustrated with the help of following experiment:

Set up a series of tubes each containing a constant amount of antibody. Add decreasing amounts of antigen to the tubes in the row. Observe the precipitation which would commence in the form of haziness (Fig. 21.1).

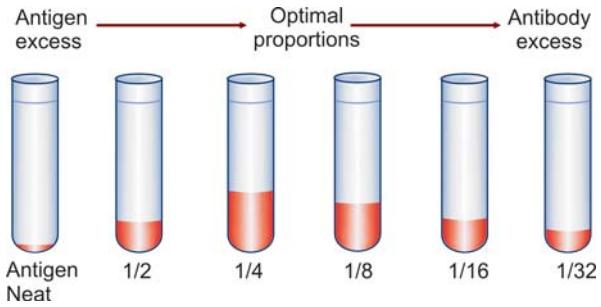


Fig. 21–1. Precipitation reaction

The haziness starts to appear in the tubes gradually increasing to visible aggregates or precipitates. It is observed that precipitation increases along the row, reaches to a maximum where antigen and antibody meet at optimal proportion and then tapers off with the lower antigen concentration.

When the amount of precipitates in the different tubes are plotted, resulting curve will show three phases (Fig. 21.2).

Zone of antibody excess. In this zone uncombined antibody shall be present. This is called zone of antibody excess or *prozone.* The prozone is of importance in clinical serology since a serum sample having large amount of antibody may give a false negative precipitation result until several dilutions are tested.

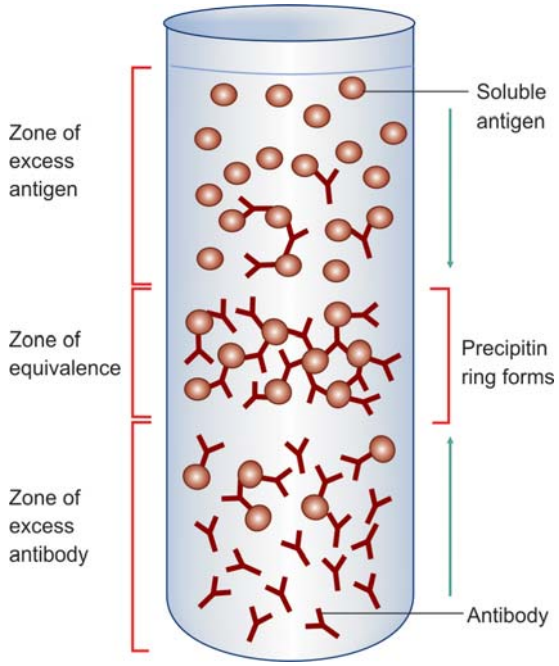


Fig. 21–2. Zone phenomenon

Zone of equivalence. In this zone both antigen and antibody are completely precipitated and no uncombined antigen or antibody is present. Maximum complement fixation also takes place in this zone.

Zone of antigen excess. In this zone all antibody has combined with antigen and additional uncombined antigen is present. In this zone, precipitation is partially or completely inhibited because soluble antigen-antibody complexes form in the presence of excess antigen.

Mechanism of Precipitation

A lattice hypothesis in support of mechanism of precipitation was put forth by Marrack in 1934. Large aggregates of antibody and antigen form best when these meet in optimal proportions. Under such conditions, after initial combination of the molecules, free antigen binding sites and antigen determinant groups remain. These link up and help in forming lattices of antigen-antibody complexes (Figs 21.2 and 21.3).

In antibody excess all the free determinants of the antigen molecule are soon taken up with antibody and hence little linking takes place between the complexes (Fig. 21.3). Similarly in situations where antigen is in excess, less precipitation appears because of the inability of the antigen-antibody complexes to link up to other complexes and produce a large aggregate or lattice.

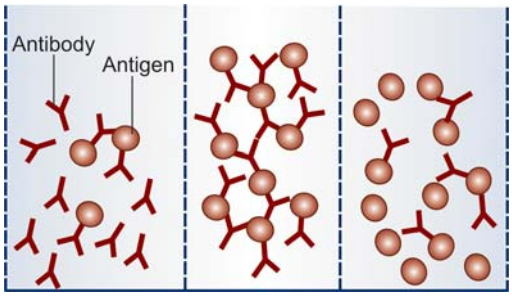


Fig. 21–3. Lattice formation

The phenomenon of lattice formation holds true for agglutination also.

Applications of Precipitation Reactions

Both qualitative determination as well as quantitative estimation of antigen as well as antibody can be performed with precipitation tests. The qualitative tests are much more widely used for detecting and identifying antigens. Detection of antigen with this technique has been found to be more sensitive than estimation of antibody. The precipitation tests are capable of detecting as little antigen protein as 1µg.

Some of the types of precipitation and flocculation tests which have found applications in diagnostic bacteriology are as under:

Ring test. This is performed by layering an extract of the organism over antiserum in tubes. After a short while, a ring of precipitation forms at interface (Fig. 21.4). Typing of streptococci and pneumococci, C-

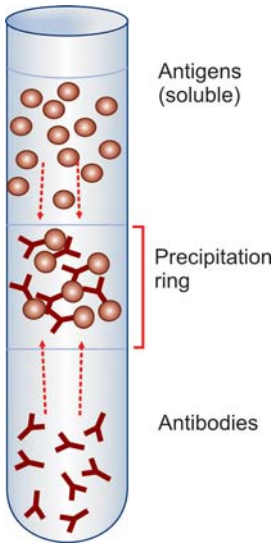


Fig. 21–4. Ring test

reactive protein test and detecting adulteration of foodstuffs are some of the applications of ring test. This test also finds applications in forensic sciences where it is employed for identification of blood and seminal stains.

Slide test. This is an example of flocculation which occurs when a drop of antigen and antibody are mixed and shaken for sometime. The reaction appears in the form of floccules. VDRL—one of the widely performed test for diagnosis of syphilis is an example.

Tube flocculation test. Flocculation test can be done in tubes also. Kahn test for syphilis is an example of this technique which is also used for standardization of toxins and toxoids.

Immunodiffusion. This term denotes precipitation in gel which provides more sensitive and specific results. The reaction is in the form of bands of precipitation and can be stained for better viewing as well as preservation. If a large number of antigens are present, each antigen-antibody reaction will give rise to a line of precipitation. This technique also indicates *identity, cross reaction* and *nonidentity* between different antigens.

Various types of immunodiffusion tests are in practice. These are based upon number of diffusions and dimensions in which these take place. Following four combinations can result:

Single diffusion in one dimension. This is also known as *Oudin procedure*. Agar gel having antibody is taken in a tube and antigen solution is layered over it. With the migration of antigen in gel with antibody, line of precipitation appears at the point where these meet in optimal concentration. A large number of bands may appear if different antigens are present (Fig. 21.5).

Double diffusion in one dimension. This is also called as *Oakley-Fulthorpe procedure*. After putting some agar gel incorporated with antibody in tube, a column of plain agar is added. On top of this, the antigen is layered as in Oudin procedure. Antigen moves downwards in column of plain agar and antibody also migrates into

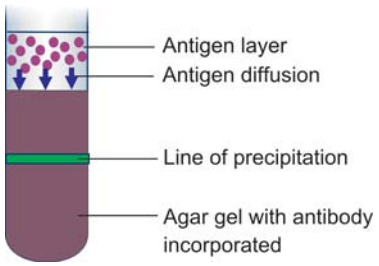


Fig. 21–5. Single diffusion in one dimension

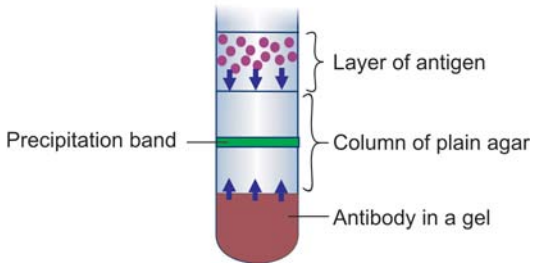


Fig. 21–6. Double diffusion in one dimension

same column. Precipitation band appears when these meet in equivalent ratio (Fig. 21.6).

Single diffusion in two dimensions. This procedure is also called as *radial immunodiffusion.* This is done in petridishes or slides having layer of agar in which antibody is incorporated. Wells are cut in layer of agar and antigen is added to these wells. The antigen diffuses and concentric bands of precipitation can be seen around the well. The diameter of the ring of precipitation is used to estimate the concentration of the antigen (Fig. 21.7).

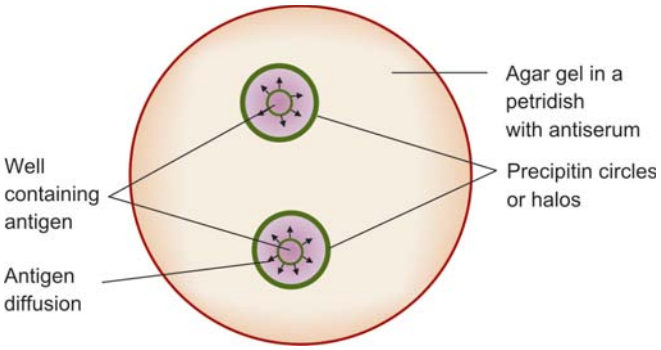


Fig. 21–7. Single diffusion in two dimensions

Double diffusion in two dimensions. This technique is better known as *Ouchterlony procedure* and is the most widely used technique which helps to compare different antigens and antisera directly. Wells are cut in a layer of agar in petri dish. Antiserum and antigen solutions are placed opposite each other in the wells (Fig. 21.8) and after allowing a few days for diffusion to take place precipitation bands will form where antigen and antibody meet in suitable proportion.

As shown in Figure 21.8 no reaction takes place with antigen C and D as the antiserum in the central well contains antibody only against antigens A and B. *Lines of identity* formed between the two A wells enable the technique to be used for identifying unknown antigen *(reaction of identity).* When the adjacent antigens

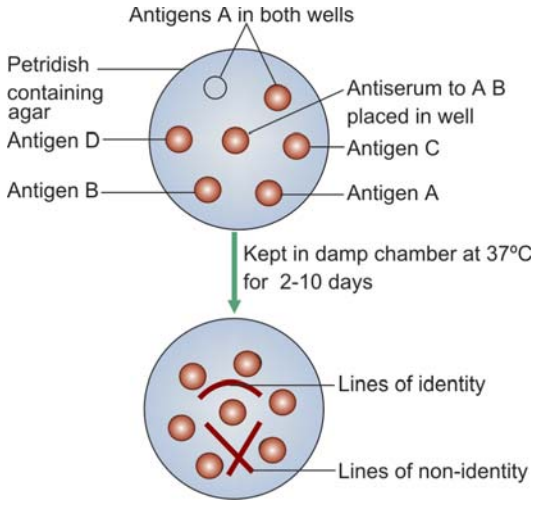


Fig. 21–8. Double diffusion in two dimensions

are unrelated the lines will cross each other *(reaction of nonidentity)*. Cross reaction or partial identity is indicated by spur formation *(reaction of partial identity)*. A special variety of this test is Elek test for toxigenicity of diphtheria bacilli.

Immunoelectrophoresis. The use of electrophoresis in conjunction with immunodiffusion has greatly increased the utility. The antigen is first subjected to electrophoresis in agar. After electrophoresis, a trough is cut longitudinally in the agar and an antiserum against the electrophoresed antigen is placed in the trough. The two components diffuse towards each other and precipitation bands form. For better visibility and preservation these bands can be stained. Steps of immunoelectrophoresis are given in Figure 21.9.

Electroimmunodiffusion is basically of two types:

Cross immunoelectrophoresis. This is one dimensional single electroimmunodiffusion which is also called as *Rocket electrophoresis* because of the shape of the bands of precipitation. In this procedure the proteins in the antigen are first separated by agarose gel electrophoresis after which they are electrophoresed at right angles to the original direction into an antibody containing agarose gel. The precipitate that forms shows up as sharp peaks. The height of the peak is determined by the concentration and mobility of the protein. This method also shows up heterogeneity of antigens or identity of various components by fusion or overlapping patterns (Fig. 21.10).

Counter current immunoelectrophoresis (CIE). In this procedure the antiserum is placed in one well and the antigen under test in another. At pH 8.2, the antibody

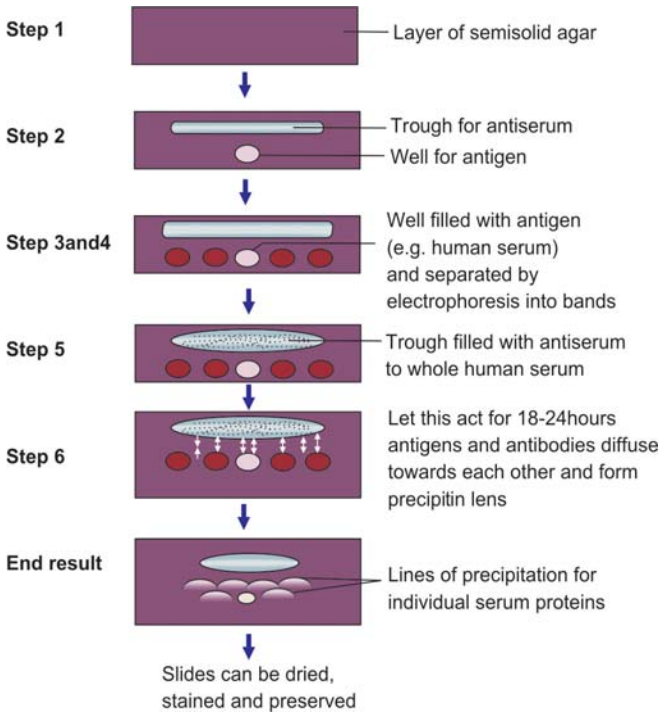


Fig. 21–9. Steps of immunoelectrophoresis

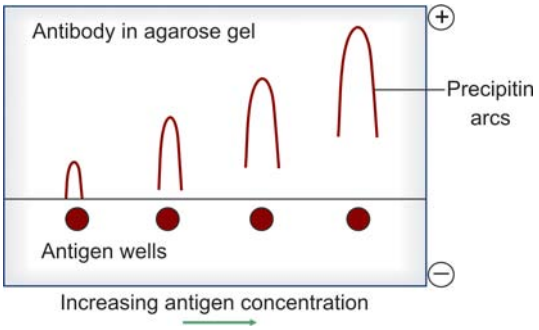


Fig. 21–10. Crossed immunoelectrophoresis

moves towards the cathode because of electroendosmotic flow and if well containing the antigen is placed on the cathode side of antibody well, the antigen which normally would move towards the anode will meet the antibody somewhere in between the two wells and precipitation of antigen-antibody complex shall take place (Fig. 21.11). The procedure is carried out under same conditions as for immunoelectrophoresis. The advantage of this technique over simple immunodiffusion includes rapidity by which results can be obtained (less than one hour) and requirement of lesser quantity of antigen and antibody and atleast 10 times more sensitive than simple immunodiffusion. CIE has

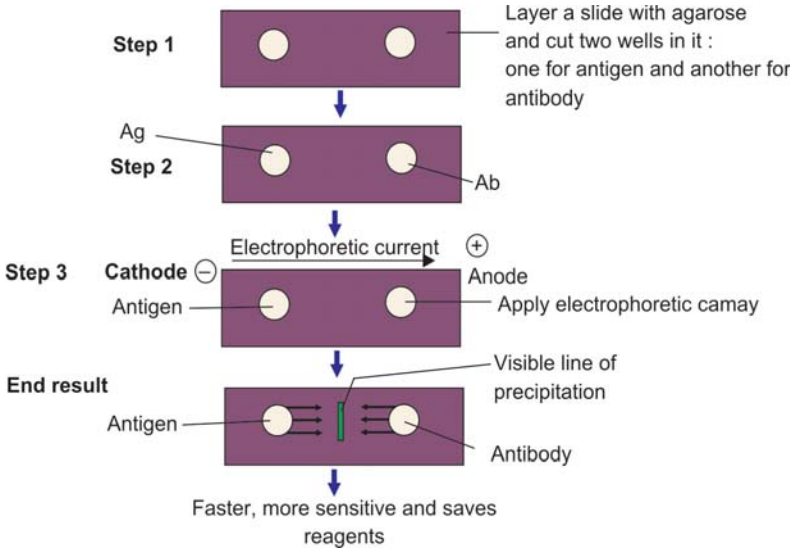


Fig. 21–11. Steps in counter current immunoelectrophoresis

been used for detection of HBsAg, alpha fetoproteins in serum and specific bacterial and cryptococcus antigen in cerebrospinal fluid in meningitis.

AGGLUTINATION

Unlike precipitation, in agglutination reaction the antigen is a part of the surface of some particulate material such as erythrocyte, bacterium or an inorganic particle e.g. polystyrene latex which has been coated with antigen. Antibody added to a suspension of such particles combines with the surface antigen and links them together to form clearly visible aggregate which is called as agglutination.

Principle

Principle of lattice formation which explains precipitation is valid for agglutination also. Agglutination is mainly, but not always used for the detection of antibodies for which it is more sensitive.

Factors Influencing Agglutination Reaction

The agglutination reaction is aided by elevated temperature and by movements (e.g. shaking, stirring, centrifugation etc.) which increase the contact between antigen and antibody. The aggregation of clump requires the presence of salts. Incomplete or monovalent antibodies do not cause agglutination, though they combine with the antigen. These may act as blocking antibodies, inhibiting agglutination by the complete antibody added subsequently.

Applications of Agglutination Reaction

Tube agglutination. This is done in round-bottomed test tubes or *perspex* plates with round-bottomed wells and series of doubling dilutions of antiserum is made up in the tubes. After adding particulate antigen, the mixture is incubated. The last tube (or the well) showing clearly visible agglutination is the endpoint of the test and dilution of antiserum at endpoint is called as its *titre*. It is a measure of the number of antibody units per unit volume of serum. In this type of tests care should be taken to avoid prozone phenomenon.

Tube agglutination test is routinely employed for:

- The serological diagnosis of typhoid,
- Brucellosis and typhus fever,
- Weil Felix test is performed for typhus fever and
- Widal test for serological diagnosis of typhoid.

The tube agglutination test for brucellosis may be complicated by prozone phenomenon and presence of blocking antibodies. Hence, several dilutions of serum should be tested to avoid false negative results due to prozone. Incomplete or blocking antibodies can be detected by performing the test in hypertonic saline or by *Coomb's antiglobulin test*.

Coomb's test was devised in 1945 by Coomb's, Mourant and Race for the detection of anti-Rh antibodies that do not agglutinate Rh-positive erythrocytes in saline. On mixing, sera having incomplete anti-Rh antibody with Rh-positive erythrocytes no agglutination takes place since surfaces of red blood cells are covered by antibody globulin. To have agglutination wash these

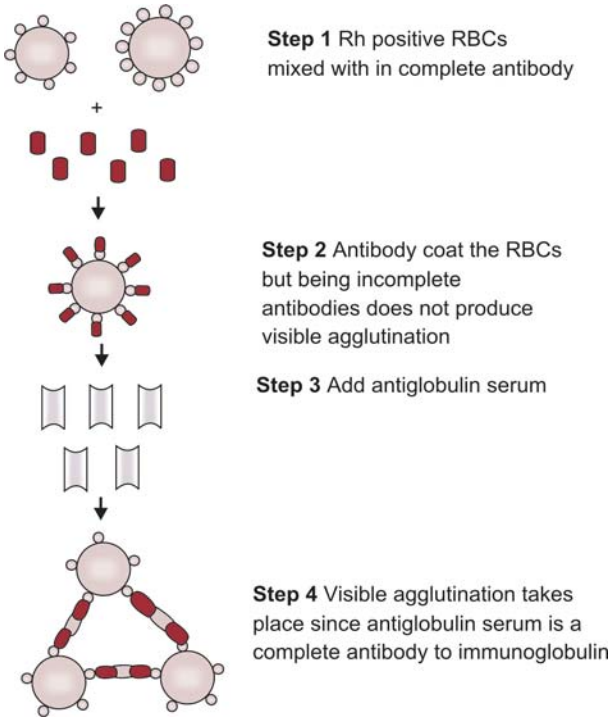


Fig. 21–12. Coomb's (Antiglobulin) test

erythrocytes to get rid of unattached proteins and then mix with rabbit antiserum (Coombs serum) against human gamma globulins (Fig. 21.12). Coomb's test is of direct and indirect type. In the direct Coomb's test, the sensitisation of the erythrocytes with incomplete antibodies takes place *in vivo* as in the haemolytic disease of the newborn due to Rh-incompatibility. In the indirect test, sensitisation of red cells with the antibody globulin is performed *in vitro*.

In vivo sensitisation of red blood cells occurs with incomplete antibodies. This occurs in erythroblastosis foetalis. When these cells are washed to remove unattached proteins and Coomb's serum is added, agglutination occurs. In the indirect Coombs test, sensitization of red blood cells with the antibody globulins is performed *in vitro*. Coomb's test is also used for demonstrating incomplete antibodies in other diseases, for example, in brucellosis.

Slide agglutination. This method is useful where only small quantities of culture are available; where agglutination is carried out with undiluted serum and this is applicable only when agglutination occurs within a minute or so. Some of the important examples of application of this technique are:

- Identification of *Bordetella pertussis*,
- Typing of pneumococci and streptococci
- Confirmatory diagnosis of organisms of *Salmonella* and *Shigella*.
- This method is also used for blood grouping and cross-matching.

The test can be carried out readily on a glass slide. To a drop of saline is added a small amount of culture and emulsified in it. With a platinum loop, a drop of serum is placed on the slide just beside the bacterial suspension. Both are mixed and examined. Agglutination when occurs is rapid and can be seen with the naked eye, but the use of some form of magnification is an advantage. Suitable controls must be incorporated on the slide.

While the slide agglutination test is rapid and convenient, it carries limitations of occasional nonspecificity especially when high titre serum is used in undiluted form. Any ambiguity in slide agglutination should be cross checked by tube agglutination.

Agglutination absorption tests. When a serum containing antibody against a specific organism is mixed with homologous organism and mixture is centrifuged, it is seen that the antibody is 'absorbed' or removed by the organisms from the serum. This absorption is of use while raising antisera in animals. On immunizing an animal with a particular bacterium, 'group antibodies' for allied organisms are developed. In some cases these may be present in high titre. Absorption with a heterologous strain would remove only the group specific antibodies without affecting specific antibodies. These effects are examplified in the *Salmonella* and *Brucella* groups.

Coagglutination. This method was introduced in 1973 by Kronvall for serological typing of pneumococci. The test is based upon the presence of protein A on the surface of some strains of *Staphylococcus aureus* (especially Cowan I) which can bind nonspecifically any IgG molecule through its Fc portion. Thus Fab portion remains free to bind to specific antigen. Whenever Fab combines with specific antigen, the reaction becomes visible because of clumping of *Staph.aureus* (Fig. 21.13).

The coagglutination reaction is performed as a simple slide test with suspension of presumptive homologous antigen prepared from a bacterial culture or, alternatively, the test can be used directly for detecting:

- Presence of bacterial antigens in serum and urine.
- Commercial reagents are now available for the identification of *Neisseria gonorrhoeae* serogrouping of *Staphylococcus aureus*.
- Similarly reagents are available for detecting meningococcal, pneumococcal and haemophilus

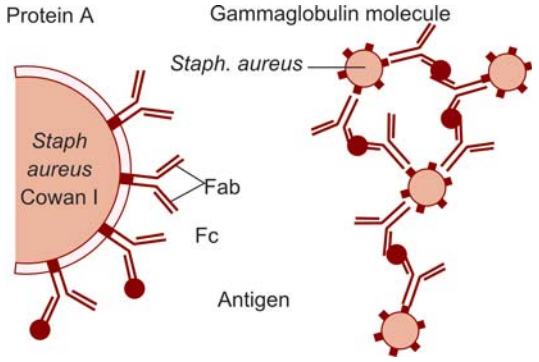


Fig. 21–13. Coagglutination

antigens in CSF. But CSF has to be absorbed with stabilized staphylococci to prevent nonspecific agglutination with human IgG.

Passive agglutination. A precipitation can be converted into agglutination reaction by coating soluble antigen onto surface of carrier particles such as polystyrene, latex or erythrocytes. Such tests are more convenient and more sensitive for detection of antibodies.

- Latex polystyrene beads coated with denatured human IgG were first used to detect rheumatoid factor in serum.
- Latex particles coated with globulin from antisera to meningococci, *Haemophilus influenzae* type b or from pneumococcal omni-serum can be used to detect the corresponding antigen in cases of pyogenic meningitis.
- Most widely used passive agglutination tests employing erythrocytes are *Treponema pallidum* haemagglutination (TPHA) test for serological diagnosis of treponemal infection and
- Rose-Waaler test to detect rheumatoid factor.
- *Latex agglutination test*s are widely used for detection of hepatitis B, ASO, CRP, rheumatoid factor, hCG and many other antigens.
- This type of test can be used for the detection of antigen also by coating carrier particles with antibody. This technique is known as *reverse passive agglutination.*

COMPLEMENT FIXATION TEST

Complement is normal component of the serum and is activated when it couples with antigen-antibody complex. This coupling does not have any visible effect. Hence, it is necessary to use an indicator system consisting of sheep red blood cells coated with anti-sheep red cell antibody. Complement has the property of lysing antibody coated cells.

In a test the antibody, complement and antigen are mixed together and after a period of incubation the indicator system-antibody coated sheep cells, is added. Complement would, however, have been taken up during the incubation stage by original antigen-antibody complex and will not be available to lyse the red cells. Thus, a positive complement fixation test is indicated by the absence of lysis of the red cells while a negative test, with unused complement, is shown by lysis of red cells (Fig. 21.14).

The antigen in this test may be soluble or particulate. Prior to commencement of test the serum should be inactivated by heating it at 56°C for 30 minutes to destroy any complement activity the serum may have and also to remove some nonspecific inhibitors of complement. The best source of complement for laboratory use is guinea pig serum.

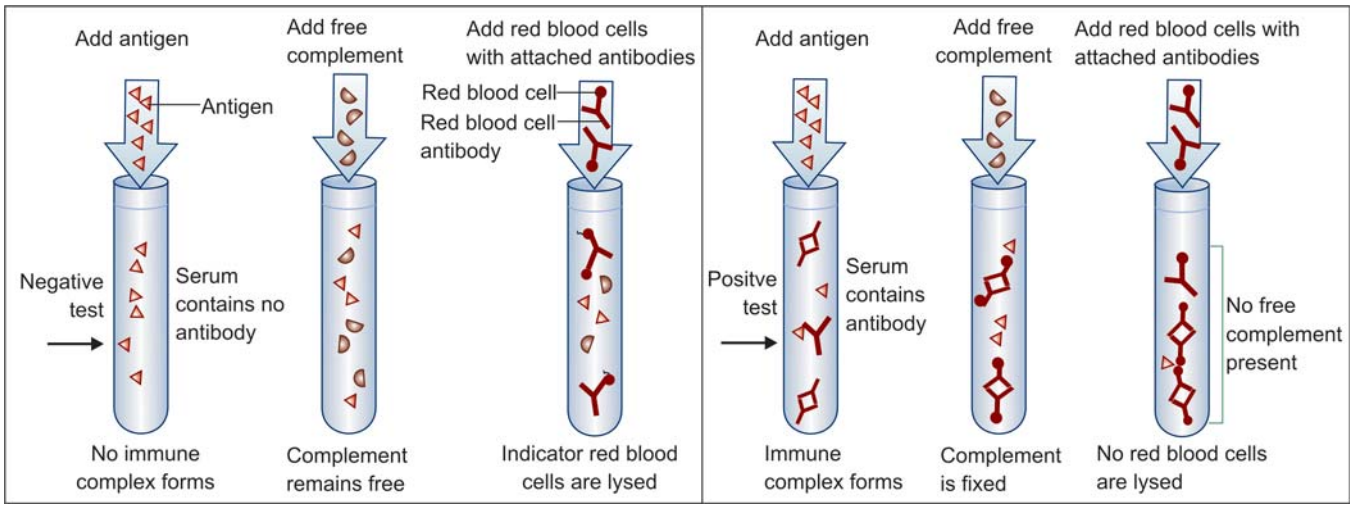


Fig. 21–14. Complement fixation test

The classical example of complement fixation test is the Wassermann reaction which was used for serodiagnosis of syphilis. Some of the variations of complement dependent tests are:

Indirect Complement Fixation Test

Guinea pig complement is not fixed by some animal and bird sera. While examining such sera indirect complement fixation test is employed. The test is set up in duplicate. After incubation of antigen, complement and antibody (as in standard test), the serum known to fix complement is added to one set. If the antigen-antibody test complex gets formed in the first step, no complement shall be attached to known serum (because it attaches only antigen-antibody-complex) and hence, would be available free in system and produce haemolysis. Hence, in indirect test, haemolysis indicates a positive test.

Conglutination

Instead of indirect complement fixation test, conglutination can be done for those sera which do not fix guinea pig complement. Horse complement which is non-haemolytic is employed in this test where use is made of conglutinin- a beta globulin present in bovine serum and behaves as antibody to complement. The indicator system in this test is sensitised sheep erythrocytes mixed with the bovine serum.

Conglutinins cause agglutination of sensitized sheep RBCs if they have combined with complement.

Other Tests Employing Complement

These include *complement lysis* and *immobilisation tests.* In complement lysis which is also known as cytocidal or cytolytic test, in the presence of complement antibody combines with a microbial surface antigen causing lysis of bacterium or enveloped virus. The test is used for organisms like *Vibrio cholerae, Esch.coli* and *Neisseria gonorrhoeae*. This test can also be used to assay anticholera antibodies by vibriocidal antibody test.

The *immobilisation test* depends on the principle that antibody combining with antigen on locomotor organ in the presence of complement shall result in inhibition of motility. The classical application of this test is the *Treponema pallidum* immobilisation test for the serodiagnosis of syphilis.

NEUTRALISATION TESTS

These are basically of two types (Fig. 21.15):

- Toxin neutralisation tests
- Virus neutralisation tests.

In toxin neutralization homologous antibodies prevent the biological effect of toxin as observed *in vivo* in experimental animals (e.g. intracutaneous adminstration of a mixture of *Clostridium perfringens* toxin and antitoxin to guinea pigs or mice) or with special culture media *in vitro* (e.g. Nagler reaction and anti-streptolysin O test)

In virus neutralisation tests various methods are available by which identity of virus can be established as well as antibody against a virus can be estimated.

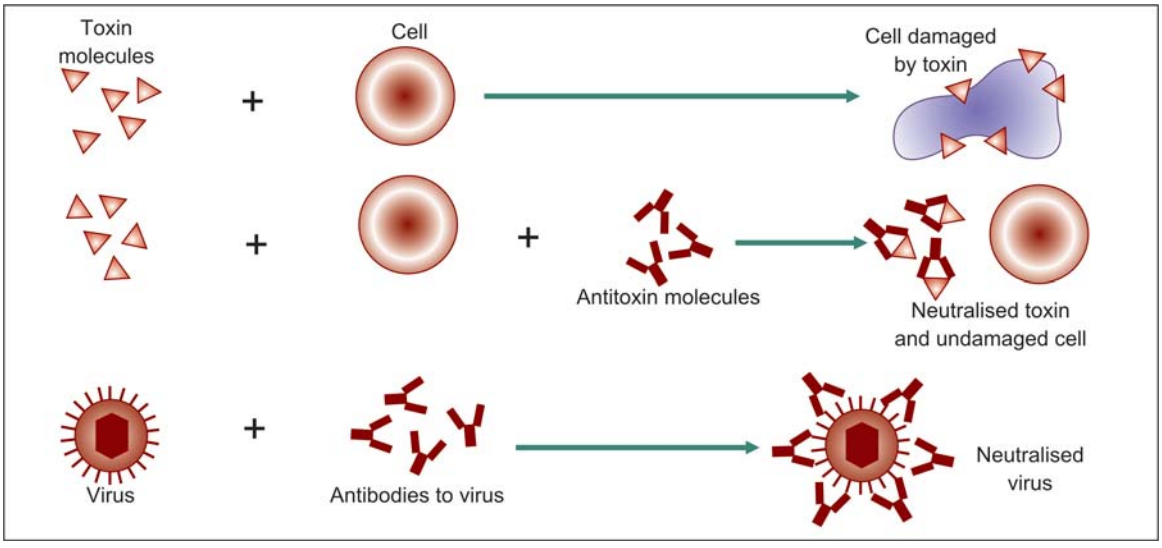


Fig. 21–15. Neutralization reactions

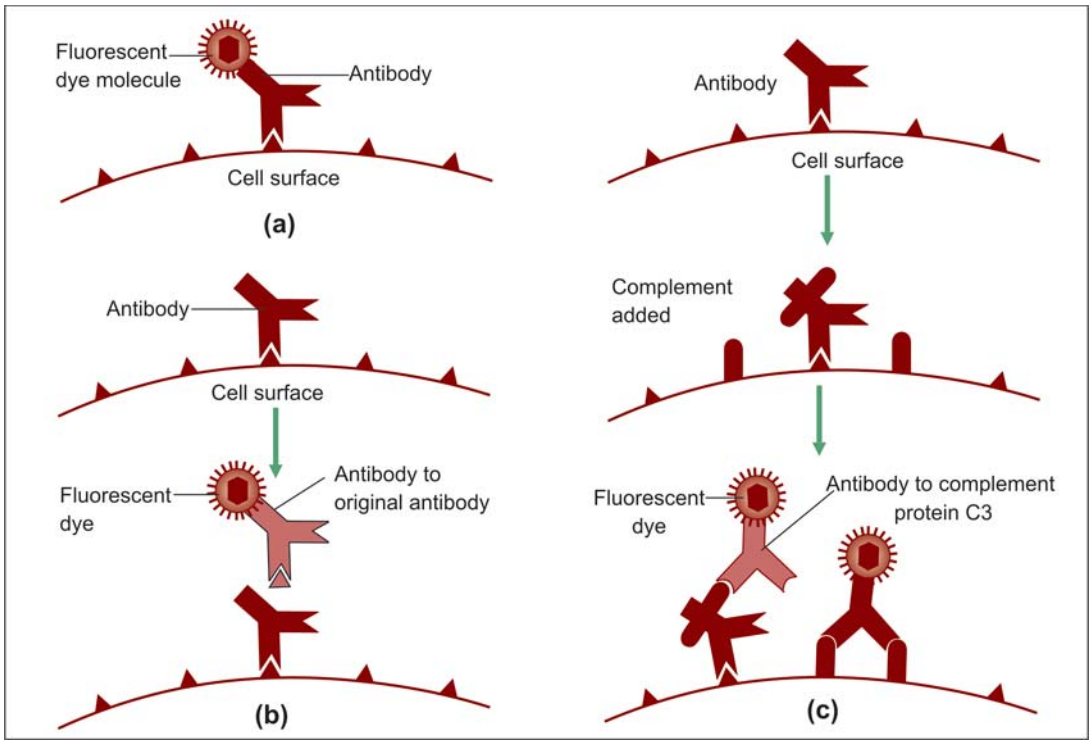


Fig. 21–16. Immunofluorescence

These tests can be performed in animals, eggs as well as in cell cultures.

IMMUNOFLUORESCENCE

An extremely high sensitivity of the detection of antigens, antibodies as well as antigen-antibody complexes has been achieved by the introduction of the use of fluorochrome labelled proteins by Coons and his colleagues in 1942. With the passage of time, fluorescent antibody or immunofluorescence technique has found several diagnostic and research applications.

The fluorescent dyes commonly used are fluorescein isothiocynate (FITC) and lissamine rhodamine. These dyes exhibit fluorescence by absorbing UV light between 290 and 495 nm and emitting longer wave length coloured light of 525 nm which gives shining appearances (fluorescence) to protein labelled with the dye. Blue-green fluorescence is seen with fluorescein isothiocynate and with lissamine rhodamine, it is orange-red.

This technique is more sensitive than precipitation or complement fixation techniques. The technique can easily detect at concentration around 1 µg protein/ml body fluid. Major disadvantage with this technique is the frequent occurrence of non-specific fluorescence in tissues and other materials.

There are two main procedures in use. The *direct method* consists of bringing fluorescein tagged antibodies into contact with antigens fixed on a slide, allowing them to react, washing off excess antibody and examining under a fluorescent microscope. The site of union of the labelled antibody with its antigen can be seen by apple green fluorescence on the slide (a in Figure 21.16.

The indirect method can be used both for detecting specific antibodies as well as for identifying antigens. A non-labelled antiserum is first layered. The reaction of this antiserum with material on slide is shown by means of a fluorescein tagged antiglobulin serum specific for the globulin of the antiserum applied first (b in Fig. 21.16). This test is more sensitive than direct method. Suitable conjugates are widely available commercially. Similarly antigen-antibody complex can be detected by demonstrating the presence of complement, which gets attached to Ag-Ab complex, using FITC conjugated antibody to complement protein C3 (c in Fig. 21.16).

Instead of labelling with fluorescein dye, *enzyme labelled antibody* have also been used. Rest of the prin-

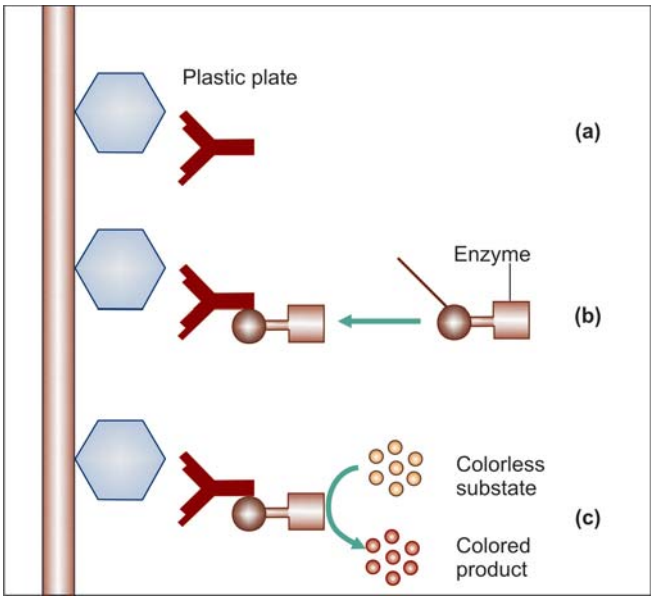


Fig. 21–17. ELISA

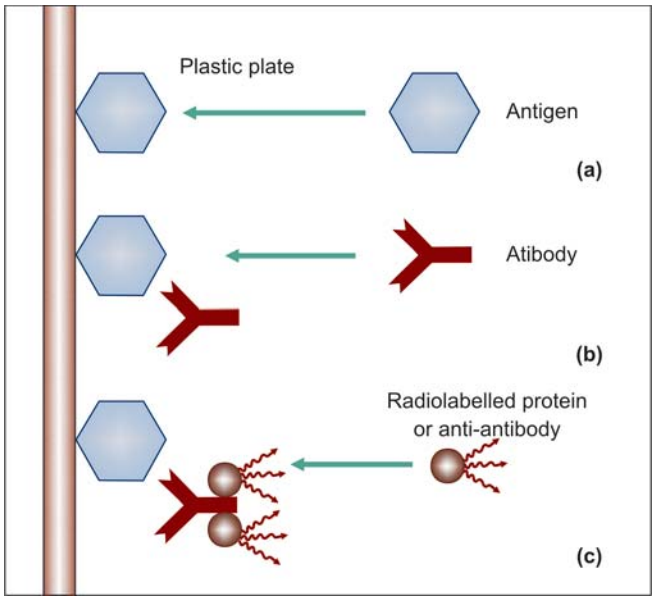


Fig. 21–18. Radioimmunoassay

ciple remains same as with immunofluorescence. Commonly used enzyme is horse radish peroxidase. This technique has advantage of prolonged storage whereas fluorescein preparations fade after exposure to UV light.

ENZYME IMMUNOASSAYS

These are commonly called as enzyme linked immunosorbent assays or ELISA. This is simple and versatile technique which is as sensitive as radioimmunoassays. ELISA is perhaps now the most widely employed technique for detection of antigens, antibodies, hormones, toxins and viruses.

Antibodies are conjugated with enzyme by addition of glutaraldehyde so that resulting antibody molecule has both immunological and enzyme activities and quantified by their ability to degrade as suitable substrate. The commonly used enzymes are alkaline phosphatase and horse radish peroxidase. There respective substrates are p-nitrophenyl phosphate and O-phenyl diamine dihydrochloride. Enzymatic activity results in a colour change which can be assessed visibly or quantified in a simple spectrophotometer (Fig. 21.17).

ELISA can be performed with sensitized carrier surfaces in the form of polystyrene tubes (macro-ELISA) or polyvinyl microtitre plates (micro-ELISA) or even beads.

Several variations of ELISA techniques are now available with the aim to provide simple diagnostics for clinical and bedside utilities. These include card and dipstick methods.

RADIOIMMUNOASSAY

It is an extremely sensitive technique in which antibody or antigen is labelled with a radioactive material (I^{125}). The amount of radioactive material in the antigen-antibody complex can be measured with which concentration of antigen or antibody can be assayed (Fig. 21.18). The radioimmunoassay was first described in 1959 by Berson and Yalow. The modifications made in the test help in detecting proteins upto picogram quantities.

This test is also called as binder-ligand assay where *binder* is the component to which radioactive material is labelled and *ligand* (or analyte)- is the component (antigen or antibody) which is to be assayed or detected.

In radioimmunoassay, fixed amount of antibody and radioactive material labelled antigen react in the presence of unlabelled antigen (test antigen). After the reaction 'free'- and 'bound' fractions of antigen are separated and their radioactivity measured. The concentration of test antigen can be calculated from the ratio of the bound and total antigen label using appropriate standards.

Table 21–3. Antigen-antibody reactions

Test	*Basic Principle*	*Modifications*	*Examples/applications*
Precipitation	Utilizes only soluble antigens	Ring test Flocculation Immunodiffusion Immunoelectrophoresis	Grouping of streptococci, typing of pneumococci VDRL test for syphilis, Kahn test for syphilis Elek's test for diphtheria toxin Detection of HB_sAg in serum, cryptococcal antigen in CSF
Agglutination	Utilizes particulate/isoluble antigen or the antigen which is part	Tube test Slide test	Widal test for typhoid fever Standard tube test for brucellosis Confirmation of identity of isolates and serotyping of *Salmonella, Shigella, Escherichia coli* etc.
Complement fixation test	Detection of antigen or antibody by demonstration of binding of complement to Ag-Ab complex	Coagglutination	Detection of antigens (such as neisseriae in CSF) Wasserman test for syphilis
Neutralization test	Demonstrates absence of activity of toxin or virus after neutralization of same by specific antibody	Toxin neutralization Virus neutralization	Naegler reaction, tetanus toxin detection Detection of rabies virus or atibodies against rabies
Immunofluorescence	Detection of antigen/antibody by using Ab coated with fluoresceing dyes such as FITC	Direct Indirect	Detection of rabies antigen, Pertussis, Anthrax Myco. tuberculosis, etc Detection of various viruses, antibody to *Toxoplasma, Leishmania*
ELISA	Ab coated with enzymes are utilized which on combining with Ag and specific substrate brings about change in colour	Micro-ELISA using plates and Macro-ELISA using tubes	Detection of various antigens and antibodies, hormones, drug, Example: IgMHAV, IgGHCV, IgMHEV, IgM Rubella, Rabis antigen
RIA	Ag/Ab labelled with a radioactive material the quantity of which can be measured in Ag-Ab complex		Detection of various antigens and antibodies, hormones, drugs

MISCELLANEOUS TESTS

Immune Electron Microscopy

Antibody combining with viral or surface antigens causes clumping of virus particles which can be visualised under the electron microscope. This technique is mainly used to detect viruses of diarrhoea and hepatitis.

Capsule Swelling Tests

When capsulated bacteria are mixed with homologous antiserum, the complex becomes easily visible under microscope. Pneumococcus and *Klebsiella* are two bacteria which give this reaction which was earlier thought to be *quellung* or swelling reaction.

Immunoferritin Test

Ferritin is an electron dense substance which is obtained from horse spleen and can be conjugated with antibody. This antibody is allowed to combine with antigen and electrodense nature of label facilitates visualization under electron microscope.

Detection of Immune Complexes

Many diseases are now known to be caused by immune complexes. Focal nephritis and endocarditis in streptococcal infection and liver damage and polyarthritis in hepatitis B infection are few important examples. These immune complexes can be detected by various methods which include cryoprecipitation, precipitation by polyethylene glycol and detection of complement component in the immune complexes. For the diagnosis of some of the clinical conditions e.g. bacterial endocarditis, commercial kits are also available.

A brief summary of all Ag-Ab reactions has been shown in Table 21.3.

OPSONISATION

Opsonisation is the process where particles such as microorganisms become coated with molecules which allow them to bind to receptors on phagocytes. Antibodies (especially IgG) and complement proteins like C3b can opsonise and are therefore referred to as "opsonins". An opsonin is any molecule that acts as a binding enhancer for the process of phagocytosis.

The IgG antibodies bind to the antigens with the Fab region leaving the Fc region sticking out. Phagocytes have Fc gamma receptors and therefore they can bind to the coated molecules and internalize them. The complement fragment, C3b, nonspecifically binds to foreign organism. Phagocytes also have receptors for C3b, on their surface. The antibodies and C3b tag the microorganisms for destruction by phagocytes.

Examples of opsonins are:

- antibodies: IgG and IgA
- components of the complement system: C3b, C4b.

Antibody opsonisation is when antibodies opsonise a pathogen, making it ready for digestion.

22 Hypersensitivity

An intact immune system is essential for defending an individual against microorganisms and thus for survival; however, inappropriate or excessive activation of the immune system can be harmful to the host. These detrimental responses (usually the heightened responses) are referred to as *hypersensitivity* reactions. These are of five types (Table 22.1).

Table 22–1. Classification of hypersensitivity reactions (Modified from Gell and Coombs)

Antibody mediated hypersensitivity	
Type I	Anaphylactic: immediate, IgE mediated
Type II	Cytotoxic
Type III	Immune complex; Arthus: Serum sickness
Type V	Stimulatory hypersensitivity
Cell mediated hypersensitivity	
Type IV	Delayed type hypersensitivity

Type I immediate hypersensitivity reactions tend to occur quickly following the interaction of antigens with IgE antibodies on the surface of tissue mast cells and blood basophils. A variety of potent mediators are released that increase vascular permeability and constrict smooth muscle tissues.

Type II cytotoxic hypersensitivity reactions are initiated by antibodies combining with the surface of cells or with antigens attached to tissues. Secondary damage to cells may result from activation of the serum complement system or effector mononuclear cells (T lymphocytes and macrophages).

Type III immune complex hypersensitivity reactions occur when antigens combine with antibodies in circulation forming aggregates referred to as immune complexes. If these complexes are deposited in the microcirculation, they may induce intense inflammatory response through complement activation.

Type IV delayed hypersensitivity occurs when T lymphocytes respond to appropriate antigens by releasing various lymphokines. These lymphokines activate other cells to induce delayed hypersensitivity reaction. Antibodies are not required.

Type V stimulatory hypersensitivity where antibody reacts with a key surface component such as hormone receptor and 'switches on' the cell.

TYPE I HYPERSENSITIVITY

Type I hypersensitivity is also known as *immediate hypersensitivity* or *anaphylactic hypersensitivity* because of the speed with which the immunologic reactions take place which may culminate into anaphylaxis within minutes following antigen challenge. The most common type of immediate hypersensitivity reaction is seasonal rhinitis, or hay fever. The symptoms of hay fever develop following exposure of the individual to ragweed or other pollens. Other types of immediate hypersensitivity reactions are bronchial asthma, allergic dermatitis and some food allergies. Insect venoms and injected drugs are associated with systemic reactions involving the cardiovascular system (anaphylactic shock).

For immediate hypersensitivity reactions to occur, an individual must first come in contact with an antigen and produce IgE antibody in response to that antigen. A genetic factor may play role in determining whether a given individual will produce IgE or antibody of any other class in response to an antigen.

Once IgE antibody is formed, it gets fixed to mast cells in the tissues and basophils in the blood. These cells possess high affinity receptors specific for the Fc portion of the IgE antibodies. Thus, Fab portion of IgE remains exposed. The interaction of IgE with the mast cells surface is a relatively stable one and found to persist upto 12 weeks.

Table 22–2. Mediators of immediate hypersensitivity reactions

Mediator	*Biological effects*
Preformed mediators (present in mast cells and basophils)	
Histamine	Vasopermeability, bronchoconstriction
Heparin	Complex with proteases, anticoagulant
Tryptase	Digestion of basement membranes
Chymase	Digestion of basement membranes
Eosinophil chemotactic factor (ECF)	Influx of eosinophils
Neutrophil chemotactic factor (NCF)	Influx of neutrophils
Membrane-derived mediators (formed de novo after cell activation)	
Prostaglandin D2	Vasopermeability, bronchoconstriction
Leukotrienes C4, D4, E4	Vasopermeabilty, bronchoconstriction
Platelet activating factor (PAF)	Vasopermeability, bronchoconstriction chemotaxis, platelet aggregation

Upon reexposure to the sensitizing antigen, the IgE molecules on the mast cell surface become cross linked, thus activating the mast cells. The mast cell activation is accompanied by the release of intracellular granules that contain potent mediators of inflammation (Fig. 22.1). It is these mediators which are responsible for symptoms in type I hypersensitivity. In addition to mast cells, the blood basophils also bind IgE and participate in immediate hypersensitivity reactions.

Mediators of Immediate Hypersensitivity

These can be divided into two groups: The substances that are preformed and packaged into granules and those that are membrane derived lipids (Table 22.2).

The first group includes heparin, histamine, proteases (tryptase, chymase), eosinophil chemotactic factor and neutrophil chemotactic factor. The cumulative biological effects of these include constriction of smooth muscles in bronchioles, vascular dilatation with increased permeability and increase in nasal secretions.

The second class of mediators released by mast cells as a result of allergic exposure are the newly formed mediators, which are produced by the metabolism of phospholipids within the mast cells. These induce vasodilatation, increase in vascular permeability and bronchoconstriction. The leukotrienes C4, E4 and D4 (previously known as slow reacting substances-SRS) also mediate bronchoconstriction, vasopermeability, and mucus secretions.

Detection

It can be tested by determining the cutaneous reactivity. Antigen is injected intradermally and a wheal and flare response determined within 15-30 mts. The reaction is caused by mediators, especially histamine released from skin mast cells and is correlated with sensitivity to antigen. A skin test of this sort should be conducted if it is necessary to administer an antigen systemically to a person, because it will reveal if the person is susceptible to anaphylaxis.

PCA and P-K Reactions

To perform the passive cutaneous anaphylactic (PCA) reaction, serum from an anaphylactically sensitized animal is injected into the skin of a normal animal. Several hours later, when antigen is injected syste-

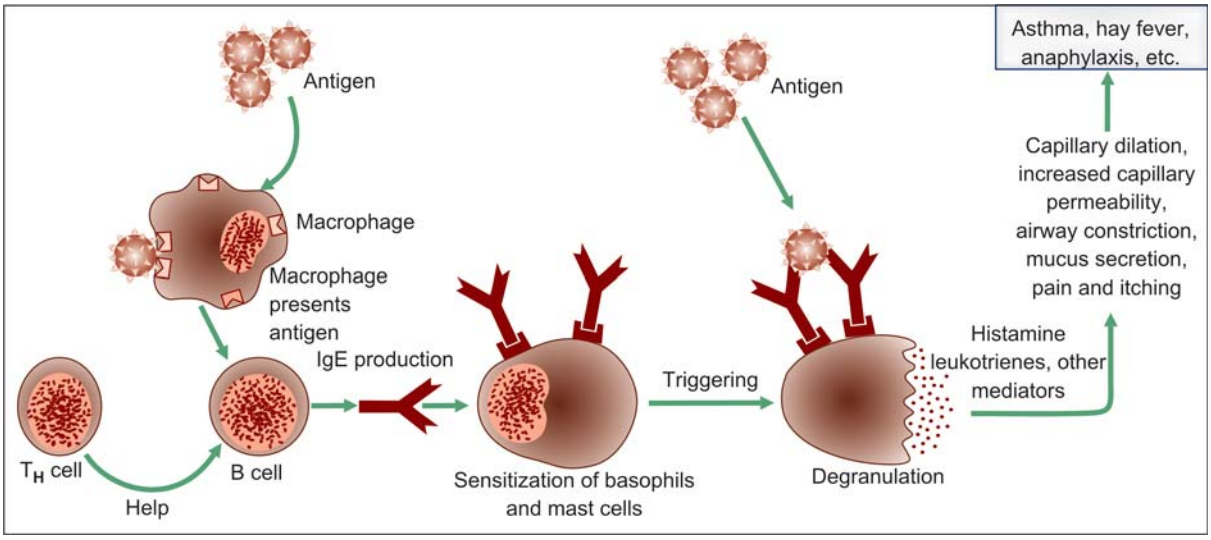


Fig. 22–1. Mechanism of type I hypersensitivity

PART IV

mically, an oedematous and erythematous reaction develops at skin site. The antigen injection can be delayed for several days and still immediate skin reaction can be elicited. When the PCA reaction is conducted in man, it is known as Prausnitz-Kustner (P-K) reaction, named after the German physicians who first performed this test.

P-K test for immediate hypersensitivity is performed in a normal subject who has been passively sensitized by immunoglobulin from the allergic individual. In the original version of this test Kustner was allergic to cooked fish. Kustner's serum was injected into the skin of the nonallergic Prausnitz, and this was followed the next day by the injection of an extract of cooked fish into the same site in Prausnitz (rather than systemically), an immediate skin reaction developed. The extracts of the raw fish, however, did not elicit this reaction, which indicates that Kustner was allergic to a heat-denatured antigen.

Schultz-Dale Reaction

The original experiment that illustrated the necessity of cell bound (cytotropic) antibody for anaphylaxis was the Schultz-Dale reaction. Schultz and Dale found that the uterus or a segment of ileum removed from a sensitized guinea pig, if heavily perfused, can be washed free of circulating Ig. When suspended between a fixed and movable pole in an isotonic bath solution, muscles in these tissues will contract when the sensitizing antigen is added to the bath. These contractions can be recorded on kymograph. The Schultz-Dale reaction is thus an *in vitro* anaphylactic reaction that proves the need of cell bound antibody to produce anaphylaxis.

Type I hypersensitivity can also be demonstrated by measurement of increased serum IgE antibodies against a particular antigen.

Anaphylactoid Reaction

An anaphylactoid reaction may be defined as any reaction having the characteristics of anaphylaxis but not based on immunologic phenomena. These are acute, life-threatening reactions that follow the intravascular administration of nonantigenic materials. These substances include starch, organic iodine, bromophenol blue and contrast media used in urographic analysis. The reactions with contrast media are unpredictable and rare.

Atopy

Atopy (*atopy*, a strange disease) is defined as an IgE-dependent allergy often arising from unknown exposure to an antigen or autocoupling hapten.

These allergies can be divided into:

a. Inhalant allergy
b. Ingestant allergy
c. Injectant allergy
d. Miscellaneous

TYPE II HYPERSENSITIVITY

Type II hypersensitivity is often referred to as *cytotoxic* hypersensitivity because in these reactions IgG or IgM antibody directed against cell surface components causes damage to, or lysis of, the affected cell. Complement can participate in these reactions by affecting cell lysis or through opsonisation of the antibody coated cell. The incompatible blood transfusion reaction and autoimmune haemolytic anaemia are important examples.

Mechanism

The critical event in any of the type II cytotoxic reactions is the binding of IgG or IgM antibody to the cell surface antigens. If the antibody is IgM, IgG1, IgG2 or IgG3, then C1q component of the complement is bound and activated. This initiates complement activation by classical pathway and terminates in lysis of the affected cell (Fig. 22.2).

Damage can also be caused by means which are independent of complement. For example, an antibody coated cell can lead to destruction of target cell by the

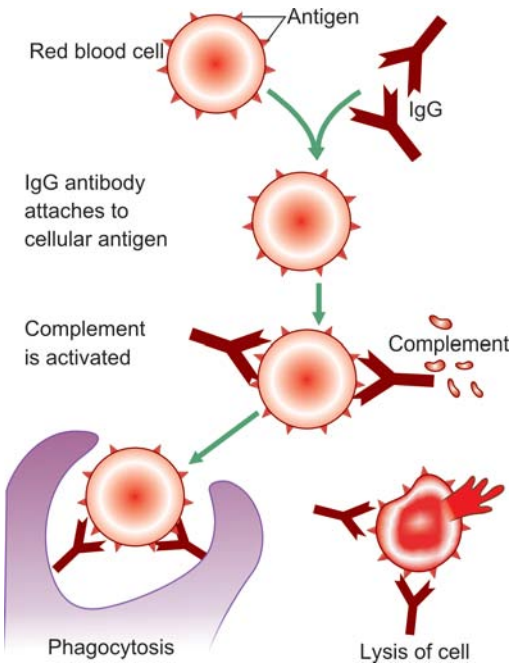


Fig. 22–2. Mechanism of type II hypersensitivity

killer cell through antibody dependent cell mediated cytotoxicity.

A classic type II cytotoxic hypersensitivity disease is erythroblastosis fetalis, also known as haemolytic disease of the newborn.

TYPE III HYPERSENSITIVITY

This is also known as *immune complex hypersensitivity* since the tissue damage results from the deposition of immune complexes in tissue. Complement participates in the tissue damage that occurs in these reactions through the anaphylactic properties of the split products of complement, C3a and C5a. Some examples of type III hypersensitivity include SLE, rheumatoid arthritis, glomerulonephritis and cutaneous vasculitis.

Mechanism

In this type of hypersensitivity, following the formation of Ag-Ab complex, complement is bound and activated through classical pathway (Fig. 22.3). This results in the release of anaphylatoxins. These induce basophils to release histamine and increase vascular permeability. Polymorphs bind to immune complex through Fc and C3b receptors and phagocytoze the immune complexes. In the process of phagocytosis, polymorphs release some of its enzymes which cause local tissue damage. The entire process may take place within the blood vessel walls or along the glomerular basement membrane in the kidney.

Immune complexes which are continuously being formed in body are removed from the circulation by macrophages located primarily in the liver, spleen and lungs. There is some evidence that individuals who develop type III hypersensitivity have defect in the system of macrophage clearance of immune complexes.

Factors which influence the immune complex mediated tissue damage include:

a. Size of immune complex
b. Local vascular permeability
c. Deposition of immune complexes at certain favoured sites in the body. Sites where biological filtration takes place such as renal glomerulus and choroid plexus are particularly susceptible to immune complex mediated damage.

The lesions which are usually seen in type III hypersensitivity are shown in Table 22.3.

Arthus Reaction

It was first described in 1930 by Arthus who named it *local anaphylaxis*. In this an extensive zone of erythema and oedema appears around the bleb created by intradermal injection of antigen. Within a few hours a cyanotic centre develops within an erythematous ring. Later this assumes a deep purplish black cast indicative of cellular necrosis. Over the succeeding day or

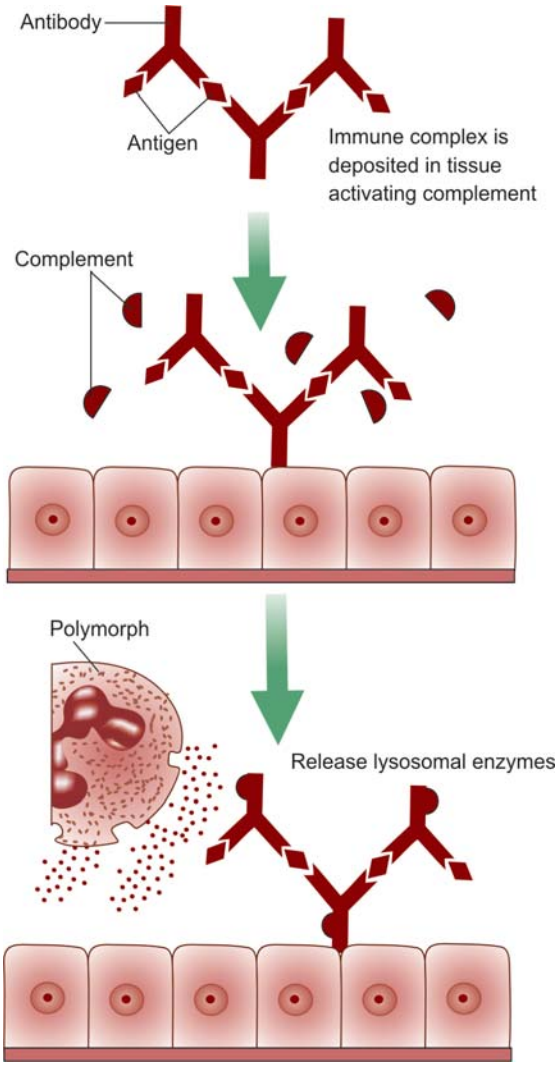


Fig. 22–3. Mechanism of type III hypersensitivity

Table 22–3. Lesions of type III hypersensitivity

Due to locally formed complexes
• Arthus reaction
• Reaction to inhaled antigens
• Reaction to internal antigens
Due to circulating complexes
• Serum sickness
• Immune complex glomerulonephritis
• Complex at other sites.

two this necrotic zone may enlarge to a few centimeters in diameter. The dead tissue dries and over a period of a week or more healing becomes complete.

This local reaction is caused by deposition of an intravascular precipitate and thombosis. Diffusion of the antigen into the vascular bed surrounding the injection site creates a zone of sufficient high concentration of antigen and antibody that a precipitate forms. This precipitate becomes so extensive as to physically blockade the small venules which results into tissue destruction. With the appearance of complement and associated changes, further damage occurs which is followed by gradual healing.

Variants of the Arthus reaction include the passive Arthus and the reversed passive Arthus (RPA) reactions. The first of these is simply the provocation of Arthus reaction in an animal that has been passively immunized. In RPA reaction, the antiserum is injected into the skin (rather than systemically) and the antigen is then given systemically (rather than intradermally). Both injections are made at about the same time so that a sufficient amount of antibody will remain near the injection site to precipitate with the antigen and cause local necrosis.

The differences between PCA and RPA have been summarized in Table 22.4.

Table 22–4. Differences between PCA and RPA

PCA	*RPA*	
Quantity of antibody required	Little	Large
Cytotropic antibody required	+	–
Latent period after transfer	+	–
Histamine release	+	–
Antihistamine effective	+	–
Complement required	–	+

Serum Sickness

Serum sickness develops in approximately 50% of normal human beings who receive a single injection of bovine or horse antitoxin against tetanus, gas gangrene, rabies, diphtheria or other toxins for prophylactic or therapeutic purposes.

The symptoms of serum sickness viz. *hives*, extensive oedema, joint pain, malaise and fever appear after about 7–10 days of injection, persist for several days after which they gradually subside. It is caused by mutual presence of antigen and antibody in the blood following primary immunisation by about fifth to eighth day of immunisation, antibody starts appearing in the blood and at the same time some antigen is also circulating. This results into formation of immune complexes which are deposited at various locations in the body. These complexes further activate complement and biological activities of complement also get manifested. All these ultimately result into hypocomplementemia (decreased complement levels) oedema, joint pain and eosinophilia seen in patients with serum sickness.

Since the symptoms of serum sickness do not appear until several days after the injection of the antigen, the disease has also been called as protracted anaphylaxis.

Immune complex glomerulonephritis. Many cases of glomerulonephritis are associated with circulating complexes and biopsies stained by fluorescent method confirm this. Well known example is glomerulonephritis following infection with nephritogenic streptococci where complexes of infecting organisms have been implicated.

The choroid plexus being a major filtration site is also favoured for immune complex deposition and this could be responsible for frequency of central nervous system disorders in SLE. Apart from SLE, subacute sclerosing panencephalitis (SSPE) and vasculitis skin rashes may also be caused by immune complexes.

Detection of Immune Complex Formation

The techniques which can be employed to detect immune complexes are shown in Table 22.5.

Table 22–5. Detection of immune complexes

- Measurement of C3 and C3c
- Cryoprecipitation of sera at 4°C
- Immunofluorescence
- Precipitation and measurement of complex IgG
- Binding of C3b with conglutinin
- Estimation of binding of I^{125}-C1q
- Detection with radiolabelled anti Ig of serum complexes capable of binding to bovine conglutinin or C3b

TYPE IV HYPERSENSITIVITY

Type IV hypersensitivity is also known as delayed hypersensitivity since the signs of reactions are observed 24 hours or more after contact with antigen. Unlike types I to III, this hypersensitivity reaction is mediated by T-lymphocytes and macrophages (Table 22.6). Allergic contact dermatitis is a common example. More severe forms of delayed type hypersensitivity reactions have been observed in diseases like leprosy and tuberculosis where an antigen is persistent and cannot easily be eliminated by the macrophages.

T-lymphocytes, sensitized by prior contact with antigen, become activated upon reexposure to allergen and release soluble mediators known as *lymphokines*.

Table 22–6. Characteristics of four types of hypersensitivity

	Type I	*Type II*	*Type III*	*Type IV*
Characteristic	Immediate	Cytotoxic	Immune complex	Delayed
Main mechanism	IgE	IgG, IgM	IgG, IgM	T cells
Other mediators	Mast cells Basophils Anaphylactic factors	Complement	Complement Eosinophils Neutrophils	Lymphokines Macrophages
Antigen	Soluble or particulate	On cell surface	Soluble or particulate	On cell surface
Reaction time	Seconds to minutes	Variable, usually hours	3-8 hours	24-48 hours
Nature of reaction	Local flare and wheal	Clumping of RBCs	Acute inflammation	Cell mediated, cell destruction
Therapy	Desensitization	Steroids	Steroids	Steroids
Example	Anaphylaxis	Thrombocytopenia Haemolytic anaemia	Serum sickness	Tuberculin test

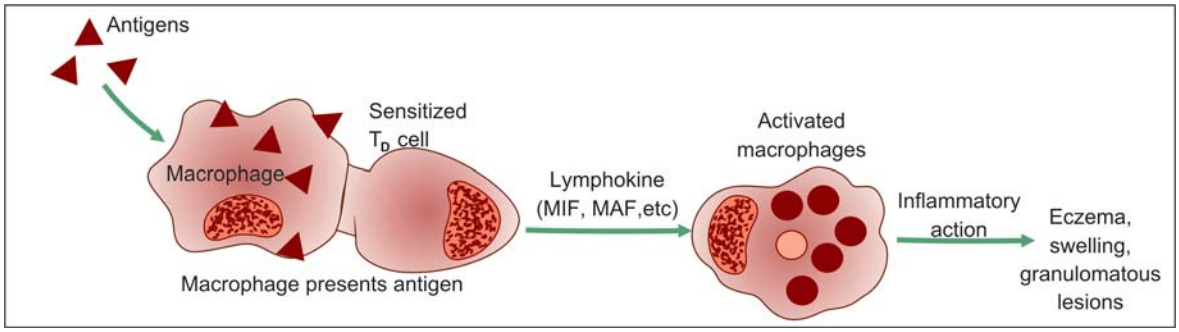


Fig. 22–4. Mechanism of Type IV hypersensitivity

One of these lymphokines, macrophage activating factor or gamma interferon causes differentiation of responsive macrophages into activated macrophages which are now capable of enhanced phagocytosis and increased microbicidal activity. Another lymphokine acts to amplify immune response by increasing the number of T cells in the vicinity. In this way lymphocytes and activated macrophages recruited by lymphokines accumulate at local reaction sites.

It is often the case that following infection with certain bacteria such as *Mycobacterium tuberculosis* and *Myco. leprae*, the host cannot adequately eliminate these agents and they persist within macrophages or in the tissues. This results into an intense area of inflammation around infection. The inflammatory response is characterized by a core of macrophages and epithelioid cells, the latter being the characteristic cells of a granuloma. Giant cells may also be present. Epithelioid and giant cells are derived from macrophages and represent terminal stages of differentiation. An area of actively proliferating lymphocytes is usually seen surrounding the core area of the granuloma. This picture is mainly due to the inability of the host macrophages to eliminate the infecting organism. The end product of the interaction between the T-lymphocytes and macrophages is tissue death, called necrosis (Fig. 22.4).

TYPE V STIMULATORY HYPERSENSITIVITY

Many cells have receptors on their surfaces which when come in contact with an appropriate and specific agent get activated and transmit the signal to interior of cell

Table 22–7. Differences between immediate and delayed hypersensitivity

Character	*Immediate hypersensitivity*	*Delayed hypersensitivity*
Appearance	Rapid	Slow
Duration	Short	Longer
Induction by	Antigen or hapten	Infection
Antibody mediated reaction	+	–
Cell mediated reaction	–	+
Passive transfer with	Serum	Lymphocytes
Desensitization	Easy but of short duration	Difficult
Lesions show	Acute exudation around blood vessels	Mononuclear infiltration
Maximum reaction in	6 hours	24-48 hours

to commence or augment cellular activity. This is what happens in thyroid when thyroid stimulating hormone (TSH) of pituitary origin combines with receptors for TSH on surface of thyroid cells. In the sera of patients with thyrotoxicosis an autoantibody directed against an antigen on thyroid surface exists which stimulates the cell and produces same changes which are brought about by TSH. The situation is similar to stimulation of B lymphocytes with Ig receptors which can be stimulated by changes induced through receptor molecules either by binding of specific antigen or antibody to the Ig.

The differences between immediate and delayed hypersensitivity are shown in Table 22.7.

23 Immunodeficiency

Immunodeficiency (or immune deficiency) is a state in which the immune system's ability to fight infectious disease is compromised or entirely absent. A person who has an immunodeficiency is said to be immunocompromised. An immunocompromised person is very vulnerable to opportunistic infections.

An efficient immune system provides protection to body against a wide range of agents. Defects in these mechanisms may increase the chance of acquiring infection or increase the severity of eventual lesion. Such defects may be genetically determined (congenital or *primary immunodeficiency)* and present from birth or may be acquired by way of some disturbance in the internal or external milieu of the individual (acquired or *secondary immunodeficiency)* (Fig. 23.1).

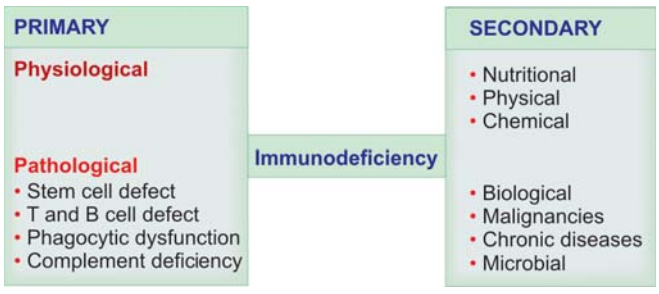


Fig. 23–1. Types of immunodeficiency

Primary Immunodeficiency

Physiological Primary Immunodeficiency

This presents as hypogammaglobulinaemia of newborn who have to commence synthesis of their own immunoglobulin by the age of 4-5 months with the waning of passive acquired antibody from mother.

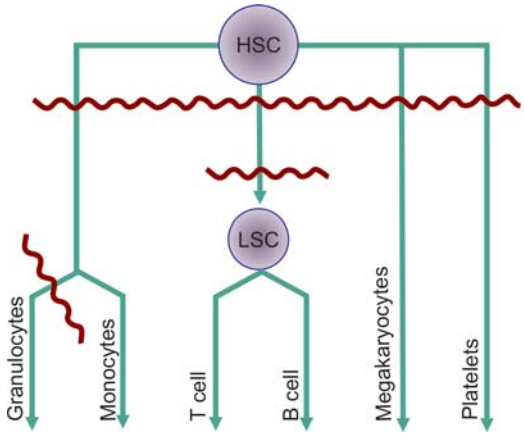


Fig. 23–2. Stem cell deficiencies

Pathological Primary Immunodeficiency

This is rare, but can result from defects in either natural or adaptive immunity. Deficiencies can occur at various levels (Fig. 23.2), from stem cells to more differentiated precursors resulting in neutropenias or, for lymphocytes, severe combined immunodeficiencies which include following syndromes:

- Swiss type agammaglobulinaemia
- Nezelof's syndrome
- Adenosine deaminase deficiency
- Reticular dysgenesis

Classification of primary immunodeficiency syndromes is given in Table 23.1.

Secondary Immunodeficiency (Acquired)

Nutritional

Immune responsiveness can be depressed nonspecifically by many factors. Cell mediated immunity in particular may be impaired in a state of malnutrition.

Table 23–1. List of congenital immunodeficiencies as per the immune component that is impaired or missing

- B cell deficiencies
  - X-linked agammaglobulinaemia
  - Selective immunoglobulin deficiency
- T cell deficiencies
  - DiGeorge's syndrome (Thymic aplasia)
  - Chronic mucocutaneous candidiasis
  - Hyper-IgM syndrome
  - Interleukin-12 receptor deficiency
- Combined T cell and B cell abnormalities
  - Severe combined immunodeficiency disease (SCID)
  - Wiskott-Aldrich syndrome
  - Ataxia-telangiectasia
- Complement deficiencies
  - Hereditary angioedema or hereditary angioneurotic edema
  - Recurrent infections
  - Autoimmune-like diseases
- Phagocyte deficiencies
  - Leucocyte adhesion deficiency
  - Chronic granulomatous disease (CGD)
  - Chédiak-Higashi syndrome
  - Job's syndrome (Hyper-IgE syndrome)
  - Cyclic neutropenia
  - Myeloperoxidase deficiency
  - Glucose-6-phosphate dehydrogenase deficiency
  - Interferon-γ deficiency
- Various/unknown
  - Common variable immunodeficiency (CVID)

Physical Immunosuppression

Exposure to X-rays result into an extended latent period for production of immune response, prolonged antigen elimination and decreased antibody titre. B cells are more sensitive to irradiation than T cells.

Chemical Immunosuppression

A number of chemicals act as immunosuppressants. These include corticosteroids, anticancer agents (cyclophosphamide), antibiotics, enzymes and plant alkaloids. The immunosuppressant antibiotics include actinomycin D, mitomycin C, puromycin, cyclosporin and chloramphenicol. Ribonuclease and asparaginase enzymes have also got immunosuppressive actions.

Biological Immunosuppression

Antilymphocyte serum (ALS) also known as antilymphocyte globulin (ALG), antithymocyte serum or globulin (ATS or ATG) is prepared by immunization of a heterologous species with cells from the lymph nodes, blood, thymus or spleen of donor. ALS is administered as a passive immunization of the donor species before, at the end or after the exposure to the antigen.

Microbial Infections

Various infections frequently produce immunosuppression (Table 23.2). Most important example is measles in man which results into direct cytotoxic effect of virus on the lymphoid cells. Macrophage functions get aberrant in malaria and diminished immune response is a feature of lepromatous leprosy. The most notorious of all microbes is HIV which causes acquired immunodeficiency syndrome (AIDS) in which attack by virus on CD4 helper cells destroys the ability to give a CMI response and leaves the patient open to infection with opportunistic organisms wuch as *Pneumocystis carinii* and CMV which can kill.

Table 23–2. Infections that cause immunosuppression

Viral	*Bacterial*
Measles	*Mycobacterium tuberculosis*
Mumps	*Mycobacterium leprae*
Congenital rubella	*Brucella species*
Epstein Barr virus	
Cytomegalovirus	
Human immunodeficiency virus	

Malignancies

Lymphoproliferative disorders like chronic lymphatic leukaemia, myeloma and Waldenstrom's macroglobulinemia are associated with varying degrees of hypogammaglobulinaemia and impaired antibody response.

Chronic Diseases

Many a times it is difficult to pinpoint the site of the deficiency but association with certain ailments has been seen too often. These diseases include diabetes mellitus, renal failure, autoimmune diseases and various malignancies.

Treatment Induced Deficiency

Many of the components of treatment have been described earlier, e.g. corticosteroids, immunosuppressants, antilymphocytic globulin and splenectomy.

Infections and Immunodeficient Patients

Apart from known pathogens, the *opportunistic* organisms also produce diseases in patients who are immunologically compromised (Table 23.3). Infections of skin and soft tissues are common.

Infections of respiratory tract and central nervous systems are also common whereas septicaemia is the most serious complication and may prove fatal.

Table 23–3. Microbes producing infections in hosts with defective immunity

Bacteria
- Gram-negative aerobic bacilli, e.g *Esch coli*
- *Klebsiella* sp., *Pseudomonas aeruginosa*
- *Streptococc*us and *Staphylococcus*
- Mycobacteria including atypical mycobacteria
- *Listeria monocytogenes, Nocardia*

Viruses
- Herpes simplex
- CMV, EB virus
- Varicella zoster
- Vaccinia virus
- Measles virus
- Papovavirus, Papilloma virus

Fungi
- *Candida albicans*
- *Cryptococcus neoformans*
- *Histoplasma capsulatum*
- *C. immitis*
- *Blastomyces dermatitidis*
- *Aspergillus, Mucor, Rhizopus*

Parasites
- *P. carinii*
- *Toxoplasma gondii*
- *Cryptosporidium*
- *Isospora belli*
- Strongyloides infection (extra intestinal)

Table 23–4. Important points regarding immunodeficiency

- Immunodeficiency may lead to repeated microbial infections.
- Enhanced susceptibility to malignancies.
- It may involve AMI, CMI or both and also non-specific immune mechanisms.
- Though primary immunodeficiencies are classified as affecting B-cells and T-cells, but there is always an overlap.
- Secondary immunodeficiencies are much more common than primary deficiencies.
- Nutritional deprivation and ageing also cause waning in the efficiency of acquired immunity.

Diagnosis of Immunodeficiencies

Repeated occurrence of acute infections or isolation of opportunistic organisms should raise suspicion of immunodeficiency and investigations should proceed to assess various components of the immune response

Defects in immunoglobulins can be assessed by several immunological quantitative estimations. The humoural immune response can be assayed by screening serum for natural antibody. T cell deficiency can be confirmed by the absence or reduced response in skin tests to antigens like tuberculin, *Candida*, and dinitrochlorobenzene (DNCB).

The important features regarding immunodeficiency are summarised in Table 23.4.

24 Autoimmunity

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

By virtue of genetic make-up, every individual has unique antigens on the cell surfaces. Some of these are called as histocompatibility (*histo*: tissue) antigens. These are responsible for determining whether one tissue is compatible with another. In human beings these antigens are called as human leukocyte antigens (HLA).

Expression of histocompatibility antigens is controlled by genes or groups of genes (loci) located close together (linked) on the same chromosomal strand. This segment of chromosome is called major histocompatibility complex, or MHC. In humans MHC is called as HLA complex and is located on chromosome 6. This is large, complex genetic region that controls not only the exchange of tissues but also diverse cellular interactions of human cells, production of certain serum proteins,and production of few cytokines and enzymes.

Certain HLA antigens are also related to increased susceptibility to specific diseases (Table 24.1). With the help of standard antisera, HLA types of an individual can be ascertained. An important medical application of HLA typing is transplantation where the donor and the recipient must be matched. This is done by tissue typing by various serological methods. Two main classes of HLA antigens are important in such matches: class I antigens (HLA-A, HLA-B and HLA-C) and class II antigens (HLA-DR, HLA-DP and HLA-DQ). Every individual will have a unique pattern of HLA type.

The existence of HLA antigens in humans denotes the presence of a system which recognises these as self and does not reject these.

AUTOIMMUNITY

The purpose of the immunological response is to protect the host and to discriminate between what is *self* and what is *non-self* for the host. The primary means by which the immune system recognises *foreign* antigens is through comparison with self major histocompatibility complex (MHC) determinants. Therefore, MHC serves as a 'yardstick' against which foreign determinants can be measured. This implies that a mechanism exists in the host which can recognise its own self-antigens. At the same time mounting an immune response against self-antigens shall be devastating. Recognition of self-determinants is normal and occurs during the generation of nearly all immune responses, whereas immunologic reactivity against self-determinants can lead to autoimmune disease.

The emergence of autoimmune disease is postulated to be due to mutation of a non-self reactive lymphocyte into self-reactive one, the activation of previous tolerant helper T cell or the inhibition of suppressor T cells.

Criteria for Autoimmune Disease (Witebsky's Postulates)

The disease must fulfil following criteria before it can be called as autoimmune disease. After the name of the scientist who suggested these, the criteria are known as Witebsky's postulates. These are:

a. The autoimmune response must be regularly associated with the disease.
b. A replica of the disease must be inducible in laboratory animals.
c. Immunopathologic changes in the natural and experimental diseases should parallel each other.
d. Transfer of autoimmune illness should be possible by the transfer of serum or lymphoid cells from the diseased individual to a normal recipient.

Relationship of HLA Types and Autoimmune Diseases

On the basis of various studies in animals, it has been shown that a relationship exists between MHC and autoimmune diseases. A link between HLA types and autoimmune diseases has been suggested by studies in families and population with disease vis a vis normal healthy subjects.

A few of the diseases having demonstrable relationship with various HLA types have been depicted in Table 24.1.

Table 24–1. Relationship of HLA and autoimmune human diseases

Disease	*HLA type*
Ankylosing spondylitis	B 27
Reiter's syndrome	B 27
Yersinia arthritis	B 27
Salmonella arthritis	B 27
Autoimmune thyroiditis	Bw 35
Anterior uveitis	B 27
Sjögren's syndrome	Dw 3
Addison's disease	B 8
Graves' disease	B 8
Multiple sclerosis	B 7

Ankylosing spondylitis is the commonest diseases and an individual with HLA-B27 type is at higher risk of contracting this disease. Some of the diseases (Table 24.1) are infectious but the pathology does not stem directly from infection but are sequelae which occur even after the infection has been eliminated.

Immunological Features and Diagnosis of Autoimmune Diseases

Many of the following immunological findings are associated with autoimmune diseases:

a. General hypergammaglobulinaemia
b. Specific cell directed immunoglobulins
c. Hypocomplementaemia–general or in specific complement component
d. Increase in activities associated with the activation of complement
e. Increase in chemotactic attraction to sites where gammaglobulin and complement are bound to tissues
f. Appearance of T lymphocytes with self-directed activities.

The serological tests directed toward identification of these unusual immunologic manifestations are useful screening procedures for the diagnosis of the autoimmune diseases.

A few of the major autoimmune diseases falling at two ends of spectrum are mentioned in Table 24.2 alongwith the self-antigen recognised by the immune system in each disease.

Table 24–2. Autoimmune diseases

Autoimmune disease	*Self-antigen*
Organ specific diseases	
Hashimoto's thyroiditis	Thyroglobulin
Pernicious anaemia	Gastric parietal cells
Graves' disease	Thyroid stimulating hormone receptor
Juvenile insulin dependent diabetes	Pancreatic islet cells
Multiple sclerosis	Central nervous system myelin
Non-organ specific diseases	
SLE	DNA, RNA, RBCs, platelets, lymphocytes
Rheumatoid arthritis	Gamma globulin
Goodpasture's syndrome	Basement membrane

Autoimmune Diseases

Hashimoto's Thyroiditis

This was the first disease that satisfied Witebsky's criteria for an autoimmune disease. Hashimoto's disease is characterised physiologically by a deficiency in thyroid hormone and anatomically by an enlarged thyroid gland infilterated with plasma cells and lymphocytes.

Thrombocytopenic Purpura

Thrombocytopenic purpura is an illness characterised by thrombocytopenia and the appearance of purpuric or petechial haemorrhages in the skin and tissues. In infants, this condition can arise through alloimmunisation in much the same way that erythroblastosis fetalis develops, that is the transplacental migration of maternal antifetal thrombocyte globulins. An autoimmune form of thrombocytopenic purpura is seen in adults. Invariably the afflicted person is on a continued drug regimen of some sort (Fig. 24.1). The offending drug may be aspirin, sulfonamide, quinine, antihistaminics.

Myasthenia Gravis

This is a disease in which a gradual progressive weakness of striated muscle is a prominent external sign and which becomes so severe that even eating is laborious. Myasthenia gravis patients exhibit a number of immunologic aberrations. Antinuclear antibodies,

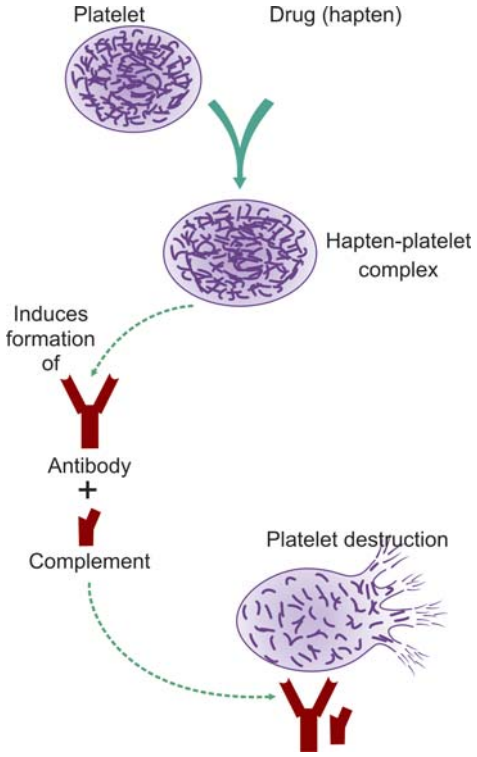


Fig. 24–1. Drug-induced autoimmune thrombocytopenia

rheumatoid factors and antibodies that react with striated muscles are frequently detected. Commonest immunopathological feature is presence of antibody to acetylcholine receptor.

Multiple Sclerosis

Partial loss of vision, nystagmus, facial palsy and muscular incoordination are a few of the varied symptoms of multiple sclerosis. Remissions and exacerbations are characteristics of this disease. The major pathologic feature is an inflammatory lesion of the myelin in the central nervous system.

Glomerulonephritis

There are three forms of immune diseases that involve the glomerulus. One is associated with antecedent group A streptococcal infection, second is involved with heterologous antibodies versus glomerular basement membrane antigens and third is based on immune complex formation with foreign antigens, as in serum sickness or alloantigens, as in SLE. The autoimmune glomerulonephritis, in which oedema, haematuria and other features of renal failure become manifest, is an immune complex disease in which globulins precipitate within the kidney following infection with group A streptococci, staphylococci, pneumococci or even malarial parasite.

Rheumatoid Arthritis

It is inflammatory disease of joints and connective tissue, amyloid deposition of tissues and permanent deformity of the joints may result. An autoantibody seen in this condition is an agglutinating agent known as rheumatoid factor (RF) which is a 19S Ig compatible in all respects with IgM.

Systemic Lupus Erythematosus

An association of this disease with HLA-B8 has been observed in a large number of patients.

Graves' Disease

This disease is a form of hyperthyroidism and more common than Hashimoto's disease. It is now believed to be caused by a long acting thyroid stimulator (LATS)—an immunoglobulin found in the sera of majority of patients with Graves' disease. The LATS autoantibody is a type of enhancing antibody that operates at the cellular level, much like the thyroid stimulating hormone, to stimulate thyroid hormone release and thyrotoxicosis.

Important features about autoimmunity are summarised in Table 24.3.

Table 24–3. Important features about autoimmunity

- Autoimmunity is a condition in which structural or functional damage is produced by the action of immunologically competent cells or antibodies against the normal components of the body.
- Autoimmunity literally means "protection against self" but it actually implies "injury to self"
- Presence of autoantibody is n't always the cause of the disease, it could be incidental.
- Autoimmune disease could be transitory (e.g. anaemia, thrombocytopenia) or chronic.
- Once initiated, most autoimmune responses tend to be self perpetuating.
- Their progress can be arrested by immunosuppresive therapy.
- Autoimmune disease could be localised (organ specific) such as affecting only thyroid gland, skin, eye, nervous system or be non-organ specific such as SLE, Rheumatoid arthritis, polyarteritis nodosa, etc.
- Their incidence is generally more in females.
- By and large they are chronic and non-reversible.
- Associated with elevated levels of immunoglobulins

25 Transplantation and Malignancy

It has been the endeavour of medical profession since long to transplant a healthy organ as a replacement of the diseased tissue. Till recent past these attempts always resulted into failure because of the inbuilt mechanism of human host to reject such graft or transplantation.

The classification of grafts in depicted in Figure 25.1.

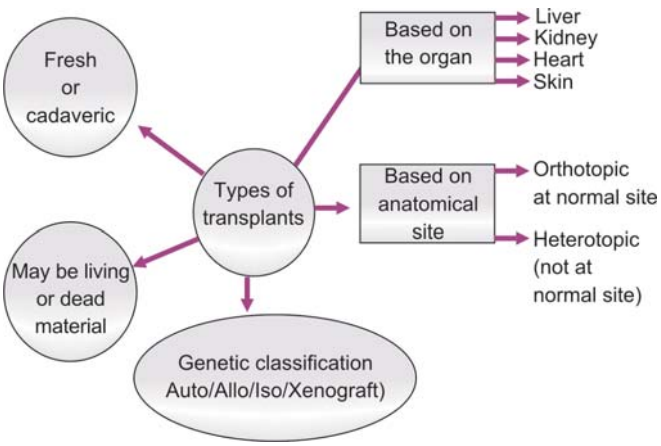


Fig. 25–1: Classification of grafts

Genetic Types of Graft

Autograft. Tissue transplanted back on to the original donor.

Isograft. Graft between two individuals with same genetic make-up, i.e. between twins.

Allograft. Graft between allogeneic individual or i.e. members of same species but of different genetic constitution, e.g. man to man and one mouse strain to another.

Xenograft. Graft between two different species, e.g. horse to man.

Types of Graft Rejection

Hyperacute Rejection

It occurs within minutes of transplantation and occurs in individuals with pre-existing humoural antibody.

Acute Early Rejection

This takes place within 10 days of transplantation and is characterised by dense cellular infiltration. It appears to be a cell mediated hypersensitivity reaction involving T-cells.

Insidious and Late Rejection

This is seen in rejection of kidney allografts when sub-endothelial deposits of Ig and C3 take place on glomerular basement membrane.

Graft Versus Host (GVH) Rejection

The rejection of the graft by the host (HVG) is a common and extensively studied phenomenon. The reverse of this is also a distinct possibility and is known as graft versus host (GVH) reaction. This reaction may develop when immunocompetent tissues are transferred to an immunologically handicapped host. This type of adoptive immunization results in a host-directed rejection process.

GVH may occur under natural circumstances when maternal lymphoid tissues are transferred to the foetus during pregnancy. These can also be induced artifically when adult tissues are injected into unborn or newborn animals or when lymphoid tissue is transferred to adults who have been irradiated or heavily immunosup-

pressed with chemical agents. The situation in newborn or foetal animal results into failure of the animal to grow, splenomegaly, diarrhoea and anaemia. This is called as *runt disease* and is often fatal.

Mechanism of Graft Rejection

There is considerable complexity of action and interaction of cellular and humoural factors in graft rejection. Lymphoid cells play primary role in first set rejection. This is consistent with the histology of the early reaction.

Prevention of Graft Rejection

Matching tissue types. The rejection can be minimised by matching graft and recipient in terms of MHC antigen in much the same way the individuals are cross-matched for blood transfusion. Of the different loci, matching of DR locus is more important than any other locus. This matching is also known as *histocompatibility testing* which can be performed by:

- Microcytotoxicity test
- Mixed lymphocyte culture.

Use of immunosuppressants. Graft rejection can be delayed or avoided by the use of agents which interfere with the induction or expression of immune response.

Antigen specific depression of allograft reactivity. To avoid generalised immunsuppression and its resultant complications, efforts are being made to develop approaches by which only specific immune response against graft is reduced significantly leaving rest of the immunological apparatus intact.

Immunological Enhancement

This mechanism operates in the survival of kidney grafts. It is based upon the observation that deliberate immunisation of animals with irradiated tumour cells produces enhancing antibodies which prolong the life of the tumour. Comparable manipulations can also enhance the survival of kidney graft through this anti-idiotype enhancing antibodies.

Clinical Experience in Grafting

Privileged sites. Corneal grafts survive without the need for immunosuppression. Because they are avascular they do not sensitize the recipient although they become cloudy if the individual had been pre-sensitised. Grafts of cartilages are successful in the same way.

Kidney grafts. Kidney transplantation has now become a common practice. If HLA-D loci of donor and recipients match, a graft survival of 5 years or more has been observed. It is believed that if HLA-B and HLA-A loci also get matched, the survival rate can be prolonged. Administration of multiple blood transfusions prior to grafting has been shown to have a significant beneficial effect on survival. The exact reason for this is, however, not known.

Heart transplant. More than 80% of heart transplants now survive for more than one year. A good HLA-D matching (with not more than single DR mismatch) can give survival of 3 years or more.

Liver transplant. With the combined use of cyclosporin and steroid therapy, success rate of liver transplants is now as good as that of heart transplants.

Bone marrow grafting. Successful results with bone marrow transfers have been obtained in certain immunodeficiency disorders and aplastic anaemia. An extremely high compatibility between donor and recipient is a must otherwise a fatal graft versus host reaction may take place. Siblings offer the best chance of finding a matched donor.

TUMOUR IMMUNOLOGY

For the host, there should not be any difference between a graft and a tumour because both present with a set of antigens which is different than that of the host. In transplantation the whole immune system comes into action to reject the graft. Paradoxically, in malignancy, the host's immune response is either not fully activated and fails to reject the tumour tissue or develops in such a way that growth of the tumour is not only permitted it is even encouraged (Fig. 25.2).

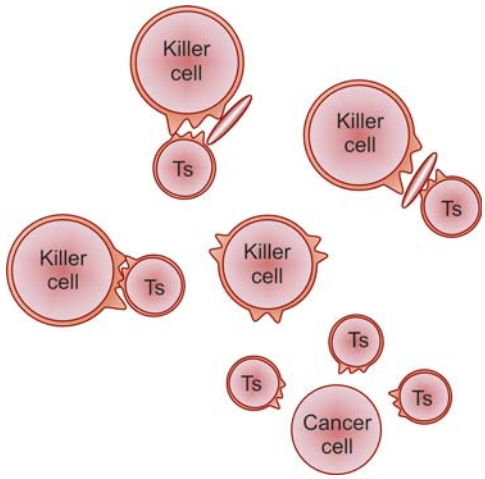


Fig. 25–2. Growth of cancer cells by the blockage of killer cells by Ts lymphocytes

Tumour Antigens

Followings are considered the immunological expression of tumours:

- Antigen induced by chemicals
- Antigen induced by virus
- Carcinofoetal antigens
- Carcinofoetal enzymes

Antigens Induced by Chemicals

With chemically induced tumours different antigens appear. These new antigens are different for each tumour. Even two anatomically distinct tumours induced on a single mouse by the same chemical carcinogen will be antigenically distinct.

Antigens Induced by Viruses

Viral induced tumours express unique antigens which unlike those induced by chemicals, are antigenically constant from specimen to specimen. This constant expression of identifiable antigens is a useful diagnostic aid. The DNA viruses which can code for new antigens in hosts are herpes viruses, adenoviruses and the papova viruses. The RNA viruses include Rous Sarcoma virus and mouse mammary tumour virus.

The antigens associated with viral transformed cells are of three types.

a. Those which are associated with infective virion
b. The tumour (T) or nuclear antigen
c. The tumour specific transplantation antigen (TSTA) or cytoplasmic membrane antigens.

The T antigens are located in the nucleus and are specific for inducing virus and not the malignant cell. The TSTA are important as these come in contact with immune system. Antibodies to these antigens are found in circulation in tumour bearing animals. However, the titre of these antibodies does not correlate well with resistance to tumour or regression of tumour. It is not yet clear as to how tumour cells escape the rejection process.

Carcinofoetal Antigens

The antigens are synthesised by the body when there is a cancerous growth to express a metabolic shift from an adult to an *immature* pathway of protein synthesis. The following antigens have been studied in detail:

a. Carcinoembryonic antigen
b. Alpha fetoprotein
c. Alpha 2 hepatic protein

Carcinoembryonic antigen. This antigen has been detected by radioimmunoassay (RIA) in tumours of colon, small intestine, liver, stomach, etc. but not in normal tissues surrounding these growths. This antigen was also found in human embryonic gut and gut associated organs during the first two trimesters, after which the antigen becomes more difficult to detect. Because of these facts, it was named as carcinoembryonic antigen (CEA).

The normal adult blood level of CEA is about 2.5 mg/L. Levels significantly higher than these are a good index of cancer. CEA gets elevated almost three months prior to the development of clinical features of cancer. Continuous high levels, even after therapy, denotes a poor prognosis.

Presence of CEA is not diagnostic of tumour and this is because of two reasons: (i) its presence in many non-malignant conditions, e.g. cirrhosis liver, cigarette smoking and chronic lung disease and (ii) its absence in a large number of cases with confirmed carcinomas of the digestive tract.

Alpha fetoprotein (AFP). This is present in foetal serum in very high concentration of 3000 mg/ml. In pregnant woman the level may be upto 500 mg/ml whereas in normal adult it is 5-10 mg/ml. The detection of AFP, like that of CEA is of potential diagnostic and prognostic significance in human oncology. Another successful use of AFP is in the monitoring of neural tube malformations (spina bifida) in fetuses by measuring the AFP level in amniotic fluid which is normally 1.5 to 26 mg/ml at the fifteenth week of gestation. Excesses of this concentration correlate well with serious defects including anencephaly.

Alpha 2 hepatic protein (AHP). This is a globulin with high iron content and was earlier found to be associated with hepatoma. Subsequently it could be extracted from the liver of patients who had malignancies at anatomical sites other than liver. Although 50% of patients with malignancies have detectable AHP in their serum, 20% of patients with non-malignant diseases are also positive.

Carcinofetal Enzymes

Several enzymes described as carcinoplacental or carcinoembryonic enzymes and originating from trophoblastic tissues have been associated with a broad range of cancers. Regan isozyme of alkaline phosphatase is the best known of these which is present in sera of individuals with various forms of cancers and is not restricted to those with placental or trophoblastic tissue tumours. Other isozymes associated with cancerous states include aldolase, glycogen phosphorylase, glucosamine 6-phosphate synthetase and amino acid transaminase.

Table 25–1. Clinical evidence of immune response in malignancy

- Higher incidence of malignancies in immunodeficient individuals.
- Spontaneous regression of established tumours such as neuroblastoma or malignant melanoma.
- Histological evidence of immune response is provided by the presence of lymphocytes, plasma cells and macrophages in the infiltrating tumours.
- Dramatic cures following chemotherapy shows the contributory role of immune response.

Table 25.1 sumsup the clinical evidences of immune response in malignancy.

Immunological Surveillance

Immunological surveillance means that cell mediated immunity should "seek and destroy" malignant cells that arise by somatic mutation. Hence immune system is expected to maintain a constant 'vigil'. Indefficiency of this immune surveillance due to any reason leads to an increased incidence of cancer. This was opined by Ehrlich in 1906 and later revived by Levis Thomas and Burnet in 1950s. But if this was true, tumors should not develop. The development of tumours appears to be a lapse in immune surveillance. The possible explanations of this immunological lapse could be:

a. Due to faster rate of tumour growth some cells might sneak through the immune surveillance mechanism.
b. Once the tumour reaches a particular size, it may be beyond the capacity of immune surveillance.
c. The tumour antigens may be covered by antigenically neutral substance and may not be detected by immune system.
d. Circulating tumour antigens may coat the lymphoid cell and prevent their action.
e. Some tumours may be of low immunogenicity
f. Some tumours may form cytokines which supress cell-mediated immunity.

Immunotherapy of Cancers

Following approaches have been followed for the immunotherapy of cancers.

a. *Passive immunotherapy:* Using antisera prepared by immunising animals with tumour biopsy proved unsuccessful. Monoclonal antibodies to tumour may play a role as carriers in transporting cytotoxic or radioactive drugs specifically to the tumour cells.
b. *Specific active immunotherapy:* By the injection of tumour cell vaccines was tried early in the last century but without success.
c. *Nonspecific active immunotherapy:* Employing BCG vaccine and non-living *Corynebacterium parvum* have proven useful. Intralesinal BCG in malignant melanoma has been reported to induce complete remission.
d. *Specific adoptive immunotherapy:* Has been tried with transfer factor, lymphocytes and immune RNA. The donors have been the persons who have been cured of these neoplasms.

Immunotherapy is not very effective in the presence of large tumour cells. It is best used along with surgery, radiotherapy and chemotherapy.

26 Blood Group Antigens

Before the 1900s, it was thought that all blood was the same, a misunderstanding that led to frequently fatal transfusions of animal blood into humans and hazardous transfusions of blood between people. Human blood is not the same—people belong to different blood groups, depending upon the surface markers found on the red blood cell.

At the beginning of the 20th century an Austrian scientist, Karl Landsteiner, noted that the RBCs of some individuals were agglutinated by the serum from other individuals. He made a note of the patterns of agglutination and showed that blood could be divided into groups. This marked the discovery of the first blood group system, ABO, and earned Landsteiner a Nobel Prize.

Landsteiner explained that the reactions between the RBCs and serum were related to the presence of markers (antigens) on the RBCs and antibodies in the serum. Agglutination occurred when the RBC antigens were bound by the antibodies in the serum. He called the antigens A and B, and depending upon which antigen the RBC expressed, blood either belonged to blood group A or blood group B. A third blood group contained RBCs that reacted as if they lacked the properties of A and B, and this group was later called "O" after the German word "Ohne", which means "without". The following year the fourth blood group, AB, was added to the ABO blood group system. These RBCs expressed both A and B antigens.

Currently more than 400 erythrocyte antigens are recognized (Table 26.1). Most of these are, however, poorly immunogenic and do not stimulate immune system upon transfusion. Clinical significance is attached to only those antigens which are strongly immunogenic. To this group belong ABO and Rh blood group systems. Testing blood group antigen has applications in transfusion medicine, transplantation, disputed paternity, forensic pathology and anthropology.

Table 26–1. Blood group systems

Blood group system	*Antigen*	*Discovered in*
ABO	A, B, AB	1900
Rhesus	C, D, E	1940
Duffy	Fya, Fyb	1950
Kidd	Jk, jk	1951
MN	M, N, S, s, U	1926
Lewis	Lea, Leb	1946
P	P, Pi, p	1926
Kell	K, k, Kp, Js	1946
Xg	Xg	1962
Lutheran	Lu, Lu	1945
Dombrock		1965
Colton		1967

The functions of many of the blood group antigens are not known, and if they are missing from the red blood cell membrane, there is no ill effect. But the presence or absence of red blood cell antigens becomes extremely important when blood from different people mixes, e.g. when a patient receives a blood transfusion from a blood bank. This also happens when a mother becomes pregnant because during labour, a small amount of foetal blood enters her circulation. In these circumstances, exposure to the foreign antigens on the red blood cells can trigger immune reactions.

Chemical composition of red blood cell antigen. Blood group antigens are either sugars or proteins, and they are attached to various components in the red blood cell membrane. The antigens of the ABO blood group are sugars. They are produced by a series of reactions in which enzymes catalyze the transfer of sugar units.

In contrast, the antigens of the Rh blood group are proteins. The RhD gene encodes the D antigen, which is a large protein on the red blood cell membrane. Some people have a version of the gene that does not produce D antigen, and therefore the RhD protein is absent from their red blood cells.

ABO Blood Group System

This system consists of two antigens which divide humans into four groups. These two antigens - A and B are detected with corresponding antibodies (anti-A and anti-B) obtained from sera of normal individuals. The composition of four groups is shown in Table 26.2.

Table 26–2. ABO blood group system

Group	*Ag on RBC*	*Ab in Serum*	*Genotype*
A	A	Anti-B	AA or AO
B	B	Anti-A	BB or BO
AB	AB	None	AB
O	None	Anti-A and Anti-B	OO

Genotypes of ABO groups. RBCs antigens are inherited. The ABO blood group antigens are encoded by one genetic locus, the ABO locus, which is located on chromosome 9 and has three alternative (allelic) forms—A, B, and O. A child receives one of the three alleles from each parent (Table 26.3), giving rise to six possible genotypes and four possible broad blood types (phenotypes).

ABO phenotypes. The four basic ABO phenotypes are O, A, B, and AB. After it was found that blood group A RBCs reacted differently to a particular antibody (later called anti-A1), the blood group was divided into two phenotypes, A_1 and A_2. Thus there are six pheonotypes: O, A_1, A_2, A_1B, A_2B, and B. As far as transfusion purposes are concerned A1 and A2 are interchangeable.

H antigen. The A and B antigens have their precursors in H antigen which is present in all red blood cells.

Table 26–3. ABO genotypes in offsprings

ABO genotype in the offspring		ABO alleles inherited from the mother		
		A	B	O
ABO alleles inherited from the father	A	A	AB	A
	B	AB	B	B
	O	A	B	O

Individuals who are homozygous for null alleles at this locus (h/h) do not produce H antigen, and because the H antigen is an essential precursor to the ABO blood group antigens, they cannot produce A and B antigens. Therefore, their serum contains anti-A and anti-B, in addition to potent anti-H. This rare phenotype of H-deficient RBCs is called the "**Bombay phenotype**" (O_h) after the city in which it was first discovered. Individuals with the Bombay phenotype are healthy, but if they ever needed a blood transfusion, the antibodies in their serum would place them at a high-risk of having an acute haemolytic transfusion reaction. This can be avoided by using only blood products from a donor who also has the Bombay phenotype (usually a relative).

Each human RBC expresses about 2 million ABO blood group antigens. Although the ABO blood group antigens are regarded as RBC antigens, they are actually expressed on a wide variety of human tissues and are present on most epithelial and endothelial cells.Other blood cells, such as T cells, B cells, and platelets, have ABO blood group antigens that have been adsorbed from the plasma. In individuals who are "secretors", a soluble form of the ABO blood group antigens is found in saliva and in all bodily fluids except for the cerebrospinal fluid.

Function of the A and B Antigens

The functions of the ABO blood group antigens are not known. Individuals who lack the A and B antigens are healthy, suggesting that any function the antigens have is not important, at least not in modern times.

Clinical Significance of ABO Antibodies

ABO antibodies are of major clinical significance for two reasons: they are naturally occurring and are found universally, and, they are highly reactive.

If a recipient who has blood group O is transfused with non-group O RBCs, the naturally occurring anti-A and anti-B in the recipient's serum binds to their corresponding antigens on the transfused RBCs. These antibodies fix complement and cause rapid intravascular haemolysis, triggering an acute hemolytic transfusion reaction that can cause disseminated intravascular coagulation, shock, acute renal failure, and death.

Rh Blood Group System

The nomenclature Rh to the human blood group system has been derived from the fact that it was discovered with the aid of rabbit antibody to rhesus (Rh) monkey

erythrocytes. These antigens are integral part of erythrocytes and are protein in nature. The antigen is present on almost all erythrocytes. The red blood cells that lack Rh antigen (Rh null phenotype) have a shortened life span.

The Rh system consists of antigen C, D and E. Of these D is highly immunogenic and of considerable clinical significance. Majority of the individuals who lack D antigen shall develop anti D antibody after transfusion of blood having red blood cells that contain D antigen. Therefore, in addition to determining the ABO blood group of the donor and recipient, it is important to type their blood for D antigen also. Individuals with D antigen on their red blood cells are considered Rh positive, and those who lack it are considered Rh negative. Patients who are Rh negative should receive blood transfusion only from donors who are Rh negative.

The D antigen is responsible for the haemolytic disease of the newborn, also known as **erythroblastosis foetalis.** The disease occurs in a child who has Rh positive father and Rh negative mother. During first pregnancy, particularly at the time of delivery, foetal red blood cells may enter the maternal circulation and stimulate the production of anti-D antibody in the mother. Nothing happens during the first pregnancy. In the subsequent pregnancies, the maternal anti-D antibody of IgG class can cross the placenta and cause haemolysis of the foetal Rh positive erythrocytes. This leads to anaemia, congestive heart failure and death. The disease can be prevented by the passive administration of specific antibody which will block the antibody response to the antigen. Therefore, administration of Rh immune globulin to the mother in late pregnancy and again just after the birth prevent the development of maternal antibody against Rh positive foetal red blood cells.

Blood Transfusion

To avoid a transfusion reaction, donated blood must be compatible with the blood of the patient who is receiving the transfusion. More specifically, the donated RBCs must lack the same ABO and Rh D antigens that the patient's RBCs lack. For example, a patient with blood group A can receive blood from a donor with blood group A (which lacks the B antigen) or blood group O (which lacks all ABO blood group antigens). However, they cannot receive blood from a donor with blood group B or AB (which both have the B antigen).

Before a blood transfusion, two blood tests known as a "type and cross-match" are done. First, the recipient's blood type is determined, i.e. their ABO type and Rh D status. In theory, once the recipient's blood type is known, a transfusion of compatible blood can be given. However, in practice, donor blood may still be incompatible because it contains other antigens that are not routinely typed but may still cause a problem if the recipient's serum contains antibodies that will target them. Therefore, a "cross-match" is done to ensure that the donor RBCs actually do match against the recipient's serum.

To perform a cross match, a small amount of the recipient's serum is mixed with a small amount of the donor RBCs. The mixture is then examined under a microscope. If the proposed transfusion is incompatible, the donor RBCs are agglutinated by antibodies in the recipient's serum.

Transfusion Reactions

Immune-mediated transfusion reactions occur when incompatible blood products are transfused into a patient's circulation, triggering a response from the patient's immune system. The destruction of incompatible RBCs is called a haemolytic transfusion reaction, which may occur immediately (acute) or after a period of days (delayed). The destruction of incompatible donor white blood cells (WBCs) causes a febrile non-haemolytic transfusion reaction (FNHTR), and the destruction of incompatible donor platelets causes post-transfusion purpura (PTP).

Haemolytic Transfusion Reaction: Red Blood Cell Incompatibility

Haemolytic transfusion reactions (HTRs) are reactions in which donor RBCs are destroyed by antibodies in the recipient's circulation. They occur when antigen-positive donor RBCs are transfused into a patient who has preformed antibodies to that antigen. The donor RBCs may be destroyed immediately (a potentially serious reaction) or may have a shortened or even normal survival time (milder reactions).

Red blood cell incompatibility may also occur when the patient's RBC antigens are attacked by antibodies from the donor's plasma. This tends to be a minor problem because of the small amount of antibody present in the donated plasma, which is further diluted on transfusion into the recipient's circulation.

Acute haemolytic transfusion reactions occur within 24 hours of the transfusion and often occur during the transfusion. Delayed haemolytic transfusion reactions may occur as soon as 1 day or as late as 14 days after a blood transfusion. The donor RBCs are destroyed by the recipient's antibodies, but the haemolysis is

"delayed" because the antibodies are only present in low amounts initially.

The final outcome can be intravascular or extravascular haemolysis. *Intravascular haemolysis* is complement mediated and results in hypotension, renal failure, diffuse intravascular coagulation, bleeding and death. Anti-A and anti-B antibodies, capable of causing complement mediated intravascular haemolysis, are the antibodies most commonly responsible for fatal haemolytic transfusion reactions.

Extravascular haemolysis is mediated by molecular phagocytic cells of the reticuloendothelial system in which red blood cells are lysed or injury produced by these results into conversion of erythrocytes into rigid spherocytes which are subsequently trapped and destroyed in spleen. Such erythrocytes may also be destroyed by antibody dependent cell mediated cytotoxicity. The clinical features are usually fever, anaemia and jaundice.

Apart from haemolytic transfusion reactions, several other reactions can take place in case of mismatch. These are:

- Febrile non-haemolytic transfusion reaction (FNHTR): White blood cell incompatibility.
- Post transfusion purpura (PTP): Platelet incompatibility.
- Anaphylaxis: IgA anti-plasma protein antibodies.
- Transfusion associated lung injury (TRALI): Donor anti-leukocyte antibodies attack.
- Transfusion associated graft-versus-host disease (TA-GVHD): Donor T cells attack.

Transfusion Transmissible Infections

More than 500 microorganisms can be transmitted through blood transfusion. Some of these are life-threatening especially HIV, hepatitis B and hepatitis C viruses. Though the risk of blood transfusions transmitting infectious diseases has been greatly reduced, but a small risk still remains. Bacteria can also contaminate blood products while they are being stored unless proper precautions are taken.

To minimize the risk of infection, blood donors are now screened, and people who are at risk of infectious diseases are excluded from donating blood. In addition, all donated blood is tested for infectious agents.

PART V
GRAM POSITIVE BACTERIA

27 Staphylococcus

Staphylococci and streptococci are two genera of gram positive cocci which are of great medical importance. Staphylococci were first seen by Koch in 1878; cultivated in liquid medium by Pasteur in 1880 and named so by Alexander Ogston in 1881. The name *Staphylococcus* was derived from the Greek noun *staphyle* (a bunch of grapes) and *coccus* (a grain or berry). Staphylococci differ from streptococci in their ability to produce catalase.

SPECIES OF STAPHYLOCOCCUS

Currently 32 species of *Staphylococcus* are recognized of which three are of medical importance

- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Staphylococcus saprophyticus*

Identification tree for Gram-positive cocci of medical importance is given in Figure 27.1.

Various tests are now available to differentiate between three species of *Staphylococcus* (Table 27.1).

Table 27–1. Characters distinguishing species of Staphylococcus

Character	*S. aureus*	*S. epidermidis*	*S. saprophyticus*
Coagulase production	+	–	–
Fermentation of mannitol	+	–	–
Production of DNase	+	–	–
Novobiocin resistance	–	–	+
Colony colour	Yellow to orange	Usually white	Usually white
β haemolysis	+	–	–

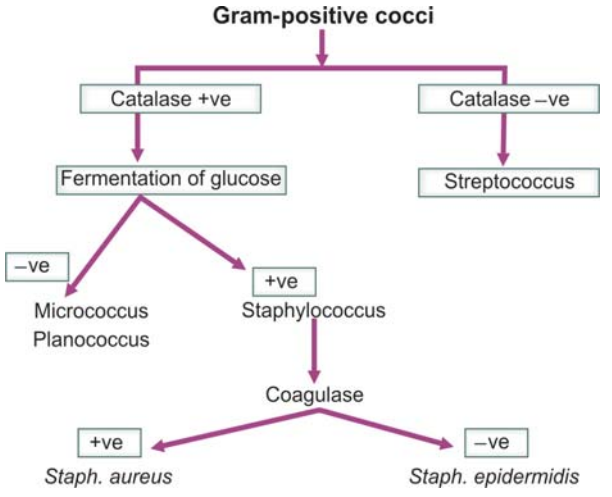


Fig. 27–1. Identification of Gram-positive cocci

STAPHYLOCOCCUS AUREUS

Habitat

Staphylococci are associated with skin, skin glands and mucous membranes of almost all the warm blooded animals. This bacterium is also widely present in the environment. Nearly one-third of the human population supports the colonization of *Staphylococcus aureus* and are designated as carriers. Nasal carriage of

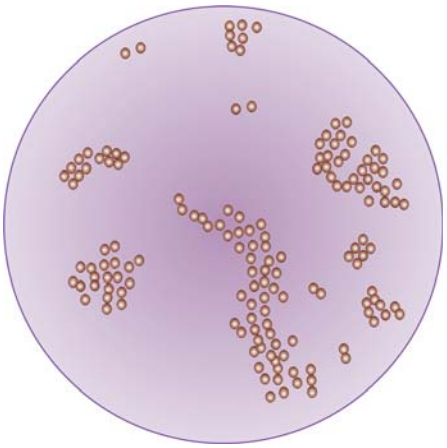


Fig. 27–2. Staphylococci

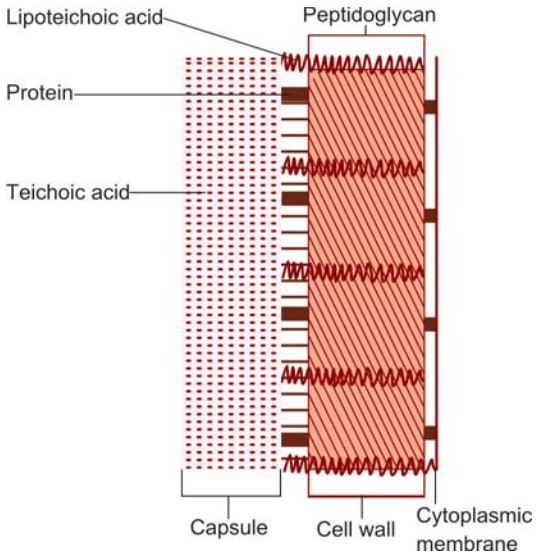


Fig. 27–3. Cell wall of staphylococci

Staph. aureus occurs in 40-50% of humans. Major habitat in man is anterior nares or on skin elsewhere. Hospitalised patients as well as medical and paramedical staff show higher incidence of carriage of *Staph.aureus*. Several coagulase negative species of staphylococci are present on the skin as commensal normal flora.

Morphology

These are nonmotile, nonspore forming, Gram positive cocci which measure around 0.7 to 1.2 µm in diameter. After phagocytosis or in old cultures, these may appear as gram-negative. Young cocci stain strongly Gram-positive. They are non-motile and don't form spores.

These are characteristically grouped in irregular clusters that resemble bunches of grapes (Fig. 27.2). This appearance is because of incomplete separation of cells after successive cell divisions which take place in perpendicular planes. Instead of residual attachment along the plane of division, the attachment point is usually eccentric to the plane of division. This results in irregular aggregates of cocci. The clusters are usually seen in growth obtained on solid media or in pathological materials.When cultivated in liquid medium, staphylococci usually form short chains. Single cocci, pairs or tetrads are also seen in liquid cultures.

The generation time of *Staph. aureus* is about 20 minutes.

Cell Wall

The cell wall of staphylococci is rich in peptidoglycan (Fig. 27.3) which is characterised by unique pentaglycine bridges that link the tetrapeptides attached to the muramic acid residues. Peptidoglycan is destroyed by strong acid or exposure to lysozyme. It elicits production of Interleukin-1 (endogenous pyrogen).

Cultural Characteristics

These are easy to grow organisms. They prefer aerobic environment but can also grow in the absence of oxygen; range of temperature for growth is 6-44°C (optimum 37°C) and the range of pH is 4.2-9.3 (optimum 7). The organisms grow easily in simple liquid media especially nutrient broth and peptone water. In both these media the growth becomes visible as uniform turbidity. They grow best at 37°C but form pigment best at room temperature (20-25°C).

Growth on Nutrient Agar

Colonies of *Staph. aureus* are sharply defined, round, convex and measure around 4 mm in diameter. These have a smooth, glistening surface and butyrous consistency. On nutrient agar, these look like *oily paint* colonies. *Staph. aureus* usually forms grey to deep golden yellow colonies. No pigment is produced in broth or when incubated anaerobically.

Growth on Blood Agar

Primary isolation is best done on blood agar medium on which most strains of *Staph. aureus* produce a characteristic golden yellow (*aureus*) carotenoid pigment. This pigment does not diffuse into the medium. The production of pigment is optimum at 22°C in the presence of oxygen. On blood agar medium the appearance and size of colonies is same as those on nutrient agar.

On blood agar however these colonies are usually surrounded by a zone of clear (beta) haemolysis.

Growth on Media that Enhance Pigment Production

The pigment production can be enhanced by cultivating the organisms on:

a. Milk agar
b. 1% glycerol monoacetate agar
c. Glucose-peptone-yeast extract agar and incubating at 30°C for 5 days.

Growth on MacConkey agar

On MacConkey agar, the colonies are small to medium in size and pink in colour because of the fermentation of lactose.

Growth on Mannitol Salt Agar

Mannitol salt agar is an indicator as well as selective medium. It contains mannitol, NaCl (7.5%) and phenol red in nutrient agar. *Staph. aureus* strains form colonies surrounded by yellow zones due to fermentation of mannitol. NaCl inhibits the growth of other bacteria.

Susceptibility to Physical and Chemical Agents

Staphylococci are extremely hardy organisms and can survive in adverse environment for a very long time. Some strains can even withstand temperature of 60°C for 30 minutes. As compared to other bacteria these are more resistant to the action of disinfectants. *Staph. aureus* is very sensitive to aniline dyes and a concentration of 1:500,000 of crystal violet can inhibit their growth. Most strains can grow in the presence of 10% NaCl. Fatty acids inhibit the growth of staphylococci.

Biochemical Properties

There is no consistent pattern of biochemical reactions for *Staph. aureus*. The single most important test to differentiate *Staph. aureus* from *Staph. epidermidis* is the production of coagulase. Approximately 97% of staphylococci isolated from pathological lesions elaborate this enzyme. For remaining three percent, one has to resort to testing of DNAse to label it as *Staph. aureus*. They ferment a number of sugars producing acid but no gas. Fermentation of mannitol is of particular use since it is positive only for *Staph. aureus* strains.

Biotypes

On the basis of certain biochemical reactions staphylococci have been divided into six biotypes designated as A,B,C,D,E and F. The strains pathogenic to human beings fall in biotype A only.

Genetics

Plasmid mediated drug resistance has acquired special significance in *Staph. aureus*. Salient features of genetics of *Staph. aureus* are summarised in Table 27.2.

Table 27–2. Salient features of genetics of *Staph. aureus*

- Almost all strains are lysogenic
- Plasmids are responsible for:
  - — Resistance to antibiotics
  - — Resistance to heavy metals
  - — Production of bacteriocin
  - — Production of enterotoxin
  - — Production of epidermolytic toxin B
- Plasmids are transferred by transduction

Staphylococcin

This is a plasmid-mediated bacteriocin which is produced by many strains of *Staph. aureus*, most of which belong to phage group II. It is thermostable and active against many Gram-positive bacteria.

Antigenic Structure

More than 30 antigens have been detected from *Staph. aureus* so far. A few which have been characterised in detail include: capsular antigen, polysaccharide A, protein A, clumping factor and colony compacting factor (Fig. 27.4).

Capsular Antigens

A few strains of *Staph. aureus* are encapsulated and these tend to be more virulent than the noncapsulated strains.

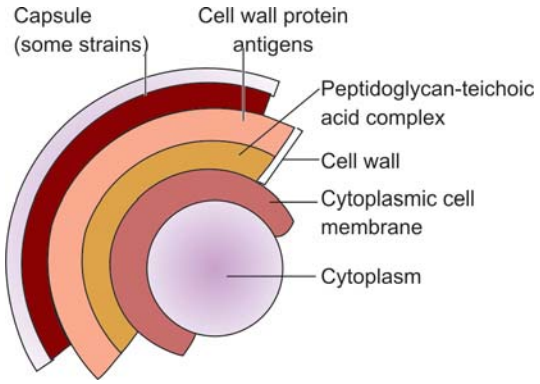


Fig. 27–4. Antigenic structure of *Staph. aureus*

PART V

Polysaccharide A and B

A group specific polysaccharide resides in the cell wall of bacterium. Polysaccharide A occurs with mucopeptide in the cell wall in an insoluble form and requires the lytic enzymes for release. This group specific polysaccharide is also present in strains of *Staph. epidermidis* but differs from polysaccharide A of *Staph. aureus* and is designated as polysaccharide B.

Protein A

This is a group specific antigen present on the surface of the bacterium and also appears in culture medium. More than 90% strains of *Staph. aureus* possess this antigen. The essential properties of protein A are listed in Table 27.3.

Table 27–3. Properties of protein A of *Staph. aureus*

- Antigenic; mol wt : 13,000-42,000
- Anticomplementary
- Antiphagocytic
- Causes hypersensitivity reactions
  - — Arthus phenomenon
  - — Local and systemic anaphylaxis
- Causes induction of T and B cells
- Precipitated by all normal human sera
- Reacts with Fab portion of specific antibody
- Combines nonspecifically with a wide variety of mammalian antibodies (Fc portion)
- Used for coagglutination

The nonspecific adsorption of immunoglobulin to protein A has been exploited as a means of detecting antigen-antibody reaction. The test has been named as coagglutination in which protein A forming staphylococci coated with immunoglobulin are mixed with antigen (bacterial suspension, cellular extract or pathological specimen) and in case of specific interaction between antigen and antibody aggregation occurs which becomes visible. This is shown in Figure 27.5.

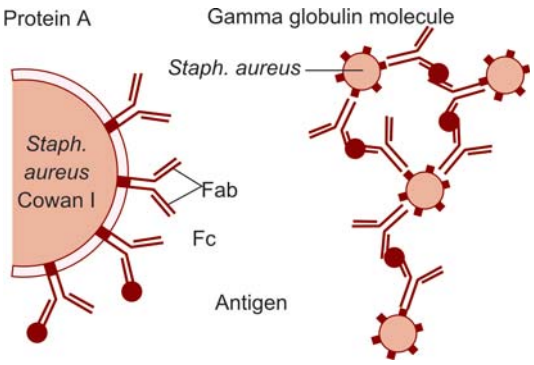


Fig. 27–5. Coagglutination

Clumping Factor (Bound Coagulase)

It was observed long ago that staphylococci that coagulated plasma were also clumped by it. This phenomenon was detected in noncapsulated strains of *Staph. aureus* when these were suspended in plasma or in fibrinogen containing solution. Clumping factor is surface component that reacts with both alpha and beta chains of fibrinogen and causes cross-linking. This factor is detected by performing the test on a slide and hence the test is erroneously also known as *slide coagulase test*. Almost all clumping factor producing strains also liberate coagulase whereas about 12% of coagulase producing strains may not liberate clumping factor. This factor is also not detectable in capsulated strains of *Staph. aureus.*

Colony Compacting Factor

In soft agar medium containing plasma, some strains of *Staph. aureus* produce compact spherical colonies. Though precise nature of this colony compacting factor is not known, it may be bound coagulase, protein A or teichoic acid.

Determinants of Pathogenicity

Staph.aureus has unmatched versatility and exceptional adaptability to survive, propagate and produce disease in the host because of a large number of antigens, toxins and enzymes liberated by this organism (Table 27.4).

Table 27–4. Determinants of pathogenicity of *Staph. aureus*

Surface antigens	*Enzymes*	*Toxins*
Capsule Polysaccharide A Protein A	Coagulase Staphylokinase Hyaluronidase Nuclease Lipase Phosphatase Penicillinase Proteases	Haemolytic toxins • Alpha-lysin • Beta-lysin • Gamma-lysin • Delta-lysin Leucocidin Enterotoxins Epidermolytic toxins Toxic shock syndrome toxin (TSST)

Haemolytic Toxins

The membrane of red blood cells is lysed by four toxins produced by *Staph. aureus*. These have been designated as alpha, beta, gamma and delta lysins.

Leucocidin

This staphylococcal toxin acts selectively on leucocytes. Apart from destroying leucocytes, it also brings about necrosis of skin. Its role in pathogenesis is uncertain.

Enterotoxin

This is produced by one-third of all the strains of *Staph. aureus*. In a protein rich food contaminated with enterotoxigenic *Staph. aureus* sufficient enterotoxin is formed at room temperature within four hours to cause clinical features of food poisoning in man. Patient does not die of this poisoning but may wish he had because the reactions are so violent. Some of the salient characteristics of staphylococcal enterotoxin are mentioned in Table 27.5.

Table 27–5. Staphylococcal enterotoxin

- Protein in nature, mol wt: 30,000
- Seven antigenic types: A, B, C1, C2, C3, D, E
- Type A commonly causes food poisoning
- Some strains may produce more than one enterotoxin serotype
- Resistant to:
  - — Boiling
  - — Trypsin
  - — Chymotrypsin
  - — Pepsin
- Inactivated by autoclaving (121°C, 30 mts)
- Biological activities:
  - — Induce vomiting
  - — Pyrogenic
  - — Mitogenic
  - — Cause hypotension
- No immunity is conferred
- Can be detected by:
  - — Gel precipitation
  - — Haemagglutination inhibition
  - — Radioimmunoassays
  - — ELISA

Epidermolytic Toxins

These toxins separate the outer layer of epidermis from the underlying tissues. These toxins are of two types: epidermolytic toxin A (ETA) and epidermolytic toxin B (ETB). Both are proteins with approximate molecular weight of 30,000 daltons. ETA is heat stable while ETB is heat labile. Production of ETA is under chromosomal control whereas ETB is plasmid mediated. Most of the strains belong to phage group II.

Toxic Shock Syndrome Toxin-1 (TSST-1)

This toxin was earlier designated as enterotoxin F because of its capability of producing enteritis. However, unlike other enterotoxins it does not cause food poisoning. It is a protein of molecular weight of 22,000 daltons. Toxin production is pH dependent and occurs at pH 7-8. Purified toxin is pyrogenic and releases interleukin-1 when injected into animals. The toxic shock syndrome is characterised by fever, hypotension, myalgia, vomiting and diarrhoea alongwith a rash. Most of the strains producing this toxin belong to phage group I.

Coagulase

Staph.aureus has the unique ability to clot a variety of mammalian plasmas. Clotting is caused by an extracellular product, coagulase (or free coagulase). Virtually all naturally occurring strains of *Staph. aureus* produce coagulase and since tests for coagulase detection are simple to perform, *Staph. aureus* species is usually identified by coagulase production rather than by serological or biochemical tests. *Coagulase producing strains of staphylococci are, by definition, Staphylococcus aureus.* Nevertheless, there is no conclusive evidence that coagulase producing strains are more virulent. No loss of virulence is seen in those mutants of *Staph. aureus* which do not produce coagulase.

Mechanism of action. Extracellular coagulase reacts with a component of plasma designated as coagulase reacting factor (CRF) to produce coagulase-CRF complex. This substance is clinically indistinguishable from thrombin. It reacts with fibrinogen to form fibrin which results in clotting of plasma.

Seven antigenically different extracellular coagulases have been identified from various staphylococci, but the only pathogenic role suggested for the enzyme is the coating of the organism with fibrin to inhibit their phagocytosis.

Tube test for coagulase determination. About 1.0 ml of an overnight broth culture or broth suspension from an agar plate is mixed with 1 ml of 10% (v/v)dilution of rabbit, human or pig plasma. The mixture is incubated at 37°C for 3-6 hours. If no clot appears it is left overnight at room temperature and reexamined (Fig. 27.6).

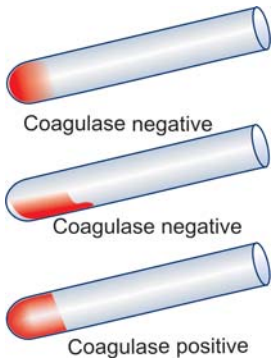


Fig. 27–6. Coagulase causing clotting of plasma

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As far as possible human plasma should not be used since it may contain variable amount of coagulase reacting factor and antistaphylococcal antibodies.

False results with tube coagulase test can be obtained when citrated plasma is employed for the test. *Pseudomonas*, *Serratia* and *Enterobacter* utilise citrate and cause clotting of plasma. Hence, plasma having EDTA is considered superior to the one which has been obtained from citrated blood. False negative reactions can be obtained when *Staph. aureus* strain produces so much staphylokinase that it lyses the fibrin clot.

Other coagulase positive staphylococci: Besides *Staph. aureus*, *Staph. intermedius* and *Staph. hyicus* which are animal parasities are also coagulase positive.

Slide coagulase detection test. It detects bound coagulase or clumping factor which has a good correlation with extracellular (or free) coagulase. This is done by mixing colonies of suspected *Staph. aureus* on a glass slide with undiluted plasma. Immediate clumping indicates presence of bound coagulase. False negative results are seen in as many as 15% of strains and hence strains which are negative for bound coagulase should be tested by tube method for free or extracellular coagulase.

Slide coagulase test should not be performed on colonies picked up from media having high salt content since high salt causes some of the strains to autoagglutinate.

Commercially available kits for latex agglutination and passive haemagglutination are also used for the detection of coagulase.

Important differences between clumping factor and coagulase are described in Table 27.6.

Table 27–6. Differences between clumping factor and coagulase

Clumping factor	*Coagulase*
Present on surface	Released extracellularly
Heat stable	Heat labile
One serotype	Seven serotypes
Detectable by slide test	Detected by tube test
All clumping factor +ve strains produce coagulase	All coagulase +ve strains may not produce clumping factor

Staphylokinase

Many strains of *Staph. aureus* that do not produce betalysin may produce staphylokinase. It can digest fibrin in the plasma of man. Its activity on a plate of blood agar may result into appearance of tiny spots of clearing at some distance from colonies of *Staph. aureus*. This phenomenon is known as *Muller phenomenon.*

Hyaluronidase

This enzyme is produced by almost all the strains of *Staph. aureus* albeit in differing quantities. It is postulated that this enzyme hydrolyzes the hyaluronic acid present in the intercellular ground substance of connective tissue, thereby, facilitating spread of infection. Since inflammation antagonizes the spreading action by hyaluronidase, its importance is limited to early stages of infection.

Nuclease

Production of a heat-resistant nuclease by *Staph. aureus* is a character which is unique to this species in the genus. It has been purified and well characterised. It is a compact globular protein consisting of single polypeptide chain.

Lipases

A large number of lipases are produced by *Staph. aureus* which act on an array of lipid substrates.The utilization of these substrates is of survival value to the organism. This explains the colonization of this bacterium in the sebaceous areas of greatest activity. A correlation between production of lipase and ability to cause boils has been demonstrated.

Pathogenesis and Clinical Features

The pathogenesis of staphylococcal diseases relates to resistance to phagocytosis, to the action of several staphylococcal enzymes, to the development of delayed hypersensitivity and to the activities of toxins. Man is constantly exposed to staphylococci from birth until death. Even so, the most virulent staphylococci seldom cause serious infections in a human host unless resistance is drastically lowered by events such as burns, trauma or surgery.

Two types of diseases are produced by *Staph. aureus*: invasive and toxigenic (Table 27.7).

The invasive lesions are suppurative whereas toxinoses are nonsuppurative.

Laboratory Diagnosis

a. *Specimens:* Clinical sample as per the site of lesion is collected. These could be pus, blood, surface swab, CSF or tracheal fluid, etc.
b. *Smears:* The diagnosis of staphylococcal disease is suggested by the finding of Gram positive bacteria in clumps in the pathological material such as cerebrospinal fluid, but final diagnosis can be

Table 27–7. Clinical entities caused by *Staph. aureus*

Invasive (suppurative)	*Toxinoses (Nonsuppurative)*
Carbuncle/furuncle	Food poisoning
Microabscesses	Toxic shock syndrome
Abscesses in deep organs	Scalded skin syndrome
Osteomyelitis	Enterocolitis
Pneumonia	
Septicaemia	
Endocarditis	
Pyoarthritis	
Meningitis	
Urinary tract infection	
Pharyngitis	
Endodontitis	
Styes and conjuctivitis	
Wound infection	
Impetigo	

achieved by culture and appropriate biochemical tests.

c. *Culture:* Pus, purulent fluid, sputum and urine can be directly streaked onto the blood agar. Specimens from patients who have been administered penicillin should be treated first with penicillinase to inactivate residual penicillin in that specimen. Heavily contaminated specimens such as faeces, dust or vomitus shall require use of selective media such as salt-milk agar or mannitol agar. The media are incubated aerobically at 37°C for 24 hours. For blood culture 10 ml of venous blood should be inoculated into 50 ml of tryptose-phosphate or glucose broth.

d. *Identification of Staph. aureus* depends upon its colony characters on media such as blood agar. The characteristic haemolysis and yellow golden pigmentation strongly point towards *Staph.aureus*. A positive coagulase test confirms the identity to be that of *Staph. aureus.*

E. *Serological diagnosis* has not been established in this infection. Many normal human beings carry antibodies to *Staph. aureus* and many a times infection is present without any antibody response in the patient. Serology can, however, provide clue to deep-seated hidden staphylococcal infection by the detection of antistaphylolysin antibodies in the serum of the patient.

F. *Antibiotic sensitivity* of these strains is determined on nutrient agar or Mueller-Hintons agar. Both disk diffusion and broth microdilution testing should be done routinely.

G. *Phage typing* If epidemiological tracing is to be conducted, use of phage typing technique is to be made.

Treatment

When penicillin was introduced almost all the strains of *Staph. aureus* were sensitive to this drug. Now more than 50% isolates show resistance to it. The resistance is due to the production of penicillinase (beta lactamase) by the organism which inactivates penicillin. Penicillinase resistant penicillins such as methicillin, oxacillin and cloxacillin can be used in treating patients having infection with penicillinase producing strains. Cephalosporins are the drugs of choice in those who are hypersensitive to penicillin.

Epidemiology

Staph. aureus is ubiquitous. Almost one third of adult population is asymptomatic carrier. The principal reservoir of staphylococci in nature is man. Direct cross infection from one human to another is very common and may either be airborne or result from direct contact. Staphylococcal disease may follow exogenous or endogenous infection.

Staphylococcal infections can never be completely eradicated because of carrier state in man. Control of spread of infection both in home and in the hospital requires proper hygiene care and proper disposal of pus contaminated material.

Hospital infection due to *Staph. aureus* is a worldwide phenomenon. These strains are resistant to penicillins and methicillin.

Bacteriophage Typing

Cultures of *Staph.aureus* are classified according to their susceptibility to a set of phages having relatively narrow host range at predetermined dilutions. Phages are chosen so as to make as many epidemiological valid distinctions as possible between the strains. It is, therefore, a method of bacterial classification based on a single class of character. The one thing that this typing system can never do is to show that the two isolates are "the same". What it can establish with varying degree of success is that the isolates are "different". Its use in the field investigations is to narrow down the field of enquiry by the exclusion of alternative sources of infection.

Most strains of *Staph. aureus* are lysogenic and they carry phages to which they themselves are immune but which will lyse some of the other members of the species. Susceptibility of *Staph. aureus* strains to the various temperate phages provides the basis for a phage typing system. The phage patterns of different strains fall into four broad groups (Table 27.8).

Table 27–8. Typing phages of *Staph. aureus*

Group	*Phages*
I	29, 52, 52A, 79, 80
II	3A, 3C, 55, 71
III	6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
Not Allocated	81,94,95,96

On a nutrient agar plate a "lawn" of *Staph. aureus* is prepared by spreading a four hours growth of bacterium and inoculating it with routine test dilution (RTD) of phages of basic set in a quantity of 0.2 ml each. The lysis of bacterium by phage or a number of phages denotes the phage type of that particular strain of *Staph. aureus* (Fig. 27.7).

A strain of *Staph. aureus* can be of phage type 3A or 47 or 85 or may be lysed by a large number of phages and the pattern is denoted by 81/94/95 or 3A/3C pattern and so on.

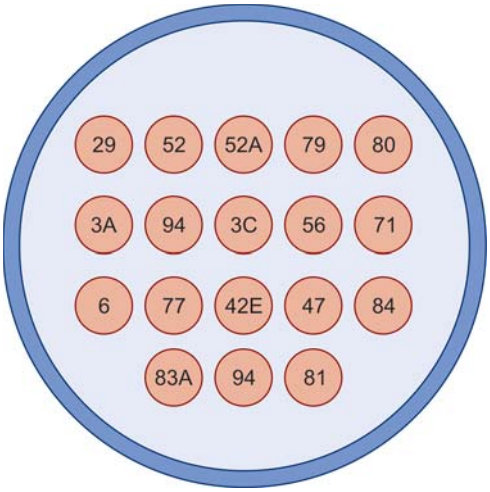

Fig. 27–7. Phage typing of *Staph. aureus*

COAGULASE NEGATIVE STAPHYLOCOCCI

Apart from *Staph. aureus* there are few other species of the genus *Staphylococcus*. Coagulase-negative staphylococci comprise a large group of related species which are commonly found on the surface of healthy persons in whom they are rarely the cause of infection. More than 30 species are recognised. Of these, *Staph. epidermidis* and *Staph. saprophyticus* are now being increasingly associated with certain pathologies in human beings. The types of infections commonly associated with coagulase negative staphylococci are shown in Table 27.9.

Coagulase-negative staphylococci are opportunistic pathogens that cause infection in debilitated or compromised patients. The organisms cause particular problems after cardiac surgery (prosthetic valve endocarditis), in patients fitted with CSF shunts or in immunocompromised patients.

Table 27–9. Lesions caused by coagulase negative staphylococci

Urinary tract infections	• Nosocomial • Community acquired
Infections of indwelling devices	• Prosthetic heart valves • Intravenous catheters • Joint prostheses • Haemodialysis and cerebrospinal shunts • Pacemakers • Peritoneal dialysis catheters
Bacteraemia in compromised hosts	• Premature infants • Patients with cardiovascular or neoplastic diseases • Burn patients • Transplant recipients • Patients with congenital defects
Osteomyelitis	• Postsurgical infections • Prosthesis associated infections
Postsurgical endophthalmitis	

Unlike *Staph. aureus*, coagulase-negative staphylococci produce few, if any toxins. Their pathogenicity is believed to be related to their ability to adhere to biomaterials consisting of synthetic polymers and production of extra-cellular slime.

STAPHYLOCOCCUS EPIDERMIDIS

This species is also known as *Staph.albus*. Colonies of this organism are usually white and rarely yellow or orange. It can be differentiated from *Staph.aureus* on the basis of coagulase production and certain biochemical tests (Tables 27.1 and 27.10).

STAPHYLOCOCCUS SAPROPHYTICUS

This organism was earlier considered as nonpathogenic, but recent studies have shown it to be capable of causing urinary tract infection in women during reproductive years. Unlike other staphylococci, this species is resistant to novobiocin. Its other characters have been described in Table 27.10.

Identification of Coagulase Negative Staphylococci

Apart from the tests mentioned in Table 27.1, these two species can be identified by following two methods:

Resistance to Novobiocin

Only *Staph. saprophyticus* is resistant to novobiocin. This can be determined by using a novobiocin disc having 5 µg of drug and measuring the zone of inhibition in a lawn prepared with test strain. A zone of inhibition of less than 12 mm indicates resistance.

Trehalose-Mannitol-Phosphatase Agar

This medium makes use of some properties of *Staph. saprophyticus* and *Staph. epidermidis* (Table 27.10) in identifying these.

Table 27–10. Differentiation between *Staph. epidermidis* and *Staph. saprophyticus*

Species	*Trehalose and mannitol fermentation*	*Alkaline phosphatase production*	*Changes in medium*
Staph. epidermidis	–	+	Colonies turn pink when spotted on filter paper moistened with 1N ammonium hydroxide
Staph. saprophyticus	+	–	Colour of medium changes from purple to yellow

MICROCOCCUS

Micrococci are larger in size than staphylococci with diameter of upto 2 µm. These are usually arranged in tetrads or cubical packets (Table 27.11). Nine species of this genus are now recognised. They produce circular colonies which may have yellow, pink or red pigment. They have doubtful pathogenicity in patients whose immunological defences are compromised.

PLANOCOCCUS

These are Gram positive flagellated cocci which are found in sea water, shrimps and prawns. These have the ability to grow in a medium having 12% NaCl and on the basis of this character, these can be differentiated from other Gram positive cocci (Table 27.11). These are nonpathogenic to humans.

Table 27–11. Differentiation between Staphylococcus, Micrococcus and Planococcus

Feature	*Staphylococcus*	*Micrococcus*	*Planococcus*
Motility	–	–	+
Arrangement	Clusters	Clusters	Tetrads
Fermentation of glucose	+	+	–
Production of brown pigment	–	–	+
Presence of Teichoic acid	+	–	–

PART V

28 Streptococcus

Streptococci are gram-positive cocci which grow in chains (*strept*) in liquid medium. These were discovered in 1874 by Bilroth.

Man is most susceptible of all the animals to streptococcal infections. No organ or tissue of the body is completely immune to infection by streptococci. In addition, infections caused by it may lead to post-infection syndromes of acute rheumatic fever, rheumatic heart disease and acute glomerulonephritis.

Classification

The genus *Streptococcus* is the only genus of medical importance in the family *Streptococcaceae*. These organisms are gram-positive, spherical to ovoid in shape and less than 2 µm in diameter. Their inability to produce catalase is an important feature that distinguishes these from staphylococci. These organisms divide in one plane to form chains or pairs (Fig. 28.1).

The genus *Streptococcus* is the only one of the five genera of family *Streptococcaceae* that contains organisms pathogenic to man. Two classifications are currently in vogue. The classification which is widely accepted at present is shown in Figure 28.2. The second classification is based upon haemolysis produced by organism on blood agar medium.

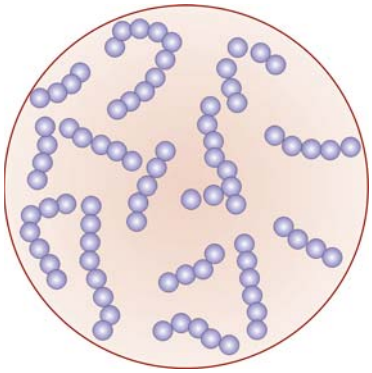

Fig. 28–1. Streptococci

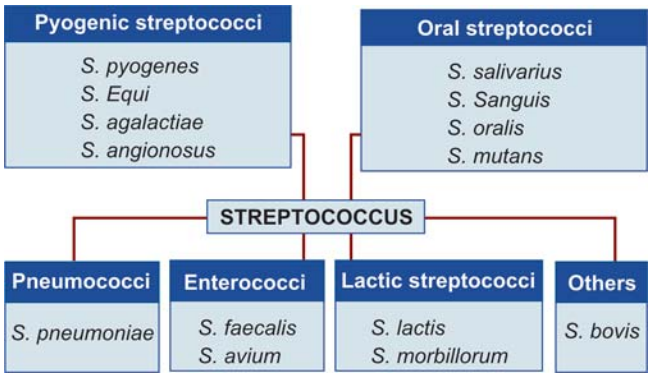

Fig. 28–2. Classification of streptococci

Classification Based Upon Haemolysis

On the basis of their action on blood agar medium and lysis of red blood cells, streptococci have been classified into three groups:

Alpha Haemolytic Streptococci

The colonies produced by this group on blood agar medium are surrounded by a narrow zone of haemolysis, with unhaemolysed RBCs persisting in the inner zone and complete haemolysis in the outer zone. A greenish discolouration takes place because of the formation of reductase of haemoglobin. The greenish discoloration gives the name *viridans* to these streptococci (*viridan*:green). The most common species belonging to this group is *Strept. salivarius.*

Beta Haemolytic Streptococci

These organisms produce complete haemolysis of RBCs and colonies are surrounded by a clear zone of haemo-

lysis (beta haemolysis). This lysis of RBCs is due to the production of two types of streptolysins by the organisms: *streptolysin O* and *streptolysin S*. To this group belongs the most important species for man: *Streptococcus pyogenes*.

The beta haemolytic streptococci have further been subdivided into a number of *immunologic groups* designated by latters A to V. These groups are known as Lancefield groups after the name of Rebecca Lancefield who introduced this type of serological method of classification.

Lancefield Grouping

The beta haemolytic streptococci have been separated into 20 immunologically distinct groups which have been designated as A, B, C, D, E, F, G, H, K, L, M, N, O, P, Q, R, S, T, U and V. This grouping depends upon the presence of *group specific carbohydrate antigens* (C- carbohydrate) in their cell wall. These carbohydrates can be extracted by any of the following methods:

a. Acid extraction with HCl (Lancefield's method)
b. Formamide extraction at 150°C (Fueller's method)
c. Autoclaving (Rantz and Randall's method)
d. Enzyme extraction (Maxted's method).

After the extraction of carbohydrate moiety it is reacted with type specific antiserum by a precipitation reaction. Most haemolytic streptococci that are pathogenic to man fall in group A, also called as *Streptococcus pyogenes*.

Gamma Haemolytic Streptococci

These do not produce any haemolysis or discolouration on blood agar. *Strept. faecalis* is the typical non-haemolytic species.

STREPTOCOCCUS PYOGENES

Morphology

These are gram-positive, nonmotile-catalase negative cocci that are usually arranged in chains (Fig. 28.1). Individual cocci measure 0.6 to 1.0 µm in diameter. Growth occurs by elongation on the axis parallel to the chain and division is at right angles in the equatorial plane. Whereas pneumococci and oral streptococci grow in pairs or short chains in liquid medium, *Strept. pyogenes* grows in longer chains (5 cocci or more). However, these fail to grow indefinitely since most of streptococci liberate a *dechaining factor*.

Many strains produce capsule. In group A strains the capsule is hyaluronic acid in nature. In addition,

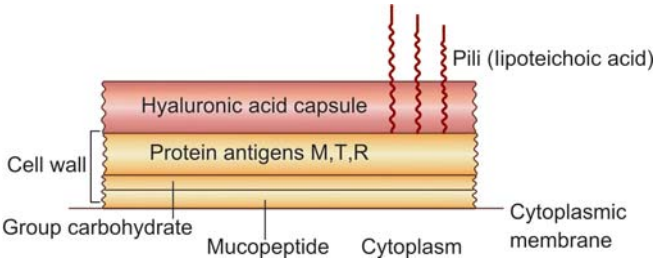

Fig. 28–3. Surface structures on streptococci

three major proteins, M,T and R, are present on the surface of the bacterium (Fig. 28.3).

The M protein is of major importance since it is antiphagocytic and hence directly involved in virulence. The other two (T and R proteins) are not related to virulence.

Cultural Characters

Strept.pyogenes is facultative anaerobe and best growth is achieved at pH 7.4-7.6 and at temperature of 37°C.

Growth on Blood Agar

Growth on ordinary media is poor and blood or blood products are required for the luxurious growth of these bacteria. For the primary isolation, blood agar prepared with sheep blood is preferred. Human blood should not be used unless proven to be free of inhibitory substances.

Growth is profuse on media having blood, yet the colony size on blood agar seldom exceeds 1 mm after 24 hours of incubation. Growth can be enhanced by incubating at reduced oxygen tension.

Strept.pyogenes is invariably beta haemolytic and the zone of haemolysis is far greater than the size of colony. The colonies are around 1 mm in diameter, surrounded by a zone of clear haemolysis, semi-transparent, low convex, discrete and vary in appearance.Three types of colonies are encountered:

Mucoid colonies are produced by strains forming large amounts of hyaluronic acid. Strains with M protein give rise to **matt** colonies whereas those without it produce **glossy** colonies.

Growth on Selective Media

These organisms are capable of growth in blood agar having 1mg/litre of crystal violet. At this concentration of crystal violet, other Gram positive cocci are inhibited. Crystal violet blood agar is hence used as a selective medium for streptococci.

Growth in Liquid Media

In liquid media enriched by glucose or serum, streptococci grow profusely giving powdery turbidity and granular deposits at the bottom as well as along the sides of the tube.

Biochemical Reactions

These do not produce catalase and oxidase enzymes, but ferment a large number of sugars with the production of acid alone.

Susceptibility to Physical and Chemical Agents

Strept. pyogenes is killed at 55°C within 30 minutes, by usual concentration of disinfectants but resists daylight for weeks. The organism is sensitive to penicillins and a wide variety of antimicrobial drugs. It is naturally resistant to aminoglycosides and has acquired resistance to sulfonamides, tetracyclines and to some extent to clindamycin.

Antigenic Structure

Several antigenic substances are found in group A streptococci (Fig. 28.4). These have been enumerated in Table 28.1.

Table 28–1. Antigenic structures on *Strept. pyogenes*

- Capsule
- Group specific cell wall antigen
- Type specific cell wall antigens
- M protein
- T protein
- R protein
- Nucleoprotein
- Lipoteichoic acid

Capsule

The streptococcal cell wall is encompassed by a hyaluronic acid capsule that is also antiphagocytic and serves as an accessory virulence factor. The degree of encapsulation varies greatly among strains of group A streptococci. Colonies of organisms that have particularly prominent capsules have a highly mucoid appearance on blood agar plates. Group A streptococcal strains that are rich in M proteins and are heavily encapsulated are readily transmitted from person to person and tend to produce severe infections.

Group Specific Cell Wall Antigen

The cell walls of pyogenic streptococci contain a polysaccharide antigen. The group polysaccharide is nontoxic and is hapten in rabbits. Human infection may be followed by the appearance of antibody to group polysaccharide. Group A polysaccharide consists of chain of rhamnose molecules with little branching.

Type Specific Cell Wall Antigens

M Protein

M protein is the chief virulence factor of group A streptococci. Organisms that are rich in M protein resist phagocytosis by polymorphonuclear leukocytes and multiply rapidly in fresh human blood. Group A streptococci may be divided into serotypes based on

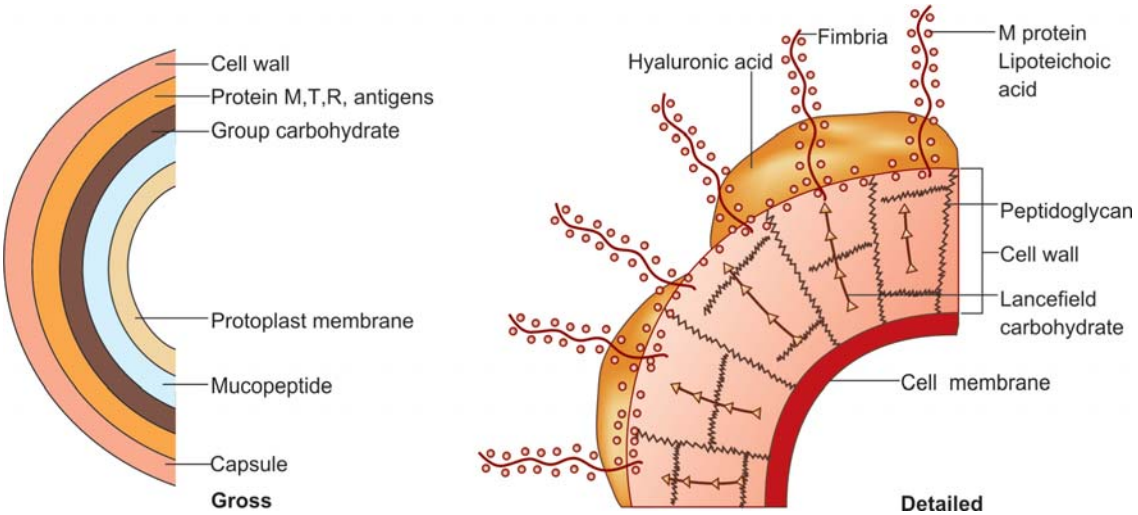

Fig. 28–4. Antigenic structure of *Strept. pyogenes*

the M protein molecule. More than 80 such serotypes are currently recognised.

T Proteins

T antigens are resistant to trypsin and pepsin but are acid and heat labile. This antigen has no relationship with virulence of group A streptococci.

T typing scheme has been developed as an important epidemiological surveillance tool. This is done by slide agglutination test, using trypsin treated whole streptococci. Some T antigens are restricted to a single M type, while others may be shared by several M types.

R Proteins

The R antigens are a series of antigens that can cause cross reaction in grouping or typing antisera that contain antibodies to one or other of them. These are nonprotective. It is also believed that R antigens may be biologically defective or inactive forms of M proteins.

Nucleoproteins

These are also called as P substances and can be obtained by alkali extraction of streptococci. These do not have any serological specificity and perhaps make up most of the streptococcal cell body.

Lipoteichoic Acid

In addition to M proteins, lipoteichoic acid is expressed on the surface of group A streptococci and plays a critical role in the host-bacterial interaction. Lipoteichoic acid is the adhesion responsible for the binding of the organism to the surface of oral epithelial-cell membranes and is thus critical in colonization of infection.

Determinants of Pathogenicity

More than 20 soluble antigens are produced by streptococci of group A which help the organism in producing large array of clinical conditions. Important amongst these are given in Table 28.2.

Table 28–2. Toxins and enzymes liberated by group A streptococci

- Haemolysins (Streptolysin): Streptolysin O, Streptolysin S
- Erythrogenic toxin (Pyrogenic exotoxin)
- Streptokinase (Fibrinolysin)
- Deoxyribonuclease (Streptodornase)
- Hyaluronidase
- Proteinase
- Serum opacity factor
- Neuraminidase
- Nicotinamide adenine dinucleoitidase

Haemolysins

Strept.pyogenes elaborates two haemolysins. One of these is oxygen labile and hence named as streptolysin O (SLO) and the other being stable to the action of oxygen and soluble in serum is designated as streptolysin S (SLS).

Streptolysin O. It is responsible for haemolysis in the deeper layers of blood agar where streptococci grow in the absence of oxygen.

SLO is toxic for red blood cells, white blood cells, as well as myocardial cells in tissue cultures. On intravenous administration in animals it may cause death due to cardiac failure. High titres of antibodies against this antigen (ASO) are seen following recent systemic streptococcal infections or as an exaggerated immune response to an earlier exposure in a hypersensitive person. A titre of more than 300 is usually considered as high.

Streptolysin S. This agent is responsible for the haemolytic zone around the streptococcal colonies growing on the surface of blood agar in the presence of oxygen. It is nonantigenic because of its low molecular weight.

Erythrogenic Toxin (Pyrogenic Exotoxin)

It is a soluble product which cannot withstand boiling for more than 60 minutes. Four erythrogenic toxins are known and most strains of *Strept. pyogenes* produce one or more.

Streptococcal pyrogenic exotoxins exert profound effect on immune system, including enhancement of susceptibility to endotoxic shock, blockade of reticuloendothelial system and alterations in the T cell function.

These toxins are produced only by those strains of group A streptococci which are carrying temperate phage in their genome.

Classically it was thought that this toxin caused red reaction in the skin of nonimmune individual (*positive Dick test*) and no reaction in individuals with immunity (*negative Dick test*). Antitoxin injected into the skin of a patient with scarlet fever causes localised blanching due to neutralization of erythrogenic toxin (*Schultz-Charlton reaction*).

Streptokinase

This is a fibrinolysin which is produced by the group A streptococci. Strains from groups C and G also produce it. It transforms the plasminogen of human plasma into plasmin, an active proteolytic enzyme that digests fibrin and other proteins. Anti-streptokinase

which is a specific antibody against streptokinase inhibits this activity.

The streptokinase is now extensively used intravenously for the treatment of pulmonary and coronary artery emboli and venous thrombosis.

Streptococcal Deoxyribonuclease (Streptodornase)

All the strains of *Strept. pyogenes* produce at least one deoxyribonuclease (DNase). Various strains of streptococci produce 4 types of DNase of which type B is the predominant nuclease in *Strept. pyogenes.* An antibody to DNase develops after infection with these organisms and can be measured for diagnosis.

Hyaluronidase

Hyaluronic acid is an important component of connective tissue. Hyaluronidase liberated by group A streptococci splits it and thus helps the organism in spreading. Sometimes this is also referred to as spreading factor. Nearly all the strains of *Strept. pyogenes* produce this enzyme.

Hyaluronidase is antigenic and gives rise to antibodies which can be measured for serological evidence of infection.

Serum Opacity Factor

Most of the strains of group A streptococci produce a substance which will produce opalescence of horse serum when allowed to grow in a medium having this serum and broth. Apart from group A, some strains of group L streptococci also produce it.

Nicotinamide Adenine Dinucleotidase

This is formed by many, but not all, strains of group A streptococci as well as some strains of groups C and G.

Neuraminidase

Many strains of groups A, B, C, G and L have been shown to produce this enzyme. In some of the isolates from babies with invasive streptococcal diseases, greater enzyme production capacity has been detected which makes it a candidate virulence factor for streptococci that survive on mucosal surfaces.

Other Enzymes

Some strains of group A streptococci produce diphosphopyridine nucleotidase enzyme which is related to the ability of streptococcus to kill leukocytes. An **endopeptidase** is also produced by some group A streptococci which attacks the complement cleavage product, C5a which induces the migration of leukocytes into an area of complement activation. Proteinases and amylases are also produced by certain bacteria.

Pathogenesis

The commonest *Streptococcus* that causes diseases in human beings is *Strept. pyogenes.* The most frequent primary infections are sore throat and pyoderma whereas acute glomerulonephritis and rheumatic fever are late sequelae. This organism is a human pathogen and does not cause any disease in animals.

Group A streptococci possess numerous factors that enhance virulence and allow the organism to establish themselves in the host. The cell wall of these organisms is resistant to lysozyme and persist in cells or tissues indefinitely.

Clinical Manifestations

The major types of clinical conditions associated with group A streptococci are shown in Figure 28.5 and Table 28.3.

