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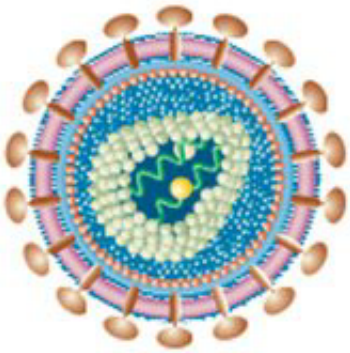
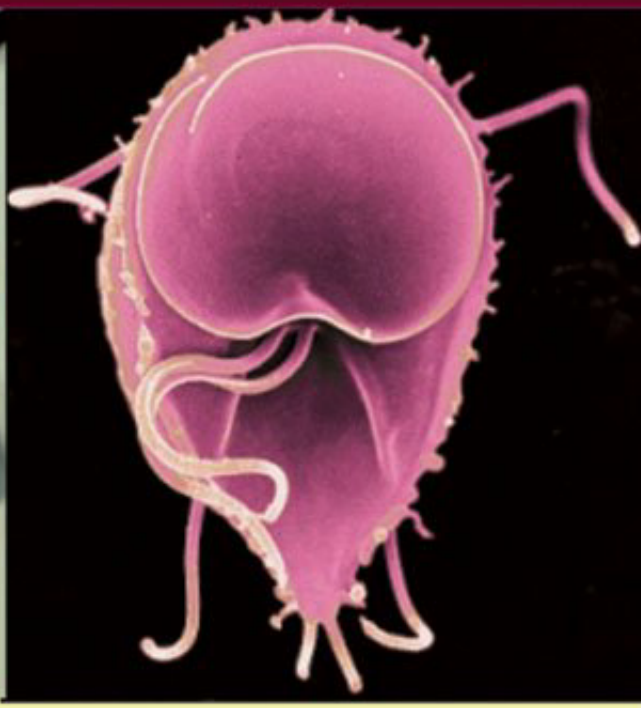


The Short Textbook of Medical Microbiology (Including Parasitology)

Satish Gupte



JAYPEE

The Short Textbook of
Medical Microbiology
(Including Parasitology)

The Short Textbook of Medical Microbiology

(Including Parasitology)

Tenth Edition

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Foreword

Dr Satish Gupte has done wonderful job in updating his book to tenth edition. The simplicity of language and scholarly work done in the book shows the effort of author for updating the book.

I wish more undergraduate and postgraduate students will read this book for updating their knowledge and skills.

I wish the book to be a landmark in subject of Microbiology.

Dr AS Sekhon MD
Principal
Gian Sagar Medical College and Hospital
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Distt. Patiala, Punjab
India

Preface to the Tenth Edition

Over 27 years back first edition of *The Short Textbook of Medical Microbiology* was brought out in 1982. Since then the volume is galloping while passing through silver jubilee year landing right into revised, enlarged and updated tenth edition decorated elegantly and colorfully with entirely fresh and new get up. Indonesian edition of this volume was published in 1990 with collaboration of Binarupa Aksara under the title MICROBIOLOGI DASAR earning fame and name in that region.

It has been kept in mind to condense and include the latest major meaningful advances in medical microbiology in this volume. Distinct feature of this volume is inclusion of updated Medical Parasitology with Entomology. All the chapters are thoroughly revised and updated as highlighted by esteemed readers from time to time. New chapters are included namely; microbiology in the service of human being, molecular techniques in microbiology, emerging and re-emerging infections and laboratory and hospital wastes. Also included in this volume: Biofilm, new methods of disinfection and sterilization, super antigen, Ogawa medium, adenosine deaminase activity, immunological investigations of *Mycobacterium tuberculosis*, Napah virus, pandemic of swine flu (H_1N_1) breakthrough 2009, laboratory diagnosis of septicemia/bacteremia and infective endocarditis and Nobel prize in Medicine etc.

The challenge was to keep the volume comprehensive, profusely colored, beautifully illustrated and still handy maintaining emphasis on the needs of MBBS and BDS students. Several colored figures and photographs, tables and colored illustrations (adjusted appropriately between texts) are included to enhance and intensify the utility of this volume. The chapters pertaining to clinical microbiology are especially addressed to budding doctors (interns and house surgeons/physicians) who usually face problems in collection and transportation of clinical samples for laboratory investigations.

It is the privilege to offer my sincere gratitude to my respected teachers: Professor TD Chugh, Professor Uma Sabharwal, Professor RK Arya and Professor DR Arora for their blessings, encouragement, inspiration and good will. My special thanks to Professor Aruna Aggarwal, Professor BL Sherwal, Professor Jagdish Chander, Professor Prem Narwan, Professor Raj Kumar, Professor Anjum, Professor Bella Mahajan and many others for their best wishes and valuable suggestions in upbringing the current edition of this volume.

I am thankful to Dr GM Warke of Hi Media Laboratory, Mumbai, for kind permission to use some photographs.

I acknowledge the help rendered by faculty of Department of Microbiology, Gian Sagar Medical College and Hospital, (Banur), Patiala in the preparation of this edition. Thanks to Mr Gurpreet Singh for taking responsibility of secretarial work of this volume.

Professor AS Sekhon, Dean and Principal, Gian Sagar Medical College, (Banur), Patiala has encouraged me and enriched me with his good wishes to accomplish the commitment of bringing up this edition to the contentment and expectations of readers. From the core of my heart, I am grateful to him indeed.

My sincere thanks to Shri Jitendar P Vij (Chairman and Managing Director), Jaypee Brothers Medical Publishers (P) Ltd., New Delhi and his dedicated and dynamic team for publishing this book elegantly and speedily.

In true sense I derived much inspiration and blessings from my mother, respected Late Smt. Kaushalya Gupte and respected father Late Shri Charanjit Gupte. Also, I take opportunity to express my thanks to all my family members especially to my wife Mrs Jyotsna Gupte and dear son Anubhav Gupte who actually helped me sincerely while I was simply engrossed in preparing and also during finalizing this manuscript.

Comments, suggestions and constructive criticisms towards improvements of future editions of this volume are very much welcomed. In fact, they underline the shortcomings which are looked into and rectified in the interest of our esteemed readers.

Satish Gupte

"Gupte House"
60, Lower Gumat,
Jammu 180016
September, 2009

Preface to the First Edition

In India as also in other countries of the Third World, despite advances in the field of microbiology, there has been an acute dearth of well-written textbooks on the subject for the undergraduate medical students.

The *Short Textbook of Medical Microbiology* attempts to fill that long-left gap. In spite of great deal of expansion in all branches of microbiology, I have tried to make it concise yet comprehensive, handy and up-to-date. The stress has been on contemporary medical microbiology's relevance for today's needs in our set-up. A large number of line diagrams and tables are included to simplify the points. Also, the language has been simple, easy and straightforward. In line with our requirements more emphasis has been laid on practical aspects. A strong point of the digest is a special stress on the mechanism of production of disease by the microbes and laboratory diagnosis.

In writing this volume, I have considerably counted on the superb teaching of my teachers, namely Prof TD Chugh, Prof RK Arya and Prof (Mrs) Uma Sabharwal. The goodwill and wise counsels of Dr DR Arora, Dr Ashok Malik, Dr (Mrs.) Santhosh Saini, Dr Satish Walia, Dr RL Kaul, Dr (Mrs) Sudershan Kumari Gupta and Dr Satish Sharma proved a source of much stimulation.

Lastly, I express my appreciation for the various favors extended by the family folk at various stages of publication.

With this, let me hope that this little book makes its modest contribution by presenting the subject of medical microbiology in an easy, simple and straightforward form helping the undergraduates to imbibe basic and applied things on the subject.

"Gupte House"
60, Lower Gumat,
Jammu
July 1982

Satish Gupte

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General Bacteriology

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1 Glossary of Microbiology

Readers may find it useful to go through the following definitions, or at least indications of their meanings.

A

Abscess: A localized collection of pus.

Acid fast: Resistant to decolorization by acid after staining with hot carbol fuchsin and hence retaining a red color when stained by Ziehl-Neelsen method.

Accessory: Something added which helps.

Active immunity: Dependent upon stimulation of person's own immunological mechanisms.

Active: To make something work.

Adenine: A purine constituent of nucleic acid.

Adenosine triphosphate (ATP): Compound in which energy is stored in high energy phosphate. bonds; its components are the purine adenine, D-ribose and three phosphoric acid groups.

Adjuvant: Insoluble materials which act to keep antigens in tissues for longer period, thus cause a longer stimulation of antibody production.

Aerobe: An organism which requires oxygen to live and reproduce.

Agglutinate: To join together to form clump.

Allele: One of a group of genes which can occupy a given place on a pair of identical chromosomes.

Allergy: An abnormal sensitive reaction.

Anaerobe: A microorganism not requiring oxygen to live or reproduce.

Anamnestic reaction: A rise of an existing antibody level in response to irrelevant stimulus.

Anhydrous: Containing no water.

Anode: A positive electrode.

Antagonism: Impairment of the efficacy of drug in the presence of the other.

Antibiotic: A substance used to kill microorganisms. It is a product of microorganism

Antibody: A globulin produced in the body in response to the antigen or foreign bodies.

Antigen: Any substance which can cause the production of antibodies.

Antiserum: A serum containing antibodies against particular organism.

Antitoxin: An antibody against particular toxin.

Asepsis: Without infection.

Atmospheric pressure: The pressure of air on earth.

Attenuated: Reduced virulence but retaining antigenecity for host.

Atypical: Unusual.

Auto-infection: Infection of oneself.

Automatic: Doing something by itself.

B

Bacilli: Stick-like or rod-like bacteria.

Bacteria: Single celled organisms containing both RNA and DNA which reproduce by binary fission.

Bacteriology: The study of bacteria.

Bacteremia: Presence of bacteria in bloodstream.

Bacteriocide: A chemical used to kill bacteria.

Balantidium coli: A protozoan ciliate causative agent of balantidiasis, a type of dysentry.

Beaded: Staining at intervals along the length of bacillus.

Binary fission: Simple cell division by which the nucleus and cytoplasm divides into two.

Biologic oxidation: Any chemical reaction occurring within a cell that results in the release of energy respiration.

Biologic oxygen demand (BOD): A measurement of the amount of oxygen required for the microbial decomposition of the organic matter present in water.

Biopsy: The removal of small piece of tissue during life for examination.

Bio-type: A classification or a group of genetically similar organisms.

Bi-polar: The staining of bacillus at both ends.

Blepharoplast: Basal body structure in hemoflagellates from which axoneme arises.

Blister: A small swelling in the skin filled with serum.

Bovine: Associated with cattle.

Booster dose: Additional infection of antigen to maintain antibody production at its peak.

Brittle: Easily broken.

Brownian movement: Passive to and fro movement of bacteria suspended in a fluid, due to irregular bombardment of molecules in fluid.

Budding: An asexual form of reproduction of unicellular organisms, e.g. yeast cells.

Buffer: A solution, the reaction of which is not easily altered by adding an acid or alkali.

C

Candling: Inspection of an unbroken egg by holding it in front of bright light source to know whether embryo is alive or dead.

Capsid: The protein coat surrounding the genome of virus.

Capsomere: One of the units of which virus capsid is composed.

Capsule: A coating outside cell walls of some bacteria and fungi.

Carrier: One who is harboring but not currently suffering any ill-effect from pathogenic organism.

Cathode: A negative electrode.

Cell: A microscopic mass of protoplasm containing nucleus.

Cell line: An *in vitro* culture of cells of known origin.

Cellulitis: The result of a spreading infection of pyogenic bacteria in the subcutaneous tissues.

Characteristic: A quality which distinguishes something, i.e. typical.

Chemotherapeutic agent: A synthetic chemical suitable for systemic administration and effective in treatment of microbial infections.

Chitin: Polysaccharide containing glucosamine, characteristic of cell walls of some fungi and also found in insects.

Chromatin: Darkly staining nuclear material.

Chromosomes: Thread-like structure in the cell nucleus which contains genes carrying inherited characteristics.

Chronic: Slowly developing.

Clone: Cell derived from single cell.

Clinical disease: The ways by which a disease shows itself in a patient.

Clue cells: These are epithelial cells covered with large clumps of coccobacilli. They are good markers of infection.

Coccus: A rounded or ovoid bacterium.

Colony: A number of organisms living or multiplying together on culture media and they result from multiplication of a single organism.

Commensal: Deriving nourishment from a host without causing any harm or benefit to host.

Complement: A group of substances present in fresh serum and necessary for completion of some processes like lysis that result from anti-antibody interactions.

Control: A check.

Constitutive enzyme: Produced under all circumstances, not dependent upon the presence of appropriate substrate.

Conjugation: Exchange of genetic material between bacteria.

Culture: The growth and multiplication of microorganism.

Culture media: The material used in a culture to nourish the growth of microorganism.

Cytopathic effect: Degenerative changes occurring in tissue culture cells as a result of microorganism infection.

Cytoplasmic streaming: Continuous movement of cytoplasm within the cell which results in constant distribution of intracellular contents. It provides amoebic motility to some types of cells.

D

Darting: A fast jerky movement.

Decolorize: To remove color.

Deaminase: An enzyme which catalyzes the removal of an amino group from a molecule with liberation of ammonia, the process is called deamination.

Delicate: Fragile, which can be damaged easily.

Deposit: The sediment at the bottom of tube.

Desiccation: Drying or removal of water.

Detergent: A surface active agent used in cleaning.

Diplococci: Cocci which occur in pairs.

Disinfectant: A substance of chemical nature used for destroying pathogenic microorganisms.

Distorted: Altered or changed.

E

Effective: Producing a result.

Electrolyte: A chemical which helps to keep correct water balance between the fluid in the cell and that which surrounds it.

Elementary bodies: Single chlamydiae particle visible by ordinary microscope after staining.

Embed: To penetrate into tissue.

Emergency: A serious situation requiring immediate attention.

Encapsulate: To surround with a capsule.

Endemic: A disease constantly present in an area.

Endogenous: Originated by organisms or factors already present in the patient's body before onset of disease.

Endotoxin: A toxic component of microorganism (Gram negative), largely dependent on the death or disruption of the organism for its release.

Enriched medium: A culture medium to which an extra nourishing substance is added.

Enrichment medium: A liquid medium used to encourage preliminary growth of an organism so as to enhance the chances of growing it on subsequent plate culture.

Enzyme: A chemical produced by living organism to help some particular reaction.

Epidemic: A disease that temporarily has a high frequency in a given community.

Eradicate: To get rid of.

Excision repair: An enzyme system which can repair DNA by the elimination of ultraviolet light induced thymine dimers.

Exogenous: Originated by organisms or factors from outside the patient's body.

Exotoxin: A toxin released by living microorganisms into the surrounding medium or tissues.

Exudate: A fluid, often from formed elements of blood, discharged from tissue to a surface or cavity.

F

Factor: A substance with a special purpose, e.g. blood clotting factor.

Facultative: Able to behave in a specified way, with the implication that is not, however, the usual behavior.

Fermentation: The slow decomposition, for example by microorganisms, of organic material such as the decomposition of a sugar solution by yeast to form alcohol.

Filament: A fine thread-like structure.

Fimbria: Hair-like protrusion from bacterial cells.

Flagellum: Whip-like organ of motion.

Flocculant: A mass of cells floating or settled to the bottom in a liquid medium.

Flocculation: Precipitation in small cloudy mass.

Fluctuation test: It is to determine the development of mutants in culture. The test is based on the concept that mutation is a completely random event, and spontaneous mutation would result in the number of mutants in a series of identical cultures.

Fluorosis: Mottling of tooth enamel due to excessive amount of fluoride in water.

Fomites: Objects contaminated with pathogenic microorganisms.

Fragment: A piece broken off.

Fungus: A simple unicellular or multicellular structure which reproduces by forming spores.

Fusiform: Spindle shaped.

G

Gel: To become solid, to set.

Gene: A unit of inheritance found on chromosomes.

Genetic: The study of inheritance.

Genome: The total genetic material of an organism.

Genotype: The inherited characters of a particular individual.

Genus: A group of closely related species of plant, animal or microorganism.

Globulin: A plasma protein divided into the alpha, beta and gamma fraction.

Gram-negative: Staining red by Gram's method.

Gram-positive: Staining violet by Gram's method.

Growth factor: An ingredient of which at least small amount must be present in a culture medium so that it supports the growth of given organism.

Granules: Small grains or particles, e.g. metachromatic granules of diphtheria bacilli.

H

Halophilic: Salt loving.

Hapten: A substance which acts as an antigenic stimulus only when combined with protein but capable of reacting with resultant antibody in uncombined state even.

Hemolysis: The destruction of red cells with the release of hemoglobulin.

Hereditary: Transmitted from one generation to the other.

Hetero: Different.

Heterologous: Related to different kind of organism.

Hfr strain: A high frequency mating strain.

Homologous: Related to same kind of organism.

Host: The organism from which a parasite takes its nourishment.

Hypertonic: A condition in which the fluid surrounding a cell is more concentrated than that within it.

Hypotonic: A condition in which the fluid surrounding a cell is less concentrated than that within it.

I

Identical: Exactly same.

Immune: Protected from disease by the presence of antibodies.

Immunoglobulin: Globulins which act as antibodies.

Inactive: The heating of serum at 56°C to destroy its complement and inhibitory factors.
Inclusion: Something which is enclosed.
Incubate: To keep at the same temperature for a given length of time.
Infection: The entry and multiplication of pathogenic organisms within the body.
Inoculate: To introduce a living organism into a culture medium.
Intra: Inside.
In vitro: In laboratory apparatus.
In vivo: In a living animal or human being.

L

L-form: Cell wall deficient mutant.
Ligases: Enzymes that catalyze the linking together of two molecules.
Locus: The definite place of a gene on a chromosome.
Lyophilization: Combined freezing and desiccation (freeze drying).
Lysis: Disruption.
Lysogenic conversion: Alteration of the property of bacterium as a result of lysogeny.
Lysogeny: A temporary stable relationship between bacteriophage and its bacterial host, in which the phage is reproduced in step with the bacterium and, thus, handed on to succeeding generation of bacteria.

M

Macrophage: A large mononuclear phagocytic cell.
Medium: A nutrient substance used to grow microorganisms.
Metabolism: The process of building up chemical compounds in the body and their breaking down during activity.
Microaerophile: An organism which grows best in subconcentration of oxygen.
Misense mutation: A change in cell's DNA with the effect that a wrong amino acid has been put into essential protein.
Molecule: The smallest part of an element or compound which can exist in normal way.
Monolayer: A sheet of tissue culture cells one cell thick.
Morphology: A study of the form of cells and organisms.
Motile: Capable of movement.
Moult: The shedding of skin.
Mutation: An alteration in genetic material.

N

Neutral: Neither acidic nor alkaline with pH of 7.0.
Nodule: A small rounded swelling.
Normal: Usual or ordinary.
Normal solution: A solution in which the equivalent weight in grams of a chemical is dissolved in one liter of solution.
Nucleoid: Genome.
Nucleocap: The genome and capsid.
Nucleus: An essential part of the living cell, containing the chromosomes and controlling cell activity.
Nutrition: Food.

O

Obligatory anaerobe: An organism which cannot live in oxygen.
Occult: Hidden.
Opaque: Not allowing light to pass.
Optimum: The most suitable.
Oxidation: Combination with oxygen.

P

Pandemic: World-wide epidemic.
Parasite: An organism which takes its food from another organism without giving anything in return.
Passage: Administration of micro-organism to a host and its subsequent recovery from the host. This way pathogenicity of organism is modified.
Passive immunity: Dependent upon injection of readymade antibodies and not upon the subject's own immunological mechanisms.

Pathogen: An organism which can cause disease.

Petri dish: A shallow circular flat bottomed glass or plastic dish used as a container for solid media.

Phage type: The identity of a bacterial strain as indicated by its sensitivity or resistance to the lytic action of bacteriophages.

pH: The symbol that indicates the acidity or alkalinity of a solution. pH less than 7 is acid and more than 7 is alkali.

Phagocyte: A cell which ingests microorganisms.

Phenotype: The expression of genotype.

Pigment: A coloring substance.

Pilus: Fimbria.

Plasmid: An extrachromosomal portion of genetic material.

Polymerase: General name of enzymes concerned with synthesis of nucleic acid.

Prophage: Bacteriophage in a lysogenic relationship with its host.

Prophylactic: A medicine to prevent disease.

Prozone: The occurrence of an antigen-antibody reaction only when serum is adequately diluted but not when it is used at higher concentration.

Protoplast: A bacterium deprived of its cell wall.

Puerperal fever: Acute infection following child birth due to introduction of infectious agent into the uterus.

Purulent: Containing pus.

Pus: A thick yellowish green fluid containing phagocytic cells collecting in tissues infected with pyogenic bacteria.

Pyogenic: Pus forming.

R

Rack: A stand for holding tubes.

Rash: A skin reaction, usually seen as small reddened or raised area.

Reaction: An action which takes place in response to something.

Reagin: Antibodies associated with certain types of hypersensitivity reactions. It may be contained in serum causing Wassermann reaction and related reactions.

Recombinant: A cell or clone of cells resulting from conjugation.

Reduction: The removal of oxygen from a chemical compound.

Replication: Virus reproduction.

Reticuloendothelial system: The system of phagocytic cells in the body.

Retraction: Shrinking.

Ribonucleoprotein: Material in the cytoplasm and nucleus of cell.

Rodent: A gnawing animal, e.g. rat, mouse.

Room temperature: Usually 18 to 20°C.

Routine: Carried out regularly.

S

Sanitize: To reduce microbial number to safe levels.

Saprophytic: Living on dead organic matter.

Saturated solution: A solution which has dissolved as much as it can of a substance.

Satellitism: Enhancement of bacterial growth on a solid medium around a source of growth factor.

Selective medium: A solid culture medium on which all but the desired organisms are wholly or largely inhibited.

Septicemia: Presence and multiplication of bacteria in blood stream resulting severe disease.

Serology: The study of serum especially antibody contents.

Serotype: Antigenic type.

Specific antibody: An antibody which react with one particular antigen only.

Spheroplast: Similar to protoplast with the difference that cell wall damage is partial and reversible.

Spirochete: Genera of spiral bacteria.

Sterilization: The process of killing all living microorganisms including spores.

Symbiotic: Living in mutually with the host.

T

Technique: The method by which something is done.

Temperate phage: A phage capable of a lysogenic relationship with its bacterial host.

Thermostat: An instrument to control the temperature.

Tissue: A group of similar cells.

Titer: The highest dilution of antibody in a serum which will react with its specific antigen.

Tolerogen: An antigen that induces tolerance.

Toxoid: Toxin rendered harmless but retaining antigenicity.

Transduction: Transfer of genetic material from bacterial strain to another by means of bacteriophage.

Transformation: Acquisition of genetic characters of one bacterial strain by a related strain grown in presence of DNA from the first strain.

Transport medium: A medium which increases the chances of survival of microorganism during transportation from patient to laboratory.

Turbid: Cloudy.

Typical: Showing usual features.

U

Undulating: Up and down, i.e. wavy.

Unicellular: Single celled.

Unstable: Easily changed.

Urticaria: A skin rash caused by allergy.

V

Vaccinate: To introduce into the body killed or mildly pathogenic organisms to produce resistance against disease.

Vaccine: Material used in vaccination.

Variation: A change from the usual.

Vector: An insect which carries microorganism or parasite and is capable of transmitting this.

Viable: Able to live.

Vibrio: A comma shaped microorganism.

Viremia: Presence of viruses in the bloodstream.

Virion: A virus particle.

Virulent: Harmful.

W

Working solution: A solution used in a test, made up from concentrated stock solution.

Y

Yeast: A unicellular fungus.

Z

Zygote: The cell formed by the fertilization of a female cell by a male cell.

2 History and Scope

INTRODUCTION

The first simple forms of life appeared on earth more than three billion years ago. Their descendants have changed and developed into the several million types of animals, plants and microorganisms recognized today. Of course, thousands more remain to be discovered and officially described.

Microscopic forms of life are present in vast numbers in nearly every environment known, i.e. soil, water, food, air, etc. Since the conditions that favor the survival and growth of many are the same as those under which people normally live. It is not unusual to find out these microscopic forms on the surface of our bodies and in mouth, nose, portions of the digestion tract and other body regions. Fortunately, the majority of these microorganisms are not harmful to humans.

Many microscopic microorganisms or microbes occur as single cell, others are multicellular, and still others such as viruses, do not have a true cellular appearance. Some organisms called anaerobes are capable of carrying out their vital functions in the absence of free oxygen; whereas other organisms can manufacture the essential compounds for their physiological needs from atmospheric sources of nitrogen and carbon dioxide. Other microorganisms such as viruses and certain bacteria are totally dependent for their existence on the cells of higher forms of life. The branch of science known as microbiology embraces all of these properties of microorganisms and many more.

As we progressed towards conquering over many infectious diseases like smallpox, anthrax, plague, etc. and campaign is in progress with varied vaccines and other efforts on war footing hopes we dashed to the ground with reemergence of diseases like malaria, influenza, tuberculosis, plague, dengue and many more. Not only this, but we were further discouraged with the appearance of new microbes causing disastrous diseases like AIDS (1981), Lyme's disease, Ebola fever, Marburg disease, Hepatitis-C, Hepatitis E, Hepatitis F, Hepatitis G, SARS bird flu, swine flu etc.

On the positive side microbiology has its share in contributing as a model in studies of genetics, molecular biology in addition to suggesting diagnostic methods, prevention and control of various diseases of microbes origin. Genetic engineering is another magnificient gift of microbes with other technique of genetic manipulations.

One of the attractive features of microbiology is the amount of investigations and work remaining to be done. Many decisions affecting the future of the world may depend upon and involve the activities of microorganisms in areas like food production, pollution control, energy production and the control and treatment of diseases. In short, microbiology has assumed a position of great importance in modern society.

Medical microbiology (Gr. mikros-small, bios-life, logos-science) is the study of causative agents of infectious disease of human beings and their reactions to such infections. In other words it deals with etiology, pathogenesis, laboratory diagnosis, treatment, epidemiology and control of infection.

Obviously, medical microbiology has close link with other disciplines, i.e. pathology, clinical medicine, surgery, pharmacology cum therapeutics and preventive medicine.

Microbiologists are enthusiastic to confirm the diagnosis and cause of macroscopic changes by taking smears and preparing cultures from the lesions to demonstrate microorganisms, e.g. circumscribed boil of staphylococcus, the spreading cellulitis of streptococcus, the red-liver-like appearance of lung in pneumococcal pneumonia, the tubercles followed by syphilis and intestinal ulceration of salmonella organism, etc. The pathology of infection is quite fascinating as it includes affinity of pathogens for certain tissue and initiation of infection and characteristic tissue reaction. Each investigation is regarded as research project in miniature. The clinician by requesting it is asking a question and the laboratory will attempt to reply it as reliably, rapidly and economically as possible.

Historical Events

Long before the discovery of microorganisms certain processes caused by their life activities, such as fermentation of wine juice, milk, yeast, etc. were known to mankind. In ancient times at the beginning of civilization, man by using these processes learned to prepare koumurs, sour milk and other products.

In the works of Hippocrates (460-377 BC), Verona (2nd century BC), Lucretins (95-55 BC), Pliny (23-79 AD), Galen (131-211 AD) and other scientists of that time, the hypotheses of living nature (contagium vivum) of contagious diseases was described.

Varo and Columella (first century BC) suggested that diseases were caused by invisible organisms (animalia minuta) inhaled or ingested.

Fracastorius of Verona (1546) felt that contagium vivum may be the cause of infectious disease.

Kircher (1659) could find minute worms in the blood of a plague patient. It is more likely that he observed perhaps only blood cells with the apparatus which he had at his disposal at that time.

Antoni van Leeuwenhoek (1683) could give description of various types of bacteria. He also invented the simple microscope.

As microbes are not visible to the unaided eye, the observation had to wait till microscopes were developed. Jensen (1590) happened to be the first fortunate person who successfully magnified object using hand lens. The credit for observation and description of bacteria is attached to Antony van Leeuwenhoek of Holland (1683). An amateur lens grinder and was first person to make glass lenses powerful enough for observation and description of bacteria. He suggested that population of increase of animalcules were progeny of few parenteral organisms and described many different living and parasitic protozoa, filamentous fungi and globular bodies (yeast cells) from human and animal stools. He introduced meat and vegetable infusions in culture medium and 250 single lensed microscopes. Robert Hook, a contemporary of Leeuwenhoek developed compound microscope in 1678 and confirmed Leeuwenhoek's observation.

Von Plenciz (1762) proposed that each disease was caused by a separate agent.

Augustino Bassi (1835) proposed that muscardine disease of silk worm was caused by fungus.

Oliver Wendell Holmes (1843) and Ignaz Semmelweis in Vienna (1846) independently put forward the view that puerperal sepsis was transmitted by the contaminated hands of obstetricians and medical students. Washing of hands in antiseptic solution was suggested for its prevention.

Pasteur (1857) established that fermentation was the result of microbial activity. Different types of fermentation were associated with different kinds of microorganisms. He introduced techniques of sterilization and developed steam sterilizer, hot air oven and autoclave. He started his work on pebrine, anthrax, chicken-pox, cholera and hydrophobia. He attenuated culture of anthrax bacilli by incubation at high temperature (42°C to 43°C) and proved that inocculation of such culture in animal induced specific protection against anthrax. Pasteur's development of vaccine for hydrophobia was the greatest breakthrough in medicine. He is remembered as a man who laid the foundation of microbiology. He is also known as father of microbiology.

Robert Koch (father of bacteriology) perfected bacteriological techniques during his studies on the culture of anthrax bacillus (1876). He introduced staining techniques and also methods of obtaining bacteria in pure culture using solid media. He discovered bacillus tuberculosis (1882), *Vibrio cholerae* (1883) and also demonstrated Koch's phenomena which is the expression of hypersensitivity phenomena of *Mycobacterium tuberculosis.*

He also suggested criteria before blaming the organism responsible for disease. It goes by the name of Koch's postulate, according to which:

1. Organism should be present in the pathological lesion and its demonstration from this lesion.
2. We must be able to culture the organism from the lesion.
3. These cultured organisms must be able to produce same lesion when injected into animals.
4. Again these organisms should be cultured from animal lesion.
5. Antibodies against these organism should be demonstrable (added subsequently).

Lord Lister (1854) used carbolic acid spray on wound during operation. He is also called father of antiseptic surgery.

Hansen (1874) described leprosy bacillus.

Neisser (1879) described gonococcus.

Ogston (1881) discovered staphylococcus.

Loeffler (1884) isolated diphtheria bacillus.

Nicolaier (1884) observed tetanus bacilli in soil.

Fraenkel (1886) described pneumococcus.

Schaudin and Hoffman discovered the spirochaete of syphilis.

Roux and Yersin (1888) described mechanism of pathogenesis when they discovered diphtheria toxin.

Loeffler and Frosch (1898) observed that foot and mouth disease of cattle was caused by a microbe, i.e. filter passing virus.

Walter Reed (1902) observed that yellow fever was caused by filterable virus and that it was transmitted through the bite of mosquitoes.

Landsteiner and Popper (1909) showed poliomyelitis was caused by filterable virus.

Towert (1951) and Herelle (1917) discovered lytic phenomenon in bacterial culture. The agent responsible was termed as bacteriophage (viruses that attack bacteria).

Fleming (1925) made the accidental discovery that the fungus penicillium produces a substance which destroys staphylococci.

Ruska (1934) introduced electron microscope and hence detailed study of morphology of virus was possible.

Jerne (1955) proposed natural selection theory of antibody synthesis.

Burnet (1957) put forward clonal selection theory.

Burnet (1967) introduced concept of immunological surveillance.

New agent of infectious diseases continue to emerge, e.g. HIV (identified in 1980). The outbreaks of plague in 1994, cholera in 1995, and dengue hemorrhage fever in 1996.

As such many workers in medicine have been awarded Nobel Prizes for their outstanding contributions in microbiology (Table 2.1)

The methods of many infectious diseases and vaccine production have been revolutionized, e.g. recombine DNA technology, PCR, nuclear anaprobes, radioimmunoassay, ELISA, etc.

MICROBIOLOGY

In short this is the science dealing with the study of microorganisms.

APPLICATION TO HEALTH

Scientific names of microorganisms in laboratory investigation reports can be quite informative for the physician who may take appropriate decision in treating the patients accordingly. Certainly the knowledge of various microorganism can be helpful in understanding their activities in the laboratory studies.

Of course genetics and the adoptability of microorganisms to chemotherapeutic agents is a must for correct administration of doses of antibiotics at properly spaced intervals.

The student will very soon start to associate names of various organisms with morphology and then with specific disease they cause. Needless to say that scientific names of

TABLE 2.1: Noble prize winners in microbiology

1901	Behring	Antitoxins
1902	Ross	Malaria
1905	Koch	Bacteriology
1907	Laveran	Malaria
1908	Ehrlich and Metchnikoff	Theories of immunology
1913	Richet	Anaphylaxis
1919	Bordet	Immunology
1928	Nicolle	Typhus fever
1930	Landsteiner	Blood groups
1939	Domagk	Sulfonamide
1945	Fleming, Florey and Chain	Penicillin
1951	Theiler	Yellow fever vaccine
1952	Waksman	Streptomycin
1954	Ender	Cellular culture of polio virus
1958	Lederberg, Taitum and Beadle	Genetics
1959	Ochoa Kornberg	Genetics RNA
1960	Burnet and Medawar	Theories of immunity
1961	Watson and Crick	Genetic code, structure of DNA
1965	Jacob, Mond and Lwoff	Genetic episome and prephage
1966	Rous	Viral etiology of cancer
1968	Nirenberg, Holley and Khuranna	Synthesis of DNA
1969	Dulbeco, Luria and Delbruck	Genetics, mutations
1972	Porter and Edelman	Structure of immunoglobulin
1974	Christian	Lysosomes
1975	Dulbeco, Baltimore and Teonin	Genetics and mutations
1976	Gajdusck and Blumberg	Slow virus and Australia antigen
1977	Rosalyn Yalow	Radioimmunoassay
1978	Arber, Nathans and Smith	Restriction enzyme
1980	Snell, Dausset and Benacerrof	Major histocompatibility complex (MHC) and genetic control of immune response
1983	Barbara McClintoch	Mobile genetic element
1984	Georges Koehler and Danish Niele Jerne	Monoclonal antibodies
1987	Tonegawa Susuma	Generation of immunoglobulin diversity
1988	Gertrude Elion, George Hitchings and James Black	Discoveries of important principles that have resulted in the development of a series of new drugs including Acyclovir for herpes and AZT for treating AIDS
1989	Michael Bishop and Harold Varmus	Discovery of cellular origin of viral oncogenes
1993	Richard J Robert	Split genes
1997	Stanley B Prusiner	Prion
2005	Barry J Marshal and Roben Warren	*Helicobacter pylori* as causative agent of gastritis and peptic ulcer
2006	Andrew Fire and Craig Mello	RNA interference—gene silencing by DS RNA

microorganisms are clues to the characteristic properties of each organism. However, it would be useless to learn the name of microorganisms without knowing the meaning and significance of these names.

Recovery of *Bacillus subtilis* from sterilized packages in operating room is quite dangerous because its survival indicates that sterility is not attained. However recovery of the *Bacillus subtilis* from dust in the hospital wards would not be a source of alarm, since this organism is not pathogenic.

There is a widespread notion that staphylococcal infections to be seriously viewed. As such the beginning student may take with great concern reports of skin cultures that show the presence of *Staphylococcus epidermidis*. The more experienced and

knowledgeable investigator, however, is able to discriminate and recognize only those strain of staphylococcus that produce a golden yellow (aureus) pigment, the enzyme coagulase, that are the main causes of dangerous cases of staphylococcal infections.

One of the best ways of learning microbial nomenclature is to look up the characteristics of each genus and species of organism that is encountered in the classroom, in the laboratory, or in practice. With this practice, a lot of information will quickly become a part of working knowledge, on which one can build the sound practice of the profession.

Branches of Microbiology

1. Medical microbiology
2. Industrial microbiology
3. Food microbiology
4. Soil microbiology
5. Plant microbiology.

Here we are concerned with medical microbiology. It is studied under following headings:

a. Parasitology deals with the study of parasites causing diseases in human being.
b. Mycology deals with the study of fungi causing diseases in human beings.
c. Immunology is concerned with mechanism involved in the development of resistance by body to infectious diseases.
d. Bacteriology deals with the study of bacteria.
e. Genetics is the study of heredity and variations.
f. Virology is the study of viruses.

Scope of Microbiology

1. Diagnostic, e.g. isolation and identification of causative organism from the pathological lesions. We can also diagnose typhoid fever by doing Widal's test.
2. Prognosis of disease, e.g. in Widal's test rising titer signifies active disease and ineffective treatment. Falling titer means effective treatment and curing of disease.
3. Guidance in treatment, e.g. by culturing the organism in pure form and then performing drug sensitivity test we can suggest the effective drug for the treatment of that particular infection.
4. Source of infection, e.g. in sudden outbreak of infectious disease we can find out the source of infection.
5. Detection of new pathogens and then development of vaccines.

Free Living Nature of Bacteria

They are:

1. Some bacteria improve the natural environment by giving away carbon, nitrogen, oxygen and other elements in the air produced during metabolism and thereby a favorable atmosphere for other forms of life is created.
2. Some bacteria are useful in souring milk and ripening cheese.
3. Many free living bacteria produce acetic acid, citric acid, butanol and antibiotics as end product of their metabolism. The metabolic property of bacteria is utilized in certain cases for manufacturing of these substances.
4. The resident flora of the body that are constantly present on the body surface are known as commensals. They play an important role in maintaining health and normal function of the body, e.g. *Staphylococcus epidermidis* of skin and *Escherichia coli* of intestinal tract.
5. Natural water organisms present in the air find their way into the natural water through rains. Soil organisms may also be found in natural water. In addition, water may be polluted by human and animal stools. The principal water organisms are coliform, *Pseudomonas aeruginosa*, Bacillus subtilis, clostrudia, actinomyces, micrococcus, chromobacter and achromobacter.

BIOLOGICAL WEAPONS

Now it is quite clear and understandable that biological warfare is not new at all. Biological warfare was resorted by early Romans who polluted water sources of their enemies by dumping animal carcasses. Then British distributed blankets to Indians in 1763. These blankets had been used by smallpox patients. Hence Indian users contracted smallpox. British had detonated an experimental anthrax bomb in Gruinard Island in second world war.

In 1984, 750 people fell ill to food poisoning in Oregaon because of spread of salmonella that had been cultured in the laboratory.