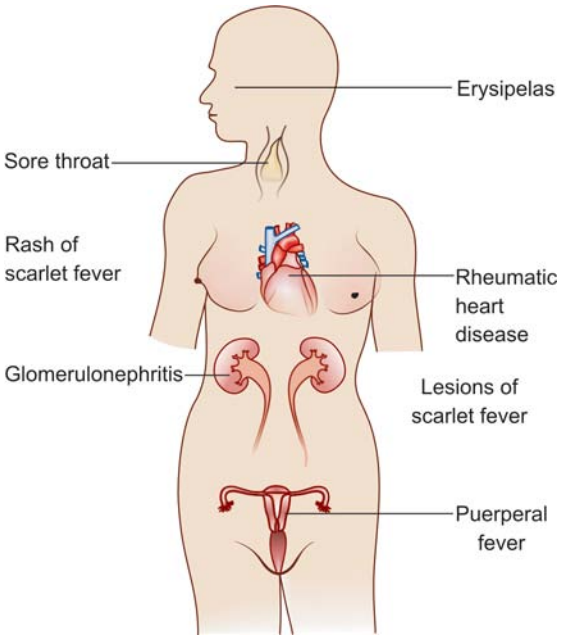

Fig. 28–5. Major clinical diseases due to group A streptococci

Post-streptococcal Diseases

Glomerulonephritis and rheumatic fever are the sequelae of streptococcal infections and usually occur 1 to 4 weeks after the acute infection. These two conditions are not infectious but occur as a direct result of previously untreated streptococcal pharyngitis or pyoderma.

Table 28–3. Diseases due to group A streptococci

Local infections	• Sore throat • Pyoderma
Invasive diseases	• Erysipelas • Puerperal fever • Sepsis • Toxic shock like syndrome
Infective endocarditis	• Acute endocarditis • Subacute endocarditis
Other infections	• Meningitis
Post-streptococcal disease	• Rheumatic fever • Glomerulonephritis

In spite of much research, the mechanism and pathogenesis of these diseases are not well understood. Some evidence indicates that these are immune complex diseases. The immune complexes stimulate inflammatory response and inflammation results in formation of scar tissue, which replaces some of the normal body tissue. The antibodies involved in these two diseases are those produced in response to streptococcal infection.

Acute Rheumatic Fever

This entity consists of diverse clinical manifestations and results in almost 0.5% of all children who suffer from acute streptococcal pharyngitis. During epidemic by any type of group A streptococcus, this figure may go up to 3%. The major mortality and morbidity associated with rheumatic fever are due to the subsequent development of rheumatic valvular heart disease.

Clinical manifestations of acute rheumatic fever can be divided into *major manifestations* and *minor manifestations* (Table 28.4). On the basis of these manifestations certain criteria were established by Ducket Jone which are now called as Jone's modified criteria for the diagnosis of this condition.

Table 28–4. Modified Jone's criteria

Major manifestations	*Minor manifestations*
Carditis	Fever
Arthritis	Arthralgia
Chorea	Elevated ESR or CRP
Erythema marginatum	ECG changes
Subcutaneous nodules	Previous history of rheumatic fever or rheumatic heart disease

According to Jone's criteria two major or one major and two minor manifestations with evidence of previous streptococcal infection indicate a high probability of rheumatic fever.

The *pathogenesis* of rheumatic fever is poorly understood. A variety of theories have been put forth which include antigenic cross reactivity between cardiac tissue and streptococcal antigen, direct toxicity of streptococcal exotoxin, actual invasion of heart by streptococci and localization of antigens within damaged tissue or valves.

It is now known that the streptococcal strains causing acute rheumatic fever and acute glomerulonephritis in the same population are different. The M serotype represents at least one marker of rheumatogenicity. With rare exceptions, the M serotypes most strongly related epidemiologically with acute rheumatic fever and postpharyngeal and postpyodermal acute glomerulonephritis appear to be distinct (Table 28.5).

Table 28–5. M serotypes associated with post-streptococcal sequelae

Sequelae	*M type*
Acute rheumatic fever	1, 3, 5, 6, 14, 18, 19, 24
Acute glomerulonephritis	
Post pharyngeal	1, 4, 12
Post pyodermal	49, 55, 57, 60

Acute Poststreptococcal Glomerulonephritis

This condition is most often seen in children and is characterised by acute onset of edema, oliguria, hypertension, congestive heart failure and seizures. Serological evidence of recent streptococcal infection confirms clinical findings. There are certain M types which are called as *nephritogenic* M types (Table 28.5) and infections with carry more chances of development of these sequelae. Immunofluroescent examination of biopsy material from kidneys shows complement components with or without immunoglobulins. The inflammatory response is possibly due to deposition of immune complexes within the kidney, although the localization of streptococcal cellular components or exotoxins may also play a part.

Essential comparative features between acute rheumatic fever and acute glomerulonephritis are given in Table 28.6.

Laboratory Diagnosis

For the laboratory diagnosis of a suspected streptococcal infection, the clinical sample shall depend upon the nature of the infection. For the direct demonstration and isolation of streptococci a throat swab, pus or blood is obtained whereas evidence of antibody production can be assayed from the examination of serum.

Table 28.6: Essential comparative points between acute rheumatic fever and acute glomerulonephritis

	Acute rheumatic fever	*Acute glomerulonephritis*
Site of infection	Throat	Throat or skin
Prior sensitisation	Essential	Not necessary
Immune response	Marked	Not so marked
Complement level	Not lowered	Lowered
Repeated attacks	A feature	Not a feature
Antibiotic prophylaxis	Required	Not required
Course of disease	Progressive or static but never spontaneous resolution	Spontaneous resolution
Prognosis	Variable	Good

Direct Demonstration

Smears prepared from specimens such as pus, wound exudate, and CSF shall show single cocci or pairs rather than definite chains. On Gram staining these exhibit Gram positive character. If smear shows Gram positive cocci, but the culture fails to yield anything, infection with anaerobic streptococci should be suspected. Smears from sites such as throat shall not be of much use because of the presence of morphologically resembling normal flora.

Culture and Identification

Cultures are made on blood agar having 5-10% blood and incubated at 37°C for 24 hours. Incubation in the presence of CO_2 or by slicing the inoculum onto blood agar gives better haemolysis. The degree and the kind of haemolysis helps in identifying the organism.

Group A streptococci do not grow on MacConkey agar. If the clinical specimen is from a site suspected to be having contamination with other Gram positive cocci, crystal violet blood agar should also be inoculated which will permit the growth of beta streptococci and inhibit rest of the bacteria.

Group A streptococci from the plates can be confirmed with the help of fluorescent antibody test. Bacitracin sensitivity is also a feature of group A streptococci which are otherwise oxidase and catalase negative. A presumptive diagnosis can be made on the basis of these features.

For grouping of isolated streptococci, Lancefield's technique or any of its modifications can be adopted.

Antigen Detection Tests

Several kits are now available commercially for the identification and grouping of streptococci. Though these kits are rapid and sensitive, their high cost prohibits their use in routine. Latex particle agglutination kit as well as coagglutination kits are available.

Typing of Group A Streptococci

These tests are undertaken in specialised laboratories and use precipitation and agglutination techniques of M typing and T typing. Some laboratories are using typing schemes for serum opacity factor.

Serological Tests

These tests detect antibody to extracellular products liberated by *Strept. pyogenes*. These are preferably performed on paired serum samples to detect rise in antibody titre to one or other products. These tests can be used to confirm the presumed bacteriological diagnosis as well as for the diagnosis of post streptococcal sequelae where isolation of streptococci or detection of their antigens is not possible.

Test for the detection of antistreptolysin O antibody is most frequently done. The upper limit of antibody level in the normal individuals is around 200 Todds unit/ml. Similarly anti-DNase B estimation has been found to be useful in these conditions. Other antibodies which can be measured are against streptokinase and NADase.

Antibody to M protein can also be measured by several tests but these are rarely performed.

Treatment

All groups of streptococci are sensitive to penicillin G and most are sensitive to erythromycin. Penicillin is effective in more than 90% of cases and should be the drug of choice. In patients who are allergic to penicillin, erythromycin is recommended. Tetracylines and sulfa drugs are generally not recommended because streptococci are quick to develop resistance to these agents.

OTHER PATHOGENIC STREPTOCOCCI

Streptococcus agalactiae (Group B streptococci)

Morphology and Physiology

These bacteria are part of normal flora of female genital tract and increasingly recognised as important causative agents of neonatal sepsis and meningitis.

Morphologically these organisms are identical to group A streptococci. In liquid medium these grow as diplococci or form short chains. On blood agar medium, haemolysis is produced which may be complete (beta)

in nature or incomplete (alpha) or no haemolysis. The lysis of erythrocytes is the result of a haemolysin which is similar to streptolysin O of *Strept. pyogenes.* The colonies are bigger than that of group A streptococci but the zone of haemolysis is smaller. When grown on blood agar having rabbit blood these produce double zone of haemolysis, if refrigerated after initial incubation.

Practically all strains of group B, whether haemolytic or otherwise, give a positive CAMP (**C**hristie, **A**tkins, **M**unch-**P**eterson) reaction. They produce a diffusible substance that completes the lysis of sheep erythrocytes when exposed to sphingomyelase Cl—the staphylococcal beta lysin or the alpha toxin of *C. perfringens.*

Antigenic Structure

The group specific antigen is composed of D-galactose, D-glucosamine and L-rhamnose. L-rhamnose is the major antigenic determinant. Five serotypes have been recognised of which four are polysaccharides and designated as Ia, Ib, II and III and the fifth is protein in nature and known as Ic. Two more protein antigens have been detected on the surface. These are R and X-antigens.

Pathogenesis and Clinical Picture

Group B streptococci are commonly found in the flora of female genital tract, pharynx and gastrointestinal tract. About 5-10% of pregnant women are carriers of this organism and are believed to transmit the organism to newborns. The determinants of pathogenicity are poorly understood but a large number of products of group B streptococci are potential candidate virulence factors. The diseases produced by this bacterium are shown in Table 28.7.

Table 28–7. Clinical diseases produced by group B streptococci

Age group	*Disease*
Newborn	
Early onset infection	• Pneumonia • Meningitis • Respiratory distress
Late onset infection	• Meningitis • Osteomyelitis
Adult	• Septicaemia • Endocarditis • Meningitis • Arthritis • Wound sepsis • Pyoderma

Infections in adults are mainly in women after childbirth or abortion and in adults in any gender who have any underlying immunodeficiency.

Treatment

Penicillin G is the drug of choice for the treatment of infections due to group B streptococci.

GROUP C AND G STREPTOCOCCI

These are basically animal pathogens. Rarely these have been isolated from human infection such as impetigo, abscesses, pneumonia and pharyngitis as well as endocarditis. These often look like group A streptococci on blood agar medium where these also produce beta haemolysis. They are identified by reaction with specific antisera for groups C and G.

These, however, differ from group A by causing neither glomerulonephritis nor rheumatic fever and by possessing a group specific carbohydrate polymer of L-rhamnose in group C and galactose, galactosamine and rhamnose in group G.

GROUP D STREPTOCOCCI

The organisms belonging to this group are also known as **enterococci** and seven species have been grouped under this genus. These are oval cocci which are seen in pairs or short chains. Some of the saprophytic members are motile. Colonies of these streptococci are larger than those of group A streptococci. It can cause alpha, beta or gamma haemolysis on blood agar. Colonies on MacConkey agar are minute, pin point and magenta coloured. Some of the important differentiating characters have been shown in Table 28.8.

Table 28–8. Differentiating features of groups A, B and D streptococci

Feature	*Group A*	*Group B*	*Group D*
Haemolysis	Beta	Beta	Alpha, beta, gamma
Bacitracin sensitivity	+	–	–
Hippurate hydrolysis	–	+	–
Esculin fermentation	–	–	+
Growth in 6.5% NaCl	–	+	+
CAMP test	–	+	–
Resistant to 60°C/30 mts	–	–	+

PART V

Epidemiology and Clinical Features

Enterococci are common inhabitants of skin, upper respiratory, gastrointestinal and genitourinary tracts. Man to man transmission has been documented and disease results because of the invasion of these bacteria from normal flora.

These bacteria are frequently associated with urinary and biliary tract infections, septicaemia, endocarditis, wound infection and intra-abdominal abscesses. They also cause bacterial endocarditis especially in those who have an underlying valvular disease.

Treatment

Most of the isolates of enterococci are resistant to penicillin. However, clinical response may be observed if penicillin is given alongwith aminoglycosides. Ampicillin and erythromycin are also useful in these infections.

VIRIDANS STREPTOCOCCI

This is a heterogenous group of very poorly defined alpha haemolytic streptococci that are present in abundance in the mouth and pharynx. About 50% of bacterial endocarditis is caused by these bacteria. Since they do not have a defined group carbohydrate antigen, no correlation exists between serologic and physiologic characteristics of these strains.

The viridans streptococci comprise of six species viz. *Strep. pneumoniae, Strept. mutans, Strept. sanguis, Strept. salivarius, Strept. milleri* and *Strept. mitior.*

Clinical Significance

When isolated from mouth, throat or respiratory tract, viridans streptococci are generally regarded as harmless commensals. The medical importance of this group is because of its association with dental caries, sepsis and bacterial endocarditis. *S. milleri* is associated with deep sepsis, including liver and brain abscesses. *S. mutans, S. mitior* and *S. sanguis* are involved in the production of dental caries. Endocarditis can be caused by *S. mitior* and *S. sanguis*. Nothing much is known about the pathogenicity of *S. salivarius* but is believed to be capable of causing dental caries. Streptococcus MG group is one of the viridans group which share certain antigens with *Mycoplasma pneumoniae*.

Dental Caries

It is a multifactorial disease that results only when all dependent factors coincide (Fig. 28.6).

Three important factors that cause it are:

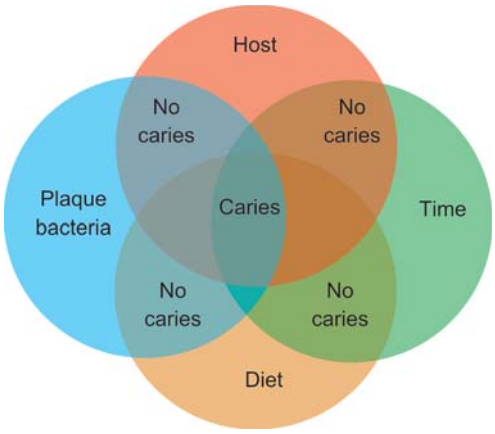

Fig. 28–6. Factors causing dental caries

1. Susceptibility of host and teeth. Pits, fissures and spaces tend to entrap food and let microflora flourish.
2. Excessive dietary sucrose intake which can be fermented by bacteria to produce acid which can demineralize the dental enamel.
3. Cariogenic microflora which comprises of various species of streptococci and lactobacilli. Among the streptococci, *S. mutans, S. mitior* and *S. sanguis* have been shown to cause dental caries even in experimental animals. Species of *Lactobacillus* which are generally present in oral cavity and have been incriminated as possible causative agents of dental caries are *L. casei, L. fermentum, Bifidobacterium bifidum* and *L. acidophilus.*

A brief summary of identification of streptococci is shown in Figure 28.7.

Infective Endocarditis

Viridans streptococci account for about 40% of the cases of infective endocarditis. *Strep. sanguis* and *Strep. mitis* are most commonly incriminated followed by *Strep. mutans* and *Strep. milleri.*

Factors predisposing to infective endocarditis include:

i. Cardiac factors
   - Rheumatic heart disease
   - Congenital heart disease
   - Cardiac surgery
   - Prosthetic heart valves

ii. Non-cardiac factors
   - Dental manipulations
   - Sepsis

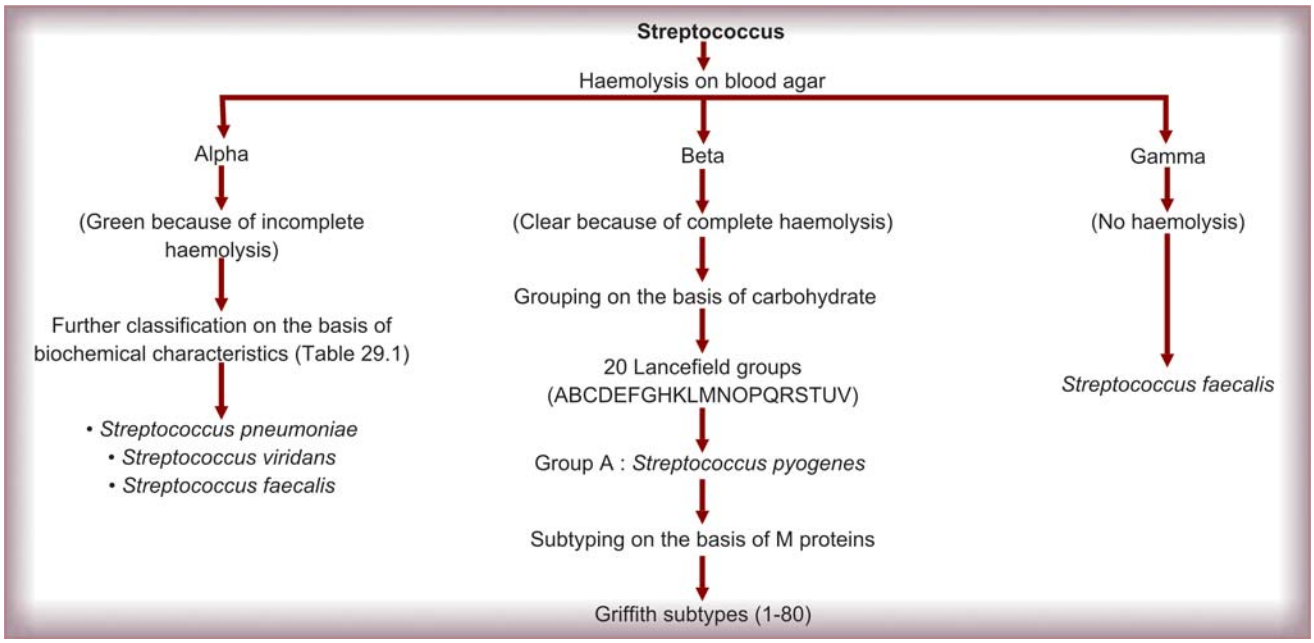

Fig. 28–7. Brief summary of identification of streptococcus

- Intravenous drug abuse
- Intravenous shunts
- Endoscopy

Diagnosis

Repeated blood cultures be performed to establish the diagnosis and aetiological role played by the organism isolated.

29 Pneumococcus

Pneumococcus *(Streptococcus pneumoniae)* was discovered by Pasteur in France and Sternberg in the USA in 1881.

Habitat

Pneumococci are normally present in the nasopharynx of many healthy persons. Carriage rate may be upto 30% in some communities. The organism usually does not cause any illness itself unless a viral infection or some other predisposing factor provokes it to spread to lower respiratory tract, middle ear, sinuses and blood and cause a large number of clinical entities.

Morphology

Pneumococci are gram-positive cocci which are usually seen in pairs and hence sometimes called as diplococci. These are oval or spherical in shape whereas when seen in clinical material these look like lancet shaped, in pairs or in short chains (Fig. 29.1). They measure 0.5 to 1.25 µm, are nonmotile and nonsporing. The capsule can be readily demonstrated by India Ink method or by the use of type specific antiserum in the quellung reaction.

Continued laboratory cultivation, especially on unfavourable media, leads to formation of longer chains as well as loss of gram-positivity. Ageing cultures also show gram-negative reaction.

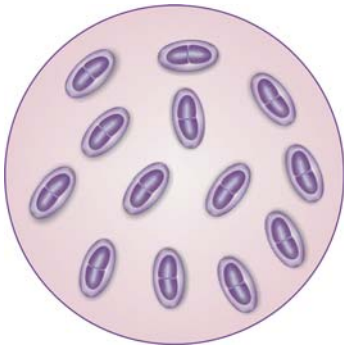

Fig. 29–1. Pneumococci

Cultural Characters

These are facultative anaerobes and can grow at temperatures between 25 and 40°C (optimal: 37°C).

Growth on Blood Agar

Growth is better in an environment of 5-10% carbon dioxide and on media enriched with blood, serum or heated blood. These substances provide essential nutrients as well as enzymes such as catalase which can neutralise the toxic hydrogen peroxide liberated by pneumococci.

Young cultures show small, smooth and transparent colonies which are low convex but become flattened or depressed centrally showing draughtsman form (Fig. 29.2) as they grow to a diameter of 1 mm or more. The central flattening is due to production of amidase which lyses many organisms in the colony.

When incubated aerobically the colonies are surrounded by greenish (alpha) haemolysis. Under anaerobic incubation, the haemolysis is clear (beta). The beta haemolysis is due to the production of heat labile pneumolysin.

Because of the production of hydrogen peroxide and autolytic enzymes such as amidase, the organisms tend to die quickly in cultures. This happens more so in cultures, in media without blood or blood products.

Growth in Liquid Media

An aerobic culture in a liquid medium without blood may show growth in the form of turbidity after 6-

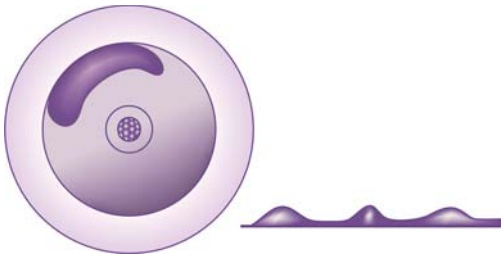

Fig. 29–2. Draughtsman colony: Cross-sectional view

12 hours of incubation but may become absolutely clear after another 24-36 hours.

In broth, pneumococci occur as short chains or isolated cocci. In common with other streptococci, lactic acid formation on prolonged incubation causes a fall in pH which is inhibitory to growth.

Biochemical Reactions

Pneumococci are oxidase and catalase negative organisms; ferment glucose, lactose, sucrose with the production of acid alone. Since some other streptococci *(Strept. faecalis, Strept. viridans)* also produce greenish haemolysis on blood agar, help of certain tests is taken to differentiate them (Table 29.1).

Of all the biochemical tests, bile solubility and optochin (ethyl hydrocuprein hydrochloride—0.001%) sensitivity are easy to perform and give consistently good and reliable results in differentiating pneumococci from other viridan streptococci.

Bile Solubility

Bile solubility is assessed by adding a few drops of 10% sodium deoxycholate solution to 1 ml of overnight broth culture. The culture clears due to lysis of the pneumococci. This test can be done on blood agar medium by putting a few drops of 10% bile solution on colonies of pneumococci and observing them for lysis within a few minutes.

Table 29–1. Differentiating features of alpha haemolytic streptococci

Feature	*S. pneumoniae*	*S. faecalis*	*S. viridans*
Presence of capsule	+	–	–
Quellung reaction	+	–	–
Draughtsman colonies	+	–	–
Uniform growth in broth	+	–	–
Growth on MacConkey agar	–	+	–
Resistance to 60°C (30 mts)	–	+	–
Growth in 6.5% NaCl	–	+	–
Optochin sensitivity	+	–	–
Bile solubility	+	–	–
Inulin fermentation	+	–	–
Virulence in mice	+	–	–

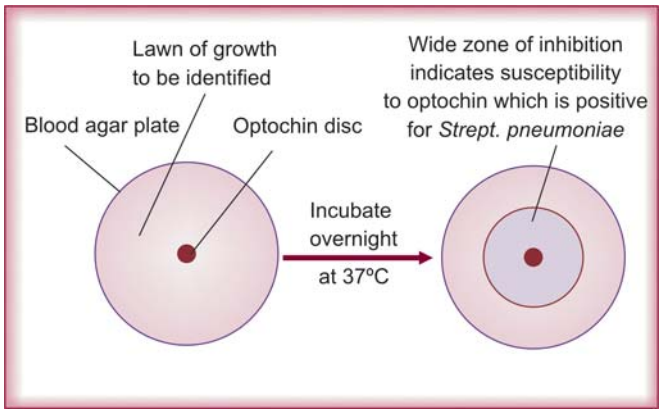

Fig. 29–3. Optochin sensitivity test

Optochin Sensitivity

Optochin sensitivity can be ascertained by applying a disc of optochin onto blood agar medium inoculated with the test organism. A wide zone of inhibition occurs around pneumococci after incubation (Fig. 29.3).

Susceptibility to Physical and Chemical Agents

Pneumococci are extremely fragile organisms; readily killed by most disinfectants as well as moist heat at 55°C within 10 minutes. It is difficult to maintain the cultures of this organism in the laboratory. Long-term preservation is possible only with freeze drying method.

Genetic (S to R) Variation

The pneumococci which are capsulated and give rise to smooth colonies are referred to as **S forms**. If property of capsule generation is lost due to mutation, rough colonies **(R forms)** are seen. The R forms are nonvirulent. In liquid medium, S forms produce smooth and uniform growth whereas R forms give rise to rough and granular growth.

S to R variation in pneumococci showing mutation and their role in virulence can be demonstrated easily. Repeated subcultures of a smooth capsulated strain of *Strept. pneumoniae* on solid media or incubation in the presence of specific antiserum, may result in the production of rough, granulated colonies due to uncapsulated or rough (R) mutants present in the culture. Conversely when the broth cultures of R strains are injected into

Table 29–2. Substances that cross react with pneumococcal serotypes

- Other pneumococcal serotypes
- Klebsiellae
- Rhizobium
- Enterobacteria
- Fungi
- Vegetable gums
- Group A human erythrocytes

mice, R strains are replaced by S strains which are smooth, capsulated and virulent. This is because of the destruction of avirulent organisms in animal and selection of virulent capsulated strains.

Antigenic Structure

Capsular Antigen On the basis of capsular polysaccharide antigen pneumococci have been separated into 83 serotypes. A few more types are awaiting confirmation. These polysaccharides are high molecular weight gels whose sugar compositions vary with the serotype. Cross reaction is a common phenomenon with these serotypes (Table 29.2).

For the identification of pneumococci an omniserum is employed which contains antibodies to all recognised types. Further typing can be done with monovalent antisera by latex agglutination or capsular swelling phenomenon called as quellung reaction (Fig. 29.4).

Somatic Antigen A species specific carbohydrate, the C polysaccharide, is a major structural component of the cell wall of all pneumococci. Chemically, it is choline containing ribitol teichoic acid. The choline can be replaced with ethanolamine and the resultant cells are resistant to the pneumococcal autolysin, form chains, become resistant to several phages and lose the ability to undergo genetic transformation.

Forssman Antigen This antigen is also called as F antigen and it cross reacts with the Forssman series of antigens present on mammalian cell surfaces. This antigen is lipoteichoic acid in nature and contains choline.

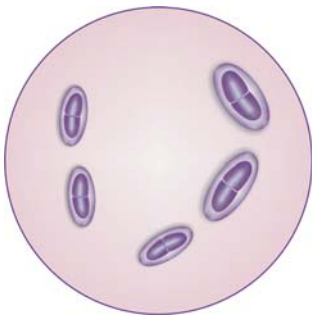

Fig. 29–4. Quellung reaction

M. Antigens These are type specific antigens analogous to those present on the surface of *Streptococcus pyogenes*. These are proteins and differ antigenically from M proteins of *Strept. pyogenes*. These have been extracted from both capsulated as well as noncapsulated strains of pneumococci.

Determinants of Pathogenicity

No significant toxin is produced by pneumococci. They produce disease by their capacity to multiply in the tissues and avoiding the defence mechanism of the host. Uncapsulated strains are ingested and destroyed by the polymorphs whereas capsulated strains remain protected. The role played by some of the extracellular products such as haemolysin, immunoglobulin, A1 protease, neuraminidase and hyaluronidase is not clear.

Clinical Features

Pneumonia is the most important and frequent manifestation of pneumococcal infection. Almost 25% of all pneumonias in adults are due to this organism and 75% of these are due to only 9 serotypes. Pneumonia is most often seen in infants, elderly persons, alcoholics or persons with chronic diseases. Overall mortality from penumococcal pneumonia is around 15%. This is increased by age, underlying disease, bloodstream involvement, metastatic infection and certain types of pneumococci such as serotypes 3 and 7.

Other clinical entities attributed to pneumococci include:

- otitis media
- septicaemia
- wound infection
- meningitis especially neonatal meningitis.

Pneumococcal meningitis is the most virulent of major bacterial meningitides with mortality rate of approximately 20%.

Laboratory Diagnosis

Clinical Specimens

Sputum, blood, wound exudates and cerebrospinal fluid are the specimens that are commonly collected.

Direct Demonstration

This is possible in a smear made from sputum, wound exudate or deposit of the CSF which can be stained by

Gram's method. The presence of gram-positive diplococci is suggestive. This is particularly useful for diagnosis of pneumococcal meningitis.

Culture

Culture using sputum, blood, CSF or other clinical sample is done on blood agar or heated blood agar (chocolate agar) with incubation in an environment of 5-10% carbon dioxide. Culture results are good only if there is significant number of viable bacteria in clinical specimen. The suspected colonies on blood agar are subjected to various tests which are mentioned in Table 29.1 for confirmation of the identity of the isolate.

Detection of Antigen

In the absence of such number of organisms **detection of pneumococcal antigen** can be performed by coagglutination, latex agglutination, or countercurrentimmunoelectrophoresis with polyvalent or type specific antiserum. All these tests are equally sensitive and specific but coagglutination and latex agglutination are easy and quick to perform. There are commercial kits available for detection of antigens.

Capsule Swelling Tests

Fresh emulsified sputum mixed with antiserum gives capsule swelling (the quellung reaction, shown in Figure 29.4) for identification of pneumococci and possible typing of the prevalent serotype also. Peritoneal exudates can also be used for such test.

Animal Pathogenicity

Animal inoculation is also of great help. The intraperitoneal inoculation of organisms (or clinical specimen) into mice generally causes peritonitis, septicaemia and death of the animal within 1-3 days and post-mortem demonstration of pneumococci in various organs confirms the identity. But this method is not used in practice.

Treatment

For all practical purposes, pneumococci are sensitive to penicillin and chemotherapy should be started as early as possible. In persons allergic to penicillin, any broad spectrum antibiotic can be given.

Immunity

Immunity to infection with pneumococci is type specific and depends both on the antibodies to capsular polysaccharide and on intact phagocytic function. Vaccines can induce production of antibodies against the serotypes used in the vaccine.

Prevention

Avoidance of predisposing factors and maintaining healthy dry lungs are the best preventive measures. A polyvalent pneumococcal vaccine having antigens from 23 of the most commonly occurring serotypes is available in developed countries (now available in India also) and is recommended for use in young children, elderly patients or persons with predisposing factors to this infection.

30 Corynebacterium

The bacterium causing diphtheria was described for the first time by Kleb (1883) and Loeffler in 1884 demonstrated its aetiological significance (Kleb-Loeffler's bacillus or KLB).

Classification

The causative agent of diphtheria exhibits pleomorphic club shape and gram-positive reaction. Similar characters are manifested by few other bacteria also. All these have been collectively called as **coryneform** (earlier known as **diphtheroids**) bacteria.

CORYNEBACTERIUM

These are gram-positive, non-motile and non-sporing bacilli. Five species of this genus are capable of causing disease in human beings (Table 30.1).

Table 30–1. Diseases caused by species of Corynebacterium

Species	*Disease*
C. diphtheriae	Diphtheria
C. haemolyticum	Pharyngitis
C. xerosis	Endocarditis
C. pseudotuberculosis	Tuberculosis-like illness
C. ulcerans	Pharyngitis

CORYNEBACTERIUM DIPHTHERIAE

Corynebacterium diphtheriae is the causative agent of diphtheria. It is a pleomorphic organism. The bacterium measures 3-6 µm × 0.6-0.8 µm, is slender and sometimes has swollen ends giving it "club-shaped" appearance. It contains 2-3 granules at the swollen ends which give reddish purple colour when stained with Loeffler's alkaline methylene blue. Rest of the bacterium is unevenly stained with this dye. The granules are also known as **metachromatic granules, Babes Ernst's granules** and **volutin granules**. These granules store energy sources for the bacterium.

C. diphtheriae is gram-positive and nonacid fast. With Albert's method the granules stain bluish black and the cytoplasm green. These bacilli exhibit characteristic arrangement in smear preparations. Adjacent bacteria lie at various angles to each other giving V or L appearances which collectively resemble arrangement of Chinese letters. This unusual arrangement is because of incomplete separation of daughter cells at the moment of division (Fig. 30.1).

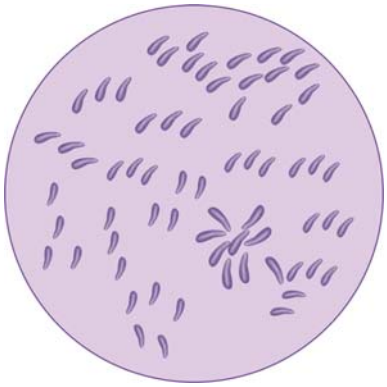

Fig. 30–1. *Corynebacterium diphtheriae*

Culture Media

Corynebacterium require serum for their growth. Several media which have been enriched with serum have been used. The selectivity of the media can be increased by the addition of sodium tellurite.

Growth in Hiss's serum water

The organism grows easily in this liquid medium. A pellicle forms on the surface of the otherwise uniformly turbid growth.

Growth on Loeffler's Inspissated Serum Medium

Loeffler's inspissated serum medium is a solid **enriched** medium without agar. It has been used extensively to grow *C. diphtheriae*. It gives luxurious growth of bacterium in a short period of 6-8 hours. The colonies are small, circular, creamy and glistening. However, differences in colonial morphology cannot be recognised on it. This medium is of great use to obtain large growth in short periods. The morphology of the bacterium is best developed in this medium.

Growth on Blood Tellurite Agar Medium

Mcleod's heated blood tellurite agar medium is the medium of choice (**selective medium**) to study the colony characters. Potassium tellurite present in the medium in the concentration of 0.04% provides it selectivity. Detailed description of all colonies are given in Table 30.2.

Table 30–2. Salient characters of three biotypes of *C. diphtheriae*

Character	*Gravis*	*Intermedius*	*Mitis*
Morphology			
Size	Short rods	Long rods	Long rods
Pleomorphism	++	+++	++++
Granules	Few or no	Few	Prominent
Special forms	Tear-drop	Cigar shaped	Wispy
Staining reaction	Uniform	Irregular	Irregular
Colonies on blood agar			
Size	3-5 mm	Small	Variable
Shape	Convex	Convex	Smooth
Opacity	Opaque	Translucent	Opaque
Haemolysis	–	–	Weak
Special character	Daisy head	Frog's eggs	Poached-egg
Consistency	Dry, friable	Weak butyrous	Butyrous
Colour	Dull grey	Grey	Grey
Colonies on tellurite agar			
Size	1.5-2 mm	0.5-0.75 mm	1.5-2 mm
Colour	Gun-metal grey	Grey	Grey
Surface	Matt	Shining	Glossy
Consistency	Brittle	Weak buttery	Buttery
Growth in broth			
Pellicle on surface	+	–	+
Deposit	Granular	Granular	Nil
Turbidity	Little or no	+ at 24 hrs	Diffuse at 48 hrs
Starch Fermentation	+	–	+
Phage types	14	3	4
% strains toxigenic	95	99	85
Antigenic types	13	4	40

Colony Characters

All but few strains of *C. diphtheriae* fall into one of the three colony types (**biotypes**) which are designated as *gravis, intermedius* and *mitis*. The salient differences between these biotypes have been depicted in Table 30.2. A fourth biotype of *C. diphtheriae* namely belfanti has also been described.

Susceptibility to Physical and Chemical Agents

C. diphtheriae is extremely sensitive to heat and suspensions are killed at 60°C in less than 10 minutes. It however, resists dry environment for months together. Most strains are highly susceptible to various disinfectants and chemotherapeutic agents.

Toxin

The pathogenicity of *C.diphtheriae* is attributed to release of a potent **exotoxin** by the bacteria.The production of toxin is dependent upon the presence of *tox* gene in the chromosome of the bacterium (Lysogenic conversion). *In vitro* production of this toxin depends largely on the concentration of iron. Toxin production is optimal at 0.14 µg of iron per ml. of medium but is virtually suppressed at 0.5 µg/ml. The factors that control toxin production *in vitro* are not well understood.

Properties of Toxin

Diphtheria toxin is extremely potent and lethal dose for a guinea pig weighing 250 grams is 0.0001 mg. With the help of trypsin it can be fragmented into two dissimilar fragments called A and B.

Mechanism of Action

Neither fragment A nor B is toxic on its own, even in very high concentrations. Fragment A gains entry into the cell and catalyses ADP ribosylation of diphthamide, a novel amino acid on elongation factor 2 (EF2). This leads to inhibition of protein synthesis and death of cell which clinically manifests as the necrotic lesion of diphtheria. On its own, fragment A cannot enter into cell cytoplasm. It needs fragment B for the same.

Animal Toxigenicity

Guinea pigs and rabbits are susceptible to the action of diphtheria toxin and death results within 96 hours of inoculation of toxigenic strain or toxin itself.

Formation of Toxoid

The diphtheria toxin can be converted into toxoid wherein it retains its immunogenicity but loses the viru-

lence by prolonged storage, incubating it at a temperature of 37°C for 4-6 weeks or by subjecting it to the action of formaldehyde or acidic pH. This toxoid is extensively used to induce active immunity in children against diphtheria.

Detection of Toxigenicity

Both *in vivo* and *in vitro* methods are available for detection of diphtheria toxin.

In vivo Tests

In the *in vivo* methods inoculation can be by intradermal or subcutaneous routes.

i. *Subcutaneous method:* In this test, two normal guinea pigs are taken and one of these is protected with adequate dose of antitoxin (500-1000 IU) which is injected intraperitoneally about 12 hours prior to the test. For test both the guineapigs are inoculated subcutaneously with the suspension of 18 hours growth of bacilli in Loefflers's serum slope. If the strain is toxigenic, the unprotected animal will die within 96 hours and postmortem will show pathological changes. The protected guinea pig shall remain normal.

ii. *Intradermal test:* In this test, 0.2 ml of 18 hours growth of test bacterium on Loeffler's serum slope is injected intradermally into the shaven side of an albino guineapig. Simultaneously 20- 40 IU of antitoxin is injected intraperitoneally to prevent the death of animal from toxaemia. The guineapig is observed for 48 hours for the development of local erythematous lesion. A known virulent and an avirulent strains of *C. diphtheriae* are also injected intradermally as controls. This method has the advantage of testing a large number of strains on single animal. Unlike the subcutaneous test which is wasteful of the animals used, in this test the inoculated animal doesn't die and more than one isolate can be tested in the same animal.

In Vitro Tests

i. *Elek's test:* The *in vitro* method employs the immunodiffusion technique and is also known as **Elek's test**. A horse serum agar medium plate is implanted with a filter paper strip impregnated with 100–200 I.U. of antitoxin before it gets solidified. Once the medium has set, the test strains of *C.diphtheriae* are streaked at right angles to the filter paper strip and incubated at 37°C for 24–48 hours. A positive and negative control are also put up. If toxin is produced by the strains, a precipitate appears in the form of double arrow-headed lines (Fig. 30.2).

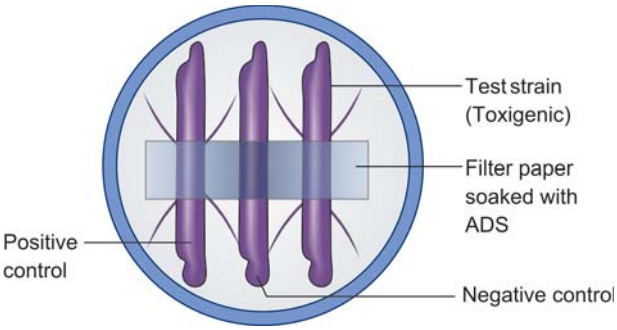

Fig. 30–2. Elek's test

ii. *Tissue culture test:* The toxigenicity can also be demonstrated by incorporating the strains in the agar overlay of cell culture monolayers. The toxin produced diffuses into the cells below and kills them.

iii. *PCR test:* A PCR test has also been developed for the detection of diphtheria toxin.

Pathogenesis

Man (clinical case and asymptomatic carrier) is the only source and reservoir of *C. diphtheriae*. In classical diphtheria the site of infection is nasopharynx. The bacilli multiply here and produce exotoxin. The toxin causes local necrosis. The combination of cell necrosis and exudative inflammatory response leads to accumulation of red blood cells, necrosed cells, bacteria and fibrin and these all mesh together to form the characteristic *pseudomembrane* which is white to grey in colour. This membrane first appears on tonsils, and may spread to larynx and trachea (Fig. 30.3). Toxaemia

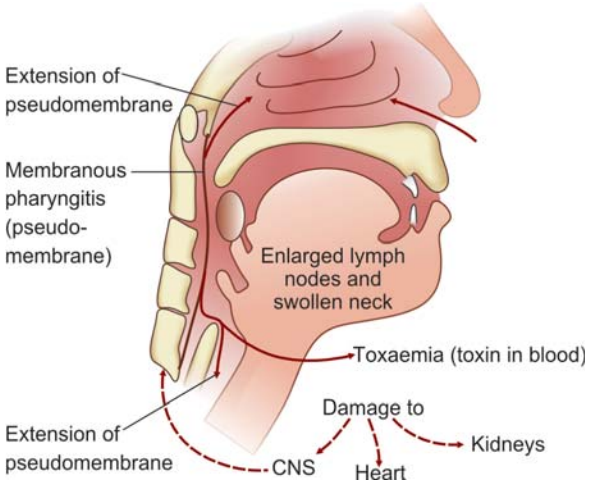

Fig. 30–3. Pathogenesis of diphtheria

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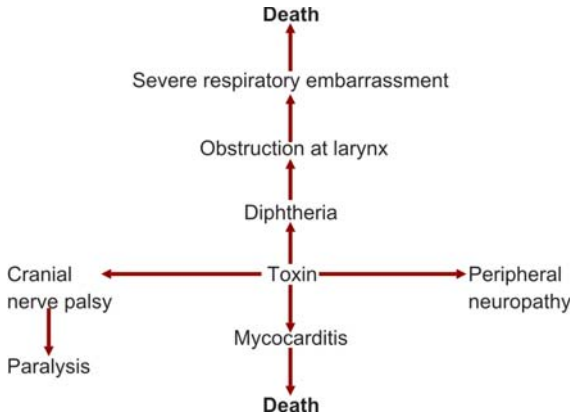

Fig. 30–4. Pathogenesis of diphtheria

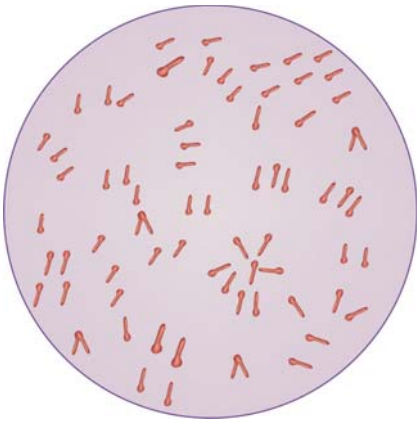

Fig. 30–5. Microscopic appearance of *C. diphtheriae*

and systemic manifestations of diphtheria result due to absorption of toxin from the site of membrane. Toxin can result in death of cells because of damage to the protein synthesis. Clinical manifestations and death are usually due to neural and cardiac involvement (Fig. 30.4).

Primary cutaneous involvement is also not infrequently seen in diphtheria. Other sites where diphtheritic membrane can be formed include lips, conjunctivae, ears, vagina and rarely uterine cavity.

Clinical Features

The clinical features include high fever due to toxaemia and pain in the throat because of the stretching membrane. Very severe cases of diphtheria are often termed as *malignant or hypertoxic* in which there is striking cervical adenopathy (bull neck), extreme toxaemia and a poor response to antitoxic treatment. Death in diphtheria occurs due to circulatory failure.

Laboratory Diagnosis

The diagnosis of diphtheria is basically clinical. The laboratory diagnosis is based upon:

a. Demonstration of organism
b. Isolation of organism and
c. Confirmation of toxigenicity of isolate.

Clinical Sample: Collection and Transportation

Two throat swabs are collected from the patient—one for making smears for Gram and Albert staining and the other for culture purposes. The swabs are taken from the pseudomembrane formed in the throat and preferably should peel off a part of it. Collection of swab requires good illumination and hence tongue is depressed while taking the sample. Swabs are immediately transported to the laboratory.

Demonstration of Organism

Two smears are prepared using one of the throat swabs on two clean, grease free glass slides. One smear is stained with Gram's stain and the other with Albert's stain. These slides are examined immediately under the microscope and characteristic features of *C. diphtheriae* observed. These are:

a. Thin, slender, gram-positive bacilli with clubbing at ends
b. Metachromatic granules (seen better with Albert's stain)
c. Bacilli arranged at acute angles giving the appearance of *Chinese letters* or *cuneiform writing* (Fig. 30.5).

The Gram's stained smear is examined first. The Albert's stained smear is examined only if Gram's staining shows gram-positive bacilli. Since some of the gram-negative bacilli such as *Escherichia coli* and *Pseudomonas* also contain metachromatic granules, examining only Albert's smear for such bacteria can give false positive diagnosis of diphtheria. Smears showing gram-negative bacilli should not be stained for *C. diphtheriae* by **Albert's method**. Other coryneform bacteria (previously known as diphtheroids) differ morphologically from *C. diphtheriae* in being thick and stumpy; lacking metachromatic granules and not having any specific arrangement. If morphology is suggestive of *C. diphtheriae*, a provisional report should be immediately sent to the treating physician to enable him to commence specific treatment using antibiotics and antidiphtheric serum.

Isolation of Organism

The second throat swab should be used to culture on different media. The advantages and disadvantages of various media that should be employed are given in Table 30.3.

Table 30–3. Media used for growth of *C. diphtheriae*

Medium	*Advantages*	*Disadvantages*
Blood agar	Useful for isolation of all pathogens	Isolation rate low for *C. diphtheriae*
Loeffler's serum slope	Rapid growth with best morphology of organism	Overgrowth of commensals as compared to pathogen. Biotyping not possible
Blood tellurite (0.04%) agar	Higher isolation rate	Morphology not well developed
	Biotyping possible on colony characters	Delayed growth

The organism grows best at 37°C and growth is examined after 24 hours (6-18 hours for Loeffler's medium) and 48 hours. The colonies are subjected to Gram's and Albert's staining and biochemical tests done.

Confirmation of Toxigenicity

It is not essential for diagnosis and treatment purposes in a routine hospital laboratory. However, for academic interest, it must be pursued. These tests can be performed *in vitro* (Elek's test) or *in vivo* either by subcutaneous or intradermal inoculation of culture material into guinea pigs.

Antimicrobial Susceptibility Testing

There is no need for routine antimicrobial susceptibility testing. The organism is sensitive to penicillin and erythromycin.

Steps in laboratory diagnosis of diphtheria are summarised in Figure 30.6.

Susceptibility determination

The susceptibility of the individual can be assayed with the help of Schick test.

Schick Test

It is no longer in use and is being described for academic interest only.

Principle

Schick test operates on the principle that when diphtheria toxin is injected intradermally into a susceptible person, it causes a local reaction, while in an immune individual, no reaction ensues as the toxin is neutralised by the antitoxin in circulation.This test was introduced by Schick in 1913 and is performed to assess the immunity against diphtheria in children above 2 months of age. The test comprises of injecting intradermally 0.2 ml of diphtheria toxin which contains 1/50 MLD of toxin in the left forearm. Similar dose of heat inactivated toxin is injected in the right forearm. Readings are taken after 24-48 hours and then after 5-7 days of inoculation. Any of the following types of reactions may be observed:

In **negative reaction** there is no reaction of any kind in either forearm. This indicates that person is immune to diphtheria and the antitoxin concentration in the

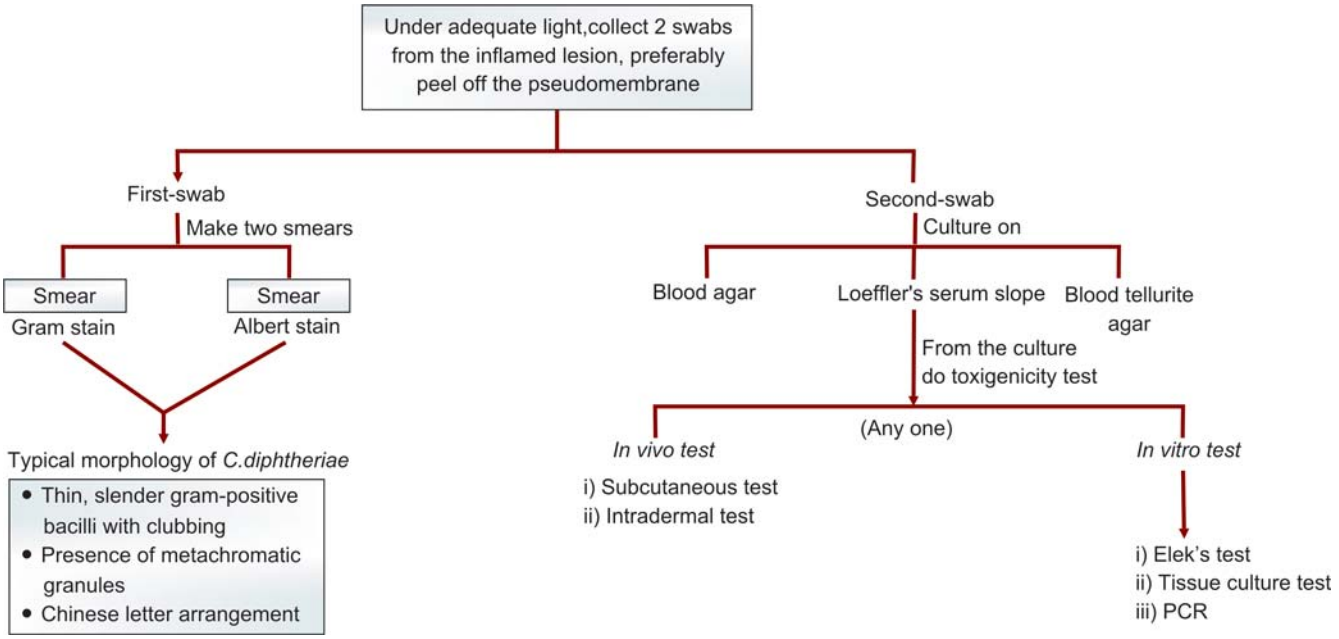

Fig. 30–6. Steps in laboratory diagnosis of diphtheria

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serum of the individual is 0.01 unit or more/ml. In positive reaction an erythematous reaction appears in the test arm within 24-36 hours (1-3 cm diameter) and persists for 7 days whereas there is no reaction on the control arm. This status is indicative of susceptibility of the individual to diphtheria.

The **pseudoreaction** develops in both the arms in less than 24 hours, is not sharply circumscribed and usually fades away within four days. This is also indicative of immunity to diphtheria.

In combined reaction both the arms show reaction during first 24 hours, after which in test arm, reaction continues to develop whereas in control arm it fades. By fourth day, a clear distinction can be seen in two arms. This status is **indicative of susceptibility to diphtheria**.

Similarly a localised swelling may occur after the subcutaneous injection of diphtheria toxoid in some individuals. This test is known as **Moloney** test and such individuals should not be given injections of diphtheria toxoid since:

a. They may have violent local or systemic reaction to injection
b. Most are already immune
c. The test itself would have stimulated more anti-toxin production.

Epidemiology of Diphtheria

The essential feature of epidemiology of diphtheria are summarised in Table 30.4.

Table 30–4. Epidemiological features of diphtheria

- A disease primarily of non-immune children under the age of 15 years
- Being detected more and more in adults due to waning of the clinical disease which served as a source of subclinical infection
- Humans—both carriers and cases are the reservoir
- Infection usually spreads by contact with a patient or carrier or rarely contact with soiled articles with discharges from the infected patient
- Usual incubation period 2-5 days
- Effective antibiotic therapy immediately reduces infectivity
- Passive immunity received transplacentally protects newborns upto 6 months of age
- Disease or subclinical infection, usually, but not always induces life long immunity
- Immunization with toxoids leads to prolonged but not life-long immunity
- Most important source of infection are asymptomatic carriers.

Terms used in Relation with Toxins and Antitoxins

Minimum lethal dose (MLD): of the diphtheria toxin is defined as the least amount of the toxin required to kill a guinea pig weighing 250 grams within 96 hours after subcutaneous inoculation.

One unit of antitoxin: was defined as the smallest amount of anti-toxin required to neutralise 100 MLD of toxin.

L_O *(Limes null)* dose of the diphtheria toxin is the largest amount of toxin that when mixed with one unit of anti-toxin and injected subcutaneously into a 250 gram guinea pig will on average produce no or minimal local oedema.

L^+ *(Limes tod):* dose of diphtheria toxin is the smallest amount of toxin that when mixed with one unit of anti-toxin and injected subcutaneously into a 250 gram guinea pig will on an average kill the animal within 96 hours.

Minimum reacting dose (MRD): is the least amount of toxin that when injected intradermally in a guinea pig, causes an erythematous flush 5 mm in diametre visible after 36 hours.

Lf unit: The flocculating or Lf unit of diphtheria toxin is the amount of toxin which flocculates most rapidly with one unit of anti-toxin. It is the only method used for titration of toxoids.

Treatment

The mainstay of treatment are diphtheria anti-toxin and antibiotic therapy at the first clinical suspicion without waiting for the laboratory confirmation. The dosage of ADS varies between 20000 to 100000 units. Alongwith this a course of penicillin therapy is given. For treatment of carriers, erythromycin is more effective.

Immunization

Active immunization using diphtheria toxoid is the mainstay. Passive immunization alongwith antibiotic therapy is given in the clinical cases. Diphtheria toxoid is usually given in children as a triple vaccine "DPT". 10-25 Lf units of diphtheria toxoid (indicated as D-dose) is used and for older children and adults smaller dose of 1-2 Lf units (indicated as d dose) is used. 3 doses are given with an interval of 4 weeks between the doses beginning at 6 weeks of age followed by boosters a year later and at school entry. ADS, after skin testing, is given in clinical cases. Ideally all individuals recovering from clinical disease should receive active immunization.

OTHER CORYNEBACTERIA

Apart from *C. diphtheriae*, four other species of this genus are known to produce diseases in human beings.

C. haemolyticum and *C.ulcerans* may cause pharyngitis; *C.xerosis* produces endocarditis and *C. pseudotuberculosis* can cause tuberculosis like illness in some individuals.

Differences between *C.diphtheriae* and corynebacterium bacteria are shown in Table 30.5.

Propionibacterium acnes

Propionibacterium acnes is a coryneform bacterium and probably the most common bacterium on human skin. It is often isolated from the lesions of acne, but its precise role in causation of this disease is yet to be established. The organism produces propionate which is cytotoxic to Vero and human diploid fiibroblast cells. It also produces histamine and these products may be responsible for causing inflammation in acne.

Table 30–5. Differences between *C.diphtheriae* and coryneform bacteria (diphtheroids)

Character	*C. diphtheriae*	*Coryneform organisms*
Gram reaction	Gram positive	Gram positive
Morphology	Thin and long	Thick and short
Metachromatic granules	+++	+/–
Pleomorphism	+	–
Chinese letter arrangement	+	–
Can be cultured on	Enriched media	Ordinary media
Fermentation of sucrose	–	+
Production of toxin	+	–
Pathogenic	+	–

31 Mycobacterium

MYCOBACTERIUM

The Mycobacteria are named so because of the mold like pellicular growth of these organisms in liquid medium (*myco:* fungus; *bacterium:* bacteria). The true bacterial nature of these organisms was, however, soon established.

An important character of the mycobacteria is their ability to resist decolourisation by a weak mineral acid after staining with one of the aryl-methane dyes. This gives the name *acid fast bacteria* to these organisms.

Classification

Mycobacteria have been classified in a variety of ways. However, a clinical classification is more practical and is described

Group 1. Obligate pathogens
- — *M. tuberculosis*
- — *M.leprae*
- — *M. bovis*

Group 2. Skin pathogens
- — *M. marinum*
- — *M. ulcerans*

Group 3. Opportunistic pathogens
- — *M. kansasii*
- — *M. avium-intracellulare* (MAC)

Group 4. Non or rarely pathogenic
- — *M. gordonae*
- — *M. smegmatis*

Group 5. Animal pathogens
- — *M. paratuberculosis*
- — *M. lepraemurium*

Tuberculosis is an infectious disease caused by multiplication of bacilli belonging to the genus Mycobacterium. The principal bacterium is *Mycobacterium tuberculosis* (also called Koch's bacillus) and to a lesser extent *M. africanum* and *M. bovis*. These 3 species collectively are called as "tuberculous mycobacteria". Non-tuberculous mycobacteria can also cause infections simulating tuberculosis.

MYCOBACTERIUM TUBERCULOSIS

Morphology

The tubercle bacilli are slender, straight or slightly curved rod shaped organisms measuring 2-4 µm in length and 0.2-0.8 µm in breadth occurring singly, in pairs or in small groups. The bacilli are non-sporing, non-motile and non-capsulated. In suitable liquid culture media, virulent human and bovine tubercle bacilli form characteristic long, tight, serpentine cords (Fig. 31.1) in which organisms are alligned in parallel.

The bacilli are Gram positive though they do not take the stain readily. These organisms resist decolourisation by 25% sulphuric acid and absolute alcohol for ten minutes and hence these are called acid and alcohol fast.

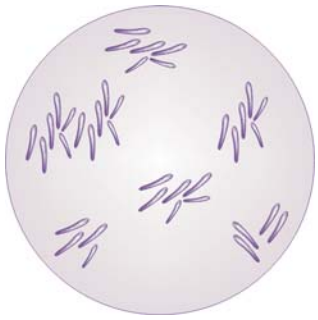


Fig. 31–1. Serpentine cords of *Mycobacterium tuberculosis*

Table 31–1. Media used for the growth of *M.tuberculosis*

Solid media	*Liquid media*
Functions	
Isolation of organism	Sensitivity testing
Antigen preparation	
Chemical tests	
Examples	
Lowenstein-Jensen's (LJ) medium*	Dubos' medium
Loeffler serum slope	Middlebook's medium
Pawlowsky's medium (potato medium)	Sula's medium
Tarshis medium (blood medium)	Sauton's medium

*most widely used

Cultural Characters

Culture Media

Various types of media that are commonly used have been summarised in Table 31.1.

Colony Characters

On solid media human type of tubercle bacilli give rise to discrete, raised, irregular, dry, wrinkled colonies which are creamy white to begin with and then develop buff color (Fig. 31.2). By contrast the bovine type grows as flat, white, smooth, moist colonies which "break up" more readily when touched.

Tubercle bacilli will grow on top of liquid medium as a wrinkled pellicle (Fig. 31.3) if the inoculum is carefully floated on the surface and flask left undisturbed otherwise they will grow as floccules throughout the medium.

Mycobaterial Cell Wall

It has high lipid content which accounts for about 60% of the dry weight of the cell. The structure of the cell wall is depicted in Figure 31.4.

Virulence in Animals

Under natural conditions *M. tuberculosis* infects man, monkeys, pigs, dogs and occasionally parrots. Under experimental conditions, it is virulent to guinea pigs and mice and less virulent in rabbits and avirulent in chicken.

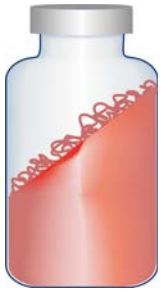


Fig. 31–2. Growth of *M. tuberculosis* on LJ medium

Fig. 31–3. Growth of *M. tuberculosis* in liquid medium

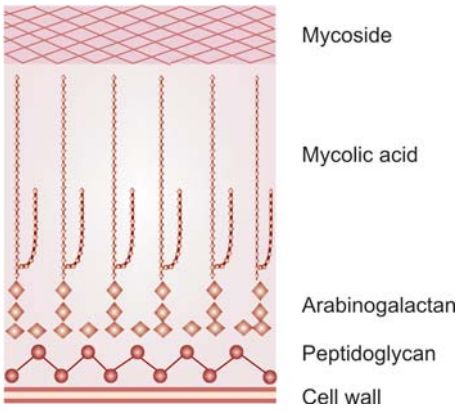


Fig. 31–4. Cell wall of mycobacteria

Susceptibility to Physical and Chemical Agents

The best method to inactivate tubercle bacilli is by heat and chemical methods are all relative to it.The thermal death time at 60°C is 15-20 minutes. They are more resistant to chemical agents than other bacteria because of the hydrophobic nature of the cell surface and their clumped growth.

Antigenic Structure

Mycobacteria being complex unicellular organisms, contain many antigenic proteins, lipids and polysaccharides. The exact number of antigenic determinants is unknown. The mycobacterial antigens have been broadly classified as:

- Soluble (cytoplasmic) and insoluble (cell wall lipid bound)
- Carbohydrates or proteins
- By their distribution within the genus.

Soluble antigens have been extensively used to *classify*, *identify* and *type* the mycobacteria.

Biochemical Properties

M. tuberculosis has distinctive biochemical properties, some of which are utilized for identification of various species (Table 31.2).

Table 31–2. Biochemical tests to differentiate mycobacteria

Test	*M. tuberculosis*	*M. bovis*	*Atypical mycobacteria*
Production of niacin	+	–	–
Binding of neutral red	+	+	+/–
Hydrolysis of Tween 80	–	–	+
Production of enzymes:			
• Nitrate reduction	+	–	+/–
• Arylsulphatase	–	–	–/+
• Catalase at room temp	–	–	+
at 68°C	–	–	+
• Catalase-Peroxidase	Weak +	Weak +	Strong +
Nicotinamidase	+	–	–
• Pyrazinamidase	+	–	+/–
Susceptibility to:			
• Pyrazinamide	+	–	–
Uptake of iron	–	–	–/+

Pathogenesis

The first event in the pathogenesis of tuberculosis, whether inapparent or overt, is the implantation of bacilli in tissues. The most frequent portal of entry is lungs, resulting from the inhalation of airborne droplets containing a few bacilli which are expectorated by an open case of tuberculosis. Less frequently the bacilli may be ingested and lodged, in the tonsil or in the wall of the intestine, which may follow consumption of raw contaminated milk. A third but rare mode of infection is direct implantation of bacilli into the skin, such as in workers working with infected materials or handling cultures of tubercle bacilli.

The production and development of lesions and their healing or progression are determined chiefly by the number of mycobacteria in the inoculum and their subsequent multiplication and resistance and hypersensitivity of the host. The essential pathology of tuberculosis consists of the production, in infected tissues of a characteristic lesion, the tubercle.

Dissemination of bacilli from the site of implantation occurs via the lymphatics to the regional lymph nodes to form the *primary complex* (Fig. 31.5).

Mycobacteriophages

Mycobacteriophages have been used for subdivision of some species of mycobacteria. *M. tuberculosis* has been divided into 4 phage types-A, B, C and I (I stands for intermediate between A and B).

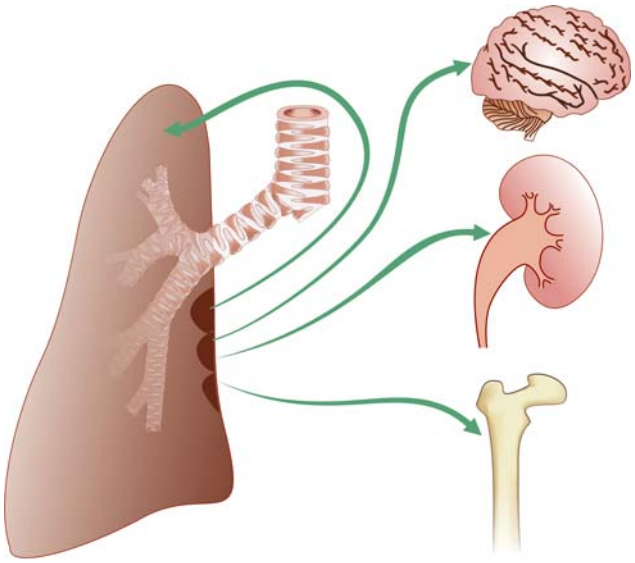


Fig. 31–5. Primary tuberculosis

Bacteriocins

There is limited evidence that some strains of mycobacteria liberate substances that inhibit the growth of other species. *M. tuberculosis* is divisible into eleven types by means of bacteriocins produced by rapidly growing mycobacteria.

Immunity and Hypersensitivity

Infection with *M. tuberculosis* induces delayed hypersensitivity (allergy) and resistance to infection (immunity). Unless a host dies during the first infection with tubercle bacilli, there is an increased capacity to localize tubercle bacilli, retard their multiplication, limit their spread and reduce lymphatic dissemination. This can be attributed to the development of cellular immunity during the initial infection.

In the course of primary infection the host also acquires hypersensitivity to the tubercle bacilli. This is made evident by the development of a positive tuberculin reaction.

Koch's Phenomenon

The contrast between primary infection and reinfection is shown experimentally in Koch's phenomenon. When a guinea pig is injected subcutaneously with virulent tubercle bacilli, the puncture wound heals quickly, but a nodule forms at the site of injection in 2 weeks. This nodule ulcerates and the ulcer does not heal. The regional lymph nodes develop tubercles and caseate massively. When the same animal is later injected with tubercle bacilli in another part of the body, the sequence of events is quite different. There is rapid necrosis

of the skin and tissue at the site of injection, but the ulcer heals rapidly. Regional lymph nodes either do not become infected at all or do so only after a delay. These differences are attributed to immunity and hypersensitivity induced by the primary infection. The Koch's phenomenon has 3 components-a local reaction, a focal response in which there occurs acute congestion around tuberculous foci in tissues and a "systemic" response of fever which may at times be fatal.

This effect is not caused exclusively by living tubercle bacilli, but also by killed ones, no matter whether they are killed by low temperatures or prolonged periods, or by boiling or by certain chemicals.

Tuberculin Test

This is performed with purified protein derivative (PPD) of old tuberculin (OT) or a still purer form known as **new tuberculin**. PPD is standardised in terms of its biologic reactivity as *tuberculin units* (TU). TU is defined as the activity contained in a specified weight of reference PPD. Bioequivalency of PPD products is not based on weight of the material but on comparative activity.

Method. The skin testing is begun with 1TU (equivalent to 0.01 mg OT or 0.00002 mg PPD) to 100 or 250 TUs. If a large dose is injected in a hypersensitive host, it may give rise to several local reactions and a flareup of inflammation, hence the reason of starting in a graded way. The volume injected is 0.1 ml given intradermally.

Reaction. In an individual who has not had contact with mycobacteria there is no reaction to PPD. An individual who has had a primary infection with tubercle bacilli develops induration, oedema, erythema in 24-48 hours and with very intense reactions, even with central necrosis. The diameter of induration is measured transversely to the long axis of the forearm. Erythema is ignored. It is considered positive if the injection of 5 TUs is followed by induration of 10 mm or more in diametre. Weak reactions may disappear very rapidly while positive tests tend to persist for several days.

False negatives (anergy). It can be negative in overwhelming tuberculosis, measles, Hodgkin's disease, sarcoidosis, immunosuppression or malnutrition.

False positives. These may be seen in infections with related mycobacteria such as atypical mycobacteria. These are usually low grade reactions and can be differentiated by testing with tuberculins prepared from those mycobacteria.

Hypersensitivity reaction. The dermal changes elicited by tuberculin are usually considered due to a single immunological phenomenon namely a type IV or delayed hypersensitivity reaction. However there is now clear evidence that it could induce atopic (type I), Arthus (type III) as well as type IV reactions.

Interpretation and applications of tuberculin testing. These have been summarised in Table 31.3.

Table 31–3. Interpretation of tuberculin test

- Positive in <2 year's old child indicates active tuberculous lesion even if there are no manifestations
- No longer considered as a very sensitive epidemiological tool for measuring the prevalence of infection in community
- A negative test indicates possibility of absence of active tuberculosis in body
- Size of reaction may be directly proportionate to risk of developing disease at a later stage
- Repeat test exerts booster effect
- Positive test may revert to negative with INH therapy
- It is not correlated with protective immune response

Despite these limitations, the tuberculin test continues to be the only tool for measuring the prevalence of tuberculosis infection in a community.

Laboratory Diagnosis

The laboratory diagnosis is based on *demonstration and isolation* of tubercle bacilli and no other test is of much use. The essential steps for diagnosis are:

Clinical Sample: Collection and Transportation

In pulmonary tuberculosis, sputum is the sample of choice. If tuberculosis of any other organ of the body is suspected, sample has to pertain to that specific organ or system such as urine for renal tuberculosis and cerebrospinal fluid for tubercular meningitis. Tubercular bacilli are most numerous in lesions showing rapid caseation.

The sample is collected in sterile container. It is a common misassumption that as mycobacterial samples are *decontaminated* before culture, cleanliness of the container is not important. Unsterilized containers may be contaminated with environmental mycobacteria.

The sputum is collected in wide-mouth containers which are free from antiseptics, screw-capped and preferably disposable. It is preferable to obtain at least three early morning samples of sputum and these are transported to the laboratory at the earliest. If the delay is unavoidable, sample can be stored at refrigerator temperature for about seven days. If refrigerator is not available and sample is to be transported in hot climate then it should be preserved by adding equal volume of 1% cetyl pyridinium chloride in 2% saline.

CSF and pus are placed directly in sterile containers and sent to the laboratory without any delay.

The laboratory diagnosis is based only on the demonstration/isolation of tubercle bacilli and no other test is of much use. The essential steps for diagnosis are:

- Direct demonstration by microscopy
- Isolation by culture
- Animal pathogenicity
- Immunodiagnosis
- Tuberculin test
- Molecular techniques
- Histopathological examination.

Direct Demonstration

Use of microscopy in diagnosis of tuberculosis is of paramount importance as culture takes a long time before the results are ready. Microscopy is also helpful in the detection of open or infectious cases. Stained smears are examined directly from the sputum and after concentration.

New glass slides should be used for making smears as acid-fast bacilli are not always removed from the old slides. Only those reagents and diluents should be used which have been shown to be free of environmental mycobacteria to avoid false positive smears. Direct examination is performed by selecting a purulent-looking portion of sputum and spreading it thinly on a glass slide with a bacteriological loop or a wooden stick. The watery part of sputum is less likely to contain bacilli.

The smears are air dried, fixed and stained with Ziehl-Neelsen technique and examined under the microscope. Acid fast bacilli are seen as bright-red rods against the blue, green or yellow background (depending upon the counterstain used in staining). A negative result does not exclude tuberculosis since at least some 100,000 tubercle bacilli must be present in per ml of sputum for the demonstration of a positive microscopic finding. Before declaring a slide negative it is essential that at least 100 fields are examined taking at least 10 minutes. Smears can be graded according to the number of bacilli seen (Table 31.4).

Fluorescent Staining

An alternate to Ziehl-Neelsen staining method is fluorescent staining with auramine or rhodamine. Although the equipment required is expensive, it is less tiring and results can be given quickly for a large number of slides. Smears are examined under a lower magnification thereby increasing the chances of detecting small number of acid fast bacilli. Since fluorescent staining is less specific, it is suggested that smears positive by fluorescent staining should be confirmed by Ziehl-Neelsen staining.

Table 31–4. Grading of AFB smears

Number of AFB	*Grading*
More than 10/oil immersion field	3+
1-10 per oil immersion field	2+
10-99 per 100 oil immersion fields	1+
1-9 per 100 oil immersion fields	Scanty (Record exact no.)
No AFB in 100 fields	Negative

Samples of urine, CSF and other body fluids are centrifuged and the deposit is stained and examined.

Drawbacks of Microscopy

For diagnostic purposes, following drawbacks have been observed with microscopy:

a. It is not a very sensitive method. It is positive in only in open cases of tuberculosis when the bacterial count is more than 100,000 per ml.
b. Mere presence of acid fast bacilli may not be diagnostic of tuberculosis since these may belong to saprophytic species. Though most saprophytic species stain uniformly without giving any beaded appearance, these are acid fast without being alcohol fast. A list of false positives and false negative results is given in Table 31.5.

Table 31–5. False results in sputum microscopy

False positive	*False negative*
Use of old slide	Poor quality specimen
Not stained with filtered stain	Incomplete staining
Inadequate decolourization	Too intense decolourization
Improper labelling	Improper labelling
Food particles/fibres in sputum	Wrong recording of results

To increase the sensitivity of microscopic examination, various methods for concentrating the bacillary content of sputum and other clinical samples are used. The most widely used method which concentrates the bacilli without inactivating them is Petroff's method.

Petroff's Method

In this method sputum is incubated with an equal volume of 4% sodium hydroxide at 37°C with frequent shaking till it becomes clear. This takes an average of

15-20 minutes. It is centrifuged at 3000 rpm for 30 minutes. The deposit is neutralised with dilute hydrochloric acid using neutral red as indicator. This deposit can be used for making smear, culture and animal inoculation.

All patients with chest symptoms (i.e., three weeks of cough) or other symptoms suggestive of TB are advised to undergo three sputum examinations for acid-fast bacilli. Patients with two or three positive smear results are diagnosed as having sputum smear-positive pulmonary TB and are started on the appropriate treatment. Those with only one positive result of the three smear examinations performed, are advised to get a chest X-ray done and, if found to be compatible with TB, are also treated as sputum smear-positive pulmonary TB cases. Patients, in whom all three samples are smear-negative, are prescribed broad-spectrum antibiotic, such as co-trimoxazole, for 10-14 days. If not suffering from TB, most patients are likely to improve with antibiotics. However if the symptoms persist after the course of broad-spectrum antibiotics, the patient is re-evaluated on the basis of repeat sputum examination and X-ray. Thereafter, if in the opinion of the treating physician, the patient is suffering from tuberculosis, treatment is initiated accordingly. The diagnostic algorithm based on this is shown in Figure 31.6.

Isolation by Culture

Cultures are very sensitive for detection of tubercle bacilli and may be positive with as few as 10-100 bacilli per ml of sputum. Most commonly used medium is Lowenstein Jensen (LJ) medium. It contains eggs, asparagine, glycerol and some mineral acids.

The clinical sample as such, or after concentration, is inoculated onto two bottles of LJ medium and incubated at 37°C. Cultures are examined initially after 3-4 days to rule out the presence of rapid growing mycobacteria and contaminant fungi and bacteria. Thereafter, cultures are examined twice weekly. A negative result is given, if no growth appears after 8-12 weeks. If growth is obtained, then a Ziehl-Neelsen stained smear

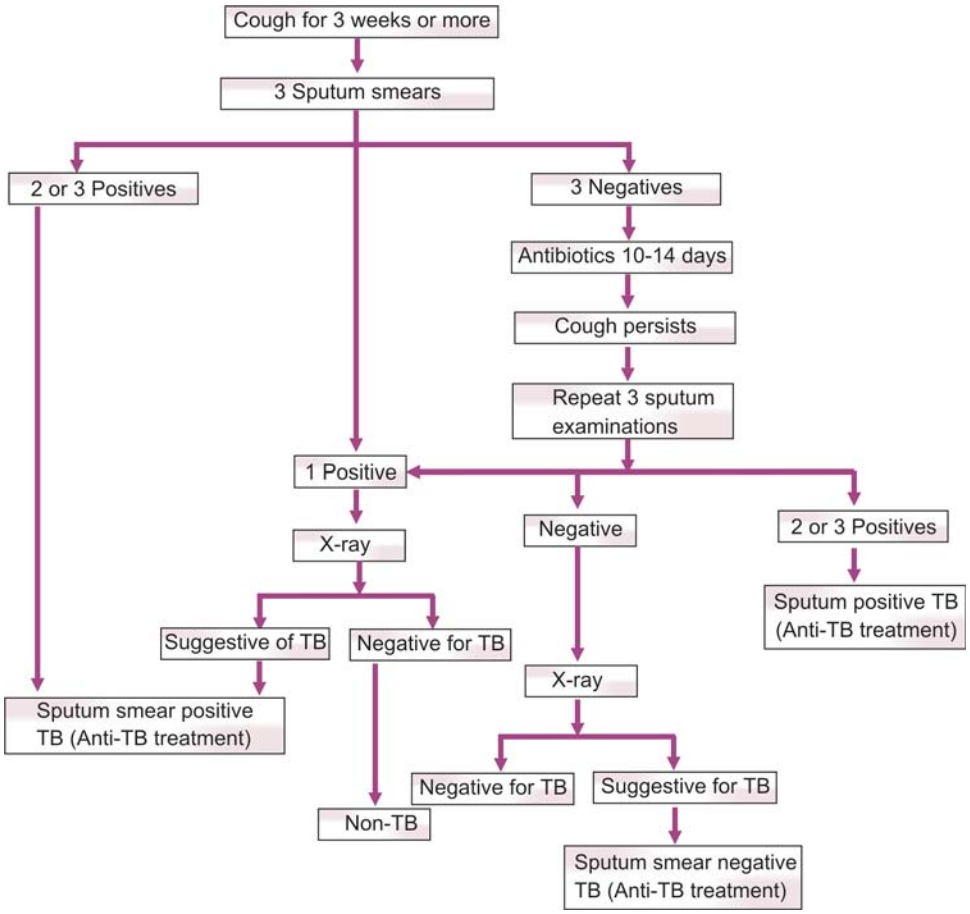


Fig. 31–6. Diagnostic algorithm for pulmonary TB

made from the same is examined and routine biochemical tests put up. For practical purposes an organism fulfilling following criteria is taken as *M. tuberculosis*.

- slow growing
- nonpigmented
- niacin positive
- acid-fast bacillus

TESTS FOR CONFIRMATION OF IDENTITY

To differentiate *M. tuberculosis* from *M. bovis* a battery of tests is available (Table 31.6).

Table 31–6. Salient characters of *M. tuberculosis* and *M. bovis*

Character	*M. tuberculosis*	*M. bovis*
Growth at 24°C	–	–
Pigment production	–	–
Niacin	+	–
Nitrate reduction	+	–
68°C catalase	–	–
Tween hydrolysis	–	–
Iron uptake	–	–
Arylsulfatase test	–	–
Growth on MacConkey agar	–	–
Urease	+	+
Pyrazinamidase	+	–

Animal Inoculation

Guinea pig inoculation was once a popular way of diagnosing tuberculosis but should now be regarded as obsolete. It has been clearly demonstrated that the use of this animal offers no practical advantage over *in vitro* culture. In addition to humane considerations, animal inoculation is costly and generates many biohazards. However, in some laboratories it is still used. The animal test can be done by intradermal, subcutaneous or intramuscular method. Two healthy, preweighed, about 12 weeks old guinea pigs free from tuberculous infection are taken. The concentrated material is injected intramuscularly. Progressive loss of weight is an indication of tuberculosis. One animal is sacrificed 4 weeks after inoculation and if found negative for tuberculosis, the second animal is sacrificed at 8 weeks. Autopsy findings of the animal positive for tuberculosis show:

a. Caseous lesion at the site of inoculation
b. Enlarged caseous draining lymph nodes
c. Enlarged spleen showing irregular necrotic areas
d. Tubercles in the peritoneum and also, to a lesser extent, in lungs.

Diagnosis is confirmed by the demonstration of acid-fast bacilli in these tissues by Ziehl-Neelsen staining. Organisms with low virulence do not produce typical lesions in the guinea pigs.

Histopathological Examination

It is important for extra pulmonary TB. The sample taken by Fine needle or as excision biopsy be subjected to histopathology for diagnosis.

Tuberculin Test

It is of limited value in diagnosis of tuberculosis, especially in children. A positive test indicates past exposure or vaccination with BCG. A negative test rules out tuberculosis with the exception of miliary tuberculosis. A positive test in children with more than 10 mm induration is of diagnostic value.

Immunodiagnosis or Molecular Techniques

No clinically acceptable immunological test has emerged so far though availability of monoclonal antibodies against *M. tuberculosis* has improved the prospects of immunodiagnosis. Latex particles coated with antibody to plasma membrane antigen of *M. tuberculosis* has proved useful in diagnosis of tuberculous meningitis by antigen detection.

DNA probes have been developed for the detection of tubercle bacilli in sputum and other specimen. These have proved rapid and accurate. ELISA test kit has also been used for the detection of tubercle antigen in CSF. PCR has been found useful for the diagnosis of tubercular meningitis.

In spite of great strides in laboratory diagnosis, many cases fail to be diagnosed by the laboratory. In such cases clinical acumen and radiological evidence may provide vital diagnostic clues.

Recent Advances in Diagnosis of TB

i. Enhanced sputum smear microscopy by proper training, maintenance of microscope, quality control and techniques to enhance the sedimentation of mycobacteria for better yield in microscopy.
ii. Radiometric BACTEC 460 TB method has shortened the time to half of that of conventional methods for detection of mycobacteria and determination of susceptibility.
iii. There are many other systems available such as MGIT 960, Septicheck AFB method, TK, medium, etc. which have shortened the time for detection of mycobacteria.

iv. There have been advances for rapid identification of mycobacterium isolates such as probe-based identification, DNA chips, analysis of lipids by gas chromatography, etc.
v. As an alternative to sputum microscopy both genotypic (PCR and NAA—Nucleic acid amplification assays) and Phenotypic methods (FAST Plague TB) have been developed for demonstration of mycobacteria directly from the clinical samples.
vi. Serological tests by and large have not been very promising.
vii. Genotypic methods have been developed for rapid diagnosis of MDR-TB strains.
viii. PCR has been found out to be very sensitive and specific test for diagnosis of Tubercular meningitis.

Sensitivity testing. With the emergence of multi-drug resistance in mycobacteria it is essential to perform sensitivity test on the tubercle bacilli isolates as an aid and guide to treatment. Drug-resistant mutants continuously arise at a low rate in any mycobacterial population. The purpose of sensitivity testing is to determine whether the great majority of the bacilli in the culture are sensitive to the antituberculous drugs currently in use. The sensitivity testing may be direct (performed on the original specimen) or indirect (performed on a subculture).

Several methods of drug susceptibility testing have been standardised. These are:

1. The absolute concentration method
2. The resistance ratio method
3. The proportion method
4. The BACTEC-460 radiometric method
5. Epsilinometer testing (E-test)
6. Luciferase mycobacteriophage testing.

Prevention

Primary prevention: Prevention could be primary or secondary. Best measure for primary prevention of TB is treatment of infectious cases and through good public health practice to reduce the chances of infection in institutions by adequate ventilation and isolation of the patients.

Secondary prevention: Refers to prevention of disease in already infected persons which involves two technical measures: (a) Chemoprophylaxis for groups at risk, (b) BCG vaccination.

Groups at risk: "Groups at risk" are the population groups whose risk of contracting tuberculosis is 5-10 times higher than that of general population either because they have a greater risk of being infected or because they have a greater likelihood of progressing to disease once infected. These include (a) family contacts of a smear positive case, (b) immunosuppressed individuals hospitalised at the same time as drug-resistant TB patient, (c) groups with lowered immunity such as AIDS patients and (d) underprivileged migrants, refugees. Preventive chemotherapy can be targeted for children less than 5 years of age who are contacts of a smear positive patient using isoniazid is a dose of 5 mgm/kg for 6 months.

BCG Vaccination

BCG vaccine consists of live bovine tubercle bacilli whose virulence has been attenuated by multiple passages through glycerinated potato. The bacilli of the vaccine are therefore alive, but have lost some of their virulence. BCG is the most widely used vaccine in the world.

Efficacy of the vaccine: The protection conferred by BCG when it is administered correctly at birth acts mainly on the severe extrapulmonary forms in children. It is currently estimated at between 60-90%.

Indications: In countries with a high prevalence of TB, BCG vaccination should be administered to infants as soon as possible after birth and in any case before the age of one year.

Availability of vaccine: The vaccine is available in dry, lyophilised powder that is sensitive to heat, so the vaccine should be kept in a cold chain and away from light. To avoid exposure to light the vaccine is delivered in coloured vials. The vial is accompanied by another vial containing a solvent that must be used cold, by refrigerating it for at least 24 hours before use. After reconstitution the mixture must be kept in the refrigerator and used within 3-4 hours.

Dose: The dose of vaccine is 0.05 ml for newborns and children aged upto one year. It is 0.1 ml for children aged over one year.

Requirements: 1 ml syringe and intradermal needle.

Site: Usually same site is recommended for use in the whole of the country so that it is easy to detect the vaccination scar. Usually it is the front of the left upper arm.

Route: Vaccine is given intradermally. If by chance the needle goes beyond the dermis, the needle should be withdrawn and inserted at an adjacent spot. The injection should raise a wheal and the skin takes an

"Orange peel" aspect. After injection the arm should be wiped and left open to the air for several minutes.

Combination with other vaccinations: It is possible to give several vaccinations at the same time. BCG and polio zero dose are given at birth. If this vaccine is missed then BCG can be given at 6 weeks of age when 1st dose of DPT and OPV are given. Similiarly if this is also missed then it can be combined with second or third dose of DPT and OPV given at 10 weeks and 14 weeks respectively or at 9 months along with measles vaccine.

Developments at vaccine site: The weal from the vaccination disappears within half an hour. After 3 to 4 weeks a small red induration appears which swells to 6-8 mm in diametre and can persist for one or two months. It may ulcerate and ooze fluid. This stops after 2-8 weeks, a scab forms and later a scar develops which is round, lightly depressed and approximately half a centimetre in diametre. The parents of the children receiving vaccination should be explained this process as a normal course of events.

Contraindications: Infants having congenital or acquired immunodeficiency should not be given BCG vaccination. However infants who are only HIV-seropositive can be given BCG vaccination as the reste of tuberculosis in such children is greater than the risk of complications from the vaccine. Infants born to HIV-positive mothers should also be vaccinated, unless they present symptoms of AIDS.

Complications: It is unusual for complications to occur if vaccine is given correctly. In about one in 1000 children lymphadenopathy may develop in the axilla or inside the elbow which may become fluctuant and fistulise. Simple incision and drainage and dressing will heal it within days. Occasionally local complications may occur if the vaccine is administered in a faulty way.

Revaccination: The protective effect of BCG lasts for 10-15 years but revaccination has no proven benefit.

Multi-Drug Resistant Tuberculosis (MDR-TB)

Emergence of strains resistant to Isoniazid and Rifampicin–the two most important drugs for treatment of TB is known as MDR-TB.

Types: Drug resistance is broadly classified as primary and acquired. Drug resistance in a patient who has never received anti-TB treatment previously is termed as primary resistance. Acquired resistance is that which occurs as a result of specific previous treatment.

Causes: The causes of drug resistance including MDR-TB include:
- Inadequate treatment administration
- Use of substandard drugs
- Irregular drug supply or intake
- Interruption of chemotherapy
- Non-adherence to prescribed norms
- Wrong prescription giving incorrect doses of medicine
- Massive bacillary load
- Illiteracy and ignorance

Detection: The conventional methods of isolation, identification and susceptibility testing require 8-10 weeks. This leads to delay in diagnosis and permits transmission of drug resistant bacteria. There are newer methods available which are quicker but are costly and not widely available.

Extensively Drug Resistant Tuberculosis (XDR-TB)

XDR-TB which is an abbreviation for extensively drug-resistant TB, was the term coined by WHO in October 2006. TB can easily be treated with a course of four standard or first line, anti-TB drugs. If these drugs are misused or mismanaged, multi-drug resistant TB (MDR-TB) can develop. MDR-TB is defined as TB bacteria that are resistant to at least isoniazid and rifampicin—the two most powerful anti-TB drugs. XDR-TB is TB that in addition to MDR-TB is resistant to any fluoroquinolone and at least one of the three injectable second-line drugs (capreomycin, kanamycin and amikacin). Important features are:
- XDR-TB can either be acquired as primary infection or an MDR-TB can get converted to XDR-TB.
- There is no difference in the spread of XDR-TB from any other form of TB.
- The prevention of XDR-TB can be done by timely and complete treatment of drug-sensitive TB cases so as to prevent MDR-TB and eventually XDR-TB.
- In places where XDR-TB is most common, people living with HIV are at greater risk of becoming infected with XDR-TB as compared to people without HIV.
- Symptoms of XDR-TB are not different from ordinary or drug-susceptible TB.
- The diagnosis of XDR-TB is not clinical, it is always laboratory based.

Tuberculosis and HIV Infection

HIV infection is the most powerful risk factor that increases the likelihood of development of TB in a

person previously infected with *M. tuberculosis.* HIV-associated tuberculosis is included in the current international AIDS definition. An HIV infected person co-infected with *M.tuberculosis* has a 50% lifetime risk of developing TB disease, whereas an HIV non-infected person infected with *M. tuberculosis* has only a 10% risk of developing TB. Though in the early phase of HIV infection the presentation of TB is similar to the uninfected individuals, it differs in the late phase as follows: (a) More frequency of extra pulmonary TB (b) often smear negative, (c) lesions are infiltrative and not cavitary which is the reason for smear negativity.

Impact of HIV on TB Programme

- Under diagnosis of smear positive and over diagnosis of smear negative cases
- High mortality rates during treatment
- High default rate due to adverse drug reactions
- High rates of TB recurrence
- Nosocomial transmission.

Impact of TB on HIV/AIDS Programme

- TB is the most common opportunistic infection in AIDS
- Untreated TB shortens the survival of patients with HIV infection
- TB accelerates the progression of HIV
- TB is leading cause of death in AIDS patients
- Late TB diagnosis contributes to death.

Early diagnosis and effective treatment of active TB disease in HIV infected patients are critical for curing TB, minimising its negative effects on the course of HIV and interrupting the transmission of TB to other persons in the community. It is essential to prevent TB disease in TB/HIV co-infected individuals.

MYCOBACTERIUM LEPRAE

Morphology

Mycobacterium leprae also known as Hansen's bacillus, is the bacterium that causes leprosy (Hansen's disease).

Leprosy bacilli are straight or slightly curved rod-like bacteria about the size of 5× 0.5 µm with pointed, rounded or club-shaped ends. These are non sporing. The organisms show many morphological variations-clubbed forms, lateral buds or branching may be observed. *M. leprae* is Gram-positive and stains more readily than tubercle bacilli. It is acid fast but less strongly acid fast than *Mycobacterium tuberculosis*. 5% sulphuric acid is employed for decolourisation after staining with carbol fuchsin. In stained skin smears or sections, they are seen lying singly as pink lines, in clumps or in bright pink compact masses known as *globi*. The parallel rows of bacilli in the globi present a **cigar bundle** appearance (Fig. 31.7).

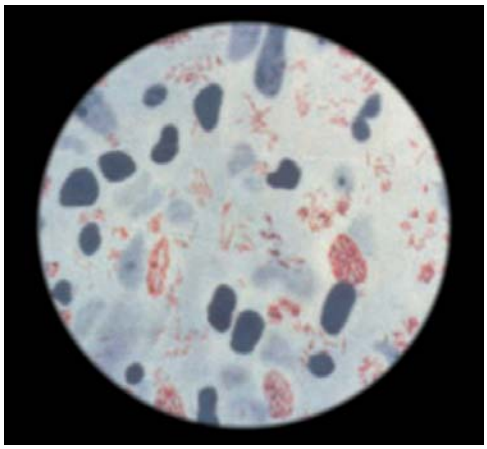


Fig. 31–7. Microphotograph of *Mycobacterium leprae* taken from a skin lesion

Morphological index (MI). It is calculated as number of regularly stained bacilli per hundred of total organisms examined. Only the solid staining bacilli are viable.

Bacteriological index (BI). Skin-slit stain smears are prepared from scrapings from a skin incision of the lesion particularly earlobes. After Ziehl-Neelsen staining the smear is graded on a 0 to 6+ logarithmic scale according to Ridley's scale. This is given as in Table 31.7.

Table 31–7. Ridley's scale for grading *M. leprae* slides

BI	*AFB*	*Oil immersion fields*
6	1000	1
5	100-1000	1
4	10-100	1
3	1-10	1
2	1-10	10
1	1-10	100
0	None	100

SFG index. Ridley has devised an index which is known as SFG index. In this, bacilli are divided into three classes: *solid* **(S)** *fragmented* **(F)** and *granular* **(G)**. A value is assigned to the bacilli of each class in a smear: 2 if they appear numerous (over 20% of all bacilli); 1 if few (1-20%); 0 if less than 1%. Thus, the relative proportion of bacilli in the three classes SFG (in this order) are represented by one of the permutations of 2-1-0. These

combinations are placed in order of descending granularity from 2-0-0 (all solid) to 0-0-2 (all granular) to give an index.

If several smears are available the mean index is taken. An SFG index of 2 or less signifies that there are no solid-staining unbroken rods.

Cultivation

It has not been possible to culture *M. leprae* in bacteriological media or tissue cultures.

The leprosy bacillus can be grown in the footpad of a mouse to some extent. This was used for determining viability of this bacillus in tissue biopsies and for performing drug sensitivity tests. Small inocula (100-1000 bacilli) replicate upto a ceiling of 10^6 organisms.

Growth in Armadillo. In 1971, Kirchheimer and Storrs (USA) reported a disseminated experimental leprosy bacillus infection in the nine banded armadillo *(Dasypus novemcinctus)* (Fig. 31.8). The use of the armadillo as an experimental source of *M. leprae* has been limited by:

Fig. 31–8. Nine banded Armadillo

a. The failure of these animals to breed in captivity.
b. Some of the armadillos caught from wild life are naturally infected with a leprosy like disease.
c. Some animals contain acid fast bacilli which are unrelated with *M. leprae*.

Susceptibility to Physical and Chemical Agents

Lepra bacilli have been found to remain viable in warm humid environment for 1-2 weeks and in moist soil for one and a half months. It has been observed that *M. leprae* remain viable for 24 hours outside the body. It can survive exposure to UV light for 30 minutes and sunlight for 2 hours.

Clinical Features

The three cardinal diagnostic signs of leprosy are:

a. Inability to feel touch, heat and/or pain in the affected area.
b. Enlargement and/or tenderness of peripheral nerves associated with sensory loss and/or paralysis.

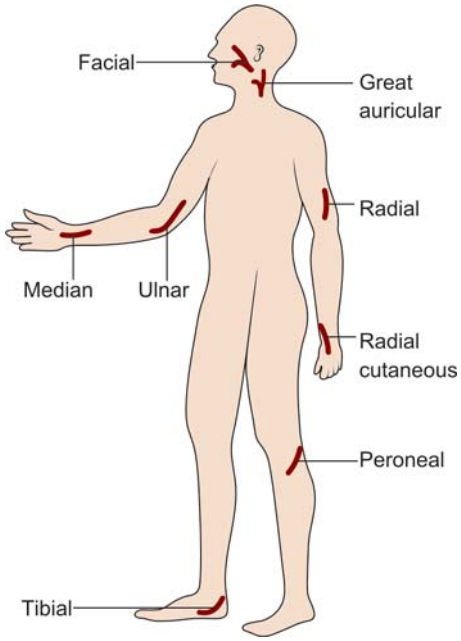


Fig. 31–9. Sites of predilection for nerve enlargement

The nerves commonly involved are shown in Figure 31.9.

c. Finding non-cultivable bacilli in skin smears taken from the affected areas.

The polar lepromatous leprosy patient presents with diffuse or nodular lesions (lepromas) containing many acid fast *M. leprae* bacilli. On the other hand, polar tuberculoid leprosy consists of a few well-defined anaesthesized lesions containing only a few acid-fast bacilli.

Clinical Classification

Leprosy is broadly classified in 2 main types i.e., *tuberculoid type* (representing high resistance) and *lepromatous* type (representing low resistance). Since both these types of leprosy represent two opposite poles of host immune response, these are also called as *polar forms* of leprosy. Cases not falling in either of the two poles are classified as *indeterminate* or *borderline* types.

In persons with fairly good resistance the invading leprosy bacilli can be destroyed to a great extent and such individuals develop paucibacillary (PB) leprosy. On the other hand, persons having poor or no resistance cannot contain the infection and develop multibacillary (MB) leprosy. MB leprosy is a generalised disease.

Spectrum of immunity in leprosy is depicted in Figure 31.10.

Leprosy is classified into 6 groups according to *modified Ridley and Jopling's* classification. These groups are

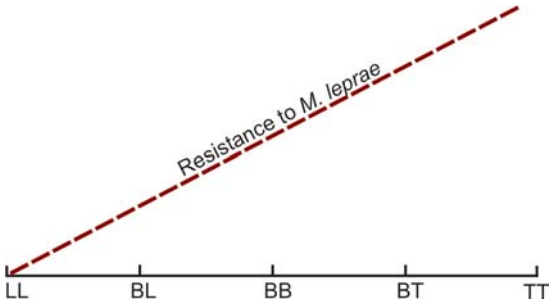


Fig. 31–10. The spectrum of immunity in leprosy

TT (Tuberculoid Polar), BT (Boderline Tuberculoid), MB (Mid Borderline), LI (Lepromatous Indeterminate), BL (Borderline Lepromatous), LL (Lepromatous Polar).

Incubation Period

- Leprosy can occur in a few weeks following exposure, however, cases developing 30 years after exposure have been described.

Transmission

- *M. leprae* is not highly infectious
- Transmission is more common among household contacts
- Transmission is believed to occur through inhalation of infectious organisms
- Transmission through insect bites and inoculation through broken (or intact) skin have not been excluded
- Infectious people are thought to shed the organism from the nasal mucous membranes, especially if there is ulceration
- *M. leprae* can survive in nasal secretions for more than 36 hours
- Reinfection may account for cases of leprosy in older people
- At present there is conflicting information on the effect of HIV/AIDS on the incidence of leprosy.

Pathogenesis

Since *M. leprae* has never been cultured *in vitro*, it appears to be an obligate intracellular pathogen that requires the environment of the host macrophage for survival and propagation. Estimates of the replication rate *in vivo* are on the order of 10 to 12 days. The bacilli resist intracellular degradation by macrophages, perhaps by escaping from the phagosome into the cytoplasm, and accumulate to high levels (10^{10} bacilli/g of tissue) in lepromatous leprosy. The peripheral nerve damage appears to be mediated principally by the host immune response to bacillary antigens. Tuberculoid leprosy is characterized by self-healing granulomas containing only a few, if any, acid-fast bacilli.

Immunology

Leprosy is a disease in which almost all the clinical manifestations are due to the immune response of the host to the presence of *M. leprae*. It is a classical example of a disease which demonstrates a spectrum-clinical, histological and immunological. This does not depend on the variations of properties of the bacillus but rather on host's immune system. Infection with *M. leprae* induces both antibody-mediated and cell-mediated immune (CMI) response. Of these only CMI is protective. There is no increase in the immunoglobulin levels and albumin: globulin ratio in the serum is normal.

Lepromin Test

Principle. Test is based on the principle of delayed hypersensitivity.

Lepromins. The lepromins, used as antigen in lepromin test may be of human origin (Lepromin-H) or Armadillo origin (Lepromin-A).

Test method. The test is performed by injecting 0.1 ml of lepromin intradermally into the inner aspect of the forearm of the individual. As a routine, the reaction is read at 48 hours and 21 days.

The response to the intradermal injection of lepromin is typically biphasic-early reaction (Fernandez) and late (Mitsuda reaction).

Early reaction (Fernandez reaction). An inflammatory response develops within 24-48 hours which tends to disappear after 3-4 days. There is redness and induration at the site of inoculation. The criteria used for taking the reading are given in Table 31.9. An infiltration greater than 20 mm is considered strongly positive.

Late reaction (Mitsuda reaction). The reaction becomes apparent in 7-10 days following the injection and reaching its maximum in 3-4 weeks. The test is read at 21 days. The reaction is read as shown in Table 31.8.

Significance of lepromin test. The following are important pieces of information that can be provided by this test:

a. Lepromin test is not used to diagnose leprosy. Neither it is used to find out the prevalence of leprosy.
b. It is used to classify the lesions of leprosy patients. This test is positive in tuberculoid and negative in lepromatous leprosy.

Table 31–8. Interpretation of lepromin test

Fernandez reaction		*Mitsuda reaction*	
Induration (mm)	*Interpretation*	*Induration (mm)*	*Interpretation*
<5	-ve	0	No reaction
5-10	+/–	<3	+/–
10-15	+	3-5	+
15-20	++	6-10	++
20	+++	10	++++

c. Prognosis of the disease can be found out. A positive test is indicative of good prognosis.
d. Conversion to lepromin positivity during treatment is evidence of improvement.
e. For recruitment of persons to work in leprosy homes, only lepromin positive persons are recommended to be appointed.
f. To verify the identity of candidate lepra bacilli, cultivable acid fast bacilli claimed to be lepra bacilli should give matching results when tested in parallel with standard lepromin.

Laboratory Diagnosis

i. *Demonstration of organism in smears:* Bacteriological diagnosis is easy in lepromatous leprosy where bacilli are abundant but may be difficult in tuberculoid cases. Acid fast bacilli can occasionally be found in the skin in conditions other than leprosy and it must be remembered that they are usually not found in tuberculoid leprosy. However sensory loss is invariably present in tuberculoid skin lesions though it may not be found in lepromatous macules which however contain acid-fast bacilli.

The diagnosis consists of demonstration of acid fast bacilli in the lesions. It is demonstrated in "slit-skin smears" or in skin biopsies.

Slit skin smears. Slit skin smears are taken from lesions and from standard sites such as the ear, chin and elbows. The skin is pinched between thumb and forefinger and a small cut is made. This cut should reach the dermis but without causing bleeding as blood causes acid-fast artefacts. Tissue fluid is scraped out with the tip of the scalpel blade held at 90 degrees to the line of incision and smeared on a glass slide. Nasal scrappings are taken from lower turbinate or septum with a nasal curette. After Z-N staining, the slides are examined under oil immersion and bacterial index and morphological index or SFG index determined as described earlier.

ii. *Skin testing (Lepromin test)* is of no diagnostic value in leprosy.
iii. *PCR:* Recently PCR for defection of *M. leprae* DNA in environmental and clinical samples has been standardised.

Treatment and Control

A variety of combinations of the following drugs (so-called multi-drug therapy or MDT) are used to treat leprosy: dapsone, rifampin, clofazimine, and either ethionamide or prothionamide. Paucibacillary cases (tuberculoid and borderline tuberculoid) can be treated in 6 months, although dapsone alone is usually given for up to 3 years after disease inactivity. Therapy for patients with lepromatous or borderline lepromatous leprosy may require primary treatment for 3 years, with dapsone alone continued for the rest of the patient's life. Although some public health officials believe that MDT alone will result in eradication of leprosy in the near future, other experts are much more cautious. Drug resistance has been documented in *M. leprae.* In many cases patient management must include anti-inflammatory therapy to alleviate the immunologic sequelae. Irreversible nerve damage leading to loss of sensation may result in paralysis or occult wounds and deformities. Wound prevention techniques and proper wound care are important.

ATYPICAL MYCOBACTERIA

Mycobacteria other than human or bovine tubercle bacilli that cause human disease resembling tuberculosis are called *atypical mycobacteria*. These are also *known as anonymous, unclassified, tuberculoid, paratubercle* or MOTT (Mycobacteria Other Than Tubercle Bacilli). The important features of this group of organisms are given in Tables 31.9 and 31.10.

Table 31–9. Runyon classification of atypical mycobacteria

Runyon group	*Species*	*Disease in man*
I Photochromogens	*M. kansasii* *M. marinum* *M. simiae*	Pulmonary disease Swiming pool granuloma
II Scotochromogens	*M. scrofulaceum* *M. gordonae* *M. szulgai*	Lymphadenitis in children
III Nonphoto-chromogens	*M. avium* *M.intracellulare* *M. xenopi*	Pulmonary disease in immunocompromised
IV Rapid growers	*M. chelonae* *M. fortuitum*	Superficial and systemic diseases

PART V

Incidence of diseases due to atypical mycobacteria is on the rise in most countries of the world where incidence of tuberculosis has come down and there is increased incidence of AIDS cases. Disease almost certainly arises as a result of contact with bacilli in the environment. The probable vehicle is water and infection may result from drinking, washing or indulgence in aquatic games.

Classification

Atypical mycobacteria have been classified in 4 groups by **Runyon** in 1959 based on production of pigment and rate of growth. These are:

- Group I : Photochromogens
- Group II : Scotochromogens
- Group III : Nonphotochromogens
- Group IV : Rapid growers

Photochromogens produce pigment in light, but not in darkness, scotochromogens develop pigment when growing in dark and nonphotochromogens develop various degrees of pigmentation unrelated to exposure to light. Various organisms included in these 4 groups are given in Table 31.10.

Salient characters of atypical mycobacteria have been summarised in Table 31.10.

Table 31–10. Salient features of atypical mycobacteria

- Normally present as saprophytes in environment
- Opportunistic pathogens
- Carry low virulence
- Not readily transmissible from man to man
- Some produce pigment in culture
- Can grow at 22°C
- Fail to give niacin reaction
- Form enzyme aryl sulphatase
- Give negative neutral red reaction
- Usually resistant to INH and streptomycin
- Nonpathogenic to guinea pigs
- Incapable of giving rise to progressive disease in man

Group I Photochromogens

This group of photochromogens form yellow or orange pigment when their cultures are grown with exposure to light, but not when they are kept in dark from soil, water and milk.

M. kansasii, M. marinum (syn. *M. balnei*), and *M. simiae* are important photochromogens. *M. marinum* causes swimming-pool granuloma or fish tank granuloma. The infection is acquired through water. *M. simiae* has been isolated both from monkeys and man.

Group II Scotochromogens

The mycobacteria in this group form pigmented colonies (yellow, orange, red) even in dark. These are widely distributed in the environment and sometimes contaminate cultures of tubercle bacilli. The intensity of the colour of the colonies may increase on exposure to light. *M. scrofulaceum* is the only important member of this group which may cause scrofula (cervical adenitis) in children.

Group III Nonphotochromogens

These strains don't form pigment even on exposure to light. The colonies may resemble those of tubercle bacilli. Medically important species such as *M. avium*, *M. intracellulare* and *M. xenopi* are all non-photochromogens.

Group IV Rapid Growers

The rapidly growing mycobacteria are those species that from an inoculum give well seperated colonies within 5 days at 37°C, usually in 2-3 days. The important species are *M. fortuitum*, *M. chelonei* and *M. szulgai*. All the chromogenic rapid growers are saprophytes.

MYCOBACTERIA PRODUCING SKIN ULCERS

Two conditions where skin ulceration occurs are *M. ulcerans* infection causing what is known as *Buruli ulcer* and infection with *M. marinum* which causes swimming pool granuloma. In addition to these two there are some other inoculation-associated infections.

M. ulcerans

This was first described in Australia and the organism was named as *M. ulcerans* in year 1948. Later, a similar disease was seen in the Buruli country of Uganda and hence the name Buruli ulcer was given. Epidemiological investigations suggest that the organism is inoculated into the skin by thorny vegetation.

M. marinum

This is natural pathogen of cold blooded animals. The skin disease produced is known as "swimming-pool granuloma" or "fish tank granuloma" or "fish fancier's finger". The differences between *M. ulcerans* and *M. marinum* are given in Table 31.11. In the absence of treatment many *M. marinum* infections will eventually resolve. Surgical excision or antimicrobial therapy is usually resorted to with minocycline, co-trimoxazole and rifampicin.

Other inoculation-associated mycobacterial diseases include:

a. Superficial warty lesions: occasionally caused by *M. kansasii*, *M. szulgai* and *M. chelonei*
b. Post-injection mycobacterial abscesses: usually caused by the rapid growers *M. chelonei* and *M. fortuitum*. Corneal lesions due to these two organisms have followed abrasions or penetrating injuries.

SAPROPHYTIC MYCOBACTERIA

Saprophytic mycobacteria are non-pathogenic acid-fast bacilli found in milk, butter, water, manure, grass and smegma of human beings and animals.

Table 31–11. Differences between *M. marinum* and *M. ulcerans*

Feature	*M. marinum*	*M. ulcerans*
Distribution	Temperate zone	Tropics
Ulcer	Self limiting	Progressive
Pigment on growth	Present	Absent
Rate of growth	1-2 weeks	4-8 weeks
Number of bacilli in ulcers	Scanty	Abundant
Growth at 25°C	Positive	Negative
Growth at 37°C	Abundant	Negative
Cord formation in smears	No	Yes
Lesions in mouse foot pad	Inflammation with purulent ulcers	Oedema only

32 Bacillus

The family *Bacillaceae* comprises of gram-positive bacilli which are characterised by the formation of endospores. This family consists of two genera of medical importance: *Clostridium* and *Bacillus*. The clostridia are obligatory anaerobes but the members of the genus *Bacillus* are aerobic organisms.

The aerobic spore bearers are widely distributed in the environment. Most of these are nonpathogenic. Two species viz. *B. anthracis* and *B. cereus* are known pathogens for man and animals.

BACILLUS ANTHRACIS

Morphology

Thr organisms belonging to species *B. anthracis* are rod shaped, straight with rounded or square ends and exhibit great pleomorphism in size. The size varies from 3-10 µm × 1-1.6 µm)Young cultures are gram-positive while old cultures may become gram-negative. *B. anthracis* grows *in vitro* in long chains that look like a joined bamboo rod. *In vivo*, the chains are shorter and single bacilli or pairs of organisms may also be seen (Fig. 32.1).

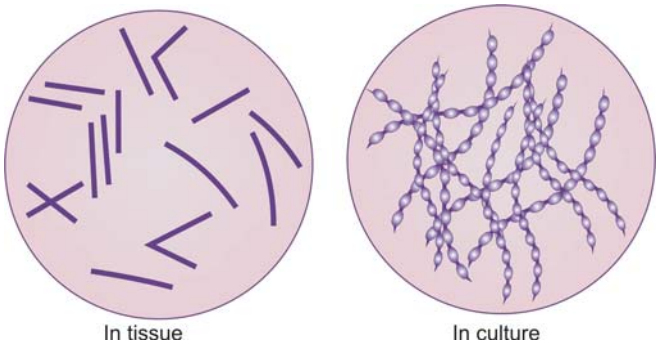


Fig. 32–1. *Bacillus anthracis*

B. anthracis is capsulated organism. Chemically the capsule is polypeptide (poly-D-glutamic acid).

The endospores are formed in the presence of oxygen. These spores are usually located centrally but may also be found subterminally or terminally. Since spores are formed only in the presence of free oxygen, they are not seen in the blood of animals infected with *B. anthracis*.

Staining of anthrax bacilli with polychrome methylene blue gives rise to a characteristic microscopic appearance known as McFadyean's reaction. This is used for the presumptive diagnosis of anthrax in animals. In a smear that has been stained for 10-15 seconds, an amorphous purple material is visible around the bacilli. The material comprises of disintegrated bacterial capsule.

Susceptibility to Physical and Chemical Agents

Because of their ability to produce spores, *B. anthracis* is extremely resistant to adverse chemical and physical environments. In the dry state or in soil the spores may survive for many years. The vegetative bacilli are killed in moist heat at 60°C in 30 minutes. With dry heat spores are killed at 150°C in 60 minutes.

Cultural Characters

The anthrax bacilli are aerobes and facultative anaerobes. These grow well on most laboratory media. Maximal growth is obtained at 37°C but the organisms are capable of growing in range of 12 to 45°C. Optimum pH is 7 to 7.4. The media commonly employed for the growth of these bacilli are nutrient agar, blood agar (using blood which is free of antibiotics) and a selective medium known as PLET (consisting of Polymyxin, Lysozyme, Ethylene diamine tetra acetic acid, Thallus acetate) medium.

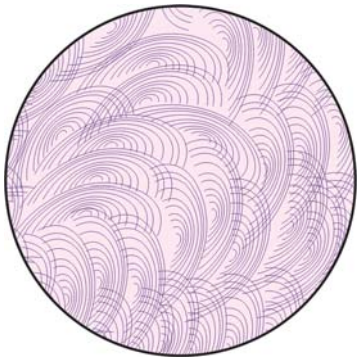


Fig. 32–2. Medusa head appearance of the colonies of *B. anthracis*

Growth on Nutrient Agar

On nutrient agar, colonies are greyish, granular discs, 2-3 mm in diameter after 24 hours of incubation. The colonies possess an irregular, fringelike edge; uneven surface and wavy margins which gives them the appearance of tangled masses of long hairlike structure under the microscope. This appearance is called as "medusa head" appearance (Fig. 32.2).

Colonies have membranous consistency and are difficult to emulsify.

Growth on Gelatin Agar

A stab culture on gelatin agar slope results in growth of organisms in a manner that gives the appearance of inverted fir tree. This is because of gradual decrease in the growth with reduced aerobic environment.

Growth on Blood Agar

On blood agar usually no haemolysis is produced. The size and characteristics of colonies are same as seen on nutrient agar.

Growth on Selective (PLET) Medium

The PLET medium consists of heart infusion agar as the basal medium to which are added polymyxin, lysozymes, EDTA and thallous acetate. Small smooth colonies are formed on this medium by *B. anthracis* whereas other species of this genus including *B. cereus* are inhibited.

Growth in the Presence of Penicillin

In presence of penicillin, the anthrax bacilli become large, spherical and grow in form of chains on the surface of the medium. This phenomenon is called as "string of pearl" reaction and differentiates *B.anthracis* from *B.cereus*.

B. anthracis differs from *B. cereus* in many other aspects also (Table 32.1).

Table 32–1. Differences between *B. anthracis* and *B. cereus*

Character	*B. anthracis*	*B. cereus*
Colonies on blood agar		
— Rough and flat	+	+
— Comma shaped outgrowths	Many	Few
— Haemolysis	None	Beta
— Tenacity	+	–
Fluorescent antibody test	+	–
Susceptibility to gamma phage	+	–
Pathogenicity to animals	+	–
Reduction of		
— Litmus milk	–	+
— Methylene blue	–	+
Motility	–	+

Biochemical Reactions

Glucose, maltose and sucrose are fermented with production of acid but no gas. Nitrates are reduced to nitrites and catalase is formed.

Antigenic Structure

Three antigens of *B. anthracis* have been partially characterised. These are the capsular polypeptide, somatic polysaccharide antigens and a complex protein toxin.

Capsular polypeptide. This is a high molecular weight substance which consists exclusively of d-glutamic acid. There appears to be single antigenic type.

Somatic polysaccharide antigen. This contains d-glucosamine and d-galactose in equimolar amounts. It is a constituent of cell wall. It gives rise to antibodies in the host but these antibodies are not protective.

Anthrax toxin. Anthrax toxin is a complex protein which consists of three parts designated as oedema factor (EF), protective antigen (PA) and lethal factor (LF). These are also designated as factor, I, II and III respectively. These are believed to act synergestically.

On entering the cell, EF is activated by calmodulin in the cytoplasm and adenylate cyclase activity produces altered water and ion exchange in the cells of the tissue resulting in edema at the site of infection.

Determinants of Pathogenicity

Only capsulated strains of *B. anthracis* that produce toxin are fully virulent. The capsule interferes with the

phagocytosis and helps the organism's survival especially during the early stages of infection. Antibodies produced against the capsule are not protective. The signs and symptoms of anthrax are due to the production and action of a toxin.

Pathogenesis and Clinical Picture

Anthrax is primarily a disease of sheep, cattle, horses and many other animals. Humans are rarely affected. There are three possible modes by which a man may get infected by *B. anthracis* and produce 3 types of disease.

i. **Cutaneous anthrax:** The spores gain access through small abrasions or cuts and multiply locally. This localised infection is called as cutaneous anthrax or malignant pustule.
ii. **Pulmonary anthrax:** It is also called as the hide Porter's disease. They may also gain access through inhalation (Woolsorter's disease). This results in germination of spores in the lungs or in tracheobronchial lymph nodes. Production of haemorrhagic mediastinitis, pneumonia, meningitis and sepsis which are usually fatal conditions may follow.
iii. **Intestinal anthrax:** A rare method of acquisition of infection is through ingestion of infected meat with resultant invasion of gastrointestinal tract and ulceration.

Anthrax infection in man provides permanent immunity and second attacks are extremely rare.

Anthrax as a Biological Weapon

Many characteristics of this organism have made this as a potential biological weapon. It is easily available, can be grown luxuriously, is without any smell, can survive in environment for a long period, can be converted into dried powdered form with which it can be sprayed to enable it to enter human body through respiratory tract leading to a severe illness after a very short incubation period. Nonavailability of an efficient immunoprophylactic agent and requirement of extensive antimicrobial therapy are additional features that make it an attractive organism for biological warfare or bio-terrorism.

Laboratory Diagnosis

The clinical material suspected to be having *B. anthracis* must be handled with utmost care in a biosafety cabinet because of its tremendous capacity to infect the handler.

i. **Collection of clinical samples:** The specimens are collected as per the type of lesion. In malignant pustule, the best specimen is fluid from an unbroken vesicle from the edge of lesion. In pulmonary anthrax, sputum and in septicaemic clinical stage blood are the samples which should be processed. In malignant pustule, the fluid from the lesion can be collected on a swab. In early stages of lesion, scrapings can be obtained with the help of a needle.
ii. **Examination of smear:** Two smears are made of which one is stained by Gram's method and the other by Giemsa's technique. Gram-positive bacilli shall be visible in cases of anthrax in Gram staining wherein with Giemsa stain, blue stained bacilli surrounded by irregular, red purple capsular material can be seen. These slides must be autoclaved before discarding because the spores are not killed by the methods of fixation and hence can be a potential health hazard.
iii. **Culture:** Inoculate the material on blood agar and nutrient agar and incubate these at 37°C for 18-24 hours. Typical "medusa head" colonies are seen which can be confirmed to be Gram-positive bacilli, some with central, oval spores, by staining procedure. Cultures can be confirmed by gamma phage lysis and direct fluorescent antibody test. The cultures have to be carefully autoclaved before final discard.

Sputum in cases of pulmonary anthrax should be examined on the same lines as for fluid from cutaneous anthrax. For blood culture 5-10 ml of the blood is inoculated into blood culture medium routinely used for isolation of aerobic bacteria. After incubation, subcultures are made onto blood agar and nutrient agar media and rest of the process is same as described above.

iv. **Animal pathogenicity:** The broth culture should be diluted 1:100 and then 0.1 ml of the same be injected subcutaneously into a guinea pig or mouse. Within 72 hours the animal dies if anthrax bacilli are there and the same can be demonstrated from the heart blood and spleen to confirm the presence of *B. anthracis*.
v. **Serological diagnosis:** Though techniques are now available to detect antibodies against anthrax bacilli, these are rarely found in cases with cutaneous anthrax. However, in cases where septicaemia occurs, antibody detection is possible. Both *in vitro* (gel diffusion) as well as *in vivo* (neutralization test in rabbits) tests are available.

In addition, high affinity dot ELISA based upon monoclonal antibody is now available as a screening test. Anthrax antigen can be detected in tissue extracts by Ascoli's thermoprecipitin test

vi. **PCR:** Polymerase chain reaction test has been standardised for the diagnosis of anthrax, which has replaced the animal pathogenicity test.

Treatment and Prevention

Doxycycline and ciprofloxacin are effective in treatment and prophylaxis.

ANTHRACOID BACILLI

There are many aerobic spore bearers which are saprophytic and do not cause any disease in human beings. These, however, resemble *B. anthracis* morphologically and are known as pseudo-anthrax bacilli or **anthracoid** bacilli. The important species included in this group are *B. subtilis*, *B, licheniformis*, and *B. mycoides*.

Unlike anthrax bacilli, anthracoid bacilli are generally motile, noncapsulated, grow in short chains, do not produce medusa head colonies, cause beta haemolysis on blood agar medium, are not susceptible to gamma phage and not pathogenic to laboratory animals (Table 32.2).

Rarely some of the species of this genus can produce disease in human beings. The best example is that of *B. subtilis* which may, on occasions, cause eye infection and septicaemia. Inclusion of *B. cereus*, which is well known pathogen of humans in this group by some scientists is debatable.

Table 32–2. Differences between anthrax and anthracoid bacilli

Character	*Anthrax bacillus*	*Anthracoid bacilli*
Motility	–	+
Capsule	+	–
Size of chains formed	Long	Short
Medusa head colony	+	–
Haemolysis on blood agar	–	+
Turbidity in broth	–	+
Growth on penicillin agar	–	+
Growth at 45°C	–	+
Inverted fur tree growth in gelatin	+	–
Sensitivity to gamma phage	+	–
Fermentation of salicin	–	+
Pathogenic to man	+	–
Animal pathogenicity	+	–

BACILLUS CEREUS

Bacillus cereus is widely present in soil, vegetables and a large variety of food and poultry products. It has been recently incriminated as an important causative agent of food poisoning in humans.

Morphology

These are gram-positive bacilli which have straight or rounded ends. Spores are formed in the presence of oxygen but their location within the bacillus is not characteristic. These are motile by means of peritrichous flagellae. Flagellar antigens have also been used for strain differentiation.

Culture Characters

The organisms can grow on ordinary nutrient media at 37°C at a pH of 7-7.4 under aerobic conditions. It does not grow on desoxycholate agar medium but produces large, irregular pale colonies on MacConkey agar. A relatively selective medium for *B. cereus* is phenol red egg yolk polymyxin agar. The colonies are recognised by their capacity to produce opacity in the egg yolk and their pink colouration with phenol red because they do not ferment mannitol present in the medium.

The important differences between *B. cereus* and *B. anthracis* have been shown in Table 32.1.

Antigenic Structure

All the strains of *B. cereus* share a common spore antigen which they also share with *B. thuringiensis*. On the basis of vegetative somatic (O) antigen, 13 serotypes of *B. cereus* have been identified. Flagellar (H) antigens have found wider application in serological studies in *Bacillus*. With its help a typing scheme has been developed for *B. cereus*. More than 17 H serotypes have been recognised and these have been employed in sero-epidemiological studies of food poisoning outbreaks.

Pathogenicity

Four groups of virulence factors for this organism have been detected.

i. *Phospholipase C group of enzymes*. These are three in number and act on phospholipase C and sphingomyelin of biological membranes.

ii. *Haemolysins*. Two types of haemolysins are produced. However, their role in pathogenesis is not known.

iii. *Diarrhoeal enterotoxin*. It is multicomponent protein of molecular weight 38000-46000 daltons. It causes

fluid accumulation in ligated loops as well as severe mucosal damage.

iv. *Emetic toxin*. This induces emetic type of food poisoning in monkeys and man.

Clinical Features

Food poisoning is the most important clinical syndrome produced by this organism. Two distinct forms of poisoning have been detected.

Emetic (Food Poisoning of Short Incubation Period) Type

The emetic type is associated with rice dishes. The emetic form begins 1-6 hours after ingestion of contaminated food.

Diarrhoeal (Food Poisoning of Long Incubation Period) Type

The diarrhoeal type manifests after 1-24 hours of ingestion of essentially meat dishes.

Mere presence of *B. cereus* in the stool of patient is not sufficient to make diagnosis since the bacteria may be present even in normal stool. Same serotype of organism should be isolated from the incriminated food and the number of bacteria in food should also be more than 100,000 per gram of food.

B. cereus has been associated with many other opportunistic infections in immunologically compromised hosts as well as certain invasive diseases in otherwise healthy individuals.

Laboratory Diagnosis

Food, faeces and vomitus are inoculated on DCA, MacConkey agar as well as selective mannitol-phenol red-egg yolk-polymyxin medium and typical colonies studied and further examined. The organism can be differentiated from *B.anthracis* on the basis of characters mentioned in Table 32.1.

BACILLUS STEAROTHERMOPHILUS

Bacillus stearothermophilus can withstand temperature of 121°C for 12 minutes and is one of the most heat resistant organisms known. This organism is used to test autoclaves which run on a time-temperature cycle designed to ensure the destruction of spores. Strips containing this organism are included with the material being autoclaved, and subsequently examined by culture for surviving spore. To test the ethylene oxide sterilizers, a red pigment producing variant of *B. subtilis* designated now as *B. gobigi* is used. Similarly, efficacy of ionizing radiations can be tested with *B. pumilis*.

OTHER BACILLUS SPECIES

B. subtilis, *B. pumilis*, and *B.licheniformis* have been implicated in causing food poisoning similar to one due to *B. cereus*. Instead of producing any enterotoxin or enteroinvasiveness, these organisms seem to cause disease through the production of an antibacterial peptide which facilitates their growth in intestine.

33 Clostridium

The anaerobic bacteria are easily separated into two groups, those with and without spores. The spore forming anaerobes constitute the genus *Clostridium*. These are gram-positive bacilli, produce powerful exotoxins and occur across a wide variety of habitats. The important member of the non-spore bearing anaerobes is Bacteroides. Anaerobic infections are generally polymicrobial. These bacteria are found in mixed infections with other anaerobes, facultative anaerobes and aerobes.

Habitat

Clostridia are widely distributed in nature. Their main habitat is soil. Some of these, however, are commensals of intestinal canal of man and animals. *Clostridium perfringens* is uniformly present in the faeces of human beings whereas faeces of breast-fed infants commonly has *Clostridium difficile* in it. *C.tetani* has been isolated from 10-40% of faecal specimens of domestic animals.

Nomenclature and Classification

The spores in this genus are wider than the body of the bacterium thus giving it a swollen appearance which resembles a spindle and hence the name clostridium (*kloster*: spindle). The possible location of spores has been shown in Figure 33.1.

The clostridia can be classified on the basis of three simple criteria:

a. Location and shape of spores
b. Pathogenicity to human beings
c. Biochemical reactions

a. Location and shape of spores. The appearance and location of spores are characteristic for some species of this genus (Table 33.1).

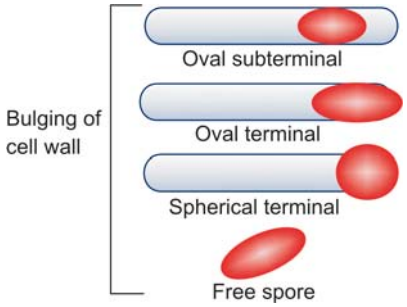


Fig. 33–1. Location of spores of Clostridium

Table 33–1. Location of spores in various clostridia

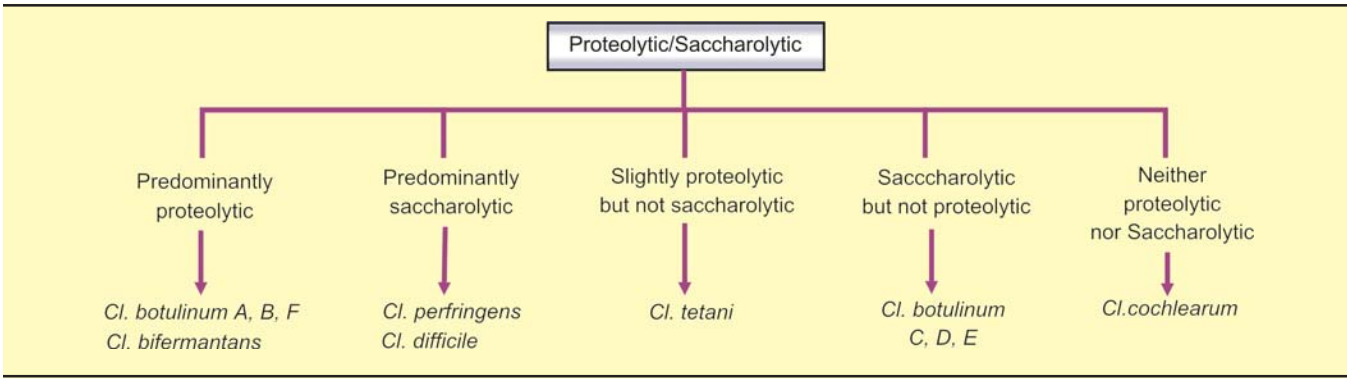
Spore	Species
Central or equatorial	*C.bifermentans*
Subterminal	*C.perfringens*
Oval or terminal	*C.tertium*
Spherical and terminal	*C.tetani*

b. Pathogenicity to human beings. Though clostridia usually lead a saprophytic existence, several species are causative agents of recognised diseases in human beings. On the basis of their pathogenicity, clostridia have been classified into four groups (Table 33.2).

Table 33–2. Classification of clostridia according to pathogenicity

Group	*Species*
Tetanus	*C.tetani*
Acute colitis	*C.difficile*
Food poisoning	
• Gastroenteritis	*C.perfringens* type A
• Botulism	*C.botulinum*
Gas gangrene	*C.perfringens*
	C.septicum
	C.novyi
	C.histolyticum
	C.fallax

Table 33–3. Classification of clostridia based on biochemical tests



c. Based on biochemical reactions. Based on their property of proteolytic and saccharolytic the clostridia can be classified as shown in Table 33.3.

CLOSTRIDIUM BOTULINUM

Morphology

Organisms belonging to this species are large, stout rods with straight axis, parallel sides and rounded ends. These measure 4-6 µm × 0.9 µm. Spores are situated at or near the ends and are oval in shape (Fig. 33.2). Spores are wider than the body of the bacillus and are formed better in sugar-free media.

C. botulinum is sluggishly motile and possess 4-8 peritrichate flagellae. These organisms do not have capsule.

Resistance to Physical and Chemical Agents

Spores of different serotypes show variable response to heat. Spores of type A, B and F are highly resistant, those of C and D intermediate and of type E of low resistance. These are destroyed by dry heat at 180°C in 5 minutes. Moist heat at 100°C destroys them in 5 hours, at 105°C in 100 minutes and at 120°C in 5 minutes.

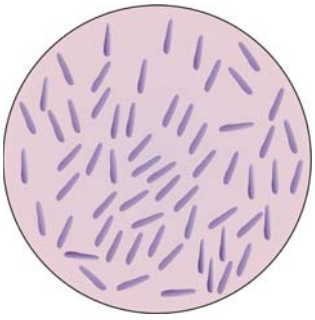


Fig. 33–2. *Clostridium botulinum*

Cultural Characters

Under anaerobic conditions this organism can be grown in various media including blood agar, egg-yolk agar medium, MacConkey's agar, and cooked meat medium.

On the basis of colony characters *C.botulinum* can be divided into four groups:

Group I. Colonies are 3-8 mm in diameter, rounded, opaque compact centre, matt or semiglossy surface and growth tends to spread over the entire surface of the medium. This group includes strains from type A and proteolytic strains of types B and F.

Group II. Colonies are 1-3 mm in diameter, irregularly circular, greyish, semitranslucent and with a ground glass surface. To this belong strains of type E and non-proteolytic strains of types B and F.

Group III. These colonies are of same size as are colonies of Group II, circular, entire, raised, greyish white and semitranslucent with a smooth matt surface. Non-proteolytic strains of types C and D fall into this group.

Group IV. These measure around 0.5-1 mm in diameter, greyish and translucent, entire and with a shiny surface. Occasionally these give characteristic appearance of **fried eggs**. Strains of type G are included in this group.

Narrow zone of haemolysis is produced on blood agar and all strains except those of type G produce lipolytic reaction on the egg-yolk agar.

Antigenic Types

Seven toxigenic types designated A-G have been established for *C. botulinum*. These fall into three distinct serological groups on the basis of heat-stable somatic antigen (Table 33.4).

Table 33–4. Serological and toxigenic types of *C.botulinum*

Serological group	*Toxigenic types*
I	Type A Proteolytic strains of types B,F
II	Type E Non-proteolytic strains of types B,F
III	Types C and D
Unclassified	Type G

Toxigenic types A and B can be divided into several subtypes on the basis of agglutination reaction. Types C and D share some minor antigens and their toxin production is phage mediated. Types A, B, E and F cause botulism in human beings.

Pathogenicity and Toxin

Pathogenicity entirely depends upon the toxin production. *C.botulinum* is rarely a parasite because of its inability to grow easily in tissues. Its pathogenic effects are usually determined by the formation of toxin in food substances before their ingestion. However, in infants growth of the organism alongwith the production of toxin may take place in the gut of the child. This phenomenon rarely occurs in adults.

Botulinum toxin. Toxin produced by *C.botulinum* is one of the most poisonous substance known to mankind. All antigenic types produce neurotoxin which has similar pharmacological activity. Purified neurotoxin of *C. botulinum* type A contains, per mg, about 30 million LD_{50} for mice. A fatal dose for man has been estimated to be between 0.1 and 1.0 µg. It is million times more toxic than strychnine.

It is usually made up of two components. Toxin produced by types A, B and D is 4-10 times more toxic to mice as compared to the toxin of C,E and F types.

The potent neurotoxin is released as prototoxin which is acted upon by proteases of the organism or the environment to be converted into active toxin. It is absorbed from the alimentary tract through gastric and upper intestinal mucosa. It can be demonstrated in the blood via which it reaches peripheral nervous system. The primary site of action of toxin is the cholinergic nerve terminal where it blocks the release of neurotransmitter, acetylcholine.

The toxin produced by different types exhibits differences in susceptibility to inactivation by heat. Boiling rapidly destroys the toxin. The purified toxins are detoxified by formalin with the production of immunogenic toxoid.

Natural immunity to these toxins does not develop because lethal dose of toxin is less than that required to elicit an antibody response. The salient features of botulinum toxin have been summarised in Table 33.5.

Table 33–5. Salient features of botulinum toxin

- Formed *in vitro* in food except in infants in whom it is formed even *in vivo* in gut
- Protein and one of the most poisonous substance Million times more toxic than strychnine. Fatal dose for man is < 1 µg
- Toxins produced by different antigenic types:
  - Are immunologically similar
  - Carry variable pathogenicity
  - Exhibit difference in susceptibility to heat
- Released by bacterium as *prototoxin* which is converted to active toxin by protease
- Primary site of action is cholinergic nerve terminal where it blocks the release of acetylcholine
- Boiling inactivates
- Formalin converts into nontoxic but antigenic toxoid
- Natural immunity does not develop
- Can be detected by specific neutralization test in mice

Transmission and Clinical Features

Spores of *C.botulinum* are often present in food. Production of botulinum toxin results when the microorganisms grow in an anaerobic environment in foods stored at room temperature. Alkaline foods favour the growth of *C.botulinum* and the development of exotoxin. Man to man transmission is not possible. Small outbreaks may result if the same food is consumed by a large number of people.

In most cases, botulism results from the ingestion of preformed toxin. The toxin produces complete paralysis of the nerve impulse by preventing the release of acetylcholine (Fig. 33.3). Death occurs from the paralysis

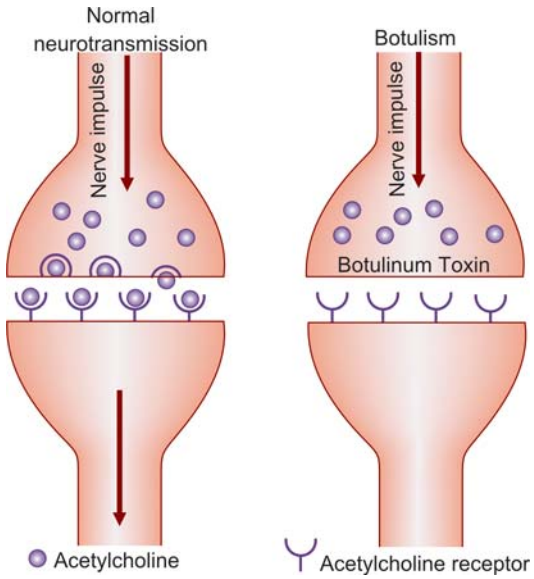


Fig. 33–3. Mechanism of action of botulinum toxin

PART V

of respiratory functions. Symptoms may appear as soon as 12 to 36 hours after the ingestion of contaminated food. First symptoms are usually weakness and dizziness. Diplopia, and dysphagia are important features. Pupils get dilated. Fever is rare. Muscle weakness progresses, leading to paralysis as the disease progresses. The mortality rate varies between 20-70%.

Wound botulism is an extremely rare disease arising from contamination of a wound or devitalised tissue with *C. botulinum*. The toxin gives rise to neurological symptoms after 4-14 days.

Laboratory Diagnosis

i. **Clinical samples.** Material suspected to be contaminated with botulinum toxin must be handled with extreme precautions. Specimens that can be collected shall include faeces, food, vomitus, gastric fluid, serum, environmental samples and occasionally wound exudates.
ii. **Direct demonstration of organism.** The organism can be detected in the clinical specimen with the help of a fluorescent antibody procedure. It is a difficult technique and culture of the specimen is usually preferred over this method.
iii. **Culture.** The culture is done on egg-yolk agar, blood agar and two bottles of cooked meat broth. One of these bottles is heated to 80°C for 10 minutes to act upon the resistant spores. These cultures are incubated anaerobically at 30°C and incubated for 3-5 days. In between periodic screening for the presence of toxin is done from cooked meat broth in mice. For the presence of organisms, fluorescent antibody test can act as a screening test. Absence of any toxin production during 5 days of incubation usually rules out botulism.
The colony characters on solid media have already been described. Presence of suggestive organisms can be confirmed with fluorescent antibody test and the organisms can be put into cooked meat medium for testing of specific toxin after appropriate incubation.
iv. **Neutralisation test.** The ideal method for diagnosis of botulism is detection of toxin in the blood of the patient by specific neutralisation test in mice. However, since it is not always possible one has to resort to isolation, identification and detection of toxigenicity of the causative agent. Toxin can also be detected in the clinical material such as vomitus, food, faeces, etc.

Treatment

Once botulism is suspected, antitoxin should be given as soon as possible. Because any one of the three most common toxin serotypes may cause the disease, a polyvalent antitoxin containing antitoxin to types A, B and E is used. The antitoxin will not reverse the effects of toxin already affecting the nerves but will neutralize the circulating toxin. Supportive care particularly in maintaining respiratory functions is very important.

CLOSTRIDIUM DIFFICILE

C. difficile is responsible for antibiotic-associated pseudomembranous colitis, and for many of the cases of antibiotic-associated diarrhoea. This organism was detected for the first time in 1935 by Hall and O'Toole. It can be isolated from faeces of 15-70% of normal adults. Sometimes, the antibiotics suppress other bacteria of intestine resulting in overgrowth of *C. difficile* and the resultant enteropathogenicity.

Pre-existing disease of gastrointestinal tract and presence of these bacteria in environment predisposes to colonisation of gut.

Morphology

C.difficile organisms are large gram-positive bacilli measuring 6-8 µm in length and 0.5 µm in width. Young cultures give uniform Gram reaction but the older cultures are frequently gram-negative. The spores are terminal, elongated and slightly wider than the body of the bacillus.

Culture

These bacteria are obligatory anaerobes. They grow easily on blood agar without producing any haemolysis. The colonies are 2-3 mm in diameter, irregularly circular, flat to slightly raised, semitranslucent and white with a glossy but rough and often pitted surface. These colonies after incubation for 48 hours and on exposure to UV rays usually show bright green fluorescence.

For epidemiological studies, *C. difficile* has been divided into several types with the help of slide agglutination, bacteriophages and bacteriocines as well as polyacrylamide gel electrophoresis of cell proteins.

Sensitivity to Chemical and Physical Agents

The vegetative cells of *C. difficile* are sensitive to oxygen whereas the spores are resistant. Spores can also withstand temperature of 75°C for 10-20 minutes.

Pathogenicity

Two thermolabile toxins are produced by many, but not all the strains of *C. difficile.* These have been designated as toxin A which is an enterotoxin and toxin B which is a cytotoxin. The enterotoxin causes fluid accumulation in the gut and cytotoxin causes extensive damage to cells especially of mammalian origin.

Both the toxins can be detected by ELISA and latex agglutination tests. For the detection of cytotoxin, CIEP as well as assays in tissue cultures have been developed.

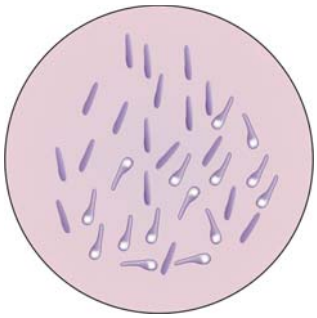


Fig. 33–4. Clostridium perfringens

Laboratory Diagnosis

A fresh specimen is very essential since vegetative forms are rapidly killed on exposure to oxygen. Swabs must be immediately processed. The faecal supernate can be investigated for the presence of cytotoxin by testing for its activity (rounding of cells) on human embryo fibroblasts in microtitre plates and then testing for neutralization of this activity by specific antitoxin.

The isolation of *C. difficile* has been greatly facilitated by the introduction of cycloserine cefoxitin fructose agar (CCFA) which is a selective medium. Increased yields of *C. difficile* may be obtained by the use of this medium with reduced (almost half) concentrations of antibiotics and pretreatment of sample with alcohol to reduce the number of nonsporing bacteria. The medium is incubated anaerobically and examined after 24 hours.

The cooked meat broth (CMB) can be used as enrichment medium to increase the yield. After incubation, subculture is made onto CCFA medium. Colonies are then studied and identification made. Production of cytotoxin can be assayed by the method described above.

Treatment

The use of offending agent should be discontinued immediately and fluid and electrolytes loss caused by diarrhoea should be replenished. Most of the cases recover within 48 hours. However, if no recovery takes place or the disease is extremely severe, vancomycin, bacitracin or metronidazole can be used.

CLOSTRIDIUM PERFRINGENS

C. perfringens was also known as *C. welchii* after the name of one of the two workers (Welch and Nuttall) who isolated it for the first time in 1892. Since then it has been found to be ubiquitous and in the intestinal tract of man and animals.

Morphology

The organisms of species *C. perfringens* are stout nonmotile bacilli with blunt or square ends. These measure around 4-8 µm × 0.8-1.0 µm and are usually arranged singly often side by side forming small bundles. The spores are large, oval and subterminal (Fig. 33.4). Sporulation is favoured by alkaline medium and rarely occurs at pH less than 6.6. Sometimes capsules are formed in animals.

Culture

Under anaerobic conditions bacteria grow easily on blood agar and produce beta haemolysis around the colonies. Two types of colonies may be formed. One is round, 2-4 mm in diameter, greyish yellow, amorphous, opaque and with entire edge. These are butyrous and easily emulsifiable. The other type is light in colour with brownish centre and crenated edges. These organisms grow rapidly in cooked meat medium and produce lot of gas. The meat particles are converted into pink colour.

Antigenic Types

C. perfringens is divisible into five serologic types, A to E. This is according to the type and proportion of soluble antigens (exotoxins) produced by the organisms.

Pathogenic Properties

Types A, C and D are pathogenic for man. Type A is responsible for gas gangrene and food poisoning and type C for enteritis necroticans. The diseases produced by different types have been shown in Table 33.6.

Table 33–6. Diseases produced by serotypes of *C. perfringens*

Serotype	*Diseases in man*
A	Gas gangrene Puerperal infection Septicaemia Food poisoning Wound infection
B	None (pathogenic for animals)
C	Enteritis necroticans
D	Gangrenous appendicitis
E	None (? pathogenic for animals)

Toxicology

C perfringens produces at least 20 exotoxins. The most important exotoxins and their biologic effects are as given in Table 33.7.

Table 33–7. Important exotoxins and their biologic effects

Type of toxin	*Activity*
Alpha toxin (lecithinase)	Lethal,*, necrotising, haemolytic, cardiotoxic
Beta toxin	Lethal,* necrotising
Epsilon toxin	Lethal,* permease
Iota toxin	Lethal,* necrotising
Delta toxin	Lethal,* haemolysin
Phi toxin	Haemolysin, cytolysin
Kappa toxin	Lethal,* collagenase, gelatinase, necrotising
Lambda toxin	Protease
Mu toxin	Hyaluronidase
Nu toxin	Lethal,* deoxyribonuclease, haemolytic, necrotising

*Lethal as tested by injection in mice

The precise role of these exotoxins in the pathogenesis of gas gangrene is not entirely clear; however, alpha-toxin is apparently of utmost importance.

Alpha Toxin

The alpha toxin is a 370-residue zinc metalloenzyme that has phospholipase-C activity (i.e. lecithinase) and causes cell destruction by hydrolysis of key cell membrane components. This toxin can cause lysis of erythrocytes, leukocytes, platelets, fibroblasts, and muscle cells. Strains that do not produce this toxin are less virulent, underscoring its importance. Purified alpha-toxin has a myocardial suppressant effect *in vitro* and causes shock when injected into animals.

Phi Toxin

The phi toxin is a hemolysin. Although it does not directly suppress myocardial function *in vitro*, it contributes to myocardial suppression *in vivo*, possibly by increasing the synthesis of secondary mediators which do suppress myocardial function *in vitro*. At higher concentrations, the phi-toxin can cause extensive cellular degeneration and direct vascular injury.

Kappa Toxin

The kappa toxin produced by *C. perfringens* is a collagenase responsible for destruction of blood vessels and connective tissue. Alpha, beta, epsilon and iota are the major lethal toxins for mice. The lecithinase activity of alpha toxin splits lecithin to phosphorylcholine and a diglyceride and can be demonstrated by production of opalescent clostridia on egg yolk agar (Nagler reaction) and by lysis of sheep or mouse erythrocytes.

An enterotoxin is produced by sporulating cells of types A and C which are responsible for food poisoning and is produced and released in the large intestine during sporulation.

Nagler Reaction

This is performed on a agar medium containing 5% egg-yolk. On one half of the plate, 2-3 drops of *C. perfringens* antitoxin, also called AGGS (Anti-gas Gangrene Serum) is spread and plate is allowed to dry. It is inoculated with the test strain. The inoculum starts from antitoxin-free portion of the medium and extends to part having antitoxin.

Incubation at 37°C is done in anaerobic environment. On the side containing no antitoxin, *C.perfringens* colonies show a surrounding zone of opacity (Fig. 33.5) which is called as Nagler reaction after the name of the scientist who devised the medium and the test for the first time. This reaction is due to the production of phospholipase. Apart from *C.perfringens*, there are three

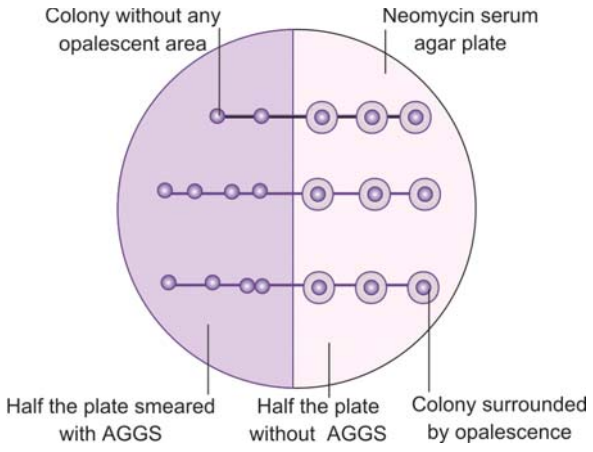


Fig. 33–5. Nagler reaction

Table 33–8. Characteristics of Nagler-positive Clostridium species

Species	*Indole*	*Gelatin*	*Urease*	*Motility*	*Reverse CAMP*
C. perfringens	–	+	–	–	+
C.baratii	–	–	–	+	–
C. bifermentans	+	+	–	+	–
C. sordelli	+	+	+	+	–

other species of this genus which show positive Nagler reaction because of the production of serologically related phospholipases by them. However, these can be distinguished by other tests (Table 33.8).

Reverse CAMP test. This test is similar to the CAMP test for identifying group B beta haemolytic streptococci except that *Clostridium* species replaces *Staphylococcus aureus* and a known group B beta haemolytic streptococcus is used. Although group B streptococci may exhibit some enhanced haemolysis with other clostridia, it is only with *C.perfringens* that the characteristic arrow head form is demonstrated (Fig. 33.7).

Pathogenesis

Contamination with clostridial spores in posttraumatic or postoperative lesions establishes the initial stage of infection. Local wound conditions are more important than the degree of clostridial contamination in the development of gas gangrene. Disrupted or necrotic tissue provides the necessary enzymes and a low oxidation/reduction potential, allowing for spore germination. Foreign bodies, premature wound closure, and devitalized muscle reduce the spore inoculum necessary to cause infection in laboratory animals.

The typical incubation period for gas gangrene frequently is short (i.e. <24 h), but incubation periods of 1 hour to 6 weeks have been reported. Self-perpetuating destruction of tissue occurs by the presence of a rapidly multiplying microbial population and the production of locally and systemically acting exotoxins. Local effects include necrosis of muscle and subcutaneous fat and thrombosis of blood vessels. Marked edema may further compromise blood supply to the region. Fermentation of glucose probably is the main mechanism of gas production in gas gangrene.

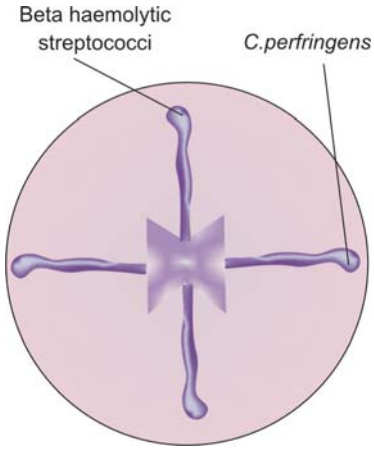


Fig. 33–6. Reverse CAMP test

Systemically, exotoxins may cause severe hemolysis. Hemoglobin levels may drop to very low levels and, when occurring with hypotension, may cause acute tubular necrosis and renal failure. A rapidly progressive infection can cause a patient to progress quickly into shock. The mechanism of shock is poorly understood. Unconcentrated filtrate from *C perfringens*, purified alpha-toxin, and purified phi-toxins cause hypotension, bradycardia, and decreased cardiac output when injected into laboratory animals. Because alpha-toxins and phi-toxins are lipophilic and may remain locally bound to tissue plasma membranes, the toxins may stimulate synthesis of secondary mediators, which cause cardiovascular abnormalities.

Clinical Picture

Many wounds that occur in wartime or road accidents get contaminated with clostridia. When a niche of devitalised tissue or foreign body exists in the wound, it may create an anaerobic area in which the clostridia can proliferate. As the toxin and enzymes of proliferating clostridia are released, the tissues adjacent to the wound are devitalised and supply of oxygenated blood is stopped. The situation creates an expanded area of anaerobiasis in which clostridia can grow and liberate more toxins and enzymes. The damage thus keeps on progressing.

Gas gangrene is a more severe form of the infection with toxic destruction of the adjacent muscle tissues and an ever-widening expansion of the lesion. The swollen tissues have a dark yellowish discolouration and produce a foul smelling, dark fluid exudate. Gas is formed by the bacteria, causing some distension of subcutaneous tissue and considerable pain. Symptoms of gas gangrene begin to appear 12 to 72 hours after the injury. Along with the local tissue involvement, a

generalised toxic reaction including fever, toxaemia and shock may be seen in these patients. Death results if proper treatment is not given.

Food poisoning. In addition to producing gas gangrene, *C.perfringens* is a common cause of food poisoning. Infection is most commonly associated with consumption of contaminated meat dishes. The spores may survive the normal cooking process.

Laboratory Diagnosis

The bacteriological diagnosis of gas gangrene should be done in conjunction with diagnosis of any other bacteria from the wound.

i. **Clinical samples.** It is advisable to collect sample from the deeper recesses of the wound where anaerobiasis is suspected. Surface swabs because of their constant exposure to environmental oxygen shall not act as good specimen for isolation of clostridia. Pus, excised tissue or necrosed tissue should be preferred over swab. To obviate the danger of anaerobic bacteria getting killed in transit, it is better to inoculate one bottle of cooked meat medium with the sample at the bed side itself. If only swabs can be collected, at least three should be obtained: one for staining and one each for aerobic and anaerobic cultures.

ii. **Direct demonstration of organism.** Stain smear with Gram's method. If gas gangrene is present, gram-positive bacilli which are thick and stubby shall predomi–nate in case of infection due to *C.perfringens*. Organisms of *C.septicum* shall be pleomorphic and boat- or leaf shaped. *C.novyi* appear as large rods with oval subterminal spores but these are scanty in number.

iii. **Direct immunofluorescence method** is now available to detect *C.novyi* and *C.septicum* but not *C.perfringens.*

iv. **Culture.** In addition to media used for the isolation of aerobic bacteria, cooked meat broth, blood agar and blood agar with neomycin should be inoculated for the isolation of anaerobes. These should be incubated at 37°C for 24 hours in an environment of 10% carbon dioxide.

Examine growth in cooked meat broth (enrichment medium) and make subculture from here onto solid media and incubate anaerobically. Examine subcultures for typical colonies and perform biochemical and other identification tests as discussed earlier.

C.perfringens is present in the environment and may also be present in the clinical sample as an environmental contaminant. This will grow profusely in enrichment medium of cooked meat broth. It can be distinguished from organisms from animal or man by the absence of capsules in environmental *C.perfringens*.

v. **Gas chromatography** is now available to confirm these clostridia.

Treatment

Removal of dead tissue from the wound is the first step in treatment. Penicillin or other antibiotics alongwith antitoxin should be given and are helpful if all dead tissue is removed.

CLOSTRIDIUM NOVYI

Morphology

This are long bacilli which may measure as 4-20 µm × 1-2 µm. with rounded ends and are usually seen single, in pairs or in chains. Spores are large, oval or subterminal. With the help of 20 or more peritrichate flagella, the organism is motile under anaerobic conditions. The organism does not form capsule and young cultures are gram-positive but turn gram-negative after repeated subcultures.

Culture

Colonies are 2-3 mm in diameter, irregularly round, glistening, translucent, butyrous and easily emulsifiable. Haemolysis is produced on horse blood agar by most strains.

Antigenic Structure

Of the four types, types A, B and C share two O antigens in varying proportions. Strains of type D differ from other types but within the type share same somatic antigens.

Pathogenicity

Types A, B and D produce potent exotoxin. Culture filtrates contain at least six antigens. These types are pathogenic for man and produce gas gangrene.

CLOSTRIDIUM SEPTICUM

These bacteria are widely present in the soil and also in the intestinal tract of animals and man.

Morphology

These organisms resemble *C.novyi* but are smaller in size. Spores are readily formed and are oval, subterminal and slightly wider than the body of the bacillus.

These are motile but do not produce any capsule. As with other clostridia, young cultures are gram-positive and ageing cultures become gram-negative.

Culture

After 36 hours of incubation large sized colonies measuring about 10 mm in diameter are produced. These are irregularly round, greyish, translucent, butyrous and easily emulsifiable.

Antigenic Structure

On the basis of two somatic (O) and five H antigens, the organism is divisible into six groups. Culture filtrates contain at least 4 antigens, alpha, beta, gamma and delta and haemolysin.

OTHER HISTOTOXIC CLOSTRIDIA

Apart from the important species of *Clostridium* which can produce gas gangrene and which have been described earlier, few more species can act as histotoxic. These include *C.histolyticum*, *C.bifermentans* and *C.fallax.*

C.histolyticum is a normal inhabitant of human intestinal tract as well as soil. This organism is capable of growing even aerobically on blood agar medium. However, incubation under anaerobiasis gives better yield. Several soluble antigens are produced, of which alpha and beta toxins are lethal and necrotizing. Beta toxin is a collagenase that causes the destruction of collagen fibres and marked disruption of tissues is observed which leads to myonecrosis.

C.bifermentans was earlier considered to be synonymous with *C. sordelli* but later on important differences between the two were detected giving *C.bifermentans* an independent species status. This organism is also normally present in soil as well as intestinal tract of humans. It produces proteolytic enzymes and lecithinase which is serologically related to alpha toxin of *C.perfringens*.

C. fallax has been rarely recorded to be pathogenic. It is a strict anaerobe and rapidly loses virulence after isolation and artificial cultivation.

CLOSTRIDIUM TETANI

C.tetani is the causative agent of tetanus which is one of the important killer infections in the developing countries. Both neonatal tetanus which results because of unhygienic care of umbilical cord as well as adult tetanus are widely prevalent and are diseases of paramount public health importance.

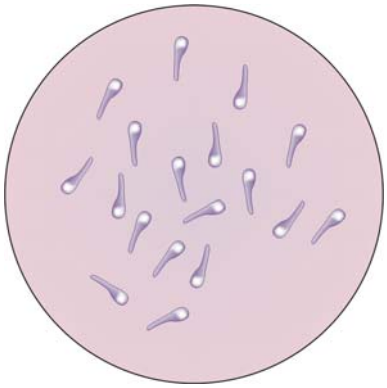


Fig. 33–7. *Clostridium tetani*

The bacilli of tetanus are common saprophytes occurring in cultivated soil throughout the world. The first isolation of this organism was made in 1889 by Kitasato.

Morphology

The bacilli are long as compared to other members of genus, thin and straight. Individual bacilli range from 2-5 µm in width and 3-8 µm in length. The bacilli have rounded ends. In young cultures bacteria stain strongly Gram positive. Even spores during early stages are Gram positive and stain solidly. In older cultures and smears made from wounds, the bacilli are usually Gram negative. Under appropriate culture conditions the organism produces a spore terminally located and of considerably greater diameter than the vegetative cell, giving the characteristic drum stick appearance (Fig. 33.7).

These organisms do not have capsule and are motile with the help of peritrichate flagella, the motility albeit is sluggish.

Cultural Characters

C. tetani is an obligate anaerobe and moderately fastidious regarding its requirements for anaerobiasis. The optimal temperature for growth is 37°C and the optimal pH is 7.4. Like other clostridia, this organism has also got complex nutritional requirements. Blood agar and cooked meat broth, however, adequately meet these requirements. Colonies are irregular, 2-5 mm in diameter, glistening, translucent and greyish yellow. These are butyrous and emulsify quickly. Isolated colonies of motile strains are extremely difficult to obtain. Usually a fine spreading growth on the medium is seen which may not be apparent on cursory examination. On blood agar, haemolysis is evident under

initial confluent growth. Isolated colonies are produced by nonmotile variants. In cooked meat broth a small amount of growth is seen after 48 hours of incubation without any digestion of meat.

Sensitivity to Physical and Chemical Agents

The spores of *C. tetani* show variable resistance to environmental agents. Some are killed by boiling water within 5-15 minutes but certain resistant strains resist boiling for upto 3 hours before these are killed. Dry heat (150°C) for one hour, and exposure to 5% phenol or 0.1% mercuric chloride for upto 2 weeks may not kill the spores. The spores are, however, sensitive to the action of 1% watery solution of iodine and hydrogen peroxide. Vegetative cells are sensitive to penicillin and various other antibiotics including clindamycin and metronidazole.

Determinants of Pathogenicity

C. tetani fails to produce invasive cellulitis when present all alone in the human body. Other factors which stimulate the production of pathogenicity are not known. It is hypothesized that other bacteria present in the lesion play an important role in reducing the oxidation-reduction potential at the site of injury and only then tetanus bacilli produce their soluble products.

Two products liberated by *C.tetani* are the classical neurotoxin (*tetanospasmin*) and a haemolysin (*tetanolysin*). The haemolysin is heat labile and inactivated by oxygen. It is liberated by those strains which do not produce neurotoxin. Like tetanospasmin, tetanolysin produced by different serotypes is similar.

All the symptoms in tetanus are attributable to an extremely toxic neurotoxin. The purified neurotoxin is a simple protein containing no carbohydrate and with a molecular weight of 150000 daltons. The toxin exists in two stages—the toxin monomer and a dimer of about twice that molecular weight. The dimer is nontoxic but still antigenic.

Tetanus toxin is one of the most poisonous substances known. There is no *in vitro* test to detect its activity. Toxicity is observed by assaying the toxin in experimental animals. The toxin is relatively heat labile being destroyed at 65°C in 5 minutes. It is stable in dry state. It is not absorbed from the gastrointestinal tract and may be inactivated by gastric juice.

Treatment of toxin with formaldehyde results in polymerization of toxin and results into formation of a product (**toxoid**) which is non-toxic but antigenic.

Mode of Action. The molecular basis for the action of tetanospasmin is not known. The site of action is the synaptosomes (nipped-off nerve endings) that are high in toxin fixing capacity. This binding of toxin takes place because of gangliosides in synaptic membranes. The gangliosides are water soluble mucolipids containing residues of stearic acid, glucose, galactose and sialic acid. The toxin specifically affects the synaptic junction of nerves by preventing the inhibition or erasing of nerve impulses once they have crossed the synaptic junctions. The nerve continues to send impulses, a condition that results in spasmodic contraction (tetany) of the involved muscles. Early symptoms are muscle stiffness with the muscles of the jaw often developing spasms first. This condition gives the disease its common name of **lockjaw**. As the disease progresses, spasms develop in other muscles. The spasms may be brief but can occur frequently and cause great pain and exhaustion. Respiratory complications are many and death rate is high, especially in young children and elderly persons. In nonfatal cases, recovery takes several weeks but is often complete.

The essential features of tetanus neurotoxin are summarised in Table 33.9.

Table 33–9. Salient features of tetanus neurotoxin

- Protein in nature, without any carbohydrate
- Exists either as monomer or as dimer
- Monomer is toxic and antigenic, dimer is only antigenic
- Extremely poisonous
- Heat labile (destroyed at 65°C within 5 minutes)
- Toxicity is detected in animals only
- Site of action is synaptosomes
- Prevents inhibition of nerve impulse once they have crossed the synaptic junction
- Formalin converts it into toxoid

Pathogenesis

Because of the wide distribution of *C.tetani* (commonly found in soil and manures), wounds are often contaminated with these spores. However, the disease tetanus does not develop in a large majority of cases. The condition of the wound must be such that an anaerobic environment exists with some dead tissues present. These conditions allow the spores to germinate, bacteria to proliferate and toxin to be produced. Such conditions are often seen in puncture wounds. Other kinds of wounds or conditions resulting in tissue damage may also offer a suitable environment for the growth of *C.tetani*. The most important example of this is unhygienic care of umbilical cord in developing countries which gives rise to high incidence of tetanus neonatorum. Once the toxin is formed it gains access to central nervous system by ascending the motor nerves. Lot of evidence has accumulated in favour of this mode of transport. Moreover, toxin does not appear to be delivered directly to central nervous system by the bloodstream.

Laboratory Diagnosis

Diagnosis of tetanus is mainly clinical based on its typical clinical features. The steps in laboratory confirmation are:

i. **Clinical specimen.** The specimen would be wound exudate or tissue removed from the wound. The laboratory diagnosis has three components: direct smear examination, culture and animal pathogenicity tests.

ii. **Direct staining.** Direct smear made from exudate and stained with Gram's method shall demonstrate a few drum stick appearance bacilli. All the bacilli shall not be of this appearance. The presence of drum stick bacilli, however, should not be considered as conclusive evidence of tetanus as bacilli of tetanus can be present in any wound which has been contaminated with soil but the patient may not necessarily have tetanus. It may not be possible to distinguish by microscopy *Cl. tetani* and morphologically similar bacilli such as *Cl. tetanomorphum and Cl. sphenoides.*

iii. **Direct immunofluorescence test** can also be employed for the demonstration of tetanus bacilli. Conjugated immunoglobulins are now commercially available.

iv. **Culture examination.** The specimen is inoculated onto cooked meat medium as well as blood agar. One blood agar plate with antitoxin control can also be employed. In this half of the freshly prepared blood agar plate is smeared with tetanus antitoxin. If toxin producing *C.tetani* are present in the culture, haemolysis produced by them on blood agar shall be inhibited in half of the plate by the antitoxin.

Plates are incubated anaerobically and examined with a hand lens for spreading colonies. Simultaneously examine the cooked meat medium broth daily microscopically. If *C.tetani* are suspected heat part of broth at 80°C for 10 minutes and then subculture heated as well as unheated broth onto blood agar. At 80°C spores of tetanus bacilli get killed.

The growth obtained on solid medium can be confirmed by Gram's staining as well as immunofluorescence.

v. **Animal pathogenicity.** It can be checked once the pure growth of organisms is obtained on solid culture medium. Alternatively it can be done with supernate of cooked meat broth. A pair of mice is used for test. One of the animals can be protected with intraperitoneal injection of 500-1500 units of tetanus antitoxin administered one hour prior to test. Inject 0.1 ml of cooked meat broth supernate of the organism intramuscularly into the hind limb of the test as well as control mice. Signs of ascending tetanus develop in test animal and the protected mouse remains normal. If large doses are injected, death of mice may take place within 18-24 hours without any clinical features of tetanus.

Prophylaxis

Tetanus can be prevented by good management of the wound, passive and active immunization. A combination of these steps proves effective even in the worst contaminated wounds.

Passive immunisation using antitetanus serum (ATS) obtained from hyper-immune horses and given in dosage of 1500 IU subcutaneously or intramuscularly in non-immune persons after a tetanus prone injury has been in practice since long. However the equine ATS had two disadvantages (a) hypersensitivity reactions and (b) short duration of protection by it.

In most of the developed countries, tetanus immunoglobulin raised in humans has replaced the antitetanus toxin raised in horses. The latter is however, still in use in developing countries. The tetanus immune globulins (TIG) have the advantage of longer half-life, freedom from the risk of adverse reactions and of early elimination. The minimum concentration of antitoxin in the blood that is adequate to provide the protection is 0.01 unit/ml and it can be achieved usually by administration of 1500 units of horse antitoxin or 750 units of TIG.

Passive immunisation is used once only. Its repeated use is risky and also wasteful. A previously immunised individual does not need any ATS/TIG and if required the immunity can be boosted up using tetanus toxoid only.

Active immunization is achieved through tetanus toxoid prepared from tetanus toxin released by the bacilli in liquid culture in large capacity fermenters. The crude toxin is purified before being adsorbed with a suitable adjuvant. It is used alone, in combination with diphtheria toxoid; both diphtheria toxoid and pertussis antigen and in some places alongwith inactivated polio vaccine.

Injecting into man of 2-3 doses of toxoid (0.5 ml each) at properly spaced duration raises the antitoxin level of the blood within a few weeks to protective status. The immunity provided by one full course of three injections is long lasting and may persist life long. It has been seen in many studies that circulating antitoxin level after immunization with toxoid falls gradually for 10 years and then remains more or less stationary.

In severely contaminated wounds, combined active and passive immunization should be attempted.

34 Actinomycetes

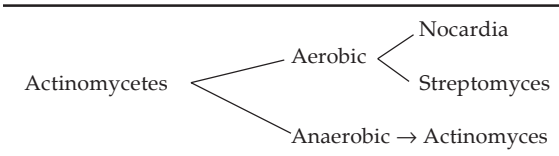
The actinomycetes are gram-positive, non-motile, non-sporing, non-capsulated filaments that break up into bacillary and coccoid elements. These are a heterogenous group of bacteria related to corynebacteria and mycobacteria and superficially resembling fungi. These are also known as fungus-like bacteria or higher bacteria. These are traditionally considered to be transitional forms between bacteria and fungi. There are various characters which distinguish these bacteria from fungi (Table 34.1).

Table 34–1. Features distinguishing actinomycetes from fungi

Prokaryotes	:	Lack nuclear membrane
Cell wall	:	Contains muramic acid
	:	Lacks chitin and glucans
Growth	:	Inhibited by conventional antibacterial drugs
	:	Unaffected by conventional antifungal agents
Infected by phages (actinophages)		
Colonies are typical of bacteria		
Thin mycelia or filaments resemble bacterial forms		

Classification

The human pathogenic actinomycetes are classified in the order *Actinomycetales*. These can be broadly classified as follows:



Three medically important genera of Actinomycetes are: *Nocardia, Streptomyces* and *Actinomyces.* Some Actinomycetes are acid-fast. Most are free living particularly in soil. The anaerobic species are part of the normal flora of the mouth. Some of the aerobic species found in soil may cause disease in humans and animals. Various clinical entities produced by different species of these genera have been depicted in Table 34.2.

Table 34–2. Diseases produced by actinomycetes

Disease	*Species*
Actinomycosis	*Actinomyces israelii*
	Actinomyces bovis
	Actinomyces naeslundi
Nocardiosis	*Nocardia asteroides*
	Nocardia brasiliensis
	Nocardia caviae
Actinomycotic mycetoma	*Actinomadura madurae*
	Streptomyces somaliensis
	Actinomadura pelletierii

ACTINOMYCOSIS

Actinomycosis is a chronic, suppurative disease that spreads by direct extension, forms draining sinus tracts. It is caused by *Actinomyces israelii* and related anaerobic filamentous bacteria including *Arachnia sps.* These form part of the normal flora of the oral cavity and it is not clear what transforms carriage of the organism into invasive disease.

Epidemiology

In man it is an endogenous infection. The disease occurs throughout the world but its incidence has been declining in the last few decades probably as a result of the widespread use of antibiotics. Young males are most commonly affected. Majority of the cases (60%) are of cervicofacial type.

Clinical Features

The characteristic appearance of actinomycosis is hard, red, relatively nontender swelling that usually develops slowly. It becomes fluctuant, points to a surface and eventually drains forming a chronic sinus tract with little tendency to heal. Lesions spread by contiguity. Dissemination via the bloodstream is very rare.

Based on site of involvement this is of three types:

a. *Cervicofacial:* producing what is called "lumpy jaw"
b. *Thoracic type*
c. *Abdominal type*

In over half of the cases the initial lesion is cervicofacial involving face, neck, tongue or mandible. About 20% of the cases show predominant involvement of the lungs with empyema formation. In most cases the primary lesion is in caecum, appendix or pelvic organs and may develop multiple draining fistulae. Pelvic actinomycosis has occurred particularly in women wearing intrauterine contraceptive devices.

Characteristic of lesion. The typical lesion consists of an abscess with central necrosis, surrounded by granulation and fibrous tissue. The pus often contains "sulphur granules" and may drain to outside through sinuses. In early lesions a mixed flora is always seen.

Laboratory Diagnosis

Collection of specimen. Specimen is collected aseptically from lesions (pus), sinus tract or fistulae and sputum or tissue biopy material.

Microscopic examination. The typical "sulphur granules" should be actively searched. Granules can be obtained by applying gauze pads over discharging sinuses or collecting the pus or sputum in saline which is centrifuged and granules are obtained in the sediment. On gross examination, the granules are white or yellowish in colour and range in size from minute specks to about 5 mm. These are crushed in between two slides and stained with Gram's stain. The granules are found to consist of a dense network of thin gram-positive filaments, surrounded by a peripheral zone of swollen radiating club shaped structures, presenting a "sun ray appearance" (Fig. 34.1). The clubs are gram-negative and are of host origin. If no granules are found, stained smears of gram-positive branching rods and filaments are suggestive of diagnosis.

Culture. The granules are washed repeatedly with sterile saline crushed and inoculated by streaks in plates of brain heart infusion agar and incubated under anaerobic conditions at 37°C for atleast 2 weeks.

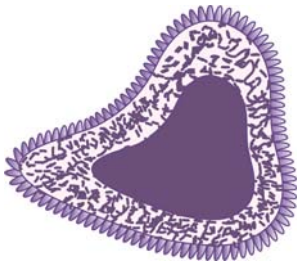


Fig. 34–1. Sulphur granules

Colonies are small, white and flat or centrally indented, resembling a "molar tooth". In thioglycollate broth pure cultures present a "diffuse appearance". Microscopically long or short branching filaments and diphtheroid forms are observed. No conidia are produced.

Animal inoculation, skin test and serological tests are not useful.

Treatment

Most cases respond to antibiotic treatment given over long period. However, the drugs may penetrate the abscess poorly and some of the tissue destruction may be irreversible, hence surgical drainage and removal are accepted forms of treatment.

NOCARDIOSIS

This infection is being seen with increasing frequency in patients who are immunologically compromised. it is an opportunistic pulmonary pathogen that may spread to other organs. These organisms can also produce mycetoma.

Three species of this genus are important: *N.brasiliensis, N.asteroides and N.caviae.*

Morphology

These organisms are thin, gram-positive, branching filaments that may fragment into bacillary or coccoid forms. Many isolates are acid fast when decolourised with 1% sulphuric acid. The acid fastness does not persist when decolourisation is done with 20% sulphuric acid. Because of this *Nocardia* are also called as partial acid-fast. Apart from this, nocardia differ from actinomycetes in various aspects (Table 34.3). These do not produce granules.

Clinical Features

Nocardiosis begins as a pulmonary infection that may be subclinical or produce pneumonia. After breathing in the organism, manifestations are initially pulmonary, resembling tuberculosis or bacterial pneumonia.

Table 34–3. Differences between genera actinomyces and nocardia

Feature	*Actinomyces*	*Nocardia*
Growth atmosphere	Facultative anaerobe	Obligate aerobe
Catalase	Variable	+
Acid-fastness	–	Partial
Habitat	Mouth and skin	Soil
Growth temperature	35-37°C	Wide range
Source of infection	Endogenous	Exogenous

Purulent sputum contains acid-fast branching filaments. Early spread of the disease results in scattered subcutaneous draining abscesses involving the brain and the skin. Less frequently the pleura and heart are also affected. Nocardiosis should be suspected whenever there is suspicion of tuberculosis since both manifest similarly. Brain and kidney lesions may also develop.

Nocardiosis is commonly seen in immunosuppressed patients such as AIDS cases, leukaemia and lymphoma patients.

Laboratory Diagnosis

Collection of specimen. Specimen consist of sputum, spinal fluid, pus and biopsy material.

Microscopic examination. Gram stained smears show coccal or bacillary forms or tangled masses of branching rods. Some strains are partially acid-fast.

Culture. *Nocardia* species grow on most laboratory media but may be inhibited by the presence of antibacterial quantities of antibiotics in media. These survive mycobacteria concentration procedures and grow rapidly on media recommended for mycobacteria. The colonies are chalky, brittle, verrucose and white to orange to pink to red. Usually no conidia are seen but in older cultures, the conidia may be produced.

Histopathology. *Nacardia* species does not take up the H and E stain. Results with PAS and Gridley stains are also not reliable. However, Gomori methenamine silver stain effectively demonstrates these organisms.

Treatment

The sulphonamides are the drugs of choice. Co-trimoxazole is also very effective. Surgical drainage or resection may also be required. Treatment of the underlying disease should be attempted.

ACTINOMYCOTIC MYCETOMA

Mycetoma resulting from puncture or abrasion with contaminated material is usually limited to the feet but may be seen in hands and buttocks. Mycetoma could be produced by true fungi (eumycotic mycetoma), fungi like bacteria (actinomycetoma, Table 34.2) and bacteria (botryomycosis). The establishment of correct diagnosis of mycetoma is of paramount importance because of different line of treatment for diverse aetiology.

PART VI
GRAM-NEGATIVE BACTERIA

35 Neisseria

The genus *Neisseria* is one of the five genera included in the family *Neisseriaceae.* Other genera in this family include *Branhamella*, *Moraxella* and *Acinetobacter*.

Man is the only known reservoir of these organisms. Apart from *N.gonorrhoeae* and *N.meningitidis* which are pathogenic to man there are several other species which are either commensals or form part of normal bacterial flora of upper respiratory tract and other mucosal surfaces of the body (Table 35.1).

Table 35–1. Characteristics of species of Neisseria

Species	*Acid from*				H_2S
	G	*M*	*L*	*S*	
N.gonorrhoeae	+	–	–	–	–
N.meningitidis	+	+	–	–	–
N.lactamica	+	+	+	–	–
N.sicca	+	+	–	+	+
N.subflava	+	+	–	v	+
N.flavescens	–	–	–	–	+
N.mucosa	+	+	+	+	+

v=variable G = Glucose, M = Maltose, L = Lactose, S = Sucrose

NEISSERIA MENINGITIDIS

Neisseria meningitidis, commonly called as meningococcus, was isolated for the first time in 1887. Meningococcus is the causative agent of meningococcal meningitis.

Morphology

These are oval, gram-negative diplococci, with flattened or concave opposing edges and long parallel axis. These measure around 0.6 µm in diameter. Fresh isolates are usually encapsulated (Fig. 35.1). These do not possess flagellae. Individual cocci are kidney-shaped, when the organisms occur in pairs, the flat or concave sides are adjacent.

Growth Requirements

The meningococci are fastidious organisms with complex nutritional requirements. Their growth is improved by addition of blood or serum.

Growth of primary isolates is enhanced by the incubation in the presence of 2 to 8% CO_2.

All pathogenic *Neisseriae* have an optimum growth temperature that ranges from 36 to 39°C. The minimum temperature at which growth can take place is about 24°C. The Neisseriae produce oxidase and give positive oxidase reactions. It is a key test in its identification.

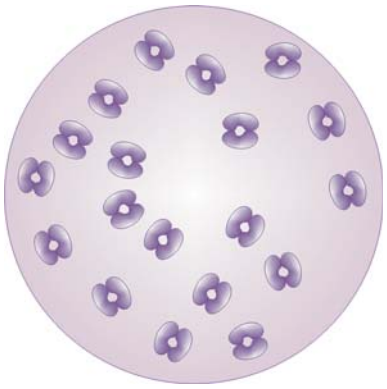


Fig. 35–1. Neisseria meningitidis

Susceptibility to Physical and Chemical Agents

These are extremely fragile organisms and cannot withstand gross variations in requirements of environmental factors. In cultures most of them die out in few days probably because of the large quantities of alkali produced which kills them. These are destroyed at 55°C within 5 minutes. They are very susceptible to desiccation and oxidation. The organisms are rapidly killed by drying, sunlight, moist heat and many disinfectants.

Culture

Meningococci grow poorly on unenriched medium. Strains will, however, grow on Mueller Hinton medium without the addition of blood or serum. After 24 hours of growth at 37°C, the colonies on blood agar are 1-2 mm in diameter, convex, grey and translucent. After 48 hours the colonies are larger with an opaque raised centre and thin transparent margins which may be crenated. No haemolysis is seen on blood agar medium. Larger colonies are obtained on heated blood agar (chocolate agar). These are oxidase positive organisms.

Antigenic Structure

On the basis of capsular polysaccharide, thirteen serogroups of meningococci have been established. These have been designated as A, B, C, D, H, I, K, L, X, Y, Z, Z1 (29E) and W-135.

Strains belonging to serogroups A, B and C have been found to be responsible for epidemics of the disease. Because of this reason these 3 serogroups are classified as major serogroups while remaining are classified as minor serogroups. Capsular polysaccharides are important antigens in immunity to meningococcal disease and the polysaccharides of groups A, C, Y and W-135 have been incorporated into effective vaccines.

Determinants of Pathogenicity

The capsular polysaccharide and endotoxin are two important determinants of pathogenicity for this organism. The capsular polysaccharide contributes to the invasive properties of the meningococci by inhibiting phagocytosis. In the presence of specific antibody, organisms are readily ingested and destroyed by the phagocytic leucocytes. No intracellular growth of bacteria has been documented.

The endotoxin resembles the endotoxin of other gram-negative bacteria and is responsible for extensive vascular damage. This endotoxin has been shown to have two antigenic determinants, one of which is a protein and the other a carbohydrate.

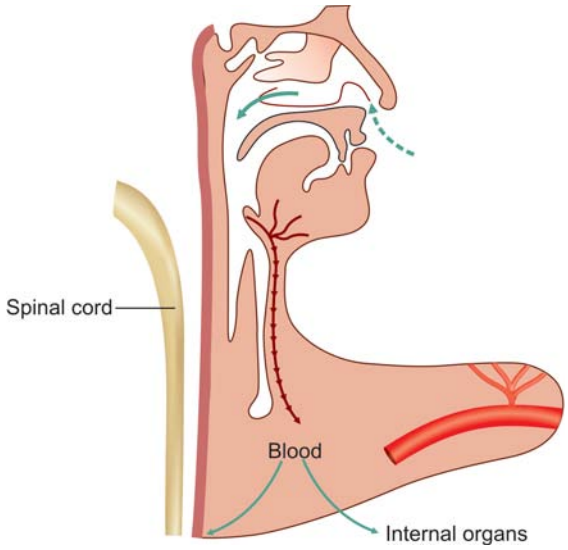


Fig. 35–2. Pathogenesis of meningococcal infection

Pathogenesis and Clinical Manifestations

Meningococci gain entry into human body through nasopharynx (Fig. 35.2) and with the help of pili attach themselves to the epithelial cells. For an indeterminate period they constitute a part of normal flora of that site and then enter the bloodstream. What prompts a transient flora of nasopharynx to cause meningococcaemia is as yet not known, but clinical picture manifests with bacteraemia. **Fulminant meningococcaemia** is a severe condition with high fever, haemorrhagic rashes and circulatory shock. The haemorrhages may take place in different parts and sites of body including skin and adrenals. The former result into skin rashes characteristic of this bacterial infection whereas the latter causes **Waterhouse Friderichsen syndrome**. Disseminated intravascular coagulation (DIC) is a frequent complication of this disease.

Nevertheless, the most common complication of bacteraemia is meningitis. It has a sudden onset with intense headache, vomiting and stiff neck and may progress to coma within a few hours. Meningococcaemia in the absence of meningitis carries a poor prognosis.

Laboratory Diagnosis

i. **Clinical samples:** Meningococcal infection is specifically diagnosed by the identification of *N.meningitidis* in materials obtained from the

patient. The specimens may include cerebrospinal fluid (CSF), blood, aspirate from skin lesion or pus. Swabs can be obtained from nasopharynx for determining the carrier stage. The specimens must be processed immediately, but if the delay is inevitable, the swabs should be transported in Stuart's transport medium.

ii. **Cerebrospinal fluid cytology:** The cell count in the CSF shall indicate the type of meningitis. In cases with meningococcal meningitis, the response is primarily polymorphonuclear. From the deposit of the centrifuged CSF, Gram's stained smear shall show gram-negative diplococci. All these cocci may not be intracellular, some may be extracellular. If fluorescein isothiocyanate conjugated antisera against different serogroups are available, identification of serogroup can be done in a smear from this deposit.

The supernatant can be used for performing biochemical tests as well as detection of antigen of *N.meningitidis*. The biochemical reaction shall show increase in proteins and chlorides and a fall in glucose level of the CSF.

iii. **Immunodiagnosis:** The availability of rapid immunodiagnostic tests such as coagglutination, countercurrent immunoelectrophoresis (CIEP) and latex agglutination have made the diagnosis much easier and sensitive. Commercial kits of latex agglutination against common groups of meningococcus are now widely available and produce result within minutes of the testing of supernatant.

iv. **Culture:** CSF is cultured on blood agar and heated blood agar (chocolate agar) and incubated at 37°C in an environment of 10% CO_2 for 24 hours after which colonies are stained by Gram's method and oxidase test performed.

Further diagnosis can be based upon the results of biochemical tests (Table 35.1). Antibiotic sensitivity against penicillin, sulphonamides, chloramphenicol, tetracycline and rifampicin can be determined by agar diffusion techniques.

Serogrouping can be performed by slide agglutination with hyperimmune serum. This can also be performed by incorporating the specific antiserum in a concentration of 5% in the Mueller Hinton agar plate and inoculating the same with the strain to be serogrouped. In a positive reaction a halo of precipitate appears in the medium around the colonies after incubation of 24-48 hours.

Although intravenous injection of viable meningococci into rabbits shall prove fatal within 24 hours, animal tests are not recommended for diagnosis.

Blood. It is inoculated into blood culture bottles and after incubation subcultures are made onto blood agar and chocolate agar medium. The incubations, examination and further processing of these plates is done as described under CSF.

Pus, aspirate and swabs. These can be examined by making direct smear and staining with Gram's as well as FITC conjugated antisera. Cultures are made onto chocolate agar and processed as described earlier.

v. **Demonstration/isolation from petechiae:** The organism can be demonstrated from the irrigated fluid taken from the petechial skin rashes. The same can be cultured to yield *N. meningitidis* in positive cases.

vi. **PCR:** PCR has been standardised for diagnosis but is not widely available in many laboratories.

Treatment

Penicillin remains the drug of choice for therapy of meningococcal infections. In penicillin sensitive individuals, chloramphenicol or a third generation cephalosporin such as cefotaxime or ceftriaxone may be used.

Prevention

Vaccines have been developed against serogroups A and C Y, W-135. These vaccines use purified capsular antigens and a single dose produces a good antibody response. With the help of group C vaccine, meningitis due to this serogroup has been virtually eliminated from the military recruits of the USA. These vaccines seem to be less effective in children under 2 years of age.

Chemoprophylaxis/immunoprophylaxis: Close surveillance of household, day care and other intimate contacts of an index case for early signs of illness, specially fever is essential. All such contacts can be given chemoprophylaxis for 4-5 days using rifampicin, ceftriaxone or ciprofloxacin. Mass chemoprophylaxis or prolonged chemoprophylaxis is not recommended. Health care workers are rarely at risk even when caring for infected patients, only intimate exposure to nasopharyngeal secretions warrants prophylaxis.

When an outbreak occurs, major emphasis must be placed on careful surveillance, early diagnosis and immediate treatment of suspect cases. When large scale epidemics occur, mass vaccination of the entire population should be considered when vaccine supply and administrative facilities allow. Epidemics may develop in situations of forced crowding - in such

situations a special watch should be kept. Though vaccination with meningococcal vaccines is not covered under International Health Regulations, some countries may require a valid certificate of immunisation against meningococcal meningitis, e.g. Saudi Arabia for Haj pilgrims.

NEISSERIA GONORRHOEAE

Morphology

Gonococcus resembles morphologically meningococci with certain subtle differences. These bacteria are also diplococci with adjacent sides flattened. In pus obtained from a case with gonorrhoea, gram-negative diplococci, most of which shall be within the pus cells, can easily be seen (Fig. 35.3). Autolysis in cultures gives rise to many variations in staining reactions. These are non-motile and freshly isolated organisms may be capsulated. Pili are formed on the surface of gonococci which help them in adhesion. Three antigenically distinct types of pili have been observed from cultures.

Culture

Gonococci are more delicate and nutritionally exacting organisms as compared to meningococci. These are aerobic organisms and grow well on enriched media such as chocolate agar. Primary isolation on unlysed blood agar does not give good results. In case the clinical material is highly contaminated it is advisable to use a selective medium for the isolation of gonococci. For incubation a moist environment with 5-10% carbon dioxide is must. Optimum growth occurs at 37°C and no growth takes place if the temperature is less than 25°C or more than 38.5°C.

The colonies are 0.6 to 1.4 mm in diameter, convex, translucent with entire edge, amorphous with granular surface.

The biochemical reactions manifested by gonococci have been depicted in Table 35.1. The gonococci are oxidase positive and utilise glucose but not maltose, sucrose, lactose or fructose. These do not reduce nitrate and H_2S is also not produced.

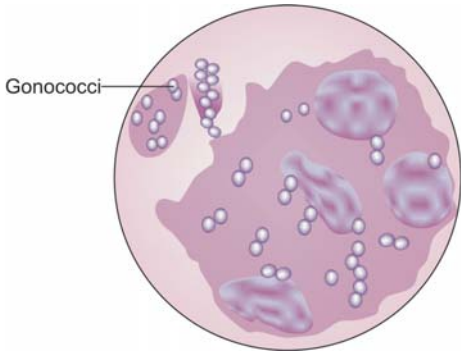


Fig. 35–3. Neisseria gonorrhoeae in pus

Sensitivity to Physical and Chemical Agents

Though gonococci are extremely sensitive organisms which cannot tolerate inimical agents, yet these can survive in pus contaminating linen or other fabrics. Cultured organisms die in a few days at room temperature. These are readily killed by drying, soap and water, and many other disinfectants and antiseptics in proper dilutions. Freeze drying is the most effective method for long storage of gonococci but keeping these at –70°C or in liquid nitrogen is also very useful.

Antigenic Structure and Virulence Factors

N.gonorrhoeae is antigenically heterogenous.To avoid host defenses these can change their surface structures both *in vitro* as well as *in vivo*. Surface structures include the followings:

Capsule. The capsule is most evident in recent isolates or on isolates grown *in vivo.* It is loosely associated with cell surface and can easily be sheared. Its synthesis is dependent upon environmental and nutritional factors. The capsule is antiphagocytic but anticapsular antibodies promote phagocytosis.

Pili. These are hair like structures which extend from the surface. These have a diameter of about 7 nm. The pili are proteins (pilin) in nature. The pilins of almost all the strains of gonococci are antigenically different.

Pili play an important role in attachment of gonococci to the host cells. Pili appear to recognise specific receptors on the surface of the host cells.

Lipopolysaccharide (LPS). It differs in its chemical structure from LPS of gram-negative bacilli. It does not have long O- antigenic side chains and is sometimes called as lipo-oligosaccharide. The molecular weight of LPS of gonococci is between 3000 to 7000 daltons. This LPS endows the endotoxic effect on gonococci.

Other proteins. There are six such proteins. These are: Lip(H8), Fbp(iron binding protein) and IgA1 protease, protein I (Por), protein II (Opa) and protein III (Rmp). The precise role of all these in pathogenesis of infection due to gonococci is not clear.

The structure and virulence factors of *N.gonorrhoeae* have been diagrammatically presented in Figure 35.4.

Pathogenesis and Clinical Picture

The primary infection begins at the columnar epithelium of the urethra and periurethral ducts and glands

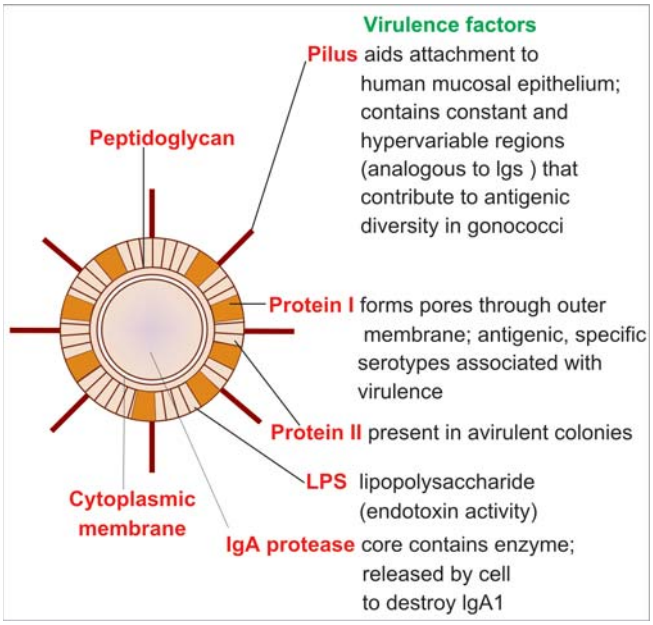


Fig. 35–4. Virulence factors of gonococci

of either sex. Cervical, conjunctival and rectal mucosa may also serve as portal of entry. The infection gets established within one hour and bacteria get anchored to surface of host cells through pili. Spread is facilitated by various virulence factors (Fig. 35.4). These organisms reach the subepithelial connective tissue within 72 hours by traversing intercellular spaces. The host responds in an intense inflammatory reaction which is rich in polymorph cells. This exudate can obstruct the ducts and glands and cause abscesses as well as cysts. Spread occurs by direct extension or through lymphatics but rarely through blood vessels.

Though gonococcal infections have short incubation period (1-2 days) but the disease is not highly contagious. An unprotected male has approximately 22% chances of acquiring gonorrhoea from intercourse with an infected female in a single exposure.

Certain strains of gonococci are associated with disseminated infection. The characters of these strains are summarised in Table 35.2.

Table 35–2. Bacterial features associated with dissemination

- Resistance to bactericidal action of serum
- Marked susceptibility to penicillin
- Growth in laboratory requires:
  - — Arginine
  - — Uracil
  - — Hypoxanthine

The diseases produced by gonococci in male, female and children have been shown in Table 35.3.

Table 35–3. Clinical manifestations of gonococcal infection

Group	*Common diseases*	*Less common diseases*
Male	Acute gonorrhoea Urethral stricture Epididymitis Prostatitis	Septicaemia Peritonitis Meningitis Dermatitis Arthritis Pharyngitis Proctitis
Female	Acute gonorrhoea Pelvic inflammatory disease	Proctitis Arthritis Dermatitis DIC Meningitis Pharyngitis
Children	Gonorrhoea Conjunctivitis Ophthalmia neonatorum	Arthritis Vulvovaginitis

Immunity

Inspite of repeated infections with *N.gonorrhoeae*, immunity to gonorrhoea does not develop. This is because of the antigenic variety of gonococci. Antibodies of IgG and IgA classes can be easily demonstrated in serum as well as mucosal surfaces but these are either highly strain specific or have little protective ability.

Laboratory Diagnosis

i. **Clinical specimens:** The specimens required for the isolation and identification of gonococci include pus and secretions taken from the urethra, cervix, rectum, conjunctiva, throat or synovial fluid. In generalised infections blood culture should be resorted to but the blood culture medium should be free of polyanethol sulfonate. Best results are obtained if the clinical samples are inoculated onto media at the bedside of the patient. When direct plating and incubation are not practical, specimen should be transported in a suitable transport medium. Amie's modification of Stuart's transport medium gives good results for transportation.

The sites from where samples are collected have been mentioned in Table 35.4.

Table 35–4. Sites* sampled for gonococci

Men	Urethral meatus Rectum Pharynx
Women	Urethral meatus Cervical os Rectum Pharynx
Newborns	Conjunctival secretions

*Smears for Gram's staining are prepared from all the sites except pharynx from where only culture is made.

ii. **Direct smear examination:** Presumptive diagnosis of infection can be made by examining the Gram stained smear and detection of gram- negative diplococci *some of which must be intracellular.* If such an evidence becomes available, no further processing is required in male but culture is required even in these circumstances when patient is a female.

When used by properly trained personnel, the direct Gram staining is approximately 95% sensitive and 100% specific for the diagnosis of gonococcal urethritis in symptomatic men. The sensitivity of endocervical smear in detecting gonorrhoea in symptomatic women is only 60%.

iii. **Culture:** Blood agar (or chocolate agar) and Thayer Martin medium with antibiotics are the media required. Incubation is done at 37°C in the presence of 5-10% carbon dioxide and a moist environment. Plates are examined after 24 hours of incubation. Apart from colony characters, positive oxidase test is strongly suggestive of infection.

Biochemical tests can be performed on the bacteria grown on solid media. A **coagglutination test** is now available which uses monoclonal antibody reactive with Por (Protein I). This test has a sensitivity of 99.7% and 100% specificity.

Antibiotic sensitivity specially against penicillin and production of enzyme beta lactamase (penicillinase) should also be ascertained for instituting an effective therapy.

In suspected disseminated gonococcal infection blood culture can be put up in biphasic medium.

iv. **Immunological tests:** Antibody of the classes IgG and IgA against pili, outer membrane proteins and LPS appear in the serum as well as genital fluid. Though not of much diagnostic importance these antibodies can be detected by ELISA, immunoblotting and radioimmunoassays. These tests are not useful as diagnostic aids for several reasons (a) heterogeneity of gonococcal antigenicity (b) delay in the development of antibodies in acute infection (c) high level of back ground antibodies in the sexually active population.

Treatment

Penicillin is the drug of choice for susceptible strains. The alternate drugs in penicillin resistant infection or penicillin sensitive individuals include spectinomycin, tetracycline, doxycycline and ceftriaxone. Resistance has already been reported for spectinomycin and tetracycline.

Gonococcal ophthalmia neonatorum is prevented by local application of 0.5% erythromycin ophthalmic ointment or 1% tetracycline ointment. Resistance of the gonococcus to common antimicrobials is due to the widespread presence of plasmide that carry genes for resistance. Thus strains of gonococcus are resistant to penicillin (PPNG), tetracycline (TRNG) and fluoroquinolones (QRNG).

OTHER NEISSERIAE

Other members of genus *Neisseria* rarely cause disease. Most of these constitute part of normal bacterial flora, mostly of respiratory tract. Some of these resemble biochemically the pathogenic species of the genus and can be mistaken as variants of gonococci or meningococci. *N.lactamica,* as the name suggests, ferments lactose unlike other neisseriae.

Moraxella Catarrhalis

This organism was earlier placed in the genus *Neisseria (N. catarrrhalis)* and later on shifted to genus *Branhamella* (*B.catarrhalis)*. It is now placed in genus *Moraxella.*

In a large number of children this organism is present as normal flora. However, it is capable of causing respiratory tract infections including bronchitis, pneumonia, sinusitis, otitis media as well as conjunctivitis. This organism has gained importance as a potential pathogen of individuals with immunologically compromised status. Most strains of *M. catarrhalis* from clinically significant infections produce β-lactamase. It can be differentiated from other Neisseriae spp by its lack of carbohydrate fermentation and by its production of DNase.

36 Haemophilus

The members of the genus *Haemophilus* are small gram-negative bacilli which are strict pathogens. The genus derives its name from its essential growth requirement of certain factors present in the blood (*Haemophilus: Haem*: blood; *philos*: loving).

The first member of this genus was isolated by Koch in 1883 from a case with conjunctivitis in Egypt and is known as *H.aegyptius*. The type species of *Haemophilus;* i.e. *H.influenzae* was isolated in 1892 by Pfeiffer from a patient of influenza and was erroneously considered as causative agent of this disease. The aetiologic agent of influenza is now known to be a virus.

Many other gram-negative bacteria have been included in this genus because of their requirement of one or both of the growth factors present in blood and essential for the growth of organism. These factors are designated as X (hemin or other porphyrins) and V (coenzyme 1, NAD or NADP).

A large number of species of this genus are known to be isolated from various clinical samples. However, a few are of significant medical importance. Some of the salient features on the basis of which these can be differentiated have been shown in Table 36.1.

Table 36–1. Salient features of species of Haemophilus

Species	*Requirement of*			*Haemolysis on horse blood agar*
	X	*V*	CO_2	
H.influenzae	+	+	–	–
H.aegyptius	+	+	–	–
H.ducreyi	+	–	–	–
H.haemolyticus	+	+	–	+
H.aphrophilus	–	–	+	–
H.parainfluenzae	–	+	–	–
H.parahaemolyticus	–	+	–	+

HAEMOPHILUS INFLUENZAE

Haemophilus influenzae is found on the mucous membrane of the upper respiratory tract in humans. It frequently causes meningitis in children and also produces respiratory tract infections in children as well as adults.

Morphology

These are small gram-negative bacilli measuring 0.3-0.5 µm × 1-2 µm. When freshly isolated these are short coccoid in shape. Old cultures, however, show pleomorphism and filamentous as well as swollen forms are seen in these. These are non-motile, non-sporing and non-acid fast. Virulent strains form capsule which can be detected by India ink preparation or by capsular swelling reaction using type specific antiserum. Some strains form fimbriae.

Cultural Characters

The organisms do not grow at a temperature less than 22°C but the optimal growth is seen at 35-37°C. Better growth is obtained under aerobic conditions as compared to anaerobic environment. Presence of CO_2 improves the growth. This organism requires both X and V factors for growth. These factors are present in blood and hence the organism grows on the media containing blood but fails to grow on nutrient agar medium.

X Factor

X factor is a heat stable factor present in blood. Earlier it was thought that this factor is haemoglobin but now it is known that requirements of X factor can be met by various iron containing compounds or haems or protoporphyrin IX. X factor is required for the synthesis

of iron containing enzymes cytochrome oxidase, peroxidase and catalase.

V Factor

V factor is a thermolabile factor and was earlier thought to be a vitamin and hence the name 'V' factor. This has been now ascertained to be NAD and NADP or coenzyme I. This is present inside the erythrocytes and hence not available to bacteria for their growth. Heating blood till it acquires chocolate colour lyses the erythrocytes thus releasing V factor and at the same time elevated temperature destroys NADase activity. V factor is required in oxidation-reduction processes in the growing bacterial cell.

Growth on Solid Media

On ordinary blood agar the colonies of *H.influenzae* are small because of suboptimal concentration of V factor. Larger colonies can be obtained by any of following methods:

a. Using heated blood agar (chocolate agar)
b. Supplementing blood agar with 10-20 mg/litre of NAD at 55°C
c. Using a transparent medium having digested blood such as Fildes agar or Levinthal's agar
d. Growing *H.influenzae* in the vicinity (1-3 mm) of colonies of *Staph.aureus* which excrete an excess of V factor thus promoting the growth of the organisms. This phenomenon is known as **satellitism** (Fig. 36.1).

On a medium with sufficient V factor, the colonies of noncapsulated strains, after 24 hours incubation at 37°C, are 0.5-1 mm in diameter, circular, low convex, smooth, pale grey and transparent. The colonies of capsulated strains are larger (1-3 mm in diameter), high convex in shape and mucoid. *H.influenzae* does not produce any haemolysis on blood agar. For the isolation of *H.influenzae* from sites which have normal bacterial flora such as staphylococci, addition of penicillin and bacitracin to chocolate agar is done.

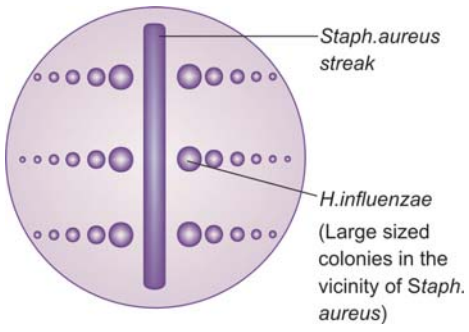


Fig. 36–1. Satellitism

Filde's agar, even without additives, is moderately selective for haemophilus because the large content of haemin provided by the blood digest is inhibitory to viridans streptococci, pneumococci and some neisseriae, though not to enterococci and coliforms.

Growth in Liquid Media

In **liquid media** such as Filde's broth and Levinthal's broth, most strains of *H.influenzae* produce uniform turbidity.

Biochemical Characters

All strains of *Haemophilus* reduce nitrate to nitrite. Other biochemical tests have been given in Table 36.2.

Table 36–2. Biochemical characters of Haemophilus

Species	*Catalase*	*Oxidase*	*Fermentation of*			
			G	*S*	*L*	*M*
H.influenzae	+	+	+	–	–	–
H.parainfluenzae	+	+	+	+	–	–
H.ducreyi	–	+	–	–	–	–
H.haemolyticus	+	+	+	–	–	–
H.parahaemolyticus	+	+	+	+	–	–
H.aphrophilus	–	–	+	+	+	–

G: Glucose; S: Sucrose; L: Lactose; M: Mannitol

Eight **biotypes** of *H.influenzae* have been identified on the basis of indole, urease and ornithine decarboxylase tests.

Sensitivity to Physical and Chemical Agents

Moist heat at 55°C kills *H.influenzae* within 30 minutes. It dies within a few days at 4°C as well as on culture plates. In dried secretions the bacilli die in less than 2 days. *H.influenzae* is very susceptible to the common disinfectants and to desiccation. Cultures are difficult to maintain in laboratory because of autolysis. Proper maintenance requires frequent subcultures on chocolate agar and long-term preservation can be done by lyophilisation.

Strains of this organism are now becoming resistant to ampicillin and amoxycillin. Resistance against sulphonamides and tetracyclines is also being reported.

Antigenic Structure

The capsular antigen. The major antigenic determinant of encapsulated *H.influenzae* is the capsular polysaccharide. This polysaccharide confers type specificity on the organism and is the basis of the grouping of the organism into six serotypes designated as a, b, c, d, e

PART VI

and f. Serotype e strains can be divided into two groups: majority of e serotype strains possess e1 and e2 antigens whereas a few strains possess only e2 antigen. The six serotypes are chemically different. Type b which is commonest is polyribose-ribitol phosphate (PRP).

The type b antigen can be detected in the body fluids by various techniques which include: latex agglutination, coagglutination, countercurrent immunoelectrophoresis, ELISA and radioimmunoassay.

Encapsulated *H.influenzae* may be typed by quellung reaction using antisera prepared against the type specific polysaccharide. Typing may be done on fresh sample from infected patients or on primary isolates. Agglutination and fluorescent antibody techniques can also be used but must be interpreted with caution.

A DNA probe has been developed that hybridizes with a fragment of chromosomal DNA concerned in the production of capsular antigen and can be used for typing of this organism.

The type b teichoic acid (PRP) is a host protective antigen and a number of PRP vaccines and PRP-protein conjugate vaccines have been prepared and lipo-oligosaccharide (LOS) also shows antigenic variation.

Phase Variation

Extracellular production of a polysaccharide capsule is strongly associated with virulence of *H.influenzae* strains. However, at times loss of capsule production yields a selective advantage for this agent at certain times, for example, during adherence to epithelial cells. This loss occurs because of mutation in the locus of the gene that is responsible for extracellular transport of the capsule. Thus such spontaneous variation produces phenotypically uncapsulated strains although the capsule antigen is made and accumulated intracellularly.

Bacteriophages and Bacteriocins

Four phages have been reported for *H.influenzae.* These are HP1, HP3, S2 and N3. *H.influenzae* type b produces a bacteriocin which is called as **haemocin**. This is active against capsulated strains of other types of this organism, other species of this genus, and certain enterobacteria such as *Esch.coli.*

Virulence Factors

Capsular Polysaccharide

The type b capsular polysaccharide is the major virulence determinant of *H.influenzae* type b. It allows the organism to resist the bactericidal activity of serum complement and thus permits longer survival and eventual multiplication of the organism in blood. The fact that the type b capsular material is poorly immunogenic in young children may also play a role in the pathogenicity of the organism.

Pili

The attachment of the organism to the surface of respiratory tract is a prerequisite for the pathogenicity. This property is endowed by the pili (fimbriae).

Outer Membrane Protein

The outer membrane protein facilitates adhesin and protects the organism from the action of various harmful agents.

Protease

Haemophilus produce a protease that inactivates the IgA present on surface of respiratory tract.

Pathogenesis and Clinical Picture

The *Haemophilus* bacteria are widespread in humans; most persons develop active antibody immunity against them before they reach adulthood. Newborns receive passive immunity from their mothers and hence valuable protection during the early months of life. Because antibody to the capsular polysaccharide is protective, as long as infants are protected by this transplacental antibody from their mothers they are at little risk of serious *H.influenzae* type b infection. However, as the antibody begins to be lost, there is a definite and increasing risk of meningitis until the child develops its own antibody.

H.influenzae colonizes in the respiratory tract and as many as 50% of children may be carriers. Only a small number of people who carry this bacterium develop clinical disease. Thus, *H.influenzae* generally functions as an opportunistic pathogen. Inspite of opportunistic character, it is a leading cause of systemic disease. The respiratory tract serves as area for invasion of bloodstream and spread to other parts of body where distinct clinical diseases are caused by this organism (Table 36.3).

Laboratory Diagnosis

Specimens. Blood and cerebrospinal fluid are the samples required for diagnosis of meningitis due to *H.influenzae*. For ear infections, empyema and pyarthrosis, pus from lesion is collected for bacteriological examination and from infections of respiratory tract sputum or throat swabs constitute the clinical material

Table 36–3. Diseases caused by *H.influenzae*

- Meningitis
- Pneumonia
- Acute bacterial epiglottitis
- Otitis media
- Otitis externa
- Childhood pyarthrosis
- Cellulitis
- Pericarditis
- Empyema
- Conjunctivitis
- Nasopharyngitis

for the demonstration and isolation of *H.influenzae*. It must be remembered that samples collected from sites of respiratory tract may have commensal, and usually uncapsulated, *H.influenzae* even if the disease is caused by some other organism.

Direct demonstration. This is possible from sputum, deposit of cerebrospinal fluid and pus.

- Gram stained preparation of these material may show gram-negative coccobacilli.
- Immunofluorescence or Quellung's reaction can be employed for direct demonstration of this organism in clinical material.

Culture isolation and identification. These are done on blood agar supplemented with NAD, charcoal agar or Filde's digest agar and incubated at 37°C in an atmosphere of 10% carbon dioxide. The media can be made selective for isolation from sample taken from upper respiratory tract by adding penicillin and bacitracin to media.

Suspected colonies are stained by Gram's method and confirmation can be obtained by using any of the known immunological tests such as immunofluorescence, latex agglutination, capsular swelling reaction or coagglutination. Otherwise requirement of growth factors X and V coupled with biochemical tests can help in diagnosis and reaching at species as well as biotype level of the isolate.

Tests for X and V factors requirements can be done in several ways. The *Haemophilus* species that require V factor grows around paper strips or discs containing V factor placed on the surface of the agar that has been autoclaved before the blood was added. Autoclaving destroys the V factor since it is heat labile. Alternatively a strip containing X factor can be placed in parallel with one containing V factor on agar deficient in these nutrients. Growth of *Haemophilus* in areas between these two strips indicates requirements for both these factors.

Detection of antigen. Various techniques are now available to detect *H.influenzae* type b antigen in clinical material especially CSF and these include latex agglutination and coagglutination and commercial kits are available for the same.

Treatment and Prevention

For meningitis due to *H.influenzae*, chloramphenicol is the drug of choice. Newer cephalosporins such as cefotaxime are as effective as chloramphenicol. Ampicillin was once considered as an alternative to chloramphenicol but emergence of drug resistance to this antibiotic has restricted its utility.

H.influenzae type b disease can be prevented by the inoculation of *Haemophilus* b conjugate (Hib) vaccine to children aged 18 months or older. 3 doses at 6, 10, 14 weeks of age of vaccine are given.

OTHER HAEMOPHILUS SPECIES

Haemophilus aegyptius

This organism was formerly called as Koch-Weeks bacillus and some people regard as *H.influenzae* biotype III but differs from this biotype in its pathogenicity. *H.influenzae* biotype III causes sporadic conjunctivitis which is mild in intensity whereas *H.aegyptius* causes acute contagious conjunctivitis. It also differs in its nutritional requirements which are more exacting and its failure to ferment xylose. It produces small (0.5 mm) colonies on chocolate agar.

Haemophilus haemolyticus

This organism produces a zone of beta haemolysis around its colonies on blood agar which is more pronounced if sheep or ox blood is used. It is present as a commensal in the throat. Colonies produced by it on blood agar sometimes resemble those of *Strept.pyogenes* but can be differentiated by anaerobic incubation when much reduced haemolysis is seen with *H.haemolyticus* whereas that of streptococci becomes stronger. This organism has not been associated with any illness.

Haemophilus parainfluenzae

This organism differs from *H.influenzae* in its non-requirement of X factor, ability to ferment sucrose but not D-xylose and low pathogenicity. It is present as a commensal in throat, mouth and vagina. It may act as an opportunistic pathogen in causing endocarditis, conjunctivitis and bronchopulmonary infections. The colonies formed by this organism on chocolate agar are bigger than those of *H.influenzae* and are opaque yellowish white.

Haemophilus parahaemolyticus

This organism resembles *H.parainfluenzae*, but in addition causes beta haemolysis around its colonies on blood agar. It is present as a commensal in mouth and throat and may occasionally be responsible for causing endocarditis in patients who are immunologically compromised.

Haemophilus aphrophilus

This organism is present as a commensal in mouth and throat and sometimes may cause endocarditis, jaw infection and brain abscess. It forms high convex colonies upto a diameter of 1.5 mm on chocolate agar within 24 hours. The colonies are yellowish in colour. It does not require V factor for its growth.

Haemophilus ducreyi

H.ducreyi is not present in healthy individuals and instead is the causative agent of a sexually transmitted disease called as *chancroid* or *soft sore*. It is an obligate parasite and very sensitive to desiccation. *H.ducreyi* grows poorly on most media, requires X factor but not V and is biochemically virtually inactive. The primary isolation of this organism is very difficult. Colonies on heated blood agar are less than 0.5 mm in diameter after 72 hours of incubation in an atmosphere of CO_2.

Clinical picture emerges after an incubation of 7-14 days and causes exudative ulcer and bubo. Gram smear from these can show pleomorphic gram-negative bacilli.

The disease responds to therapy with sulphonamides and tetracyclines. Aminoglycosides also yield good results.

GARDNERELLA VAGINALIS

This is a small gram-negative bacillus which was earlier known as *Haemophilus vaginalis.* Because of the variability exhibited by it in its Gram reaction which tended to be gram-positive at most of the times and also because of the presence of volutin granules, it was classified as *Corynebacterium vaginalis.* However, recent electron microscopic studies and DNA homology have resulted into creation of a new genus called as *Gardnerella.* Till date it contains only one species—*G.vaginalis.*

This organism is present as a commonesal in the vagina of almost 50% of normal women. Under the effect of certain hitherto unknown factors, this organism is incriminated as responsible for anaerobic vaginosis, a condition which is characterised by foul smelling vaginal discharge.

Morphology

These are small, pleomorphic rods which show Gram variability. These can be stained with Albert's stain to show metachromatic granules. These are non-motile and nonsporing organisms.

Cultural and Biochemical Characters

These are facultatively anaerobic, grow best at 37°C (range 25-40°C) and the yield can be improved by growing these in an environment of CO_2 under anaerobic conditions. Incubation has to be continued for 48 hours. *Gardnerella vaginalis* does not grow on nutrient agar but can be cultivated on moist blood agar or chocolate agar.

Colonies on blood agar are very small (0.5 mm in diameter) and show beta haemolysis if human or rabbit blood has been used. Growth is slightly better on chocolate agar.

Sensitivity to Physical and Chemical Agents

It is very unstable in cultures and requires subcultures every 48 hours for preservation. It fails to grow in media having more than 2% concentration of NaCl. *G.vaginalis* is resistant to colistin, gentamicin and nalidixic acid but is sensitive to penicillin, ampicillin and trimethoprim. Metronidazole has been found to be the drug of choice against this organism.

Laboratory Diagnosis

Specimen required for bacteriological diagnosis is high vaginal swab or endocervical swab soaked in exudate. For transportation, Stuart's medium can be used. When stained with Gram's method, Gram variable small bacilli may give a clue towards the disease to be due to *G.vaginalis*. Swab is to be plated onto blood agar as well as another medium which has been made selective by the addition of antibiotics. Incubate anaerobically at 37°C for 48 hours and examine the colonies. Confirm the identity of the isolate by Gram staining, biochemical characters such as catalase and hydrolysis of hippurate.

Treatment

Metronidazole is the drug of choice. Almost all the strains of *G.vaginalis* are sensitive to this drug albeit the inhibitory concentration may vary.

37 Bordetella

The members of the genus *Bordetella* are small Gram-negative coccobacilli which are obligate respiratory tract pathogens of warm-blooded animals including birds. These are unable to survive outside their hosts.

Four species of this genus are recognised.

- *B.pertussis*
- *B.parapertussis*
- *B.bronchiseptica*
- *B.avium*.

All these except *B.avium* are pathogenic for human beings and cause pertussis (whooping cough). *B.pertussis*—the commonest agent of whooping cough, was isolated by Bordet and Gengou in 1906. While *B.parapertussis* is believed to cause mild disease and *B.bronchiseptica* has been isolated from very few cases of whooping cough.

Morphology

The bordetellae demonstrate pleomorphism in morphology. These may appear as coccobacilli or bacilli arranged singly, in pairs or in small groups. Their size varies from 0.3-0.5 µm × 0.5-2.0 µm. In virulent strains of *B.pertussis*, bacillus forms predominate whereas in avirulent strains coccobacilli forms are seen in majority. When freshly isolated *B.pertussis* may possess a poorly defined capsule. With toludine blue stain bipolar metachromatic granules can be demonstrated.

Growth and Cultural Characters

The bordetellae are aerobes with an optimum temperature of growth of 35°C. Primary isolation is not possible on conventional media such as blood agar and nutrient agar and requires an enriched medium.

Growth on Bordet and Gengou's Medium

Bordet and Gengou's medium which contains glycerol, potato, agar and blood is the medium of choice. The blood concentration may be upto 15-20% which facilitates examination of haemolysis around the colonies. Charcoal agar with 10% blood has also been used successfully for the primary isolation of bordetellae. The other members of this genus except *B.pertussis* grow easily on MacConkey's agar.

Colonies of *B.pertussis* on Bordet-Gengou medium are smooth, convex, glistening, almost transparent and pearl-like or mercury like in appearance. Zone of haemolysis around the colonies can be easily seen. The growth becomes visible after minimum of 72 hours of incubation. The subcultures from the exacting media can be made onto less exacting media such as nutrient agar to which charcoal or starch has been added.

Biochemical Activities

The bordetellae have a respiratory metabolism. These do not produce indole, H_2S and acetylmethylcarbinol. *B.pertussis* is weakly oxidase positive. Some of the important characters of various species have been shown in Table 37.1.

Sensitivity to Physical and Chemical Agents

A temperature of 56°C kills the bordetellae within half an hour. Otherwise these bacteria can survive in dried droplets for upto 5 days. Standard disinfection practices readily destroy this bacterium. Even on culture plates it dies within a few days. Bordetellae are resistant to penicillins, streptomycin and bacitracin but sensitive to ampicillin and erythromycin.

Table 37–1. Cultural and biochemical characters of bordetellae

Character	*B.pertussis*	*B.parapertussis*	*B.bronchiseptica*
Motility	–	–	+
Growth on peptone agar	–	+	+
Browning of peptone agar	–	+	–
Growth on MacConkey agar	–	+	+
Reduce nitrates	–	–	+
Utilize citrate	–	–	+
Produce urease	–	+	+
Visible growth on Bordet-Gengou medium	4 days	2 days	2 days
Production of exotoxin	+	–	–
Pili	+	–	–
Filamentous haem-agglutinins	+	–	–
Oxidase	+	–	–

Animal Pathogenicity

Mice is highly susceptible to small intracerebral inoculation with virulent strains. This procedure has been used to test effectiveness of *B.pertussis* vaccine. Intranasal inoculation of *B.pertussis* results into severe interstitial pneumonia.

Antigenic Structure

Two types of antigens are known to exist on bordetellae. These are somatic (O) and capsular antigen (K). The O antigen is heat stable and single antigen is present on most of the strains of bordetellae. This is a protein which can easily be extracted from cells.

The K–antigen is heat-labile. These are often referred to as 'factors' and designated in arabic numerals. Of the 14 K–antigens that are known, 7 is common antigen in almost all the strains of this genus and for other species different antigens are specific (Table 37.2).

Table 37–2. K– antigens of bordetellae

Species	*Common antigen*	*Species-specific antigen*	*Other antigens*
B.pertussis	7	1	2-6
B.parapertussis	7	14	8-10
B.bronchiseptica	7	12	8-11

Determinants of Pathogenicity

Exact knowledge of pathogenic mechanism in various *Bordetella* infections of man is still in primitive stage. Pili present in *B.pertussis* probably play a role in adherence of bacteria to the ciliated cells of upper respiratory tract. Various virulence factors are produced by different species (Table 37.3) and these alone or together are responsible for producing clinical manifestations. Of these pertussis toxin is considered most important.

Table 37–3. Virulence factors produced by bordetellae

Factor	*B.pertussis*	*B.parapertussis*	*B.bronchiseptica*	*B.avium*
Heat labile toxin	+	+	+	+
Tracheal cytotoxin	+	+	+	+
Endotoxin(LPS)	+	+	+	+
Adenylate cyclase	+	+	+	–
Pertussis toxin	+	–	–	–
Haemolysins	+	+	+	–
Filamentous haem-agglutinin	+	+	+	–

Pertussis Toxin (PT)

PT is produced only by *B.pertussis* and is thought to play a central role in the pathogenesis of whooping cough. It has diverse biochemical and biological activities (Table 37.4). Its important features are:

Table 37–4. Biological properties of pertussis toxin

In mouse	*At cellular level*
• Acute toxicity • Histamine sensitivity • Leucocytosis promotion in neutrophils • Hypoproteinaemia • Protection by detoxified PT against intracerebral challenge cells	• Agglutination of chicken erythrocytes • Inhibition of oxidative burst • Inhibition of macrophage migration • Lymphocyte mitogenesis • Clustered growth of CHO

- PT has a molecular weight of 117 000 daltons and comprises of six polypeptide units designated as S1 to S5 with two copies of S4.
- S1 unit is also known as A-unit which is enzymatically active whereas remaining five polypeptides constitute B-unit which is needed to cross the membrane (Fig. 37.1).
- Within the target cell, PT causes increased cAMP with consequent disturbances in the normal cell function.

Pathogenesis and Clinical Picture

B.pertussis is found only in humans and is transmitted, in most cases, only by persons with an active infection. Upto 90% of the unimmunized household contacts of a clinical case may develop whooping cough. Histori-

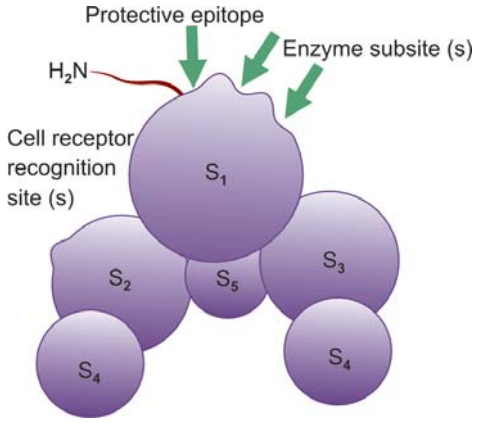


Fig. 37–1. Pertussis toxin

cally whooping cough has been one of the prominent childhood diseases and even today, found worldwide. There are three stages of illness:

Catarrhal Stage

B.pertussis is aerosolized from the throat of a person with whooping cough and transmitted to others by airborne route. This bacterium selectively attaches to the epithelial cells of the respiratory tract and growth is limited to the superficial tissues. After an incubation period of 10 days, generalised symptoms of an upper respiratory tract infection occur which are collectively called as *catarrhal stage* and include sneezing, running nose and coughing. The first stage or prodrome lasts a week or two.

Paroxysmal Stage

The second stage progresses into episodes of uncontrollable cough. Each paroxysm consists of 5-20 rapid coughs with the patient unable to breathe between the coughs. At the end of paroxysm a forced inspiratory breath causes the 'whooping' sound and the disease has derived its name from this feature. Such prolonged coughing may lead to anoxia, expelling of mucus and vomiting. The second stage may continue for 1-6 weeks.

Convalescence Stage

Third stage is that of convalescence and may last for several weeks. Some cough may persist during this stage also. Respiratory distress and secondary bacterial pneumonias complicate the disease progress. Central nervous system also gets affected and contributes to the mortality and morbidity associated with this disease.

Several types of adenovirus and chlamydia pneumoniae can produce a clinical picture resembling that caused by *B. pertussis.*

Immunity

The immunity that develops from an attack of whooping cough prevents a second attack except in rare instances.

Laboratory Diagnosis

Three methods are available:

a. Direct microscopy
b. Culture
c. Serology

For the demonstration of organism or its isolation by culture, specimen must be collected during early phase of illness since chances of its recovery decrease with the progress of disease. At any time all the three methods given above may not be positive and hence two or preferably all three should be applied collectively to obtain best results.

i. **Collection of specimen.** Four methods are available for the collection of material. These are:
- Per-nasal swab,
- Cough plate,
- Post-nasal secretions and
- Post-nasal swab.

Of all these, pernasal swab has generally replaced all others. In this method a sterile cotton-wool swab on a flexible wire is passed gently along the floor of the nose until it meets resistance. The swab thus collects mucopus (Fig. 37.2). It is withdrawn and either immediately plated onto bacteriological media or placed in transport medium. Even in transport media these bacteria cannot survive for long and hence culture must be performed within a few hours of collection.

Single swab taken from patient usually does not yield bordetellae and hence upto six swabs may be taken during 2-3 days of illness before this organism is considered negative.

ii. **Direct microscopy.** Ordinary staining methods do not help in diagnosis of pertussis. A rapid diagnosis can be made by the fluorescent antibody technique applied directly to nasopharyngeal secretions (as collected by pernasal swab)on a slide. In suspected cases of whooping cough, this method may have a sensitivity of upto 75%. It is a sensitive, rapid and accurate test and can produce results in patients who have been partially treated with antibiotics where the organisms present in secretions are not viable. However, nonspecific reaction may lead to false positive results and some reaction may be blocked by the host antibody in the secretions.

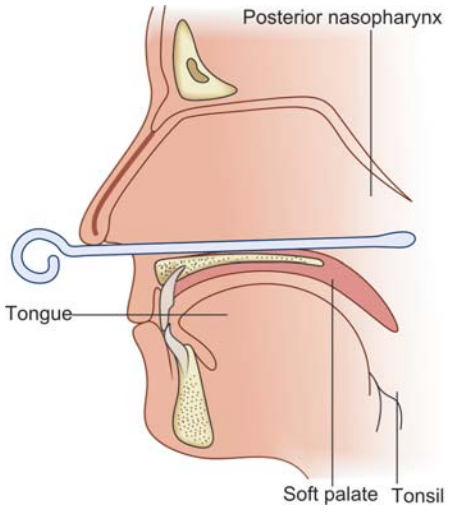


Fig. 37–2. Collection of material from a patient of whooping cough

iii. **Culture.** The preferred media are modified Bordet-Gengou medium and charcoal blood agar to which cephalexin has been added. The latter gives larger colonies. The media plates must be adequately thick and incubated in humid environment because of the prolonged incubation (upto 5 days) that is required for these organisms. The colonies (bisected pearl or mercury drop) should be first examined by Gram staining and then confirmed by slide agglutination test using specific antiserum. The final confirmation can also be done by subjecting the isolate to fluorescent antibody test.

iv. **Serology.** The antibodies against these organisms appear after 2-3 weeks of onset of disease and this is the time at which the first sample should be collected. Since only one sample shall not yield diagnostic result because of previous immunization or whooping cough, second sample should be collected 2-3 weeks later. In many instances when culture is negative, serology provides diagnosis.

The tests which have been used include CFT, direct agglutination, indirect haemagglutination and ELISA with varying success.

v. **Polymerase chain reaction (PCR).** PCR is the most sensitive method to diagnose pertussis. Primers for both *B. pertussis* and *B. parapertussis* are available. Wherever available, PCR should replace both culture and direct fluorescent antibody tests.

Treatment and Prevention

Although no antibiotic is universally successful against *B.pertussis*, erythromycin has been the drug of choice and most effective chemotherapeutic agent. Tetracyclines and chloramphenicol have also been used but are somewhat less effective than erythromycin. Treatment after onset of the paroxysmal phase rarely alters the clinical course. Removal of respiratory secretions, oxygen therapy to aid breathing and general supportive measures are of value in treating patients with severe symptoms.

A killed pertussis vaccine has been widely used for many years and is associated with a steady decline in the number of cases of whooping cough. The vaccine is usually given in combination with tetanus and diphtheria toxoids as triple (DPT) vaccine.

Safer vaccines which do not react with the brain tissue have been developed. Such vaccines are currently licensed in Japan and USA which are called as acellular pertussis vaccines and contains PT and FHA products of organisms.

BORDETELLA PARAPERTUSSIS

This organism produces a disease similar to whooping cough in children but with mild intensity. Often this infection remains subclinical. This organism grows rapidly as compared to *B.pertussis* and produces larger colonies. It also grows on blood agar and MacConkey agar.

BORDETELLA BRONCHISEPTICA

This is a motile species which is responsible for a miniscule (0.1%) cases of whooping cough in humans. It is otherwise normally present in the respiratory tract of many animals. It produces disease in canines, rabbits and swine.

BORDETELLA AVIUM

This organism does not cause any illness in humans and is basically a pathogen of turkey poultry where disease produced is called as turkey coryza, turkey rhinotracheitis or turkey bordetellosis.

38 Brucella

Till 1887, the causative agent of a disease characterised by fever which had fairly regular remissions and which was known by names such as Malta fever, Mediterranean fever, undulant fever or Gibraltar or Rock fever was not known. Sir David Bruce isolated gram-negative coccobacilli from such patients in 1887 and subsequently the genus was named as *Brucella* and all the clinical manifestations became known as brucellosis. Brucellosis is now recognised as a major problem of animals and an important zoonosis.

Brucellae are mammalian intracellular parasites and pathogens with a relatively wide host range.

Morphology

Brucellae are small, nonmotile bacilli, usually coccobacillary in shape and range in size from 0.5 to 0.7 µm by 0.6 to 1.5 µm. These are arranged singly, sometimes in pairs, short chains or small clusters. The members of genus *Brucella* do not produce spores, or flagella. Capsules, when formed, are small and can be demonstrated only in smooth and mucoid variants. These are gram-negative but counterstain poorly and require longer time for uptake of stains.

Cultural Characters

Growth requirements. Brucellae have complex nutritional requirements. Defined media having amino acids, vitamins, salts, and glucose have been used for their growth. Blood agar and trypticase soya agar are media of choice. Other media which have been used include tryptose agar, serum-potato infusion agar, and serum dextrose agar. Addition of bacitracin, polymyxin and cycloheximide to these media makes them selective for brucellae. *B.abortus* requires 5-10% CO_2 for growth whereas the remaining three human pathogenic species can grow in air.

All brucellae are aerobic and no growth occurs in anaerobic environment. Growth can occur in range of 20-40°C but optimal growth takes place at 37°C. The optimum pH is near neutrality and excess of acidity or alkalinity tend to kill brucellae.

Sensitivity to dyes. Different species of *Brucella* exhibit differing sensitivity to various dyes. Basic fuchsin and thionin are important dyes which can be used to differentiate between various species of this genus and their biogroups. On the basis of CO_2 requirement, H_2S production and sensitivity to certain dyes brucellae can be identified (Table 38.1).

Colony characters on solid media. On solid culture media colonies take 2-3 days to develop. These are small, smooth, transparent, low convex with an entire edge. Smooth, mucoid and rough variants are recognised by colonial appearance and virulence. The typical virulent organism forms a smooth, transparent colony upon culture, it tends to change to the rough form, which is avirulent.

Growth in liquid media. In liquid media, growth is uniform and in old cultures a powdery or viscous deposit is formed. Brucellae can grow in chorioallantoic membrane of chick embryos and bring about their death in a few days.

Table 38–1. Characteristics of brucellae

Species	*CO_2 requirement*	*H_2S production*	*Growth in presence of*	
			Thionine (1: 25000)	*Basic fuchsin (1: 50000)*
B.abortus	+	++	–	+
B.melitensis	–	–	+	+
B.suis	–	+	+	–
B.canis	–	–	+	–

Biochemical Reactions

Catalase and oxidase are usually produced by strains of this genus and nitrates are reduced to nitrites. Hydrogen sulphide is produced by many strains. Though brucellae utilise carbohydrates they produce neither acid nor gas in amounts sufficient for classification.

Biotypes. On the basis of biochemical reactions 7 biotypes of *B.abortus* (1, 2, 3, 4, 5, 6, 9); 3 of *B.melitensis* and 5 of *B.suis* have been identified.

Sensitivity to Physical and Chemical Agents

These bacteria are killed at 60°C within 10 minutes. In milk they are readily destroyed by holder as well as flash methods of pasteurisation. In agar media kept at 4°C they survive upto one month. *B.melitensis* can survive in urine for 6 days, in dust for 6 weeks and in water or soil for 10 weeks. Brucellae are very sensitive to sunlight.

Antigenic Structure

On the basis of O chains of the LPS complexes of the cell envelope, two antigenic determinants have been found. These are A (abortus) and M (melitensis). The quantity of each antigen present and their spatial distribution allows for the antigenic variation observed between the species. B.abortus contains almost 20 times as much A as M whereas B.melitensis contains 20 times as much M as A. *B. suis* has an intermediate antigenic pattern. *B.abortus* is sensitive to phage Tiblis.

Phages

Numerous DNA phages lytic for *Brucella* have been isolated and these have been grouped into 6 groups. Phages of group 1, typified by Tbilisi (Tb) phage replicate only in B.abortus and to some extent in B.neotomae. This phage has been extensively used for identification of brucellae and their biotypes.

Determinants of Pathogenicity

Brucellae are obligatory intracellular parasites with distinct host preferences. *B.abortus, B.melitensis* and *B.suis* show distinct host preferences but can establish infection in a wide range of host species, including man. It is believed that virulence is a function of O side-chain of LPS. LPS and other cell envelope components have also been implicated in the blocking of bactericidal action of phagocytes thus permitting the survival of these organisms in mononuclear and polymorph cells. *B.abortus* has been shown to release a low molecular weight nucleotide fraction as well as low molecular weight RNA which protect the bacteria from the host defenses. No exotoxin has been detected to be produced by *Brucellae.*

Pathogenesis and Clinical Picture

Large number of *Brucella* organisms are shed in urine, placental fluids, milk and other secretions of infected animals. Transmission among animals occurs by direct contact with contaminated materials. Similarly transmission to humans is by contact with contaminated animal products. Thus infections are commonly seen in persons who come in close contact with animals or animal products. This group includes veterinarians as well as other animal health care workers. Drinking of unpasteurised milk is a major transmission route of brucellosis in developing countries of the world.

The *Brucella* organisms enter the body via lesions or cuts, ingestion or inhalation. The bacteria are readily phagocytosed by white blood cells; however, they are able to survive inside both polymorphs and macrophages and much of the pathogenesis of brucellosis is associated with this intracellular survival.

Bacteria are carried with the phagocytic cells through the lymphatic system to the blood and into such organs of the reticuloendothelial system as the liver and spleen, which may, in turn, become enlarged. Circulating antibodies are produced but are unable to neutralise the bacteria sequestered inside the white blood cells.

The onset of clinical features of brucellosis is usually gradual and often occurs weeks or months after exposure. Clinical symptoms are generalised and include fever, weakness, malaise, bodyache, headache and sweating. The fever may occur in cycles with febrile periods alternating with afebrile periods. This fever pattern had given the disease the name of undulant fever. With the development of cell mediated immunity body is better able to contain and eliminate *Brucella* organisms. Recovery from the disease is gradual. Some infections remain subclinical.

Laboratory Diagnosis

Various clinical findings in persons most likely to be exposed are suggestive of the disease in humans. The infected material must be handled with utmost caution in the laboratory because of its capability of causing infection to handlers.

Direct demonstration of the organism by culture or indirect evidence in the form of specific antibody can be shown to establish the diagnosis.

i. Cultivation of the organism: Diagnosis can be confirmed by the cultivation of *Brucella* organisms from various specimens viz.

- Blood
- Tissues such as lymph nodes, bone marrow and liver biopsy of the patient.
- Cerebrospinal fluid, urine and abscesses. Sometimes sputum, breast milk, vaginal secretions and seminal fluid may also need culture for the isolation of brucellae.

However, culture is difficult and positive in only 20% of cases. Moreover, brucellae can be isolated from patients only during the acute stage of the illness or during recurrence of the disease activity.

Blood Culture

Blood or tissues are incubated in trypticase soya broth by inoculating 5 ml of blood in 4 bottles of medium. Castaneda blood culture technique is useful in reducing contamination during long incubation period. In this method both solid and liquid media are available in the same culture bottle. The blood is inoculated into the broth and bottle incubated in the upright position. For subculture, one does not have to open the bottle and the bottle is just tilted so that broth flows over the solid medium slant (Fig. 38.1). It is again incubated in upright position. Colonies shall appear on slant. With this method chances of contamination are reduced as well as incidence of laboratory acquired brucellosis is also minimised. Subcultures are made on solid media twice a week upto 8 weeks after which culture is discarded as negative. When *B.abortus* infection is a possibility incubation should be done in an environment with 10% CO_2.

Identification of Organisms

If organisms resembling brucellae are isolated, they are identified and typed by H_2S production, dye inhibition, agglutination by absorbed sera and reaction to Tb phage.

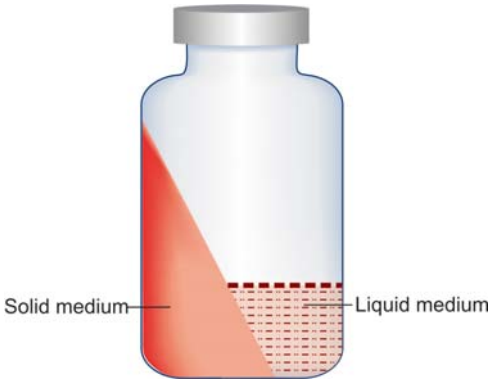


Fig. 38–1. Castaneda blood culture bottle

Table 38–2. Serological tests for brucellosis

- Tube agglutination test
  - — Standard tube test
  - — 2-Mercaptoethanol agglutination
  - — Antihuman globulin(Coomb's test)
- Complement fixation test
- ELISA
- RIA

ii. Serodiagnosis: Infection with *Brucella* organisms gives rise to IgM antibodies which persist for upto 3 months after which their level wanes off. IgG and IgA appear after about 3 weeks of infection and persist for a long time. The tests which can be employed for detection of antibrucella antibody have been shown in Table 38.2.

Tube agglutination test is performed with standardised heat-killed, phenolized, smooth *Brucella* antigen to which is added patient's serum in different dilutions and incubated at 37°C for 24 hours. IgG agglutinin titre of more than 1:80 is usually suggestive of active infection. The addition of 2- mercaptoethanol (2ME) destroys the IgM antibody and leaves IgG for agglutination. By adding 2-ME, the sensitivity of the test decreases but the results correlate better with chronic active disease. If the tube agglutination test is negative but the clinical picture is strongly suggestive of brucellosis, test must be made for the presence of 'blocking antibody'. These can be detected by adding anti-human globulin to the antigen-serum mixture. ELISA and RIA tests are usually performed by the reference laboratories. Table 38.3 summarises the interpretation of serological tests.

Table 38–3. Interpretation of serological tests in brucellosis

Test	*Type of brucellosis*		
	Acute	*Chronic*	*Past infection*
Tube agglutination	+	–/weak +	–/weak +
Complement fixation	+	+	–
Coomb's test	+	+	–/weak +

False positive serological tests. In some rural communities, the sera from a proportion of the normal population agglutinate brucellae in low dilutions because of previous inapparent or latent infection. This is more true in developing countries with predominant rural population which constantly comes in contact with the animals which are reservoirs of this organism. The sera of persons who have been immunised against

cholera and of those who have antibodies against *Francisella tularensis* may give false-positive reactions in the agglutination test against brucella organisms.

iii. Animal pathogenicity. Guinea pig is the laboratory animal that is most susceptible to brucellae. A progressive infection sets in the animal. The clinical sample is inoculated into the thigh of the animal. It is killed after 6-8 weeks. Culture is made from the enlarged lymph nodes near the site of inoculation. Similarly culture can be made from blood and spleen for brucellae.

iv. Detection of antibody in milk. This is done by **milk ring test**. It is a screening test to detect presence of infection in infected cattle. It is sufficiently sensitive to be used for testing the bulk of milk supply from a herd. A drop of concentrated suspension of *B.abortus* stained with haematoxylin is added to 1 ml column of well mixed milk in a narrow test tube. If agglutinins are present in the milk the bacteria are *agglutinated* and rise with the cream to form a blue line above the white skim-milk (Fig. 38.2). In the absence of agglutinins the cream line is white above a blue milk column.

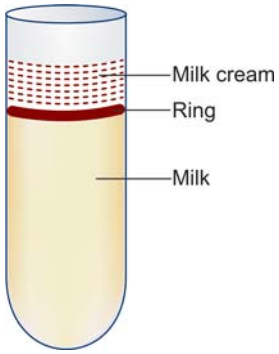


Fig. 38–2. Milk ring test

Treatment and Control

Tetracyclines are the most effective antibiotics against brucellosis and generally cure the clinical disease within a few days. Prolonged therapy for 3-4 weeks is recommended to ensure killing of bacteria trapped within the cells and to prevent relapses.

Following preventive measures are recommended:

a. Educate the public regarding the risks associated with drinking of raw milk.
b. Educate farmers and workers in slaughter houses about the risks in handling of potentially infected animals.
c. Testing of cow's milk by ring test to eliminate infected source.
d. Pasteurise or boil the milk before use.
e. Exercise care in handling and disposal of placenta, discharge and foetuses.

39 Enterobacteriaceae

The family *Enterobacteriaceae* consists of a large number of closely related bacterial species that inhabit large intestine of man and animals, soil, water as well as decaying material. These have also been referred to as enteric bacteria or coliform bacilli. Some of the most important intestinal pathogens for humans are included in this family. These include causative agents of typhoid fever (enteric fever), bacillary dysentery and enteritis. Many members of this family do not cause any disease so long as they are confined to the gut but when their habitat is changed their pathogenicity is also manifested.

Definition

The members of family *Enterobacteriaceae* are gram-negative bacilli that are either motile with peritrichous flagella or non-motile, grow both aerobically and anaerobically on simple laboratory media including MacConkey's agar. These are oxidase negative, catalase positive and reduce nitrates to nitrites. They ferment glucose in peptone water with the production of either acid or acid and gas, and they breakdown glucose and other carbohydrates both fermentatively under anaerobic conditions and oxidatively under aerobic conditions.

Inspite of an exhaustive definition, there are certain commonly occurring bacteria which may be confused with the family *Enterobacteriaceae.* These include *Acinetobacter, Pseudomonas, Vibrio, Aeromonas* and *Plesiomonas.* However, there are certain important differences between all these (Table 39.1).

Classification

Two classifications are currently in vogue.

Table 39–1. Important characteristics of aerobic gram-negative bacilli

Genus/family	*Motility*	*Anaerobic growth*	*Catalase reaction*	*Oxidase reaction*	*Utilisation of glucose*
Acinetobacter	–	–	+	–	–
Pseudomonas	+	–	+	+	O
Vibrio	+	_	+	+	F
Aeromonas	+	+	+	+	F
Plesiomonas	+	+	+	+	F
Enterobacteriaceae	+/–	+	+	–	F

O = oxidative; F = fermentative

Classification Based Upon Lactose Fermentation

Reactions with lactose are of great practical importance in this family (Table 39.2). Almost all the gram-negative bacilli which are of medical importance to human beings and which ferment lactose are included in this family in addition to many other genera which fail to ferment lactose.

Table 39–2. Classification on the basis of lactose fermentation

Rapid fermenters	*Slow fermenters*	*Non-fermenters*
Escherichia	*Edwardsiella*	*Shigella spp* other than *sonnei*
Enterobacter	*Serratia*	*Salmonella*
Klebsiella	*Citrobacter*	*Proteus*
Salmonella Group VI	*Arizona*	
	Providencia	
	Erwinia	
	Shigella sonnei	

On the basis of Bergey's manual,the family *Enterobacteriaceae* has been divided into various groups (or

tribes) which consist of related species of various genera (Table 39.3).

Table 39–3. Classification of enterobacteriaceae

Group I Escherichieae	*Group II Klebsielleae*	*Group III Proteeae*	*Group IV Yersinieae*	*Group V Erwinieae*
Escherichia *Edwardsiella* *Citrobacter* *Salmonella* *Shigella*	*Klebsiella* *Enterobacter* *Hafnia* *Serratia*	*Proteus*	*Yersinia*	*Erwinia*

Antigenic Structure

O Antigens

These are also called as somatic antigens and are the most external part of cell wall lipopolysaccharide. It consists of repeating subunits of polysaccharide. These are resistant to heat and alcohol and are usually detected by agglutination. These give rise to predominantly IgM antibodies.

Each of the main genus of family *Enterobacteriaceae* can be subdivided by means of agglutination test into a number of O-antigenic groups. Some are characterised by single antigen whereas others may have a combination of such factors, which may appear in different combinations in other members of the genus. There is also extensive sharing of O antigen between otherwise quite unrelated organisms. Thus, most shigellae share one or more O antigens with *Esch.coli*. *Esch.coli* may cross react with some species of *Providencia, Klebsiella* and *Salmonella*.

K Antigens (Capsular Antigen)

These are located external to O antigen in some, but not all the members of family *Enterobacteriaceae* (Fig. 39.1) Some are polysaccharide in nature whereas others are proteins. Some people prefer to call these as surface antigens because of their location and the fact that they may be present on the surface in some of the noncapsulated strains.

The VI antigen of salmonellae, most of the K antigens of *Esch.coli* and *Klebsiella* are polysaccharides but K88 and K99 antigens of *Esch.coli* are filamentous proteins. These are also sometimes referred to as the F antigens.

The K antigen polysaccharide enhances the virulence of invasive bacteria since they counteract the bactericidal action of complement and phagocytes.

When K antigens are well developed they form microscopically distinct capsules and give rise to large mucoid colonies on solid media. Klebsiellae are the best example of this type of organism. The capsule can be delineated by quellung reaction. In these bacteria, K antigen can cover O and H (flagellar) antigens.

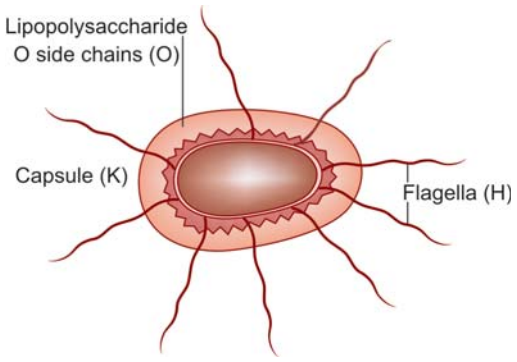


Fig. 39–1. Antigenic structure of *Enterobacteriaceae*

Cross sharing of K antigens between genera of this family as well as with genera which do not belong to this family has been encountered. The type 2 capsular polysaccharides of Klebsiellae is similar to polysaccharides of type 2 pneumococci. Some K antigens cross react with the capsular polysaccharides of *Haemophilus influenzae* or meningococci. Thus, antibodies raised against one can cross react with some other distinct species.

H Antigens

These are located on the flagella. The H antigen can be removed or destroyed by alcohol or heat. These can be preserved by treating them with formalin. The type of antibodies that are predominantly generated by H antigens is IgG. The H antigen is based upon the sequence of amino acids in its units called as flagellin. Within the same bacterium H antigen may be present in two phases. Phase I is designated with small letters such as a:, c:, g: and m whereas H antigen in phase II is designated with Arabic numerals such as 1, 5, 8. The organism may manifest only one phase or both the phases simultaneously. Moreover, organism may change itself from one phase to the other and this phenomenon is called as phase variation. These phases are serologically distinct and serologic determination is performed by agglutination reaction.

Biochemical Reactions

It was recognised quite early that differentiation within the family can be easily done with the help of biochemical tests. The earliest classification of family *Enterobacteriaceae* was also based upon the biochemical reaction of fermentation of lactose. Classifications given by Ewing or the one accepted by Bergey's manual have also been based upon biochemical reactions as the

differentiating features. Table 39.4 presents the salient biochemical reactions of most of the medically important genera of family *Enterobacteriaceae.*

ESCHERICHIA COLI

This genus was first described, in 1885, by Escherich who isolated a bacterium from the faeces of an infant who was suffering from diarrhoea. Only one species, *Escherichia coli* is recognised and it is further divided into serotypes.

Esch. coli is an extremely versatile organism which is capable of survival as a commensal in the human body and also causes a wide spectrum of diseases in all the age groups of human beings and various animals.

Morphology

Esch.coli are gram-negative nonsporing bacteria which are usually (80% strains) motile. They vary in size between 1-3 µm × 0.4-0.7 µm. Strains which are responsible for extraintestinal infections are usually capsulated. The capsule is polysaccharide in nature. Many strains form abundant loose slime when grown on sugar containing media at 15-20°C.

The fimbriae are present on almost 80% of strains. In most of the strains these are of type 1. In some strains different types of fimbriae are formed which are mannose resistant and yet in some of the strains both the types of fimbriae are produced.

Cultural Characters

This organism can grow easily on ordinary medium and does not have complex nutritional requirements. It grows over a wide range of temperature but 37°C is the optimal temperature. It grows equally well under aerobic and anaerobic conditions. Within 18-24 hours luxuriant growth occurs even on selective media. The colony size is 1-1.5 mm in diameter, and these are smooth, circular, glossy and translucent.

Growth on Blood Agar

On blood agar, the colonies of some strains are surrounded by zone of haemolysis.

Growth on MacConkey Agar

On MacConkey agar the colonies are pink in colour because of the fermentation of lactose.

Growth on Selective Media

Growth does not take place on desoxycholate citrate agar. This organism is unable to grow in the presence

Table 39–4. Key identification characteristics for Enterobacteriaceae

Genus/species	*Fermentation of*				*Gas*	*MR*	*VP*	*Indole*	*Citrate*	*Urease*	H_2S	*Motility*
	G	*L*	*S*	*M*								
Escherichia coli	+	+	+	+	+	+	–	+	–	–	–	+
Shigella	+	–	–	+	–	+	–	–/+	–	–	–	–
Shigella sonnei	+	+	–	+	–	+	–	–	–	–	–	–
Edwardsiella tarda	+	–	–	+	+	+	–	+	–	–	+	+
Salmonella	+	–	–	+	+	+	–	–	+	–	+	+
Citrobacter freundii	+	–	–	+	+	+	–	–	+	+/–	+	+
Klebsiella pneumoniae	+	+	–	+	+	–	+	–	+	+	–	–
Enterobacter	+	–	+	+	–	+	–	+	–	+	+	+
Hafnia	+	–	–	+	+	–	+	–	–	–	–	+
Serratia	+	+	–	+	+	–/+	+	–	+	–	–	+
Proteus	+	–	–	+	+/–	+	–	+	–/+	+	+	+
Morganella	+	–	–	+	+	+	–	+	–	+	+	+
Providencia	+	–	–	+	–	+	–	+	+	+	–	+
Yersinia	+	–	–	+	–	+	–	+/–	–	+/–	–	+

G: glucose, L: lactose, S: sucrose, M: mannitol, MR: Methyl Red, VP: Voges Proskauer,

of sodium selenite, sodium tetrathionate, brilliant green and other substances used in various media to make them selective for salmonellae and shigellae. *Esch.coli* also fails to grow in the presence of 7% NaCl, a concentration at which *Staph.aureus* grows.

Biochemical Activities

Most strains of *Esch.coli* produce acid and gas from a large number of carbohydrates which are attacked fermentatively (Table 39.4). The species include certain late lactose as well as certain nonlactose fermenter strains. The saprophytic coliforms can be distinguished from *Esch.coli* by their inability to form gas from lactose.

Antigenic Structure

Esch.coli possesses all three types of antigens, H, O and K.

H antigen. The H antigen is usually monophasic in this organism. Rarely biphasic strains are also encountered. More than 50 H types of *Esch.coli* have been recognised. Not many of these H types cross react with H antigens present on other bacteria.

O antigen. The O antigens can be detected by boiling or autoclaving the strains to overcome inagglutinability caused by K antigen. More than 160 O antigens have been detected so far. Numerous cross reactions occur between individual *Esch.coli* antigens and the O antigens of *Citrobacter, Providencia, Salmonella, Shigella* and *Yersinia.* These antigens have been found to be associated with various types of pathogenic *Esch.coli* (Table 39.5). Six major categories of *Esch. coli.* strains cause diarrhoea. Important features of these are described in Table 39.6.

Table 39–5. Association of O serotypes with pathogenicity

Group of Esch.coli	*O serotypes*
Enteropathogenic *Esch.coli* (EPEC)	O26, O55, O86, O111, O119 O125, O126, O127, O128, O142
Enterotoxigenic *Esch. coli* (ETEC)	O6, O8, O15, O25, O27, O63, O78, O115, O148, O153, O159, O167
Enteroinvasive *Esch. coli* (EIEC)	O28ac, O11ac, O124, O136, O143 O144, O152, O164
Enterohaemorrhagic *Esch. coli*	O157
Vero-cytotoxic *Esch. coli* (VTEC)	O157
Esch. coli causing urinary tract infections	O1, O2, O4, O6, O7, O9, O11 O18, O39, O75

K antigen. More than 100 different K antigens of *Esch. coli* are known. In the past the K antigens were divided into three groups on the basis of effect of heat on agglutinability, antigenicity and antibody binding power of bacterial strains that carry them. These three groups are:

- **The A type of K antigen** is associated with the presence of a capsule, is heat resistant (121°C for 2 hours) and even after subjecting to this heat treatment the antigen retains the ability to bind to specific antibody.
- **The L antigen** is destroyed at 100°C within one hour after which it loses its ability to remove specific antibody from serum.

Table 39–6. Categories of *Esch. coli* causing diarrhoea

1. Enterohaemorrhagic strains (EHEC)	These are also called as Shiga toxin producing *Esch. coli* also called as Verotoxin producing strains. Typical example is *Esch. coli* 0157:H7. The most severe manifestation is haemolytic uraemic syndrome (HUS). These strains produce Shiga toxin 1 and 2. Shiga toxin 1 is identical to the one produced by Shigella dysentriae 1. The diagnosis is made by isolation and serotyping and also by the use of DNA probes.
2. Enterotoxigenic strains (ETEC)	These strains elaborate a heat labile entero toxin (LT), a heat stable toxin (ST) or both toxins (LT/ST). The infection is usually through contaminated food. It leads to serotype specific immunity. Diagnosis is confirmed by LT/ST assays using immunoassays, bioassays and DNA probes.
3. Enteroinvasive strains (EIEC)	These strains can invade and multiply within the epithelial cells like Shigella. Clinically it produces watery diarrhoea. Mainly endemic in developing countries. Diagnosis is confirmed by detecting enteroinvasive plasmid by DNA probes or by bioassays.
4. Enteropathogenic strains (EPEC)	These strains primarily produce diarrhoea in children under 1 year of age. EPEC causes dissolution of the microvilli of enterocytes and initial attachment of the bacteria to enterocytes. The diagnosis is confirmed by demonstrating localised adherance to HEP-2 cells in cell culture and by agglutination tests.
5. Enteroaggregative *Esch. coli* (EAgg EC)	It causes persistent diarrhoea in infants in developing countries. In animals, they have been shown to adhere in aggregates to the enterocytes. The diagnosis is made by HEP-2 assay and DNA probe. Examples are *Esch. Coli* 03:H2, 044:H18.
6. Diffuse adherence *Esch. coli* (DAEC)	The name is derived from the typical pattern of adherence of these bacteria to HEP-2 cells in tissue culture. DAEC is the least well-defined category of diarrhoea producing *Esch. coli*. It is more pathogenic in children of pre-school age.

- **The B antigen** also loses its antigenicity if treated at 121°C for 2 hours but retains the ability to bind to specific antibody.

With few exceptions a strain can possess only one of the three kinds of K antigens, i.e. L, A or B.

The serotype of any strain of *Esch. coli* is defined by its full antigenic formula such as *Esch. coli* O86:K59:H6.

Susceptibility to Physical and Chemical Agents

Esch. coli are killed by moist heat at 60°C usually within 30 minutes. These organisms may remain alive in water for several weeks or months but do not seem to multiply in natural water outside the body. When dried on clothing or in dust these can survive for weeks. Pathogenic serotypes are known to survive in the hospital environment for many days.

Determinants of Pathogenicity

K antigens. These are the surface polysaccharides that help the organism in its pathogenicity by interfering with the phagocytosis. A large number of cases of neonatal meningitis are due to *Esch. coli* K1.

There are certain K types which are more commonly associated with infections in the urinary tract. These include K1, K2, K3, K5, K12 and K13.

Colonisation factor. Fimbriae (or pili) are present on the surface of many strains of *Esch. coli* and play important role in pathogenesis because of their adhesive nature. Apart from providing an anchorage to the bacterium on the host cell, this structure also makes it possible for enterotoxin producing *Esch. coli* to deliver their toxin close to the host cell. The production of these antigens (fimbriae) is mediated by the plasmids.

K88 and K99 are two serotypes in which the colonising factor has been greatly characterised.

Four colonisation factor antigens (CFA) have been detected from enterotoxigenic strains that cause disease in human beings. These have been designated as CFA/I, CFA/II, CFA/III and CFA/IV. These are differentiated on the basis of their antigenicity as well as capability to agglutinate human or bovine erythrocytes.

Endotoxin. All the members of the family *Enterobacteriaceae* produce endotoxin. Most of the biologic activity of endotoxin resides in lipid A moiety. Many of the symptoms associated with invasiveness of *Esch. coli* are due to endotoxin. Among these effects are pyrogenicity; utilisation of complement; consumption of coagulation factors and shock. Injection of large doses of LPS causes hypotension, irreversible shock and death. Similar symptoms result when large number of organisms of *Esch. coli* are present in the blood.

Haemolysin. A haemolysin is produced by some strains of *Esch. coli* which can bring about lysis of RBCs of some species. The exact role played by this haemolysin in production of disease is not known.

Enterotoxin. Some of the strains of *Esch. coli* produce mild diarrhoea or malabsorption syndrome because with the help of their colonising factor antigens they stick to the small bowel in large numbers. However, the enterotoxigenic strains produce either one or two plasmid mediated enterotoxins. These have been designated as **heat stable enterotoxin (ST) and heat labile enterotoxin (LT).**

Disease can result because of the action of either of two but the strains which produce both the types of enterotoxins simultaneously give rise to severe illness.

Both LT and ST, either collectively or individually, cause net secretion of fluid and electrolytes in the lumen of the bowel. Most of the ETEC strains possess two distinct plasmids, one encoding the CFA and the other encoding one or both enterotoxins. The LT and ST genes may reside on separate plasmids.

Heat Labile Enterotoxin (LT)

LT is structurally and functionally similar to cholera toxin and has a molecular weight of 86000 daltons. It is protein in nature. It is composed of an enzymatically active A subunit surrounded by five identical binding (B) subunits. The A subunit has a molecular weight of 25000 and each of the B subunits is of 11500 daltons. Subunit A is usually isolated as a single unnicked polypeptide but can be activated to yield A1 and A2. After binding to the host cell, translocation occurs across the membrane of intestinal epithelial cells, A1 catalyses the NAD dependent activation of adenylate cyclase to cause an increase in the concentration of cAMP. In the intestinal villus cells cAMP inhibits the absorption of sodium and therefore of chloride and water. The resulting water and electrolyte outlfow into the lumen of the small intestine leads to profuse watery diarrhoea (Fig. 39.2).

Heat Stable Enterotoxin (ST)

ST enterotoxin has a low molecular weight and is poorly immunogenic. Two different types have been recognised. These are STA and STB. Both are polypeptides with size ranging from 18 to 50 amino acids. STA has been found to be of two types : STA1 and STA2.

Both STA and STB have been shown to be plasmid mediated but they differ in their biologic activity as well as certain other physicochemical characteristics. Various techniques are now available for the detection of enterotoxins (Table 39.7).

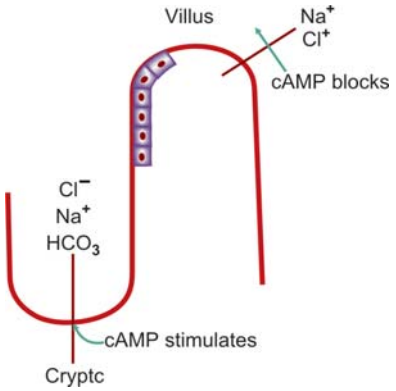


Fig. 39–2. Mode of action of enterotoxin

Table 39–7. Methods of detection of ETEC enterotoxins

Assay	*LT*	*ST*
Ligated rabbit ileal loop		
• At 6 hours	+/–	+
• At 18 hours	+	–
Infant rabbit bowel	+	+
Infant mouse intragastric	–	+
Adult rabbit skin (vascular permeability factor)	+	–
Steroid production in Y1 mouse adrenal cell culture	+	–
Morphological changes in Chinese hamster ovary (CHO) cells	+	–
Solid phase radioimmunoassay (RIA)	+	–
Enzyme-linked immunosorbent assay (ELISA)	+	–

EPEC adherence factor. Though the precise nature of EPEC adherence factor (EAF) is not known, it does not appear to be fimbriae. It is associated with those serotypes which are responsible for disease caused by enteropathogenic *Esch. coli*. The genes associated with EAF have been cloned and a DNA probe developed for the detection of such serotypes.

Cytotoxin. This is an enterotoxic substance which is similar to the one released by some shigellae. Some EPEC strains release it and in association with EAF it is believed to cause diarrhoea.

Vero cytotoxin. This is also known as 'Shiga-like toxin' (SLT) because it has similar biological properties, physical characters and antigenicity. It is of two types VT1 and VT2, the former is neutralised by antishiga toxin whereas the latter resists this neutralisation. These toxins are cytotoxic for Vero cells as well as Hela cells, enterotoxic in ligated rabbit gut loops and can produce mouse paralytic lethality. In several of the strains the capacity to produce VT has been found to be phage encoded.

Clinical Features

Various diseases are produced by *Esch. coli* in human beings (Table 39.8).

Table 39–8. Clinical infections due to *Esch. coli*

- Urinary tract infection
- Meningitis (especially neonatal)
- Septicaemia
- Wound infections
- Pneumonia
- Dysentery
- Diarrhoeas

Enteropathogenic strains of *Esch. coli* are responsible for an acute diarrhoea in children under 2 years of age. With the help of other mechanisms available with this bacterium (production of enterotoxin, invasiveness and production of cytotoxin as well as VT) this bacterium is now known to be an extremely important pathogen for different types of diarrhoeas and dysenteries in adults all over the world.

The importance of *Esch. coli* K1 in causing neonatal meningitis has already been discussed.

Laboratory Diagnosis

Laboratory diagnosis of any illness due to *Esch. coli* shall require direct demonstration of the organism from samples such as cerebrospinal fluid and pus where presence of gram-negative bacilli may suggest the presence of coliform bacilli. Specimens likely to be having mixed bacterial flora including *Esch. coli* should be cultured onto solid media such as MacConkey agar and blood agar. Isolation fom blood, where the organisms are present in proportionately reduced numbers, shall require first incubation in blood culture medium (glucose broth or taurocholate broth) and subsequent subcultures on the suitable media.

Esch. coli can grow well on ordinary laboratory media. The colonies are easily seen on unselective medium. On blood agar haemolysis is a routine feature. Typical colonies are, however, seen on MacConkey agar which acts as not only a selective medium but also differential medium for this organism. The MacConkey medium also enhances the mucoid character of Klebsiellae, prevents the spread of proteus and inhibits the growth of many non-coliform bacilli.

Biochemical tests shall confirm the identity of the isolate to be *Esch. coli* if it conforms to the reactions mentioned in Table 39.4. Further serotyping can be attempted for O, K and H antigens. Various tests are also available for detection of enterotoxin (Table 39.7) to reach at a conclusion that the isolate is toxigenic or non-toxigenic.

Treatment

Esch.coli isolated from community acquired infections are usually sensitive to most antibiotics except penicillin. Multiple resistant isolates have been obtained from hospital acquired infections. There is no set pattern for sensitivity and therapy has to be based upon the results obtained by *in vitro* determination.

KLEBSIELLA

This genus differs from other members of the family *Enterobacteriaceae* on the basis of certain characters. All the strains of *Klebsiella* are non-motile, do not liquefy gelatin and do not produce ornithine decarboxylase as well as phenylalanine deaminase. The first isolation of this organism was from the sputum of a patient by Friedlander and for a long time these bacilli were known as Friedlander's bacilli. Four species of this genus have been well characterised (Table 39.8).

Morphology

Klebsiellae are gram-negative bacilli which are thick and short. These are non-motile and tend to produce large capsules on primary isolation. The capsules are retained even after repeated subcultures in the laboratory.

Cultural Characters

This organism grows well on ordinary nutrient media in a temperature range of 12 to 43°C with optimum growth at 37°C. Colonies are large, raised, moist and viscid which are typically designated as mucoid. The mucoid character is dependent upon the ability of bacterium to produce polysaccharide capsule as well as availability of polysaccharide in the medium. Since large number of strains ferment lactose, they give rise to pink coloured colonies.

Biochemical Reactions

The reactions of different species of *Klebsiella* are shown in Table 39.9.

Of the four important species of this genus, *K. rhinoscleromatis* is least active biochemically. It is anaerogenic, does not ferment lactose, is MR +ve and VP -ve, citrate negative but malonate positive.

Antigenic Structure

The klebsiellae have been differentiated into 80 serotypes on the basis of their K antigens. The detection of antigen is done by the microscopical demonstration of capsule swelling in wet films with capsular antiserum. Five different O antigens have been distinguished. On the basis of O antigens it is possible to divide klebsiellae into five groups which can further be divided on the basis of K antigens. R antigens have been recognised in the rough variant strains of *Klebsiella.* M antigen has been detected in the bacteria free supernatant of cultures and has been found to be loose slime polysaccharide.

Table 39–9. Biochemical reactions of different species of Klebsiella

Reaction	*K. aerogenes*	*K. pneumoniae*	*K ozaenae*	*K. rhinoscleromatis*
Gas from glucose	+	+	+/–	–
Lactose (acid)	+	+	–	–
Sucrose (acid)	+	+	+/–	+
Dulcitol (acid)	+/–	+	–	–
ONPG	+	+	+	–
Methyl red	–	+	+	+
Voges-Proskauer	+	-	–	–
Citrate	+	+	+/–	–
Urease	+	+	+/–	–
Malonate	+	+	–	+
Lysine decarboxylase	+	+	+/–	–

Pathogenicity and Clinical Features

The virulence of klebsiellae shows tremendous variation. Capsule does not seem to play an important part in causing the disease. The spectrum of diseases caused by this bacterium includes bronchopneumonia, wound sepsis, bacteraemia, meningitis and urinary tract infections. Its role as an important pathogen in nosocomial infections is gaining recognition.

PROTEEAE

The members of the tribe *Proteeae* are characterised by one extremely characteristic feature of oxidative deamination of phenylalanine in the PPA test. Three genera are now included in this tribe: *Proteus, Morganella* and *Providencia.* Earlier there were two genera of medical importance in this tribe, *Proteus* and *Providencia* but with some rearrangement of erstwhile species of *Proteus,* a new genus of *Morganella* was created and one species of *Proteus (P. rettgeri)* was transferred to genus *Providencia.*

Genus *Morganella* has only one species—*M. morganii.* The members of this tribe are free-living saprophytes in soil, vegetation, water and sewage and are found in the intestine of many healthy individuals. They are capable of causing infections of urinary tract, wound, meningitis and sometimes bacteraemia.

Morphology

The members of proteeae are gram-negative, non-sporing rods which exhibit pleomorphism in their size and shape which varies from coccobacillus form to filamentous forms. Non-motile variants are also encountered but most of the strains are richly flagellated and highly motile. '*Swarming*' is the characteristic feature of some of the strains. In this phenomenon a group of cells at the edge of developing microcolony migrate to an uninoculated area over the whole plate. The swarming is due to the production of multinucleate cells which synthesize vast number of flagella and an acidic polysaccharide slime which helps in spread.

Various methods are available to prevent or retard swarming of these strains. Easiest and the best method is to increase the concentration of agar in solid media to 3-4%. Bile salts present in medium such as MacConkey and bismuth sulphite in Wilson Blair medium also inhibit swarming. Addition of antiflagellar serum in the medium, ethanol, boric acid, detergents, sulphonamides, chloral hydrate, neomycin, barbiturates and sodium azide can also check swarming and produce isolated colonies. Among the chemicals, addition of which inhibit the swarming, best results are obtained with incorporation of p-nitrophenyl-glycerol to the nutrient agar base before autoclaving.

Dienes phenomenon. This phenomenon states that when two identical strains of *Proteus* are inoculated at different points on same agar plate without any swarming inhibitory substance, they will swarm and growth shall coalesce without any sign of demarcation whereas two different strains of this genus shall be separated by a narrow but easily visible gap. This phenomenon can be applied for epidemiological matching of the strains.

Cultural Characters

The members of tribe proteeae grow easily on almost all nutrient media. These can easily be identified by swarming and fishy smell. *M. morganii* does not cause swarming on ordinary media but does so on 'soft agar' media which have a reduced content of agar. On MacConkey agar the colonies are pale and small, circular and without any characteristic other than fishy smell.

Biochemical Reactions

The biochemical reactions of this tribe have been mentioned in Table 39.10.

Table 39–10. Biochemical reactions of tribe Proteeae

Reaction	*P. vulgaris*	*P.mira-bilis*	*M. morganii*	*Pr. rettgeri*	*Pr. alcali-faciens*	*Pr. stuartii*
PPA	+	+	+	+	+	+
Urease	+	+	+	+	–	–
Indole	+	–	+	+	+	+
H_2S	+	+	–	–	–	–
Lysine	–	–	–	–	–	–
Ornithine	–	+	+	–	–	–
Glucose (Gas)	+	+	+/–	+/–	+/–	–
Esculin	+	–	–	+	–	–
Inositol	–	–	–	+	–	+
Maltose	+	–	–	–	–	–
Mannitol	–	–	+	+	–	+/–
Mannose	–	–	+	+	+	+
Adonitol	–	–	–	+/–	+	–
Swarming	+	+	–	–	–	–
G+C (mole%)	39	39	50	39	41	41

Antigenic Structure

Serotype specific O antigens have been detected in various species of *Proteus*. There are now 32 specific for *M. morganii,* 22 for *P. vulgaris* and 5 are found in both the species. 42 serotypes of *M. morganii* have been established. Similarly for *Providencia,* 46, 17 and 93 serotypes have been detected for *Pr. alcalifaciens, Pr. stuartii* and *Pr. rettgeri* respectively.

Certain O antigens of Proteus cross react with antibodies that are produced during infection with some rickettsiae. These strains of Proteus are designated as X19, X2 and XK. These correspond respectively to Proteus vulgaris O1, Proteus vulgaris O2 and M. morganii O3. Strain X19 has two receptors and one each is shared by Proteus and Rickettsia prowazekii. These strains are used as source of antigen for serological test done to detect antibody to Rickettsia and the test is known as **Weil-Felix reaction**.

Clinical Manifestations

Urinary tract infections (UTI) due to *Proteus* are more common in boys and elderly of either sex and are usually more severe than the UTI caused by *Esch. coli.* UTI due to *Proteus* may lead to the formation of renal stones and in rare cases may lead to hyperammonaemic encephalopathy and coma. Superficial septic infection, bacteraemia and pneumonia are also caused by *Proteus.*

Treatment

One of the most resistant organisms in this tribe is *Pr. stuartii.*In contrast *M. morganii* strains are usually

sensitive to carbenicillin, nalidixic acid, chloramphenicol and aminoglycosides. Other strains of this tribe show variations in their antibiograms which largely depend upon their microbial and antimicrobial agents environment.

OTHER MEMBERS OF ENTEROBACTERIACEAE

Apart from klebsiellae and proteeae, certain other genera of the family *Enterobacteriaceae* can act as opportunistic pathogens. These include *Citrobacter, Enterobacter, Edwardsiella, and Serratia*. Though there are few other such genera, it will be beyond the scope of this book to discuss all of them in details.

All these organisms fulfill the basic criteria for inclusion in the family. Their biochemical tests have been described earlier. Most *Enterobacter* species give positive test for motility, citrate and ornithine decarboxylase and produce gas from glucose. *E. aerogenes* has a small capsule. *Serratia* produces DNAse, lipase and gelatinase and both these genera are VP +ve.

Citrobacter is typically citrate positive and differ from salmonella in that they do not decarboxylase lysine.

Table 39–11. IMViC characteristics for common Enterobacteriaceae

Genus/species	*Indole*	*MR*	*VP*	*Citrate*
Escherichia coli	+	+	–	–
Shigella	–/+	+	–	–
Shigella sonnei	–	+	–	–
Edwardsiella tarda	+	+	–	–
Salmonella	–	+	–	+
Citrobacter freundii	–	+	–	+
Klebsiella pneumoniae	–	–	+	+
Enterobacter	–	–	+	+
Hafnia	–	–	+	–
Serratia	–	–/+	+	+
Proteus	+	+	–	–/+
Morganella	+	+	–	–
Providencia	+	+	–	+
Yersinia	+/–	+	–	–

They ferment lactose very slowly, if at all. *Serratia* also produces colicins similar to *Esch. coli* and *Proteus.* Most of these bacteria possess complex lipopolysaccharides in their cell wall which acts as a determinant of pathogenicity.

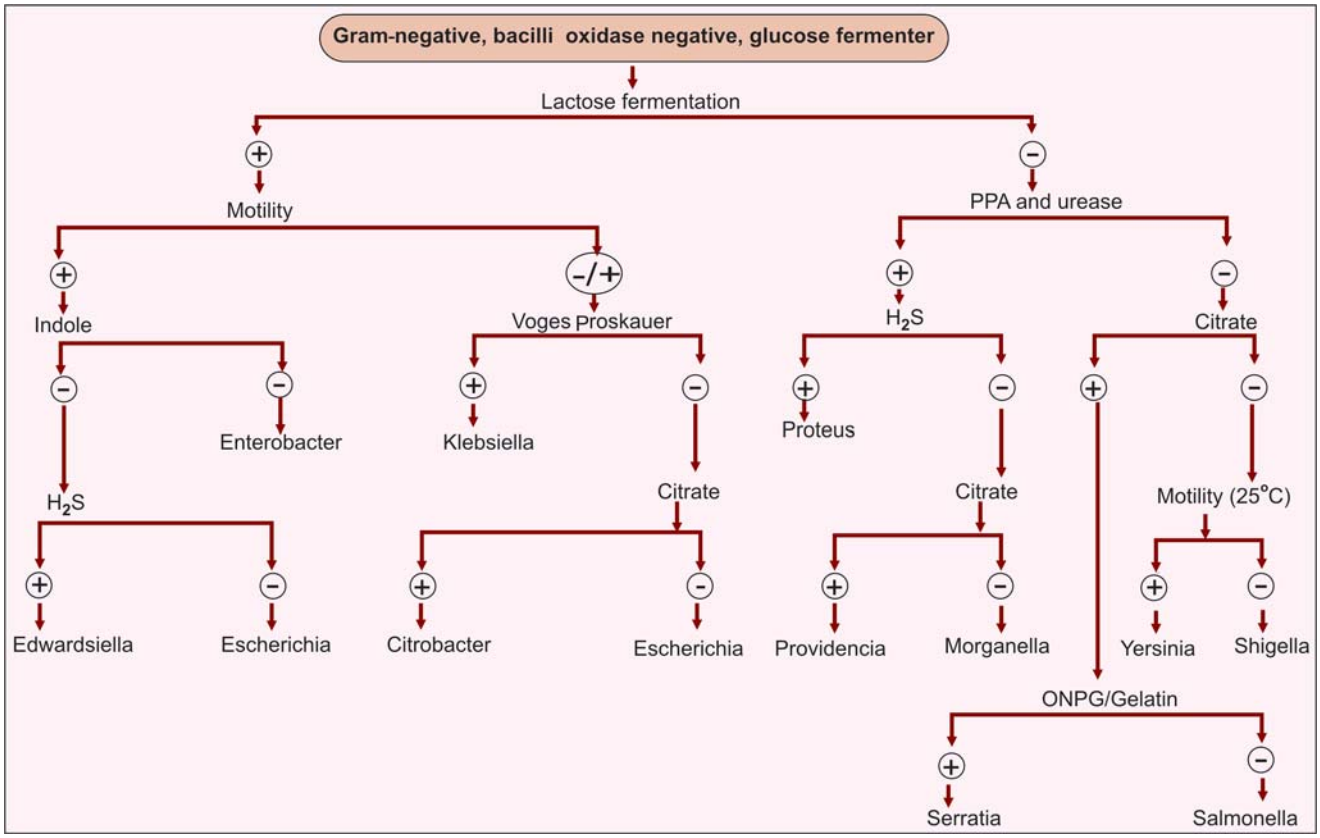


Fig. 39–3. Flow chart for identifying important enteric genera

PART VI

Pathogenesis and Clinical Findings

Enterobacter aerogenes has a small capsule and is usually found free-living as well as in the intestinal tract. It may cause urinary tract infection and sepsis. *Serratia marcescens* is a common opportunistic pathogen in hospitalised patients. It can cause pneumonia, bacteraemia and endocarditis. This organism is usually multidrug resistant. *Citrobacter* also causes UTI and sepsis.

Treatment

No single specific therapy is available and determination of antibiogram in the laboratory is essential especially because multidrug resistance is a common feature in these bacteria.

A simple flow chart for the identification of members of enterobacteriaceae has been shown in Figure 39.3 and Table 39.11 showing few important biochemical reactions (IMViC) shall facilitate identification.

40 Salmonella

The genus *Salmonella* includes a large number of pathogens of human beings as well as other mammals which are antigenically related to one another. Currently 2324 serotypes (earlier designated as species) of *Salmonella* are known. With the exception of a handful of these (such as *S.typhi, S.paratyphi*) all are primary pathogens of animals with capability to infect human beings also. The genus has been named after Salmon. Eberth isolated it for the first time in 1885.

Morphology

The members of genus *Salmonella* resemble other members of the family *Enterobacteriaceae*. These are gram-negative bacilli which are non-acid fast, non-capsulated and non-sporing. These measure 2-4 µm × 0.6 µm. With the exception of Gallinarum-Pullorum serotypes these are motile with the help of peritrichous flagellae. Most strains of *S.paratyphi A* are non-fimbriated and few of *S.paratyphi B, S.typhi*, and *S.typhimurium* also fail to produce fimbriae.

Cultural Characters

Growth requirements. Salmonellae grow readily on ordinary media under aerobic as well as anaerobic conditions. Some strains, however, require enrichment with specific nutritional factors. *S.typhi* strains require tryptophan and some strains of *S.typhimurium* require cysteine, nicotinamide or some other growth factors. Though the optimal growth temperature is 37°C, salmonellae are capable of growing in a temperature range of 15-45°C.

Colony characters. After an incubation of 18-24 hours, colonies on nutrient agar and blood agar are 2-3 mm in diameter, grey white, moist, circular and with smooth convex surface. Rough (R) strains form opaque and granular colonies with an irregular surface. Due to production of loose polysaccharide slime, some strains of *S.paratyphi B* produce large mucoid colonies. These rough strains produce granular deposit and thick pellicle in peptone water and nutrient broth in contrast to smooth strains which produce uniform turbidity.

Pale yellow (non-lactose fermenting) colonies are produced by most of the strains on MacConkey agar. *S.typhi* fails to grow on but other salmonellae do grow and produce pale-green translucent colonies. *Esch.coli* also does not grow on this medium but late lactose fermenting strains of *Salmonella* give rise to blue-purple colonies.

On desoxycholate agar, the colonies are similar to those produced on MacConkey agar. Sometimes these colonies develop a black centre after an incubation of 48 hours or more. Wilson and Blair's brilliant green bismuth sulphite agar is the ideal medium for the growth of salmonellae especially *S.typhi*. Growth of coliforms, proteeae and shigellae is inhibited on this medium. Salmonellae form small colonies (1 mm diameter) on this medium which are green or pale brown in colour. These colonies are surrounded by metallic sheen because of the production of hydrogen sulphide. On XLD medium, salmonellae result into pink colonies which resemble those of shigellae but differ from them by the black centres in pink colonies produced as a result of H_2S production.

Various media have been used for the enrichment of salmonellae from the clinical material. Tetrathionate broth enriches salmonellae and sometimes shigellae but also permits the growth of proteeae. Tetrathionate broth with brilliant green inhibits the growth of *Proteus* but is also inhibitory, to some extent, for shigellae and *S.typhi.* Selenite F is usually preferred but it may also be inhibitory for some salmonellae especially *S.paratyphi*

B and *S. choleraesuis*. Subcultures from enrichment media should be made onto selective media within 6-24 hours except for *S.typhi* for which better isolation can be done if enrichment medium is incubated at 42°C for 48 hours.

Biochemical Reactions

The biochemical reactions which differentiate salmonellae from other genera of the family *Enterobacteriaceae* have been shown in Table 39.4 in previous Chapter. A few biochemical tests can be made use of in differentiating commonly isolated serotypes (Table 40.1).

Table 40–1. Biochemical reactions of common serovars of Salmonella

Reaction	*S.typhi*	*S.paratyphi A*	*S.cholerasuis*	*S.enteritidis*
Citrate	–	–	(+)	+
Ornithine	–	+	+	+
Lysine	+	–	+	+
Glucose(gas)	–	+	+	+
Trehalose	–	–	+	+

Antigenic Structure

The salmonellae carry a complex antigenic structure. The antigens which have been detected on these organisms include somatic (O) antigen, flagellar (H) antigen, surface antigens (such as Vi), fimbrial (F) antigen, M and N antigens as well as antigens present on rough strains and designated as R antigen.

O Antigens

These are heat stable polysaccharide antigens that are part of lipopolysaccharide of the cell wall. These antigens are resistant to boiling upto 2 hours and 30 minutes and alcohol—stable, withstanding treatment with 96% ethanol at 37°C for 4 hours. These also remain unaffected in suspensions having 0.2% formaldehyde.

Till date 67 somatic (O) antigens have been described and with their help salmonellae have been divided into 46 O serogroups.

H Antigens

These flagellar antigens are heat and alcohol labile but are well preserved in 0.04 to 0.2% formaldehyde. The production of this antigen can be biphasic in some, but not all, the strains. Phase 1 is also called as specific phase. A large number of flagellar antigens have been found in this phase (more than 80). These are designated as small letters of alphabet a to z and subsequently as z1 to z68. The presumptive identification of serotypes therefore depends on the identification of the H antigen in phase 1. A different set of H antigens expresses when the bacterium is in phase 2 which is also called as group phase. This phase is also known as nonspecific phase because numerous salmonellae share the same antigens when these are in phase 2. Earlier all antigens detected in phase 2 were designated with Arabic numerals but soon it was found that certain phase 1 antigens, especially e, n, x, z, and w were found in phase 2 of some of salmonellae.

The complete identification of a strain of *Salmonella* requires the detection of serological structure in both the phases. A culture of biphasic bacteria may consist of bacteria entirely in one phase at a time. It then becomes necessary to convert the given culture into the second phase also. This can be done by Modified Craigie's method in which a tube containing semisolid agar (0.2-0.3% agar) is used. Within the agar a small tube which is open at both the ends is placed with its upper end projecting well above the agar. To the agar is added antiserum against nonspecific H antigen and isolated bacterium is inoculated into the inner tube with both ends open (Fig. 40.1).

Within 8-16 hours, the bacteria which are in nonspecific phase are immobilised by the specific antiserum but those which are in specific phase swarm out from the bottom end of the inner tube to the semisolid agar outside. A subculture can be done onto solid medium from here and the resultant isolate shall be the bacterium in specific phase.

Surface Antigens

These include Vi, M and N, F as well as R antigens which are located on the surface of the salmonellae and at times render the bacteria inagglutinable by antibodies against O antigen.

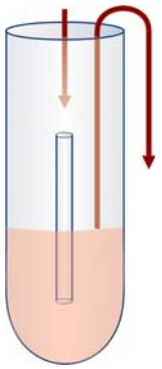


Fig. 40–1. Craigie's tube for phase conversion

Vi antigen. The Vi (virulence) antigen is a surface polysaccharide which is formed by most of the strains of *S.typhi* and some strains of *S.paratyphi C, S.dublin* as well as *Citrobacter freundii*. The production of Vi antigen is chromosomally mediated in all these bacteria. When fully expressed, it renders the bacterium inagglutinable by O antiserum but agglutinable by anti, Vi serum.

The Vi antigen is heat labile and destroyed at 100°C within 60 minutes. Mutants deficient in Vi antigen also arise spontaneously and convert the strain into Vi lacking. These mutants are also called as W forms and the variation as V to W variation.

M and N antigens. M antigen is responsible for mucoid character of some of the colonies of salmonellae. These are present on the surface and are polysaccharide in nature. These also prevent agglutination by O antiserum. Boiling for two and a half hours destroys this antigen. Non-mucoid and motile mutants can be selected by repeated subcultures. These mutants are called as N forms and the variation as M to N.

F antigens. Salmonellae carry antigens on fimbriae which are called as F antigens. Many salmonellae are in two phases, one with F antigens and the other without F antigen (N form).

R antigens. When smooth to rough variation occurs, the O antigen is lost and instead appears a new R antigen. These are same for salmonellae but differ from R antigens of other enterobacteria.

Antigenic Variations (Table 40.2)

Plasmid Conversion

Some of the salmonellae lose some O antigen if a particular plasmid is removed from them. This is best exemplified by the somatic antigen O54. *S.tonev* has antigenic structure of 21,54:b:e,n,x. This organism also carries a small plasmid which if removed causes loss of O54 antigen and converts the antigenic configuration of plasmidless variant to 21:b:e, n, x. This antigenic formula is of *S.minnesota*. Thus, with the loss of a small plasmid, *S.tonev* becomes *S.minnesota*. Similarly loss of this plasmid from *S.winnipeg* (54:e,h:1,5) not only results in disappearance of O54 but also causes expression of O8 and thus the organism becomes *S.ferruch* (8:e,h:1,5).

Antigenic Notation

There is international agreement over antigenic notation for any salmonella serotype. It consists of three parts which describe the O antigen, phase-1 H antigen and phase-2 H antigen. These three parts are separated by colons and components of each part by commas. The O antigens are denoted by arabic numerals, phase-1 H antigens by a to z and z1 to z68 whereas phase-2 H antigens are mentioned by arabic numerals (1-12) but may also contain certain antigens of e and z series. Types e, l, w and some z types may appear in both phases. Those somatic antigens which are determined by phage are underlined and those which are present only in few strains of that serotype are enclosed within square bracket. Thus antigenic formula for *S.typhi* is 9, 12 (Vi):d:- and that for *S. typhimurium* 1, 4, (5), 12:i: 1, 2.

Kauffmann-White Diagnostic Scheme

This scheme was first devised by Kauffmann and White in 1934 and classifies salmonellae into different O serogroups each of which contains a number of serotypes

Table 40–2. Antigenic variation

H → O variation	This variation converts a motile organism to non-motile. It occurs in environment which inhibits flagella. Usually it is a temporary phenomenon and organism regains motility as soon as the inhibitory environment is removed. Rarely, there is a permanent loss of H antigen
H phase variation	H antigen manifests in either of two phases. Phase 1 antigens are specific or shared between limited numbers of species. Phase 2 is called as non-specific or group phase. Species that manifest only one phase are called as monophasic while those which possess both phases are known as biphasic. However, in any culture there will be only one predominant phase and to detect other phase one has to perform phase conversion (Fig. 40.1)
V → W variation	Pathogenic isolates from patients will have a Vi antigen on its surface. This antigen covers the O antigen making anti-O antisera ineffective in agglutinating the strain. After several subcultures Vi antigen gets lost and O antigen becomes detectable
S → R variation	The organisms when grown on culture media usually give rise to smooth colonies. Expression of rough variant generates rough colonies. This is primarily because of mutation induced changes that produce either partial or no O antigen
O antigen variations	These can be due to lysogenisation with phages or because of certain unspecified influences in nature.

possessing a common O antigen not found in other O groups. Where more than one O antigen is present, one of them is the major O antigen and is regarded as determining the group to which the strain shall belong. The O groups first defined were designated by capital letters A to Z and some were subdivided into subgroups C1-C4, E1-E4. Thus serogroups A to Z represented O antigens 2 - 50. O antigens described thereafter were numbered O51-O67. Now each group is designated by its O factor or factors (Table 40.3). Previously known group A was characterised by O antigen 2, group B by O antigen 4 and group D by antigen 9.

O antigen 29 is characteristic of *Citrobacter* and O61-64 of *Arizona*. Many a times two different serotypes share same antigenic formula such as *S.paratyphi* B and *S.java* as well as *S.paratyphi* C and *S.decatur*. These are distinguished from each other by biochemical tests.

Almost 60% of 2324 known serotypes of *Salmonella* belong to seven serogroups (O2 to O1,3,19) which were formerly designated as A to E4. Almost all pathogens of humans belong to these seven groups (Table 40.3).

Table 40–3. Some illustrations of Kauffmann-White scheme

O serogroup	*Previous name*	*Serovar*	*O antigen*	*Phase-1 H*	*Phase-2 H*
2	A	*paratyphi A*	1,2,12	a	(1,5)
4	B	*paratyphi B*	1,4(5),12	b	1,2
		agona	1,4,12	f,g,s-	
		typhimurium	1,4(5),12	i	1,2
7	C1	*choleraesuis*	6,7	c	1,5
		paratyphi C	6,7(Vi)	c	1,5
		virchow	6,7	r	1,2
8	C2	*newport*	6,8,20	e,h	1,2
9	D1	*typhi*	9,12(Vi)	d	–
		enteritidis	1,9,12	g,m	(1,7)
		dublin	1,9,12(Vi)	g,p	–
3,10	E1	*anatum*	3,10(15),(15,34)	e,h	1,6
1,3,19	E4	*senftenberg*	1,3,19	g,(s),t	–

Pathogenicity

Salmonellae are often pathogenic to humans or animals when acquired by oral route. These are transmitted from animals and animal products to human beings. Broadly speaking the disease may present in three ways—enteric fever, septicaemia and enteritis. The enteric fever and septicaemia (bacteraemia with focal lesions) are caused by those salmonellae which possess invasive properties whereas an extremely large number of serotypes of salmonella (upto 2000) can result into enterocolitis. The enterocolitis was earlier designated as gastroenteritis but stomach is seldom involved. Many people prefer to call this condition as enteritis only. Important differences between these entities have been shown in Table 40.4.

Table 40–4. Clinical diseases caused by salmonellae

Feature	*Enteritis*	*Septicaemia*	*Enteric fever*
Onset	Abrupt	Abrupt	Insiduous
Incubation period	<48 hrs	Variable	7-21 days
Fever	Low	Rapidly high	Gradually high
Duration	<5 days	Variable	Few weeks
Early constipation	–	–	+
Diarrhoea	+	–	Occurs late
Blood culture	–	+	+ in early-stage
Stool culture	+	–/+	+ in late stage

ENTERIC FEVER

This is a septicaemic disease caused by members of certain salmonella serovars (Table 40.5). The commonest causative agent of enteric fever is *Salmonella typhi*. The clinical manifestations caused by this bacterium are also called as typhoid fever and those due to *S.paratyphi* A and B as **paratyphoid fever**.

Table 40–5. Causative agents of enteric fever

Common agents	*Uncommon agents*
S.typhi	*S.sendai*
S.paratyphi A	*S.dublin*
S.paratyphi B	*S.bareilly*
S.paratyphi C	*S.enteritidis*
	S.saintpaul
	S.panama
	S.eastbourne
	S.oranienberg

Since typhoid fever is the commonest presentation, the following description is limited to it.

Pathogenesis and Clinical Features

The primary mode of transmission of the typhoid bacillus is the fecal-oral route through contaminated food and water. The incubation period is usually 14 days but may range between 5 to 20 days. The organisms on entering the gastrointestinal tract attach to and penetrate the epithelial lining of the small intestine. Following penetration the bacteria are phagocytosed by macrophages but are unfortunately not destroyed. These organisms multiply within the macrophages. The macrophages carry these to the reticuloendothelial system. These events occur during the first week of infection and may be accompanied by fever, malaise, lethargy and aches and pains. During the second week extended bacteraemia is present and the foci of infection may occur in various tissues; often the gallbladder

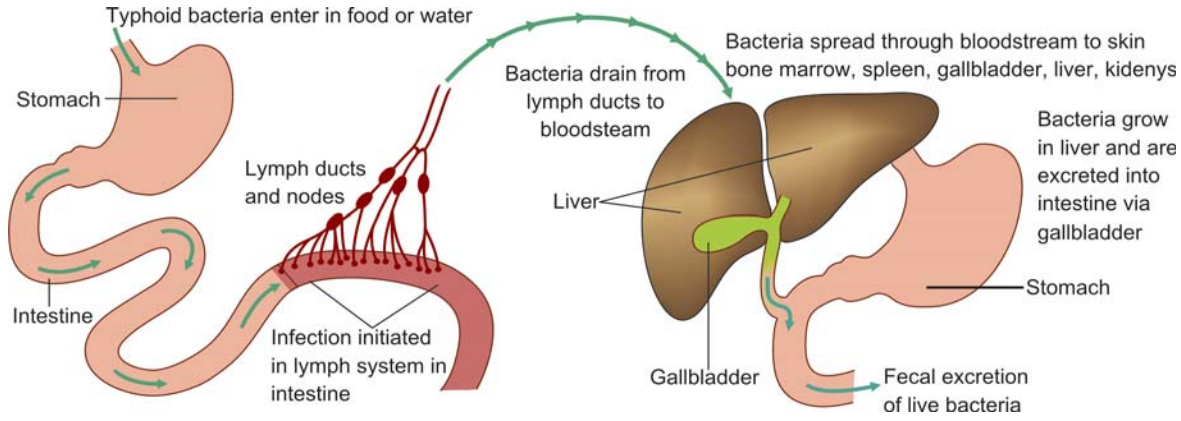


Fig. 40–2. Pathogenesis of typhoid fever

becomes infected. Bacteria may be shed from the gallbladder back into the intestinal lumen. During this time ulcerative lesions of Peyer's patches may develop and the patient is often severely ill with a constant fever as high as 40°C, abdominal tenderness, diarrhoea or constipation, and vomiting. By the third week the patient may be exhausted but still be febrile. Death may occur in 10% of untreated patients. After recovery, multiplication of these bacteria in the gallbladder has been seen in 3% of the patients. These persons may become chronic carriers and serve as source of future outbreaks. This pathogenesis has been shown in Figure 40.2.

The typhoid fever may occur in two epidemiological types. The first is the endemic or residual typhoid which occurs throughout the year and the other one is the epidemic typhoid fever which may occur in endemic or non-endemic areas. This epidemic may be water, food or milk borne. Rarely the source of infection in an epidemic may be shellfish, dried or frozen, eggs, meats and meat products, recreational drugs such as marihuana, animal dyes such as carmine used in preparation of drugs, food and cosmetics as well as household pets.

Laboratory Diagnosis

Laboratory diagnosis of typhoid fever depends upon following three parameters:

a. Isolation of causative agent
b. Detection of microbial antigen
c. Titration of antibody against causative agent.

Specimens for isolation. Blood(or bone marrow or clot), stool and urine are the possible clinical samples from which the causative agent can be isolated in cases with typhoid/enteric fever. However, the isolation rate is different at different stages of the disease (Fig. 40.3).

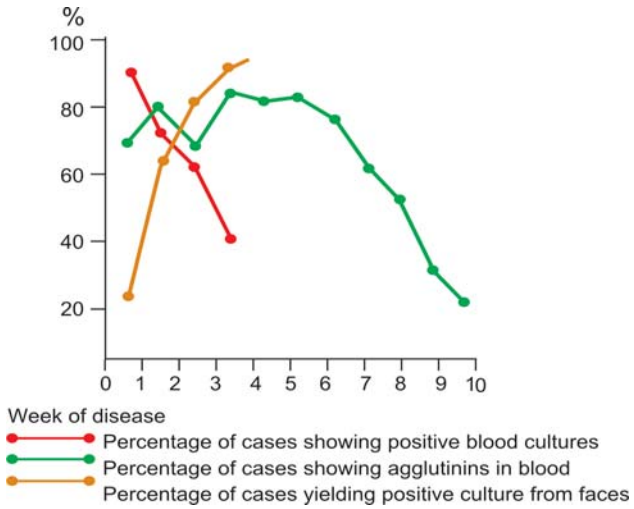


Fig. 40–3. Bacteriology of typhoid fever

Since the onset of symptoms is associated with bacteraemia, blood culture is often positive during the first week of illness. From the first week onwards, the frequency with which causative agent can be isolated from the blood falls. By the end of 3rd week, it can be found in the blood of almost 50% of patients. Isolation becomes infrequent after 4th week of illness.

Typhoid bacilli are most easily isolated from the faeces between 3rd to 5th week of illness. During the first week of illness organisms can be isolated from the faeces in almost half the cases. The number of organisms in the faeces increases from 1st to 3rd week. Thus there is an inverse relationship between frequency of isolation at different stages between blood and faeces.

Urine culture may be positive after second week of illness. Isolation of salmonellae from urine or duodenal

aspirate establishes the presence of salmonellae in the urinary or biliary tract in carriers.

Cultural methods. The method of choice for isolation of causative agents of typhoid/enteric fever is blood culture.

Blood culture. With all aseptic precautions, about 10 ml of blood should be withdrawn. This large quantity of blood is required because in many cases number of bacteria in blood is too little (just 1 organism/ml). As far as possible the sample should be collected prior to the administration of any antibacterial therapeutic agent to the patient. For some of the antibiotics, such as those belonging to beta lactam group or sulfa group, antibiotic inactivating enzymes can be added to the blood culture bottles immediately after the introduction of blood. Penicillinase is added in concentration of 0.2 ml to 50 ml of blood culture medium. In case of other antibiotics, if possible, collection of blood for culture should be delayed for at least 24 hours after the administration of last dose.

To maximise the chances of isolation of scanty bacteria present in blood, it is preferred to inoculate two bottles of blood culture media having different media which are ideal for different groups of bacteria. Five ml of blood is added to 50 ml of the medium in each bottle. The commonly used set of blood culture bottles consists of glucose broth and taurocholate broth.

Depending upon the number of bacteria in bloodstream, the growth in these media shall take place after as little as few hours or may take a period of one to two weeks. As it is desired that the bacteria should be isolated at the earliest, the subcultures can be commenced after 4 hours of incubation and can be done upto 2 weeks. However, this is not a practical solution and repeated opening of bottles for subcultures gives rise to chances of bottles getting contaminated. Experience has shown that two subcultures (one done after overnight incubation and the other after 7 days) detect almost all the positive cultures.

Subcultures from these bottles are made onto two plates of blood agar (from glucose broth) and MacConkey agar (from taurocholate broth). Of the two blood agar plates, one is incubated in an environment of CO_2 and all the three plates are incubated overnight at 37°C after which the colony characters are studied.

Clot culture. Blood from the patient is separated into serum and clot. While the serum is used for detection of antibody (Widal test), clot is cultured. The clot is broken with a sterile stick/rod and added to the bile broth to which 100 units/ml of streptokinase have been added. The streptokinase digests the clot and bacteria are released in medium.

Bone marrow culture. It may give a positive result when blood culture fails, particularly in patients admitted to hospitals after prolonged antibiotic therapy. *Blood clot* from which serum has been removed often gives a positive result when similar volume of whole blood yields no growth. If the clot is dissolved with streptokinase, not only does growth occur more rapidly but the total number of positive cultures also increases.

Stool culture. A spoonful of faeces should be collected in a clean container. In case delay in processing is inevitable, especially so in warm weather, the faeces can be placed in a container with about 5 ml of buffered glycerol saline transport medium. Though *rectal swabs* are convenient to collect, the results obtained with them for isolation are far inferior to those obtained from faeces. The *faecal swabs* are the swabs which have been dipped into and heavily charged with faeces after it has been passed, are good specimens. These swabs can be easily transported in Stuart's transport medium.

Numerous selective and enrichment media are available. Few of these are equally good for the growth of all salmonellae. It is, therefore, unwise to depend upon a single medium. Most bacteriologists favour direct inoculation of faeces onto desoxycholate citrate agar (DCA) and Wilson and Blair's bismuth sulphite brilliant green agar, and enrichment cultures in selenite F broth with subsequent plating on the same two solid selective media.

Urine culture. It can be cultured either as such or the deposit obtained after centrifugation, duodenal fluid, and bile can be processed in the same way as a faecal specimen is processed.

After overnight incubation of culture medium inoculated with various specimens colonies on the DCA or other primary plates are examined for 'salmonella like colonies'. If there are no such colonies, the plates on which subcultures have been made from the enrichment media should be examined the next day for salmonella like colonies.

If there are salmonella like organisms these are to be tested for biochemical reactions to confirm the genus and if possible biogroup.

Slide agglutination test. If on the basis of biochemical reactions the organism has been identified as *Salmonella*, its identity can be confirmed with slide agglutination test and serotype ascertained. The identity of the genus can be confirmed by observing for agglutination with polyvalent O antisera and polyvalent H antisera against salmonellae. For identification of sero-

type the isolate is reacted with group specific antisera followed by monovalent O specific antiserum. Similarly H antigens in phase 1 or phase 2 can be determined to reach at the final antigenic structure of the organism. All this is attempted on the basis of the Kauffmann White scheme.

Detection of microbial antigen. The circulating salmonella antigen may be detected in the blood of the patients with typhoid fever by the coagglutination method. ELISA has also been attempted by some workers to detect Vi antigen in the urine of patients. However, diagnostic value of these tests is yet to be established.

Titration of antibody. A large number of serological tests have been devised (Table 40.6) to detect and titrate the antibody against common agents of typhoid/enteric fever. The tube agglutination test (Widal test) has great historical but little diagnostic importance and yet, unfortunately, this is one of the most extensively used test in the practice of bacteriology.

Table 40–6. Serological tests for typhoid fever

- Widal tube agglutination test
- Widal slide agglutination test
- Indirect haemagglutination test
- Counterimmunoelectrophoresis
- Solid phase radioimmunoassay
- ELISA
- IgM-ELISA

Widal Test

In this test the patient's serum is tested by tube agglutination for its titres of antibodies against H, O and Vi suspensions of enteric fever bacteria that are commonly encountered such as *S.typhi* and *S.paratyphi* A India. These suspensions are commercially available from Central Research Institute, Kasauli (Himachal Pradesh).

Patient's serum is diluted in a series in test tubes and to these are added the antigen suspension. Four rows of such dilutions are prepared and to these are added suspension of *S.typhi* O antigen (TO), H antigen (TH) and corresponding O and H antigens of *S.paratyphi* A designated as AO and AH. After incubating the mixtures at 37°C, the agglutination is checked by examining the bottom of the tube for sediment. When agglutination does not take place, a small compact deposit ("button") is formed and in agglutination one sees irregular clump ("shield") at the bottom. The titre of patient's serum for each salmonella suspension is read as the highest dilution of serum giving visible agglutination, e.g. if the dilution is 1 in 320, the titre will be 320 for that particular antigen. Similarly titre for Vi antigen can be ascertained.

Interpretation. The Widal test can be considered diagnostic (showing active infection) if a four-fold or more rise in the antibody titre can be demonstrated between the two samples that have been collected at a gap of 10-14 days or say during the first week of illness and third week of illness. It can also indicate disease if a four-fold or more fall can be demonstrated in two samples collected during acute illness and convalescence stages.

Factors influencing Widal test. Various studies have shown limited utility of this test in diagnosis of enteric fever. The factors which can influence it are summarised in Table 40.7.

Table 40–7. Factors influencing results of Widal test

- Repeated subclinical infections
- Past clinical infection
- Infection with organisms antigenically cross reacting with salmonellae
- Immunization against typhoid fever
- Antibiotics therapy in early stages of illness
- Healthy carriers

1. Repeated subclinical or past clinical infections in areas endemic for salmonellosis result into repeated boosting up of immune system and the otherwise healthy individuals in the community may have high and variable titres of antibodies against causative agents. The frequency distribution of agglutinins in the population may be of help in giving a baseline data regarding antibody profile. However, this also cannot be a permanent standard since in endemic areas, the situation shall be continuously changing.
2. O antigens of salmonellae cross react with certain other bacteria especially members of the family enterobacteriaceae. Infection with any of these organisms shall result into formation of antibodies that shall react with antigen suspension prepared from causative agents of enteric fever.
3. Immunisation with vaccines against typhoid fever with either TAB or the newer vaccines shall give rise to antibodies which may persist for long time.
4. The early treatment of cases of enteric fever with antibiotics has a profound effect on the antibody response. If agglutinins have not appeared when treatment is begun they are unlikely to do so subsequently and if they are already present no further rise in titre occurs.

5. The Widal test gives significant reading in healthy carriers. Although a negative reaction does not exclude the carrier state, a positive reaction especially a Vi titre of 10 or greater is said to be of help in identifying carriers.
6. Bacterial suspensions used as antigens should be free from fimbria, otherwise false positive results may occur.

False positives and false negatives in Widal test are described in Table 40.8.

Table 40–8. False positives and negatives in Widal Test

False +ve	*False –ve*
• Repeated subclinical infection • Post-clinical infection • Immunisation with typhoid Vaccine • Chronic carrier of *S. typhi* • Anamnestic response • Patients with cirrhosis and hepatitis • Poor quality of antigen used in test	• Too early in the disease sample collection • Patients on antibiotics • 5-10% patients does not respond by antibody formation

Laboratory diagnosis of carriers. To label a person to be carrier of *S. typhi*, isolation of causative agent is a must. The clinical samples that can be examined for the isolation of organism include faeces and urine. If these are repeatedly negative, bile or duodenal aspirates can be tested. Serological methods have been widely used as screening procedure to short list possible carriers. Antibody against H and O antigens may be present in low titre, but greater significance is attached to the demonstration of Vi antibody. Antibodies against Vi antigen are found in a titre of more than 5 in almost 85% of carriers. It is obvious that all the carriers will not have these antibodies and that limits the utility of this screening procedure.

The number of salmonellae in the faeces of an asymptomatic carrier may be far fewer than that in the faeces of a patient. The excretion of these bacteria is also intermittent and hence bacteriological examination of such individual requires repeated use of more than one plating medium and one enrichment medium and to increase the efficiency of enrichment by incubating the enrichment media at 43°C. If a number of specimens have given negative results and the suspicion of carriage remains, the duodenal bile should be collected, preferably by a nylon string, and examined bacteriologically.

While examining urine for carriage state, the concept of significant bacteriuria is not valid. Like faeces, repeated cultures may have to be attempted and urine as such or its centrifuged deposit can be used.

The search of a human carrier of salmonellae has been facilitated by Moore's sewer swab technique. Samples are collected from the main sewer, its tributaries and finally drains of individual houses and examined for the isolation of salmonellae. By this it becomes possible to identify the house in which the carrier is living.

Bacteriophage Typing

The bacteriophage typing of various salmonellae has helped in establishing the epidemiology of disease as well as carriers. The phage typing techniques are now available for a large number of salmonellae (Table 40.9).

The phage typing of *S.typhi* is done by determining the sensitivity of culture to a series of variants of single phage (Vi-phage) which have been adapted to different types of typhoid bacilli. The extraordinary adaptation of the Vi phages is due in part to the selection of spontaneously occurring host-range mutants of the phage by the bacterium and in part to the non-mutational phenotypic modifications of the phage by the host strain.

Table 40–9. Phage typing schemes available for salmonellae

Well established	*Occasionally used*
S. typhi	*S.agona*
S. paratyphi B	*S.bareilly*
S. typhimurium	*S.enteritidis*
	S.hadar
	S.newport
	S.panama
	S.thompson
	S.virchow
	S.welteverden
	S.wien

Biotyping

On the basis of 15 biochemical characters Duguid in 1975 could subdivide *S.typhimurium* into a large number of biotypes which have been found to be discriminatory as well as stable. Uptil now 24 primary and about 200 full biotypes have been recognised for *S.typhimurium*.

Plasmid Typing

A large number of plasmids are present in various salmonellae. These can be separated on the basis of molecular weight by electrophoresis and their plasmid profile ascertained. This technique is gaining wide acceptance and compares quite well with phage typing.

Treatment

Till around two decades ago, strains of *S.typhi* were universally sensitive to many antibiotics especially chloramphenicol which was considered the drug of choice in typhoid fever. Indiscriminate and irrational use of drugs has resulted into emergence of antibiotic resistance in this organism. Strains of *S.typhi* resistant to many drugs including chloramphenicol are now widely prevalent in India as well as many other countries.

Other serotypes of *Salmonella* also frequently demonstrate multidrug resistance. Outbreaks due to *S.typhimurium* which were resistant to almost all the known antibiotics have occurred in various parts of the world.

Treatment of carriers. No methods are available by which development of carrier state can be prevented. Most of the patients who excrete salmonellae even after three months of recovery become chronic carriers. Chemotherapy can cure them of carrier state but success in all the cases cannot be guaranteed. Ampicillin, amoxycillin and cotrimoxazole have been successfully used in the treatment of carriers. More than 75% carriers were cured by a three months regimen of ampicillin given orally. Amoxycillin in doses of 6 gm per day orally can also induce same effect in four weeks. Carriers who do not respond to chemotherapy should be encouraged to get their cholecystectomy done.

Vaccines Against Typhoid Fever

Late nineteenth century saw the development and availability of first vaccines against typhoid fever. Since then various vaccines have been prepared to be administered orally as well as parenterally with variable success.

Parenteral killed whole cell vaccine. This is inactivated by heat-phenol or acetone and provide protection against typhoid fever with an efficacy of 50-80%.

Ty21a, live oral typhoid vaccine. Ty21a is a chemically induced mutagenic strain of *S.typhi* that is safe and protective as oral vaccine. This vaccine is available in enteric coated capsule or in liquid form. Three doses are given on alternate days. It provides protection upto 3 years after vaccination.

Purified Vi polysaccharide vaccine. This vaccine is also commercially available in many countries of Europe and India. It contains purified Vi which is injected parenterally in a dose of 25 µg. Only one dose is required which gives immunity for 3 years. Revaccination is recommended every 3 years.

SALMONELLA GASTROENTERITIS

This is the most common manifestation of *Salmonella* infection. It can be caused by any of the serotypes of this genus. Many serotypes are found as normal flora in the intestinal tract of animals and birds. However, when ingested by humans, salmonellae proliferate in the intestines and symptoms of gastroenteritis may begin within 18 to 36 hours. These symptoms include fever, nausea, abdominal pain and diarrhoea. This condition is usually self-limiting and complete recovery occurs within few days. In serious cases severe dehydration may also occur. These infections usually limit themselves to the lumen of the intestine but in rare instances may progress to enteric fever.

Inflammatory lesions are seen in large and small intestines. Bacteraemia may occur in immunodeficient individuals. Blood cultures are usually negative but stool cultures are positive for salmonellae and may remain positive after several weeks of recovery.

SALMONELLA BACTERAEMIA WITH FOCAL LESIONS

Many a times generalised infection with salmonellae present with a picture of pyaemia with localization in various internal organs. Important serotypes which can give rise to this type of picture are *S.choleraesuis*, *S.paratyphi C*, *S.dublin* and *S.enteritidis*. Immunodeficient individuals, patients of sickle cell anaemia and those suffering from diseases such as malaria are more prone to manifest these clinical features. Osteomyelitis and salmonellosis of bones and joints may occur. The causative agent can be isolated from blood, pus and sometimes from faeces also.

41 Shigella

The members of genus *Shigella* are important causative agents of bacillary dysentery in human beings.The genus has been named so after Shiga who isolated this organism for the first time from the stool of an individual suffering from dysentery in Japan. The members of this genus are enterobacteria, oxidase negative, gram-negative rods that are non-lactose fermenting, non-motile, mostly anaerogenic, urease negative and do not utilize citrate. These fail to grow in medium containing KCN.

Morphology

Shigellae are non-sporing, uncapsulated gram-negative bacilli which measure around 3 µm × 0.6 µm. They are non-motile and non-flagellated.

Cultural Characters

Growth requirements. *Shigella* organisms are aerobic and facultative anaerobic. They can grow over a wide range of temperature but optimal growth temperature is 37°C. *Shigella sonnei* grows well at 10°C as well as 45°C. These organisms can grow on ordinary media provided glucose and nicotinic acid are added to the medium.

Colony characters. Various media are suitable for the growth of shigellae. Some of these are:

- Nutrient agar
- MacConkey agar
- Desoxycholate agar
- XLD medium
- Salmonella-Shigella medium

On simple media such as **nutrient agar** and blood agar, colonies measure 2-3 mm in diameter. These are smooth, greyish or colourless, translucent colonies. Some strains of *S. sonnei* give rise to rough colonies with irregular margins and matt surface. These are irreversible variants of those strains which produce smooth colonies.

On **MacConkey agar,** colonies are pale. If the incubation is prolonged beyond 24 hours, some strains of *S.sonnei* may produce pink colonies because of their fermentation of lactose. Colonies similar to those of salmonellae are seen on desoxycholate agar (DCA). However, these do not form a black centre.

Shigellae do not grow on Wilson and Blair's medium. The best selective medium for the growth of this organism is xylose lysine desoxycholate (XLD). Red colonies without black centre are produced on this medium.

Salmonella-Shigella agar is a selective medium for the isolation of both *Salmonella* and *Shigella*. This medium contains bile salts, sodium citrate, ferric citrate, lactose and neutral red. Gram-positive bacteria and coliforms are inhibited on this medium. Lactose fermenting organisms produce red colonies and those which produce H_2S give rise to colonies with black centres. Shigellae produce colourless colonies on this medium while those of salmonellae have black centre.

In liquid media (peptone water and nutrient broth) shigellae give rise to uniform turbidity. Tetrathionate broth and brilliant green media are inhibitory and unsuitable for enrichment cultures. Selenite F is the only recommended medium for enrichment but in this medium also only *S.sonnei* and *S.flexneri* serotype 6 are enriched. Rest of the shigellae are inhibited.

Biochemical Reactions

All the strains of genus *Shigella* are oxidase negative. All produce catalase with the exception of *S.dysnteriae* serotype 1 and a few strains belonging to serotype 4a of *S.flexneri*. Glucose is fermented by all the strains.

Most strains of *S.sonnei* are late lactose fermenters. Mannitol is fermented by strains belonging to groups B, C and D whereas those in group A fail to ferment it. On the basis of biochemical reactions, the genus has been divided into four groups A, B, C and D (Table 41.1).

Susceptibility to Physical and Chemical Agents

Shigellae are not considered very resistant organisms. These are destroyed by moist heat at 55°C in less than one hour. Phenol (1%) kills it in 15 minutes. It can survive for few days at room temperature in dried conditions. Prolonged survival has been observed in naturally infected faeces if the pH remains alkaline. These do not survive for more than few hours in acidic faeces. *S.sonnei* is more resistant to adverse environmental conditions as compared to other species.

Antigenic Structure

O antigens. A close relationship exists between the O (somatic) antigens of many shigella serotypes and those of serotypes in other species of same genus as well as certain serotypes of *Esch.coli.*

Shigellae are differentiated by the O antigens into serotypes identified by agglutination tests with absorbed antisera.

Group A (S.dysenteriae). This group consists of 12 serotypes of which serotype 1 is also known as **Shiga's bacillus** and serotype 2 as **Schmitz's bacillus**. Serotype 2 shares a minor antigen with serotype 10.

Group B (S.flexneri). There are six main serotypes of this group and each is characterised by a different specific antigen. These may be further subdivided into subserotypes (e.g. 1a and 1b). Thus serotypes 1,2 and 4 have two subserotypes each; designated a and b whereas serotype 3 has three subserotypes designated as 3a, 3b and 3c.

Group C (S.boydii). Eighteen serotypes are now recognised in this group.

Group D (S.sonnei). This group is antigenically homogenous.

K antigens. Shigellae possess surface (K) antigens which are heat labile and cultures possessing them remain inagglutinable for O antigen unless heated at 100°C for one hour.

Fimbrial antigens. The strains of *S.flexneri* produce antigens on fimbriae which are identical in all the strains of this group. Minor sharing occurs between this species and *Esch.coli* as well as *Klebsiella.*

Virulence Factors

Three toxins are considered fundamental to cause pathology. These are:

Lipopolysaccharide

All shigellae release endotoxin after autolysis. This toxin brings about increase in motility of the intestine due to the irritation caused by it.

Exotoxin

Infections due to *S.dysenteriae* are always potentially more serious than due to other species. The Shiga's bacillus forms a toxic product which is called as a neurotoxin (an exotoxin). This was earlier found to have two components, a thermostable glycolipid and a thermolabile protein which was thought to be an exotoxin having a neurotropic effect. This exotoxin has now been found to be immunologically identical to verocytotoxin 1 (VT1).

Verocytotoxin

This toxin is also known as Shiga-like toxin (SLT1 and SLT2). All strains of *S.dysenteriae* type 1 produce VT1 and the genes responsible for production are located on chromosome. DNA probes have shown that these genes are not present in species other than *S.dysenteriae* type 1.

Table 41–1. Biochemical reactions of genus Shigella

Species	*Acid from*				*Indole*	*Lysine*	*Ornithine*	*Catalase*	*Serotypes*
	G	*L*	*M*	*S*					
S.dysenteriae (Gr. A)	+	–	–	–	–	–	–	–	12
S.flexneri (Gr. B)	+	–	+	–	v	–	–	+	6
S.boydii (Gr. C)	+	–	+	–	v	–	–	+	18
S.sonnei (Gr. D)	+	(+)	+	(+)	–	–	–	+	1

v : variable; (+) : delayed positive; G : glucose; L:lactose; M : mannitol and S: sucrose.

VT1 comprises of two subunits, A(one copy) and B (five copies). The B subunit is believed to be responsible for toxin binding to susceptible cells while the A subunit inhibits protein synthesis. This cytotoxic action has been shown on human colon and ileal cells in tissue cultures. The actions of Shiga toxin have been summarised in Table 41.2.

Table 41–2. Actions of Shiga toxin

Neurotoxicity	• Due to action on blood vessels of central nervous system. • No direct action on neurons
Cytotoxicity	• Mammalian cells • Colon and ileal cells
Enterotoxicity	• Causes fluid secretions in ligated loop of rabbit ileum

Pathogenesis

Shigellae infect man and other primates. The infecting dose is small, between 10 and 100 organisms, and the bacteria seem relatively unaffected by gastric acid or bile.

Following ingestion, the shigellae are usually unable to penetrate the intestine into the deeper body tissues or into the blood, but multiply in the small intestine. The bacteria are mechanically carried to the large intestine.

The primary step in the disease process is the invasion into the cells of the colonic epithelium by the pathogen. To reach the epithelial cells, the organism must traverse both the mucous and glycocalyx layers, which coat the epithelium (Fig. 41.1). On coming in contact with the colonic intestinal epithelium, the bacterium is engulfed by an invagination of the cell membrane. After invasion, the pathogen is observed in a vacuole (consisting of host cell membrane) within the epithelial cell. The integrity of plasma membrane is not compromised during the process of invasion.

Lysis of the vacuole precedes multiplication and intercellular spread of the organisms. The organisms spread throughout the cytoplasm and invade an adjacent cell through lateral membrane. The result of this process is the formation of ulcers through which blood and inflammatory cells reach the lumen of the intestine. Shigellae that reach the lamina propria evoke an intense inflammatory reaction and rarely penetrate further to submucosa.

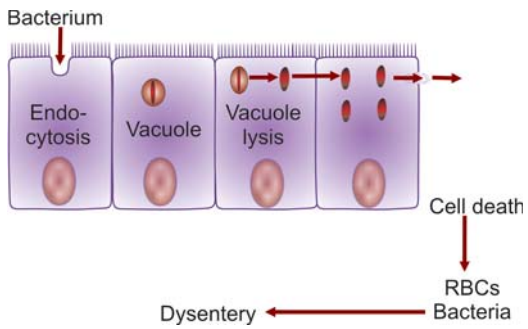


Fig. 41–1. Pathogenesis of dysentery

Inflammation, together with sloughing of the epithelial cells, results in ulcerative lesions. After 1-3 days of incubation, the patient experiences a sudden onset of symptoms-abdominal cramps, fever and diarrhoea. The diarrhoeal stool frequently contains blood and mucus. In otherwise healthy persons the disease is self limiting and complete recovery takes place in 3-7 days.

Genetics of Virulence of Shigellae

The infectious phenotypes of shigellae result from the coordinate expression of a number of virulence genes encoded on the chromosome and on a large non-conjugative plasmid. Loss of this plasmid is associated with avirulence since such strains fail to invade the epithelium. This plasmid is also referred to as invasion plasmid.

Shigella Carriers

During recovery from an attack of bacillary dysentery due to shigellae, the organisms are excreted in the stools. This excretion usually lasts for few weeks, but a few patients become chronic carriers especially so if they develop chronic dysentery too. Excretion for longest duration is seen with *S.dysenteriae* wherein the period may extend to months during which the patient suffers from chronic dysentery. The period is shorter and excretion intermittent in infections due to other groups of *Shigella.*

Treatment

Different strains of shigellae differ in their antibiotic sensitivity to commonly used agents. No specific therapy is available. Treatment with antibiotics tends to prolong the excretion of bacilli and should be avoided unless illness is very severe and in such instances also it should be based upon the results of antibiotic sensitivity determination.

Laboratory Diagnosis of Bacillary Dysentery

Dysentery is defined as a serious diarrhoea which is accompanied by blood and mucus in the stool alongwith severe abdominal cramps. Various micro-

bial and chemical causes can result into dysentery in human beings. Among the microbial aetiology are included both protozoa and bacteria. Various species of microbes are recognised causes of bacillary dysentery. These include:

- *Shigella*
- *Escherichia coli*
- *Vibrio parahaemolyticus*
- *Campylobacter jejuni* (rare)
- *Yersinia enterocolitica* (rare)

Clinical Specimen

Freshly passed stool is the ideal clinical sample for the isolation of shigellae and establishment of diagnosis. Since faeces contains a large number of commensal bacteria, collection of sample in a sterile container is not mandatory. Preferably it should be collected in a clean container.

Rectal swabs are convenient to collect but do not yield as good results as can be obtained with fresh faeces or faecal swab. The faecal swab is collected by dipping and heavily charging the swabs with the faeces of the patient. However, these also suffer from the disadvantage that microscopic examination is not possible. If delay is expected in the processing and arrival at laboratory, swabs should be transported in Stuart's medium. For the transport of faeces suspected to be having shigellae, selenite F is the medium of choice.