Biological warfare may be defined as intentional use of doses to harm or kill an adversary military forces, population, food or livestock and includes any living, or nonliving organisms or its bioactive substance (toxin). Hence, germ warfare can be spearheaded by bacteria, viruses, fungi, toxins, etc.

Some of the characteristics of biological warfare agents include low infective dose, high virulence, short incubation period, little immunity in target population, ease of production, ease of delivery, rebustness, stability and availability with aggressor, etc.

Organisms which can be used for biological war are *Bacillus anthracis* (causing pneumonia with a mortality rate 95%, if untreated), smallpox (contagious with high mortality rate), *Yersinia pestis* (plague causing bacteria), Francisella tularensis (tularemia), Ebola and Marburg viruses (hemorrhage fever), *Clostridium botulinum* (botulinism), etc.

Since organisms are capable of infecting widespread illness together with their low cost, their use as biological war weapons seems to be next possibility. The organisms likely to be used for this purpose may be rendered resistant to antibiotic agents, or even to vaccine by genetic means, etc.

Molecular Epidemiology

Molecular epidemiology is defined as a science that focuses on the contribution of potential genetic and environmental risk factors, identified at molecular level, to the etiology, distribution and prevention of diseases within families, countries and continents. Genetic variability within and between infectious agents/disease pathogens forms the foundation of molecular microbiology.

Epidemic Prone Diseases

Ancient

- Smallpox
- Plague
- Kala-azar
- Anthrax.

Middle Era (killer diseases)

- Cholera
- Malaria
- Typhoid
- Tuberculosis.

Middle Era (emerging/reemerging)

- O_{139} *V. cholerae*
- Dengue
- HDRTB
- Leptospira.

Current Era

- Viral hepatitis
- HIV.

Threat of Rare Diseases

- Ebola virus
- Yellow fever
- Rift vallgry
- Kango.

Application of Molecular Epidemiology

- Tracing the source and origin of infection
- Tracking the routes of pathogen transmission
- Identifying reservoirs sustaining transmission
- Identifying new, emerging and reemerging pathogens
- DNA finger printing in actual diagnosis of pathogens
- Characterizing drug resistant strains
- Monitoring the progress of disease and central activities
- Identifying links between cases and infections
- Linking pathogen variants to endemicity and epidemicity
- Monitoring impact of immunization program, e.g. polio eradication.

3 Morphology of Bacteria

Bacteria are unicellular free living organisms without chlorophyll having both DNA and RNA. They are capable of performing all essential processes of life, e.g. growth, metabolism and reproduction. They have rigid cell wall containing muramic acid. They were originally classified under plant and animal kingdoms. This being unsatisfactory a third kingdom PROTISTA was proposed by Hackel in 1866 for them. Protista is again divided into 2 groups: (a) Prokaryotes, and (b) Eukaryotes. Bacteria and green algae (photosynthetic and possess chlorophyll which can exhibit gliding movement like photosynthetic bacteria) are prokaryotes while fungi, algae, slime moulds and protozoa are eukaryotes. It is worth mentioning to enumerate the differences between prokaryotic and eukaryotic cells (Table 3.1).

TABLE 3.1: Difference between prokaryotic cell and eukaryotic cell

	Character	*Prokaryotic cell*	*Eukaryotic cell*
I	**Nucleus**		
	Nuclear membrane	Absent	Present
	Nucleolus	Absent	Present
	Histones	Absent	Absent
	Chromosome	One	More
	Mitotic division	Absent	Present
	DNA	Circular	Linear
II	**Cytoplasm**		
	Cytoplasmic streaming	Absent	Present
	Cytoplasmic ribosome	70s	80s
	Pinocytosis	Absent	Present
	Mitochondria	Absent	Present
	Lysosomes	Absent	Present
	Golgi apparatus	Absent	Present
	Endoplasmic reticulum	Absent	Present
III	**Chemical composition**		
	Sterol	Absent	Present
	Muramic acid	Present	Absent
	Diaminopimelic acid	May be Present	Absent
IV	**Miscellaneous**		
	Diameter	1 µm	10 µm
	Oxidative phosphorylation site	Cell membrane	Chloroplast
	Cilia	Absent	Present
	Pili	Present	Absent

Size of bacteria: Most of bacteria are so small that their size is measured in terms of micron.

1 micron (µ) or micrometre (µm) =
One-thousandth of millimetre.

1 millimicron (mµ) or nanometre (nm) =
One-thousandth of micron.

1 Angstrom units (Å) =
One-tenth of nanometre.

Generally cocci are about 1 µ in diameter and bacilli are 2 to 10 µ in length and 0.2 to 0.5 µ in width. The limit of resolution with unaided eye is about 200 µ. Obviously bacteria can be visualized only under magnification.

Shape of bacteria: On the basis of shape, bacteria are classified as under:

(A) Cocci (from kakkos, meaning berry):

They are spherical. On the basis of arrangement of individual organisms, they are described as staphylococci (clusters like bunches of grapes), streptococci (arranged in chains), diplococci (forming pairs), tetrads and sarcina are cocci arranged in groups of four and cubical packet of eight cell respectively (Fig. 3.1).

(B) The cylindrical or rod shaped organisms are called *bacilli* (from baculus, meaning rods). They are of following types (Fig. 3.2):

1. In some of the organisms length may approximate the width of the organisms. These are called coccobacilli, e.g. brucella.
2. Chinese letter arrangement is seen in corynebacteria.

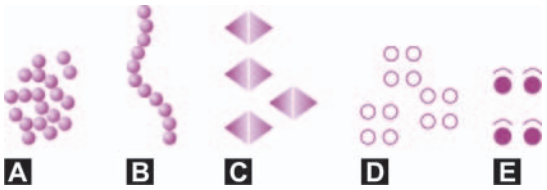


Fig. 3.1: Arrangement of bacilli

(a) Cocci in cluster—staphylococci
(b) Cocci in chain—streptococci
(c) Cocci in pair—diplococci
(d) Cocci in groups of four—tetrad
(e) Cocci in group of eight—sarcina

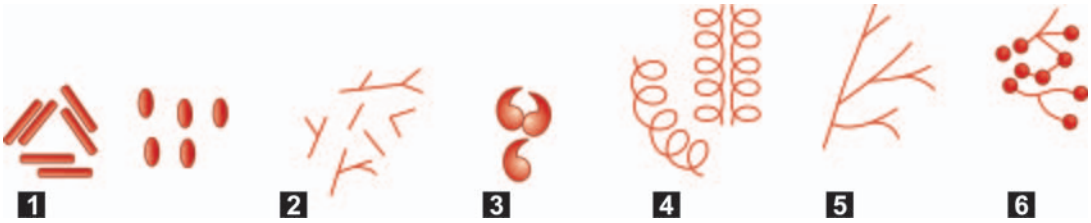


Fig. 3.2: Types of bacilli

3. Vibrio: These are comma shaped, curved rods and derive the name from their characteristic vibratory motility.
4. Spirochaetes (from speria meaning coil, chaete meaning hair). They are relatively longer, thin, flexible organisms having several coils.
5. Actinomycetes (actis meaning ray, mykes, meaning fungus) are branching filamentous bacteria, so called because of resemblance to radiating sunrays.
6. Mycoplasma are organisms which lack cell wall and so do not possess a stable morphology. They are round or oval bodies with interlacing filaments.

Many a time cell wall synthesis becomes defective either spontaneously or as a result of drug, e.g. in presence of penicillin bacteria lose their distinctive shape. Such organisms are called protoplast spheroplast or L forms.

Bacterial Anatomy

The outermost layer consists of two components—(a) a rigid cell wall, and (b) cytoplasmic membrane or plasma membrane. Inside this there is protoplasm comprising of the cytoplasm, cytoplasmic inclusions such as ribosomes, mesosomes, granules, vacuoles and nuclear body. The cell may be enclosed in a viscid layer which may be loose slime layer or organised as a capsule. Nowadays all substances containing polysaccharides lying external to cell wall (both slime layer and capsule) are called glycocalyx. Apart from this some bacteria carry filamentous appendages protruding from cell surface; the flagella, organ of locomotion and the fimbriae which seem to be organ of adhesion.

The important structural features of bacterial cell as found under electron microscope are described below (Figs 3.3 and 3.4).

SLIME LAYER

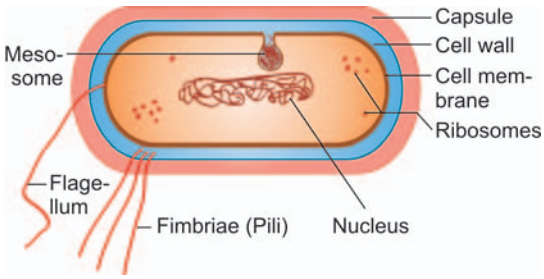


Fig. 3.3: Structure of bacterium

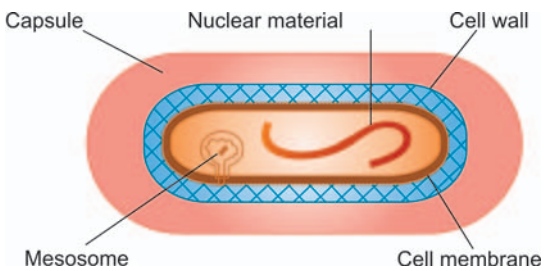


Fig. 3.4: Capsulated bacterium showing nuclear material, a mesosome and relation of cytoplasmic membrane to cell wall

Some bacteria secrete viscid substance which may diffuse out into surrounding media or remain outside cell wall. This viscid carbohydrate material is called slime layer. Its presence can be shown only on immunological ground. Bacteria secreting large amount of slime produce mucoid growth on agar with stringy consistency when touched with loop, e.g. klebsiella. Slime has little affinity for basic dye and so not visible in Gram stained smear.

CAPSULE (FIG. 3.4)

It is the gelatinous secretion of bacteria which gets organized as a thick coat around cell wall and is known as capsule. Capsulated bacteria are usually nonmotile as flagella remains unfunctional in the presence of capsule. Development of capsule is dependent on the existence of favorable environmental conditions such as presence of high sugar concentration, blood serum or growth in a living host. The capsules which are thinner than true capsules are called microcapsule, e.g. Meningococci, *Streptococcus pyogenes* and *Haemophilus influenzae.* It may be composed of complex polysaccharide (pneumococci and klebsiella) or polypeptide (*Bacillus anthracis*) or hyaluronic acid (*Streptococcus pyogenes*). Capsules have no affinity for dyes and so they are not seen in stained preparations.

Demonstration of Capsule

a. Negative staining with India ink: In this procedure, bacterial bodies and spaces in between are filled with India ink and capsule is seen as halo around cell.
b. Special capsule staining technique using copper salt as moderant.
c. Serological methods: If suspension of capsulated bacterium is mixed with its specific serum and examine under microscope, capsule becomes prominent and appears swollen due to increase in refractivity (Quellung reaction).

Functions

1. Protection against deleterious agents, e.g. lytic enzymes.
2. Contribute to the virulence of pathogenic bacteria by inhibiting phagocytosis.
3. Capsular antigen is hapten in nature and specific for bacteria.

CELL WALL

Cell wall is elastic and porous and is freely permeable to solute molecules of less than 10,000 molecular weight. The mucopeptide component of cell wall possesses target site for antibiotics lysozymes and bacteriophages. The cell wall is the outermost supporting layer which protects the internal structure. It is about 10 to 25 nm in thickness and shares 20 to 30 percent of dry weight of the cells.

Chemical Structure of Cell Wall

Cell wall is composed of mucopeptide (muerin), scaffolding formed by N. acetyl glucosamine and acetyl muramic acid molecules alternating in chain cross-linked by peptide chain (Fig. 3.6). Cell wall antigens of Gram negative organisms act as endotoxin. A comparison of cell walls of Gram positive and Gram negative bacteria is enumerated in Figure 3.7 and Table 3.2 (page 18).

Gram-negative cell wall: It is a complex structure (Fig. 3.5) comprising of:

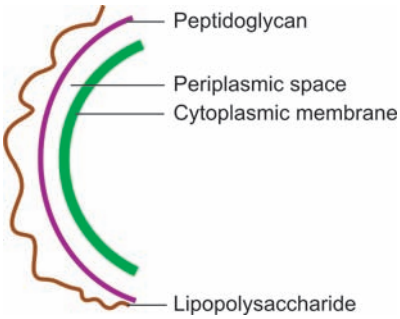


Fig. 3.5: Cell wall (Gram negative bacteria)

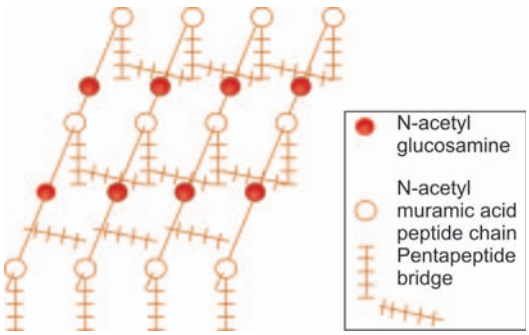


Fig. 3.6: Chemical structure of bacterial cell wall

TABLE 3.2: Difference between Gram-positive and Gram-negative organisms

		Gram positive	*Gram negative*
1.	Thickness	15 to 25 nm	10 to 15 nm
2.	Variety of amino acid	Few	Several
3.	Aromatic and sulfur containing amino acids	Absent	Present
4.	Lipids	Low 2 to 4%	High 15 to 20%
5.	Teichoic acids	Present	Absent
6.	Periplasmic space	Absent	Present
7.	Result of enzyme digestion	Protoplast	Spheroplast

i. Lipoprotein layer which connects outer membrane to peptidoglycan.
ii. Outer membrane is a phospholipid bilayer containing specific proteins. These specific proteins form porins and hydrophilic molecules are transported through these porins. Other proteins are target sites for phages, antibiotics and bacteriocins.
iii. Lipopolysaccharide consists of a complex lipid, called lipid A, to which is attached a polysaccharide. Lipopolysaccharide is the endotoxin of Gram negative bacteria—The toxicity is associated with lipid portion (lipid A) and the polysaccharide represents a major surface antigen, the O antigen. The polysaccharide consists of a core and a terminal series of repeat units. These terminal repeat units confer antigenic specificity to the bacterium.
iv. The periplasmic space is the space in between the inner and outer membranes and contain a number of important proteins (such as enzymes and binding proteins for specific substrates) and oligosaccharides (which play an important role in osmoregulation of the cells).

Gram-positive cell wall:

i. The peptidoglycan layer of Gram positive bacteria is much thicker (15 to 25 nm) than that in Gram negative (10 to 15 nm). The periplasmic space is absent and the peptidoglycan is closely associated with cytoplasmic membrane.
ii. Specific components of Gram positive cell wall include significant amount of teichoic and teichuronic acids which are soluble polymer containing ribotol or glycerol polymers and maintain a level of divalent cations outside cell membrane. The teichoic acids constitute major surface antigens of Gram positive bacteria.
iii. Other components of Gram positive cell wall contain antigens such as the polysaccharide and protein.

Cell wall synthesis may be inhibited or interfered by many factors. Lysozyme enzyme present in many tissue fluid causes lysis of bacteria. It acts by splitting cell wall mucopeptide linkages. When lysozyme acts on Gram positive organism in hypertonic solution a PROTOPLAST is formed consisting of cytoplasmic membrane and contents. Characteristic features of PROTOPLAST are:

1. Unstable structures.
2. Very sensitive to the influence of osmotic pressure, mechanical action and aberration.
3. Unable to synthesize the component parts of cell wall (diaminopimelic and muramic acids).
4. Resistant to phage infection.
5. Not capable of active motility.

With Gram negative bacteria the result is SPHEROPLAST which differs from protoplast in that some cell wall material is retained.

Protoplast and spheroplast are spherical regardless of original shape of the bacterium. Such organisms might have a role in certain persistent chronic infections such as pyelonephritis.

Demonstration

1. When intact bacteria are placed in a solution of very high solute concentrations and osmotic pressure, protoplast shrinks thus retracting the cytoplasmic membrane from cell wall. The process is called plasmolysis and is useful in demonstrating cell wall.
2. Cell wall may also be demonstrated by a special technique called microdissection.
3. Reaction with specific antibody is also a way to study cell wall.
4. Electron microscope gives detailed structural information of even very minute particles like parts of cell wall.

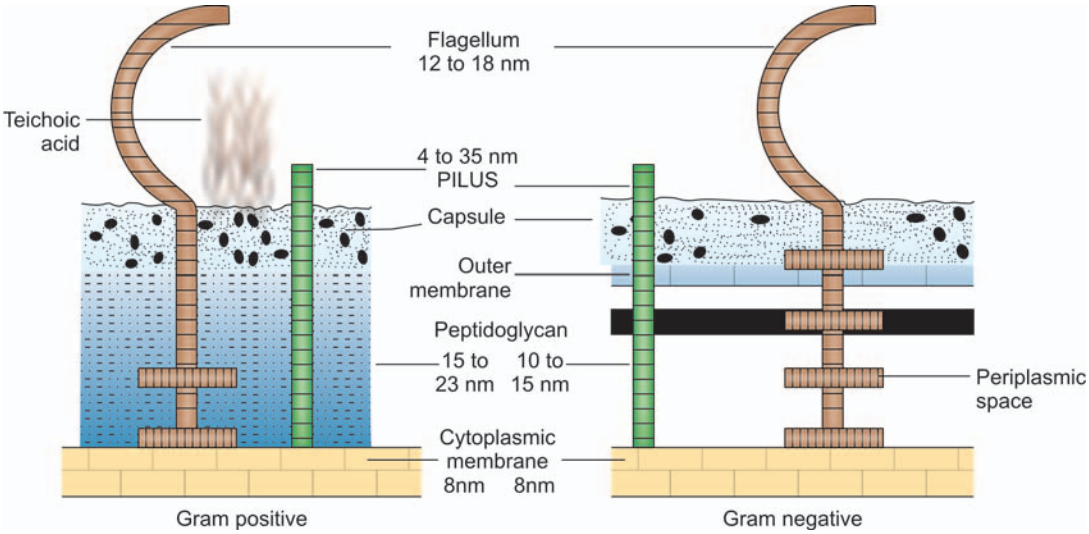


Fig. 3.7: Differences between Gram positive and Gram negative cell wall

Functions

1. Protection of internal structure (supporting layer).
2. Gives shape to the cell.
3. Confers rigidity and ductility (mucopeptide).
4. Role in division of bacteria.
5. Offers resistance to harmful effect of environment.
6. Contains receptor sites for phages and colicin.
7. Provides attachment to complement.

CYTOPLASMIC MEMBRANE

It is thin semipermeable membrane which lies just beneath the cell wall. The whole bacterial cytoplasm is bound peripherally by a very thin, elastic and semipermeable cytoplasmic membrane also known as cell membrane. It is 5 to 10 nm in width. Electron microscope shows the presence of three layers constituting a unit membrane structure. Chemically the membrane consists of phospholipid with small amount of protein. Sterol is absent except in mycoplasma (Figs 3.8 and 3.9).

Demonstration

1. The separation of membrane from the cell wall is achieved readily in Gram negative bacteria when they are suspended in medium of high osmotic tension. Such a phenomenon is called plasmolysis.
2. Electron microscope.

Functions

1. It controls inflow and outflow of metabolites to and from protoplast.
2. Presence in the membrane of specific enzyme (permease) which plays important role in passage through membrane of selective nutrients.
3. The cytoplasmic membrane contains some other enzymes, e.g. respiratory enzymes and pigments (cytochrome system), certain enzymes of tricarboxylic acid cycle and perhaps, polymerizing enzymes that

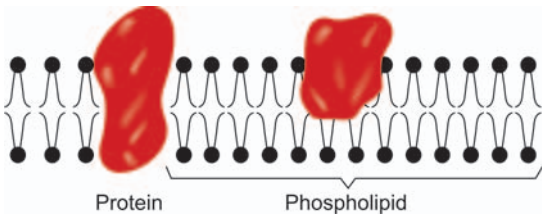


Fig. 3.8: Chemical structure of cytoplasmic membrane

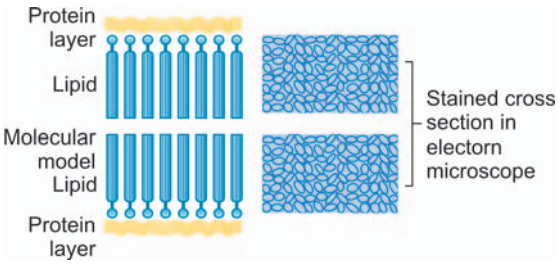


Fig. 3.9: A unit cell membrane with a lipid bilayer with hydrophylic regions towards a protein layer at each surface with characteristic appearance of stained cross-section in electron microscope

manufacture the substance of cell wall and extracellular structure.
4. It provides little mechanical strength to bacterial cell.
5. It helps DNA replication.
6. It concentrates sugar, amino acids and phosphatase creating 300 to 400 fold gradient across osmotic barrier.

CYTOPLASM

The bacterial cytoplasm is suspension of organic and inorganic solutes in viscous watery solution.

It does not exhibit protoplasmic streaming (internal mobility) and it lacks endoplasmic reticulum or mitochondria. It contains ribosomes, mesosomes, inclusions and vacuoles.

Ribosomes: Ribosomes appear as small granules and pack the whole cytoplasm. These are strung together on strands of mRNA to form polymers. The code of mRNA is translated into peptide sequence at this place. The ribosomal particles become linked up and travel along the mRNA strand. It determines the sequence of amino acids brought to the site on tRNA molecule and build up the polypeptide.

Ribonucleoprotein granules measure 10 to 20 nm units in diameter and their sedimentation coefficient is 70 svedberg units. The 70s ribosome is composed of two smaller units of 50s and 30s.

Functions: They are the sites of protein synthesis.

Polysomes: They are groups of ribosomes linked together like beads of chain by messenger RNA.

Mesosomes: They are vesicular, convoluted or multilaminated structures formed as invaginations of plasma membrane in the cytoplasm. They are prominent in Gram positive bacteria. There are two types of mesosomes, i.e. septal mesosome and lateral mesosome. The septal mesosome is attached to bacterial chromosome and is involved in DNA segregation and in the formation of cross walls during cell division. They are also called chondroids and are visualized only under electron microscope. They are more prominent in some Gram positive bacteria.

Functions

1. They are the sites of respiratory enzymes in bacteria.
2. Coordinate nuclear and cytoplasmic division during binary fission.
3. Responsible for compartmenting DNA at sporulation.

Intracytoplasmic Inclusions

Three types of cytoplasmic granules are encountered, i.e. glycogen (enteric bacillium) poly-betis hydroxybutyrate (bacillus and pseudomonas) and Babes Ernst (corynebacterium, *Yersinia pestis*).

a. **Volutin granules** (Metachromatic or Babes-Ernst granules) are highly refractive, basophilic bodies consisting of polymetaphosphate.
   Demonstration: Special staining techniques such as Albert, or Neisser, demonstrate the granules more clearly. They are characteristically present in diphtheria bacilli and *Yersinia pestis.*
   Function: They are considered to represent a reserve of energy and phosphate for cell metabolism.
b. **Polysaccharide granules** may be demonstrated by staining with iodine. They appear to be storage products. These granules are used as carbon sources when protein and nucleic acid synthesis is resumed.
c. **Certain sulfur oxidizing bacteria** convert excess H_2S from environment into intracellular granules of element sulfur.
d. **Lipid inclusion** are storage product and demonstrated with fat soluble dyes such as sudan black.
e. **Vacuoles** are fluid containing cavities separated from cytoplasm by a membrane. Their function and significance is uncertain (Table 3.3).

NUCLEUS

It is a long filament of DNA tightly coiled inside the cytoplasm. The bacterial nucleus is not surrounded by nuclear membrane and is Feulgen positive. It is an ill-defined homogenous pale area of cytoplasm. The nuclear

TABLE 3.3: Cytoplasmic granules In bacteria

Granules	*Bacteria*
Glycogen	Enteric bacteria
Poly beta hydroxyl butyrate	Bacillus pseudomonas
Babes Ernst	Corynebacterium *Yersinia pertis*

DNA does not appear to contain some basic protein. It does not have nucleolus. Nucleus cannot be demonstrated under direct light microscope. It appears as oval or elongated body, generally one per cell.

The genome consists of a single molecule of double stranded DNA arranged in the form of circle. It may open under certain conditions to form long chain about 1000 µ in length. Bacterial chromosome is haploid and replicates by simple fission. Genes are arranged along the length of chromosome in fixed order and bear hereditary characters.

Bacteria may sometimes have extranuclear genetic material. These are called plasmid or episomes. They may be transmitted to daughter cells:

a. during binary fission.
b. by conjugation or
c. through agency of bacterial phages.

Plasmids are not essential for the life of cell. They may confer certain properties like toxigenecity virulence and drug resistance.

FLAGELLA

These are long, sinnous contractile filamentous appendages known as flagella. These are organs of locomotion, e.g. *Escherichia coli*, salmonella, vibrio, pseudomonas, etc. The number of flagella varies up to 10 to 20 per cells according to species of bacteria. *Escherichia coli* has a motility of about 30 µ and that of *Vibrio cholerae* about 60 µ per second. These are extremely thin 12 to 30 nm, helical shaped structure of uniform diameter throughout their length. These are 3 to 20 µ long. Each flagellum (Fig. 3.10) consists of hook and basal body. It originates in a spherical body (basal granule) located just inside cell wall. These are antigenic and are composed of protein called flagellin which has properties of fibrous protein, keratin and myosin. Around 40 gene products are involved in its assembly and function.

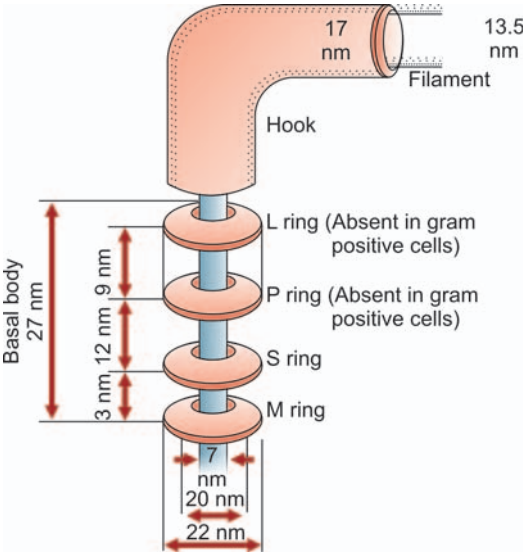


Fig. 3.10: Basal structure of bacterial flagella

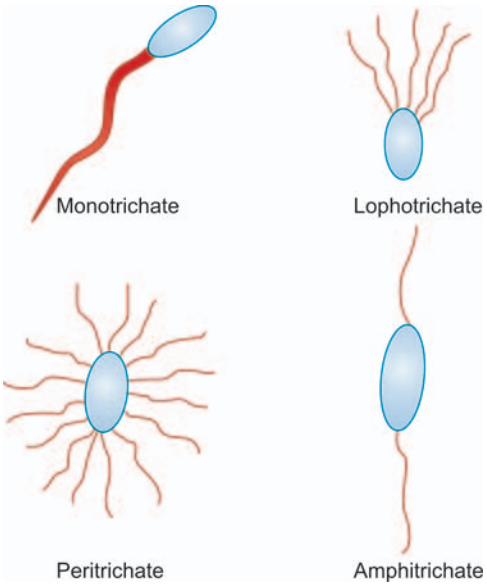


Fig. 3.11: Arrangement of flagella in bacteria

The number and arrangement of flagella are characteristic of each bacteria. Flagella may be arranged on bacterial body in following manner (Fig. 3.11).

Monotrichate: One flagellum at one end of the organism, e.g. vibrio, pseudomonas, spirillum, etc.

Amphitrichate: One flagellum at both poles, e.g. *Alcaligenes fecales.*

Lophotrichate: A tuft of flagella at the end, e.g. pseudomonas.

Peritrichate: Several flagella present all over the surface of bacteria, e.g. *Escherichia coli*, salmonella.

Function: It is responsible for bacterial motility. Motility may be observed microscopically (hanging drop preparation) or by detecting the spreading growth in semi solid agar medium. It has been suggested that heat formed as a result of metabolism is given off to the environment through the flagella, while ATP serves as an energy source. The created difference in temperature causes a stream of water along the flagella, and the bacterium moves in an opposite direction.

Demonstration

1. Dark ground microscopy
2. Special staining techniques in which their thickness is increased by mordanting.
3. Electron microscope.
4. Hanging drop preparation
5. Craigie's tube method

FIMBRIAE

Fimbriae (fringes) are filamentous, short, thin, straight, hair-like appendage 0.1 to 1.5 µ long and less than 4 to 8 nm thick. They are also called pili (hair). Fimbriae are seen only in some Gram negative bacteria. Each bacterium may have 100 to 500 fimbriae peritrichously all over the body of bacteria. They project from cell surface, as straight filaments. They are best developed in freshly isolated strains and in liquid culture. They tend to disappear following subcultures on solid media. Fimbriae are composed of protein known as pillin (molecular weight 18000 daltons).

Pili or fimbriae are as under of following forms:

i. Common pili.
ii. F (fertility) pili.
iii. Col I (colicin) pili.

Common Pili

They are numerous, short in size (1.5 µ) and peritrichous in distribution. They are considered organs of adhering to the surfaces of other cells, e.g. red cell of various animal species. This forms the basis of hemagglutination.

Common pile are of 6 types based on their morphology number per cell, adhesive properties and antigenic nature. The virulence of certain pathogenic bacteria depends both on toxins as well as "colonization antigens (pili)" which provide adhesive properties.

Types of common fimbriae:

Type 1 fimbriae relatively thick and involved in hemagglutinating activity. They can be inhibited by mannose (mannose sensitive).

Type 2 fimbriae are like type 1 fimbriae but they are non-hemagglutinating.

Type 3 fimbriae are thin and are mannose resistant. They confer hemagglutinating activity on parent cells.

Type 4 fimbriae thinner than type 3 fimbriae and are mannose resistant with hemagglutinating activity on fresh red blood cells.

Type 5 fimbriae are monopolar which have been found only in one species of pseudomonas.

Type 6 fimbriae are very long and few of them are seen in one species of klebsiella.

F Pili

They are associated with fertility (F^+) and help in bacterial conjugation processes. They are longer (20 µ length) than common and Col I pili.

Col I Pili

They are about 2 µ in length and associated with colicin factor I.

Demonstration

1. Electron microscope.
2. Hemagglutination.
3. Fimbriated bacteria form pellicle in liquid media.

Functions

1. Organ of adhesion.
2. Hemagglutination.

3. Conjugation tube through which genetic material is transferred from the donor to recipient cell.
4. They are antigenic.
5. Agglutination and pellicle formation.

TABLE 3.4: Differences between flagella and fimbriae

	Flagella	*Fimbriae*
1.	Size larger and thicker	Smaller and thinner
2.	Arise from the cytoplasm or cytoplasmic membrane but not attached to the cell wall	Attached to the cell wall
3.	Organ of movement	Organ of adhesion and conjugation
4.	They are never straight	They are always straight
5.	Not required for conjugation	Required for conjugation

SPORES (FIG. 3.12)

They are highly resistant dormant state of bacteria found in certain genera, e.g. bacillus, clostridium, sporosarcina (Gram positive coccus) and *Coxiella burnetii*. They are not destroyed by ordinary methods of boiling for several hours. They are killed when autoclaved at 15 lb pressure per square inch at 121°C for 20 minutes. The spores are characterized by the presence of 5 to 20 percent dipicolinic acid which is not found in vegetative cell and by their high calcium content. The resistance to destruction by physical or chemical method is ascribed to factors like impermeability of spores, cortex and coat, low contents of water, low metabolic activity, low enzymatic activity and high contents of calcium and dipicolinic acid. Spores of different species of bacteria are antigenically distinguishable. Spores are highly refractile and require special staining for demonstration, *e.g.* (1) Modified Ziehl-Neelsen method, (2) Gram stain, (3) Moller stain.

Function: They make survival of certain organisms possible under unfavorable condition like dry state. Spores are resistant to heat, drying, freezing and toxic chemicals.

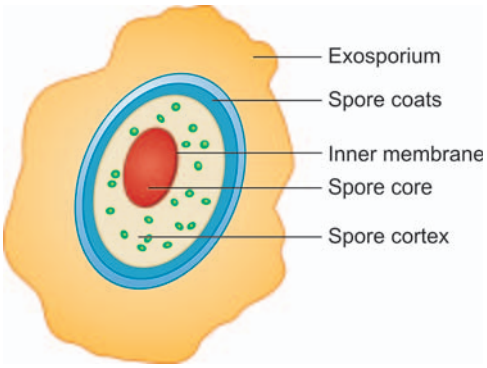


Fig. 3.12: Structure of bacterial spore

Laboratory application: Can be used as sterilization control

i. Bacillus stearothermophilus is killed at 121°C in 15 to 30 minutes.
ii. Bacillus subtilus may be destroyed at 105°C in 5 minutes.

Formation of Spores (Fig. 3.13)

Exact stimulus for sporulation is not known. Perhaps it is related to depletions of exogenous nutrient. Sporulation involves the production of many new structures, enzymes and metabolites along with the disappearance of many vegetative cell components. Sporulation can be induced by depleting PO_4, S, C, N and Fe from culture medium. A series of genes whose products determine the formation and final composition of spore are activated. Another series of genes involved in vegetative function are inactivated. These changes involve alterations in the transcriptional specificity of RNA polymerase, determined by the support of the polymerase core protein with promoter specific protein called sigma factor. Different sigma factors are produced during vegetative growth and sporulation. During the process of sporulation, some bacteria may release peptide antibiotics which may play a role in regulating sporogenesis.

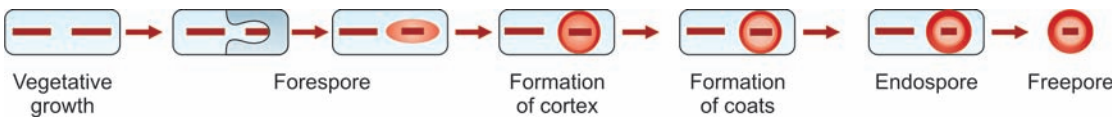


Fig. 3.13: Formation of spore

Sporulation is initiated by appearance of clear area near one end of cell which gradually becomes more opaque to form forespore. The fully developed spore (Fig. 3.12) has its core nuclear body surrounded by spore wall, a delicate membrane (future cell wall). Outside this is spore cortex which in turn is enclosed by multilayered spore coat. Spore cortex contains an unusual type of peptidoglycon sensitive to lysozyme. The spore coat is formed by keratin like protein which is imprevious to antibacterial chemical agents. Exosporium is a lipoprotein membrane with some carbohydrate residue. Young spores remain attached to parent cell. Some spores have an additional outer covering called exosporium having ridges and grooves.

Spores may be (Fig. 3.14):

1. Spherical central.
2. Oval central.
3. Oval subterminal not bulging.
4. Oval subterminal bulging.
5. Terminal spherical bulging.
6. Oval terminal bulging.

Spore forming bacteria:

a. Gram positive bacilli
   1. Obligatory aerobic
      Bacillus anthracis
      Bacillus subtilis
   2. Obligatory anaerobic
      Clostridium tetani
      Clostridium perfringens
      Clostridium botulinum

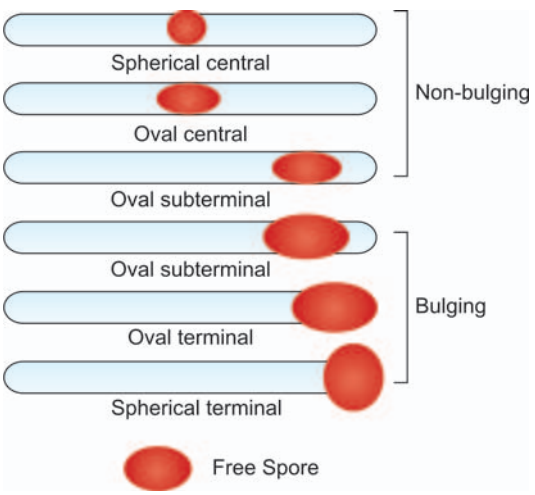


Fig. 3.14: Types of bacterial spores

3. Other bacteria
   Gram positive coccus—Spore sarcina
   Gram negative bacillus—*Coxiella burnetii*

When transferred to favorable conditions of growth, spores germinate. The spore loses its refractility and swells. The spore wall is shed and germ cell appears by rupturing the spore coat. The germ cell elongates to form vegetative bacterium.

Germination of Spore

It is the process of conversion of spore into a vegetative cell under suitable condition. It occurs in about 2 hours under ideal conditions. It occurs in following phases:

a. *Activation* The activation process requires agents like heat (60°C for 1 hour), low pH, compound containing free sulfhydryl, abrasion, etc. Above mentioned agents damage the coat of the spore and help in germination by growing in nutritionally rich environment.
b. *Initiation* Species of bacteria after getting activated recognize effectors as signalling a rich medium like L-alanine for one species and adenosine for other species and so on. Binding of the effector substance to spore coat, activates an autolysin which destroys peptidoglycan of the cortex. After this water is taken up and calcium dipicolonic acid is released. Thus, a number of hydrolytic enzymes degrade the spore constituent.
c. *Outgrowth* Spore coat breaks open and single germ cell comes out and starts growing to new vegetative cell (consisting of spore protoplast with its surrounding wall). Now active biosynthesis takes place producing an outgrowth. Outgrowth is a stage right from germination to the formation of first vegetative cells just before first cell division.

Pleomorphism: Some species of bacteria show great variation in shape and size of undivided cell. This is called pleomorphism. It may be due to defective cell wall synthesis.

Involution: Certain species (e.g. plague bacillus, gonococcus) show swollen aberrant forms in aging cultures especially in presence

of high salt concentration. It may be due to defective cell wall synthesis or due to the activity of autolytic enzymes.

L forms: The name L form is after the Lister Institute, London where swollen and aberrant morphological forms from the culture of *Streptobacillus moniliformis* were studied. They are seen in several species of bacteria developing either spontaneously or in the presence of penicillin or other agents that interfere with cell wall synthesis.

L form resembles mycoplasma in several ways including morphology, type of growth on agar and filterability. Possibly mycoplasma represent stable L forms of as yet unidentified parent bacteria. L form of bacteria have been isolated from patients of chronic urinary and suppurative infections. Yet their role is not clarified.