Microscopic Examination

This will help in differentiating bacillary dysentery from amoebic dysentery. Presence of numerous RBCs, pus cells and some macrophages with few non-motile bacteria is suggestive of bacillary dysentery due to shigellae (Fig. 41.2). Gram staining is of no use.

Culture

MacConkey agar, XLD agar and DCA are the selective media which can be directly plated and incubated. Simultaneously enrichment can be attempted in Selenite F medium from where after 8-12 hours, subcultures are to be done on the selective media as done in primary isolation. Shigellae shall produce non-lactose fermenting pale yellow colonies on DCA and MacConkey agar and red colonies on XLD medium.

Biochemical Tests

The isolated colonies are subjected to biochemical tests as enumerated in Table 41.1 to reach at a tentative diagnosis of *Shigella* and its species.

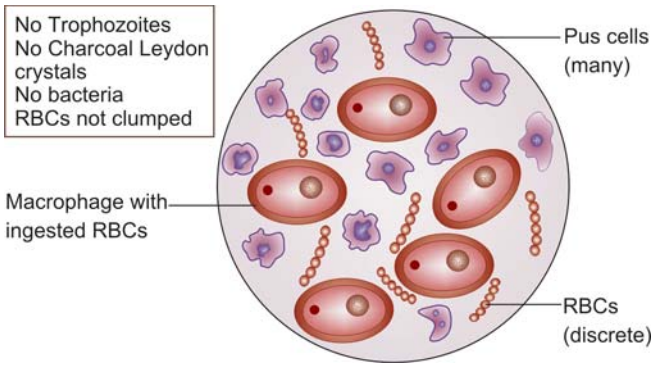


Fig. 41–2. Microscopic appearance in bacillary dysentery

Slide Agglutination

If biochemical tests suggest the organism to be *Shigella*, the diagnosis can be confirmed by performing slide agglutination test with specific antiserum. Polyvalent antisera of three groups (A, B, and C) of *Shigella* and antiserum against group D (*S.sonnei*) are used one by one against the isolate. Monovalent antisera are used for the group for which agglutination is observed with the polyvalent and group specific antiserum.

Sometimes it is difficult to get agglutination with somatic antisera because of the masking of O antigens by the K antigens. The K antigen can be removed by heating a suspension of bacteria for 60 minutes at 100°C. After cooling the suspension, it is retested with shigella antisera by slide agglutination test.

If there are some ambiguities in the results obtained with slide agglutination tests, tube agglutination test can be performed to arrive at a final diagnosis.

Serodiagnosis

Antibody production by shigellae is erratic in bacillary dysentery and hence tests for it have no diagnostic or prognostic value.

In case the strains have been isolated from an outbreak due to *S.sonnei*, help of colicin typing should be taken to trace the source of infection.

In all severe cases of shigella dysentery, antibiotic sensitivity pattern of the isolate should be ascertained because of the prevalence of multidrug resistant strains.

Prevention and Control

Prevention measures are same as for typhoid fever, except that no vaccine is available for protection against Shigella infection.

42 Yersinia

History records that more humans have been killed by plague than by any other disease. Till the advent of antibiotics, plague was considered to be the greatest scourge of mankind. In India alone, during the first two decades of this century more than 12.5 million people died of plague—a figure which has not been surpassed by any other disease or even the two Great Wars put together.

The causative agent of plague, *Yersinia pestis* was isolated simultaneously but independently by Yersin and Kitasato in 1884.

The genus *Yersinia* is placed in the family *Enterobacteriaceae* and *Y.pestis* is the type species.

YERSINIA PESTIS

The plague bacillus is primarly a pathogen of rodents. It can infect more than 200 species of rodents. It is transmitted between rodents through rat-fleas, several species of which act as vectors of this organism. The disease is maintained in rodents and man gets accidentally caught in its cycle of transmission and then can carry the infection to distant places and to other humans (**pneumonic plague**).

Morphology

The members of this species are gram-negative coccobacilli or rods with rounded ends and straight or convex sides, 1-3 µm long and 0.5-0.8 µm wide. When grown under unfavourable conditions, pleomorphism is exhibited and diverse morphological forms become evident. These are non-acid fast and non-sporing.

When grown *in vivo* or under favourable conditions in the presence of serum, a capsule develops around the bacterium which can be seen under dark-ground illumination or immunospecific staining. This capsule corresponds to Fraction 1 (F1) antigen of *Y.pestis* and is glycoprotein in nature. It confers antiphagocytic property and activates complement.

When stained with methylene blue bipolar staining is commonly seen with freshly isolated strains (Fig. 42.1). This appearance has been given the name of **safety pin appearance** of plague bacilli and provides important clue to presumptive diagnosis on direct examination of clinical material specially under field conditions.

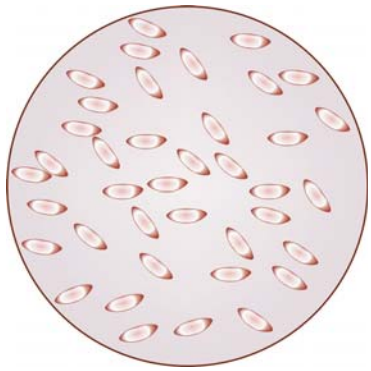


Fig. 42–1. *Yersinia pestis*

Cultural Characters

Y. pestis can grow over a wide range of temperature from 14 to 37°C. Optimal temperature for growth is 27°C. This is an aerobic and facultative anaerobic organism. It can grow on ordinary laboratory media even from small inocula.

Growth on Solid Media

On **nutrient agar medium** small mucoid colonies may appear in one to two days. On **blood agar** after 24 hours of incubation colonies are very small, transparent,

white circular discs, 1 mm or less in diameter but later enlarging to 3-4 mm and becoming opaque. In older cultures a mixture of opaque and transparent colonies may be seen. No haemolysis is produced on blood agar medium. *Y.pestis* grows on MacConkey agar but the colonies disappear after 2-3 days probably due to autolysis. On desoxycholate agar very small red colonies may be seen on the second day of incubation.

Growth on Liquid Media

In liquid medium, *Y.pestis* results in granular deposit at the bottom and on the sides of the tube. If a drop of oil is allowed to float on the surface of liquid medium, a characteristic growth develops which consists of stalactites hanging down from the oil drop.

Biochemical Reactions

Y.pestis grows poorly in peptone water and hence takes longer time to exhibit fermentation of sugars. Some of the important biochemical tests which differentiate between species of *Yersinia* as well as between genera of *Yersinia,Francisella* and *Pasteurella* have been shown in Table 42.1.

Within the species *Y.pestis*, three biogroups have been identified which can be differentiated on the basis of reduction of nitrate and fermentation of glycerol and melibiose. The names of these three biogroups and scheme of their differentiation are given in Table 42.2.

The biotype antiqua is prevalent in Central Asia and Central Africa; medievalis in Iran, Iraq, Turkey and erstwhile USSR and orientalis in South America, USA, Africa and many countries of Asia.

Sensitivity to Physical and Chemical Agents

Y.pestis is extremely sensitive to heat and drying. It is readily killed at 55°C. If moisture and low temperature are provided, the bacterium can remain viable for months. Phenol (0.5%) destroys this bacterium within 15 minutes. Exposure to sunlight for 3-4 hours is also lethal. It can survive for many months in the soil of rodent burrows. If repeatedly subcultured virulent strains tend to lose their virulence as well as virulence associated plasmids.

This microbe is sensitive to tetracycline, chloramphenicol, cotrimoxazole, gentamicin and streptomycin but resistant to penicillin.

Table 42–2. Biogroups of *Y. pestis*

Biogroup	*Nitrate reduction*	*Fermentation of*	
		Glycerol	*Melibiose*
Antiqua	+	+	–
Medievalis	–	+	+
Orientalis	+	–	–

Antigenic Structure

Outer membrane proteins. *Y.pestis* forms no O-specific side chains and hence only one serotype of plague bacillus exists. However, the **outer membrane proteins** play an important role in pathogenesis. At least 8 such proteins have been recognised of which E protein of *Y.pestis* has been identified as a porin.

F1 envelope protein complex. This complex is formed by *Y.pestis*. It has two components, F1A and F1B. F1A is a heat labile glycoprotein fraction and F1B is antigenically related carbohydrate free protein. It is nontoxic but stimulates protective immunity in man.

V and W proteins. Virulent strains of *Y.pestis* form **V** and **W** proteins. V antigen is a protein whereas W antigen is a lipoprotein. The production of V, W and outer membrane proteins is mediated by a plasmid at low calcium concentration.

Table 42–1. Differentiation between *Yersinia,Pasteurella* and *Francisella*

Test	*Y.pestis*	*Y.pseudotuberculosis*	*Y.enterocolitica*	*P.multocida*	*F.tularensis*
Growth on nutrient agar	+	+	+	+	–
Growth on MacConkey agar	+	+	+	–	–
Motility at 37°C	–	–	–	–	–
Motility at 22°C	–	+	+	–	–
Acid from sucrose	–	–	+	+	–
Acid from maltose	+	+	+	–	+
Indole production	–	–	–	+	–
Urease production	–	+	+	–	–
Oxidase test	–	–	–	+/–	–
ONPG	+	+	+	–	–
Ornithine decarboxylase	–	–	+	+	–

Other toxins/enzymes. Other antigens elaborated by *Y. pestis* include murine and guineapig toxins and some virulence associated factors such as pesticin 1, fibrinolysin and coagulase. The production of virulence associated factors is mediated by a single plasmid.

Determinants of Pathogenicity

Five determinants of pathogenicity have been identified (Table 42.3).

Table 42–3. Determinants of pathogenicity of *Y. pestis*

- V and W antigens
- Fraction 1 antigen
- Virulence associated factors:
  - pesticin 1
  - coagulase
  - fibrinolysin
- Ability to absorb certain pigments
- Purine synthesis

The V and W antigens appear to confer on *Y.pestis* the ability for small number of bacilli to establish infection in animals. Once infection is established, F1 antigen is reported to enable *Y.pestis* to resist phagocytosis and consequent killing by phagocytosis. Virulent organisms can thus survive and multiply in macrophages and other phagocytic cells. Resistance to intracellular killing is also influenced by V and W proteins as well as outer membrane proteins. Murine and guinea pig toxins are known to cause clinical features in rodents and guinea pigs but their role in causing plague in humans is not clear. The LPS of *Y.pestis* provides typical activities of endotoxin of gram-negative bacilli.

Pathogenesis and Clinical Features

The natural cycle of plague is found in wild rodents and is called as sylvatic plague. It is transmitted among rodents by rat-fleas. The most frequent carriers of plague are squirrels, mice, prairie dogs and chipmunks. Some animal species die of plague whereas some suffer from subclinical infections only.

The second cycle occurs when plague is spread to urban rodents, mainly rats that live in close proximity to humans. This cycle is called urban or domestic plague. Urban rats frequently die of plague, which causes their fleas to seek new hosts and result in an increased chance of spread to other animals as well as humans.

The third cycle is called as human plague and starts when humans are bitten by an infected flea from either the sylvatic or urban cycles. Generally the flea-bite occurs on the leg or on arms and the bacteria spread to regional lymph nodes in the groin or axilla where excessive multiplication and swelling occurs. The swollen lymph nodes are called as **buboes** and this form of disease is called as **bubonic plague.** The buboes usually appear after about a week of flea-bite. Fever, chills, nausea, malaise and pain may precede and accompany the bubo. The spread of bacteria is not stopped by the lymph nodes and hence bacteraemia occurs. The presence of *Y.pestis* in the blood is called **septicaemic plague**. Massive involvement of blood vessels occurs resulting in purpuric lesions in the skin. This manifestation was responsible for the disease being given the name of **black death.** Bacterial emboli may become trapped in the lungs, where the lesion erodes into the air sacs and causes **pneumonic plague.** Pneumonic plague gives an added dimension to the disease for the bacteria can now be readily transmitted from the patient to the other person (human to human) by the airborne route. Those who contract primary pneumonic plague develop severe symptoms of the disease and die within 2-3 days if not treated vigorously. The epidemiology and pathogenesis of plague is shown in Figure 42.2.

The death rate from untreated bubonic and septicaemic plague is 50-75% whereas that from pneumonic plague is almost 100%.

Laboratory Diagnosis

Specimens. The clinical specimen for the demonstration of plague bacilli shall include aspirate from bubo, sputum, blood and biopsy from body organs such as spleen.

Direct demonstration of organism. Smears prepared from aspirate or sputum are stained with Gram's and methylene blue staining techniques. Characteristic gram-negative bacilli and bacilli with bipolar staining with methylene blue give a presumptive diagnosis of plague. Direct demonstration of such bacteria is never diagnostic and hence the laboratory report should only indicate "bacteria morphologically resembling *Y.pestis* seen." Direct demonstration is also possible by direct immunofluorescent test using FITC conjugated specific antibody.

Culture. The blood culture bottles are incubated and subculture is made onto blood agar. Similarly aspirate and sputum are cultured onto blood agar medium and incubated at 27°C for 24-48 hours. The organisms thus grown can be confirmed to be *Y.pestis* by various biochemical tests (Table 42.1) as well as their property of forming stalactites when drop of oil is allowed to float onto liquid medium.

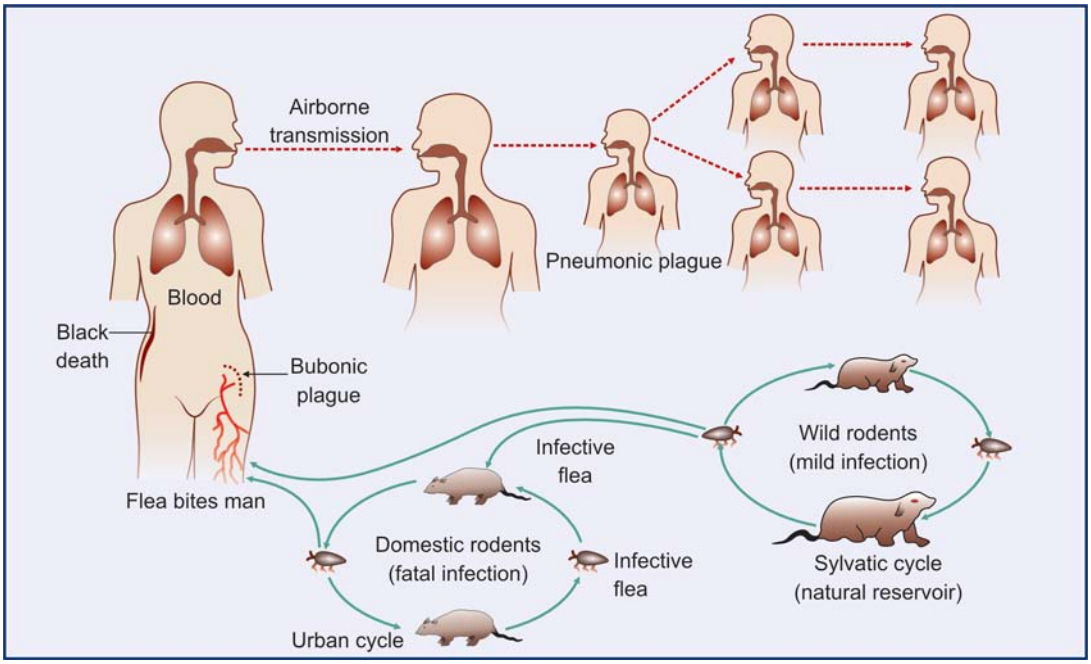


Fig. 42–2. Pathogenesis of plague

Animal pathogenicity. The diagnosis of isolated strain can be confirmed by animal pathogenicity test using guinea pigs or white rats. The animals are injected subcutaneously with exudate from bubo or with 24 hours growth in broth. Infected animals die within 2-5 days. Postmortem shows local necrosis with oedema, enlargement of regional lymph nodes and spleen. Impression smears prepared from the organs shall show the presence of plague bacilli.

Presumptive diagnosis through lysis by phage. A Y. pestis specific phage can lyse the colonies of the organism grown on brain heart infusion agar. This lyis can occur even at room temperature within 6-8 hours.

Serology. For the serological diagnosis and sero-epidemiological studies antibody against fraction 1 antigen are detected in the serum of the individual. The World Health Organisation has standardised an indirect haemagglutination test for this purpose. Complement fixation test as well as ELISA can also be employed.

Epidemiology

Plague is a zoonotic disease which has killed more than 12 million people in India during this century. Availability of antibiotics dramatically reduced the number of cases. By 1966, the incidence of plague in humans came down to zero. However, being a cyclic disease, plague hit Surat (Gujarat) and Beed (Maharashtra) in 1994 and caused tremendous panic and economic loss. The panic was also because of the doubts expressed by some regarding the diagnosis of outbreak. However, isolation of *Y. pestis* strains and their confirmation by WHO Collaborative Centres in USA, Russia and France established the diagnosis.

Another outbreak of plague occurred in Himachal state of India in 2002. However, rapid clinical and laboratory diagnosis and implementation of efficient control measures could contain this outbreak in a very short period. This demonstrates that plague is no longer the dreadful infection it used to be and good public health measures and reliable laboratory tests can tackle the outbreaks without much morbidity and mortality.

Treatment and Prevention

Y.pestis is highly sensitive to tetracyclines, chloramphenicol and streptomycin but resistant to penicillin because of the production of penicillinase. Early treatment is essential since it reduces mortality to a significant extent. Repeated immunisation gives rise to higher incidence of side effects.

In persons at high risk chemoprophylaxis with 1 gm tetracycline per day in divided doses is recommended. The antibiotic should be taken till the person is at risk. An alternative for tetracycline is sulphonamide.

YERSINIA PSEUDOTUBERCULOSIS

Morphology and Cultural Characters

These are small, pleomorphic coccobacilli varying greatly in size and shape. These may be arranged singly,

in groups or short chains. At 22°C this organism is motile but is non-sporing, non-capsulated and non-acid fast. On nutrient agar , after 24 hours of incubation at 37°C, colonies that appear are upto 1 mm in diameter, umbonate, granular, translucent, greyish yellow with entire edge and butyrous consistency. Growth in broth is without any turbidity but a viscous deposit is formed which disintegrates on shaking.

In sensitivity to physial and chemical agents it resembles *Y.pestis*.

Biochemical Reactions and Antigenic Structure

Y. pseudotuberculosis takes about 14 days to produce only acid from glucose, mannitol, salicin, melibiose, aesculin and some other sugars. It is MR +, catalase + and urease + but oxidase -, citrate - and indole -. Some of the important tests that differentiate this from other species of *Yersinia* have been given in Table 42.1.

It possesses a heat labile flagellar antigen which is produced only at a temperature of less than 25°C. The somatic antigen is heat stable and can be divided into 6 major O groups and four subgroups some of which cross react with other members of *Enterobacteriaceae*. Many internal diffusible antigens are also produced and in addition *Y.pseudotuberculosis* produces virulence associated factors such as adhesin, V and W proteins, and invasin.

Pathogenicity and Clinical Features

Usually non-toxigenic and rare strains produce a lethal toxin. It causes pseudotuberculosis in rodents, guinea pigs, mammals and birds. In infected guinea pigs the lungs, spleen and liver show multiple nodules resembling those of tuberculosis and hence the species was named as 'pseudotuberculosis'. Human infection is rare but may present as a typhoid like septicaemic illness or mesenteric lymphadenitis simulating acute appendicitis. Rarely it causes gastroenteritis in man. If not treated early, the infection may take a fatal course.

YERSINIA ENTEROCOLITICA

On the basis of DNA-DNA pairing and biochemical reactions it was proposed to divide *Y. enterocolitica* into different species such as *Y.enterocolitica*, *Y.intermedius*, *Y. frederiksenii* and *Y. kristensenii* but yet there were many strains which could not be placed into any species and showed considerable overlapping. Hence, it was decided that till better parameters become available all these should be retained in *Y. enterocolitica* which should be referred as *Y. enterocolitica* group. However, for ease of description we shall use the term *Y. enterocolitica.*

Morphology and Cultural Characters

These are gram-negative coccobacilli which show pleomorphism in old cultures. At 22°C these are motile and are non-sporing and nonacid fast. After 24 hours of incubation at 37°C on nutrient agar small colonies (1-2 mm in diameter) are formed with glistening surface and entire edges. CIN agar (cefsulod-inirgasan-novobiocin agar) is an excellent medium for the cultivation of this organism.

The colonies are easily emulsifiable. Unlike *Y.pseudotuberculosis*, uniform turbidity is produced by this organism in broth with a slight powdery deposit.

Y. enterocolitica is a facultative anaerobe and psychrotroph (an organism that can grow at refrigerator temperatures) and hence it can multiply in vaccum-packaged food under refrigeration. Thus, two effective methods to control microbial growth, namely anaerobic conditions and cold temperature storage, do not control *Y.enterocolitica*.

Y. enterocolitica is sensitive to heat but can survive for years on freezing. It is sensitive to usual concentrations of disinfectants and almost all the antibiotics except penicillin against which it produces penicillinase enzyme.

Biochemical and Antigenic Structure

It shows considerable disparity in biochemical reactions. Acid is produced from a large number of carbohydrates and few may even produce gas. All strains are catalase +, MR + and many are ornithine decarboxylase +. These are oxidase +ve, H_2S +ve, arginine and lysine -ve and demonstrate variable VP reaction.

Y. enterocolitica has a complex antigenic structure. More than 60 O-antigenic groups have been identified some of which cross react with other bacteria. O:9 group has maximum cross reaction with other bacteria. About 20 flagellar antigens have been demonstrated and these are produced at temperatures of less than 30°C. A few O serotypes are associated with human disease. These include O:3, O5,27; O:6,30; O:8 and O:9.

Determinants of Pathogenicity and Clinical Picture

Virulent strains produce V and W proteins as well as certain outer membrane proteins in addition to adhesin, invasin and pili like structures. A heat stable enterotoxin is produced which is identical with ST enterotoxin of *Esch.coli*. This toxin is produced only at a temperature of less than 30°C. Hence, this is seen in contaminated food which has been stored at low temperature.

Y. enterocolitica is associated primarily with domestic animals but may be transmitted to humans through

contaminated food stuffs or directly from such animals as poultry and swine. The organism is frequently associated with large outbreaks of disease. This is typical of point-source food-borne infections. Unpasteurised milk is another vehicle through which this organism can be transmitted to a large number of people.

The infection is characterised by acute abdominal pain, profuse (sometimes bloody) diarrhoea and headache. Vomiting may occur in some patients. The clinical picture simulates that of acute appendicitis. Mesenteric lymphadenitis is seen in older children and young adults. Sometimes adults may develop meningitis, arthralgia or bacteraemia due to this organism.

The isolation of *Y.enterocolitica* for diagnosis can be made from stool. However, when present in low numbers it can be cultured by a cold-enrichment procedure in which the culture specimen is held in saline at 4°C for upto 3 weeks. Periodic cultures are made from the cold enrichment to regular media and incubated at 35°C to recover the organism.

Treatment is easy because the bacterium is sensitive to almost all antibiotics except penicillin and in most of the cases enteritis is self-limiting.

43 Vibrio

Vibrios are short and curved gram-negative bacilli which are motile, nonsporing and oxidase positive organisms. This genus comprises of 33 well-defined species of which 11 have been isolated from clinical specimens obtained from human beings. *Vibrio cholerae* and *V. parahaemolyticus* are two important species of this genus.

Classification of Vibrios

Various classifications of vibrios are currently in vogue. Two of these are:

Heiberg Classification

Heiberg classification is based upon fermentation reactions of mannose, sucrose and arabinose. Vibrios have been divided into eight groups in this classification (Table 43.1).

Table 43–1. Heiberg classification

Group	*Fermentation of*		
	Mannose	*Sucrose*	*Arabinose*
I (*V.cholerae*)	+	+	–
II	–	+	–
III	+	+	–
IV	–	+	+
V	+	–	–
VI	–	–	–
VII	+	–	+
VIII	–	–	+

Gardner and Venkatraman Serological Classification

This classification is based upon flagellar (H) and somatic (O) antigens. This classification divides vibrios into two broad groups-A and B, on the basis of similar biochemical reactions and common H antigen. Based upon O antigen, group A is further subdivided into six O serogroups or serovars. These are designated as serovars I to VI. The cholera vibrios fall into O-I serovar. All other vibrio serovars are considered non-pathogenic and called as non-cholera vibrios (NCV).

VIBRIO CHOLERAE

Morphology

The bacteria of this species are gram-negative and in young cultures are usually curved or comma shaped. S forms or spirals may also occur (Fig. 43.1). These measure about 2 µm × 0.5 µm. Pleomorphism is seen in old cultures. The structure of the cell envelope is similar to that of other gram-negative bacilli.

In liquid media, *V. cholerae* are often seen in pairs or short chains giving S shaped appearance. These organisms show sheathed polar flagellum. The sheath is continuous with outer membrane of cell envelope. Fimbriae have occasionally been seen in *V. cholerae*.

Cultural Characters

All species of *Vibrio* require NaCl for their growth in a range of 1-3%. *V. cholerae* can, however, grow even in the absence of NaCl. They can grow at a temperature

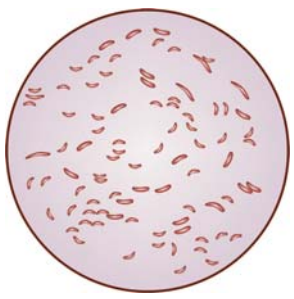


Fig. 43–1. Morphology of *V. cholerae*

of 20-37°C. Most strains grow at pH 6.5-9 and some, notably those of *V. cholerae*, can grow even at pH 10. These organisms do not have any exacting nutritional requirements and can grow rapidly in alkaline peptone water with a generation time of around 11 minutes.

Growth on Solid Media

Nutrient agar. After 12-18 hours, on nutrient agar *V. cholerae* produces colonies that are 1-2 mm in diameter, glistening, translucent with a bluish or greenish appearance in obliquely transmitted light.

MacConkey agar. Colonies on MacConkey agar, after 18 hours of incubation, are smaller than those on nutrient agar but prolonged incubation gives rise to pink and finally dark red coloured colonies. Some strains of *V. cholerae* fail to grow on desoxycholate citrate agar.

Blood agar. On blood agar a zone of clearing (2-3 mm) is seen around the colonies of *V. cholerae*. True haemolysis is produced by some strains of EI Tor biotype whereas pseudohaemolysis is caused by classical *V. cholerae*.

Gelatin agar. In a gelatin stab, there occurs a white line of growth along the track of the wire. Liquefaction in this stab begins from the top and spreads downwards giving a funnel-shaped appearance (Fig. 43.2).

Growth in Liquid Media

In peptone water, this organism grows as a surface pellicle because of its affinity for oxygen and the surface pellicle becomes visible within 6-9 hours of incubation.

Selective Media

Numerous selective media have been formulated for the isolation of vibrios and almost all of these are based upon the tolerance of vibrios for alkaline conditions, bile salts, tellurite, thiosulphate and citrate. To improve differentiation sucrose, starch or mannose can be added alongwith a suitable indicator. Addition of polymyxin B makes a medium highly selective for EI Tor biotype.

Fig. 43–2. Growth of *V. cholerae* in gelatin stab

Alkaline peptone water (APW) with pH 8.6. APW is useful for preliminary enrichment of vibrios from faeces and other contaminated material. It is an enrichment medium.

TCBS medium. The most selective medium used now a days is thiosulphate citrate bile sucrose (TCBS) agar medium with a pH 8.6. This medium was devised by Kobayashi et al in 1963 and since then has attained worldwide acceptance. After 24 hours of incubation the colonies are 2-3 mm in diameter and yellow in colour for those organisms which ferment sucrose. These include *V. cholerae, V.alginolyticus* and *V. fluvialis.* Vibrios which do not ferment sucrose give rise to green coloured colonies. These are *V.parahaemolyticus,* and *V.mimicus*.

Monsur's tellurite taurocholate gelatin agar has also been used successfully for the isolation of *V. cholerae* from faeces and *V. parahaemolyticus* from food stuff. It is an enrichment medium.

Sensitivity to Physical and Chemical Agents

Vibrios are killed by moist heat at 55°C in 15 minutes as well as by drying and pH. Normal gastric juices also destroy these bacteria within minutes. In clean, non-acid fresh or sea water it may survive for 1-2 weeks. In soil at a temperature of around 25°C, vibrios can survive for upto 2 weeks provided adequate moisture is also made available.

Almost all vibrios are sensitive to compound O/129 (2,4-diamino-6, 7-diisopropylpteridine) and this is employed for the identification of these microbes. The El Tor biotype is more resistant to polymyxin B than the classical biotype. Most vibrios are sensitive to tetracycline, gentamicin, chloramphenicol and nalidixic acid. Plasmid mediated resistance to tetracycline, and some other antimicrobials, appeared in *V. cholerae* in 1976 and since then it has spread to various parts of the world. Drug resistance is rare in other vibrios. However, all are resistant to penicillin.

Biochemical Characters

V. cholerae ferments glucose, maltose, mannose, sucrose and mannitol with the production of acid only. These may ferment lactose after a prolonged incubation. On the basis of certain biochemical parameters vibrios can be differentiated from other similar organisms (Table 43.2). They are catalase positive, oxidase

Table 43–2. Differentiation of vibrios from other aerobic Gram-ve bacilli

Character	*Vibrio*	*Aeromonas*	*Plesiomonas*	*Pseudomonas*	*Enterobacteriaceae*
Oxidase	+	+	+	+	–
Glucose fermentation	+	+	+	–	+
Gas from glucose	–	+/–	-	–	+/–
Polar flagellum	+	+	+	+	–
Sheath around flagellum	+	–	–	–	–
Sensitivity to O./129	+	–	+	–	–

positive, urease negative, liquefy gelatin and give cholera red reaction (Table 43.3). Cholera red reaction is because of production of indole and reduction of nitrates to nitrites. This is done by adding few drops of concentrated H_2SO_4 to an overnight growth of organisms in peptone water. A reddish pink colour develops due to formation of nitroso-indole. Apart from *V. cholerae*, all other organisms which produce indole and reduce nitrates also give positive cholera red reaction. These include *Esch. coli*, *Proteus*, etc.

Table 43–3. Biochemical properties of *V. cholerae*

Test	*Reaction*
Reaction on triple sugar iron	Acid utilization
Growth at 4°C	–
Growth without NaCl	+
Sucrose fermentation	+
O/129 sensitivity	+
Lecithinase production	+
Indole	+
Oxidase	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Arginine dihydrolase	–

Within the genus *Vibrio*, differentiation between *V.cholerae* and *V. parahaemolyticus* can be done on the basis of certain characters, some of which have been depicted in Table 43.4.

Table 43–4. Differentiation between *V. cholerae* and *V. parahaemolyticus*

Character	*V. cholerae*	*V. parahaemolyticus*
Growth without NaCl	+	–
Growth in 8% NaCl	–	+
Swarming	–	+
Beta galactosidase	+	–
Voges Proskauer reaction	+	–
Resistance to O/129 (10 mg)	–	+
Acid from arabinose	–	+
Acid from sucrose	+	–

Various tests are now available to differentiate between classical biotype of *V. cholerae* and EI Tor biotype (Table 43.5).

Table 43–5. Distinguishing features of classical and EI Tor *V. cholerae*

Character	*Classical*	*EI Tor*
Haemolysis of sheep RBCs	–	+
Haemagglutination of chicken erythrocytes	–	+
Resistance to polymyxin B	–	+
Voges Proskauer reaction	–	+
Resistance to lysis by phage IV	–	+
Lysis by phage 5	–	+

Antigenic Structure

V. cholerae has heat stable somatic (O) antigens and heat labile flagellar (H) antigen. At least 139 O-groups are now known and all these share same flagellar antigen. These are referred to as serovars or serogroups.

The classical as well as EI Tor biotypes of *V. cholerae* belong to serogroup O1. This serogroup can further be subdivided into three serotypes (Table 43.6).

- Ogawa,
- Inaba and
- Hikojima

Ogawa and Inaba strains agglutinate with their respective specific and absorbed antisera whereas Hikojima strains agglutinate with both these antisera. Variation from Ogawa to Inaba serotypes occasionally takes place.

The serovars O2 to O139 are found among the non-cholera vibrios and termed non-O1 *V. cholerae* since these are not agglutinated by O1 antiserum.

The H antigen present in all the O serogroups is identical and hence of no use in identifying and serotyping the strains. Its location in the core of sheathed flagella however, causes some problems in obtaining clear agglutination unless the suspension is treated with phenol or some other detergent such as triton which exposes the H antigen.

Table 43–6. Serotyping of El Tor biotype

Serotype	*O antigens*
Ogawa	AB
Inaba	AC
Hikojima	ABC

Heiberg (1934) classified vibrios based on the fermentation of mannose, sucrose and arabinose (Table 43.7). A serological classification given by Gardner and Venkatraman (1935) is given in Table 43.8.

Table 43–7. Heiberg grouping of Vibrios

Group	*Mannose fermentation*	*Sucrose fermentation*	*Arabinose fermentation*
I	A	A	—
II	—	A	—
III	A	A	A
IV	—	A	A
V	A	—	—
VI	—	—	—
VII	A	—	A
VIII	—	—	A

Phage Typing

Lysogeny is widespread in *V. cholerae* O1. Various attempts have been made to type these strains with phages. Lee and Furniss (1981) have devised a scheme with 14 phages. These phages include four of Mukherjee et al and four isolated by Basu and Mukherjee. This scheme has been used in epidemiological studies. Individual phages have been used to differentiate EI Tor from classical strains. A vibriocin typing scheme has been put forth by Mitra et al in 1980.

Determinants of Pathogenicity

The important clinical features of cholera are as a result of host reaction to an extracellular enterotoxin. The toxin is called as cholera toxin (CT), cholera enterotoxin or choleragenoid. It has been purified and its pharmacology has been studied in details. The CT gene has been cloned and sequenced. *V. cholerae* produces this toxin after it has attached to the epithelium of the intestine through a *pilus colonization factor*.

The CT has a molecular weight of about 84000 daltons and comprises of two major regions or domains: A (comprising of A1 and A2) and B. The B region has a molecular weight of about 56000 daltons and is made up of five identical noncovalently associated B subunits (Fig. 43.3), each with a molecular weight of about 11500. The B region is responsible for binding of the toxin to the host cell and may also facilitate the entry of A1 component of A region to enter the cell. The synthesis, assembly and transport of A and B units is coordinated

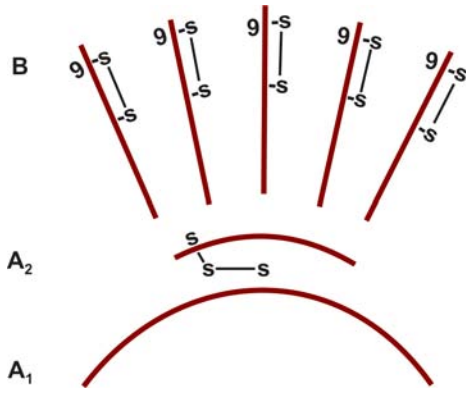
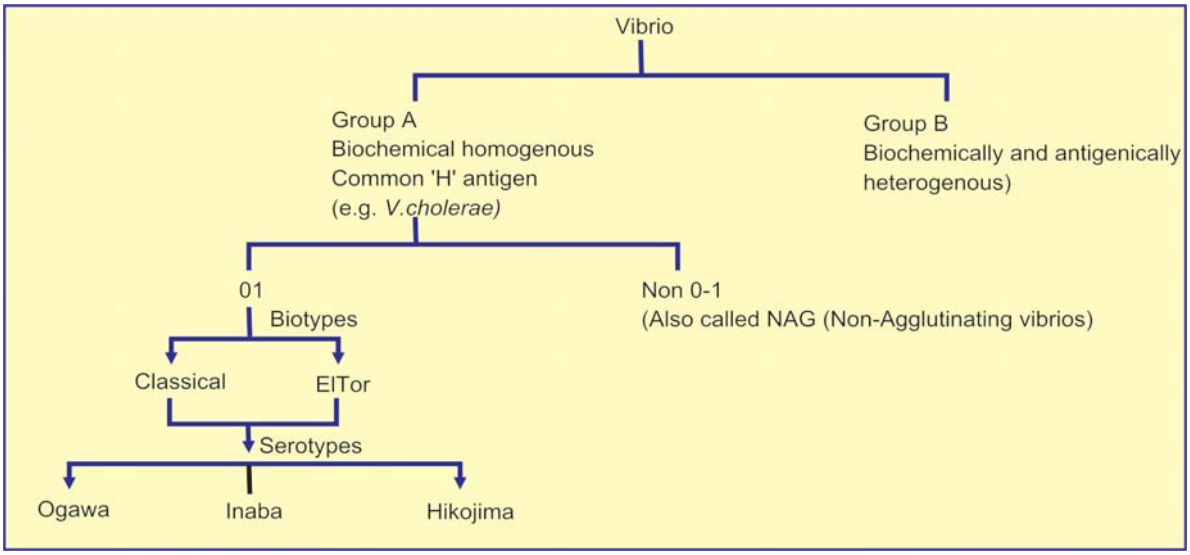


Fig. 43–3. Structure of CT

Table 43–8. Gardner and Venkatraman's classification



PART VI

in such a way in the bacterium that the entire molecule is secreted. The pathogenesis is due to A component and resembles that of LT of *Esch. coli.* The latter has similar pharmacological activities and is immunologically cross reactive with cholera toxin.

Pathogenesis and Clinical Features

Cholera is transmitted through faeco-oral route by contaminated food or water. In a normal individual gastric acidity acts as a moderate barrier for *V. cholerae*. Food and alkaline materials facilitate bacteria to cause infection even in small dose of upto 1000 to 10000 bacteria. The mucus barrier of the small intestine prevents the attachment of bacteria to epithelium. To overcome this, it is believed that bacteria make use of their motility, chemotaxis, and production of mucinase and other proteolytic enzymes. An association between motility and virulence has been established. Once *V. cholerae* adhere to the epithelium, diarrhoea is produced by cholera toxin (Fig. 43.4). This bacterium does not invade the tissues.

After an incubation period of 2–3 days, an abrupt onset of disease occurs. The initial signs are vomiting and diarrhoea. The solids in the intestinal tract are purged early in the disease and the subsequently voided fluid is watery, without odour and contains such electrolytes as sodium chloride, potassium and bicarbonate. The patient develops sunken eyes and cheeks and the skin becomes wrinkled. Death may result from rapid dehydration and resulting electrolyte imbalance. Without treatment the case fatality rate may reach 60% and with treatment around 1%.

V. cholerae can remain in intestine for a long time after recovery of patient. These carriers may continue to shed bacteria for upto one year. Persons over 50 years of age may become chronic carriers and intermittently shed the organism for many years.

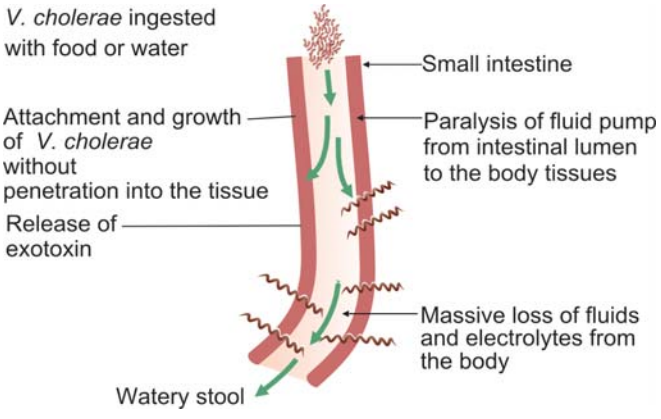


Fig. 43–4. Pathogenesis of cholera

Laboratory Diagnosis

Specimen for the laboratory diagnosis of cholera shall be faeces which should preferably be collected in a sterile container. Rectal swabs are of use provided the same can be cultured immediately. In cases of delay not exceeding 6 hours in processing of sample, samples should be placed in alkaline peptone water to prevent overgrowth of vibrios by other organisms, if the delay is expected to exceed six hours then sample should be placed in VR fluid.

Microscopic Examination of the sample can yield valuable information if it can be performed on sample collected during the first or second day of infection when large number of bacteria is present in it. A wet preparation, preferably seen under dark ground illumination, may show vibrios *darting* (moving like arrows). The test can be made more specific by immobilizing the bacteria with the addition of vibrio antiserum to the wet suspension of clinical sample.

A Gram staining may show gram-negative curved bacilli in different forms and shapes.

Culture can be done by inoculating 2 ml of faeces into 20 ml of alkaline peptone water and spreading a loopful of faeces onto TCBS medium. These are incubated aerobically at 37°C. After incubating for 6 hours, subculture a loopful from the surface of the alkaline peptone water onto TCBS medium and incubate it. The alkaline peptone water can be tested microscopically for the presence of darting vibrios.

The TCBS plates are examined for the colonies of *V. cholerae* which are yellow in colour, and 2-3 mm in diameter.

Test bacteria from yellow colonies for slide agglutination with *V. cholerae* O1 antiserum. If the test is negative or the bacteria show autoagglutinability, the help of biochemical tests (Table 43.3) is taken to establish the identity of the isolate. If vibrio colonies are found not to be *V. cholerae* O1, test them against other O-antisera, if available, to establish their identity as belonging to O2-O139 serogroups.

Serological diagnosis can also be attempted on a pair of sera and by demonstrating a rising titre of antibodies in an agglutination test.

Treatment

Death of a cholera case is not due to tissue damage but is caused by loss of fluids. Effective treatment of cholera, if initiated early, is amazingly simple. Replacement of fluids and electrolytes lost is the key to successful management. Recovery in severe cases can be seen within hours by the intravenous administration of fluids.

Prevention and Control

Proper sewage treatment and water purification systems are the most important preventive measures. Countries with adequate systems have few, if any, outbreaks of cholera. Rapid detection, isolation and treatment of patients and carriers are also important.

A killed vaccine has been used for many years but has not been found to be highly effective.

V. PARAHAEMOLYTICUS

This organism was isolated for the first time in Japan in 1951 as the causative agent of food poisoning due to sea fish. It was named as *V. parahaemolyticus* in 1963 by Sakazaki because of the true haemolysis produced by this organism on blood agar. It has now been known to be responsible for a large number of cases of gastroenteritis and infections of eye and ears in those who ingest or handle the sea products.

Morphology and Culture Characters

V. parahaemolyticus resembles *V. cholerae* in its morphology and staining reaction as well as most of the growth requirements. Important differences have been shown in Table 43.4. *V. parahaemoyticus* strains are capsulated especially when grown in medium with 3% NaCl; have peritrichous flagella and fail to grow if the medium does not contain at least 2% NaCl. These can tolerate salt concentration upto 8% but fail to grow if it exceeds 10%. These organisms produce green coloured colonies on TCBS medium which are opaque with raised centre and flat translucent periphery.

V. parahaemolyticus is extremely susceptible to moist heat and is killed at 60°C within 15 minutes. Drying as well as making suspension in distilled water or vinegar also destroy this organism. It does not multiply at 4°C but can survive at this temperature as well as freezing for a very long period.

Biochemical reactions show this organism to be oxidase, catalase, nitrate, indole and citrate positive. It ferments glucose, maltose, mannitol, mannose and arabinose with the production of acid only.

Antigenic structure. The O and K antigens are useful for the serologic typing of *V. parahaemolyticus*. There is an established serotyping scheme based on 11 thermostable O antigens and 65 thermolabile K antigens. Some of these antigens are shared with *V. alginolyticus*. No correlation has been found beteween antigenic type and pathogenicity.

Determinants of Pathogenicity

No enterotoxin has been demonstrated from *V. parahaemolyticus*. The only significant difference that has been seen in virulent and avirulent strains is the production of *thermostable direct haemolysin*. These strains produce haemolysis on Kanagawa medium and the process is called as Kanagawa phenomenon. These strains lyse human or rabbit erythrocytes but not horse erythrocytes. This haemolysin has been characterized and found to be a protein of mol wt. 21000 which is cytotoxic, enterotoxic and cardiotoxic. Though a correlation has been seen in the ability of the organism to produce haemolysin and to cause disease, the role of toxin in the pathogenesis of disease is still not clear. Other possible virulence factors include a factor that causes elongation of Chinese hamster ovary cells, a heat labile protein that causes diarrhoea in mice and an adhesin.

Clinical Disease

V. parahaemolyticus causes gastroenteritis which may range from self-limiting diarrhoea to cholera like illness. Diarrhoea is explosive and watery with no mucus or blood. Cases with Shigella like picture have also been reported.

Laboratory Diagnosis and Treatment

These are essentially on the same lines on which cholera is to be diagnosed and treated. The laboratory diagnosis is clinched by the biochemical tests and production of true haemolysin by the pathogenic strains. Treatment is also based upon correction of fluids and electrolyte balance.

As compared to cholera or gastroenteritis due to *V. parahaemolyticus*, the remaining vibrios spp. do not carry significant public health importance and are isolated rarely.

AEROMONAS

Aeromonas are gram-negative bacilli or coccobacilli which are straight with rounded ends which are oxidase and catalase positive and hence sometimes mistaken as vibrios or *Pseudomonads* (Table 43.2). Two groups of these organisms have been recognised : motile and nonmotile. The nonmotile strains are primarily pathogenic for fishes and include the species of *A. salmonicida*. There is no consensus opinion regarding taxonomy of motile aeromonads. Only one species *A. hydrophila*

(which is considered by some as a group having three species : *A. hydrophila, A. sobria* and *A. caviae*) is a recognised human pathogen.

Morphology

These are gram-negative bacilli or coccobacilli with rounded ends and occur singly, in pairs or short chains. These are motile with single polar flagellum. *A. hydrophila* is nonsporing and noncapsulated.

Cultural Characters

These are facultatively aerobic and grow well at 30°C as well as 37°C. Colonies on nutrient agar after 24 hours of incubation are 1-3 mm in diameter, circular, smooth, convex, whitish and translucent. Most strains produce haemolysis on blood agar. Because of resistance of most of the strains to ampicillin, a selective blood agar has been prepared for this organism by incorporating ampicillin in the medium.

Biochemical Reactions

A. hydrophila metabolizes glucose both fermentatively as well as oxidatively and gas may be produced by some strains. Nitrates are reduced to nitrites.

Some important biochemical reactions which can be made use of in differentiating this organism from morphologically resembling *Plesiomonas shigelloides* have been given in Table 43.9.

Antigenic Structure

All motile strains share same H antigen but differ in their O antigenic configuration. 44 serogroups on the basis of variations in somatic antigen have been described.

Pathogenicity and Clinical Features

A large number of substances are produced by this organism which may be responsible for pathogenicity. These are:

i. Cytotoxic beta haemolysin which causes diarrhoea in mice

Table 43–9. Biochemical characters of *A. hydrophila* and *P. shigelloides*

Test	*A. hydrophila*	*P. shigelloides*
Oxidase	+	+
Fermentation of glucose	+	+
• lactose	–	+/–
• mannitol	+	–
• inositol	–	+
Lysine decarboxylase	–	+
Ornithine decarboxylase	–	+/–
Arginine dihydrolase	+/-	+
Citrate	+/–	–
Voges Proskauer reaction	+/–	–
Hydrolysis of aesculin	+	–

ii. Heat labile enterotoxin pathogenic for rabbits and rats
iii. Cytotoxic enterotoxin effective in suckling mice only
iv. Enteroinvasive factor for HEp-2 cells
v. Haemagglutinin
vi. Protease and elastase

There is disagreement on exact pathogenicity of *A. hydrophila* though it has been frequently isolated from the faeces of many cases with diarrhoea. Treatment is symptomatic.

PLESIOMONAS

This genus has only one species which, because of antigenic relationship with shigellae, is designated as *Plesiomonas shigelloides*. These are gram-negative straight rods with rounded ends which are motile by polar flagella. These can grow easily on nutrient agar and blood agar and produce colonies which are 1-1.5 mm in diameter, greyish and opaque with raised centre and smooth surface. These do not grow on TCBS medium. The biochemical reactions have been given in Table 43.9. A total of 50 somatic (O) antigen groups and 17 H antigens have been described.

P. shigelloides has been isolated from various natural sources, fishes, mammals as well as from faeces of the human beings suffering from diarrhoea. No enterotoxin has been detected.

44 Pseudomonas

Pseudomonads are gram-negative and nonfermentative bacilli that are widely distributed in nature as saprophytes or as commensals and pathogens for man. Gessard in 1882 isolated *Pseudomonas aeruginosa.*

More than 100 species of Pseudomonads have been isolated so far. The most frequently encountered species of *Pseudomonas* in humans is *P. aeruginosa*. This is a classical opportunist pathogen with innate resistance to many antibiotics and disinfectants. It is invasive and toxigenic, produces infections in patients with abnormal host defences and is an important nosocomial pathogen.

PSEUDOMONAS AERUGINOSA

Morphology

P.aeruginosa strains are gram-negative bacilli with variable length. These measure 1.5-3.0 µm × 0.5 µm. These have parallel sides and rounded ends and are arranged singly, in small bundles or short chains. The organism is motile with monotrichate flagellum (Fig. 44.1). The cell wall and other structural components are similar to other Gram negative bacilli. Fimbriae present on *P.aeruginosa* differ from those on other gram-negative bacilli in their inability to cause haemagglutination.

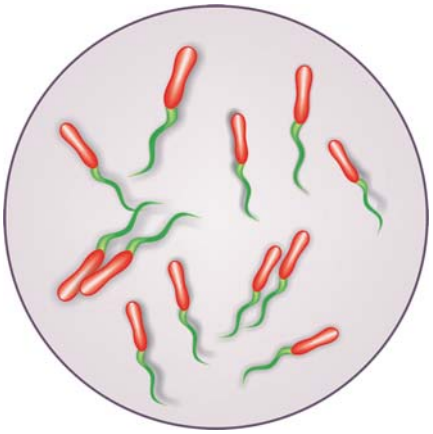


Fig. 44–1. Stained flagella of *P. aeruginosa*

Cultural Characters

P.aeruginosa is capable of growing in a wide range of temperature but optimal growth occurs at 28°C. This organism can grow even at 41°C. pH below 4.5 is not conducive for its growth. It is an obligatory anaerobic species.

P.aeruginosa is an extremely adaptable organism that can utilize over 80 different organic compounds for growth. It can easily grow on various media used for the cultivation for enterobacteria as well as on media recommended for growth of vibrios because of its ability to tolerate alkaline environment. Clinical isolates grown on blood agar are frequently haemolytic.

Growth on nutrient agar. On nutrient agar *P.aeruginosa* can produce three types of colonies which are 2-3 mm in diameter with a matt surface, a floccular internal structure and butyrous consistency.

Strains which are isolated from cases of cystic fibrosis are **mucoid** in nature and produce copious amounts of extracellular polysaccharide on nutrient agar.

Production of pigment on media. Four types of pigments can be produced by *P.aeruginosa* strains.

- Pyocyanin
- Pyoverdin
- Pyorubin
- Pyomelanin

Most commonly produced is blue-green pigment called as **pyocyanin** which diffuses into the surrounding

medium. This pigment is not produced by other species of this genus and hence its detection becomes diagnostic for *P.aeruginosa*. Many strains produce a green fluorescent pigment known as **pyoverdin** whereas some strains produce red pigment (**pyorubin**) and few produce **pyomelanin** (black) pigment. Use of certain specialised media such as Pseudomonas P agar is made to enhance the production of pyocyanin.

Most of the strains of *P.aeruginosa* cultures give a characteristic fruity odour due to the production of aminoacetophenone from tryptophan.

Growth on Other Media

- **On blood agar** the colonies are similar to those on nutrient agar. Many strains produce haemolysin.
- Non-lactose fermenting pale coloured small colonies are produced on **MacConkey agar.**
- **Cetrimide agar** is a selective medium for Pseudomonas and growth of almost all other bacteria is inhibited.
- **Peptone water** or nutrient broth show Pseudomonas as a uniform turbidity growth with a pellicle near the surface.

Sensitivity to Physical and Chemical Agents

Temperature of 55°C for 60 minutes exerts lethal effect upon *P.aeruginosa.* This microbe is resistant to drying. It survives well in wet environment and can remain alive in water at ambient temperature for months. It can also multiply in water with minimal nutrients. To reduce surface contamination due to this organism on instruments and in hospitals and drains, disinfectant must be selected with great care. *P.aeruginosa* is resistant to many disinfectants including quaternary ammonium compounds and hence dettol and cetrimide have been used in preparing selective media for this organism.

Biochemical Characters

P. aeruginosa is oxidase positive. Although a few other gram-negative genera are also oxidase-positive, none reacts as swiftly as *P.aeruginosa*, which gives a positive reaction in less than 30 seconds. It does not ferment carbohydrates, but many strains oxidatively utilize glucose.

Antigenic Structure

Somatic antigens. 17 somatic antigens designated as O1 to O17 have been accepted by the International Antigenic Typing System. These divide the strains of *P.aeruginosa* into 17 serogroups.

Flagellar antigens. Two flagellar antigens have been isolated from strains of *P.aeruginosa*. One of these is uniform and the other is complex and may consist of 5-7 factors.

Determinants of Pathogenicity

P.aeruginosa is pathogenic only when introduced into areas devoid of normal defences such as disruption of skin and mucous membrane by direct tissue damage; use of intravenous or urinary instrumentation or in immunologically deficient individuals. With the help of fimbriae, the organism attaches to the surface of the host, colonises there and after local invasion produces systemic disease. A large number of enzymes and toxins are liberated by this organism (Fig. 44.2).

Pathogenesis and Clinical Picture

Several exotoxins and enzymes produced by *P.aeruginosa* along with endotoxic cell wall and fimbriae as well as occasionally a mucoid capsule probably account for the virulence associated with this organism. The organism is so ubiquitous in wet hospital environment that some people prefer to call it as **water bug**. In a hospital setting no open wound, burn or immunologically deficient patient is free from exposure. The major body defence against *Pseudomonas* infection appears to be functioning phagocytic system. In any condition which causes leukopenia, *Pseudomonas* assumes an opportunistic pathogenic role.

Several clinical conditions which are highly correlated with *P.aeruginosa* infection include cystic fibrosis, burns, urinary catheterization, lumbar puncture and cancer chemotherapy. From any such condition the patient may develop *Pseudomonas* pneumonia or bacteraemia (Fig. 44.3). These conditions are serious and

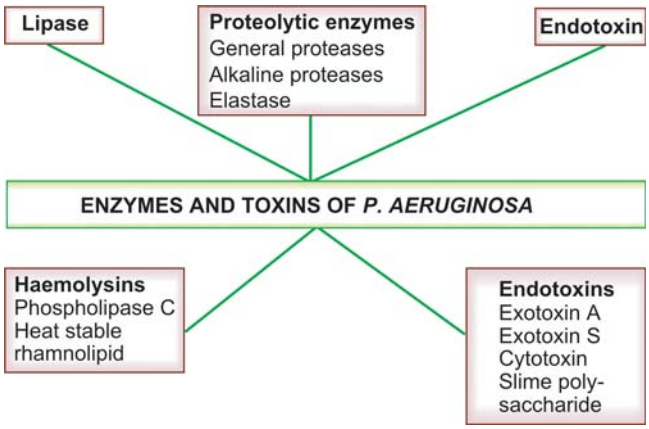


Fig. 44–2. Enzymes and toxins of *P.aeruginosa*

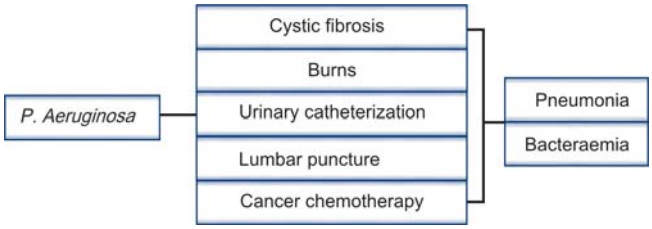


Fig. 44–3. Spread of *P.aeruginosa* in hospitalised patients

have mortality rates in the order of 60-70%. This organism also frequently causes otitis which is sometimes referred to as swimmer's ear. It may cause invasive malignant otitis externa in diabetic patients. Infection of the eye which may lead to rapid destruction occurs most commonly after injury or surgical procedure on eye. The septicaemia may lead to ecthyma gangrenosum which is haemorrhagic necrosis of skin.

Laboratory Diagnosis

The specimens to be collected shall depend upon the site of lesion which is usually organ specific in these infections. Commonly collected samples include urine, blood, cerebrospinal fluid, sputum and pus. The smears made from these specimens do not show any typical character which may be of help in diagnosis.

The sample is cultured onto ordinary laboratory media such as blood agar, nutrient agar and MacConkey agar and help is taken of the cultural characters and biochemical tests to reach at a diagnosis.

Bacteriocins and Bacteriophages

Three types of bacteriocins are produced by *P.aeruginosa* which are known as R, F and S. Pyocins R or F are produced by almost 90% of strains of *P.aeruginosa*. These pyocins are active against other members of this species and occasionally against fluorescent pseudomonads but not against non-fluorescent pseudomonads.

The pyocin typing is employed for epidemiological studies. It involves streaking of 8 indicator strains (numbered 1-8) on the surface of agar that had previously supported the growth of test strain. The pattern of inhibition of indicators determines the type of strain. Further discrimination between same types is done with the help of five additional indicator strains (A-E). Recently the technique has been modified by incorporating the indicator strains in the soft-agar overlay over spots of growth of the producer strain. With this technique at least 105 pyocin types of *P.aeruginosa* are recognised.

Almost all the strains of *P.aeruginosa* are lysogenic for phages which may be RNA or DNA. These phages belong to 10 serogroups. No effective phage typing scheme is currently in practice.

Treatment and Prevention

P.aeruginosa infections are often serious and very resistant to therapy.

Clinically significant infections with this organism should not be treated with single drug. One of the penicillins such as mezlocillin can be used in combination with an aminoglycoside, usually gentamicin, amikacin or tobramycin. Ciprofloxacin has been found to be effective against *P.aeruginosa.*

OTHER SPECIES OF PSEUDOMONAS

BURKHOLDERIA MALLEI

This is the causative agent of glanders which is primarily a disease of horses from where it can be transmitted to humans. It is a small, nonmotile, nonpigmented, gram-negative rod that grows easily on various bacteriological media. In horses the disease is primarily pulmonary but in humans it begins as an ulcer of the skin or mucous membrane followed by lymphangitis and sepsis. Inhalation of this organism may cause primary pneumonia. High mortality has been reported in human beings. Tetracycline in combination with an aminoglycoside is the treatment of choice.

BURKHOLDERIA PSEUDOMALLEI

This organism is usually present in soil, water and plants and produces infection in rodents from where human beings can also get infection and suffer from **meliodosis**. This disease has been reported from Myanamar, Thailand, Vietnam, Phillipines and some countries in the West. It may manifest as abscess or pulmonary infection and carries high mortality, if remains untreated. Latent infection with this organism may get activated in immunological deficiency states.

B.pseudomallei is a small, motile, aerobic, gram-negative rod, that resembles other nonpigmented pseudomonads but is antigenically distinct.

BURKHOLDERIA CEPACIA

It is a small gram-negative motile bacillus which is multitrichous. It accumulates poly beta hydroxybutyrate as granules and hence stains irregularly. The colonies after prolonged incubation are coloured reddish purple because of the formation of phenazine. It is a low grade human pathogen but an important cause of nosocomial infection and resistant to a large number of antibiotics.

PART VII
SPIROCHAETES AND OTHER BACTERIA

45 Treponemes

Treponemes belong to the family Spirochaetaceae which includes organisms that cause syphilis, pinta and yaws. The spirochaetes are slender, undulating, cork screw like, relatively flexible filamentous organisms, measuring 2-500 µm in length, ubiquitous, occurring in nature in soil, decaying organic matter, in and upon the body of plants, animals and man. They range from saprophytic to commensals to pathogenic to man.

Classification of Spirochaetes

The spirochaetes are grouped under the order Spirochaetales, which has two families: Spirochaetaceae and Treponemataceae. Each family has 3 genera, which are shown in Figure 45.1.

One of the unusual morphological features of spirochaetes is presence of axial fibrils and an outer coating, the sheath. These fibrils, or axial filaments, are flagella like organelles that wrap around the cell wall. These provide locomotion to spirochaetes. These are attached within the cell wall by *insertion discs* which are plate like structures.

Differentiation of genera within the family spirochaetaceae is based on the number of axial fibrils as well as the number of insertion discs present (Table 45.1).

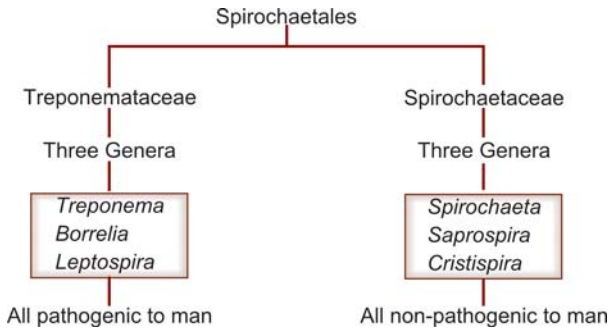


Fig. 45–1. Classification of spirochaetes

Table 45–1. Classification of pathogenic spirochaetes

Genus	*Axial fibrils*	*Insertion discs*
Treponema	6–10	1
Leptospira	2	3–5
Borrelia	30–40	2

The spirochaetes can also be loosely classified into genera with the help of gross morphology (Fig. 45.2). *Treponemes* are slender with tight coils; *Borrelia* are somewhat thicker with fewer and looser coils and *Leptospira* resemble the *Borrelia* except for their hooked ends.

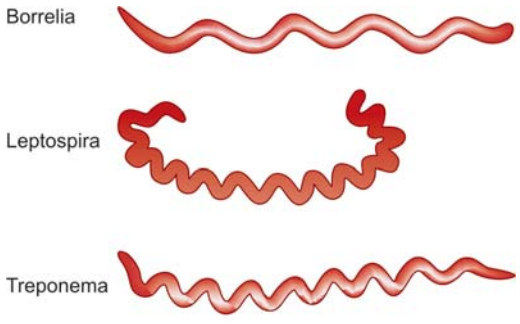


Fig. 45–2. Classification of pathogenic spirochaetes on the basis of morphology

TREPONEMA

Treponemes (*trepos*, meaning to turn and *nema* meaning thread) are both pathogenic and non-pathogenic. Pathogenic treponemes cause the following diseases in man:

T. pallidum	:	Venereal syphilis
T. pertenue	:	Yaws
T. carateum	:	Pinta
T. pallidum (variant)	:	Endemic syphilis

TREPONEMA PALLIDUM

T. pallidum is the causative agent of venereal syphilis in man. *T. pallidum* is now termed *T. pallidum* subspecies (or biotype) *pallidum*, *T. pertenue* is termed *T. pallidum* subspecies *pertenue* and *T. pallidum* variant is termed *T. pallidum* subspecies *endemicum*. *T. carateum* retains its original name. For the sake of convenience *T. pallidum* shall be used in this chapter as the causative agent of venereal syphilis.

Morphology

Typical organisms are slender spirals measuring about 0.2 µm in width (in wet preparations) and 6-15 µm in length. The spiral coils are regularly spaced at a distance of 1 mm from one another. In dry preparations the width is about 0.13 µm. The two ends of the spirochaete are tapering. It is actively motile, exhibiting rotation round the long axis, backward and forward movements and flexion of the whole body (Fig. 45.3).

During motion the primary spirals are maintained though secondary curves appear and disappear in succession. When dark field microscopy is not available, the organism can be detected in wet films of the exudate mixed with India ink.

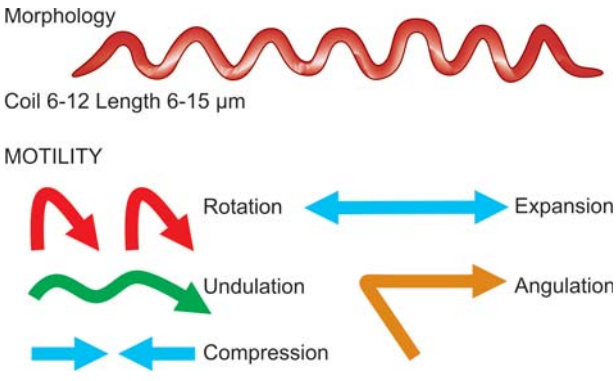


Fig. 45–3. Morphology and motility of *T.pallidum*

Morphologically it is indistinguishable from *T. pertenue* and some commensal spirochaetes. Most saprophytic spirochaetes show lashing motility and lack uniform spirals.

Staining. *T. pallidum* cannot be stained by simple aniline dyes or by Gram's method. In films stained with Giemsa's prolonged method for 24 hours the organisms appear as delicate pink threads. It can be stained by silver impregnation methods. Fontana's method is useful for smears and Levaditi's for tissue sections.

Multiplication. The spirochaetes multiply by transverse binary fission. Divided organisms may adhere to one another for sometime. The division time is 30 hours, while the division time of cultivable Reiter's strain is about 10 hours.

Treponema pallidum, carateum and *pertenue* have not been cultivated in artificial media, not even in chick embryo or tissue culture where they appear only to persist for a long time. In proper suspending fluids and in the presence of reducing substances *T. pallidum* may remain motile for 3-6 days at 25°C. In whole blood or plasma stored at 4°C, organisms remain viable for atleast 24 hours, which is important in blood transfusion services. Virulent *T. pallidum* can be maintained by serial passage in rabbit testis. One such strain called as Nichol's strain has been maintained by this way since 1912.

Non-pathogenic strains such as Reiter's strain can be cultivated in enriched heart infusion broth.

Resistance

Drying kills the spirochaetes rapidly as does the elevation of the temperature to 42°C for about 60 minutes. When stored at referigerator temperatures, the organism is killed in 1-3 days. Stored frozen at –70°C in 10 percent glycerol or in liquid nitrogen the organism remains viable for 10-15 years. Resistance to penicillin has not been demonstrated in syphilis.

Antigenic Structure

The antigens of *T. pallidum* have not been defined. The infection in human beings shows at least three distinct antibodies but the antigens that evoke them are not clearly understood. These are:

Nonspecific antibodies. The antibodies reacting in VDRL or Wassermann's reaction. In this antibody reacts with a lipid hapten that can be extracted by alcohol from beef heart. These lipidophilic antibodies are known as *reagin*. This lipid hapten is called as *cardiolipin*. It is not known whether cardiolipin is contained in

T. pallidum itself or whether it is released as a hapten from tissues damaged by infection. Hence detection of these antibodies is not specific.

Group specific antigen. The second antigen is contained solely within the treponemes themselves where it can be demonstrated by specific immunofluorescence. This appears a group antigen. This can be used to confirm the syphilis diagnosis in sera that react positive with cardiolipin antigens.

Species specific antigen, which is probably polysaccharide in nature is species-specific.

Animal Pathogenicity

All attempts to culture *T. pallidum, in-vitro* have failed, but it is possible to propagate the spirochaetes by inoculating living treponemes from the pathological lesions into the testis, scrotum, skin or eye of the rabbit. Serial passage in rabbits does not appear to reduce the virulence of spirochaetes to man.

Pathogenesis

Most cases of syphilis are contracted during sexual intercourse. The treponemes are present in the superficial genital lesions and pass from one partner to other through intact mucous membranes or through minor skin abrasions. In a small number of cases the primary lesion can be extragenital, i.e. in and around mouth, or on the hands of medical or nursing staff after investigation or treating cases of syphilis. Since the organism is very delicate and does not survive at outside temperatures, it cannot spread through fomites, etc. *T. pallidum* can cross the placental barrier in a syphilitic mother particularly in the secondary stage and produce congenital syphilis in the foetus. If blood is transfused fresh from an infected patient to another person, the infection can follow the transfusion. But if the donated blood is stored at refrigerator temperatures for 3-4 days, the treponemes die.

Clinical Features

When the spirochaete has penetrated its new host it begins to multiply at the site of its entrance, the generation time being less than 5 hours, during the next 9-90 days the primary syphilis appears.

The course of venereal syphilis in man assumes several well-defined clinical stages and these are as follows:

Primary Stage

The primary lesion in syphilis is the *chancre*. Spirochaetes multiply locally at the site of entry and some spread to the nearby lymph nodes and then reach the blood stream. Within 3-6 weeks, whether treated or not, the chancre heals and leaves a thin atrophic scar. The infected lymph nodes have a very *rubbery appearance* on palpation. Clinically, syphilis in the primay stage is a localized infection, but bacteriologically it is a generalised infection.

Secondary Stage

Secondary syphilis sets in 6-8 weeks after the primary lesion heals, during which period the patient is asymptomatic. Occasionally these lesions may develop 3-6 months afterwards. Spirochaetes spread to the skin and mucosal surfaces via haematogenous route to produce widespread mucocutaneous, eruptive macular or papular lesions that occur almost anywhere on the trunk, extremities, specially the palms and soles.

Latent Stage

Untreated patients enter the latent stage of the disease within a few months after primary infection. During latency there is no readily apparent clinical evidence of infection. However the presence of spirochaetes can be determined by reactive serological tests.

Tertiary Stage

About 40 percent of untreated syphilitic patients in the latent stage of the disease proceed to final tertiary stage with its accompanying catastrophic pathology. Tertiary syphilis reflects a destruction of tissues as an immunological response to previous spirochaetal infection and may affect any tissue or body organ. Because the manifestations of syphilis may mimic a variety of unrelated diseases, syphilis has long been known as the great imitator. The cardiovascular and central nervous system are most frequently involved in tertiary syphilis.

Congenital Syphilis

A pregnant syphilitic woman can transmit *T. pallidum* to the foetus through the placenta beginning in the 10th to 15th week of gestation. It can cause abortion or still birth. Others are born live but develop the signs of congenital syphilis in childhood which include interstitial keratitis, Hutchinson's teeth, saddle-nose, periostitis and a variety of central nervous system anomalies. In congenital infection the child makes IgM anti-treponemal antibodies.

Immunity

A person with active or latent syphilis or yaws appears to be resistant to superinfection with *T. pallidum*. A

patient is said to be refractory to reinfection so long as the original infection persists, which is called as premunition or infection immunity. The patient becomes again susceptible to infection after the primary disease is cured. This is called *pingpong syphilis*. Though the patients develop IgM, IgG and IgA antibodies following infection these are not protective in nature. Cell-mediated immunity may play an important role in protection, which appears after the secondary stage of syphilis. Various immune responses usually fail to eradicate the infection or arrest its progression.

Epidemiology

Worldwide, syphilis continues to be one of the major sexually transmitted diseases, though syphilis is not a major contributor to the current epidemic of sexually transmitted diseases in the world which is dominated by AIDS. Syphilis is becoming a serious health problem in urban male homosexual communities and continues to cause congenital lesions in the newborns of the infected mothers. Like most other STDs, syphilis is usually acquired during the years of frequent sexual activity. Data indicate that the risk of acquiring syphilis from only one sexual encounter with an infected partner is approximately 33 per cent. With the availability of very effective treatment and the absence of an extrahuman reservoir, it should be possible to eradicate syphilis, but changing social customs, habits and values have contributed towards the existence or rise in the number of cases.

Laboratory Diagnosis

The diagnosis of syphilis can be established in one of the following ways:

a. Demonstration of *T. pallidum* by microscopy which is possible in primary and secondary stages and congenital lesions.
b. Demonstration of reaginic and treponemal antibodies in serum or CSF.

Direct Examination

Following points are important while considering the direct demonstration of spirochaetes:

a. Samples should be collected before antibiotics are started.
b. The exudates are rich in spirochaetes and all precautions should be taken to prevent contracting infection.
c. Non-pathogenic treponemes may be part of the normal flora which should be kept in mind while interpreting the results.

The detection of treponemes within lesion material is a key aid to diagnosis. Although these organisms can be stained, their thinness makes them difficult to be seen with light microscopy. Dark-field microscopy is recommended. The organisms are white against a black background (Fig. 45.4). Phase-contrast microscopy may also be used, but the organisms are not as readily apparent. Exudates from the lesions of each treponematosis contain both motile and nonmotile treponemes. Depending on the development of the lesion, the numbers may vary from 1 organism per 20 fields to 50 organisms per field.

For most specimens, motile organisms rotate around their longitudinal axis and also bend, snap, and flex along their length. With specimens from well-developed syphilitic lesions, the exudate may contain large amounts of mucoid material. Besides rotating, the organisms in these specimens exhibit a smooth, translational backward-and-forward movement. This directed motility is also occasionally observed in specimens from yaws lesions.

Samples for dark-field microscopy are obtained in different ways. If multiple lesions are present, choose the youngest one available. The chances of visualizing treponemes decrease with increasing lesion development. Clean the surface of the lesion with saline, and blot it dry. Gently remove the crusts if the lesion which has already ulcerated. Superficially abrade the lesion until very slight bleeding occurs. The object is to obtain the clear serum exudate from the lesion subsurface. Apply gentle pressure at the base of the lesion and wipe away the first few drops of blood. Touch a glass slide to the clear exudate, place a cover slip on the specimen, and immediately examine the slide by dark-field microscopy. The exudate may also be removed with a capillary pipette and then transferred to a glass slide. If no fluid exudes, a small drop of saline can be added to the lesion.

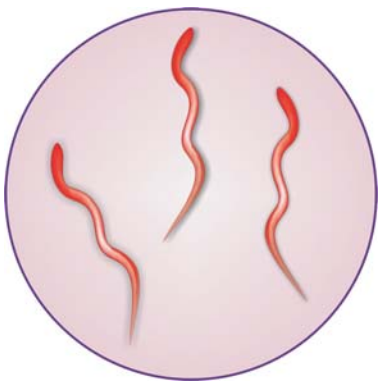


Fig. 45–4. *T. pallidum* organism

Method of examination. Place the slide containing specimen on the mechanical stage with cover-slip on. With the dark field condenser in position and the substage iris diaphragm open, adjust the mirror to reflect the maximum amount of light into the objective lens. Put one drop of immersion oil on the condenser lens and then raise the condenser lens until it just touches the lower surface of the microscope slide. Low-power objectives (10 x, 40 x) should be used to bring the field into focus. Lighting will be scant but cellular debris can be identified. By raising and lowering the dark-field condenser very gently, bring a uniform circle of light into view. This light should be centred in the field of vision by using the adjustment screws on the sides of the substage condenser. Place a drop of immersion oil on the coverslip and lower the oil-immersion objective into the oil. Finally, fine focusing and minor adjustments in the height of the substage condenser and in the position of the slide may be necessary to achieve a uniformly dark field in which brilliant objects can be seen.

If the chancre is healing, spirochaetes may not be demonstrable in it, but the examination of fluid aspirated from lymph node may be positive. If it is not possible to examine the sample immediately, the exudate should be collected in capillary tubes, the ends sealed and transported to the laboratory.

A negative result in dark field microscopy does not rule out the presence of *T. pallidum*. Repeated examinations are sometimes necessary.

Immunofluorescence. Tissue fluid or exudate is spread on a glass slide, air dried and sent to the laboratory. It is fixed, stained with a fluorescein-labelled anti-treponeme serum and examined by means of immunofluorescence microscopy for typical fluorescent spirochaetes. This gives a higher positivity rate than direct microscopy.

Serological Tests

These tests form the mainstay of laboratory diagnosis. An ever enlarging list of serological tests is available with varying degrees of specificity and sensitivity. These tests can be divided as shown in Figure 45.5.

The non-treponemal tests are of greatest value for:

a. Screening procedures
b. To determine response to treatment by quantitation of antibodies
c. To detect reinfection
d. Diagnosis of neurosyphilis
e. Practical and widely available

Treponemal tests are most commonly used:

a. To determine if a reactive non-treponemal test is due to syphilis or some other condition
b. To detect latent syphilis
c. To detect syphilis in patients with negative nontreponemal tests but with clinical evidence for late syphilis

Drawbacks of treponemal tests include:

a. Treponemal tests do not indicate the response of the patient to the treatment
b. Quantitative treponemal tests are of no value in diagnosis or prognosis
c. They are of doubtful diagnostic value in active neurosyphilis

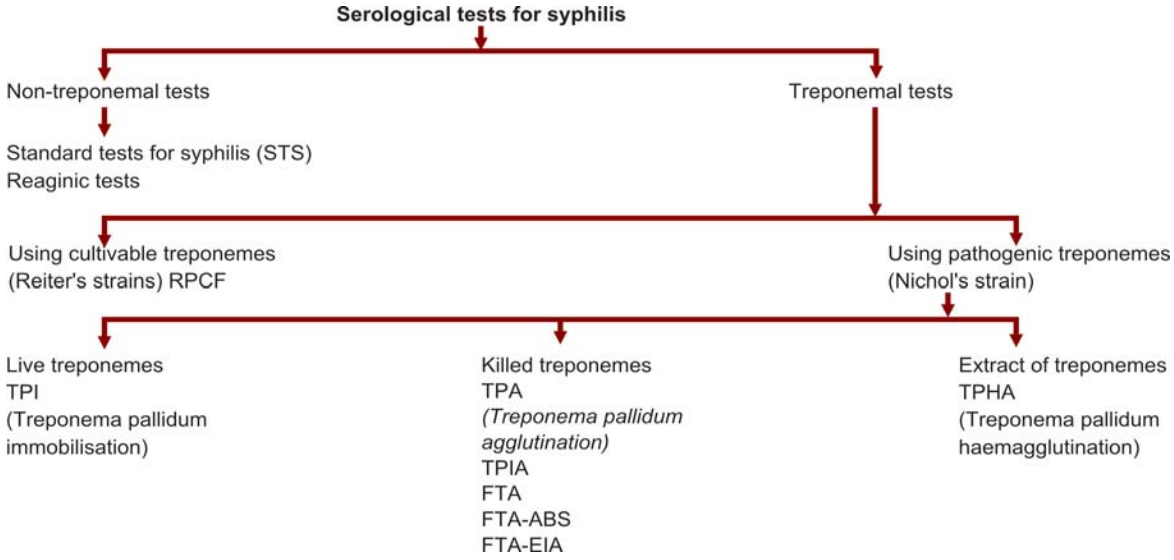


Fig. 45–5. Serological tests for syphilis

PART VII

d. These should not be used in reagin-negative sera, in the absence of clinical suspicion, since false-positive treponemal reactions can occur in such situations.

Drawbacks of non-treponemal tests are:
a. The level of reactivity of such tests is a compromise between sensitivity and specificity
b. Chances of acute and chronic biological false positive are always there
c. Of limited diagnostic value in primary syphilis and latent syphilis.

Standard tests for syphilis (STS). Bordet in 1898 first clearly described serum haemolysis in the test tube. At the turn of the century Wasserman described complement fixation test. In 1931 Kahn introduced a flocculation test which required no complement and could be read macroscopically in a few hours. Many modifications of these tests subsequently appeared with each one having positive and negative points. The non-treponemal tests most fequently used are:
a. Venereal Disease Research Laboratory (VDRL) test
b. Rapid Plasma Reagin (RPR) Card test
c. Complement fixation test (Wasserman and Kolmer)

Wasserman used livers from congenital syphilitics as an antigen, in the belief that it was specific. Later it was demonstrated that other tissues, particularly alcoholic extracts of beef heart could be used equally well as an antigen. The purified cardiolipin from the beef heart is a diphosphatidylglycerol. It requires the addition of lecithin and cholesterol or other "sensitizers" to react with syphilitic "reagin". Reagin is a mixture of IgM and IgA antibodies directed against some antigens widely distributed in normal tissues. It is found in patient's serum after 2-3 weeks of untreated syphilitic infection and in spinal fluid after 4-8 weeks of infection. Brief description of tests used to detect these "reagins" are as follows :

Complement fixation tests (Wasserman test and Kolmer test). These two tests are based on the principle of complement fixation, i.e. reagin-containing sera will fix complement in the presence of cardiolipin "antigen". It is essential to ascertain that the serum is not anticomplementary, i.e. it does not destroy complement in the absence of antigen. This test can be quantitated by using two-fold dilutions of serum. Both these tests are no longer popular, because of their comparative complexity in performance.

Kahn test. In 1931 Kahn introduced a flocculation test which required no complement. It is a tube flocculation test. In this test 0.15 ml of serum taken in 3 tubes is mixed with different amounts (0.05, 0.025, 0.0125 ml) of freshly prepared antigen dilution, shaken in Kahn shaker at 280 oscillations per minute and examined after addition of saline. The negative test shows uniform opalescence. Floccules appear in the positive test, which can be graded based upon the degree of flocculation.

Kahn verification test. To obtain more reliable results the test is observed both at 1 and 37°C. The antibodies due to syphilis generally give a stronger reaction at 37°C and the non-specific antibodies at 1°C. However, even with this test false positive reactions occur.

VDRL and RPR card test. These tests are based on the fact that the particles of lipid antigen remain dispersed with normal serum but form visible clumps when combining with reagin. Results develop within a few minutes, particularly if the suspension is agitated.

VDRL has several advantages, because of which this is the most popular test used today. The advantages are:
a. It is simple and rapid test
b. Reasonably sensitive and specific
c. Requires small quantity of serum
d. Can be easily quantitated
e. The results are reproducible
f. The reagents can be easily obtained, and have good shelf life
g. Quality control of the test can be done.

In a *slide flocculation test*, 0.05 ml of inactivated patient's serum is taken in special slides with depressions or slides prepared with paraffin rings. One drop of freshly prepared antigen is added with a syringe delivering 60 drops from one ml. The slide is shaken in a VDRL shaker for 4 minutes at 180 rotations. It is then examined under the microscope with the low power objective. Formation of clumps indicates positive reaction while uniform distribution of crystals in the drop indicates that serum is non-reactive. The reactive sera can be diluted and results quantified. VDRL test can also be done as a tube flocculation test.

RPR card test. It is also called RPR 18 mm circle card test. In this test stabilised suspension of standardised VDRL antigen and charcoal particles are used as the RPR card antigen. The antigen is added to the serum of the patient, mixed on a mechanical rotator and examined macroscopically for degree of flocculation. Any degree of reactivity obtained in the undiluted serum indicates a need to perform the quantitative test to determine the endpoint titre.

This test has all the plus points of VDRL test and in addition has the following advantages:

a. It is a kit test, contains all needed reagents, controls and prepared antigen
b. Unheated serum is used
c. The reaction is read macroscopically
d. A finger prick blood is sufficient and hence can be used in the field

The only disadvantage with RPR card test is that it can not be used with CSF specimens.

Biological false positive (BFP). The main disadvantage of STS is that the antigen used is non-specific and hence may react with sera of patients who may not have syphilis. The occurrence of false positive reactions due to these inherent reasons and not due to any technical error is known as "biological false positive" reaction. BFP reactions can be classified as acute and chronic-in the former they disappear within 6 months whereas in the latter they persist indefinitely. In ruling out BFP, the use of treponemal tests is recommended. The list of BFPs is given in Table 45.2.

Table 45–2. Biological false positives in STS

- Systemic lupus erythematosus
- Malaria
- Lepromatous leprosy
- Infectious mononucleosis
- Tropical eosinophilia
- Relapsing fever
- Hepatitis
- Rheumatoid arthritis
- Collagen disorders
- Severe trauma
- Coronary artery disease
- Repeated blood loss
- Menstruation
- Vaccination
- Pregnancy
- Haemolytic anaemia
- Heroin addiction
- Tissue regeneration
- Upper respiratory infections
- Certain antihypertensive drugs

Treponemal Tests

Treponemal antigen tests are more specific, since they measure antibodies to *T. pallidum.* Their greatest value lies in their aid in distinguishing specific from nonspecific (BFP) seroreactions and in the diagnosis of late syphilis syndromes where the STS may have become nonreactive, e.g. late cardiovascular syphilis or tabes dorsalis.

Tests using Reiter Treponemes. The only test used in this group is Reiter Protein Complement Fixation (RPCF) test. It is based on the same principle as Wasserman test except that antigen used is extract of the Reiter treponeme. The test was initially thought to be as specific as *T. pallidum* immobilisation (TPI) but subsequent evaluation has proved it to be far less specific than the TPI and perhaps no more sensitive than VDRL. Hence, its use is no longer encouraged.

Tests using *T. pallidum* (Nichol's strain). A few of these tests are:

***Treponema pallidum immobilisation test (TPI) (Test using live T. pallidum*).** It is a complement fixation test using the Nichol's strain of spirochaete as the antigen. This strain is obtained from testicular syphiloma of infected rabbits. Live treponemes are harvested and placed in a survival medium, the patient's serum and complement are added and incubated. The percentage of treponemes immobilized compared to controls determines the results. The test is considered reactive if more than 50 per cent of the treponemes are immobilised, doubtful if 20-50 per cent are immobilised and non-reactive if less than 20 per cent are immobilised. The test is difficult and is not practical. It requires availability of rabbits and expert technicians. It cannot be recommended as a routine diagnostic tool. Test is reactive in yaws.

Treponema pallidum agglutination (TPA): (using killed T. pallidum). This employs a suspension of Nichol's strain inactivated by formalin. When the serum and the antigen are incubated and examined under dark ground illumination, the treponemes are found agglutinated in the presence of antibodies. False positive reactions are common, test is not very specific.

Treponema pallidum immune adherence (TPIA) test (using killed T. pallidum). In this test, suspension of treponemes is mixed with test serum, complement and fresh heparinised whole blood from a normal individual and incubated. The treponemes will be found to adhere to the erythrocytes in the presence of antibodies. In the absence of antibodies immune adherence will not occur. This test is not very widely used.

Fluorescent antibody test (FTA) (using killed T. pallidum). This is an indirect immunoflourescence test using Nichol's strain of *T. pallidum* in the form of smear as antigen. The slides prepared once can be stored for months. The patient serum diluted 1 : 5 is made to react with this antigen, followed by layering of anti-human immunoglobulin flourescent conjugate over it. After proper washing the smear is examined under fluorescent microscope. In a positive test fluorescent treponemes will be observed.

There have been modifications of this method. To remove false positive tests, serum dilution was increased from 1 : 5 to 1 : 200 (hence test called as FTA 200). Since still false positive results were obtained, the patient's serum was first absorbed with an extract of Reiter treponeme and then test performed (FTA-ABS test). FTA-ABS test is as specific as TPI. IgMFTA-ABS detects only IgM antibodies and can be very successfully used in diagnosis of congenital syphilis.

Treponema pallidum haemagglutination (TPHA) test (using. T. pallidum extract). Red blood cells are treated to adsorb treponemes on their surface. When mixed with serum containing antitreponemal antibodies, the cells become clumped. This test is similiar to FTA-ABS test in specificity and sensitivity, but becomes positive somewhat later in the infection. Microhaemagglutination—*T. pallidum* (MHA-TP) is automated version of this test and so also is HATTS (Haemagglutination Treponemal Test for Syphilis) test.

MHA-TP and HATTS are considered satisfactory substitutes for the FTA-ABS. The chief advantages of these two tests over the latter are:

- Technical simplicity
- Economical
- Good quality control
- But these two tests lack sensitivity for diagnosing primary syphilis.

Quality control. The reliability and accuracy of tests for syphilis is dependent upon the quality of reagents used and tight control of technical factors in the test procedure. The quality of reagents can be controlled by adequate testing before use. New lots of reagents must always be tested in parallel with reference reagents or with other lots of the reagents known to be satisfactory.

Interpretations of Serological Tests

Results of serological tests for syphilis must be interpreted in relation to the stage of syphilis suspected, the presence of other underlying diseases or conditions, a history of previous infection or treatment, and epidemiological findings.

The nontreponemal tests are useful as screening tests, in monitoring therapy, and in detecting reinfection. They are biologically nonspecific and are known to react in a variety of diseases and conditions other than syphilis. The more specific treponemal tests are helpful in confirming the specificity of reactive nontreponemal tests.

Once reactive, treponemal tests tend to remain reactive. They are helpful in the detection of late infections and the confirmation of reactive nontreponemal test results. They are not useful in following therapy or in the detection of reinfection.

Currently used serologic tests lack sensitivity in the early stages of infection. Since *T. pallidum* is present in lesions before the appearance of detectable serum antibody, conventional and fluorescent-antibody dark-field tests are frequently helpful in the detection of early infection. Negative results do not necessarily exclude syphilis, since the ability to demonstrate *T. pallidum* can be affected by the age and condition of the lesion and the adequacy of the specimen.

If adequate treatment is given immediately after the primary lesion appears, the patient may never develop demonstrable antibodies and all serological tests will be non-reactive. If treatment is given in secondary stage, the tests become non-reactive in 2-6 months. If treatment is delayed serological tests may not become non-reactive despite, treatment.

A "battery of tests" is recommended to rule out BFP reactions. FTA-ABS and TPHA have found wider applications. TPI is no longer being used for diagnosis except in reference laboratories.

VDRL and FTA-ABS tests can also be performed on spinal fluid. Antibodies do not reach the CSF from the bloodstream but are probably formed in the CNS in response to syphilitic infection.

Congenital syphilis can be diagnosed by differentiating passively transferred antibodies to the foetus from those of actively produced in the newborn. The repeated tests on infant's blood sample will show declining titre in passively received antibodies while the antibody level will rise in case of active infection. Passively acquired VDRL antibodies in baby are usually negative by 3 months. If baby's titre is equal or more than 4 times mother's, congenital syphilis is very likely. Majority of mothers have titres 2-4 times that of baby's. Demonstration of IgM-antibodies in the infant is also diagnostic of congenital syphilis as IgM antibodies normally don't cross the placental barrier.

The percentage frequency of reactive serological tests in cases of untreated syphilis are given in Table 45.3.

Table 45–3. Frequency of reactive serological tests in untreated syphilis cases

Stage	*VDRL*	*TPHA*	*FTA-ABS*
Primary	65-70%	80-85%	50-55%
Secondary	100%	100%	100%
Latent or late	65-70%	upto98%	upto98%

Chemotherapy

Penicillin in concentrations of 0.003 units/ml has definite treponemicidal activity and penicillin is the drug of choice. In syphilis of less than 1 year duration, penicillin levels are maintained for 2 weeks by a single injection of benzathine penicillin G, 2.4 million units given intramuscularly. In older or latent syphilis, benzathine penicillin G 2-4 million units IM is given 3 times at weekly intervals.

YAWS

In areas where yaws has long been endemic, there are names for it in the local language or dialect. Some of its synonyms are: *pian* (French); *framboesia* (German Dutch); *buba* (Spanish); *bouba* (Portuguese), *parangi* (India).

Causative Agent

The organism responsible for yaws is *Treponema pertenue*. It is identical in appearance to *T. pallidum* and *T. caraeteum*. *T. pertenue* does not cause congenital infections because it cannot cross the placenta. It produces lesions in the skin, bone and cartilage, but not in deeper tissues or organs. Like other pathogenic treponemes, it is easily killed by drying, exposure to oxygen, and elevated temperature. The organism multiplies very slowly (once every 30-33 hours) in man and experimentally infected animals. It does not grow in culture.

Occurrence

Yaws occurs primarily in the warm, humid, tropical areas of Africa, Central and South America, the Caribbean and the equatorial island of south-east Asia. Persistent low levels of yaws are reported from Indonesia and Sri Lanka. In India yaws cases used to occur in the states of Andhra Pradesh, Madhya Pradesh and Orissa. Last human case in India was in 2003.

Reservoir

Children aged 2-15 years and latent cases serve as the reservoir of infection. There is no extra-human reservoir. The problem of yaws is one of either *residual yaws* or *recrudescence*.

Mode of Transmission

Yaws is transmitted by direct (person-to-person) non-sexual contact with the exudate or serum from infectious yaws lesions. Late yaws lesions are not infectious. Indirect transmission by insects and contaminated utensils (fomites) is generally of limited significance.

The spread of yaws may be facilitated by crowding and poor community sanitation. The lack of water and soap for bathing and washing, of shoes and clothing for children between the ages of 5 and 15 years are said to favour yaws transmission. Transplacental, congenital transmission does not occur.

Incubation period

It is 3-5 weeks.

Clinical Features

The primary lesion or *mother yaws* appears at the site of inoculation after incubation period of 3-5 weeks. It is an extragenital papule which enlarges and breaks down to form an ulcerating granuloma. *Late yaws* appears in the form of destructive and often deforming lesions of the skin, bone and periosteum. CVS and CNS involvement is infrequent.

Laboratory Diagnosis and Treatment

T. pertenue is indistinguishable from *T. pallidum* in morphology, motility, staining properties ability to provoke anti-cardiolipin and treponemal antibodies and also in its susceptibility to antibiotics. Diagnosis and treatment is same as for venereal syphilis. Yaws has been eliminated from India.

PINTA

Casuative Agent

Treponema carateum is the causative organism for pinta. This is also called as *T. herrejoni*. The disease was thought to be a fungal infection until 1938, when serous exudate from a lesion in a Cuban patient was shown to contain treponemes indistinguishable from those causing yaws and syphilis. *T. caraeteum* is pathogenic only in man and the higher Apes, and it provides some cross immunity to yaws and syphilis.

Occurrence

Formerly, it was highly prevalent in the semi-arid regions of Brazil, Colombia, Cuba, southern Mexico, and Venezuela, but today only scattered foci remain in northern South America and Mexico.

Like yaws and endemic syphilis, pinta is found in remote, rural communities.

Age Group Affected

It differs from yaws and endemic syphilis in that it affects children and adults of all ages. The disease appears to be restricted to dark-skinned races.

Reservoir

The main reservoirs of infection are thought to be young adults of 15-30 years of age who have skin lesions of long duration.

Mode of Transmission

The precise mode of transmission is not known, but repeated direct, lesion-to-skin contact is the likely mechanism. Treponemes are abundant in early lesions and persist through to the late dyschromic stage. It is transmitted by nonsexual means either direct or through the agency of flies or gnats.

Course of Infection

The usual incubation period is 2-3 weeks. The initial lesion is a papule or an erythemato- squamous plaque. It is almost always located on uncovered parts of the body, usually the legs, the dorsum of the foot, the forearm, or the back of the hands.

The papule enlarges slowly by local extension or by merging with satellite lesions to form a hyperkeratotic, pigmented lesion accompanied by an enlargement of the lymph nodes, draining the lesion.

Disseminated lesions identical to the initial lesions develop 3-9 months after infection. These "pintids" vary in number and location. They may slowly enlarge and merge to reach a diameter of 7-25 mm. The lesions become pigmented with age, changing slowly from a copper colour to lead-grey to slate-blue as a result of photo sensitisation.

Late pinta is characterized by pigmentary changes, from dyschromic treponeme-containing lesions to achromic treponeme-free lesions.

Diagnosis

T. carateum can be demonstrated in the skin lesions and in the lymph nodes. It is morphologically indistinguishable from *T. pallidum.* The methods used for the laboratory diagnosis are same as for yaws and syphilis.

Treatment

Treatment is same as that for yaws and syphilis.

ENDEMIC SYPHILIS

Some of the common synonyms of endemic syphilis are: bejel (Arabic), njovera, dichuchwa (Zimbabwe), endemic syphilis of Bosnia, and non-venereal or childhood syphilis.

Causative Agent

Endemic syphilis is caused by *Treponema pallidum (subspecies endemicum*). This organism is closely related, if not identical, to the *T. pallidum* of venereal syphilis. Many believe that the subtle antigenic and pathogenic differences between endemic and venereal syphilis represent only strain variations of the same organism. Endemic syphilis acquired in childhood protects against later infection with venereal syphilis.

Reservoir

Children aged 2-15 years with early infections, as well as latent cases of the disease, serve as the reservoir of infection.

Mode of Transmission

Because most common initial lesion of endemic syphilis occurs on the oral mucosa, indirect transmission of the disease via contaminated drinking vessels is thought to be the most common mode of transmission. Direct lesion-to-skin contact among children and contact with fingers contaminated with saliva containing treponemes also important transmission mechanisms. The disease tends to occur in family groups, the infection being acquired first by children who spread it to susceptible adults.

Course of Infection

A primary lesion is rarely seen in endemic syphilis. The first lesions to appear are mucous patches on the oropharyngeal mucosa, which may be followed by a variety of secondary-type rashes or lesions. The latter prefer the moist body surfaces such as the axillary and genital areas.

The early disease may be followed by a latent period of variable duration. It is not known if infectious relapses occur.

Most patients develop some lesion of late endemic syphilis such as a granulomatous ulcer or nodule. The most common are rhinopharyngitis mutilans (gangosa) and osteoperiostitis. These complications disable and deform the individual, but they are seldom a cause of death.

Laboratory Diagnosis and Treatment

It is same as for venereal syphilis.

46 Leptospira

The leptospires belong to family spirochaetaceae. The genus *Leptospira* comprises of many serogroups which are further subdivided into serovars which possess related serological characteristics. Morphologically and culturally the leptospires cannot be differentiated. However, they can be classified serologically. Till today, 20 serogroups comprising of 176 independent serovars have been isolated from human beings as well as the animals.

Morphology

The organisms of this genus are well described morphologically by the name *lepto* (thin, refined) *spira* (spiral). These are cylindrical organisms about 0.1 µm or even less in thickness and 4-12 µm in length. The length is around 30-40 times the thickness of the bacterium. The organisms are convulated and form regular and tight spirals.

Leptospira consists of three major cell components– an outer sheath or envelope, two independent axial filaments originating from opposite ends of the cell and a cytoplasmic membrane beneath these filaments.

The leptospires are characterised by very quick movement of the bacterium around its own axis.

Pathogenesis

The site of entry into the host is through mucosal surfaces. Important portals of entry are fresh or partially healed abrasions of the skin and intact mucosa of the buccal cavity, nasal passages or conjunctiva. No lesion is caused at the site of entry or in the regional lymph nodes by the leptospires. The organisms quickly enter the bloodstream where they multiply and this process is accompanied by the developement of transient fever. Simultaneously, the bacteria start acting upon other organs and subsequent symptoms pertain to the affected organ.

Clinical Features

The clinical features of leptospirosis in human beings as well as in animals are extremely variable with fever being a common and consistent feature. Because of such presentation, it is considered as one of the possibilities in almost every case which presents with pyrexia of unknown origin (PUO).

After an incubation period of 2-17 days and a short prodromal phase disease may progress to cough, diarrhoea or myo-pericarditis. In severe cases jaundice and renal failure may set in within one week.

In Weil's disease all these features are present but are also supplemented by abdominal pain, nausea, and vomiting. Haemorrhages are seen in various organs and epistaxis may be the presenting feature.

Laboratory Diagnosis

There are three lines of investigations:

- Microscopic demonstration
- Isolation of bacterium
- Serological examination.

Microscopic Demonstration

Leptospires can be demonstrated from the infected material by dark field microscopy, silver impregnation methods and fluorescent antibody techniques.

Dark-field microscopy. Leptospires are routinely examined under a magnifiction of 100-600x with dark field microscopy. They are slender, averaging 0.05 to 0.1 micron in diameter and more tightly coiled. They have elongated structures with terminal segments that

may curve to resemble the letters 'C', 'S' and 'J' (Fig. 46.1).

Silver impregnation method. Tissue specimens obtained on autopsy can also be stained by silver impregnation staining technique to demonstrate the presence of leptospirae. This technique gives best results in paraffin tissue sections.

Fluorescent antibody technique. Demonstration of leptospirae in fluids and tissues by the fluorescent antibody technique is helpful in the diagnosis of the disease. This test is particularly useful in urinary shedders. By application of this method living, dead or even fragmented leptospirae can be demonstrated. This test carries a very high sensitivity and specificity.

Isolation

The strains of *Leptospira* can be isolated on laboratory media which may be solid, liquid or semisolid. *Liquid media* are extensively used in laboratories for the primary isolation of cultures and for maintaining strains. *Semisolid media* are prepared by the addition of 0.2-0.5 percent agar to the liquid medium. Leptospirae grow more rapidly and abundantly in semisolid media than liquid media. These media should preferably be used for the primary isolation. *Solid media* contain 0.8-1.3 per cent of agar have not yet found wide application for diagnostic purposes.

Several leptospirae media are commercially available. Some of them are Stuart's, Fletcher's, EMJH media, PLM-5 and Leptospirae medium 5x.

Blood culture. A suitable inoculum may be conserved for this purpose by mixing 5 ml of acute phase blood specimen with 1 ml of 1 percent liquid—an anticogulant that also counteracts the natural bactericidal action of the blood.

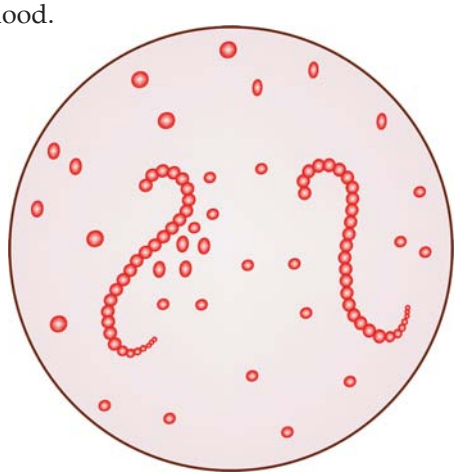


Fig. 46–1. Morphology of leptospires

Urine culture. Leptospirae appear in the urine from about 14th day onwards and the same method of examination as for blood is used.

Animal Pathogenicity

For isolation of leptospirae, blood and CSF are collected during acute illness for inoculation into experimental animals. Guinea pigs, hamsters, gerbils, etc. are very sensitive to infection with leptospirae and thus serve as good indicators for virulence and pathogenicity of the organisms. Animals develop rapidly fatal illness characterised by fever, haemorrhages and loss of weight. Death of the animal may occur within 4-7 days.

Serological Diagnosis

These tests have been classified into two groups:
a. Genus specific tests
b. Serogroup and serotype specific tests

Genus specific tests. These tests are based upon the use of a single antigen common for the genus *Leptospira*. Such tests distinguish leptospirae from other microbial causes of febrile illness. Genus specific tests include a sensitized erythrocyte lysis (SEL), a complement fixation test, a biflexa agglutination test and an indirect immunofluorescence test.

Serogroup Specific Tests

Microscopic agglutination test is a very sensitive and highly specific test for detecting acute infections and is useful for epidemiological screening. The test is applicable only in specialised laboratories where number of prevalent and important serovars are routinely maintained.

Macroscopic agglutination tests are performed on slides or plates. Formalin killed antigens are mixed with serum to be tested. This mixture of serum and antigen is allowed to react and then viewed with naked eye for presence or absence of agglutination.

Epidemiology and Control

Leptospirosis is amongst the most widespread of zoonotic diseases. Pathogenic leptospires survive for long periods in the convoluted tubules of the kidney in natural hosts (such as rodents), multiply and are shed in the urine. Animal carriers can excrete upto 100 million leptospires per ml of urine. The infected urine when contaminates the alkaline or neutral pH water or mud, the leptospires can survive for weeks. When people come in contact with this infected water, the

leptospires enter the body through abraded skin or mucosa and initiate infection Leptospires can also be shed in the milk of the lactating animal. They are not shed in the saliva. No vector-borne transmission takes place. Rats and dogs have been mainly incriminated as carriers. Human beings are dead end hosts. There is no evidence of human to human transmission. It is primarily a rural problem but has started affecting urban areas. Epidemics of jaundice due to leptospirosis following monsoons are frequently reported from the states of Gujarat, Maharashtra, Kerala, Karnataka, etc. Vaccine against selected serovars for humans has been tried. Treatment should be started at the earliest. The organism is sensitive to penicillin and tetracycline Doxycycline 200 mgm taken orally once a week is effective in prophylaxis.

47 Borrelia

The genus *Borrelia* contains the spirochaetes which cause relapsing fever in man (*Br.recurrentis*). It also includes *Br. vincenti* and *Br. buccale*, members of the symbiotic group of organisms which cause Vincent's angina and fuso-spirochaetal symbiotic disease.

Br. recurrentis is a delicate spiral thread which measures 8-20 µm in length and about 0.3 µm in thickness, with one or both ends pointed. It is actively motile and lashing movements may be observed in blood films from patients.

Cultivation

The borreliae are strict anaerobes. For primary isolation, the best method is to inoculate mice or rats intraperitoneally. Multiplication is rapid in chick embyro when blood from patients is injected on to the chorioallantoic membrane.

Antigenic Structure

The borreliae readily undergo antigenic variations *in vivo* and this is believed to be the reason for the occurrence of relapse in the disease.

Pathogenicity

All forms of relapsing fever are clinically identical. The incubation period varies from 2-10 days. The onset is sudden, with chills, fever, severe headache, muscular and joint pain, moderate enlargement and tenderness of spleen and commonly jaundice. The fever ends by crisis in 3 or 4 days. Successive relapses occur at intervals of 2-14 days, and the period of relapses varies from a few hours to longer than primary fever.

Laboratory Diagnosis

The diagnosis is made by demonstration of the spirochaete in the blood during onset of relapse by direct darkfield microscopy or animal inoculation.

Serological tests are now becoming popular and easily available. An indirect immunofluorescence test is available. ELISA is widely used and immunoblotting is used to confirm the diagnosis. Specific IgM antibodies develop within 3-6 weeks of infection.

White mice or young rats are inoculated intraperitoneally with blood. Stained films of tail blood are examined for spirochaetes 2-4 days later. Patients suffering from epidemic (louse-borne) relapsing fever may develop agglutinins for *Proteus* OXK and also a positive VDRL test. The morphology of *Br. recurrentis* in blood smear is depicted in Figure 47.1.

Tick Borne Infection

This represents transmission from animal reservoir of infection to man. The tick vector *Ornithodorous turicate* and *O. hermsi* are known to tansmit disease. Infection may persist for long periods in the tick. Furthermore the spirochaetes may be transmitted to the offspring of the tick.

Louse borne European Relapsing Fever

This is transmitted from man to man by the body louse *Pediculus vestimenti*. In contrast to tick-borne infection,

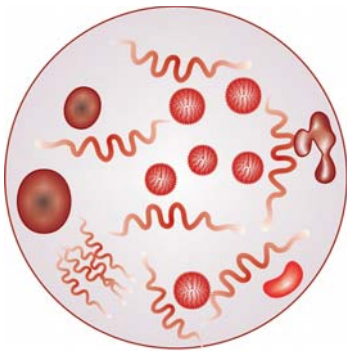


Fig. 47–1. Morphology of *Br.recurrentis*

in the louse borne infection, the bite is not infected by the secretions of the lice rather the infected louse must be crushed on the skin and the spirochaetes present in the body fluid of lice contaminate the bite.

BORRELIA VINCENTI

Morphology

Br. vincenti also called as *T. vincenti* is a motile spirochaete which is longer and coarser than the treponemes. It is about 17-18 µm long and 0.2-0.6 µm thick. There are 3-8 loose coils of very variable size. It is stained readily with weak carbol fuchsin and is gram-negative.

Cultivation

It is only cultivated with difficulty and requires an enriched medium and anaerobic conditions.

Normal Habitat

It is a normal mouth commensal. Along with fusiform bacillus it forms a symbiotic combination that is found inhabiting the healthy gums.

Pathogenesis

The number of these bacteria increases enormously when the resistance of the local tissues is reduced. This happens when tissue is damaged by trauma, deficiency of vitamins such as vitamin C and niacin or infections such as herpes simplex and infectious mononucleosis.

Clinical Features

It causes ulcerative gingivostomatitis or oropharyngitis (Vincent's angina). It is an opportunistic disease. It is also called as *fusospirochaetal disease* because fusiform bacilli *(Fusobacterium fusiforme)* is always associated with it.

Laboratory Diagnosis

In clinically suspected cases of Vincent's infection smears are made directly from the ulcerative lesions or from swabs and stained with dilute carbol-fuchsin. A clinical diagnosis is confirmed when very large number of both spirochaetes and barred fusiform bacilli are seen together with many pus cells as shown in Figure 47.2. Culture is not easy hence not of great diagnostic value.

LYME DISEASE

Lyme disease is caused by *Borrelia burgdorferi*, named following Burgdorfer's discovery of the aetiological agent.

Fig 47–2. Smear from Vincent's angina

Clinical Features

The most common sign of the initial phase of Lyme disease is the characteristic skin rash. The rash first appears as a red, raised area which occurs within several days or weeks after a tick bite. The circular or oblong rash may increase in size and develop well-defined margins with central clearing, giving it a characteristic "target" appearance. The rash may be itchy, warm, tender, and/or "doughy". In about one-half of all Lyme disease cases, the rash may be preceded or accompanied by fever, headache, fatigue, malaise, muscle and joint pain, and stiff neck. If not promptly treated, chronic arthritis, heart, and/or nervous system disorders may develop in some patients.

Mode of Transmission

Transmission of *Br. burgdorferi* from vertebrate to vertebrate depends on blood-feeding arthropods. Infected vertebrate hosts are lightly spirochaetemic also. The disease spreads by tick-bite.

The ticks involved are Deer tick *(Ixodes dammini)* and the Lone star tick *(Amblyomma americanum)*.

Like other pathogenic spirochaetes, it is probably transmissible via the placenta to the foetus.

Laboratory Diagnosis

Br. burgdorferi can be detected directly in tissue or body fluid, isolated in culture medium or identified in experimental animals inoculated with patient's specimens. Alternatively the aetiological agent can be used as the basis of a serologic test. The successful immunoassays used include IFA or ELISA, both incorporate either whole cells or a crude fraction of the spirochaetes as the test antigen. Specificity of the currently available assays is not high.

Treatment

The *in vitro* susceptibility studies have shown that this spirochaete is susceptible to penicillin, ampicillin, erythromycin, tetracycline, doxycycline, minocycline and chloramphenicol.

48 Mycoplasma

Mycoplasma were earlier known as pleuropneumonia like organism (PPLO) and Eaton agent.

Classification

The mycoplasmas belong to the class Mollicutes which literally means *soft skin* in reference to lack of rigid cell wall in this organism (Fig. 48.1).

The ureaplasma was previously also known as T-mycoplasma because of the *tiny* colonies formed by them as compared to those of mycoplasma and acholeplasma.

Though mycoplasmas have some similarities in colony characters with L forms, these differ from them (Table 48.1).

Table 48–1. Comparison between mycoplasmas and L-forms

Property	*Mycoplasma*	*L-forms*
Reversion to cell-wall containing forms	–	+
Require high salt medium to maintain integrity	–	+
Sterols in plasma membrane	+++	–
Inhibition of reproduction by penicillin	–	+

Morphology

Mycoplasmas are gram-negative bacteria but stain poorly with this technique. Giemsa staining done overnight gives better results. Under light microscope these bacteria demonstrate pleomorphism and show various morphologies. Locomotion in mycoplasmas is 'gliding' in nature. The mycoplasma multiply by binary fission.

Mycoplasmas are facultatively anaerobic and grow best in an environment of 95% nitrogen and 5% carbon dioxide.

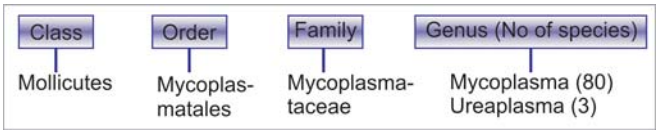


Fig. 48–1. Classification of mycoplasmas

Colony Characters

M.pneumoniae usually produces colonies without a translucent peripheral zone that are mullberry-like in appearance. Some of the mycoplasmas from animals may produce colonies upto 2 mm in diameter whereas those of ureaplasmas may be as small as 15-60 µm.

Most of the colonies are umbonate in appearance and under transmitted light, these give typical **fried egg** appearance representing an opaque central zone of growth deep in agar and a translucent periphery on the surface (Fig. 48.2).

Staining of colonies. The most generally used is Diene's stain and Giemsa method. While the former gives purple violet stain to colonies, the latter stains them red-purple.

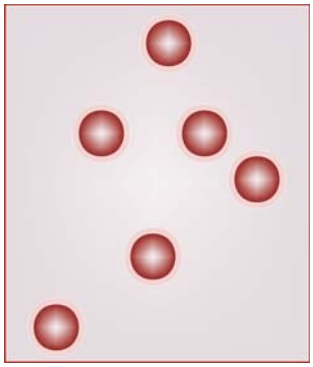


Fig. 48–2. Fried egg appearance of colonies of mycoplasma

Certain distinctive features of mycoplasmas have been shown in Table 48.2.

Table 48–2. Distinctive features of mycoplasmas

- Smallest free living organisms that can grow on artificial media
- Highly pleomorphic because of absence of cell wall
- Bounded by membrane, containing sterol
- Resistant to penicillin
- Can reproduce in cell free media
- Growth inhibited by specific antibody
- Do not revert to forms with rigid cell wall
- Have an affinity for mammalian cell membrane
- Require sterol for growth in artificial media
- Resistant to thallium in concentrations at which other bacteria are inhibited

Sensitivity to Physical and Chemical Agents

Mycoplasmas cannot withstand temperature of 56ºC for more than 30 minutes. These survive for six weeks at 37ºC and for three months at 0ºC.

Clinical Features

The best known human disease caused by mycoplasmas is called **primary atypical pneumonia** and the causative microbe is *M.pneumoniae*.

The **extrapulmonary manifestations** of *M. pneumoniae* infections include myocarditis, pericarditis, pancreatitis, tubo-ovarian abscess, haemolytic anaemia, thrombocytopenia, intravascular coagulation, myalgia, arthralgia, meningitis and meningoencephalitis.

Ureaplasmas are strongly incriminated as causing nongonococcal urethritis (NGU). The urethral syndrome in women is also caused by ureaplasmas.

In virology laboratories many mycoplasmas cause contamination of cell lines.

Laboratory Diagnosis

The **specimens** from which mycoplasmas can be isolated include throat swab, sputum, inflammatory exudates and respiratory, urethral or genital secretions.

Direct examination of smear by staining does not yield any diagnostic information. The clinical sample is to be **cultured** on media and incubated at 37ºC in microaerophillic environment with 5% CO_2. Prolonged incubation or subcultures are required before the growth becomes suitable for staining or immunofluorescence examination. Colonies yield typical fried egg appearance after incubation of 48-72 hours.

The identity can be confirmed by the following two methods:

Immunofluorescence on Slides

Colonies from a solid medium are transferred onto chemically treated slides and subjected to reaction with fluorescein conjugated antimycoplasma antiserum.

Serology

The development of antibody in humans to mycoplasmas can be measured by a range of techniques of widely differing sensitivity and specificity (Table 48.3).

For all these tests a rising titre of antibody is to be demonstrated to have any diagnostic significance because of the high incidence of positive serologic tests in normal population. *M.pneumoniae* and *M.genitalium* are serologically cross-reactive.

A nonspecific reaction in the form of **c**old agglutination carries historical importance for serological diagnosis of mycoplasma infections. In almost 50% of patients with infection due to mycoplasmas agglutinins develop which will agglutinate erythrocytes of their own group or O group at 4ºC. Some people produce antibodies in response to mycoplasma infections which can agglutinate strain of *Streptococcus* MG. This reaction may be due to similarities between glycolipid haptens in the mycoplasma membrane and carbohydrate determinants in the streptococcal cell wall.

Table 48–3. Serological tests for mycoplasmas

Serological test	*Sensitivity*	*Specificity*
Latex agglutination	++	+
Complement fixation test	++	+
Immunofluorescence	++	+++
Microimmunofluorescence	+++	+++
Passive haemagglutination	+++	++
ELISA	+++	+++

Epidemiology and Control

Mycoplasma pneumoniae causing pneumonia is an established pathogen while *M. hominis* and *Ureplasma urealyticum* are presumed pathogens. There are many non-pathogenic and saprophytic speceis. Mycoplasmas tend to cause more severe and prolonged infections in the HIV infected and other immunodeficient-subjects. Mycoplasmas are also a nuisance as cell culture contaminants. Eradication of mycoplasmas from the infected cells in difficult. Mycoplasmas are susceptible to tetracyclines, doxycycline, newer macrolides and quinolones.

49 Chlamydia

The chlamydiae have been known, till recent past, as *Bedsonia*, the PLT (psittacosis-lymphogranuloma-trachoma) group as well as TRIC (trachoma and inclusion conjunctivitis) agents.

Classification

These organisms belong to the order *Chlamydiale* which contains one family *Chlamydiaceae*. The family comprises of one genus *Chlamydia* in which three species have been recognised. These are *C.trachomatis*, *C.psittaci* and *C.pneumoniae*. The third species (*C.pneumoniae*) has been recognised in 1989 only. It comprises of the strains which were earlier designated as TWAR strains. The acronym TWAR was derived from initial letters of the designation of early isolates from Taiwan (TW) and acute respiratory (AR) infection. The important distinguishing features for these two species are shown in Table 49.1.

Table 49–1. Differences between *C.trachomatis* and *C.psittaci*

Character	*C.trachomatis*	*C.psittaci*
G+C content of DNA	45 mol%	41 mol%
Inclusion body	Single	Multiple
Glycogen in inclusion	+	–
Resistance to sulpha	No	Yes
Infections caused	Localised	Systemic
Predominant host	Man	Birds

Morphology

Chlamydiae exist in two forms known as reticulate body (RB) which was earlier known as initial body and the elementary body (EB). EBs are derived from RB by a process of fission. The RB is the noninfective vegetative form which is rich in ribosomes. The essential differences between EB and RB have been shown in Table 49.2.

Table 49–2. Differences between EB and RB

Character	*EB*	*RB*
Morphology	Small, dense centred	Large and homogenous
RNA:DNA	1:1	3:1
Sonication	Resistant	Sensitive
Resistance to trypsinization	+	–
Infectivity	+	–
Toxicity	+	–
Haemagglutinin	Present	Absent
Permeability	Slight	Marked
Envelope	Present	Absent
Location	Extracellular	Intracellular

EBs are round particles with a diameter ranging between 200 and 300 nm. These are rich in DNA and the cell wall resembles that of gram-negative bacteria. Accordingly the chlamydiae are gram-negative. The reticulate body of *C.trachomatis* gives basophillic appearance when stained with Giemsa. On staining with acridine orange dye, RB appears orange and EB yellow green under UV rays. Mature inclusions of *C.trachomatis*, because of the presence of glycogen, stain coppery-brown with iodine. Inclusions of *C.psittaci* do not stain with iodine.

A helical projection protrudes from the surface of the elementary body through a flower-like structure (Fig. 49.1). Within the cell it is attached to the cytoplasmic membrane and the strands of DNA appear to connect to this adhesion point.

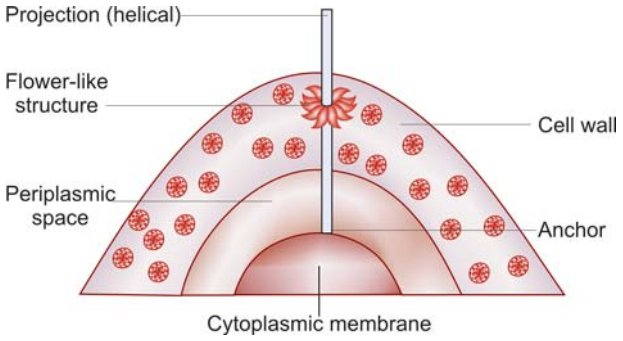


Fig. 49–1. Projection from the surface of EB

Replication

Chlamydiae go through a unique developmental cycle inside the intracytoplasmic vacuole of the host cell (Fig. 49.2). The elementary body is infectious, metabolically inert and enters the host cell by phagocytosis. The replication occurs in the phagosome, which becomes enlarged and is called as vacuole. Before replication can occur EB changes into a large structure of diameter (0.7-1.0 µm) that is called reticulate body. The reticulate body is not infectious, metabolically active, survives only intracytoplasmically and is the replicative form of chlamydiae. It divides by binary fission to fill the vacuole with new particles. These next change into smaller elementary bodies that are not able to multiply.

The growth of chlamydiae may cause the death and breaking up of the host cell that results in the release of elementary bodies. The released elementary bodies enter new host cells and the cycle is repeated. Each such cycle takes around 48 hours.

Antigens

C.trachomatis has been divided into 15 serotypes. These are A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2 and L3.

Table 49–3. Diseases caused by chlamydiae

Chlamydia	*Host*	*Disease in humans*
C.psittaci	Birds	Psittacosis Ornithosis
C.pneumoniae (TWAR)	Human	Acute respiratory disease
C.trachomatis serogroups:		
A,B,Ba,C	Humans	Trachoma
L1,L2 and L3	Humans	Lymphogranuloma venereum
D, E, F, G, H, I, J, K	Human	Oculogenital diseases
Adult male	*Adult female*	*Infants*
Conjunctivitis	Conjunctivitis	Conjunctivitis
Urethritis (NGU)*	Cervicitis	Pneumonia
Epididymitis	Salpingitis	Bronchiolitis
Arthritis (Reiter's syndrome)(NGU)	Urethral syndrome	
	Postpartum endometritis	

*NGU: non-gonococcal urethritis

Their association with various human diseases has been well established (Table 49.3).

Pathogenesis and Immunity

Chlamydiae do not produce demonstrable exotoxin. The cell envelope of these organisms, though similar to other gram-negative bacteria does not possess endotoxic activities. But they do have several properties of significance in pathogenesis, one of which is toxicity of live elementary body preparation.

Hypersensitivity has also been incriminated as playing an important role in influencing clinical severity of infection. Individuals who had prior exposure to chlamydial antigen, on reinfection show severe clinical symptoms.

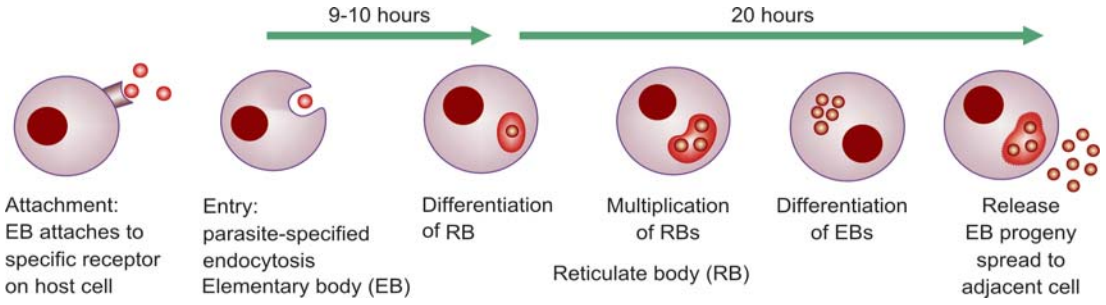


Fig. 49–2. Replication of chlamydiae

PART VII

Diseases Caused by Chlamydiae

A variety of diseases are caused by different serotypes of *Chlamydia*. These have been depicted in Table 49.3.

PSITTACOSIS

Psittacosis is a zoonotic disease. Large number of birds and animals are infected by this organism. The name **psittacosis** was given because in the beginning this disease was seen only in the birds belonging to the family Psittacine. It is now also known as **ornithosis** because many birds other than those of family Psittacine are also infected by these microbes. Many domestic animals also get infected with *C.psittaci* and act as reservoir for disease in human beings.

Birds usually develop diarrhoea and excrete bacteria in their faeces. Persons who come in close contact with the birds stand the greatest risk of being infected. Exposure is usually by airborne route via contaminated dust.

TWAR strains of this species, which have now been grouped into a new species of *C.pneumoniae* cause pneumonia and pharyngitis in human beings but not in animals. It is believed that this may be a human strain that can spread from man to man without animal contact.

TRACHOMA

Trachoma ranks among the major infectious disease problems of mankind and is an infection of the conjunctiva (conjunctivitis) and in some cases that of cornea (keratitis). Cases of varying degrees of severity appear and range from asymptomatic carriers to those showing extensive scarring of the cornea with resultant blinding.

LYMPHOGRANULOMA VENEREUM

Lymphogranuloma venereum (LGV) is caused by three serogroups (L1, L2 and L3) of *C.trachomatis* and is transmitted through sexual contact. A variety of nonspecific symptoms may be experienced in the early stage of illness with lesions on the skin and mucous membrane of the genital organs. This is followed by characteristic large lymph nodes in inguinal area which may suppurate. Patients often have fever, nausea, headache and conjunctivitis or skin rash. If untreated, disease can lead to permanent obstruction of the lymphatic or rectal stricture.

Many other conditions are caused by different serotypes of chlamydiae. In many cases these infections remain asymptomatic while only mild symptoms are manifested which remain undiagnosed. One of these conditions is nonspecific urethritis (NSU) which is also known as nongonococcal urethritis (NGU). This is the most common sexually transmitted condition. Infection spreads to other reproductive organs in both males and females and may cause infertility and sterility.

Through an infected cervix newborn also acquires this infection and may present with pneumonitis or conjunctivitis. Inclusion conjunctivitis may also occur in adults who acquire the infection from contaminated towels, fingers and similar items.

Laboratory Diagnosis of Chlamydial Infections

For cytologic studies, impression smears of involved tissues or scrapings of involved epithelial sites should be appropriately fixed (cold acetone for immunofluorescence and methanol for Giemsa stain). For isolation studies samples are to be collected from affected sites by vigorous swabbing or scraping. These can be transported in chlamydial transport medium with aminoglycosides and fungicides.

Direct Cytologic Examination

C.trachomatis infections of the conjunctiva, urethra or cervix can be diagnosed by demonstrating typical intracytoplasmic inclusion bodies (Halberstaedter-Prowazek body-Fig. 49.3). The cytologic procedures are usually less sensitive than isolation in tissue culture. For the detection of these inclusion bodies, fluorescent antibody, iodine staining and Giemsa staining techniques are employed.

Fluorescent antibody technique can be employed either in direct or indirect methods. Using monoclonal antibody the sensitivity and specificity of the technique have increased manifold. The procedure is somewhat less sensitive than culture but it is faster and far less expensive.

Iodine staining technique requires air-drying of scrapings. The smear is then fixed with absolute metha-

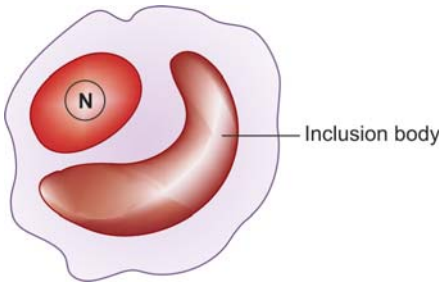


Fig. 49–3. Halberstaedter-Prowazek body

Table 49–4. Detection of Chlamydia inclusions

Method	*Appearance*	*Advantages*
Giemsa stain	*Brightfield*: Reddish purple nucleus	Simple to perform Permanent preparation Bluish purple cytoplasm Differentiates EB from RB
	Dark-field: EB and RB stain bright golden-yellow	
Iodine stain	Dark to reddish brown inclusions, cells yellow	Rapid and simple Easy to read Methanol decolorizes for restaining with Giemsa or IF
Immuno-fluorescence	Bright yellow inclusion within cytoplasm	Sensitive, rapid and confirmatory

nol and stained with Lugol's iodine for 3-5 minutes. Slides are examined as wet mounts. Though it is the least sensitive technique, its simplicity and speed have made it popular for examination of *C.trachomatis* infected tissue culture.

Giemsa staining technique employs Giemsa stain for one hour over the air-dried and absolute methanol-fixed smear. The slide is then rinsed rapidly in 95% ethyl alcohol and examined microscopically. EB stains reddish purple whereas RB being more basophilic stains bluish.

The appearance of chlamydial inclusions by different stains and the advantages of various staining techniques have been summarised in Table 49.4.

Enzyme immunoassays are now available to detect antigen of chlamydiae from clinical specimens.

Isolation of Organism

Chlamydiae can be isolated in tissue culture as well as chick embryos. The most common technique involves inoculation of clinical specimens into cycloheximide-treated McCoy cells. The infected cells are stained with iodine to detect the glycogen-positive inclusions. Fluorescent antibody may allow earlier detection of inclusion.

For the identification of the organisms fluorescent antibody technique is employed.

For isolation of chlamydiae from clinical material, yolk sac inoculation has been superceded by cell culture methods, but because of the high yield it is still sometimes used for preparing suspensions for chemical or antigenic analysis. Chlamydiae grow well in the yolk sac of 6-8 day embryos, which die from about 4 days onwards after inoculation. Impression smears are prepared from the endothelial cells and organisms can be detected by Giemsa's stain. Some chlamydiae produce small pock like lesions on the chorioallantoic membrane and propagation in the allantoic cavity has been employed to obtain pure suspension of the adapted organisms.

Serodiagnosis

The most widely used serologic test for diagnosing chlamydial infections is the complement fixation test. It is useful in diagnosing psittacosis, in which paired sera often show fourfold or greater increase in titre. It is also useful in diagnosis of LGV wherein single raised titre of 1: 64 or more may be diagnostic. The micro-immunofluorescence (micro-IF) method is a more sensitive and rapid method for detection of anti-chlamydial antibodies. Antibody-capture enzyme immunoassays are now available which are easy to perform, sensitive and rapid methods for the detection of antichlamydial antibodies. Serology has been found to be of great use in diagnosis of chlamydial pneumonia in neonates.

Treatment

For the treatment of trachoma, tetracycline given topically as well as orally is the drug of choice. An alternative drug especially in younger children in whom tetracycline may cause harm is erythromycin.

50 Rickettsia

Rickettsiae are named after Howard T Ricketts, who identified them as causative agents of typhus and Rocky Mountain spotted fever.

The family rickettsiaceae consists of microorganisms that cause diseases which fall mainly in four major groups namely *typhus fevers, spotted fevers*, *Q-fever* and *trench fever*. Because of their obligate intracellular parasitism, they were thought to be close to viruses, but they are true bacteria. Salient features of rickettsiae are summarised in Table 50.1.

Table 50–1. Salient features of rickettsiae

- Highly fastidious
- Obligate intracellular parasites except *Rochalimaea quintana*
- Pleomorphic, usually seen as rods and in pairs
- Most species are gram-negative *except C.burneti* which is gram-positive
- Possess multilayered limiting membrane
- Limiting membrane digested by lysozyme
- Cell wall contains muramic acid
- Some organisms are capsulated
- Possess both RNA and DNA
- Multiply by binary fission
- Cannot pass through filters which hold conventional bacteria
- Susceptible to antibiotic therapy
- Primarily parasitic in arthropods

They are thus true bacteria, specially adapted to obligate intracellular parasitism. Rickettsiae appear as pleomorphic coccobacillary forms. They vary in length from 0.25 to 2 µm. These are sometimes observed in pairs or chains or filaments. *C.burneti* is the smallest, while the spotted fever rickettsiae are the largest. The organisms are found within the nuclei or cytoplasm. They are non-motile and non capsulated, are by and large gram-negative though they do not take the stain well.

Cultivation

Rickettsiae do not grow in cell free media with the exception of *Rochalimaea quintana* which can grow on blood agar. They are readily cultivated in the yolk sac of developing chick embryo. They also grow on continuous cell lines such as Hela, Hep2, Detroit 6; etc. but the growth is not satisfactory. Laboratory animals such as guinea pigs and mice are useful for the isolation of rickettsiae from patients.

Growth in Animals and Eggs

Male guinea pigs or the mice are used for primary isolation of the rickettsiae, where the typhus rickettsiae can be maintained without change in their antigenic structure or pathogenicity. Scrub typhus rickettsiae have been grown in the anterior chamber of the rabbit eye. The cultivation of the rickettsiae in the fertile hen's egg occurs in the chorio allantoic membrane but method of yolk sac inoculation has been more widely used. Rickettsiae can also be propagated in arthropods.

Susceptibility to Physical and Chemical Agents

Rickettsiae are readily inactivated by physical and chemical agents. In general, rickettsiae are quickly destroyed by heat, drying and bactericidal chemicals. Although rickettsiae are usually killed by storage at room temperature, dried faeces of infected lice may remain infective for months at room temperature. The organism of Q-fever is most resistant to drying.

Antigenic Structure

Three types of antigens have been demonstrated.

Group specific soluble antigen is present on the surface of the rickettsiae.

Species specific antigen is associated with the body of the organism . In the case of scrub typhus it is strain specific.

Polysaccharide antigen is the third antigen.It is an alkali stable polysaccharide found in some rickettsiae and in some strains of *Proteus* bacilli. This sharing of antigens between rickettsiae and *Proteus* is the basis for the Weil-Felix reaction, which is used for diagnosis of rickettsial infections by demonstration of agglutinins of *Proteus* strains OX19, OX2 and OXK.

Pathogenesis

Rickettsial diseases develop after infection through skin or respiratory tract. Ticks and mites transmit the agents of spotted fever and scrub typhus by inoculating the rickettsia directly into the dermis during feeding. The louse and flea, which transmit epidemic and murine typhus respectively, deposit infected faeces on the skin and the infection occurs when organisms are rubbed into the puncture wound made by the arthropod. The rickettsiae of Q-fever gain entry through the respiratory tract when infected dust is inhaled. Although organisms probably multiply at the original site of entry in all instances, local lesions appear with regularity only in certain diseases such as scrub typhus, etc. After multiplication at the local site these enter the blood stream. Rickettsiae multiply in endothelial cells of small blood vessels and produce vasculitis. The cells become swollen and necrotic, there is thrombosis of the vessel leading to rupture and necrosis. Vascular lesions are prominent in the skin but vasculitis occurs in many organs such as muscles, heart, lung and brain (Fig. 50.1). In the brain, aggregations of lymphocytes, polymorphonuclear leucocytes and macrophages are associated with the blood vessels of the grey matter, these are called typhus nodules.

Classification

Rickettsiae are classified into 4 genera based on the antigenic relationship, vector of transmission, growth properties, and resistance to physical and chemical agents. These genera are *Rickettsia, Rochalimaea, Ehrlichia* and *Coxiella.*

a. The genus *Rickettsia* contains agents causing typhus fevers, spotted fevers and scrub typhus.
b. *Rochalimaea* (*Ro. quintana*) causes trench fever. This genus has two important properties of being usually extracellular in the arthropod host and its cultivability on cell free media such as blood agar.
c. The genus *Coxiella* (named after Cox who first isolated it) causes Q-fever. It differs from other rickettsial agents by being smaller in size, not transmitted by arthropods and more resistant to physical and chemical agents.

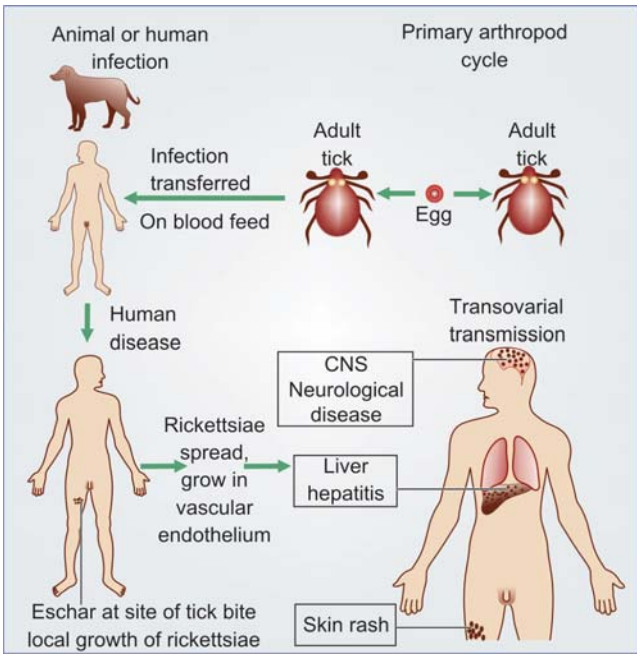


Fig. 50–1. Pathogenesis of rickettsiosis

The classification of important rickettsial diseases of man is given in Table 50.2.

Table 50–2. Rickettsial diseases of man

Disease	*Organism*	*Insect vector*	*Animal reservoir*
Spotted fever group			
RMSF*	*R.rickettsi*	Tick	Wild rodents, dogs
Boutonneuse fever	*R.conori*	Tick	Wild rodents, dogs
Rickettsial-pox	*R.akari*	Mite	Mouse
Typhus group			
Epidemic typhus	*R.prowazeki*	Body louse	Man
Brill Zinsser Disease	*R.prowazeki*	None	Man
Murine typhus	*R.typhi*	Flea	Small rodents
Scrub typhus	*R.tsutsugamushi*	Mice	Wild rodents
Trench fever	*Ro. quintana*	Louse	Man
Q-fever	*C.burneti*	?Ticks	Cattle,sheep, goat
Ehrlichia	*E. sennetsu*	Japan	?
	E. canis	USA	

*RMSF: Rocky Mountain Spotted Fever

Clinical Features

Except for Q-fever in which there is no skin lesion, rickettsial infections are characterized by fever, headache, malaise, prostration, skin rash and enlargement

of spleen and liver. Q-fever resembles influenza and primary atypical pneumonia. In epidemic typhus the rash is maculo-papular and appears on the fourth or fifth day of illness. Endemic typhus presents same clinical picture but in a mild way. An important feature of scrub typhus is the presence of punched out ulcer (eschar) covered with a blackened scab which indicates the site of bite. The spotted fever group resembles typhus fever clinically.

EPIDEMIC TYPHUS

Louse-borne typhus is the prototype of the typhus group of rickettsial diseases. The primary illness and its recrudescent form (Brill-Zinsser's disease) is caused by *Rickettsia prowazeki*. Louse-borne typhus is also known as epidemic typhus, classic typhus, typhus exanthematicus, and jail fever.

Pathogenesis and Pathology

Following proliferation locally at the site of the louse bite, the organism presumably spreads haematogenously. *R.prowazeki*, like most rickettsiae, produces a vasculitis by infecting the endothelial cells of capillaries, small arteries, and veins. The process results in fibrin and platelet deposition, followed by occlusion of the vessel. Perivascular infiltration with lymphocytes, plasma cells, histiocytes, and polymorphonuclear leukocytes occurs with or without frank necrosis of the vessel. The angitis is most marked in the skin, heart, central nervous system, skeletal muscles and kidneys. In local thrombosis, extensive gangrene of skin and/or distal extremities occurs.

Clinical Features

Following an incubation period of approximately one week, an abrupt onset with intense headache, chills, fever, and myalgia is characteristic. There is no eschar. The fever worsens quickly (102-104°F), becomes unremitting, and the patient is soon prostrated by the illness. A rash begins in the axillary folds and upper trunk on about the fifth day of illness. Within several days, the rash becomes maculopapular, darker, petechial, fixed and confluent and involves the entire body, sparing the face, palms, and soles.

Treatment

The antibiotics-chloramphenicol and tetracycline, are both effective against louse-borne typhus.

Prevention

Control of the human body louse and the conditions that foster its proliferation are the mainstay of prevention of louse-borne typhus.

BRILL-ZINSSER DISEASE

Brill-Zinsser disease occurs as a recrudescence of a previous infection with *R. prowazeki*. Its pathogenesis is unknown, but recurrence is presumed to be precipitated by stress or a waning immune system. The illness is similar to louse-borne typhus but is usually milder and more closely resembles murine typhus.

ENDEMIC OR MURINE TYPHUS

The aetiologic agent of murine typhus is *Rickettsia typhi* (formerly *R. mooseri)*, an obligate intracellular bacterium that shares common soluble antigens with *R. prowazeki*.

Clinical Features

After an incubation period of 1 to 2 weeks, the illness is characterized by headache, myalgia and fever. Onset is variable being gradual less often sudden. There is no eschar. Frequently a nonproductive cough occurs early in the course. Rash occurs in 60-80 per cent of the patients and first becomes evident on the third to fifth day of illness. The rash of murine typhus becomes maculopapular, and remains for 4-8 days. The rash may vary greatly in duration and intensity.

Treatment

Therapy with tetracycline or chloramphenicol produces defervescence in 2 days.

SCRUB TYPHUS

Scrub typhus is a mite-borne illness characterized by fever, headache, rash, and an eschar at the site of the inoculating chigger bite. It is endemic in southern and eastern Asia, northern Australia, and the Western Pacific Islands.

Aetiologic Agent

Scrub typhus is caused by *Rickettsia tsutsugamushi* which is an obligate, intracellular bacterium seen best with the Giemsa stain in infected tissues.

Antibodies against Weil-Felix OX-K but not OX-19 or OX-2 strains of *Proteus* are found in about 50 per cent of the patients with the disease.

Pathogenesis and Pathology

The infected chigger, while feeding, deposits rickettsiae into the tissue of the human host. Multiplication takes place locally resulting in a cutaneous multiloculated vesicle which eventually ulcerates and forms a characteristic eschar with a black crust. A rickettsemia has been demonstrated late in the incubation period. Endothelial cells are infected during the haematogenous spread of the organism, and perivascular inflammation with mononuclear cells develops. Lymphadenopathy and splenomegaly are common. Severe illness has been associated with pneumonia and myocarditis.

Clinical Manifestations

Six to 20 days after the infecting chigger bite, the patient suddenly becomes ill with fever, chill, headache, and myalgia. Although usually on the extremities, the eschar may be found in other areas, particularly where skin surfaces come together, e.g. the axilla and scrotum.

If untreated, the fever will persist for 2 weeks or more and, among survivors, may fall gradually over several days. Complications leading to death include pneumonia and circulatory collapse. Convalescence may be prolonged.

Treatment

Scrub typhus is even more responsive to tetracycline and chloramphenicol than the other rickettsiosis, patients becoming afebrile within 24-36 hours of starting therapy.

TRENCH FEVER (Quintana Fever)

Trench fever is a nonfatal, febrile, bacterial disease varying in manifestations and severity. It is characterized by headache, malaise, pain and tenderness, especially on the shins; onset is either sudden or slow, with a fever which may be relapsing, typhoid-like, or limited to a single febrile episode lasting for several days. Splenomegaly is common, and a transient macular rash may occur. Causative agent is *Rochalimaea quintana (Rickettsia quintana).*

Reservoir is man. The intermediate host is the body louse, *Pediculus humanus.* The organism multiplies extracellularly in the gut lumen for the duration of the insect's life, which is approximately 5 weeks after hatching. No transovarian transmission occurs.

The disease is not directly transmitted from person to person. Man is infected by inoculation of the organism in louse faeces through a break in the skin, either from the bite of the louse or other means. Infected lice begin to excrete infectious faeces 5-12 days after ingesting infective blood and continue for the rest of their life-span. Nymphal stages may become infected. The disease spreads when lice leave abnormally hot (febrile) or cold (dead) bodies in search of a normothermic clothed body. Incubation period is generally 7-30 days. Organism may circulate in the blood (by which lice are infected) for weeks, months or years and may recur with or without symptoms. A history of trench fever is a permanent contraindication to blood donation.

ROCKY MOUNTAIN SPOTTED FEVER

(New World spotted fever, Tick-borne typhus fever, Sao Paulo fever)

This prototype disease of the spotted fever group is characterized by sudden onset with moderate to high fever, which ordinarily persists for 2-3 weeks in the untreated cases, significant malaise, deep muscle pain, severe headache, chills and conjunctival injection. In about half the cases, a maculopapular rash appears on the extremities on about the third day; this soon includes the palms and soles and spreads rapidly to much of the body; petechiae and haemorrhages are common. Absence or delayed appearance of the typical rash contributes to delay in diagnosis and increased fatality.

This clinical syndrome, especially early Rocky Mountain Spotted Fever (RMSF), may be confused with meningococcemia and enteroviral infection. Causative agent is *Rickettsia rickettsi*.

The disease is transmitted ordinarily by bite of an infected tick. Several hours (4-6) of attachment of the tick and feeding on blood are required before the rickettsiae become reactivated to become infectious for man. Contamination of skin with crushed tissues or faeces of the tick may also cause infection.

Incubation period ranges from 3 to about 14 days. The disease is not directly transmitted from person to person. The tick remains infective for life, commonly as long as 18 months. One attack probably confers lasting immunity.

Q-FEVER

An outbreak of a febrile disease in animals in Australia in 1937 due to an unknown cause prompted Derrick to name it as **Q-fever** (Q:query). Since then cases of this disease have been reported from more than 50 countries.

Q-fever is an acute illness caused by *Coxiella burneti* and is characterized by sudden onset of fever, chills, headache, myalgia,and often pneumonitis. *C. burneti* is an unusual rickettsia in that its antigens do not elicit antibodies to the X strain of *Proteus vulgaris* and in that it is highly resistant to drying and other physical assaults.Moreover there is no arthropod involved in transmission to man, and the route of infection is characteristically inhalation rather than cutaneous inoculation. Finally, unique among the rickettsiae, *C. burneti* does not cause a rash in man.

Aetiologic Agent

Coxiella burneti is a pleomorphic, obligate intracellular bacterium which is seen with Gimenez or Giemsa stains. Although rickettsia-like, because of the differences noted above, it has been placed in a separate genus, *Coxiella*. It multiplies in cytoplasmic vacuoles of endothelial and serosal cells. It can be grown in yolk sacs and injection into rats, mice, and guinea pigs elicits serum antibody responses.

Clinical Features

Q-fever commonly manifests as an acute febrile systemic illness or, rarely, as a subacute endocarditis. The acute form occurs after an incubation period of about 20 days (range 14-28 days) and typically is of sudden onset with temperature elevations up to 104°F, shaking chills, drenching sweats, myalgia, and anorexia. A severe frontal headache is usually present.

Although often considered to be a pulmonary infection, Q-fever is more accurately termed a systemic infection that involves the pulmonary parenchyma in some instances.

Hepatitis is commonly present, evident by liver function abnormalities or by hepatic enlargement but usually not severe enough to cause clinical jaundice.

The untreated febrile illness typically persists for 5-14 days. The duration of the febrile illness increases with age. Convalescence may be long, even lasting several months. Occasionally recurrence or relapse occurs. Even in untreated cases, mortality is low.

Treatment

Tetracyclines and chloramphenicol have proven effective as rickettsiostatic agents. Penicillin, erythromycin, and streptomycin have been ineffective.

LABORATORY DIAGNOSIS OF RICKETTSIAL DISEASES

Rickettsial disease may be diagnosed in the laboratory either by isolation of the rickettsiae or be serology.

Isolation

The difficulties and dangers of working with live pathogenic rickettsiae are great enough to make it impossible for many laboratories to even attempt it. Since it can cause fatal infections in laboratory workers, the isolation should be attempted with utmost care and in laboratories equipped with appropriate safety provisions.

Rickettsiae may be isolated by injecting whole blood or emulsified blood clot in guinea pigs, mice or eggs. Rickettsiae are recovered most frequently from blood drawn soon after onset. If the guinea pigs fail to show disease (fever, scrotal swelling, haemorrhagic necrosis, death) serum is collected for antibody test to determine if the animal has had an inapparent infection. Some rickettsiae can infect mice and rickettsiae are seen in smears of peritoneal exudate.

Rickettsia rickettsi, the agent of spotted fevers forms characteristic plaques in monolayers of primary chicken embryo cells or Vero monkey cells within 6-8 days after inoculation. *Rickettsia typhi* and *R. prowazeki* plaques appear at 8-11 days and *C. burneti* plaques appear at 12-14 days after inoculation.

Demonstration of antigen. It can be demonstrated in the tissues by *immunofluorescence* technique. The test carries almost cent percent specificity and 70% sensitivity.

Serological Diagnosis

Laboratory diagnosis in most cases depends upon demonstration of specific antibodies in serum specimens and an increase in the antibody titres as the disease progresses. If possible 3 samples of blood should be taken from the patient, one as soon as possible after onset of illness, one during second week of illness and another during fourth week.

The single most important diagnostic criterion in the identification of rickettsial diseases is the demonstration of a rise in serum antibody when paired acute and convalescent phase sera are titrated simultaneously in the same test. A significant rise in titre is defined as a four fold increase. The initial antibody response to rickettsial disease typically is IgM or a mixed IgM-IgG type. Thus for early diagnosis a test which can readily detect IgM antibody is a test of choice.

Various serological tests used for the diagnosis of rickettsial diseases are summarised in Table 50.3.

Weil-Felix Reaction

Principle. The Weil-Felix reaction is an agglutination test in which sera are tested for agglutinins to the 'O'

Table 50–3. Serological tests in rickettsial diagnosis

- Complement fixation test
- Immunofluorescence
- Weil-Felix reaction
- Agglutination
- Micro-agglutination
- Indirect haemagglutination
- Latex agglutination
- ELISA
- Immunoperoxidase
- Radio-isotope precipitation
- Toxin neutralization test

Table 50–4. Weil-Felix reaction in rickettsial diseases

Disease	*OX-19*	*OX-2*	*OX-K*
Epidemic typhus	++++	+	0
Murine typhus	++++	+	0
Brill-Zinsser disease	Variable	0	0
Scrub typhus	0	0	++++
Spotted fever group	++++/+	+/++++	0/0
Q fever	0	0	0
Rickettsial pox	0	0	0
Trench fever	0	0	0

antigens of certain non-motile *Proteus* strains OX-19, OX-2 and OX-K.

The test was developed from the chance finding of Weil and Felix that a *Proteus* strain isolated from the urine of a patient of epidemic typhus was agglutinated by the patient serum as well as by the sera of the other typhus patients.

The basis of the test is the sharing of an alkali stable carbohydrate antigen by some rickettsiae and by certain strains of *Proteus* (*Proteus vulgaris* OX-19, OX-2 and *Proteus mirabilis* OX-K).

Method. The test is done as macroscopic agglutination in tubes mixing the antigen with dilutions of serum. Serum dilutions of 1:10 to 1:640 are made to which equal amounts of antigens are added. Positive and negative controls are always run with each test. The tubes are incubated in a water bath at 37°C for 2 hours followed by incubation overnight at 4°C. Complete agglutination is shown by complete clearing of the supernatant fluid and the formation of smaller masses of agglutinated antigen in the bottom of the tube. The test can also be performed in microtitre plates.

Results. If properly performed, Weil -Felix test is capable of establishing useful presumptive diagnosis in diseases caused by the typhus and spotted fever groups of rickettsiae. The summary of usual results encountered is given in Table 50.4.

False positives. (i) Infection with *Proteus* sps. and relapsing fever caused by spirochaetes of the genus *Borrelia* also cause a rise in Weil-Felix agglutinins. (ii) Reagents used are not standardised.

EHRLICHIA

The organisms of this genus are small gram-negative and coccobacillary in shape. These attack leucocytes. Their multiplication occurs within vacuoles and they appear in clusters termed **morulae**. Not much is known about the pathogenicity and determinants of virulence as well as epidemiology of these organisms. However, two species have been recently incriminated as capable of causing human diseases.

Ehrlichia sennetsu is primarily a pathogen of animals but a disease resembling infectious mononucleosis in humans has been caused by this organism in Japan. Similary *E.canis* has been implicated in human disease in the USA.

ROCHALIMAEA

These are also gram-negative and small coccobacillary organisms which differ from other rickettsiae in their capability of growing in cell free medium. They share many properties with rickettsiae to which they are genetically also very closely related.

51 Non-sporing Anaerobes

The anaerobic bacteria can survive only can be bacilli as well as cocci, gram-positive as well as gram-negative. Some of these may form spores and some do not. In this chapter only anaerobic cocci and nonspore forming gram-negative bacilli are described (Fig. 51.1).

The isolation of anaerobic organism from a clinical specimen depends entirely upon the proper collection and transportation of the clinical sample. Since oxygen is toxic to these bacteria, utmost care has to be taken to maintain reduced oxygen environment throughout the process. The common features suggestive of anaerobic infections are depicted in Table 51.1.

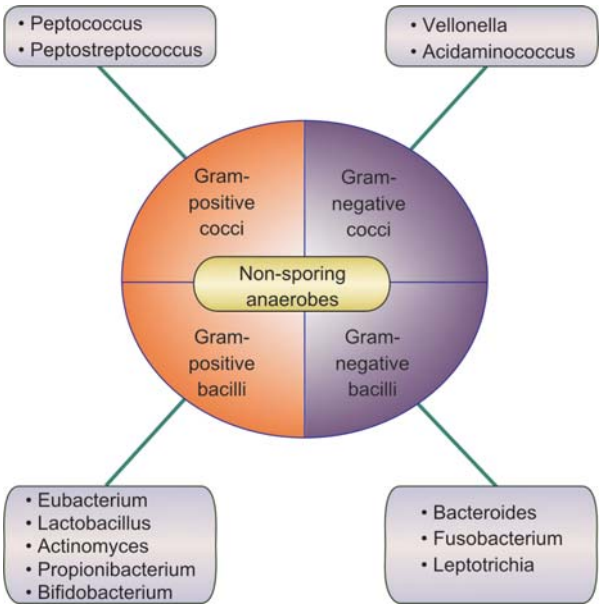


Fig. 51–1. Non-sporing anaerobes

Table 51–1. Features suggestive of anaerobic infections

- Foul smelling discharge
- Necrotic tissue and gangrene
- Gas in tissue and discharges
- Malignancy associated infections
- Septic thrombophlebitis
- Infection following human or animal bite
- Presence of sulphur granules in discharge
- Bacteraemic picture with jaundice
- Negative aerobic cultures

ANAEROBIC GRAM-NEGATIVE BACILLI

Classification

The gram-negative anaerobic non-sporing bacilli of medical importance are essentially contained in the family *Bacteroidaceae*. This family has several important genera, *Bacteroides, Prevotella, Porphyromonas, Fusobacterium* and *Leptotrichia.*

BACTEROIDES, PREVOTELLA AND *PORPHYROMONAS*

A large number of heterogenous group of anaerobic gram-negative bacilli, some of which are pathogens for man and animals, earlier constituted the genus *Bacteroides*. With a major revision in its taxonomy, only those species have been retained in this genus which were saccharolytic and grew in the presence of 20% bile. The moderately saccharolytic species which are inhibited by 20% bile and are indigenous to the oral cavity have been placed in a newly constituted genus, *Prevotella*. The asaccharolytic and pigmented species have been reclassified into another new genus, *Porphyromonas* (Table 51.2). Many other former *Bacteroides* species that

were isolated from non-human sources have been reclassified and several new genera have been proposed for them. The species of this genus can be distributed into four groups (Table 51.2).

Morphology

These gram-negative bacilli exhibit great pleomorphism and bacilli ranging from short rods to filamentous and fusiform shapes can be detected. These do not produce spores and most of these are non-motile.

Cultural Characters

Most of the organisms grow well on blood agar which should be freshly prepared or pre reduced. Some species shall require haemin and vitamin K for their growth. Incubation upto 48 hours is required to have optimum growth. Better growth is achieved if the incubation is done in an environment of 10% carbon dioxide. Variations in colony characters are noticed depending upon the species and the medium used. Usually the colonies are large, grey, circular or irregular.

Clinical Features

Infection with gram-negative anaerobic bacilli, as with anaerobic infections generally, is characterised by abscess formation and tissue destruction. The most common infections in which gram-negative anaerobic rods participate are pleuropulmonary, intraabdominal and female genital tract infections, but infections of any type anywhere in the body may involve these organisms (Fig. 51.2).

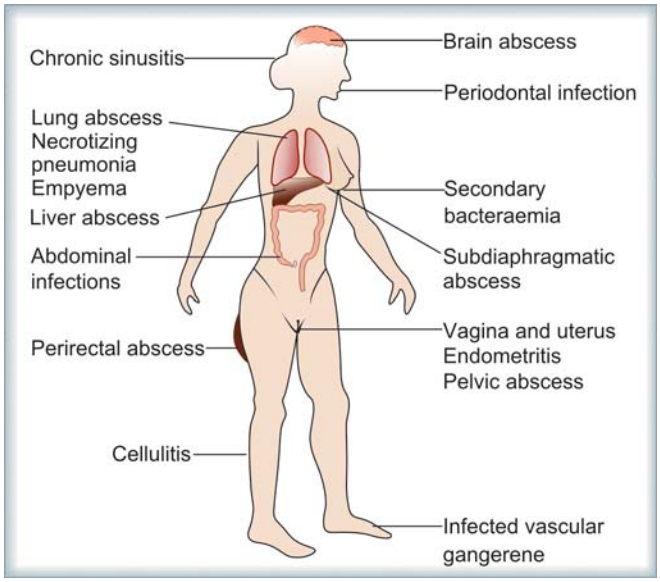


Fig. 51–2. Common sites of anaerobic infections

Sensitivity to Physical and Chemical Agents

These organisms are sensitive to oxygen. Generally, these are resistant to penicillins because of the production of potent beta lactamase. The drug of choice is metronidazole.

Laboratory Diagnosis

The specimen should ideally be pus, wound exudate, tissue or blood which has been transported to the laboratory in a closed syringe or in a container from which oxygen has been removed by flushing it with nitrogen. In laboratory specimen must be processed

PART VII

Table 51–2. Reclassification of former genus Bacteroides

Former Group	*Current genus*	*(No. of species) Important species*	*Diseases in man*
Fragilis	*Bacteroides*	(12) *B.fragilis* *B.vulgatus* *B.uniformis*	Bacteraemia Soft tissue abscess Abdominal abscesses Head and neck infections
Melaninogenicus oralis	*Prevotella*	(16) *P.melaninogenicus* *P.buccalis* *P.oralis* *P.corporis*	Normal oral flora Abscesses and soft tissue infections in various parts of body
Asaccharolytic	*Porphyromonas*	(10) *P.asaccharolyticus* *P.gingivalis* *P.endodontalis*	Normal oral flora Periodontitis Endodontitis Abscesses and soft tissue infections
Unclassified	*Capnocytophaga*		

without delay. Freshly poured plates or pre-reduced plates should be used and incubated anaerobically in an environment of 10% carbon dioxide. Direct smear can be prepared and apart from staining by grams method it can be exposed to UV rays for brick red fluorescence which suggests the presence of *B.melaninogenicus.*

Specimen should be cultured on two blood agar plates and two selective blood agar plates having gentamicin, kanamycin or vancomycin. Cooked meat medium should also be inoculated. One set of blood agar plates is incubated aerobically and the other anaerobically with 10% carbon dioxide. After 24 hours, blood agar plates are examined and the enrichment of cooked meat broth is subcultured onto solid medium (blood agar) and incubated. After another 24 hours, plates are examined for colonies and with the help of battery of biochemical tests, diagnosis is confirmed.

Treatment

Metronidazole is the drug of choice. Clindamycin and chloramphenicol can also be used. Penicillin, aminoglycosides and tetracyclines are of no use.

FUSOBACTERIA AND *LEPTOTRICHIA*

Fusobacteria are gram-negative anaerobic rods of various sizes and morphology with a typical gram-negative cell wall. They range from short to long filamentous forms. Many have tapered or pointed ends. These are usually nonmotile. These do not survive at 53°C for more than an hour and are even lost on repeated subcultures. They are generally sensitive to all the antibiotics.

These bacteria have been divided into 10 species. Many of these constitute normal flora of the body whereas some such as *F.necrophorum* are important pathogens.

Leptotrichia genus has only one species designated as *L.buccalis*. These are long Gram negative bacilli with considerable width and with terminal tapering. It grows on blood agar under anaerobic conditions and after 48 hours of incubation produces colonies of the size of 2-3 mm in diameter, very irregular and often striate.

L.buccalis was earlier thought to be a commensal of mouth but now its association with certain pathological conditions is being appreciated. It is sensitive to metronidazole.

ANAEROBIC GRAM-POSITIVE BACILLI

This group comprises of a large number of genera of bacteria including *Eubacterium, Propionibacterium, Lactobacillus, Actinomyces* and *Bifidobacterium*. Of all these strains belonging to the genera *Eubacterium* and *Propionibacterium* are isolated more frequently from clinical specimens. As compared to clostridia, which are spore bearers, these organisms are slow growing.

Among the gram-positive anaerobic bacilli, *Eubacterium lentum* is the most common isolate from clinical samples and is seen frequently in association with *B.fragilis*. It is part of normal flora and its precise role in causing disease is not known.

Propionobacterium acne is a common inhabitant of normal skin and sometimes incriminated as causing acne. It has been described in Chapter on *Corynebacterium.*

Strains of lactobacilli form part of normal flora in the mouth and gastrointestinal tract and may be the predominant flora in urogenital tract. *L.catenaforme* has been incriminated as a possible causative agent of pleuropulmonary infection in humans. Similarly *Bifidobacterium* is a part of normal flora of the gastrointestinal tract.

ANAEROBIC COCCI

These include both gram-positive as well as gram-negative cocci(Table 51.3).

Table 51–3. Anaerobic cocci

Gram-positive	*Gram-negative*
Peptococcus	*Veillonella*
Streptococcus	*Acidaminococcus*
Peptostreptococcus	*Megasphaera*
Ruminococcus	
Coprococcus	

PEPTOSTREPTOCOCCUS AND *PEPTOCOCCUS*

Peptococci are seen as single, in pairs or in clumps in the clinical material and young cultures stain gram-positive. With age, gram-positivity fades. Peptostreptococci may also be in singles, pairs or chains just like aerobic streptococci. The colony characters of these genera on blood agar are just like that of their counterparts in aerobic groups viz. staphylococci and streptococci. These produce haemolysis on blood agar which may be alpha or beta in nature.

These cocci are present as normal flora in the mouth, urogenital tract as well as gastrointestinal tract. Both genera have been found to be capable of producing disease in human beings. These are important in causing brain abscess, pleuropulmonary diseases and

obstetrics and gynaecologic infections. These have been isolated from a variety of infections and clinical specimens (Table 51.4).

Table 51–4. Infections from which Gram-positive anaerobic cocci are isolated

- Bacteraemia
- Nervous system abscesses
- Head and neck infections
- Dental abscesses
- Periodontitis
- Pleural infections
- Intra-abdominal abscesses
- Genito-urinary tract infections(especially in females)
- Foot ulcers
- Decubitus ulcers

VEILLONELLA AND *ACIDAMINOCOCCUS*

Veillonella organisms are small cocci occurring in pairs, short chains or clumps. These are present in normal flora of the mouth, gastrointestinal tract and urogenital tract. Their role in causation of disease is still unknown. Acidaminococci cells are larger and may partially stain gram-positive. These are present as part of normal flora in gastrointestinal tract and urogenital tract. Their pathogenicity, if any, is not known.

Other genera of anaerobic cocci are found as part of normal flora. Their association with disease process in man is yet to be established.

SPIRAL SHAPED MOTILE ANAEROBES

Two genera have been found to contain spiral shaped motile anaerobes which are associated with diseases in human beings. Several species of *Treponema* are found normally in oral cavity and an increase in their number occurs in diverse oral pathologies. These have been isolated, alongwith fusobacteria and *Prevotella intermedius* from acute necrotizing ulcerative gingivitis. Similarly motile, spiral-shaped Gram negative anaerobes of the genus *Anaerobiospirillum* have been isolated from patients with diarrhoea and bacteraemia.

52 Legionella

The genus owes its name to a common source outbreak of respiratory infection by an organism which was not known till 1976 when a large number of people attending American *Legion* convention in Philadelphia got infected with it. The organism was named as *Legionella pneumophila*. During last 15 years more than 30 species of this genus have been isolated of which around 10 are being incriminated as causative agents of pneumonia in man.

LEGIONELLA PNEUMOPHILA

Morphology and Growth Characters

Legionellae are fastidious gram-negative bacilli that measure 1 × 2-50 µm. To stain them properly with Gram's method counterstaining with basic fuchsin, instead of safranin, is done. These do not grow on ordinary laboratory media but require iron and cysteine for primary isolation. Buffered charcoal yeast extract (BCYE) agar medium is the ideal medium and colonies on this medium after 2-3 days of incubation at 37°C are 1-2 mm in diameter, circular, convex with irregular edge,and shining and smooth surface. *Legionella* takes around two weeks to grow from blood culture which can be done in biphasic BCYE medium.

Legionellae are catalase positive, oxidase positive, hydrolyse hippurate and some strains produce gelatinase.

Antigenic Structure

Legionellae possess both somatic and flagellar antigens. The serogroup specific antigen of legionellae of various species are heat stable, and can be recognised by immunofluorescence, ELISA as well as agglutination reaction. There are 14 serogroups of *L.pneumophila* of which serogroup 1 was the cause of 1976 outbreak. Three monoclonal antibody typing schemes have been developed for *L.pneumophila* serogroup 1. In legionella infection, specific antigen may be present in the urine of the patient early in disease and may persist for sometime even after the recovery. The detection in urine can be done with ELISA or RIA techniques.

Determinants of Pathogenicity

No toxin has been detected. The ability of the organism to survive and multiply intracellularly seems to be the most important virulence factor.

Mode of Transmission

The natural habitat of legionellae is water and these can survive in sterile tap water for upto one year. Once an artificial water system has been colonized it becomes very difficult to eradicate legionellae from that source. Since these organisms are hydrophobic, they tend to become concentrated in the foam that develops in some cooling tower ponds and can easily become the source of aerosols which are presumed, on epidemiological grounds,to be the principal means of infection. The infectious aerosols can travel a distance of 1 km or more thus infecting more water sources.

Clinical Features

Legionellae cause disease in human beings only. No person to person spread has been seen with this organism. Most infections are caused by *L.pneumophila* serogroup 1. Two main types of diseases may be caused:

- Pontiac fever
- Legionnaire's disease

The former is a brief, non-fatal illness in which legionellae antigen has been detected in the urine whereas

the latter is a pneumonic disease with variable severity in which the organism can be isolated from sputum, bronchial washings as well as lung tissues or blood.

Most cases of Legionnaire's disease are seen in older or immunosuppressed persons, although all the ages may be affected. Between 3 and 6% of all hospital-acquired-pneumonias are believed to be due to *L.pneumophila*. Risk factors include alcoholism, advanced age, smoking and immunosuppressive therapy. Most sporadic cases occur in renal transplant or cancer patients.

Laboratory Diagnosis

Direct Demonstration

For the diagnosis in laboratory, specimens from respiratory tract are collected. It is difficult to detect *L. pneumophila* in clinical samples with the help of Gram's staining. Direct immunofluorescence staining may detect the bacterium in a large number of specimens. DNA probe that detects *L.pneumophila* is diagnostic and carries almost 100% sensitivity.

Culture

Culture is made on BCYE agar and colonies can be confirmed by immunofluorescent test. The antigen can be detected in the urine of patients with ELISA and RIA.

Serodiagnosis

Serological tests are useful in seroepidemiology but are not of much diagnostic value since their sensitivity does not exceed 65%.

The essential steps in the laboratory diagnosis of legionnaire's disease are shown in Table 52.1.

Table 52–1. Diagnostic tests for Legionella infection

Detection of	*Test*	*Specimen required*
Whole organism	Culture Immuno-fluorescence DNA probes	Sputum and other specimens from respiratory tract
Soluble antigen	ELISA	Urine
Antibody	Immunofluorescence ELISA Rapid microagglutination test	Serum

Treatment

L.pneumophila is sensitive to erythromycin even in immunologically compromised patients. Rifampicin has also been used. Supportive therapy in maintaining ventilation is extremely essential.

Some important features about Legionella infections are given in Table 52.2.

Table 52–2. Important features about Legionella infections

- Acute bacterial disease with 2 distinct clinical and epidemiological manifestations: Legionnaire disease (pneumonic) and Pontiac Fever (Non-pneumonic).
- Epidemic Pontiac Fever has shown very high attack rates (about 95%) in many outbreaks.
- Reservoir is primarily aqueous: hot water systems, air-conditioning cooling towers etc.
- Transmission is predominantly air-borne.
- Person-to-person transmission has not been documented.
- Erythromycin is the drug of choice for treatment
- Decontamination of the implicated sources by chlorination or superheating has been effective.

53 Campylobacter and Helicobacter

For a very long time campylobacters were grouped with vibrios. These were assigned the generic name of *Campylobacter* in 1963.

More than 13 species of *Campylobacter* have been isolated. Some of these are pathogenic to man, though their reservoir is in animals (Table 53.1).

Table 53–1. Medically important campylobacters and their reservoirs

Species	*Reservoir*	*Human Disease*
C.jejuni	Animals and birds	Diarrhoea
C.fetus	Cattle and sheep	Septicaemia
C.coli	Pigs	Diarrhoea
C.laridis	Birds and animals	Diarrhoea
C.cinaedi	Man	Infection in homosexual men
C.hyointestinalis	Man	Infection in homosexual men
C.fennelliae	Man	Infection in homosexual men

Morphology

Campylobacters are spiral bacteria with single unsheathed polar flagellum and most species are motile. These are slender bacteria which measure 0.2-0.5 µm in diameter with varying length and can easily pass through filters with pore size of 0.65 µm. These organisms are non-sporing.

Cultural Characters

Campylobacters of medical importance are strictly microaerophilic. These fail to grow in air but require oxygen concentration of 5-10% for their growth. Some of these are capable of growing under anaerobic conditions.

Under ideal conditions these bacteria take around 48 hours to grow. Three species prefer to grow at 42-43°C and are called as thermophile organisms. These include *C.jejuni, C.coli* and *C.laridis.* The thermophile organisms produce flat and effuse colonies which may swarm on moist agar. Remaining species produce circular and convex colonies.

Biochemical Reactions

Campylobacters are biologically not very active (Table 53.2). These are oxidase positive and most of the strains are catalase positive.

Biotyping

On the basis of DNAse production and detection of H_2S *C.jejuni* has been divided into four biotypes.

Sensitivity to Physical and Chemical Agents

Campylobacters are killed at temperature of 60ºC. These can survive in water at 4ºC for several weeks. All campylobacters are quite sensitive to ultraviolet rays

Table 53–2. Biochemical reactions of campylobacters

Character	*C.fetus*	*C.jejuni*	*C.coli*	*C.laridis*
Growth at 43°C	–	+	+	+
Monotrichous flagella	+	–	–	–
Catalase	+	+/w	+	+
Nitrate reduction	+	+	+	+
Hippurate hydrolysis	–	+	–	–
Urease	–	–	–	+/–
H_2S production	–	–	+	+
Nalidixic acid	R	S	S	R

w:weak, R:resistant; S:sensitive

as well as gamma irradiation. Disinfectants such as hypochlorites, phenols, iodophores and quaternary ammonium compounds destroy *C.jejuni* within one minute. These organisms are also killed at pH 9.0. Most of these fail to grow if NaCl concentration in the medium is more than 1.5%.

Antigenic Structure

Campylobacters have a complex antigenic structure. Outer membrane protein (omp) antigens along with flagellar antigens (heat-labile antigens) have been used by Lior to define 112 serogroups within the combine species of *C.jejuni*,*C.coli* and *C.laridis* and these are designated as numerals with LIO as prefix.

Cytotoxins and Enterotoxin

Most strains of *C.jejuni* produce two types of cytotoxins-cytotoxin 1 and 2 and an enterotoxin. The enterotoxin is similar to cholera toxin and heat labile enterotoxin of *Esch.coli* and is neutralised by antisera against these toxins. So far no heat stable (ST) enterotoxin has been detected from campylobacters. Cytotoxins are heat and trypsin stable. No association between serogroups, biogroups and ability to produce enterotoxin or cytotoxin has been observed.

Pathogenesis and Clinical Features

Campylobacters are widely present in nature and responsible for infectious enteritis in large number of people. Statistics show that campylobacters are responsible for enteritis in as many cases as are caused by *Salmonella* and *Shigella* combined. Infection can occur in all age groups.

C.jejuni is the commonest campylobacter that causes acute gastroenteritis in humans. The disease is characterised by fever, bloody diarrhoea, headache and abdominal pain. It is usually self limiting and may last upto 10 days.

Laboratory Diagnosis

Direct Demonstration

Wet preparation of faeces when examined under dark-ground microscope or phase contrast microscope shows characteristic motility and morphology of campylobacters and helps in establishing a presumptive diagnosis.

Culture

The sample of faeces is cultured directly on selective medium which contains antibiotics to inhibit the growth of competitive faecal flora. Skirrow's medium or Preston medium are suitable. Incubation of inoculated plates is done in an environment of 1-10% CO_2 at a temperature of 42-43°C. Cultures are incubated for 48 hours though the growth of some species may become visible within 24 hours. Colonies are typically flat and effuse, with a tendency to spread on moist agar.

Serology

Complement fixation test (CFT) and ELISA are currently in use to diagnose patients with aseptic arthritis or Guillain-Barré syndrome due to campylobacters.

Treatment

Therapy is usually recommended to reduce the length and severity of disease. A large number of antibiotics are effective including erythromycin, tetracyclines and quinolones.

HELICOBACTER PYLORI

This organism was earlier known as *Campylobacter pylori* and is believed to be the causal agent of chronic gastritis. Strong evidence has accumulated regarding its close association with gastric and duodenal ulcers. It is also being incriminated as responsible for initiating the process of metaplasia in gastric epithelium which ultimately may lead to carcinoma of stomach.

Morphology

In stomach this organism takes the form of short spirals or S shaped gram-negative bacteria which are about 3 µm long, 0.5-1.0 µm wide with a wavelength of about 2.5 µm. After growth on laboratory media *H.pylori* are visible under the microscope as curved gram-negative rods (Fig. 53.1).

Fig. 53–1. *Helicobacter pylori*

H.pylori possesses lophotrichate flagellae which are sheathed. These organisms acquire coccal shape if exposed to air at room temperature.

Cultural Characters

Microaerophilic environment with 5-20% CO_2 and high humidity are required for the growth of *H.pylori.* To provide exacting nutrition to this organism blood, haemin, serum or charcoal are needed. Growth is best on moist freshly prepared chocolate agar. Colonies appear only after 3-5 days of incubation at 37ºC. These are less than 2 mm in diameter, circular, convex, and translucent. Weak haemolysis is produced on horse blood agar.

Biochemical Reactions

H.pylori is biochemically rather inactive. It produces catalase, DNAse, alkaline phosphatase and glutamyl aminopeptidase. All strains produce significant amount of urease and this enzyme is considered as an important determinant of pathogenicity.

Mechanism of Pathogenicity

The mechanism by which this organism causes mucosal inflammation and damage in stomach is not known. The bacteria invade the epithelial cells to some degree. Lipopolysaccharide may damage the mucosal cells and the ammonia produced by the urease activity may directly damage the cells also. An adhesin that helps *H.pylori* in attaching itself to epithelium cells has been conclusively shown.

Laboratory Diagnosis

The diagnosis of gastritis and *H.pylori* infection can be made histologically as well as bacteriologically.

Microscopic Examination

Routine stains can demonstrate gastritis and Giemsa or Silver stain shall show the organisms. The bacteria may not be easily visible in stained preparations. They can be seen in freshly made smears of biopsy material examined either wet by dark ground or phase-contrast microscopy, or dried and stained with Gram's stain.

Culture

Material obtained on biopsy of gastric mucosa should be immediately cultured onto Skirrow's campylobacter medium and incubated at 37°C. The incubation in an humid environment has to continue for upto one week. *H.pylori* forms discrete domed colonies unlike the effuse colonies of *C.jejuni* and *C.coli*.

Urease Tests

Two types of tests are now available:

- Biopsy urease test
- Urea breath test

This organism produces urease in abundant quantity. To detect it, biopsy material suspected to be having this organism is put into a solution of urea which also contains an indicator. If *H.pylori* is present , the pH changes from acidic to alkaline within 2 hours and a change in colour occurs.

In urea breath test, urea containing an isotope of carbon (either -14 or -13) is fed to the patient and emission of the isotope as carbon dioxide in the breath is measured. Patients with *H.pylori* give high readings of carbon dioxide.

Treatment

H.pylori is sensitive to penicillin, cephalosporins, tetracycline, erythromycin, rifampicin, aminoglycosides and nitrofurans.

GASTROSPIRILLUM HOMINIS

Gastrospirillum hominis is morphologically similar to *H.pylori* but tightly spiralled. This organism was isolated for the first time in 1989 and is incriminated as another possible causative agent of gastritis and ulcer. It has not been possible to culture this organism so far.

54 Miscellaneous Bacteria

PASTEURELLA MULTOCIDA

This organism is a small, ovoid non-motile gram-negative bacillus which measures around 1-2 to 0.3-1 µm and shows bipolar staining. These may be arranged single, in pairs or in short chains. Some of the strains are capsulated and give rise to mucoid colonies.

P. multocida grows fairly well on nutrient agar forming circular colonies of about 1 mm diameter. The colonies may be smooth, rough or mucoid in appearance. The smooth colonies are highly virulent. On blood agar with sheep blood, these colonies are surrounded by a narrow zone of haemolysis.

All strains of *P.multocida* produce acid, but no gas, from glucose, sucrose, mannitol, maltose and galactose. They produce indole, reduce nitrate and produce small quantity of H_2S. Both MR and VP tests are negative whereas oxidase and catalase are weakly positive. Citrate and urease tests are negative.

On the basis of capsular antigen four types have been identified for *P.multocida.* These are A, B, D and E. These along with 11 O antigens give rise to 15 serotypes.

P. multocida is pathogenic for a wide variety of animals, producing a wide spectrum of clinical illness which may vary from a harmless latent infection to fatal haemorrhagic septicaemia. Man is an occasional victim. It is perhaps the most common organism in human wounds inflicted by bites from cats and dogs. It can also produce human infections in many systems and may at times be part of normal human flora.

ACTINOBACILLUS

Actinobacillus is present as normal flora of oral cavity of human beings.

These are very small gram-negative, non-motile, non-sporing bacilli which may be present in clinical lesion in coccal form also. These are noncapsulated. On agar the organism gives rise to colonies that are tough, adhere to medium and give the appearance of 'crossed cigars'. Better growth is seen under anaerobic conditions. Growth is improved in the presence of blood. This organism does not grow on MacConkey agar. It ferments glucose, fructose and xylose with the production of acid only. It is indole negative; urease negative and reduces nitrates to nitrites.

With the help of six agglutinating antisera, the strains of this species can be grouped into 24 patterns and with 3 precipitating antigens into three types designated as a,b and c.

This organism is often found in lesions of actinomycosis and can also cause severe periodontal disease in adolescents, endocarditis, abscesses, osteomyelitis and other infections. Tetracyclines and chloramphenicol are the drugs of choice for treatment.

FRANCISELLA TULARENSIS

This is a gram-negative coccobacillus which measures around 0.2-0.7 µm. This is a non-motile and nonsporing organism but possesses a capsule. Growth does not occur on ordinary bacteriological media and cysteine is required for the same. Glucose cysteine blood agar (GCBA) is the ideal medium for the isolation of *F.tularensis*. This organism should not be cultured in laboratory without proper isolation facilities to avoid laboratory-acquired infection. The colonies on GCBA are 1-3 mm in diameter, greyish, viscous and produce a greenish pigment.

The identification of this organism can be made by growth requirements, immunofluorescent staining or agglutination by specific antiserum.

F.tularensis is widely found in animal reservoirs and is transmissible to humans by biting arthropods, direct contact with infected animal tissues, inhalation of aerosols or ingestion of contaminated food or water. Inhalation of even 50 organisms can cause the disease. After an incubation period of 2-6 days **tularaemia** manifests. It is characterised by regional lymphadenopathy, peribronchial inflammation and localised pneumonitis. All cases are accompanied by fever, malaise, headache and pain in the involved region.

Diagnosis can be made by isolation of organism but is difficult. Serology provides a simpler answer and presence of antibody in an agglutination test in a titre exceeding 1:160 is highly suggestive of disease. Streptomycin and gentamicin therapy results into cure within 10 days.

STREPTOBACILLUS MONILIFORMIS

This organism is responsible for two human diseases both of which are zoonoses. Rat bite fever is caused by the bite of rats who deposit Streptobacillus moniliformis, a normal flora of their throats, into the human blood causing fever, rashes and arthritis. The other disease is by ingestion of unpasteurized milk and is called as Haverhill fever which has the potentiality of causing epidemics.

S. moniliformis is a fastidious highly pleomorphic gram-negative bacterium which occurs in coccobacillary or bacillary form with characteristic yeast-like swellings. The L forms were recognised for the first time in this bacterium by Klieneberger. It is a nonmotile and nonacid fast organism.

S.moniliformis grows on serum or blood agar giving rise to low convex, circular and greyish colonies with entire edge and butyrous consistency. In serum broth, 'puff ball' floccules are seen. It fails to grow on MacConkey medium.

All the strains of this organism are antigenically similar. A serum agglutination test is available to diagnose the clinical disease. This organism is sensitive to penicillin, erythromycin, cephalosporins, tetracyclines and aminoglycosides which can be effectively used in treatment.

Rat bite fever is also caused by another bacterium known as *Spirillum minus*.

SPIRILLUM MINUS

This organism was isolated for the first time from a rat in 1888 but till date it has not been possible to assign it a universally acceptable taxonomic status. Most of the other species of the genus *Spirillum* are saprophytes and it has not been possible to culture these satisfactorily in artificial media. Morphologically it resembles *Campylobacter jejuni.*

S.minus is 2-5 µm long and 0.2 µm in diameter with 2-3 rigid spirals and one or more flagella attached to each pole. Methylene blue and Giemsa stains readily stain it.

This organism is a natural parasite of rats which act as carriers of *S.minus*. It gives rise to rat bite fever in man.

LISTERIA MONOCYTOGENES

Morphology

These are small gram-positive coccobacilli measuring around 1-3 µm in length and 0.5 µm in width. These are nonmotile at 37°C but become motile at 30°C due to peritrichous flagella. The motility is called as *tumbling* motility.

Cultural Characters

Any ordinary bacteriological medium will support the growth of *Listeria* if it contains serum, blood or a fermentable carbohydrate.

After overnight incubation at 37°C, the smooth forms of *Listeria* give rise to colonies 1-2 mm in diameter which are round, translucent and slightly raised as well as easily emulsifiable. The rough forms produce large, flatter colonies which are umbonate and difficult to emulsify. All cultures have a characteristic sour, butter, milk-like smell. Growth in broth is usually poor but enhanced by the addition of 0.5 to 1% glucose. This organism multiplies rapidly in milk. The important biochemical characters have been shown in Table 54.1.

Antigenic Structure

On the basis of somatic and flagellar antigens, *L.monocytogenes* has been divided into 13 serotypes which are also known as serovars or serovariants. These have been designated as 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. The somatic antigens are designated as I, II and III whereas the flagellar antigens are denoted with A, B, C. Immunological cross reaction between *Listeria* and many other bacteria has been reported. These include *Staphylococcus, Streptococcus, Erysipelothrix, Bacillus, Corynebacterium and Escherichia*.

Pathogenesis and Clinical Features

The common infections produced by *L.monocytogenes* are given below:

Table 54–1. Salient features of Listeria and Erysipelothrix

Feature	*Listeria*	*Erysipelothrix*
Motility at 30°C	+	–
Growth at 4°C	+	–
Growth on MacConkey agar	+	–
Haemolysis on blood agar	+	–
Acid from:		
• glucose	+	+
• lactose	–	+
• trehalose	+	–
• salicin	+	–
Catalase	+	–
Acetoin production	+	–

- Neonatal sepsis
- Neonatal meningitis
- Spontaneous abortion or stillbirth
- Sepsis in immunocompromised patients
- Meningitis in immunocompromised patients
- Puerperal sepsis.

Of all the above mentioned, meningitis is the most common presentation and pregnant women, newborns, or organ transplant recipients are most likely to be involved.

Treatment

All strains are sensitive to ampicillin and this drug, either alone or in combination with aminoglycosides, remains the treatment of choice. This organism is resistant to cephalosporins.

ERYSIPELOTHRIX RHUSIOPATHIAE

Erysipelothrix are rod-shaped organisms with a tendency to form long filaments. These are gram-positive, non-motile and non-sporing. These are present either in smooth or in rough form. In the smooth form the organisms are small, straight or slightly curved with rounded ends and measure about 0.8-2.5 µm long and 0.3-0.6 µm broad, arranged singly, in small packets or groups or short chains. In rough forms long filamentous bacteria, which may measure upto 60 µm, are seen.

Growth of this bacterium on ordinary media is scanty. It is improved by glucose, blood or serum.

After an incubation of 18 hours at 37°C, the smooth organisms give rise to small, circular, convex and amorphous colonies whereas the rough colonies are flat and large and with matt surface. Some of the important characters and features which differentiate it from *Listeria* are mentioned in Table 54.1.

Erysipeloid in man is an occupational hazard especially for fish workers, butchers and poultry workers. It follows the introduction of the organism through the skin and causes the formation of a painful swelling of purplish erythematous area which is slightly raised. The hands are the usual sites of infection. Sometimes septicaemia may also occur. Rare cases of endocarditis have also been reported.

E.rhusiopathiae is resistant to sulfonamides and neomycin and susceptible to penicillin, streptomycin, erythromycin and tetracycline.

CALYMMATOBACTERIUM GRANULOMATIS

This organism is the causative agent of Donovanosis which is also known as granuloma inguinale or granuloma venereum. This organism is transmitted by sexual as well as nonsexual but intimate contact also. Man is the only reservoir.

C.granulomatis is a small, thick and capsulated gram-negative bacillus which may show polar staining. In smears from lesions it is seen within the large mononuclear phagocytes. This organism does not grow easily on artificial laboratory media. Growth can be achieved on yolk sac of a developing chick embryo (age 5 days) and confirmed after 3 days of incubation by Gram's or Leishman's stain.

It causes a chronic granulomatous disease which is prevalent in warm humid tropical and subtropical countries. Initial lesions are on genitals or site of intimate contact and may be accompanied by bubo. The patient can be treated with ampicillin or tetracycline.

EIKENELLA

Eikenella corrodens is present as commensal in the human mouth and intestine. This is a small gram-negative bacillus which shows the unusual characteristic of 'jerking' or 'twitching' motility which is not due to flagella but is caused by fimbria-like filamentous appendages.

E.corrodens requires haemin for growth and produces on blood agar, after 5 days of incubation at 37°C in an environment of CO_2, colonies of less than 1 mm diameter with spreading edges because of the twitching motility of bacteria. Because of this irregularly spreading edges, the species has been given the name *corrodens*.

Some of the salient features of this species and that of *Cardiobacterium hominis* and *Chromobacterium violaceum* have been shown in Table 54.2.

Table 54–2. Characteristics of Eikenella, Cardiobacterium and Chromobacterium

Feature	*Eikenella*	*Cardio-bacterium*	*Chromo-bacterium*
Motility by flagella	–	–	+
Twitching motility	+	–	–
Haemin for growth	+	–	–
Growth on MacConkey agar	–	–	+
Haemolysis on blood agar	–	–	+
Catalase	–	–	+
Oxidase	+	+	+
Acid from glucose	–	+	+

E.corrodens is an opportunistic pathogen which can cause endocarditis as well as mixed infections with anaerobic bacteria in infections of mouth and periodontal diseases. Wound infections, liver and brain abscesses, meningitis and osteomyelitis may also be rarely caused by this bacterium. It is sensitive to ampicillin, benzylpenicillin, chloramphenicol, tetracycline and cephalosporins but always resistant to clindamycin and metronidazole.

CARDIOBACTERIUM

Cardiobacterium hominis is a commensal in the nose and throat of humans and can rarely cause endocarditis. It is a pleomorphic gram-negative bacillus which is nonmotile. It grows in an humid environment in the presence of CO_2 on nutrient agar as well as blood agar. Colonies are smooth, 1-2 mm in diameter, circular and opaque. In cases with endocarditis, it can be isolated from blood and patient can be treated with tetracycline, gentamicin or chloramphenicol. Important features have been shown in Table 54.2.

CHROMOBACTERIUM

Chromobacterium violaceum is a saprophyte of soil and water and a rare pathogen of man where it causes suppurative or septicaemic illness. It is a large (2-4 mm) gram-negative bacillus which is motile because of single polar flagellum. Grows on ordinary media including MacConkey agar, produces haemolysis on horse blood agar and colonies are violet coloured and hence the species name of 'violaceum'. Few characteristic of this organism have been shown in Table 54.2. Tetracycline is the drug of choice for treatment. It is resistant to penicillin.

ACHROMOBACTER

Only one species of this genus, *Achromobacter xylosoxidans* has been isolated from many sites in human body but has not been incriminated so far as the sole causative agent of any lesion in man. This is an oxidase positive gram-negative bacillus.

CAPNOCYTOPHAGA

The members of this genus are part of normal flora of oral cavity. These are fastidious gram-negative gliding bacteria which are fusiform in appearance.

Capnocytophaga is facultative anaerobe. Growth under aerobic conditions occurs only in the presence of CO_2. Though it is a commensal, sometimes it may cause systemic disease in immunologically compromised patients.

DF-2 BACTERIA

This is a group of gram-negative, oxidase positive and catalase positive bacteria. The name DF is derived from their ability to act as dysgonic fermenters and do not show their fermentation pattern on routine media.

DF-2 bacteria form part of normal flora of oral cavity of dogs. In patients whose defenses have been compromised such as asplenic patients or alcoholics, this bacterium may cause a fulminant infection. Healthy individuals are rarely affected.

FLAVOBACTERIUM MENINGOSEPTICUM

F.meningosepticum has been isolated from cases of hospital-acquired neonatal meningitis.

The organism is a small rod when seen in clinical material such as CSF and changes to filamentous forms on artificial media. Some of the strains are motile with single flagellum. It is nonsporing and noncapsulated.

On agar medium colonies are 1-2 mm in diameter with yellowish pigment which is produced at temperatures of 37°C. 15 serotypes have been identified and designated as A to O. Neonatal meningitis is usually due to serotype C and bacteraemia in adults due to serotype F.

Flavobacteria are resistant to a large number of antibiotics and sensitive to cotrimoxazole, rifampicin, clindamycin and novobiocin.

KINGELLA

The members of this genus are short gram-negative rods which at times are mistaken for neisseriae. These are catalase negative and saccharolytic and differ from *Moraxella* on the basis of these features.

Kingellae are oxidase positive and can grow on Thayer Martin medium and hence can be misdiagnosed as neisseriae.

ACINETOBACTER

Acinetobacters were earlier known as *Mimapolymorpha* as well as *Herellea.* This genus is now recognised to have one species, *A.calcoaceticus* which has two variants: *anitratus* and *lwoffi*.

Acinetobacters are free living saprophytes which are gram-negative and coccobacilli in shape. These grow well on ordinary laboratory media. Their important characteristics and features which differentiate these from *Moraxella* and *Neisseria* are given in Table 35.1.

A.calcoaceticus colonies are white or cream coloured, smooth and circular with an entire edge. Some strains are haemolytic on blood agar. These organisms are oxidase negative, catalase positive and indole negative. These do not ferment sugars. All strains are penicillin resistant.

Acinetobacters are present as commensals of man and animals. These are also present in the hospital environment from where these can cause infection in compromised patients. Clinical manifestations include bronchopneumonia and septicaemia.

Hospital strains of this organism are multiple drug resistant and therapy for opportunistic infections caused by them should be based upon testing for antimicrobial sensitivity in the laboratory.

ALCALIGENES

Alcaligenes faecalis derives its name from the alkaline reaction produced by it in milk and its isolation from faeces. The genus is now characterised and contains motile gram-negative bacilli which are strict aerobes and generally do not utilize glucose. These are non-capsulated and grow easily on nutrient agar giving rise to greyish white colonies which are either umbonate with smooth centre or high convex and circular. Young cultures emit an aromatic or fruity smell.

A.faecalis is catalase and oxidase positive; indole negative, H_2S -ve, urease -ve and PPA -ve. It is resistant to penicillin and streptomycin but sensitive to many other antibiotics.

BARTONELLA

Bartonella bacilliformis is the causative agent of Oroya fever and verruga peruana. These stain poorly with aniline dyes and are gram-negative. Giemsa stain gives better results. This organism shows pleomorphism but are usually short rods arranged in pairs, chains or clumps. It is motile with the help of 10 flagella at one pole. In man the bacterium is present not only in red blood cells but also in cytoplasm of endothelial cells of spleen, liver and lymph nodes.

B.bacilliformis do not grow in the absence of serum, blood or glycerol. Growth is slow and colonies take upto 4 days to become visible. These are minute, circular, mucoid and clear colonies. No haemolysis is produced. The organisms do not ferment any sugar. Antigenically all the strains are homogenous. It is sensitive to penicillin, streptomycin, tetracycline and many other drugs.

55 Normal Microbial Flora of the Human Body

The term normal microbial flora refers to the population of microorganisms that inhabit the skin and mucous membranes of healthy normal persons.

The normal body flora can be of two types: resident flora and transient flora.

Resident Flora

This consists of relatively fixed types of microorganisms regularly found in a given area at a given age and if it is disturbed it promptly reestablishes itself.

Transient Flora

This consists of nonpathogenic or potentially pathogenic microorganisms that inhabit a particular body location for limited period. If the normal flora is intact, there is very little significance of the transient flora. But if the normal flora is disturbed, the transient microorganisms may colonize, proliferate and produce disease.

Proper knowledge of normal body flora is essential for clinical microbiologist for correct interpretation of the results of microbiological laboratory investigations.

Functions of Resident Flora

Resident flora plays an important role in maintaining proper health by performing various functions (Table 55.1). Its presence is, however, not essential for life.

The normal bacterial flora can also act as a source of spread of bacterial pathogens from one area of world to another. For example, various serotypes of *Neisseria meningitidis* are normally carried in nasopharynx. Congregation of people from different regions at one place, e.g. Haj pilgrims in Saudi Arabia have always the risk of bringing back a different serogroup. These situations can sometimes lead to outbreaks of disease.

Table 55–1. Beneficial and harmful functions of resident flora

Beneficial
• Help in absorption of nutrients
• Synthesize vitamins especially vitamin K
• Prevent colonization by pathogenic organisms
• Elevate immune status against pathogens having related or shared antigens
• Bacteriocins produced, inhibit growth of pathogens
• Endotoxin released by it augment host defences by triggerring the alternative complement pathway.
Harmful
• Opportunistic pathogens
• Produce disease if introduced in large number in foreign locations with predisposing factors
• Penicillinase produced can antagonize therapy
• Excessive production of endotoxin may cause shock
• May transfer organisms from one part of world to another
• Ubiquitous presence creates problems in establishing accurate diagnosis

Hence, for collection and analysis of any clinical sample, following points must be kept in mind:

a. The specimen submitted for microbiological analysis must be obtained from the suspect lesion and not from an adjacent, non-involved area
b. The site of sampling should be properly prepared and all precautions taken not to contaminate the sample with the normal flora.

The normal flora of the human body can be considered under two heads (Table 55.2): sites which are normally sterile and sites which normally harbour microorganisms.

Usually Sterile Sites

The larynx, trachea, bronchi, bronchioles, alveoli and the accessory nasal sinuses are usually sterile. These organs remove any offenders by various defense mechanisms.

Table 55–2. Sites which are normally sterile and sites with normal flora

Sites normally sterile	*Sites with normal flora*
Bone marrow	Skin
Blood	Mouth and upper respiratory tract
Cerebrospinal fluid	Gastrointestinal tract
Serous fluids	Female genital tract
Tissues	Conjunctiva
Urine	
Lower respiratory tract	
Middle and inner ear	

Oesophagus and stomach are contaminated with bacteria whenever food is ingested. The microbial population does not survive well at these two sites. Proximal small intestine, liver, and gallbladder are usually free from contaminating organisms. Peritoneum is also normally sterile.

Excepting external genitalia, anterior urethera, and vagina, other structures of the genito-urinary tract are without permanent microbiota.

CSF is usually sterile. Blood is also normally sterile. Occasionally, bacteria may be isolated from blood cultures of asymptomatic individuals. Often these bacteria represent low level contamination of the circulation from inapparent skin lesion or other sources.

Tissues and various serous fluids (peritoneal, pleural and pericardial) are normally sterile.

Urine is normally sterile, but it can be contaminated with bacteria located in the anterior urethera.

SITES WITH NORMAL FLORA

The distribution of normal flora in human body is shown in Figure 55.1.

Skin

Because of its constant exposure to and contact with the environment, skin is constantly populated with microorganisms that reflect the contacts, habits and profession, etc, of the host. Certain areas of the skin, especially those adjoining the various body openings reflect the microbiota of these adjacent sites. The normal flora of the skin is given in Table 55.3.

Important factors which help in elimination of non-resident microorganisms from the skin include low pH, fatty acids in sebaceous secretions and presence of lysozyme. The number of superficial skin microorganisms can be diminished by vigorous daily scrubbing with soap containing disinfectants. The nature of the skin microbial flora is also dependent on one's occupation and environment and degree of clothing used. The pathogenic organisms harboured on the skin can play a significant role in nosocomial infections.

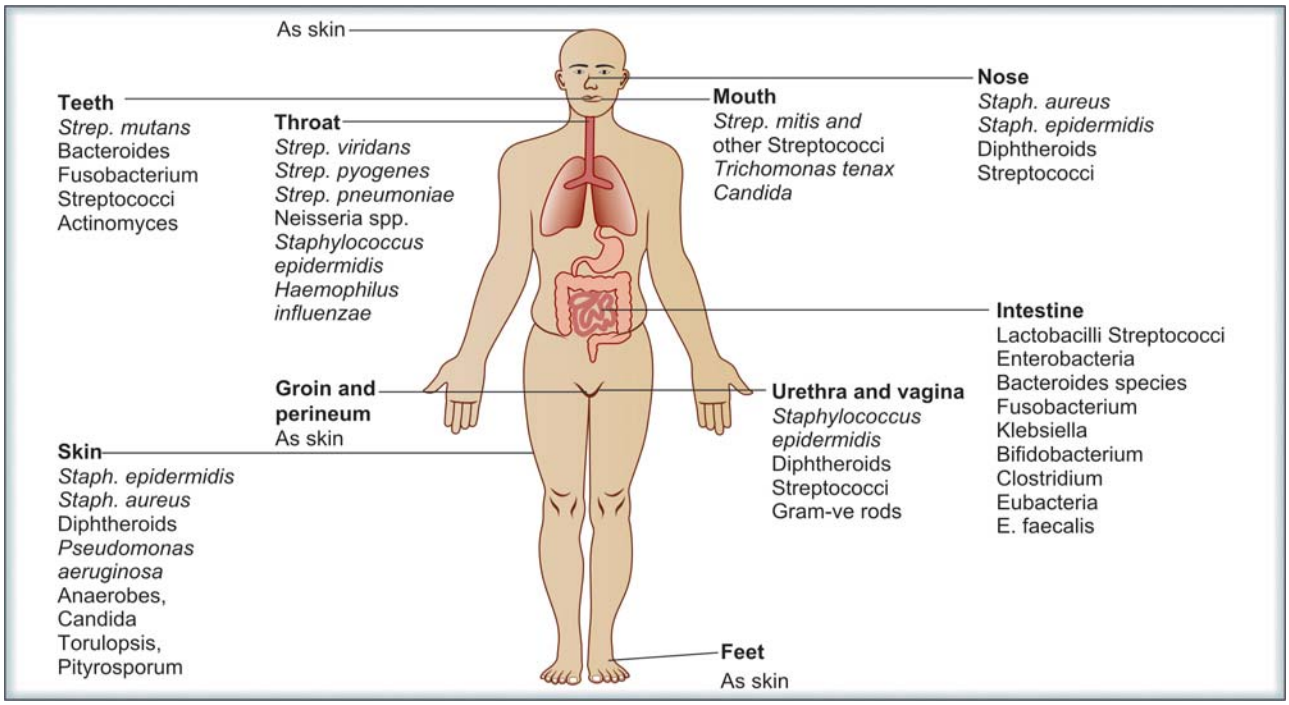


Fig. 55–1. Normal flora

Table 55–3. Normal flora of skin

- *Corynebacterium*
- *Propionibacterium*
- *Staphylococcus epidermidis*
- *Staphylococcus aureus* (occasionally)
- *Peptococcus*
- Aerobic gram-positive spore bearers
- *Streptococcus viridans*
- *Streptococcus faecalis*
- *Acinetobacter*
- Nonpathogenic mycobacteria
- Fungi and yeasts in the skin folds

The external auditory canal usually reflects the flora of the skin, with the exception of *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* which are isolated frequently.

Conjunctiva

The conjuctival flora is normally held in check by the flow of tears, which contain antibacterial lysozyme. The predominant organisms encountered as normal flora in conjunctiva are given in Table 55.4.

Table 55–4. Normal flora of conjunctiva

- *Corynebacterium xerosis*
- *Staphylococcus epidermidis*
- *Neisseria*
- *Moraxella*
- *Haemophilus influenzae*
- *Candida albicans*
- Non-haemolytic streptococci

Intestinal Tract

At birth the intestine is sterile. In breast-fed children the intestine contains large number of lactic acid streptococci and lactobacilli. In bottle-fed children a mixed flora exists in the bowel and lactobacilli are less prominent. Diet has a marked influence on the composition of intestinal and faecal flora. Bowels of newborns in intensive care nurseries tend to be colonized with pathogenic organisms such as *Klebsiella, Citrobacter* and *Enterobacter.*

In oesophagus and stomach the number of organisms is that swallowed with the food and it is temporary because the acidity of the stomach is protective. In conditions where there is decreased acidity of the stomach there are chances of increased number of organisms present in the stomach. The number of bacteria increases progressively beyond the duodenum to the colon, being comparatively low in the small intestine. The relative numbers of microorganisms in different parts of the intestine have been enumerated (Table 55.5).

Table 55–5. Number of bacteria (million per gram content)

Stomach	0.001-0.1
Duodenum	0.001-1.0
Jejunum and ileum	0.1-100
Caecum and transverse colon	100-10,000
Sigmoid colon and rectum	100,000

In the upper intestine, lactobacilli and enterococci predominate, but in the lower ileum and caecum the flora is faecal. In normal adult colon the resident flora is *predominantly anaerobic* ranging between 96-99% consisting of *Bacteroides, Fusobacterium, Anaerobic lactobacilli, Clostridium sp.* and *anaerobic streptococci.* Only 1-4% are facultative aerobes consisting of gram-negative coliform bacteria, *Enterococci, Lactobacilli, Proteus, Pseudomonas, Candida* and *others*. More than 100 distinct types of organisms occur regularly in normal faecal flora. Prevalence of drug resistant organisms in the intestine serves as a source of plasmid-mediated drug resistance in the bacteria.

Urethra

The anterior urethra of both sexes contains small numbers of the same types of organisms found on the skin and perineum. The organisms regularly appear in the normal voided urine. The commonly encountered organisms are listed in Table 55.6.

Table 55–6. Microbial flora of urethra

- Coagulase negative staphylococci
- Enterococci
- Non-pathogenic neisseriae
- Non-pathogenic mycobacteria
- Various enteric gram-negative rods
- *Corynebacterium*
- *Trichomonas vaginalis**
- *Candida albicans**
- *Staphylococcus aureus**

*Occasionally present

Vagina

The usual microflora of the vagina from the menarche to the menopause is dominated by lactobacilli designated as Doderlien's bacilli. The nature of the flora in the vagina depends on the pH of its secretions and its

PART VII

Table 55–7. Normal microbial flora of vagina

- Anaerobic streptococci
- Beta haemolytic streptococci
- Bacteroides
- *Gardnerella vaginalis*
- *Ureaplasma urealyticum*
- *Listeria monocytogenes*
- *Staphylococcus aureus*

enzyme content. At birth the vagina is sterile. In the first 24 hours it is invaded by micrococci, enterococci and diphtheroids. With the appearance of glycogen the pH changes to acidic and the nature of the flora changes. The organisms normally encountered in the vagina are listed in Table 55.7.

Vaginal organisms present at the time of delivery may infect the newborn.

Mouth and Upper Respiratory Tract

The flora of the nose consists of prominent coryne bacteria, staphylococci (*Staph. epidermidis* and *Staph. aureus*) and streptococci. The mucous membrane of the mouth and pharynx are often sterile at birth but may be contaminated by passage through the birth canal. Within 4-12 hours after birth, viridans streptococci become established as the most prominent members of resident flora and remain so for life. Early in life, aerobic and anaerobic staphylococci, gram-negative diplococci, diphtheroids and occasional lactobacilli are added. When teeth begin to erupt anaerobic spirochaetes establish themselves alongwith anaerobic vibrios and lactobacilli. Actinomyces spp. are normally present in tonsillar tissue and various protozoa may also be present. Candida spp. can also be present in mouth. In the pharynx and trachea, a similar flora establishes iteself. A few bacteria are found in normal bronchi. Small bronchi and alveoli are normally sterile. The predominant organisms in the upper respiratory tract, particularly the pharynx are non-haemolytic and alpha haemolytic streptococci and neisseriae. Staphylococci, diphtheroids, haemophilus, pneumococci and mycoplasma may also be encountered.

PART VIII
GENERAL VIROLOGY

56 Nature and Classification of Viruses

Viruses are infectious agents too small to be seen with a light microscope. These were discovered in 1898 when bacteria free filtrate was shown to be causing disease. Since no particulate material was present in the filtrate, the agent was named as *virus* which in Latin means *poison*.

Definition

Viruses are *obligate intracellular parasites* which means that they replicate (or multiply) only inside a living host cell. These have very simple structural organization but are characterized by certain distinctive features (Table 56.1).

Table 56–1. Distinctive features of viruses

- Contain a single type of nucleic acid, either DNA or RNA but *never both*
- Contain a protein coat that surrounds the nucleic acid
- Multiply inside the living cells using the synthetic machinery of the host cell, and
- Cause the synthesis of specialised elements that can transfer the viral nucleic acid to other cells.

Host Range of Viruses

Apart from human beings the viruses can infect vertebrate and invertebrate animals including mammals, birds, reptiles, amphibia, helminths, arthropods, plants, algae, fungi, protozoa, and even bacteria. There are even certain *satellite viruses* which depend upon other viruses for their survival and replication.

The *host range* of a virus refers to the different kinds of organisms it can infect. The polioviruses can cause infection only in humans. In contrast, the rabies virus attacks cells of the central nervous system in humans and a wide variety of warm-blooded animals. The *specificity* of a virus refers to the specific kinds of cells the virus can infect. Virus specificity is determined mainly by whether a virus can attach to a cell or not. This depends upon the presence of specific receptors on the surface of the virus.

Diseases Caused by Viruses in Human Beings

The diseases caused by viruses in the human beings fall into a wide spectrum. Certain diseases carry invariably a fatal outcome. These include rabies and acquired immunodeficiency syndrome (AIDS). Some of the viral diseases such as hepatitis, encephalitis, ebola haemorrhagic fever and yellow fever frequently turn out to be fatal. Tremendous morbidity results from worldwide prevalent diseases such as measles, mumps, rubella, common cold, influenza and chickenpox. Some of these usually appear in epidemic forms (e.g. measles) and some may even acquire pandemicity (e.g. influenza). Rubella virus is well known to induce teratogenic effects and evidence is accumulating at a rapid pace to incriminate viruses as carcinogenic in human beings.

Classification of Viruses

Before much was known about the structure or chemical properties of viruses, they were classified by where

they were found or what organs they infected. Thus, they have been classified as *bacterial viruses, plant viruses,* or *animal viruses*. They have also been classified as *dermotropic* if they infect the skin, *neurotropic* if they infect nerve tissue, *viscerotropic* if they infect organs of the digestive tract or *pneumotropic* if they infect the respiratory system. Some people even made the mode of transmission of virus from one host to another as the basic character and classified viruses as *enteric, respiratory* and *arboviruses*. As more has been learnt about the structure of viruses at chemical level, a classification has been developed based upon physicochemical parameters.

Classification Based on Physicochemical Criteria

The viruses have been divided into families which are split into genera and finally species. The primary criteria employed are :

a. Kind of nucleic acid and strategy of viral replication
b. Morphology of virion which includes size, shape, symmetry of nucleocapsid and presence of envelope.

The presently accepted methodology of classification of viruses is shown in Figures 56.1 and 56.2 by depicting classification of viruses. The classification of various viruses has been shown in Table 56.2.

Nucleic Acid and Classification

Apart from the structure and shape, the classification is primarily based upon type, polarity and shape of the nucleic acid (Fig. 56.2). The DNA can be double stranded or single stranded and either linear or circular in appearance. All DNA viruses are double stranded except parvoviruses which are single stranded.

RNA of the viruses may be single stranded or double stranded; in influenza viruses and reoviruses this is segmented and some may have positive polarity and some negative. A nucleic acid that encodes the information for making proteins needed for the virus is called a *positive sense nucleic acid.* A nucleic acid made up of bases complementary to those of positive sense nucleic acid is called a *negative sense nucleic acid.* Host cell ribosomes reading the base sequence of a negative sense nucleic acid also contain an enzyme called transcriptase. Inside the host cell this enzyme uses the negative sense nucleic acid as a template and makes a complementary positive sense nucleic acid.

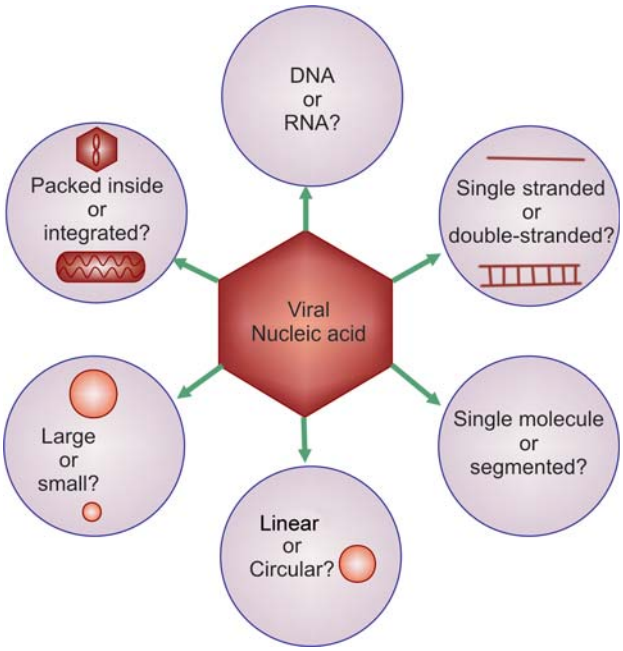


Fig. 56–2. Classification criteria for viruses

Nomenclature of Viruses

The nomenclature of viruses, like their classification,is decided by the International Committee on Taxonomy of Viruses. The viruses are grouped into families which are named with a suffix-*viridae.* Subfamilies, if any,

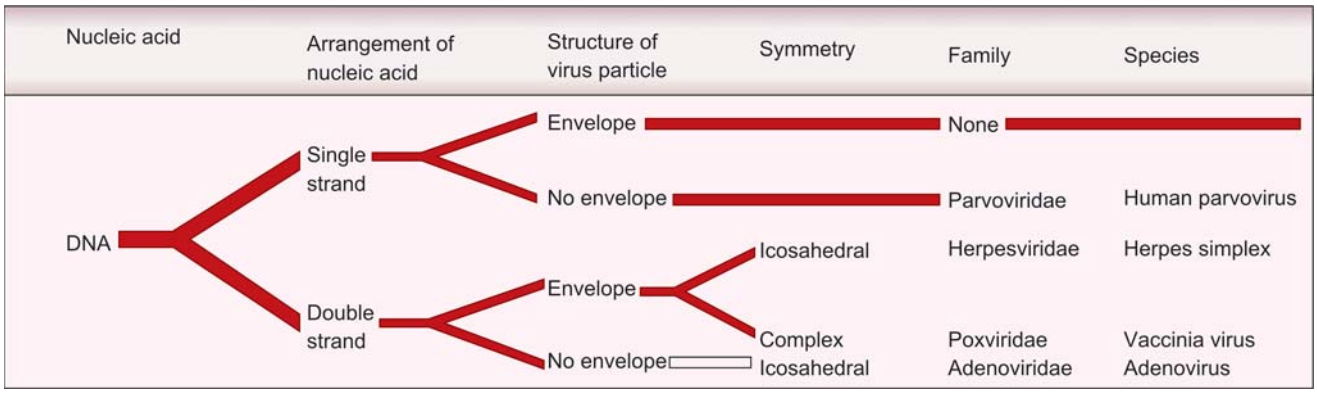


Fig. 56–1. Parameters used to classify viruses

Table 56–2. Classification of viruses

Class	Nucleic acid	Envelope	Shape	Example
RNA Viruses				
Ia	(+), ssRNA	–	Icosahedral	Picornaviruses
Ib	(+), ssRNA	+	Icosahedral	Togaviruses
II	(–), ssRNA	+	Helical	Paramyxoviruses Rhabdo viruses
III	(–), ssRNA*	+	Helical	Orthomyxoviruses Arenaviruses
IV	ds, RNA*	–	Icosahedral	Reoviruses
V	(+), ssRNA	+	Helical	Retroviruses
DNA Viruses				
Ia	ds, linear DNA	–	Icosahedral	Adenoviruses
Ib	ds, linear DNA	+	Icosahedral	Herpesviruses
Ic	ds, linear DNA	+	Complex	Poxviruses
II	ds, circular DNA	–	Icosahedral	Papovaviruses
III	ss, linear DNA	–	Icosahedral	Parvoviruses

* segmented; ds:double stranded; ss:single stranded

shall end with suffix-*virinae* and the genera will have suffix of *-virus*. The prefix may be another latin word or a *sigla*, i.e. an abbreviation derived from some initial letters. The latinized names are written in italics whereas vernacular names are written in roman letters.

PART VIII

Unconventional Infectious Agents

In a group of diseases characterised by transmissible spongiform encephalopathies and examples of which include Creutzfeld-Jakob disease, kuru and scrapie, no conventional agent or agent specific nucleic acid has ever been isolated. This has led to the belief that causative agent may be the pathogenic nucleic acid coated with host protein and called as **virino.**

VIROIDS

Some diseases of the plants have been found to be caused by a new class of subviral agents which do not exhibit any extracellular dormant phase and carry very small nucleic acids. These have been designated as *viroid.* These are RNA molecules which are resistant to heat but destroyed by nuclease. Till date, no viroid has been shown to cause human disease, but there is no reason to suppose they cannot infect animals.

Viroids differ from viruses in four essential ways (Table 56.3).

Table 56–3. Features distinguishing viroids from viruses

- Each viroid consists of a single specific RNA
- Viroids exist inside cells as particles of RNA without capsids
- Viroid particles are not apparent in infected tissues without using special techniques to identify nucleotide sequences in RNA
- Compared to viral nucleic acids, the RNA of viroids is a low-molecular weight material.

PRIONS

The name prion has been derived from proteinaceous infectious particles. These are sensitive to proteases but resistant to nucleases and UV rays. Strong evidence is now available to incriminate these in causing transmissible spongiform encephalopathies (TSE) which is a group of neurological degenerative diseases of man. These proteins are self-replicating and when come in contact with normal proteins convert them into infectious elements. The Nobel Prize for 1997 was given to Prussner for his work on prions and their possible association with transmissible spongiform encephalopathies in animals (*mad cow disease*) and humans. Some of important properties of prions are given in Table 56.4.

Table 56–4. Properties of prions

- Very resistant to chemicals, radiations, and heat
- Do not exhibit virus morphology in electron microscopy
- Not associated with foreign nucleic acid isolated from infected host cells
- Proteinaceous, filterable
- Multiply in cell culture but no CPE
- Do not elicit inflammatory reaction in host
- Do not elicit antibody formation in host
- Responsible for plaques and abnormal fibres forming in brain of host
- Very difficult to transmit

57 Morphology of Viruses

Size of Viruses

The viruses are measured in units of nanometre (nm) which is one-thousandth part of a micrometre or a one-millionth part of a millimetre. An Angstrom unit (A) is one-tenth of a nm. Viruses vary in diameter from 3 to 20 nm. Most viruses can be measured under electron microscope by comparing their size with that of known sized latex particles. By employing various techniques it has been found that the poxviruses are largest in size and measure around 330 × 230 × 100 nm. Paramyxo- and herpesviruses vary in their diameter from 120-300 nm whereas influenza and adenoviruses have diameter of 60-120 nm. The reo, toga and papova viruses have a diameter of 40-60 nm. The smallest viruses are picorna and parvoviruses which measure between 20-40 nm. The diversity in sizes and shapes of viruses is illustrated in Figure 57.1.

Morphology

The viruses are composed of nucleic acids and proteins. The *genome* consists of single nucleic acid which stores all the vital information required by the virus for its multiplication. The genome is surrounded by a shell or coat made of protein and called as *capsid*. The genome and capsid are collectively designated as *nucleocapsid*. Most of the viruses have an additional covering of lipid around their nucleocapsid which is known as the *viral envelope*. The complete virus particle is also called as *virion*. The term virion indicates that the virus is infectious as well as structurally intact.

Viral Nucleic Acids

Though the viruses possess single nucleic acid, they show a large diversity in this respect. Some are DNA,

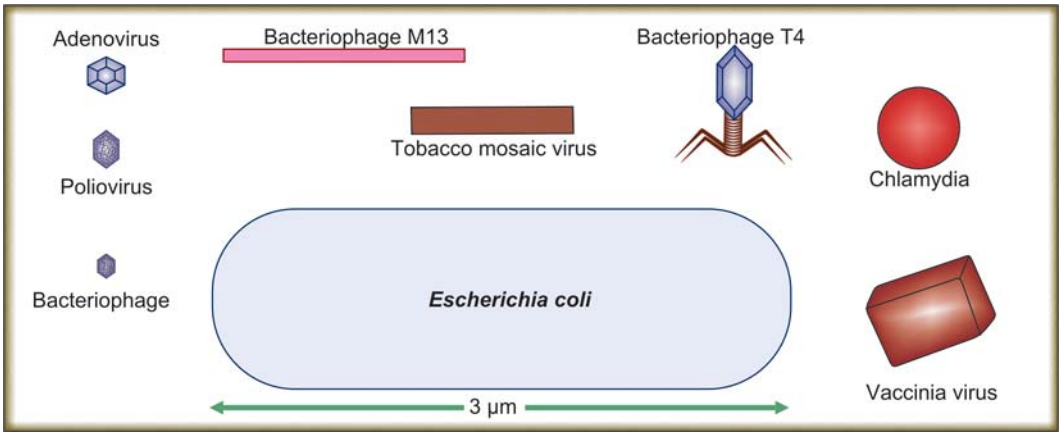


Fig. 57–1. Variations in shapes and sizes of viruses as compared to *Esch. coli*

other RNA; some are double stranded (ds), others are single stranded (ss), some are linear, others circular; some have plus polarity others have minus polarity. A nucleic acid that encodes the information for making proteins needed by a virus is called a *positive sense nucleic acid.* A nucleic acid made up of bases complementary to those of a positive sense nucleic acid is called a *negative sense nucleic acid.*

Content and Size of Nucleic Acid

There is wide variation in content of nucleic acids in viruses. In poxviruses, the DNA constitutes only 5% of the virus whereas in picorna virion the RNA constitutes about 25%. The largest weighing nucleic acid is that of poxviruses and smallest of picornaviruses, parvoviruses and togaviruses.

Structure of Viral Nucleic Acid

Both double stranded as well as single stranded DNA or RNA can act as genome of viruses. Most viral nucleic acids are linear but papovavirus DNA exists in the form of double stranded supercoiled circles. Most of the viruses have genomes which consist of unbroken strands of nucleic acids with the exception of reoviruses which have 10 segments of double stranded RNA and influenza virus which consists of eight segments of ssRNA.

Infectivity of Nucleic Acid

The information required for the replication of virus is lodged in the nucleic acid. The naked nucleic acid of some families of viruses such as Picornaviridae, Togaviridae, Papovaviridae, Adenoviridae, and Herpesviridae is infectious. The infectivity of naked nucleic acid is usually less than that of the virion because the naked nucleic acid is quickly acted upon by nucleases which are present in extracellular fluids as well as on outer cell membranes and also because of the fact that uptake of naked nucleic acid is poor by the host cell.

Capsids

These are the aggregates of repeating subunits arranged in a defined pattern. The subunits are called as the capsomers. The capsomers may be made up of a few molecules of protein or, in their simplest form, may comprise one protein molecule. The presence of repeating subunits helps the virus in two ways. One, the genetic information required to synthesize such molecules is minimum and secondly the assembly of similar units occurs more efficiently.

The capsid has two functions:

a. To protect the genome from external harmful factors or agents
b. To introduce the viral genome into the host cells.

Similar functions are also performed by the lipid envelope of the virus.

Envelope

In some viruses the capsid is covered by a lipid envelope (Fig. 57.2). Sometimes proteins are also present in the envelope. The envelope proteins are invariably coded by the virus.

Depending on the virus, envelopes may or may not be covered by spikes (Fig 57.2). The spikes are carbohydrate-protein complexes that project from the surface of the envelope. The ability of certain viruses, such as the influenza virus, to clump red blood cells is associated with spikes. Such viruses bind to red blood cells and form bridges between them. The resulting clumping is called as *haemagglutination* and is the basis for several useful laboratory tests.

Viruses whose capsid is not covered by an envelope are known as *naked viruses* (Fig. 57.2). In naked viruses, it is the capsid that protects the nucleic acid from nuclease enzymes in biological fluids and that promotes attachment to susceptible host cell.

Symmetry of Viruses

The viruses have been shown to demonstrate two types of symmetries—helical and icosahedral (or cubic or polyhedral). The helical nucleocapsids measure 10-20 nm in diameter whereas the icosahedral capsids show considerable diversity.

Icosahedral Symmetry

An icosahedron is a platonic solid of geometry and consists of 12 vertices, and 20 faces each an equilateral triangle. The capsomers are arranged precisely according to icosahedral patterns characterized by 5:3:2- fold

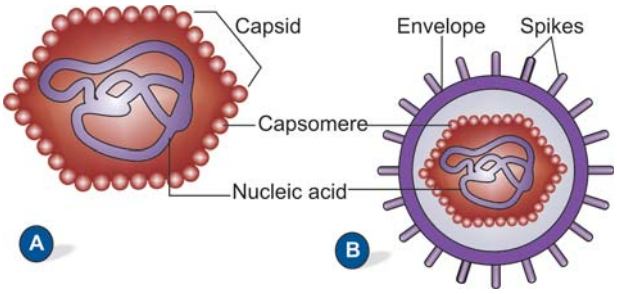


Fig. 57–2. Naked virus (A) and enveloped virus with spikes (B)

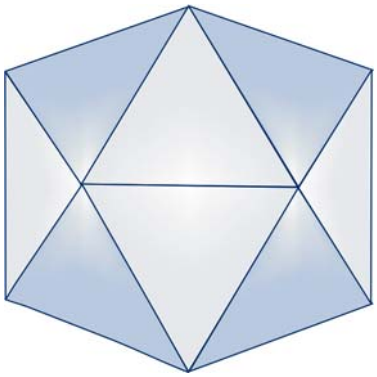


Fig. 57–3. An icosahedron

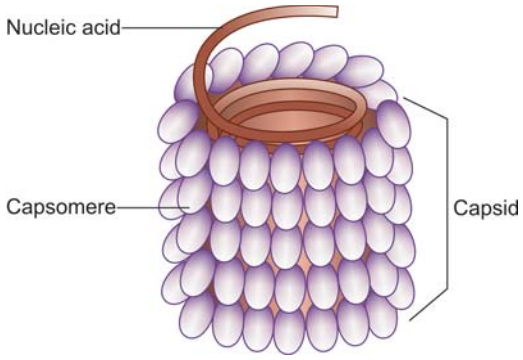


Fig. 57–4. Morphology of helical virus

rotational symmetry. Seven such patterns are found among animal viruses. The icosahedral pattern gives a strong structure to enclose a maximum volume in capsid (Fig. 57.3).

Helical Symmetry

Helical viruses resemble long rods that may be rigid or flexible. This is best exemplified by tobacco mosaic virus in which a nucleic acid of the shape of a filament is surrounded by protein molecules arranged helically so as to yield a structure with a single rotational axis (Fig 57.4). Rabies virus is an important example of this type of symmetry.

Complex Viruses

Some viruses have a very complicated structure and are referred to as complex viruses. Examples of complex viruses are poxviruses, which do not contain clearly identifiable capsids, but have several coats around the nucleic acid.

Viral Proteins

Viral proteins can be broadly divided into two groups:

a. Structural proteins
b. Non-structural proteins (functional proteins).

Structural proteins are the proteins present in the coat of the virus whereas the enzymes and haemagglutinins are known as *non-structural proteins*.

Haemagglutinins

These proteins are present in certain viruses such as picornaviruses, togaviruses, reoviruses, myxoviruses, adenoviruses and papovaviruses and agglutinate erythrocytes of various animal species. This property is endowed by the glycoproteins present on envelope.

Enzymes

Four groups of enzymes have been detected in the viruses. These are:

a. Neuraminidase
b. RNA polymerase
c. Reverse transcriptase
d. Enzymes of cellular origin

Viral Lipids

The lipids present in the viruses originate from the cell membrane of the host and hence their composition shall be same as that of the cell membrane. Lipids constitute about 35% of the dry weight of enveloped viruses.

Viral Carbohydrates

Carbohydrates are present in viruses as parts of nucleic acids, glycoproteins on the surface, glycoproteins in the core (as in poxviruses) and in the outer capsid proteins of rotaviruses.

Preservation of Viral Infectivity

Viruses are more sensitive to environmental changes than other organisms. Temperature, pH and lipid solvents affect viruses and their infectivity.

Temperature

As a general rule viruses can survive for seconds at 60°C, minutes at 37°C, hours at 20°C, days at 4°C and years at –70°C or lower temperature. Accordingly, virus preparations must be stored at low temperature to maintain their infectivity. Viruses can be kept for few days at 4°C (ordinary refrigerator) but longer preservation shall require storage at sub-zero temperature. Deep freezers with temperatures upto –80°C are

available. Temperatures of –70°C can be obtained by dry ice (*frozen carbon dioxide*) and –80°C is the temperature of liquid nitrogen. Enveloped viruses are more heat labile than nonenveloped viruses.

pH

The viruses prefer physiological pH and any alteration in this results in deterioration of infectivity of virus.

Lipid Solvents

Ether or chloroform, detergents and any lipid solvent on coming in contact with viruses (especially enveloped viruses) destroy them.

Measuring the Size of the Viruses

Because some bacteria may be smaller than the largest viruses, hence filterability is no longer regarded as a unique feature of viruses. The methods used for determining the size of the viruses and their components include:

i. Direct observation in an electron microscope. Electron microscopy is the most widely used method for estimating particle size.
ii. By filtering through membranes of graded porosity.
iii. By sedimentation in an ultracentrifuge where particles settle to the bottom at a rate that is proportionate to their size.
iv. By comparative measurements, e.g. comparing the size with that of Staphylococcus, etc.

58 Replication of Viruses

Viruses are obligatory intracellular parasites. They do not possess any machinery which may be of help to them in synthesizing their nucleic acids or proteins the genetic information for which is present in the genome of the virus. The viruses make use of the metabolic machinery of the host cell to undertake these processes. The replication of viruses takes place in a systematic manner and the whole event can be divided into a number of stages:

- Attachment of virus to host cell
- Penetration
- Uncoating
- Transcription of early mRNA
- Translation of early proteins
- Replication of viral genome
- Transcription of late mRNA
- Translation of late proteins
- Assembly of virions
- Release.

Attachment of Virus to Host Cell

This process is also called as binding or adsorption. This is a specific process wherein the structures present on the surface of the virus (sometimes called as ligands) bind to the receptors on the surface of the host cell. The presence of appropriate receptors on the cell surface is a prerequisite for virus attachment and subsequent infection.

Penetration

There are four possible mechanisms which the viruses use to enter a host cell. These are:

1. *Translocation* is the process by which the whole nonenveloped virus enters the host cell by moving across the cell membrane.
2. *Endocytosis* is the engulfment of the virus by the invagination of a section of plasma membrane. This is a common mode of penetration of nonenveloped viruses.
3. *Fusion* is the endocytosis of enveloped viruses in which the envelopes of the viruses fuse with the membrane of the endosome.
4. *Direct fusion of the virion envelope* with the surface membrane of the cell also takes place in some of the families of viruses.

Certain naked viruses (e.g. *enteroviruses*) are taken into the cells by *viropexis*, a process that is similar to phagocytosis.

Some of the common mechanisms of entry of viruses in host cells have been shown in Figure 58.1.

Uncoating

Immediately after entry into the host cell, interaction of the virion with host components and enzymes leads to a breakdown of the virion and exposure of the genome so that it can express.

Transcription of Early mRNA

Transcription of early mRNA is required to produce certain enzymes and proteins which are necessary for the replication of viruses and that of late mRNA is for viral structural proteins. The DNA viruses which replicate in the host nucleus, use the host DNA dependent RNA polymerase to perform this function. All other viruses require virus coded transcriptase to synthesize required mRNA (Fig. 58.2).

Translation of Early Proteins

The viral mRNAs use the ribosomes of the host cell to synthesize proteins in the same way as the host mRNA.

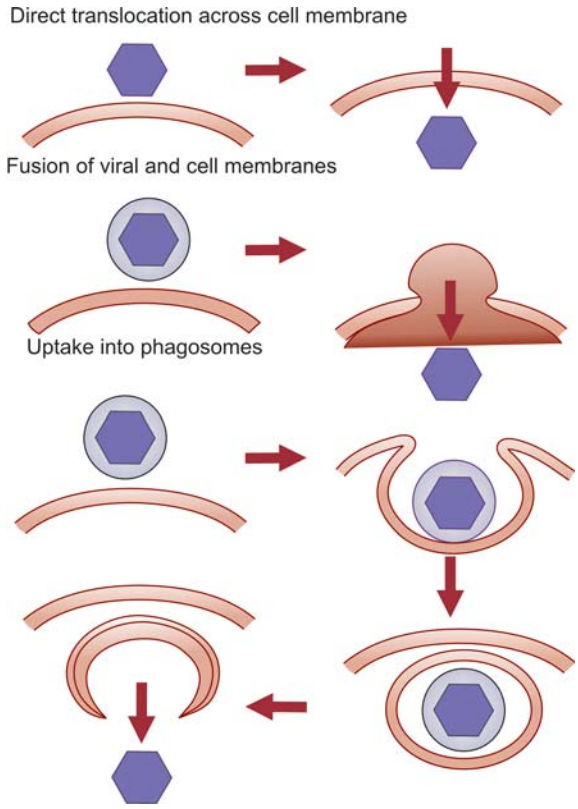


Fig. 58–1. Entry of viruses into host cells

A wide variety of proteins are synthesized and their production is regulated by a mechanism at the level of translation. Two types of proteins are synthesized: *early* and *late*, depending upon their time of appearance.

Replication of Viral Genome

Viruses reproduce from their nucleic acid, which directs not only its own replication but also the synthesis of virus specific proteins. Some of these are structural components of the virion, while others are proteins leading to the selective shutdown of synthesis of cellular macromolecules or enzymes involved in the synthesis of viral components. The viral nucleic acids replicate by Watson-Crick base pairing.

Transcription of Late mRNA

This is required for the production of structural proteins. The cytoplasmic double stranded DNA carries DNA dependent RNA polymerase where ssRNA viruses make use of their own ssRNA dependent RNA polymerase to transcribe late mRNA.

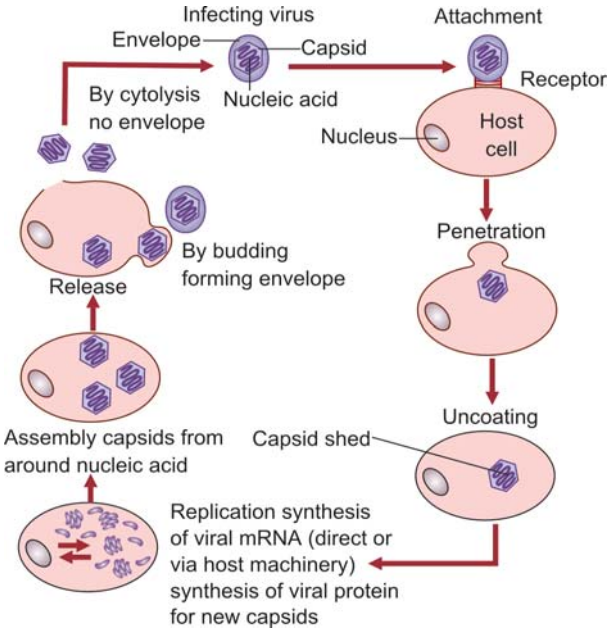


Fig. 58–2. Infection and replication of viruses

Translation of Late Proteins

These result after the synthesis of late mRNA and are usually the result of instructions of the newly formed progeny of the virus in the host cell.

Assembly of Virion

Virion assembly may take place in the host cell in the cytoplasm or the cell nucleus. Herpesviruses and adenoviruses are assembled in the nucleus whereas the picornaviruses and the poxviruses are assembled in the cytoplasm of the host cell.

The association of nucleic acid and capsid (which is called as *procapsid* till this stage) is a complex mechanism which has been studied in details for adenoviruses. The viral DNA has a packaging sequence which controls this process and enables the virus to enter the procapsid.

The acquisition of envelope by the viruses is through the process of budding through the cellular membrane which is regarded as a nonphysiological form of exocytosis (Fig. 58.3).

RELEASE

There are three mechanisms by which mature virions can escape from the host cell and start their cycle once again in new host cells. These are:

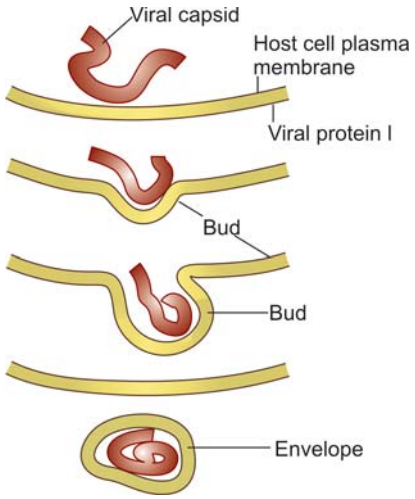


Fig. 58–3. Budding of an enveloped virus

Cell Lysis

This occurs with most of the nonenveloped viruses when the cell lysis occurs after the completion of the replication of the viruses. This cell lysis is not the result of natural death of the cell but is because of the large number of viruses in the host.

Cell Degeneration

Many viruses such as parvoviruses accumulate within the nucleus of the host and are released only after the death of the cell which follows the degeneration of the cell.

Budding

This is the mechanism by which most of the enveloped viruses exit from the host cell without damaging the cell (Fig. 58.3). This process takes a long time and does not kill the host cell.

Some of the enveloped viruses are also lytic in nature (alpha herpesviruses) and certain viruses may be released in enveloped form by budding or in non-enveloped form by cell lysis. Both these forms are infectious.

Abortive Viral Infections

Viral infections of the host cells are not always productive. Infection of a cell with a virus shall not necessarily result into production of large number of viruses. Either the viral coded products are not synthesized or these are not properly assembled. Such processes are known as *abortive infections.* These may be due to defect in the host cell which is unable to manufacture products as per the demands of the virus or because of an inherent fault in the virus itself.

DEFECTIVE VIRUSES

Certain viruses which are unable to replicate themselves in a responsive host cell are called as defective viruses. Some of these can replicate in the presence of a *helper virus.* Adeno-associated viruses are important examples because these cannot multiply unless adenovirus is present in the same host cell.

The extreme example of defectiveness is exhibited by papillomaviruses which on infecting a cell either replicate as *episomes* or get integrated into the chromosome of the host and replicate with it. The virus may express its genetic information but fails to produce its progeny on its own.

General points regarding viral replication are given in Table 58.1.

Table 58–1: General features about viral replication

- Occurs only in living cells
- Involves many host cell enzymes and functions
- Follows a sequential pattern as described above
- May be incomplete in some cells (abortive infection)
- May lead to the death of host cells (virulent viruses)
- May occur without apparent damage to the host cell (moderate viruses)
- Is very similiar for all viruses in a specific family

59 Cultivation and Assay of Viruses

Since viruses are obligate intracellular parasites, a living cell system is required to cultivate them. Three kinds of such systems have been developed over the years.

- Laboratory animals
- Embryonated eggs
- Cell lines (Tissue cultures)

Laboratory Animals

The first method that was used to grow viruses was their inoculation into animals. This method has now gradually disappeared because of inherent disadvantages (Table 59.1) and availability of cell cultures which are simple to handle and much more versatile. However, animals are still used for various functions in virological studies.

Embryonated Eggs

The eggs have been found to be much simpler to handle than animals, are inexpensive and easily available. Suitable cells for the growth of viruses are present in the embryo and its membranes. Since these eggs do not have a well developed defence mechanism of their own, they usually do not interfere with the growth of viruses.

The chick embryo used are usually 8-11 days old. After inoculation these are incubated for 2-9 days, the duration depending upon the virus type and the route of inoculation.

Viruses may kill the chick embryo or produce specific evidence of viral activity such as production of pocks on chorioallantoic membrane which helps not only in isolation and diagnosis of poxviruses but also in enumerating the same since one pock is the result of activity of one viable virus. The haemagglutinating activity in amniotic or allantoic fluids may reveal the presence of influenza or related viruses (Table 59.2).

The allantoic and amniotic cavities are used even today for the research and production of vaccine of influenza virus because of the high yield provided by this cavity (Fig. 59.1).

Despite many advantages with embryonated eggs, this method of cultivation does not permit a controlled study of virus-cell interaction and there are many viruses which fail to grow on primary inoculation into the eggs. However, the embryonated eggs remains one of the best host systems for influenza A virus.

Cell Lines (Tissue Cultures)

Various types of techniques and cultures (e.g. whole organ, explant cultures etc.) have been used in the past.

Table 59–1. Use of animals in virology

Disadvantages	*Advantages*	*Animals still in-use*	*Function*
• Expensive • Difficult to handle • Require meticulous maintenance • Show biological diversity • May be infected with latent viruses • Pressure from animal friends groups	• Isolation of those viruses which do not grow in cell lines/eggs • Understanding pathogenesis • Understanding immune response • Studies on efficacy of vaccines and drugs studies	• Suckling mice • Rabbits • Hamsters • Monkeys	• Isolation of dengue/other arboviruses • Basic research on various viruses

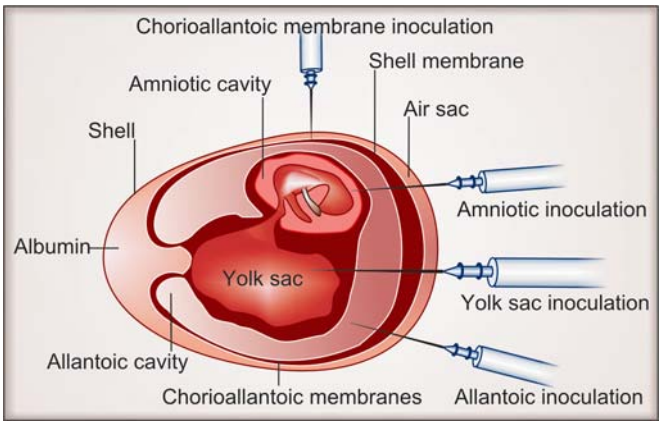


Fig. 59–1. Embryonated egg showing routes of inoculation

Nowadays most of diagnostic and research work is carried out in cell cultures—usually in monolayers. Monolayers are produced from desired tissues by cutting them into small pieces and treating with a proteolytic enzyme, e.g. trypsin. The cells disperse into single cell suspension and these are settled onto the surface of glass or plastic container where nutrition to these is provided by a growth medium which contains all the essential nutrients and serum.

Types of Cell Lines

The cell lines have been classified according to the number of divisions which a cell line can undergo *in vitro* before dying out. At present three broad types of cell lines are in use.

Primary cell cultures. These cell cultures are capable of only 5-10 divisions at the most. Since these contain tissues taken directly from animals, a large variety of cells are present in it which makes this type of cell cultures as very sensitive to the growth of numerous viruses. These cell cultures suffer from the drawbacks of having latent viruses present in the animal tissues; limited number of divisions, i.e. short life and high cost.

Diploid cell strains. These cells can undergo many more cell divisions than primary cell cultures before dying out. The number of divisions is roughly related to the life span of the species of animal from which it has been derived. The human diploid cells undergo around 50 divisions. These cells have been widely used for the diagnostic purposes as well as for the commercial production of some vaccines such as rabies vaccine.

Continuous cell lines. These cell lines are also called as *immortal* cell lines and these originate from malignant cells or by the spontaneous transformation of a diploid cell strain. These cell lines do not resemble their cells of origin because of numerous mutations that these undergo during their prolonged culture.

Continuous cell lines have been derived from monkeys (vero cell line), dogs, cattle, pig, cat, mouse, hamster and rabbits. These carry the great advantage of indefinite propagation by subculturing at regular intervals. At –196°C these remain viable for years. When large number of cells are required the continuous cells can be grown in suspension culture. Recently evolved useful method of growing cells on a large scale for vaccine production is on plastic or Sephadex beads (*microcarriers*) maintained in suspension in large fermentation tanks. Various cell lines that have been utilized for isolation of viruses are described in Table 59.3.

Uses of Cell Cultures

Cell cultures serve three important purposes:
1. Isolation of viruses from clinical samples for diagnosis
2. Production of vaccines and antigens
3. Biochemical studies of viral replication.

Table 59–2. Viruses grown in chick embryo

Route of inoculation	*Virus*	*Lesion on CAM*	*Haemagglutination*
Chorioallantoic membrane (CAM)	Poxviruses	+	–
	Herpes simplex	+	–
	Herpesvirus B	+	–
Amniotic cavity	Influenza virus	–	+
	Mumps virus	–	+
Allantoic cavity	Newcastle disease	–	+
	Influenza virus	–	+
	Mumps virus	–	+
Yolk sac	JE virus	+	–
	West Nile virus	+	–

PART VIII

Table 59–3. Isolation of viruses from cell lines

Type of cell line	*Virus isolated*
Primary	
African green monkey	HSV, RSV, Mumps, Rubella
Chick embryo fibroblast	Rabies, poxviruses
Diploid Cell	
Human foetal lung (WI-38, MRC-5)	Rabies, Adeno, CMV
Continuous	
HeLa	Polio, Pox, Reo, RSV
HEp-2	Adeno, RSV
MDCK	Influenza
RD	Polio, enteroviruses
Vero	Polio, Rabies, Measles

Recognition of Viral Growth in Cells

These can be detected by the following four techniques:
1. Cytopathic effect (CPE)
2. Haemagglutination and haemadsorption
3. Immunofluorescence
4. Interference

Cytopathic effect (CPE). Many viruses kill the cells in which they grow and some bring about detectable changes in the morphology of the host cells. All these effects are collectively called as cytopathic effects. Some viruses, however, do not produce detectable CPE. CPEs can be visualised under the microscope in both stained and unstained preparations. Followings are the main types of CPEs:
- Rounding of cells (picorna viruses)
- Syncytium formation in which the membranes of adjacent cells fuse to form giant cells containing several nuclei (measles and respiratory syncytial viruses)
- Rounding and aggregation of cells into grape like clusters (adenoviruses)
- **Inclusion bodies** which are intracytoplasmic or intranuclear aggregates of viral replication and can be seen only after staining (Fig. 59.2).

Intranuclear inclusion bodies are produced by adeno- and herpesviruses whereas vaccinia and rabies viruses produce intracytoplasmic inclusions. Inclusion bodies of measles are seen in both the locations.

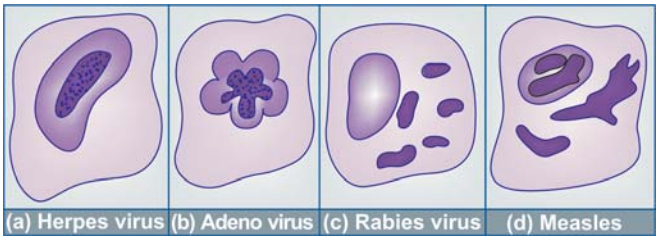


Fig. 59–2. Viral inclusion bodies

Haemagglutination and haemadsorption. The property of adsorbing to erythrocytes is exhibited by myxoviruses and togaviruses and can be demonstrated after 24 hours of infecting the cell. Haemagglutination is a related phenomenon and virion as well as haemagglutinin are demonstrable in the supernatant fluid of an infected culture.

Some viruses cause little or no CPE. However, during the process of infection the cell membranes become altered in such a way that erythrocytes of some animal species will firmly attach to them when added to the culture. This phenomenon is called as haemadsorption and is commonly seen with members of families orthomyxoviridae and paramyxoviridae.

Immunofluorescence. By this technique newly synthesized viral antigen can be detected by employing specific antiviral antibody coated with a fluorescent dye. Rabies antigen is quickly detected by this method in specimens of brain and spinal cord.

Interference. The phenomenon of interference is sometimes used for diagnosis of some viruses which do not multiply in the presence of another virus in the same host (interfering virus). However, now such cell lines are available which permit the growth of these viruses.

Assay of Viral Infectivity

The measurement of the infectivity of the virus is an important condition for any scientific work on viruses. Various techniques are now available and used according to the facilities available. Some of these are:
- Electron microscope count
- Quantitative assay
  - — In eggs
  - — In cell cultures
  - — By plaque formation
- Haemagglutination assay

Electron Microscope Count

This is the ideal method for counting the virus particles and most sensitive of all the available techniques. Negative staining with potassium phosphotungstate makes it possible to count the number of particles in viral suspension by electron microscopy.

Quantitative Assay in Eggs

This technique is sometimes used to titrate poxviruses on the chorioallantoic membrane of the chick embryo. Each viable infecting particle gives rise to a pock.

Quantitative Assay in Cell Cultures

Serial dilutions of virus are inoculated into cell cultures and adequate time is allowed for virus to replicate and produce its effect (death or CPE). The end point of a quantal titration is taken to be that dilution of virus which infects 50% of the inoculated cells ($TCID_{50}$).

Quantitative Plaque Assay

Plaque assay is a standard procedure for the quantitation of most animal viruses. Viral suspension is inoculated onto monolayer and sufficient time is given to the virions to adsorb to the cells. The infected cells are overlaid by agarose to restrict the spread of virus to adjacent cells. After some time each infective particle gives rise to a localised focus of infected cells that soon becomes large enough to be seen with naked eyes as area of cytopathology. Infection with a single viable virus is sufficient to form such a focus which is called as plaque (Fig. 59.3) and the infectivity titre is expressed in terms of plaque forming units (PFU) per ml.

Haemagglutination Assay

Some viruses have certain substances on their surfaces (usually in the envelope) which cause agglutination of erythrocytes obtained from different animal species.

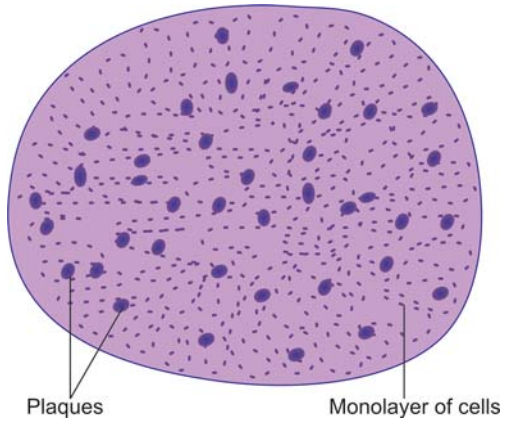


Fig. 59–3. Quantitative plaque assay

This property is made use of as a crude method to assay the infectivity of some viruses such as influenza virus. About 10^7 influenza virions are required to produce haemagglutination which indicates that this is not a good indicator for the presence of small number of virions.

If a given preparation of virus suspension is titrated by all the methods described above, the results will not be uniform. Electron microscopy shall provide the best and most accurate results and haemagglutination most crude result.

60 Genetics of Viruses

Viruses can vary their genomic structure by either of the following two methods:

- Mutations
- Recombination

In addition interaction between viral gene products can give rise to new phenotypes of viruses.

MUTATIONS

As with other living organisms, most important and frequent changes in the nucleic acids are due to mutations. Viruses multiply into millions of progeny and while the genome of parent is being copied into that of progeny, chances of error do occur. Such mutated viruses may not survive if the mutation proves to be lethal. Survival of the progeny with mutation is facilitated if the mutation provides a selective advantage and if it occurs early in the course of infection. General principles of mutation are same as have been described in Chapter 5 on Bacterial Genetics.

GENETIC RECOMBINATION BETWEEN VIRUSES

Four types of genetic recombinations may be seen whenever a host cell is infected by two virions. These are:

- Intramolecular recombination
- Reassortment
- Reactivation
- Marker rescue or cross reactivation

Intramolecular Recombination

This is a common occurrence in double stranded DNA viruses and retroviruses. Rarely it has been seen in poliomyelitis virus also. This usually occurs between two closely related viruses. It has not been seen in single stranded DNA viruses.

Reassortment

This occurs only in those viruses which have segmented genomes such as *Arenaviridae* and *Birnaviridae* (2 segments each), *Bunyaviridae* (3 segments), *Influenza-virus* A and B (8 segments) and *Reoviridae* (10-12 segments). An exchange of segments occurs between various viruses resulting into synthesis of various stable reassortments. This phenomenon also occurs in nature and results in genetic variation in viruses.

Reactivation

In this phenomenon two virions of same strain infect a host. Both the virions are having lethal mutation in different genes and coinfection may result into viable recombinants. This has been seen in poxviruses, orthomyxoviruses and reoviruses.

Cross Reactivation or Marker Rescue

It occurs when an infectious virion and an inactivated virus infect a host cell simultaneously. By the processes of gene transfer, discussed above, some infectious virion may be produced which has got active genes from both viruses.

Once a recombinant has been produced it continues to produce its yield. Recombinants may occur between two infectious viruses; one infectious and another non-infectious virus and two noninfectious viruses.

INTERACTION BETWEEN VIRAL GENE PRODUCTS

These can be of three types:

- Complementation
- Phenotypic mixing
- Interference
- Polyploidy

Table 60–1. Viral genetic interactions in host cells

Interaction	*Description*
Complementation	When two viruses with defective genes or mutations cannot multiply alone, they may complement each other to produce progeny comprising both mutants. Permits survival of viruses with nonfunctioning or defective genes.
Interference	In mixed infections, there is usually replication of one virus
Reactivation	The damaged viruses cooperate with each other to produce intact genomes
Cross reactivation	Simultaneous infection with infectious and inactivated virus can yield progeny with mixed genotype
Phenotypic mixing (Transcapsidation)	When two closely related viruses infect the same cell, the resultant capsid may be hybrid or may be of the other virus (masked or transcapsidated)
Recombination	Usually occurs in ds DNA viruses; involves reassortment of nucleic acid segments so that some progeny has a unique combination of genes derived from both parents

Complementation

This includes all those interactions in which when two viruses infect a cell, their gene products such as proteins and enzymes complement the action of each other in such a way so as to have increased yield of one or both viruses. This can occur even between unrelated viruses. There is no genetic interaction and the progeny are like the parent viruses.

Phenotypic Mixing

Following infection of a host cell by two genetically different virions, some of the progeny may have phenotypic characters of both the parents though their genome may be true copy of the parent. Envelopes of some of the viruses have been shown to have antigens derived from both the parents. The phenomenon of *transcapsidation* in nonenveloped viruses is also an example of this type of exchange. In it, both partial or complete exchange of capsids may occur.

Interference

Interference indicates inhibition of growth of one virus in a host when it is simultaneously infected with another virus. This is usually mediated by interferon. In contrast, mixed infections sometimes increase the yield of one virus. This phenomenon is called as *enhancement*.

Polyploidy

All the viruses of the vertebrates are haploid except retroviruses which are diploid. But sometimes within one envelope many nucleocapsids get enclosed and this phenomenon is known as polyploidy.

Genetic interactions that occur between viruses and hosts are summarised in Table 60.1.

GENETIC VARIATION IN NATURE

The large number of viruses in nature and evolution of new viruses is believed to be the result of mutation and genetic exchange through the processes of reassortment, reactivation and intramolecular recombination. Reassortment is now considered to be the major mechanism for changes that are seen in nature in a wide variety of viruses including rotaviruses, influenza virus A and bunyaviruses.

VIRAL VECTORS

These can be constructed with recombinant DNA technology and allow gene transfer into cells. These are usually defective viruses that cannot replicate but can infect cells. These have been used for the production of some vaccines, e.g. hepatitis B vaccine.

61 Viral Pathogenesis and Host Response

Pathogenicity of a virus (sometimes also called as its virulence) is the capacity to produce the disease. This encompasses entry into the host, multiplication therein and production of harmful effects which may prove fatal for the host at times. All the viruses, in spite of being obligatory intracellular parasites, are not pathogenic. Some are absolutely nonpathogenic *(avirulent)* and a few are poorly pathogenic *(attenuated)* because they lack ability to infect the host or produce the harm. Clinical manifestations of viral infections are also diverse.

PATHOGENICITY OF VIRUSES

Unlike bacteria, viruses do not produce toxins and enzymes to damage the host cells. To cause disease a virus has to clear a number of hurdles that vary somewhat in type and number according to the virus concerned and its host. The following sequence of events is typical. The virus must:

- *Invade* the host
- *Establish a bridgehead* by replicating in susceptible cells at the site of inoculation
- *Overcome the local defenses,* e.g. lymphocytes and interferon
- *Spread* from the site of inoculation to other areas often via bloodstream
- *Undergo further replication* in target areas which can be localised or generalised
- *Exit* from the host in large enough numbers to infect other susceptible hosts.

Viral Entry into Host

Most of the viruses enter the host through skin or mucous membrane of the respiratory tract and gastrointestinal tract. Few viruses also gain access to body through urogenital tract and conjunctiva (Table 61.1, Fig. 61.1).

Table 61–1. Routes of entry of viruses

Route	*Virus*
Skin	
— After break in continuity	Rabies
— Through insect bite	JE, dengue
— Through infected needle	HIV, Hepatitis B
— Biological means	Papova viruses
Respiratory tract	Influenza
	Measles
Gastrointestinal tract	Enteroviruses
	Rotavirus
	Hepatitis A and E
Conjunctiva	Adenovirus 8
Genitourinary tract	HIV, Hepatitis B
	Herpes simplex

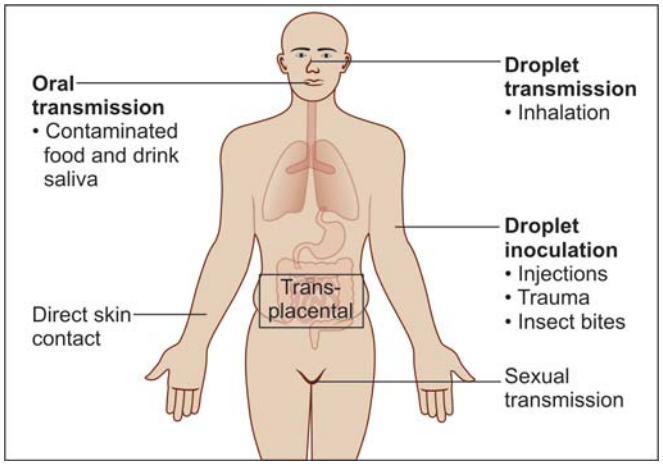


Fig. 61–1. Routes by which viruses enter the body

Systemic Spread of Viruses

Viruses may remain localised to the body surface through which they entered (i.e. skin, respiratory tract, digestive tract, etc.) or may cause generalised infections which are associated with *viraemia* and subsequent localisation in *target organs*.

Viraemia

After traversing the surface epithelium from their portal of entry, viruses reach subepithelial tissues where they are exposed to tissue macrophages and can subsequently enter the lymphatics. Through lymphatics most of the viruses reach the blood stream. This stage is called as *viraemia*. In blood, viruses may move freely in plasma or may associate with various types of leucocytes, platelets and erythrocytes. Viraemia is usually characterised by fever. Interval between the first entry of infective virus and the first onset of fever and clinical features is called *incubation period*.

Target Organs

The important target organs are skin and central nervous system. Other organs such as liver, muscles and heart may also get infected.

Skin. Skin is one of the principal target organs for viral attacks and rashes are characteristic of many acute viral infections. A few important viruses which cause generalised infection with rashes have been shown in Table 61.2.

Central nervous system. The virus can reach the central nervous system either through haematogenous route or through neural route. The commonly encountered such viruses are shown in Table 61.3.

Table 61–2. Generalised viral diseases with rashes

Type of rash	*Viral infection*
Maculopapular	Measles Echovirus Rubella Coxsackie A
Vesicular	Herpes simplex Varicella Zoster Vaccinia Coxsackie virus
Haemorrhage	Smallpox Measles Arboviruses

Table 61–3. Viruses which attack CNS in man

Haematogenous route	*Neural route*
Poliovirus	Rabies
Enteroviruses	B virus
Coxsackie virus	
ECHO	
Herpes simplex (in children)	
Rubella (in foetus)	
Cytomegalovirus (in foetus)	

Liver. Liver is another organ which is targetted by circulating virions. Infective virions of hepatitis, some poxviruses and probably the Epstein-Barr virus of infectious mononucleosis have been shown with the help of immunofluorescence to be phagocytosed by Kupffer cells where they multiply before penetration and generalised invasion of the substance of the liver.

Damage to Host

Once the virus has overcome the defense mechanisms of the host it can damage the host tissues and produce a mild, severe or fatal illness. Although cells can be damaged by a passive role of virus, two main mechanisms probably account for cell damage:

1. Direct cytotoxicity
2. Immunopathological reactions

There is no evidence that viruses produce circulating toxins, as do bacteria. There is, nevertheless, increasingly accumulating evidence that infected cells are damaged by the purely local action of virion components and possibly virus specified products, both of which might therefore be called viral cytotoxins. There can be various reactions of host cells to viral infection (Table 61.4).

It is well known that immune response can destroy virus infected cells. Usually the number of cells involved is small and the damage is minimal. Sometimes a large number of cells are destroyed which result into illness or even death of the host. Virus infected cells may be damaged by either type II (antibody mediated) or type IV (immune cell mediated) cytotoxic reactions. Uninfected host cells also get damaged by the immune response evoked by host antigen in viral envelopes or by type I (anaphylaxis) and type III immune reaction.

HOST RESPONSE TO VIRAL INFECTIONS

The host response to viral infections can be:

- Specific immune response
- Non-specific resistance.

The specific immune response has two arms: antibody mediated immunity and cell mediated immunity.

Table 61–4. Reaction of host cell to viral infection

Reaction	*Representative viruses causing these effects*
Death of host cell	Most viruses
Proliferation of host cells	Poxvirus Papovaviruses Papillomavirus
Fusion of membranes of adjacent cells to form multinucleate cells	Respiratory syncytial virus Measles virus Herpesvirus Human immuno-deficiency virus (HIV)
Transformation of normal cells into malignant cells	Polyomaviruses Herpesvirus Adenovirus Retroviruses
No histologic change in host-cell appearance	Rubella virus Some adenoviruses

All these processes play their role in protecting the human body from the viral infection.

Specific Immune Response

This response is also known as specific response and is of two types (Fig. 61.2):

Antibody mediated immunity. The important immunoglobulins which protect against viral infections are IgG, IgM and IgA. The antiviral activity of IgG and IgM is due to their ability to neutralise the processes of viral attachment, penetration and uncoating. IgA is produced locally by the plasma cells in the mucous membranes of respiratory and digestive tracts. Although its major activity is protection at local level, it is also transported as a dimeric unit into bronchial secretions, saliva and intestinal fluids. IgA plays a major role in providing a barrier to the systemic spread of viruses from localised surface areas.

Cell mediated immunity. This is primarily mediated by T cells which have virus specific receptors on their surface. On coming in contact with the virus, T cell gets activated and converts itself into lymphoblast and secretes specific as well nonspecific soluble factors known as *lymphokines*. Certain other cells such as NK cells, killer cells and macrophages directly attack the viruses.

There are three important mechanisms through which immune system asserts itself. These are:

1. Destruction of infected cells
2. Production of interferon
3. Neutralisation of viral infectivity.

After some virus infections a life long immunity may result whereas with few it may last only for a few weeks. When there has been an extensive phase of viraemia, a prolonged immunity may result specially so if the virus is antigenically stable and monospecific. This is true for smallpox, measles, mumps and yellow fever and hence second attack with these diseases is rare.

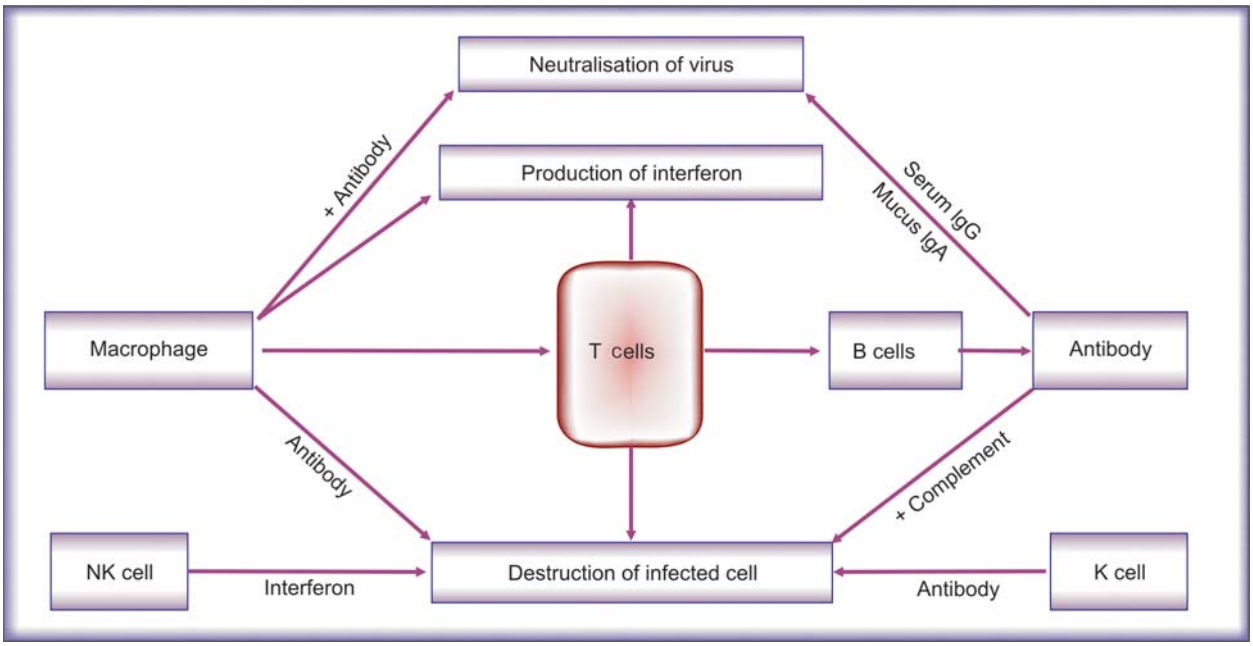


Fig. 61–2. Immune response to viral infections

Nonspecific Resistance

These include many important physiological defenses (Table 61.5).

Table 61–5. Nonspecific resistance

- Phagocytosis
- Interferon
- Body temperature
- Hormones
- Nutrition
- Age
- Stress

Outcome of Viral Infections

The interplay between the virus and the host can result into different effects. Viruses like measles, smallpox and varicella can produce disease in almost all the susceptible people. In many other infections, most of the infected people will not manifest any clinical disease. Perhaps most outstanding example is that of Japanese encephalitis where for every clinically apparent case, there may be hundreds of infected people without any signs and symptoms. The *inapparent* or *subclinical* infection can be detected by serological assays.

Latent infections are of long duration and usually inapparent. They result when an equilibrium has been established between the virus and the host. *Slow viral infections* are characterised by progressive tissue damage continuing throughout a long incubation period and are probably sequence to acute infections.

Patterns of infections caused by viruses can be categorised in three broad groups, within each of which there are different clinical patterns (Table 61.6).

Laboratory Diagnosis of Viral Infections

Specific diagnosis of viral infections can be achieved by one of the following three methods:

- Isolation and characterisation of causative virus.
- Direct demonstration of virion, viral antigen or viral nucleic acid in tissues, secretions or excretions.
- Detection and measurement of specific antibodies.

Virus Isolation

This is the benchmark against which other methods are measured. This process requires minimum of a week and is expensive. The virus from the clinical material is cultivated in the laboratory on cell cultures, embryonated eggs or laboratory animals. Cultured cell lines are preferred nowadays and one has to be careful in selecting suitable cell line. The growth of the virus in cell line may exhibit typical cytopathic effect (CPE). Help of tests like immunofluorescence and haemagglutination is taken to confirm the diagnosis. Neutralisation of the isolate can be done with specific antiserum and if it occurs, it can be considered as the final proof of isolation of that particular virus.

Direct Demonstration of Virus

This includes demonstration of virion, viral antigen and viral nucleic acid. The techniques currently available are shown in Table 61.7.

Detection and Measurement of Antiviral Antibodies

The principles which are applicable in serology of bacterial infections are applicable for viral diseases also. Serology can be used for the detection of antibody for

Table 61–6. Patterns of viral pathogenesis

Group		*Clinical patterns*	*Examples*
I Acute non-persistent infections Acute onset usually with clinical features	**Ia**	Rapid recovery	Influenza, hepatitis A
	Ib	Apparent recovery with central nervous system complications	Mumps, measles
	Ic	Rapid death	Rabies
II Primary infection usually with physical signs and followed by persistent infections	**IIa**	Symptom free period punctuated by reactivation	Herpes simplex
	IIb	Long symptom free period followed by illness and death	AIDS EBV infection followed by cancer
	IIc	Chronic disease with periodic exacerbations	Chronic active hepatitis B, warts
III Insidious infections with fatal outcomes	**IIIa**	Long incubation period followed by illness of long duration	Slow viruses
	IIIb	Congenital infection followed late in life by acute illness	No human pathogen known

PART VIII

Table 61–7. Direct demonstration of virus

Demonstration of	*Technique*
Virion	Electron microscopy
Viral antigen	Immunofluorescence
	Immunoperoxidase staining
	Radioimmunoassay
	ELISA
	Immunodiffusion
	Complement fixation test
Viral nucleic acid	*In situ* hybridisation

diagnosis in individual as well as prevalence of infection in community. For diagnosis of infection in individual, paired serum samples are to be collected and a rising titre clinches the diagnosis in acute phase of disease. If facilities for testing IgM antibodies are available, diagnosis can be established on the basis of a single sample since presence of IgM antibodies indicates recent infection. The advantages and disadvantages of commonly used diagnostic techniques are given in Table 61.8.

Prevention of Viral Diseases

Immunoprophylaxis against a large number of viral diseases is now possible by the use of vaccines and antisera or immunoglobulins. Two types of vaccines are currently available:

- Live vaccines
- Non-replicating vaccines (killed or inactivated vaccines).

Live Viral Vaccines

These are prepared from attenuated strains which are devoid of capability of producing the disease but retain their immunogenic properties. Since these can multiply in the host in which these have been inoculated, a continuous source of viral antigen is provided to the body thus giving better immunity. A large number of live viral vaccines are currently licenced. These include vaccines for yellow fever, measles, mumps, rubella and poliomyelitis (Oral polio vaccine or Sabin vaccine).

Non-replicating Viral Vaccines

These vaccines contain non-replicating viral material and to be effective, must contain more antigen than live vaccines. The traditional agent for inactivation of virus is formalin. One has to be very careful in inactivating because excessive inactivation shall destroy the immunogenicity whereas incomplete inactivation shall leave infectious virus capable of causing disease (Table 61.9).

Table 61–8. Diagnostic techniques advantages and disadvantages

Techniques	*Advantages*	*Disadvantages*
1. Virus Isolation	• Sensitive • Can catch all types of viruses • Detects only viable viruses • Provides isolate for further study	• Slow • May require multiple cell-lines • Requires a lot of labour
2. Antigen detection by immuno flourescence	• Rapid • Sensitive for some viruses such as Respiratory Syncytial virus	• Requires skilled staff • Sensitivity variable • Dependent on quality of the specimen
3. Electron microscopy	• Can detect all types • Can detect unculturable viruses • Can also confirm cytopathic effects	• Large capital investment • Not widely available • Requires skilled staff • Sensitivity not very high
4. Serology	• Can detect both recent and past infections • In the absence of acute phase specimen allows retrospective diagnosis • Rapid • Can be automated • Can utilise non-invasive samples, e.g. saliva or urine	• Cross-reactivity • Not appropriate for immuno compromised • Delayed results – may require paired samples • Insensitive for some infections
5. Molecular techniques (e.g. PCR)	• Diagnosis of both infection and disease • Staging of infection • Monitoring therapy • Virus genotyping • Confirming transmission events	• Not widely available in all the laboratories • Need trained staff • Need continuous availability of reagents • Need of good quality control

Table 61–9. Non-replicating viral vaccines

Vaccine	*Vaccine type*
Rabies	Killed whole virus
Inactivated polio	Killed whole virus
Influenza	Killed whole virus or surface antigen
Hepatitis B	Plasma derived HB_sAg
	Genetically engineered
Japanese B encephalitis	Killed whole virus
Tickborne encephalitis	Killed whole virus

Antisera and Immunoglobulins

Immunity of short duration in the form of readymade antibody can be provided to a host by antiserum and hyperimmune immunoglobulins. Such products are available against a large number of viral diseases. These antibodies can be raised in a species other than to which these are administered (*heterologous*). Heterologous antisera for therapeutic uses in human beings are usually raised in horses (e.g. rabies). The antisera raised in human beings, by repeatedly immunising healthy volunteers with antigen, are called as hyperimmune immunoglobulins or *homologous antisera* (e.g. rabies immunoglobulins, hepatitis A immunoglobulins). In most of the viral infections, antisera are given intramuscularly.

62 Oncogenic and Teratogenic Viruses

Apart from being responsible for a large variety of clinical infectious diseases, the viruses have been incriminated as causative agents of cancers in human beings and animals (*oncogenesis*). These have also been shown to cause infection of developing foetus *in utero* which may result into congenital deformities (*teratogenesis*).

ONCOGENIC VIRUSES

Both RNA and DNA viruses have been shown to cause cancers in animals and gradually evidence is accumulating regarding their probable role in producing malignancies in man. *In vitro* evidence for demonstrating capability of viruses to cause malignancies can be generated by inducing transformation in the cells.

Transformation of Cells by Viruses

Viruses are unique among carcinogens in that their tumorigenic activity is expressed efficiently *in vitro* and can therefore be studied under controlled conditions. Several viruses can change the growth characters of cultured cells, the process is called as *transformation.*

The important changes in transformed cells are:

- increased mitotic rate
- unrestricted multiplication, and
- capacity to produce tumours in mice which have defective cellular immunity (*nude mice*).

Both DNA and RNA viruses can transform cells. In most of the tumorigenic viruses, viral DNA in transformed cells is integrated into the cell DNA and is designated as *provirus.* Exception to this rule is papillomavirus and herpesvirus DNAs which persist as plasmids.

Viruses Considered as Oncogenic

Diverse viruses are being increasingly incriminated as causes of cancers in vertebrate animals. Some of these have been given the importance of capability of causing neoplasms in man also (Table 62.1).

Table 62–1. Viruses directly implicated in human cancers

Virus	*Tumour*
RNA virus	
Human T-cell lymphotropic virus type 1(HTLV-1)	Adult T-cell leukaemia/lymphoma
DNA virus	
Epstein-Barr virus (EBV)	Burkitt's lymphoma Nasopharyngeal carcinoma
Herpes simplex (HSV)	Carcinoma cervix (?)
Papillomavirus	Skin carcinoma Carcinoma cervix (?)
Hepatitis B virus	Carcinoma liver
Hepatitis C virus	Carcinoma liver

Mechanism of Viral Oncogenesis

There are many ways in which a virus can be incriminated to be causing cancers. For the sake of discussion these can be divided into three:

- Virus infects the cell and persists in it
- Virus infects the cell but persists for a transient period
- Virus does not infect the cell, yet causes neoplasms.

Virus Infects the Cell and Persists

It has been observed that some of the viruses carry a gene which is responsible for the causation of cancer in

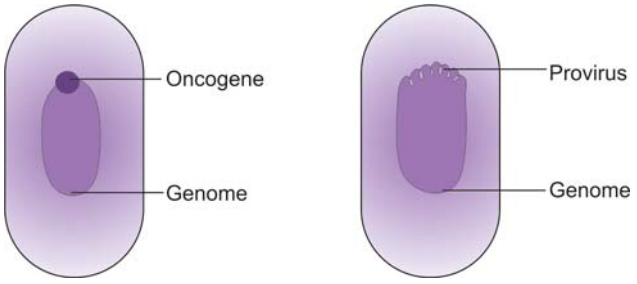


Fig. 62–1. Oncogene and provirus

host cell. This gene (Fig. 62.1) has been designated as *oncogene*. If this gene is removed from the virus, the tumorigenic property is also lost. The viral oncogenes (v-*onc*) produce various proteins which can transform the cells.

Some of the viruses do not possess the oncogenes but their genome gets incorporated into the genome of the host cell as provirus in a specific region of the chromosome (Fig. 62.1). This insertion proves to be mutagenic and changes the sequence of DNA in that region resulting into activation of some gene or its ablation. In some cases the activated gene may be responsible for causing cancer while in others the ablated gene may remove the normal repressor from the oncogene which is present in almost all the cells.

Virus Infects the Cell but Persists for a Short Time

In some of the tumours where viral aetiology is suspected virus isolation has been rewarding. It is believed that such cells had transient infection with the virus and the genes which were required for tumour evolution acted only in the initial stage and unknown selective pressure against the virus eliminated it during tumour progression. It is very difficult to find conclusive proof for such causations because of the hit and run nature of the infection.

Virus Does not Infect the Host Cell

It is also very difficult to study such mechanism. In this type of theory, neither the host cells nor their ancestors need ever been infected by the causative virus. Tumour arises in response to viral infections of other cells. The cancer can be produced as a consequence of cell death or with survival of the cell after viral infection.

TERATOGENIC VIRUSES

Teratogenesis is the induction of defects during embryonic development. A teratogen is an agent that induces such defects. Certain viruses are known to be transmitted across the placenta and infect foetuses and few act as teratogens. The earlier in gestation period the embryo is infected, the more extensive the damage is likely to be. Three major groups of viruses (Table 62.2) are known teratogens in human beings.

Table 62–2. Congenital viral infections in man

Virus	*Syndrome*
Smallpox	Foetal death and abortion
Cytomegalovirus	Severe neonatal disease
Rubella	Congenital defects
Herpesvirus	Rubella syndrome
	Severe neonatal diseases

The teratogenic virus may affect the foetus in such a way that it causes the death of foetus and resultant abortion. In many cases, however, the infection is insufficient to cause death, but severe enough to interfere with the normal development of the growing cells of foetus resulting into congenital infections.

During first trimester of pregnancy, infection of mother with rubella virus may result into abnormalities which include deafness, blindness, congenital heart and brain defects. This is perhaps due to the reduced number of cells observed in many organs of this type of children. There is evidence that rubella virus-infected cells multiply somewhat less rapidly than normal cells. Rubella may also cause severe neonatal disease (*rubella syndrome*) which is characterised by hepatosplenomegaly, purpura, jaundice and such cases are often fatal soon after birth.

Mother suffering an inapparent infection with cytomegalovirus during pregnancy may deliver a baby with clinical features of hepatosplenomegaly, thrombocytopenic purpura, hepatitis and jaundice as well as microcephaly and mental retardation.

PART IX
DNA VIRUSES

63 Adenoviruses

The first isolation of an adenovirus was made in 1953 by Rowe from the human adenoid tissue. Since then 41 serotypes of adenoviruses of humans have been isolated. Not all of these serotypes are pathogenic. The appearance of adenoviruses resembles that of an orbiting satellite.

Classification

The family *Adenoviridae* consists of non-enveloped icosahedron virions which carry double stranded DNA that mature in the nucleus of the infected cell. The family is composed of four genera: Mastadenovirus, Aviadenovirus, Atadenovirus and Siadenovirus. The human pathogens are specific to man only and fall in genus Mastadenovirus.

Adenoviruses from man have been further subdivided into six subgenera (Table 63.1) on the basis of certain characteristics.

Properties

- Non-enveloped viruses, 70-76 nm in diameter
- Double stranded DNA, mol. wt. of 25,000
- Icosahedral symmetry
- Capsid consists of 252 capsomeres in an icosahedron with 20 triangular faces and 12 vertices. A fibre with knob at distal end projects from each vertex (Fig. 63.1)
- The structural polypeptides comprise 87% by weight of the virus
- Stable between pH 6-9.5
- Retains infectivity for 70 days at 4°C
- Resistant to chloroform, ether
- Sensitive to acetone, chlorine at 1 part per 10 million in water.

Antigenic Structure

The adenoviruses carry a complex antigenic configuration. The hexon, penton, fibre as well as major core

Table 63–1. Classification of adenoviruses of man

Subgenus	*Serotypes*	*Haemagglutination of RBCs of*	*Target organs*
A	12, 18, 31	Rat	Gastrointestinal tract
B	3, 7, 11, 14, 16, 21, 34, 35	Monkey	Pharynx, lungs, urinary tract, conjunctiva
C	1, 2, 5, 6	Rat	Pharynx
D	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 29, 30, 32, 33, 36, 37, 39	Rat	Eye
E	4	Rat	Upper respiratory tract
F	40, 41	Rat	Gastrointestinal tract

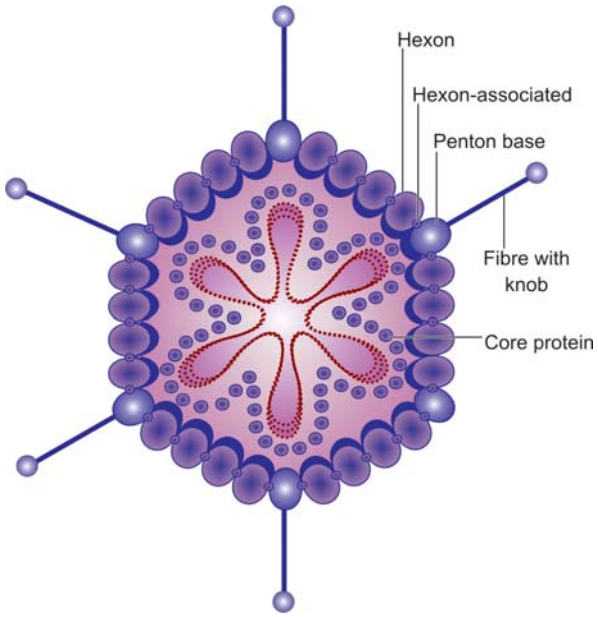


Fig. 63–1. Adenovirus

proteins are all antigenic. The hexon carries four antigenic specificities: a group specific activity demonstrable by complement fixation or indirect fluorescent, a type specific activity and intrasubgroup and intersubgroup activities shown by neutralization. 41 serotypes of adenoviruses are known (Table 63.1).

Clinical Manifestations

Adenovirus infections occur throughout the world. Though only a few serotypes are known to be pathogenic, a large population is affected by these. The main clinical syndromes associated with adenovirus infections in man are shown in Table 63.2.

Pathogenesis and Immunity

Adenoviruses multiply initially in the pharynx, conjunctiva or small intestine and do not spread beyond the draining lymph nodes. Incubation period is short (5-7 days) but excretion of virus in faeces occurs for a long time. The acquired immunity to adenovirus infections is long lasting and second attacks with the same serotype are rare.

Table 63–2. Adenovirus infections of humans

Serotype	*Clinical syndrome*
1,2,5,6	Acute respiratory disease (ARD) of children
3,4,7,14,21	Sore throat, pneumonia, febrile cold
8,9,37	Epidemic keratoconjunctivitis (Shipyard's eye)
4,7,21	Respiratory disease in army recruits
2,11,21	Acute haemorrhagic cystitis
7	Meningoencephalitis
3,7	Pharyngoconjunctival fever (Swimming pool conjunctivitis)

Laboratory Diagnosis

Isolation

Virus may be recovered from the throat, faeces or conjunctiva by inoculating specimens into cell cultures of human epithelial origin such as human embryonic kidney, human diploid cells (WI-38, MRC-5), HEp-2 and HeLa. The human embryonic kidney cells have been found to be superior to WI-38 fibroblasts in displaying CPE earlier.

CPE

CPE can be visualized in the first week of incubation and is generally apparent after 14 days. Viral CPE in cell culture is evidenced by increased acidity of the medium, and development of enlarged, round cells in grape like clusters.

Confirmation and Serotyping

Identification can be achieved by using antiserum to the common group antigen (i.e. antisera to hexon antigen) in either a complement fixation or indirect immunofluorescent test. Serotyping can be done with neutralization test. Nucleic acid hybridization has been successfully employed to diagnose adenoviral infections.

Electron Microscopy

Some of the isolates from faeces are difficult to grow and electron microscopy is the ideal method for detecting such serotypes.

Epidemiology

Adenovirus infections occur early in life and by 5 years of age almost all children are infected by at least one serotype. The endemic serotypes (1, 2, 5) circulate widely among young children whereas the epidemic serotypes (3, 4, 7) are found more frequently in older children and adults. Adenoviruses cause less than 10% of viral upper respiratory tract diseases in civilian population. However, for unknown reasons, among military recruits the proportion of adenovirus respiratory infections exceeds 50%, while college students in the same group rarely suffer from adenoviral infections.

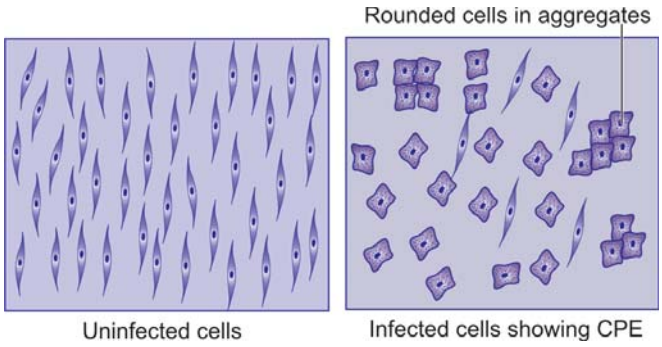


Fig. 63–2. CPE of adenoviruses

Prophylaxis

A number of antiviral agents have been found to be capable of inhibiting the adenoviruses *in vitro* but an effective chemotherapy against these viruses is yet to be developed.

ADENOVIRUSES ASSOCIATED DEFECTIVE VIRUSES

A virus is considered to be defective in a given host when its replication in that cell falls short of the production of infectious virus. Two such viruses are associated with adenoviruses.

Adeno Associated Viruses (AAV)

These are small single stranded DNA parvoviruses of four serotypes which are defective in human cells. Their growth is complemented by adenoviruses with which they cohabit in the human throat. So far they have not been found to be causing any illness in human beings.

Adenovirus-SV 40 Hybrids

Human adenoviruses do not multiply in simian cells unless they are complemented by a simian adenovirus or by SV 40. Sometimes when simian cells are simultaneously infected with an adenovirus and SV 40, the progeny may contain hybrids of SV 40 and adenovirus. These are laboratory artefacts but have generated considerable interest because of relevance of such hybrids in vaccine production and oncogenesis.

Important features of Adenoviruses are summed up in Table 63.3.

Table 63–3. Important features of adenoviruses

- Are naked viruses with an icosahedral nucleocapsid composed of hexons, pentons and fibres.
- Contain double stranded DNA that replicates asymmetrically.
- Produce localised infections of the eye (pink eye), respiratory tract, gastrointestinal tract and urinary bladder (Haemorrhagic cystitis)
- Frequently produce subclinical infections
- Adenovirus infections are transmitted by direct contact, small droplet aerosols, faeco-oral route, water, ocular instruments and fomites
- Infections occur most frequently during childhood where they tend to be self-limited and induce type specific immunity following recovery
- They can cause fatal infections in immunocompromised individuals and also in bone-marrow transplant receipients
- At present there are no proven effective therapies for treatment
- Prevention at the community level not possible
- Adenoviral infections spread in hospitals can be reduced by isolating infected patients, frequent hand washing and cleaning of equipment and instruments.

64 Herpesviruses

The term *herpes* (Greek: *to creep*) has been used since the earliest epoch of Greek medicine to include spreading cutaneous lesions of varied aetiology. Infection with herpes simplex virus type 1 (HSV-1) is virtually universal, regardless of geography or race. This infection occurs most frequently during childhood and usually affects body sites above the waist. HSV-2 infections, on the other hand, occur most often during adolescence and young adulthood and involve body sites below the waist, primarily the genitals. Most neonatal infections are also caused by HSV-2.

The herpesviruses are a unique group of viruses with a host range that includes invertebrates and vertebrates of all species examined thus far. The family *Herpesviridae* consists of more than 80 viruses. There is great diversity in pathology and biology of herpes viruses and the only common feature is their property of establishing latent infection.

Classification

The family *Herpesviridae* has been divided into three subfamilies:

Alpha herpesvirinae (e.g. *Herpes simplex virus).* These are primarily neurotropic viruses with a variable host range, a short growth cycle and spread rapidly with destruction of infected cells. These viruses establish latency by infecting ganglia.

Beta herpesvirinae (e.g. *Cytomegalovirus).* These establish latency in secretory glands and lymphoreticular cells. These viruses have narrow host range. These spread slowly and have a long reproductive cycle. These are also capable of setting up carrier cultures.

Gamma herpesvirinae (e.g. *Epstein-Barr virus).* All members of this subfamily can infect lymphoblastoid cells *in vitro* and are associated with lymphoproliferative diseases in the natural hosts.

Till date seven different herpesviruses have been isolated whose natural host is man. The nomenclature for these viruses, as per the recommendations of the International Committee on Taxonomy of Viruses, is Human herpesvirus 1 to 7. However, with the exception of human herpesvirus (HHV) 6 and 7 the remaining are generally known by their common names (Table 64.1). The eighth human herpesvirus is associated with some forms of human neoplasia. It has discovered in Kaposi's sarcoma, with which it is strongly associated and hence the designation Kaposi's sarcoma associated herpes virus.

Table 64–1. Human herpesviruses

Designation	*Subfamily*	*Common name*	*Abbreviation*
Human herpesvirus 1	Alpha	Herpes simplex virus type 1	HSV-1
Human herpesvirus 2	Alpha	Herpes simplex virus type 2	HSV-2
Human herpesvirus 3	Alpha	Varicella-zoster virus	VZV
Human herpesvirus 4	Gamma	Epstein-Barr virus	EBV
Human herpesvirus 5	Beta	Human cytomegalovirus	CMV
Human herpesvirus 6	Beta	Human B cell lymphotropic virus	HHV-6
Human herpesvirus 7	Beta	RK virus	HHV-7

Morphology of Herpesviruses

Envelope

The lipid envelope has many spikes of glycoproteins. The diameter of enveloped virus is about 120 nm. Within the envelope is the capsid.

Capsid

It has a diameter of 100 nm, icosahedron symmetry with 12 pentavalent capsomers present at the vertices and a total of 162 capsomers.

Tegument

This is the electron-dense material present between the envelope and the capsid. It contains many proteins but its precise functions are yet to be elucidated.

Core

This is the densly staining region within the capsid and it is believed to be a cylindrical protein plug around which the DNA is wound in the form of a torus.

Genome

Herpesviruses are DNA viruses with seven structural forms. There are more than 30 virion proteins of *Herpes simplex* type 1 virus which include major capsid protein, the major tegument protein and glycoproteins.

HERPES SIMPLEX VIRUS

Herpes simplex virus exists in two serologically related but distinguishable types. Type 1 virus (HSV-1) infects regions of body above the waist and type-2 (HSV-2) affects genital and anal regions. Usual sites for latency for HSV-1 and HSV-2 are trigeminal ganglion and sacral ganglion respectively.

Clinical Manifestations

The diseases caused by HSV fall into a large number of clinical entities (Table 64.2).

Reactivation

The property of producing recurrent lesions in healthy individuals is unique to HSV-1. The reactivation does not occur in most of the people who harbour this virus. The commonest lesion associated with reactivation is *cold sores*. These are classically seen at mucocutaneous junctions around nose and mouth. These can be sometimes present at other sites also. Various non-specific stimuli can provoke the attack of cold sores.

Table 64–2. Diseases caused by Herpes simplex virus

- Stomatitis
- Herpes labialis
- Primary herpetic dermatitis
- Eczema herpeticum
- Traumatic herpes
- Acute herpetic rhinitis
- Keratoconjunctivitis
- Keratitis
- Neonatal herpes
- Meningitis
- Encephalitis
- Herpetic hepatitis
- Arthritis
- Disseminated rash
- Autonomic system dysfunction
- Genital lesions
- Proctitis
- Acute necrotic cervicitis

These lesions are far more in immunologically compromised subjects. The less common form of recurrence of herpes is *dendritic ulcers of cornea*. This condition progressively involves deeper layers of cornea and infection may spread to uveal tract. Both cold sores and dendritic ulcers of cornea arise from the spread of reactivated virus latent within the trigeminal ganglion.

Reactivation of genital herpes is also common and is due to reactivation of virus latent in the sacral ganglion. Recurrent genital herpes is also painful, though less in intensity than cold sores but nevertheless a source of distress to patient.

Pathogenesis and Pathology

The primary infection occurs through skin or mucous membrane and results in formation of a vesicle under the layer of keratinized squamous epithelial cells. The vesicle fluid consists of characteristic multinucleated giant cells, inflammatory cells, and cellular debris. Many cells contain eosinophilic intranuclear inclusions. Virus spreads from the local lesion to the draining lymph nodes causing their enlargement. The lesion heals with residual scarring. The reactivated lesions are usually less severe in inflammatory reaction and lymphadenopathy.

After primary infection the virus travels to sensory root ganglia which innervate the area of infection (Fig. 64.1). Commonly involved ganglia are trigeminal and sacral. Sometimes superior cervical and vagal ganglia also get involved. The virus travels to these ganglia by retrograde intra-axonal flow and settles within the neurones in the ganglia. The herpesviruses do not persist in ganglia as whole virion but as viral

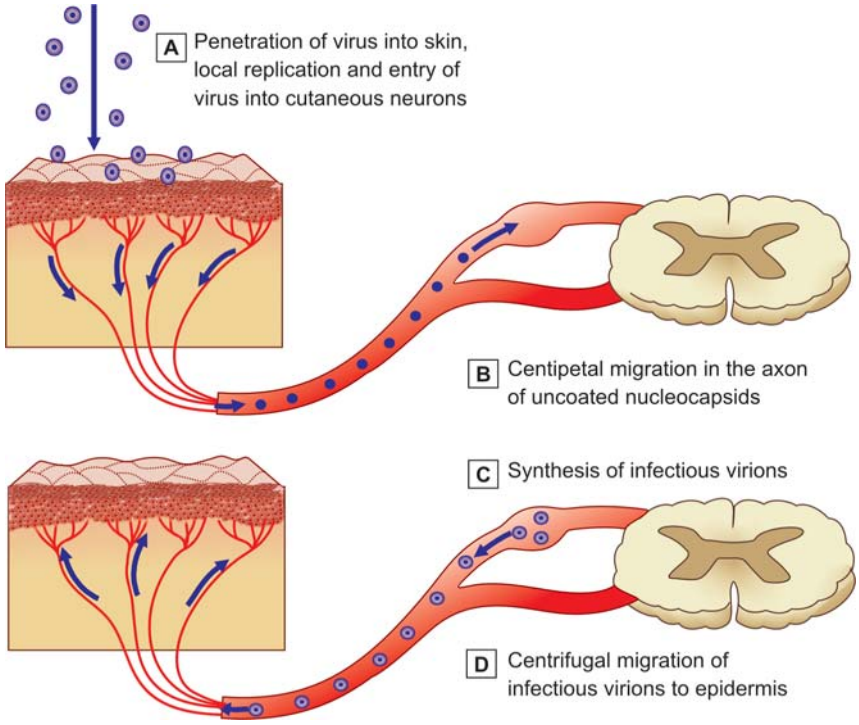


Fig. 64–1. Pathogenesis of herpesvirus infection

DNA integrated into the cellular genome. These get reactivated after provocation by various stimuli.

Immune Response

Primary infection with these viruses evokes both humoral and cell-mediated immune responses. The neutralizing antibodies against these viruses can be detected in the serum of the patient. These antibodies do not protect the individual from reactivation. This may be because of the 'protected' route of travel (intraaxon) or localisation of virus in ganglia. The antibodies do protect the individual from an exogenous attack with herpesviruses.

Herpes simplex viruses also induce cell-mediated immunity which is much more demonstrable in reactivation than in primary infection. CMI as well as antibody dependent cell-mediated cytotoxicity play an important role in containing the herpes infections.

Laboratory Diagnosis

Clinical specimens. Herpes simplex virus can be isolated from various clinical specimens:

- oral and genital vesicle fluid
- throat swab and washings
- swab from oropharynx
- CSF.

Growth in cell cultures. These viruses can grow in primary cell cultures as well as primate and nonprimate cell lines. The most sensitive cell culture systems are rabbit kidney cells and human diploid cells. Other cell cultures which also provide good results include HeLa, HEp-2, and Vero cell lines.

CPE is manifested in the form of characteristic rounding of cells which contain eosinophilic intranuclear inclusions. Most rapid results are obtained with human amnion cells where extensive cellular destruction occurs in 2-3 days. Neutralization, complement-fixation or immunofluorescence tests with specific HSV-immune serum can be performed for final identification.

Growth in animals. Newborn mice are particularly sensitive to HSV. When inoculated with the virus intracerebrally or intraperitoneally, the animals will develop encephalitis within a short period. Staining of mouse brain tissue shall exhibit *Cowdry type A* inclusion bodies.

Growth in developing chick embryo. Types of HSV can be recognised using chorioallantoic membranes of embryonated eggs. HSV-1 shall produce well defined pinpoint pocks on the membrane within 3-4 days after inoculation whereas HSV-2 produces easily recognisable, large and clear pocks.

Differences between HSV-1 and HSV-2. Apart from the growth characters on developing chick embryo,

HSV-1 is sensitive to bromodeoxyuridine whereas HSV-2 is relatively insensitive. The use of this differential sensitivity is being made now to confirm the type of isolate of HSV.

Immunofluorescence. This is the method most widely used to diagnose suspected herpetic mucocutaneous lesions.

Treatment

Herpes simplex is one of the few viruses against which specific and effective drug therapy is available. The anti-viral agents which have been in use for last few years are idoxuridine, vidarabine and cytarabine. The latest and the most effective as well as safe drug to be licensed is acyclovir (acycloguanosine).

Epidemiology

Herpes simplex viruses are distributed worldwide. There are no known seasonal patterns of infection. Since infection with virus is followed by development of antibody that persists for life, incidence of infection can be determined by antibodies studies. The prevalence of antibodies is inversely related to socioeconomic status. In developing countries, almost 100% of adults have antibody to HSV, whereas in higher socioeconomic groups, this proportion falls to 30 to 50%. The time of maximal acquisition of infection varies with the two types. The prevalence of antibody to HSV type 1 rises during childhood, whereas the major period of infection with HSV type 2 follows puberty.

VARICELLA-ZOSTER VIRUS

Varicella-zoster virus (Human Herpesvirus 3) is the aetiological agent of two diseases of man, varicella and herpes zoster. Varicella follows primary exogenous contact with the causative virus, and zoster reflects endogenous activation of varicella-zoster virus infection that has survived in latent form following an attack of varicella.

Varicella is a ubiquitous, contagious, generalised exanthematous disease of seasonally epidemic propensities that follows primary exposure of a susceptible person, most often a child. The disease is commonly known as chickenpox: the origin of word has remained obscure till date, since the virus is neither a derivative of any pathogen of chickens nor itself has been ever shown to produce pocks in chickens.

Chickenpox is one of the more infectious diseases of childhood: and also one of the mildest. Many a times it is little more than a nuisance. In otherwise healthy young children it is much less dangerous than common cold. However, it is one of the most deadly infections in children with leukaemia or other immune defects.

Herpes zoster (shingles) is an endemic sporadic disease, most frequent in elderly people, characterised by the appearance of a unilateral, painful, vesicular eruption localised to the dermatome innervated by a specific dorsal root or extramedullary cranial ganglion. *Zoster* is a Greek word which means the belt with which a warrior used to secure his armour similar to a girdle worn by women and signifies spread or appearance of the vesicles.

Clinical Features of Varicella

It is one of the common childhood exanthemata, usually affecting children in early years at school. The incubation period ranges from 10-21 days. Disease begins with fever followed by sudden eruptions of crops of skin lesions distributed mainly centripetally and appearing predominantly on covered parts of the body (e.g. trunk) rather than on face and limbs. The rashes induce intense itching which tempts the child to scratch the lesions, leading to bacterial superinfection and permanent scarring.

Clinical Features of Zoster

The disease is an endogenous reactivation of virus latent in sensory ganglia following primary varicella. Virus probably persists just like *Herpes simplex* virus but the provoking stimuli are not known. Clinically, zoster presents as a crop of painful vesicles which appear within the dermatome served by the sensory nerve from the affected ganglion. The eruptions are just like those of varicella but excruciating pain differentiates zoster from varicella. The most commonly affected area is skin extending from vertebral column to the ventral midline. Cranial zoster is also common and ophthalmic nerve is commonly involved. In immunocompromised patients, zoster sometimes becomes disseminated with vesicles appearing throughout the skin.

Pathogenesis and Pathology

Varicella virus enters the body through respiratory route followed by a lengthy incubation period during which the virus is demonstrable in blood. Subsequently virus gets disseminated to most organs and tissues including the skin. Infected skin cells show 'ballooning'. Intranuclear inclusions and multinucleated giant cells are present along with predominantly lymphocytic

infiltration. The histology of skin lesions in zoster is similar to that in varicella and infiltration with polymorphonuclear leukocytes is a prominent feature. During an attack of zoster infectious virus has been demonstrated in the corresponding ganglion. The pathogenesis of herpes zoster infection has been shown in Figure 64.2.

Immune Response

Primary infection with varicella-zoster virus always results into long lasting immunity to exogenous reinfection with varicella. Immunoglobulins of class IgG, IgA and IgM are detectable after varicella. IgM is not always produced as a response to zoster and whenever produced is in low titres as compared to varicella. Infection is also followed by cell-mediated immunity.

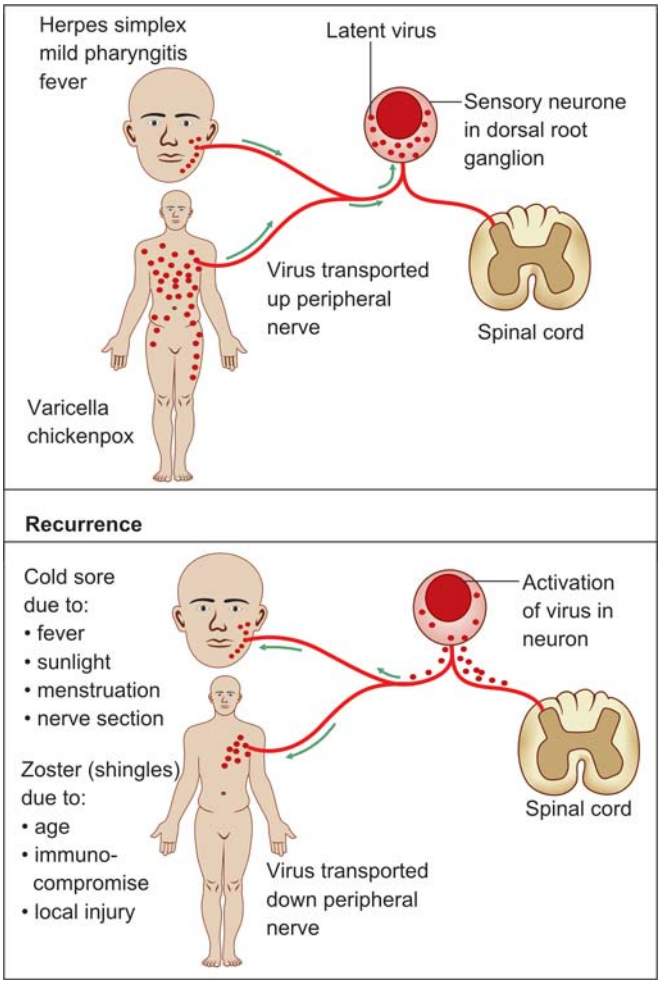


Fig. 64–2. Pathogenesis of herpes-zoster

Laboratory Diagnosis

The best method of isolating this virus begins with a sterile aspiration of fresh vesicles. Human diploid cell cultures or primary human cell cultures are most sensitive host system for the isolation of this virus. Inoculated cell cultures are incubated at 36-37°C in roller drums and examined microscopically for evidence of CPE over next 30 days. The CPE can be confirmed by immunofluorescent staining of the infected cells, electron microscopy and the recently introduced technique of dot blot DNA hybridisation.

Treatment and Prevention

The specific therapy is mainly by acyclovir. This virus is, however, less sensitive to the action of acyclovir as compared to herpes simplex virus. Acyclovir, in oral form, is now extensively used against zoster but the dosage required is considerably higher than that for herpes simplex. Topical application of this drug has also been found to be useful in treating ophthalmic zoster. Idoxuridine and vidarabine are also used for local application, though these have been superceded in clinical use to a great extent by acyclovir.

A live attenuated vaccine prepared from Oka strain is now available. The vaccine has been found to confer protection in healthy children against chickenpox with minimum side effects. Passive immunisation with specific zoster immunoglobulins is also indicated in children born to mothers suffering from varicella around the time of delivery.

Epidemiology

Varicella is a highly contagious disease. In household exposures almost all the susceptible children get infected. The subclinical attack rate is not more than 4%.

CYTOMEGALOVIRUS

Cytomegalovirus (CMV) was cultured for the first time in 1956 by Rowe, Smith and Weller, all of whom worked independently. This virus is now believed to be one of the most common parasites of man. The cells with huge cytoplasm, which give the virus its name, are found in salivary glands or renal tubules of many asymptomatic children.

Clinical Features

Most of the infections remain asymptomatic. In rare cases when the disease manifests, the outcome is usually

fatal. The disease has various manifestations (Table 64.3).

Table 64–3. Clinical manifestations of CMV infections

Group	*Clinical features*	*Mode of infection*
Congenital infections (CMID)	Hepatosplenomegaly Jaundice Thrombocytopenic purpura Haemolytic anaemia Microcephaly Permanent mental abnormalities	Transplacental
Childhood infections	Insidious hepatitis Pneumonitis	Respiratory route
Reactivation	Hepatitis Interstitial pneumonia	Postimmuno-suppression
Postperfusion syndrome	Fever Increase in atypical mononuclear cells	Repeated transfusions

Pathogenesis

Mode of transmission of CMV infection varies depending upon the age group of the patient (Table 64.4). Congenitally infected children do not show immunological tolerance. It is assumed that the foetus is infected during maternal viraemia. However, it is not clear how viraemia can occur in mothers already possessing antibodies against cytomegalovirus. The typical CMV infected cell is greatly swollen with an enlarged nucleus distended by a huge acidophilic inclusion which is separated from the nuclear membrane by a nonstaining halo. This cell gives the appearance of **owl's eye**. The transplacental transmission of the virus may give rise to a condition called as *cytomegalic inclusion disease (CMID)* of newborn, which often proves fatal within few days or weeks.

Immune Response

IgG antibodies are produced against CMV infection and can be detected easily by ELISA or immunofluorescence. Production of IgM in primary infection has been demonstrated. With repeated infections, it becomes difficult to detect IgM antibodies. It has been reported that pregnant women delivering babies congenitally infected with CMV have impaired CMI.

Laboratory Diagnosis

Most useful specimens for CMV isolation are throat washings and urine. The virus is shedded in urine for months after the clinical features have subsided. CMV can also be isolated from saliva, breast milk, cervical secretions, blood and semen as well as various biopsy materials. CMV displays strong specificity for cell line. Human diploid cell cultures such as WI-38 and MRC-5 are most sensitive. CPE appears slowly and generally takes 2-3 weeks to manifest in typical picture. Immunofluorescence test or immunoperoxidase staining using specific antisera can be employed to confirm the results of CPE.

A highly specific technique has been developed recently which is as sensitive as tissue culture technique but requires less than 48 hours to perform. It involves dot blot hybridizations using molecularly cloned CMV-DNA probes.

Table 64–4. Transmission of CMV infections

Category	*Mode of infection*
Foetus	From mother across placenta
Infant	Contact with maternal body fluids during birth and breastfeeding
Young child	Contact with saliva or urine of other children
Adolescents, adults	Kissing, sexual intercourse and blood transfusion
Transplant recipient	Donated tissues, blood transfusion; reactivation due to immunosuppression

EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) is named after its discoverers Epstein and Barr and is known to cause a broad spectrum of diseases. This virus has been incriminated in various malignancies also (Table 64.5).

Table 64–5. Epstein-Barr virus infections

Syndrome	*Age group affected*	*Distribution*
Infectious mononucleosis	Adolescents, young adults	Global
Burkitt's lymphoma	4–12 years	Sub-Sahara Africa
Nasopharyngeal carcinoma	20–50 years	Southern China
B cell lymphoma	Children and adults	Seen in immunodeficiencies

Clinical Features

EBV can cause glandular fever (*infectious mononucleosis)* which is characterised by pyrexia, sore throat, lymphadenopathy, malaise and fatigue. The acute phase can persist for several weeks. Disease in mild form may persist chronically. Relapses may occur after apparent recovery.

In immunocompromised individuals EBV reactivation may trigger B cell lymphoma. Male children with *Duncan syndrome* may succumb to an overwhelming EBV infection.

Burkitt's lymphoma is seen in children 5-8 years of age. It affects multiple sites such as jaw, orbital cavities and gastrointestinal tract. It progresses rapidly but is sensitive to antitumour chemotherapy. The primary tumour of nasopharyngeal carcinoma manifests only after it has spread to regional lymph nodes and carries a bad prognosis.

Epidemiology

EBV infection spreads by salivary transmission as well as blood transfusion. Burkitt's lymphoma is restricted to areas of equatorial Africa and New Guinea where malaria is hyperendemic. Malaria, being immunosuppressive, may act as a co-factor by interfering with the immune responses that normally prevent reactivation of EBV from B cells to enable its full oncogenic potential to be expressed. Undifferentiated nasopharyngeal carcinoma is mainly restricted to southern China or people of Chinese descent. Genetic predisposition is believed to be important.

Pathogenesis

Epithelial cells of pharynx and cervix are the major initial sites for EBV infection (Fig. 64.3). When B cells pass through these sites, they become infected. EBV expresses neoantigens on B cells as well as epithelial cells against which immune response is mounted by the body and the resulting damage manifests as infectious mononucleosis. Thus, glandular fever has an immunopathological basis.

Laboratory Diagnosis

It is extremely difficult to isolate EBV and establish and confirm the identity of cells transformed by EBV. Most EBV infections are diagnosed serologically. Past infections can be shown by the presence of IgG antibody against viral capsid antigen and a negative result for IgM antibody. Test for detection of heterophile agglutinins goes after the name of Paul and Bunnell and is widely used.

Paul-Bunnell Test

Paul-Bunnell antibody is a heterophile antibody that appears in the serum of the patient with glandular fever during early stages of disease and disappears within about two months. This antibody agglutinates sheep erythrocytes. Since similar antibody appears after injection of some sera or even in the blood of normal persons, it has to be differentiated by absorption tests (Table 64.6).

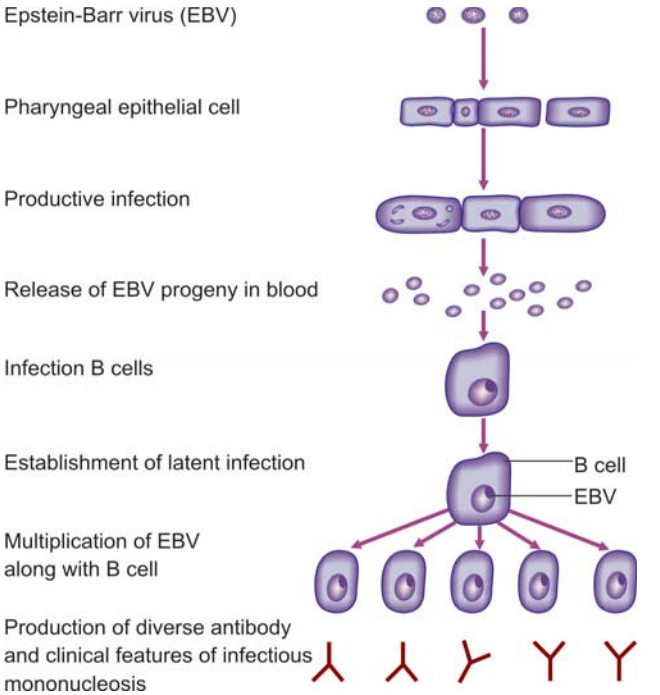


Fig. 64–3. Persistent EBV infection

Table 64–6. Absorption of Paul-Bunnell antibody

Serum of	*Absorption with Guinea pig kidney*	*Ox RBCs*
Patient with glandular fever	–	+
Patient given serum therapy	+	+
Normal individuals	+	–

This test is performed by taking inactivated serum in doubling dilutions and mixing it with equal volumes of sheep RBCs. After incubation the tubes are examined for agglutination. A titre of more than 100 is suggestive of infectious mononucleosis. The diagnosis is confirmed by subjecting to the absorption tests as mentioned in Table 64.6.

Prophylaxis and Therapy

At present no vaccine is available against EBV. Even the clinical use of acyclovir has been disappointing. Administered in early stages of lymph nodes proliferation, this drug may be of some use.

HUMAN HERPESVIRUS 6

This virus was isolated for the first time in 1986. It was earlier known as human B-lymphotropic virus. Though this virus has a typical herpesvirus morphology, serologically it is distinct. It is widely prevalent as is evidenced

by the presence of antibodies against this virus in almost 70% of children. The infection is perhaps acquired during infancy. Its role in human diseases is still not known. The virus can, however, remain latent within blood leucocytes and other tissues. The infection has been found to be associated with exanthem subitum and myalgia, encephalomyelitis (*post viral fatigue syndrome)*. The role of this virus in causing these diseases is yet to be confirmed.

HUMAN HERPESVIRUS 7

Human herpesvirus 7 was discovered in 1990. This is also referred to as RK. This has been cultivated from the cord blood and adult peripheral blood lymphocytes. The virus seems to be prevalent widely and many children and young adults show antibody against it. So far no clinical disease has been associated with HHV-7.

65 Poxviruses

The family *Poxviridae* includes several viruses of veterinary and medical importance. The history of poxviruses has been dominated by smallpox—the only infectious disease that has been eradicated from the earth with the use of a simple live virus vaccine prepared from vaccinia virus. Though smallpox has been eradicated, vaccinia virus is still the subject of intense scientific study as a vector for recombinant vaccines, for both medical and veterinary use.

Classification

Poxviruses pathogenic to human beings fall in two genera–Orthopoxvirus and Parapoxvirus (Table 65.1).

Table 65–1. Poxviruses pathogenic to human beings

Genus	*Virus*	*Primary host*	*Clinical features in human*
Orthopoxvirus	Variola	Man	Smallpox
	Vaccinia	Man	Vesicular vaccination lesions
	Cowpox	Cattle, cat rodents	Lesions on hands
	Monkeypox	Monkeys, squirrels	Resembles smallpox
Parapoxvirus	Pseudocowpox	Cattle	Milker's nodes
	Orf	Sheep, goat	Localised lesions
Unclassified	Tanapox	Monkeys	Vesicular skin lesions
	Molluscum	Man	Multiple small skin nodules

Morphology

Poxviruses that cause human infections are relatively large, brick shaped or ovoid virions which possess an external coat containing lipid and tubular or globular protein structures. Since the nucleocapsid of poxviruses does not conform to either of the two types of symmetries found in most other viruses, these are called as complex virions. Most genera are 300 × 240 × 100 nm with regular arrangement of tubules on outer membrane.

The nucleic acid is dsDNA, linear in nature and molecular weight varies from 130–280 million daltons. There are over 100 polypeptides in the virion. The core protein includes a transcriptase and several other enzymes. The virion also contains around 20 antigens which can be recognised by immunodiffusion. These replicate only in cytoplasm.

The description of some of the diseases caused by poxviruses is as under :

SMALLPOX

The disease was believed to have originated 10,000 BC in Asia and Africa. Because of the intensified effort for global smallpox eradication by the WHO and member countries, the world saw the last case of endemic smallpox which occurred in Somalia, in October, 1977.

Smallpox was transmitted to humans by person-to-person contact or by fomites. The incubation period for human infections ranges between 7 to 17 days. The virus enters through respiratory tract. The prodromal phase is characterised by fever, chills, headache, backache, vomitting, pain in the limbs and prostration. Through haematogenous route, the virus reaches internal organs, multiplies there and again appears in blood. The skin eruptions appear on 3rd to 4th day. The eruption develops through the stages of macule, papule, vesicle and pustule within 5 to 6 days. The distribution of the eruption characteristically involves the face and

the limbs. The lesions begin to dry up by 10th day of appearance and scabs are shed by third week.

During the outbreak of smallpox, two types of clinical pictures could be encountered: variola major and variola minor. Of the two, variola major was severe form of disease with 15-40% case fatality rates. Variola major (also known as alastrim or Kaffir pox) appeared in 19th century. The last case of natural smallpox in Somalia was of variola minor.

Laboratory Diagnosis

The use of chorioallantoic membrane (CAM) of embryonated egg of 11-13 days age gives the best results for the isolation of smallpox virus as well as helps in identification of the virus because of the characteristic morphology of the pocks. Smallpox virus pocks are about 1 mm in diameter when examined three days after the inoculation. They are greyish white, opaque, convex, raised above the CAM surface, regular, round with a smooth outer edge, are not haemorrhagic and all the pocks are nearly of the same size.

The viruses of variola major, variola minor and vaccinia can be differentiated by employing 'ceiling temperature' phenomenon in which the pocks are not produced if the virus is grown at higher than a particular (ceiling) temperature. The ceiling temperature for variola major, variola minor and vaccinia is 38°C, 37.5°C and 41°C respectively.

The variola and vaccina viruses can be grown in tissue cultures of monkey kidney, HeLa and chick embryo cells. The CPE is produced within 48 hours by these viruses.

Electron microscopy is the ideal method for the identification of the viruses. This technique can be used for the identification of all poxviruses. Immunofluorescence sometimes gives false positive results when a field specimen has been frozen for more than 7 days. CF test and examination of stained smears was used in earlier days but have now been replaced by better tests.

Indirect immunofluorescent test has now become the test of choice for serological analysis and all the reagents are commercially available.

Immunity

Recovery from smallpox gives life-long immunity. The immunity following vaccination with vaccinia virus is less prolonged. Reinfection is extremely rare.

Factors which Favoured Eradication of Smallpox

Smallpox could be eradicated because of certain favourable features of disease and control activities (Table 65.2) along with the strong political will of the Governments of all of the countries.

Table 65–2. Factors that favoured smallpox eradication

- Long incubation period
- Low communicability
- No subclinical infection
- No carrier state
- No zoonotic reservoir
- Single serotype
- Induction of life-long immunity
- Potent single dose vaccine
- Aggressive surveillance-containment measures
- Effective strategy
- International cooperation

HUMAN MONKEYPOX

Monkeypox was first discovered as a disease of captive monkeys in 1958. This was found to be a disease entity of human beings in 1970 in Zaire. Since then around 400 cases of monkeypox have been reported in the world.

Clinically the disease is indistinguishable from smallpox and laboratory help is needed to establish the diagnosis. Usually the patient recovers in 2-4 weeks but monkeypox also carries a case fatality rate of 15% in human beings.

Monkeypox is of public health importance not because of the magnitude of the problem but because of its similarity in clinical appearance with that of smallpox.

COWPOX

Cowpox was known to Edward Jenner who used this virus in his early smallpox vaccine. Cowpox virus infection in humans is transmitted by direct contact with infections on the skin of the udder and teats of cows. The lesions in human are found on the fingers, with reddening and swelling which develops into papules that become vesicular in 4-5 days and heal in 2-4 weeks.

Earlier, it was thought that the disease could be transmitted only by direct contact. Recent studies have implicated rodents as capable of transmitting the disease.

BUFFALOPOX

Buffalopox virus has been responsible for outbreaks of disease in buffaloes in India in 1960s and 1970s. During

an outbreak of the disease in buffaloes, it is transmitted to humans by direct contact with an infected buffalo. The lesions are localised on the fingers, hands and sometimes on the face of the humans. No generalised infection has been seen so far and no man-to-man transmission has been reported. The scabs fall within 2 weeks and the patient recovers fully.

TANAPOX

The disease is also called as 'river smallpox' and gets the name Tanapox from the river Tana in Kenya from where initial outbreaks of this poxvirus in humans were reported in 1957 and 1962. The disease can begin in human beings as a febrile illness with backache, headache, and prostration. Lesions seen on uncovered areas are few, usually one or two. These lesions appear on the skin of the upper arms, face, neck or trunk. It is difficult to extract fluid from the vesicles of this disease. The lesions heal without becoming pustules. Healing time extends to 7 weeks. The disease is believed to be transmitted by mosquitoes. The reservoir of infection is unknown but monkeys are suspected.

MILKER'S NODULES

Milker's nodules were referred to by Edward Jenner as *spurious cowpox* suggesting that it was different from smallpox and cowpox. This is also called as pseudo-cowpox. Milker's nodule is transmitted to humans by direct contact with an infected animal, and the lesions are usually located on the abraded skin area of the fingers and hands. The lesion starts as an erythematous papule which soon becomes firm, elastic, bluish red and semiglobular nodule, which measures about 1-2 cm and has a central depression. The lesions become flat as these heal and disappear in 4-6 weeks.

ORF

It is primarily an infection of sheep and goat which is also called as contagiosa ecthyma, contagious pustular stomatitis, contagious pustular dermatitis, or sore mouth. The disease is transmitted to human beings by direct contact with the infected sheep. Infection sites are usually on the fingers, hands and arms. Rarely these are seen on face and neck. The lesions appear in maculopapular stages. A red centre then develops in the lesions surrounded by a white ring and a red halo. A nodular stage with red and weeping surface follows, often with central umbilication. The lesions become granulomatous or papillomatous in 3-4 weeks and some become ulcerated and superinfected with bacteria. Healing occurs in around 2 months.

MOLLUSCUM CONTAGIOSUM

This disease has a worldwide prevalence. Two types of diseases are known to occur. One, found in childhood, manifests itself with lesions on the face, trunk and limbs and is transmitted by direct contact from skin to skin or by fomites. This type is common in tropics. The second type is seen in young adulthood. It manifests as lesions in the lower abdominal area, pubis, inner thighs and genitalia and is transmitted by sexual contact. The lesions take the shape of umbilicated papules which are pink to white, measuring 2-8 mm. A semisolid material can be expressed from lesions. Sections of lesions show large eosinophilic inclusion bodies which displace the nuclei to the margin. These molluscum bodies are composed of large number of virus particles embedded in a protein matrix. This virus cannot be grown in laboratories and man is the only susceptible host. The disease is self-limiting but may last from several months to several years.

66 Papovaviruses

The viral aetiology of human warts is known since long. The papillomaviruses are now considered as potential carcinogens for malignancies of genital tract in human beings. On the basis of morphology, such viruses have been grouped along with polyomavirus of mice and vacuolating agent (SV40) of monkey. The name papovaviruses (Papilloma, Polyoma and Vacuolating agent) was thus coined. These are small, naked, icosahedral viruses that have a double stranded DNA genome and that multiply in the nucleus. The family *Papovaviridae* consists of two genera:

- Papillomaviruses
  - — Types 1 to 57
- Polyomaviruses
  - — BK virus
  - — JC virus

Viruses of both genera are widely distributed in nature and affect human beings also.

HUMAN PAPILLOMAVIRUSES

Papillomaviruses cannot be grown in cultures and existence of a large number of distinct human papillomaviruses became evident only after the development of recombinant DNA technology. Different types of human papillomaviruses (HPV) are associated with lesions of specific morphology and at specific anatomic sites (Table 66.1).

Morphology

The virion is non-enveloped and has a diameter of 55 nm, icosahedral symmetry and 72 capsomers. The viral genome is a double stranded, circular DNA molecule with molecular weight of 5.2 million daltons.

The viral capsid proteins consist of a major polypeptide and a number of minor polypeptides. Purified virion contains four histones of host origin.

Pathogenesis

Human papillomaviruses infect only epithelium of skin and mucous membranes. The virus probably infects cells of the lower layers of the epithelium which undergo proliferation and form the warts. Histologically, a wart is localised epithelial hyperplasia with a defined boundary and an intact basement membrane. Clinically warts can be of various types (Table 66.1).