Study of Morphology of Bacteria

It is of considerable importance that identification of bacteria be made. For this purpose following methods are employed:

Optical Methods

a. **Light microscopy** is useful for the motility, size, shape and arrangement of bacteria. Due to lack of contrast, details cannot be appreciated.
b. **Phase contrast microscopy** makes evident structure within cells that differs in thickness and refractive index. In the phase contrast microscope "phase" differences are converted into differences in intensity of light producing light and dark contrast in the image.
c. **Dark ground microscopy** in which reflected light is used instead of direct transmitted light used in ordinary microscope. With its help extremely thin slender organism like spirochaetes can clearly be seen.
d. **Oil immersion microscopy** in which magnification produced by oil immersion objective of light microscope makes visible majority of bacteria.
e. **Fluorescence microscope** in which bacteria are stained with auramine dye or rhodamine dye. Dye changes the wavelength of bacteria stained with above dye and become visible as shining objects against a dark background. Here ultraviolet light is used as a source of light
f. **Electron microscope** where beams of electron is used instead of beams of light used in optical microscope. The electron beam is focussed by circular electromagnets, analogous to the lenses in light microscope. The object which is kept on path of beam scatters the electrons and produces an image which is focussed on fluorescent viewing screen. Table 3.5 depicts some points of difference between light microscope and electron microscope. Resolving power of electron microscope is 0.1 nm. Scanning electron microscope is a recent development and provides a 3 dimensional image of the surface of the object.
g. **Interference microscope** can reveal cell organelles and also enable quantitative measurement of chemical constituents of cell, e.g. lipids, protein, nucleic acid, etc.
h. **Polarization microscope** enables the study of intracellular structure using differences in birefrigence.
i. **Autoradiography** where cells that have incorporated radioactive atoms are fixed on a slide, covered with photographic

TABLE 3.5: Differences between light and electron microscope

Properties	*Light microscope*	*Electron microscope*
• Image formation	Visible light	Electron
• Medium through which radiation travels	Air	Vacuum
• Specimen mounting	Glass slides	Thin film of collodion
• Nature of lenses	Glass	Electrostatic lenses or magnetic fields
• Focussing	Mechanical	Electrical
• Magnification	Changing of objectives	Changing the current of the projector lens cool
• Mean of providing specimen contrast	Light absorption	Electron scattering

emulsion, and stored in the dark for a suitable period of time, and then tracks appear in the developed film emanating from the sites of radioactive disintegration. If the cells are labelled with a weak emitter such as tritium the tracks are sufficiently labelled. This procedure, called auto-radiography, has been particularly useful in following replication of DNA, using tritium labelled thymidine as a specific tracer.

Other types of microscope available are scanning electron microscope, X-ray, dissecting microscope, laser microscope (vaporize minute part of tissue *in vivo* or *in vitro* or biopsy specimen which is then subjected to emission spectrography) and operation microscope for conducting delicate surgical operations.

UNSTAINED PREPARATIONS

The wet preparations of bacterial suspensions are mainly used for (a) bacterial motility, and (b) for demonstration of spirochaetes.

Four to eight hours growth in fluid media is examined in hanging drop preparation or cover slip preparation. For the study of spirochaete, e.g. *Treponema pallidum,* dark ground microscopic examination is done.

STAINING OF BACTERIA

The bacterial nucleic acid contains negatively charged phosphate group which combines with positively charged basic dye. Acidic dye does not stain bacterial cells and are used to stain background material. Most commonly used stains to study bacterial morphology are as under:

GRAM'S STAIN

First described by Gram in 1884. It is used to study morphologic appearance of bacteria. Gram's stain differentiates all bacteria into two distinct groups:

a. Gram positive organisms.
b. Gram negative organisms.

Principle: Some organisms are not decolorized and retain color of basic stain, e.g. gentian violet (Gram positive organisms) while the others lose all gentian violet when treated with decolorizing agent and take up the counter stain, e.g. dilute carbol fuchsin or sufranin (Gram negative organisms).

Procedure: Bacterial suspension is spread in the form of thin films on the surface of clean glass slide and allowed to dry in air. It is called smear. Smear is fixed by passing the slide over the flame two or three times. Now proceed as follows:

1. Cover the slide with gentian violet for 1 to 2 minutes.
2. Wash the smear with Gram's iodine and keep Gram's iodine on the slide for 1 to 2 minutes.
3. Decolorize the slide with acetone or alcohol carefully and immediately wash the smear with water.
4. Counter stain with 0.5 percent aqueous sufranin solution or dilute carbol fuchsin (1 : 20) for 1 to 2 minutes.
5. Finally wash the smear, allow it to dry in air and put a drop of oil and see under oil immersion lens.

Mechanism of Gram Staining: Different theories are put forward regarding mechanism of Gram reaction as exact mechanism is not known. They are:

1. **Acidic protoplasm theory**: As per this theory Gram positive bacteria have more acidic protoplasm as compared to Gram negative bacteria. So Gram positive bacteria tend to retain, the primary stain (basic in nature) more than Gram negative bacteria. Additionally because of iodine, the cytoplasm becomes more acidic and acts as mordant. Hence it enhances the attraction of the primary stain to the cell cytoplasm. As a result it helps to fix the stain in bacterial cell.
2. **Cell wall permeability:** Gram positive bacterial cell wall contains more mucopeptide. It makes the cell wall thicker and stronger. As a result due to iodine complex cannot come out of Gram positive cell wall. On the contrary Gram negative cell wall contains less mucopeptide and so its cell wall is thin and less strong. It is the reason that dye iodine complex diffuses out of cell and color of the counter stain is taken up.

3. **Magnesium ribonucleate theory:** As per the theory magnesium ribonucleate is present in Gram positive bacteria. Hence there is formation of magnesium ribonucleate dye iodine complex which is isoluble in alcohol. In Gram negative bacteria magnesium ribonucleate is absent. And so only dye iodine complex is formed which is soluble in alcohol.

ACIDIC ACRIDINE ORANGE STAIN

Routine Gram stains require at least 10^5 bacilli/ml (almost close to the limit of visible turbidity in liquid media) to make its detection microscopically possible. Acidic acridine orange stain may detect bacteria much earlier with bacilli count much less. Bacteria fluoresce under ultraviolet light as red orange forms against greenish leukocytes and unstained erythrocytes. In short, this stain may be substituted for early detection of bacilli.

ALBERT STAIN

Some bacteria may have metachromatic black granules, e.g. *Corynebacterium diphtheriae* which stain dark bluish or black with methylene blue or a mixture of taludine blue and malachite green.

Procedure: Heat fixed smear is covered with Albert stain I. After 5 to 7 minutes it is replaced by iodine solution (Albert stain II) and is kept there for 5 minutes. Smear is washed with water and studied under oil immersion lens. The body of bacilli look green while granules take bluish-black color.

ZIEHL-NEELSEN STAIN

This is also called acid-fast stain.

Principle: Some organisms retain carbol fuchsin even when decolorized with acid. Such organisms are called acid fast organism. However, mycolic acid is thought to be responsible for acid fastness.

Procedure: Take heat fixed smear and add concentrated carbol fuchsin. Now gently heat it till steam comes out. Do not allow the stain to boil or dry. Keep it up for 8 to 10 minutes. Now decolorize the smear with 3 percent solution of hydrochloric acid in 95 percent ethyl alcohol or 20 percent aqueous sulfuric acid. Now wash the slide with water and counter stain it with methylene blue or melachite green. Acid fast organisms take red stain.

Acid-fast organisms are:

1. Mycobacteria (tubercle bacilli, leprabacilli).
2. Bacterial spores.
3. Nocardia.
4. Ascospore of certain yeast.
5. Actinomyces club.
6. Inclusions in lungs from cases of lipid pneumonia.
7. Ceroids in liver of rat.
8. Exoskeleton of insects (Table 3.6).

SPORE STAIN

Spores are resistant to ordinary method of staining. In Gram stain spores appear as clear areas in deeply stained body of bacilli. Methods of spore staining are:

1. **Modified acid-fast stains:** Treat the heat fixed smear with steaming carbol fuchsin for 3 to 6 minutes. Decolorize with 0.5 percent sulfuric acid or 2 percent nitric acid in absolute alcohol. Wash and counter stain with 1 percent aq. methylene blue. Wash with water and study it under oil immersion lens. Spores are stained bright red and vegetative part of bacilli blue.
2. **Moller methods:** Here heat fixed smear is kept over beaker with boiling water. As

TABLE 3.6 :Grading of slides in AFB microscopy

Examination	*Result*	*Grading*	*No. of fields to be examined*
More than 10 AFB per oil immersion field	Positive	3+	20
1-10 AFB per oil immersion field	Positive	2+	50
10-99 AFB per 100 oil immersion fields	Positive	1+	100
1-9 AFB per 100 oil immersion fields	positive	Scanty 1-9 (Record exact number seen)	100
No AFB per 100 oil immersion fields	Negative	-	100

soon as steam starts condensing over under surface of slide, add malachite green. Keep it up for 1 to 2 minutes. Wash it and counter stain it with dilute carbol fuchsin.

CAPSULAR STAIN

They are not stained with ordinary aniline dyes. In Gram stain they are shown as area of halo around bacterium. Methods of capsule staining are:

1. **India ink method:** Emulsify small amount of culture in a loopful of India ink on a slide and cover it with coverslip. Capsule is seen as clear halo between refractile outline of cell wall and greyish background of India ink. It is the best and rapid method of capsule demonstration.
2. **Hiss's method:** Treat thin freshly prepared smear with hot crystal violet for one minute. Now wash with 20 percent solution of copper sulfate and blot.

The capsule is stained blue and the body of the bacteria stains deep purple.

BIOFILM

Definition :	Biofilm is obiquitous and medically important complex structure consisting of microbial associated cells embedded in self produced extracellular matrix of hydrated extra polymeric substances which are irreversibly attached to biological or non biological surface. Microbes that reside as biofilms are resistant to traditional antibiotics.
Formation of biofilms:	Basically biofilms formation comprises of : (a) Initial attachment of microbes and microcolony formation. (b) Maturation of attached microbes into differentiated biofilm. (c) Detachment and dispersal of planktonic cells, from biofilm.
Clinical importance of biofilm	1. Biofilm persisters are well protected from host defense because of exopolysaccharide matrix. 2. Bioflim is relevant because the mode of growth is associated with chronicity and persistence of diseases. 3. Biofilm associated diseases are periodontitis, bacterial keratitis, chronic lung infections in cystic fibrosis. 4. Nosocomial infections connected with use of central venous catheters, urinary catheters, prosthetic heart valves, intraocular lenses and orthopedic devices are in fact closely associated with biofilms that adhere to biometrical surfaces. 5. Microbes in biofilm evade host defenses and withstand antimicrobial agents. 6. Important microorganisms capable of forming biofilms are; *Staphylococcus epidermides; Staphylococcus aureus*, *Pseudomonas aeruginosa, Vibrio cholerae, Bacillus subtilis* etc. 7. Biofilm can easily be, studied in a better way using atomic force microscope. Thus application of new microscopic and molecular techniques has revolutionized our understanding of biofilm structure, composition, organization and activities resulting in significant advances in prevention and treatment of biofilm related diseases.

4 Nutritional Requirements of Bacteria

Bacteria may require adequate nutrition, optimum pH, temperature and oxygen for multiplication and growth. Bacteria can be classified into following types on the basis of nutritional requirement:

I. On the basis of energy sources:
   a. *Phototrophics* which get energy from photochemical reaction.
   b. *Chemotrophics* which get energy from chemical reaction.

II. On the basis of their ability to synthesize essential metabolites.
   a. *Autotrophic:* These are the organisms in which all essential metabolites are synthesized from inorganic sources. They use carbon dioxide as the main source of carbon and simple inorganic salts, e.g. nitrates, nitrites, ammonium sulfate, phosphates, etc. to form new protoplasm of the cell.
   b. *Heterotrophic:* Here some of the essential metabolites are not synthesized. Organic compounds, e.g. protein, peptones, amino acids, vitamins and growth factors are supplied from outside. Most of the bacteria producing disease in man are heterotrophic.

GROWTH REQUIREMENTS

Nutritional Requirements

1. *Essential elements* The essential elements required for synthesis of bacterial structural components (carbohydrate, lipid, protein nucleic acid) are hydrogen, carbon and nitrogen. Of course phosphorous and sulfur are also required for bacterial growth.

   Hydrogen and oxygen are made available from water added to culture medium. Heterotrophic bacteria require organic carbon for growth in a suitable form and can be assimilated by the bacteria. Carbohydrate is principal source of carbon which is degraded by the bacteria either by oxidation or by fermentation. Thus providing energy in the form of adenosine triphosphate.

   Nitrogen is a major component of protein and nucleic acids and its main source is ammonia usually in the form of ammonium salt. The ammonium salt is made available either from environment or it may be produced by deamination of amino acids by bacteria.

   Sulfur forms part of the structure of several coenzymes cysteinyl and methionyl side chains of proteins. Most bacteria use sulfate (SO_4) as a sulfur source and reduce it to hydrogen sulfide.

   Phosphorus is required as a component of nucleic acid ATP coenzyme NAD, and flavins. It is always assimulated as free inorganic phosphate.

2. *Mineral salts* They are potassium, calcium, magnesium, iron, copper, manganese, molybdenum and zinc required in traces for enzyme function and can be provided in tap water or as contaminant of other medium ingredients.

3. *Growth factor* These may be essential for some who do not grow in their absence. Following a gene mutation of bacterium, there results in failure of one of the enzymes to synthesize amino acids or purines or pyrimidines from simpler compounds. In such case exogenous

supply of relevant factor is required called accessory growth factors.

The other nutritional requirements are as under:

Gas Requirements

a. **Oxygen:** The capacity of bacteria to grow in the presence of oxygen and to utilize it depends on possession of a cytochrome oxidase system.

Aerobes: The aerobe organisms grow only in the presence of oxygen, e.g. pseudomonadaceae, bacillus, nitrobacter, sarcina, etc. They require oxygen as hydrogen acceptor.

Facultative anaerobes: They are the organisms that can live with or without oxygen, e.g. vibrio, *Escherichia coli*, *Salmonella*, *Shigella* and *Staphylococcus*. The microaerophilic organisms grow well with relatively small quantities of oxygen, e.g. *Hemophilus*.

Obligate anaerobes: The strict anaerobes multiply only in the absence of oxygen, e.g. *Bacteroides*, *Clostridium*. They require a substance other than oxygen as hydrogen acceptor.

The toxicity of oxygen results from its reduction by enzymes in the cell (e.g. flavoprotein) to hydrogen peroxide and even more toxic free radical superoxide. Aerobes and aerotolerant anaerobes are protected from these products by the presence of superoxide dismutase, an enzyme and the presence of catalase. There is one exception to this rule, i.e. lactic acid bacteria; aerotolerant anaerobes that do not contain catalase. This group however, relies instead on peroxidases. All strict anaerobes lack superoxide dismutase, catalase and peroxidase. Superoxide dismutase is indespensable for survival in presence of oxygen.

Hydrogen peroxide owes much of its toxicity to the damage it causes to DNA. DNA repair deficient mutants are exceptionally sensitive to hydrogen peroxide. The rec A gene product has the function of both genetic recombination and repair. It is said to be more important than either catalase or superoxide dismutase in protecting *Escherichia coli* against hydrogen peroxide toxicity.

b. **Carbon dioxide:** The metabolic activities of some organisms like *Neisseria gonorrhoeae*, *Brucella abortus* are greatly enhanced by the presence of extra-amount of carbon dioxide (capnophilic bacteria) in atmospheric air.

Moisture

Bacteria require water for their growth. Desiccation may kill most of bacteria.

Accessory Nutritional Requirements

Most often the accessory growth factors are vitamins. The requirement of growth factors differ widely in various bacteria, e.g.

Organisms	Growth factors
N. gonorrhoeae	Glutathione
C. diphtheriae	B-alanine
S. aureus	Nicotinic acid, thiamine
H. influenzae	Hematin (Co-enzyme I)

They are not synthesized by bacteria and so supplied in media.

Bacterial growth *in vivo* depends upon:

- Availability of nutrition with human body
- Generation time of bacteria
- Cellular and humoral defence of host, i.e. human body
- Redox potential
- pH

GROWTH CURVE

When organisms are cultured in appropriate fluid media, there would be increase in the size of bacteria without any multiplication for some time (lag phase). This is followed by multiplication and increase in number of bacteria to the extent that media look turbid to the naked eye (log phase). After some time growth rate becomes stationary and later on declines. Counting of bacteria at different periods after inoculation and then events of sequences are represented on a graph which is called growth curve (Fig. 4.1).

Lag Phase

It has short duration (1 to 40 hours) and during this phase there occurs:

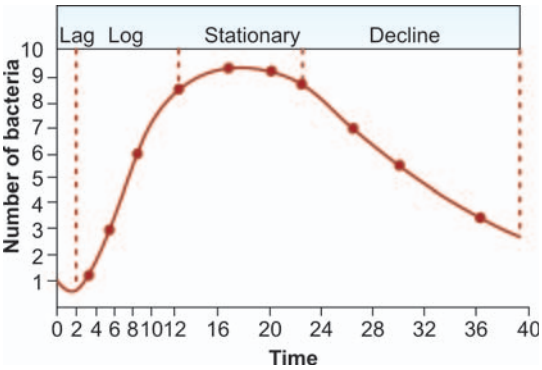


Fig. 4.1: Growth curve of viable bacteria

1. Increase in size of cell.
2. Increase in metabolic rate.
3. Adaptation to new environment and necessary enzymes plus intermediate metabolites are built up for multiplication to proceed.
   The length of lag phase depends upon:
   a. Type of bacteria.
   b. Better the medium, shorter the lag phase.
   c. The phase of culture from which inoculation is taken.
   d. Size of inoculum.
   e. Environmental factors like temperature.

Log Phase

Following lag phase (8 hours duration) the cells start dividing and their number increase by geometric progression with time. Logarithms of viable count plotting time gives straight line. During this period:

i. Bacteria have high rate of metabolism.
ii. Bacteria develop best morphologically with typical biochemical reactions.
iii. Bacteria are more sensitive to antibiotics. Control of log phase is brought about by:
   a. Nature of bacteria.
   b. Temperature.
   c. Rate of penetration of the medium depends on the concentration of material in the medium.

Stationary Phase

After some time (few hours to few days) a stage comes when rate of multiplication and death becomes almost equal. It may be due to:

a. Depletion of nutrient.
b. Accumulation of toxic products. Sporulation may occur during this stage.

Decline Phase

During this phase (few hours to few days) population decreases due to death of cells. Factors responsible for this phase are:

i. Nutritional exhaustion.
ii. Toxic accumulation.
iii. Autolytic enzymes. Involution is common in phase of decline.

Survival Phase

When most organisms have died, a few survive for several months or years.

TABLE 4.1: Bacterial changes during growth curve

Bacterial changes	*Phase of growth curve*
1. Maximum size	1. End of lag phase
2. Uniform staining and bacteria are more sensitive to antibiotics	2. Log phase
3. Irregular staining, variable Gram reaction, exotoxin production and sporulation	3. Stationary phase
4. Involution form	4. Phase of decline

CONTINUOUS CULTURE

This method of culture is being used for both research and industrial purposes. Continuous culture may correspond with situation occurring in some diseases of man and animals. Continuous culture may be obtained by following methods:

1. **Chemostat:** Here rate of growth is controlled by rate of addition of fresh nutrient which may be pumped from medium vessel. This in turn controls the rate of removal of cells via the overflows device into the collector vessel. This rate of production of new cells by multiplication is equal to rate of removal of grown bacteria into the collector vessel. This process may be continued indefinitely (Fig. 4.2). The disadvantage of chemostat is that growing cells are always in a state of semistarvations for one nutrient and must be grown at less than maximum rate to achieve good result.

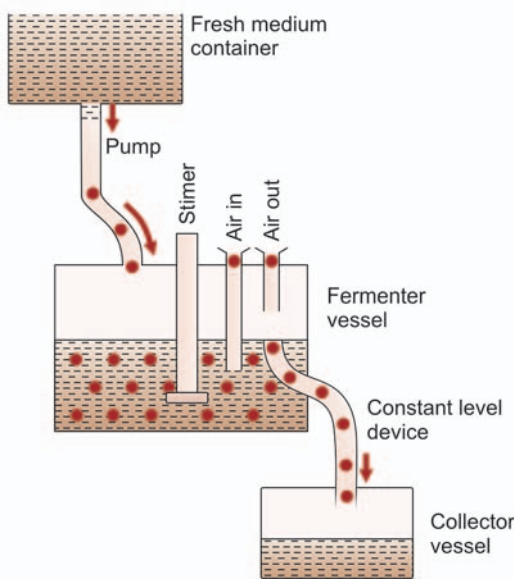


Fig. 4.2: Chemostat

2. **Turbidostat:** Here addition of a measured volume of fresh medium is triggered when the turbidity in the growth vessel reaches a predetermined value that is monitored by photoelectric cell. As a result of addition of fresh medium, bacteria are diluted and continued to grow until they reach critical turbidity.

Factors Influencing Growth

1. **Temperature:** The temperature range at which an organism grows best is called optimum temperature. In human parasitic organisms, optimum temperature ranges between 30° and 37°C.

   There are three groups of bacteria related to regards the temperature of growth.
   a. *Psychrophilic:* These are the organisms growing between 0° and 25°C. They are mostly soil and water bacteria.
   b. *Mesophilic:* They grow between 20° and 44°C. This group includes bacteria producing disease.
   c. *Thermophilic:* Some organisms grow between 50° and 60°C, e.g. bacillus and algae. The upper range of temperature tolerated by them correlates well with the thermal stability of the species protein as measured in cell extract.
2. **Hydrogen ion concentration:** Most of pathogenic bacteria grow best at pH 7.2 to 7.6. However, *Lactobacillus* and *Thiobacillus thiooxidans* grow at acidic pH while *Vibrio cholerae, Alcaligenes fecalis* grow at alkaline pH.
3. **Moisture:** Water is quite essential for the growth of bacteria. Organism like *Neisseria gonorrhoeae* and *Treponema pallidum* die almost at once on drying. However, *Mycobacterium tuberculosis* and *Staphylococcus aureus* survive for quite a long time even on drying.
4. **Osmotic pressure:** Bacteria are usually resistant to changes of osmotic pressure. However, 0.5 percent sodium chloride is added to almost all culture media to make environment isotonic.
5. **Light:** Darkness is usually favorable for the growth and viability of all the organisms. Direct light exposure shortens the survival of bacteria. Photochromogenic mycobacteria form pigment on exposure to light. Organisms are sensitive to ultraviolet and other radiations.
6. **Mechanical and sonic stress:** Bacteria have tough cell walls. Vigorous shaking with glass beads, grinding and exposure to ultrasonic vibration may cause rupture or disintegration of cell wall.

BACTERIAL REPRODUCTION

The bacteria reproduce by a sexual binary fission. The DNA is a double helix with complementary nucleotide sequences in the two strands. At replication the strands separate and new complementary strands are formed on each of the originals so that two identical double helices are produced. Each of them has the same nucleotide sequence and so the same genetic information as the original one. The sequence of cell division includes:

a. Formation of initiator of chromosome replicator.
b. Chromosome duplication.
c. Separation of chromosomes.
d. Formation of septa and cell division.

GENERATION TIME

Time required for bacterium, to give rise to two daughter cells under optimum condition is called generation time or generation gap.

Generation time of:

a. Coliform bacteria is 20 minutes.
b. *Mycobacterium tuberculosis* is 20 hours.
c. Lepra bacilli is 20 days.

SYNCHRONOUS GROWTH

In ordinary cultures, the cells are growing non-synchronously at any moment and cells are present in every possible stage of the division cycle. When all the bacterial cells divide at the same time, growth thus obtained is called synchronous growth. Synchronous growth is a way to study biochemical events related to cell division, i.e. study of DNA synthesis, stability of genetic material, susceptibility of lethal agents and transfer of genetic material.

Synchronous growth may be obtained by: (i) delayed initiation of DNA replication and then restore the condition required, (ii) repeated alternation between short periods of incubation at 37°C and at 25°C, (iii) depriving an auxotrophs of thymine followed by its restoration, (iv) some organisms go through one or two synchronous growth when diluted from stationary phase culture to fresh medium.

The main drawback of these methods is that cell composition and their state of balance is drastically altered. Hence, there is no reliable information regarding mechanism responsible for balanced growth.

However, physiological synchronization is achieved by mechanical separation of smaller recent products of normal division in other stages of cycle. For this purpose culture may be filtered through layers of filter paper. Larger cell on the 'verge of division' remains on filter paper. Smaller cells come down through filter paper. Both fraction on further incubation gives synchronous growth. Membrane filter may also be used for this purposes. Reverse flow through filter provides continuous supply of newborn cells. Centrifugation may be applied to get synchronous growth. Synchrony only persists for 1 to 4 cycles. After that time the cells become more and more out of phase until their division become completely random.

BACTERIAL COUNT

1. Total bacterial count includes living as well as dead bacteria. It can be obtained by following methods:
   a. Direct counting under microscope using counting chamber.
   b. Counting may be in an electronic device called Coulter counter.
   c. Direct counting using stained smears prepared by spreading known volume of culture over measured area of a slide.
   d. Comparing relative number in smear of the culture mixed with known number of other cells (erythrocytes).
   e. By opacity measuring with nephalometer or absorption meter.
   f. Measuring wet and dry weight of cells after centrifugation or filtration.
   g. Chemical assay of nitrogen.
2. The viable count measures number of living organism. It can be obtained in the manner described ahead.
   a. *Dilution method* Several tubes are inoculated with varying dilution and viable count calculated statistically from the number of tubes showing growth. This method does not give accurate values. This method is used for presumptive coliform count in drinking water.
   b. *Plating method* Here appropriate dilutions are inoculated on solid media either on the surface of plate or as pour plates. The number of colonies that develop after incubation gives an estimate of viable count.

5 Bacterial Metabolism

The processes of breakdown and utilization of food material in bacteria are basically similar to higher organisms with following differences:

1. Bacteria are metabolically more active due to relatively large surface through which nutrients are readily absorbed and metabolic end products are released into the environment.
2. The substrates utilized for the synthesis of cell material and production of energy, are different in different types of bacteria, e.g. lactose is fermented by a few bacteria among enterobacteriaceae.
3. The metabolic end products from a given substrate are different in different organism.

BACTERIAL METABOLISM

The living organisms are similar in their component chemical units as well as in the mechanism by which these components are formed—the process of metabolism. It comprises of (a) catabolism and (b) anabolism.

Catabolism

All microorganisms seem to have certain fundamental metabolic pathways concerned in the interconversions necessary for the production of the basic building blocks. Enzymes concerned in glycolytic pathway, the pentose phosphate cycle and the tricarboxylic acid cycle are synthesized irrespective of environmental conditions and form a group of constitutive enzymes. On the other hand, there is a group of enzymes produced only in the presence of inducer and are called inducible enzymes.

Microorganisms differ considerably in the way in which the energy source is broken down to provide energy. This difference mainly concerns the involvement of oxygen as a terminal electron acceptor in the system and accordingly organisms may be facultative anaerobes (grow aerobically and anaerobically), obligate aerobes (grow only in the absence of oxygen). Yet there is another group of organisms that grow best in the presence a trace only of free oxygen and often prefer an increased concentration of carbon dioxide; these are called microaerophilic.

Aerobes obtain energy by a series of coupled oxidoreductions in which the ultimate electron acceptor is atmospheric oxygen. In this aerobic respiration, the carbon and energy source may be completely oxidized to carbon dioxide and water. Here energy is obtained by a process called oxidative phosphorylation, i.e. production of energy rich phosphate bonds and their transfer to adenosine diphosphate to form adenosine triphosphate.

Anaerobes oxidize compound at the expense of some electron acceptor other than oxygen. Anaerobic growth perhaps occurs by a process in which carbon and energy source acts both as the electron donor and the electron acceptor in series of oxidoreduction. This process is called fermentation. Fermentation leads to formation of ethanol, acid and alcohols. Fermentation is accompanied with production of acid and gas (carbon dioxide or hydrogen). During process of fermentation energy rich phosphate bonds are produced which are transferred on to adenosine diphosphate to form adenosine triphosphate in the presence of appropriate phosphorylation enzyme. Facultative anaerobes may obtain

their energy exclusively by fermentation (streptococci) or either by fermentation or respiration (enterobacteria). The amount of energy produced from given amount of carbon and other energy source under anaerobic conditions is considerably less than under aerobic conditions; hence growth of facultative anaerobe is usually much more abundant under aerobic conditions.

Redox Potential

Oxidizing agents may be considered as substances capable of taking up electrons. On the other hand, reducing agents are substances which are able to part with electrons. The capability of a substance to take-up or lose electron is called oxidation reduction or redox or Eh potential. It is measured in millivolts. The higher is redox potential value in oxidized substances whereas this value is lower in reducing agents.

The Eh, i.e. redox potential of most of media in contact with air is +0.2 to 0.4 volt at pH 7. The strict anaerobes are unable to grow unless Eh is 0.2.

Anabolism

There are wide differences in the ability of cells to carry out the individual biosynthesis of essential monomers and coenzymes. Some are capable of synthesizing all their amino acids, nucleotides, monosaccharides, coenzymes, etc. from the building blocks produced by catabolism. Others almost completely lack such power of biosynthesis and depend entirely on their nutrient environment for the provision of such substances in readymade form. Within these two extremities, there is a wide spectrum of different biosynthetic abilities. Microorganisms vary widely in their possession of enzymes that catalyze the biosynthesis of essential low molecular weight compounds. However, all cellular forms of life must have a certain range of enzymes to catalyze the required polymerizations as it is not possible to incorporate an extracellularly provided polymer directly into cell structure.

Carbohydrate metabolism A large number of molecules are known to be attacked by bacteria. Glucose obtained after the breakdown of polysaccharides by exoenzymes is easily penetrated into the bacterial cell where it is acted upon by endoenzymes. Degradation of glucose varies in different species of bacteria. Glucose fermentation is the same up to the point of pyruvic acid. Differences occur in the manner in which pyruvic acid is utilized.

The end product of carbohydrate fermentation are formed by various pathways from pyruvic acid. Important end products of glucose fermentation are: gases (CO_2, H_2), acids (acetic, formic, lactic, pyruvic, etc.) and neutral products (alcohol). Production of acid with or without gas is used in the identification of bacteria.

METABOLISM OF NITROGENOUS COMPOUNDS

Metabolism of nitrogenous compounds makes available enzymes, coenzymes and nucleic acids, etc. With enzyme protease, protein is converted into peptide which is further broken down into amino acids. This occurs outside the cell. The amino acids enter the cells where they are degraded by the methods like deamination, decarboxylation or splitting (e.g. indole production from tryptophan). The amino acids entering cells may be used for protein synthesis. The main steps of protein synthesis are as under:

1. Amino acids are activated and form a complex with enzyme synthetase.
2. The activated amino acids are transferred to soluble RNA or transfer RNA (low molecular weight). These reactions are catalyzed by amino acyl RNA synthetase.
3. mRNA (high molecular weight) is synthetized on a template of chromosomal DNA through the activity of RNA polymerase.
4. mRNA migrates to ribosomes to form polysome and serves as a template for assembly of amino acids into polypeptide chain. The genetic code is incorporated in the mRNA.

6 Media for Bacterial Growth

Culture media gives artificial environment simulating natural conditions necessary for growth of bacteria. The basic requirement of culture media are:

1. Energy source.
2. Carbon source.
3. Nitrogen source.
4. Salts like sulphates, phosphates, chlorides and carbonates of sodium, potassium, magnesium, ferric, calcium and trace elements like copper, etc.
5. Satisfactory pH 7.2 to 7.6.
6. Adequate oxidation-reduction potential.
7. Growth factor like tryptophan for *Salmonella typhi*, glutathione for gonococci, X and V factors for hemophilus.

The characteristics of an ideal culture medium are:

1. Must give a satisfactory growth from single inoculum.
2. Should give rapid growth.
3. Should be easy to grow.
4. Should be reasonably cheap.
5. Should be easily reproducible.
6. Should enable to demonstrate all characteristics in which we are interested.

Media used for obtaining the growth of bacteria are:

FLUID MEDIA

Bacteria grow very well in fluid media in 3 to 4 hours. Hence, they are used as enriched media before plating on solid media. They are not suitable for the isolation of organism in pure culture. We cannot study colony characters as well. Examples of fluid media are nutrient broth, peptone water, etc.

Types of Liquid Media

Broth

It is a clear transparent straw colored fluid prepared from meat extract or peptone. Following types of broth are in common use:

a. **Infusion broth:** Fat free minced beef meat is added to water and kept in refrigerator overnight. Fluid obtained after removal of meat is boiled for 18 minutes. To it peptone and 0.5 percent sodium chloride is added.
b. **Meat extract broth:** This is commercially available as Lab Lemco.
c. **Digest broth:** It is prepared from meat by enzymatic action. Nutritionally, it is more rich than infusion and extract broth. Addition of peptone is not required in digest broth. Hence, it is more economical. Enzymes used are trypsin, pepsin, etc.

Peptone

It is a protein partially digested with hydrolytic enzymes like pepsin, trypsin, papain, etc. Peptones supply nitrogenous material and also act as a buffer. Several bacteria can grow in 1 percent peptone water. Constituents of peptone are proteoses, polypeptides and amino acids.

Yeast extract: It is prepared by extracting autolyzed yeast with water. It has high contents of vitamin B.

The other examples of liquid media are sugar media (1% sugar in peptone water), glucose broth (1% glucose in nutrient broth), bile broth (0.5% bile salts in nutrient broth), Hiss serum (1 part serum and 3 parts glucose broth), liquid MacConkey, glycerol saline and enrichment media (tetrathionate and selenite).

LIQUID MEDIA

	Medium	*Uses*
1.	Peptone water	• Routine culture • Demonstration of motility • Sugar fermentation Indole test
2.	Nutrient broth	• Routine culture • Methyl red test • Voges Pauskaur test
3.	Glucose broth	• Blood culture Culture of bacteria like streptococci, etc.
4.	Enrichment media	
	a. Glycerole alkaline peptone water	Transport media for stool samples
	b. Selenite F broth	Culture of stool for Salmonella and Shigella
	c. Tetrathionate broth	Culture of stool for Salmonella
	d. Robertson cooked meat broth	Culture of anaerobic bacteria

SOLID MEDIA

	Medium	*Uses*
1.	Nutrient agar	• Routine culture • Antibiotic sensitivity test
2.	Blood agar	• Routine culture
3.	Chocolate agar	• Culture of fastidious bacteria like Neisseria, *H. influenzae*, etc.
4.	Loeffler serum slope	Culture of *C. diphtheriae*
5.	Deoxycholate citrate agar	Culture of Salmonella and Shigella
6.	MacConkey's medium	Culture of intestinal bacteria
7.	Bile salt agar	Culture of *V. cholerae*
8.	TCBS	Culture of *V. cholerae*
9.	Lowenstein-Jensen medium	Culture of *Mycobacterium tuberculosis* and atypical mycobacterium

SOLID MEDIA

They are used to study colonies of individual bacteria. They are essential for isolation of organism in pure form.

a. **Agar:** It is important constituent of solid media. It is complex polysaccharide obtained from seaweeds (Algae geledium species). It melts at 80 to 100°C and solidifies at 35 to 42°C. It does not provide any nutrition to the bacteria. It acts only as solidifying agent. It is not metabolized by any pathogenic bacteria.

b. **Gelatin:** It is protein prepared by hydrolysis of collagen with boiling water. It is in liquid form at 37°C. It forms transparent gel below 25°C. The main use of gelatin is to test the ability of bacteria to liquefy it. This feature is important in the identification and classifications of bacteria. Blackening of media indicates hydrogen sulfide production.

Classification of Media

Media have been classified in many ways:

A. (i) Solid media
   (ii) Liquid media
   (iii) Semisolid media.

B. (i) Simple media
   (ii) Synthetic media or defined media
   (iii) Complex media
   (iv) Semidefined media
   (v) Special media.

Special medias are further divided as under:

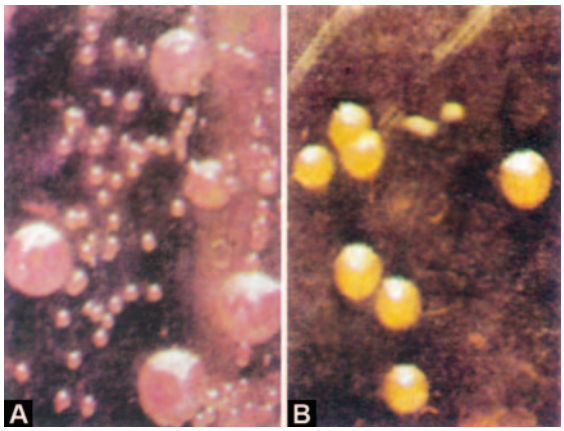
(i) Enriched media
(ii) Enrichment media
(iii) Selective media
(iv) Indicator and differential media
(v) Sugar media
(vi) Transport media.

C. Aerobic media and anaerobic media.

1. **Simple media:** It is also called basal media. It consists of meat extract, peptone, sodium chloride and water.

a. *Peptone water:* It is prepared by adding 1 gram peptone, 0.5 gram sodium chloride to 100 ml of distilled water.
b. *Nutrient agar:* Addition of 2 percent agar in nutrient broth constitute nutrient agar.

2. **Complex media:** These are ingredients for special purposes or for bringing out certain characteristics or providing special nutrient required for the growth of certain organisms.
3. **Synthetic or defined media:** These media are prepared solely from pure chemical substances and the exact composition of medium is known. They are used for research purposes.
4. **Special media**
   a. *Enriched media:* In these media substance like blood, serum or egg is added to basal medium, e.g. blood agar, chocolate agar, egg media and Loeffler serum slope.
   b. *Enrichment media:* Some substances are added to liquid media with the result that wanted organism grow more in number than unwanted organism. Such media are called, enrichment media, e.g. selenite F broth, tetrathionate broth.
   c. *Selective media:* This is like enrichment media with the difference that inhibiting substance is added to solid medium, e.g. deoxycholate citrate medium which contains nutrient agar, sodium deoxycholate, sodium citrates lactose and neutral red.
   d. *Indicator media:* The media contain an indicator which changes color when bacterium grow in them, e.g. *Salmonella typhi* reduces sulphite to sulphide in Wilson and Blair medium (colonies of *Salmonella typhi* have black and metallic sheen).
   e. *Differential media:* A medium which has substance enabling it to bring out differing characteristic of bacteria thus helping to distinguish between them, e.g. MacConkey's medium (peptone, lactose, agar, neutral red and tauro-cholate). It shows lactose fermenter as red colonies while nonlactose fermenter as pale colonies (Figs 6.1A and B).