Table 66–1. Lesions caused by human papillomaviruses (HPV)

Type of lesion	*Site*	*Predominant HPV type*	*Benign/ liable to cause malignancy*
Common wart	Skin, various sites	2, 4	Benign
Palmar and plantar wart	Feet, hands	1, 2, 4	Benign
Genital wart	Cervix, vulva, penis	6, 11	Benign
Laryngeal papilloma	Larynx	6, 11	Benign
Flat warts	Skin	10	Malignant(?)
Carcinoma	Cervix, penis	16, 18	Malignant(?)
Papilloma/carcinoma	Larynx	16	Malignant(?)
Pre-malignant intra-epithelial neoplasia	Cervix, penis	6, 11, 16, 18, 40, 44	Malignant(?)

Diagnosis

It is essentially clinical. History can confirm the diagnosis of warts but it does not indicate the type of HPV responsible. No serological tests are available for virus identification. Human papillomaviruses cannot be grown in culture.

A broadly cross-reactive genus-specific antiserum is available, which is capable of recognising capsid antigen of all human and animal papillomaviruses by immunoperoxidase or immunofluorescent tests. Identification of viral genotype is possible by DNA hybridisation methods.

Treatment

Most skin and genital warts regress spontaneously. The patient seeks treatment for cosmetic reasons, pain, discomfort, and disability depending on the location and size of warts. The treatment includes application of caustic agents such as podophyllin and salicylic acid, cryotherapy, surgical removal, and antimetabolites (e.g. 5-fluorouracil).

HUMAN POLYOMAVIRUSES

The first conclusive evidence of human infection with polyomavirus was obtained in the mid-1960s with the detection of virus particles in the brain tissue of a patient with progressive multifocal leukoencephalopathy (PML). The causative agent JC virus (JCV) could be isolated from brain in 1971. Similarly, another polyomavirus (BKV) was isolated from urine of a patient who had undergone renal transplantation. Subsequent studies showed that both viruses—JCV and BKV persist in the kidney following clinically inapparent primary infection in childhood. This infection gets reactivated as and when CMI of the body gets lowered. Diseases due to JCV and BKV are seen only in immunodeficient individuals.

Morphology

The virion is non-enveloped with a diameter of 44 nm, icosahedral symmetry and 72 capsomers. The viral genome is a double stranded circular DNA molecule with molecular weight of 3.2 million daltons. Each of the two DNA strands carries about 50% of the genetic information.

Both JCV and BKV haemagglutinate human erythrocytes. The capsid consists of three virus-specified proteins (VP1, VP2 and VP3) and three cellular histones (VP4, VP5 and VP6). The major capsid protein, VP1, accounts for 70% of the virion mass.

Pathogenesis

In immunocompetent hosts, both JCV and BKV persist in kidney following primay infection during childhood which remains inapparent. Reactivation of these viruses in immunologically deficient hosts results in disease. Conditions in which viruses get reactivated include pregnancy, diabetes mellitus, organ transplantation, anti-cancer therapy and immunodeficiency diseases.

Progressive multifocal leukoencephalopathy (PML) is a rare, fatal, subacute demyelinating disease of the nervous system that results from JCV infection of the oligodendrocytes in the brain. This disease has unique pathologic features. The affected area of the brain contains foci of demyelination, which have at their edges enlarged oligodendrocytes. The nuclei of the affected oligodendrocytes contain abundant number of JCV particles. The diagnosis of PML can be conclusively established only by pathologic examination of a biopsy or at postmortem. Its incidence is higher in immunocompromised individuals.

Role of Polyomaviruses in Human Malignancies

Both JCV and BKV are oncogenic for laboratory animals and they transform cultured cells. Sporadic reports of existence of DNA of these viruses in genome of cells from cancers have appeared, but no reproducible and consistent association of JCV or BKV with any human malignancy has ever been demonstrated.

Diagnosis

Evidence of multifocal brain disease in an immunocompromised individual suggests the possibility of PML, but definitive diagnosis can be established only by examination of affected tissue obtained by biopsy or at autopsy. The virus can be specifically identified by an immunoperoxidase test using monospecific anti-JCV serum. Alternatively, the viral genome in the lesion can be identified by hybridisation of the total DNA extracted from the affected tissue or by *in situ* hybridisation of paraffin sections with a JCV probe. Serological studies are not helpful in the diagnosis of PML.

67 Parvoviruses

The *Parvoviridae* comprises a well defined family of naked, single stranded DNA viruses. Several of the viruses of this family have been found to be persistent contaminants of cell cultures.

Classification

The family *Parvoviridae* has been divided into three genera:

1. *Densovirus* genus comprises autonomous viruses which infect only invertebrates.
2. *Dependovirus* genus contains viruses which are defective and depend on a helper virus, (hence the name) usually an adenovirus, for their replication, and
3. *Parvovirus* genus wherein the members are capable of productive replication without the aid of a helper virus. Some of the members of this genus have been known to cause illness in humans.

Properties of Parvoviruses

The properties of parvoviruses have been depicted in Table 67.1.

Table 67–1. Properties of parvoviruses

- Icosahedral virion, 20 nm diameter, 32 capsomers
- (–) sense single stranded DNA
- Intranuclear replication
- Large eosinophilic inclusion bodies
- pH range 3-9—thermostable (60°C × 60 mts)
- Most members haemagglutinate

The parvoviruses capsids are simple and comprise at most four structural proteins. No parvovirus group antigen has been detected.

The parvoviruses associated with human beings constitute a small number of total parvoviruses (Table 67.2).

Table 67–2. Viruses of Parvoviridae family

- *Parvovirus*
  - — B19
  - — HB virus (?)
- *Dependovirus*
  - — Adeno-associated type 2
  - — Adeno-associated type 3
  - — Adeno-associated type 5

Pathology and Clinical Features

Dependovirus infections have not been associated with any specific pathological effect in either man or animals. In contrast autonomous parvoviruses have been frequently associated with specific diseases. Of these only B19 has so far been definitely associated with disease.

The common manifestations of B19 infection is a mild fever with maculopapular rash of variable intensity. In children first sign of illness is erythema of the cheeks ('slapped cheek' appearance). In adults, instead of rash, arthritis appears. These are the symptoms of erythema infectiosum, or 'fifth disease'. Two important complications are: aplastic crisis that occurs in patients with underlying haemolytic anaemia and arthropathy.

Infection with B19 virus in first trimester of pregnancy can lead to foetal loss, missed and spontaneous abortion. Infections during second trimester is associated with hydrops foetalis.

Diagnosis

B19 virus can be detected in blood by any of the following techniques:

- Electron microscopy
- Counterimmunoelectrophoresis
- Radioimmunoassay
- ELISA
- DNA-DNA hybridisation.

The simplest and most rapid of these tests is counterimmunoelectrophoresis of the patient's serum against anti-parvovirus antibody. A positive result should be confirmed with direct or immune electron microscopy.

Class specific antibody may be detected by ELISA or RIA.

Treatment and Prevention

There is no specific antiviral treatment for B19 infection. Cases of aplastic crisis require blood transfusions till a satisfactory haemoglobin level is attained. There is no vaccine for parvovirus B19.

PART X RNA VIRUSES

68 Picornaviruses

The family *Picornaviridae* is one of the largest and most important families of human pathogens. These viruses are also the smallest RNA viruses hence the name *pico* (small) *RNA* virus. Members of this family include polioviruses, coxsackieviruses, echoviruses, hepatitis A and few other viruses.

Classification

The picornaviruses are small RNA-containing viruses causing a variety of diseases in men and animals. The family consists of four genera (Table 68.1) of which *Enterovirus* and *Rhinovirus* are pathogenic to man whereas *Aphthovirus* is responsible for foot and mouth disease in animals and *Cardiovirus* can produce encephalomyelitis in mice.

POLIOVIRUS

Global eradication of poliomyelitis by 2000 AD was the target set by the World Health Assembly in its forty-first meeting held in 1988. It continues to be an enormous challenge.

The term acute anterior poliomyelitis was first used by Erb in 1875. The word comes from *polios* meaning grey and *muelos*—the marrow.

Table 68–1. Classification of picornaviruses

Genus	*Group*	*Serotypes*	*Major diseases*
Enterovirus	Polioviruses	1-3	Paralytic poliomyelitis Aseptic meningitis
	Coxsackievirus A	1-22,24	Aseptic meningitis Herpangina Conjunctivitis
	Coxsackievirus B	1-6	Aseptic meningitis Fatal neonatal disease Pleurodynia Myocarditis/pericarditis
	Echovirus	1-9,11-27 29-34	Aseptic meningitis Rashes Febrile illness
	Enterovirus	68-72	Conjunctivitis (Type 70) Polio-like illness Hepatitis A (Type 72)
Rhinovirus		1-100	Encephalomyocarditis (71) Respiratory infections
Aphthovirus and Cardiovirus			Nonpathogenic to man

The infectious nature of the disease was shown for the first time by Karl Landsteiner and Erwin Popper in 1909 in Vienna when they could succeed in transmitting the disease to monkeys. One of the most important discoveries in virology was made in 1949 when Enders, Robbins and Weller could successfully propagate poliovirus in non-neural tissue of human beings. For this discovery they shared a Nobel Prize in 1954.

Morphology

Polioviruses are small, spherical, naked viruses with icosahedral symmetry and share many properties with other enteroviruses (Table 68.2). The capsid of poliovirus is composed of 32 capsomers, each being made up of one molecule each of the four virion proteins VP1, VP2, VP3 and VP4.

Table 68–2. Properties of polioviruses

- 18-30 nm diameter, ss RNA (positive sense)
- RNA constitutes 31% of virion
- Mol wt of genome 2.2 million daltons
- Four virion proteins: VP1, VP2, VP3 and VP4
- Resistant to low pH (pH 3)
- Resistant to various proteolytic enzymes
- Resistant to 70% alcohol, 5% lysol, or 1% quaternary ammonium compound, ether, bile
- Sensitive to 0.3% formaldehyde, 50°C/60 mt 0.3-0.5 ppm free residual chlorine in the absence of organic material

Replication

Replication of viral RNA starts within one hour of infection of a cell and has two stages. The parental positive sense RNA-strand is transcribed into a negative sense strand which serves over and over again as a template for transcription into new progeny positive sense RNA strands. Assembly of nucleic acid and structural proteins takes place in the host cell cytoplasm.

Antigens

There are three antigenic types of poliovirus (1, 2 and 3). Originally these were called as Brunhilde, Lansing and Leon respectively. Further antigenic analysis showed presence of D antigen which corresponds to the infectious particles and C antigen which corresponds to empty non-infectious capsid. Type 1 is isolated from paralytic cases most often and type 3 less so.

Pathogenesis

Despite a member of *Enterovirus*, polioviruses do not produce an enteric disease. They are so named because the alimentary canal is their predominant site of replication.

Polioviruses have a very restricted host range. Man is the only natural reservoir. The portal of entry is mouth or oropharynx. Following ingestion, poliovirus multiplies first in the pharynx or small intestine. Virus infects the mucosal tissue of pharynx and gut and enters blood as well as gains access to reticuloendothelial cells. Virus gets widely disseminated in body but affects nervous system only in exceptional cases. Through blood virus reaches anterior horn cells of the spinal cord and motor cortex of the brain (Fig. 68.1). The incubation period is usually 7-14 days with extremes of 2-35 days.

The probability of involvement of nervous system depends upon certain factors (Table 68.3). Most striking is influence of age. If an adult is nonimmune and gets affected by poliovirus the resulting paralysis shall be very severe.

Table 68–3. Factors increasing CNS involvement by poliovirus

- Elder age
- Pregnancy
- Trauma
- Tonsillectomy
- Fatigue
- Inoculations
- Absence of antibody against poliovirus

Clinical Features

Infection of an individual with poliovirus may present in any of the four types (Table 68.4). Paralytic poliomyelitis occurs in very few cases but yet remains the dominant viral manifestation. Acute flaccid paralysis (AFP) is hallmark of poliomyelitis and sometimes is also seen in Guillain-Barré syndrome. Any case of AFP requires immediate confirmation with laboratory support.

The paralysis of poliomyelitis is usually asymmetric with fever present at the outset, with maximum extent of paralysis reaching in 3-4 days. The most frequent cause of acute flaccid paralysis (AFP) that must be distinguished from poliomyelitis is Guillain-Barré syndrome (GBS). Paralysis in GBS is typically symmetrical and may progress for periods as long as 10 days.

Laboratory Diagnosis

i. **Specimens.** Specimens for virus isolation are usually stool and rectal swabs, throat swabs and washings and cerebrospinal fluid. Since virus concentration is very high in stool, isolation of virus from this specimen is most promising. Virus is most readily isolated from throat shortly after infection and upto 15 days or more from stool and rectal swabs upto 4 weeks after infection and from the CSF during the manifestations of clinical features and 2-3 weeks after infection.

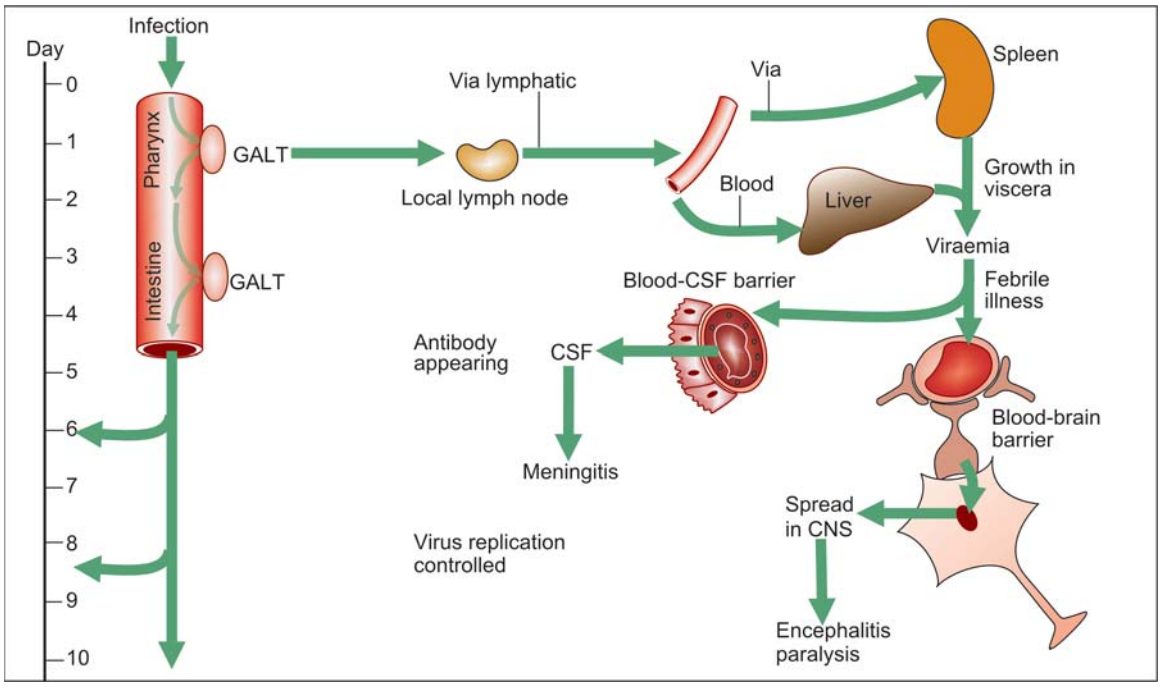


Fig. 68–1. The pathogenesis of poliomyelitis

Table 68–4. Presentations of poliovirus infections

Presentation	*Incidence*	*Clinical features*
Inapparent infection	90-95%	No clinical feature. Virus present in stool, or throat or both.
Abortive or minor	4-8%	Symptoms of respiratory or intestinal infection. Virus present in stool or antibody present.
Non-paralytic poliomyelitis	1-2%	Illness similar to aseptic meningitis, lasts for 2-10 days, rapid recovery.
Paralytic poliomyelitis	0.1-2%	Flaccid paralysis; spinal bulbar and bulbospinal; postrecovery residual paralysis may persist.

ii. **Growth in cell lines.** Polioviruses are strongly cytolytic and result in destruction of cells by lysis. Most of the infected cells detach from the culture vessel. The remaining cells withdraw from adjacent cells, round up and are attached to the substratum by long filapodia.

iii. **Confirmation of the isolate.** Neutralization test is done to confirm the identification of the virus isolate as well as to ascertain the type with nucleic acid probes and/or ELISA. Both ELISA and nucleic acid probes are used to differentiate between vaccine and wild strain.

iv. **Direct demonstration of virus.** Direct virus visualization as well as immune electron microscopy can also be performed to test the stool specimens.

v. **Serological diagnosis** can be made by neutralisation test using tissue cultures. Four-fold or greater rise in antibody titre is now less helpful in diagnosis of wild poliomyelitis infection, since type specific antibodies may already be present when paralysis develops and significant rise may not be demonstrated in paired sera. The antibody response following OPV administration mimicks the antibody response following wild virus infection.

Vaccines Against Poliomyelitis

There are two types of vaccines for immunisation against poliomyelitis: a vaccine made of noninfectious virus particles (killed or inactivated virus vaccine; administered by injection, referred to as killed poliovaccine-KPV or inactivated poliovaccine-IPV or Salk vaccine) and a vaccine made of infectious virus particles attenuated in neurovirulence (live attenuated virus vaccine; administered orally; referred to as live polio vaccine-LPV or oral polio vaccine-OPV or Sabin vaccine).

Table 68–5. Differences between IPV and OPV

	IPV	*OPV*
Nature of vaccine	Killed	Live attenuated
Route of administration	Intramuscular	Oral
Induction of Ab—Humoral IgG	+	+
—Local IgA	–	+
Prevention of paralysis	+	+
—Reinfection with wild virus	–	+
Useful in control of epidemics	–	+
Commercial production	Difficult	Easy
—Cost	Expensive	Economical
Shelf life	Longer	Shorter
Sensitivity to heat	Less	Extreme
Vaccine associated paralysis	–	+

WHO has recommended use of OPV. The OPV content of virus for each of the three types is 10^6 $TCID_{50}$ for type 1, 10^5 $TCID_{50}$ for type 2 and $10^{5.5}$ $TCID_{50}$ for type 3 virus per dose. Three doses are recommended.

The salient differences between IPV and OPV have been shown in Table 68.5.

Epidemiology

Poliomyelitis can occur sporadically, endemically or epidemically. Behaviour of the disease has changed during the past 100 years. Originally, it was a sporadic disease and has gradually assumed epidemic proportions of different degree and severity. It used to predominantly affect children (especially infants) but is now affecting higher age groups and whereas poliomyelitis was earlier confined to countries with temperate climates it is now increasingly reported from tropical countries too.

Prevention and Control

Public should be educated on the advantages of immunisation in early childhood. OPV simulates natural infection by inducing both circulating antibody and resistance to infection of the pharynx and intestine, and also immunises some susceptible contacts through secondary spread. WHO recommends the use of OPV alone for immunisation programmes in developing countries because of low cost, ease of administration and superior capacity to provide immunity through community spread. IPV like OPV, provides excellent individual protection by inducing circulating antibody that blocks the spread of the virus to CNS. Although IPV also protects against pharyngeal infection, it does not induce intestinal immunity of the level induced by OPV. WHO recommends four doses of OPV at 6, 10 and 14 weeks of age and 4th one at 9 months of age when measles vaccination is given. WHO also recommends the use of national supplemental immunisation campaigns administering 2 doses of OPV, one month apart to all children under 5 regardless of prior immunisation status. Diarrhoea is not a contraindication to OPV. It can be given to even HIV infected children. OPV causes paralytic poliomyelitis in vaccine recipients or their healthy contacts at a rate approximately one in every 2.5 million doses administered. Two approaches have been recommended to overcome this (a) replacement of OPV with IPV (b) introduction of a mixed OPV / IPV schedule where IPV is used for routine immunisation and OPV for mass campaigns to control possible outbreaks.

Poliomyelitis is a disease under surveillance by WHO and even a single case is immediately reported. Primary isolation of the virus is done in a laboratory under Global Polio Eradication Laboratory network and it is tested for it to be a wild type.

Routine immunisation of adults is not considered necessary. Primary immunisation is advised for previously non-immunised adults travelling to endemic countries.

The Global Polio Eradication Initiative

WHO launched the Global Eradication Initiative in 1988 with the objectives:

a. To interrupt transmission of the wild poliovirus as soon as possible.
b. To achieve certification of global polio eradication.
c. To contribute to health system development and strengthening of routine immunisation and surveillance for communicable disease.

Four strategies recommended to stop transmission of the wild poliovirus include:

i. High infant immunisation coverage with 4 doses of OPV in the first year of life.
ii. Supplementary doses of OPV to all children under 5 years of age.
iii. Surveillance for wild poliovirus through reporting and laboratory testing of all AFP cases among children under 15 years of age.
iv. Targeted "mop up" campaigns once wild poliovirus transmission is limited to a specific focal area.

COXSACKIEVIRUSES

Coxsackieviruses are named so because of the first isolation of these viruses from a village Coxsackie in state

Table 68–6. Clinical features associated with enteroviruses

Syndrome	*Coxsackie A*	*Coxsackie B*	*Echoviruses*
Asymptomatic infections	+	+	+
Aseptic meningitis	+	+	+
Herpangina	+	–	–
Pleurodynia	–	+	–
Respiratory disease	+	+	+
Myocarditis	–	+	–
Pericarditis	–	+	+
Congenital anomalies	+	+	–
Rash diseases	+	+	+
Hepatitis	–	–	+

of New York. These have been divided into two groups A and B, on the basis of pathological changes induced by these in suckling mice. These viruses do not infect adult mice.

General Features

The morphology and susceptibility to various physical and chemical agents is exactly same as for other enteroviruses including poliovirus. In suckling mice coxsackieviruses of group A produce a generalised myositis and flaccid paralysis. Death results within a week. A patchy focal myositis, spastic paralysis and necrosis of brown fat are produced by viruses of group B.

Serotyping of these viruses with the help of neutralisation test has divided coxsackieviruses of group A into 24 serotypes which were numbered 1 to 24. Type 23 was subsequently found to be same as ECHO 9 and hence coxsackie group A consists of only 23 serotypes. Similarly type B has got six serotypes.

Clinical Features

The major serotypes of this genus producing human diseases have been mentioned in Table 68.1. However, a wide spectrum of diseases is caused by coxsackieviruses (Table 68.6).

Laboratory Diagnosis

i. *Isolation of the Virus*

Though some of the coxsackieviruses can grow in cell cultures, the best way to isolate them from clinical specimens (lesions or faeces) is to inoculate suckling mice and examine the histopathological changes. The identification can be confirmed and typed by neutralisation tests.

In view of the many viruses involved, this is best done by using several pools of reference sera, each containing antibodies to some viruses but not to others. By noting which pools do or do not neutralise infectivity, it is possible to identify the virus in the specimen. These reagents are also known as LBM pools (after Lim, Benyesh and Melnick, who devised them). Such tests can be done only in reference laboratories.

ii. *Detection of Antibody*

A rising titre of antibody in paired sera taken early in infection and 10-14 days later is good evidence of infection, but such tests are of little value in practice, both because of time involved and many possible antibodies. They are now being superceded by IgM antibody capture ELISA tests especially in circumstances where a specific virus is suspected.

Treatment and Prophylaxis

No vaccine is available against any coxsackievirus. Antiviral therapy has also not made much progress against any of the enteroviruses.

ECHOVIRUSES

ECHO is a sigla that has been derived from four words: enteric, cytopathic, human and orphan. This name was given to these viruses because after their initial isolation from faeces of humans it was not possible to associate them with any specific disease. Soon these were placed in the family Picornaviridae because of morphological resemblance and other characters of picornaviruses.

Neutralisation tests had shown existence of 34 serotypes of echoviruses but subsequently types 10 and 28 were taken out of this genus. The former was found to be a reovirus and the later a rhinovirus.

Echoviruses infect only human beings. These do not grow in laboratory animals but grow well in human and monkey kidney cultures producing cytopathic effects.

Clinical Features

Though initially the echoviruses were thought to be orphan, i.e. not producing or associated with any illness, it has been shown now that these are capable of a large number of diseases in man (Table 68.6).

Laboratory Diagnosis

Specimens of faeces, CSF or throat swabs can be inoculated directly onto monkey kidney tissue culture and growth of virus detected by examination of CPE. The identification of virus and its typing can be performed with the help of neutralisation tests. Serological tests for diagnosis are not recommended.

Table 68–7. New enteroviruses and human disease

Serotype No.	*Disease*
68	Pneumonia and bronchitis
69	—
70	Acute haemorrhagic conjunctivitis
71	Meningitis Encephalitis
72	Hepatitis A Virus (HAV)

Treatment and Prophylaxis

No effective chemotherapy against echoviruses is available. Similarly a vaccine is yet to be made available for use.

ENTEROVIRUSES TYPES 68-72

Of the five types of enteroviruses, four are associated with disease in human beings (Table 68.7).

Acute Haemorrhagic Conjunctivitis

Two enteroviruses (coxsackievirus A24 and enterovirus 70) have resulted in two massive epidemics of acute haemorrhagic conjunctivitis during the past two decades.

The epidemic due to coxsackievirus A24 started from Singapore in 1970 where 60,000 cases were reported. Similar epidemics again occurred in South East Asia in 1975 and 1985 and in America in 1986. The disease carried a very short incubation period of 18-48 hours, caused mild to severe conjunctivitis with subconjunctival haemorrhage and complete recovery took place within 1-2 weeks. Virus could be isolated from conjunctival swabs, scrapings or throat swabs.

During 1970, another virus (*Enterovirus 70)* caused similar pandemic of acute haemorrhagic conjunctivitis which started from Ghana and spread to Indonesia, Japan, Moscow, London, Brazil and reached North America by 1981. The incubation period averaged 24 hours and acute subconjunctival haemorrhage was the most striking clinical feature. Corneal involvement was transient and disease carried a good prognosis. Rare neurological complications included radiculomyelitis, palatal paresis and Bell's palsy.

Enterovirus 70 and coxsackievirus A24 are antigenically unrelated.

ENTEROVIRUS 71

Enterovirus 71 (EV-71) has all the typical features of enteroviruses but also carries extended pathological spectrum with potential to cause large scale epidemics. This virus can cause:

- Meningitis
- Paralytic disease
- Encephalitis
- Respiratory tract infection
- Hand, foot and mouth disease.

EV-71 has become important because of its potential to cause large scale outbreaks. The biggest outbreak occurred in Taiwan in 1998 in which around 300,000 children were affected and 55 died. A similar outbreak hit Hong Kong in 1998. The laboratory diagnosis is similar to other enteroviruses. Earlier outbreaks were reported from Malaysia (1977) and Bulgaria (1975).

RHINOVIRUSES

Rhinoviruses constitute one of the four genera of family Picornaviridae and are named so (*Rhine:* nose) because of special adaptation of these viruses to grow in the nose. Like other picornaviruses, these viruses are also naked, ether-resistant with icosahedral symmetry and single stranded RNA of molecular weight 2.5 million daltons. This genus is differentiated from other genera of the family primarily by their instability below pH 5.6 and the optimum temperature for growth which in case of rhinoviruses is 33°C.

More than 100 serotypes of rhinoviruses have been authenticated so far. Rhinoviruses exist in two antigenic forms: the native N (or D) form which is infectious and H (or C) which may be produced by heating mature virions which lack VP4 protein.

Pathogenesis

Rhinovirus remains localised to the point of entry, i.e. nasal mucosa where they provoke inflammation with oedema and copious secretions within 48 hours. The localisation to nose is perhaps due to their adaptability to grow at 33°C. The virus runs its course for 4-5 days and unless complicated by superadded bacterial infection subsides completely within one week.

Clinical Features

Common cold is too common to need description. Irritation in nose, feeling of fullness and a profuse watery discharge characterise infection with rhinoviruses. Fever may manifest only in children. Sinusitis and otitis media are possible complications. The infection may exacerbate chronic bronchitis and asthma.

Laboratory Diagnosis

Rhinoviruses are most consistently isolated from nasopharyngeal washings. Best results are obtained with rapid inoculation of specimen in tissue cultures. The best cell cultures for isolation of rhinovirus from clinical

specimens are human embryonic kidney, human diploid cell strain and HeLa cells. Optimal conditions for rhinovirus isolation include incubation of cell cultures at 33°C, the use of roller cell cultures and medium maintained at neutral pH. CPE consisting of cell rounding and refractility is usually observed in the first week and often within 48 hours.

The methods which differentiate rhinoviruses from other viruses include demonstration of lability at pH 3 and a resistance to inactivation at 56°C for 30 minutes in the presence of magnesium chloride. Due to multiplicity of serotypes, serological diagnosis is not possible.

Treatment and Prophylaxis

A wide variety of compounds with powerful activity against rhinoviruses *in vitro* are available but effective therapy remains elusive.

At least three monospecific inactivated vaccines against serotypes 1A, 2 and 13 have been prepared in past 25 years and administered to volunteers who were protected against challenge by live virus. But no large scale production was undertaken because of the multiplicity of the serotypes with little or no cross-protection.

69 Paramyxoviruses

The family *Paramyxoviridae* comprises of three genera. *Paramyxovirus*, *Morbillivirus* and *Pneumovirus* which are usually transmitted by the airborne route. The human pathogens belonging to three genera of *Paramyxoviridae* include agents of measles, mumps, and certain other respiratory infections (Table 69.1).

Table 69–1. Paramyxoviruses pathogenic to human beings

Paramyxovirus
- Parainfluenza viruses
- Mumps virus

Morbillivirus
- Measles virus

Pneumovirus
- Respiratory syncytial virus

Morphology

The paramyxoviruses are enveloped viruses with pleomorphic forms and variable dimensions. The infectivity is associated with filamentous forms. The nucleocapsid is around 18 nm in diameter. Though all these viruses share most of the properties (Table 69.2) a major difference is in the length of the spikes. These spikes are longer in pneumoviruses as compared to spikes of paramyxoviruses and morbilliviruses.

Table 69–2. Properties of paramyxoviruses

- Pleomorphic virion with diameter of 150-300 nm
- Helical symmetry
- Haemagglutinin present in envelope
- ssRNA, single molecule, mol. wt. 7 million daltons
- Transcriptase in virion
- Sensitive to lipid solvents, non-ionic detergents, formaldehyde, oxidising agents

Differentiation of Genera

There is no paramyxovirus group antigen. Antigenic sharing does occur within the members of same genus which may be pathogens of different species. The salient differences have been shown in Table 69.3.

The genome of all paramyxoviruses is negative sense single strand RNA and virion transcriptase first synthesises monocistronic RNA molecule.

Table 69–3. Differentiation between members of Paramyxoviridae

Character	*Para-influenza*	*Mumps*	*Measles*	*RSV*
Serotypes	4	1	1	1
Nucleocapsid diameter	18 nm	18 nm	18 nm	14 nm
Spike length	8 nm	8 nm	8 nm	12 nm
Haemagglutinin	+	+	+	–
Neuraminidase	+	+	–	–
Haemadsorption	+	+	+	–
Cytoplasmic inclusions	+	+	+	+
Nuclear inclusions	-	-	+	–
Growth in eggs	+	+	+	–
Pathogenic to monkey	+	+	+	–
Pathogenic to chimpanzee	–	–	–	+

MEASLES

Measles is a highly contagious, acute, febrile and exanthematic illness. In the developing countries it has the highest morbidity and mortality among all vaccine preventable illnesses. Measles is one of the most ubiquitous and persistent of human viruses. Its distribution is worldwide and it causes disease in any climate and under any conditions, provided enough susceptible human beings are brought together to enable it to spread.

Clinical Features

Measles is one of the most important childhood infections. After an incubation period of 10-12 days, the disease manifests with prodromal symptoms of fever and upper respiratory tract infection marked with coryza, cough and conjunctivitis. Early diagnosis can be made by detecting Koplik's spots, which are red macules or ulcers with a bluish white centre, seen on the mucous membrane of the inside of cheek.

Rashes appear on different parts of the body starting from head followed by chest, trunk and then limbs. After a few days they start fading and then recovery is rapid and complete. Measles can cause severe and multiple complications (Table 69.4) in a large number of patients (10-20%).

Table 69–4. Complications of measles

- Otitis media
- Pneumonia
- Croup
- Bronchitis
- Giant cell pneumonia
- Encephalomyelitis
- SSPE

Encephalomyelitis has an incidence of less than one out of 1000, but carries a mortality of 15%. It also has sequelae of epilepsy and personality changes.

SSPE

Subacute sclerosing panencephalitis (SSPE) is a delayed complication of measles and occurs few years after primary infection. A progressive loss of cerebral functions ending in spasticity, coma and inevitable death within one year are the features of this rare complication. It can be diagnosed by the demonstration of antibody in CSF and by fluorescent antibody test on neural tissue.

Isolation of virus from the brain of a patient with SSPE is difficult because the virus is defective. It can, however, be accomplished by elaborate **cocultivation** technique involving layering of brain cells from affected patients onto monolayers of monkey kidney or other susceptible cells.

Pathogenesis

Measles, like mumps, is a typical systemic viral infection. Virus gains entry through respiratory tract, multiplies in the epithelial lining and then spreads to lymph nodes where another phase of replication occurs. Further spread to organs takes place and skin, brain and lungs get involved (Fig. 69.1). Lesions produced are characterised by the presence of multinucleated giant cells with well defined intranuclear and intracytoplasmic inclusions.

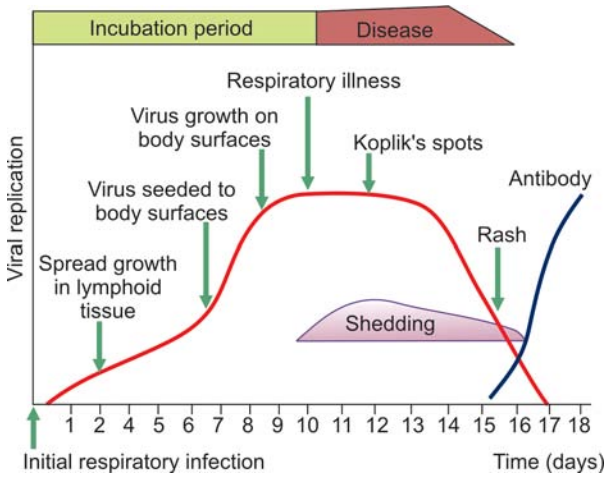


Fig. 69–1. Pathogenesis of measles

It is now well established that the maculopapular rash of measles is mediated by immunopathological mechanism. Such rashes do not appear in immunologically compromised patients who develop pneumonia and in case of adults, the disease proves fatal.

Immune Response

High titres of IgG, IgM as well as secretory IgA are seen after primary infection with measles virus. IgM and IgA disappear after sometime but IgG persist lifelong making the individual immune to reinfection.

Though a strong immune response is mounted by the body on getting infected with measles virus, the disease has got an immunosuppressive action.

Laboratory Diagnosis

i. *Clinical Samples*

Diagnosis of a typical case of measles can be made based upon clinical symptoms. However, demonstration of the virus or seroconversion against the virus is necessary to confirm the diagnosis. Best results for isolation of virus are obtained when specimens are taken during the first few days of illness. Measles virus may be isolated from blood, throat, conjunctivae and urine.

ii. *Growth in Cell Cultures*

Measles virus grows very slowly in primary human kidney, monkey kidney and human amnion cell cultures. The virus produces characteristic multinucleate giant cells after 7-10 days of incubation. Cultures can then be tested for haemadsorption with monkey erythrocytes.

The confirmation can ideally be made by immunofluorescent staining using monoclonal antibody.

iii. *Demonstration of Giant Cells*

Rapid diagnosis can be achieved by direct method of demonstration of giant cells (*Warthin-Finkeldey* cells) in respiratory secretions or affected tissues.

iv. *Demonstration of Measles Antigen*

More reliable method shall be demonstration of measles antigen in these tissues by immunofluorescent method using monoclonal antibody.

v. *Serodiagnosis*

The most practical method to make a laboratory diagnosis of measles is to obtain acute and convalescent phase sera and demonstrate a greater than four-fold rise in specific antibody using haemagglutination inhibition, neutralisation, complement fixation, RIA or ELISA techniques. The presence of specific IgM antibody can be used to diagnose recent infection.

High titres of measles complement fixing antibody in serum and particularly CSF strongly suggest SSPE. To show that these antibodies have been produced in the brain itself and have not crossed the blood-brain barrier because of inflammation or haemorrhages, the CSF should be tested for antibodies present in the serum as well as albumin. Albumin and these antibodies will be present in high titre in CSF in cases of haemorrhages and inflammation but not in cases with SSPE.

Epidemiology

Measles and its complications kill almost two million children every year in the world, one every 15 seconds. The six diseases included in Expanded Programme on Immunisation (EPI) together kill 10 children per minute and disable 10 more: measles alone is responsible for one-third of these deaths. Another two million children are permanently disabled by this disease as a result of blindness (with associated vitamin A deficiency), deafness or brain damage. These are as a result of the encephalitis which occurs in one out of every 2000 cases.

Prevention and Control

Extensive use of live attenuated vaccines have brought down the incidence of measles quite significantly throughout the world. The vaccine is prepared from any of the following strains:

- Edmonston
- Schwartz
- Edmonston-Zagreb

The Edmonston Zagreb strain has been found to be superior to the other two strains.

Measles vaccine is given when the child is 9 months of age or more to prevent inactivation by the maternal antibody. Measles vaccine is available in combined form in which antigens of mumps and rubella viruses have also been incorporated. WHO recommends measles immunisation of all infants and children regardless of HIV status because of greater risk of severe measles in such persons. Children with measles be kept out of school for 4 days after appearance of rash. The contact of a case can also be vaccinated within 72 hours of exposure. During measles infection vitamin A reserves fall rapidly and hence vitamin A supplements be given. Vaccination given at the beginning of an epidemic limits its spread.

MUMPS

Mumps is predominantly, but not exclusively, a disease of childhood. Attacks in adult life are probably much more frequent than of measles, chickenpox and other common infections. No age is immune and there is no difference in the incidence between the two sexes although the disease appears to be more frequent in young male adults than in females. This difference is perhaps more apparent than real and due mainly to high incidence of orchitis which draws attention to what might otherwise be a short and soon-forgotten illness.

Like measles mumps virus is a member of the family *Paramyxoviridae*. Important features of this virus have been presented in Tables 69.2 and 69.3.

Pathogenesis

Mumps is a typical systemic viral infection (Fig. 69.2). The portal of entry of the virus is thought to be the upper respiratory tract. The time interval after exposure to virus before the appearance of the clinical features ranges from 14 to 21 days, with the usual incubation period being 16-18 days. After entering the host, the virus replicates and viraemia results. It leads to secondary invasion of several organs. Tissues such as the salivary glands (predominantly the parotids), meninges, testes, pancreas, ovaries, thyroid and heart may show evidence of infection. Virus is also excreted in urine and transient abnormalities in renal functions have been found. Pathogenesis of damage to nervous

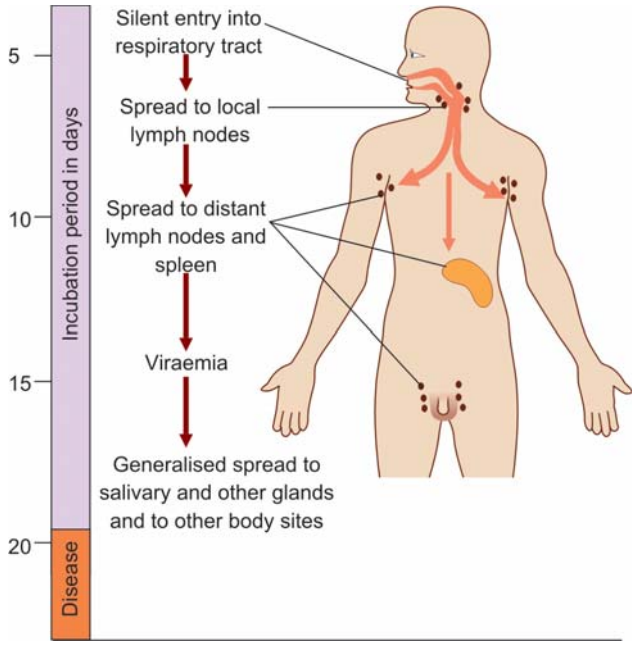


Fig. 69–2. Pathogenesis of mumps

system is poorly understood but may be due to direct lysis of cells and immunopathological mechanisms.

All classes of specific immunoglobulins are induced by primary infection due to mumps virus. The IgM response persists for a few days but the IgG response is life-long.

Clinical Features

Mumps is a common contagious disease of children and young adults. It is characterised by the inflammation of salivary glands. Bilateral involvement of parotid glands is the commonest occurrence. Unilateral involvement of this gland can also occur. Similarly inflammation and swelling of submaxillary glands as well as sublinguals can also get infected. The disease is considered more notorious because of its complications of which orchitis and sterility in adult males is important.

Infection with mumps virus during the first trimester of pregnancy may result into abortion. Though no teratogenicity has been demonstrated because of this virus, ample evidence for transplacental transmission is available.

Laboratory Diagnosis

In the presence of typical clinical picture the diagnosis of mumps is very simple and does not require any support from the laboratory. However, diagnosis by the virus isolation or serological techniques is most useful when the patient presents with an atypical or asymptomatic infection.

i. *Clinical Specimens*

Virus isolation from the spinal fluid, blood, saliva and urine confirms the diagnosis of recent mumps infection.

ii. *Isolation of the Virus*

Primary monkey kidney cell cultures are the most sensitive substrates for the isolation of this virus. These cells may be of rhesus or cynomolgus monkey origin. Continuous human cell lines such as HeLa and primary cell cultures of human amnion or human embryonic kidney can also be used for the growth of the virus. The virus produces a characteristic cytopathic effect (CPE) with large syncytia. Some strains may not produce CPE and their adsorption with guinea pig erythrocytes should be attempted to identify them.

Rapid identification of mumps isolates can be achieved by immunofluorescence staining.

iii. *Serodiagnosis*

Serological diagnosis of mumps infection can be very important, especially in those cases of meningitis or encephalitis that occur in the absence of parotitis. Serological methods for the diagnosis of mumps infection include complement fixation, haemagglutination inhibition, neutralisation, and ELISA. Use of ELISA to detect IgM is particularly suitable for early diagnosis of mumps infection with one serum specimen.

Treatment and Prevention

A live attenuated vaccine prepared from Jeryl Lynn strain of mumps virus was licensed for human use in the USA in 1967 and since then more than 100 million doses have been administered to children. Till recent past vaccines were prepared from following strains of mumps virus:

- Jeryl Lynn strain
- Urabe strain
- Rubini strain

However, a higher incidence of vaccine associated meningitis because of the vaccine prepared from Urabe strain has forced the authorities in United Kingdom and Europe to discontinue the use of mumps vaccine prepared from this strain. The other two strains have not exhibited this kind of adverse reactivity.

Mumps vaccine can be given along with or in combination with antigens of measles and rubella in combined form of measles-mumps-rubella (MMR). There is no specific treatment

INFECTIONS CAUSED BY PARAINFLUENZA VIRUSES

Clinical Features

The parainfluenza viruses cause upper respiratory tract infection especially in young children. The disease may remain mild in nature or assume severe form. Type 1 and 2 may also cause croup in which inflammation of vocal cords occurs.

No life-long immunity is conferred by these viruses and hence reinfection is common.

Pathogenesis

Not much is known regarding the pathogenesis of this disease. The virus enters through the respiratory tract and produce localised disease. The virus multiplies in the epithelium of the respiratory tract. The damage caused to the epithelium results into an inflammatory response. Virus infection induces production of local IgA and systemic IgG. These antibodies as well as CMI combine together to cause termination of illness.

Laboratory Diagnosis

i. *Clinical Samples*

These viruses are present in the respiratory secretions and maximum isolation has been seen if the samples were collected from posterior pharynx or nasopharynx. Prompt inoculation of specimens into the cell cultures is critical for successful virus isolation.

ii. *Virus Isolation*

The inoculated cell lines are incubated at 33-36°C and for better results a roller apparatus is employed. Type 2 strains produce a typical CPE but the CPE in type 3 strains is ill defined and those of types 1 and 4 are virtually non-existent. Virus in cultures is thus detected by haemadsorption.

iii. *Serological Tests*

Rapid detection of parainfluenza virus in cell smears of clinical specimens can be made by using indirect immunofluorescence. This test can be highly specific and sensitive if good quality antisera are used. Serology can be of help in undertaking retrospective epidemiological studies.

INFECTIONS CAUSED BY RESPIRATORY SYNCYTIAL VIRUS

Respiratory syncytial virus (RSV) is a large paramyxovirus that is sufficiently different from the parainfluenza viruses to be classified in a separate genus *Pneumovirus*. The differences include a smaller ribonucleoprotein helix, and the absence of haemagglutinin, neuraminidase and haemolysin activities associated with the envelope glycoproteins.

Clinical Features and Pathogenesis

RSV is the major cause of bronchiolitis and pneumonia of infancy, although these may also result from infection with parainfluenza type 3, influenza A and B viruses and occasionally other respiratory viruses. Respiratory syncytial virus infection occurs in annual winter epidemics which last 2 to 5 months. Recent reports suggest that there are at least two strains of RSV.

Characteristically, illness occurs in a male child aged less than six months. Rarely children aged less than two years may also be affected. Child experiences respiratory difficulty manifested by hyperventilation and retractions. Examination of the chest reveals expiratory prolongation wheezes and rhonchi. There may or may not be pulmonary infiltrate. In more severe cases anoxia may be present and child must be hospitalised.

Laboratory Diagnosis

i. *Clinical Samples*

Both viral isolation and direct demonstration of RSV are widely used. Nasal washes yield the best specimens for virus isolation. Specimens should be transported and stored at 4°C because viral infectivity decreases rapidly at room temperature or after freeze-thawing. Best results are obtained when the specimen is inoculated onto cell culture as soon after collection as is possible.

ii. *Virus Isolation*

HEp-2 and HeLa cells are most sensitive to RSV infection and are widely employed for its isolation. Typical CPE consists of the formation of large multinucleated syncytia, which are detectable in an average of 4 to 5 days. Formation of large syncytia in these cell lines is strong presumptive evidence for RSV isolation.

Definite identification can be performed by immunofluorescence or virus neutralisation.

iii. *Serological Tests*

For the direct identification of RSV in clinical specimens both direct and indirect immunofluorescence have been described. Reverse passive haemagglutination also has been described for the detection of this virus in the nasopharyngeal secretions.

A variety of serologic tests can be used to confirm the past RSV infection. These include complement fixation, neutralisation or ELISA assays. In addition, indirect immunofluorescence can be employed for the study of specific immune response to primary and subsequent RSV infection.

Therapy and Prophylaxis

Ribavirin has been found to be effective in treatment of children suffering from acute bronchiolitis due to RSV. Inactivated vaccine against RSV has proved not only unsuccessful but also positively harmful.

70 Orthomyxoviruses

Influenza is the popular name for a short-lasting fever associated with acute respiratory infection; a disease which is considered trivial by an individual but is important as a cause of severe morbidity and mortality especially amongst young children and old people; and a virus which has defied control till date. Excess mortality often accompanies influenza epidemics, the vast majority affected being the elderly people.

The viruses capable of causing influenza belong to the family Orthomyxoviridae. Members of families Orthomyxoviridae and Paramyxoviridae are mainly pathogens of respiratory tract and derive their name from their affinity for mucin (Greek *myxo* : mucin). Yet there are many differences between these two families (Table 70.1).

Table 70–1. Differences between orthomyxoviruses and paramyxoviruses

Feature	*Orthomyxovirus*	*Paramyxovirus*
Virion size	80-120 nm	100-300 nm
Diameter of nucleocapsid	9 nm	18 nm
Filaments	+	–
Haemolysis	–	+
No of segments in genome	8	1
Ribonucleoprotein synthesis takes place at	Nucleus	Cytoplasm
Genetic recombination	+	–
Antigenic stability	Variable	Stable
Requirement of DNA dependent RNA	+	–
Inhibition by actinomycin-D	+	–

General Features

Influenza viruses have a wide range of hosts. These include human beings, lower terrestrial mammals and aquatic mammals and birds. Genetic reassortment readily occurs between different strains of influenza viruses in laboratory as well as in nature.

A and B are separate genera whereas because of some morphological and antigenic differences. Influenza C has been accorded provisionally a separate genus status.

Morphology

The orthomyxoviruses are pleomorphic in shape, usually spherical and rarely filamentous in appearance. The RNA is present in a helical nucleocapsid surrounded by a lipid containing envelope (Fig. 70.1). Particles have an external diameter of 80-120 nm.

Densely arranged radial projections (spikes) cover the influenza viruses. In influenza A and B, these spikes have two distinct morphologies which represent the *haemagglutinin* (HA) and the *neuraminidase* (NA) components of the virus.

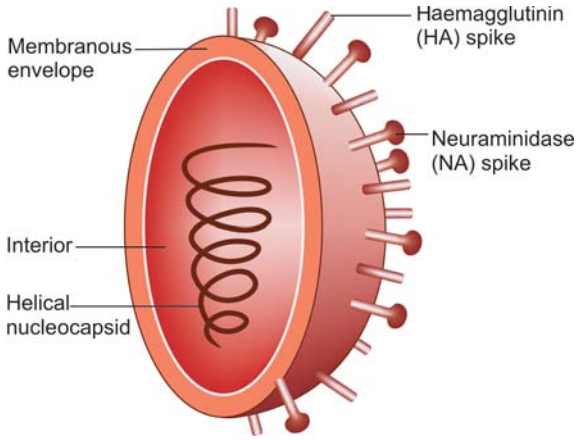


Fig. 70–1. Influenza virus

The haemagglutinin protein exists as 500 spikes which are 12 nm in length. Each spike has two ends-one narrow which is inserted into the lipid membrane of the virus and the other broad which has a breadth of 5 nm. The neuraminidase consists of 100 spikes per virion and are mushroom shaped. The tails of neuraminidase and haemagglutinin pierce the lipid layer. Three polymerase polypeptides designated as PA, PB1 and PB2 are also present.

The genome is a segmented single stranded RNA of length of 13,600 nucleotides and molecular weight of 5 million daltons. The RNA genome comprises of only 2% of the mass of the virion. The eight segments code for different proteins. Gene 7 and 8 are large in size and code for more than one proteins. These proteins are NS1, NS2, NP, M1, M2, M3, HA, and NA.

Resistance to Physical and Chemical Agents

Exposure to heat for 30 minutes at 56°C inactivates most strains. These viruses are also inactivated by 20% ether, phenol, formaldehyde, salts of heavy metals, detergents, soaps and many other chemicals.

Influenza virus withstands slow drying at room temperature. It can remain viable in the dust even upto two weeks. It can be preserved for long periods in allantoic cavities as well as at –70°C. It remains viable indefinitely after freeze drying.

Polypeptides

Various polypeptides are synthesised by the genome of the virus. Of these haemagglutinin and neuraminidase have been subjected to intensive research.

Haemagglutinin

A cross-section of haemagglutinin shows it to be triangular in shape. The tail of the haemagglutinin (HA) contains 25-28 amino acids by which it is inserted into virus membrane.

Five antigenic sites have been detected on the distal tip of the HA molecule. These have been designated as HA1 to HA5. The distal end also contains sites for binding to the host cells.

During major antigenic changes in the HA molecule (**antigenic shift**), nucleotide and amino acid sequences of the polypeptides HA1 and HA2 change radically, whereas in minor antigenic changes (**antigenic drift**) only small modifications of the amino acids composition of these components are detected.

The haemagglutinin enables the virus to adsorb to glycoprotein receptors present on the surface of erythrocytes of many mammalian and avian species. The association is usually stable at 4°C but at 37°C the virus rapidly elutes as a result of destruction of the receptors by the viral neuraminidase.

Neuraminidase

The enzymic action of neuraminidase (NA) is the hydrolysis of N-acetyl neuraminic acid (sialic acid) residues from specific glycoprotein substrates. Such glycoproteins form specific receptor sites for haemagglutinin on the surface of the host cell or the RBC. Role of neuraminidase in viral maturation has also been suggested but needs confirmation. Just like haemagglutinin, the molecule of NA also possesses two ends—one hydrophobic end is in contact with and embedded into the lipid membrane whereas the other end contains enzymatically active sites as well as antigenic determinants.

Types and Strain Designation

The influenza viruses A has been further subdivided into 16 *subtypes* (A1 to A16)), and the subtypes into strains. A1, A2 and A3 subtypes are primary pathogens of humans whereas remaining A subtypes can infect poultry and other animal species.

All strains of influenza viruses of a genus (A, B or C) share common internal proteins (the nucleoproteins, membrane proteins) but differ in their surface proteins, the haemagglutinin and neuraminidase. Thus, the major pandemic subtypes of Influenza in 1957 was A/Singapore/1/57 (H2N2) which indicates that it was influenza A which arose in Singapore and isolated for the first time in 1957. The bracketed letters indicate its haemagglutinin and neuraminidase antigens which were H2 and N2. In 1968 another pandemic started from Hongkong and the strain was designated as A/Hongkong/1/68 (H3N2). This designation indicates that the strain underwent change in haemagglutinin but the neuraminidase antigen remained the same as was in Singapore strain of 1957.

Since 2003, a novel subtype H5N1 has been spreading in poultry all over the world. This subtype is also called as high pathogenic avian influenza (HPAI) virus because of the rapid and extensive mortality in chicken. HPAI virus has occasionally jumped to human beings causing mortality in more than 50% of cases. Fears have been expressed that a recombinant between HPAI and any of the primary human pathogen can result into a strain with high pathogenisity and extreme transmissibility.

Antigenic Shift and Antigenic Drift

Major changes in HA or NA antigen are called as *antigenic shift*. Minor changes which occur at frequent intervals within the same HA or NA antigenic types are called as *antigenic drifts*.

Genetic Basis of Antigenic Shift and Antigenic Drift

Single mutation affecting an amino acid in a key antigenic determinant of the HA glycoprotein is sufficient to bring about significant antigenic drift, whereas antigenic shift results from the replacement of the gene for HA with one coding for a totally different amino acid sequence. By and large, changes in the HA are of greater epidemiological importance than changes in NA because antibodies directed against HA are considerably more effective in neutralising the infectivity of the virion.

In antigenic shift virtually all the antigenic determinants are altered whereas in antigenic drift only certain epitopes change, while others are conserved and enable the HA to be identified as belonging to a particular subtype.

Antigenic shift occurs independently in the HA and NA and appears to be a requirement for the appearance of pandemic influenza. At the same time though each episode of antigenic drift is minor in nature but it can produce additive effect and over a prolonged period may result in appearance of strains differing considerably from the original pandemic virus.

Pathogenesis

The influenza viruses enter through the respiratory tract where these may be inactivated by the IgA antibody produced in response to earlier infections with influenza viruses. If it does not occur, inflammation of upper respiratory tract results. Due to this infection, necrosis and widespread desquamation of the respiratory epithelium results. Lower respiratory tract is rarely involved. Influenza viruses lower the resistance of the respiratory tract thus permitting the bacterial infections to supervene.

Immune Response

Following infection with influenza virus, antibodies develop to the major structural proteins HA, NA, NP and M as well as to NS1. Antibody to HA constitutes an important part of the immune response because of the capability of these antibody to inactivate the influenza virus. The precise mechanism by which these antibodies neutralise influenza virus is not known.

Acquired immunity to a given strain is adequate for a year or two. This depends upon the titre of the neutralising antibody (IgA) in the respiratory mucosa. Because of continuous emergence of mutants as a result of antigenic drift, the immunity induced by previous infection may not be sufficient to protect the individual. The response to second infection shall depend upon the previous exposure. Because of some antigenic overlap, an anamnestic response results. Hence, repeated infections with influenza viruses shall increase the immune response to those determinants which have been presented to immune system in previous exposures. Obviously, the individual will have maximum antibody against those epitopes which he first experienced and variants of which have been attacking his body subsequently. This phenomenon has been called as doctrine of **original antigenic sin**. In this phenomenon, the antibodies produced as a result of second or 'reinforcing' infection react more readily with the virus encountered in the first or 'priming' infection than with the reinforcing virus that stimulated the response.

Antibodies against neuraminidase are also raised in the human body subsequent to an infection with influenza virus. There is ample evidence to suggest that these antibody are also protective in nature albeit play a role which is not as great as that of HA antibody.

The role of cell-mediated immune response in protection against influenza is uncertain.

Clinical Features

After an incubation period of 2-3 days, the first symptoms of headache, shivering and a dry cough accompanied by fever appear. These symptoms may subside within 24 hours in children or may take a prolonged course in which temperature remains high for upto 5 days. Adults may also experience bodyache and malaise. In the absence of complications, patient may recover fully in 7-10 days but some persons may experience weakness for a longer time. The complications that may result, especially in children have been shown in Table 70.2.

Laboratory Diagnosis

Virus isolation and identification is the standard laboratory method to diagnose influenza virus infections, although many methods to directly examine clinical specimens for virus antigen have been described and are becoming commercially available.

Table 70–2. Complications of influenza

Respiratory system
• Bronchitis
• Bronchiolitis
• Pneumonia
• Secondary infection with *Staphylococcus* and *Pneumococcus*
Cardiovascular system
• Nonspecific ECG changes
• Cardiomyositis
Central nervous system
• Reye's syndrome

Clinical Samples

Frequently collected specimens are nasal and throat washes, or swabs of nasopharynx and throat. Optimal viral isolation is obtained from a nasopharyngeal wash but a combined nose-throat swab is acceptable. Freezing of specimens results in reduction of virus number by 60% with a single freeze-thaw cycle.

Growth in Cell Lines

Because of different sensitivities of influenza types, subtypes and strains, no single cell line can be adopted. Rhesus and cynomolgus monkey kidney cells, and continuous cell lines such as Madin-Darby canine kidney (MDCK) and rhesus kidney (LLC-MK2) are used to isolate influenza virus.

Because of their uniform sensitivity and easy availability cell lines are widely used. Inoculated cell cultures are incubated in the absence of serum (which may have viral inhibitory factors) and in the presence of trypsin. Most influenza isolates produce little or no cytopathology. Vacuolation and cell lysis may be seen with influenza B but not with influenza A. The detection of influenza virus in cell culture is performed by haemadsorption using guinea pig, human type O or chicken erythrocytes. Influenza type A and B haemadsorb both at 4ºC and 22ºC whereas type C haemadsorbs chicken RBCs only at 4°C due to the presence of a receptor destroying enzyme which is active at 22ºC.

Growth in Embryonated Eggs

Classically, embryonated eggs and primary kidney cells have been the methods of choice. Embryonated eggs are inoculated in the allantoic and amniotic cavities and the fluids collected from these cavities is tested for viral haemagglutination after a three days incubation period.

Methods for further typing and subtyping influenza isolates include haemagglutination inhibition, haemadsorption inhibition, complement fixation and neutralisation.

Immunofluorescence

Direct examination of clinical specimens using a direct immunofluorescent technique can be done and in experienced hands this technique gives as good results as are seen with isolation in cell culture. ELISA and RIA have been developed for the detection of influenza antigens in the clinical specimens.

Serodiagnosis

Diagnosis of infection can also be made by detecting a serologic response when sera obtained during the acute and convalescent phase of infection are compared.

Treatment

Amantidine has been found to be extremely useful against infection with influenza A. Paradoxically, this drug is almost ineffective in infections with influenza B virus. The drug has been found to have prophylactic as well as therapeutic activity against various subtypes of influenza A (H1N1, H2N2 and H3N3).

Ribavirin has also been found to have prophylactic and therapeutic use against influenza virus but the toxicity of the drug prevents its use in routine.

Outbreaks of Influenza

Though influenza was first described by Hippocrates in 412 BC the first well described pandemic of influenza-like illness occurred in 1580. Since that time 31 such possible influenza pandemics have been documented, with three occurring in this century: in 1918, 1957 and 1968. There is evidence that the viruses which caused these epidemics originated from animals (1918 = swine, 1957 and 1968 = avian strains). In 1976, a new influenza virus from pigs caused human infections and severe illness. A vaccine against swine influenza was developed and administered in some countries, although no pandemic in fact occurred.

The most devastating of the 20th century influenza pandemics was that known as Spanish Flu which killed more than 20 million around the globe between 1918 and 1920. The virus responsible for this pandemic is now known as influenza A(H1N1).

AVIAN INFLUENZA (BIRD FLU)

Introduction

Avian influenza, or "bird flu", is a contagious disease of animals caused by viruses that normally infect only

birds and, less commonly, pigs. Avian influenza viruses are highly species-specific, but have, on rare occasions, crossed the species barrier to infect humans.

In domestic poultry, infection with avian influenza viruses causes two main forms of disease, distinguished by low and high extremes of virulence. The so-called "low pathogenic" form commonly causes only mild symptoms (ruffled feathers, a drop in egg production) and may easily go undetected. The highly pathogenic form is far more dramatic. It spreads very rapidly through poultry flocks, causes disease affecting multiple internal organs, and has a mortality that can approach 100%, often within 48 hours.

Causative Agent

Influenza A viruses have 16 H subtypes and 9 N subtypes. Only viruses of the H5 and H7 subtypes are known to cause the highly pathogenic form of the disease. However, not all viruses of the H5 and H7 subtypes are highly pathogenic and not all will cause severe disease in poultry.

On present understanding, H5 and H7 viruses are introduced to poultry flocks in their low pathogenic form. When allowed to circulate in poultry populations, the viruses can mutate, usually within a few months, into the highly pathogenic form. This is why the presence of an H5 or H7 virus in poultry is always cause for concern, even when the initial signs of infection are mild.

Role of Migratory Birds

Considerable circumstantial evidence suggests that migratory birds can introduce low pathogenic H5 and H7 viruses to poultry flocks, which then mutate to the highly pathogenic form. Recent events make it likely that some migratory birds are now directly spreading the H5N1 virus in its highly pathogenic form.

Risks of Avian Influenza to Human Health

The widespread persistence of H5N1 in poultry populations poses two main risks for human health. The first is the risk of direct infection when the virus passes from poultry to humans, resulting in very severe disease. Of the few avian influenza viruses that have crossed the species barrier to infect humans, H5N1 has caused the largest number of cases of severe disease and death in humans. The disease caused by H5N1 follows an unusually aggressive clinical course, with rapid deterioration and high fatality. Primary viral pneumonia and multiorgan failure are common. A second risk, of even greater concern, is that the virus—if given enough opportunities—will change into a form that is highly infectious for humans and spreads easily from person to person. Such a change could mark the start of a global outbreak (a pandemic).

Mode of Transmission

Direct contact with infected poultry, or surfaces and objects contaminated by their faeces, is presently considered the main route of human infection. To date, most human cases have occurred in rural or periurban areas where many household keep small poultry flocks, which often roam freely, sometimes entering homes or sharing outdoor areas where children play. As infected birds shed large quantities of virus in their faeces, opportunities for exposure to infected droppings or to environments contaminated by the virus are abundant under such conditions.

In areas experiencing outbreaks, poultry and poultry products can also be safely consumed provided these items are properly cooked and properly handled during food preparation. The H5N1 virus is sensitive to heat. Normal temperatures used for cooking (70°C in all parts of the food) will kill the virus. Consumers need to be sure that all parts of the poultry are fully cooked (no "pink" parts) and that eggs, too, are properly cooked (no "runny" yolks).

Risk of Pandemic

A pandemic can start when three conditions have been met: a new influenza virus subtype emerges; it infects humans, causing serious illness; and it spreads easily and sustainably among humans. The H5N1 virus amply meets the first two conditions: The virus can improve its transmissibility among humans via two principal mechanisms. The first is a "reassortment" event, in which genetic material is exchanged between human and avian viruses during co-infection of a human or pig. The second mechanism is a more gradual process of adaptive mutation, whereby the capability of the virus to bind to human cells increases during subsequent infections of humans.

Diagnosis

Laboratory identification of human influenza A virus infections is commonly carried out by direct antigen detection, isolation in cell culture, or detection of influenza-specific RNA by reverse transcriptase-polymerase chain reaction.

Rapid antigen detection results can be obtained in 15-30 minutes by immunofluorescence assay and

enzyme immunoassay for influenza A nucleoprotein (NP). Virus culture provides results in 2-10 days. Both shell-vial and standard cell-culture methods may be used to detect clinically important respiratory viruses. Positive influenza cultures may or may not exhibit cytopathic effects but virus identification by immunofluorescence of cell cultures or haemagglutination-inhibition (HI) assay of cell culture medium (supernatant) is required. Polymerase chain reaction and real-time PCR assays have also been developed.

Status of H5N1 Vaccine

Vaccines effective against a pandemic virus are not yet available. Vaccines are produced each year for seasonal influenza but will not protect against pandemic influenza. Although a vaccine against the H5N1 virus is under development in several countries, no vaccine is ready for commercial production and no vaccines are expected to be widely available until several months after the start of a pandemic.

Treatment

Two drugs (in the neuraminidase inhibitors class), oseltamivir (commercially known as Tamiflu) and zanamivir (commercially known as Relenza) can reduce the severity and duration of illness caused by seasonal influenza. The efficacy of the neuraminidase inhibitors depends, among others, on their early administration (within 48 hours after symptom onset). For cases of human infection with H5N1, the drugs may improve prospects of survival, if administered early, but clinical data are limited.

71 Retroviruses

Retroviruses are unique in the sense that the flow of genetic information is reverse to the universal flow. In all other organisms, DNA is the store house of genetic information and from here it is transmitted to RNA. In retroviruses the sequence is RNA to DNA. Genetic information is passed on from RNA to DNA through RNA-dependent DNA polymerase (*reverse transcriptase)*. The viral genetic information in DNA form is called as *provirus*. This is capable of integration into the host genome. On activation of provirus, virus specific proteins are manufactured.

Retroviruses are known to cause cancers in various animals and in 1980 relationship between a retrovirus and a human malignancy was documented that led to characterisation of human T cell leukaemia virus.

Classification

The family Retroviridae comprised of three subfamilies: *Oncovirinae*, *Lentivirinae* and *Spumavirinae*. The human pathogens in this family have been shown in Table 71.1.

Table 71–1. Human pathogens in family retroviridae

Subfamily	*Human pathogen*
Oncovirinae	Human T cell leukaemia virus I, II
Lentivirinae	
Human lentiviruses	HIV-1 and HIV-2
Spumavirinae	Human foamy viruses

Retroviruses of vertebrates are now classified into 7 Genera (Table 71.2).

The *human foamy viruses* are so-called because of their characteristic lace like CPE in cell cultures. These infect a large number of mammalian species. Though antibody to these viruses have been detected in human sera, their association with any disease is yet to be ascertained.

Table 71–2. Classification of retroviruses of vertebrates

Genus	*Example*
1. Alpha-retroviruses	*Rous sarcoma virus* *Avian leukosis virus*
2. Beta-retroviruses	*Murine mammary tumour virus* *Simian retrovirus type I*
3. Gamma-retroviruses	*Murine leukaemia virus*
4. Delta-retroviruses	*Human T cell leukaemia virus*
5. Epsilon retroviruses	*Fish dermal sarcoma virus*
6. Lentiviruses	*Human immunodeficiency virus*
7. Spumaviruses	*Primate foamy virus*

HUMAN T CELL LEUKAEMIA VIRUSES

Morphology

The virion of human T cell leukaemia virus (HTLV) comprises icosahedral core containing the RNA genome and surrounded by an envelope acquired as the virion buds through the host cell membrane. Virus specific envelope glycoproteins are inserted within the membrane that surrounds the virus.

Antigens

Three structural antigens are recognised in HTLV. These are *core antigen, envelope antigen* and *RT antigen.* The RT (reverse transcriptase) antigen is derived from a polypeptide precursor which on being cleaved results into RT, protease and endonuclease. RT is quite antigenic and sera from infected patients contain antibody to it.

Clinical Features

No acute illness is caused by HTLV. The chronic form may manifest as leukaemia or as neurologic disease.

An overwhelming number of infections remain asymptomatic.

The leukaemias include adult T cell leukaemia-lymphoma (ATLL); secondary immune paresis and hairy cell leukaemia. The neurological disorder is associated with upper motor neuron lesion which produces progressive symmetrical spastic paraparesis.

Laboratory Diagnosis

Antibodies to HTLV can be detected by immunofluorescence, particle agglutination and enzyme linked immunosorbent assays (ELISA). For confirmatory tests, assays such as Western blotting and radioimmunoprecipitation can be used.

Viral antigen can be occasionally detected in CSF but is not a reliable marker of infection. Gene amplification with polymerase chain reaction (PCR) has been successfully applied to lymphocytes from patients with antibody to HTLV.

Treatment

No specific therapy or vaccine against HTLV is available.

HUMAN IMMUNODEFICIENCY VIRUS

Origin and Discovery of Causative Agent

First few cases of AIDS were detected in June, 1981, when the US. Centers for Diseases Control and Prevention (CDC) reported a cluster of *Pneumocystis pneumonia* (PCP) caused by a form of *Pneumocystis carinii,* now recognised as a distinct species *Pneumocystis jiroveci*, in five homosexual men in Los Angeles. The disease was originally dubbed GRID, or Gay-Related Immune Deficiency, but health authorities soon realized that nearly half of the people identified with the syndrome were not homosexual men. In 1981, the CDC introduced the term AIDS to describe the newly recognised syndrome, though it was still casually referred to as GRID.

In 1983, scientists led by Luc Montaginer at the Pasteur Institute in France first discovered the virus that causes AIDS. They called it lymphadenopathy associated virus (LAV). A year later a team led by Robert Gallo of the United States confirmed the discovery of the virus, but they renamed it human T lymphotropic virus type III (HTLV-III). The dual discovery led to considerable scientific disagreement, and it was not until President Mitterrand of France and President Reagan of the USA met that the major issues were resolved. In 1986, both the French and the US names for the virus itself were dropped in favour of the new term, human immunodeficiency virus (HIV).

Global Scenario

HIV infection in humans is now pandemic. As of January 2006, the Joint United Nations Programmes on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognized on December 1, 1981, making it one of the most destructive pandemics in recorded history. In 2005 alone, AIDS claimed an estimated 2.4-3.3 million lives, of which more than 570,000 were children. It is estimated that about 0.6% of the world's living population is infected with HIV. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth and increasing poverty. According to current estimated, HIV is set to infect 90 million people in Africa, resulting in a minimum estimate of 18 million orphans.

Indian Scenario

Since the first detection of HIV infection in commercial sex workers (CSW) in Chennai in 1986, the infection has now spread to all parts of the country. There are an estimated 2 million to 3.1 million HIV infected person at the end of 2006. Although the magnitude of disease has been found to vary in different parts of the country, the states of Maharashtra, Tamil Nadu, Andhra Pradesh, Karnataka, Nagaland and Manipur are the hard hit high prevalence states. Heterosexual transmission has been found to be the commonest route of transmission, accounting for 85% of the total reported cases. Sentinel surveillance data also suggest that HIV has begun to spread in several rural areas. The epidemic varies from state to state with heterosexual transmission predominating in the southern states and transmission due to injecting drug use concentrated in the northeastern states. India remains a low prevalence country with overall HIV prevalence of 0.36% in adults 15 years or above.

Structure and Genome

HIV is different in structure from other retroviruses. It is about 120 nm in diameter (around 60 times smaller than a red blood cell) and roughly spherical (Fig. 71.1).

It is composed of two copies of positive single-stranded RNA that codes for the virus's nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24. The single stranded RNA is

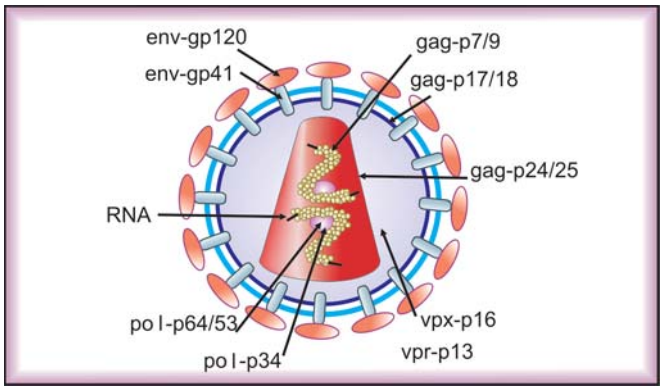


Fig. 71–1. Structure of HIV virus

tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by the viral envelope which is composed of two layers of fatty molecules called phospholipids taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle. This protein, known as env, consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle. Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines against HIV.

Of the nine genes that are encoded within the RNA genome, three of these genes, *gag*, *pol,* and *env,* contain information needed to make the structural proteins for the new virus particles. env, for example, codes for a protein called gp160 that is broken down by a viral enzyme to form gp120 and gp41. This six remaining genes, *tat, rev, nef, vif, vpr and vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease. The protein encoded by *nef,* for instance, appears necessary for the virus to replicate efficiently, and the *vpu*-encoded protein influences the release of new virus particles from infected cells.

The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell.

Mode of Transmission

Epidemiological studies throughout the world have shown four modes of HIV transmission. Unsafe sexual contact, transfusion of contaminated blood, use of contaminated syringes and from infected mother to the newborn child are the major routes of transmission of HIV.

i. **Sexual intercourse:** Whether heterosexual or homosexual, is the major route of transmission. HIV can be transmitted through any individual act of unprotected sexual intercourse, that is any penetrative sexual act in which a condom is not used where one partner is infected with HIV. The risk of becoming infected through an act of unprotected sexual intercourse depends on four main factors.
   a. *The likelihood that the sex partner is infected*: The probability that a person has become infected with HIV is in general proportionate to the number (frequency) of unprotected sex acts and the number of high-risk partners with whom the person has had sexual contact in recent years.
   b. *The type of sex act:* All unprotected acts of sexual penetration (anal, vaginal,oral) carry a risk of HIV transmission because they bring sexual secretions directly into contact with exposed mucous membrane. Injury to the mucous membrane of the rectum, the vagina or the mouth may help the virus to enter into the bloodstream. "Receptive" partners are thus at a greater risk than "Insertive" partners in acts of intercourse. However, HIV can be transmitted even through unbroken mucous membrane.
   c. *The amount of virus present in the blood* or sexual secretion (semen, vaginal or cervical secretions) of the infected partner. Individuals with HIV infection become more infectious as they progress to HIV related disease and AIDS. There is also an early period of high infectiousness around the time of seroconversion.
   d. The *presence of other sexually transmitted diseases* and/or genital lesions in either partner. It is important to be aware that HIV can be transmitted sexually even when neither partner has any of the other sexually transmitted disease. However, there is strong

Table 71–3. Estimated per act risk for acquisition of HIV-1 by exposure route

Exposure route	*Estimated infections per 10,000 exposurs to an infected source*
Blood transfusion	9,000
Childbirth	2,500
Needle-sharing injection drug use	67
Receptive anal intercourse without condom	50
Percutaneous needle stick	30
Receptive penile-vaginal intercourse without condom	10
Insertive anal intercourse without condom	6.5
Insertive penile-vaginal intercourse without condom	5

evidence that men and women with genital ulcer disease or urethral discharge are at 5-9 times increased risk of acquiring and transmitting HIV.

ii. **HIV infected blood, blood products**, transplanted organs or tissues and the use of improperly sterilized needles and syringes that have been in contact with infected blood can transmit HIV.

iii. **HIV infected women can transmit HIV to her** foetus or infant before, during, or after birth. A pregnant women with HIV infection has an approximately 20-40% chance of passing the virus to her foetus or newborn baby. There is evidence that infection can occur as early as the first 12-15 weeks of gestation. 50% of perinatal infections are *in utero* or during the birth process. It is estimated that a large number of perinatal infections occur through breastfeeding.

Estimated risk per act of exposure by various routes is depicted in (Table 71.3) and transmission cycle has been briefly summed up in (Fig. 71.2).

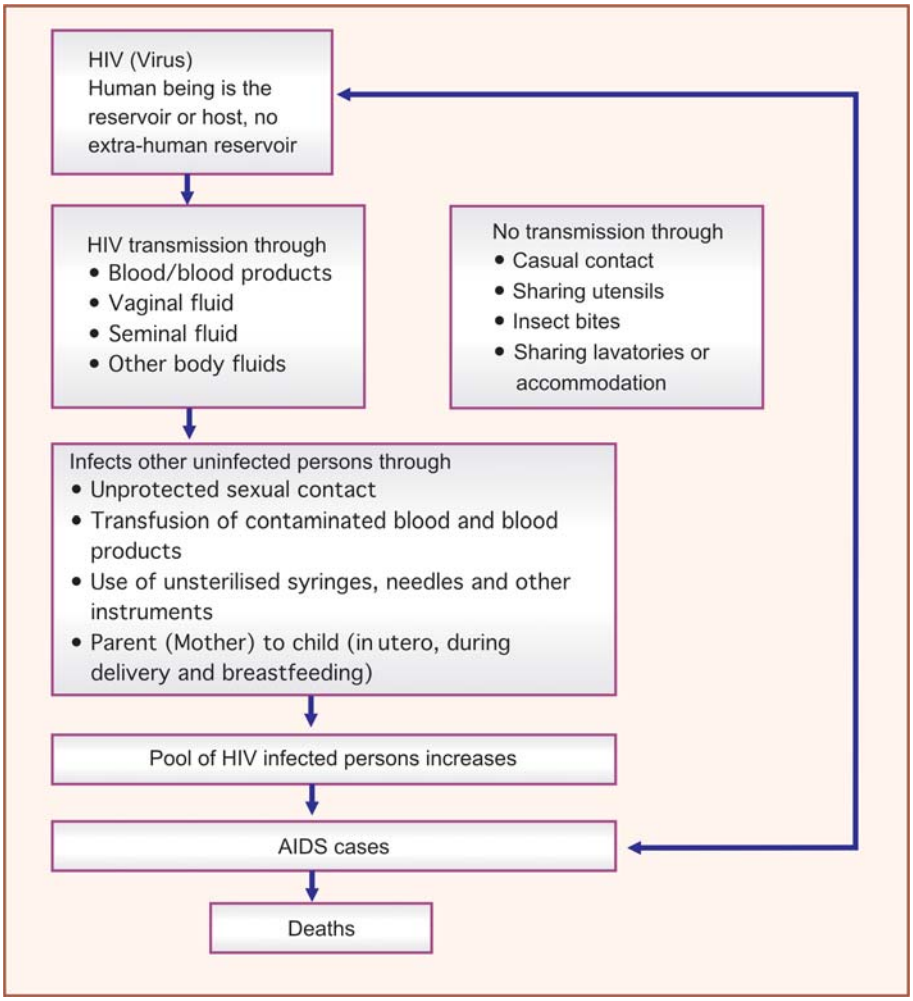


Fig. 71–2. Transmission cycle of HIV

Susceptibility of HIV

Fortunately HIV is a very fragile virus. It is susceptible to heat, a temperature of 56°C for 30 minutes or boiling for a few seconds kills the virus. Most of the chemical germicides used in hospital/laboratories and health care settings kill HIV at much lower concentrations. Thus 0.5 to 1% sodium hypochlorite, 70% ethanol, 2% glutaraldehyde, acetone, ether, beta propiolactone (1:400 dilution) and sodium hydroxide (40 m Mol/litre) inactivate the virus.

Sterilization and disinfection – summary of methods which kill HIV.

Sterilisation

- Autoclaving at 121°C, 15 lbs pressure for 20 minutes.
- Dry heat 170°C for 1 hour.
- Boiling for 20-30 minutes.

Chemical Disinfection

- *Sodium hypochlorite:* 5 gm/litre (0.5 to 1% ordinarily, 5-10% for high organic matter content, e.g. discarding tissues, etc.)
- *Calcium hypochlorite:* 1.4 gm/litre
- *Chloramine:* 20 gm/litre (Available chlorine 0.1%)
- Ethanol 70%
- Formaline 3-4%
- *Glutaraldehyde:* 2% for 30 minutes
- Polyvidone iodine (PVI).

Replication

Glycoprotein 120/140 of HIV binds to a receptor/receptors on HIV permissive host cell. Predominant receptor is the CD4 molecule present on T lymphocytes and macrophages, though others such as galactosyl ceramide (gel C) have also been proposed. Receptors are molecules (proteins and or glycoproteins) present on the surface of host cells which facilitate the attachment and entry of viruses in to the cell. Entry of virus into the host cell requires certain cellular coreceptors/factors, e.g. CCR-5, CXCR-4, CCR-2 and CCR-3, etc. designated collectively as cell infectivity factors (CIF). CIF may be a coreceptor or enzyme helping in virus interaction with host cell. Most convincing candidate is the chemokine receptor related protein, fusin (CXCR-4). Once the gp41/36 of the virus fuses with the host cell membrane the capsid is uncoated and a ribonucleoprotein complex capable of reverse transcription is formed. During the process of reverse transcription cDNA is formed under the effect of viral enzyme, the reverse transcriptase. Reverse transcription is inefficient in quiescent cells suggesting the involvement of host components in the process. The nucleoprotein complex formed after transcription comprises of linear double stranded DNA, the gag matrix (MA) protein, the accessory vpr protein and the viral integrase (IN). This is called preintegration complex and is transported into the host cell nucleus. IN mediates a complex series of enzymatic steps and integration occurs at cellular loci with open chromatin structure. Integration probably is an essential step for viral replication. The integrated virus is called provirus. The virus may not be expressed in many cells and is considered latent. Virus expression can be stimulated by many viral, cellular and exogenous factors. Other, co-existent viral infections, e.g. cytomegalovirus, herpes virus, etc. can make the non-permissive cells permissive. Maturation of virus also takes place after virus assembly and budding.

Various human cells and tissues susceptible to HIV infection are given in Table 71.4.

Table 71–4. Human cells/cell lines and tissues susceptible to HIV

HIV practically multiples in all cells but the extent of replication varies in different cells.

Haematopoietic system T lymphocytes, B lymphocytes, Macrophages, NK cells. Megakaryocytes. Dendritic cells, Promyelocytes, Stem cells, Thymic epithelium, Follicular dendritic cells.

Brain Capillary endothelial cells, Astrocytes, Macrophages, Microglia, Oligodendrocytes, Choroid plexus, Ganglia, Neuroblastoma cells, Glioma cells lines and Neurons.

Skin Fibroblasts and Langerhans cells

Bowel Columnar and Goblet cells, Enterochromaffin cells and Colon carcinoma cells

Others Myocardium, Renal tubular cells, Synovial membrane, Hepatic sinusoid epithelium, Hepatic carcinoma cells, Kupffer cells, Pulmonary fibroblasts, Foetal adrenal cells, Adrenal carcinoma cells, Retina, Cervix epithelium (?) Prostate, Testes, Osteosarcoma cells, Rhabdomyosarcoma cells, Foetal chorionic villi, Placental trophoblast cells.

Genetic Variability

HIV differs from many other viruses as it has very high genetic variability. This diversity is a result of its fast replication cycle, with the generation of 10^9 to 10^{10} virions every day, coupled with a high mutation rate of approximately 3×10^5 per nucleotide base per cycle of replication and recombinogenic properties of reverse transcriptase. This complex scenario leads to the generation of many variants of HIV in a single infected patient in the course of one day. This variability is

compounded when a single cell is simultaneously infected by two or more different strains of HIV. When simultaneous infection occurs, the genome of progeny virions may be composed of RNA strands from two different strains. This hybrid virion then infects a new cell where it undergoes replication. As this happens, the reverse transcriptase, by jumping back and forth between the two different RNA templates, will generate a newly synthesised retroviral DNA sequence that is a recombinant between the two parental genomes. This recombination is most obvious when it occurs between subtypes.

Tropism

The term viral tropism refers to which cell types HIV infects. HIV can infect a variety of immune cells such as CD4+ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and CD4+ T cells is mediated through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells and also with chemokine coreceptors.

Clinical Course of Infection

Infection with HIV-1 is associated with a progressive decrease of the CD4+ T cell count and an increase in viral load. The stage of infection can be determined by measuring the patient's CD4+ T cell count, and the level of HIV in the blood.

The initial infection with HIV generally occurs after transfer of body fluids from an infected person to an uninfected one. The first stage of infection, the primary, or acute infection, is a period of rapid viral replication that immediately follows the individual's exposure to HIV leading to an abundance of virus in the peripheral blood with levels of HIV commonly approaching several million viruses per mL. This response is accompanied by a marked drop in the numbers of circulating CD4+ T cells. This acute viraemia is associated in virtually all patients with the activation of CD8+ T cells, which kill HIV-infected cells, and subsequently with antibody production, or sero-conversion. The CD8+ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4 +T cell counts rebound to around 800 cells per mL (the normal values is 1200 cells per mL). A good CD8+ T cell response has been linked to slower disease progression and a better prognosis, though it does not eliminate the virus. During this period (usually 2-4 weeks post-exposure) most individual (80 to 90%) develop an influenza or mononucleosis-like illness called acute HIV infection, the most common symptoms of which may include fever, lymphadenopathy, pharyngitis, rash, myalgia, malaise, mouth and oesophageal sores, and may also include, but less commonly, headache, nausea and vomiting, enlarged liver/spleen, weight loss, thrush, and neurological symptoms. Infected individuals may experience all, some, or none of these symptoms. Symptoms have an average duration of 28 days and usually last at least a week although duration of symptoms may vary. Because of the nonspecific nature of these illnesses, it is often not recognized as a sign of HIV infection. Even if patients go to their doctors or a hospital, they will often be misdiagnosed as having one of the more common infectious diseases with the same symptoms. Consequently, these primary symptoms are not used to diagnose HIV infection as they do not develop in all cases and because many are caused by other more common diseases. However, recognizing the syndrome can be important because the patients is much more infectious during this period.

A strong immune defense reduces the number of viral particles in the bloodstream, marking the start of the infection's clinical latency stage. Clinical latency can vary between two weeks and 20 years. During this early phase of infection, HIV is active within lymphoid organs, where large amounts of virus become trapped in the follicular dendritic cells (FDC) network. The surrounding tissues that are rich in CD4+ T cells may also become infected, and viral particles accumulate both in infected cells and as free virus. Individuals who are in this phase are still infectious. During this time, CD4 + CD45 RO + T cells carry most of the proviral load.

When CD4 + T cell numbers decline below a critical level, cell-medicated immunity is lost, and infections with a variety of opportunistic microbes appear. The first symptoms often include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis, otitis media, pharyngitis), prostatitis, skin rashes, and oral ulcerations. Common opportunistic infections and tumors, most of which are normally controlled by robust CD4 + T cell-mediated immunity then start to affect the patient. Typhically, resistance is lost early on to oral Candida species and to *Mycobacterium tuberculosis*, which leads to an increased susceptibility to oral candidiasis (thrush) and tuberculosis. Later, reactivation of latent herpes viruses may cause worsening recurrences of herpes simplex eruptions, shingles, Epstein Barr virus-induced B-cell lymphomas, or Kaposi's sarcoma, a tumor of endothelial cells that occurs when HIV proteins such as Tat interact with Human Herpesvirus-8. Pneumonia caused

by the fungus *Pneumocystis jirovecii* is common and often fatal. In the final stages of AIDS, infection with cytomegalovirus (another herpes virus) or *Mycobacterium avium* complex is more prominent. Not all patients with AIDS get all these infections or tumors, and there are other tumors and infections that are less prominent but still significant.

Mechanism of Cell Death

- Increase in cell permeability due to budding of virus. Virus punches holes and kills the cell.
- Increase in cell permeability due to toxic effects of virus replication.
- Syncytia formation – involving uninfected cells.
- Apoptotic cell death of activated T cells.
- Autoimmune phenomenon involving CD4 molecule.
- ADCC, i.e. antibody dependent cell cytotoxicity.

Mechanism of CD4 Cells Depletion and Dysfunction

CD4 cells are the main targets of HIV and progressive destruction of these cells is characteristic of all stages of HIV disease. CD4 cells serve as surrogate markers to monitor the progression of HIV infection. These cells can be destroyed by two mechanisms:

i. Direct damage by the virus
ii. Immune mechanism triggered during the course of HIV infection.

HIV can kill cells singly or after giant cell and syncytia formation. Single cell killing occurs due to accumulation of unintegrated viral DNA and inhibition of cellular protein synthesis. Syncytium formation is induced by virulent strains of HIV in a multi-step mechanism. CD4 cells expressing viral antigens on the surface attract CD4 uninfected cells and the membranes of these fuse producing giant cells and syncytia. One such HIV infected cell can eliminate hundreds of uninfected cells by syncytium formation. Glycoprotein 120 and other intracellular adhesion molecules bring about the cellular adhesion and subsequent damage.

The non-virologic mechanisms which can damage/destroy CD4 cells include autoimmune mechanism, anergy, super antigens, apoptosis (programmed cell depth) and virus specific immune response. A number of hypothesis and complex immune mechanisms have been postulated for CD4 cell depletion involving one or more of above mentioned pathways.

Human immunodeficiency virus type 1 (HIV-1) infects and destroys leucocytes that express the CD4 surface receptors, as a result, depletes its hot of CD4 + T-lymphocytes is essential to assess and manager persons infected with HIV. Depletion of CD4 + T-lymphocytes has been linked to the immunopathogenesis of HIV infection and the progression of the disease. From the early days on, CD4 + T-lymphocyte assay has been recognised as the hallmark clinical surrogate marker for staging HIV disease progression. A CD4 + T-lymphocyte count of 200 cells/ml and less in presence of HIV infection is regarding as an AIDS defining event.

CD4 + T-lymphocytes in HIV infected individuals are monitored for the following reasons:

- To estimate the level of immune competence of an individual
- To stage HIV disease
- To make decision of ART
- Monitoring response to anti-retroviral therapy / to estimate rate of progression of HIV disease
- To initiate chemoprophylaxis against opportunistic infections.

Factors Affecting CD4+ T-lymphocyte Counts

There are certain other factors including some physiological states that may adversely affect CD 4+ T-lymphocyte counts. These include:

Transient reductions	• Diurnal variations in normal individuals • Physiological stress (postoperative immediately after exercise) • Exogenous steroids, cytotoxic drugs • Intercurrent infections (urinary tract and influenza)
Transient increases	• Women in follicular phases of menstrual cycle
Long-term reductions	• Systemic Lupus Erythematosus • Sarcoidosis (margination into tissues) • Primary immunodeficiencies
Long-term increases	• Smoking

The Course of Progression HIV Infection

Three dominant patterns of HIV disease progression have been described. These are based on the kinetics of immunologic and virologic events described above.

i. 80-90% of HIV infected are 'typical progressors' with a median survival time of 10 years approximately.
ii. 5 to 10% of HIV infected individuals are "rapid progressors" with a median survival time of 3-4 years approximately.

iii. About 5% to HIV infected individuals do not experience disease progression for an extended period of time and are called "long-term non-progressors" (LTNPs).

Laboratory Diagnosis

Window period (WP) is the time that elapses between entry of HIV into the body and the detection of HIV specific antibodies. Usually WP is three weeks to three months. At times, the same may get extended up to six months.

Purpose of HIV Testing

- Information is useful for prophylaxis, medical management and treatment of HIV and related illnesses.
- To assure blood safety and donation safety.
- To assess the efficacy of targeted intervention in a defined cohort.
- To monitor trends of epidemic (sentinel surveillance, etc.)
- Identification of asymptomatic individuals (practising high-risk behaviour).
- To plan personal and family's future if the result is positive.
- To motivate for behaviour modification through counselling amongst those who test negative and who practice high-risk behaviours.
- To induce behaviour change and prevent transmission by conselling in those who test positive.
- To diagnose clinically suspected cases.
- For peace of mind of individuals practising high-risk behaviour.

Laboratory Investigations

HIV infection can be detected in the laboratory either by detection of antibodies to HIV, or by detection of the virus, its antigen and its DNA. Detection of specific antigens, viral nucleic acid, isolation/culture of virus are all confirmatory tests in that the presence of the virus is detected. But they are risky because of the danger of infection to laboratory workers, are very laborious and difficult to perform, require skilled expertise and hence are to be done only in specified laboratories.

The indirect predictors of HIV infection (CD4 cell count, β2 microglobulin, etc.) are monitors of immunity status of patients and are to be done at routine intervals to monitor the progression of disease.

The specimens which can be utilized to detect various markers of HIV infection are given below:

Antibody Detection

- Blood/serum/plasma
- 3-5 ml of blood is collected in clean, screw capped plain vial for ELISA and for the supplemental tests. Saliva and urine have been used to detect antibodies to HIV but the assays have not as yet been validated in India.

Antigen Detection

- Serum/plasma
- Cerebrospinal fluid
- Cell culture supernatant (i.e. the tissue culture fluid).

Virus Isolation

Virus isolation can be attempted on HIV infected tissues. It may be isolated from blood, semen, vaginal/cervical specimen, tissue, CSF and plasma. It is less successful on other body fluids like saliva, urine, breast milk, tears and amniotic fluid. Virus isolation is done for research purposes only and never for diagnosis in India.

Detection of Specific Antibodies

This is done by performing initial screening tests, which if positive, are followed up by supplemental tests to confirm the diagnosis.

Screening Tests

The screening assays can be either ELISA and or rapid HIV tests. ELISA is the preferred test at blood banks and the bigger laboratories where a large number of samples are tested at a time. Rapid tests are preferred at sites where the number of samples to be tested is less and where same day/emergency testing is required.

ELISA (Enzyme linked immunosorbent assay) is the most commonly performed test at blood banks and tertiary laboratories to detect HIV antibodies.

There are various kinds of ELISAs based on the principle of test:

- Indirect ELISA
- Competitive ELISA
- Sandwich ELISA
- Immune capture ELISA

ELISA is also classified on the basis of the antigens utilized into:

1st generation: Infected cell lysate is used as the antigen.

2nd generation: Glycopeptides (recombinant antigens) are used as the antigen.

3rd generation: Synthetic peptides are used as the antigen.

4th generation: Antigen and antibodies are detected simultaneously. The assays may use a combination of recombinant and synthetic peptides as antigens.

Rapid Tests

- Dot blot assays (immunoconcentration based).
- Particle agglutination (gelatin, RBC, latex, microbeads).
- Dip stick and comb tests, etc. (ELISA technology based).
- Immunochromatography based tests.

When a serum samples tests reactive once by a system of ELISA/Rapid (E/R) test, the test is to be repeated immediately by a different system in order to confirm the diagnosis. The sample is then to be taken up for supplemental tests to confirm the diagnosis. Supplemental tests may be E/R/ WB, IFA, RIPA, etc.

ELISA takes up to three hours to yield results. It has a major advantage of being economical. Although rapid tests give result within minutes these are far more expensive. Commercial kits are available for ELISA and rapid tests.

Tests which detect antibody to both HIV 1 and 2 and all the subtypes are to be employed.

Supplemental Tests

- Second and third ELISA/Rapid
- Western blot.

Surrogate Markers

Surrogate markers are the in direct-predictors of HIV infection and these include:

- Decreased CD4 cells
- Increased β2 microglobulin
- Increased serum neopterin
- Indicator diseases for HIV.

Diagnosis of HIV Infection in Newborn (Congenital HIV Infection)

Transplacental transmission of HIV can occur from infected pregnant mother to the foetus as early as 8 weeks of gestation or may be even earlier. It is estimated that >80% of AIDS cases in infants <1 year old are due to perinatal transmission of HIV-1. Diagnosis of HIV infection in infants born to seropositive mothers is difficult because maternal antibody (IgG) to HIV-1 crosses the placenta and can persist for up to 18 months making the distinction between maternal and neonatal IgG difficulty. The tests which can be undertaken to diagnose HIV infection in neonates before 18 months of age are detailed below:

Detection of IgA and/or IgM Anti-HIV Antibodies

This class of antibodies do not cross placenta. The IgA class of HIV antibody assay using Western blot technique in infected children at 3 months of age has a sensitivity of 97.6% and specificity of 99.7% as reported in a study. IgM class of antibodies are produced by infected infants by six months of age. Production of IgM is erratic, false positive results are obtained due to rheumatoid factor and polyvalent nature of IgM which leads to nonspecific binging.

Estimation of p24 Antigen (Core Antigen)

The immune – complex dissociation assays which involve pretreatment of serum/plasma to liberate p24 antigen complexed with p24 antibody prior to performance of ELSIA are sometimes used to identify HIV-infected infants. However, the test is not sensitive. p24 antigen detection is undertaken in the following conditions:

- To detect infection in the newborn
- To detect infection during early window phase
- To resolve equivocal Western blot results
- To monitor response to anti-retroviral-therapy
- To diagnose CNS disease.

Polymerase Chain Reaction (PCR)

The technique specifically amplifies viral DNA sequences of interest. Theoretically it is possible to identify one infected cell in the specimen as also latent HIV infection. Various reports indicate the specificity of PCR to be invariably>95% regardless of age of testing while sensitivity ranges from 15% in neonates (within 48 hours after birth) to more than 95% in infants over 1 month of age. This test is recommended to detect infection in children born to HIV infected women.

Differences in Paediatric and Adult HIV Infection

- Overall progression of disease is more rapid in children
- Higher CD4+ counts in children
- Recurrent invasive bacterial infections are more common in children
- Disseminated CMV, Candida, varicella zoster and herpes simplex infections are more common in children

- Lymphoid interstitial pneumonitis occurs almost exclusively in children
- Peripheral neuropathy, myopathy are rarer in children.

Diagnosis during Window Period

HIV infection during window period can be detected by demonstrating the presence of virus and virus components. PCR and detection of p24 antigen may be helpful. However, in case of accidental occupational exposure the virologic test if undertaken must be substantiated by serological test for a positive diagnosis.

Strategies of HIV Testing in India

Because of the enormous risk involved in the transmission of HIV through blood, safety of blood and blood products is of paramount importance. Since the Positive Predictive Value (PPV) is low in populations with low HIV prevalence, WHO/GOI have evolved strategies to detect HIV infection in different population groups and to fulfill different objectives. The various strategies, so designated, involve the use of categories of tests in various permutations and combinations.

i. ELISA/Rapid/Simple tests (E/R/S) used in strategy I, II and III (Fig. 71.3).
ii. Supplemental tests like Western Blot and Line Immunoassays are used in problematic cases, e.g. in cases of indeterminate/discordant results of E/R/S.

Strategy I

Serum is subjected once to E/R/S for HIV. If negative, the serum is to be considered free of HIV and if positive, the sample is taken as HIV infected for all practical purposes. This strategy is used for ensuring donation safety (blood/blood products, organ, tissues, sperms, etc.). The unit of blood testing reactive (positive) is discarded. Donor is not informed.

Strategy II

A serum sample is considered negative for HIV if the first ELISA report is so, but if reactive, it is subjected to a second ELISA which utilizes a system different from the first one. It is reported reactive only if the second ELISA confirms the report of the first. This strategy is used for surveillance and for diagnosis only if some AIDS indicator disease is present.

Strategy III

It is similar to strategy II, with the added confirmation of a third reactive ELISA test being required for a sample to be reported HIV positive. The test to be utilized for the first ELISA is one with the highest sensitivity and for the second and third ELISAs, tests with the highest specificity are to be used.

Strategy II and III are to be used for diagnosis of HIV infection. ELISA 2 and ELISA 3 ought to be tests with the highest PPV possible to eliminate any chances of false positive results. Strategy III is used to diagnose HIV infection in asymptomatic individuals indulging in high risk behaviour.

The follow-up sample from patients with indeterminate result should be collected two weeks after the first sample collection. If the second sample also shows indeterminate result, it should be tested by a confirmatory assay (e.g. western blot). However, if the confirmatory test fails to resolve the serodiagnosis, follow-up testing should be undertaken at four weeks, three months, six months, and 12 months. After 12 months, such indeterminate results should be considered negative.

An individual reactive in three different systems of testing is confirmed to be having HIV infection. The other supplemental tests like Western blot are used to resolve discordant results of ELISA and research, as far as India is concerned, as these tests are expensive, time consuming and need expertise. Whichever commercial kits is selected, it should be ensured that it detects antibodies against both HIV-1, HIV-2 and their subtypes. Presence of HIV antibodies indicates the individual is infected with HIV and can transmit infection to others through unsafe risk behaviour.

Types of Testing

Unlinked Anonymous Testing

Such type of screening or testing is not directed to the individuals, but has as its objective, the public health surveillance of HIV infection. It is a method for measuring HIV prevalence in a selected population with the minimum of participation bias. Unlinked anonymous screening offers a distinct advantage over mandatory or voluntary testing. Unlinked anonymous testing involves use of blood already collected for other purposes; therefore, the effect of selection bias will remain minimal, and will depend upon time, location and other details of blood collection.

Voluntary Confidential Testing

Testing is often done for diagnostic purposes. Here it is important that the issues related to confidentiality receive great attention. Since this method is based on voluntary HIV testing or testing for diagnosis of HIV/AIDS cases, it is imperative to respect the individual's

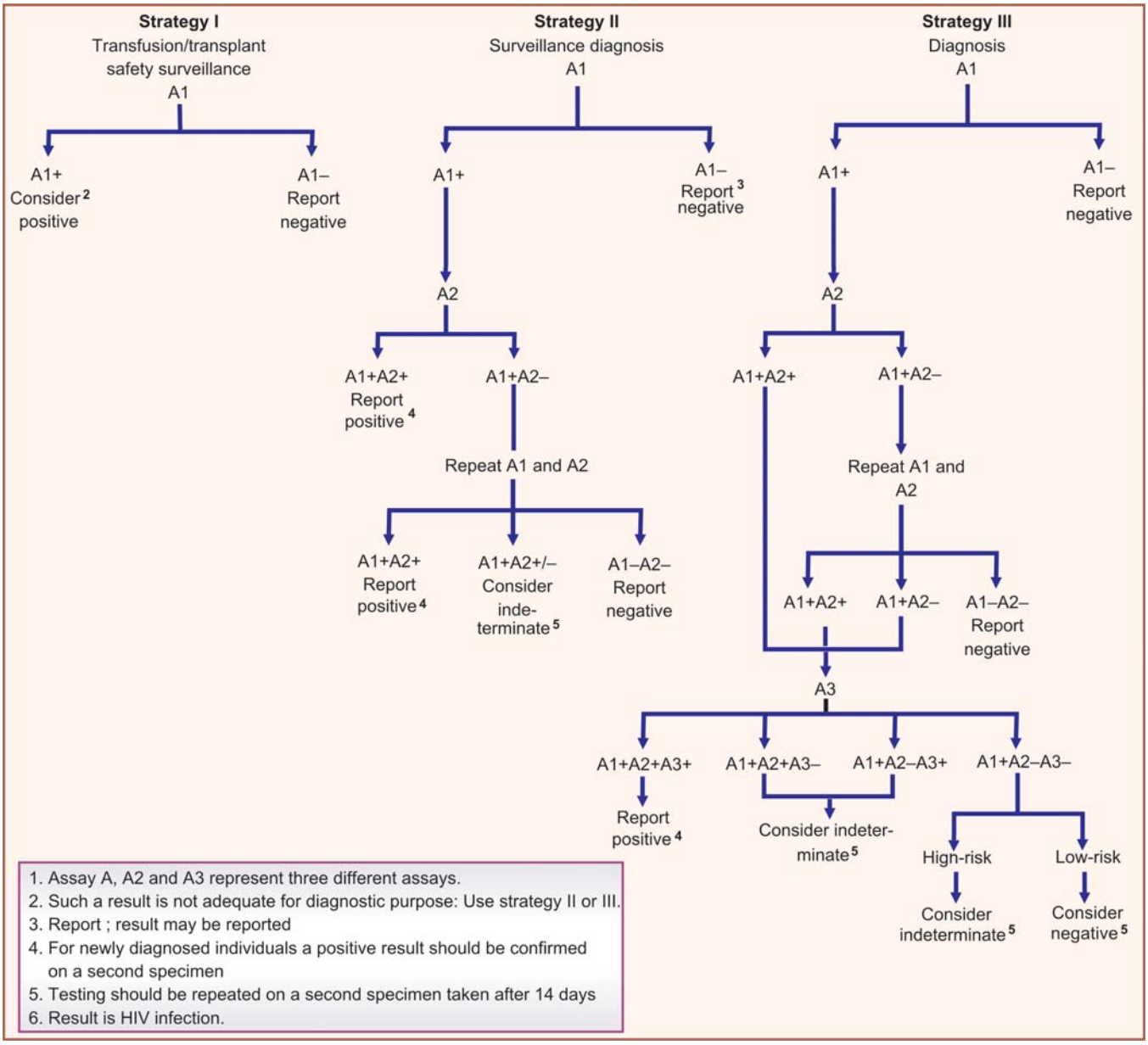


Fig. 71–3. Schematic representation of HIV testing strategy

need to maintain confidentiality. By maintaining confidentiality, it will not only instill faith in the individual about the health care system in the community but also encourage more and more people practicing risk behaviour to come forward for an HIV test. This testing is done after counselling and informed consent of the client.

Mandatory Testing

When testing is done without the consent of the patient and when the data could be linked to identify the person, it is called 'mandatory testing". Mandatory testing is recommended only for screening donors of semen, organs or tissues (to ensure transplantation safety) in order to prevent transmission of HIV to the recipient of the biological products.

National HIV Testing Policy

- No individual should be made to undergo mandatory testing for HIV.
- Mandatory HIV testing should not be imposed as a precondition for employment or for providing health care services and facilities.

- Any HIV testing except that undertaken for blood safety and transplantation safety must be accompanied by pretest and post-test counselling services and informed consent. Confidentiality of the result should be maintained.

HIV Testing in Health Care Settings

The fear and apprehension that exists among health care workers in managing HIV infected individuals and AIDS patients is largely due to the minimal risk that exists of HIV transmission due to a needle stick or other sharp injury. Thus the demand for mandatory HIV testing of patients admitted in hospitals or undergoing surgery, etc is not rational or appropriate. A mandatory HIV test is no substitute for standard work precautions that need to be adopted for every patient in a hospital or any other health care setting. On the other hand testing without explicit consent of the patient has been proven to be counter productive in the long run. In the control of the HIV epidemic, such testing can drive the target people underground and make it more difficult for launching interventions.

Essential Steps of Biosafety

It is important to strictly adhere to the practice of universal precautions. The essential biosafety measures in relation to various steps of activities in a health care setting are listed in Table 71.5.

Table 71–5. Essential preventive steps in biosafety

	Steps	*Preventive measures*
1.	Pre use screening	Avoiding the following: • Unnecessary risky procedures, e.g. mixing, grinding • Use of sharp objects • Allowing persons with broken skin, weeping skin lesions, etc.
2.	Barrier precautions	• Use of gloves to prevent contact with blood/body fluids, mucous membranes, broken skin • Mask, protective eye wear, face shield to prevent droplet infections • Gowns, aprons to prevent splash of blood/body fluid
3.	While in use precautions	Prevention of injuries by sharp objects, e.g. needles, scalpel set Needles are never recapped.
4.	After use precautions	Placing all used instruments in disinfectant jar. Placing the jar as close as possible to the working areas.

Management of Accidental Exposure to Blood for HIV Prophylaxis

Steps to be taken on accidental exposure to blood or body fluid containing blood) are:

i. Wash wound immediately with soap and running water.
ii. Inform the laboratory/hospital management and document occupational accident.
iii. Consult with nearest ART centre/resource for post-exposure prophylaxis, evaluation, and follow-up (as per the National guidelines on PEP).
iv. Counselling and collection of blood for testing from the exposed person with written informed consent must be done.
v. Whenever possible confidential counselling and testing of source for Hepatitis, HIV, etc. must be done. A history should be taken as well to ascertain likely risk of the source. (PEP should be provided to the exposed person until report of source is available and confirmed negative).
vi. Risk of infection and transmission must be evaluated.
vii. Never delay start of therapy due to debate over regimen. Begin with basic 2-drug regimen, and change if warranted, once expert advice is obtained.
viii. Re-evaluation of the exposed person should be considered within 72 hours post-exposure, specially as additional information about the exposure or source person becomes available. The exposed person is advised to seek medical evaluation for any febrile illness that occurs within 12 weeks of exposure.
ix. Administer PEP for 4 weeks. PEP should be provided until result of the source's test is available and confirmed negative or until course completed, if source positive or unknown.
x. A repeat HIV test of the exposed individual should be performed at 6 weeks, 12 weeks and 6 months post-exposure, regardless of whether or not PEP was taken.

The steps in HIV post-exposure prophylaxis are listed in Table 71.6.

Prevention of Parent of Child Transmission Centres (PPTCT)

Mother (parent) to child transmission of HIV is a major problem worldwide. Most of the children (90%) who are HIV positive were born to HIV-infected mother. HIV-1 may be transmitted from an infected mother to the baby during pregnancy, i.e. *in utero*, during delivery

Table 71–6. HIV post-exposure prophylaxis

Exposure	*Status of source (see below)*			*HIV status negative*
	HIV + and low-risk	*HIV + and high-risk*	*HIV status unknown*	
Mucous membrane/non-intact skin; small volume (drops)	Consider 2-drug PEP	2-drug PEP	Consider 2-drug PEP	No PEP is required if the source blood is confirmed HIV negative.
Mucous membrane/non-intact skin; large volume (major blood splash)	2-drug PEP	3-drug PEP	Consider 2-drug PEP	
Percutaneous exposure: Not severe solid needle, superficial	2-drug PEP	3-drug PEP	Consider 2-drug PEP	
Percutaneous exposure: Severe large bore hollow needle, deep injury, visible blood in device, needle in patient artery/vein	3-drug PEP	3-drug PEP	Consider 2-drug PEP	

and during breastfeeding. The transmission rates through this route range from 20 to 40%. Transmission of HIV-1 from mother to child depends upon various viral, host and obstetric factors. There are a number of interventions which reduce the parent to child transmission of HIV-1. These include early identification of the mother who is HIV-1 positive through counselling and testing, administration of antiretroviral drugs to reduce the viral load in the mother, appropriate obstetric and pre natal care of mother, counselling about breast feeding and administration of antiretroviral drugs to the HIV- exposed newborn. Primary prevention would focus on the steps to protect women of child bearing age from becoming infected with HIV and provision of family planning services including pregnancy termination wherever legal to enable women to avoid unwanted births.

Stages of HIV Disease

Sings and clinical features associated with various stages of HIV are given in (Table 71.7).

Tests for Monitoring Progression of HIV Infection

Three approaches are used for monitoring the progression of HIV infection:

i. *Enumeration of CD4 positive T cells* using flow-cytometry. Declining CD4 counts despite therapy probably indicate failure of ART.
ii. *Estimation of viral load:* It is predicted that with successful therapy, a fall of 1.5 to 2 log in plasma viral load occurs within 4-6 weeks with successful ART, it should become undetectable in 4-6 months of therapy.
iii. *HIV p24 antigen detection by ELISA* has recently been used for estimating viral load. The cost of this test is about 1/5th of the conventional viral load assays.

Determination of HIV Drug Resistance

The increased use of ART is expected to lead to emergence of drug-resistant mutants of HIV. Drug-resistance should be suspected if the plasma viral load does not show a greater than 1 log fall within 8 weeks

Table 71–7. Stages of HIV disease

Signs and clinical features	*Typical duration*	*CD4 T-cell counts range/mm^3*
Acute primary HIV infection	1-2 weeks	1000-500
Asymptomatic, no signs/symptoms other than lymphadenopathy	10 years	750-500
Early symptomatic (non life-threatening infections of chronic or intermittent symptoms)	0-5 years	500-100
Late symptomatic (increasingly severe symptoms, life-threatening infections, malignancies)	0-3 years	200-50
Advanced AIDS (serious opportunistic infections, increasing hazard of death)	1-2 years	50-0

of therapy. Two types of antiretroviral drug resistance assays are available: Phenotypic and genotypic assays.

Opportunistic Infections in HIV/AIDS

As the body resistance goes down the opportunistic infections start surfacing. Opportunistic infections could be due to agents of low pathogenicity which are generally non-pathogenic in immuno-competent individuals or even the established pathogens can start behaving in more severe or recurrent fashion. Many times an AIDS infection is suspected based on the presence of such infections. A list of common opportunistic infections and malignancies associated with AIDS are depicted in Table 71.8.

Prevention and Control

HIV is not transmitted through casual social contact. Solutions against the epidemic are based on direct interruption of transmission of HIV and also by tackling the contributing factors. Solutions are listed below:

- Awareness about transmission modes
- Practice of safe sexual behaviour
- Mandatory screening of blood/blood products
- Using sterilized needles/syringes
- Reducing infection from mother to child
- Political commitment (for implementation of HIV/AIDS control measures)
- Care and support for the infected and affected pesons
- Reducing stigma and discrimination so that more and more people access the VCCTC/ICTC and PPTCT services.
- Voluntary confidential counselling and testing.

Table 71–8. Opportunistic infections and malignancies typically associated with HIV infection

1. **Parasitic**
   - *Pneumocystis carinii* (now known as *Pneumocystis giroveci)* pneumonia
   - Neurotoxoplasmosis
   - Cryptosporidiosis
   - Isosporiasis
   - Generalised strongyloidosis
2. **Mycotic**
   - Oral candidiasis (thrush)
   - Cryptococcosis (meningitis)
   - Aspergillosis
   - Disseminated histoplasmosis
   - Coccidioido mycosis
3. **Bacterial**
   - Mycobacterial infections (Tuberculosis and non-tuberculous infections)
   - Salmonellosis
   - Campylobacter infection
   - Nocardia and actinomycetes
   - Legionellosis
   - Pneumococcal pneumonia
4. **Viral**
   - Disseminated CMV infection
   - Disseminated herpes simplex
   - Herpes zoster
5. **Malignancies**
   - Kaposi's sarcoma
   - Lymphomas – Hodgkin's and non-Hodgkin's types
   - Oral hairy leucoplakia

Treatment

There is currently no vaccine or cure for HIV or AIDS. The only known method of prevention is avoiding exposure to the virus. However, an antiretroviral treatment, known as post-exposure prophylaxis is believed to reduce the risk of infection if begun directly after exposure. Current treatment for HIV infection consists of highly active antiretroviral therapy, or HAART. Current HAART options are combinations (or "cocktails') consisting of at least three drugs belonging to at least two types, or "classes," of anti-retroviral agents. Typically, these classes are two nucleoside analogue reverse transcriptase inhibitors (NARTIs or NRTIs) plus either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Because AIDS progression in children is more rapid and less predictable than in adults, particularly in young infants, more aggressive treatment is recommended for children than adults.

HAART allows the stabilisation of the patients's symptoms and viraemia, but it neither cures the patients, nor alleviates the symptoms, and high levels of HIV-1, often HAART resistant, return once treatment is stopped.

AIDS Vaccine

Significant progress has been made towards the goal of developing a safe and effective HIV vaccine, although many challenges remain. While a vaccine that prevents HIV infection has always been and still remains the aim of HIV vaccine research efforts, years of research have led us to expand our thinking about how an HIV vaccine might work. Specifically, development of a vaccine that does not prevent infection completely but that slows the progression of disease in people who get infected despite vaccination could still serve as a positive intermediate step in the search for a safe and effective AIDS vaccine that completely prevents infections.

Most "classical" vaccines against viral diseases – seasonal influenza, polio, and rabies – stimulate antibodies that control the virus, prevent disease and ultimately rid the virus from the body. However, inducing such effective antibodies against HIV is an extraordinary challenge because the virus has multiple ways of evading the body's immune defenses such as:

a. HIV rapidly mutates; and these variants of HIV change continually, and an infected person's immune system invariably cannot keep up with all of them.
b. Within days of infection, HIV begins to destroy critical immune cells that would normally protect against the virus.
c. HIV inserts itself into the DNA of human cells where it can remain undetected by the immune system. Even with extended drug therapy that reduces virus in the blood to undetectable levels, HIV is not eradicated from the body.

A "non-classical" approach to HIV vaccine development is evolving. Research in animal models of AIDS has demonstrated that stimulate immune cells, called T cells, which do not attack the virus directly, but can recognize and kill HIV infected cells, might be beneficial even if the vaccine does not completely prevent HIV infection. In animal models of HIV disease, vaccines that induce these types of T cells and that were given before the animals were exposed to virus blunted the magnitude of the initial infection, preserved immune-fighting cells, and resulted in a longer disease-free period.

Thus, a non-classical HIV vaccine given before on individual is exposed to the virus may not prevent initial infection or eliminate HIV or prevent disease entirely, but it might induce T cells that control HIV, contain the virus at low levels, and substantially delay the need to start drug therapy. In addition, because the efficiency of transmission of HIV from one person to another is directly related to the level of virus in the transmitting partner, restricting the virus to very low levels could lessen or even halt person-to-person HIV transmission.

If such a non-classical vaccine proves to be effective in clinical trials, the vaccine would need to be delivered as part of a multifaceted, comprehensive HIV prevention program so that recipients minimize or eliminate behaviors that expose them to HIV. Otherwise, any beneficial effect of the vaccine could be eliminated.

The current approach is on the development of a therapeutic vaccine rather than a preventive vaccine. These are over two dozen vaccines under different stages or phases of human clinical trials:

a. Live attenuated vaccines are not currently being developed for use in humans because of safety concerns.
b. The first AIDS vaccines developed and tested were designed using the subunit concept. The first AIDS vaccine to go through complete testing in humans the AIDSVAX gp120 was a subunit vaccine which failed to protect against HIV infection in an efficacy trial.
c. DNA vaccines and recombinant vector vaccines are the strategies being currently tested.

72 Rhabdoviruses

No sound invokes more terror in mankind than the bark of a rabid dog. It has terrified man since time immemorial. The fear by no means is unfounded because the disease transmitted by such animal in humans is perhaps the most painful and horrible of all the communicable diseases in which the sick person is tormented with thirst and the fear of water (*hydrophobia*) at the same time.

Classification

Rabies virus belongs to the family *Rhabdoviridae* and genus Lyssavirus which contains four related but distinct virus types (Table 72.1).

Table 72–1. Members of genus Lyssavirus

Virus	*Serotypes*	*Distribution*
Rabies	1	World wide
Lagos bat	2	Nigeria, Africa
Mokola	3	Nigeria, Africa
Duvenhage	4	Africa, Europe

Isolation of new Lyssaviruses has now lead to creation of 7 genotypes. The rabies virus belongs to genotype 1, serotype 1.

Morphology

The rabies virus is bullet shaped, round at one end and flat at the other (Fig. 72.1). Though the dimensions vary, it ranges between 200 × 80 nm. The membrane at the flattened end of the virion often appears invaginated. Atypical elongated filaments and occasional V- or Y-shaped and conical forms are observed in rabies virus harvested from cell cultures late after infection. Truncated bullets (T-particles) encountered in many rhabdovirus preparations are non-infectious.

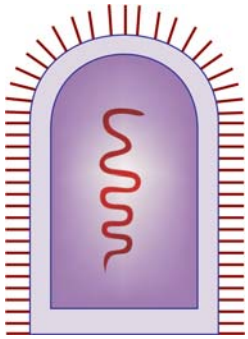


Fig. 72–1. Rabies virus

Chemical Composition

The RNA is an unsegmented, single, negative strand which is non-infectious. The RNA is closely associated with a major protein, N and two smaller proteins, L and NS. This structure is tightly coiled and helical in appearance and constitutes the nucleocapsid of the virus. It is enveloped in a lipoprotein membrane through which protrude a large number of spikes with knobs at their distal ends. The inner surface of the envelope is lined by a genome coded M protein. Associated with the envelope is a glycoprotein (G) of which the surface projections are composed.

Susceptibility to Physical and Chemical Agents

The rabies virus is highly resistant to cold, dryness, decay, etc. It can remain infectious for weeks in the cadavers. Rabies virus is thermolabile, with a half life of approximately 4 hours at 40°C and 35 seconds at 60°C. However, thermal inactivation is diminished in the presence of serum proteins and chelating agents. The virus remains stable for several days at 0-4°C, indefinitely at –70°C and when freeze dried. This virus

cannot withstand pH less than 4 or more than 10. It is also susceptible to the action of oxidising agents, most organic solvents, surface acting agents, quaternary ammonium agents, soaps and detergents. Proteolytic enzymes, ultraviolet rays and X-rays rapidly inactivate rabies virus.

Street and Fixed Virus Strains

The street virus which was first designated by Pasteur as *virus des rues* refers to the virus isolated from animals and which has not undergone any modification in the laboratory. The strains of this type are characterised by a variable incubation period and by their capacity to invade salivary glands. The fixed virus, by contrast, refers to strains adapted to laboratory animals by serial intracerebral passages which have a short incubation period of only 4-6 days and do not invade the salivary glands. Fixed virus remains neurotropic and its virulence for the central nervous system may get enhanced; it produces paralytic rather than furious symptoms. Its infectivity by peripheral inoculation is diminished, as its ability to induce inclusion bodies in the central nervous system.

Pathogenesis

Compared to other mammals man is somewhat less susceptible to the rabies virus. However, the probability of contagion depends on the virulence of the particular virus strain, the quantity of virus transmitted by the bite and location of the bite. Whereas a bite on the face has a contagion index of almost 100% that on the body or leg only about 2%. Once the disease is established there is no cure.

After a bite on the human body the neurotropic virus spreads mainly along the endoneural lymphatic ducts, thus reaching the brain and the spinal cord where it multiplies intracytoplasmically in the grey matter. The movement of the virus from peripheral sites to the CNS is effected by passive transport. The rate of movement for some strains has been estimated to be 3 mm/hour.

Extensive replication occurs within the brain and terminally neurons in all the parts may be affected. The symptomatology in cases with rabies is dependent upon the parts of brain affected. Pronounced localisation of virus in limbic system is responsible for furious and aggressive behaviour.

The pathogenesis of rabies in dog has been shown in Figure 72.2.

Man to man transmission of rabies does not take place but corneal transplants from donors with undiagnosed rabies provide bizarre examples of human to human transmission.

Clinical Features

The average incubation period is 30-90 days. It is shorter in case the bite is closer to brain.

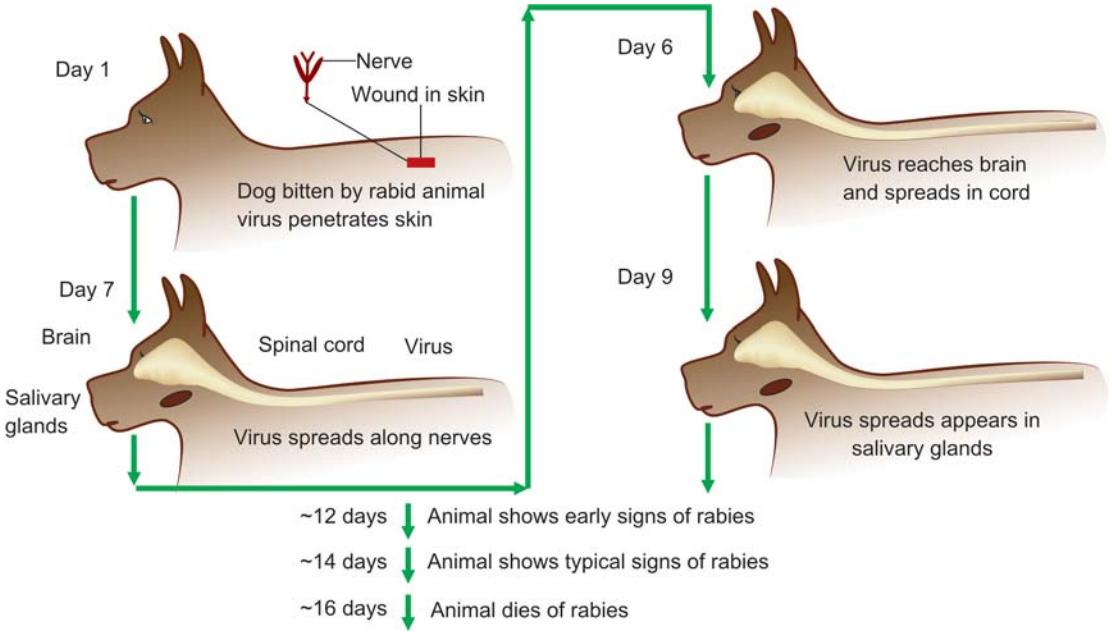


Fig. 72–2. Pathogenesis of rabies

In a typical course of the disease, the uncharacteristic prodromal stage is followed by the excitation stage with high motor activity, tremors, tonic clonic spasms, opisthotonus, increased salivation, shortness of breath etc. The sight of water, sudden exposure to bright light or loud noise or subjecting him to a current of fast air, trigger violent spasms of the gullet making it impossible for the patient to drink water (hence the name *hydrophobia*). Unless prolonged by intensive care, disease progresses to coma and death ensues within 3-7 days after its onset.

Aggression, delirium and intolerance to stimuli are among the behavioural changes frequently seen in patients with rabies. Hydrophobia does not manifest in all the cases but if present, leaves no doubt about the diagnosis.

Death in rabies is usually due to respiratory paralysis while the patient is fully conscious.

More rarely the disease presents as dumb rabies which begins with uncharacteristic prodromal features but is characterised by ascending paralysis, absence of hydrophobia and a more prolonged illness.

Rabies in dogs can be furious or dumb. The clinical features prior to death do not persist beyond seven days in dogs and cats. These animals are infectious 2-3 days prior to appearance of clinical features thus making them infectious for a maximum of 10 days prior to death. This scientific fact is made use of in deciding the treatment to a person bitten by a dog or a cat. If the animal has survived for a period exceeding 10 days from the date of bite, it can be safely presumed that the animal was not secreting rabies virus in its saliva at the time of bite and hence no prophylactic treatment is required.

PART X

Laboratory Diagnosis

Laboratory diagnosis of rabies in man and animals is carried out on the same principles.

Antemortem Diagnosis

Antemortem diagnosis with varying degree of success has been attempted in man. For this purpose corneal smear, skin biopsy, hair follicles and saliva have been tested by immunofluorescence. The sensitivity of this method does not exceed 25%.

Postmortem Diagnosis

Any animal suspected to be having rabies should be sacrificed immediately and brain specimen sent to the laboratory for examination.The brain, spinal cord and salivary glands are the organs from which the virus can be demonstrated or isolated.

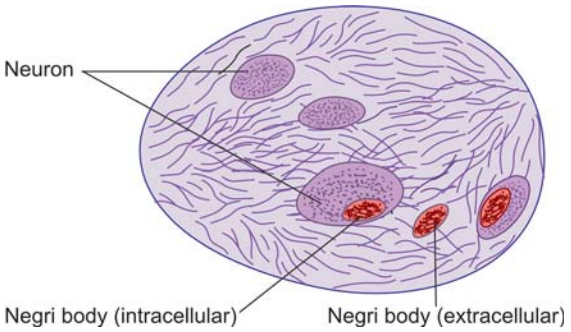


Fig. 72–3. Negri body

Following tests have achieved wide acceptance for the diagnosis of rabies:

a. Detection of Negri body by Seller's stain.
b. Detection of viral antigen by immunofluorescence and EIA.
c. Mouse pathogenicity or biological test.
d. Rabies tissue culture infection test (RTCIT)
e. PCR.

i. Detection of Negri body. The neurons of animals or man infected with rabies street virus form characteristic *intracytoplasmic* inclusion bodies (Fig. 72.3) which have been designated as Negri bodies and have been known since 1903 to be pathognomonic of rabies. These can be detected by a simple staining technique known as Seller's staining. The sensitivity of this test has been found to be 75% in best of hands. It indicates that this test shall not be able to detect rabies virus in 25% of rabies cases. In such cases, better results (upto 95%) have been obtained with fluorescent staining. Salient features of Negri bodies are given in Table 72.2.

Table 72–2. Salient features of Negri bodies

- End product of viral invasion of brain
- Eosinophilic and intracytoplasmic
- Presence confirms diagnosis of rabies but absence does not rule out the diagnosis
- Pathognomonic of rabies
- Oval, spherical or elongated in shape
- Size varies from 1-27 mm, smallest in rabbits and largest in cows
- May be present in upto 20% of infected neurons
- More than one may be present in one neuron
- Diagnostic sensitivity is about 75%
- More frequent and greater in number in areas of infected brain with little inflammation
- More commonly seen in Ammon's horn of hippocampus
- Larger bodies show fine inner basophilic granules
- Smaller inclusions may be uniformly acidophilic (Lyssa bodies)
- Less refractile than other inclusions

ii. Fluorescent antibody test (FAT). In this test rabies specific antibody is conjugated with FITC and smear prepared from brain of animal is treated with this. Presence of fluorescence under UV rays indicates presence of rabies antigen in clinical material. It is mainstay of rabies diagnosis.

iii. Biological test. The mouse pathogenicity test is considered as the ideal test for the diagnosis of rabies. It suffers from the disadvantage of taking a very long time (2-3 weeks) before the diagnosis can be established. The test involves intra-cerebral inoculation of clinical material into laboratory mice and their observation for the development of clinical features of rabies and death. This can only detect live viruses.

iv. Rabies tissue culture infection test (RTCIT). The cell lines are now available to grow virus in the laboratory from the clinical material.Vero, BHK and mouse neuroblastoma cell lines can be used. Since rabies virus does not produce any CPE, the presence of virus is confirmed by fluorescent antibody test after 18-24 hours of inoculation of cells.

v. PCR. PCR has been standardised for the diagnosis of rabies. It is probably not going to be a widely used test for the diagnosis of rabies. It's main use will be for strain differentiation while using it as an epidemiological marker.

The advantages and limitations of various tests used for diagnosis of rabies are given in Table 72.3.

Table 72–3. Common tests used for rabies diagnosis

Test	*Advantages*	*Limitations*
Seller's staining for inclusion bodies (Negri bodies)	• Simple • Rapid (1 hour) • Easy to perform • No special equipment required • A small training required	• Positive in 50-70% of cases • Sensitivity dependent on the quality of samples and stage of disease
Fluorescent antibody test (FAT) for antigen detection	• Specific • Sensitive • Rapid (over night) • Easy to perform	• Expensive • Good fluorescent microscope required • Good quality conjugate required • Trained manpower needed
Mouse inoculation test (MIT) for virus isolation	• Can detect very small quantity of virus • Confirmatory	• Takes long time (2-3 weeks) hence not useful in deciding treatment • More of academic value • Use of laboratory animals (mice). Thus laboratories should have a well maintained animal house • Ethical issues involved in use of laboratory animals • Dead virus not detected
Rapid tissue culture infection test (RTCIT) for virus isolation	• Relatively rapid (4 days) • Sensitive	• Special cell culture laboratory needed • Expensive • Trained manpower required
Polymerase chain reaction (PCR)	• Rapid (few hours) • Sensitive	• Possible only in big laboratories • Training and reagents required • Not a practical method for diagnosis

Treatment

Treatment of rabies patient consists only of symptomatic therapy. Utmost attention should be given to proper nursing care. Patient has to be protected from external stimuli such as draughts, noise or bright light. To reduce psychomotor excitation, parentral diazepam can be given. If it is administered alongwith antihistaminics and analgesics, patient's agony is considerably reduced.

The dramatic dehydration observed in all cases due to aversion of drinking water and excessive sweating and salivation demands a special care for the water salt balance. As a rule, patients require massive intravenous infusions. Prednisolone and mannitol may be administered in the patients with high intracranial pressure. Infusions of blood or blood substitutes are not required for patients with rabies.

Intensive therapy in the form of respiratory and cardiac support may be given to alleviate the sufferings and ensure peaceful death to the victim.

Prevention of Rabies

Prevention of rabies in man comprises of following three steps:

- Management of wound
- Passive immunoprophylaxis
- Active immunisation.

Management of Wound

Since rabies virus enters the body through a scratch or bite, it is imperative to remove as much saliva as is possible by an efficient wound toilet that should not

involve additional trauma. This can be done by washing the wound with soap and water. If soap is not available, the best course is to flush the wound with copious quantity of water. After the removal of soap, any quaternary ammonium compound may be applied as an antiseptic. Cauterisation of wounds is no longer recommended as it leaves bad scars and does not provide any additional advantage over application of soap and water.

The wounds inflicted by animals should not be stitched at least within 24-48 hours of injury. If suturing is unavoidable, antirabies serum should be first infiltrated around the wound followed by application of minimum stitches. Administration of tetanus toxoid and antibiotics should be as per the merit of particular case.

Passive Immunoprophylaxis

Both antirabies serum of equine origin (crude or purified) and rabies immunoglobulins can be used for passive immunisation. The dose of equine antirabies serum is 40 IU per kg body weight of the patient and is given after a sensitivity test upto a maximum dosage of 3000 IU. The dose of human rabies immunoglobulin is 20 IU per kg body weight with a maximum of 1500 IU. The human rabies immunoglobulin does not require any prior sensitivity testing. Part of this is infiltrated around the wound and remaining injected intramuscularly. It is indicated in all cases with severe bites. The severity of the bite is decided based on the classification of the exposure (Table 72.4).

Table 72–4. Guidelines for post-exposure treatment against rabies

Category	*Type of contact with suspected animal*	*Recommended treatment*
i.	Touching or feeding of animals Licks on intact skin	None, if reliable case history is available
ii.	Nibbling of uncovered skin Minor scratches or abrasions without bleeding Licks on broked skin	Administer vaccine immediately, stop if during 10 day observation The biting dog or cat remain normal (10 days period valid only for dogs and cats) or Laboratory techniques confirm suspect animal to be rabies negative
iii.	Single or multiple transdermal bites or scratches Contamination of mucous membrane with saliva	Administer rabies Immunoglobulin and vaccine immediately and stop if suspected animal confirmed as rabies negative

Table 72–5. Currently available vaccines against rabies

Vaccine	*Substrate*	*Type*
Semple (No longer used)	Brain: sheep, goat	Phenol-inactivated BPL-inactivated
HDCV	Human diploid cell	BPL-inactivated
PCECV	Chick embryo cells	BPL-inactivated
PVRV	Vero cells	BPL-inactivated
PDEV	Duck embryo	BPL-inactivated

Active Immunisation

The currently available vaccines can be grouped into three on the basis of the substrate used to grow the vaccine virus strain: neural tissue derived vaccines, duck embryo derived vaccines and tissue culture derived vaccines (Table 72.5). The vaccines that are currently available in India are:

- Human diploid cell strain (HDCS) vaccine
- Purified chick embryo cell (PCEC) vaccine
- Purified Vero cell rabies vaccine (PVRV)
- Nervous tissue derived vaccine.

The tissue culture vaccines give rise to high titres of neutralising antibodies in a short period and hence carry extremely good immunogenicity. These do not contain any foreign virus or oncogenic material and provide excellent general and local tolerance. Five doses of tissue culture vaccine are given in one month on day 0, 3, 7, 14 and 28. Day 0 being the day on which vaccination is started. This is called as Essen schedule.

Though tissue culture vaccines vaccines are virtually free of side effects, their high cost has kept these out of the reach of millions of people.

Intradermal Inoculation of Anti-rabies Vaccines

Intradermal regimens consist of administration of a fraction of intramuscular dose of certain rabies vaccine on multiple sites intradermally. The vaccines used are same; however, route, dose and site administration differs. The use of intradermal route leads to considerable savings in terms of total amount of vaccine needed for full pre- or post-exposure vaccination, thereby reducing the cost of active immunization.

Single bolus dose (1 ml) of rabies vaccines/antigen when given by IM route gets deposited in the muscles. There after the antigen is absorbed by the blood vessels

and is presented to antigen presenting cells which triggers immune response. Small amount (0.1 ml) of rabies vaccines/antigen is deposited in the layers of the skin at multiple sites. The antigen is directly presented to the antigen presenting cells (without circulation/dilution in blood) at multiple sites triggering a stronger immune response.

Multiple clinical trials since early eighties have proven that ID route of inoculation of Tissue Culture Anti-rabies Vaccines (TCARV) is efficacious, safe and economical. It requires less quantity of vaccine which brings down the cost of immunisation and allows wider coverage in the existing quantity of vaccine. WHO recommended use of ID route of inoculation of TCARV in 1992. Thialand, Sri Lanka and Phillipines have successfully adopted ID route of inoculation.

There are two schedules approved by WHO for ID route:

1. **Updated Thai Red Cross Schedule (2-2-2-0-2):** Regimen: 2-2-2-0-2, i.e. one dose of vaccine, in a volume of 0.1 ml is given intradermally at two different lymphatic drainage sites, usually the left and right upper arm, on days 0,3,7 and 28.
2. **Eight site intradermal regimen (8-0-4-0-1-1):** Regimen: 8-0-4-0-1-1 i.e. one dose of 0.1 ml is administered intradermally at eight different sites (upper arms, lateral thighs, suprascapular region and lower quadrant of abdomen) on day 0. On day 7, four 0.1 ml injections are administered intradermally into each upper arm (deltoid region) and each lateral thigh (Fig. 72.4). Following these injections one additional 0.1 ml dose is administered on 28 and 90.

General Guidelines for Use of Intradermal Vaccines

- Vaccines to be applied by intradermal route of administration should meet the WHO requirements for production and control related to vaccines for intramuscular use including an NIH potency test of at least 2.5 IU per single dose (intramuscular).
- Immunogenicity and safety of vaccine in question should be demonstrated in appropriate human trials using WHO post-exposure regimens.
- Intradermal injections must be administered by staff trained in this technique.
- Vaccine vials must be stored at 2°to 8°C after reconstitution.
- The total content of reconstituted vial should be used as soon as possible, but atleast with in 8 hours after which it should be discarded.

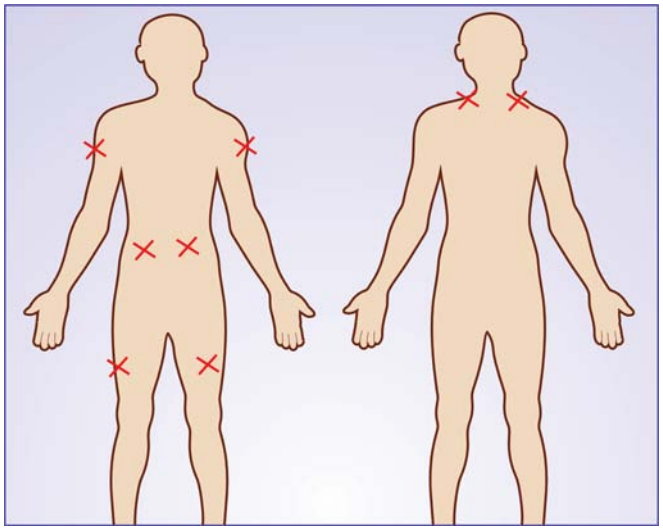


Fig. 72–4. Sites for 8-site intradermal regimen

- Rabies vaccines formulated with an adjuvant should not be administered intradermally.
- Vaccine when given intradermally should raise a visible and palpable bleb in the skin.
- In the event that the dose is inadvertently given subcutaneously or intramuscularly, a new dose should be given intradermally.

Pre-exposure Prophylaxis

Pre-exposure prophylaxis may be offered to high-risk groups like laboratory staff handling the virus and infected material, clinicians and paramedicals attending to hydrophobia cases, veterinarians, animal handlers and catchers, wildlife wardens, quarantine officers and travellers from rabies free areas to rabies endemic areas. Pre-exposure immunisation should be three full IM dose of TCV given on day 0, 7 and 28 or 0, 28 and 56 followed by booster at one year and then a booster every three years.

Laboratory staff and others at high continuing risk of exposure should have their neutralising antibody titres checked every 6 months. If it is less than 0.5 iu/ml a booster dose of vaccine should be given. Such individuals on getting exposed to rabies virus after successful pre-exposure immunisation require only two booster injections of vaccine given on days 0 and 3 without any antirabies serum.

Prevention and Control

Rabies is primarily a disease of animals. In developed countries it is wild animals while in the developing

countries it is mainly the stray dogs. Strategies to control rabies in developing countries include four components:

a. *Epidemiological surveillance:* To generate data for planning interventions.
b. Mass vaccination compaigns using parentral vaccines for dogs to vaccinate atleast 75% of the stray dogs. Oral vaccines for dogs have also been developed.
c. *Dog population management:* The practical methods include (i) movement restriction of dogs during immunisation campaigns (ii) Habitat control by restricting access of stray dogs to food, water and shelter (iii) Reproduction control by sterilisation of male dogs.
d. Community participation is essential for prevention and control measures.

PART XI MULTIPLE VIRAL COMPLEXES

73 Viral Hepatitis

Classification

In true sense viral hepatitis is inflammation of liver by viruses. Various viruses which cause damage to liver are:

- Epstein-Barr virus
- Cytomegalovirus
- Herpes simplex virus
- Rubella
- Mumps
- ECHO viruses
- Yellow fever
- Hepatitis viruses.

Of all these only hepatitis viruses primarily attack hepatocytes and hence these are considered as agents of viral hepatitis.

Viral hepatitis refers specifically to a primary infection of the liver by one of the seven aetiologically associated but different hepatotropic viruses. These types are designated as types A, B, C, D, E, F and G. The clinical manifestations of almost all these types are similar. Hepatitis B and C viruses are also responsible for hepatocellular cancer. Status of hepatitis F virus is yet unclear.

Classification of Hepatitis Viruses

This is shown in Table 73.1.

Table 73–1. Classification of hepatitis viruses

Virus	*Abbreviation*	*Genus*
Hepatitis A virus	HAV	Enterovirus 72
Hepatitis B virus	HBV	Hepadna virus
Hepatitis C virus	HCV	Togavirus
Hepatitis D virus	HDV	Defective virus
Hepatitis E virus	HEV	Calici virus
Hepatitis G virus	HGV	?

HEPATITIS A VIRUS (Also Known as Infectious Hepatitis, Epidemic Hepatitis, Epidemic Jaundice, Catarrhal Jaundice)

Classification and Morphology

The morphological and physiochemical properties of HAV are similar to those of picornaviruses and hence it has been classified as a member of family *Picornaviridae* and designated as enterovirus type 72. The virus is spherical, non-enveloped particle, 27 nm in diameter with an icosahedral symmetry (Fig. 73.1).

Fig. 73–1. Hepatitis A virus

The capsid structure of HAV closely resembles that of poliovirus and rhinovirus and image enhancement techniques suggest the presence of 32 capsomers. The unit structure of the capsid antigen consists of four polypeptides: viral proteins 1 through 4 (VP1-VP4).

The genome of HAV is a single stranded RNA molecule with (+) polarity which has a length of approximately 7500 nucleotides. Naked RNA is infectious. The molecular weight is around 2.25 million daltons.

HAV has only one serotype and only one neutralisation site is immunodominant.

Sensitivity to Physical and Chemical Agents

Hepatitis A virus is stable to treatment with ether (20%), acid (pH 2.4) and heat (60°C for one hour). It is destroyed by autoclaving, boiling in water for 5 minutes, dry heat, by UV irradiation and treatment with formalin (1:4000 for 3 days at 37°C). Its infectivity can be preserved for at least one month after lyophilisation and storage at 25°C and 42% relative humidity or for years at –20°C. It is inactivated by chlorine (free residual chlorine concentration of 2.0 to 2.5 mg/litre for 15 minutes) and iodine (3 mg/litre for 5 minutes).

Pathogenesis

HIV is transmitted from person to person by faeco-oral route. Occasionaly it is acquired through sexual contact and blood transfusion. The average incubation period of HAV infection is about one month. The pathogenesis is not fully understood. It appears that HAV enters the portal blood from the intestine and is transported to liver. It is presumed that an early phase of intestinal replication occurs. After two weeks of experimental infection in mormosets and chimpanzees, HAV has been detected in the cytoplasm of the hepatocytes. Viral antigen is detectable one week prior to the elevation of liver enzymes or detection by histopathology.

Clinical Manifestations

Onset of hepatitis A is heralded by a variety of nonspecific symptoms which include fever, chills, headache, fatigue and vague pains. These are followed by anorexia, nausea, vomiting and pain in right upper quadrant of abdomen. Appearance of dark urine, clay coloured stool and jaundice of sclera and skin help in establishing clinical diagnosis of hepatitis. Jaundice usually deepens during the first few days and persists for 1-2 weeks. It gradually diminishes over next 2 weeks or so. Convalescence is prolonged and recovery in adults takes place in few months. There is no evidence of progression of hepatitis A to chronic liver disease.

Inapparent or subclinical infections or infections without jaundice are common in children. Clinical picture may range from mild anicteric illness to acute disease with jaundice, to severe prolonged jaundice.

Epidemiology of Hepatitis A

HAV infection is endemic globally. It is not possible to estimate the incidence because of a large number of asymptomatic and anicteric cases. Infection spreads by faeco-oral route and is particularly common in places with overcrowding and poor sanitation. Common source outbreaks result from contamination of water (as in developing countries) and food (as in developed countries). All age groups are susceptible to HAV. The highest incidence is seen in children of school age. In developed countries more cases are now seen in adult population. In many tropical countries, the peak of infection tends to occur during the rainy season, with a lower incidence during the dry period.

Hepatitis A virus generally causes less severe disease than some of other hepatitis viruses and never progresses to chronicity. It is, however, the cause of significant human morbidity and loss of productivity.

HAV continues to be highly endemic in many underdeveloped countries and, like many other enterically transmitted viruses, infects almost 100% of the population by the age of 5 to 10 years. Such infections are often unrecognised because hepatitis A is milder in infants and children than in older individuals.

Laboratory Diagnosis

Laboratory diagnosis can be supported by histologic, biochemical and haematological tests apart from specific viral detection techniques.

i. *Biochemical Features*

ALT levels in acute viral hepatitis range between 500 to 2000 units and are almost never below 100 units. A sharp elevation of ALT within a short duration (4-20 days) is more suggestive of HAV infection and a gradual rise with a longer duration (35-200 days) is more consistent with HBV infection. Fluctuating levels of ALT indicate hepatitis due to agents other than HAV and HBV. Serum albumin levels are decreased and total serum globulin is increased. In many patients with HAV infection, there is an abnormally high level of total IgM 3 to 4 days after ALT begins to rise.

ii. *Haematological Features*

Leucopenia is typical in preicteric phase and may be followed by lymphocytosis. Large atypical lymphocytes (not exceeding 10% of total cells) are occasionally seen in smears.

iii. *Demonstration of HAV*

HAV has been isolated in cell culture also. It has been grown in primary, secondary and continuous kidney cells of primates and diploid fibroblasts. The virus

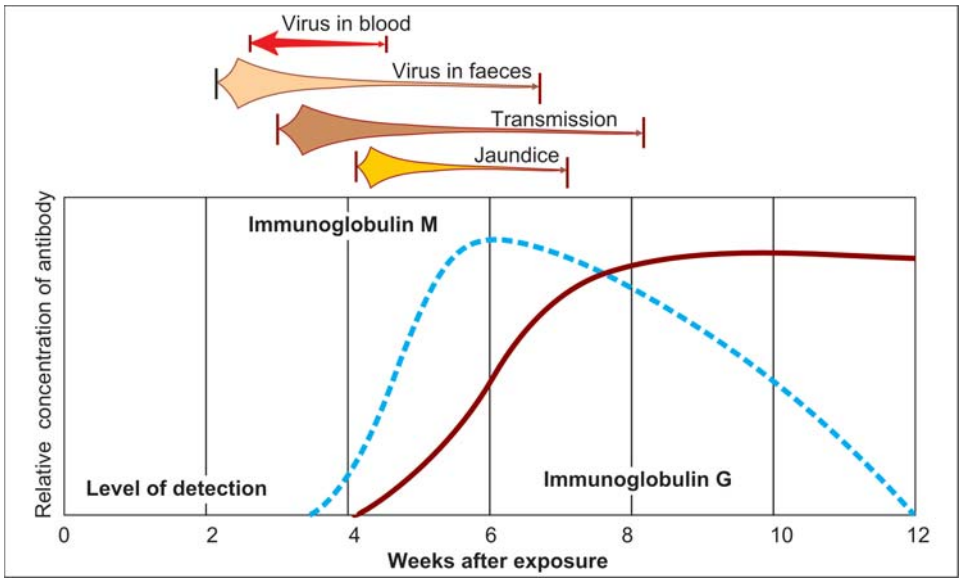


Fig. 73–2. Virology and immunology of hepatitis A

usually remains cell associated and can be detected by immunofluorescence. This technique is still beyond the reach of a large number of laboratories.

The presence of HAV in stool and the humoral immune response of the host vis a vis the presence of clinical symptoms and liver enzyme elevation are illustrated in Figure 73.2.

iv. *Serodiagnosis*

Presence of IgM antibody to HAV is diagnostic. ELISA and RIA provide reliable tests. These tests are sensitive, specific and practical for diagnosis of hepatitis A infection. Viral antigen in faeces can also be detected. Presence of IgG antibody to HAV indicates past infection.

Prevention and Control

i. Educate the public about good sanitation and personal hygiene with special emphasis on handwashing and sanitary disposal of faeces.
ii. Provide proper water treatment and distribution and sewage disposal.
iii. There are atleast 4 inactivated good quality vaccines available in the market for HAV. Clinical trials have shown them to be safe, immunogenic and efficacious. Protection begins 2-3 weeks after a single injection of vaccine. A second dose is felt necessary for long-term protection. The indications of hepatitis A vaccination differ in developing and developed countries.
iv. For proven hepatitis A patients, enteric precautions during the first 2 weeks of illness, but no more than one week after the onset of jaundice are necessary.
v. There is no specific treatment for HAV infection.
vi. Outbreaks of hepatitis A are usually controlled by sanitary and hygienic practices to eliminate faecal contamination of food and water. Outbreaks in institutions may warrant mass prophylaxis with hepatitis A vaccine and immunoglobulins.

HEPATITIS B VIRUS (Also Known as Serum Hepatitis, Australia Antigen Hepatitis, Type B Hepatitis, Homologous Serum Jaundice)

During early 1960s, Blumberg and his colleagues while examining sera of patients who had been repeatedly transfused came across an unique antigen. Since this antigen was seen for the first time in the serum obtained from an Australian aborigine, it was designated as Australia antigen and identified by the symbol Au. During 1968, this antigen was found to be associated with acute viral hepatitis. Subsequently the antigen was isolated from the sera of patients who suffered from 'long incubation period serum hepatitis'. Within a few years from this momentous discovery, the hepatitis B virus (HBV) was structurally characterised, its serologic determinants defined, and its aetiological role in acute and chronic hepatitis recognised.

Classification

Hepatitis B virus (HBV), also referred to as Dane particle belongs to a new family called as *Hepadnaviridae*.

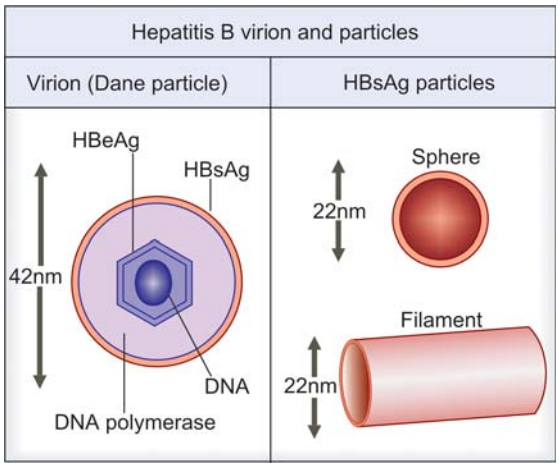


Fig. 73–3. Virion of hepatitis B virus

The virion of HBV (Fig. 73.3) is a complex 42 nm double shelled particle. The outer surface or envelope contains hepatitis B surface antigen (HBsAg) and surrounds a 27 nm inner core that contains hepatitis B core antigen (HBcAg). Inside the core is the genome of the HBV, a single molecule of circular DNA. The DNA molecule consists of 3200 base pairs and has a molecular weight in excess of 2 million daltons.

Of the two strands of DNA, one strand represents incomplete synthesis on (–) DNA template during the final phase of replication. The resulting single-stranded gap covers about 22% of the whole genome of HBV. It has been proposed that this unique structure of viral core may be biologically relevant for integration of the DNA chromosome into the chromosome of hepatocytes which may eventually be responsible for development of hepatocellular carcinoma. In addition, the HBV core also contains DNA dependent DNA polymerase as well as hepatitis e antigen (HBeAg). Presence of HBeAg in the serum of an individul indicates its high infectivity. In the serum of an individual, this may be present in a concentration of 10^{13} particles per ml.

The DNA genome is organised into four overlapping open reading frames (ORFs) in such a manner that the DNA is utilised approximately 1.5 times. These ORFs encode viral polymerase, the X protein, the core protein and the enveloped proteins which form the coat of infectious virus. The ORF that encodes the envelope proteins is demarcated into three domains, each of which begins with an in-frame initiation *codon* and encodes three proteins referred to as PreS1+PreS2+S (the large-L-protein), PreS2+S (the middle-M-protein) and S (the major protein, HBsAg). These envelope proteins are all found in varying proportions in intact virus as well as noninfectious 22 nm particles. These proteins form the basis of all vaccines successfully developed todate, as well as being the targets for vast majority of efforts aimed at development of future vaccines.

Sensitivity to Physical and Chemical Agents

HBV is stable at –20°C for more than 20 years and stable after repeated freezing and thawing. Infectivity is not destroyed by incubation at 37°C for 60 minutes and the virus remains viable after dessication and storage at 25°C for one week. The Dane particle, but not HBsAg, is inactivated at high temperatures (100°C for one minute) or when incubated for longer periods (60°C for 10 hours) depending on the amount of virus present in the sample. HBsAg is stable at pH 2.4 for upto 6 hours with loss of viral infectivity. Sodium hypochlorite in concentration of 0.5% destroys antigenicity within 3 minutes at low protein concentrations. HBsAg is not destroyed by UV irradiation of plasma or any other blood product.

Viral Proteins

HBsAg

This is also known as Australia antigen, SH antigen, Au/SH antigen or hepatitis-associated antigen (HAA). It is antigenically complex. A group specific *a* is shared by all the strains. Various subtypes are known (Table 73.2) which provide valuable epidemiological markers for distinguishing the source of any particular infection.

HBcAg and HBeAg

HBcAg is called as hepatitis B core antigen because of its enclosure within the virus by an outer coat of HBsAg-reactive material. An additional antigen is found in some HBsAg positive sera and is specific for HBV. It has been designated as HBeAg (Table 73.3).

Table 73–2. Subtypes of HBsAg

Subtype	*Distribution*
adw2	Worldwide
adw4	Worldwide
adr	Asia
ayw1	Africa, India, USSR
ayw2	Africa, India, USSR
ayw3	Africa, India, USSR
ayw4	Africa, India, USSR
ayr1	Japan, Papua New Guinea
adyw	
adywr	

Table 73–3. Hepatitis B antigens and antibodies

HBsAg	Envelope (surface) antigen of HBV particle also occurs as free particle (spheres and filaments) in blood; indicates infectivity of blood
Anti-HBs	Antibody to HBsAg; provides immunity; appears late (not in carriers)
HBcAg	Antigen in core of HBV
Anti-HBc-IgM	Antibody to HBcAg; appears early
HBeAg	Antigen derived from core; indicates transmissibility
Anti-HBeAg	Antibody to HBeAg; indicates low transmissibility

Mode of Transmission

Body substances capable of transmitting HBV include blood and blood products, saliva, CSF, peritoneal, pleural, pericardial, synovial and amniotic fluids, semen, vaginal secretions and unfixed organs and tissues. The transmission occurs by percutaneous and permucosal exposure to infective body fluids. Major modes of transmission other than blood and blood products include:

- Sexual contacts: Sexual transmission from infected men to women is about 3 times more efficient than women to men.
- Mother to infant transmission (called as vertical transmission).
- Injecting drug use.
- Nosocomial exposure to needle injury, haemodialysis, acupuncture, etc.
- Community used razor and toothbrushes.

Pathogenesis

The incubation period of HBV is usually about three months, with a range between 45-180 days. Although HBV is transmitted predominantly by the percutaneous route, it is also recognised that it can be transmitted by other means, because HBsAg has been detected in almost every body fluid. Spread of HBV by oral and genital contact has been demonstrated.

HBV infection may result in a variety of syndromes, including subclinical infection with or without HBsAg in blood, fulminant hepatitis or subacute hepatic necrosis with possible death within three months, chronic active hepatitis frequently resulting into cirrhosis, chronic persistent hepatitis or a silent carrier state with minimal or no liver damage or even primary hepatocellular carcinoma. These differences in the outcome of HBV infection cannot be explained on the basis of single parameter. Certain host factors such as age, sex, genetic background, physiologic state, immune response of the host are believed to play important role. Broadly speaking three factors of host play important role in determining the response to HBV. These are:

a. Immunogenetic predisposition
b. Immunocompetence
c. Iatrogenic immunosuppression

It is also believed that virus strain, dose and route of inoculation or cofactors such as drugs, play a role in determining severity and chronicity of the disease.

Clinical Features

The clinical manifestations of hepatitis B are identical to those of hepatitis A. HAV does not persist in the host, nor is there any evidence of progression to chronic liver damage. Hepatitis B virus may give rise to persistent infection, prolonged carrier state and progression to chronic liver disease. Substantial evidence is now available regarding association between HBV and hepatocellular carcinoma.

Outcome of Hepatitis B

The outcome of acute hepatitis B in terms of clinical entities is shown in Figure 73.4.

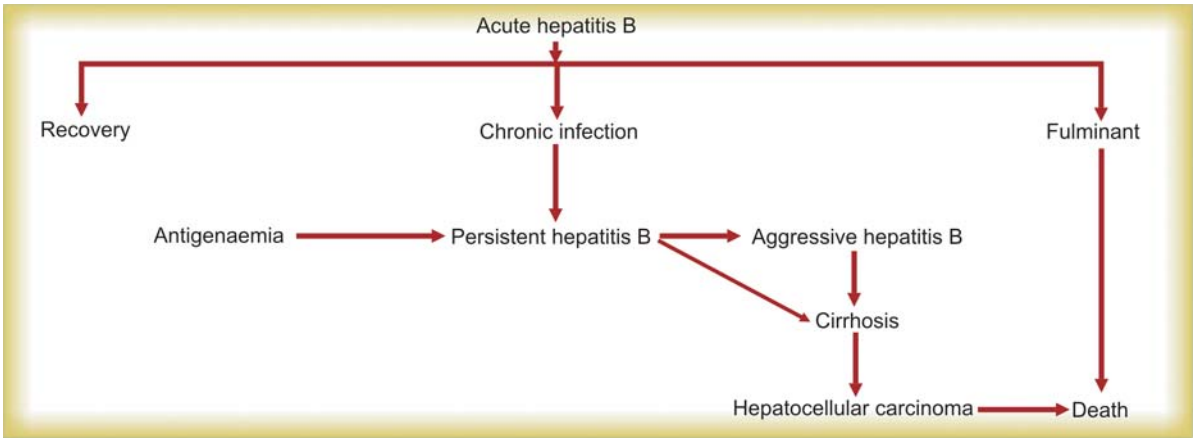


Fig. 73–4. Outcome of acute hepatitis B

Table 73–4. Serodiagnostic profile of HBV infection

Interpretation	*Serological markers*					
	HBsAg	*HBeAg*	*IgM anti-HBc*	*Total anti-HBc*	*Anti-HBe*	*Anti-HBs*
Acute Infection						
Incubation	+	+	–	–	–	–
Acute phase	+	+	+	+	–	–
Early convalescent phase	+	–	+	+	+	–
Convalescence	–	–	+	+	+	–
Late convalescent phase	–	–	–	+	+	+
Past infection	–	–	–	+	+/–	+
Chronic Infection						
Active hepatitis	+	+/–	+/–	+	+/–	–
Persistent hepatitis	+	+/–	+/–	+	+/–	–
HBV carrier	+	+/–	+/–	+	+/–	–
HBsAg Immunization	–	–	–	+	–	+

Laboratory Diagnosis

The histological, biochemical and haematological picture in viral hepatitis has been described under laboratory diagnosis of hepatitis A.

The present unavailability of cell culture for isolation of HBV limits the laboratory diagnosis of hepatitis B to the detection of serologic markers by sensitive immunoassays. These include solid phase immunoassays, latex agglutination and haemagglutination tests which are commercially available. These tests can detect six serological markers of HBV infection. The relatively complicated system of serologic markers of HBV infection may help in establishing the stage of the disease, degree of infectivity, prognosis as well as immune status of the individual. Various phases of HBV can be ascertained with these markers (Table 73.4).