Figs 6.1A and B: (A) Lactose fermenter colonies on MacConkey media, (B) Nonlactose fermenter colonies on MacConkey media

Blood agar is an enriched medium but also differentiates between hemolytic organisms and non-hemolytic organisms. So it also acts as a differential medium.

   f. *Sugar media:* The usual sugar media consist of 1 percent sugar concerned, in peptone water along with appropriate indicator. A small tube (Durham's tube) is kept inverted in sugar tube to detect gas production (Fig. 6.2).
   g. Hiss's serum (25% serum) is used for organisms which are exacting in their growth requirement, e.g. pneumococci.
   h. *Transport media:* Delicate organisms like gonococci which may not survive the time taken for transporting the specimen to the laboratory or may be overgrown by nonpathogen (dysentery or cholera organism) special medium is required called transport medium, e.g. Stuart medium for gonococci and glycerol saline for stool.

Anaerobic media: These media are used to grow anaerobic organisms, e.g. Robertson's cooked meat medium.

Fig. 6.2: Sugar media with inverted Durham's tube

Anaerobic indicators used is reduced methylene blue and it contains NaOH, methylenes blue, glucose.

Storage media: Lyophilization (freeze drying in vacuum) is the best method of preservation and storage of bacteria Dorset egg and semisolid agar may be used to preserve and store bacteria for a few months. For this loop charged with bacteria is inoculated on these media. After giving adequate incubation bacterial growth appears on the media which can be stored in the refrigerator. Robertson cooked meat media can also be used for preservation.

Culture Techniques

In clinical laboratory indications for culture are:

a. Isolation of bacteria in pure culture.
b. To demonstrate their properties.
c. To obtain sufficient pure growth for preparation of antigen and for other tests.
d. For typing of bacterial isolate by method like bacteriophage and bacteriocin susceptibility.
e. To determine sensitivity to antibiotics.
f. To estimate viable count.
g. To maintain stock culture.

METHODS OF ISOLATING PURE CULTURE

1. Surface plating.
2. Use of enriched and selective media.
3. Pretreatment of specimens with appropriate bacteriocidal agents.
4. By heating liquid medium.

Methods of Culture

1. **Streak culture** (surface plating) is the method routinely employed for the isolation of bacteria in pure culture. A platinum loop with 2½" long wire and loop with diameter 2 mm (Fig. 6.3) is charged with specimen to be cultured and is placed on the surface of dried plate of solid media towards peripheral area (Fig. 6.4). The inoculum is spreaded thinly over the plate in series of parallel lines in different segment of the plate. On incubation we may find confluent growth at the site of primary inoculum. Well-separated colonies are obtained over the final series of streaks.
2. **Lawn or carpet culture:** Lawn cultures are prepared by flooding the surface or plate with suspension of bacteria. It provides uniform surface growth of bacteria. It is useful for bacteriophage typing and antibiotic sensitivity test.
3. **Stroke culture**: It is made in tubes containing agar slopes. It is used for

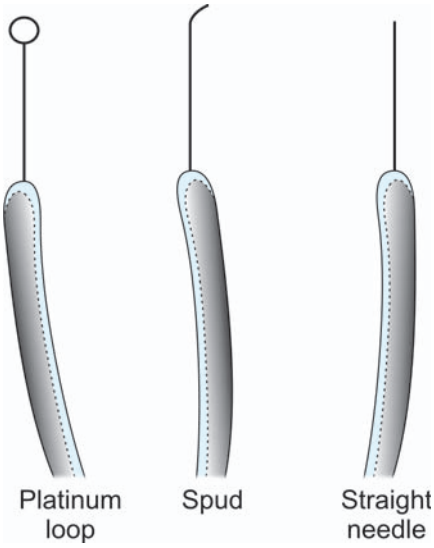


Fig. 6.3: Types of inoculation loops

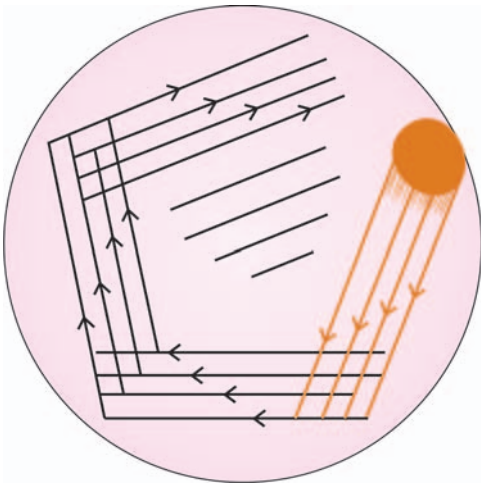


Fig. 6.4: Method of streaking culture plate

providing a pure growth of bacterium for slide agglutination.

4. **Stab culture:** It is prepared by puncturing with charged long, straight wire (4" long). Stab culture are employed mainly for demonstration of gelatin liquefactions and for maintaining stock culture.
5. **Pure plate culture:** 15 ml of agar medium is melted and left to cool in water bath at 45°C to 50°C. Appropriate dilution of inoculum is added in 1 ml volume to molten agar and mixed well. Content of tube is poured in Petri dish. It is allowed to set and after incubation colonies will be seen distributed throughout the depth of medium. This method gives viable bacterial count in a suspension. It is the recommended method for quantitative urine culture.
6. **Liquid culture** in a tube, bottle or flask may be inoculated by touching with a charged loop. Liquid cultures are preferred when large and quick yield is required. The major disadvantage of liquid culture is that it does not provide pure culture from mixed inocula.

Description of colonies of bacteria: Colonies of bacteria are described as follows:

Shape: Circular, irregular, radiate or rhizoid.

Surfaces: Smooth, rough, fine or coarsely granular, papillate, glistening, etc.

Size: Surface of colony is measured in millimeter. It measures 2 to 3 mm and if very small then 0.5 to 1 mm.

Elevation: Raised, low convex dome, umblicate (Fig. 6.5).

Some bacteria produce spreading growth, e.g. proteus, clostridia, etc.

Edges: Mostly edges are entire, e.g. Klebsiella, *Escherichia coli,* Staphylococcus. Sometimes edges may be crenated, fimbriated (*B. subtilis*) or effuse (Fig. 6.6).

Color: Some organisms may produce pigmented colonies, e.g. staphylococci, *Pseudomonas aeruginosa.*

Opacity: Colonies on nutrient agar may be transparent, translucent or opaque.

Consistency: Colonies may be hard or firm, e.g. *Mycobacterium tuberculosis*, friable and membranous, e.g. *B. subtilis.* Mostly they are soft and butyrous, e.g. *Escherichia coli.*

Changes in the medium: Some organisms produce beta type of hemolysis around the colony, e.g. *Staphylococcus aureus* and *Streptococcus pyogenes.* Few bacteria produce soluble pigment that diffuses into the medium, e.g. *Pseudomonas aeruginosa.*

Emulsifiability: Growth of bacteria like *Escherichia coli,* Salmonella is easily emulsifiable whereas growth of *Neisseria catarrhalis* is not emulsifiable.

Growth in liquid media is described as:

1. *Turbid*
2. *Deposit:* Growth of *Streptococcus pyogenes* is characterized by deposits at the bottom of tube.

Fig. 6.5: Elevation of colonies

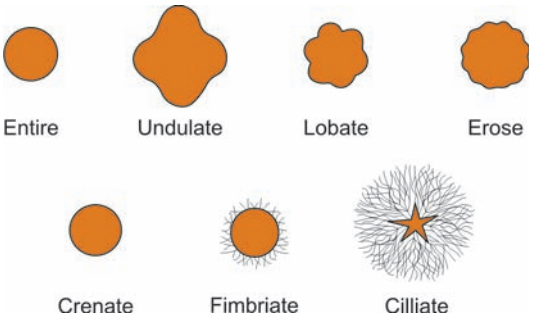


Fig. 6.6: Edges of colonies

3. *Surface growth:* Surface growth is related to aerobic, nature of organism.
4. *Color changes:* Some organisms produce water soluble pigment which after diffusion change the color of medium, e.g. *Pseudomonas aeruginosa.*

METHODS OF ANAEROBIC CULTURE

Obligate anaerobes grow only in absence of free oxygen. These bacteria lack mechanism of oxidation through respiratory enzymes like cytochrome oxidase, catalase and peroxidase resulting in H_2O_2 accumulation. This H_2O_2 is toxic for the growth of anaerobic bacteria.

Clostridium tetani are strictly anaerobic. A number of methods are described for achieving anaerobiosis on the basis of following principles:

a. Exclusion of oxygen.
b. Production of vacuum.
c. Displacement of oxygen with other gases.
d. Absorption of oxygen by chemical or biological means.
e. Reduction of oxygen.

1. Cultivation in vacuum was tried by incubating culture in vacuum desiccator. It has disadvantages:
   a. Some oxygen is always left behind.
   b. Fluid culture may boil over and media get detached.
2. Displacement of oxygen with gases like hydrogen, nitrogen, helium or carbon dioxide was also tried. Anaerobiosis is never complete.

   Candle jar is again ineffective. Here inoculated plates are placed, inside airtight container and lighted candle is kept before lid is sealed. A burning candle should use up all the oxygen before it gets extinguished. Unfortunately some oxygen is always left behind. It also provides concentration of carbon dioxide which stimulates growth of bacteria other than anaerobes.
3. Absorption of oxygen by chemical or biological mean:
   a. Pyrogallic acid and sodium hydroxide.
   b. Chromium and sulfuric acid.
   c. GASPAK is now the method of choice consisting of an envelope and jar. Envelope is placed inside jar. GASPAK envelope contains 3 tablets, one each of citric acid, sodium carbonate and sodium borohydrate. It generates hydrogen and carbon dioxide on addition of water. Hydrogen combines with oxygen to produce water, and thus creation of anaerobiosis.
   d. McIntosh and Fildes anaerobic jar (Fig. 6.7):

      It consists of glass or metal jar with metal lid which can be clamped airtight with screw. The lid has two tubes, one acts as a gas inlet and the other one as outlet. Additionally lid has two terminals which can be connected to electrical supply.

      Inoculated culture plates are placed inside jar. Outlet tube is connected to vacuum pump and air inside is evacuated. The outlet tap is closed and in-

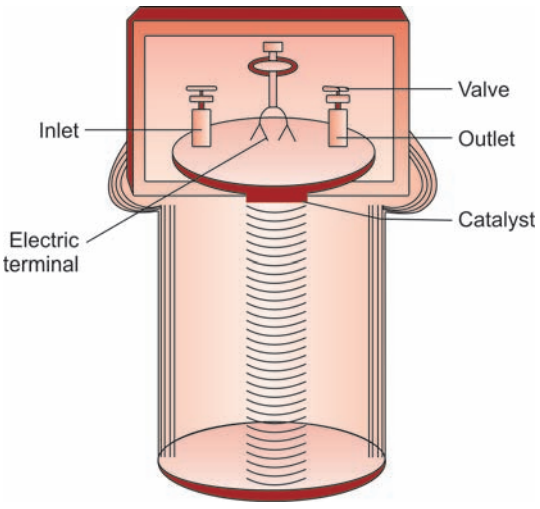


Fig. 6.7: McIntosh and Fildes jar

let tube is connected with hydrogen gas cylinder. After filling of jar with hydrogen, electric terminals are connected so that palladinised asbestos is heated. This acts as catalyst for combination of hydrogen and residual oxygen. It ensures complete anaerobiosis. At the same time it also carries risk of explosion.

An indicator should also be kept for verifying anaerobic condition in jar. Reduced methylene blue is used for this purpose. It is colorless anaerobically and regains blue color on exposure to oxygen.

4. *Anaerobic glove box:* This is a self-contained anaerobic system with provisions of circulation of hydrogen, nitrogen and carbon dioxide within it and catalytic conversion of residual oxygen to water. It is expensive. It is recommended for total anaerobic gut flora studies.
5. *Reduction of oxygen* in medium is achieved by using various reducing agents:
   a. 1 percent glucose.
   b. 0.1 percent thioglycolate.
   c. 0.1 percent ascorbic acid.
   d. 0.05 percent cystine.
   e. Broth containing iron pieces flamed red hot.
   f. Broth containing fresh animal tissue, e.g. rabbit kidney, spleen, etc.
   g. Robertson cooked meat medium produces anaerobiosis as under:
      (i) Unsaturated fatty acids present in meat utilize oxygen for autoxidation.
      (ii) Glutathione and cystein are reducing agents of meat and use oxygen.
      (iii) Sulfahydryl compounds of cystein also precipitate for reduced oxidation reduction.

It consists of fat free minced meat in broth. It permits growth of even strict anaerobes. The meat itself contains reducing substances, particularly glutathione, which helps in the growth of anaerobes. Further fresh entry of oxygen into the medium is prevented by layering the top with sterile liquid paraffin. It indicates sacchrolytic (meat being red) and proteolytic (meat being black) activities.

7 Classification and Identification of Bacteria

Bacterial classification presents special problem. A number of criteria have been employed to group them, e.g.

a. *Energy source* Phototrophic, chemotrophic, autotrophic and heterotrophic.
b. *Nutrient requirement* Simple or complex.
c. *Ability to grow* in living tissue—saprophytes and parasites.
d. *Temperature of growth* Psychrophilic, mesophilic and thermophilic.
e. *Oxygen requirement* Aerobic and anaerobic.

None of these seem satisfactory. Following systems are used to classify bacteria:

1. **Biological classification:** It is based on observable characters like physiological, immunological and ecological.

Division Protophyta

Class: Schizomycetes.

Orders: 1 Pseudomondales.
2 Eubacterales.
3 Actinomycetales.
4 Spirochaetales.
5 Mycoplasmaetales.

On the basis of main characters of each order, further families and genera are classified.

2. **Morphological classification:** All the organisms are classified into two groups.
   - **A. Higher bacteria:** They are filamentous and grow by branching to form mycelium, e.g. antinomycetes. Organisms producing true mycelium among actinomycetales are further classified into:
     a. Vegetative mycelium fragments into bacillary or coccoid element. Of course they are Gram-positive. They may be of following types:
        i. Anaerobic, acid fast, e.g. nocardia.
        ii. Anaerobic non-acid fast, e.g. *Actinomyces israelii, Actinomyces bovis.*
     b. Vegetative mycelium does not fragment into bacillary or coccoid form. Conida are formed in chain from aerial hyphae, e.g. streptomyces.
   - **B. Lower or true bacteria:** They are unicellular and never form mycelium. They are grouped on the basis of their shape.
     a. Cocci—spherical
     b. Bacilli—rod shaped
     c. Vibrio—Comma shaped
     d. Spirilla—Spiral twisted non-flexous rods
     e. Spirochaetes—Thin spirally twisted, flexous rods.

Cocci: Following types of arrangement is seen:

Diplococcus: Binary fission occurs in one plane, e.g. pneumococci.

Streptococcus: Cocci are arranged in chain, e.g. *Streptococcus pyogenes, Streptococcus viridans.*

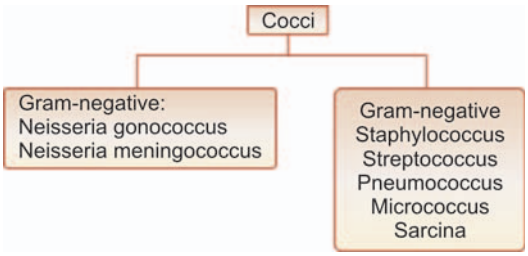
Staphylococcus: Cocci are arranged in cluster, e.g. *Staphylococcus aureus, Staphylococcus albus.*

Tetracoccus: Arrangement of cocci in group of four, e.g. *Micrococcus tetragena.*

The cocci are further classified into Gram-positive and Gram-negative. Gram positive are again divided on the basis of arrangement of cells.

Vibrio: They are curved, non-flexible, Gram-negative, highly motile, e.g. *Vibrio cholerae.*

Spirilla: Consists of coiled, non-flexous motile cells, e.g. *Spirilum minus.*

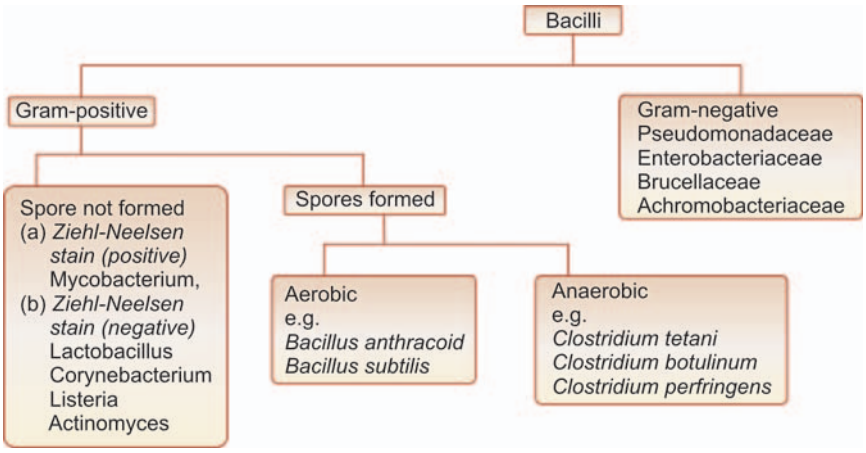


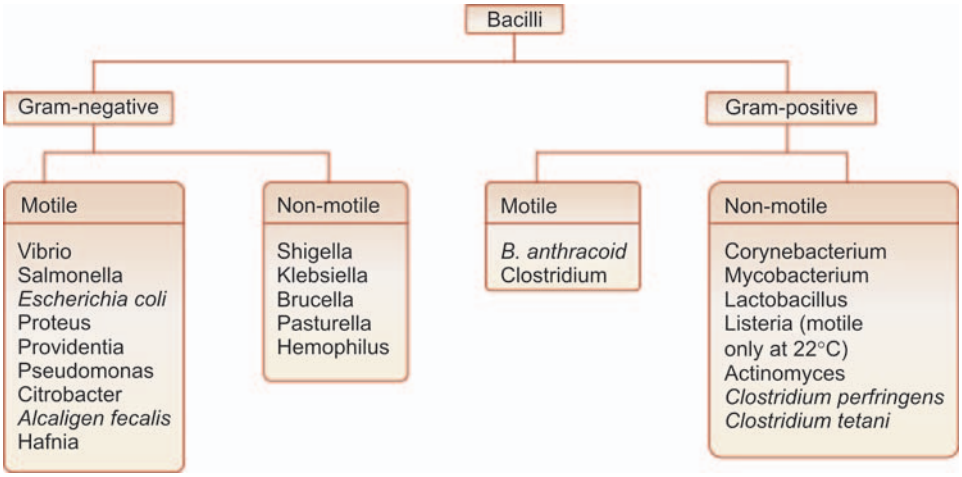
Bacilli: They may be Gram-positive or Gram-negative. Gram positive, may be acid fast on the basis of staining reaction to Ziehl-Neelsen stain. Gram negative organism are further identified on the basis of biochemical reaction and antigenic analysis.

Spirochaetes: They are slender, refractile and spiral filaments. The pathological species are classified into 3 genera.

a. Treponema, e.g. *Treponema pallidum.*
b. Leptospira, e.g. *L. icterohemorrhagica.*
c. Borrelia, e.g. *Borrelia recurrentis.*

3. **Adansonian or numerical classification:** This system determines the degrees of relationship between strains by a statistical coefficient. It includes similar and different characters, all of which are given equal importance. The different organisms are compared by scoring for a large number of phenotypic character (score+1 if the character is present, –1 if it is absent). The classification thus depends upon total scores of as many similarities and differences as can be observed. Thus in a collection of strains in which the results of large number of tests are compared with one strain and another, the degree of similarity of strain A to B is indicated by similarity index (S).





$$S = \frac{\text{Ns (Number of shared, i.e. positive characters)}}{\text{Ns (Number of shared)—Nd (Number of differences detected)}}$$

4. **Biochemical classification:** Chemical composition of cell wall of prokaryotic cell is different from eukaryotic cell. N-acetyl muramic acid does not occur in eukaryotic cell. Further prokaryotic cell wall membrane lacks sterols which typically occur in the membrane of eukaryotic cells. Gram-positive bacteria contain glycerol techoic acid and ribitol techoic acid and some monosaccharides like rhamanose and arabinose.
5. **DNA composition as a basis for classification:** By measuring G + C (guanine and cytosine) content of bacterial preparation it can be shown that there is wide range of the G + C components of DNA in bacteria, varying from 25 to 80 moles percent in different genera. For any one species, the G + C is relatively fixed or fall within a very narrow range and this provides a basis for classification.

Identification of Bacteria

For the identification of organism we proceed as under:

1. *Microscopic examination:* It helps to find out whether the bacteria is cocci, bacilli, vibrio, spirillum or spirochaete. On Gram staining we can have two groups of organism: Gram-positive and Gram-negative organisms.
2. *Motility:* Pathogenic cocci are non-motile. Among Gram-negative bacilli, salmonella, *Escherichia coli*, proteus, pseudomonas, *Alcaligens fecalis, Vibrio cholerae* are motile. Among Gram-positive bacilli clostridia and bacillus are motile. Hanging drop preparation, dark ground microscopy, phase contrast, electron microscope help in their study.

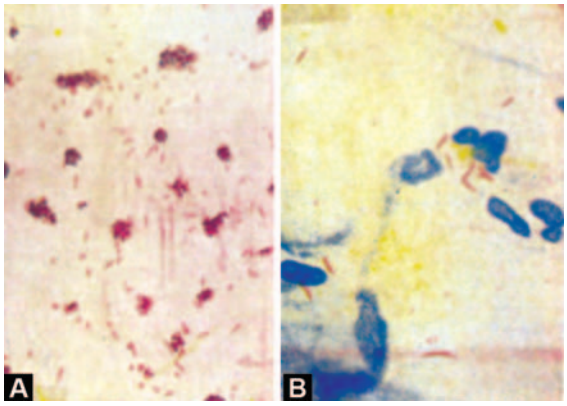
Common Staining Techniques

1. Simple stains where watery solution of single basic dye such as, methylene blue or basic fuchsin are used as simple stain.
2. Negative staining bacteria are mixed with dyes (India ink nigrosin). The background gets stained leaving the bacteria contrastingly colorless. The technique is useful in demonstration of bacterial capsule.
3. Impregnation methods where bacterial cells and appendages that are too thin and delicate cannot be seen under ordinary microscope. These delicate structures are thickened by impregnation of silver on the surface to make them visible under light microscope, e.g. demonstration of spirochaetes and bacterial flagella.
4. Differential stains impart different colors to different bacteria or their structures. In a stained film, bacterial shape, arrangement and presence of other cells (pus cells) are noted. The two commonly used differential stains are Gram's stain and acid fast stain (Fig 7.1A and B).

Gram Stain (Fig. 7.1A)

The Gram stain is named after the Danish scientist, Hans Christian Gram who originally devised the staining technique. Gram stain is the most widely used stain in microbiology that differentiates bacterial species into two large groups (Gram positive and Gram negative) based on the physical and chemical properties of their cell walls.

The basic steps of Gram stain include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine),



Figs 7.1A and B: (A) Gram-stained smear showing Gram-positive cocci (cluster) and Gram-negative bacilli, (B) Ziehl-Neelsen stained smear showing acid-fast bacilli

rapid decolorization with alcohol or acetone, and counterstaining with safranin or basic fuchsin.

Depending on the results, the two broad categories of bacteria include:

- Gram positive bacteria- resist decolorization and retain the color of primary stain i.e. violet
- Gram negative bacteria- are decolorized by acetone/alcohol, thereby take up the counterstain and appear red.

Ziehl-Neelsen stain (Fig. 7.1B)

The Ziehl-Neelsen (ZN) stain or the acid fast stain was first described by a bacteriologist, Franz Ziehl and a pathologist Friedrich Neelsen. It is a differential bacteriological stain used to identify acid fast bacteria, especially Mycobacterium. The reagents used are Ziehl-Neelsen carbolfuchsin (primary stain), acid alcohol and methylene blue (counterstain). 5% sulphuric acid is used for staining *Mycobacterium leprae* and 20% for *Mycobacterium tuberculosis.*

A positive smear typically contain pink colored, rod shaped bacteria that are slightly curved, sometimes branching, sometimes beaded in appearance, present singly or in small clumps against a blue background of other cells. Acid fastness of a bacterium is attributed to the high content of lipids, fatty acids and higher alcohols found in the cell wall. Mycolic acid, acid fast, waxy material is present in all acid fast bacteria. Apart from lipids, integrity of the cell wall also contributes to the acid fastness of a bacterium.

Staining Reaction

Ziehl-Neelsen stain into acid fast and non-acid fast bacilli, Albert stain for the demonstration of metachromatic and fluorescent dye to bring out special character.

Study of morphology and staining characteristics helps in preliminary identification.

Culture character: Growth requirement and colonial characteristics in culture are useful for the identification of organism, e.g. *Staphylococcus aureus* shows beta type hemolysis with pin-head colonies whereas *Staphylococcus albus* is without any hemolysis. *Streptococcus pyogenes* are pin-point colonies with β hemolysis whereas *Streptococcus viridans* show α type hemolysis.

Resistance: Resistance to heat, concentration of disinfectant, antibiotic, chemotherapeutic agent and bacitracin help in differentiating and identification, e.g. resistance of *Streptococcus fecalis* to heat at 60°C for 30 minutes and clostridial spores to boiling for various period.

Metabolism: Requirements of oxygen, need of carbon dioxide, capacity to form pigment and power of hemolysis is helpful for classification of bacteria and to differentiate species.

Biochemical Reactions

The more important and widely used tests are as under:

1. **Sugar fermentation:** This is tested in sugar media having Andrade's indicator. Acid production changes the color of medium into pink. Gas produced collects in Durham's tube.
2. **Indole production:** This test demonstrates production of indole from tryptophane. This tryptophane is present in peptone water. In 48 hours peptone water culture 0.5 ml Kovac reagent is added. Red colored ring indicates positive test.
3. **Methyl red test:** It is to detect the production of acid during fermentation of glucose and maintenance of pH below 4.5. Glucose phosphate culture is taken and few drops of 0.04 percent methyl red are added. Red color is positive while yellow color means negative test.
4. **Voges-Proskauer test:** It depends on production of acetyl methyl carbinol from pyruvic acid. 48 hours growth of glucose phosphate culture is taken. To it we add 40 percent KOH (1 vol.) and 3 volumes of a naphthal. Deep pink color in 2.5 minutes which deepens into magenta or crimson color means positive tests.
5. **Citrate utilization:** Some organisms use carbon as sole source of energy. Koser citrate medium (liquid) is taken for this test. Turbidity in this medium means citrate has been used up. In Simmon's medium (solid) after overnight incubation color of medium changes from green to blue if citrate is used up by the organism.

6. **Nitrate reduction:** Organism is grown in broth containing 1 percent KNO_3 for 5 days. To it is added 1 to 2 drops of mixture of sulfanilic acid and naphthalamine (mixed in equal proportion). Red color appears within few minutes if test is positive.
7. **Urease test:** It is done in Christensen's urease medium. Inoculate heavily the slope and incubate at 37°C. Urease producing organism produce pink color. Urease producing bacteria reduce urea to ammonia and hence pink color.
8. **Hydrogen sulphide production:** Some of the organisms decompose sulphur containing amino acid producing H_2S among the products. It turns lead acetate paper strip into black. Instead of lead acetate we may use ferrous acetate or ferric ammonium citrate.
9. **Catalase production:** Pour a drop of 10 vol H_2O_2 on glass slide. Now touch straight wire charged with bacterial colony. In positive reaction gas bubbles are produced.
10. **Oxidase reactions:** The reaction is due to cytochrome oxidase. One percent solution of tetra methyl-p-phenylene diamine hydrochloride is made. The colony to be tested is smeared (5 mm line) overpaper soaked in above-mentioned solution. Smeared area turns dark purple in 5 to 10 seconds in positive cases.
11. **Growth in KCN:** 1/13000 dilution of KCN is used to identify Gram-negative bacilli.
12. **ONPG (O-nitrophenyl-beta-galactopyranoside) test:** In peptone broth 0.15 percent ONPG is added and pH is adjusted at 7.5. This test is used to detect enzyme betagalactosidase present in lactose fermenters like *Escherichia coli.* This enzyme may break down ONPG to release yellow-O nitrophenol, a color change seen within 3 hours. Test is negative if no color change occurs after 24 hours.
13. **Hippurate hydrolysis:** The principle of the test is based on hydrolysis benzyl glycine (hippurate) to benzene and glycine detectable by addition of ferric chloride.

Ferric chloride reagent (12% $FeCl_3$. $6H_2O$ and 2.5% HCl in water) may be mixed to 1 ml of uninoculated medium until precipitated hippurate just redissolves. At the same time, same quantity of ferric chloride reagent is added to 1 ml of clear supernatant of an incubated culture. Now the development of precipitate means test is positive for hippurate hydrolysis. This test is positive in *Klebsiella aerogenes.*

Antigenic analysis: By using specific sera we can identify organism by agglutination reaction, e.g. beta hemolytic streptococci is differentiated into 18 serological groups (A to T except I and J) on the basis of polysaccharides component. Likewise, pneumococcus is divisible into 77 capsular types by capsular swelling reactions with anticapsular sera.

Bacteriophage typing: Viruses that parasitize bacteria are called bacteriophage or phage. Phage brings about lysis of susceptible bacterial cells. Phage typing is useful in distinguishing strain among salmonella and staphylococcus. There is correlation between bacteriophage type and epidemic source.

Pathogenecity: For pathogenecity test commonly used laboratory animal models are guinea pig, rabbit, rat and mouse. The route used may be subcutaneous, intramuscular, intraperitoneal, intracerebral, intravenous, oral or nasal spray.

Resistance to antibiotic and other agents: Information about sensitivity pattern of strain is useful for selecting choice of drug. This may be useful as an epidemiological marker in tracing hospital infection, e.g. *Staphylococcus aureus* sensitivity to mercury salt.

In other cases sensitivity of bacteria to agents help in identification of organism, e.g. *Streptococcus pyogenes* are sensitive to bacitracin and pneumococcus to optochin.

Plasmid profile and bacitracin typing also further help in identification of bacteria.

DEVELOPMENT OF RAPID METHODS FOR IDENTIFICATION OF ORGANISMS

The last few years have seen exciting advances in finding out new ways of identifying microorganisms within a few minutes of arrival of a specimen in the laboratory. Specific microbial components may be detected by countercurrent immunoelectrophoresis also designated as CIE. Another nice method for identification of

microorganism is gas-liquid chromatography (GLC) in which specific metabolic products are detected.

Microcalorimetry may be used to find out rapidly the number of bacteria in liquid specimen like urine, by detecting their heat output.

Luminescent biometry is again a useful method in which amount of bacterial adenine triphosphate is detected and thus a number of bacteria in a given amount of liquid can be known by measuring the brightness of flash of light when that amount is mixed with standard preparation of luciferase (lighting system of fire flies). Radiorespirometry using C^{14} glucose in basal nutrient medium with different antibiotics is used for antibiotic sensitivity and thus recommending appropriate antibiotic for an infection in only 3 hours time.

Genetic probes become another latest upcoming technique which is added in the list of rapid methods for the identification of organisms. Some other methods are calorimetry (detection of small amount of heat produced when organisms grow) limulus lysate (small amount of endotoxin produced by Gram-negative organisms can clot blood from horse shoe crab) and impedance (large molecules are broken into smaller molecules by the growth of bacteria so that electrical impedance is lowered which may be detected by a sensitive meter).

8 Sterilization and Disinfection

The process of sterilization finds application in microbiology for prevention of contamination by extraneous organisms, in surgery for maintenance of asepsis, in food and drug manufacture for ensuring safety from contaminating organism and many other situations.

Sterilization: It is a process by which articles are freed of all microorganisms both in vegetative as well spore state.

Disinfection: It is a process of destruction of pathogenic organisms capable of giving rise to infection.

APPLICATION OF DISINFECTANTS

In Bacteriology

- For disposal of culture (3% Lysol)
- For preservation of sera, agar or phenol agar, vaccine, etc.

In Surgical Procedures

- Washing the hand
- To prepare and clean the area of operation
- To collect the blood under aseptic precautions
- For safe disposable of excreta and surgical dressing
- Cleaning of infected wounds
- For disinfection of used instrument.

In Hospitals

- To disinfect the operation theaters
- To disinfect costly equipments like endoscopes and cystoscopes etc.
- To control the spread of cross infection
- To disinfect linen and surgical dressing.

In Public Health Services

- For providing safe drinking water (e.g. chlorinated water)
- For disinfection of sewage before its disposal into the fields.

Antiseptic: It means prevention of infection by inhibiting growth of bacteria.

Bacteriocidal agents: These are those which are able to kill bacteria.

Bacteriostatic agents: Only prevent multiplication of bacteria and they may remain alive.

Mainly there are two methods of sterilization.

Physical

1. Sunlight
2. Drying
3. Dry heat
4. Moist heat
5. Filtration
6. Radiation
7. Ultrasonic vibrations.

Chemicals

1. Acids
2. Alkalies
3. Salts
4. Halogens
5. Oxidizing agents
6. Reducing agents
7. Formaldehyde
8. Phenol
9. Soap
10. Dyes
11. Aerosol, etc.

Physical Methods

1. **Sunlight:** It possesses appreciable bacteriocidal activity. The action is due to ultraviolet rays. This is one of the natural methods of sterilization in case of water in tanks, river and lakes.
2. **Drying:** Drying in air has deleterious effect on many bacteria. Spores are unaffected by drying. Hence it is a very unreliable method.
3. **Heat:** The factors influencing sterilization by heat are:
   i. Nature of heat
   (a) dry (b) moist
   ii. Temperature and time.
   iii. Number of organisms present.
   iv. Whether organism has sporing capacity.
   v. Type of material from which organism is to be eradicated.

Dry Heat

Killing by dry heat is due to:
1. Protein denaturation.
2. Oxidative damage.
3. Toxic effect of elevated levels of electrolytes.

(a) Red heat: It is used to sterilize metallic objects by holding them in flame till they are red hot, e.g. inoculating wires, needles, forceps, etc.

(b) Flaming: The article is passed over flame without allowing it to become red hot, e.g. mouth of culture tubes, cottonwool plugs and glass slides.

(c) Incineration: This is an excellent method for rapidly destroying material, e.g. soiled dressing, animals carcasses, bedding and pathological material, etc.

On the negative side incinerators send toxic material like dioxin and mercurial products into the environment and cause pollution. Dioxin is released from half burnt chlorin based plastics, e.g. PVC. The source of mercury and its products may be broken thermometers, blood pressure apparatus and other diagnostic products. Mercury is known to cause irreversible poisoning. Dioxin may cause cancer and hormone mimicking, i.e. it replaces the natural hormones and can cause serious disturbances in reproductive process.

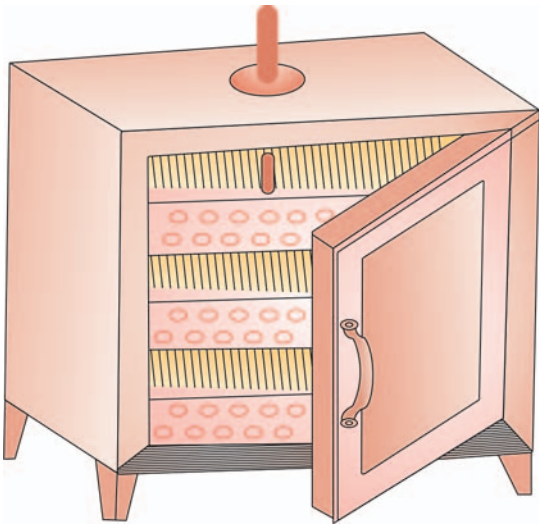


Fig. 8.1: Hot air oven

(d) Hot air oven (Fig. 8.1)*:* Sterilization by hot air oven requires temperature of 160°C for one hour. We can sterilize all glass syringes, petridishes, test tubes, flask, pipettes, cotton swabs, scalpel, scissors, liquid paraffin, dusting powder, etc.

Precautions in Use of Hot Air Oven

1. It must be fitted with fans to ensure distribution of hot air.
2. It should not be overloaded.
3. Oven must be allowed to cool for about 2 hours before opening the doors otherwise glasswares are likely to get cracked.

Sterilization Control of Hot Air Oven

1. The spores of non-toxigenous strain of *Clostridium tetani* are used to test dry heat efficiency.
2. Browne's tube (green spot) is available for sterilization by dry heat. A green color is produced after 60 minutes at 160°C.
3. Thermocouples may be used.

Moist Heat

The lethal effect of moist heat is by denaturation and coagulation of protein.

Temperature below 100°C

(i) *Pasteurization of milk:* Temperature employed is either 63°C for 30 minutes (holder method) or 72°C for 15 to 20 seconds (flash method). Heating is always followed by sudden and instant cooling of milk. Organisms like mycobacterium, salmonellae and brucellae are killed. *Coxiella burnetii* is relatively heat resistant and hence, may survive the holder method.

(ii) *Vaccine bath* (Fig. 8.2)*:* It is used for killing non-sporing bacteria which may be present in vaccine. In vaccine bath the vaccine is treated with moist heat for one hour at 60°C.

(iii) *Inspissation:* The slow solidification of serum or egg is carried out at 80°C in an inspissator, e.g. serum slopes, Lowenstein-Jensen's medium, etc.

Temperature at 100°C

(i) *Tyndallization:* This is the process by which medium is placed at 100°C in flowing steam for 30 minutes each on 3 successive days. The mechanism underlying this method is that vegetative cells get destroyed at 100°C and remaining spore which germinate during storage interval are killed on subsequent heating. This method may be used for sterilization of egg or serum containing media.

(ii) *Boiling:* Most of vegetative form of bacteria, fungi and viruses are killed at 50 to 70°C in short time. For needles and instruments boiling in water for 10 to 30 minutes is sufficient to sterilize them. Addition of little acid, alkali, or washing soda, markedly increases the sterilizing power of boiling water. Spores and hepatitis viruses are not readily destroyed by such procedure.

(iii) *Steam at atmospheric pressure* (100° C): Here free steam is used to sterilize culture media which may decompose if subjected to higher temperature. A Koch or Arnold steamer (Fig. 8.3) is used. This is a cheap method of sterilization.

Temperature above 100°C

Steam under pressure: For bacteriological and surgical work boiling is not sufficient because spores survive boiling. Hence high pressure sterilizer or autoclave is used.