A typical serologic profile of HBV-associated markers after onset of acute hepatitis is illustrated in (Fig. 73.5).

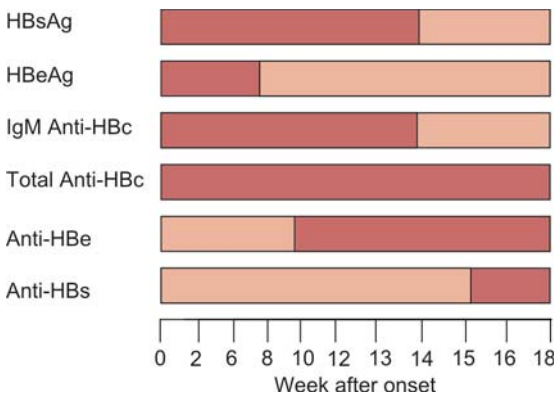


Fig. 73–5. HBV markers in hepatitis B

HBsAg appears prior to anti-HBc and, therefore, the presence of HBsAg with a negative test for anti-HBc indicates very early infection in nonvaccinated individuals. Testing for HBeAg and anti-HBe provides information about the relative infectivity of the patient. The presence of HBeAg correlates with abundant circulating Dane particles. It has also been shown to have a strong correlation with increased risk of transmitting infection upon accidental needle stick injury, and the transmission of HBV from mother to baby during the perinatal period or following exposure in household settings. Conversely, a positive result for anti-HBe indicates a low number of Dane particles and reduced risk of transmitting infection. Persistence of HBeAg as detected by RIA at 10 weeks or more after the onset of symptoms has the same prognostic value as persistence of HBsAg at 4-6 months, and both may be useful in predicting development of the carrier state. Detection of anti-HBe indicates that recovery from infection is underway and complete resolution of infection is likely.

There are certain features on the basis of which HAV and HBV infections can be differentiated (Table 73.5).

Epidemiology

Hepatitis B

The most frightening statistics regarding hepatitis B are the tremendous number of chronic carriers of this virus (estimated 300 million globally) and the immense mortality (25-30%) that would result among the carriers who may progress to cirrhosis or hepatocellular carcinoma.

Table 73–5. Differences between HAV and HBV infections

	HAV	*HBV*
Diameter of virion	27 nm	42 nm
Symmetry	Icosahedral	Icosahedral
Nucleic acid	RNA	DNA
Growth in cell cultures	+	–
Infection in chimpanzees	Subclinical	Hepatitis
Animal model	Mormoset	Rhesus
Viraemia	Brief	Prolonged
Virus in faeces	+	–
Chronic carriers	–	+
Persistence	–	+
Primary transmission	Faeco-oral	Parenteral
Other routes of infection	–	Oral, contact
Sexual transmission	–	+
Perinatal transmission	–	+
Australia antigen in blood	–	+
Incubation period	2-4 weeks	6-24 weeks
Onset	Usually acute	Insidious
Extrahepatic lesions	–	+
Affliction in children and young adults	Common	All ages
Elevated ALT	1-3 weeks	4-30 weeks
Elevated serum IgM	+	–
Chronic active hepatitis	–	+
Mortality	<1%	1-10%
Seasonal variation	+	–
Availability of vaccine	+	+

The HBV infection is global. It has been shown by various serological studies that HBV has penetrated and persisted throughout the world, even among remote and insular populations. Antigen carrier rates vary widely among different populations. Rates are higher in tropical countries (upto 20% or greater) than in most temperate areas (<0.1%). It is low in Europe and the USA. China and many countries of south-east Asia have an incidence of 6-12% whereas some countries in Africa have reported an incidence exceeding 20%. In India, it is estimated that at least 40-45 million people are carriers of HBV.

Acute hepatitis B completely resolves in 90% cases; 1 to 2% patients develop fulminant hepatitis and upto 10% become chronically infected with varying degree of progressive liver damage ranging from asymptomatic through chronic persistent to chronic active disease. In contrast, HBV infection leads to chronic carrier state in at least 85% of infected neonates.

The HBV is present in patient's or carrier's bloodstream in very high concentration and an amount of blood as small as 0.0001 ml may contain an infective dose. This amount may easily leak back into the syringe used for an injection, especially an intravenous one, and if the same syringe is used for another patient, HBV may be conveyed to him. The high incidence of hepatitis among the injectable drug addicts is explained on this basis only. Among the drug addicts attempts at asepsis are usually rudimentary and it is not uncommon to know that syringes have been shared with addicts already jaundiced.

Staff in hospitals in contact with patients or their blood are especially liable to be infected. Doctors, especially surgeons, and dentists are in contact with blood of patients, and this contact may be reflected in their own blood if they are not aware of the danger.

HBsAg has been detected in the saliva, breast milk, urine, and other body fluids and the virus has been shown to be transmitted from mother to offspring, among family members sharing same toothbrush, by sexual activity and tattooing.

Prevention Against Hepatitis B

Hepatitis B transmission can be effectively prevented by appropriate application of environmental control measures and immunization. The environmental control measures include use of barriers and personal hygiene, particularly hand washing and practising universal precautions by health workers.

Immunization

Two types of vaccines are currenly available. The vaccine is recommended for groups considered to be at high-risk for HBV infection (Table 73.6).

Plasma derived HBV vaccine. This vaccine consists of purified, noninfectious HBsAg derived from plasma of

Table 73–6. High-risk groups requiring HBV vaccine

Pre-exposure

Persons for whom vaccine is recommended

- Health care workers having blood or needle-stick exposure
- Haemodialysis patients
- Homosexually active men
- Illicit injectable drug users
- Patients receiving clotting factor concentrates
- Household and sexual contacts of HBV carriers
- Special high-risk populations

Persons for whom vaccine should be considered

- Inmates of long-term correctional facilities
- Heterosexually active persons with multiple sex partners
- International travellers to HBV endemic areas

Post-exposure

- Infants born to HBsAg positive mothers
- Health care workers having needle-stick exposures to human blood

chronic carriers of HBV. Following purification of the HBsAg, three inactivation steps are undertaken consisting of treatments with pepsin at pH 2, 8 mol urea and 1:4000 formalin. This treatment inactivates not only HBV but any other virus that may be present in plasma of the donor. The vaccine is administered as three dose series.

Genetically engineered HBV vaccine. This vaccine is produced from HBsAg derived from *Saccharomyces cerevisiae* into which a plasmid containing the gene for HBsAg subtype adw has been inserted. The purified HBsAg protein is treated with formalin.

The only precaution for administering HB vaccine relates to the use of recombinant vaccine which should not be used in individuals who are hypersensitive to yeast, haemodialysis patients or other persons with immunodeficiencies. Pregnancy is not a contraindication to vaccination.

The immunization against HBV has been summarised in Table 73.7.

Table 73–7. Immunisation against HBV

Vaccines available
• Plasma derived vaccine • Recombinant DNA vaccine
Pre-exposure dose
• Three intramuscular injections with second and third doses given 1 month and 6 months after the first dose
Post-exposure schedule in non-immunised persons
• Hepatitis B immunoglobulins plus • HB vaccination at 0, 1 and 6 months

Till a few years back the virus detected from 90% cases of post-transfusion hepatitis and 20% cases of sporadic hepatitis could not be confirmed to be either HBA or HBV. Such hepatitis was labelled as non A non B hepatitis (NANB). The diagnosis of NANB hepatitis remained a diagnosis of exclusion because all direct means of identification were unsuccessful. Now it has been found that this group of hepatitis was caused by two distinct viruses, hepatitis C and hepatitis E viruses, the former caused hepatitis through parenteral route whereas the latter caused the enterically transmitted hepatitis.

Treatment of HBV Infections

No specific treatment is available for acute hepatitis B infection. Alpha interferon and lamivudine have been licensed for treatment of chronic hepatitis B in many countries.

HEPATITIS C (Also Known as Parenterally Transmitted Non-A, Non-B Hepatitis (PT-NANB) and Non-B Transfusion Associated Hepatitis)

Parenterally transmitted non-A, non-B hepatitis (PT-NANB). Non-B transfusion associated hepatitis, HCV infection).

Hepatitis C virus is a small, enveloped, positive sense, single stranded RNA virus. It is related to human flaviviruses and has been placed in the genus hepatitis C virus. So far this virus has not been fully characterised in terms of its morphology and biochemical parameters. However, by electron microscopy the virus core particles have been shown to be 33 nm in diameter. Intact virion has a diameter of 55-65 nm with spike like projections.

The HCV genome encodes for four structural proteins, core, E1, E2 type A and E2 type B as well as at least six nonstructural proteins (NS2, NS3, NS4a, NS4b, NS5a and NS5b).

Infection of human T cell lines (MOLT-4 and HPB-Ma) has been achieved with HCV, but the replication efficiency of this virus seems to be very low.

Genetic Heterogeneity

On comparison of genomic nucleotide sequence of a large number of isolates from different parts of the world it has been shown that HCV is highly heterogenous. This is because of the presence of reverse transcriptase enzyme which lacks the normal mechanism of genetic proof reading. HCV is therefore genetically endowed to be heterogenous. The spontaneous nucleotide substitution rate of HCV is very high and has an annual frequency of 1:100 to 1:1000 substitutions at each nucleotide site. So far six HCV genotypes and more than 80 subtypes have been recognised. Various implications of genetic heterogeneity are:

- Severity of disease shall depend upon infecting genotype
- Response to interferon may be influenced by genotype
- Vaccine development shall require inclusion of multiple subtypes
- Epidemiology of disease may be influenced by different genotypes.

Reservoir of Infection

Although HCV has been transmitted experimentally to chimpanzees, human beings are the only natural reservoir for this virus. About 170 million carriers in the world constitute the reservoir for continuous trans-

mission of HCV. In addition to blood, HCV-RNA has been detected in semen, urine, tears and ascitic fluid of HCV infected persons.

Mode of Transmission

Percutaneous exposure to blood and plasma derivatives, use of contaminated needles and syringes, needle stick injury in health care settings, sharing of needles by intravenous drug users, tattooing and skin piercing are the most common methods of transmission of HCV. Sexual transmission does not appear to be a substantial contributor to the spread of HCV infection.

Pathogenesis and Pathology

The virus has an average incubation period of 5-12 weeks, with peak of onset of disease 7-8 weeks after infection. The majority of acute cases are asymptomatic. Fatigue is common, but it is rarely clinically significant. Jaundice manifests in less than 25% of patients.

Fulminant hepatitis C is rare. A common feature of HCV infection is an episodic, fluctuating pattern of ALT (SGPT) activity.

The striking feature of HCV-induced liver disease is its tendency towards chronicity and slowly progressive liver damage. The disease may remain clinically silent for years or even decades. Cirrhosis may develop after 20-30 years of infection.

Various factors have been implicated as determinants of the progression from chronic hepatitis to cirrhosis. These are:

- Patients's age at exposure
- Duration of liver damage
- Level of liver damage in initial exposure
- Host immunity
- Alcohol intake
- Concomitant infection with HIV, HBV or HDV.

Laboratory Diagnosis

HCV diagnosis is currently achieved by detecting specific antibody against HCV or RNA of the virus. The antibodies can be detected by enzyme immunoassays (ELISA) as well as recombinant immunoblot assays (RIBA). Anti-HCV IgM is detectable intermittently in acute and chronic HCV infections. Both the systems use antigens derived from cloning of HCV genome. If both antibody tests are positive, there is high likelihood that the patient is infected.

Tests for HCV-RNA may be used to help diagnose cases before seroconversion or if immunoblot test is indeterminate. However, a single negative HCV-RNA test does not indicate that an individual is uninfected or has responded to treatment. RNA can be detected by using reverse transcription polymerase chain reaction (RT-PCR).

Treatment and Control

To-date recombinant interferon is the only therapeutic agent approved by the United States' Food and Drug Administration for the treatment of chronic hepatitis C. There is no vaccine against HCV. General control measures against HBV infection which include safe blood and injections also apply to HCV. All HCV positive persons should be considered infectious and these persons should not be allowed to donate blood or organs. General control measures against HBV infection apply.

HEPATITIS E (Also Known as Enterically Transmitted Non-A, Non-B Hepatitis (ET-NANB) Epidemic Non-A, Non-B Hepatitis, Faeco-oral Non-A, Non-B, HEV Infection)

Hepatitis E virus (HEV) is small, round, nonenveloped, positive strand RNA virus. When examined from stool specimens it measures 29 nm in diameter. HEV has been suggested to be classified as a calici-virus. Though there is minor heterogeneity in isolates of HEV from different geographical locations, there are evidences to suggest existence of only one serotype of this virus.

This virus has great public health importance since it can cause explosive outbreaks of water-borne viral hepatitis in communities. Salient features of hepatitis E infection are:

- Clinico-epidemiological picture resembles hepatitis A
- Affects predominantly adult population
- Usually occurs in the form of explosive outbreaks
- Sewage contaminated water is usual reservoir
- Mortality is high if women affected in third trimester of pregnancy
- No chronicity or sequelae
- Major public health problem in developing countries
- No vaccine available as yet.

Natural Host

Humans are the only natural host for HEV. Monkeys and chimpanzees have been experimentally infected with this virus. Natural infections have been described in pigs, chicken and cattle in endemic areas.

Mode of Transmission

Primarily by faeco-oral route. Faecally contaminated water is the most common vehicle. Person to person transmission probably occurs through faeco-oral route.

Clinical Significance

HEV is responsible for much of the viral hepatitis in areas where it is endemic. Individuals can be affected in large epidemics, focal outbreaks and as sporadic cases. Almost all the outbreaks of hepatitis in India have been found to be due to HEV.

Hepatitis E is predominantly a disease of young male adults. Fulminant hepatitis E is rare except in pregnant women where the mortality rate has been found to be upto 25% and is highest during the third trimester.

Pathogenesis

How HEV travels from gastrointestinal tract to liver is not known. Hectic viral replication is usually the first event and occurs before hepatocellular changes and inflammatory alterations manifest. Hepatitis and anti-HEV antibody appear to develop concurrently. The pathogenesis of fulminant hepatitis in pregnant women is not understood.

Laboratory Diagnosis

This can be accomplished by the detection of IgM or IgG antibody to HEV. Commercial kits are available to detect these by ELISA techniques. However, immune response to HEV is unique. In majority of patients IgM response disappears fairly early. Its level fluctuates even during acute phase and fades within three weeks of infection. Therefore, diagnosis of acute HEV infection poses problems. IgG titres also tend to recede but remain detectable in normal population in endemic areas.

There are no routine methods for detecting HEV in clinical materials. Moreover, HEV is present in faeces only during first week after onset of jaundice. HEV concentration in clinical specimens is usually too low for detection by immunoassays or direct nucleic acid hybridization. RT-PCR for HEV is under development.

Treatment and Control

There is no specific treatment and in the absence of an effective immunizing agent, the mainstay of prevention of hepatitis E remains provision of safe drinking water. The outbreaks of HEV have been effectively curtailed with the restoration of safe water supply to communities. No vaccine is available as yet against HEV.

HEPATITIS D (Also Known as Delta Agent Hepatitis, Hepatitis Delta Virus)

Hepatitis D virus (HDV) was earlier considered as a defective virus which needed HBV for its survival. Subsequently, it was also considered as an associated virus. However, the recent evidence suggests that HDV is a satellite hepatitis B virus.

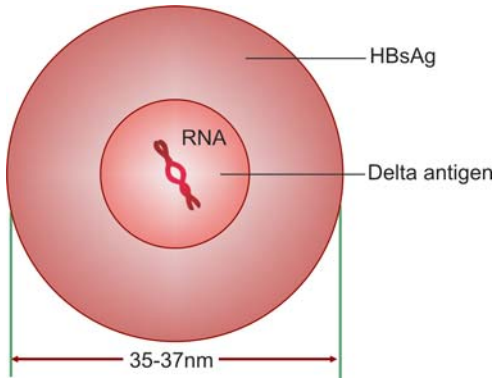


Fig. 73–6. Hepatitis D virus

The hepatitis D virus (HDV) was first detected in Italy in 1977. It was called as delta agent and subsequently named as hepatitis D virus.

HDV has a unique RNA genome which is smaller than the genome of conventional viruses but larger than that of the viroids of plants.

Morphology

Hepatitis D virus is a 35-37 nm particle. It contains HBsAg on the surface and delta antigen and a small RNA genome in the interior (Fig. 73.6). The molecular weight of genome is 0.55 million daltons. The particle consists of an organised structure that prevents hydrolysis of the internal RNA, thus, protecting the infectivity of the virus.

Delta antigen can be detected in the nuclei of the infected hepatocytes by immunofluorescence. HBV specific HBcAg and delta antigen never appear together in the same cell. The replication of HDV takes place in the nucleus of infected cells whereas HBV replicates in the cytoplasm.

HDV is resistant to treatment with EDTA, detergents, ether, nuclease, glycosidase and acid but partial to complete inactivation follows treatment with alkali, thiocyanate, guanidine hydrochloride, trichloroacetic acid and proteolytic enzymes.

Clinical Features

Since presence of HBV is necessary for the replication of HDV, it can cause disease only in the presence of HBV. There are three situations where it can happen. These are:

a. Patient gets simultaneous infection with HBV and HDV (coinfection). Replication of HDV shall depend

upon the spread of progeny of HBV in sufficient number to ensure dual infection of susceptible liver cells. The disease shall resemble typical acute HBV infection and may progress to fulminant hepatitis.

b. Patient is already having HBV infection and gets superinfection with HDV (superinfection). A serious acute delta related hepatitis superimposes on the existing disease.
c. An asymptomatic carrier of HBV gets infected with HDV and acute hepatitis results. This can be confirmed by the absence of an HBV specific anti-HBc-IgM response.

Pathogenesis

The incubation period of hepatitis D varies from 2-12 weeks and is shorter in those who are carriers of HBV. The liver damage in hepatitis D has been associated with a virus mediated cytopathic mechanism, thus explaining the failure of conventional immunosuppressive therapy to alter the course of disease. The activity of chronic hepatitis correlates with the presence but not with the amount of delta antigen in liver. It has been observed that children who develop mild HBV infection, often suffer severe and even fatal attacks of HDV infection.

Laboratory Diagnosis

Initially the detection of delta antigen in the nuclei of hepatocytes was achieved by direct immunofluorescence using either frozen unfixed or fixed embedded liver biopsy specimens. Delta antigen can also be visualised in the liver biopsy specimens by indirect immunoperoxidase staining. Enzyme immunoassays and RIA kits are now commercially available for the detection of antibodies to delta agent, thus reducing the need for liver biopsy in many cases.

Acute HBV and acute HDV coinfection is diagnosed when HBsAg, anti-HBc IgM, HBeAg and anti-HD IgM are positive.

Prevention and Control

No specific therapy and immunoprophylaxis are currently available against HDV. Preventive measures against HBV also prevent HDV since HDV cannot survive in the absence of HBV.

HEPATITIS G

Epidemiology

A new flavivirus-like agent was identified in the second half of 1994 which causes hepatitis in humans. This virus is distinct from HCV and has been labelled as hepatitis G virus (HGV). The transmission of HGV through blood transfusion and by other parenteral routes of exposure, such as through intravenous drug use, may occur.

Limited clinical studies in developed countries revealed that HGV is widely distributed and has been found in blood donors in a frequency of upto 1.5%. Its circulation in India has also been demonstrated. HGV may result in persistent infection. The majority of individuals infected with HGV do not have clinical evidence of liver disease. Although HGV has been linked to acute and chronic hepatitis, it is yet to be proved whether HGV is the causal agent for viral hepatitis.

Table 73–8. Properties of hepatitis viruses

Property	*HAV*	*HBV*	*HCV*	*HDV*	*HEV*
Virus group	Enterovirus72	Flavivirus	Togavirus	Very small	Calicivirus
Type of virus	ssRNA	dsDNA	ssRNA	ssRNA	ssRNA
Stability in environment	+	++	–	–	–
Mode of infection	Faecal-oral	Blood and sex	Blood	Blood	Faecal-oral
Propagation					
In vitro	+	–	+	–	–
In vivo	+	+	+	+	+
Incubation period (weeks)	2-4	6-24	5-12	2-12	6-8
Persistence in liver	–	+	+	+	–
Severity of hepatitis	+	++	+	+	+/–
Extrahepatic replication	+	+	Not known	–	+
Hepatic carcinogenicity	–	+	+	–	–

Prevention and Control

Many HGV positive persons are also co-infected with HBV and HCV, because of shared risk factors of infection. Screening of blood for HBV and HCV also removes HGV infected blood thereby reduces the incidence of HGV related post-transfusion hepatitis (PTH), if it really occurs.

HEPATITIS F

During early 1990s, a new virus was incriminated as yet another causative agent of hepatitis. It was tentatively designated as HFV. However, subsequent evidence has not supported the existence of HFV.

OVERVIEW OF VIRAL HEPATITIS

Of the seven known types of hepatitis viruses, five have been found to be of great public health importance. Their important features have been summarised in Table 73.8.

The diagnosis of some of these conditions is possible with serological tests but a few are diagnosed by the process of exclusion.

74 Arboviruses

DEFINITION

An arbovirus is one that multiplies in a blood sucking arthropod and is transmitted by the bite to a vertebrate. According to the World Health Organization arboviruses are defined as those viruses which are maintained in nature principally through biological transmission between susceptible vertebrate hosts by haematophagus arthropods. Such *arthropod-borne viruses* comprise a convenient epidemiological grouping and comprise of members belonging to five different families (Table 74.1). Arboviruses as such do not have any taxonomic significance.

Table 74–1. Families having arboviruses as members

Family	*Genus*
Togaviridae	Alphavirus
	Arterivirus
	Pestivirus
	Rubivirus
Flaviviridae	Flavivirus
Bunyaviridae	Bunyavirus
	Nairovirus
	Phlebovirus
	Uukuvirus
Reoviridae	Orbivirus
Rhabdoviridae	Vesiculovirus
	Lyssavirus

Almost all the arboviruses—which now number more than 500, are zoonoses and these are maintained in nature by hosts other than man (Fig. 74.1). Two important exceptions are dengue and O'nyong-nyong.

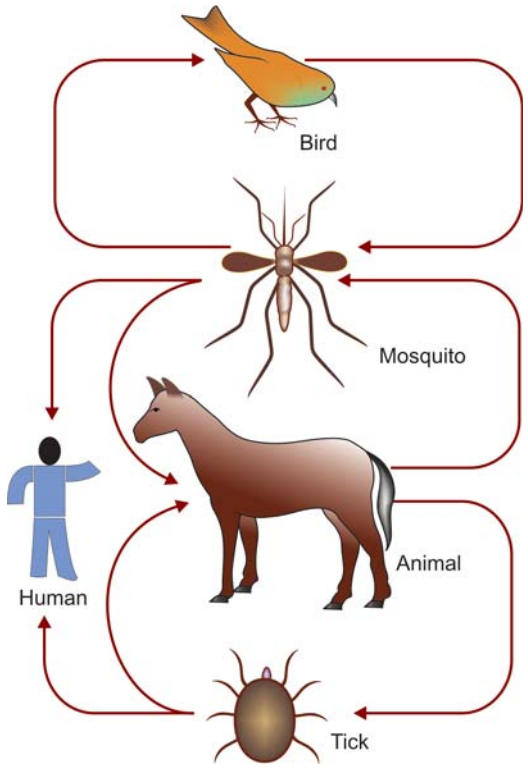


Fig. 74–1. Arbovirus life cycle

VIROLOGY OF COMMON ARBOVIRUSES

Flaviviridae

The family name of Flaviviruses has been derived from the Latin word *flavus* which means yellow and the virus of yellow fever is the type species of this genus. More than 70 viruses are members of this genus of which around 50 are arboviruses. Most of the human

Table 74–2. Flaviviruses which cause disease in humans

Virus	*Vector*
Banzi	Mosquito
Dengue*	Mosquito
Japanese encephalitis*	Mosquito
Kunjin	Mosquito
Murray valley	Mosquito
St Louis	Mosquito
Sepik	Mosquito
Usutu	Mosquito
West Nile	Mosquito
Yellow fever	Mosquito
Zika	Mosquito
Kyasanur Forest*	Tick
Louping-ill	Tick
Omsk	Tick
Powassan	Tick
Eastern encephalitis	Tick

* Cases occur in India

pathogens are transmitted by mosquitoes and a few by ticks (Table 74.2). Yet there are a few flaviviruses for which the vector is yet to be ascertained. Only one genus—*Flavivirus* is currently accepted in the family *Flaviviridae*.

Morphology

Virion has a diameter of about 45 nm inclusive of lipid bilayer envelope. The nucleocapsid has a spherical symmetry and diameter of 30-35 nm. A single stranded, positive sense RNA constitutes the genome. It has a length of 11 kb.

Antigenic Structure

The envelope contains E antigen, the matrix or membrane protein possesses M antigen and the nucleocapsid has C antigen. Between 5 and 12 non-structural proteins have been described.

All flaviviruses are antigenically related. Cross reactions are seen when haemagglutination, haemagglutination inhibition or plaque reduction neutralisation tests are performed.

TOGAVIRIDAE

The family name is derived from Latin word *toga* which means cloak—a reference to the viral envelope. The family Togaviridae comprises of following four genera:

- Alphavirus
- Arterivirus
- Pestivirus
- Rubivirus

The genus *Rubivirus* contains only one member which is the causative agent of rubella. Of the currently described 32 members of the genus *Alphavirus*, a few are capable of causing disease in human beings (Table 74.3). All these are transmitted through mosquitoes.

Table 74–3. Human pathogens belonging to genus Alphavirus

- Barmah forest
- Chikungunya*
- Eastern encephalitis
- Igbo-Ora
- Mayaro
- O'nyong-nyong
- Ross river
- Sindbis
- Venezuelean encephalitis
- Western encephalitis

* Reported from India

Morphology

The members of this family are enveloped and have a diameter of 50-70 nm. Alphaviruses demonstrate icosahedral symmetry. The genome is comprised of single molecule of single stranded positive sense RNA which is almost 12 kb in length and 4 million dalton molecular weight.

Antigens

Alphaviruses carry at least three distinct antigenic molecules. These include a subgroup specific antigen designated as E1 and a virus specific antigen called E2. C antigen is a cross reactive antigen.

BUNYAVIRIDAE

More than 300 viruses have been identified so far as the members of this family. Not all are arboviruses and just a few are pathogenic to man. Five genera have been recognised but still more than 50 viruses in this family need to be placed in some of the recognised genus (Table 74.4). The largest group is *Bunyavirus* which comprises of about 150 viruses.

Morphology

The external diameter of these viruses is around 100 nm including the lipid envelope. The virion exhibits helical symmetry. Some of the viruses have surface projections 8-10 nm long. Virion contains three circu-

Table 74–4. Classification of Bunyaviridae

Genus	*Serogroup/virus*
Bunyavirus	Bunyamwera California encephalitis
Hantavirus	Hantaan virus
Nairovirus	Crimean Congo Ganjam
Phlebovirus	Phlebotomus Rift Valley fever
Uukuvirus	Uukuniemi
Unassigned	50 viruses

lar, helical, internal nucleocapsids, each containing one species of RNA. These three have been designated as L (large), M (medium), and S (small). A virion transcriptase is also present.

Antigens

All members have three major structural proteins. Two are associated with envelope glycoproteins called as G1 and G2. The nucleocapsid is associated with a nucleoprotein N. Two nonstructural proteins and another large proteins are also present. G1 and N proteins are the principal antigens reacting respectively in neutralisation and complement fixation tests.

GENERAL CLINICAL FEATURES

A large number of arboviruses do not produce clinical disease in human beings. A few cause minor manifestations such as fever, yet there are around 50 arboviruses which are capable of producing serious ailments, and about half of these are potentially lethal. On the basis of usual clinical picture, arboviral infections can be grouped into four clinical syndromes. These are:

- Arboviral fever—arthralgia rash syndrome
- Haemorrhagic fever
- Haemorrhagic fever with hepatitis and nephritis
- Encephalitis.

Arboviral Fever—Arthralgia Rash Syndrome

This is the most common manifestation of arboviral disease. Many viruses, which are known to cause serious ailments also, sometimes manifest with vague clinical features of fever, arthralgia and rash. However, commonly these features are caused by few arboviruses.

Dengue fever is the commonest of all these infections. The characteristic features are sudden onset of fever, chills, headache, conjunctivitis and lymphadenitis. These are accompanied by pain in back, muscles and joints (hence the name *break bone disease)*. A rash may appear after 3-4 days of onset of disease.

Haemorrhagic Fever

Thrombocytopenia is a characteristic feature of this syndrome and this results into haemorrhage which may manifest as petechiae and ecchymoses on skin and mucous membranes as well as bleeding from various orifices. These diseases may be clinically similar but epidemiologically distinct (Table 74.5).

Table 74–5. Arboviral haemorrhagic fevers

Virus	*Vector*	*Reservoir*
Chikungunya*	Mosquito	Monkey ?
Crimean HF-Congo	Tick	Mammals
Dengue*	Mosquito	?
Kyasanur Forest disease*	Tick	Monkey
Omsk	Tick	Mammals
Yellow fever	Mosquito	Monkey

* Cases occur in India

Haemorrhagic Fever with Hepatitis and Nephritis

Haemorrhagic fever with nephritis and hepatitis is produced by yellow fever. It was first demonstrated to be caused by a mosquito bite by Walter Reed. It manifests as fever, chills, headache, backache, vomiting, gastrointestinal haemorrhages, hypotension, and jaundice which may lead to death. Case fatality rate has been reported to be 10%. Yellow fever is an internationally notifiable disease.

Encephalitis

A large number of arboviruses are capable of giving rise to encephalitis (Table 74.6). Apart from usual features of fever, and bodyache, central nervous system involvement results into neck rigidity, drowsiness and delirium. The worst cases progress to confusion, paralysis, convulsions, coma and death.

Many of these cases suffer from sequelae of encephalitis which include mental retardation, epilepsy, paralysis, deafness or blindness.

Pathogenesis

After the introduction of virus in a subcutaneous capillary by the vector, local multiplication of virus occurs followed by viraemia. Viral multiplication also takes place in organs of reticuloendothelial system which precipitates the systemic phase of illness. The disease process may halt here or may progress to

Table 74–6. Arboviruses causing encephalitis

Virus/disease	*Vector*	*Reservoir*
Australian encephalitis	Mosquito	Birds
California encephalitis	Mosquito	Rodents Rabbits
Eastern equine encephalitis	Mosquito	Birds
Japanese encephalitis*	Mosquito	Birds
Louping ill	Tick	Sheep
Powassan	Tick	Rodents
Russian Spring Summer encephalitis	Tick	Rodents
St.Louis encephalitis	Mosquito	Birds
Tick-borne encephalitis	Tick	Mammals
Venezuelan equine encephalitis	Mosquito	Rodents
West Nile encephalitis	Mosquito	Birds
Western equine encephalitis	Mosquito	Birds

* Cases occur in India

involve more organs causing rash, arthritis, hepatitis, nephritis or encephalitis. Fever is associated with viraemic phase.

Skin rash is due to swelling of capillaries and perivascular oedema; haemorrhages are due to thrombocytopenia; liver atrophy followed by hyaline necrosis of liver which occurs in yellow fever. Two types of inclusion bodies are seen in this condition—*Torres bodies* and *Councilman bodies*. In encephalitis inflammatory foci are distributed throughout the brain and are marked by necrosis of neurons, perivascular cuffing, capillary thrombosis and variable degree of meningitis.

LABORATORY DIAGNOSIS OF ARBOVIRUSES

Virus Isolation

Virus is rarely found in the plasma or serum of encephalitis cases, but it is readily isolated during the first 3-4 days of systemic illnesses such as dengue fever.

Virus can be isolated from liver, spleen and kidney in systemic illnesses and at necropsy, it has been isolated from brain of cases with encephalitis.

Intracerebral inoculation of mice (1-3 days old) is the isolation method of choice for those arboviruses which cause encephalitis. Alternate isolation systems include Vero, BHK-21 and primary chicken or duck embryo cells. The mosquito cells obtained from *Aedes albopictus* are highly susceptible to the encephalitis virus as well as to dengue and yellow fever viruses. These cells, however, do not always express CPE and hence presence of virus has to be confirmed with immunofluorescence or by subculture into another host.

Isolation of virus takes time. Mice inoculated with encephalitis causing viruses die within 48 hours to 7 days. The time for appearance of plaque or CPE in cell cultures is shorter than the incubation time in mice. Virus is identified by complement fixation, haemagglutination inhibition, immunofluorescence, neutralisation or ELISA. Type specific monoclonal antibodies against four types of dengue viruses are now commercially available.

Detection of Antigen

The antigen detection is now replacing isolation of virus for certain arboviral infections. In Colorado tick fever, antigen can be detected in the RBCs within first two weeks of infection by immunofluorescence. This test can also be used for detection of viruses in the brain tissue on necropsy. Antigen detection by ELISA is also being attempted.

Detection of Antibodies

The method of choice for the diagnosis of arboviral encephalitis should be detection of IgM antibody. With ELISA, a reliable diagnosis can be established in first 5 days after onset of CNS features. Acute phase blood and CSF should be taken for antibody detection. If a diagnosis is not made during acute phase, then a second serum sample should be collected one week or later.

Many public health and hospital laboratories still rely on demonstration of a four-fold or higher rise in antibody titre using paired sera. Complement fixation, haemagglutination inhibition and neutralisation tests are employed. Of these, neutralisation test is the best but expensive and not widely used.

ARBOVIRAL DISEASES IN INDIA

Japanese Encephalitis

Both animals and birds have been known to play important role in natural cycle of transmission of JE though the precise nature of this relationship is yet to be fully elucidated. A working hypothesis which is agreed to by various scientists has been shown in Figure 74.2.

The disease cycle between the birds in nature is also called as primary cycle whereas some of the animals such as pigs, on getting infected from the birds through vectors maintain not only a cycle within themselves but also augment the availability of virus to vectors. Such *amplifier hosts* are considered to play a vital role in the epidemiology of JE.

More than 40,000 cases of JE were reported from India during 1980s with about 15,000 deaths and a case

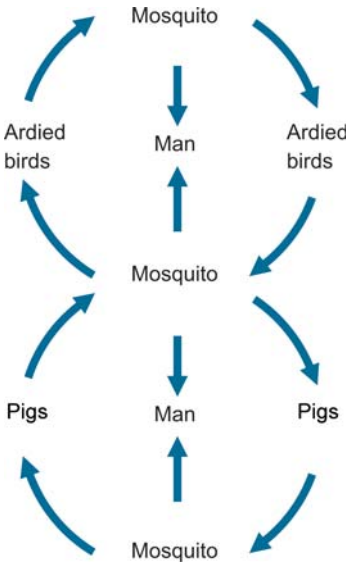


Fig. 74–2. Epidemiology of JE

fatality rate of 37%. Even these figures are based upon institutional data and there are reasons to believe that these represent gross underreporting.

Essential clinical features of JE are described under clinical features of arboviruses causing encephalitis. The isolation of JE virus is difficult. Detection of IgM antibody by ELISA or rising titre of IgG antibody by ELISA and haemaglutination inhibition are commonly used methods of diagnosis. The virus can also be isolated from CSF and autopsied brain tissue and antigen can be demonstrated by fluorescent antibody test.

Treatment

There is no specific treatment of JE. However, supportive treatment and good nursing care can significantly reduce case fatality rate. Fluid and electrolyte balance should be maintained. Antipyretic and anticonvulsants should be given to alleviate the suffering. Corticosteroids do not confer any beneficial effects. Physiotherapy is necessary to avoid or reduce neurological sequelae.

Vaccine

A killed JE vaccine is produced in India at Control Research Institute, Kasauli (HP)from the brain of suckling mice. Two doses of 1 ml each (0.5 ml for children under three years of age) should be administered subcutaneously at an interval of 7-14 days. A booster injection of 1 ml should be given after a few months of primary immunisation. Revaccination may be given after three years. JE vaccination is not included in national immunisation schedule. A live attenuated chinese vaccine SA-14-14-2 is being used for mass vaccination in the JE endemic districts of the country.

DENGUE FEVER AND DENGUE HAEMORRHAGIC FEVER

Dengue virus infections are significant causes of morbidity and mortality in many parts of the world, including India. The dengue virus is believed to cause two forms of clinical syndromes, namely, classical dengue fever (DF) and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS).

Dengue fever is a self-limiting disease and represents the majority of cases of dengue infection. In some situations, it manifests in severe forms as haemorrhagic (DHF) and shock syndrome (DSS).

In India, dengue infection is known to exist in endemic proportions for a very long time. In 1996, an outbreak of dengue fever in Delhi caused more than 10,000 cases and 423 deaths and there was another outbreak which affected Delhi in Sept-Oct. 2003 and in 2005.

Causative Agent

DF/DHF is caused by a group B arbovirus (Flavivirus) and include serotypes 1, 2, 3 and 4 (Den-1, Den-2, Den-3 and Den-4).

Mode of Transmission

The incubation period is usually 5-6 days but may vary from 3 to 10 days. Man is the only definitive host. The infection is transmitted by the bite of an infected female mosquito *Aedes aegypti*. The mosquito usually bites during day time. The mosquito becomes infected by biting a patient with dengue infection. Once the mosquito becomes infected, it remains so for life. The female mosquitoes can survive upto 3 weeks under normal temperature and humidity.

Female mosquitoes get infected after feeding on a viraemic host. They can transmit the virus to human host after an extrinsic period of 8-10 days. The ambient temperature range for dengue transmission is 16°C to 40°C. Below 16°C *Aedes aegypti* ceases to bite.

High-risk Areas

Usually urban areas having high population density, poor sanitation and large number of desert coolers, overhead tanks, discarded buckets, tyres, utensils etc. containing water which promote mosquito breeding, are at high-risk. Dengue fever/DHF can also occur in rural areas where the environment is friendly for

mosquito breeding. Mosquito breeding can occur, for example, in large containers used for collecting rain water, which are not emptied and cleaned periodically.

Clinical Manifestations

The symptoms of dengue fever are similar to acute fevers of viral origin. There is sudden onset of fever, headache, bodyache, joint pains and retro-orbital pain. Other common symptoms include anorexia, altered taste sensation, constipation, colicky pain, abdominal tenderness, dragging pain in the inguinal region, sore throat and general depression.

Patient may or may not have rash. Some of the patients may also show signs of bleeding from the gum, nose, etc.

Dengue Haemorrhagic Fever (DHF)

DHF is a severe form of dengue fever. Typically, it begins abruptly with high fever accompanied by headache, anorexia, vomiting and abdominal pain. During the first few days, the illness resembles classical dengue fever, but a maculopapular rash is less common. There are signs of haemorrhage (bleeding), such as easy bruising and bleeding at the venepuncture sites. The liver is usually enlarged, soft and tender. Approximately 50% of patients have generalised lymphadenopathy.

The critical stage is reached after 2-7 days, when the fever subsides. Accompanying or shortly after a rapid drop in body temperature, varying degree of circulatory disturbances occur. The patient is usually restless and has cold extremities. Sometimes there may be sweating.

DHF is clinically confirmed by the positive tourniquet test (a blood pressure cuff is used to impede venous flow and the test is considered positive if there are more than 20 petechiae/square inch).

Thrombocytopenia and haemoconcentration are constant findings in DHF. Haemoconcentration, indicating plasma leakage is always present.

Pathogenesis and Pathophysiology

The mechanism of DHF is not clear, but two main pathophysiologic changes occur:

- Vascular permeability increases which results in plasma leakage, leading to hypovolaemia and shock
- Abnormal haemostasis, due to vasculopathy, thrombocytopenia and coagulopathy, leading to various haemorrhagic manifestations.

The severity of DHF as compared with dengue fever may be explained by the enhancement of virus multiplication in macrophages by heterotypic antibodies resulting from a previous dengue infection. There are evidences suggesting that cell mediated immune response may also be involved in the pathogenesis of DHF.

Immunity

Infection with one serotype provides life-long homologus immunity but does not provide protection against other serotypes, and instead may exacerbate subsequent infection.

Laboratory Diagnosis

Serodiagnosis

The diagnosis of DF/DHF can be confirmed by serological tests. The tests include detection of IgM antibodies by ELISA test which appear around the end of first week of onset of symptoms and are detectable upto 1-3 months after the acute episode. A rising titre of IgG antibody in paired sera taken at an interval of ten days or more is confirmatory. Haemagglutination inhibition and ELISA tests are available for the detection of IgG antibody.

IgG antibodies indicate previous infection and are useful for conducting sero-epidemiological studies to determine the extent of silent infection and immunity levels in the local population.

Isolation/Detection of Virus/Antigen

The virus can be isolated by growing the clinical material in cell lines such as Vero or CC636 or by intracerebral inoculation of suckling mice. Viral genomic sequences can be detected in CSF serum or autopsy tissue sample by using PCR.

Treatment

The management of dengue fever is symptomatic and supportive and comprises of:

- Bed rest is advisable during the acute febrile phase
- Antipyretics or sponging are required to keep body temperature below 39°C. Salicylates should be avoided. Paracetamol may be prescribed
- Analgesics or a mild sedative may be required for those with severe pain
- Home available fluids and ORS solution are recommended for patients with excessive sweating, nausea, vomiting or diarrhoea to prevent dehydration.

KYASANUR FOREST DISEASE

In early 1957, a disease broke out in Sagar-Sorab area in South India and was provisionally diagnosed as ente-

ric fever. The zoonotic nature became apparent because it was, from the beginning, associated with the death of monkeys in the area. Hence, the disease was often called by the natives as *monkey disease*. The disease is caused by a virus which has been named after the Kyasanur forest area in Sagar Taluka of Shimoga district of Karnataka state.

At present the disease is restricted to some districts of Karnataka state only. However, it is gradually spreading. In 1957, when it was first recognised, it was active in an area of about 800 sq. km. By 1982, the area had increased to more than 4000 sq km. The maximum number of cases occur during dry months particularly January to June. This period coincides with the peak nymphal activity of ticks. This period also coincides with the period of maximum human activity in the forest.

Clinical features. KFD has sudden onset with chills, headache, fever, pain in lower back and limbs. Cervical lymphadenopathy and conjunctival suffusion are usually present. Confusion and encephalopathic symptoms may occur. Severe infections are associated with bleeding from gums, nose, GI tract, etc.

Mode of transmission. This infection is transmitted through the bite of infected ticks *(Haemaphysalis spinigera)*. The disease has an incubation period of 3-8 days. Man to man transmission does not occur. However, disease occurs in all ages and both genders. Previous infection leads to immunity.

Laboratory diagnosis. Virus persists in blood upto 10 days after onset of infection and can be cultivated in suckling mouse brain as well as cell lines such as Vero and BHK-21 lines. Haemagglutination inhibition and ELISA tests are also employed for serodiagnosis.

Treatment and prevention. The treatment is entirely symptomatic and a vaccine is currently in experimental stages.

CHIKUNGUNYA VIRUS

Chikungunya virus belongs to the Alphavirus genus of the Togaviridae and is primarily transmitted by bites of mosquitoes of the genus *Aede*, the same mosquito that transmits dengue fever. Infection with chikungunya virus results in chikungunya fevers and associated symptoms.

The disease has an abrupt onset, with high fever, myalgia and sudden intense pains in one or more joints. Accompanying signs and symptoms include headache, sometimes nausea and vomiting, coryza, conjunctivitis, photophobia and retro-orbital pains. Maculopapular rash may develop on the 2nd and 5th days, sometimes accompanied by petechia. The clinical manifestations of chikungunya fevers have to be distinguished from dengue fever, with which it often occurs.

Epidemics of fever, rash and arthritis, resembling chikungunya fever have been recorded as early as 1824 in India and elsewhere. Outbreaks of chikungunya fever have been subsequently detected in Africa and Asia. After a long period of absence, outbreaks of chikungunya fevers occurred in Indonesia in 1999. Recent upsurge of chikungunya fevers were noted in India especially in the states of Orissa, Maharashtra and Tamil Nadu. During 2006, more than 1.3 million cases of chikungunya fever were reported in India.

Three main laboratory tests are used for diagnosing chikungunya fevers: Virus isolation, serological tests and molecular technique of Polymerase Chain Reaction (PCR). Virus isolation is the most definitive test. Recently, a reverse transcriptase, RT-PCR technique for diagnosing CHIK virus has been developed using nested primer pairs amplifying specific components of three structural gene regions, Capside (C), Envelope E-2 and part of Envelope E1. PCR results can be available from within 1-2 days. Serologic diagnosis can be made by demonstration of four-fold increase in antibody in acute and convalescent sera or demonstrating IgM antibodies specific for the virus using ELISA.

75 Miscellaneous Viruses

RUBELLA

Rubella is plural of a Latin word *rubellus* which means red. This is also known as 'German measles'.

The disease gained wide importance when, on the basis of observations of McAlister Gregg—an Australian ophthalmologist, it was found that it was responsible for severe congenital malformations in children born to mothers who had rubella during the first trimester of pregnancy. These congenital deformities pertained to heart, brain, eyes, ears and low birth weight and high incidence of still births.

Classification

Rubella virus is a member of genus *Rubivirus* which is a nonarthropod-borne virus belonging to the family *Togaviridae*. No antigenic relationship has been found between rubella virus and 200 or more alphaviruses and flaviviruses.

Morphology

Rubella virus is an RNA virus with a diameter of 58 nm and a lipoprotein envelope. The nucleocapsid is 30 nm in diameter and contains a single stranded RNA which has a length of 10-11 kb. Though it has not been confirmed, the symmetry of rubella virus is believed to be icosahedral. The viral envelope is a three layered structure which has been derived from the host membrane. Because of non-rigid delicate structure of the envelope, the virus particles manifest pleomorphism. Elliptical and oblong virus particles have been seen. The envelope also bears ill-defined 5-6 nm surface projections which are composed of glycoproteins.

Chemically, the virion is composed of 75% proteins, 18.8% lipids, about 4% carbohydrates and 2.4% RNA. Apart from two major polypeptides designated E1 and E2, C polypeptide is present in the nucleocapsid.

The rubella virus is stable at 4ºC for more than 7 days and for fairly long time if kept at -70ºC. The stability is enhanced by the presence of proteins and magnesium sulphate. The virus is inactivated by lipid solvents. The virus remains stable at pH 6 to 8. It gets inactivated in ultraviolet rays within 40 seconds.

Antigenic Structure

Four types of antigens have been described in rubella virus. These are haemagglutinating antigen which is associated with the surface projections, complement fixing antigens, precipitating and platelet aggregating antigens. Rubella virus also demonstrates haemolytic activity.

Cytopathogenic Effects

Rubella virus does not produce CPE in all those cell lines in which it can grow. These include RK13, SIRC (rabbit cornea) and Vero cell lines. Whereas the virus produces CPE in RK13 and SIRC cell lines, interference is made use of in detecting the growth of virus in vero cell lines.

Pathogenesis

Pathogenesis of rubella in an adult is just like any other systemic exanthemata producing viral disease (Fig. 75.1). The foetus gets at risk whenever there is viraemia in the mother which results in placental infection also. At least two mechanisms are involved in producing foetal damage: a virus induced retardation in cell division and tissue necrosis. On infecting the cells, the rubella virus may damage the chromosomes

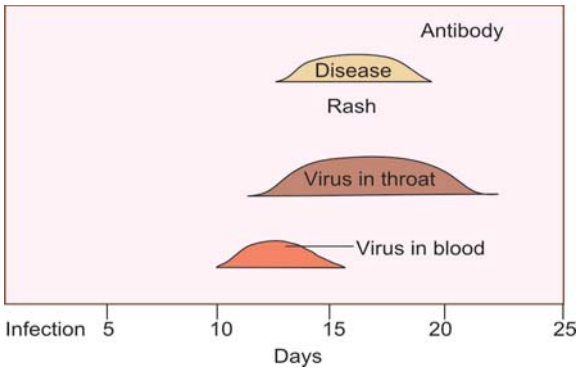


Fig. 75–1. Pathogenesis of rubella

of the host cell and result into its slow multiplication as compared to uninfected cells. When this retardation occurs during critical phase of organogenesis, it results into congenital malformation. Not all the pregnant women who contract rubella deliver congenitally deformed children. This may be due to the capacity of foetus to resist or limit the infection by a mechanism which is as yet unknown.

Clinical Features

After an incubation period of 14-21 days the characteristic features of rubella such as rash and lymphadenopathy appear. In young children, the symptoms appear suddenly. The rash is usually in the form of a pinpoint maculopapular lesion which appears first on face and subsequently on rest of the body. The patient may complain of pain in the lymph nodes before the appearance of rash.

Congenitally Acquired Rubella

The features of congenitally acquired rubella are variable. The permanent lesions seen in this syndrome are shown in Table 75.1.

Table 75–1. Clinical features of congenital rubella

- Microcephaly
- Retinopathy
- Cataract
- Deafness
- Microphthalmia
- Pulmonary stenosis
- Patent ductus arteriosus
- Ventricular septal defect
- Cryptorchidism
- Inguinal hernia
- Diabetes mellitus

A child suffering from rubella may be born with low birth weight, thrombocytopenia, hepatosplenomegaly and meningoencephalitis.

Laboratory Diagnosis

Serodiagnosis

Various serological tests (Table 75.2) are available to detect antibody against rubella virus for the purposes of diagnosis as well as seroepidemiological studies.

Table 75–2. Serological tests for rubella

- Single radial haemolysis
- ELISA
- Latex agglutination
- Passive haemagglutination
- Immunofluorescence
- Complement fixation test
- Radioimmunoassay
- Haemagglutination inhibition

For the detection of rubella specific IgM antibody, ELISA, RIA and haemadsorption inhibition test (HIT), which employs red blood cells for the detection of bound rubella antigen, have been used.

Virus Isolation

Virus isolation techniques are rarely used for the diagnosis of rubella. The virus produces CPE in RK13, and SIRC cell lines. The presence of virus can be confirmed by immunofluorescence.

Diagnosis of Congenital Rubella

The diagnosis of congenitally acquired rubella can be established by detection of rubella-specific IgM in serum samples obtained in early infancy; detection of rubella antibody after 6 months of age of infant by which time the maternal antibodies are presumed to have disappeared and by the isolation of rubella virus from the infant during early infancy.

Prevention

With the availability of potent vaccines made from RA27/3 strain of virus, incidence of rubella has been brought down appreciably. This vaccine is available in combined form with measles and mumps (MMR) antigens.

ROTAVIRUS

Rotaviruses are members of the genus *Rotavirus* within the family Rotaviridae. The family also contains genera of *Reovirus* and *Orbivirus*. The name rotavirus has been derived from the Latin word *Rota*, meaning wheel and reflecting its shape as seen under the electron microscope.

Morphology

Rotavirus exists in two forms both of which are spherical and measure about 70 and 60 nm in diameter. The large particles have smooth outer layer and are double shelled, whereas the smaller particles have rough surfaces and are single shelled.

The particles are composed of central RNA consisting of 11 segments of double stranded RNA, surrounded by a protein capsid (Fig. 75.2).

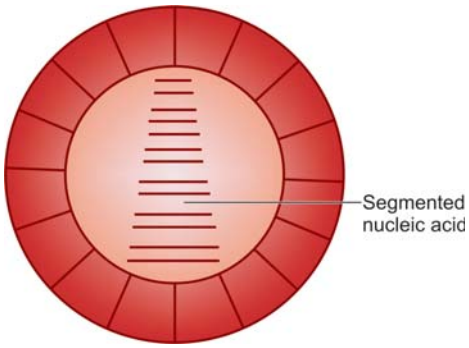


Fig. 75–2. Diagrammatic presentation of structure of rotavirus

The eleven segments (genes) have been found to perform diverse functions. These are located either in capsid or core (Table 75.3).

Table 75–3. Functions of genes of rotavirus

Segment RNA	*Protein*	*Localisation*	*Function*
1	VP1	Core	Transcription
2	VP2	Core	Polymerase
3	VP3	Core	Transcription
4	VP4	Outer capsid	Haemagglutination
			Pathogenicity
			Neutralisation specificity
5	VP5	Nonstructural	–
6	VP6	Inner capsid	Group specificity Ab
7	VP8	Nonstructural	–
8	VP7/VP8	Outer capsid	–
9	VP7	–	–
10	VP10	Nonstructural	–
11	VP11	Outer capsid	–

The virus particles contain at least two glycoproteins of which one is type specific and has neutralising specificity.

Antigenic Structure

Genomic and antigenic analysis of rotavirus have revealed four serotypes (designated 1-4) and two subgroups (I, and II) all of which possess a common group specific antigen. Recently, however, new rotavirus types (*pararota*) have been discovered which are morphologically identical to the established rotavirus, but do not share the common antigen and are consequently not detectable by immunological methods.

Sensitivity to Physical and Chemical Agents

Rotavirus is inactivated by heating at 100°C or treatment with acid (pH<3), glutaraldehyde (3%) or alcohol (>70%), while iodoforms are less active against this virus.

Pathogenesis

The pathogenesis of rotavirus infection is illustrated in Figure 75.3.

Clinical Features

After an incubation period of 2-3 days the illness begins with nausea and vomiting. Fever is commonly present and may be of high-grade. In children febrile convulsions may also accompany diarrhoea.

Dehydration is most often isotonic, but may sometimes be hypotonic. The illness lasts for 4-7 days and only in rare cases may be protracted. Many rotavirus infections remain asymptomatic.

Laboratory Diagnosis

Specimen of faeces is obtained during acute phase of illness and transported without any additive or refrigeration. Several rapid methods are now available to detect the virus or viral antigen in stools. These include:

- Electron microscopy
- ELISA
- Latex agglutination

Human rotaviruses can be cultured in few special cell lines only with great difficulties. Similarly, serological tests have not been refined to the extent to be used in routine diagnosis.

Treatment and Prophylaxis

There is no specific therapy and treatment appropriate to cases of acute gastroenteritis is given with particular

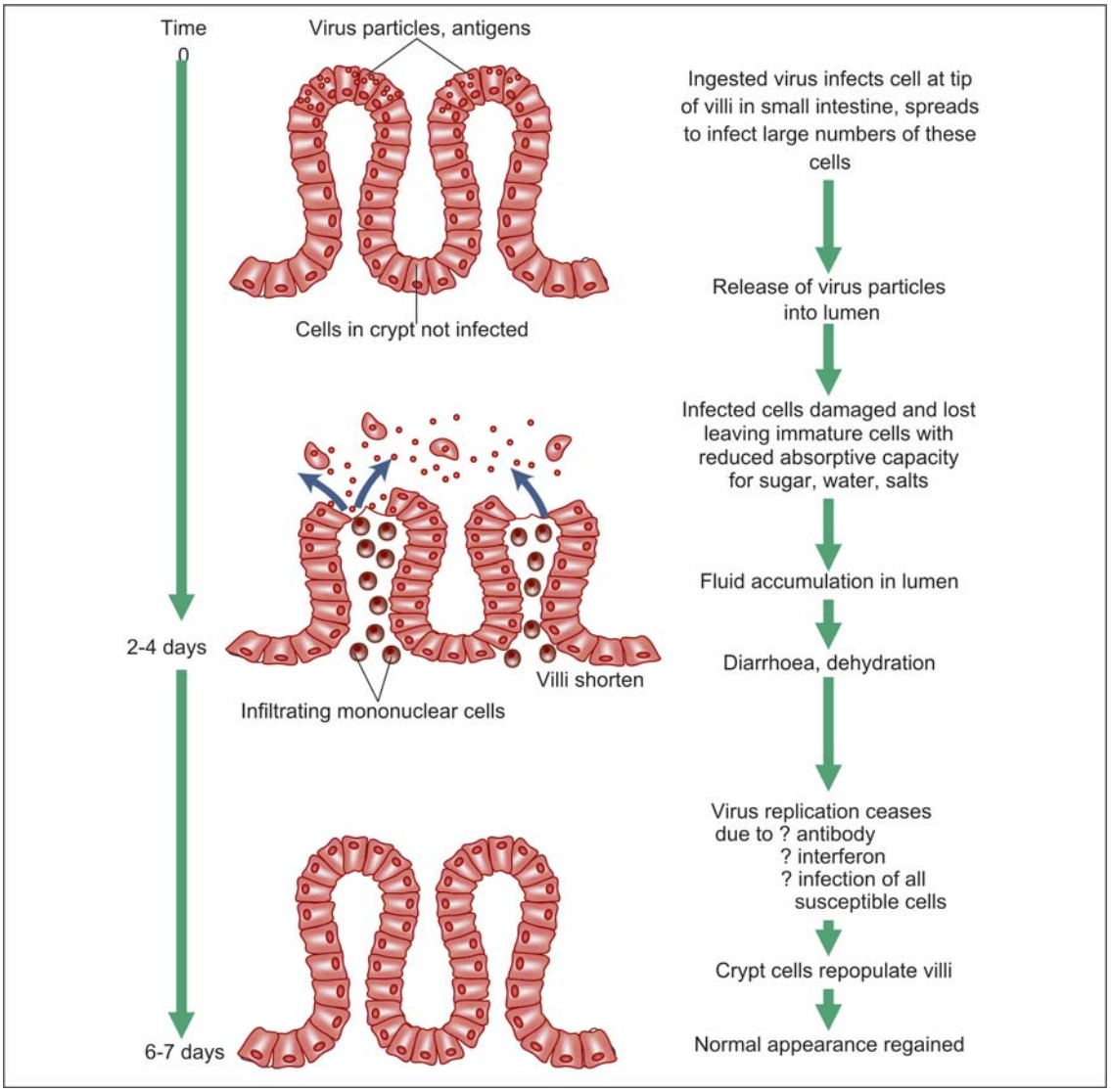


Fig. 75–3. Pathogenesis of rotavirus diarrhoea

emphasis on oral rehydration with glucose-electrolyte solution. No vaccine is currently available though some vaccines have gone to trial stage.

CORONAVIRIDAE

The coronaviruses have been put together in one genus which also constitutes the family Coronaviridae. The coronaviruses are better known after the name of the species in which they produce infection. Thus, coronaviruses causing respiratory infection in humans are known as human coronavirus. These are being described hereunder in brief.

Morphology

The virus is spherical with a diameter in the range of 80-200 nm. Pleomorphism is exhibited by the virion because of the flexibility of the envelope. The surface is covered with projections which have a size of 10-20 nm. These surface projections give the appearance of a halo around the virus which gives it the name *corona*. A single stranded RNA comprises the genome of the virus.

Polypeptide and Antigens

Most coronaviruses comprise of three to five structural proteins in addition to a nucleoprotein which is

associated with the genomic RNA. Two species of glycoproteins are located in the envelope. All the structural proteins of coronaviruses are antigenic.

Clinical Features

Clinical picture produced by coronaviruses resembles that of rhinoviruses with the difference that whereas incubation period in rhinovirus infection is shorter, the duration of illness is longer in coronavirus infection. The proportion of common cold that can be associated with coronaviruses is in the range of 2-10%. The role of coronaviruses in causing lower respiratory tract infections is not yet defined.

Laboratory Diagnosis

The laboratory diagnosis can be on the basis of isolation and identification of coronavirus as well as detection of antigen or antibody by serological techniques.

The best isolation of coronaviruses has been seen in organ cultures derived from human embryonic trachea (HETOC). Though various cell lines have also been defined, none can be recommended for the isolation of all coronaviruses.

Antibodies to these viruses can be detected in the serum by virus neutralisation, complement fixation test, indirect haemagglutination, immune adherence, haemagglutination, radial haemolysis and ELISA. The viral antigen can be detected with immunofluorescence and ELISA techniques.

Prophylaxis and Treatment

No successful vaccination has resulted against coronaviruses because of the antigenic variation in the serotypes of coronaviruses and failure of antibodies to protect against the infection. The use of antiviral chemotherapy has also been unproductive so far.

SARS-CoV

During 2003 the world was hit by a fast spreading virus that was transmitted through droplets and attacked those who came in close contact with the patients of this new clinical syndrome called as severe acute respiratory syndrome (SARS). The clinical picture was exemplified by atypical pneumonia and fever for which no other cause could be demonstrated. A coronavirus which had not hitherto affected human population was isolated from these patients and designated as SARS-CoV. The virus could be detected by PCR using specific primers. Antibody detection test kits based upon ELISA and IFAT techniques have also been developed. Use of standard precautions while handling the patients or their biological material drastically cuts short the transmission of this infection.

ARENAVIRIDAE

The family *Arenaviridae* derives its name from the Latin word *Arena* which means sand. The electron microscopic picture of the members of this family is like that of sprinkled sand and hence the name. At present there are 14 viruses in this family and with the exception of lymphocytic choriomeningitis virus (LCM) are referred to by names that reflect geographical areas from where the viruses are isolated. Almost all these viruses have rodents as their natural hosts.

Morphology

Arenaviruses exhibit pleomorphism and particles ranging in diameter from 80-150 nm are seen under the electron microscope. The virus envelope arises from the plasma membrane of the infected cell. Little is known about the internal structure of these viruses.

The genome of arenaviruses consists of two single stranded RNA segments of different sizes, designated as L and S. The L RNA strain represents about 70% of the viral genome. The S strain codes for the nucleoprotein and the envelope glycoprotein.

All arenaviruses contain a major nucleocapsid associated protein and one or two glycoproteins in the viral envelope.

Antigenic Relationship and Pathogenesis

All arenaviruses are antigenically related. Antibody to type specific envelope protein is best detected by immunofluorescence which has now become the method of choice for the diagnosis of arenaviruses. To differentiate between various serotypes monoclonal antibodies are being used.

The mechanism by which arenaviruses cause disease in man is not fully understood. It appears to be because of direct damage to the cell and not by either immunological or allergenic processes. Following four are important diseases caused by arenaviruses in man.

- Lymphocytic choriomeningitis
- Argentinian haemorrhagic fever
- Bolivian haemorrhagic fever
- Lassa fever

LYMPHOCYTIC CHORIOMENINGITIS

Man gets this infection from rodents, mice and hamsters either in nature or more commonly in the laboratory. Though the exact mode of transmission is not known it is believed to be direct contact with urine or contamination of food by the body fluids of the animals.

In a majority of infections, the disease remains inapparent. In few cases it may manifest as an influenza-like illness, aseptic meningitis or severe meningoencephalomyelitis. Death may follow meningoencephalomyelitis.

ARGENTINIAN HAEMORRHAGIC FEVER

The disease is caused by Junin virus which was isolated for the first time in 1958. The disease occurs in small outbreaks with a mortality range of 3-15%.

This disease has an insiduous onset and clinical features include headache, malaise, retrobulbar pain, fever, oedema of face, neck and upper thorax. Generalised lymphadenopathy may be followed by hypotension, oliguria and haemorrhages from gums and nose as well as other body openings. Leucopenia and thrombocytopenia also accompany the clinical features. Hypovolumia and shock may progress to death. The disease is restricted to Argentina.

BOLIVIAN HAEMORRHAGIC FEVER

This disease is caused by Machupo virus. It was reported for the first time in Bolivia in 1959. The incidence has decreased since 1970s and now very few cases of human infection are reported. The mortality had ranged between 5-30%. Clinically the disease resembles Argentinan haemorrhagic fever.

LASSA FEVER

This is caused by Lassa virus which has established itself in West Africa especially Nigeria and Liberia. Ever since its first isolation in 1969, more than 20 outbreaks have taken place with an average mortality of 27%.

The disease may remain inapparent or may manifest as a fulminant fatal infection. The clinical features include:

- High fever
- Prostration
- Chest and abdominal pain
- Sore throat with white membrane
- Dehydration
- Hypotension
- Conjunctivitis
- Faint rashes
- Haemorrhages
- Hair loss and deafness.

The disease lasts for upto three weeks after which there is a gradual recovery. Patient complains of weakness and fatigue till few weeks after recovery.

Diagnosis

Lassa virus can be grown in Vero cells from specimens of serum, throat washings, pleural fluid and urine. Direct demonstration of antigen is possible by immunofluorescence in conjunctival cells. Detection of specific antibodies is also possible with indirect immunofluorescence during the second week of illness.

Treatment

Administration of Lassa immune plasma has benefitted a few, but not all the patients. Some success has also been reported with ribavirin therapy.

FILOVIRUSES (EBOLA AND MARBURG VIRUS)

Marburg (MBG) and Ebola haemorrhagic fevers are caused by taxonomically distinct viruses that form two groups within genus Filovirus in family Filoviridae. MBG was first recognised in 1967 when 25 persons in Germany and Yugoslavia became infected following contact with monkey kidneys imported from Uganda. Seven of these died. Subsequently similar cases have been reported from Zimbabwe, South Africa and Kenya.

Ebola virus first emerged in two major disease outbreaks in Zaire and Sudan. Since the isolates from two areas were serologically distinguishable they were labelled as EBO-Z and EBO-S. A third type could be isolated from an epizootic in monkeys imported from Philippines in 1989 which were held in quarantine in Reston, USA. The third type has been called as EBO-R.

During 1996 and 1997, large number of cases of Ebola haemorrhagic fever occurred in South Africa. Other countries which have been hit by this virus are Zaire, Gabon, Nigeria, Liberia, Ivory Coast, Cameroon and Kenya.

Causative Viruses

MBG and Ebola viruses are RNA, nonsegmented, negative sense, single stranded viruses. The infectivity is associated with uniformly sized bacilliform particles 790 nm in length for MBG virus and 970 nm for Ebola virus. Each infectious particle contains a nucleocapsid

with a 20- or 30- nm central axis surrounded by a helical capsid 40-50 nm in diameter. Both viruses produce seven structural proteins.

Clinical Features

After an incubation period of 4-16 days, these viruses induce sudden fever, chills, anorexia, headache, abdominal pain, and diarrhoea. A rash appears within seven days and haemorrhagic spots appear over trunk and mucous membranes. Gastrointestinal tract bleeding is common and often profuse to lead to shock and death. Profound leucopenia and thrombocytopenia are observed.

Diagnosis

Direct immunofluorescence staining and IFA staining of impression smears or air-dried suspensions of liver, spleen and kidney are useful in detecting cytoplasmic inclusion bodies associated with MBG and Lassa virus.

Serological diagnosis has also been found to be useful with the help of IgG and IgM ELISA as well as indirect immunofluorescence methods.

Methods of Control

No vaccine and no specific treatment available as yet for either Ebola or Marburg.

PART XII
MYCOTIC INFECTIONS

76
Medical Mycology

Introduction

Medical mycology is the study of human diseases caused by fungi and **mycosis** (plural: *mycoses*) is the term given to any disease caused by a fungus. Further characterisation of a fungal disease is made by the use of a prefix which is derived either from the part of the body affected (e.g. nail-*onchyo*mycosis, skin-*dermato*-mycosis, etc.) or from the name of pathogen involved (e.g. *coccidioido*mycosis). Alternatively, the disease names are often coined by addition of the suffix *osis* to the generic name of the pathogen (e.g. aspergillosis, histoplasmosis, cryptococcosis, etc.). Sometimes the disease names bear a geographical connotation as in the case of South American Blastomycosis, North American Blastomycosis, etc.

Natural Habitat

The natural habitats of most of the fungi which cause systemic mycoses are in organic wastes or debris or in soil enriched by organic wastes.

Differences from Bacteria

Fungi differ from bacteria in a number of ways (Table 76.1).

The term fungus is a general term encompassing such diverse forms as molds and yeasts. The yeasts are oval spherical or elongated cells which reproduce by budding (Fig. 76.1) and which form moist or mucoid colonies. The molds are characterised by tubular branching cells which constitute a hypha. The hyphae are

Table 76–1. Differences of fungi from bacteria

- Greater size
- Complex morphological development; may be unicellular or multicellular
- Possess rigid cell-wall which contains chitin, mannan and other polysaccharides
- Sterols in the cytoplasmic membrane
- True nuclei with nuclear membrane and paired chromosomes
- Reproduction can be asexual or sexual or by both these modes

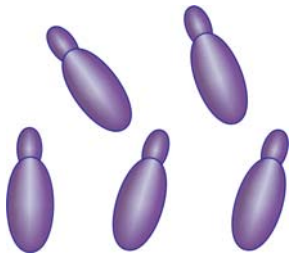


Fig. 76–1. Yeast cells

divided in most fungi by cross walls called *septa* into multicellular hyphae (Fig. 76.2). Branching, intermingled and often fused or intercommunicating hyphae constitute mycelium (Fig. 76.3) which forms the visible, usually dry colony of mold observed on natural substrates or on culture media.

Some fungi do not develop septa and have non-septate hyphae (also called *coenocytic hyphae*) which allow the protoplasm to flow uninterrupted throughout the hollow tube (Fig. 76.4).

The terms yeast and mold are not mutually exclusive. Many of the fungal pathogens of man are dimor-

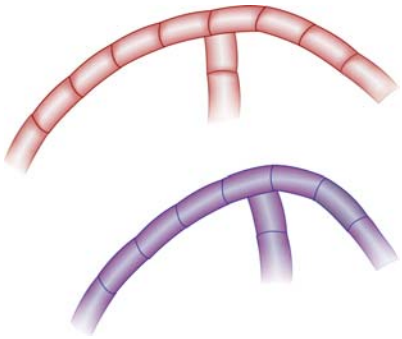


Fig. 76–2. Multiseptate hyphae

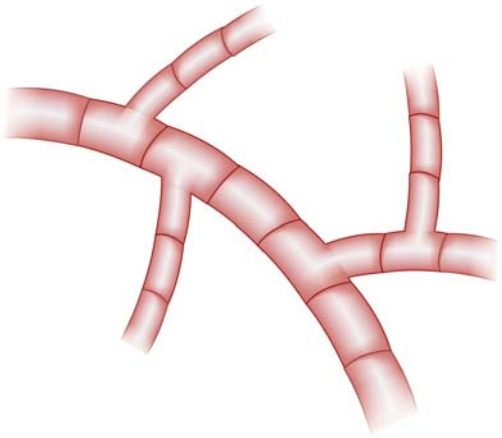


Fig. 76–3. Mycelium

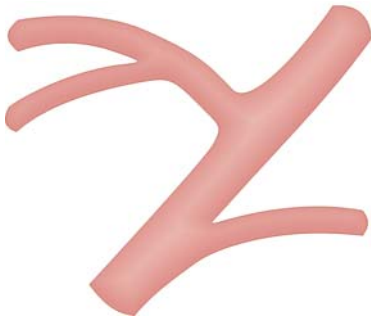


Fig. 76–4. Aseptate hyphae

phic, i.e. they are molds in their normal saprobic growth but are yeasts or yeast-like in animal tissues or when incubated on enriched media at 37°C.

Classification of Fungi

The classification of the fungi along with examples is given in Figure 76.5.

SPORE FORMATION IN FUNGI

Both sexual and asexual spores are formed in fungi. Various *sexual spores* are:

Zygospores

In certain zygomycetes, the tips of approximating hyphae fuse, meiosis occurs and large thick walled zygospores develop (Fig. 76.6).

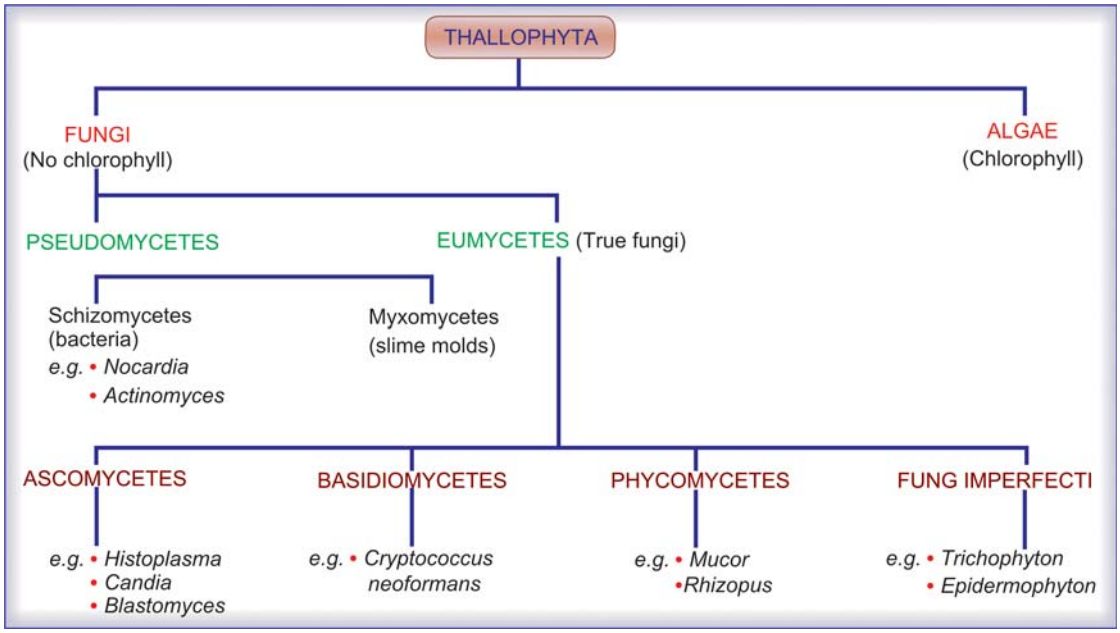


Fig. 76–5. Classification of fungi

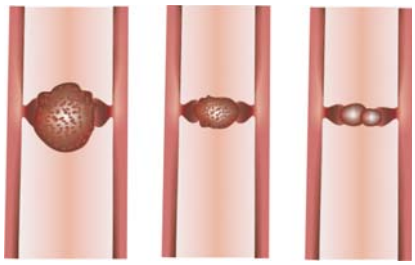


Fig. 76–6. Zygospores

Ascospores

Usually 4-8 spores form on the surface of a specialised cell called as ascus in which meiosis has taken place (Fig. 76.7).

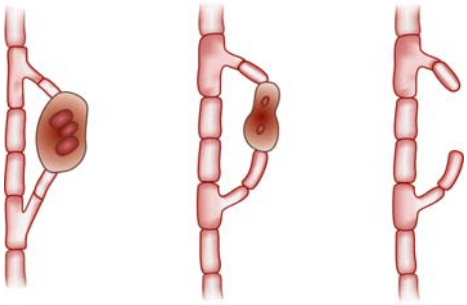


Fig. 76–7. Ascospores

Basidiospores

Following meiosis, four spores usually form on the surface of the specialised cell called as basidium (Fig. 76.8).

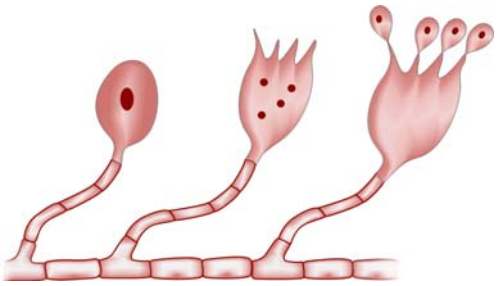


Fig. 76–8. Basidiospores

The asexual spores in fungi imperfecti are: *thallospores* which include arthrospores, blastospores and chlamydospores and conidiospores.

Arthrospores

In this type of spores the segmentation of the septate hyphae by constriction or disarticulation results in rectangular thick walled spore formation (Fig. 76.9) e.g. *Geotrichum.*

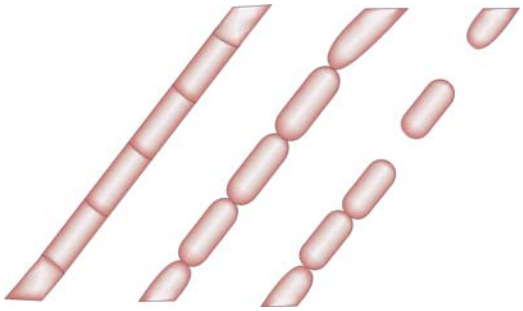


Fig. 76–9. Arthrospores

Blastospores

These are formed in yeasts by budding of one cell from a parent cell (Fig. 76.10), e.g. in *Cryptococcus* and *Candida.*

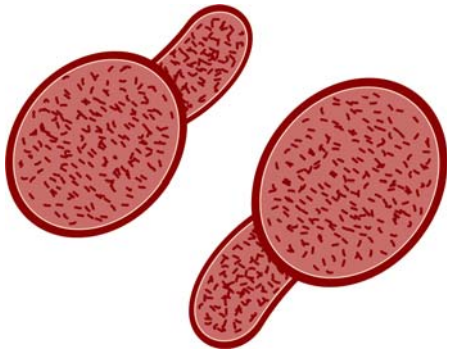


Fig. 76–10. Blastospores

Chlamydospores

These are large thick walled resting spores developed from hyphae for existence during long periods of dormancy. These could be (Fig. 76.11):

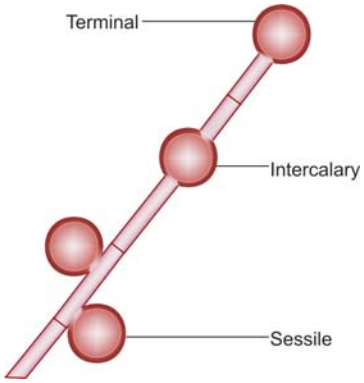


Fig. 76–11. Chlamydospores

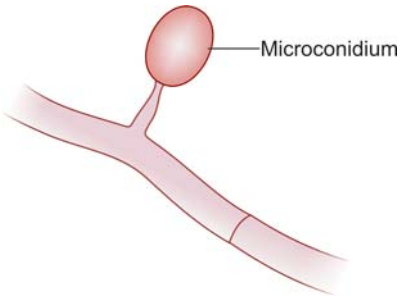


Fig. 76–12. Microconidia

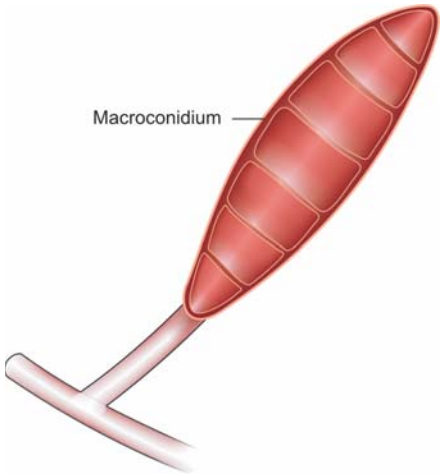


Fig. 76–13. Macroconidia

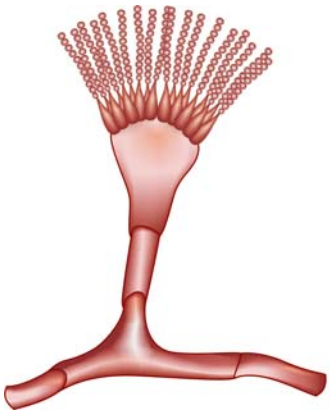


Fig. 76–14. *Aspergillus* species

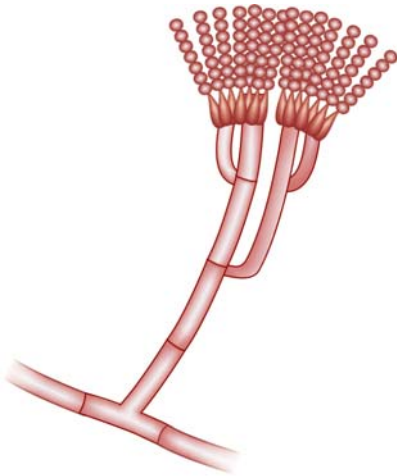


Fig. 76–15. *Penicillium species*

a. Intercalary (interposed between the septa),
b. Terminal (at the end of hyphae), and
c. Lateral (on the side of hyphae).

Conidiospores

These are spores borne externally on hyphae or specialised hyphal branches called *condiophores*. The spores produced (conidium—plural *conidia*) vary in size, shape, number, number of septations and pigmentation. Microconidia are small, single conidia (Fig. 76.12). Macroconidia are large conidia that often are multicellular (Fig. 76.13).

Aspergillus spp is characterised by a swollen end of the conidiophore called a *vesicle*, over its surface arise flask-shaped structures called *sterigmata*, from the tips of which the spores arise in long chains by cutting off of successive conidia (Fig. 76.14). As the youngest spore is near the sterigmata, there is no branching of the chain and it is said to form *basipetally*. When the spore near the sterigmata is oldest, it is called as *acropetal* mode of sporulation.

In *Penicillium* conidiophore gets divided into numerous branches each with a sterigmatum that produces a chain of spores giving a brush-like appearance to the organism (Fig. 76.15).

If conidia are produced by the conidiophores by budding and each end in turn buds, a chain will be produced if no detachment takes place. The most proximal spore in this case is the oldest and the chains developed by this method are formed *acropetally*. This is seen in *Hormodendrum* species.

Asexual spores in phycomycetes are produced inside a swollen structure called *sporangium* (plural: sporangia) by formation of cleavage planes. They develop on the ends of branches or hyphae called *sporangiophores*. The spores are *sporangiospores*. These are released by rupture or dissolution of the sporangium (Fig. 76.16).

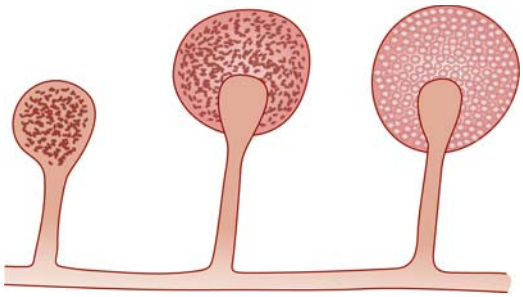


Fig. 76–16. Sporangiospores

ECONOMIC IMPORTANCE OF FUNGI

Useful

1. Fungi exist either as *saprobes* or *parasites*. Their preeminent ability to break down complex organic substrates of almost every type is an important and essential activity in the recycling of carbon and other elements in the cycle of life.
2. Edible wild or domesticated varieties of mushrooms (*Basidiomycetes*) are important as food sources.
3. Fungi have been used to alter the texture, improve the flavour, increase the palatability and digestibility of natural or processed foods. *Penicillium* species has been used for ripening of certain varieties of cheese.
4. The yeasts are used for the fermentation purposes in production of beverages and juices as well as brewing and baking.
5. Products of fermentation yield industrial alcohol, fats and proteins. A mold (*Fusarium*) can produce within 48 hours, 12-15 grams of fat from a litre of 50% glucose solution.
6. Molds (such as *Aspergillus* species) are used in the production of citric, oxalic, gluconic and itaconic acid.
7. Actinomycetes and fungi are important sources of antibiotics such as penicillin, amphotericin B, adriamycin and bleomycin, etc.
8. Ergot which is used for inducing uterine contractions, controlling bleeding and treatment of migraine is a fungal product. Ergot is the resistant over-wintering stage of *Claviceps purpurea*—a fungal pathogen of rye which transforms the seed of that plant into a compact spur-like mass of pseudohyphae.

Harmful

1. Molds can cause deterioration of fabrics, leather, electrical insulation and other manufactured goods. Extensive losses may follow failure to protect material from ravages of fungi in warm humid climates.
2. Fungi can spoil the agricultural produce, if improperly stored. These also destroy vegetables, fruits and cereals.
3. Mycotoxicoses (ingestion of toxins of fungal origin) and mycetismus (mushroom poisoning—through ingestion of fungal elements) are becoming increasingly a public health problem in tropical countries such as India.

MYCOSES

Mycoses *(diseases caused by fungi)* can be divided into four broad categories (Fig. 76.17).

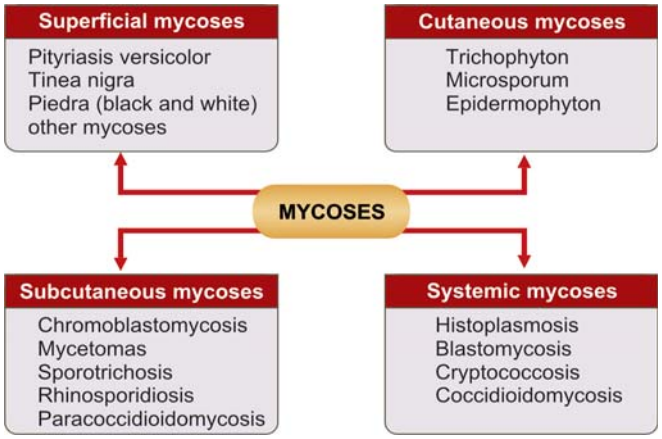


Fig. 76–17. Categories of mycoses

Mycoses can also be grouped under two heads:

Superficial mycoses. This includes the infections of skin and appendages.

Deep mycoses. These include the subcutaneous and systemic mycoses which constitute the infections of deeper tissues and visceral organs.

TINEA VERSICOLOR

Synonyms. Pityriasis versicolor, Tinea flava, Dermatomycosis furfuracea, Liver spots.

Definition

Tinea versicolor is a superficial infection of the horny layer of the epidermis characterised by white, brown or fawn coloured superficial lesions. The lesions are usually non-inflammatory, covered with thin branny or furfuraceous scales and usually sharply marginated. These are mainly present on the chest and occasionally on arms, thighs, groin, neck, axillae and face.

Causative Agent

Malassezia furfur (also called as *Pityrosporum furfur*).

Clinical Features

The lesions vary in colour depending upon the skin pigmentation and also the severity of the infection. The fungus interferes with the normal pigmentation and the lesions are lighter in colour than the surrounding skin. Infections are usually asymptomatic and patient seeks medical help for cosmetic reasons.

Laboratory Diagnosis

In typical cases the diagnosis can be made on clinical findings. The laboratory can confirm the diagnosis in following ways:

Examination of Infected Material

The fawn or brown patches are scraped with a scalpel and collected on a slide or in a container for examination. The scales are placed in a drop of 10% KOH, heated gently and examined microscopically. The organisms appear as clusters of thick-walled, round cells, 3-8 µm in diameter. Some may be in budding forms. Numerous short, straight or angular hyphae may surround the clusters (Fig. 76.18).

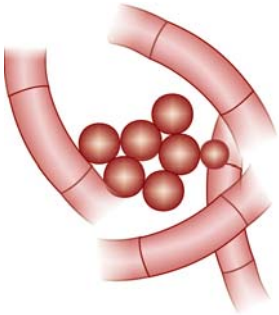


Fig. 76–18. Malassezia furfur

Culture

Routine cultures are not made as many a times growth of organism is not obtained.

Treatment

Thorough scrubbing, followed by application of mild fungicidal and keratolytic ointment will cure the infection. Clothing should be boiled to prevent reinfection. Sodium hyposulphite (20% aqueous solution) and mild sulfur ointments are also effective.

WHITE PIEDRA (PIEDRA ALBA) (Beigel's disease, Chignon disease)

White piedra is a fungal infection of the hair of scalp and beard, moustache and sometimes pubis. This is characterised by soft greyish-white nodules of variable consistency arranged in rows on the hair shaft to which they are adherent. It is caused by *Trichosporum beigelli* which is a filamentous yeast that forms arthrospores and blastospores, hyphae and pseudohyphae.

Trichosporum beigelli penetrates between the cells of the cuticle but does not invade the hair shaft itself. The fungus forms irregular small nodules, 1-1.5 mm in length, whitish-yellow or reddish-brown in colour and translucent with soft or semi-soft consistency. These nodules are firmly adherent to the hairs, particularly the midshafts. Most frequently young adults of both sexes are involved. Diabetic glycosuria appears to be a predisposing factor in the development of pubic lesions.

White piedra is chronic, benign and readily curable.

Diagnosis

The diagnosis is made by observing the presence of irregular, white, soft nodules along the hair. The nodules when pressed in a clearing solution between the slide and cover slip, show that the fungus penetrates between the cells of cuticle which it raises by means of septate hyphae between 2-4 µm in diameter. The hyphae disintegrate into rectangular, oval or round arthrospores which cluster together.

Treatment

Shaving, if possible, is the simplest method of treatment. If it is not possible various topical agents such as 5% salicylic acid, 1% iodine or imidazole derivatives can be used.

BLACK PIEDRA (PIEDRA NIGRA)

This disease of hair is characterised by the appearance of dark brown or black nodules which are gritty and adhere to the distal third of the hair.

Causative Agent

Trichosporum hortae

Clinical Features

The disease is localised to the scalp and appears in the form of small nodules, 1-2 mm in diameter which adhere to the hair, particularly to its distal third. They are spindle shaped or conical, very hard, blackish brown

in colour and enclose the hair in a sheath of variable density.

Mode of Transmission

The infection is spread by the common use of combs, hair brushes or utensils used for washing the hair.

Diagnosis

Examination of the nodules in clearing solution shows accumulation of mycelial filaments, forced into the shape of polyhedral cells.

Treatment

It is same as for white piedra.

DERMATOPHYTES

The dermatophytes are a group of closely related filamentous fungi that infect only superficial keratinised tissues, i.e. skin, hair and nails. The clinical picture produced by them is collectively called as *dermatophytoses* or *Ringworm* or *Tinea*. The term tinea (moth) was originally used to describe the dermatophyte infections that gave the hair of the head a moth eaten appearance.

Classification

The dermatophytes have been classified into three genera:

- *Trichophyton*: infects hair, skin and nails
- *Microsporum*: infects hair and skin
- *Epidermophyton*: infects skin and nails

There are 48 species of dermatophytes which are known to cause infections in man. Of these 25 belong to the genus *Trichophyton* and 22 to *Microsporum*. The genus *Epidermophyton* contains only one species.

Habitat

The dermatophytes may infect humans (*anthropophilic*), animals (*zoophilic*) or grow in soil (*geophilic*). The examples of these are given in Table 76.2.

Trichophyton

This genus produces more microconidia than macroconidia. The macroconidia vary in shape from that of a cigar to a cylinder; size being 8-50 µm × 4-8 µm (Fig. 76.19). The walls are smooth. These spores occur in chains and are localised inside the hair shafts. *Trichophyton rubrum* is the most common species that infects man.

Table 76–2. Habitat of dermatophytes

ANTHROPOPHILIC
• *Microsporum audouinii*
• *Epidermophyton floccosum*
• *Trichophyton rubrum*
• *T. violaceum*
• *T. tonsurans*
ZOOPHILIC
• *Microsporum canis*
• *Microsporum nanum*
• *Trichophyton mentagrophyte*
• *Trichophyton verrucosum*
GEOPHILIC
• *Microsporum gypseum*
• *Trichophyton ajelloi*

Fig. 76–19. Macroconidia of Trichophyton

Epidermophyton

It produces smooth, thick walled, large macroconidia that are usually composed of 2-4 cells. They are usually abundant with a size of 20-40 µm × 6-8 µm (Fig. 76.20). These occur in clusters of 2-3 and there are no microconidia.

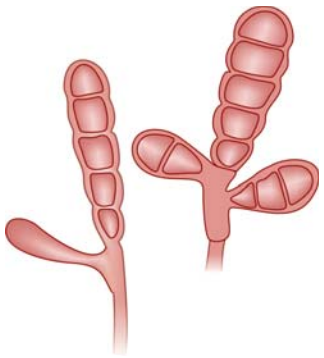


Fig. 76–20. Macroconidia of Epidermophyton

Microsporum

The number of macroconidia produced is variable. They are spindle shaped with thick roughened walls. Each

macroconidium may be divided into 5-10 cells and can be upto 100 µm long and 6-8 µm wide (Fig. 76.21). Mycelial filaments occur within the hair shaft (endothrix type) while spores occur on the outside (ectothrix type).

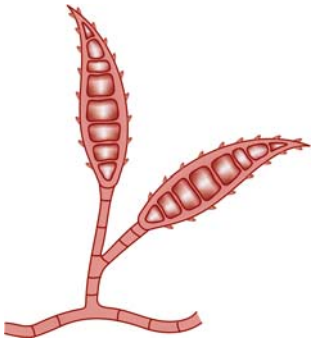


Fig. 76–21. Macroconidia of Microsporum

Clinical Features

A single species of dermatophyte may be involved in several disease types, each with a distinct pathology. Conversely, the same clinical entity may be caused by several species of dermatophytes. The clinical diseases caused by dermatophytes are well defined and are usually named after the portion of the body affected by the disease. These are:

- Tinea capitis : scalp
- Tinea corporis : body
- Tinea cruris : groin
- Tinea pedis : foot
- Tinea barbae : beard
- Tinea manum : hand

How does infection take place?

Human to human transmission is the rule for anthropophilic fungi. Transfer may be by direct contact but more often it occurs through the agency of an infected object on which dermatophytes may survive for months. Human beings are usually infected with zoophilic fungi from an animal source either by direct contact or through infected hairs left on furniture by the animal. A number of infections also originate from earth which is the natural reservoir of some of these species.

Who is likely to be infected?

Dermatophytes coming in contact with human skin do not automatically produce a dermatophyte infection. If the skin is intact, it offers a natural resistance. Development of disease depends upon the nature and size of inoculum and the reactivity of the individual.

Various factors which predispose to infection include:

- Diabetes
- Peripheral circulatory disorders
- Maceration of creases and folds of skin
- Obesity
- Poor hygiene

The salient features of some of the dermatophytoses are given in Table 76.3.

Pathogenicity

As a rule anthropophilic dermatophytes remain in the keratin of the horny layer, nails, scalp and body hair without provoking any serious inflammatory reaction in the dermis or epidermis, thus eliciting neither allergic reaction nor immunity. Intracutaneous reaction to an extract of *Trichophyton* (*trichophytin*) will, therefore, be negative and reinfection may occur.

Collection of Samples

Infected hair, nails or skin are collected for both microscopic as well as culture examination. Sufficient quantities are collected with sterilised instruments and kept in a clean container. Specimens collected are processed as early as possible, otherwise it may result in overgrowth with bacteria and other rapidly growing saprophytic fungi which make the recovery of pathogen very difficult.

Skin scrapings are collected by thoroughly sponging the infected area with ethanol and taking the scrapings from the active border areas of lesion with the help of sterile scalpel. Scrapings are kept in sterile petri dish or between two clean microscopic slides and transported to the laboratory.

Several scrapings are placed in a drop of 10% KOH and covered with a cover glass. The mount is gently warmed by passing it over the flame several times. Slide is examined under the microscope to detect fungal elements (Fig. 76.22). Negative slides are not discarded immediately but kept in a moist chamber for few hours and reexamined. Fungal elements sometimes become more obvious and clear due to the action of KOH after few hours.

The patient with scalp hair infection can be routinely examined under Wood's lamp (an ultraviolet lamp which emits radiations at 360B). The dermatophyte infected hair fluoresce under this lamp. Such hair are easily collected for examination. Hair can also be collected by scraping the lesion with sterile scalpel as

Table 76–3. Salient features of dermatophytoses

Organism	*Colonial Appearance*	*Microscopic Appearance*
Epidermophyton floccosum	Mustard yellow colonies Flat surface with radial folding White, cotton tufts may appear on surface. Reverse of colony is deep orange	Club shaped, 2-4 septate conidia Conidia present singly or in groups of 2-3 No macroconidia Smooth and thick cell walls
Microsporum audouinii	Cream or light brown colony Flat, velvety and raised in centre Reverse colony is orange	Terminal swollen cells (+/-) Microconidia usually formed Macroconidia, if formed, are large, thick and irregular
Microsporum canis	White colonies with yellow edge Silky, become cottony with irregular tufts	Spindle shaped macroconidia Thick outer and thin inner walls, Unicellular, clavate and smooth walled microconidia
Microsporum fulvum	Flat, brown colony. Powdery velvety, reverse colony is rosy	Macroconidia and microconidia similar to *M. gypseum*
Trichophyton mentagrophyte	Flat, velvety or powdery colony White to creamy. Reverse is white to reddish brown	Microconidia are round, Unicellular and hyaline; Occur in clusters, along the hyphae. Macroconidia absent
T. rubrum	White, granular or cottony colonies Reverse of colony is brown or red	Clavate microconidia Macroconidia absent
T. schoenleinii	White, raised and folded colonies Leathery, granular to velvety colonies	Conidia absent Favic chandeliers common Numerous chlamydoconidia present
T. tonsurans	Flat, compact and granular colonies Cream or yellow or rose, buff and brown Reverse colony yellow to red	Numerous microconidia Microconidia at right angle to hyphae Macroconidia rare
T. verrucosum	Dull white, grey or yellow surface Glaborous, waxy to powdery Aerial hyphae in old colonies	Chains of chlamydoconidia Microconidia present alongside of hyphae Macroconidia rare
T. violaceum	Heaped and folded colonies Smooth, waxy or velvety surface Deep purple, diffusible reverse pigment	Micro- and macroconidia rare Thiamine may stimulate conidia production

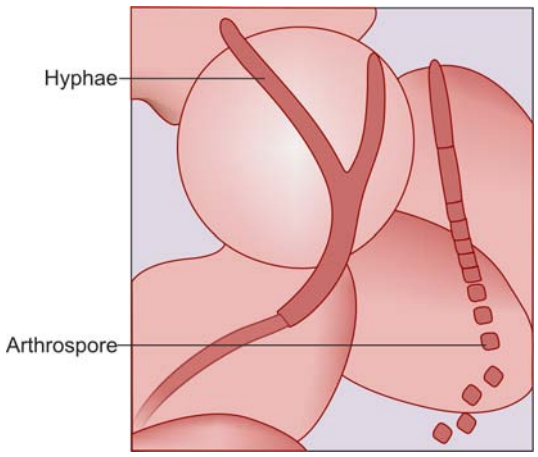


Fig. 76–22. Fungal hyphae in KOH

well as by pulling the stubs of broken hair with tweezer. Ectothrix and endothrix appearance can be seen under the microscope (Fig. 76.23).

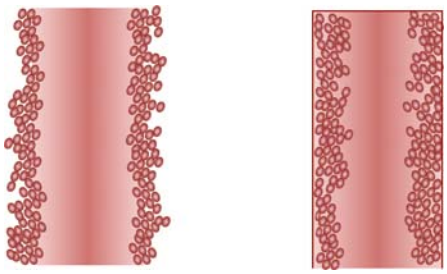


Fig. 76–23. Ectothrix and endothrix

The scrapings or clippings from infected nails can easily be collected. These are collected by shaving nails that have been cleaned with 70% ethanol. The scrapings are taken from the proximal to the distal end of the nail.

Direct Microscopic Examination

The mounted specimen is examined under low and high power of the microscope for septate hyphae and arthrospores. The hyphae appear as slightly greenish, branching threads running across the outlines of the colourless cells of the skin or nail. The size of the arthrospores in hair and their position within or outside the hair shaft may allow a presumptive diagnosis of species.

Culture

Species identification is possible on cultural characters. It is carried out by implanting fragments of specimen on Sabourad's medium containing chloramphenicol and cycloheximide at pH 6.5. The culture is incubated at 27-30°C and examined frequently for colonies. Colonies may become visible in 2-3 days but some species may take 2-3 weeks.

Serological tests are generally not useful in establishing laboratory diagnosis of dermatophytosis.

Treatment

Oral griseofulvin is the treatment of choice. Topical ointments and lotions containing clotrimazole and tolnaftate are effective.

DERMATOPHYTIDS (ID REACTION)

Synonyms: Mycids, favids, trichophytids, microsporide, epidermophytids.

Definition

This is an allergic reaction of varied morphology and occurs secondary to an inflammatory dermatophytic infection on an area far from the sites of fungal infection. These lesions (dermatophytids) do not contain any fungus.

Causative Agents

In general, the fungi retrieved from the primary lesion are those that provoke a marked inflammatory reaction, namely the zoophilic and geophilic dermatophytes. Anthropophilic dermatophytes are much less commonly involved.

Pathogenesis

The inflammatory reaction brings about large scale destruction of the dermatophytes. Antigen fractions are thus released and sensitize the host, leading to the appearance of dermatophytids.

Clinical Features

Dermatophytids are of different clinical types that may be seen simultaneously in the same patient. General systemic manifestations may be associated. These lesions make their appearance at the height of the primary inflammatory dermatophytosis.

Epidemiology

These occur in only 5-6% of all dermatophyte infections of an inflammatory character. These are principally seen in children 5-12 years of age. Adults are rarely affected. The dermatophytids may occur spontaneously or be provoked by the treatment that is either inappropriate or too energetic.

Diagnosis

These are diagnosed if the following criteria are fulfilled:

a. Existence of one or more laboratory confirmed primary foci of dermatophyte infection.
b. Absence of fungi in secondary lesions
c. Positive intracutaneous reaction to trichophytin
d. Resolution without therapy following the healing of primary lesion.

Course and Prognosis

The prognosis is good. Effective treatment of the primary lesion causes the dermatophytids to disappear in 2-3 weeks.

Treatment

No antimycotic treatment needs to be administered for dermatophytids. Symptomatic therapy may be instituted, if needed.

PARACOCCIDIOIDOMYCOSIS

Causative Agent

It is caused by a dimorphic fungus, *Paracoccidiodes brasiliensis*.

Mode of Transmission

The airways are now generally considered to be the most common portal of entry. It may also enter through

skin or mucous membrane. From the primary focus, which may be clinically active or silent the fungus spreads via the lymph tracts or bloodstream.

Clinical Features

A distinction is usually made into:

a. Mucocutaneous form
b. Lymphatic form
c. Visceral form

Mucocutaneous Form

The lesions of the skin and mucous membrane may be due to direct inoculation or by secondary manifestations. The ulcerative lesions may be in the oral cavity, larynx, conjunctiva or mucous membrane of the body orifices.

Lymphatic Form

Lymphadenopathy commonly begins in the cervical nodes, unilateral at first and then affects the other side, finally involving submaxillary, preauricular, and supraclavicular lymph nodes. If the nodes are massively enlarged there is a *bull neck*.

Visceral Form

Lung involvement is found in almost 90% of cases. In the X-ray picture the bilateral lesions are nodular, pseudotumoral or miliary (*snowstorm appearance*). The lesions may be in the central nervous system, viscera, bones and joints as well as in endocrine glands.

Laboratory Diagnosis

Source of Infected Material

Material from biopsy of lesions, pus from lymph nodes or sputum would be the most likely material for laboratory diagnosis.

Microscopic Examination

The direct smear examination may show the fungus as single and multiple budding, thick walled cells 10 to 60 µm in size. The presence of multiple buds is diagnostic.

The giant yeast cells of *P. brasiliensis* may show multiple budding in micky-mouse form (Fig. 76.24) or sometimes the buds are small and of uniform size which is called as pilot-wheel form (Fig. 76.25).

Culture

The organism grows slowly. It can be cultivated on blood agar as well as Sabouraud's agar. The yeast form appears at 37°C whereas the mycelial form grows at 30°C. For arriving at a diagnosis it is essential to have both the phases.

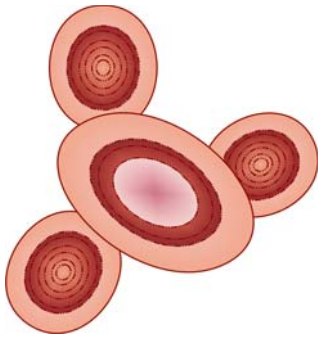


Fig. 76–24. Micky mouse form of *P. brasiliensis*

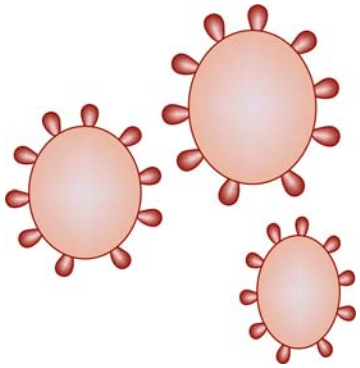


Fig. 76–25. Pilot wheel form of *P. brasiliensis*

Animal Inoculation

Infected material from lesions or saline suspension of the yeast phase can be inoculated intratesticularly into guinea pigs or intraperitoneally into mice. After 10 days or more the pus from the testicular lesions shows multiple budding cells.

Treatment

Drugs that are effective include sulfonamides, certain imidazole derivatives and amphotericin B.

BLASTOMYCOSIS

Causative Agent

The sole causative agent is *Blastomyces dermatitidis* which lives in nature as a saprophyte. Plants are apparently the principal reservoirs. The organism enters the body either by respiratory route or transcutaneously via a wound caused typically by plant thorns.

Clinical Features

Basically three clinical forms are distinguished:

a. Cutaneous form

b. Pulmonary form
c. Disseminated form.

Cutaneous Form

It is seen principally on exposed parts of the body, face, neck and hands. Trauma is an important factor in inoculation. The incubation period varies from 1-2 weeks. The lesions begin with one or more papules or nodules which break down and form a fistula. A purulent, foul smelling fluid which may be mixed occasionally with blood oozes from these fistulae. Cutaneous form is the commonest manifestation of the disease.

Pulmonary Form

The primary pulmonary syndrome has been observed in small epidemics in the USA. The clinical picture is that of a subacute infection of the airways with influenza like symptoms. The disease may remain confined to the lungs or be disseminated.

Disseminated Form

This is due to haematogenous spread of the fungus. In almost 95% of cases, the lungs are involved. Bone and CNS involvement occurs in only 30-35% cases. Urogenital system may also be affected occasionally.

Epidemiology and Mode of Transmission

Men are affected four times more than women. Blastomycosis is primarily a disease of middle adulthood. In most cases spores are inhaled. Trauma plays a dominant role in skin lesions. The dog appears to be the only animal that can contract blastomycosis.

Laboratory Diagnosis

Collection of Samples

Pus is collected from abscesses with a pasteur pipette or swab. Scale crusts are removed with blunt curette. CSF and sputum are collected in sterile containers.

Direct Microscopic Examination

It reveals round yeast cells with diameter of 8-10 µm. Their thick walls can be mistaken for a double membrane. Some cells show budding often with a single element so that they look like figure of eight (8) with one loop bigger than the other (Fig. 76.26).

Culture

The sample can be cultured on Sabouraud's dextrose agar. The growth is slow taking upto 4 weeks at a temperature of 32°C. The colonies are round, white and fluffy at first and gradually becoming smooth, creamy and yellowish in colour. Microscopic examination of a direct mount from a colony shows a mycelium consisting of fine, branched and septate hyphae. Conidia are formed opposite the septa and are sessile-either round or oval and smooth.

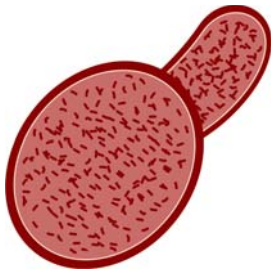


Fig. 76–26. Yeast stage of Blastomyces

Treatment

Amphotericin B is the drug of choice.

HISTOPLASMOSIS (DARLING'S DISEASE)

Causative Agent

The causative agent is *Histoplasma capsulatum*—a dimorphic fungus, which has been isolated from the soil which appears to be the source of infection for human beings. The fungus is present in the tissues in yeast form and in filamentous form in the cultures. The perfect form of *H. capsulatum* has been identified and is called as *Emmonsiella capsulata*. It is sometimes also called as *Ajellomyces capsulatum*.

Clinical Features

The disease is asymptomatic in 90-95% of the cases. It is manifested solely by a positive histoplasmin skin test and also by lung calcification detectable in later life on X-ray examination. Clinical types that are recognised in human beings are:

Acute Pulmonary Histoplasmosis

The onset resembles influenza and manifests as general malaise with fever, chills, profuse sweating, sore throat, chest pain, cough and dyspnoea.

Chronic Progressive Pulmonary Type

This is a variant of acute form. This is a primary form that remains latent for a long time and then gradually produces the same symptoms as the acute disease but

in a more pronounced form with haemoptysis and apical and subapical cavities.

Disseminated Histoplasmosis

This form may occur at any age but is seen especially in children under two years and elderly adults. The prognosis is poor. It is manifested by variable fever, anorexia, weight loss, deterioration of the general condition, anaemia, leukopenia, hepatosplenomegaly and multiple adenopathy.

Cutaneous, Subcutaneous and Mucocutaneous Type

As a rule the lesions of skin and mucous membranes are secondary and may occur in the course of one of the forms described above. These usually present as petechial or ecchymotic purpura usually on abdomen and thorax.

Epidemiology and Mode of Transmission

Adult men are affected more frequently than women but in children there are no sex related differences. The source of infection is the soil, which forms the fungal reservoir. Transfer from human to human or from animal to human is unknown. In majority of cases infection is due to inhalation of spores.

Laboratory Diagnosis

Collection of Material

Specimens of peripheral blood, sternal bone marrow, sputum, tissue from biopsy of lesion from skin, mucous membranes, lymph node or tissues from autopsies should be placed in sterile containers for laboratory examination. Smears may be made directly of blood, bone marrow, sputum or other moist specimens.

Direct Examination

Thick and thin smears should be made of peripheral blood, sternal bone marrow or other suitable infected material and stained with Giemsa's or Wright's stain. Under the microscope, the fungus appears as small oval yeast like cells, 1 to 5 µm in diameter, within the polymorphonuclear cells (Fig. 76.27).

Fluorescent antibody technique has been found to be useful in the diagnosis of histoplasmosis in tissues.

Culture

The infected material is inoculated on brain heart infusion (BHI) agar with cycloheximide and chloramphenicol and incubated at 25°C. Another set of BHI agar without antibiotics but with 6% blood is inoculated

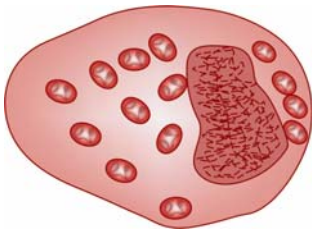


Fig. 76–27. Yeast stage of *H. capsulatum*

and incubated at 37°C. Cultures are examined upto 3 weeks of incubation.

Slides prepared from cultures grown at room temperature show septate hyphae with small (2-3 µm), smooth walled, round or pyriform conidia borne on short-lateral hyphae or sessile on the side of the hyphae as well as the large (8-15 µm) and round to pyriform tuberculate chlamydospores which are diagnostic (Fig. 76.28).

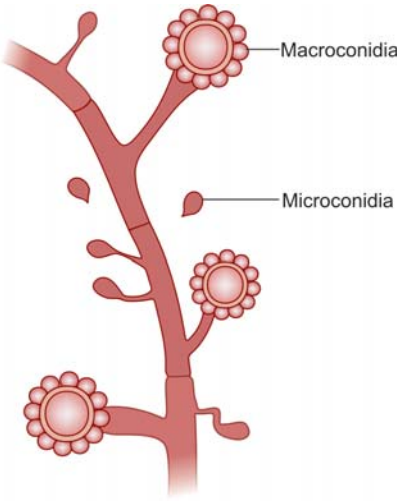


Fig. 76–28. *H. capsulatum* from culture

The yeast phase should be cultured at room temperature to verify conversion to the mycelial phase and the formation of chlamydospores.

Immunological Diagnosis

Histoplasmin skin test which is similar to tuberculin test is significant for epidemiological studies. However, a negative intracutaneous reaction turning positive is evidence of a fresh infection.

PART XII

CANDIDIASIS

Causative Agent

Of the 81 species of *Candida* there are at least seven that may be pathogenic (Table 76.4). The commonest of these is *Candida albicans*.

Table 76–4. Important species of Candida

- *Candida albicans*
- *C. tropicalis*
- *C. pseudotropicalis*
- *C. brumptii*
- *C. parapsilosis*
- *C. guilliermondii*
- *C. krusei*

The thallus of *Candida* consists of yeast cells and pseudohyphae. They reproduce by budding, ferment a number of sugars and assimilate nitrogen. Microscopic examination of pathological material shows round or oval yeast cells in the process of budding and often exhibiting pseudohyphae. Cultures on Sabouraud's agar medium produce moist, glistening creamy colonies of a dull white or greyish white colour.

Pathogenesis

Under normal conditions this fungus is not pathogenic. Many factors predispose to pathogenic effect (Table 76.5).

Table 76–5. Predisposing factors for candidiasis

- Impaired immune defences
- Spontaneous hormonal changes
- Corticosteroids
- Immunosuppression
- Long-term antibiotic therapy
- Oral contraceptives
- Pre-existing lesions of skin, mucosa or internal organs
- Pregnancy
- Menopause
- Premature birth
- Obesity
- Childbirth
- Diabetes mellitus
- Adrenal insufficiency
- Thyroid insufficiency

Clinical Features

A variety of infections are caused by *Candida* species (Fig. 76.29) though it is an opportunistic fungus.

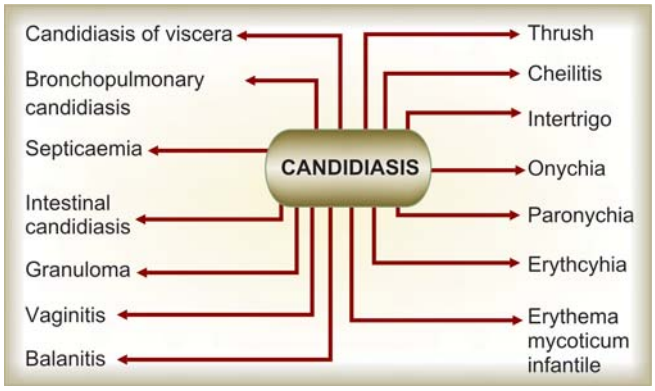


Fig. 76–29. Clinical features of candidiasis

In addition to general predisposing factors (Table 76.5), following local conditions also predispose to this infection:

- Chemical, mechanical or biological irritants
- Reduced salivation
- Digestive disorders
- Remnants of milk left fermenting in the mouth of infants.

Animal Pathogenicity

This fungus is pathogenic for rabbits, guinea pigs and mice. In rabbits or mice intravenous inoculation leads to death in 4-5 days with typical abscesses in kidney.

Laboratory Diagnosis

Collection of Infected Material

Skin or nail scrapings, mucous patches from the mouth, vagina or anus, sputum, blood, CSF or faeces may be collected for diagnosis in the laboratory. The material should be collected in sterile containers or as smears on slides.

Microscopic Examination

This process is very useful in detecting *Candida*. Skin and nail scrapings are mounted in 10% KOH with a cover slip and heated gently. Sputum or mucus material should be pressed to a thin film with a cover glass on a slide. The smear may be stained by Gram's method which may show ovoid yeasts 2.5 to 4 µm in diameter (Fig. 76.30). Presence of mycelial forms indicates colonisation and tissue invasion and is thus of great significance.

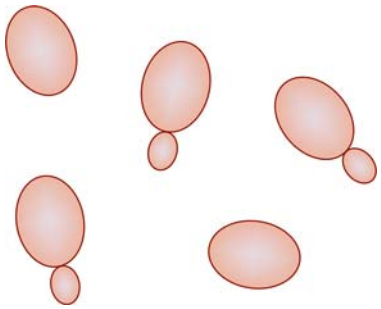


Fig. 76–30. *Candida albicans*

Culture

The clinical material is cultured on Sabouraud's glucose agar at room temperature and 37°C. The growth appears in 3-4 days as cream coloured, smooth, pasty

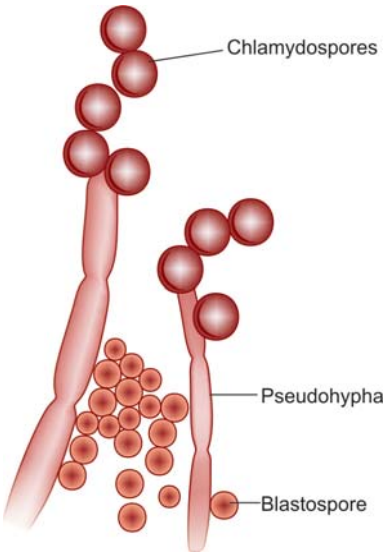


Fig. 76–31. *Candida albicans* in culure

colonies and have a yeasty odour. Microscopically, a slide mount will show oval, budding cells measuring 2.5 to 4-6 µm and some pseudomycelia may be visible if specimen is taken from submerged growth (Fig. 76.31). The species cannot be identified either by direct microscopy or by the macroscopic appearance of cultures. The species identification is based upon:

- Rapid formation of hyphae
- Production of chlamydospores
- Fermentation and assimilation of sugars
- Nitrogen utilisation.

Germ Tube Formation

This can be ascertained by inoculating 0.5 ml of rabbit, foetal calf or human serum with a small quantity of young test organism. The suspension is incubated at 37°C for 3 hours and a drop of it is examined under the microscope. Germ tubes are seen as long tube like projections extending from yeast cells (Fig. 76.32).

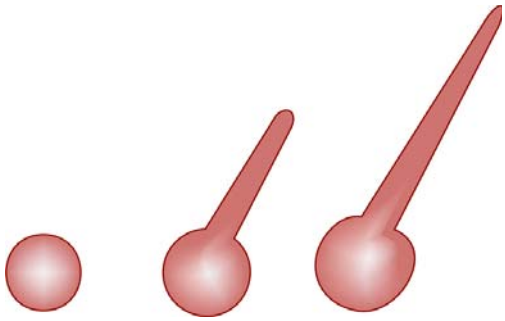


Fig. 76–32. Germ tube formation by *C. albicans*

Germ tubes differ from pseudohyphae in a few characters (Table 76.6).

Table 76–6. Differences between germ tube and pseudohypha

Germ tube	*Pseudohypha*
• Parallel sides	• Not necessarily parallel
• Nonseptate	• May be septate
• No constriction at point of attachment	• Constriction present at point of attachment

Germ tubes are formed within three hours by *C. albicans, C. stellatoidea* and rarely *C. tropicalis*. Not all strains of *C. albicans* form germ tube especially if the strain has been isolated from patient with cancer or those who are on anticandida drugs.

Animal Inoculation

C. albicans is pathogenic for rabbits, guinea pigs and mice. In rabbits or mice intravenous inoculation leads to death in 4-5 days with typical abscesses in the kidney.

Treatment

Predisposing factors should be eliminated. The affected area should be kept dry. Topical application of nystatin and systemic treatment with Amphotericin B, oral ketoconazole and fluconazole is effective.

MYCETOMA

Definition

Mycetoma is a localised, swollen lesion, usually on foot, less often on shoulders, buttocks, head or any site which is subject to trauma. It involves skin, subcutaneous tissue, fascia and bone. The lesion contains granulomas and abscesses which suppurate and drain through sinus tracts. The pus contains granules which vary from microscopic in size to more than 2 mm in diameter. Size, shape, colour and texture of the granules vary with the type of species involved. The disease was first observed in 'Madura' region of India and hence was also called as 'Madura foot'.

Geographical Distribution

Mycetomas are particularly common in regions bordering the great deserts of the tropics and subtropics. Sporadic cases have been virtually reported from all parts of the world.

Causative Agents

Mycetoma can be caused by both higher bacteria (*Actinomycetes*) as well as true fungi (Fig. 76.33).

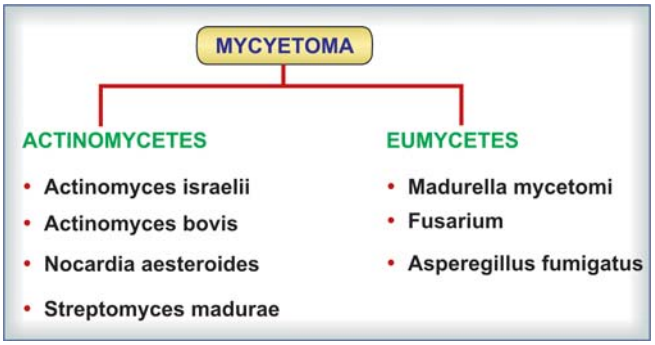


Fig. 76–33. Causative agents of mycetoma

The grains produced may be white, yellow, black or red in colour.

Epidemiology and Mode of Transmission

Mycetomas occur more frequently in men than in women: adults between 20 and 50 years of age being most commonly affected. Most of the patients belong to agriculture sector. Trauma is a critical factor in transmission. The organism is implanted at the time of injury or later as a result of secondary contamination of the wound.

Diagnosis

Diagnosis presents no problem in mycetoma of the feet, but it may be difficult in other sites. The characteristic feature of mycetoma is the presence of *granules* in a fistulated swelling which are found to contain actinomycetes or true fungi on mycological and histological examination. Biopsy examination of the developing nodules produces characteristic microscopic changes. Experimental inoculation in the animals is rarely of any diagnostic use. Immunological reactions have also not been found useful.

Treatment

Except in the case of a few actinomycetomas no conservative therapy has achieved really satisfactory results. Surgery has an important role in the treatment of mycetoma.

CRYPTOCOCCOSIS

(*Synonyms*: Buschke's disease, European Blastomycosis.)

Causative Agent

Cryptococcus neoformans is the only pathogenic species to man.

Clinical Features

The incubation period ranges between 14-25 days. The clinical picture can be described under following heads.

a. Pulmonary cryptococcosis.
b. Central nervous system cryptococcosis
c. Cutaneous cryptococcosis
d. Osseous cryptococcosis
e. Visceral cryptococcosis

Pulmonary Cryptococcosis

This is often the only portal of entry for a cerebrospinal or generalised cryptococcosis and may be acute, subacute or chronic. The clinical picture does not differ in the slightest from that of other infections of the lung. Haemoptysis is rare and infection may be quite asymptomatic. Pulmonary cryptococcosis is rarely diagnosed on the basis of clinical symptoms or sputum examinations unless such a diagnosis is suspected because of existing cryptococcosis of the central nervous system.

Central Nervous System Cryptococcosis

It may take the form of a diffuse meningoencephalitis or well-circumscribed granulomas of the brain or spinal cord. The symptoms may be those of a non-specific meningoencephalitis or brain tumour. Detection of *C. neoformans* in the CSF is mandatory for diagnosis.

Cutaneous Cryptococcosis

Skin and mucosal involvement is usually secondary and occurs in the course of systemic cryptococcosis. The skin is affected in about 10% of the cases and mucosa in 3%. The lesions are solitary or multiple, which may be dermoepidermal or in the form of mucofibrous tumour formation.

Osseous Cryptococcosis

Bone lesions occur in about 10% of the reported cases of cryptococcosis. These are frequently associated with pain and swelling of many months duration. The lesions spread slowly without periosteal proliferation. They are osteolytic and often spread to the skin by extension or following surgical exploration.

Visceral Cryptococcosis

While the lungs represent the usual site of a primary lesion and meningeal lesions of this neurotropic fungus are most often seen, any organ or tissue of the body is subject to invasion. Granulomatous lesions may bear symptomatic or even histologic resemblances to cancer.

Epidemiology and Mode of Transmission

The infection with *C. neoformans* is rare, the disease shows a predilection for persons debilitated by a serious chronic malady without distinction to age and sex. Various predisposing factors include Hodgkin's disease, Leukoses, diabetes, treatment with antibiotics, corticosteroids or immunosuppressants. The infection is acquired by inspiration or less commonly by ingestion of yeast following prolonged exposure.

Laboratory Diagnosis

Collection of Infected Material

Collect all the required samples such as CSF, sputum, visceral organs and pus from the skin lesions in sterile containers for laboratory examinations.

Direct Microscopic Examination

Examine the spinal fluid by placing directly on slide with cover glass. If no yeast cells are seen, the fluid should be centrifuged and rechecked. If the sputum, spinal fluid or blood stained exudate do not contain too much purulent or cellular materials, it is better to make a dilute (about 50%) India Ink mount and examined immediately for cells with capsule. India Ink mounts of the infected material show round to oval, budding cells ranging from 5-20 µm in diametre with thick walls. The organisms are surrounded by wide refractle gelatinous capsule which may be twice as wide as the diametre of the cell (Fig. 76.34).

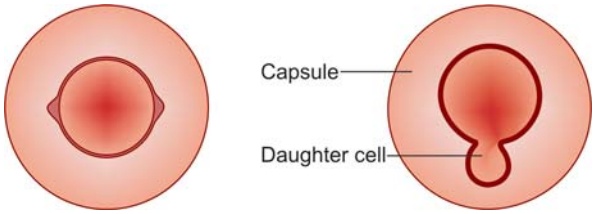


Fig. 76–34. *C.neoformans* in CSF (India Ink preparation)

Culture

Culture the infected material on Sabourads agar containing chloramphenicol at an incubation temperature of 37ºC. The growth is rapid and flat or slightly mucoid colonies, appear 4-7 days after culture has been put up. Cycloheximide should never be added to the culture medium since this inhibits the growth of *Cryptococcus* at least in the primary culture. *C. neoformans* can be distinguished from non-pathogenic species by following :

a. At 37°C *C. neoformans* will grow, while non-pathogenic cryptococci do not.
b. *C. neoformans* can assimilate nitrates while non-pathogenic species cannot.
c. It can hydrolyse urea.
d. *C. neoformans* is pathogenic to mice.

Animal Inoculation

Infected material or a saline suspension of the culture should be injected intraperitoneally in mouse or rat. In 2-4 weeks an autopsy of the animal should show gelatinous masses in the visceral cavity, spleen involvement and in more virulent strains, infection of the lung and brain. Demonstration and isolation of the fungus can be made from the lesions in the mouse.

Treatment

The treatment currently available consists of amphotericin B and flucytosine either singly or in combination.

RHINOSPORIDIOSIS

Definition

It is a chronic granulomatous disease, characterised by production of polyps or other manifestations of hyperplasia on mucous membrane surfaces.

Geographical Distribution

Rhinosporidiosis is endemic in India, Sri Lanka, in north-eastern Brazil and in the north-east of Argentina.

Causative Agent

The organism responsible for this disease is *Rhinosporidium seeberi*. It is believed to be a fungus though views are emerging that it may be a protozoan. *R. seeberi* has not been cultured so far. Though little is known about its habitat, it is believed that stagnant water may be the reservoir.

Clinical Features

It is characterised by friable polypous lesions that are richly vascularised and easily bleed. They occur predominantly on the mucous membranes, especially the nasal mucosa, which is affected in more than 70% of the cases, primarily in its posterior section. In about 14% of the cases the eye is affected, the lesions are located on the conjunctiva. The external auditory meatuses may also become involved. Sometimes these lesions can be present on genitalia. Cutaneous

rhinosporidiosis which is very rare, is generally due to spread from a neighbouring mucosal lesion. As a rule lesions are asymptomatic.

Epidemiology and Mode of Transmission

The disease is especially prevalent in a rural environment, particularly among persons working in stagnant water or in sand. The disease affects predominantly males, particularly children and young adults, all races may be equally affected.

Course and Prognosis

The course is slow and chronic, only in rare instances of visceral dissemination can the disease end fatally. On the other hand spontaneous recovery is also very rare.

Laboratory Diagnosis

Collection of Infected Material

Material from the polyps that have been removed surgically is brought to the laboratory under sterile conditions for examination.

Direct Microscopic Examination

The exudate of the material obtained is placed in water on a slide and examined under microscope. The direct slide mount if positive, shows round or oval spores 7-9 µm in diametre as well as sporangia filled with spores (Fig. 76.35).

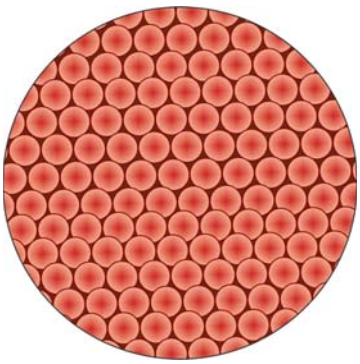


Fig. 76–35. Sporangium of *R. seeberi*

Culture

The organism has not been cultured so far.

Animal Inoculation

There is no report of successful infection of laboratory animals.

Immunological Examination

At present there is no immunological test of value.

Treatment

No drug treatment has proved effective. The disease can be controlled by radical surgery either by excision or electric cautery.

SPOROTRICHOSIS

Sporotrichosis is a chronic infection characterised by nodular lesions and ulcers in the lymph nodes, skin or subcutaneous tissues and occasionally in the internal organs. The localised lesions are usually found on the hands, arms or legs.

It is caused by a dimorphic fungus *Sporothrix schenckii* which lives as a saprophyte in the external milieu. It has been isolated from the soil, the plants and the wood.

Morphology

S. schenckii is a dimorphic fungus which produces a delicate mycelium in culture at 25°C, but is present in the form of spores and small elongated cigar shaped bodies in the tissue. The conidia arise directly from the mycelium or small stems called denticles. These arise both laterally and at the tips of delicate sterigmata on a conidiophore and are described as "palm tree like". The hyphae are considerably more slender, 2 µm in diameter, than those of most molds and often form rope-like strands.

Clinical Features

The clinical types described are:
- Cutaneous lymphatic type
- Cutaneous non-lymphatic type
- Disseminated type

Cutaneous Lymphatic Type

The commonest type of sporotrichosis follows the subcutaneous implantation of spores in a penetrating wound caused by a thorn or splinter. A small ulcerated lesion may develop at the site of injury within a week or two, develop slowly and remain localised but fail to heal under topical therapy. The typical course of the disease is characterised by involvement of the lymphatics which drain the area of the primary lesion. The lymph nodes become swollen and eventually suppurate and the connecting lymphatics become indurated and cord-like.

Cutaneous Non-lymphatic Type

There are some cases of cutaneous sporotrichosis in which lesions remain localised and do not involve lymphatics or other systems. The lesions commonly appear on the face, neck, trunk, or arm and vary widely in appearance: ulcerative, verrucose, papular, acneform or erythematoid plaques.

Disseminated Type

This may occur by haematogenous spread from the primary lesion or from suppurating lymph nodes. Dissemination may be manifested by numerous and widespread skin lesions which begin as subcutaneous nodules and become papules, pustules, gummata or confluent areas of folliculitis. Lesions of oral and nasal mucosa occur in many cases of disseminated disease. Dissemination to visceral organs is rarely observed, but pyelonephritis, orchitis, mastitis, and pulmonary disease occur.

Laboratory Diagnosis

Collection of Infected Material

Pus should be aspirated from unruptured nodules. Swabs, scrapings or biopsies of ulcerated lesions should be collected in a sterile container.

Direct Microscopic Examination

Pus or other infected material should be put in KOH on a slide and examined for *cigar bodies.* (Fig. 76.36).

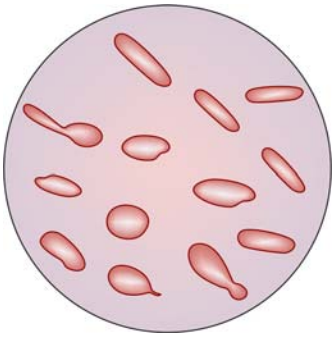


Fig. 76–36. Yeast phase of *S. schenckii* (Cigar bodies)

Culture

Pus from open lesions or from unopened nodules should be streaked on Sabourads dextrose agar and incubated at 37°C. The addition of antibiotics may be desirable if pus is contaminated. Microscopically a slide mount of a portion of a colony grown at 37°C, should show round, oval and fusiform, budding cells, commonly called as *cigar bodies*.

Animal Inoculation

Rats, mice, male hamsters, cats, dogs and monkeys are susceptible to the disease. Pus or 0.5-1 ml saline suspension of the cells from the yeast phase or mycelial fragments and conidia from filamentous cultures should be inoculated intraperitoneally into white mice or rats. Autopsy done after 3 weeks, should show peritonitis and granulomas in the mesentery of the infected animal. Male animals show severe orchitis.

Treatment

Potassium iodide given orally as saturated solution is the drug of choice for the cutaneous form while visceral and disseminated forms require treatment with amphotericin B.

COCCIDIOIDOMYCOSIS (*Synonyms* : Valley Fever, San Joaquin Fever)

Causative Agent

Coccidioides immitis is a dimorphic imperfect fungus. It is geophilic and is more abundant at a depth of 10-28 cm than on the soil surface. The agent invades the body via the respiratory tract, i.e. by inhalation of spores under natural conditions or in the laboratory.

Clinical Features

Clinical features are described under following heads:

- Primary pulmonary coccidioidomycosis
- Primary cutaneous coccidioidomycosis
- Scrofuloderma coccidiodosum
- Disseminated type

Primary Pulmonary Coccidioidomycosis

The clinical manifestations vary in severity, they range from a simple influenza like state to serious bronchopneumonia. An influenza like malady, erythema nodosum or multiforme and phlyctenular conjunctivitis together form the triad known as *San Joaquin Fever*.

Primary Cutaneous Type

The initial lesion is a nodule or plaque ulcerated at the centre and resembling a syphilitic, tuberculous or sporotrichotic chancre. The skin lesion may be accompanied by lymphangitis and regional lymph node involvement.

Scrofuloderma Coccidioidosum

Involved here are inflamed lymph nodes of the cervical region that coalesce and rupture through to the

skin via proliferative and verrucous fistulae, thus recalling the tuberculous scrofuloderma of classical pathology.

Disseminated Type

Dissemination is haematogenous starting from an active latent or residual pulmonary focus. The multiple osteoarticular lesions are of an osteomyelitic type. Involvement of central nervous system along with many organs of the body takes place.

Laboratory Diagnosis

Direct Microscopy

Sputum, gastric contents, spinal fluid exudate or pus should be examined microscopically for the presence of round, thick walled spherules, 20-80 µm in diameter with many small endospores 2-5 µm in diameter. Immature spherules are smaller and without endospores (Fig. 76.37).

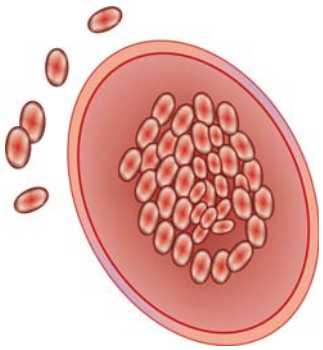


Fig. 76–37. Spherule stage of *C. immitis*

Culture

Infected material should be isolated at room temperature or 37°C and cultivated in a well-stoppered bottle or slant containing Sabourad's glucose agar with or without chloromycetein or actidione. The colony develops moderately rapid as a moist, membranous culture at first and later develops abundant aerial mycelium. Slide mounts with lactophenol cotton blue prepared from culture containing saline should show branching septate hyphae and chains of thick walled rectangular arthrospores. Spores are highly infectious and all precautions should be taken.

Immunological Diagnosis

In addition to skin test with coccidiodin, various serological reactions such as precipitin, complement fixation and immuno-diffusion are of both diagnostic and prognostic value.

Animal Inoculation Test

Saline suspension of arthrospores from cultures or clinical materials may be injected intraperitoneally in the mouse or intratesticularly in the guinea pig. Within 5-6 days the pus may be aspirated from the animal and examined for the presence of spherules.

Treatment

The drugs which have been used successfully in treatment include amphotericin B, natamycin, miconazole, ketoconazole etc. Surgery under cover of medication may be useful in some cases.

OPPORTUNISTIC FUNGI

Opportunistic is the name given to the fungi that are normally saprophytic but may become pathogenic under special conditions. The term '***asthenomycosis*** is sometimes used to describe diseases caused by such fungi. Strictly speaking most fungi are opportunistic but we will restrict the term to those organisms that become pathogenic when the host has an identifiable, pre-existing disease, which may be local or general, spontaneous or iatrogenic.

Fungi Involved

a. Yeast like fungi
- *Candidia* sps
- *Torulopsis*
- *Cryptococcus*

b. Filamentous fungi
- *Aspergillus*
- *Mucor*
- *Cephalosporium*
- *Fusarium*
- *Geotrichum*
- *Scopulariopsis*

Source of Infection

It can be endogenous or exogenous.

Endogenous. e.g. Candida albicans—a common saprophyte of intestinal tract and vagina.

Exogenous. (widespread in nature). *e.g. Mucor, Rhizopus, Absidia, Fusarium,* etc.

Transition from Saprophytism to Pathogenicity

The factors predisposing a saprophyte to pathogenicity are many and are as under:

i. **Physiological:**
- Pregnancy
- Menstruation

ii. **Various diseases:**
- Bacterial infections
- Malignant tumours
- Haemopathies
- Endocrinopathies specially diabetes and hypoparathyroidism.
- All diseases resulting in impairment of cellular and humoral immunity.
- Wetness and maceration of integument

iii. **Iatrogenic:**
- Long-term antibacterial therapy
- Anti-tuberculous therapy
- Corticosteroid therapy for long time
- Immunodepressants
- Oral contraceptives
- Indwelling intravenous catheters
- Open heart surgery

OTOMYCOSIS

Definition

Infection by normally saprophytic fungi superimposed on eczema or a seborrheic dermatitis of the external auditory canal.

Causative Agents

Fungi most commonly isolated include:
- *Candida albicans*
- *Candida tropicalis*
- *Aspergillus fumigatus*
- *Aspergillus niger*
- *Aspergillus nidulans*
- *Aspergillus flavus.*

Other agents which have been isolated include:
- *Mucor*
- *Penicillium*
- *Rhizopus*
- *Absidia*

Pathogenesis

The mycotic infection develops on a pre-existing bacterial otitis externa. Treatment with topical agents and corticosteroids predispose to fungal infection.

Clinical Features

There are no specific symptoms. It may be confused with any form of otitis externa. Otoscopy may reveal whitish patches with a surface resembling valvet or wet blotting paper strewn with grey, brown, green or black dots indicating the presence of fungal colonies.

Complications

These include:
- Bacterial otitis externa
- Eczematisation of the external canal
- Reversible conduction deafness
- Perforation of eardrum.

Diagnosis

Clinical diagnosis is not very difficult. The diagnosis can be established with confidence only by mycological examination. Microscopy shows a tangled mass of mycelial threads, yeast cells with or without pseudofilaments and also aspergillus heads.

Treatment

Nystatin in suspension form as an ointment or a gel applied twice daily heals in two or three weeks. Imidazole derivatives, particularly econazole nitrate in the form of 1% milk or cream give excellent results.

Course and Prognosis

The course is chronic with acute episodes, especially in summer, with intermittent remissions. Prognosis is good.

FUNGAL CONTAMINANTS

Contaminants are seen very often in routine fungal cultures and it is therefore important to be able to identify and differentiate them from the normally pathogenic fungi.

Common Properties

Some of the common properties of the fungal contaminants are:

a. Most are rapid growers forming mature colonies in 4-5 days
b. They are saprobic living on decaying organic matter in the soil
c. Since the conidia of fungal contaminants are inhaled constantly, their isolation in routine culture of

sputum and other respiratory secretions is of no significance.

d. Usually these organisms are non-pathogenic
e. These can act as opportunistic pathogens when body resistance goes down
f. Their growth is inhibited by the antibiotics, hence for their isolation antibiotic free media are used
g. Repeated isolations in large numbers from the same patient are required to label it as causative agent of the disease.

Descriptions about all the fungal contaminants is beyond the scope of this book. Brief description of the common contaminants is given. The classification of the contaminants is given in Table 76.7.

Table 76–7. Classification of fungal contaminants

ASEPTATE CONTAMINANTS
- *Absidia*
- *Rhizopus*
- *Mucor*

SEPTATE CONTAMINANTS

a. *Dematiaceous (Dark coloured hyphae)*
- *Cladosporium*
- *Alternaria*
- *Curvularia*
- *Drechslera*

b. *Hyaline (Light coloured hyphae)*
- *Aspergillus*
- *Penicillium*
- *Fusarium*
- *Sepedonium*

Aseptate Contaminants

All aseptate fungi, that usually do not contain cross walls, fall into the taxonomic subdivision Zygomycotina. A few of the more important ones which act as opportunistic pathogens are described here.

ABSIDIA SPECIES

Culture

On Sabouraud dextrose agar at room temperature, a woolly grey colony rapidly matures. The reverse side of the colony is colourless.

Microscopic

The mycelium is usually aseptate, with branching sporangiophores between the *rhizoid* nodes on the *stolons* (interconnecting runners). There is a slight swelling below the columella, and sporangia are pear shaped. When the sporangial wall dissolves, a collarette remains at the base of the columella (Fig. 76.38).

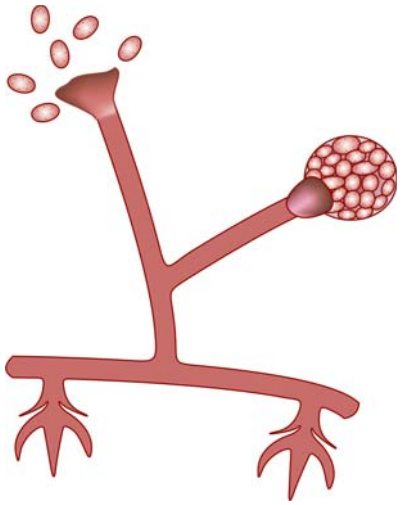


Fig. 76–38. Absidia species

Pathogenicity

Absidia may cause zygomycosis and mycotic keratitis.

MUCOR SPECIES

Culture

On Sabouraud dextrose agar at room temperature, a white, fluffy mycelium quickly forms. It becomes grey to brown with age.

Microscopic

The mycelium is usually aseptate. Single or branching sporangiophores support round, spore-filled sporangia. The columella is variable in shape and light to pigmented in colour. No rhizoids or stolons are present.

Pathogenicity

Mucor may cause zygomycosis, otomycosis and allergies.

RHIZOPUS SPECIES

Culture

On Sabouraud dextrose agar at room temperature, white, dense, cottony, aerial hyphae rapidly form, which later become dotted with brown or black sporangia.

Microscopic Picture

The hyphae are usually aseptate. Unbranched sporangiophores arise opposite rhizoids at the nodes, and each sporangiophore supports a round spore-filled

sporangium with a flattened base. Sometimes the sporangia are completely black, or they may be empty. When the sporangial wall dissolves, a bare hemispherical columella without a collarette is observed. Stolons connect the groups of rhizoids with each other (Fig. 76.39).

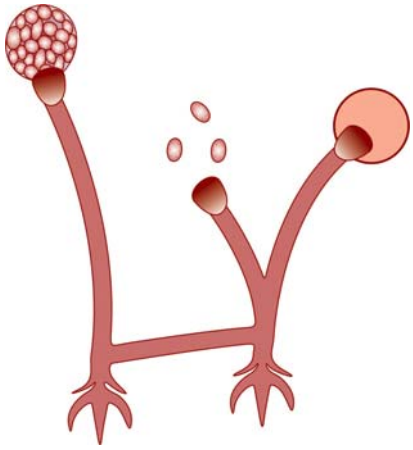


Fig. 76–39. Rhizopus

Pathogenicity

Rhizopus causes zygomycosis and otomycosis. Rhizopus, Mucor and Absidia are the species most commonly recovered from clinical cases of zygomycosis (Phycomycosis or mucormycosis).

SEPTATE CONTAMINANTS

Most contaminants contain cross-walls. Those of medical importance fall into the subdivision Deuteromycotina. The septate contaminants may be divided into those that are *dematiaceous* (dark-coloured hyphae and/or conidia), and those that are *hyaline* (light-coloured hyphae and conidia). Organisms with dark hyphae on tease mounts also have dark green to black colonies, especially on the colony reverse. The colour of the colony aids in the initial identification. Hyaline organisms exhibit light-coloured colonial aerial hyphae, but they may be covered over with brightly coloured conidia; thus, a tease mount is required. In the following descriptions, key identifying features are given.

DEMATIACEOUS CONTAMINANTS

ALTERNARIA SPECIES

Culture

On Sabouraud dextrose agar at room temperature, the light grey, woolly colony rapidly matures to dark greenish black or brown, with a black reverse.

Microscopic

Reproductive structures and hyphae are dark. The chained poroconidia, which contain horizontal and vertical septa, have club-shaped bases with tapered apices (Fig. 76.40).

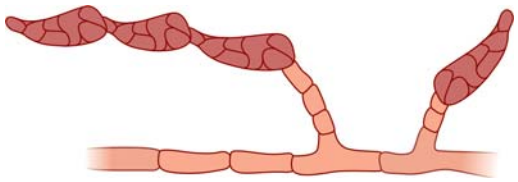


Fig. 76–40. Alternaria species

Pathogenicity

Alternaria has been reported in mycotic keratitis, skin infections, osteomyelitis, pulmonary disease, and nasal septum infection.

CLADOSPORIUM (HORMODENDRUM SPECIES)

Culture

On Sabouraud dextrose agar at room temperature, the colony is moderately slow-growing for a contaminant, requiring seven days. It is powdery or velvety, heaped and folded, and dark grey-green with the reverse black.

Microscopic

The septate hyphae are dark coloured. Short chains of dark one to four-celled blastoconidia with a distinct scar at each point of attachment are borne from repeatedly forking, shield-shaped conidiogenous cells (shield cells) (Fig. 76.41).

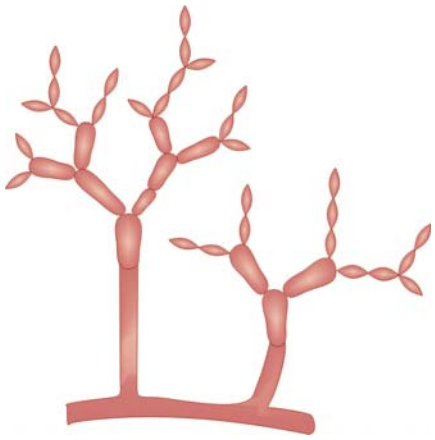


Fig. 76–41. Cladosporium

In the past, contaminant strains of the genus were differentiated from pathogenic ones by the former's ability to hydrolyze nutrient gelatin. This test is not reliable and criteria such as growth rate, microscopic morphology, and clinical picture should be used instead.

Pathogenicity

Cladosporium may cause mycotic keratitis and allergies.

CURVULARIA SPECIES

Culture

On Sabouraud dextrose agar at room temperature, the colony is moderately rapid growing, cottony, and white, light pink, orange, or green, with a brown reverse.

Microscopic

The septate mycelium is dark. Large, four to five-celled, dark poroconidia are borne on a bent-knee type conidiophore. The poroconidia are centrally distended owing to an over-enlarged central cell, and the ends are lighter than the middle (Fig. 76.42).

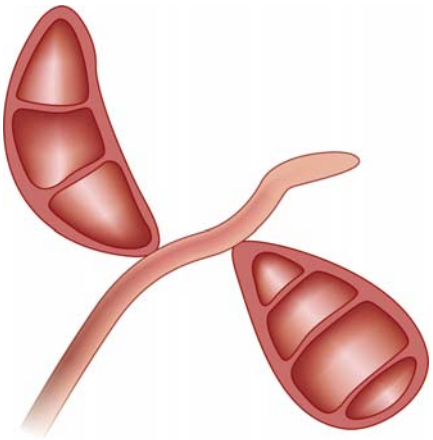


Fig. 76–42. Curvularia species

Pathogenicity

Curvularia usually causes mycotic keratitis, but occasionally it may produce mycetoma, endocarditis, pulmonary infection, allergies, and infection of the nasal septum.

PENICILLIUM SPECIES

Culture

On Sabouraud dextrose agar at room temperature, the rapid-growing colony is initially velvety and white, later becoming powdery and blue green with a white periphery and colourless reverse.

Microscopic

The mycelium is septate. *Penicillium* bears flask-shaped phialides, which in turn support chains of round phialoconidia. Conidiophores and phialoconidia may be hyaline to pigmented, and smooth to rough, depending on species (Fig. 76.43).

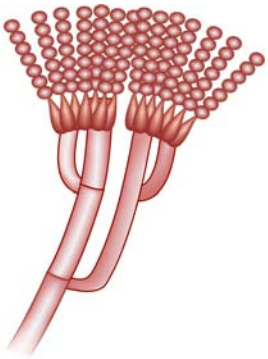


Fig. 76–43. Penicillium species

Pathogenicity

Penicillium causes mycotic keratitis, penicilliosis, otomycosis, onychomycosis, and rarely deep infections.

MYCOTOXICOSIS

Toxins produced by fungi are called as *mycotoxins* and *mycotoxicosis* is any disease that is induced by the consumption of food that has been rendered toxic by the fungal toxins. A partial list of known mycotoxins is depicted in Table 76.8.

Table 76–8. Mycotoxins and fungi producing them

Mycotoxin	*Mycotoxin-producing Fungus*
Aflatoxin	*Aspergillus flavus, A. parasiticus*
Ascladiol	*A. clavatus*
Ergot alkaloid	*Claviceps species*
Fumigatin	*Aspergillus fumigatus*
Ochratoxin	*Aspergillus ochraceus*
Muscarine	*Amanita muscaria*
Penicillic acid	*Penicillium puberulum*
Psoralens	*Sclerotinia sclerotiorum*
Rubratoxin B	*Penicillium rubrum*
Sciepenols	*Fusarium nivale*

IMMUNODIAGNOSIS OF FUNGAL INFECTIONS

The isolation of the fungal agents takes very long time and most laboratories are not well versed with the

characterisation of the fungal isolates. Immunological diagnosis, hence, plays an important role. Five types of tests are commonly used for mycoimmunological diagnosis.

Skin Tests

Cutaneous delayed hypersensitivity response is now a recognised *in vivo* method of determination of cell mediated immunity. Skin tests are available for:

- Histoplasmosis
- Paracoccidioidomycosis
- Coccidioidomycosis
- Sporotrichosis.

Some of the important features of these tests are given in Table 76.9.

Table 76–9. Salient features of skin tests for fungal diagnosis

- Cannot distinguish between past and present infection
- Positive test indicates exposure to the antigen
- Conversion of negative skin test to positive indicates *good prognosis*
- Reversion of a positive test to negative reflects *poor prognosis* and indicates *anergy*
- Mainly used in seroepidemiology

Detection of Circulating Antibody

Various serological tests are in use for diagnosis of mycoses. These include complement fixation test (CFT), immunodiffusion (ID), latex agglutination (LA), counterimmunoelectrophoresis, enzyme linked immunosorbent assay and radioimmunoassays. Of these CFT, ID and LA are the most commonly used tests. No single test fulfils the desired standard of specificity, sensitivity and rapidity of performance. ID is quite sensitive and specific for the serologic diagnosis of histoplasmosis, blastomycosis and coccidioidomycosis.

A single serum sample is usually not reliable for diagnosis unless very high titres are obtained. A four-fold or more rise in the antibody titres of two samples collected 2-3 weeks apart is considered suggestive of infection and immunocompromised hosts usually do not produce antibody.

Detection of Fungal Antigen

Two fungal diseases *Cryptococcosis* and *Candidiasis* can be rapidly diagnosed by detection of their antigen *in vivo*. Latex agglutination kits are commercially available for detection of cryptococcal antigen in CSF and blood. Circulating metabolic and structural components of *Candida albicans* have been detected in the blood of the patients with candidiasis. Detection of *mannan* (a polysaccharide constituent of candida cell wall) is suggestive of invasive candidiasis.

Exoantigen Analysis

Exoantigens are cell free antigens present in the supernatant of fungal growth media. Using specific antisera, these exoantigens can be detected in the concentrated supernatant. Exoantigen tests are available for:

- *Blastomyces dermatitidis*
- *Coccidioides immitis*
- *Paracoccidioides brasiliensis*
- *Histoplasma capsulatum*
- *Sporothrix schenckii*
- *Pseudoallescheria boydii*

Only for a few of these, reagents are commercially available.

Fluorescent Antibody Microscopy

Fluorescent antibody procedure can be used to detect either viable or dead fungal elements in tissue sections. The technique has applications for the detection of fungal antigens in pus, exudates, CSF and other samples. Reagents are available for the followings:

- Actinomycetes
- *Candida*
- *Cryptococcus neoformans*
- Endospores of *Coccidioides immitis*
- Yeast phase of *Histoplasma capsulatum*
- Yeast phase of *Paracoccidioides brasiliensis*
- Yeast phase of *Sporothrix schenckii.*

PART XIII APPLIED MICROBIOLOGY

77 Microbiology of Water, Milk and Air

Most of the diseases particularly in developing countries can be attributed to the lack of safe drinking water. Water intended for human consumption should be not only *safe* but also *wholesome*. A safe water is one that should not harm the consumer, even when ingested over prolonged periods. Even if the water is safe, but it has an unpleasant taste or appearance this may divert the people to unsafe sources. Hence, drinking water should not only be completely safe but should be wholesome. Such a water is called *potable water*. Water is said to be *contaminated* when it contains infective and parasitic agents, poisonous chemical substances, industrial or other wastes or sewage. The term *polluted* water is synonymous with contaminated water. Both pollution and contamination are the result of human or animal activity.

The hazards of water pollution may be classified into two broad groups: *biological and chemical hazards.* Chemical pollutants of diverse nature derived from industrial and agricultural wastes are increasingly finding their way into public water supplies.

Bacterial Flora in Water

The bacterial flora in water is depicted in Table 77.1.

Various factors which determine the type and number of bacteria in water are given in Table 77.2.

Table 77–1. Bacterial flora in water

Type of water	*Flora*
Natural	• *Micrococcus* • *Pseudomonas* • *Serratia* • *Flavobacterium* • *Alkaligenes* • *Acinetobacter*
With soil washed in	• *Bacillus subtilis* • *Bacillus megaterium* • *Bacillus mycoides* • *Enterobacter aerogenes*
With sewage	• *Escherichia coli* • *Streptococcus faecalis* • *Clostridium perfringens* • *Salmonella typhi* • *Vibrio cholerae*
Sewage proper bacteria	• *Proteus vulgaris* • *Clostridium sporogenes* • *Nocardia* species

Water-borne Diseases

Faecal pollution of drinking water may introduce a variety of intestinal pathogens bacterial, viral and parasitic. These organisms may cause diseases that vary in severity from mild gastroenteritis to severe and sometimes fatal disease. The list of water-borne diseases is given in Table 77.3.

Among the various water-borne pathogens there exists a wide range of minimum infectious dose levels necessary to cause human infection. The size of the infective dose also varies in different persons with age, nutritional status and general health at the time of exposure.

Table 77–2. Factors affecting number and type of bacteria in water

- Type of water
  - — Surface or deep
  - — Sea water
  - — Mineral springs
- Presence of organic matter
- Temperature
- Light
- pH
- Dissolved oxygen
- Rainfall
- Season
- Storage
- Filtration
- Protozoal contents

Microbiological Examination of Water

Microbiological examination offers the most sensitive test for the detection of recent and potentially dangerous faecal pollution, thereby providing a hygienic assessment of water quality with high sensitivity and specificity. For this reason it is important to examine a drinking water source frequently by a simple test rather than infrequently by a more sophisticated test or series of tests. It is ideal to look for individual specific pathogen but it is not practical since they are few in number than the nonpathogenic organisms and methods to detect them are costly and time consuming. Therefore, indicators of human/animal pollution are used.

Indicator Organisms

A bacterium can be used as the indicator organism if it fulfils most of the criteria mentioned in Table 77.4.

In practice these criteria are not met by any single organism though many of them are fulfilled by coliform bacteria specially *Esch. coli.* The organisms which have thus been used as indicators are:

- *Esch. coli*
- Faecal coliforms
- Faecal streptococci (*Strep. faecalis*)
- Sulfite reducing clostridia (especially *Clostridium perfringens*).

Anaerobic bacteria such as bifidobacteria and *Bacteroides* are more abundant than coliform organisms in faeces but routine methods for their detection and enumeration are not as yet available hence these are not used.

Faecal streptococci are regularly present in the faeces in varying numbers but their number is fewer than *Esch. coli* and they probably die and disappear at the same rate. The presence of faecal streptococci along with coliforms in absence of *Esch. coli* is also confirmatory of faecal pollution.

Cl. perfringens is also present in faeces in small numbers. Their spores which can resist chlorination survive for longer times. Thus, the presence of *Cl. perfringens* in natural water suggests that faecal contamination has occurred and in the absence of coliform organisms suggests that it occurred quite sometimes ago.

Water Sampling for Bacteriological Examination

Following points are considered before collecting the water sample for bacteriological examination:

a. Sampling should be properly planned and ideally, carried out with sufficient frequency to enable any seasonal variations in the quality of the water to be tested;
b. Samples should be collected, stored, and dispatched in suitable sterilised bottles;
c. The volume of water collected should be large enough to permit an accurate analysis;
d. The sampling points in the water supply system should be selected in such a way that the samples obtained are as representative as possible;
e. Great care should be taken during sampling to prevent contamination of the sample being collected;
f. In order to prevent any significant change in the composition of a collected sample prior to its

Table 77–3. Water-borne diseases

Bacterial	*Helminthic*	*Viral*	*Protozoal*
Cholera	Roundworm	Hepatitis A	Amoebiasis
Typhoid fever	Threadworm	Hepatitis E	Giardiasis
Shigellosis	Whipworm	Rotavirus diarrhoea	Balantidiasis
Diarrhoea due to:	Hydatid disease	Poliomyelitis	
Esch. coli	Guineaworm disease		
Yersinia enterocolitica	Fish tapeworm		
Campylobacter fetus	Schistosomiasis		
Leptospirosis			

Table 77–4. Criteria for indicator organisms for water bacteriology

- Present in faeces in abundant number
- Present in scanty number in other sources
- Easy to isolate, identify and enumerate
- Unable to grow in water
- Able to survive longer in water than pathogens
- More resistant to disinfectants such as chlorine

analysis, it is important to ensure that it is collected properly, and dispatched as soon as possible;

g. The sample details should be adequately described and the sample bottle properly labelled to avoid errors;

h. The testing should be undertaken in authorised laboratories, with good quality control measures.

For collection, heat sterilised bottles containing a sufficient volume of sodium thiosulphate to neutralise the bactericidal effects of chlorine or chloramine in the water are used. Each 100 ml bottle should contain 0.1 ml of a fresh 1.8% (w/v) aqueous solution of sodium thiosulphate.

Sampling of Water

Water can be divided into 3 basic types for the purpose of sampling.

a. Water from a tap or fixed hand pump
b. Water from a reservoir (River, Lake, Tank)
c. Water from a dug well

a. *Sampling from a tap or pump outlet* any attachments that may cause splashing is removed from the tap. The dirt from outside is wiped off using a clean cloth. The tap is turned on at maximum flow rate and water is allowed to flow for 1-2 minutes. Tap is sterilised for a minute with flame using gas burner, lighter or ignited cotton wool soaked in spirit. Tap is opened and water is allowed to flow at medium rate for 1-2 minutes. Container is opened for collecting the sample and water is filled by holding the bottle under the water jet. A small airspace is left to facilitate shaking at the time of inoculation prior to analysis. The cap is stoppered and brown-paper is fixed in place with the string.

b. *Sampling from reservoir* is done by opening the bottle under sterilised conditions. The bottle is filled by holding it by the lower part, submerging it to a depth of about 20 cm, with the mouth facing slightly upwards. If there is a current, the bottle should face the current. The bottle is stoppered and packed as described earlier.

c. *Sampling from a dugwell* is done by attaching a stone of suitable size to the sampling bottle with a piece of string. A 20 metre length of clean string is tied on the bottle and to a stick. Bottle is opened as described above and lowered into the well. Without touching the sides of the well the bottle is immersed completely in the water and lowered down to the bottom of the well. When the bottle is filled, it is pulled out. A little water is discarded to provide airspace. The bottle is stoppered.

The sample is examined at the earliest preferably within one hour or is quickly transported to the laboratory keeping in cool container away from sulight. It should be positively examined within 6 hours of collection. Where delay is anticipated, the water can be filtered using a membrane filter and filter can then be transported on an absorbent pad saturated with transport medium.

Methods of Analysis

Two methods have been developed for the detection of indicator bacteria in water: multiple tube method and membrane filter method.

Multiple tube method. In the multiple tube method different amounts of water to be tested are added to tubes containing a suitable culture medium. The bacteria present in the water reproduce and, from the number of tubes inoculated and the number with a positive reaction, the most probable number (MPN) of bacteria present in the original water sample can be determined statistically.

The multiple tube method is applicable to all kinds of water: it can be used with clean, coloured, or turbid water containing sewage or sewage sludge, or mud and soil particles, provided that the bacteria are homogeneously distributed in the prepared test samples.

Method. Measured volumes of water and dilutions of water are added to a series of tubes containing a liquid indicator growth medium. The indicator medium used is MacConkey broth containing bromocresol purple to indicate colour change. An inverted Durham tube is placed in each bottle to detect the presence of gas.

The media receiving one or more of the indicator bacteria show growth and a colour change which is absent in those receiving an inoculum of water without indicator bacteria. The following tubes are put up:

a. One 50 ml of water to 50 ml of double strength medium
b. 5, 10 ml quantities each to 10 ml double strength medium
c. 5, 1 ml quantities each to 5 ml single strength medium
d. 5, 0.1 ml quantities each to 5 ml single strength medium.

From the number and distribution of positive and negative reactions, MPN of indicator organisms in the sample may be estimated by reference to statistical tables. The test gives *presumptive coliform count* as the reaction observed may occasionally be due to the presence of some organisms other than coliforms.