AUTOCLAVE (FIG. 8.3)

In this apparatus, material for sterilization is exposed to 121°C for 15 to 20 minutes at 15 lb pressure per square inch. Saturated steam heats the article to be sterilized rapidly by release of latent heat. On condensation 1600 ml of steam at 100°C and at atmospheric pressure condenses into 1 ml of water and liberates 518 calories of heat. The condensed water ensures moist conditions for killing bacteria.

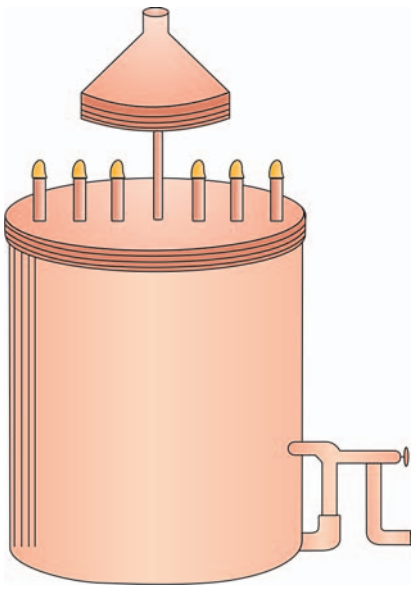


Fig. 8.2: Vaccine bath

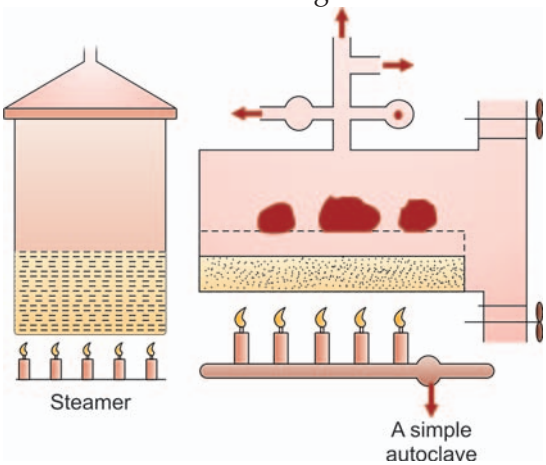


Fig. 8.3: Steamer and autoclave

Air is poor conductor of heat and must be removed from chamber. The contents must be so packed that free circulation of steam occurs.

Autoclave is used for culture media, rubber goods, syringes, gowns and dressing, etc.

Sterilization control:

1. Bacillus stearothermophilus
2. Browne's tube
3. Autoclave tapes
4. Thermocouples.

Types of Autoclave

1. Simple iron jacketed.
2. Low pressure/low temperature.
3. High pressure high vacuum type having facility to expell 98 percent of air rapidly by an electric pump and hence sterilization is done quickly.

Sterilization by Filtration

This is a method of sterilization useful for antibiotic solutions, sera, carbohydrate solution, etc. We may also get bacteria free filtrates of toxin and bacteriophages. It is also useful when we want to separate micro-organisms which are scanty in fluid.

Other Uses of Filter

- Separation of soluble products of bacterial growth, e.g. toxins.
- Sterilization of hydatid fluid
- Sterilization of serum
- Sterilization of antibiotic solution
- Sterilization of blood products
- Purification of water.

Membrane filter: They may be comprised of cellulose esters. They can be used as under:

1. Water analysis
2. Sterility testing of solution
3. Preparation of parenteral solution.

Nitrocellulose membrane filter is commonly used. It is also called multipore. Membrane filters are available in pore sizes of 0.015 um, 0.12 um and 0.22 um.

Drawback of filtration: Viruses and mycoplasma may pass through filter. Hence in filtered serum is not safe for clinical use as it may be containing viruses or mycoplasma.

Various types of filters are:

i. Earthen-ware candles, e.g. Berkefeld, Chamberland.
ii. Asbestos disk filter, e.g. Seitz.
iii. Sintered glass filters.
iv. Collodion or membranous filter.

Radiations

(i) Ultraviolet radiations: It is chief bacteriocidal factor present in sunlight. Commonly used UV lamp is of low pressure mercury vapor type whose length is 253.7 mm. It causes following changes in cell:

1. Denaturation of protein.
2. Damage to DNA.
3. Inhibition of DNA replication.
4. Formation of H_2O_2 and organic peroxide in culture media.
5. Induction of colicin production in colicinogenic bacteria by destruction of cytoplasmic repressor.

Ultraviolet lamps are used in:

a. Killing of organisms
b. Making bacterial and viral vaccines
c. Prevention of airborne infection in operation theater, public places and bacteriological laboratories.

Gram-positive bacteria show a little more resistance than Gram-negative bacteria to ultraviolet radiations. Spores are highly resistant to UV radiations.

(ii) X-rays and other ionizing radiations: Ionizing radiations have greater capacity to induce lethal changes in DNA of cell. They are useful for the sterilization of disposable material like catgut, disposable syringes, adhesive dressing, etc.

(iii) Gamma radiations

X-ray are utilized using two types of mechanics.

(i) Linear accelerator for X-ray
(ii) Cobalt-60 for gamma rays.

A dose of 2.5 M rad is sufficient to kill both vegetative and spore form of bacteria. They are used to sterilize rubber or plastic syringes, surgical catgut, bone tissue graft, adhesive dressings, etc.

Ultrasonic and Sonic Vibrations

They are bacteriocidal causing mechanical agitation and rupture of bacteria.

Chemical Methods

The chemical substances act as bacteriocidal agent as under:

a. Coagulation of bacterial protoplasm, e.g. heavy metals.
b. Disruption of cell membrane by chemical substances. They may alter physical and chemical property of cell membrane thus results in killing or inhibiting the bacterial cell growth.
c. Oxidation or burning out the bacterial protoplasm, e.g. halogens.
d. By affecting bacterial enzymes or co-enzyme system thus causing interference of bacterial metabolism.

Following chemicals are of common use:

1. *Acids and alkalies:* They are inhibitory to the growth of bacteria. Mycobacteria are more resistant to acid than alkalies. Boric acid is weak antiseptic.
2. *Distilled water:* It causes loss of viability. This action may be due to traces of metal in distilled water.
3. *Metallic ions:* $HgCl_2$ and $AgNO_3$ prevent the growth of many bacteria in concentration less than 1 part per million. This action is due to affinity of certain protein for metallic ions.
4. *Inorganic anion:* They are much less toxic to bacteria. Potassium tellurite is inhibitory to gram-negative bacteria. Fluoride inhibits many enzymes of bacteria.
5. *Halogens:* Halogen derive their name from Greek word "halos" meaning salt. Hence, halogen means salt former. Three of the halogens, i.e. chlorine, iodine and bromine are among the best bacteriocidal agents. They act mainly by forming protein halogen (salt like) compounds in living cells and they get killed quickly. Because of both toxicity and high cost bromine is not used. Halogens kill vegetative bacteria, fungi, viruses but not tubercle bacillus or bacterial spores. Iodine is used chiefly for skin. Chlorine combines with water to form hydrochloric acid which is bacteriocidal.
6. *Oxidizing agents:* They are weak antiseptic, e.g. H_2O_2, potassium permanganate.
7. *Formaldehyde:* It is useful in sterilizing bacterial vaccine and in inactivating bacterial toxin without affecting their antigenicity. 5 to 10 percent solution in water kills many bacteria. It is bacteriocidal, sporicidal and lethal to viruses also.
   Uses:
   1. Disinfection of woolen blankets, wool hides to destroy bacterial spores.
   2. Footwear of person with fungal infection (atheletic foot).
   3. Wards and operation theater.

   Fumigation: For sterilization of 100 cubic feet of air space in a room, 50 ml of 40 percent formalin is required. This gives vapor of 2 mg gas per liter of air. Formalin with sufficient water is heated in the room with its windows and doors closed and sealed. Articles and rooms are exposed to fumes for 4 hours. Alternatively, diluted formalin (50%) can be sprayed which also liberates formaldehyde gas. Ideally, humidity of air of room should be attained upto 60 percent to increase the sterilization power.
8. *Phenol:* It is used for sterilizing surgical instruments, and for killing culture accidentally split over in the laboratory. It is generally used in 3 percent solution.
9. *Soap and detergents:* They are bacteriocidal and bacteriostatic for gram-positive and some acid fast organisms. Detergent acts by concentrating at cell membrane and thus disrupting its normal function or it may denaturate protein and enzyme.
10. *Alcohol:* Ethyl alcohol is most effective in 70 percent solution than 100 percent alcohol. It is so because 70 percent alcohol has better penetration power than 100 percent alcohol. It does not kill spores.
11. *Dyes:* Gentian violet and malachite green, etc. are active against Gram-positive bacteria. They have poor penetration and hence action is bacteriostatic. Acriflavin is bacteriostatic for staphylococcus in 1:3000,000 concentration.
12. *Aerosols and gaseous disinfectant:* SO_2, chlorine and formalin vapors have been

TABLE 8.1: Sterilization methods and their applications

	Article	*Methods of sterilization*
1.	Disposable syringes	a. Gamma radiations b. Ethylene oxide.
2.	Nondisposable syringes	a. Autoclavation b. Hot air oven c. Infra-red radiation d. Boiling at 100° C
3.	Glasswares	a. Autoclavation b. Hot air oven
4.	Metal instruments	a. Autoclavation b. Hot air oven c. Infrared radiation
5.	Cystoscope, endoscope and other anesthetic equipment	a. Ethylene oxide b. Glutaraldehyde
6.	Heart lung machine	Ethylene oxide
7.	Disposable instrument	a. Gamma rays b. Ethylene oxide
8.	Thermometer	Glutaraldehyde
9.	Disposable catheter, gloves and transfusion sets	a. Gamma rays b. Ethylene oxide
10.	Nondisposable catheter, gloves and transfusion sets	Autoclavation
11.	Powder, fat and oil	Hot air oven
12.	Surgical dressing, bowl and linen	Autoclavation
13.	Antibiotic solutions and toxins	Filtration
14.	Sealed bottles, ampoules of aqueous solutions	Autoclavation
15.	Culture media containing sugars, gelatin, etc	a. Tyndallization b. Autoclavation at low pressure
16.	Other culture media without sugar or gelatin	a. Autoclavation b. Steam at 100°C, for 90 minutes
17.	Serum	Filtration
18.	Operation theater, inoculation hood and cubical entrance	Ultraviolet radiation
19.	Hospital blankets, etc.	Exposure to formalin followed by autoclavation.

used as gaseous disinfectant. Propylene glycol is powerful disinfectant.

13. *Vapor phase disinfectants:* Examples of vapor phase disinfectant are ethylene oxide and formaldehyde. Ethylene oxide can kill all kinds of microbes (Spores and tubercle bacilli). It is useful to sterilize material likely to be damaged by heat, e.g. plastics and rubber items, drugs powder, heart lung machine, etc.

Hydrogen peroxide vapors: These vapors are oxidizing by nature and so they are effective sterilants. They may be used to sterilize instruments. Hydrogen peroxide is vaporized producing reactive free radicals either with microwave frequency or radiofrequency energy. There are many variations of hydrogen peroxide vapor or gas sterilization available, e.g. plasma gas sterilization, 100s sterilizer, etc. It is felt that in near future ethylene oxide may be replaced by above mentioned methods.

Other sterilant gases are chlorine dioxide, paracetic acid, glutaraldehyde, etc.

Sterilization and their appropriate uses are shown in Table 8.1.

Uses of Disinfectant

In practice disinfectant is useful and necessary for:

1. Contaminated disposable material before incineration.

2. Surfaces like table and trolley top.
3. Cleaning material when contaminated material has been split.
4. Disinfection of instruments, not amenable to heat.
5. Disinfection of skin.

EVALUATION TESTS OF DISINFECTANT

Following tests may be used to assess the efficiency of disinfectants:-

1. *Rideal walker test:* In this case similar quantities of microorganisms are subjected to varying concentration of phenol and disinfectant to be tested. The disinfectant dilution which sterilizes the suspension in a given time is divided by matching dilution of phenol. This is phenol coefficient. This test in fact has many limitations. Actually it does not provide information about the way tested disinfectant will function in the presence of organic substance. It is no more in use.
2. *Chick Martin test:* In this test disinfectants function in the presence of organic matter to simulate natural conditions. For this 1% dried feces or yeast is added which as organic matter to simulates natural conditions. It is also not in practice now a days.
3. *Kelsey Sykes test:* Here standard micro-organisms (*Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus*) is added to the disinfectant (3 portions) at 0, 10 and 20 minutes intervals. They are allowed to be in contact for 8 minutes each. Hence they are transferred to recovery medium at 8, 18 and 28 minutes interval respectively. The disinfectant is evaluated by virtue of its ability to kill bacteria (no growth in recovery medium). In fact this test is done under clean and dirty conditions. It means this test indicates effectiveness of disinfectant in the presence of organic matter.
4. *In use test:* In this situation disinfectant solutions used in the hospital are examined quantitatively for viable microorganisms. The efficiency of disinfectant is determined by its ability to inactivate known number of standard strains of pathogenic streptococci on a given surface within certain time. The results are more useful than those of phenol coefficient.

New methods of disinfection and sterilization

1. Surfacine:	• It has antimicrobial effect of more than 13 days. • It may be used on animate and inanimate surfaces. • It is resistant to form biofilm. • It is not toxic. • Unfortunately it is costly
2. Super oxidant water	• It is not expensive as basic ingredients are saline and electricity. • Its products are not damaging to environment. • It is not toxic.
3. Endoclens	• It cleans and sterilizes auto-matically. It has rapid cycle time less than 30 minutes. It tests endoscope for channel blockage and leakage. • It is quite expensive.
4. Attest ethylene oxide rapid readout	• It is rapid and reliable and has 100% sensitivity. • It prevents recall of released sterilization loads. • It is costly.
5. Plasma sterilizer	• Use of two hydrogen peroxide diffusion plasma stage cycles is quite effective sterilization process. • It has reduced cycle time of about 45 minutes. • It leaves no toxic residue. • It is costly.

9 Infection

Infection: The lodgement and multiplication of organism in the tissue of host constitutes infection.

Classification of Infection

1. *Primary infection:* Initial infection with organism in host constitutes primary infection.
2. *Reinfection:* Subsequent infection by same organism in a host is called reinfection.
3. *Secondary infection:* When in a host whose resistance is lowered by preexisting infectious disease, a new organism may set up an infection.
4. *Focal infection:* It is a condition where due to infection at localized sites like appendix and tonsil, general effects are produced.
5. *Cross infection:* When a patient suffering from a disease and new infection is set up from another host or external source.
6. *Nosocomial infection:* Cross infection occurring in hospital is called nosocomial infection.
7. *Subclinical infection:* It is one where clinical affects are not apparent.

Saprophytes: They are free living organisms which live on decaying organic matter. They fail to multiply on living tissue and so are not important in infectious disease.

Parasites: They are organisms that can establish themselves and multiply in hosts. They may be pathogens or commensal. Pathogens are those which are capable of producing disease in a host. On the contrary, commensal microbes can live in a host without causing any disease.

Sources of Infection in Man

1. *Man:* Man is himself a common source of infection from a patient or carrier. Healthy carrier is a person harboring pathogenic organism without causing any disease to him. A convalescent carrier is one who has recovered from disease but continues to harbor the pathogen in his body.
2. *Animals:* Infectious diseases transmitted from animals to man are called zoonosis. Zoonosis may be bacterial (e.g. plague from rat), rickettsial (e.g. murine typhus from rodent), viral (e.g. rabies from dog), protozoal (e.g. leishmaniasis from dogs), helminthic (e.g. hydatid cyst from dogs) and fungal (zoophilic dermatophytes from cats and dogs).
3. *Insects:* The disease caused by insects are called arthropod borne disease. Insects like mosquitoes, fleas, lice that transmit infection are called vector. Transmission may be mechanical (transmission of dysentery or typhoid bacilli by housefly) and these are called mechanical vector. They are called biological vector if pathogen multiplies in the body of vector, e.g. anopheles mosquito in malaria.
4. Some vectors may act as reservoir host (e.g. ticks in relapsing fever and spotted fever).
5. *Soil:* Soil may serve as source of parasiting infection like roundworm and hookworm. Spores of tetanus bacilli remain viable in soil for a long time, fungi like *Histoplasma capsulatum* and higher bacteria like *Nocardia asteroides* also survive in soil and cause human infection.

6. *Water: Vibrio cholerae*, infective hepatitis virus (Hepatitis-A), guineaworm may be found in water.
7. *Food:* Contaminated food may be source of infection. Presence of pathogens in food may be due to external contamination (e.g. food poisoning by staphylococcus).

Methods of Transmission of Infection

1. *Contact:* Syphilis, gonorrhea, trachoma.
2. *Inhalation:* Influenza, tuberculosis, small-pox, measles, mumps, etc.
3. *Infection:* Cholera (water), food poisoning (food) and dysentery (hand borne).
4. *Inoculation:* Tetanus (infection), rabies (dog), arbovirus (insect) and serum hepatitis, i.e. Hepatitis-B (infection).
5. *Insects:* They act as mechanical vector (dysentery and typhoid by housefly) or biological vector (malaria) of infectious disease.
6. *Congenital:* Congenital syphilis, rubella, *Listeria monocytogenes*, toxoplasma and cytomegalic inclusion disease.
7. *Laboratory infection:* Infection may be transmitted during procedures like, injection, lumbar puncture, catheterization, etc. if proper care is not taken.

Characters of Pathogens

1. Bacteria should be able to enter the body.
2. Organism should be able to multiply in the tissue.
3. They should be able to damage the tissue.
4. They must be capable to resist the host defence.

Factors Predisposing to Microbial Pathogenicity

Before discussing factor it is worthwhile to make fine distinction between the terms pathogenecity and virulence.

Pathogenicity: It is referred to the ability of microbial species to produce disease.

Virulence: It is referred to the ability of microbial strains to produce disease, e.g. polio virus contains strain of varying degree of virulence.

Virulence is the sum of the following factors:

A. Invasiveness: It is the ability of organism to spread in a host tissue after establishing infection. Less invasive organisms cause localized lesion, e.g. staphylococcal abscess. Highly invasive organisms cause generalized infection, e.g. streptococcal septicemia.

B. Toxigenecity: Bacteria produce two types of toxins—

(a) Exotoxin: It has following characters.

1. Heat labile proteins.
2. Diffuse readily into the surrounding medium.
3. Highly potent, e.g. 3 kg botulinum can kill all the inhabitants of world whereas 1 mg of tetanus toxin is sufficient to kill one million guinea pigs.
4. They are generally formed by Gram positive organism and also by Gram negative organisms like shigella, *Vibrio cholerae* and *Escherichia coli.*
5. Exotoxin are specifically neutralized by antitoxin.
6. Can be separated from culture by filtration.
7. Action is enzymatic.
8. It has specific tissue affinity.
9. It is highly antigenic.
10. Specific pharmacological effects for each exotoxin.
11. Can be toxoided.
12. Cannot cause pyrexia in a host.

(b) Endotoxin: Endotoxin (Lipid a portion of lipopolysaccharide) has biological activities causing fever, muscle proteolysis, uncontrolled intravascular coagulation and shock. These may be mediated by production from mononuclear cells of IL-I, TNFX α 2 probably IL-6. It has following characters.

1. Proteins polysaccharide lipid complex heat stable.
2. Forms part of cell wall and will not diffuse into the medium.
3. Obtained only by cell lysis.
4. They have no enzymatic action.
5. Effect is nonspecific action.
6. No specific tissue affinity.

7. Active only in large doses 5 to 25 mg.
8. Weakly antigenic.
9. Neutralization by antibody ineffective.
10. Cannot be toxoided.
11. Produce in Gram negative bacteria.
12. Can cause pyrexia in a host.

C. Communicability: This is ability of parasite to spread from one host to another. It determines the survival and distribution of organism in a community. Highly virulent organism may not exhibit a high degree of communicability due to rapid lethal effect on hosts. Infections in which pathogen is shed in secretions as in respiratory and intestinal diseases are highly communicable.

D. Other bacterial products

1. Coagulase *(Staphylococcus aureus)* which prevents phagocytosis by forming fibrin barrier around bacteria.
2. Fibrinolysin promotes the spread of infection by breaking down the fibrin barrier in tissues.
3. Hyaluronidase split hyaluronic acid (component of connective tissue) thus facilitating spread of infection along tissue spaces.
4. Leucocidins damage polymorphonuclear leucocytes.
5. Hemolysin is produced by some organisms capable of destroying erythrocytes.
6. Ig A1 proteases: Gonococci, meningococci, *Hemophilus influenzae* pneumococci, may produce IgA1 protease which splits IgA and inactivates its antibody activity.

E. Bacterial appendages: Capsulated bacteria like pneumococcus, *Klebsiella pneumoniae* and *Hemophilus influenzae* will stand phagocytosis. Surface antigen, e.g. Vi antigen of *Salmonella typhi* and K antigen of *Escherichia coli* resist phagocytosis and lytic activity of complement.

F. Infecting dose: The minimum infection dose (MID) or minimum lethal dose (MLD) is the minimum number of organisms required to produce clinical evidence of infection or death of susceptible animal.

G. Route of infection: *Vibrio cholerae* is effective orally. No effect when it is introduced subcutaneously. Streptococci can initiate infection whatever be the mode of entry. They also differ in ability to produce damage to different organs in different species, e.g. tubercle bacilli injected into rabbit cause lesion mainly in kidney and infrequently in liver and spleen. In guinea pig, main lesion is in liver and spleen whereas kidney is spared.

10 Antimicrobial Therapy

Chemotherapeutic Agents

These are the agents which have lethal or inhibitory effect on the microbes responsible, but in therapeutic concentration have little or no toxic action on the tissues.

However, these agents used in chemotherapy are of very diverse chemical structure. They can be divided into two categories:

a. Relatively simple compounds obtained by laboratory synthesis, e.g. sulfonamides, isoniazid, PAS, trimethoprim, etc.
b. Antibiotics are the substances produced by living organisms and which are active against other living organisms. Most of them are produced by soil actinomycetes.

Antibacterial agents are divided into two classes on the basis of type of action they exhibit against bacteria:

1. Bacteriostatic drugs are drugs which, in the concentration attainable in the body, only inhibit bacterial growth, e.g. chloramphenicol, sulfonamides, tetracyclines, etc.
2. Bacteriocidal drugs are the drugs which kill the bacteria by virtue of their rapid lethal action, e.g. penicillins, cephalosporins, amino-glycosides, fucidin, nalidixic acid, etc. Bacteriocidal drugs are more effective therapeutic agents than bacteriostatic drugs.

Mode of Action

The problem can be considered from two aspects:

1. Identification of site of action of drug.
2. Its precise mechanism of action.

Site of Action

There are four major loci of action.

1. Inhibition of synthesis of cell wall peptidoglycon, e.g. penicillins, cephalosporin, cycloserine, vancomycin, ristocetin and bacitracin.
2. Damage to the permeability of the cytoplasmic membrane, e.g. tryocidin, gramicidin, polymyxin and antifungal polyene antibiotics.
3. Inhibition of protein synthesis, e.g. aminoglycosides (amikacin, netilmicin, tobramycin, gentamicin, kanamycin, neomycin, streptomycin, etc.), tetracyclines, chloramphenicol. They bind to and inhibit the function of 30 S.
4. Inhibition of nucleic acid synthesis, e.g. rifampicin inhibits the synthesis of messenger RNA by its action on the RNA polymerase whereas nalidixic acid inhibits DNA replication. Other examples, are novobiocin, pyrimethamine, sulfonamide, etc.

Mechanism of Action

There are three general mechanisms of action.

1. Competition with a natural substrate for the active site of enzyme, e.g.
   a. Action of sulfonamides to interfere competitively with the utilization of para-amino benzoic acid (PABA).
   b. Action of para-amino benzoic acid with para-amino salicylic acid (PAS).
2. Combination with an enzyme at a site sufficiently close to the active site as to

interfere with its enzymatic function, e.g. vancomycin, ristocetin and bacitracin.
3. Combination with non-enzymatic structural components, e.g. drugs which inhibits protein synthesis and drugs which act by damaging cytoplasmic membrane.

Laboratory Uses of Antibiotics

1. They may be incorporated as selective agents in culture media, e.g. penicillin may be used for isolation of *Hemophilus influenzae* from material taken from upper respiratory tract (penicillin inhibits the growth of Gram positive bacteria and neisseriae). Neomycin is used in Willis and Hobb's medium for the isolation of clostridia.
2. They are used for the control of bacterial contamination in tissue cultures used for virus isolation, e.g. penicillin, streptomycin, nystatin, etc.
3. The pattern of sensitivity of an organism to a battery of antibiotics constitute a simple method of typing which is of considerable epidemiological value.

Some Antibiotics in Current Use

1. Antibiotic mainly or exclusively active against Gram positive bacteria, e.g. penicillin (G and V), methicillin, cloxacillin, erythromycin, novobiocin, vancomycin, bacitracin and fucidin.
2. Antibiotics active against Gram negative bacteria, e.g. polymyxin, aminoglycoside.
3. Antibiotics active against both Gram positive and Gram negative bacteria, e.g. tetracycline, chloramphenicol, ampicillin, cephalosporins.
4. Antibiotics active against fungi, e.g. greseofulvin, iodides, nystatin, amphotericin B.

New Advances in Antibacterial Drugs

Azithromycin: It is a new orally administered macrolide like Roxithromycin and Clarithromycin. It has a broad spectrum of activity; improved tissue penetration and tolerability profile plus convenient once daily dose (500 mg daily). In patients with uncomplicated urethritis or cervicitis is used as first line therapy.

Cefprozile: It is a new second generation oral cephalosporin approved for the treatment of RTI and skin infection.

Ceftibuten: It is a new third generation oral cephalosporin effective against Gram negative and Gram positive organisms. It is indicated in the treatment of RTI, UTI, gynecological and skin plus soft tissue infection.

Cefetamet: It is a new third generation oral cephalosporin with properties of ceftibuten.

Cefpirome: It is a fourth generation cephalosporin indicated in severe RTI, UTI, nosocomial infection and septicemia. It is available in parenteral preparation.

Loracarbef: It is the first of a new class of beta lactam antibiotic carba cephams. It is like cephalosporin chemically and acts by inhibition of cell wall synthesis in susceptible microorganisms. It is active against both Gram positive aerobes and anaerobes and Gram negative aerobes. It is indicated in URTI, skin and soft tissue infection.

Feroxacin: It is trifluorinated quinolone, has extremely high bioavailability, low protein binding and a long serum half life. Once daily dose is adequate. It is indicated for the treatment of UTI, RTI, GIT, skin infection, soft tissue infection and STDs. Among the fluoroquinolones, it has excellent activity against enterobacteriaceae, acinetobacter and *H. influenzae.*

Antibiotic Sensitivity Tests

Drug sensitivity tests are also important in studies of the epidemiology of resistance and in studies of new antimicrobial agents. Mueller-Hinton agar media (4 mm thickness plate) is considered best because:

i. Acceptable batch to batch reproducibility for susceptibility testing
ii. It is low in sulfonamide, trimethoprim and tetracycline inhibitors
iii. It gives satisfactory growth of most non-fastidious pathogens
iv. A large body of data and experience has been collected concerning susceptibility tests performed with the medium.

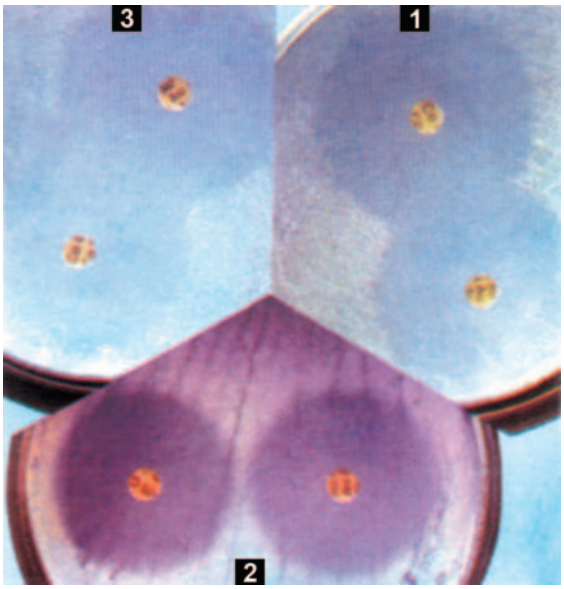


Fig. 10.1: Mueller Hinton agar. Clear zones of inhibition for combination of Trimethoprim and sulpha. 1. *Escherichia coli*; 2. *Staphylococcus aureus*; 3. *Streptococcus faecalis*

This medium should contain as low as possible thymidine or thymine (reverse the inhibitory effect of sulfonamide and trimethoprim) (Fig. 10.1).

Zones of inhibition are measured to the nearest whole millimeter using sliding calipers, ruler or template prepared for this purpose which is held on the back of inverted petri plate.

These are applied to determine the susceptibility of pathogenic bacteria to antibiotics to be used in treatment. Antibiotic sensitivity tests are very useful for clinician and hence constitute important routine procedure in diagnostic bacteriology.

Mainly sensitivity test are of three types:

1. *Diffusion tests:* The principle of it is to allow the drug to diffuse through a solid medium, concentration of drug being highest near the site of application of drug and decreasing with distance.

There are many methods for implementation of this diffusion test. The most common, simple and easy method is to use filter paper discs impregnated with antibiotics (disc diffusion method). Here filter paper discs 6 mm in diameter are charged with required concentration of drugs and are stored dry in the cold. Inoculation of pure bacterial growth in liquid medium, may be done by spreading with swabs on solid medium. After drying the plate at 37°C for ½ hour antibiotic discs are applied with sterilized forceps. After overnight incubation at 37°C, zone of inhibition of growth around each antibiotic disc is noted. Inhibition zone shows degree of sensitivity of antibiotic for that particular bacteria. The results are reported as sensitive or resistant.

Disc diffusion test is done only after the pathogenic bacteria are isolated from clinical specimen in pure form. Sensitivity tests should be done only with pathogenic bacteria and not with commensals. Further, nitrofurantin need to be tested only against urinary pathogens. Sensitivity tests on methanamine mandelate are not relevant as the drug is active only *in vivo*.

In case we require the drug sensitivity test sooner, clinical material is directly inoculated uniformly on the surface of solid media plate and discs are applied. This is done only in emergency and results are subsequently verified by testing the pure isolates.

2. *Dilution tests:* These are quite laborious for routine use. However, these are useful where therapeutic dose is to be regulated accurately, e.g. in treatment of bacterial endocarditis and to find out small degree of resistance in slow growing bacteria like tubercle bacilli. In dilution test, serial dilutions of drug are prepared and are inoculated with test bacterium. It may be done by tube dilution or agar dilution methods.
3. *E. Test:* It is known as epsilometer test. Here antibiotic with known gradient of concentration in length on absorbent strip may be used. Now above mentioned strip is kept on the petridish containing nutrient agar seeded with test microorganism. The antibiotic diffuses into nutrient agar medium. The minimum inhibitory concentration (MIC) is noted by recording the lowest concentration of the gradient which, in fact, inhibits growth of microorganisms.

Antibiotic Assays in Body Fluids

It is done by making serial dilution of specimen of body fluid and inoculating standard

suspensions of bacteria of known minimum inhibitory concentration (MIC). Lowest concentration of drug that inhibits bacterial growth is called minimum inhibitory concentration.

It is useful in verifying adequate drug concentration in blood and other body fluids. It also guards us against excessive blood levels of toxic drugs.

Drug Resistance

During treatment with drugs, bacteria may acquire resistance to them. Following are the various mechanisms of drugs resistance:

1. *Mutation:* All bacteria contain drug resistant mutants arising spontaneously once in 10^7 to 10^{10} cell divisions. It is again of two types.
   (a) Stepwise mutation in which series of small step mutations result in high levels of resistance, e.g. penicillin, chloramphenicol, tetracycline, sulfonamides, etc. However, this type of resistance can be prevented by using adequate dosage of drugs.
   (b) One step mutation in which case resistance develops suddenly even with first exposure of drug. This type of resistance is seen in tubercle bacilli developing resistance to streptomycin and isoniazid.
2. Resistance transfer by transformation may be demonstrated experimentally but its role in nature is not known.
3. Drug resistance by transduction is very commonly found in staphylococci. Penicillinase plasmid carrying determinant for resistance to mercuric chloride and erythromycin may be transmitted by transduction.
4. Drug resistance may be mediated by R factor. This is a very important mode of transferable drug resistance. There is evidence that many time R factor may lead to enhanced virulence. By this way there is simultaneous transfer of resistance to number of drugs, e.g. multiple resistance to chloramphenicol, streptomycin, tetracycline and sulfonamides, etc. Multiple drug resistance was initially seen in bacteria causing diarrhea, typhoid, urinary tract infection and so on.
5. Biochemical methods of drug resistance are:
   a. Increased destruction of the drug by bacterial products, e.g. penicillinase produced by penicillin resistance bacteria.
   b. Decreased permeability of bacterial cell to the drug.
   c. Increased formation of metabolites with which drug competes for an enzyme, e.g. increased PABA synthesis by sulfonamide resistance strains.
   d. Increased synthesis of inhibited enzymes or formation of resistant enzyme.
   e. Development of alternate metabolic pathway.

Antimicrobial chemoprophylaxis: It may be of immense help to prevent the infection. Soon after the entry and establishment of microorganism but before the development of symptoms antimicrobial chemoprophylaxis is useful, e.g. in compound fracture. Other situations where antimicrobial chemoprophylaxis is required are:

Prophylaxis in persons with susceptibility to infection:

It may be so in anatomic or functional abnormalities thus predisposing to infections. Examples are:

a. Heart diseases like valve abnormalities or with prosthetic heart valves may invite the implantation of microorganisms already in blood circulation leading to possibly bacterial endocarditis. It can be prevented if proper drug is administered during the period of bacteremia, e.g. penicillin, erythromycin for streptococcal and penicillin, gentamicin in case of enterococcal organism.
b. In respiratory tract diseases the patient may have functional or anatomic abnormalities of respiratory tract, e.g. emphysema, bronchiectasis. The most common organisms involved are pneumococci, *Haemophilus influenzae,* pseudomonas (in hospital), *staphylococcus*, proteus, etc. Chemoprophylaxis consists of administering

ampicillin, tetracycline, trimethoprim-sulfamethoxazole, etc.

c. Recurrent urinary tract infections may occur in certain females. Chemoprophylaxis comprises of daily oral intake of nitrofurantoin (200 mg) or trimethoprim (40 mg) sulfamethoxazole (200 mg). It remains effective for many months or perhaps many years till resistant microorganisms appear. Certain women are susceptible to cystitis following sexual intercourse. The ingestion of single dose of cephalexin 250 mg, nitrofurantoin 200 mg can prevent it.

Chemoprophylaxis is effective in opportunistic infection in granulocytopenia and also certain surgical procedures.

Part II

Bacterial Genetics

11 Bacterial Genetics

Genetics is the study of heredity and variation. The hereditary characters are maintained under the influence of bacterial genes. Genes correspond to specific segment of deoxyribonucleic acid (DNA) molecules of bacterial nucleus. Each bacterium contains about 1000 genes which are located in its circular chromosome. If the book of information contained is genome, paragraph is a loci, the sentences are genes, words are codons and letters nucleotides. Each specific enzyme is determined by the action of a different specific gene. Mutation of a bacterium, however, depends on the activity of modified gene.

LANDMARKS IN GENETICS

1865 Gregor Mendel, an Australian monk reported experiments in plant hybridization and inheritance in garden peas.

1927 Hermann J Müller induced mutations in the fruit fly with X-rays.

1944 DNA proved to be the substance of heredity.

1945 George W Beadle and Edward L Tatum proved that the function of most genes is to direct the synthesis of enzymes.

1953 James D Watson and FHC Crick reported a double helix structure of DNA, compatible for self duplication.

1958 J Herbert Taylor showed how DNA molecules are arranged in the chromosomes, and how they make replicas of themselves.

1961 Francois Jacob and Jacques Monod discovered the mechanisms by which hereditary information is transferred from genes to the site of protein synthesis. Their work resulted in the discovery of the genetic code by which DNA is translated into proteins.

1962 FHC Crick showed how the order of base in nucleic acid determines the order of amino acid in a protein, i.e. genetic code. He proved that each amino acid is specified by a triplet base, and that triplet should be read in simple sequence.

1966 John Cairns discovered that bacterial chromosome is a single, very long DNA molecule and also explained how it is duplicated.

1966 FHC Crick showed how protein synthesis takes place in a cell.

1967 Charles Yancesky demonstrated a linear arrangement of genes.

1968 MW Niremberg and JH Mathei studied the mechanism of protein synthesis.

1973 Hargobind Khurana produced the first synthetic functional DNA.

1974 Frank H Ruddle and Raju S Kucherlapati studied the mapping of human genes and showed how they are regulated. They also worked on fusion of human somatic cells with other mammals.

1979 Charles Weissmann identified genes responsible for synthesis of interferon — an extremely potent antiviral agent.

1980 Stanley N Cohen and James A Shapiro showed transposable genetic elements. These elements bypass the rule of ordinary genetic recombination, join together with segment of DNA that are unrelated, transferring groups of genes among plasmids, virus and chromosomes in living cells.

1980 Eli Lilly and Co., began commercial production of human insulin through genetic engineering in bacterial cells.
1980 Charles Weissmann produced human interferon in bacteria.
1982 Eli Lilly and Co., released the first man-made insulin.
1983 Dr Andrew Murray and Dr Jack Szotask constructed the first working artificial chromosome.
1983 Barbara McClintoch discovered mobile genetic elements.

STRUCTURE OF DNA

Essential material of heredity is DNA which is the storehouse of all information for protein synthesis except some viruses where genetic material is RNA instead of DNA. Unit of code consists of sequences of three bases, i.e. code in triplet. Each codon specifies for single amino acid. More than one codon may exist for same amino acid. Thus, triplet AGA codes for argenine whereas AGG, CGU, CGC, CGA and CGG also code for same amino acid. Three codons (UAA, UAG, UGA) do not code for any amino acid and are called nonsense codons. They act as a mark, terminating the message for the synthesis of polypeptides. The transfer of genetic information from DNA to RNA is called transcription and from RNA to DNA (Protein synthesis) is called translation.

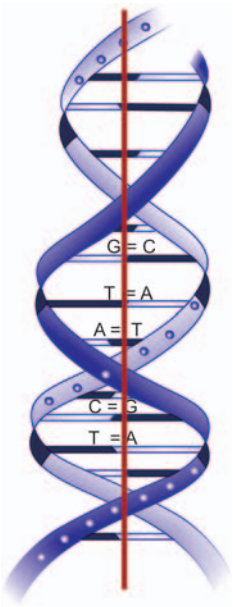


Fig. 11.1: DNA double helix

The genes are made of DNA. The structural components of a DNA molecule (Fig. 11.1) are arranged in two chains of nucleotides spiralled round a common axis. The chains are interconnected with pairs of bases at regular interval like the crossbars of a ladder. The sequence of their bases in DNA constitutes the genetic code. DNA molecules are chains of nucleotide. The four nucleotides present in DNA contain nitrogenous bases, purines (adenine, guanine) and pyrimidine (cytosine, thymine). There is a backbone of alternating deoxyribose and phosphate while purine and pyrimidine are attached to sugar. Two DNA strands are bound together to form double helix (Fig. 11.2).

Double stranded nature of the molecule is stabilized by hydrogen bonding between the bases on the opposite strands. They are held in such a manner that adenine is always linked to thymine forming a complementary base pair as do guanine and cytosine. There is equal amount of adenine and thymine in DNA and so also of guanine and cytosine. Ratio of each pair A+T/G+C varies widely from one bacterial species to another. However, this ratio is fixed for any given species. The DNA molecule replicates by first unwinding at one end to form a fork. Each strand of the fork acts as template for the synthesis of complementary strand with which it forms double helix.

RNA is structurally similar to DNA except for two major differences:

1. It contains the sugar ribose (deoxyribose in DNA).
2. One of pyrimidine base is uracil instead of thymine in DNA. There are 3 distinct types of RNA on the basis of structure and function.
   i. Messenger RNA (mRNA).
   ii. Ribosomal RNA (rRNA).
   iii. Transfer RNA (tRNA).

DNA acts as the template for the synthesis of mRNA. Adenine, guanine, cytosine and uracil in RNA will be respectively complementary to thymine, cytosine, guanine and adenine in the DNA.

Chromosome Function

Chromosome is functionally subdivided into segments, each of which determines the amino

Fig. 11.2: Diagram of a double stranded DNA

acid sequence and hence structure of a discrete protein. These proteins, as enzymes and as components of membrane and other cell structure, determine all the properties of the organism. A segment of chromosomal DNA that determines the structure of discrete protein is called a gene. The mechanism by which the sequence of nucleotides in a gene determines the sequence of amino acids on a protein is as follows:

1. An RNA polymerase forms a single polyribonucleotide strand called "messenger RNA" (mRNA), using DNA as a template; this process is called transcription. The mRNA has a nucleotide sequence complementary to one of the strands in DNA double helix.
2. Amino acids are enzymatically activated and transferred to specific adapter molecule of RNA, called "transfer RNA" (tRNA). Each adapter molecule has at one end a triplet of bases complementary to a triplet of bases on mRNA, and at the other end its specific amino acid. The triplet of bases on mRNA is called codon for that amino acid.
3. mRNA and tRNA come together on the surface of the ribosome. As each tRNA finds its complementary nucleotide triplet on mRNA, the amino acid that it carries is put into peptide linkage with the amino acid of proceeding (neighboring) tRNA molecule. The ribosome moves along the mRNA the polypeptide growing sequentially until the entire mRNA molecule has been translated into a corresponding sequence of amino acids. This process is called translation.

Thus, the nucleotide sequence of the DNA gene represents a code that determines through the mediation of mRNA, the structure of a specific protein.

Extrachromosomal genetic elements: These may be plasmid or episome.

PLASMID

It consists of DNA situated in the cytoplasm in the free state and reproducing independently. Plasmids may be conjugative, (e.g. R, F or bacteriocinogen plasmids) and nonconjugative (determinants).

Features of Bacterial Plasmids

- They are double stranded, circular DNA exist autonomously within a cell.
- Contain genes for self replication.
- Plasmids are lost spontaneously or by curing agents.
- Two members of same group of plasmids cannot coexist in the same cells.
- Some plasmids are self transferable.
- Some plasmids can integrate with host chromosome (episome).
- Self transferable plasmid can mobilize chromosomal gene or other plasmid by integration.
- They are not essential for cell survival.

Molecular Classes of Plasmid

Small Plasmids

- Nonconjugative.
- 1 to 10 megadalton.
- Multiple copies.
- Less than 0.5 percent of the size host chromosome.
- Nontransferable.
- Encodes 1 to 15 average size proteins.

Large Plasmids

- Usually conjugative.
- 25 to 300 megadalton.
- 1 or few copies.
- 1 to 10 percent of the size of host chromosome.
- Encode 50 to 500 average size proteins
- Self transferable.

EPISOMES

Genetic elements exist either autonomously in the cytoplasm or in the integrated state attached to the bacterial chromosomes.

However, plasmids and episomes are not essential for the life and function of the bacterium. They confer properties like toxigenecity and drug resistance.

A number of properties of bacteria are attributed to extrachromosomal DNA.

1. *Fertility factor (F):* The presence of F factor is determined by the fact that cell becomes genetic F^+ and synthesizes sex pilus.
2. *Colicinogenic factor (C):* It determines colicin production of *Escherichia coli.*
3. *Resistance transfer factor (RTF):* It determines drug resistance. A single factor may carry gene controlling resistance to multiple antibiotics, e.g. *Salmonella, Shigella, Escherichia coli*, etc.
4. Plasmid factor determines penicillinase production in *Staphylococcus aureus.* A number of agents are encoded by plasmid genes, including antibiotics of streptomyces, bacteriocins such as colicin and microcin (oligopeptides) produced by certain strains of *Escherichia coli.*
5. Virulence as is shown by enteropathogenic strains of *Escherichia coli* causing diarrheal illness by the liberation of enterotoxin. There are two types of enterotoxin: (i) heat labile toxin (LT) and (ii) heat stable toxin (ST) both are determined by plasmid genes.
6. *Other properties:* Plasmids are known to encode enzymes of special catabolic pathways *(Pseudomonas)*, enzymes of nitrogen fixation *(Klebsiella)* and tumorigenecity for host plants *(Agrobacterium tumefaciens).*

F, C and RTF are spontaneously transferable. Plasmid factors of penicillinase are not capable of spontaneous transfer from one cell to another. Transduction, i.e. bacteriophage may transfer it.

GENOTYPIC AND PHENOTYPIC VARIATION

Phenotypic: The manner in which these inherited traits express themselves under environmental conditions is called phenotype.

Genotypic: The total sum of determinant which are transmitted from generation is called genotype, e.g. mutation.

Phenotypic changes have following characters:

1. Changes are temporary to changed conditions of environment.
2. They are readily reversible on restoring original conditions of environment.

Some of the important properties occurring in bacteria are:

1. *Modification in morphology:* Cell from old culture may exhibit intracellular granules indicated by irregular staining and variation in size and shape. If these cells are transferred to a fresh medium, they return to normal morphology. Capsule and spore formation is again influenced by composition of medium; temperature of incubation, etc.
2. *Modification in physiological and biochemical characters:* Bacteria may exist in different physiological conditions. Activity of cell can undergo modification, e.g. young multiplying cells are more susceptible to destruction by disinfectant than old bacteria.

The other examples of environmental influences is synthesis by *Escherichia coli* of the enzyme beta galactosidase necessary for lactose fermentation. Synthesis of enzyme takes place only when it is grown in medium containing lactose.

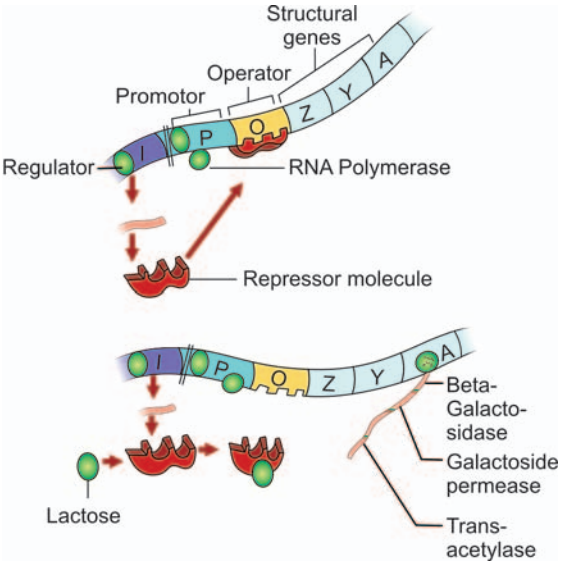


Fig. 11.3: LAC OPERON

Jacob and Monod on synthesis of beta galactosidase by *Escherichia coli* led to operon concept. It explains regulatory mechanism involving structural genes and regulatory genes. Lactose fermentation of *Escherichia coli* requires three enzymes.

a. Beta galactosidase.
b. Galactoside permease.
c. Transacetylase.

These enzymes are coded for by structural genes lac Z, lac Y, and lac A arranged linearly in sequentional unit called LAC OPERON (Fig. 11.3). The regulator gene in this case is lac I which code for repressor.

3. *Bacterial dissociation:* A number of colony variants have been described as per texture of their surface:
   1. Mucoid (M)
   2. Smooth (S)
   3. Rough (R).

Virulence is associated with smooth (S) type colony except tubercle bacilli.

Sometimes pigment producing bacteria fail to produce pigment under conditions of laboratory growth, e.g. *Serratia marcescens* produce red pigment (20° to 25°C) whereas at 37°C pigment production is nil.

Genotypic variation: It occurs by mutation and transmission of genetic material from one bacterium to another.

MUTATION

Any change in the nucleotide sequence of gene constitutes a mutation. The different forms of gene produced by mutation are called alleles.

Types of Mutation

They are broadly of 2 types:

Point Mutation

It is also called, microlesion. It is reversible and of following classes.

Base pair substitution are those mutants in which a single base pair has been substituted for another pair and is of two types as follows:

a. Transition in which there is substitution of one base pair but the purine/pyrimidine orientation is retained. Transition is the most frequent type of mutation.
b. Frame shift mutations where one or more adjacent pairs have been inserted to or deleted from DNA. This process shifts the normal translational reading frame of the coded message from point on forming an entirely new set of triplet codon.

Multisite Mutation

It is also called macrolesion. There are alterations of DNA involving large members of base pairs. It is of four types, i.e. deletion (loss), gain (addition), duplication and inversion.

Other types of mutations are:

a. Spontaneous mutation
b. Induced mutation

Effects of Mutation

1. Nonsense mutation where one or more nonsense codons (UAG, UAA, UGA) normally cause termination of polypeptide chain elongation in protein synthesis. It causes termination of synthesis of protein.
2. Missense mutation where the effect of both substitution and frame shift mutation gives rise to an altered codon. Here there may not be any apparent change in properties of protein, while in other all the protein functions may be lost.
3. Suppression mutation is a mutation that restores the function of gene inactivated by

first mutation. It may be intragenic when second mutation occurs at a site different from the first in the same gene. It is intergenic when second mutation occurs in different gene.

The change is a permanent one. Salient features of genetic mutation are:

1. It is sudden.
2. It occurs at constant rate (one mutation per 10^7 to 10^{10} divisions per cell).
3. Brings about permanent change.

Examples of common mutations are:

1. Drug resistance, e.g. sulfonamide, penicillin and streptomycin.
2. Loss of capacity to form capsule. It is usually associated with loss of virulence, antigenic type specificity and absence of mucoid colony, e.g. Pneumococcus.
3. Loss of capacity to form flagella with loss of motility, e.g. Salmonella, Proteus.
4. Attenuation of virulence.
5. Loss of sensitivity to colicin and bacteriophages.

Mutation may be spontaneous induced by mutagenic agents or silent mutation (do not express phenotypically).

Spontaneous mutation: The most common spontaneous mutation represents replication error. It is not induced by chemical mutagens or by radiations. It may arise enzymatically during DNA replication. The template mechanism of replication may function imperfectly. Frame shift mutation (small insertions and deletions) can also arise spontaneously. A mutation gene (an allele of normal gene) by virtue of its altered function may cause a general increase in the spontaneous mutation rate over the entire genome. Spontaneous mutation occurs at fairly constant rate usually in the range of once per 10^4 to once per 10^{10} cell divisions. In every infected patient, a variety of mutants arise spontaneously in the population of, say, 10^8 to 10^4 progeny.

Selection of mutants: In a patient's body or in culture, a mutant will become sufficiently numerous to be observable and to produce significant effects only if its new character makes it better fitted to grow under prevailing conditions (in culture medium or host's tissue) than parental bacteria and so enable it to outgrow and outnumber the later. Thus, mutation is significant only when conditions are selectively favorable to the mutant and bring about its natural or artificial selection. An antibiotic resistant mutant will outgrow the sensitive parental bacteria in a culture media containing antibiotics or in the body of patient receiving antibiotic therapy.

Mutagenic agents: Mutations are induced by a variety of physical and chemical agents, which are directly or indirectly the cause of general increase in mutation rate.

1. Physical agents are radiations (visible light, ultraviolet light and other ionizing radiations), heat, etc.
2. Chemical agents are of two types: (i) Acting directly, e.g. nitroso compounds, alkylating agents, base analogs, and anticancer drugs (methotrexate, dactinomycin, hydroxyurea, etc.), and (ii) The compounds requiring activation by cellular enzymes, e.g. polycyclic aromatic hydrocarbon such as benzpyrine and aflatoxins.

AMES TEST

- Useful to detect whether a substance can induce mutation or not.
- Ames test is based on hypothesis that if a substance is mutagen, it will increase the rate at which organism revert to histidine synthetizers.

Mechanism of mutation: As a result of mutation there may be alteration in colonial morphology or pigmentation, variation in cell surface antigens or insensitivity to bacteriophage or bacteriocin loss or the ability to produce capsule, spores or flagella or to utilize specific carbohydrate and changes in virulence towards particular hosts. In some cases mechanism is understood, e.g. in pneumococci a mutation leads to failure to produce a capsule. Loss of the capsule in this organism is directly related to failure to synthesize type-specific capsular antigen, alteration of normal smooth colony to a rough form and loss of virulence. Thus, single mutation produces series of biological phenomena. The mechanism of biosynthesis of pneumococcal capsular

polysaccharide is known in some detail. It is possible to ascribe this mutation to the loss of specific enzyme involved in polysaccharide synthesis. Some organisms have been subcultured in the laboratory for many generations until they have lost there virulence for man, e.g. in the production of live attenuated vaccines. Such strains have lost their virulence by a series of mutations.

To prove that mutation is responsible for variation, it is required that mutant bacteria are present in the population before exposure to selecting agent. Following tests prove the mutation:

1. *Fluctuation test:* Small inocula of sensitive bacteria are inoculated into 100 identical tubes containing media. After proper inoculation each tube is plated on medium containing phage. After required incubation number of colonies is determined. In case of any mutation before exposure to phage there is wide fluctuation in number of resistant variant in these tubes. However, control with multiple sample plated from flask would have much smaller fluctuations.
2. *Replica plating method (Fig. 11.4):* Streptomycin sensitive bacteria are plated out on streptomycin-free solid medium. After getting growth flat end of cylindrical block is pressed lightly on this plate. This charged cylindrical block is then pressed on two plates containing solid media with streptomycin. A few colonies of streptomycin resistant bacteria appear on each plate usually in coincident position. Bacteria collected from corresponding positions of the master plate with no streptomycin yield much larger portion of resistant colonies as compared to bacteria grown from other portions of the plate.
3. *Spreading experiment:* Sensitive bacteria from broth culture are plated out on nutrient agar plates. After proper incubation, half surface of the plate is rubbed with a spreader thus resulting in redistribution of bacteria. The other half of the plate is left undisturbed. Now this plate as whole is sprayed with phage suspension. The number of phage resistant colonies respread on half is more than undisturbed half.

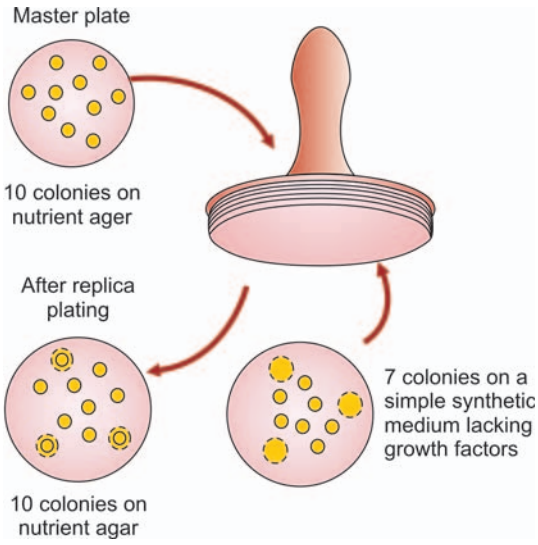


Fig. 11.4: Replica plating method

TRANSMISSION OF GENETIC MATERIAL

Genetic material transmission occurs by the following ways:

Transformation

It is the transfer of genetic information through the agency of free DNA. This process, originally discovered in Pneumococcus has now been also observed in Hemophilus, Neisseria and Bacillus. Pieces of DNA involved in transformation may carry 10 to 50 genes. The frequency and significance of transformation in bacteria is not known.

Pneumococci are capsulated bacteria and can be divided into large number of types according to the chemical composition of their capsule. Descendents of type 1 Pneumococcus would always have type 1 capsule unless they lose ability to form capsule at all. The possibility of type transformation was demonstrated by Griffith in 1928 and its mechanism elucidated by Avery, McLeod and McCarty in 1944 that transforming principle was actually DNA. Griffith injected living type II (R) bacteria mixed with large number of killed type III (S) into mice. Many of the mice died and from heart blood he obtained pure live type III (S) bacteria. Something must have passed from dead type III bacteria and change type II (R) strain into virulent type III (S) which killed mice. This process is called transformation.

There are three mechanisms of transformation:

(i) In Gram positive bacteria like pneumococci the invading DNA fragment is cut to 7 to 10 kb by endonuclease of membrane and only one strand enters the cell.

(ii) In Gram negative bacteria like Hemophilus, a membrane protein binds a sequence of about 10 nucleotides and DNA enters as double strand inside the cell.

(iii) In case of enteric group of bacteria, transformation takes place after modification of cell envelope by conversion to spheroplasts or otherwise. The modified cell surface permits taking up double stranded DNA fragments.

Transduction

It is the transfer of a portion of DNA from one bacterium to other by bacteriophages (viruses that parasitize in bacteria). Transduction is not confined to transfer of chromosomal DNA. Episomes and plasmids may also be transduced. The plasmid determining penicillin resistance in Staphylococcus is transferred from cell-to-cell by transduction.

Transduction may be generalized when it involves any segment of donor DNA. It may be restricted when specific bacteriophage transduces only a particular segment of DNA.

Several properties may be transduced.

a. Antigenic.
b. Nutritional requirements, drug resistances between Salmonella, Shigella, *Escherichia coli* and *Staphylococcus pyogenes aureus.*

Lysogenic Conversion

Some strain may get infected with temperate bacteriophage. This phage confers new properties to bacterial host. These properties are retained so long as the bacteria remain infected with phages, e.g. nonvirulent strain of *C. diphtheriae* may acquire toxigenecity (capacity to produce exotoxin responsible for virulence) by infection and lysogenization with phage derived from virulent strain. This process by which prophage DNA confers genetic information to bacterium is called lysogenic conversion.

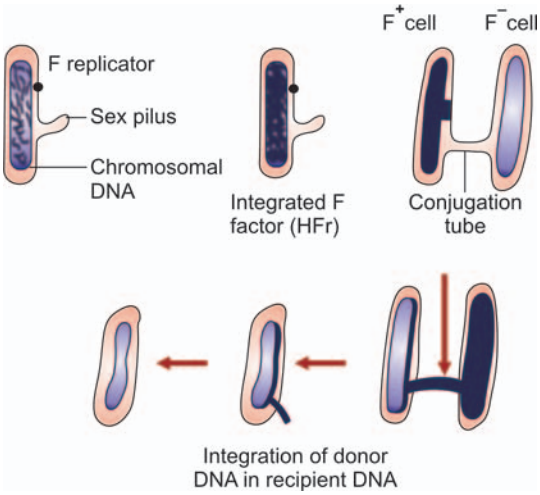


Fig. 11.5: Conjugation

Conjugation (Fig. 11.5)

It is the process by which a male or donor bacterium makes contacts with female or recipient bacterium and transfers genetic elements into it. The capacity of a strain to act as a donor is determined by sex or fertility factor F in the cytoplasm. Such cells are called (F^+) or male. The strain lacking this factor acts as recipient and this is known as female.

Before conjugation F factor migrates from cytoplasm to chromosome and becomes integrated with it. Such male cells are called (HFr). When in contact with F^+ cell genetic material passes to F^- cell through conjugate tube and F^- is converted to F^+ due to transfer of F^+ factor. This phenomena is seen in *Escherichia coli,* Shigella, Salmonella, Pseudomonas and *Vibrio cholerae.* F factor however confers certain properties such as:

1. Cell produces a specific surface antigen. Such cells tend to adhere to F^- cells.
2. It controls production of sex pilus.
3. It mobilizes F^+ chromosomes for transfer.

Protoplast Fusion

Survival of protoplast may occur in osmotically buffered environment. This facilitates protoplast fusion by uniting together cell membrane and generation of cytoplasmic bridges through which genetic material is exchanged. Fusion may occur amongst members of various kingdoms as well as unrelated cells. The gene

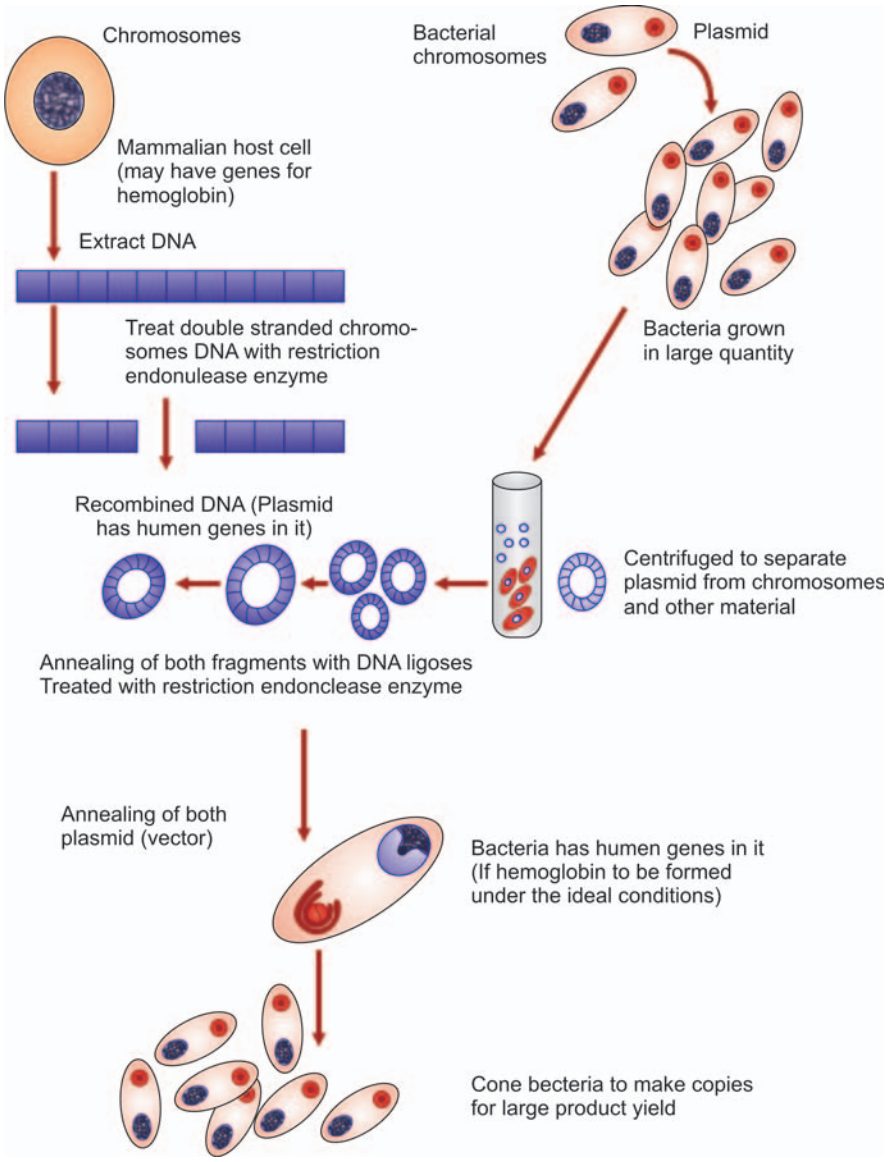


Fig. 11.6: Insertion of human hemoglobin genes into restriction enzyme treated plasmid (Vector). The vectors are used to infect bacteria, and bacteria are grown in large quantities. Vectors are purified and genes are removed. Large amounts of plasmid genes for hemoglobin production can thus be produced

transfer by this method is also named as genetic transfusion.

APPLIED BACTERIAL GENETICS

Genetic Engineering (Fig. 11.6)

New genetic material, i.e. DNA can be synthesized artifically *in vitro:* This man made gene when introduced to certain bacteria, there was alteration in genetic characters as effectively as though genetic mutation, transformation or transduction had occurred. This manipulation may cause possible biohazards. Now it is possible to insert small bits of DNA from say human cancerous tissues or certain other detrimental genes into other microorganisms, such as *Escherichia coli*. These re-engineered bacteria could perhaps enter into the intestine of laboratory workers causing great harm and also capable of spreading to other humans.

Genetic engineering may help us to overcome in borne errors of metabolism structural and metabolic defects, diabetes, arthretic diseases, conditions which may arise with aging, etc.

Techniques Established in Genetic Engineering

To extracted DNA (bacterial or human) is added restriction enzymes isolated from various bacteria, just to isolate specific genes. They cleave the polynucleotide chain of the DNA after a specific sequence of bases. Enzyme Hind III breaks the chain in front of adenine in adenine-guanine-cytosine-thymine (AGCT) sequence, otherwise ECO R_1 breaks it before AATT and Hae III breaks GGCC between guanine and cytosine. With the right enzyme, small pieces (gene size) are produced. Hydrogen bonds hold the broken pieces of DNA together at low temperature as if they had sticky end.

To prepare new gene combinations, DNA from another source (bacterial plasmids), is divided by the same restriction enzymes and mixed with the other DNA pieces. The temperature is raised. Thus, making it possible for the sticky ends to open up and receive chains of the other genetic material DNA. A ligase is used to join the DNA ends covalently and permanently.

Gene transfer from one organism to another is achieved by having bacteria taking plasmid with foreign DNA inserted in their polynucleotide structures. Cells that take up a plasmid may later be recognized by the original properties inherent in this plasmid, such as resistance to a specific antibiotic. In addition to it, the plasmid induced on the host bacteria, the properties of foreign genes that were inserted in the plasmid. These genes can be human or of any other origin.

Vectors for genetic engineering are plasmid and bacteriophages. At present bacteria are more receptive hosts.

A number of proteins are now being made by gene cloning in bacteria, e.g. somatostatin, growth hormone, insulin and interferon.

The methylotrophic bacteria produce protein from methanol and ammonia. Genetic engineering may also be useful in the production of vaccines.

PROBE

It is a labelled single stranded DNA fragment (20 to 25 nucleotide long) which will hybridize with, and thereby detect and locate (by autoradiography), complementary sequences among DNA fragment on a Southern blot. Southern blot is a technique of transferring DNA fragments, which have been separated by electrophoresis on an agar gel, to a nitrocellulose filter where they can hybridize with probe (fusion of 2 single stranded DNA by complementary base pairing).

As a diagnostic tool, gene specific probe is cloned and a labelled DNA sequence complementary to at least part of the gene responsible for particular disease.

Diagnostic DNA probes available commercially.

(a) Bacterial
- *Legionella pneumophila*
- *Mycoplasma pneumoniae*
- *Campylobacter jejuni*
- *Helicobacter pylori*
- *Mycobacterium tuberculosis*
- *Mycobacterium avium intracellulare*
- *Escherichia coli* (ST and LT).

(b) Viral
- Herpes simplex virus (1, 2 types)
- Hepatitis-B
- Rotavirus-A
- HIV-1
- HIV-2.

(c) Protozoal
- *Plasmodium falciparum.*

Application of Nucleic Acid Probes

- Detection of organism which are difficult or not cultured.
- Detection of organisms which do not have diagnostic antigen
- Detection of latent virus infection
- Identification of antibiotic resistance genes
- Development of epidemiological markers
- Rapid confirmation of cultured organisms
- Differentiation of nonpathogenic strains from pathogenic strains.

Polymerase Chain Reactions (PCR)

It is an *in vitro* method for producing large amounts of specific DNA fragment of defined length and sequences from small amount of complex template. It involves:

a. Melting of DNA to convert double stranded DNA to single stranded DNA.

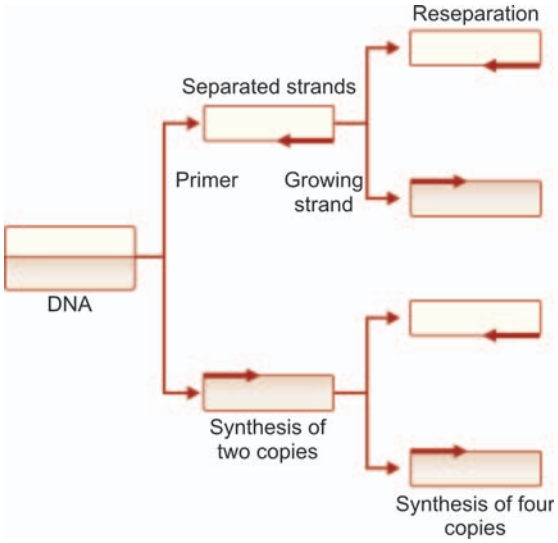


Fig. 11.7: PCR

b. Annealing of primers to target DNA.
c. Synthesis of DNA by addition of nucleotide from primers by action of polymerase.

RNA polymerase chain reactions (RNA PCR) is a modification of PCR technique which allows amplication beginning from RNA template. A complementary copy (cDNA) of desired RNA target is made by reverse transcriptase and this is followed by routine PCR which amplifies the cDNA.

PCR technique has been found useful in demonstrating number of microorganisms from clinical specimens (Fig. 11.7).

Blotting Technique

DNA fragments obtained by restriction enzyme digestion and separation on gel can be transferred from the gel by blotting to nitrocellulose or nylon membranes that bind the DNA. The DNA bound to the membrane is denatured (converted to single stranded form) and treated with radioactive single stranded DNA probes. These will hybridize with homologus DNA to form radioactive double stranded segments which can be detected on X-ray film. This technique for identifying DNA fragments by DNA: DNA hybridization is called Southern blotting. On other hand analysis of RNA is called Northern blotting.

Immunoblotting

This technique is useful for the identification of proteins. For the protein antigen mixture is separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) blotted into nitrocellular strips and identified by radiolabelled or enzyme labelled antibodies on probes.

Dot hybridization test: It is recently proved to be highly sensitive and specific. It may be used to detect viral DNA sequences in tissue samples not only from patients with acute infection but also from the patients with chronic diseases where isolation of microbes is difficult (chronic hepatitis B, hepatocellular carcinoma, Burkitt lymphoma, AIDS).

DNA of microbe in the specimen is spotted on nitrocellulose membrane, where it is bound and denatured with alkali. Then dot is hybridized with radioactive labelled DNA fragment from which DNA probes are prepared. The dot is autoradiographed next day.

In case of say rotavirus, RNA in heated denatured stool samples containing rotavirus is immobilized as described above and *in situ* hybridization is carried out with radiolabelled single stranded probes obtained *in vitro* transcription of rotavirus. However, complementary DNA probes to rotavirus and to enteroviruses may be labelled and used in dot hybridization test.

Gene Therapy

In gene therapy, the aim is to cure the disease by inserting normal genes into the body cells to restore normal function. Gene therapy was first experimented in two young girls who were suffering from severe combined immunodeficiency (SCID). Today, these young girls lead a normal life and their laboratory investigations clearly indicate that their immune systems improved considerably becasue of gene therapy which was performed over 2 decades ago. Hectic research projects are in progress to find out the possibility of role of gene therapy in other conditions like cancer, familial hypercholesterolemia, cystic fibrosis, hypertension

congenital heart diseases, NIDDM, muscular dystrophy, rheumatoid arthritis, etc.

Techniques

To introduce new genetic material into mammalian cells, following strategies are followed:

a. *Germline gene therapy:* In the germline model foreign DNA is introduced into the zygote or embryo. There are fair chance that newly introduced material will be passed on to next generation. Germline gene therapy has been developed while working on mice. In mice only 15 to 20 percent of injected eggs produce transgenic mice and of these only 20 to 32 percent actually express the introduced gene. Further, there is quite high risks including damage to resident gene by the random insertion of DNA.
b. *Somatic gene therapy:* Here normal gene is spliced into disabled cold viruses which are incapable of causing any infection, but are ideal delivery vehicles. This gene is aerosolized for delivery into the nasal passages. In short somatic gene therapy is method of introducing the genetic material which vary depending on disease being treated.

Some of the other techniques are as under:

1. Tumor infiltrating lymphocytes (TIL) plus interleukin mediate substantial tumor regression in half of the patients with advanced malignant melanoma and can survive for several months in the bloodstream and tumor sites. This method is quite safe and feasible.
2. Direct manipulation of oncogenes or tumor suppressor genes.
3. Insertion of genes is not tumors which code for proteins such as interleukins. This boost the immune system and thus make cells that normally attack the tumor that is more potent.
4. Suicide gene therapy which involves inserting a gene that makes plain tumors more susceptible to ganciclover (antiviral agent).

Future Prospects

The future possibilities for genetic intervention in cancer in the direct manipulation of oncogenes or tumor suppressor genes carry hopes. Today genetic intervention is being considered as an effective systematic treatment along with medical and surgical measures.

Unfortunately gene therapy carries certain risks and hazards, e.g. it may be potential hazard for patients plus staff and environment too. Many of the queries are still to be answered like safety, long-term efficacy and effective dosage required, etc.

Nobel Prize in Medicine—1993

Richard J Robert from UK, who works at New England Biolabs in Beverly, Mass. and Philip A Sharp from USA, who works at Massachusetts Institute of Technology Center for Cancer Research, Cambridge, Mass., won the Nobel Prize of 1993 for their discovery of SPLIT GENES. This has helped a lot in the study of cancer and other diseases.

Mr Robert and Mr Sharp independently discovered in 1977 that genes could be discontinuous and that a gene could be present in well-separated DNA segment. Before this discovery, scientists were of the view that a gene was a continuous segment within the very long double stranded DNA molecule.

The new concept of Mr Robert and Mr Sharp has changed the view on how genes in higher organisms develop during evolution. Their discovery led to the prediction of a new genetic process, splicing, the assembly of information from the gene segments, which carries much importance for expressing genetic information.

Mr Robert and Mr Sharp were working at the Cold-Spring Harbour Laboratory on Long Island, New York. There they found that an individual gene can comprise not only one but several DNA segments, separated by irrelevant DNA. Such discontinuous genes exist in organisms more complex than those studied earlier.

They studied adenovirus and tried to find where in the genome of this virus different genes were located. They found that the genetic information in the gene was discontinuously organized.

This discovery was followed by intensive research to find out whether this gene structure is present also in other viruses and in ordinary cells. It was revealed that a discontinuous (or

split) gene structure was in fact the common gene structure in higher organisms too.

The discovery of split genes has been of fundamental importance for today's basic research work in biology as well as for more medically oriented research concerning the development of cancer and other diseases. This ingeneous work carries the application also to research on hereditary diseases in which there are errors in splicing. Incidentally, the most studied of such diseases is beta thalassemia.

Nobel Prize in Medicine—2006

Nobel Prize in Medicine—2006 winner, Andrew Fire and Craig Mello discovered a mechanism that turns off or silence, the effect of certain genes thus introducing potential new opportunities for fighting diseases as varied as cancer, heart disease, HIV and hepatitis.

The process, called RNA interference (RNAI), is actually an ancient old biological mechanism developed millions of years ago by plants and animals as a defence against viral infections. When genes discover virally produced DS RNA mimicking their own naturally occurring ones, they recognize them as "outsider" and immediately shut both sets down. The procedure prevents the gene from producing protein, effectively silencing it. Meanings, if there was pre-existing defective gene overproducing blood vessels in the eye as often happens in age related blindness, it could be stopped from doing so by artificially introducing identical DS RNAs. Researchers in at least two pharmaceutical companies in the US are already working in perfecting the technique. Others elsewhere are working on using RNAI to kill HIV that causes AIDS, to block a gene involved in cholesterol metabolism, against H5N1 (avian influenza) and to fight various respiratory diseases. RNAI could also turn out to be incredible powerful research tool of the future. It can be used to turn-off or turn down a gene at will. It can ultimately lead to a complete understanding of the human genetic codes. This Nobel discovery can zap faulty genes by switching them off.

Part III

Immunology

12 Immunity

Immunology is the study of specific resistance to further infection by a particular micro-organism or its products. Immunology is the science which deals with the body's response to antigenic challenge. It is a very broad scientific discipline whose relevance to most fields of medicine has become apparent in recent years. Immunological mechanisms are involved in the protection of the body against infectious agents but periodically they can also cause damage. Immunological tests are now routinely used in clinical practice, and in some cases they are indispensable for diagnosis of disease and subsequent care of patients. The introduction of a new technology, developed only a few years ago and called hybridoma technology, is revolutionizing immunology. This technique permits the production of antibodies against single antigenic determinants (epitopes). By this technique it is now possible to obtain unlimited amounts of very homogeneous and specific antibodies. Already we can use such antibodies for diagnosis and possibly in the future they will be used to treat the patient. Currently immunology concerns itself with all reactions, processes regardless whether they are less or more vigorous than original. These reactions may be harmful or protective and in some cases they may be both at the same time. In short immunology has broad biological role involving concept of recognition, specificity and memory. In this section on immunology discussion is restricted to the most relevant topics in the field of medicine.

HISTORICAL EVENTS IN IMMUNOLOGY

Practice of variolation was in progress in India and China from time immemorial. Here protection against smallpox was obtained by inoculating live organisms from the disease pustule. Jenner (1878) used nonvirulent cowpox vaccine against smallpox infection. Later, Pasteur tried a vaccine successfully using attenuated organisms against anthrax.

Metchnikoff (1883) suggested the role of phagocytes in immunity. Von Behring (1890) recognized antibodies in serum against diphtheria toxin. Denys and Leclef (1895) suggested that phagocytosis is enhanced by immunization. Ehrlich (1897) put forward side chain receptor theory of antibody synthesis. Bordet (1899) found that lysis of cells by antibody requires cooperation of serum factor now collectively known as complement.