For highly contaminated waters smaller volumes are used. The bottles are incubated at 37ºC and examined after 24 hours. The presumptive positives are read and remaining negative bottles are reincubated for another 24 hours. Any further positives are added to the previous figures. The probable number of coliforms are read from the probability tables of McCrady.

Eijkman test (Differential coliform test or confirmed *Esch. coli* count). Eijkman test is usually done to confirm that the coliform bacilli detected in the presumptive test are *Esch. coli.*, as some spore—bearing bacteria give false-positive reactions in the presumptive coliform test. After the presumptive test, subcultures are made from all tubes showing acid and gas to fresh tubes of single strength MacConkey medium which is brought to 37°C. These tubes are incubated at 44°C in thermostatically controlled water baths and examined after 24 hours. Those tubes showing acid and gas are that containing *Esch. coli,* the number is read from the McCrady table. *Esch. coli* can be confirmed by plating on solid media and testing for indole production and citrate utilisation.

Detection of Faecal Streptococci

Faecal streptococci belong to Lancefield's group D and normally occur in human and animal faeces. Subcultures are made from all positive bottles in the presumptive coliform test into tubes containing 5 ml of glucose azide broth (great caution should be exercised in handling and discarding this medium as sodium azide is highly toxic). The presence of *Strep. faecalis* is indicated by the production of acid in the medium within 18 hours at 45°C. Further confirmation can be done by plating on to MacConkey's plates from these tubes and looking for typical colonies. Bile salt in MacConkey's medium can be replaced by 1% Teepol 610 or 0.1% sodium lauryl sulphate. Find out the most probable number from the proportion of tubes in the presumptive coliform test.

Detection of *Clostridium perfringens*

Cl. perfringens is a normal inhabitant of the intestine. It is a sulfite reducing clostridium and can be used as an indicator of faecal pollution. The organisms in this group are characterised by their ability to form spores and to reduce sulfite to sulfide. This feature is utilised in media for the presumptive detection of clostridia by the formation of a black precipitate of iron sulfide. Both multiple tube method and membrane filtration technique can be used for the detection of sulfite-reducing clostridia. The varying quantities of water in question are inoculated in litmus milk medium, and incubated at 37ºC for 5 days. A typical **stormy clot** reaction together with acidity confirms the presence of *C. perfringens.* Motility and nitrate reduction test may be done if further confirmation is required.

Membrane-Filter (MF) Method

In this method a measured volume of water is filtered through a membrane which retains the bacteria on its surface. The membrane is then incubated on a suitable selective medium, allowing the bacteria to reproduce and to form colonies. The number of colonies counted is directly related to the bacteriological content of the water sample being analysed. This method has not been as extensively used as multiple-tube method. It is not suitable for turbid water but may otherwise have several advantages (Table 77.5).

Table 77–5. Advantages and limitations of MF method

Advantages	*Limitations*
Rapid	Turbid water interferes with bacterial growth
Easy and economical	High noncoliform bacteria may interfere with counting of coliforms
Gives direct results useful in difficult rural areas	Procurement of filters
Sample can be used in field	Toxic substances present in water may be absorbed by filter and inhibit the bacterial growth

Frequency of Examination

The daily sampling of water supplies serving a population for 10,000 or less is neither required nor practical. The emphasis should be on proper control of chlorination. The water supplies serving more than 100,000 population should be examined daily.

Standards

The classification of drinking water according to bacteriological tests is given in Table 77.6.

Virological Examination

Enteroviruses, echoviruses, parvoviruses, reoviruses and adenoviruses have been found in water. Large scale water-borne hepatitis outbreaks due to enterically transmitted Non A Non B hepatitis virus (now called as hepatitis E) have been very frequently repor-

Table 77–6. Classification of drinking water based on bacteriological tests

Class	*Grade*	*Presumptive count (per 100 ml)*	*Esch. coli count (per 100 ml)*
I	Excellent	0	0
II	Satisfactory	1-3	0
III	Suspicious	4-10	0
IV	Unsatisfactory	10	0,1 or more

ted from many developing countries. Viruses enter the water environment primarily by way of sewage discharges. Sewage treatment reduces the concentration of viruses 10-100 fold but *no treatment leaves the effluent free from viruses*.

Procedures for isolation of all the viruses present in the water are not as yet available. It is not practical to test water for virological examination as frequently as bacteriological examination. The collection should be done in the same way as for bacteriological examination, but at least 2 litres of water should be collected. Since bacterial presence is taken as indicator of the presence of viruses in the water, their direct demonstration becomes unnecessary.

The water declared "safe" by coliform testing can still transmit hepatitis E virus infection, because the levels of chlorine required to kill the indicator organism is not sufficient to kill the hepatitis E virus.

Examination for Specific Pathogens

Though detection of specific pathogens has no role in routine examination of water, it may be indicated in special situations such as outbreak investigations or evaluation of a new source. These may be required to detect *Salmonella sps, Shigella sps, Vibrio cholerae,* enteropathogenic *Esch. coli., Yersinia enterocolitica,* and *Campylobacter sps.* There are defined standard procedures for each one of these pathogens, but examination will include some, if not all, of the following steps:

- Concentration of the organism in the sample
- Inoculation into enrichment broth
- Subculture on selective media and
- Biotyping and serotyping of the suspect colonies.

It is better to use more than one method while isolating these pathogens.

EXAMINATION OF MILK

Milk is an excellent vehicle for a great variety of disease agents, since it is a wonderful medium for the growth of many pathogenic bacteria. Milk contains bacteria of different types derived from the animal itself, human handler, milking machine if used and contaminated vessels, polluted water, flies and dust, etc. The number of bacteria in the milk varies from animal to animal.

Table 77–7. Types of bacteria in milk

Acid forming	*Alkali forming*	*Gas forming*	*Proteolytic*
Lactic streptococci	*Alkaligenes*	*Cl. welchii*	*B. subtilis*
Lactobacilli	*Achromobacter*	*Cl. butyricum*	*B. cereus*
Staphylococci	–	Coliforms	*Proteus vulgaris*
			Staphylococci.

Types of Bacteria in Milk

Various types of bacteria encountered in milk are given in Table 77.7

The milk even under best precautions is *never sterile*. Freshly drawn milk is both bactericidal and bacteriostatic. This activity is thermolabile being destroyed in 15 minutes at 75ºC and in 2 minutes at 80-90°C and disappears a few hours after the milk has been withdrawn. The bacteria rapidly multiply in milk in warmer climate.

Milk-borne Diseases

These can be classified into two main heads:

a. Infections of animals that can be transmitted to man
b. Infections primarily of man that can be transmitted through milk. These diseases are listed in Table 77.8.

Table 77–8. Milk-borne diseases

Infections that can be transmitted to man from animals

- Tuberculosis
- Brucellosis
- Streptococcal infections
- Staphylococcal diarrhoea
- Salmonellosis
- Q-fever
- Cowpox
- Foot and mouth disease
- Anthrax
- Leptospirosis
- Milker's nodes
- Diarrhoea due to:
  - — *Campylobacter*
  - — *Yersinia enterocolitica*

Infections that can be transmitted from man to man with milk

- Enteric fever
- Cholera
- Shigellosis
- Enteropathogenic *Esch. coli* diarrhoea
- Staphylococcal food poisoning
- Streptococcal infections
- Diphtheria
- Tuberculosis
- Hepatitis

Most of these pathogens can be destroyed either by pasteurisation or boiling of milk. These diseases can be prevented by:

a. Maintenance of dairy herds free from dangerous infections
b. Maintenance of clean conditions of collection and storage
c. Pasteurisation of milk.

Pasteurisation of Milk

It is defined as the heating of milk to such temperatures and for such periods of time as are required to destroy any pathogens that may be present while causing minimal changes in the composition, flavour and nutritive value of the milk. Pasteurisation can be achieved by any of the following 3 methods:

a. *Holder method* (*Vat Method*). In this process, milk is kept at 63-66°C for at least 30 minutes and then quickly cooled to 5°C. This is useful for smaller set ups.
b. *High temperature, short time (HTST) method*. In this, milk is heated to a temperature of 72°C for about 15 seconds and is then rapidly cooled to 4°C. This is now most frequently used method and is suitable for pasteurising large quantity of milk.
c. *Ultra high temperature (UHT) method.* Milk is rapidly heated usually in two stages to between 125°C and 145°C for a few seconds only. It is then rapidly cooled and bottled as quickly as possible.

Pasteurisation kills nearly 90% of the bacteria in milk including the more heat resistant tubercle bacilli and the Q-fever organisms, but it does not destroy thermoduric bacteria or bacterial spores. Pasteurised milk should be kept cold until it reaches the consumer. Hygienically produced pasteurised milk has a keeping quality of not more than 8-12 hours at 18°C in tropical countries. Pasteurised milk must not contain any coliform bacilli in 0.01 ml.

BACTERIOLOGICAL EXAMINATION OF MILK

Collection of Sample

From a bulk container, the contents must be mixed thoroughly and the sample is collected well below the surface with a sterile dipper and poured into a sterile stoppered or screw capped bottle of about 125 ml capacity. The sample should be kept in an insulated container and dispatched to the laboratory without delay and examined as soon as possible after arrival in the laboratory. Unopened bottle or polythene bag should be similarly transported as such. Various bacteriological tests used are listed in Table 77.9.

Table 77–9. Tests for bacteriological examination of milk

- Viable count
- Coliform test
- Methylene blue test
- Resazurin test
- Phosphatase test
- Turbidity test
- Examination for specific pathogens

Viable Count

This is done by doing plate counts with serial dilutions of the milk sample. The tests are costly in labour and materials, the results are not available for 72 hours and errors are mainly due to the common clumping of bacteria. The dilutions of milk are made as 1:10, 1:100, 1:1000 with sterile Ringer's solution. After thorough mixing 1 ml of the appropriate dilution is mixed with 10 ml of preheated and cooled yeast extract milk agar and then poured in plates which are incubated for 72 hours at 30-31°C. Count the number of colonies after 72 hours incubation and multiply by the dilution factor and report the results as the number of viable bacteria per ml of milk.

Coliform Test

This test is done by inoculating varying dilutions of milk into 3 tubes of liquid MacConkey medium (single strength). After incubation at 37°C for 48 hours production of acid and gas is looked for as the evidence of the presence of coliform bacilli. The absence of acid and gas from at least two of the three tubes is accepted as indicating that the milk has passed the test. Coliform contamination usually occurs from dirty utensils, dust and dairy workers. The presence of coliforms in the milk that passes phosphatase test indicates that contamination has taken place after pasteurisation. The coliforms should be absent in 1 ml of milk.

Methylene Blue Test

It is an indirect method for detection of microorganisms in milk and is an economical substitute for viable count testing. It depends upon the dye being reduced and so decolourised by the metabolism of any large number of viable bacteria present in the milk. The rate of reduction is taken as a measure of the degree of bacterial contamination. Under standard test conditions raw milk is considered satisfactory if it fails to decolourise the dye in 30 minutes.

Resazurin Test

It is almost identical to methylene blue dye test, but the dye resazurin on reduction passes through a series of colour changes from blue to pink to colourless, which depends upon degree of contamination.

Phosphatase Test

The enzyme phosphatase is normally present in the milk. It gets inactivated during pasteurisation of milk. Its presence in milk after pasteurisation indicates incomplete pasteurisation. The test depends upon the ability of the enzyme to liberate p-nitrophenol from disodium p-nitrophenyl phosphate and thereby produce a yellow colour that can be quantified by a colorimeter.

Turbidity Test

This is a definitive test for sterilised milk, distinguishing it both from untreated milk and pasteurised milk. The degree of heating necessary for sterilisation causes all the heat coaguable proteins in milk to become precipitable by ammonium sulphate. If the amount of heat applied to milk is insufficient for sterilisation, some of its protein will not be precipitated by ammonium sulphate and will be detected by its coagulating and giving turbidity, when a filtrate of the ammonium sulphate treated milk is boiled. Absence of turbidity indicates that the milk has been heated to at least 100°C for at least 5 minutes.

EXAMINATION OF MILK FOR SPECIFIC PATHOGENS

Tubercle Bacilli

After thoroughly mixing the milk sample centrifuge 50 ml in each of two tubes at 1500 g for 30 minutes. Make a smear from the sediment and resuspend the remainder of the deposit in 2.5 ml sterile saline solution. Inject in two guinea pigs, sacrifice one guinea pig at 4 weeks and another at 8 weeks interval after inoculation and look for evidence of tuberculosis. This obviously is not a good test and hence is not in use. Tubercle bacilli should be isolated in culture as microscopy alone is not satisfactory.

Brucellae

The brucellae can be isolated from *cream* from the milk sample by inoculating on serum glucose agar or injecting intramuscularly in guinea pigs. The animal can be sacrificed after 6 weeks and spleen used for culture of *Brucella* organisms and serum used for antibody demonstration by *milk ring test*. It is a very sensitive test for the presence of brucella antibodies in the milk of infected cows, which it may detect even in the bulk supply from a dairy herd. To confirm a positive milk ring test *whey agglutination test* can be used to detect the antibodies.

The sediment of the centrifuged milk, particularly in cases of suspected food poisoning should also be examined for *Staphylococcus aureus, Salmonella, Campylobacter* and *Yersinia enterocolitica.*

No single test is completely satisfactory for testing the quality of milk. Each test has plus and negative points. It is important to perform these tests under good quality control and on appropriate samples for them to be useful.

EXAMINATION OF AIR

i. Importance of Air

Foreign substances have always been present in the air at all times and at all places hence truly speaking there has never been pure air. The level of bacterial contamination of air is usually expressed as the number of bacteria carrying particles per m^3 or per ft^3. Conventionally ventilated rooms commonly show contamination levels between 150/m^3 to 4000/m^3. The higher levels are observed when there are many occupants, much bodily movements or other dust raising activities. An adult male inhales about 15 m^3 and a baby about 1 m^3 of air in a day, hence possibility of a person becoming infected will be greatest if he is exposed to a high concentration of airborne pathogens.

ii. Factors Affecting Air Quality

The bacterial content of air depends on location whether it is outdoor or indoor. Most of the bacteria in the open air are non-pathogenic. The factors affecting the bacterial content of the outdoor air include density of human and animal population, nature of soil, amount of vegetation, atmospheric conditions such as humidity, temperature, wind conditions, rainfall and sunlight.

iii. Droplet-nuclei and Disease Transmission

Infective materials are seldom carried for more than short distances and their capacity to cause infections is impaired except in rare instances such as foot and mouth disease. Pathogenic bacteria do not multiply in the air. In contrast to the outdoor air, indoor air may have bacteria distributed in droplet nuclei from nose and mouth. *Droplet nuclei* are a type of particles implicated in the spread of airborne infections. They vary in size from 1-10 µm. They may be formed by

evaporation of droplets coughed or sneezed into the air or by aerosols. These can also be formed accidentally in laboratories, abattoirs and autopsy rooms; particles in the range of 1-5 µm are liable to be easily drawn into the alveoli of the lungs and may be retained there. Diseases caused by droplet nuclei include tuberculosis, influenza, chickenpox, measles, Q-fever and many respiratory infections.

iv. Larger Droplets in Disease Transmission

Some of the larger droplets which are expelled during coughing or sneezing settle down by their sheer weight on the floor, carpet, furniture, bedding etc. Some of the pathogens can survive much longer under such conditions. During dusting and bed-making the dust is again released in the air and become airborne. These dust particles can also be blown by air. Important diseases transmitted by dust particles include streptococcal and staphyloccal infections, tuberculosis, Q-fever, psittacosis, and coccidioidomycosis etc.

Need of Air Quality Monitoring

Bacteriological examination of air is required in following conditions:

a. Surgical theatres
b. Premises where food articles are prepared and packed
c. Premises where pharmaceutical preparations are made
d. Hospital wards where nosocomial infections have occurred.

When a room is vacated and left undisturbed, the bacterial content of the air falls to a low level in the course of about 30 minutes.

Methods of Bacteriological Examination of Air

The methods for bacteriological examination of air are of two types.

a. Those methods which measure the rate at which bacteria-carrying particles settle down, e.g. *settle plate method*
b. Those that count the number of bacteria-carrying particles contained in a given volume of the air, e.g. *slit sampler method*.

i. *Settle Plate Method (Sedimentation Method)*

Petri dishes containing an agar medium of known surface area are left open for a measured period of time. Large bacteria-carrying dust particles settle onto the medium. The plates are incubated and a count of the colonies formed shows the number of settled particles that contained bacteria capable of growth on the medium used and under the conditions of incubation. Blood agar plates can be used to detect both pathogens and non-pathogens. Selective media can be used for special pathogens. The plates are incubated at 37°C for 24 hours. The number of colonies of bacteria is counted and the number of bacterial content of the air calculated. This method is simple but measures only the rate of deposition of large particles from air and not the total number of large and small bacteria-carrying particles suspended in it.

ii. *Slit Sampler Method*

This method overcomes the disadvantages of the sedimentation method. It draws in air from the environment at a fixed rate and causes the suspended particles to impinge on the surface of the agar plate where on incubation, each forms a colony. The efficiency even for detection of small particles is very high. The operator should avoid making unnecessary movements in the vicinity of the sampler and stand away from it while the sample is being taken, as it falsely increases the bacterial counts.

iii. *Air Centrifuge*

This involves the principles of centrifuging particles on a culture medium borne on a plastic strip. After sampling, the strip is removed from the instrument and incubated for 48 hours at 37°C when colonies can be counted. It has 2 disadvantages over slit sampler method, one, sample size cannot be accurately controlled and second it is less efficient for particles below 5 µm in size.

iv. *Sweep Plate Method*

This method is used to culture the dust particles deposited by air on personal clothing, bed clothes and domestic fabrics. An open culture plate is swept 8-10 times over the dusty surface and plate incubated and colonies counted after overnight incubation.

v. *Swab Method*

A swab soaked in nutrient broth can be used to collect sample from the dust lying on the floor.

Air Contamination Standards

The level of bacterial contamination of air is usually expressed as the number of bacteria carrying particles per mt^3 or per ft^3. It is suggested that in a surgical theatre, the bacterial count should not exceed $10/ft^3$ and in theatres catering to burns patients and surgery on central nervous system the count should not exceed $1/ft^3$. Now even more stricter standards are recommended.

78 Nosocomial Infections

Hospitals have always been considered as places which help people in getting cured of the illnesses. However, potential of these health care institutions as transmitters of diseases was recognised long time ago. Florence Nightingale is on record to caution the physicians that the hospitals should do no harm to the patients. In spite of all the precautions it has never been possible to create in the hospitals an absolute environment which is free of microorganisms which have the potential to cause disease in otherwise weakened body of the patient. Such infections which are acquired in the hospitals are known as *hospital infections* or *nosocomial infections*.

Definition

Nosocomial infections are the infections which develop during hospitalisation and were not incubating or present at the time of admission to the hospital. It includes incidence in which a single microorganism spreads from person to person or from a common source in the hospital environment. These infections may present as sporadic, endemic or in epidemic situations. Unfortunately it is only the epidemic situations which draw the attention whereas sporadic and endemic situations present far more greater challenge in terms of mortality and morbidity as well as in control or reduction of incidence.

Source of Infection

The source of causative infecting organism may be *exogenous*—from another patient or a member of hospital staff or from the inanimate environment in the hospital. It may be *endogenous* from the patient's own flora which at the time of infection may invade the patient's tissues spontaneously or be introduced into them by surgical operation, instrumental manipulations and nursing procedures (Fig. 78.1).

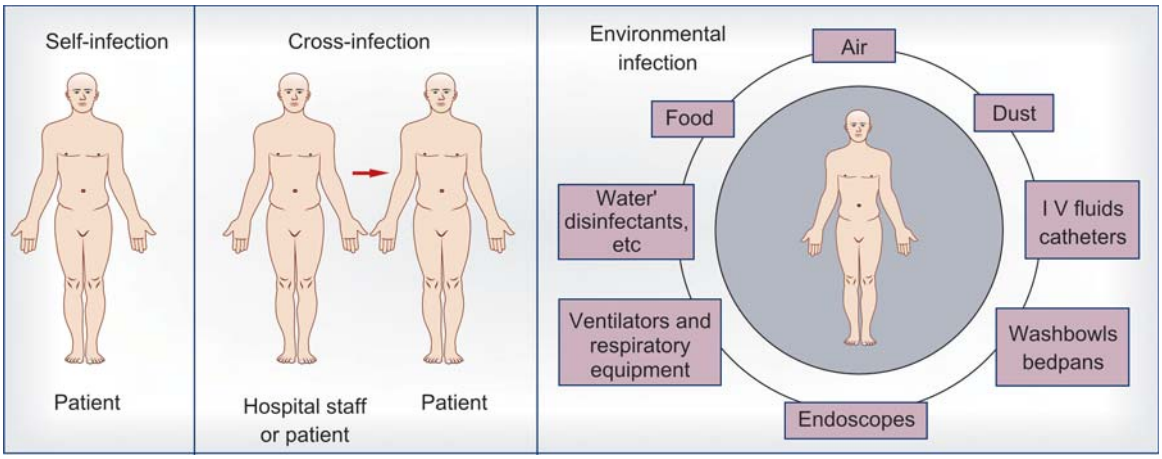


Fig. 78–1. Acquisition of hospital infections

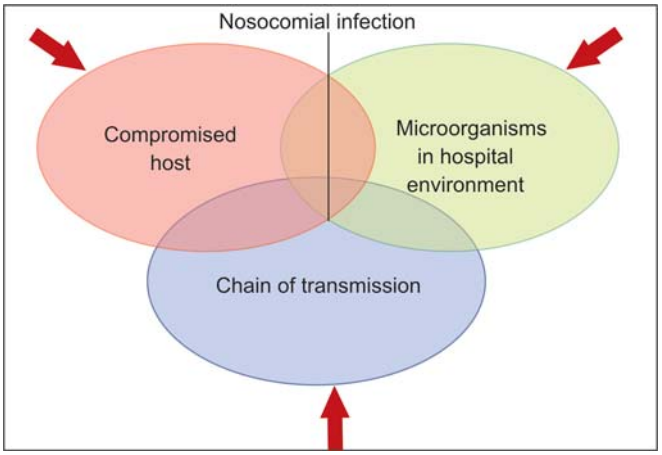


Fig. 78–2. Interplay of factor for nosocomial infections

Persons at Risk

Hospital-acquired infections may affect the discharged, inpatients, outpatients, hospital staff and subsequently it may spill over in the community.

Predisposing Factors for Nosocomial Infections

A large number of factors contribute towards deciding the severity of the infection acquired in the hospital (Table 78.1, Fig. 78.2).

Table 78–1. Predisposing factors for nosocomial infections

- Hospital environment heavily laden with a variety of pathogens. Organisms present in air, dust, antiseptic lotion, water and food or may spread from sheddings from the patients.
- Hospital microbial flora is usually multi-drug resistant. Patients have impaired defence mechanism due to: Disease therapy and investigations in the hospital.
- Instrumentation in hospitals may introduce infection.
- Blood, blood products and intravenous fluids may transmit many infections.
- Accidental inoculation of infectious material

Prevalence of Nosocomial Infections

Reliable data regarding prevalence of nosocomial infections in developing countries is very scanty. Even in developed countries, surveys of hospital infection on large scale are not undertaken frequently. However, limited data available shows the prevalence of nosocomial infections of around 5-10% in the developed countries where the patient load in the hospital is much less, and the safety precautions are used more frequently as compared to most of the developing countries. Hence, the magnitude of the problem is likely to be much more in developing countries.

Modes of Spread

Various modes of spread of nosocomial infections are:

a. Airborne
b. Infection by contact
c. Infections acquired from food
d. Infections associated with water
e. From hospital equipment
f. Infections by inoculation.

Common Agents of Nosocomial Infections

Any pathogen present in the hospital environment can cause infection because of the compromised immune status of some of the patients. However, organisms which can survive for long periods in hospital environment and in disinfectants are specially equipped to cause hospital infection. The important pathogens capable of causing nosocomial infection have been enumerated in Table 78.2.

Escherichia coli is one of the most frequently encountered bacterium in urinary tract infection. *Staphylococcus*

Table 78–2. Organisms causing nosocomial infections

Gram-positive	*Staphylococcus aureus* *Streptococcus pyogenes* *Staphylococcus epidermidis* *Streptococcus pneumoniae* *Clostridium difficile* *Clostridium perfringens* *Clostridium tetani*
Gram-negative	*Escherichia coli* *Citrobacter* *Klebsiella* *Serratia* *Enterobacter* *Proteus* *Pseudomonas* *Legionella*
Viruses	Hepatitis B Hepatitis C Hepatitis D HIV Herpesviruses Cytomegalovirus Influenza virus Enteroviruses SARS CoV
Fungi	*Aspergillus* *Candida albicans*
Parasites	*Toxoplasma gondii* *Entamoeba histolytica* *Pneumocystis jiroveci (earlier known as P. carinii)* *Cryptosporidium*

aureus continues to be one of the most important organism in nosocomial infections. The ability of *Pseudomonas aeruginosa* to grow in moist conditions with simple nutrients and its comparative resistance to antibiotics has established it as one of the foremost pathogens. Viral infections are particularly important in neonatal and pediatric patients as well as immunocompromised patients. Some viruses such as SARS CoV spread rapidly in hospital environment. Fungal infections continue to increase in hospital as more effective and broad spectrum antibacterial agents are deployed and more and more use of immunosuppressive agents is done.

Common Types of Hospital Infections

These include:
- Wound infections
- Urinary tract infections
- Respiratory infections
- Skin infections
- Bacteraemia and septicaemia
- Gastrointestinal infections.

Wound infections may be due to *Staph. aureus*, *Streptococcus pyogenes* or *Pseudomonas aeruginosa*. Nosocomial tetanus is now on decline. *Esch. coli* continues to be the commonest organism causing urinary tract infections. Burns provide a suitable site for bacterial multiplication. Burn is a richer and more persistent source of infection than surgical wound. Most hospital acquired infections of urinary tract are associated with urethral catheterisation. The risk of septicaemia may be avoided by pre-operative or perioperative administration of suitable antibiotic to patients with colonised urine. Some 15-20% of all hospital acquired infections are of the respiratory tract. Any time indwelling device may become colonised and give rise to bacteraemia and septicaemia. The source of colonising organisms is generally thought to be the skin of the patient. Hospital infections are particularly important in geriatric and long-stay facilities and neonatal units.

ROLE OF MICROBIOLOGY LABORATORY IN PREVENTION AND CONTROL OF NOSOCOMIAL INFECTIONS

The hospital laboratory has important responsibilities in surveillance, control and prevention of nosocomial infections. These include:

a. Working with other hospital personnel in infection control activities
b. Accurately identifying organisms responsible for nosocomial infection
c. Timely reporting of laboratory data relevent to infection control
d. Supporting investigation of specific hospital infection
e. Conducting studies of hospital personnel or environment.

Diagnosis of Hospital Infection

Aetiological diagnosis is made by routine diagnostic procedures depending upon the suspected diagnosis. Outbreak situation should be dealt with as rapidly as possible. Investigations of an outbreak of nosocomial infection may require isolation and identification of isolates not only in specimens from patients but also in those taken from personnel who might be colonized with the outbreak strain and from environmental objects implicated by epidemiological investigation. The cultures may have to be made from blood products, transfusion fluids, intravascular therapy equipment, from tubes, containers, surfaces, disinfectants, antiseptics, cultures from floor and equipment and water, ice and other food articles. Use of selective media may be made to reduce the workload in the laboratory. Nosocomial pathogens isolated should be stored for future reference.

Environmental or employee survey are not recommended. Monitoring of sterilisation and periodical sampling of disinfected equipment is recommended. Carriers (e.g. typhoid carriers) should be detected and suitably treated.

Use of Epidemiological Markers

An epidemiological marker is a test which establishes the similarity or difference of the organism. This is important to find out the source of infection. Various typing systems and organisms where these are used are given in Table 78.3.

Table 78–3. Typing systems for different organisms

Typing system	*Organisms*
Antibiogram or Resistogram (resistance to heavy metals)	*Staphylococcus aureus* *Staph. epidermidis* *Clostridium difficile* *Pseudomonas aeruginosa* *Salmonella* *Proteus*
Biotyping	*Haemophilus influenzae* *Escherichia coli* *Klebsiella species*

Contd...

Contd...

Typing System	*Organisms*
	Staph. aureus *Staph. epidermidis* *Proteus species*
Phage typing	*Staph. aureus* *Salmonella (typhi, paratyphi A, typhimurium, etc).* *Clostridium difficile* *Escherichia coli* *Klebsiella pneumoniae* *Staph. epidermidis* *Pseudomonas aeruginosa*
Bacteriocin susceptibility	*Klebsiella species* *Pseudomonas aeruginosa* *Streptococcus pyogenes* *Escherichia coli* *Clostridium difficile* *Shigella species* *Enterobacter species*
Serotyping	*Campylobacter* *Escherichia coli* *Pseudomonas aeruginosa* *Salmonella species* *Shigella species* *Staph. epidermidis*
Serum opacity factor	*Streptococci*
Analysis of marker proteins	*Staph. aureus* *Haemophilus influenzae* *Clostridium difficile*
Analysis of enzyme production	*Pseudomonas aeruginosa* *Staph. aureus*
RNA electrophoresis	Rotavirus
Cytotoxicity assay	*Clostridium difficile*
Diene's reaction	*Proteus mirabilis*
Reverse phage typing	*Staph. aureus*
Plasmid profile	Multiple organisms

To make the epidemiological markers a sensitive method combination of more than one system may be required.

Control and Prevention

It is necessary to remember that patients admitted with *community acquired* infection are relevant to the problem of nosocomial infections in so far as ability of their community acquired infection to spread to susceptibles in a hospital setting.

Various methods which can help in prevention of nosocomial infections are shown in Table 78.4. These are to be applied collectively and not singly because of the complex and inter-linked environment of the hospital.

Table 78–4. Prevention of nosocomial infections

- Hand washing
- Intelligent use of instrumentation
- Limitation of use of antibiotics
- Prophylactic antibiotics in specific situations for short periods
- Limitations of transfusions
- Barrier precautions
- Surveillance
- Frequent change of intravenous lines

Infection Committee

It is essential to establish an infection committee in the hospital which should consist of physicians, nurses and administrators and laboratory personnel. The committee should meet regularly and frequently and decide about hospital policy related to infection control.

79 Blood Transfusion Associated Infections

Infectious complications of blood transfusion became recognised as a significant problem in the early part of the 20th century. By World War I, donor infections which could be transmitted by blood transfusions occurred often enough so that persons with malaria, syphilis, and fever were excluded from blood donation in some countries. The rapid increase in the number of transfusions in the years during and after World War II led to a sudden awareness of post-transfusion infections such as hepatitis, cytomegalovirus (CMV) and Epstein-Barr virus (EBV). These infections may not be recognised in terminally ill patients because the underlying illness may mask or alter the clinical picture. Various infections which can be transmitted by blood transfusion are listed in Table 79.1.

Transfusion Associated HIV Infection

The first case of transfusion associated AIDS was reported in US in an infant who had received exchange transfusion in 1981. Subsequently cases were recognised among adults whose only potential risk-factor was blood transfusion in the 5 years preceding diagnosis of the disease. In 1984, the isolation of retrovirus from peripheral blood lymphocytes of a blood donor/recipient pair, both of whom subsequently became ill and were diagnosed as AIDS, proved additional evidence that this agent could cause AIDS and it could be transmitted through blood transfusion. The evidences of transmissibility of HIV infection through blood and blood products are:

a. More reports of AIDS in haemophiliacs
b. AIDS in children with history of receiving blood and blood products
c. AIDS in infants born to high risk mothers
d. Transmission pattern similar to hepatitis
e. Isolation of HIV from blood lymphocytes of transfused patients
f. AIDS cases due to transfusion traced to donors positive for HIV infection. However, risk of transmission seems to be greater with hepatitis B (Table 79.2).

Table 79–1. Organisms that can be transmitted through blood transfusion

Viruses	*Parasites*	*Bacteria*	*Fungi*
Hepatitis B virus	*Plasmodium vivax*	*Treponema pallidum*	*Aspergillus*
Hepatitis C virus	*P. falciparum*	*Brucella abortus*	*Penicillium*
Hepatitis D virus	*P. malariae*	*Proteus species*	*Hormodendrum*
HIV 1 and 2	*P. ovale*	*Esch. coli*	
HTLV I, II	*Toxoplasma gondii*	*Klebsiella species*	
Hepatitis A virus	*Trypanosoma cruzi*	*Micrococcus*	
Cytomegalovirus	*Wuchereria bancrofti*	*Enterobacter*	
Epstein-Barr virus	*Brugia malayi*	*Salmonella cholerasuis*	
Parvovirus	*Loa loa*	*Pseudomonas species*	
Ebola virus	*Leishmania donovani*	*Staph. epidermidis*	
Lassa virus	*B. microti*	*R. rickettsii*	
Yellow fever virus			
Dengue virus			

Table 79–2. Comparison of risk of transmission of HBV, HCV and HIV

Mode of transmission	*Risk of transmission (%)*		
	HBV	*HCV*	*HIV*
Perinatal			
HBeAg +ve mothers	70-90	5	14-39
HBeAg -ve mothers	10-20	–	14-39
Sexual route	30	5	10-15
Needle stick injury	10-30	3	0.3

Treponemal Infections

Transmission of *Treponema pallidum,* the causative agent of syphilis, by blood transfusion is now mainly of historic interest except where fresh blood or fresh blood components are used. *T. pallidum* does not survive for more than 72 hours in the blood stored at +4°C. No case of *T. pallidum* infection has been reported following transfusion of stored blood. Transfusion-transmitted disease begins with the secondary stage of syphilis with a generalised rash, fever and lymphadenopathy. The incubation period from transfusion to the appearance of symptoms ranges from 4 to 14 weeks.

Transfusion Malaria

The infectivity of *P. falciparum* persists for a couple of years, that of *P. vivax* for about 5 years while *P. malariae* infectivity may apparently last a life time. While infected whole blood or packed RBCs are the most common source of transfusion malaria, leucocyte concentrates, platelet concentrates and fresh plasma are known to have contained the infective agent. Haemodialysis has also become associated with transfusion malaria. Storage of blood products may reduce but does not eliminate the infectivity of malarial parasites. Cryopreserved parasitised RBCs are known to stay infective for at least two years. The degree of parasitemia in transfusion malaria can become very high with *P. falciparum* and *P. malariae* while it rarely exceeds 2% in *P. vivax* infection on account of this parasite's predilection for reticulocytes. The relative risk of acquiring transfusion malaria depends on the origin and exposure of the donors, the number of blood units transfused and eventually the use of pooled blood.

Fungal Infections

Few fungal infections have been reported in recipients of blood transfusions which could reliably be said to have been transmitted by blood. However, contamination of blood units by contaminants such as *Hormodendrum, Aspergillus* and *Penicillium* spp is reported. In addition, fungal spores are more prevalent in ambient air than bacteria in some geographic areas, and although most are not pathogenic, the risk for a blood recipient compromised by disease may be significant.

BLOOD CULTURE

Blood culture is one of the most important investigation in clinical microbiology.

Indications

Blood culture is indicated:

- where the possibility of septicaemia or bacteraemia is suggested by the clinical features
- for diagnosis of pyrexia of unknown origin.

The isolation of a bacterium from the blood of a patient with a local infection such as wound sepsis or pneumonia is valuable in:

a. Indicating the urgent need for antibacterial therapy
b. Identifying the species of bacterium against which therapy should be directed
c. Distinguishing contaminant from real pathogen.

Collection of Sample

Every care must be taken to prevent contamination of the specimen during its collection from the patient and its examination in the laboratory. The procedure to be followed include:

a. Wash, clean and dry hands before attempting to draw the blood sample. Wear gloves, if feasible, to prevent the risk of acquiring hepatitis B or HIV infection
b. Disinfect the venepuncture site on the patient's skin by applying 70% isopropyl alcohol in water with 1% iodine for at least 1 min and allow to dry
c. Collect the sample of blood, with precautions to avoid touching and recontaminating the venepuncture site. Change the needle with care to avoid touching the shaft or contaminating the operator before inoculating the required volume into each blood culture bottle.

Volume of Blood to be Cultured

In most bacterial infections of the blood in man the organisms are not numerous and it is essential for their demonstration to use relatively large amount (5-10 ml) of blood as the inoculum. As the blood's natural bactericidal or bacteriostatic action may interfere with the growth of any bacteria present, this effect should be neutralised by diluting the blood in medium. A 10 ml of blood sample in 100 ml of medium is recommended but for practical viewpoint 5-7 ml of blood can be added to 50 ml of medium. For specific neutralisation of

antibiotics in the blood specific antidotes can be added in the medium, e.g. to neutralise penicillin, penicillinase is used while para-amino benzoic acid is used to neutralise sulphonamide.

A smaller bottle is used for neonates and young children and the volumes of the blood taken from these patients are appropriately smaller.

Culture Media

It is advisable to seed more than one medium for blood culture. The wide range of available media includes brain heart infusion broth, cooked meat broth, thioglycollate broth and diphasic medium. In suspected enteric fever, glucose bile broth should be used. As acid from the glucose fermentation tends to kill bacteria, the addition of glucose to all the media is not recommended.

Incubation

One set of bottles should be incubated in an atmosphere of air with 10% CO_2, here it is essential to loosen the caps of bottles during incubation, while another can be incubated under anaerobic conditions.

Subculturing Procedures

Growth may produce a generalised turbidity or there may be discrete colonies on the surface of sedimented red cells. It is important to make subcultures from all bottles to solid media as a routine, early growth sometimes produces no visible effect in the broth. Subcultures should be done under aseptic conditions and blood cultures immediately returned to incubator. The subcultures are recommended upto 5-7 days before declaring a negative result.

The Castaneda system using biphasic medium avoids the contamination problem associated with frequent subculturing.

Clot Culture

The clot culture is useful in the following ways:

a. Where blood culture bottles are not available, the clot culture can be used
b. Serum is available as an additional sample for examination
c. The organisms are held in the fibrin network and hence can evade the action of the specific and non-specific inhibitors.

The blood collected under aseptic conditions is allowed to clot in a sterile container and then clot is transferred to the blood culture bottle. The clot is either mechanically broken or is broken enzymatically using streptokinase. Streptokinase causes rapid clot lysis with release of bacteria trapped in the clot.

Contaminants

The contaminants encountered in blood culture include:

- *Staphylococcus epidermidis* (commonest)
- Diphtheroids
- Coliform bacilli
- Anthracoid bacilli

These contaminants should be carefully evaluated in immunodeficient individuals where these can be real pathogens. In such individuals repeated cultures should be made. And if a particular organism is repeatedly isolated and the isolates have similar antibiograms, biotypes or phage types than the isolates are real pathogens.

False Negative Results

The false negative results could be due to:

a. Too early collection of blood sample in the disease
b. Patient receiving antibiotics
c. Presence of strict anaerobes for which appropriate media have not been used
d. Inadequate blood sample cultured
e. Results based only on a single culture

Thus, particularly in cases of suspected endocarditis at least six samples collected at intervals of several hours in the course of 3-6 days should be cultured.

Automated Systems

These systems employ equipment that automatically detects an early sign of bacterial growth in a special blood culture bottle. The system most commonly used is Bactec. It depends on the release of radioactive CO_2 into the atmosphere in the culture bottle by the bacterial degradation of C^{14} containing nutrients in a special culture medium. There are separate culture bottles for aerobic and anaerobic pathogens.

The advantages of automated systems include:

a. Early detection of heavy septicemia
b. Avoidance of repetitive subcultures of the negative samples.

The drawback is the cost factor involved and radiation protection devices are required.

80 Quality Assurance in Microbiology

The term *quality assurance* as applied to health laboratory results is commonly used to refer to a set of checking procedures intended to ensure a reasonable degree of consistency in the observations made and the results reported.

Quality means meeting pre-determined requirements and therefore includes appropriateness.

Quality Assurance

A wide spectrum of quality improving activities is needed to ensure the reliability and usefulness of laboratory investigations. The components of quality assurance not only contain those that are related directly to analytic methods but also to pre-analytic and post-analytic factors which emphasize appropriate collection, transportation, storage and labelling of clinical material as well as precise interpretation of the results generated by laboratory analysis.

Thus, quality assurance may be defined as the monitoring of *people*, *equipment* and *materials*. Improvements brought about by quality assurance on any link of the chain may be nullified by weakness in other limbs. It is useful to remember that "quality" we are trying to improve is the quality of the patient care and not purely the academic quality of test performance.

Quality assurance has two components: Internal quality control (IQC) and external quality assessment (EQA).

Internal Quality Control

IQC is the set of procedures undertaken by the staff of a laboratory for continuously assessing laboratory work and the emergent results, to decide whether they are reliable enough to be released. IQC procedures vary considerably between laboratories and between disciplines, depending to some extent upon whether results are reported quantitatively, semi-quantitatively or qualitatively. The main objective of IQC is to ensure day-to-day consistency (precision). IQC is *concurrent* and *regular*.

External Quality Assessment or Proficiency Testing

EQA is the term which should be used in preference to *external quality control*. This refers to a system of objectively assessing laboratory performance by an external agency. The assessment is necessarily *retrospective* and *periodic*. This has no influence on the tested laboratory's output on the day of the test.

The objectives of EQA are:

a. To establish inter-laboratory comparability (accuracy)
b. To influence the reliability of future testing
c. To ensure credibility of the laboratory
d. In stimulating performance improvements.

Both IQC and EQA have proved to be necessary for ensuring the reliability of analytical procedures in a laboratory and the results emanating from it.

Consequences of Poor Quality

The consequences of poorly performed/reported investigations can be very damaging depending upon a particular situation. Some of these consequences are listed as under:

i. It may lead to *inappropriate action* which may be in the form of over-investigations, treatment or mistreatment
ii. It may lead to *inappropriate inaction* which may be in the form of lack of investigation when required or non-treatment
iii. It may lead to *delayed action* due to lack of results or delayed results
iv. It may lead to *loss of credibility* of the laboratory.

Thus, quality ensures **right result** at **right time** on **right specimen** from **right patient** using **right technique** to test it.

Implementation of IQC

Control materials are always used in the test of each batch. The results are accepted within the appropriate acceptance limits. Incorrect results are not released. The important points for IQC are:

a. The most important contributory factor to high standards of quality is competent and motivated staff
b. Availability of up-to-date methods and procedures manuals
c. Introducing the concept of responsibility and accountability
d. Maintenance and repair of laboratory equipment

Implementation of EQA

An EQA can be organised by either of the following two ways:

Assessment by Surveys

The most popular form of EQA is the survey, whereby specimens, which are as nearly identical to clinical material as possible, are sent to participating laboratories, where they are subjected to an investigation or process, and the results are sent to the EQA organiser. The results are assessed either by reference to an absolute standard (very rare), to a result obtained by one or more reference laboratories, to a panel of assessors (for subjective evaluation), or to a *consensus mean* (for quantitative measurements). The main objections to the survey method are:

a. The distributed material may behave differently from the biological specimen that are normally investigated by the laboratory, and hence give a misleading impression of the laboratory's normal level of performance.
b. The distributed material may deteriorate in transit to a different extent for different laboratories.
c. The distributed material may be handled by the participating laboratories in a way different from routine practice, so that the impression gained in the survey bears little relation to the day-to-day level of the laboratory's performance (ideally, EQA should be "blind", with the distributed specimen received and treated in exactly the same way as a routine specimen; but this is not always easy to arrange, and it may be impossible).
d. Laboratories may consult one another about their results on the distributed specimen before submitting results to the organiser, thereby conveying a falsely favourable impression of between laboratory consistency (this may be counteracted by the distribution of different, but similar, specimens or pairs of specimens to participants).
e. There is considerable doubt as to whether either the consensus mean or the results obtained by reference laboratories constitute a scientifically impeccable base point for comparison with individual participants' results.
f. Surveys can cover only a limited number of tests, which may be such a small fraction of a laboratory's range as to be unrepresentative of the laboratory's total work (to counter this problem, the organisers try to make as representative a selection of tests as possible, within the constraints of the availability of suitable test material).

Assessment by Physical Inspection

This method is used by certain states in the USA. An inspector arrives at the laboratory under inspection without warning and requests investigation of a group of test specimens. The characteristics of the specimen have been determined shortly before (or are determined simultaneously) by a state reference laboratory. The inspectors endeavour to ensure that routine procedures are used. The test specimens are often patient specimens which have not had time to deteriorate. Of course, the system is extremely expensive and the testing necessarily takes place at very long intervals.

Common Causes of Poor Performance

Some of the common reasons of not getting quality, reliable results are:

a. Lack of commitment on part of staff performing the tests
b. Poor management and supervision
c. Poor understanding of quality assurance concepts
d. Analysts do not understand assay principles
e. Reagents used are not of high quality or are date expired
f. Poor quality instruments used
g. Procedures not followed as recommended. Short cuts devised by technical staff often give unreliable results
h. Pre- and post-analytic factors are not given due importance.

Accreditation of Laboratories

Accreditation is an approved procedure by which regulatory authorities accord formal recognition to a laboratory to undertake specific tasks provided that predefined standards are met by the laboratory.

In essence this is a process of inspection of laboratories and their licensing by a third party to ensure conformity to pre-defined criteria pertaining to various aspects of infrastructure and functioning of laboratory that comprise of a quality system.

The process of accreditation starts with the request of the laboratory for accreditation. The accreditating authority deputes inspector(s) to assess the laboratory on the basis of specific criteria. The report of the inspectors may recommend accreditation of the laboratory or may suggest some corrective measures. In either situation, the laboratory is informed of the decision of the authorities. The deficiencies pointed out by inspectors are to be rectified before formal recognition is accorded.

This system helps in ensuring good quality laboratories, since those which do not conform to the predefined criteria are not given licenses to undertake laboratory activities or a recognition of functional quality system.

81 Immunoprophylaxis Against Infectious Diseases

Rapid strides have been made in the recent past in the field of immunology as applied to prevention of infectious diseases. One of the greatest achievements of mankind has been the global eradication of smallpox. This feat was possible because of the availability of an effective vaccine. Encouraged by this, the world community has set targets for the eradication of various other diseases such as poliomyelitis and measles. WHO has established a programme designated as *Expanded Programme on Immunization* (EPI) which was an integral part of WHO's strategy to achieve health for all. Following diseases are being covered under this programme which is popularly known as EPI.

- Diphtheria
- Pertussis
- Tetanus
- Measles
- Poliomyelitis
- Tuberculosis.

The immunoprophylactic agents have been recognised world over as the most cost-effective tools with demonstrable public health results. These agents are of three types:

- Vaccines
- Immunoglobulins
- Antisera

The vaccines induce *active immunity* whereas immunoglobulins and antisera are used for confering *passive immunity*.

VACCINE

Vaccine is an immunobiological substance designed to confer specific protection against a disease. It stimulates the immune system (either humoral or cell mediated or both) to generate specific protection against an infectious agent. Vaccines may be prepared from live modified organisms, inactivated or killed organisms, toxoids or combination of these.

Currently Available Vaccines

The diseases against which vaccines are commercially available have been mentioned in Table 81.1.

Table 81–1. Diseases against which vaccines are currently available

Bacterial		*Viral*	
Attenuated	*Inactivated*	*Attenuated*	*Inactivated*
Tuberculosis	Typhoid fever*	Poliomyelitis	Poliomyelitis
Typhoid fever	Pertussis	Measles	Rabies
	Cholera	Mumps	JE
	Tetanus**	Rubella	Hepatitis B
	Diphtheria**	Yellow fever	Influenza
	Plague		Hepatitis A
	H. influenzae type b*		
	Pneumococcal infections*		
	Meningococcus A,C* + Tetravalent		

* Capsular polysaccharides; ** Toxoids

Adverse Reactions to Vaccines

No vaccine is entirely safe. A number of problems are encountered in creating safe vaccines (Table 81.2).

A variety of illnesses temporarily associated with immunization have been reported. Epidemiologic studies have shown that a few have a probable causal relationship with the vaccine. The possible adverse reactions that may occur after vaccination have been shown in Table 81.3.

Table 81–2. Problems with vaccine safety

Attenuated live vaccines	
	Insufficient attenuation
	Reversion to wild type
	Administration to immunodeficient patient
	Persistent infection
	Contaminated by other viruses
	Foetal damage
Inactivated vaccines	
	Contaminated by live organisms
	Contaminated by toxins
	Allergic reactions
	Autoimmunity
rDNA vaccines	
	Inclusion of oncogenes (?)

Table 81–3. Adverse reactions to vaccines

Normal toxicity	
Faulty production	Abnormal inherent toxicity
	Presence of foreign toxin
	Bacterial contamination
	Wrong culture used
	Viral contamination
Faulty administration	Use of nonsterile apparatus
	Contamination from operator
Allergy	Local
	Serum sickness
	Neurological illness
	General anaphylaxis
Other causes	Abnormal sensitivity of vaccinee
Indirect effects	Damage to foetus
	Provocation of disease

Some of the important pathological complications due to commonly used vaccines are summarised in Table 81.4.

Table 81–4. Pathological complications of vaccination

Vaccine	*Complications*
Measles	Hypersensitivity to egg antigen
Mumps	Hypersensitivity to viral antigen
Pertussis	Convulsions
Measles	Encephalitis
Mumps	Meningitis
Rubella	Arthritis

Live Versus Killed Vaccines

Live attenuated as well as inactivated vaccines for different diseases are available. The former are also called as *replicating* and the latter as *non-replicating vaccines*. The live vaccines are prepared from live and generally attenuated organisms which have lost their ability to induce disease but retain their immunogenicity. In general, live vaccines are more potent immunizing agents because of following reasons:

a. Can multiply in the host thus increasing the antigen dose manifold.
b. Possess all major and minor antigenic components.
c. Occupy natural niches for the pathogen in the body thus blocking colonization by the pathogen.
d. May persist for longer time in the body in latent stages.

The killed vaccines are prepared by subjecting the organism to the action of physical or chemical agents. These are usually safe but generally less efficacious than the live vaccines.

The salient differences between the two types of vaccines are summarised in Table 81.5.

Table 81–5. Differences between attenuated and inactivated vaccines

Feature	*Attenuated*	*Inactivated*
Preparation	Attenuation	Inactivation
Administration		
Route	Usually natural route	Parenteral
Dose	May be single	Usually multiple
Adjuvant	Not required	Usually required
Safety	May revert to virulence	Safe
Cold chain requirement	++++	++
Cost	Low	High
Duration of immunity	Usually long	May be long or short
Immune response		
Humoral	IgG, IgA	Mainly IgG
CMI	+	Little or no

Role of Adjuvants

To obtain optimal or still better response from the vaccine, adjuvants are administered along with the vaccine. The mechanism of action of adjuvants has been discussed in section on Immunology. The adjuvants, which are currently in use in vaccines and those which cannot be used because of their toxicity as well as those which are in experimental stages, have been listed in Table 81.6.

Contraindications to Vaccinations

WHO has recommended a limited number of contraindications to vaccinations which are summarised below:

a. Immunization should be delayed in case of severe illness with fever, so that any sign of illness will not be attributed to the vaccination.

Table 81–6. Vaccine adjuvants

Routinely used for human use
- Aluminium hydroxide
- Aluminium phosphate
- Calcium phosphate
- *Bordetella pertussis*

In experimental stage
- Liposomes
- Immune stimulating complexes (ISCOMS)
- Slow release formations
- Interleukin 1 and 2
- Interferon gamma

Too toxic for human use
- Freund's complete adjuvant
- MDP (Muramyl dipeptides)

Doubtful efficacy
- Beryllium hydroxide
- Block polymers

Malnutrition, moderate fever, respiratory infections, common diarrhoea and any other benign ailment do not constitute contraindication for vaccination.

Hospitalized children may receive necessary vaccinations before their discharge, and, in some cases, immediately following admission, particulary in the presence of nosocomial measles risk.

b. Discontinuation of DPT immunization is recommended in case of occurrence of a severe postvaccinal reaction as collapse, shock, fever above 40.5°C, convulsions and other neurological symptoms.

Diarrhoea is not considered a contraindication for oral poliomyelitis vaccination. Extra doses corresponding to those administered during the bout of diarrhoea should be given.

c. No live vaccine is to be given to a person with an immunodeficiency or undergoing immunosuppressive treatment, corticosteroids therapy, radiotherapy, antimetabolite therapy, etc.

d. Measles, mumps or rubella immunization should be delayed for at least six weeks when a recent injection of polyvalent immunoglobulin has been given.

A vaccine-a-decade was the pace of progress since Pasteur until the new biology and biotechnology revolution appeared in recent past. Of 200 infectious diseases of man caused by bacteria, viruses and parasites, there are today highly effective vaccines against about two dozen diseases. It has been predicted that with the availability of biotechnologies such as genetic engineering to design attenuated organisms or the expression and synthesis of pure antigens, new or improved vaccines can be developed for another 30 diseases within the next decade (Table 81.7).

Table 81–7. Diseases for which vaccines are needed but are not available

Viral
- AIDS
- Herpes simplex genital infections
- CMV
- Glandular fever
- Common cold due to rhinoviruses

Bacterial
- Gonorrhoea
- Syphilis
- Trachoma
- Chlamydial urethritis

Parasitic
- Malaria
- Trypanosomiasis
- Schistosomiasis
- Amoebiasis

IMMUNOGLOBULINS AND ANTISERA

Immunoglobulins (Ig)

Two types of immunoglobulins are available: *normal human Ig* and *specific human Ig*. Normal human Ig is an antibody rich fraction obtained from a pool of at least 1000 people. The preparation is rich in IgG, almost whole of which is in free form (and not in aggregates). It contains very little of IgA. Normal human Ig is administered to prevent measles in highly susceptible individuals and to provide temporary protection (upto 12 weeks) against hepatitis A infection for travellers to endemic area and to contacts of case of hepatitis A in an outbreak. There should be a gap of 3 months between the administration of normal Ig and any live vaccine.

Specific human immunoglobulins are prepared from the plasma of patients who have recently recovered from infection or who have been immunized against a specific infection. The plasma of donor should contain at least five times the amount of specific antibody as is present in standard reference serum.

The immunoglobulins are usually given intramuscularly. Peak blood levels are reached in two days after intramuscular injection. The average half-life is 20-35 days. Generally immunoglobulins should not be given just before or after active immunization. Human Ig are now available against a large number of diseases (Table 81.8).

Table 81–8. Diseases for which human Ig and heterologous antisera are available

Human specific Ig	*Heterologous antisera*
Hepatitis A	Tetanus
Hepatitis B	Rabies
Rabies	Diphtheria
Tetanus	Gas gangrene
Rubella	
Botulism	
Varicella-zoster	
Measles	
Rh-isoimmunization	

Though immunoglobulins induce passive immunity very quickly, sometimes these may cause adverse side effects. These can be *local* in the form of pain or *systemic* in the form of some allergic reaction. However, the incidence of these reactions is very low.

Antisera

Antisera are the materials that are corresponding to specific human Ig but are raised in animals, usually horses (heterologous antisera). These have been in use for many years against some diseases (Table 81.8). The half-life for antisera is half that of human Ig and there are chances of developent of anaphylaxis or serum sickness. To prevent anaphylaxis a skin test using recommended amount of antiserum should be done before administering the total dose of antiserum. Though the current trend is in favour of using human Ig for passive immunity, their high cost makes them out of the reach of numerous people in developing countries.

Combined Passive and Active Immunization

In some diseases (such as diphtheria, rabies) passive immunization (with antisera or human specific Ig) is often undertaken in conjunction with active immunization. This provides both immediate (but temporary) passive immunity and slowly developing active immunity. Caution is taken in administering these two products at two different sites on the body.

Table 81–9. National Immunization Schedule in India

Age	*Vaccine given*
At birth	BCG, OPV-O (OPV zero dose for institutional births only)
6 weeks	DPT-1 OPV-1
10 weeks	DPT-2 OPV-2
14 weeks	DPT-3 OPV-3
9-12 months	Measles
16-24 months	DPT, OPV
5-6 years (school entry)	DT
10 years	TT
For pregnant women	2 TT doses one month apart

Note:

i. By the first birth day, child should receive complete course of vaccines against 6 diseases
ii. Antenatal mothers are given TT for primary prevention of neonatal tetanus
iii. Vaccination is not denied to even those who have not adhered to recommended time schedule.

National Immunization Schedule

Every country has devised a schedule for immunizing children against common infectious diseases (which have been included in EPI of WHO) to obtain optimal results with the available resources. As per WHO recommendations (which are followed in India) one dose of BCG, three doses of combined DPT vaccine, three (if possible additional zero dose at birth) doses of oral poliovaccine and one dose of measles vaccine is to be given to the child in his first year of life to protect him against these six diseases. Of these BCG is given as soon after the birth as is possible along with a dose of OPV. DPT is injected from the age of 6 weeks onwards with a gap of four weeks each between three doses. Each injection of DPT is accompanied by a dose of OPV to reduce the contact of child with health functionary. Vaccine against measles is given on the completion of 9 months of age. Upto the age of 9 months, a child is protected against measles by the antibodies passively transferred to child from the mother. National Immunization Schedule followed in India is given in Table 81.9.

PART XIV CLINICAL MICROBIOLOGY

82 Laboratory Diagnosis of Important Clinical Syndromes

This section includes laboratory diagnosis of important clinical entities and syndromes.
These are:

- Pyogenic meningitis
- Tetanus
- Gas gangrene
- Urinary tract infection
- Pertussis
- Pyrexia of unknown origin (PUO)
- AIDS
- Syphilis
- Sore throat
- Cholera
- Diarrhoea (Gastroenteritis)
- Acute respiratory infections

LABORATORY DIAGNOSIS OF MENINGITIS

Definition

Meningitis is the infection of meninges. This infection could be due to bacteria, viruses, parasites or even fungi. This could cause the cerebrospinal fluid to become cloudy and pus like (**pyogenic meningitis**) or it may be non-pyogenic (**aseptic meningitis**).

Causative Agents

Pyogenic meningitis can be caused by many pathogenic organisms. The common agents in different situations have been shown in Figure 82.1.

Early diagnosis of meningitis is important because proper antimicrobial therapy is largely dependent upon the results of the microbiological laboratory.

Clinical Specimen: Collection and Transportation

Cerebrospinal fluid and blood are collected for the demonstration of causative agent. Cerebrospinal fluid (CSF) is more important. It should be collected under aseptic conditions and before the commencement of any antimicrobial therapy. CSF is collected in screw capped bottles and sent to the laboratory at the earliest. During transportation and storage, it should not be refrigerated because delicate organisms such as *Haemophilus influenzae* and *Neisseria meningitidis* get killed.

If delay is unavoidable, CSF is sent in a transport medium such as Amies medium, Stuart's medium or 1% glucose broth. Blood is examined microbiologically in all suspected cases of meningitis because bacteria reach meninges only via blood.

In meningococcal infections there may be petechial lesions. If present these should be irrigated by injecting 0.2 ml of sterile saline solution using a small syringe with a fine needle and fluid collected for making a slide smear and setting up culture.

Storage

Specimen should be cultured as soon as possible after receipt. Cells disintegrate and a delay may produce a cell count that does not reflect the clinical situation of the patient. Specimen should be stored at 37°C till microscopy and bacterial cultures have been done after which it can be refrigerated for further use.

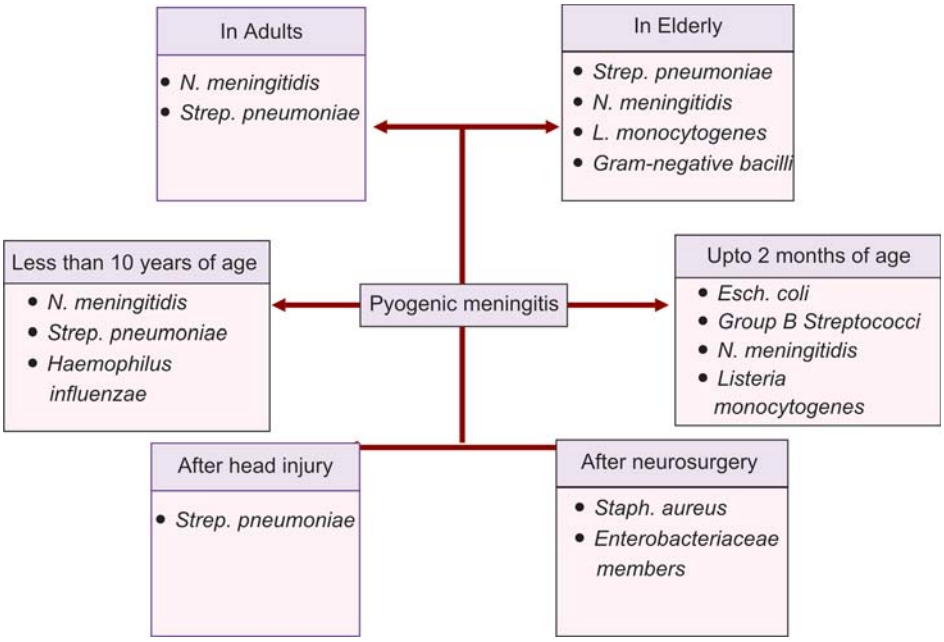


Fig. 82–1. Causes of pyogenic meningitis in various situations

Macroscopic Examination

In pyogenic meningitis, CSF is turbid and milky (pus like), in tubercular meningitis it forms a cobweb like clot on standing while it is clear watery in aseptic meningitis.

Examination for Cells

In acute bacterial meningitis there is great increase in the number of leucocytes in the CSF. It may reach upto several thousands per cubic mm and are predominantly polymorphs. In tubercular meningitis there are fewer cells in CSF (200-500/cu mm) and lymphocytes predominate. In viral infections of meninges (aseptic meningitis) total cell count is 50-1000 cells/cu mm and virtually all these are lymphocytes.

Biochemical Estimation

Estimation of glucose, proteins and chlorides in the CSF shows elevated proteins and reduced glucose in cases with pyogenic meningitis (Table 82.1).

Direct Demonstration in Stained Smear

A smear is made from the deposit of the CSF obtained after centrifuging the specimen. It is stained by Gram's staining and followings are looked for:

a. Gram-positive diplococci: *Strept. pneumoniae*
b. Gram-negative diplococci: *Neisseria meningitidis*
c. Gram-positive cocci in chains: Streptococci
d. Gram-negative coccobacilli: *Haemophilus influenzae*
e. Gram-positive cocci in clusters: *Staph. aureus.*

Table 82–1. Spinal fluid changes in meningitis

Disease	*Appearance*	*Cells per cu mm*	*Protein mg/100 ml*	*Sugar mg/100 ml*	*Pressure mm H_2O*
Normal CSF	Clear	0-10	15-45	40-60	100-200
Pyogenic	Purulent	500-5000 Polymorphs	Increased	Decreased	Increased
Tuberculous	Clear to opalescent	50-500 Lymphocytes	Increased	Decreased	Increased
Viral	Clear	50-1500	Normal or Lymphocytes slightly increased	Normal	Normal

In suspected cases of tubercular meningitis, smear is stained by Ziehl Neelsen method and acid fast bacilli are observed under the microscope.

Culture of CSF

The centrifuged deposit of CSF is seeded on blood agar and chocolate agar plates and the incubation is done at 37°C in an environment of 5-10% carbon dioxide. Simultaneously, a part of CSF is mixed with equal volume of 1% glucose broth and incubated at 37°C for extended culture. Bacterial growth, if obtained, is identified by standard procedures.

For tubercle bacilli culture is done on Lowenstein-Jensen medium and various cell lines can be used to obtain the growth of viruses.

Blood Culture

5-10 ml of blood is inoculated into 100 ml of culture medium (e.g. trypticase soya broth) and incubated at 37°C for 24 hours. From this medium, subculture is done on blood agar and chocolate agar media which are incubated at 37°C in the presence of 5-10% carbon dioxide. The incubation of inoculated trypticase soya agar is continued for 7 days after which subcultures on solid media are repeated. The bacterial growth obtained, if any, is identified by standard laboratory procedures.

Immunological Tests

For detection of dead bacteria or bacterial antigen in the CSF various immunological tests are available. These are:

a. Counterimmunoelectrophoresis (CIEP)
b. Latex agglutination
c. Co-agglutination
d. Enzyme linked immunosorbent assay (ELISA).

These tests are available for pneumococci, meningococci of Groups A and C, and *H. influenzae* type b. These tests provide rapid diagnosis and can be used in antibiotic treated patients as well as under field conditions. Latex agglutination test is shown in Figure 82.2.

In paired samples of serum or CSF, a rising titre of antibody can be diagnostic of many viral infections of meninges.

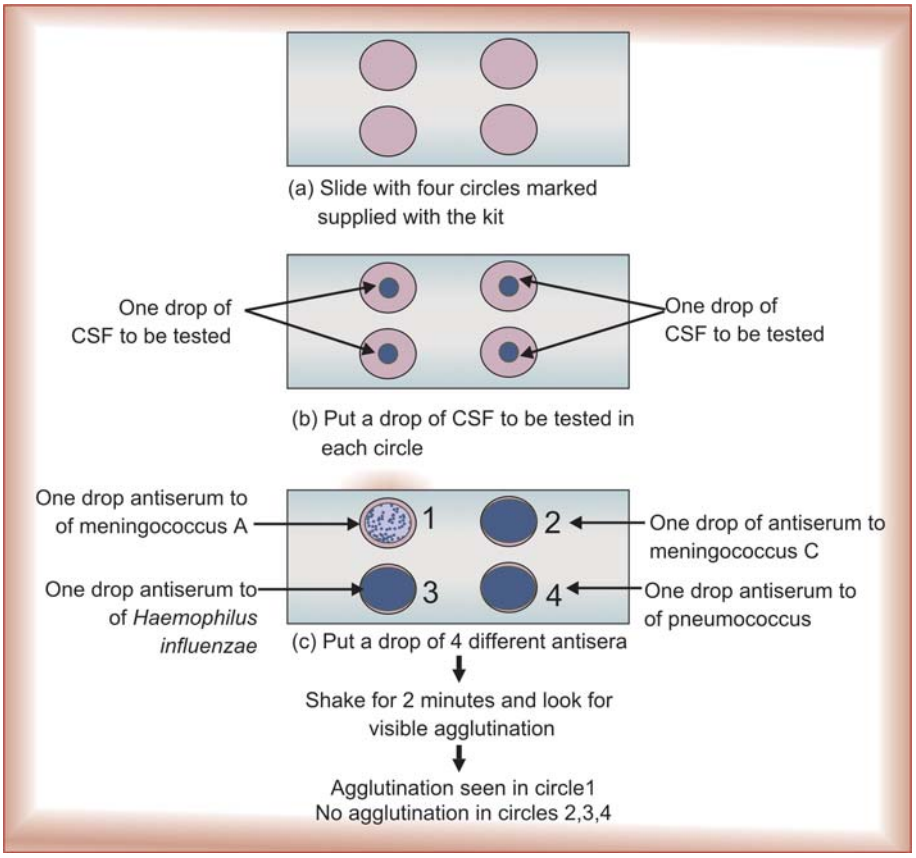


Fig. 82–2. Latex agglutination test on CSF for pyogenic meningitis

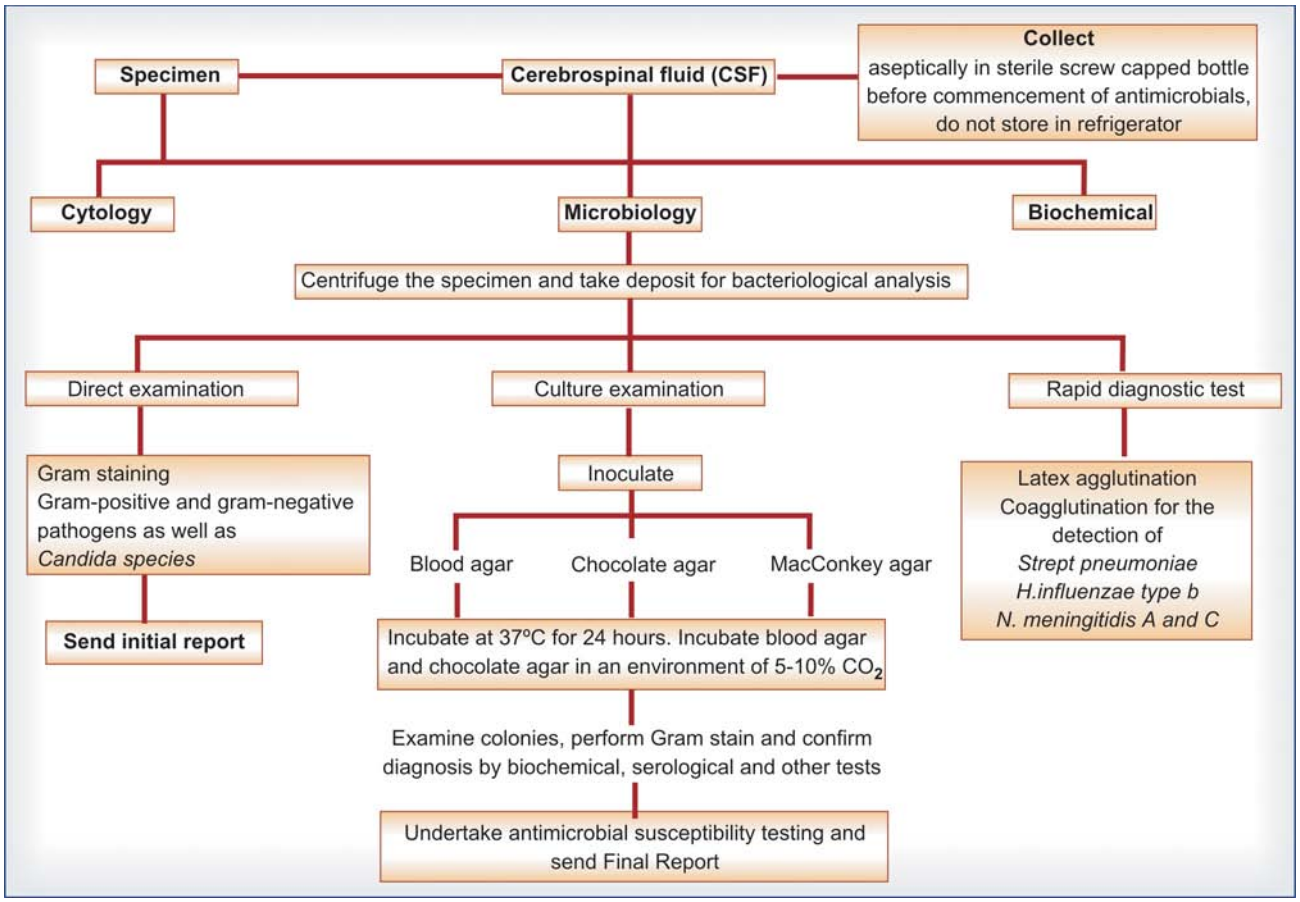


Fig. 82–3. Diagnosis of acute pyogenic meningitis

Essential differences in CSF in different types of meningitis are given in Table 82.1.

Antibiotic Sensitivity Testing

Except a few organisms, the causative agents of meningitis do not have a predictable antibiotic sensitivity pattern and hence the same is ascertained by Stoke's method or disc diffusion method. For meningitidis and beta haemolytic streptococci there is no need to perform antibiotic sensitivity testing in all the laboratories. An algorithm for diagnosis of acute pyogenic meningitis is given in Figure 82.3. Important points in diagnosis of meningitis include:

i. Process the specimens immediately because acute pyogenic meningitis is a life-threatening condition
ii. The management of the patient should start at the earliest.
iii. Notify the public health authorities when any bacterium (such as *N. meningitidis*) with epidemic potential is isolated/detected.

LABORATORY DIAGNOSIS OF TETANUS

Definition

Tetanus is caused by a spore forming anaerobic Gram positive bacillus, *Clostridium tetani*. A terminally located spore gives the organism the appearance of *drum stick*. The disease is also known as **lock jaw**.

The diagnosis of tetanus is essentially clinical. Strychnine poisoning also gives rise to similar picture. The laboratory diagnosis is indicated in post-operative tetanus cases and to confirm a clinically suspected tetanus case.

Clinical Sample: Collection and Transportation

The sample is collected from the deeper parts of the wound where the causative agent survives better because of anaerobic environment. The sample is collected in cooked meat broth and despatched to laboratory immediately. For investigation of post-operative tetanus cases, the samples are similarly collected from animate

as well as inanimate objects. The laboratory diagnosis comprises of followings:

a. Direct demonstration by Gram's staining
b. Direct demonstration by immunofluorescence
c. Isolation by culture
d. Animal pathogenicity

Direct Demonstration

Direct smear made from exudate and stained by Gram's method shall demonstrate a few bacilli with drum stick appearance. All bacilli shall not be, however, of this appearance. The presence of drum stick shaped bacilli should also not be considered as conclusive evidence of tetanus as bacillus of tetanus can be present in any wound which has been contaminated with soil and also some other species of *Clostridium* such as *Cl. tetanomorphum* and *Cl. sphenoides* can have similar morphology.

Direct immunofluorescence test can also be employed for the demonstration of tetanus bacilli. Conjugated immunoglobulins are now commercially available.

Isolation by Culture

The specimen is inoculated onto cooked meat medium as well as blood agar. One blood agar with antitoxin control can also be inoculated. One half of this plate is smeared with antitoxin serum before inoculation. If toxin producing *C. tetani* are present in culture, haemolysis produced by them on blood agar shall be inhibited in that half of the plate which has been preseeded with antitoxin.

Plates are incubated anaerobically and examined with hand lens for spreading colonies. Simultaneously, cooked meat broth is examined microscopically every day. If *C. tetani* are suspected, part of broth is heated at 80°C for 10 minutes and then subcultured onto blood agar. At 80°C the spores of tetanus bacilli get killed. The growth obtained on solid medium can be confirmed by Gram's staining as well as immunofluorescence.

Animal Pathogenicity

It can be ascertained once a pure growth of the organism has been obtained on solid medium. Alternatively it can be done with supernate of cooked meat broth. A pair of mice is used for this test. One of the animals is protected with intraperitoneal injection of 500-1500 units of tetanus antitoxin administered one hour prior to test. 0.1 ml of cooked meat broth supernate is injected intramuscularly into the hind limbs of both the animals. Signs of ascending tetanus develop in test animal and the protected mouse remains normal.

LABORATORY DIAGNOSIS OF GAS GANGRENE

Definition

Gas gangrene is a disease caused by various species of genus *Clostridium* through infection of wounds. The disease is characterised by rapidly spreading oedema, myositis, necrosis, gangrene of tissues and gas production. It is also known as **clostridial myonecrosis**.

Causative Agents

Various species of *Clostridia* can cause gas gangrene (Table 82.2).

Table 82–2. Causative agents of gas gangrene

- *Cl. perfringens*
- *Cl. septicum*
- *Cl. novyi*
- *Cl. bifermentans*
- *Cl. histiolyticum*

In more than 60% of cases gas gangrene is caused by *Clostridium perfringens*. Certain other species also produce gas gangrene albeit their incidence is extremely low.

Clinical Sample: Collection and Transportation

Pus, excised tissue or necrosed tissue obtained from the affected wound constitute the clinical sample. These are collected in cooked meat broth and despatched to laboratory. Ideally, three samples are collected: one for staining; second for aerobic incubation and third for anaerobic growth. Surface swabs of the wound are not suitable specimens because their constant exposure to atmospheric oxygen kills the causative agents which are strict anaerobes.

The bacteriological diagnosis of gas gangrene is usually combined with general bacteriological examination of infected wound with which the condition is associated.

The components of laboratory diagnosis include:

a. Direct demonstration by Gram's staining
b. Direct demonstration by immunofluorescence
c. Isolation by culture
d. Gas liquid chromatography.

Direct Demonstration

If gas gangrene is present, Gram positive bacilli with size and shape corresponding to the infecting species are seen. Thick, rectangular Gram positive bacilli are suggestive of *Cl. perfringens*. Leaf-shaped or boat-shaped bacilli are suggestive of *Cl. septicum*.

Direct Demonstration by Immunofluorescence

Reagents for direct demonstration by immunofluorescence are available for *Cl. novyi* and *Cl. septicum* but not for *Cl. perfringens*.

Isolation by Culture

In addition to media used for the isolation of aerobic pyogenic organisms, the sample is inoculated on blood agar and neomycin blood agar. These plates are incubated anaerobically at 37°C. The colonies, if any, are identified by the conventional methods.

Gas Liquid Chromatography (GLC)

GLC is available for confirmation of various isolates of clostridia. However, it is not available in routine hospital laboratories and does not have much practical use in diagnostic microbiology laboratory.

LABORATORY DIAGNOSIS OF URINARY TRACT INFECTIONS

Definition

Urinary tract infection (UTI) is the active infection in any part of urinary tract beyond distal urethera which is normally bacteriologically sterile.

Causative Agents

A large number of organisms gaining access to urinary tract are capable of causing UTI (Table 82.3). These organisms may reach the urinary tract through ascending route or by haematogenous route.

Table 82–3. Causative agents of UTI

Infection via ascending route	*Infection via haematogenous route*
Escherichia coli	*Salmonella species*
Proteus species	*Mycobacterium species*
Klebsiella species	*Schistosoma haematobium*
Staphylococcus aureus	*Histoplasma duboisii*
Staphylococcus epidermidis	*Cytomegalovirus*
Group B streptococci	*Adenovirus type II*
Pseudomonas species	
Candida species	

Factors Predisposing to UTI

Various bacterial attributes and host factors favour urinary tract infections (Fig. 82.4). Mechanical factors are important. Anything that disrupts normal urine flow or complete emptying of the bladder, or facilitates access of organisms to bladder, will predispose an individual to UTI.

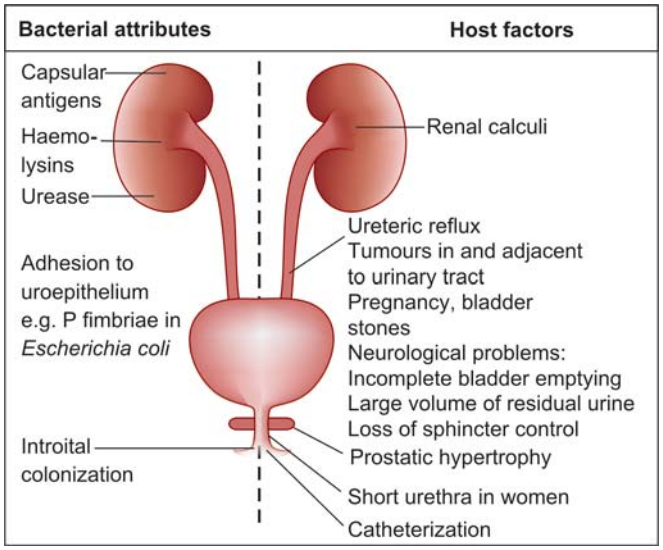


Fig. 82–4. Bacterial attributes and host factors which favour UTI

Clinical Specimen: Collection and Transportation

Mid-stream sample of urine is the ideal specimen for the diagnosis of UTI. First part of the urine washes away the surface commensals from the distal urethra and hence the midstream specimen indicates actual bacteriological picture of the urinary tract. If it is not possible to collect midstream urine, such as in young children, suprapubic aspiration of urine is done. Catheterised samples are not preferred.

The sample must be immediately cultured, otherwise stored at 4°C. Since urine is an extremely good medium for the growth of bacteria, keeping the sample at room temperature permits the unchecked and unpredictable growth of bacteria and the results obtained thus do not represent the true picture.

For diagnosis of tuberculosis of urinary tract, three consecutive early morning specimens are collected and delivered to laboratory. An alternative is collection of 24 hours sample of the urine. For tuberculosis, the urine is centrifuged and deposit is examined for acid fast bacilli.

Microscopic Examination of Urine

A small portion of urine is centrifuged and the deposit is examined for the presence of pus cells, erythrocytes

and bacteria. Both pus cells and bacteria are present in urine in UTI. Presence of bacteria in the absence of pus cells is likely to be due to contamination rather than active UTI. Detection of erythrocytes is suggestive of damage or trauma to the urinary tract making it more prone to bacterial infection.

Isolation by Culture

Blood agar and MacConkey agar are inoculated with predetermined quantity of urine. This helps in expressing the bacterial count in uniform term of per ml. A standardised loop should be used for inoculation. A loop which delivers 0.05 ml of urine is most convenient. One ml of urine shall contain 200 loopfuls. The number of colonies that are obtained after overnight incubation of inoculated plates is multiplied with 200 to get viable bacterial count per ml of urine.

Thus, if the number of colonies on a bacteriological medium is 500, the viable bacterial count per ml of urine shall be 500 × 200 = 100,000.

Kass gave a criterion of active bacterial infection of urinary tract according to which a count exceeding 100,000 bacteria per ml denotes **significant bacteriuria** and is indicative of active UTI. This count is, however, not applicable to tuberculosis of urinary tract because of slow rate of multiplication of mycobacteria. Bacterial counts in urine are, however, influenced by various factors (Table 82.4).

Table 82–4. Factors affecting bacterial counts in urine

- Urine pH
- Site of infection
- Rate of flow of urine
- Antibacterial drugs in urine
- Antibacterial substances in urine (high concentration of urea)
- Number of bacteria in one colony forming unit (staphylococci in groups)
- Rate of growth of different bacterial species

The colonies obtained on culture media are identified by standard laboratory techniques. The commonest aetiological agent, i.e. *Escherichia coli* produces pink coloured flat colonies on MacConkey agar. Klebsiellae also produce pink coloured (lactose fermenting) colonies on MacConkey agar which are mucoid.

An algorithm depicting steps for diagnosis of urinary tract infection is given in Figure 82.5.

Identification of Isolates

A scheme for the presumptive identification of common gram-negative bacteria is shown in Figure 82.6. Wherever possible, detailed biochemical tests should be put up to confirm the identity of the isolate.

Antibiotic Sensitivity Testing

The sensitivity of the isolate is determined to commonly used antimicrobial agents which include nitrofurantoin, ampicillin, sulfonamides, co-trimoxazole and nalidixic acid. Organisms resistant to these drugs are tested for newer antimicrobial agents.

LABORATORY DIAGNOSIS OF PERTUSSIS

Definition

Pertussis (*whooping cough*) is predominantly an infection of the respiratory mucosa which is characterised by severe fits of cough with typical whoop in elderly children. Cough is almost always followed by vomiting.

Causative Agent

Pertussis is caused by *Bordetella pertussis*, a gram-negative coccobacillus which shows bipolar metachromatic granules on toludine blue stain. A few mild cases of whooping cough are also caused by *Bordetella parapertussis*.

The laboratory diagnosis comprises of three steps:

- Direct microscopy
- Culture
- Serology

The specimen must be collected at the early stages of the disease when the chances of detection of causative agent are maximum.

Collection of Specimen

Four methods are available for the collection of material. These are pernasal swab, cough plate, postnasal swab and postnasal secretions. Of all these, pernasal swab is considered best (Fig. 82.7). In this method a sterile cotton wool swab on flexible wire is passed gently along the floor of the nose until it meets resistance. The swab thus collects mucopus. It is withdrawn and either immediately placed onto bacteriological media or placed in a transport medium. Single swab from patients usually does not yield the organism and hence upto six swabs may be taken during initial 2-3 days of illness.

Direct Demonstration

Ordinary staining methods do not help in diagnosis of pertussis. A rapid diagnosis can be made by the fluores-

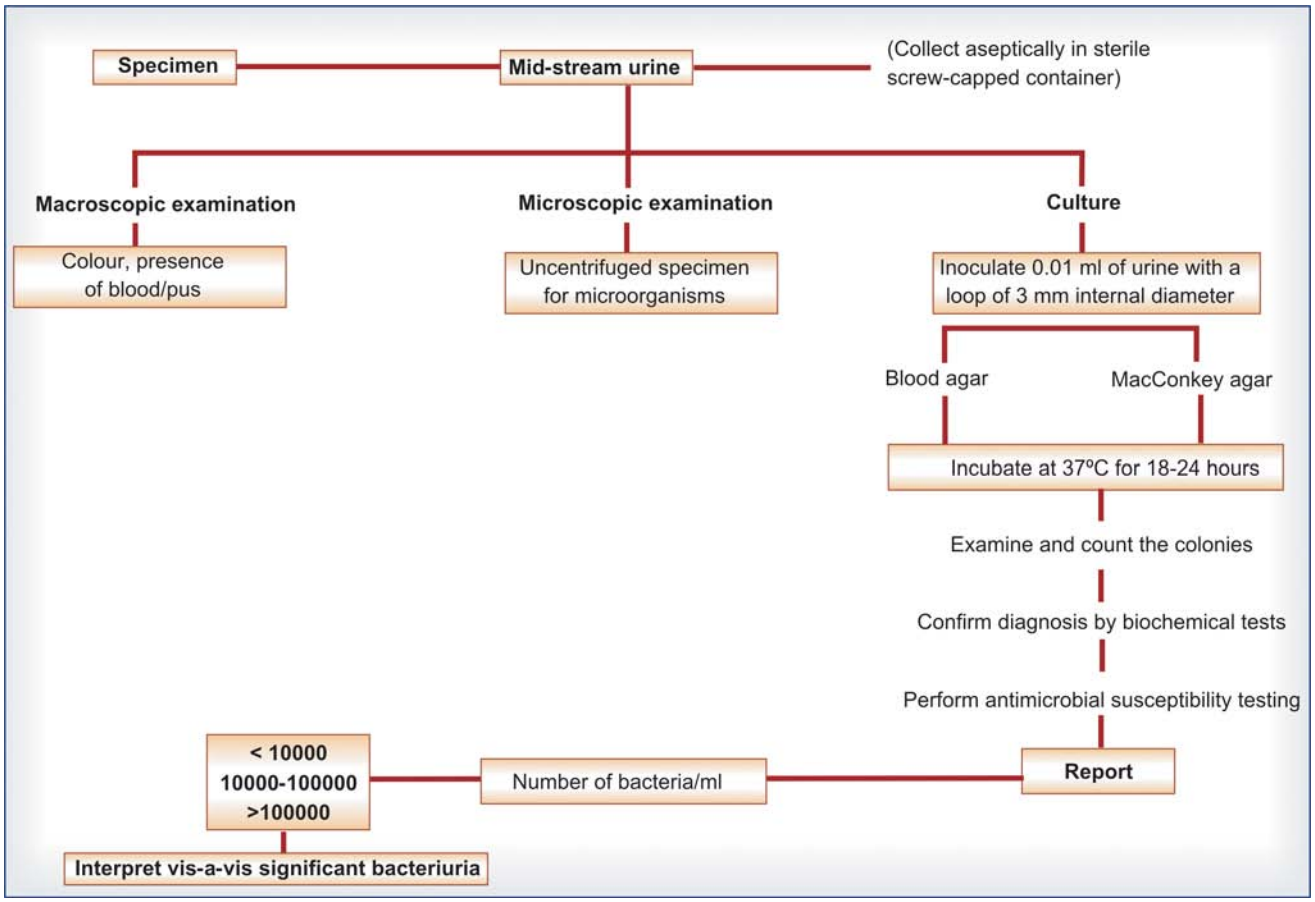


Fig. 82–5. Diagnosis of urinary tract infection

cent antibody technique applied directly to naso-pharyngeal secretions on a slide. In suspected cases of whooping cough, this method may have a sensitivity of upto 75%. It is a sensitive, rapid and accurate test and can produce results even in those patients who have been partially treated with antibiotics.

Culture

The preferred media are modified Bordet Gengou medium and charcoal blood agar to which cephalexin has been added. The latter gives larger colonies. The media plates must be adequately thick and incubated in an environment of sufficient humidity for 5 days. The colonies (*bisected pearl* or *mercury drops*) are first examined by Gram's staining and then confirmed by slide agglutination test using specific antiserum. The confirmation can also be done by immunofluorescence. Those laboratories which do not have antisera can establish the diagnosis and differentiate between three species of *Bordetella* by utilizing various other parameters (Table 82.5).

Table 82–5. Cultural and biochemical parameters of bordetellae

Character	*B. pertussis*	*B. para-Pertussis*	*B. bronchi-septica*
Motility	–	+	+
Growth on peptone agar	–	+	+
Browning of peptone agar	–	+	–
Growth on MacConkey agar	–	+	+
Nitrate reduction	–	–	+
Citrate utilization	–	–	+
Urease production	–	+	+
Appearance of growth on Bordet Gengou medium	3-4 days	1-2 days	1-2 days

Serology

The antibodies against these organisms appear after 2-3 weeks of onset of disease and this is the time when the first sample should be collected. Another serum sample is collected after a fortnight of first sample. The tests which can be performed are: CFT, direct agglu–tination, indirect haemagglutination and ELISA.

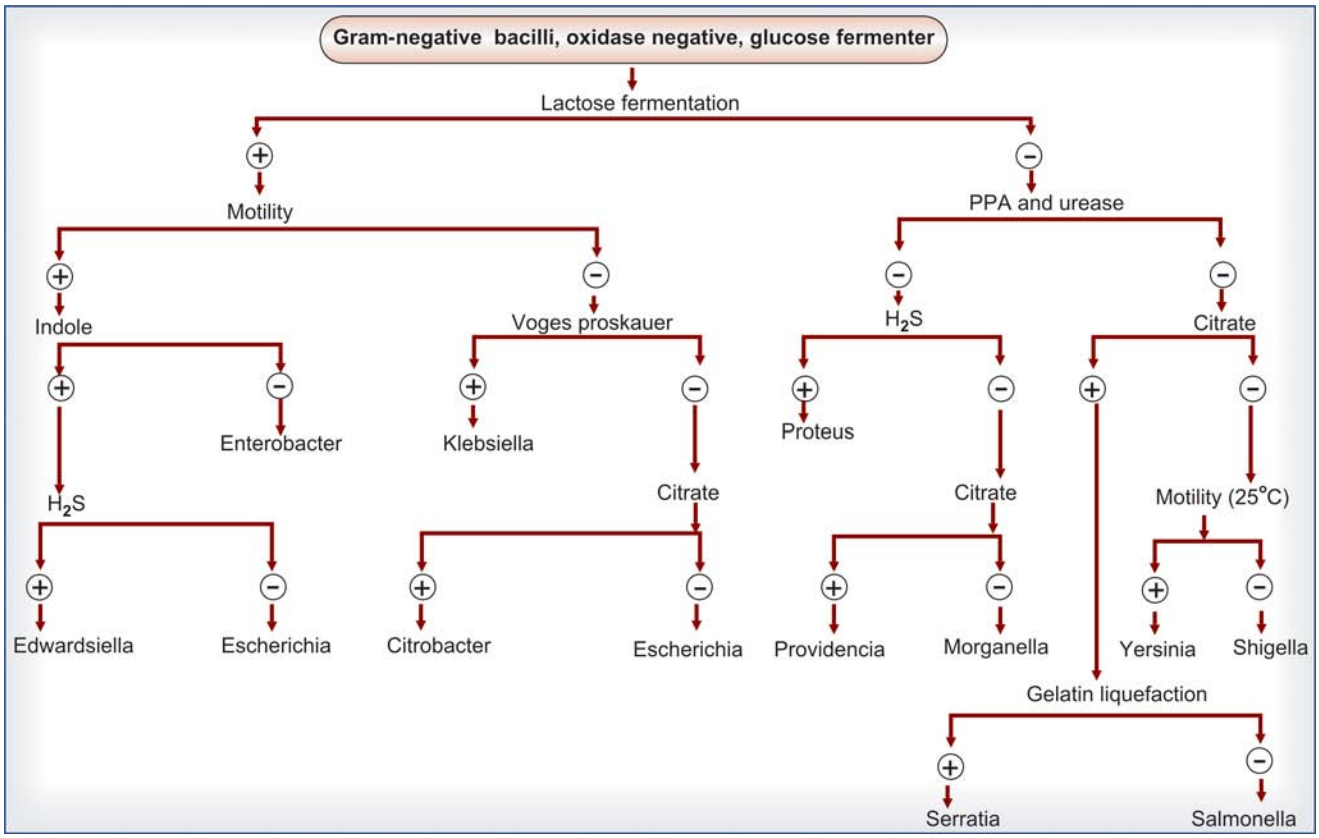


Fig. 82–6. Identification of gram-negative bacilli

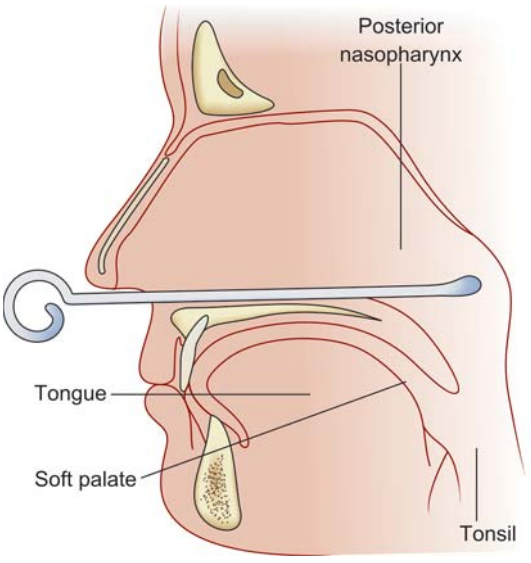


Fig. 82–7. Collection of material for whooping cough (Pernasal method)

LABORATORY DIAGNOSIS OF PYREXIA OF UNKNOWN ORIGIN

Definition

Patients who have persistent and significant (> 38.3°C or >100°F) fever the cause of which cannot be readily diagnosed on clinical examination are said to have pyrexia of unknown origin (PUO).

Classification of PUO

Four types of PUO are now identified (Table 82.6).

Causative Agents

PUO can be the result of a large number of conditions which can be grouped under infections, neoplasms, collagen vascular diseases and iatrogenic factors (Fig. 82.8). The infective causes are the commonest and have been presented in Table 82.7.

The diagnosis will depend upon the detection of pathogen in the blood or tissues. Indirect evidence for the

Table 82–6. Types of PUO

Definition	*Symptoms*
Classical PUO	Fever (>38.3°C) on several occasions and more than three weeks duration
Nosocomial PUO	Fever (>38.3°C) on several occasions in hospitalised patient; infection neither present nor incubating at the time of admission to hospital
Neutropenic PUO	Fever (>38.3°C) on several occasions; neutrophil count <500/cu mm in peripheral blood
HIV-associated PUO	Fever on several occasions (>38.3°C). More than three weeks as outpatient or more than three days as inpatient. HIV serology positive

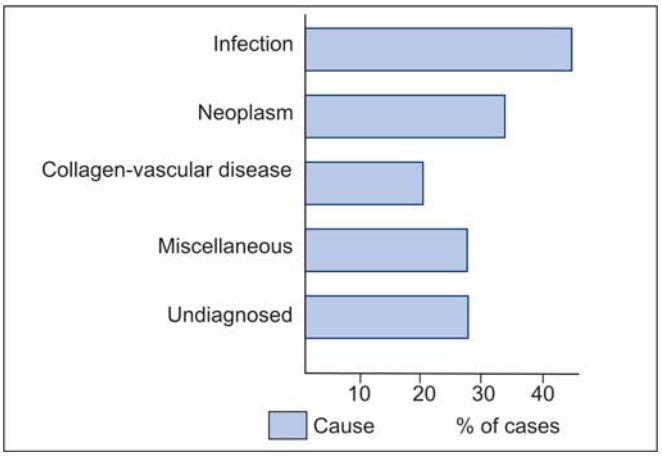


Fig. 82–8. Causes of pyrexia of unknown origin

presence of infectious agent can also be ascertained. Various methods adopted are discussed in brief hereunder:

1. Detailed History and Physical Examination

This gives clue to the system affected and also the visit to any high-risk group or area.

2. Routine Screening Tests

These include estimation of haemoglobin, total leucocyte count, differential leucocyte count, ESR and eosinophil count which may give an indication regarding the type of infection.

3. Examination of Peripheral Blood Smear

This may show the presence of malarial parasite, trypanosomes and the type of leucocytes.

Table 82–7. Infective causes of PUO

Bacterial	Tuberculosis Enteric fever Osteomyelitis Bacterial endocarditis Brucellosis Urinary tract infections Biliary tract infections Q-fever Relapsing fever
Parasitic	Amoebic hepatitis Malaria Toxoplasmosis Kala-azar
Fungal	Histoplasmosis Cryptococcosis
Viral	Hepatitis Infectious mononucleosis Cytomegalovirus infection

4. Blood Culture

Repeated blood cultures are done because the bacteria may not be continuously present in the blood, and if present, may be scanty in number. The sample is collected under aseptic conditions and immediately inoculated into blood culture bottles. Extended culture can also be done. Biphasic medium is recommended for the isolation of brucellae which also requires incubation in an environment of 5-10% carbon dioxide.

The steps in processing of blood in a patient with PUO is given in Figure 82.9.

5. Animal Inoculation

For diagnosis of leptospirosis, brucellosis and rickettsial diseases, direct blood sample may be injected into a susceptible animal.

6. Sputum Examination

Smears are made from the clinical sample of sputum, preferably after concentration, and stained by Ziehl Neelsen staining technique. Presence of acid fast bacilli is indicative of pulmonary tuberculosis.

7. Serological Tests

Paired serum samples which are collected at an interval of 2-3 weeks are tested by Widal test for typhoid fever; Weil-Felix reaction for rickettsial diseases; standard tube agglutination test for brucellosis and indirect haemagglutination test for hepatic amoebiasis. Markers for acute amoebiasis should also be studied.

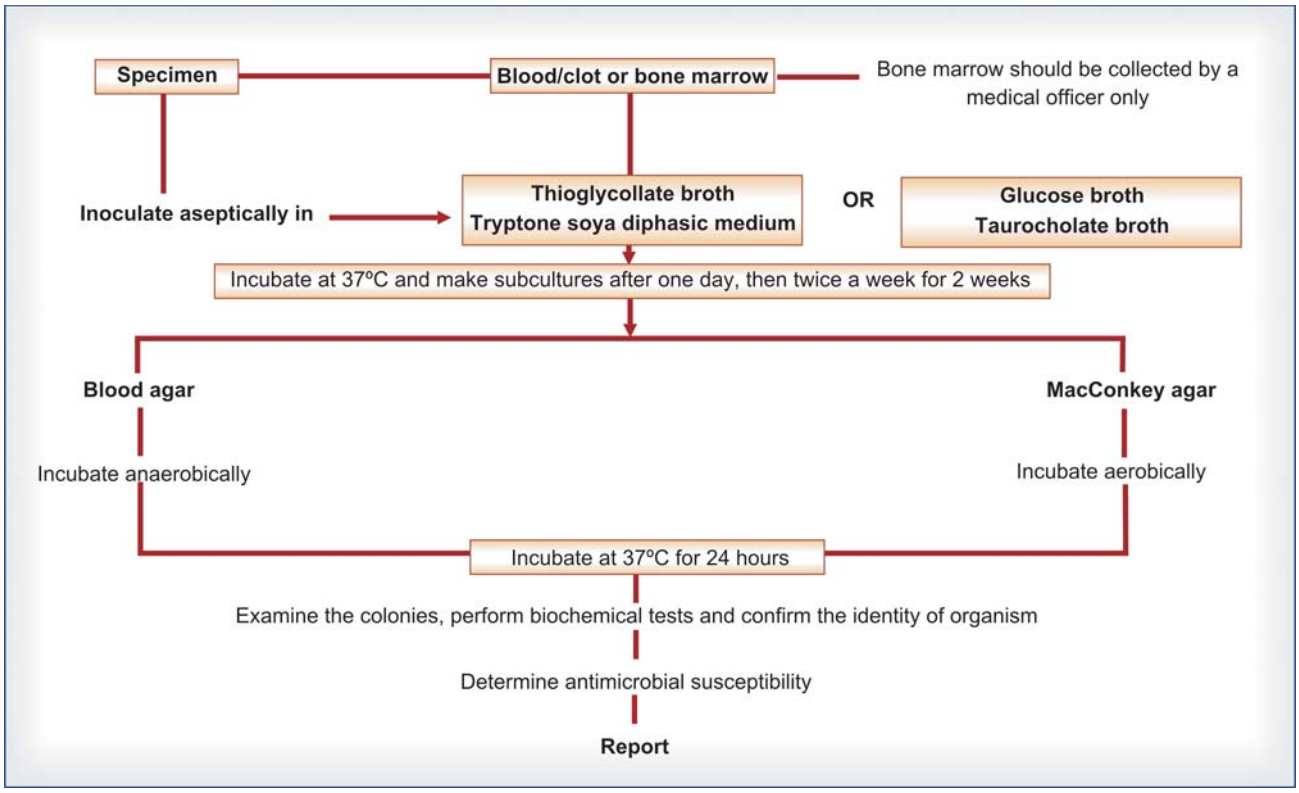


Fig. 82–9. Processing of blood in a patient with PUO

ELISA is now available for the detection of a large number of infections.

8. Urine Examination

A part of urine is centrifuged and the deposit examined under the microscope for the presence of pus cells and bacteria. It is also cultured on blood agar and MacConkey agar media as well as Lowenstein Jensen medium for the growth of pyogenic organisms and *Mycobacterium M. tuberculosis* respectively.

9. Stool Examination

Stool is examined for the presence of salmonellae, shigellae, ova of helminths and cysts of protozoa.

10. Examination of Tissues

Biopsy of the tissues such as enlarged lymph nodes, is carried out and examined histologically. The lymph node can also be grinded, homogenised and cultured, particularly for *M. tuberculosis*.

11. Examination of Body Fluids

Depending upon the suspected illness, various body fluids such as CSF, pleural fluid, bone marrow and bile etc. can be cultured for different pathogens.

12. Skin Tests

In a number of subacute and chronic infections, hypersensitivity to the constituents of the causative organism may develop. Inoculation of small quantities of suitable preparation of bacterial product such as tuberculin, brucellin or Frei antigen results in a localised delayed type of hypersensitivity reaction at the site of inoculation. The results of such tests help in confirming or excluding a specific infection.

13. Therapeutic Trials

In the absence of any laboratory confirmation, treatment based on clinical impressions can be given. Favourable response indicates specific diagnosis. This is true for conditions such as tuberculosis, amoebiasis and malaria which have specific antimicrobial treatment.

LABORATORY DIAGNOSIS OF AIDS

Definition

Acquired immunodeficiency syndrome (AIDS) is a disease characterised by the occurrence of a life threatening opportunistic infection or tumours or both. It is

caused by a human retrovirus known as human immunodeficiency virus (HIV).

CDC, Atlanta, USA has given a definition of AIDS according to which any person who is positive for HIV antibody and who has CD4 cell count of < 200/cubic ml, irrespective of his clinical status, shall be considered a case with AIDS.

Clinical Samples

Though HIV is present in many body fluids such as saliva, tears etc; the most important of all these is blood. Almost all diagnostic tests are performed on blood samples. About 5 ml of blood is collected aseptically using sterile and disposable syringe and needle. It is safely transported to the laboratory as early as possible. If delay is inevitable, sample can be refrigerated. Transportation should be done on wet ice.

The laboratory diagnosis is based on various tests which are summarised in Table 82.8. The diagnosis is based on antigen or antibody detection or showing the immunological abnormalities caused by HIV infection.

Table 82–8. Diagnosis of AIDS

TESTS FOR	DETECTION OF
Immunological abnormality	*Antibody* by ELISA or *Agglutination*
Opportunistic infections	*Antigen* by ELISA or PCR
Carcinomas and sarcomas	*Virus* by co-culture method

Tests Indicative of Immunodeficiency

These are nonspecific tests which would indicate paralysis of immune system (Table 82.9).

Table 82–9. Immunological abnormalities in AIDS

- Lowered total leucocyte count
- Lymphopenia
- Ratio of CD4:CD8 reversed
- CD4 cells <200/mL^3
- Cutaneous anergy
- Elevated IgG and IgA
- Impaired blastogenesis by T and B cells

Opportunistic Infections and Malignancies

The diseases which are currently considered suggestive of AIDS are depicted in Table 82.10.

Detection of Antibody

ELISA tests of different types are being used for antibody detection. ELISAs are available wherein reading can be taken by naked eye as well as by the use of spectrophotometer. ELISA tests are used as screening procedures for HIV infection. A positive ELISA for HIV antibody can mean any of the followings:

a. Current infection with HIV
b. Past exposure to HIV
c. False positive due to cross reaction

An ELISA which is negative for HIV antibody indicates any of the followings:

a. Absence of HIV infections
b. Failure of the test to pick up true positive and
c. Very early infection when antibodies are yet to be formed

Western blot test is used to confirm all those samples which are positive with ELISA. This test detects antibodies to various components of HIV and hence is more specific. A positive Western blot strip is shown in Figure 82.10.

Table 82–10. Diseases suggestive of AIDS

PARASITIC
- *Cryptosporidium*
- *Pneumocystis carinii* pneumonia
- Toxoplasmosis of Nervous System
- *Strongyloides stercoralis* outside GIT

FUNGAL
- Visceral candidiasis
- CNS cryptococcosis

BACTERIAL
- Disseminated infection due to
  - — *M. avium*
  - — *M. kansasii*

VIRAL
- Systemic CMV infection
- Systemic herpes simplex virus infection
- Progressive multifocal leucoencephalopathy

MALIGNANCIES
- Kaposi's sarcoma
- Primary intracerebral lymphoma

Detection of Antigen

HIV antigen can be detected in serum, plasma, CSF, etc. using indirect ELISA tests. Though it is not a widely used method because of its poor sensitivity, yet it has numerous advantages:

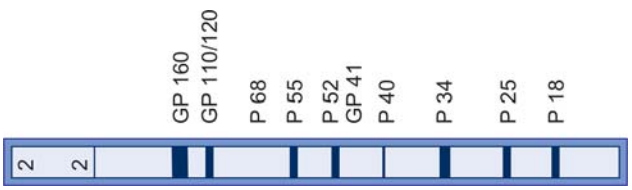


Fig. 82–10. Positive Western blot strip

a. Active infection can be detected
b. Early diagnosis can be established
c. Diagnosis in newborns and infants can be made in spite of the presence of passively transferred antibodies.
d. HIV infection can be distinguished from other immunodeficiency disorders.

Isolation of Virus

It is not used as a routine diagnostic aid. The main use is to find out the prevalent HIV strain and preparation of antigen against locally prevalent strain. It is done by the *co-cultivation* technique using peripheral lymphocytes.

MOLECULAR LEVEL DETECTION

Highly sensitive and specific tests such as **dot blot hybridisation**, polymerase chain reaction (PCR) etc are now being used by well equipped laboratories. PCR has been employed for different uses in AIDS (Table 82.11).

Table 82–11. Different uses of PCR in diagnosis of HIV infection

- Direct detection of HIV DNA
- Quantitative analysis of HIV DNA
- Quantitation of viraemia with HIV RNA-RT-PCR
- Studies on sequence variability of HIV genome
- Detection of viral load

An outline of the laboratory aspects of AIDS is presented in Figure 82.11. More details about diagnosis of AIDS can be seen in Chapter 71.

Biosafety in Laboratory

Utmost biosafety is mandatory in laboratory where material potentially infected with HIV is being handled. Use of disposable syringes and needles, avoiding mouth pipetting and handling of potentially infected material in biosafety cabinets is essential. The procedures by which HIV can be inactivated include boiling, treating with 70% ethanol, 20% gluteraldehyde, 5% formaldehyde and 0.5% lysol solution.

LABORATORY DIAGNOSIS OF SYPHILIS

Definition

Syphilis is caused by a treponeme, *Treponema pallidum*. The disease runs in stages. The diagnosis may be required in its *primary stage*, *secondary stage*, *tertiary stage* or late syphilis as well as treated and *congenital* syphilis.

Laboratory diagnosis is **indicated** for:
a. Diagnosing a suspected case of syphilis
b. Assessment of efficacy of treatment and
c. Screening of blood and preventing spread

The diagnosis of syphilis can be established by:
a. Demonstration of treponema by microscopy in congenital syphilis and primary and secondary stages

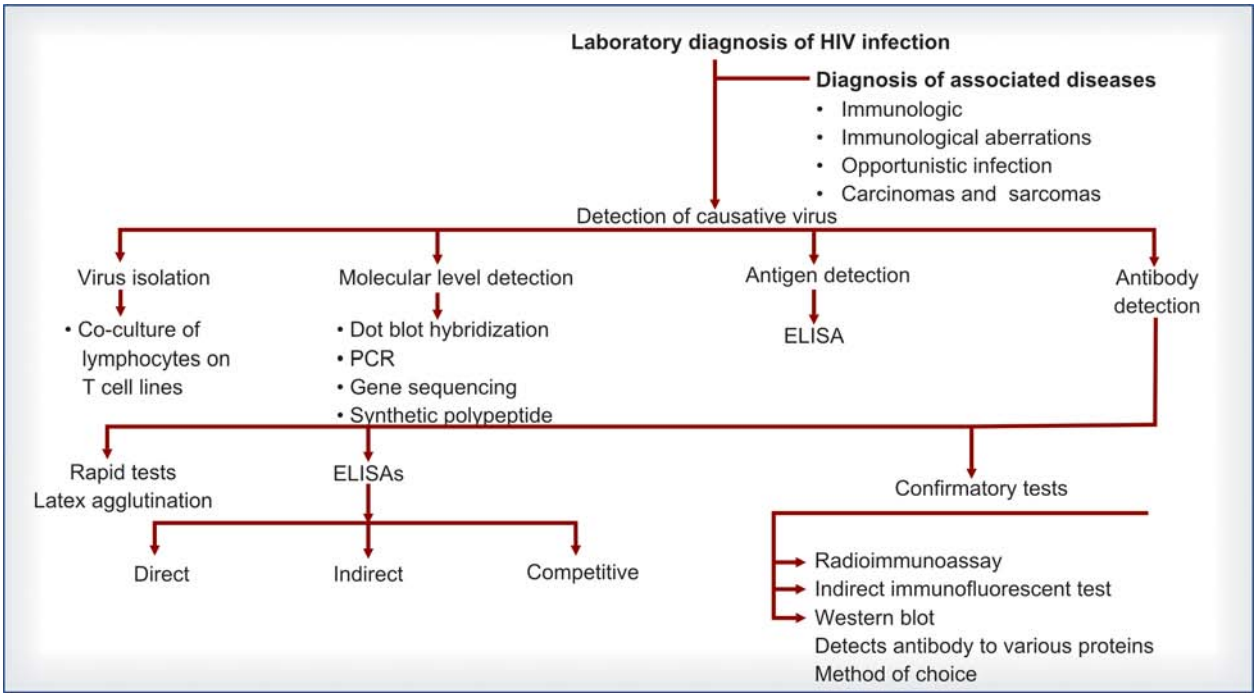


Fig. 82–11. Laboratory diagnosis of AIDS

b. Demonstration of reaginic and treponemal antibodies.

Direct Examination

Detection of treponemes from the lesion is the key aid to diagnosis. Although these organisms can be stained, their thinness makes them difficult to be seen with light microscopy. Dark-field microscopy is recommended. Samples should be collected under aseptic conditions before antibiotics are started. Wearing of gloves should be mandatory while collecting samples. Organisms of *T. pallidum* are actively motile filamentous helicals with evenly spaced coiled (Fig. 82.12).

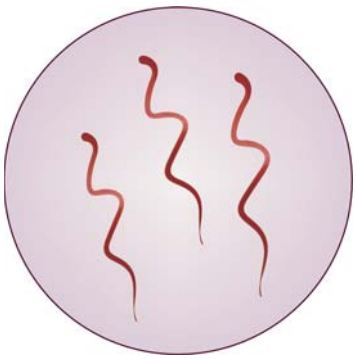


Fig. 82–12. *T. pallidum* as seen under dark-ground microscope

A negative result in dark-field microscopy does not rule out the presence of *T. pallidum*. Sometimes repeated examinations are necessary.

Immunofluorescence

Tissue fluid or exudate is spread on a glass slide, air dried and sent to the laboratory. It is fixed and stained with fluorescent labelled anti-treponeme serum and examined by means of immunofluorescent microscopy. This gives a higher positivity rate than the direct microscopy.

Serological Tests

These tests form the mainstay of laboratory diagnosis. Large number of serological tests are available with varying degree of sensitivity and specificity. It is useful to carry out at least two different tests on each specimen. Two types of antigens are available:

a. Cardiolipin: not derived from spirochaetes and used in VDRL (Venereal Diseases Research Laboratory) tests which are non-treponemal test.
b. Specific antigens derived from *T. pallidum* which are used in TPHA (*Treponema pallidum* haemagglutination test) and fluorescent treponema antibody absorbed (FTA-Abs) tests.

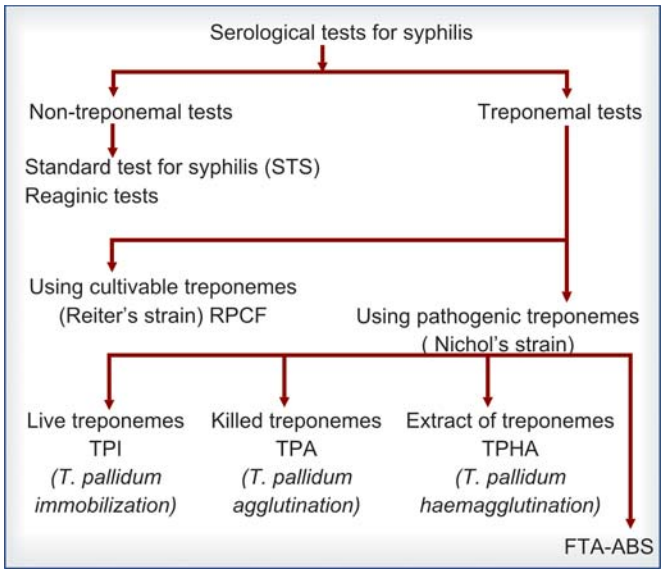


Fig. 82–13. Serological tests for syphilis

The serological tests which can be employed are shown in Figure 82.13.

In routine three serological tests (VDRL, TPHA, FTA-Abs) are used.

VDRL Test

Venereal Diseases Research Laboratory (VDRL) test is based on the fact that the particles of lipid antigens remain dispersed with normal serum but form visible clumps when combine with reagin. Results develop within a few minutes, particularly if the suspension is agitated.

VDRL is a type of flocculation test which can be performed on a slide as well as in a tube. In slide test, 0.5 ml of serum of the patient is taken in special slides with depressions (*cavity slides*) or slides prepared with paraffin rings. One drop of freshly prepared antigen is added with a syringe delivering 60 drops from one ml. The slide is shaken in a VDRL shaker for 4 minutes at 180 rotations. It is then examined under the microscope with low power objective. Formation of clumps indicates positive reaction (Fig. 82.14) designated as *reactive* while uniform distribution of crystals in the drop indicates that the serum is *non-reactive*. A weakly positive reaction may also occur (*weakly reactive*).

The reactive sera can be diluted and results quantified. VDRL test can also be done in tubes as tube flocculation test.

Advantages of VDRL Test

These include:

a. Simple and rapid test
b. Reasonably sensitive and specific

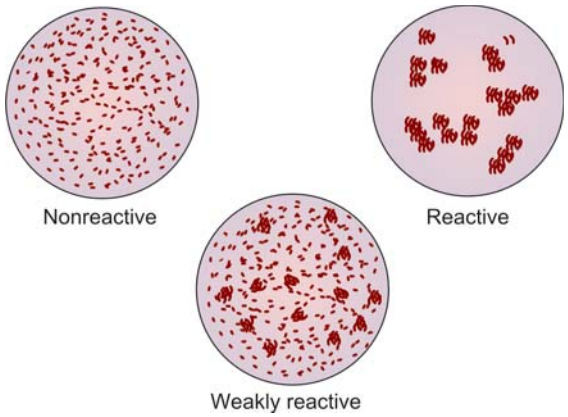


Fig. 82–14. Results of VDRL test

c. Requires small quantity of serum
d. Can be easily quantified
e. Results are reproducible
f. Reagents are cheap, easily available and have long shelf life
g. Quality control of tests can be performed.

Biological False Positive Results

In spite of simplicity of test and numerous advantages there are various biological false positive (BFP) results. These can be classified as *acute* (when they disappear in six months time) or *chronic* when they are positive for more than six months). These are shown in Table 82.12.

Table 82–12. Biological false positives in VDRL

Systemic lupus erythematosus	Coronary artery disease
Malaria	Repeated blood loss
Lepromatous leprosy	Menstruation
Infectious mononucleosis	Immunization
Tropical eosinophilia	Pregnancy
Relapsing fever	Haemolytic anaemia
Hepatitis	Heroin addiction
Tissue regeneration	
Collagen disorders	Upper respiratory infections
Severe trauma	Certain antihypertensive drugs

TPHA Test

Treponema pallidum haemagglutination (TPHA) test is performed by using extract of *T. pallidum*. Red blood cells are treated to adsorb treponemes on their surface. When mixed with serum containing antitreponemal antibodies, the cells become clumped. This test is similar to FTA-ABS test in specificity and sensitivity, but become positive somewhat later in the infection. Microhaemagglutination-*T. pallidum* (MHA-TP) is automated version of this test and so also is haemagglutination treponemal test for syphilis (HATTS). However, these two tests lack sensitivity in primary syphilis.

FTA-ABS Test

Fluorescent antibody test uses an indirect immunofluorescence test in which Nichol's strain of *T. pallidum* is employed as antigen. The patient's serum is diluted 1:5 and reacted with the antigen which has been smeared on the slide. The combination is covered with antihuman immunoglobulin fluorescent conjugate. After proper washing, the smear is examined under fluorescent microscope. In a positive test fluorescent treponemes are observed.

This test has undergone modifications to improve specificity. The patient serum is diluted to 1:200 instead of 1:5. This test is called as FTA200. Since false positive results were still obtained, the patient's serum was adsorbed with an extract of Reiter's treponemes and then the test is performed (FTA-ABS test). This is extremely sensitive and specific test.

Interpretation of Serological Tests for Syphilis

The interpretation of results obtained with these tests in different stages of syphilis is given in Table 82.13.

Table 82–13. Serological tests for diagnosis of syphilis

Stage of disease	*VDRL*	*TPHA*	*FTA-ABS*
Primary syphilis	+/–	+/–	+
Secondary syphilis	+	+	+
Tertiary syphilis	+	+	+
Late syphilis	+	+	+
Congenital syphilis	+	+	+
Treated syphilis	–	+	+

The nontreponemal tests are mainly used for:
a. screening procedures
b. to detect reinfection
c. to evaluate response to therapy
d. to diagnose neurosyphilis.

The treponemal tests are mainly used for:
a. confirmation of diagnosis of syphilis
b. detection of latent syphilis and
c. diagnosis in patients with negative nontreponemal tests.

LABORATORY DIAGNOSIS OF SORE THROAT

Definition

Sore throat is characterised by acute inflammation of the tonsillar and faucial areas with or without exudate. The exudate may be loose or adherent.

Causative Agents

The important causes of sore throat are given in Table 82.14.

Table 82–14. Causes of sore throat

BACTERIAL
- *Streptococcus pyogenes* group A
- *Corynebacterium diphtheriae*
- *Staphylococcus aureus*
- *Haemophilus influenzae*
- *Borrelia vincentii*
- *Neisseria gonorrhoeae*

VIRAL
- Rhinoviruses
- Coronaviruses
- Adenoviruses
- Parainfluenza viruses
- Influenza viruses
- Coxsackie viruses
- Epstein-Barr virus
- Herpes simplex types 1 and 2

FUNGAL
- *Candida albicans*

NONMICROBIAL CAUSES
- Agranulocytosis
- Acute leukaemia

The most common cause of sore throat may be viral infection but most important is *Streptococcus pyogenes* group A because of two reasons:

It is easily treatable. It leaves serious suppurative and nonsuppurative sequelae.

Age Group Involved

Sore throat is commonly seen in children and young adults. Infectious mononucleosis is an acute febrile illness seen most frequently in young adults who present with fever, pharyngitis and lymphadenitis. Oral candidiasis is present in any age group when the host is not immunocompetent.

Associated Conditions

Sore throat is usually accompanied by acute adenitis, sinusitis, otitis media, rhinitis, laryngitis and peritonsillar abscess.

The diagnosis of sore throat is made by:

Direct Smear Examination

Under proper light and after depressing the tongue of the patient with a tongue depressor, two swabs (made from absorbent non-bleached cotton) are collected from the inflamed areas over the tonsils and pharynx. If there is a pseudomembrane in the throat it should be peeled off by rubbing it vigorously. One swab is used for preparation of smears for staining purposes and the other for culture. The smears are stained with Gram's and Albert's methods. Since there is a variety of bacteria present in the throat, direct smear is of limited use only. It can help in following conditions:

a. Detection of *C. diphtheriae* like organisms, i.e. Gram positive bacilli with clubbed ends and metachromatic granules showing Chinese letter arrangement.
b. In case of thrush, candidal yeast cells can be seen.
c. Spirochaetes and fusiform bacilli can be seen in Vincent's angina. These are of importance only if present in large numbers only.

Culture

The second swab is immediately cultured on suitable media such as blood agar, chocolate agar and Loeffler's serum slope. In addition selective media for *H. influenzae*, *Strept pyogenes* and *C. diphtheriae* are also inoculated. The incubation is done at 37°C both aerobically and in the presence of 5-10% carbon dioxide.

Blood agar medium supports the growth of all organisms. If it shows the growth of pin-pointed, beta haemolytic colonies which are likely to be that of *Strept. pyogenes*, the same should be subjected to Lancefield grouping to distinguish Lancefield group A from other groups. If such facilities do not exist, a disc of bacitracin can be placed on a lawn of growth. Bacitracin sensitive beta haemolytic streptococci are presumed to belong to group A.

If growth of *C. diphtheriae* like organism is obtained on Loeffler's serum slope, these should be subjected to biochemical tests. Biochemically confirmed diphtheria bacilli are subjected to toxigenicity test.

C. albicans also grows on blood agar giving colonies that are slightly bigger than those of staphylococci. These can be confirmed by Gram's staining.

Antigen Detection

Tests such as agglutination and coagglutination are now developed for the rapid detection of antigen of *Strept. pyogenes* group A. In addition to their being rapid, these are simple to perform even under field conditions. These tests carry good sensitivity and specificity.

Paul Bunnel Test

If on the basis of examination of peripheral blood smear, infectious mononucleosis is suspected, Paul Bunnel test can be performed to confirm the diagnosis.

Examination of Blood

This is useful in following ways:

a. In agranulocytosis, polymorphs are significantly reduced in numbers
b. In acute leukaemia, WBC count is greatly increased and varying number of blast cells may be seen in the blood film
c. In infectious mononucleosis, there is significant increase in the number of circulating mononuclear cells.

Virus Isolation

If facilities exist, attempts at virus isolation from throat washings should be made. It is important only in an outbreak situation because in an individual case if the bacterial aetiology of sore throat is ruled out, the treatment component does not change.

LABORATORY DIAGNOSIS OF CHOLERA

Definition

Cholera is caused by *Vibrio cholerae* and typically characterised by the sudden onset of effortless and profuse watery diarrhoea. The watery stools with flecks of mucous and sweet fishy odour are characteristic of cholera. These are also popularly described as rice water stools. The frequent and profuse watery stool and vomiting lead to severe fluid loss (dehydration) and electrolyte imbalance.

Clinical Sample: Collection and Transportation

In the laboratory investigation of cholera, the categories of samples that can be collected are:

a. Stool/rectal swabs
b. Vomitus

Specimen should be collected at the earliest following the onset of clinical symptoms. The first specimen from a case of diarrhoea should, as far as possible, be collected before administration of antibiotics/chemotherapeutics.

Stool, if available, is the most rewarding specimen for laboratory diagnosis of cholera. Stool should never be collected directly from bed pans as they are likely to be contaminated from previous use and on the other hand the disinfectant commonly used to disinfect the pan may interfere with isolation of causative agent. The patient may be asked to void the stool in a sterile wide mouth paper container—something like the ones used for ice creams, and 3-5 grams transferred to a sterile screw capped bottle.

Whenever it is not possible to collect stool, **rectal swab** specimen may be collected. Though rectal swab is not as good as stool for testing, it is very useful under field conditions and in case of young babies. It absorbs 0.1 to 0.2 ml liquid faeces. Rectal swab should be made from good quality absorbent cotton.

Storage and Transport

Vibrio cholerae do not survive for more than few hours in stool if stored at room temperature and hence the specimen should be stored at +4°C and transported to laboratory with least possible delay. This may not always be possible and more so when the specimens have to be transported over long distance. To overcome this snag, the specimen should be placed in suitable transport or holding medium. The function of transport medium is to maintain viability of the causative organism present in the specimen for long period even when stored at room temperature. The transport media most commonly used for storage and transportation of stool specimen are :

a. Cary Blair medium
b. Venkataraman and Ramakrishnan fluid (VR fluid)

Cary-Blair transport medium is useful for transport of most of the diarrhoea producing bacteria. It is stable and can be stored for use in tightly sealed containers (Bijou bottles). It is a semi-solid transport medium.

Venkataraman and Ramakrishnan fluid (VR fluid) is also used for storage and transportation of stool samples. Specimens for the isolation of *V. cholerae* are to be collected in VR fluid in 1 oz. screw capped bottles (MacCartney bottles). VR fluid preserves vibrios for more than 6 weeks and has also proved to be a very convenient medium for transportation as it can be kept at room temperature after collection of specimen.

Moist Filter Paper Collection

If none of the transport medium is available, collect watery stool from suspected cholera cases by soaking blotting paper strip in the stool, sealing the strip in a polythene bag or an airtight container where the strip does not dry. The moist blotting paper strip can then be transported to the laboratory. Blotting papers have been found to be superior to the filter paper since they can retain the moisture for longer period.

The laboratory diagnosis is based upon followings:

- Direct demonstration of organism
- Culture
- Biochemical test
- Serotyping

- Biotyping
- Antibiotic sensitivity determination

Direct Demonstration of Organism

Prepare a hanging drop from 4 to 6 hr alkaline peptone water inoculated with sample and examine under high dry objective. A darting motility is suggestive of *Vibrio cholerae.*

Culture

Many media have been recommended for isolation of *Vibrio cholerae*. The two media most commonly used are *Bile Salt Agar* (BSA) and *Thiosulphate Citrate Bile salt Sucrose Agar* (TCBS).

Enrichment medium. Alkaline peptone water (pH 8.2) is ideal enrichment medium for *V. cholerae*. Alkaline peptone water with 3 percent sodium chloride is useful for halophilic vibrios such as *Vibrio parahaemolyticus*.

Inoculation of enrichment and plating media. Inoculate a loopful of stool suspension into 5 to 10 ml alkaline peptone water (pH 8.2), incubate at 37°C for 4-6 hrs. Streak a loopful of the culture taken from surface on the plating medium. At the same time transfer a small aliquot into a fresh tube containing alkaline peptone water and incubate overnight at 37°C. Inoculate on plating media.

Colony morphology. On BSA: small, translucent, raised, flat colonies.

On TCBS: Yellow, flat, smooth colonies with pale yellow periphery.

Biochemical Tests

Vibrios are oxidase positive, various tests have been mentioned in Table 82.15. Tests employed to differentiate classical *V. cholerae* from El Tor and those which help in differentiating *V. cholerae* from *V. parahaemolyticus* have been depicted in Tables 82.16 and 82.17.

Table 82–15. Salient biochemical properties of *V. cholerae*

Test	*Reaction*
Fermentation of glucose	Acid;No gas
Growth at 4°C	–
Growth without NaCl	+
Sucrose fermentation	+
O/129 sensitivity	+
Lecithinase production	+
Indole	+
Oxidase	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Arginine dihydrolase	–

Table 82–16. Differentiation between *V. cholerae* and *V. parahaemolyticus*

Character	*V. cholerae*	*V. parahaemolyticus*
Growth without NaCl	+	–
Growth in 6% NaCl	–	+
Swarming	–	+
Beta galactosidase	+	–
Voges Proskauer reaction	+	–
Sensitivity to O/129	+	–
Resistance to ampicillin	–	+
Acid from arabinose	–	+
Acid from sucrose	+	–
Growth in ethanol	–	+

Table 82–17. Differences between classical and ElTor biotypes of *V. cholerae*

Character	*Classical*	*El Tor*
Haemolysis of sheep RBC	–	+
Agglutination of chick RBC	–	+
Resistance to polymyxin B	–	+
VP reaction	–	+
Resistance to lysis by phage IV	–	+
Lysis by phage 5	–	+

Confirmation by Slide Agglutination

Suggestive colonies on bile salt agar/TCBS are tested for slide agglutination with cholera polyvalent (non-differential) antiserum (01 group antiserum). A colony is picked and emulsified in a drop of normal saline placed on a microscopic slide. A loopful of cholera 01 polyvalent antiserum is mixed into the suspension. A positive agglutination test is indicated by immediate clumping of the organism in the suspension.

Serotyping

The cultures positive for *Vibrio cholerae* 01 are further serotyped by conducting slide agglutination test using Ogawa and Inaba specific antisera.

Agglutination with Ogawa antiserum	:	Serotype 'Ogawa'
Agglutination with Inaba antiserum	:	Serotype 'Inaba'
Agglutination with both Ogawa and Inaba antisera '	:	Serotype 'Hikojima

These non 01 *V. cholerae* strains belong to serotypes other than 01 group. There are 139 different serogroups of non 01 *V. cholerae* and isolates of such strains can be

serotyped, provided the antisera for the different serotypes are available. A new serotype 0139 has emerged during 1992.

Biotyping of *V. cholerae*

Chicken Cell Agglutination

This test is performed by mixing a heavy suspension of growth from a nutrient agar slant or plate (18-24 hrs. culture at 37ºC) with a drop of 2.5 percent defibrinated washed chicken red cell suspension on a clean grease free slide. The agglutination will take place within 2 minutes, if the strain belongs to *V. cholerae* biotype El Tor. Classical strains do not agglutinate chicken red cells.

Sensitivity to Group IV Phage

One drop of group IV classical phage is to be applied on a lawn made over nutrient agar medium, prepared with a young 2-3 hour culture of the organisms in alkaline peptone water and incubated overnight at 37ºC. Phage IV causes lysis of classical strains of *V. cholerae* but Vibrio ElTor strains are resistant to it. Resistant and sensitive control strains are included with the test.

Polymyxin B Sensitivity

This test consists in using 15 µg/ml of the antibiotic, incorporated in nutrient agar medium over which spot cultures of the test strains are made with a 2 mm loopful of a young 2-4 hrs. broth culture of the organism. El Tor strains grow freely on polymyxin agar plates, while strains of classical *V. cholerae* show no growth. Both positive and negative controls should be included with the test.

An alternative method is to grow a lawn of the test culture on nutrient agar plate and place a disc containing 50 µg/disc of polymyxin B. A clear zone around the disc is suggestive of classical strains of *V. cholerae.*

Haemolysis Test

0.5 ml of a 24 hrs. culture of the vibrio in heart infusion broth (pH 7.4) grown at 35°C in a 16 mm tube containing 10 ml of the media is mixed with 0.5 ml of one percent washed sheep red cells. The mixture is incubated for 2 hrs. at 35 to 37°C and then held overnight at 4ºC, after which it is read for presence of haemolysis. A control containing only heart infusion broth should be included. Known positive and negative cultures should be included in the controls. Classical strains are non-haemolytic, while ElTor isolates may be found to be either haemolytic or non-haemolytic.

Voges Prauskauer (VP) Test

This test is often done to differentiate the VP positive ElTor vibrios from the VP negative classical *V. cholerae*. However, variable results may be obtained.

Antibiotic Sensitivity Test

Single high concentration dry disk method of antibiotic sensitivity testing may be adopted and recommended for testing the sensitivity pattern of the *V. cholerae* isolates, as laid down by the "methods of antibiotic sensitivity testing" by WHO in 1962. By this method, disks of the different antibiotics are spotted over a lawn made over nutrient agar medium, prepared from a 2-4 hours broth culture of the organism. The plates are incubated for 16-18 hours at 37°C and zones of the inhibition around the colonies are measured.

Flow chart for diagnosis of cholera is given in Figure 82.15.

LABORATORY DIAGNOSIS OF DIARRHOEAL DISEASES (GASTROENTERITIS)

Definition

Gastroenteritis is defined as inflammation of the mucus membrane of the stomach and intestines resulting in frequent loose motions with or without mucous, pain abdomen and may be associated with vomiting.

Causative Agents

Gastroenteritis could be infective or non-infective. Infective gastroenteritis could be due to bacterial, viral, parasitic or fungal agents (Fig. 82.16).

The gastroenteritis (more particularly colitis) may be associated with use of certain antibiotics such as clindamycin, lincomycin, etc wherein the organism responsible for causing colitis are *Cl. difficile* and *Staph. aureus*.

The infection with diarrhoea producing organisms can be acquired from a single common source resulting in large scale outbreaks. Agents producing common source diarrhoeas are listed in Table 82.18.

The diarrhoeas could be *invasive (dysenteric) type* or *non-invasive type (watery)* depending upon whether the lining of the intestine has been invaded or not. Invasive diarrhoea is characterised by passage of blood and mucus along with stools. Pus cells and RBCs are seen in microscopic examination. Non-invasive diarrhoea is

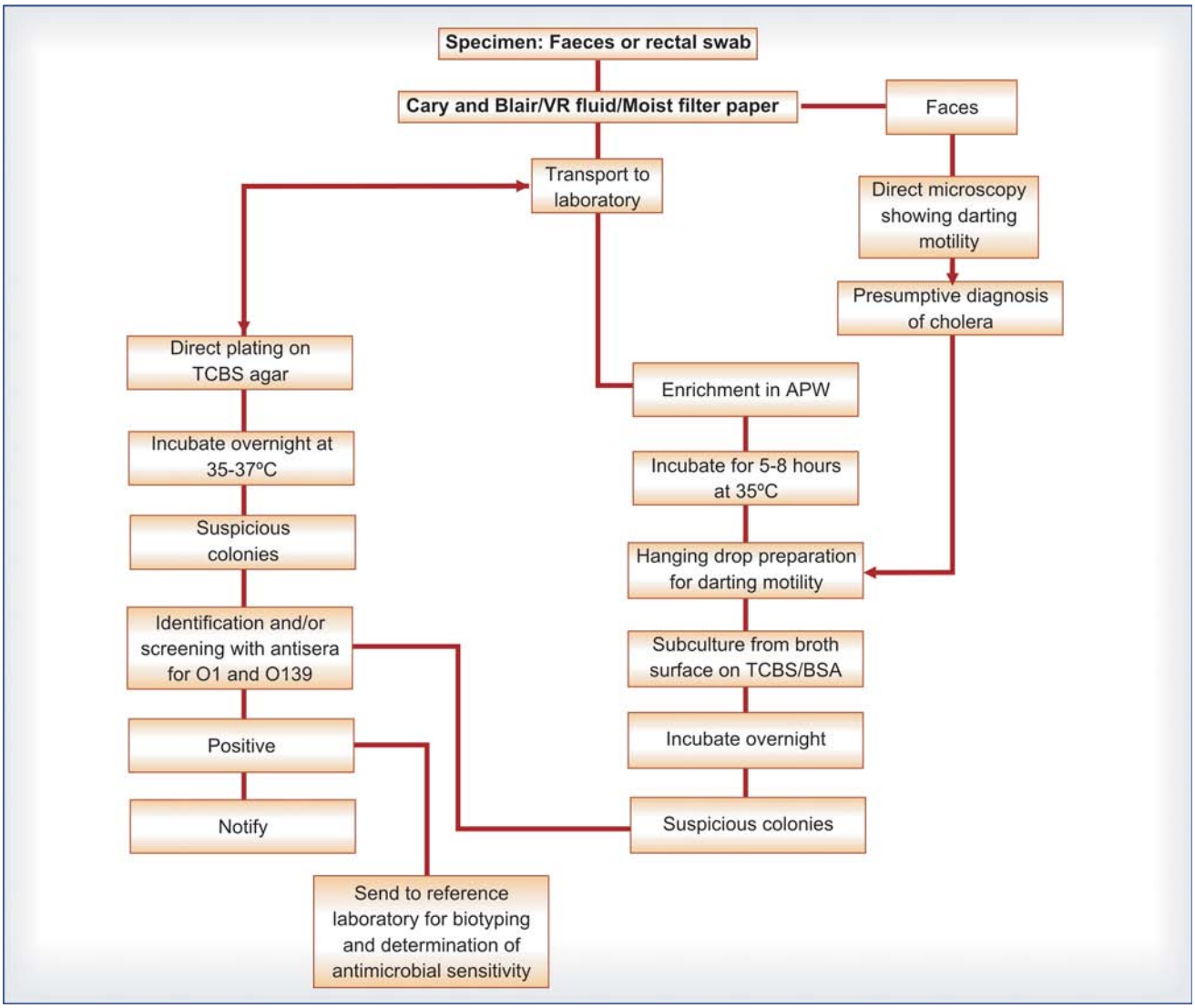


Fig. 82–15. Flow chart for diagnosis of cholera

Table 82–18. Causes of common source diarrhoea

- *Salmonella*
- *Shigella*
- *Yersinia enterocolitica*
- *Vibrio cholerae*
- *Vibrio parahaemolyticus*
- *Campylobacter*
- *Escherichia coli*
- *Staphylococcus*
- *Clostridium perfringens*
- *Bacillus cereus*
- Viruses

watery and profuse with no pus cells or RBCs seen in the direct microscopy. The common examples of non-invasive diarrhoeas are *Vibrio cholerae,* rotavirus, *Cryptosporidium* and enterotoxigenic *Esch. coli* infections, while the common examples of invasive diarrhoeas are *Shigella, Salmonella*, and entero invasive *Esch. coli* infections.

The diagnosis of cholera is given under a separate head in this chapter because of its resurgence and importance from public health viewpoint.

Collection and Transportation of Samples

Stool sample should be collected from the patient before the start of any antibiotic therapy. Specimen should be collected in any clean container free of disinfectant. Though stool sample is preferred, rectal swabs can also be taken if it is not possible to obtain stool samples.

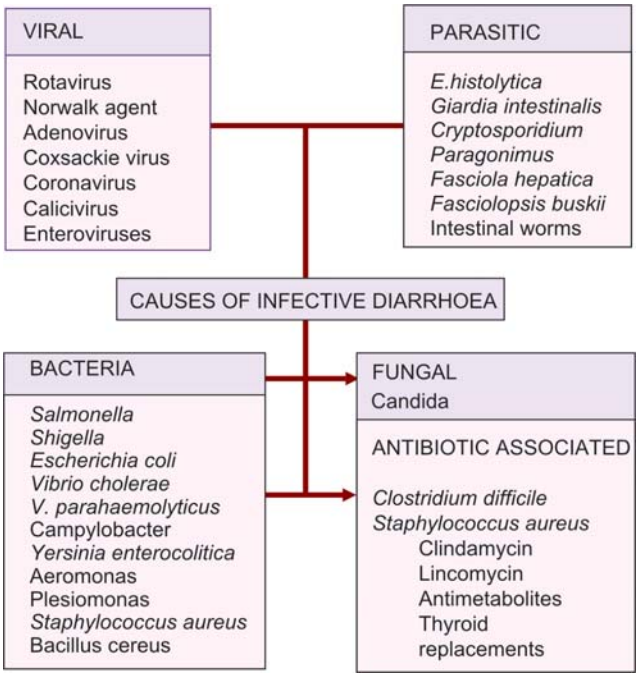


Fig. 82–16. Causes of infective gastroenteritis

Cary Blair medium is recommended for transport of the faeces or rectal swabs, if delay is unavoidable in processing the sample.

If stool sample is received it is important to do both macro- and microscopic examination.

Macroscopic Examination

The color, consistency, blood, mucus, or pus or any parasite in the stool sample are examined with naked eye.

Microscopic Examination

This is done by making a saline preparation of the stool and looking for pus cells, RBCs, or any parasitic ova and cysts. Formol ether concentration technique can be employed to concentrate parasitic ova and cysts in stool sample.

Isolation of Bacterial Pathogens

The purpose is to identify the bacterial pathogen and characterise the same. For this purpose in addition to the routine *plating media, enrichment* and *selective media* are used to inhibit the growth of normal flora. The various media used are MacConkey's agar (MA), Desoxycholate agar (DCA), Salmonella Shigella Agar (SSA) and Selenite F broth (SeF). Selenite F enrichment permits the growth of *Salmonella* and *Shigella* while inhibiting all other non-pathogenic enteric bacteria.

Usual *biochemical reactions* for presumptive identification of some of the common bacterial enteropathogens are given in Table 82.19.

An algorithm of processing stool specimens in case of diarrhoea and dysantery is given in Figure 82.17.

Newer Diagnostic Tests

The conventional tests for the diagnosis of diarrhoeal diseases are aimed at isolation and identification of the causative agent and demonstration of its pathogenic properties in animal models. The newer tests are directed towards detection of pathogenic attributes of the microorganism such as toxigenicity, adhesion, invasion, etc. or the genes responsible for these characteristics. These tests are highly sensitive, mostly repro-

Table 82–19. Biochemical reactions of bacterial enteropathogens

Test	*Esch. coli*	*Salmonella*	*Shigella (Gp A)*	*Shigella (Gp B,C),*	*Shigella (Gp D)*
Motility	+/–	+	–	–	–
Indole	+	–	v	v	–
Urease	–	–	–	–	–
Oxidase	–	–	–	–	–
Catalase	+	+	+	+	+
PPA	–	–	–	–	–
Citrate	–	+	–	–	–
Malonate	–	–	–	–	–
Mannitol	+	+	–	+	+
Lysine	v	+	–	–	–
Arginine	v	+	–/+	–/+	–/+
Ornithine	v	+	–	–	+

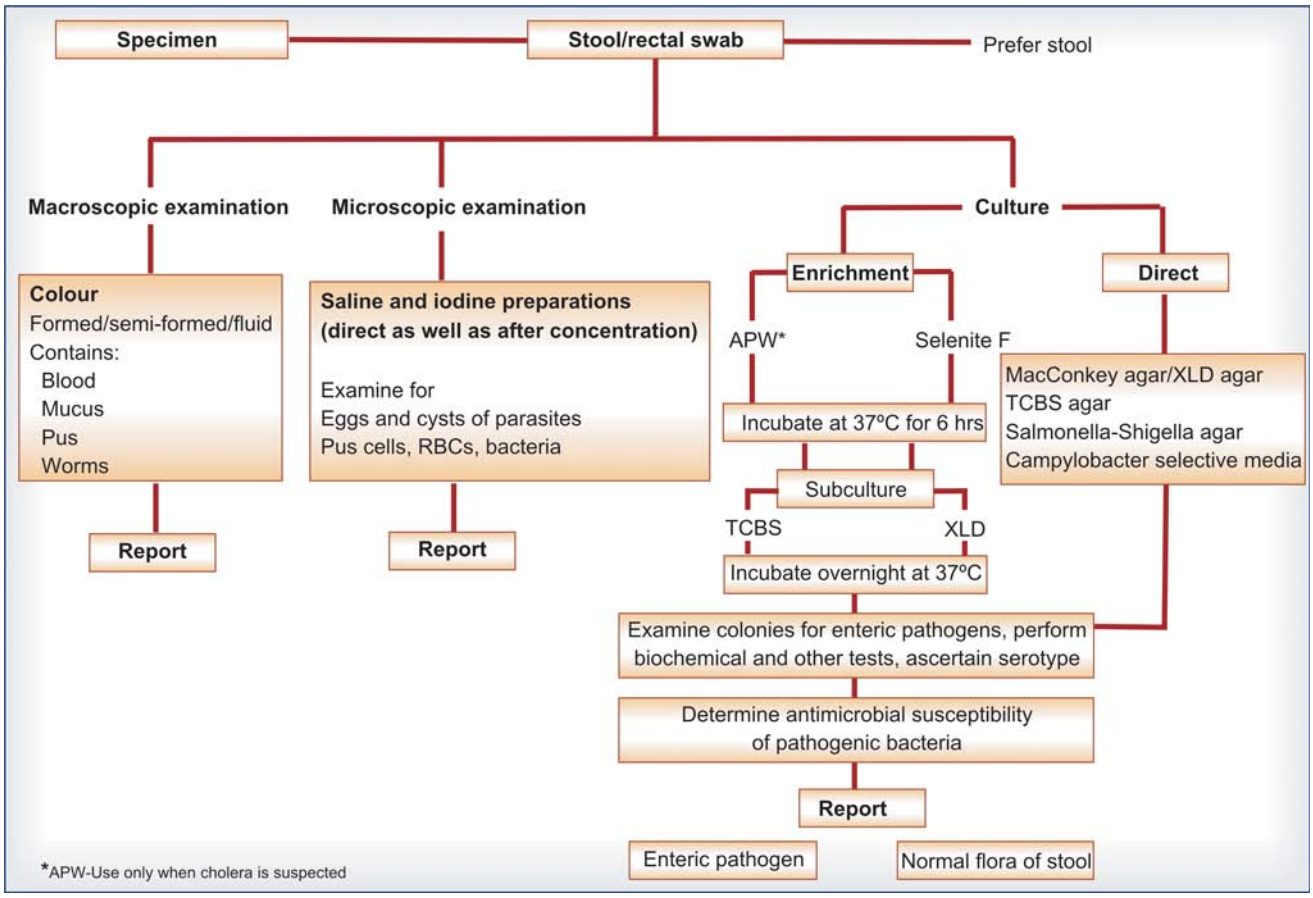


Fig. 82–17. Processing stool specimen in diarrhoea cases

ducible and do not need animal models. However, these tests identify certain properties. They fail to differentiate between the organisms showing similar properties such as *V. cholerae* and ETEC, and *Shigella* and EIEC.

Immunological Tests

These include followings:

a. Latex agglutination
b. Coagglutination
c. Biken test
d. ELISA
e. Detection of enterotoxins

Latex agglutination test has been developed for detection of cholera toxin and rotavirus. The kits are commercially available and test can be performed even in peripheral laboratories.

Coagglutination test has been adopted for LT and CT and is performed in capillary tubes.

Biken test is a precipitation test based on Elek's principle and Ouchterlony's double diffusion test. The LT toxin is liberated on culture plate by a test strain of *Esch. coli*, diffuses into the agar and reacts with LT specific antiserum which has been put into well at the centre of a cluster of colonies. If the colony produces LT, a precipitation line forms between the LT producing colony and the well containing anti-LT. The test is made sensitive by incorporation of lincomycin into the medium to further stimulate production of toxin and treatment of the colony with polymyxin B to promote release of LT from the cell.

Enzyme linked immunosorbent assay (ELISA) has successfully been used to detect diarrhoeal agents and is used for

a. Detection of cholera toxin/*Esch. coli* LT
b. *Esch. coli* heat stable toxin
c. Rotavirus

Detection of enterotoxins can be done by various methods (Table 82.20).

Antibiotic sensitivity test should be done on all the isolates so as to generate data about their sensitivity patterns in a given area.

Table 82–20. Enterotoxin assays

Assay	*ST*	*LT*	*CT*
Rabbit ileal loop			
6 hours	+	+	+
18 hours	–	+	+
Infant rabbit	+	+	+
Rabbit skin	–	+	+
Infant mouse	+	–	–
Tissue culture			
CHO cell line	–	+	+
Y-1 adrenal cells	–	+	+
Passive immune haemolysis	–	+	+
ELISA	–	+	+
Radioimmunoassay	–	+	+
DNA probes	+	+	+

DNA Probes

DNA probes for hybridisation have been developed for:

a. *Esch. coli* heat labile toxin and cholera toxin (LT/CT)
b. *Esch. coli* heat stable toxin (STa and STb)
c. *Esch. coli* cytotoxin (VT-1 and VT-2)
d. Enteroadhesive factor of *Esch. coli*
e. Enteroinvasive plasmid of *Esch. coli* and *Shigella*
f. *Campylobacter*

The advantages of DNA probes over other tests are:

a. They identify the nucleic acid content rather than the products it encodes making the method more reliable.
b. These can be developed for fastidious pathogens.
c. They can be used to detect pathogenic agents directly from the specimens.
d. The results are available in one day.
e. Large number of specimens can be processed at a time.

Though the identification and production of a nucleotide sequence is highly sophisticated and expensive, once produced, they are very cost-effective even in developing countries where large number of specimens have to be examined.

Detection of Viral Agents Causing Diarrhoea

Rotavirus is an important viral agent causing diarrhoea in children. The detection of this virus should be attempted in reference laboratories. The various methods used for demonstration of rotavirus are: (a) Latex agglutination, (b) ELISA, (c) Electron microscopy, (d) Electropherotyping. For detection of rotavirus, the stool samples should be transported to the laboratory at the earliest. If delay is anticipated the sample can be sent by suspending small amounts of faeces in 1 ml of PBS and freezing to –20°C.

LABORATORY DIAGNOSIS OF ACUTE RESPIRATORY INFECTIONS (ARI)

Respiratory tract infections are one of the most frequently encountered diseases of childhood and consist largely of acute infections. Most of these are bacterial or viral in aetiology.

Causative Agents

A number of bacteria and viruses are known to be implicated in ARI especially in childhood. A broad list of commonly occurring bacteria and viruses is given in Table 82.21.

Table 82–21. Causative agents of ARI

BACTERIAL
Streptococcus pneumoniae
Haemophilus influenzae
Staphylococcus aureus
Corynebacterium diphtheriae
Bordetella pertussis
VIRAL
Influenza A,B,C
Parainfluenza types, 1-4
Respiratory syncytial virus (RSV)
Adenoviruses
Rhinoviruses
Enteroviruses (some only)

Collection, Storage and Transport of Samples

For confirming the diagnosis of ARI and the etiologic agents involved, the proper collection, storage and transportation of clinical specimens are very important. The proper collection of clinical material for bacteriological culture provides the foundation for the successful recovery and identification of the causative agents. It is of paramount importance that the specimens are collected before the antibiotic therapy is started.

The specimens for bacterial isolations are as follows:

a. Nasal swab
b. Pernasal swab
c. Throat swab
d. Nasopharyngeal aspirate
e. Transtracheal aspirate and lung aspirate
f. Sputum
g. CSF

h. Blood
i. Middle ear aspirate

After proper collection of the clinical samples, it is imperative that these should be processed without delay. In cases where delay is expected, the specimen is transported in special transport media. The two media commonly used for this purpose are Stuart's medium and Amie's medium. For transportation of swabs of respiratory secretions or discharge, Amie's transport medium is preferable to Stuart's.

The specimens for virus isolation are as follows:

a. Nasopharyngeal specimen: nasopharyngeal aspirate, nasopharyngeal swab and nasopharyngeal wash
b. Bronchoscopic aspirate, lung aspirate or pleural fluid are rarely collected.

Specimens should be delivered to the laboratory as soon as possible after collection. During transport, these are held at 4°C on wet ice in an insulated, well-sealed container to protect them from direct sunlight and extreme temperature. If the time between specimen collection and delivery to the laboratory is expected to exceed 24 hrs, the specimens should be frozen in dry ice.

Diagnosis of Bacterial Pathogens

Gram Staining

It is particularly rewarding when the samples are from areas of the body which are normally sterile, such as the lungs, middle ear and meninges. In addition, organisms may be demonstrated that have not been detected by culture, because of antibiotic therapy or inhibition by other bacteria in sputum. It gives important clues for initiation of prompt treatment.

Culture

For initial isolation the samples are primarily inoculated on blood agar, MacConkey's agar, and chocolate agar. Material inoculated for the isolation of respiratory pathogens should be incubated promptly at a temperature of 35-36°C for 24-28 hrs. The growth of most of the respiratory bacterial pathogens is better in the presence of 5-10 percent CO_2 which is achieved by candle jar. A relative humidity of 70-90 percent is desirable which can be achieved by keeping an open water reservoir at the bottom of the incubator.

Identification of Bacterial Isolates

Streptococcus pneumoniae. Pneumococci typically produce an alpha-haemolytic low convex draughtsman shaped colonies which may be entire or undulate. Colony size is dependent on the freshness of medium, the amount of capsular material of the isolate and the atmosphere in which the cultures are incubated. The confirmation of the isolates is done by: (a) optochin sensitivity test, (b) mouse inoculation test, (c) quellung reaction, and (d) serotyping the isolate.

Corynebacterium diphtheriae. The culture is done on Loeffler's serum slope and blood tellurite agar. Colonies develop very fast in Loeffler's serum slope and also morphology is best in this medium. The characteristics of organism are (a) thin, slender, Gram positive bacilli, (b) presence of metachromatic granules, (c) clubbing of the bacteria, and (d) chinese letter arrangement of the bacteria. Toxigenicity tests and antibiotic sensitivity of the isolate should be done.

Haemophilus influenzae. It is a fastidious organism requiring media containing haemin (X-factor) and nicotinamide adenine dinucleotide (V-factor). Growth on blood agar, if it occurs, is poor, very discrete and may be alpha-haemolytic. Luxurious growth is obtained on chocolate agar. The identification is done by following tests: (a) Satellitism test, (b) X and V factor growth tests. *H. influenzae* is catalase positive, usually non-haemolytic and requires both X and V factors. After biochemical identification, the serotyping is done using type specific antisera.

Bordetella pertussis. Fluorescent antibody technique offers a promising mean of rapid and early diagnosis and a greater number of positive results, than with culture. The medium used for culture is Bordet-Gengou medium and the recommended procedure of inoculation is cough-plate method which is a bed side procedure. Plates are incubated in a moist chamber containing carbon dioxide enriched atmosphere for upto 7 days and examined each day for growth of *Bordetella*. These are gram-negative pleomorphic bacilli or coccobacilli and are poorly reactive biochemically. *B. pertussis* can be distinguished from *B. parapertussis* by its inability to grow on nutrient agar, chocolate agar and urease negativity.

Staphylococcus aureus. On blood agar usually betahaemolytic, typically golden opaque colonies are produced. Haemolysis and pigmentation are not satisfactory for differentiating *Staph. aureus* from *Staph. epidermidis*. Production of coagulase by *Staph. aureus* and its nonproduction by *Staph. epidermidis* is probably the most accurate and convenient method of differentiating the two.

Rest of the organisms are also to be identified as per the routine tests.

Antibiotic Sensitivity Test

Antibiotic sensitivity testing of isolates should be regularly done. Stoke's method, wherein control strains are used, should be preferred method of sensitivity testing. Combination drugs should be tested separately, e.g. sulfamethoxazole and trimethoprim testing in cotrimoxazole should be done separately.

Blood Culture

This gives very reliable results about the invasion by the microrganisms, especially when it is done before the antibiotic therapy is initiated.

Detection of Bacterial Antigens

The detection of bacterial antigen in the body fluids can be done even:

a. When the organism is dead before the specimen is subjected to culture,
b. When the patient has already received antibiotic therapy, and
c. Microorganism has a long replication time.

Various recommended techniques are:

a. Counterimmunoelectrophoresis (CIEP)
b. Coagglutination
c. Latex agglutination
d. Enzyme linked immunosorbent assay (ELISA)

Isolation of Viruses

Cell Culture Techniques

The cell lines that are employed for the isolation of respiratory viruses as per recommendation of WHO are shown in Table 82.22.

Table 82–22. Virus isolation in cell lines

Cell line	*Virus*
Primary monkey kidney cell	Influenza A and B, Parainfluenza, Mumps
HeLa or HEp2 cell line	RSV Adenoviruses
Human lung fibroblast cells	Enteroviruses Rhinoviruses Herpesviruses

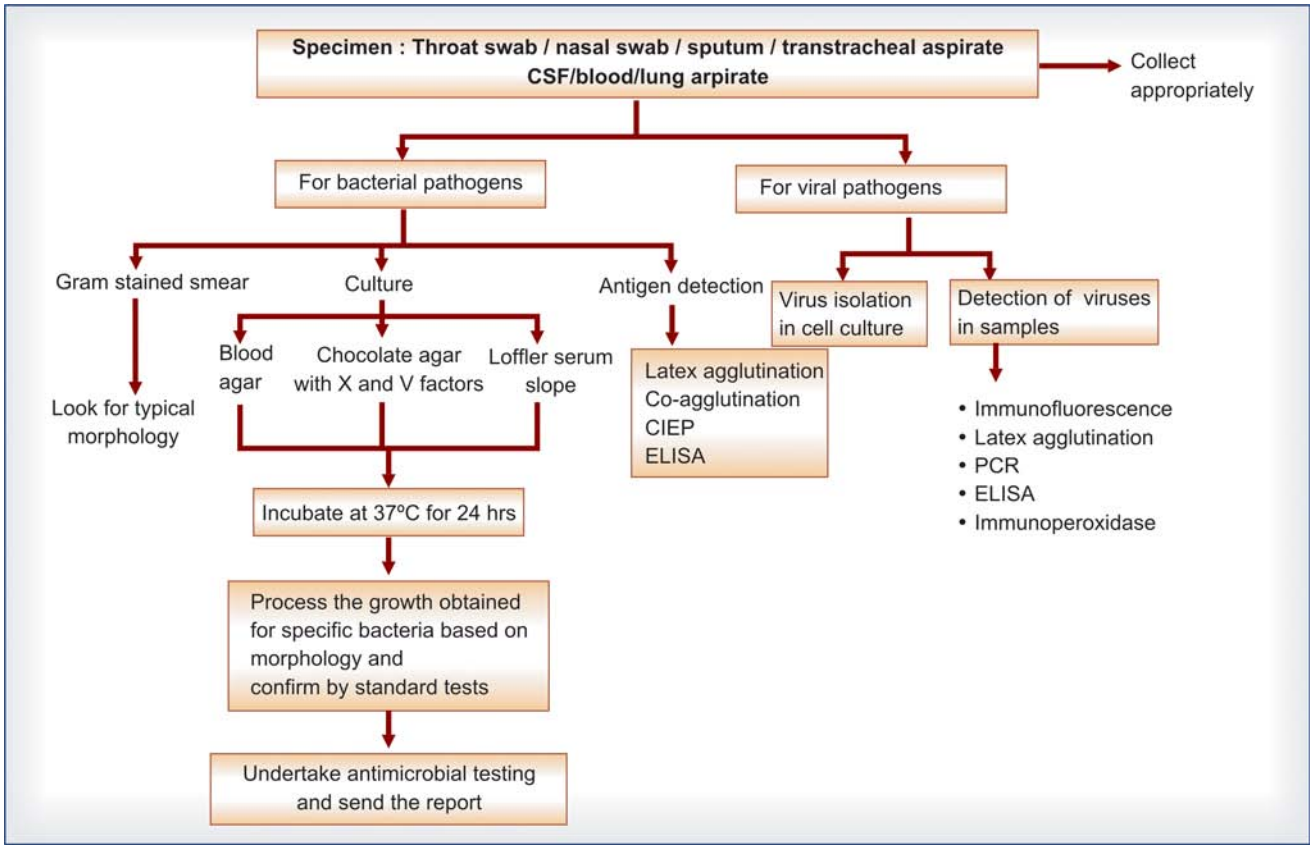


Fig. 82–18. Steps in diagnosis of acute respiratory infections

Each virus can be isolated in more than one cell line with varying sensitivities. The most sensitive cell culture for isolation of human viruses are primary human embryonic kidney (HEK) and primary monkey kidney (PMK).

Developing Chick Embryo

Influenza viruses can be isolated by inoculating nasopharyngeal washings into the amniotic cavity of 11-13 days old eggs. Amniotic and allantoic fluids are harvested after incubating eggs at 35°C for 3 days. The presence of virus is identified by haemagglutination using guinea pig and fowl cells.

Detection of Viruses

Various techniques are now available which are gradually replacing the conventional methods of isolation of viruses for diagnosis. Some of these are:

- Latex agglutination
- Coagglutination
- Counterimmunoelectrophoresis
- ELISA
- Immunofluorescence
- Immunoperoxidase test
- Dot-blot hybridization
- PCR or gene amplification.

An algorithm showing steps in diagnosis of acute respiratory infections is given in Figure 82.18.

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