Landsteiner (1900) declared human ABO groups and natural isohemagglutinin. Richest and Portier (1902) proposed the term anaphylaxis which is opposite of prophylaxis. Wright (1903) put forward opsonic activity to phagocytosis. Von Pirquet and Schick (1905) described serum sickness after injection of foreign serum. Von Pirquet (1906) correlated immunity and hypersensitivity. Fleming (1922) found lysozyme. Zinsser (1925) suggested the contrast between immediate and delayed type of hypersensitivity. Heidelberger and Kendall (1930-35) put forward quantitative precipitation studies on antigen-antibody reaction.

Coons (1942) introduced fluorescence antibody techniques. Medwar (1958) discovered acquired immunologic tolerance. Portar and Edelman (1972) determined structure of immunoglobulin. Rosalyn Yalow introduced radioimmunoassay in 1977 and Georges Koehler *et al* reported monoclonal antibody (1984).

APPLICATION OF IMMUNOLOGY

1. It helps us to understand etiology and pathogenesis of diseases, e.g. rheumatic fever, asthma, acute glomerulonephritis, etc
2. Diagnosis of disease is possible using ELISA, etc
3. Development of vaccines
4. Treatment using antibodies
5. Transplantation and blood transfusion
6. Surveillance, i.e. immune surveillance
7. It helps to find out possible future susceptibility of a person to diseases with the help of HLA typing system.

IMMUNITY

The resistance offered by the host to the harmful effect of pathogenic microbial infection is called immunity. Immunity against infectious diseases is of different types (Fig. 12.1).

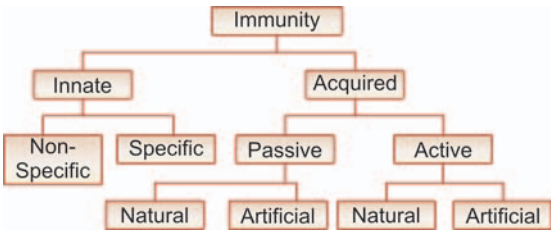


Fig. 12.1: Classification of immunity

INNATE IMMUNITY

This is basic immunity which may be genetically passed on from one generation to other generation. It does not depend on prior contacts with microorganisms. It may be nonspecific when it indicates a degree of resistance to all infection, e.g. plant pathogens, rinderpest, distemper. It is specific when it shows resistance to particular pathogens.

Innate immunity can be divided into following types:

Species immunity: Individuals of same species show uniform pattern of susceptibility to different bacterial infection. The mechanism of species immunity may be due to physiological and biochemical differences between tissue of host species which determine whether or not pathogen can multiply in them, e.g. poliomyelitis, measles, syphilis, leprosy, gonorrhea occur only in man. Many a time same species of bacteria produce different types of infection in different animals, e.g. *Salmonella typhi* produces typhoid fever in man whereas mice is resistant.

Racial immunity: Within a species different races show differences in susceptibility to infection, e.g. Negroes are resistant to yellow fever and malaria and high resistance of Algerian sheep to anthrax. Such racial differences are known to be genetic in origin principally induced by persistent environmental stimulus.

Individual immunity: Individual in population shows variation in their response to microbial infection, e.g. homozygous twins exhibit similar degree of resistance or susceptibility to lepromatous leprosy and tuberculosis. Such correlation is not seen in heterozygous twins.

Factors influencing level of innate immunity in an individual are:

1. *Age:* Two extremes of life carry high susceptibility to infectious diseases. The susceptibility of fetus to infection is related to immaturity of its immune apparatus, e.g. Coxsackie viruses cause fatal infection in sucking mice but not in adults.

   Old persons are highly susceptible to infection due to gradual waning of their immune responses.

   Many age differences in specific infections can be related to physiologic factors. Thus bacterial meningitis during first month of life is often caused by coliform bacteria because bacterial antibodies to these bacteria are IgM and thus fail to cross the placenta. Other such examples are gonococcal vaginitis in small girls, rickettsial infection more severe in advancing age, rubella infection damages the fetus severely but otherwise produces only mild disease.

   Tinea capitis caused by *Microsporum audouini* undergoes spontaneous cure with the onset of puberty. Prepubertal girls are susceptible to gonococcal infection. Some infections like poliomyelitis and chickenpox are more intense in adults than children because of hypersensitivity which may be responsible for more tissue damage.

2. *Hormonal influence:* Endocrine disorders, e.g. diabetes mellitus is related to susceptibility to infection because of increased carbohydrate levels in tissue. Corticosteroids depress host's resistance by anti-inflammatory, antiphagocytic effect and by suppression of antibody formation and hypersensitivity. The elevated steroid levels during pregnancy may have relation to increased susceptibility of pregnant women to many infections.
3. *Nutrition:* Defective nutrition depresses all types of immune response and thus increasing the risk of infection.

Mechanism of Innate Immunity

The defence mechanisms of body are related to body coverings as under:

1. *Epithelial surfaces:* The intact skin and mucous membrane covering the body confers on it considerable protection against bacteria. They provide mechanical barrier. They also provide bactericidal secretions. Their bactericidal activity is related to the presence of lactic acid, saturated and unsaturated fatty acid in sweat and sebaceous secretion. The mucous membrane of gastrointestinal tract provides protection by bactericidal enzymes secreted in lumen. The anaerobic colony bacteria produce fatty acids with antibacterial activity. The term "colonization resistance" refers to resistance offered by the predominant normal flora to infection, e.g. the intestinal anaerobic microflora prevents superinfection by coliform during antibiotic therapy. Saliva is bacteriocidal. High acidity of stomach inhibits bacterial multiplication. The normal body flora play indirect role in defence of body, e.g. intestinal flora by producing bacteriocines which are destructive to other bacteria.
2. *Tissue defenses:* If barrier of body is overcome by the organisms, a number of factors in normal tissue and body fluid, play their role. Tissue factors may be divided into:
   (a) Humoral factors.
   (b) Cellular factors.

Humoral Factors

(a) Lysozyme: It is a bacteriocidal enzyme found in nasal and intestinal secretion, seminal fluid and lacrimal secretion.

(b) Properdin: It is euglobulin present in normal serum. It causes lysis of gram negative bacteria with the help of Mg^{++} and complement. It constitutes 0.02 percent of serum protein. It is not an antibody and its level remains constant in newborn and elderly individuals in both sexes. Its molecular weight is over one million.

(c) Beta lysin: It is a relatively thermostable substance active against anthrax bacillus. It is liberated from platelets during clotting.

(d) Basic polypeptides: They are bacteriocidal substances active at high pH (7 to 8). They act upon cell wall causing cell disintegration, e.g. lukins from leukocytes and plakin from platelets.

(e) C-reactive proteins: The sera of patient with pneumococcal and other diseases give a precipitate when mixed with somatic polysaccharide C of pneumococcus in presence of calcium ions. These nonspecific substances (C-reactive protein) appear in blood of a person with tissue necrosis and inflammation. C-reactive protein does play an important role in the resolution of inflammatory process. It binds to many materials like bacterial polysaccharides and may then activate classical pathway, bringing complement components into play to potentiate bacterial killing. Recently, it is suggested that the functions of CRP is primarily to act as a binding mechanism, say for pneumococcal "C" polysaccharide which is most effectively precipitated on agglutination. The binding of such legands render them accessible to phagocytosis and as a result to clearance and metabolic breakdown. Characters of C-reactive protein are:

1. Calcium is essential for the reaction.
2. Reactive substance globulin is not detectable in normal serum. It is demonstrated in sera, pleural, peritoneal and joint fluids of patient.

The demonstration of C-reactive protein is useful in the diseases like rheumatic fever, and rheumatoid arthritis, etc.

(f) *Bactericidin:* It is non-specific serum factor active against neisseria, streptococcal hemolyticus, etc.

(g) *Complement:* It is thermolabile substance present in serum and tissue fluid. It enhances phagocytosis and kills most of gram negative bacteria sensitized by specific antibodies.

(h) *Non-specific hyaluronidase inhibitors:* In tissue damage non-specific inhibitor hyaluronidase appears in blood. It is heat labile and requires magnesium ion for its activity.

Production of antibodies against antigens of microorganisms may induce resistance because they:

1. Neutralize toxin or cellular products.
2. Have direct bacteriocidal or lytic effect with complement.
3. Block the infective ability of microorganisms.
4. Agglutinate microorganisms thus subjecting them to phagocytosis.
5. Opsonize microorganisms.

Acute Phase Proteins

Some proteins in plasma are collectively named acute phase proteins. There may be sudden increase in plasma concentration of certain proteins in response to early alarm mediator, e.g. interleukin- 1, IL- 6 and tumor necrosis factors (TNF) released as a result of infection or tissue injury. While many acute phase reactant like C. reactive proteins increase dramatically, others show more moderate increase (usually less than five folds), e.g. a proteinase inhibitors, a chymotrypsin, fibrinogen, caeruloplasmin angiotensin, mannose binding proteins, etc.

They may be involved in activating alternate pathway of complement. They may prevent tissue injury and may promote repair of inflammatory lesion.

Cellular Factors

1. *Phagocytosis:* Natural defence against invasion of blood and tissue by bacteria or others foreign particles is mediated by phagocytic cell which ingest and destroy them. The process of phagocytosis consists of:
   i. The first phase involves the approach of the phagocyte to the microbe by means of positive chemotaxis. Under the influence of the products of life activities of microbes excitation of the phagocytes occurs, which leads to a change in the surface tension of the cytoplasm and gives the phagocytes amoeboid motility.
   ii. In the second phase absorption of the microorganism on the surface of the phagocyte takes place. This process is completed under the influence of an electrolyte which alters the electrical potential of the phagocytized object (microbe).
   iii. The third phase is characterized by submergence of the microbe into the cytoplasm of the phagocyte, which seizes minute objects quite rapidly and large ones (some protozoa, actinomycetes, etc.) are engulfed in pieces.
   iv. In the fourth phase intracellular digestion of the engulfed microbes by the phagocytes takes place.

In the process of phagocytosis various changes in the microbes can be observed, e.g. the production of granules in *Vibrio cholerae*, swelling of salmonella, fragmentation of *Corynebacterium diphtheriae*, destruction of anthrax bacilli and swelling of cocci. Ultimately the phagocytosed microorganisms become completely disintegrated.

Factors which speed up phagocytosis include calcium and magnesium salts, antibodies, etc. Phagocytosis proceeds more vigorously in immunes than in non-immunes. Toxin of bacteria, leukocidin, capsular material of bacteria, cholesterol, quinine, alkaloids, and also a blockade of reticuloendothelial system inhibit phagocytosis.

Phagocytic cells may be:

a. Microphages, e.g. polymorphonuclear leukocytes.
b. Macrophages includes:
   1. Histiocytes (wandering amoeboid cells in tissue).
   2. Fixed reticuloendothelial cells.
   3. Monocytes.

2. *Inflammation:* Tissue injury or irritation initiated by entry of bacteria or of other irritant leads to inflammation. It is

important non-specific mechanism of defense. Initially constriction and then dilatation of blood vessels of affected site is followed by escape of polymorphonuclear cells into tissue. Microorganisms are phagocytosed and destroyed. Out pouring of plasma helps to dilute the toxic products. A fibrin barrier is laid, serving to wall off the site of infection.

3. *Fever:* It is a natural defence mechanism. It may actually destroy the infecting organism. Fever stimulates the production of interferon and helps in recovery from virus infections. The substances which may cause fever are endotoxins and interleukin-1.

Acute phase proteins: Because of trauma or infection there may be abrupt rise of plasma concentration of some proteins (acute phase proteins). Some of the examples of acute phase proteins are 'C' reactive proteins, mannose binding proteins, alpha-1 acid glycoproteins, etc. They may increase host resistance, help in the healing of inflammatory lesions, prevent tissue damage, activate alternative pathways ('C' reactive proteins).

Outcome of infection: Following are some possible results of infection:

1. Elimination of pathogen without any clinical lesion when immune status of host is normal.
2. Localization of pathogen with production of local lesion when there is no impairment of the patient's general health and as a rule infection is soon eradicated, e.g. small staphylococcal pustule of the skin.
3. Localization of the pathogen with production of distant lesion as happens in diphtheria and tetanus because of exotoxin.
4. Local extension of infection to surrounding tissue as in case of hyaluronidase producing *Streptococcus pyogenes*. Other examples are tuberculosis and actinomycosis.
5. General dissemination as in case of septicemia and then involves various organs of the body.

ACQUIRED IMMUNITY

The immunity acquired during the lifetime of an individual is known as acquired immunity. Acquired immunity differs from innate immunity in the following respects:

1. It is not inherent in the body but is acquired during life.
2. It is specific for a single type of microorganism.

Acquired immunity may be:

1. Active.
2. Passive.

Active immunity: It is the resistance developed by an immunity as a result of antigenic stimulus. Active immunity may be:

Natural active immunity: This is acquired after one infection or recovery from disease or subclinical infection after repeated exposure to small doses of the infecting organism.

Artificial active immunity: It may be acquired artificially by inoculation of bacteria, viruses or their products as under:

a. Living organisms: After proper attenuation, e.g. smallpox, BCG. Attenuation may be obtained as under:
   i. Subjecting the organism to drying, e.g. rabies virus vaccine.
   ii. Growing the organism at temperature higher than optimum, e.g. Pasteur's anthrax vaccine is prepared by cultivating the organism at 42°C.
   iii. By passage through animals of different species, e.g. variola virus through rabbit and calf.
   iv. By continued cultivation in presence of antagonistic substance, e.g. BCG vaccine is prepared by prolonged cultivation of tubercle bacillus (bacillus, calmette and guerin) in medium containing bile.
   v. By repeated subculture in artificial media, e.g. streptococci.
b. Organisms are killed by heat or phenol without changing the antigenic structure of bacteria, e.g. typhoid vaccine, cholera vaccine.
c. Autovaccine.
d. Non-specific protein therapy: Local cellular elements concerned with defensive mechanism respond to injection of non-specific protein substance like milk injection. They cause increased proliferation resulting in more antibody release.
e. Toxoid: Bacterial inactivated toxin (not toxigenic but retains antigenicity) is injected repeatedly in increasing doses, e.g. diphtheria and tetanus toxoid.

Passive Immunity: Here subject is immunized by prepared antibodies and body cells do not take any active part in the production of immunity. It is of the following types:

Natural passive immunity: During intrauterine life transmission of antibodies from the mother to fetus can occur through placenta. It may be by way of colostrum of mother and milk during first few months of life. Breast-fed infants resist establishment of enteroviruses in alimentary tract. These antibodies last for few weeks and protect infants from diphtheria, tetanus, measles, mumps, smallpox, etc.

Artificial passive immunity: Immunization in this case is passive and produced by injection of serum of animals that have been immunized actively. Antibodies remain in effective quantity for 10 days only. Following serum may be used:

a. *Antitoxic serum:* It is produced by injection of toxoid into horse in increasing doses till the blood is rich in circulating antibodies. The animal is bled and serum is separated. This serum contains prepared antibodies. Examples of antitoxic sera are diphtheria, tetanus, etc.
b. *Antibacterial serum:* Antibodies are produced by injection of bacteria into animals and serum is collected, e.g. pneumococcal, meningococcal, anthrax, dysentery, etc.
c. *Convalescent serum*: It is obtained from convalescent patient. It is also called convalescent serum. Such serum is used in the treatment of measles, poliomyelitis, infective hepatitis, etc.

Differences between active immunity and passive immunity are summed up as per Table 12.1.

Genetic influences: Natural and acquired immunity and predisposition towards specific disease states have a genetic component. Disease susceptibility is in some way related to genes closely associated with the major histocompatibility complex particularly the HLA-D region located on chromosome 6 in humans. This region is suspected of carrying immune response genes. An example of correlation is association of HLA-B27 with ankylosing spondylitis and juvenile arthritis in humans. The basis for this association of specific genes with specific disease susceptibility is not clear. Possible explanations are as under:

i. HLA antigens may serve as cell surface receptors for viruses or toxins.
ii. HLA antigens may be incorporated into viral coat protein.
iii. HLA antigens may not themselves be responsible but may be linked to immune response genes that do determine actual susceptibility.
iv. HLA antigens may crossreact with the antigens of bacteria, viruses or other agents to trigger autoimmune responses.

Local immunity: It has importance in infections which are either localized or where it is operative in combating infection at the site of primary entry of pathogen, e.g. influenza immunization with killed vaccine elicit humoral antibody response but antibody titer in respiratory secretion is not high enough to

TABLE 12.1: Differences between active and passive immunity

	Active immunity	*Passive immunity*
1.	Produced actively by host's immune system	Received passively by the host. No participation by host's immune system
2.	Induced by infection or by contacts with immunogen	Conferred by introduction of vaccines, e.g. readymade antibody
3.	Afford durable and effective protein	Temporary and less effective protection
4.	Immunity effective after lag phase	Immunity effective immediately
5.	Immunological memory present, i.e. subsequent challenge more effective	No immunological memory
6.	Negative phase may occur	No negative phase
7.	Not applicable to immunodeficient hosts	Application to immunodeficient hosts
8.	Used as prophylaxis to increase resistance of body	Used for treatment of acute infection
9.	Both cell mediated and humoral immunity take part	Exclusively humoral immunity is involved
10.	No inheritance of immunity	May be acquired from mother

prevent infection. Natural infection or live virus vaccine administered intranasaly provide local immunity. A special class of immunoglobulin (IgA) forms major component of local immunity.

Herd immunity: There is overall level of immunity in a community. It is relevant in the control of epidemic diseases. When herd immunity is low, epidemics are likely to occur on introduction of suitable pathogens. The term herd immunity means large proportion of individuals in a community are immune to pathogen.

VACCINATION

It is worthwhile to discuss it with the history of development of vaccine. Along with it other aspects are also taken up:

History of Development of Immunization

1721 Variolation by Lady Montague
1793 Smallpox immunization
1881 Development of Pasteur's antirabies vaccine and also demonstration of efficacy of anthrax vaccine
1891 Development of diphtheria antitoxin
1896 Vaccine against typhoid fever
1904 Production of tetanus antitoxin
1920 Diphtheria antitoxin floccules
1930 Diphtheria toxoid
1951 Yellow fever live vaccine
1955 Polio killed vaccine (SALK)
1960 Polio live vaccine (SABIN)
1970 Live vaccine of rubella and mumps. Also measles vaccine (killed as well as live).

Vaccines: Live attenuated or dead organisms when introduced into the body, artificial active immunization is produced. Killed vaccines produce relatively less immunity. The live vaccines contain major immunizing antigen and hence, more and longer antigenic stimulation resulting in prolonged immunity as compared to killed vaccine. Live vaccine is quite cheap and dose given is also small. The vaccines are classified as under (Table 12.2).

A. Live attenuated vaccines are smallpox, yellow fever, polio, BCG, plague, brucellosis, mumps, rubella and measles. Attenuation is achieved by aging of culture, culture at high temperature, passage through another host species, drying (rabies) and selection of mutant (temperature sensitive).

TABLE 12.2: Classification of vaccines available against microorganisms

	Vaccines against bacteria	*Vaccines against viruses*	*Vaccines against parasites*
Live vaccine	*Mycobacterium tuberculosis* *Vibrio cholerae* Salmonella *E.coli*	Polio Mumps Measles Rubella Vaccinia Adeno Rabies Herpes zoster Cytomegalo Influenza Yellow fever Rotavirus	
Inactivated vaccine	• *Corynebacterium diphtheriae* • Pertussis • *Clostridium tetani* • *Vibrio cholerae* Meningococcus (ACYW) • Pneumococcus • *Haemophilus influenzae* • *E. coli*	• Rabies • Polio • Influenza • Hepatitis B • Measles	• *Plasmodium falciparum* • *Trypanosomes cruzi* • *Schistosoma mansoni*

B. Killed vaccines, e.g. whooping cough, TAB, cholera, polio (SALK), antirabic and measles. For this purpose organisms are killed by heat, formalin, phenol, alcohol, ultraviolet light and photodynamic inactivation. Killed vaccines may be preserved in phenol, alcohol or merthiolate.
C. Toxoid are exotoxin which are treated with formalin to destroy their toxicity but retaining their immunogenecity, e.g. formal toxoid of diphtheria and tetanus.
D. Subunit vaccines: They consist of only relevant immunogenic material such as the capsid proteins of non-enveloped icosahedral viruses or the peplomers (glycoprotein) of enveloped proteins.

Standardization of vaccines: The purpose is to evaluate the potency of vaccine and the efficacy. In live vaccine, standardization is achieved by estimating the number of organisms in unit volume of preparation (*in vitro* viable count in case of bacterial vaccine) and tissue culture assay in viral vaccine whereas *in vivo* assessment of infectivity is determined by titration in experimental animals. Virus assay estimate is expressed in term of plaque forming unit (PFU) or tissue culture infectious dose (TCID 50). Latter one is more common.

In case of killed vaccine, potency is determined by direct challenge test or by evaluation of antibody response in laboratory animals. Similarly toxoid are evaluated by direct challenge or by determining the levels of antitoxin produced.

Hazards of vaccination: Local sepsis, serum hepatitis, fever, malaise, soreness at injection site, arthralgia after rubella vaccine, convulsions in pertussis vaccine and allergic reaction may occur as untoward side effect after vaccination.

The problem with live vaccine is its stability of attenuation. The other problem with live vaccine is drastic consequences if given to immunodeficient child. Antibodies in mother's milk may cause poor take if vaccinated infant is breastfed. Potency of vaccine may be maintained by lyophilization. The other problem is intestinal infections at subclinical levels and hence poor take of live vaccine as enteroviruses may prevent multiplication of the attenuated vaccine.

Schedule of Immunization

0 to 1 month	BCG
3 months	DPT I, oral polio vaccine I
4 to 5 months	DPT II, oral polio vaccine II
5 to 6 months	DPT III, oral polio vaccine III
1½ yrs.	Booster DPT, oral polio vaccine IV
2 yrs.	Typhoid vaccine
5 yrs.	Tetanus toxoid
School entry	Typhoid vaccine
12 to 14 yrs.	Tetanus toxoid
	Diphtheria toxoid
	Rubella vaccine

NEWER VACCINES

Nowadays, antigen identification and preparation of vaccine is achieved by recombinant DNA production of proteins and polypeptides, production of synthetic oligopeptides and epitopes by hybridoma technology, and directed mutation, selection and stabilization. Now it is possible to develop vaccines against agents that cannot even be grown.

In recombinant DNA genetic, the genes that code for immunologically important polypeptide antigens of viruses or bacteria are inserted into infectious nucleic acid vectors permitting the production of these proteins and polypeptides in new and unnatural hosts like bacterial, yeast or animal cells.

Hybridoma technology consists of the fusion of antibody producing lymphocytes with cancer cells to yield hybrid cells that can be propagated indefinitely and secrete only a single immunologic determinant a whole antigen. Monoclonal antibodies are highly specific tools for analysis of microbial antigens and allow selection of those that are of relevance to immunity. The other importance lies in their ability to define very short lengths of polypeptides that relate to specific immunity. This has opened the doors to chemical synthesis of vaccines.

Advantages of Newer Vaccines

1. Give specific immune response.
2. Rare reversion to virulence.
3. Fewer chances of contamination.

Disadvantages of Newer Vaccines

1. Limited immunogenicity of very simplified antigen.
2. Weak and inadequate immune responses.

APPLICATION OF VACCINE

- Prophylactic use for general population, e.g. BCG, polio.
- Prophylactic use for selective group of persons, e.g. Hepatitis B vaccine for doctors, nurses, infant born to HBs antigen positive mothers, etc.
- New directions in vaccine development has slowly evolved from Jenner to recombinant genetics. It has provided a possible basis for prophylactic control of essentially all the infectious diseases.

13 Antigen

Antigen is a substance which, when introduced parenterally into the body, stimulates the production of an antibody with which it reacts specifically in an observable manner. Antigenic determinant is that portion of antigen molecule that determines the specificity of antigen-antibody reaction. It is also called epitope. An epitope corresponds to an area about 6 to 10 amino acids, or of 5 to 6 doses. Antibody recognizes epitope present on the surface of native antigen in solution, which may be proteins or polysaccharides, whereas T cell receptors bind to peptide fragment processed by antigen presenting cells. In these cells, the antigen undergoes proteolytic degradation, generating peptides which are then presented in association with class I or class II proteins of the major histocompatibility complex (HLA).

Karl Landsteiner made an extensive work on the determinants of antigen and it was summarized from his various studies that specificity depends on the following factors:

1. Acid and basic groups are important in regulating the specificity of an antigenic determinant.
2. Spatial configuration of hapten is important.
3. Terminal groups in an antigen are often important determinant of specificity.
4. Interchange of nonionic groups of similar size had little effect on specificity of a determinant.

ANTIGENIC DETERMINANT

- Also called epitope
- It is smallest unit of antigencity represented by small area on antigen molecule, which determines specific immune response and reacts specifically with antibody.
- An antigen possesses several epitopes and each epitope induces specific antibody formation
- Size of epitope is 25 to 35 Å
- Molecular weight varies between 400 to 1000
- The determinant groups are:
  a. Protein antigen (Penta of hexasaccharide)
  b. Polysaccharide antigen (Hexasaccharide)
- A determinant is around 5 amino acid in size
- The site on antibody molecule, whcih combines with corresponding epitope is known as paratope

Biological Classes on Antigens

On the basis of capability of antibody formation antigens are classified as under:

1. T-Cell Independent Antigens
   - Also abbreviated as T1 antigens.
   - Can directly stimulate antibody formation by β lymphocyte cells, without involving T lymphocyte cells.
   - They are structurally simple composed of a limited number of repeating determinants, e.g. pneumococcal capsule polysaccharide, bacterial lipopolysaccharide and flagellar protein flagellin.
   - Antibody response is IgM and IgG3.
   - Do not produce immunological memory.
   - Do not require initial processing by macrophages.
   - Metabolized slowly and so remain in the body for quite a long time.
   - May cause dose dependent immune response.
2. T-Cell Dependent Antigens
   - Also abbreviated as TD antigens.
   - Need T-cell involvement to evoke an immune response.
   - Structurally more complex like red blood cells, serum proteins and wide range of hapten complex.

- Do not cause tolerance readily.
- Immunogenic over a wide dose range.
- Induce immunoglobulin isotypes, i.e. IgM, IgG, IgA and IgE.
- Produce immunological memory.
- Require initial processing by macrophages.
- Rapidly metabolized in the body.

PROPERTIES OF ANTIGEN

A number of properties have been identified which make a substance antigenic.

1. *Foreignness:* Only antigens which are foreign to the individual induce an immune response. An individual does not normally give rise to immune response against his own constituent antigen.

   Antigenecity of substance is related to the degree of foreignness. Injection of sheep RBC or rat kidney tissue extract in rabbit will cause production of antibodies. But injection of rabbit RBC or kidney tissue extract into the same rabbit will not stimulate antibody production.
2. *Size:* Antigenecity bears a relation to molecular size. Very large molecules such as hemocyanin (molecular weight 6.75 millions) are highly antigenic. Usually antigens have a molecular weight of 10,000 or more.

   Substances of less than 10,000 dalton molecular weight, e.g. insulin (5700) are either non-antigenic or weakly antigenic. However, substances with low molecular weight may be rendered antigenic by adsorbing the same on large inert particles like kaolin or bentonite.

   Depending on the size of antigen and capacity to induce antibody production antigens can be divided into:
   a. Complete antigen
   b. Partial antigen.
   a. *Complete antigen:* It is able to induce antibody formation and produce a specific and observable reaction with the antibody so produced, e.g. proteins, polysaccharide, etc.
   b. *Partial antigen* (also called hapten): Haptens are substances which are unable to induce antibody production by themselves, but are able to react specifically with antibodies, e.g. lipids, nucleic acid, sulfonamide, penicillin, etc. Clinically they are important because a number of hypersensitivity reactions may develop as a complication of drug therapy. Haptens may be of two types:
      i. Complex hapten
      ii. Simple hapten
      i. Complex hapten: They are relatively higher molecular compounds which can precipitate with specific antibodies, e.g. Wassermann antigen (cardiolipin), polysacch-arides (C substance of streptococci) and nucleic acid.
      ii. Simple hapten: They are low molecular weight compounds and are non-precipitating with specific antibodies, e.g. picric chloride, tartaric acid, para-aminobenzoic acid. Such substances when applied to skin, inhaled or injected can form compounds with proteins of skin and plasma proteins thereby producing foreign hapten group and sensitization.
3. *Chemical nature:* Most naturally occurring antigens are proteins and polysaccharides. Proteins are more effective in stimulating antibody production than polysaccharides except gelatin histone and protamines (non-antigenic protein) due to their low tyrosine contents (aromatic radicals). Not all proteins are antigenic. Gelatin is a well-known exception. Aromatic radical is a must for antigenecity. Gelatin is non-antigenic because of absence of aromatic radical.
4. *Susceptibility to tissue enzymes:* Only substances which are metabolized and are susceptible to the action of tissue enzymes behave like antigen. Substances insusceptible to tissue enzymes are not antigenic, e.g. polystyrene latex, D amino acids which are not metabolized in the body are not antigenic. Polypeptides composed of L amino acid are antigenic.
5. *Antigenic specificity:* Active sites are present at certain places in antigen molecules. These active sites are called antigenic determinants. The remaining portion of antigen molecule is antigenically inert. In antigen

antibody reaction, antigen molecule reacts specifically at determinant site with complementary combining on antibody molecule, e.g. the antigenic specificity of the Lancefield group A *Streptococcus pyogenes* depends on N acetylglucosamine present in the side chain or rhamnose backbone. Antigenic specificity is of following types:

a. *Species specificity*: Tissues of all individuals in species contain species specific antigen. It has been useful in:
   i. Tracing of evolutionary relationship.
   ii. Forensic application in identification of species of blood and seminal stains.
b. *Isospecificity:* Isoantigens are antigens found in some but not all member of a species, e.g.:
   i. Human erythrocytes antigen on which individuals can be classified into group (blood group).
   ii. Histocompatibility antigen: HL-A (human leukocyte associated antigen system). It has its application in organ transplantation from one individual to other.
c. *Auto-specificity:* A number of tissue antigen may act as autoantigen, e.g. lens protein, thyroglobulin, etc. These tissues under certain circumstances such as injury, infection or drug therapy alter the molecule so that they become foreign to one's own body and provoke autoantibody formation.
d. *Organ specificity:* They are restricted to particular organ or tissue of species. When they are restricted exclusively to an organ they are called organ specific, e.g. thyroglobulin, lens protein, brain, spinal cord and adrenal of one species share specificity with another species.
e. *Heterogenetic (Heterophile) specificity* (Table 13.1)*:* This is found in a number of unrelated animals and microorganisms. The examples are:
   i. Forssman antigen found (Table 13.2) in the tissue of guinea pigs, cat, horse, sheep, bacteria, e.g. rickettsiae. It was first described by Forssman in 1911.
   ii. Weil-Felix reaction in typhus fever.
   iii. Paul-Bunnell reaction in infectious mononucleosis.
   iv. Cold agglutinin test in primary atypical pneumonia.

SUPERANTIGEN

Certain proteins that are capable of activating a large number of T-lymphocytes irrespective of their antigenic specificities are named as super antigen. They actually bind directly to the lateral aspect of V regions of TCR beta chain. Superantigen may activate 20% of circulatory T-lymphocytes where as conventional antigenic stimuli can activate not more than 0.0001% of circulating T-lymphocytes. This excessive T-lymphocyte activation may result a huge outpouring of T-lymphocyte cytokines leading to multisystem problems. The examples are staphylococcal toxic shock syndrome and staphylococcal enterotoxin.

TABLE 13.1: Cross-reacting antigens shown by heterophile reactions

	Antigen	*Sharing antigen*
1.	Blood group B substance	*Escherichia coli*
2.	Blood group A substance	Pneumococcus type 14 (cap polys)
3.	Blood group P.I. antigen	Hydatid fluid
4.	Blood group A substance	Streptococcal extract
5.	Rickettsiae causing typhus	Proteus OX_{19}, OXK_2, OX_k
6.	Epstein-Barr virus	Sheep and Ox RBC.

TABLE 13.2: Distribution of Forssman antigen

RBC and other tissues	*Kidney*	*Bacteria cells*
Horse	Guinea pig	*Streptococcus pneumoniae*
Cat		*Shigella dysenteriae*
Mouse		Pasteurella
Chicken		*Clostridium perfringens*

14 Antibodies—Immunoglobulins

Antibody is defined as humoral substance (γ-globulin) produced in response to an antigenic stimulus. It serves as protective agent against organisms. Antibodies are found in serum, lymph and other body fluids. Sera having high antibody levels following infection or immunization is called immune sera. Antibodies are:

1. Protein in nature.
2. Formed in response to antigenic stimulation.
3. React with corresponding antigen in a specific and observable manner.

The antibody molecule is chemically indistinguishable from normal gamma-globulin. Globulin is a very complex mixture of molecules consisting of closely related proteins. Now the term immunoglobulin is used to describe these closely related proteins.

IMMUNOGLOBULINS

They are defined as proteins of animals origin endowed with known antibody activity and for some other proteins related to them by chemical structure.

Immunoglobulins are synthesized by plasma cells and also by lymphocytes. Immunoglobulins make 20 to 25 percent of the total serum proteins. The term immunoglobulins is the structural and chemical concept while antibody is biological and functional concept. All antibodies are immunoglobulins but all immunoglobulins may not be antibodies.

Based on their size, carbohydrate contents and amino acid analysis, five groups of immunoglobulins have been distinguished: IgG, IgA, lgM, IgD and IgE.

Structure of Immunoglobulin

Porter, Edelman and Nisonoff (1959-64) developed technique for fragmentation of immunoglobulin molecule by papain digestion which has led to the discovery of 5 main classes of immunoglobulin.

Papain Digestion (Fig.14.1)

Enzymatic digestion: Porter and colleagues split rabbit IgG molecule to egg albumin by papain digestion in presence of cystein into two identical Fab (Fragment antigen binding) fragments and one Fc (Fragment crystallizable) fragment.

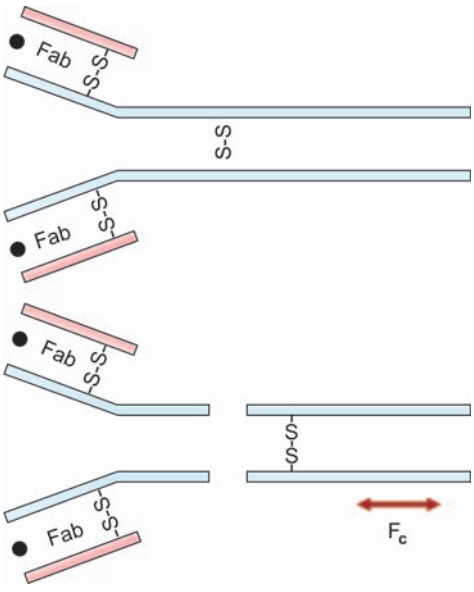


Fig. 14.1: Basic structure of an immunoglobulin molecule and papain digestion Fab and Fc fragments

Papain acts on the exposed hinge region of molecule.

1. **Fab fragments:** Each fragment of 3.5S contains a single antigen binding site. The portion of the heavy chain in the Fab fragment is called Fd region. Each Fab fragment contains a light chain.
2. **Fc fragment:** It lacks the ability to bind antigen and appears to be identical in all rabbit IgG molecules. Each Fc fragment contains parts of both H chains. As this fragment is easily crystallizable, it is so named.

Pepsin Digestion (Fig. 14.2)

Pepsin strikes at a different point of the IgG molecule and cleaves the Fc from the remainders of the molecule leaving a large SS fragment, composed essentially of 2 Fab fragments. It is divalent and still precipitates with antigen. This fragment is formulated as $(Fab)_2$.

Immunoglobulins are glycoproteins. Each molecule consisting of two pairs of polypeptide chains of different sizes held together by disulfide bonds (S—S) (Fig. 14.3). The smaller chains are called light (L) chain and larger ones heavy (H) chains. L chain has molecular weight 25,000 and H chain 50,000. The L chain is attached to the H chain by disulfide bond. The two H chains are joined together by one to five S—S bonds depending upon class of immunoglobulins. The H chains are structurally and antigenetically distinct for each class and designated by Greek letter as follows (Fig. 14.4):

IgG	γ (gamma)
IgA	α (alpha)
IgM	μ (mu)
IgD	δ (delta)
IgE	Σ (epsilon)

The L chains are similar in all classes of immunoglobulins. They may be either kappa or lambda in a molecule of immunoglobulins.

L chain: The antigen combining site of molecule is at its aminoterminus (N). Carboxyterminal portion is also called constant region. Of 214 amino acid residues that make up L chains, 107 constitute carboxyterminal half occur only in constant sequence and hence this part of chain is called constant region. Amino acid sequence in aminoterminal (N) half of chains is highly variable and so called variable region.

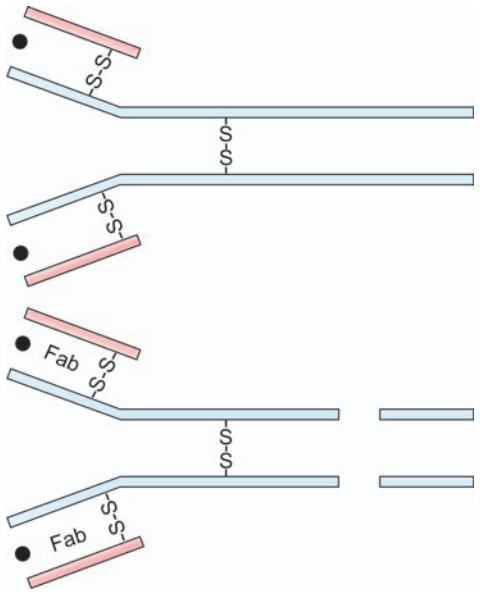


Fig. 14.2: Basic structure of an immunoglobulin molecule and pepsin digestion $(Fab)_2$

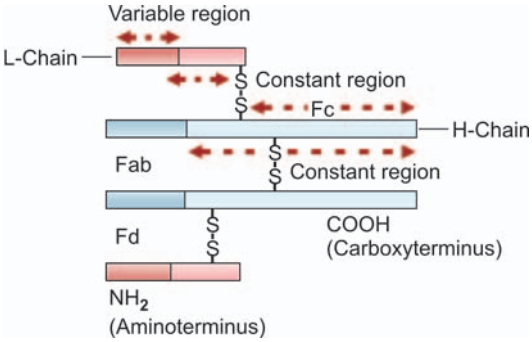


Fig. 14.3: Structure of immunoglobulin

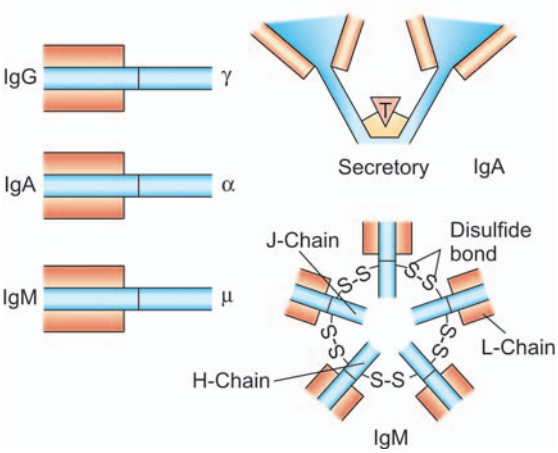


Fig. 14.4: Structure of IgG, IgA and IgM

This variability determines immunological specificity of antibody molecule. Like L chain, H chain also has constant and variable region.

The infinite range of antibody specificity of immunoglobulins depends on the variability of amino acid sequences at variable region of H chain and L chains which form antigen combining site.

Fc fragment: It is composed of carboxyterminal portion of H chain. It determines biological properties of immunoglobulins molecules like complement fixation, placental transfer, skin fixation, attachment to phagocytic cells, degranulation of mast cells and catabolic rate. It is crystallizable and contain carbohydrate.

Fd fragment: The portion of H chain present in Fab fragment is called Fd piece. Its function is not known.

Fab: It is aminoterminal half of heavy chain and one light chain. It does not crystallize. It does not contain carbohydrate and acts as antigen binding fragment.

Immunoglobulin Domains (Fig. 14.5)

Immunoglobulins are folded into globular variable and constant domains. There are 4 domains in each H chain of IgG, IgA and IgD, one in variable region (VH) and 3 in constant region (CH-1, CH-2, CH-3). In the same way L chain shows one domain in variable region (VL) and one in constant region (CL). The antigen binding site is present in the domain of hypervariable region which recognize the antigen IgM and IgE possess a fourth domain on H chain. The constant region domains mediate secondary biological functions. The area of H chain between CH-1 and CH-2 in the hinge region (exposed to enzymes and chemicals). Papain split the immunoglobulin at this site into 2 Fab and 1Fc fragment.

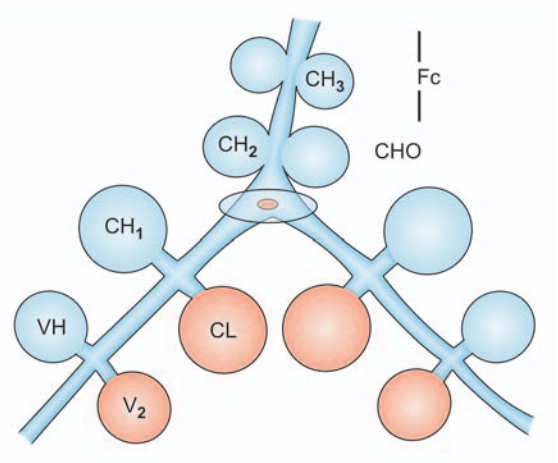


Fig. 14.5: Immunoglobulin domain

Immunoglobulin Antigen Determinants

Immunoglobulins may act as antigens when inoculated into a foreign species and so can be differentiated from each other. The type of immunological determinant occupy specific site or position on the molecules and given specific name as discussed under:

1. **Isotypic Markers (Same)**
   They are common to all members of the same species. The isotypic markers on it chains are H(α), H (δ) H (γ), H (μ), H (Σ). The light chain, L(λ) and L(Λ) are differentiated through isotypic markers.
2. **Allotypic Markers**
   They are individual specific determinant which means they exist between individuals within species. Allotypic markers are also found on the constant regions of H and L chains. They are genetically determined. The allotype markers in man are Gm on gamma heavy chains, Am on alpha heavy chains and Km (/nv) on kappa light chains. Allotype markers are useful in testing paternity and population genetics.
3. **Idiotypic Markers (Individual)**
   Idiotypes are specific for each antibody molecule and are useful in identifying markers for specific antibody produced by single clones (private idiotype), but are sometimes shared between β cell clones (public or cross-reacting idiotypes).

   Idiotype determinants are serologically defined antigenic determinants involving the hypervariable regions of immunoglobulin molecule and the antibodies produced are called anti-idiotypic antibodies.

   Application of anti-idiotype antibodies may be utilized for vaccination purposes. An antigen may induce specific immunoglobulin production in a host and this immunoglobulin may again stimulate

production of anti-idiotype antibodies. Some of these anti-idiotype antibodies contain a paratope that resemble (structure and shape) to antigen that trigerred the process.

Function of Immunoglobulins

The important functions of immunoglobulins are:

1. Complement activation causing lysis
2. Opsonization resulting in phagocytosis
3. Prevention of attachment of microbe to host cells
4. Neutralize toxins
5. Motility of microorganisms is restricted
6. Ultimately may result in agglutination of microbes.

Immunoglobulin Classes (Table 14.1)

1. **IgG:** It is the major serum immunoglobulin (Fig. 14.6). It has molecular weight of 1,50,000 and sedimentation constant is 7S. It is distributed equally between intravascular and extravascular compartments. It has half-life of 23 days. It appears to be spindle shaped 250 to 300 Å long 40 Å wide. The normal serum concentration of IgG is 5 to 16 mg/ml. It passes through placenta and provide natural passive immunity to newborn. It produces passive cutaneous anaphylaxis and participates in immunological reactions like precipitation, complement fixation, neutralization of toxin and viruses.

   Four classes of IgG have been recognized, i.e. IgG_1 IgG_2, IgG_3, IgG_4. Each is having distinct type of gamma chain identifiable with specific antisera. Their properties are enumerated in Table 14.2.

2. **IgA:** It is the fast moving alpha globulin. It constitutes 10 percent of total serum globulin. Normal serum level is 0.6 to 4.2 mg per ml. It has half-life of 6 to 8 days and molecular weight is 160,000 with sedimentation constant of 7S. It is found in high concentration in colostrum, tear, bile, saliva,

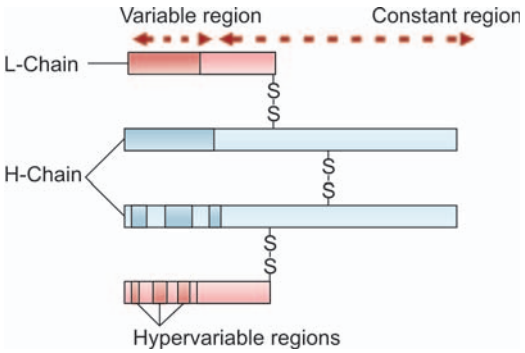


Fig. 14.6: IgG molecule

TABLE 14.1: Some properties of immunoglobulin classes

		IgG	*IgA*	*IgM*	*IgD*	*IgE*
1.	Sedimentation coefficient	7	7	19	7	8
2.	Mol. wt.	1,50,000	1,60,000	9,00,000	1,80,000	1,90,000
3.	Serum concentration (mg/ml)	12	2	1.2	0.03	0.00004
4.	Half-life (days)	23	6	5	2.8	2.3
5.	Daily production (mg/kg)	34	24	3.3	0.4	0.0023
6.	Intravascular distribution (%)	45	42	80	75	50
7.	Complement fixation	+	–	+	–	–
8.	Placental transport	+	+	–	–	
9.	Present in milk	+	+	–	–	–
10.	Secretion by seromucous glands	–	+	–	–	–
11.	Heat stability (56°C).	+	+	+	+	–
12.	Examples antibodies	Many antibodies to toxin, bacteria and viruses	Secretory antibody in mucous membrane	Antipolysaccharide antibody and cold agglutination	Mainly IgD on surface of beta lymphocytes in newborn	Antiallergic antiparasitic antibodies

intestinal and nasal secretions. Its amount is greatly increased in cases of multiple myeloma. It does not pass through placenta. IgA does not fix complement but activates alternate complement pathway. It promotes phagocytosis and intracellular killing of organisms.

IgA exists in serum as monomer H_2L_2. Two subclasses of IgA are known, i.e. IgA1 and IgA2. IgA2 in dominant form (60%) in the secretion and form only small component of serum IgA. IgA2 lacks interchain disulfide bonds between H and L chains.

IgA found in secretions contain additional structure unit called transport (T) or secretory (S) piece. T piece is synthesized in epithelial cells of gland, intestines and respiratory tract. It is attached to IgA molecule during transport across the cells. T piece links together two IgA molecule at Fc portion. J chain is also found in IgA. J chain is synthesized by lymphoid cell.

3. **IgM:** It is also called macroglobulin (Fig. 14.7). It constitute 5 to 10 percent serum globulin (0.5 to 2 mg/ml). It has half-life of 5 days. It has molecular weight of 9,00,000 to 10,00,000 with sedimentation constant of 19S. Mostly it is intravascular. Polymeric form with J chains (one J chain per 10L c hains of IgM molecule) are frequently found and help in polymerization and stabilization of IgM molecule. It appears to be of spherical shape. IgM appears in the surface (membrane) of unstimulated β-lymphocytes and acts as recognition receptors for antigens. Two subclasses of IgM are identified, i.e. IgM-1 and IgM-2. IgM appears earlier in primary response and IgG is produced later. Its half-life is 5 days and it fixes complement. It does not pass through placenta. It is more efficient in agglutination, cytolytic and cytotoxic reaction. IgM deficiency is often associated with septicemia.

   It was first detected by Rose and Fahey in 1965.

4. **IgD:** It is present in concentration of 0.03 mg per ml. It is mostly intravascular. It has half-life of 3 days. It is present on the surface of unstimulated B. lymphocytes blood. It acts as recognition receptors for antigens cell membrane bound IgD combination with matching antigen causes specific stimulation of these B lymphocytes. It results in either activation and cloning to form antibody or suppression. IgD has two subclass IgD_1 and IgD_2. Very little is known about its function. It seems likely that IgD may function as mutually interacting antigen receptor for the control of lymphocyte activation and suppression.
5. **IgE:** It is reaginic antibody responsible for immediate hypersensitive reactions. It

TABLE 14.2: Properties of subclasses of immunoglobulin G

	Properties	*IgG_1*	*IgG_2*	*IgG_3*	*IgG_4*
1.	Fixation of complement	+	+	+	+
2.	Binding of macrophages	+	+	+	+
3.	Binding the heterogenous tissue	+	–	+	+
4.	Blocking of IgE binding	–	–	–	+
5.	Crossing of placenta	+	+	–	+
6.	Percentage of total IgG in normal serum	65	23	8	4
7.	Electrophoretic mobility	Slow	Slow	Slow	Fast
8.	Spontaneous aggregation	–	–	+++	–
9.	Combination with Staphylococcal A protein	+++	+++	–	+++
10.	Sedimentation of coefficient	7	7	7	7
11.	Molecular weight	150	150	150	150
12.	Heavy chains	γ 1	γ 2	γ 3	γ 4
13.	K/λ ratio	2.4	1.1	1.4	8.0
14.	Half-life (days)	23	23	8	23
15.	Heavy chain allotype	6 gm	6 gm	6 gm	6 gm

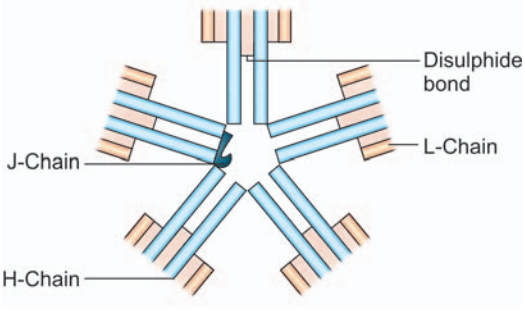


Fig. 14.7: IgM molecule

has molecular weight of 1,90,000 with sedimentation coefficient of 8S. Its half-life is 2 days. It is inactivated by heat at 56°C for 1 hour. It has affinity for surface of tissue cells (particularly mast cells) of the same species. It mediates Prausnitz-Küstner reaction. It does not pass through placenta or fix complement. It is mostly intravascular in distribution. Normally it is found in traces in serum. Elevated levels are seen in atopic condition like asthma, hay fever and eczema. Children having parasitic infection in intestine show elevated levels of IgE.

Immunoglobulin Class (Isotype Switching)

To start with all B cells matched to an antigen, are transformed into plasma cells and produce IgM in response to the antigen. Subsequently class switching occurs by gene rearrangement. The same VH assembled VH gene is sequentially associated with different CH genes. Consequently immunoglobulin produced (IgG or IgA) maintains same specificity as original IgM with different biological properties. In primary immune response, a plasma cell producing IgM earlier may later switch to IgG synthesis.

15 Antigen and Antibody Reaction

Antigen-antibody reactions are useful in laboratory diagnosis of various diseases and in the identification of infectious agents in epidemiological survey. Antigen-antibody reactions *in vitro* are called serological reactions, e.g. precipitation, agglutination, complement fixation reactions (Table 15.1).

Features of Antigens-Antibody Reactions

1. The reaction is highly specific.
2. Entire molecules react and not fragment.
3. There is no denaturation of antigen or antibody during reactions.
4. Combination occurs at surface and hence surface antigens are immunologically relevant.
5. The combination is firm but reversible. It is influenced by affinity or avidity. *Affinity* is intensity of attraction between antigen and antibody molecules. *Avidity* is strength of the bond after the formation of antigen antibody complex.
6. Both antigen and antibody participate in the formation of the agglutinates or precipitates.
7. Antigen and antibody may combine in varying proportions.

TABLE 15.1: Comparative efficiency of immunoglobulins classes in different serological reactions

Reactions	*IgG*	*IgM*	*IgA*
Precipitation	strong	weak	variable
Agglutination	weak	strong	moderate
Complement fixation	weak	strong	negative
Lysis	weak	strong	negative

PRECIPITATION

When a soluble antigen combines with its antibody in presence of electrolytes (NaCl) at a suitable temperature and pH the antigen antibody complex forms insoluble precipitate.

Uses of Precipitation Reaction

1. Identification of bacteria, e.g. detection of group specific polysaccharides substance in streptococci in Lancefield grouping, etc.
2. Identification of antigenic component of bacteria in infected animal tissue, e.g. *Bacillus anthracis* (Ascoli test).
3. Standardization of toxin and antitoxins.
4. Demonstration of antibody in serum, e.g. Kahn's test for the diagnosis of syphilis.
5. Medicolegal serology for detection of blood, semen, etc.

   Mechanism of precipitation: Lattice hypothesis explains it.

LATTICE HYPOTHESIS (FIG. 15.1)

Multivalent antigens combine with bivalent antibody in varying proportions, depending on antigen-antibody ratio in reacting mixture. Precipitation results when large lattice is formed consisting of alternating antigen and antibody molecules. This is possible only in the zone of equivalence. In zone of antigen and antibody excess lattice does not enlarge as valency of antigen and antibody is fully satisfied.

Techniques of Precipitation Reaction

1. *Ring test:* The test is very simple for detection of antigen. The antigen is layered over

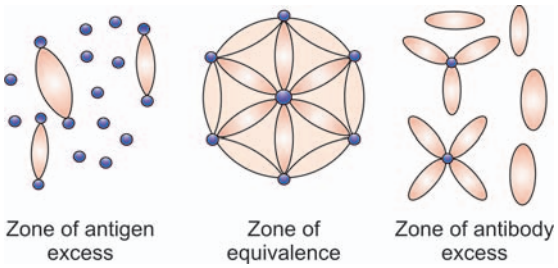


Fig. 15.1: Lattice formation

serum in a narrow tube. The reaction is visible as a white zone at the junction of two clear fluid. Examples are C-reactive protein test, Ascoli test, grouping of streptococci by Lancefield technique.

2. *Slide test:* When a drop of antigen and antiserum is placed on a slide and mixed by shaking, floccules appear, e.g. VDRL test for syphilis.
3. *Tube test:* The Kahn test for syphilis is an example of tube flocculation test.
4. *Gel diffusion:* The main advantages of this method are:
   a. The precipitate is relatively fixed by agar medium and is easily visible.
   b. If antigen or antiserum contains more than one factor then each factor produces separate precipitin line.
   c. Antigen and antibodies can be compared for common antigenic determinants.

Examples of Gel diffusion are (Fig. 15.2):

1. *Single diffusion in one dimension:* The antibody is incorporated in agar gel in a test tube and antigen solution is layered over it. The antigen diffuses downwards through the agar gel forming line of precipitation that appears to move downwards. Number of bands indicate number of different antigens present.
2. *Double diffusion in one dimension:* The antibody is incorporated in gel. Above it is placed a column of agar. Antigen is layered over agar. The antigen and antibody move towards each other through intervening agar and form a band of precipitate where they meet at optimum proportion.
3. *Single diffusion in two dimension* (radial immunodiffusion): Antiserum is incorporated in agar gel poured on slide. The antigen is added to well cut on the surface of gel. Ring shaped bands of precipitate are formed around wells. The diameter of ring gives an estimate of concentration of antigen.

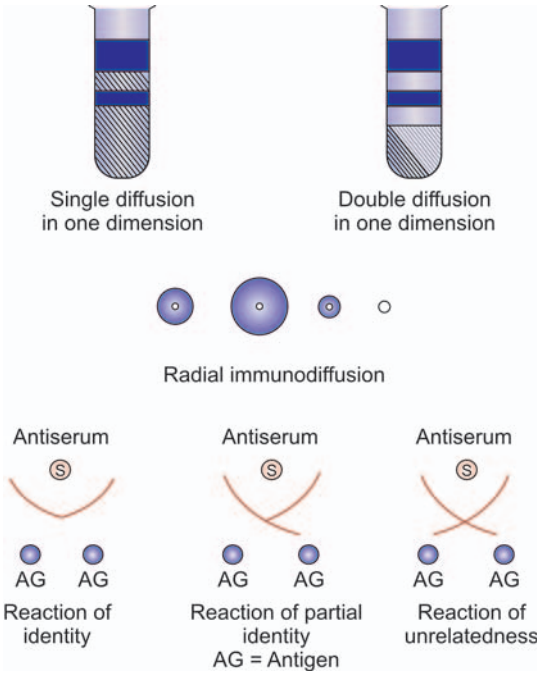


Fig. 15.2: Types of gel diffusions precipitations

4. *Double diffusion in two dimension:* Agar gel is poured on slide and wells are cut using template. Antiserum is placed on central well and different antigen in the surrounding wells. If two adjacent antigen are identical the line of precipitate will be formed by them. This method is a routine technique in the diagnosis of smallpox, Elek's test for the diagnosis of diphtheria, etc.
5. *Immunoelectrophoresis:* This is done in 2 steps. The first step is done in agar electrophoresis of the antigen. Rectangular trough is cut in agar on either side, parallel to the direction of electrophoretic migration. This trough is filled with antiserum. By diffusion lines of precipitate develop with each of the separated components of antigen. By this method over 100 different antigens are identified in human serum.
6. *Radioimmunoelectrophoresis:* This is very sensitive method for estimation of antibodies to antigens such as hormones. Pure antigen

is labelled with radioactive isotope. The antigen precipitated by specific antibody in immunoelectrophoresis is estimated by autoradiograph.

AGGLUTINATION REACTIONS

When a particulate antigen is mixed with its antibody in presence of electrolytes at a suitable temperature and pH, then the particles are clumped or agglutinated. It is more sensitive than precipitation for the detection of antibodies.

Uses

1. Identification of bacteria, e.g. serotyping of salmonella and shigella with known antisera.
2. Serological diagnosis of infection, e.g. Widal test for typhoid, etc.
3. Hemagglutination test, e.g. Rose-Waaler, Paul-Bunnell.

TECHNIQUE OF AGGLUTINATION TEST

Direct Agglutination Test

1. *Microagglutination* (Fig. 15.3)*:* It is carried on a clean slide by mixing a drop each of antiserum and antigen suspension. Reaction occurs immediately. It is used for detecting bacterial antigen, blood grouping and typing, etc.
2. *Macroagglutination:* It is carried out as quantitative test to estimate the titer of antibody and to confirm the result of microagglutination. Following type of agglutination are observed with bacterial antigen:
   a. Flagellar antigen or 'H' type of agglutination is seen when a formalized suspension of motile bacteria is treated with antiserum. It forms floccular, snowy flakes like deposit. Agglutination appears 2 to 4 hours after incubation at 52°C.
   b. Somatic 'O' type of agglutination occurs when heat killed or alcohol treated suspension of bacteria is treated with homologous antiserum. The agglutination is compact with fine granulation. The reaction appears 18 to 24 hours after incubation at 37°C.
   c. Vi agglutination is similar to O agglutination and occurs slowly at 37°C.

Co-agglutination: Here the Fc fragment of any antibody gets attached to protein A of staphylococci (Cowan strain). Thus staphylococci with a known attached antibody are agglutinated when mixed with the specific antigen (Fig 15.4).

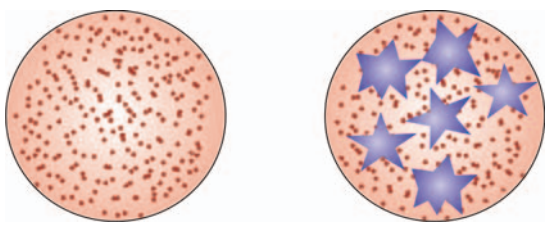


Fig. 15.3: Microagglutination test

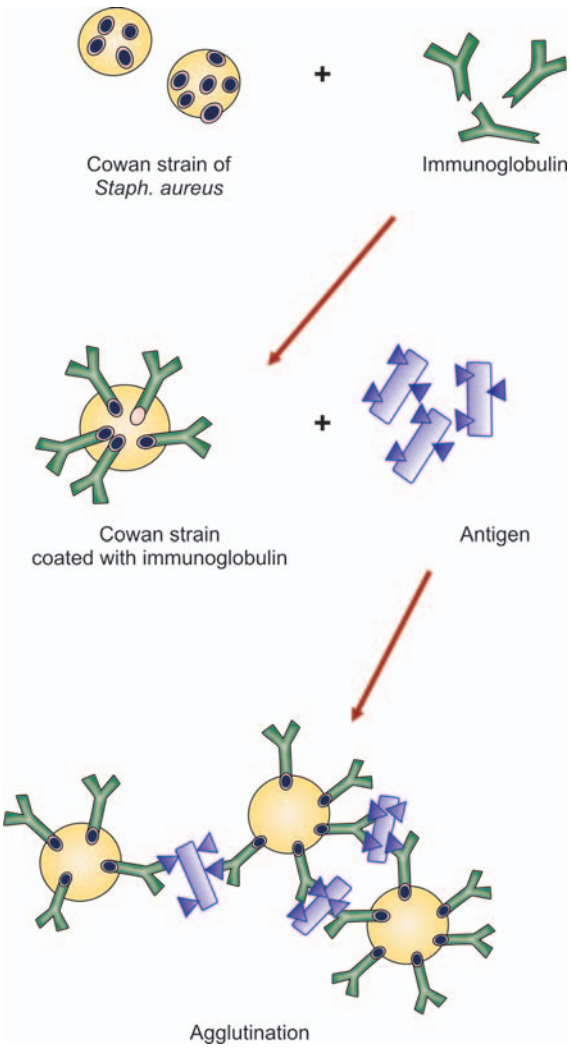


Fig. 15.4: Agglutination and co-agglutination

Indirect or Passive Agglutination Test

Recently inert particles, e.g. latex, bentonite or red blood cells have been used as carrier of antigen. It is more sensitive test. Latex particles are used for the demonstration of rheumatoid factor, CRP, etc. A special type of passive hemagglutination test is the Rose-Waaler test. In rheumatoid arthritis patient's autoantibody (RA factor) appears in serum which acts as antibody to gammaglobulin. The RA factor is able to agglutinate red cell coated with globulins. The antigen used for test is a suspension of sheep erythrocytes sensitized with subagglutinating dose of rabbit anti-sheep erythrocytes (amboceptor).

Coombs' test (Fig. 15.5)**:** It is used for the detection of incomplete antibodies (nonagglutinating anti-Rh antibody), brucella, shigella and salmonella antigen. Coombs' test may be direct or indirect (Table 15.2).

Sera containing incomplete anti-Rh antibodies is mixed with Rh positive red cells. Antibody globulin coats the surface of erythrocytes. Such erythrocytes coated with antibody globulin are washed free of all unattached proteins and treated with rabbit anti-serum against human gammaglobulin (Coombs' serum). The cells are agglutinated. This is the principle of Coombs' test.

Complement fixation test (CFT): This is a very sensitive test and is capable of detecting 0.04 mg of antibody nitrogen and 0.1 mg of antigen. It is used for serological diagnosis of diseases:

1. Bacterial diseases, e.g. gonorrhea, brucellosis.
2. Spirochetal disease, e.g. syphilis (Wassermann reaction), etc.
3. Rickettsial diseases, e.g. typhus fever.
4. Viral diseases like lymphogranuloma venereum.
5. Parasitic diseases, e.g. kala azar, hydatid cyst, amoebiasis.

Principle: The ability of antigen antibody complex to fix complement.

Technique: Heat the patient's serum at 56°C for 30 minutes to destroy its own complement. Patient serum, complement (guinea pig serum) and antigen are incubated at 37°C for 1 hour. Now sensitized sheep RBC are added as indicator system. The whole mixture is incubated at 37°C for 1 hour.

Interpretation: If complement has been used up, there would not be hemolysis. It means antigen antibody reaction has taken place. Test is reported as positive.

If sensitized RBC are lysed it means complement has not been fixed and test is reported as negative.

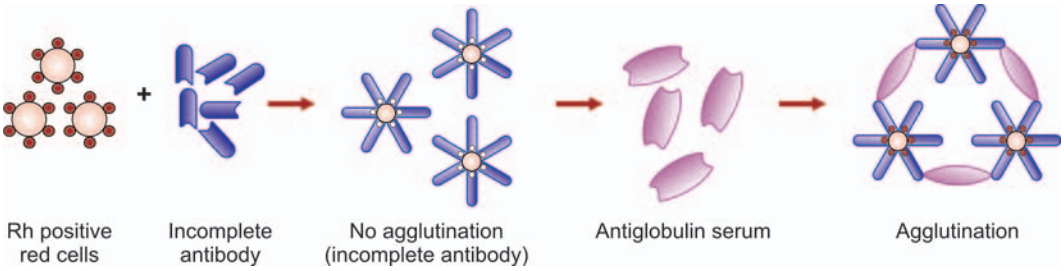


Fig. 15.5: Steps of Coombs' test

TABLE 15.2: Differences between direct and indirect Coombs' test

	Direct	*Indirect*
1.	Detects the presence of incomplete antibodies adsorbed onto erythrocyte	Detects incomplete antibody present in serum
2.	Indicated in autoimmune hemolytic anemia, erythroblastosis fetalis	Indicated for detection of anti-Rh antibody in serum of Rh negative women of Rh positive husband
3.	Test is done by washing RBC in saline (3 times). Washed RBC are mixed with AHG in presence of bovine albumin. In positive test clumping of RBC occurs	Serum of patient is mixed with saline washed Rh positive red cells (group O) and incubated at 37°C for 30 minutes. Wash red cell in saline and mix with Coombs' serum. Agglutination occurs in positive cases

Neutralization Test

Specific antibodies are able to neutralize the biological effects of viruses, toxins and enzymes.

a. *Virus neutralization:* Viruses when mixed with immune serum lose their capacity to infect fresh host, e.g. vaccinia, influenza and poliomyelitis. Neutralization may be quantified on:
   i. Chorioallantoic membrane of chick embryo (pocks formation).
   ii. By enumeration of plaques on monolayer tissue culture.

b. *Toxin neutralization:* The toxicity of endotoxin is not neutralized by antiserum. On the other hand bacterial exotoxins are good antigens. They induce the formation of antibodies, i.e. antitoxin. These antibodies protect from diseases like diphtheria and tetanus. Schick test is based on the ability of circulating antitoxin to neutralize diphtheria toxin. Anti-streptolysin "O" test (ASO) in which antitoxin present in patient sera neutralize the hemolytic activity. Nagler's reaction is another example of neutralization.

Opsonization

This is another serological reaction which sensitizes bacteria for phagocytosis. The substances in serum which promote phagocytosis are called opsonins.

The opsonic index is defined as ratio of phagocytic activity of patient's blood having bacterium, to the phagocytic activity of blood from normal individual. It is measured by incubating fresh citrated blood with bacterial suspensions at 37°C for 15 minutes. Now estimate the average number of phagocytozed bacteria per leukocyte from stained blood.

Immune Adherence Test *(Treponema pallidum)*

When a suspension of living spirochete is treated with specific antibody and complement, the spirochetes becomes sticky. The test is used in the identification of spirochaetes. Adherence reaction is also shown by protozoa, microfilaria and bacteria.

Immunofluorescence

Fluorescence is the property of absorbing light rays of one wavelength and emitting rays with different wavelengths. Serological reactions employing tagged antisera are used to detect minute amounts of weakly active antigen or antibodies. The method is suitable for only qualitative reactions. The fluorescent antibody technique is used for:

1. Rapid serological diagnosis of number of bacteria.
2. Detection of antitoxoplasma antibody.
3. Demonstration of leptospira in human and animal muscles.
4. Detection of viruses in cells.

The various modifications of fluorescent antibody methods are as under:

a. *Direct method* (Fig. 15.6): It is commonly used for the detection of antigen by using of a single layer of fluorescent labelled antibody.

b. *Indirect method* (double layer technique): It is used by treating a slide smear of the antigen with specific unlabelled serum. The preparation is thoroughly washed and is treated with fluorescence labelled gamma-globulin against the human serum.

c. Sandwich technique is used for detection of antibody in tissue. Tissue section is treated with dilute solution of antigen. After washing (remove excess of antigen) section is exposed to fluorescein labelled antibodies.

Immunoelectron Microscopic Test

a. *Immunoelectron microscopy:* This is useful in the study of some viruses, e.g. Hepatitis B virus and viruses causing diarrhea. Viral particles are mixed with specific antisera are

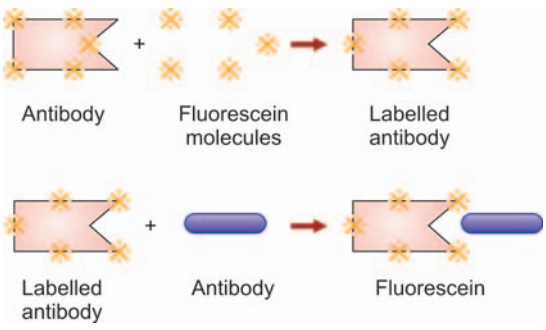


Fig. 15.6: Direct immunofluorescent technique

seen under electron microscope. Clumped virus particles are seen and studied.

b. *Immunoferritin test:* Ferritin is conjugated with antibody and such labelled antibody reacting with antigen can be visualized under electron microscope. Ferritin is electron dense substance obtained from horse spleen.

c. *Immunoenzyme system:* Some stable enzyme like peroxidase can be conjugated with antibody. Tissue section carrying corresponding antigen is treated with peroxidase labelled antisera. The peroxidase bound to the antigen can be visualized under the electron microscope by microhistochemical method. Some other enzymes such as glucose oxidase, phosphatase and tyrosinase may also be used in immunoenzyme test.

16 The Complement System

The bacteriocidal property of serum as well as of whole blood, has been recognized since 1888. These antibacterial substances were found to be inactivated by heating to 56°C for 30 minutes and function as bactericides only in the presence of specific antibody. Ehrlich discovered that antibody was also required for other activities, such as lysis of red blood cells (hemolysis) by this thermolable (heat sensitive) component of serum which he named complement. Complement is well-known for its ability to react with wide variety of antigen antibody combination to produce important physiological results. Included in this group of reaction are:

1. The destruction of erythrocytes as well as other tissue cells.
2. The initiation of inflammatory changes.
3. The lysis of bacterial cells.
4. Enhancement of phagocytosis involving some opsonized particles.

The complement refers to a series of factors occurring in normal serum that are activated characteristically by antigen-antibody interaction, and subsequently mediate a number of biologically significant consequences. Some genes controlling the production of complement components are located on human chromosome 6 in proximity to HLA locus. Some components have enzymatic activity; others are enhancers or inhibitors. Complement is heat labile. The activity of complement is lost in 30 minutes at 37°C and in few days at 4°C. This activity is also lost by bacterial contamination. It can be preserved at –25°C, lyophilization or after addition of high concentration of sodium chloride.

The concentration of complement in the serum is fairly constant for each species of animal. It constitutes 10 percent of human serum globulin. Its concentration is not increased by active immunization but is increased in abnormal conditions like carcinomatosis, coronary occlusion and rheumatic fever. Its concentration is decreased in acute glomerulonephritis, nephrosis, serum sickness, malaria, etc. Guinea pigs possess higher levels of complements than any other laboratory animals. Therefore guinea pigs serve main source of complement in complement fixation test.

Components of Complement

At present complement is known to have 9 distinct components, one of which has 3 protein subunits making a total of 11 proteins.

$C'_{1:}$ Heat labile (destroyed at 56°C for ½ hour) found in euglobulin fraction of serum.

$C'_{2:}$ Heat labile, found in alpha and gamma-globulin fractions.

$C'_{3:}$ Heat stable, inactivated by zymosan. It is found in euglobulin fraction of serum.

C'_4: Heat stable, inactivated by ammonia or hydrazine.

C_1 of human complement has the properties of proenzyme known as proesterase. C_1 component is composed of 3 fractions called C'_1q, C'_1r, C'_1s (held together by calcium ions). C'_1r is responsible for esterase activity, C'_1q attaches the whole C_1 component to antibody molecule.

The remaining components $C_{5,6,7,8,9}$ participate in cell lysis.

Genetics of Complement System

C-2, C-4 and factor B are present on chromosome-6. Some control proteins are coded for by a cluster of genes on chromosome-1, called RCA (regulation of complement activation) locus.

Regulation of Complement Activation

The lysis or damage of normal cells by excessive activity of complement is prevented by complementary regulatory proteins: plasma proteins and regulatory protein in cell membrane.

Biological Functions of Complement

1. Immune adherence and opsonization.
2. Chemotaxis, e.g. C_{5a} and $C_{5,6,7}$.
3. Anaphylatoxin, e.g. C_{3a}, C_{4a}, and C_{5a}.
4. Cytolysis, e.g. $C_{5,6,7,8,9}$.
5. Hypersensitive reaction of type II (blood transfusion) and type III (Arthus reaction and serum sickness).
6. Endotoxic shock, e.g. C_3 activation cause tissue damage by disseminated intravascular reaction. In endotoxic shock with Gram negative septicemia of damage hemorrhagic fever, endotoxin become coated with C-3b and sticks to platelets by immune adherence. $C_{5,6,7}$ generated in cascade reaction causes lysis of platelets with release of clotting factors.

Complement Receptors

Four types of complement receptors on target site are known, i.e. complement receptory type I (CR-1), CR-2, CR-3 miscellaneous for C proteins (Table 16.1).

TABLE 16.1: Types of complement receptors

Complement receptor	*Binding component*	*Target cell*
CR-1	C_{3b}, iC_{3b}, C_{4b}, C_{5b}	RBC, neutrophils, monocytes, macrophages, T and B cells
CR-2	C_{3b}, C_{3d} C-3 dg	B cells and also act as receptor of Epstein-Barr viruses
CR-3	–	Macrophages (Mac-I antigen) neutrophils, RBC

Activities of Complement

The chain of events in which complement (C′) components react in specific sequence following activation of antigen antibody complexes and culminating in immune cytolysis is known as *classical C pathway* (Fig. 16.1). The components of complement are fixed to sensitized cells in the order, $C_{1,4,2,3,5,6,7,8,9}$. Immune cytolysis is initiated by binding of C_1 to EA (erythrocyte antibody). C_1 occurs in serum as calcium chelation. With ethylenediaminetetra acetate acid (EDTA), it yields three component units C_1q, C_1r C_1s. C_1q reacts with Fc piece of appropriate immunoglobulin (IgG, IgM) bound with antigen. This activates C_1r and C_1s and activated C_1 acquires enzymatic activity.

C_1	EA + C_1qrs Ca EAC_1	
C_4	EAC_1, $_4$b,...	Virus neutralization
C_2	EAC_1, $_4$b, $_2$a	
C_3	EAC_1, $_4$b, $_2$a, $_3$b...	Phagoctosis, virus neutralization and immune adherence
C_5	EAC_1, $_4$b, $_2$a, $_3$b, $_5$b	
$C_{6,7}$	EAC_1, $_4$b, $_2$a, $_3$b, $_5$b...	Prepare cell for lysis
$C_{8,9}$	EAC_{1-9}...	Lysis of cell

Fig. 16.1: The classical pathway of complement

C_1 esterases act on C_4 and C_2. C_4 is splitted into C_4a and C_4b and C_2 into C_2a and C_2b. The $C_{4,2}$ splits C_3 into fragment C_3a and C_3b. At this stage reaction is expressed as EAC_1, $_4$b, $_2$a, $_3$b, where E is erythrocyte, A is antihemolysin. $C_{1,4,2,3}$ acts on C5 spitting it into C_5a and C_5b. C_6 and C_7 are then added. They are joined by C_8 and C_9 (EAC_1, $_4$b, $_2$a, $_3$b, $_5$b, $_{6,7,8,9}$) causing lysis of cell. The mechanism of lysis is by production of hole of 100Å in diameter in cell membrane. This disrupts osmotic integrity of membrane causing release of the contents of cell (Table 16.2).

Deficiency of complement components may be associated with diseases (Table 16.3).

TABLE 16.2: Biological activities of complement

Complement	*Activity*
C_2b	Increased vasodilatation
C_4b	Anaphylotoxin histamine release
C_3a	Anaphylotoxin
C_5a	Anaphylotoxin chemotactic factor
C_4b	Immune—adherence opsonization
C_3b	Immune adherence opsonization
$C_{5,6,7}$	Weak chemotactic factor
C_5b—C_9	Membrane distuption
C_4b, C_2	Virus neutralization

TABLE 16.3: Clinical conditions associated with deficiencies of complement components

S.No	Deficiency	Clinical condition associated
1.	C_1 inhibitor	Hereditary angioneurotic edema
2.	C_1, C_2, C_4	• Systemic lupus erythematosus • Some collagen vascular diseases
3.	C_3, C_3b inactivator	Severe recurrent pyogenic infections
4.	C_5, C_6, C_7, C_8	• Bacteremia (mainly due to Gram negative diplococci) • Toxoplasmosis
5.	Properdin	Severe meningococcal disease

Alternate Pathways of Complement

Activation of C_3 is without prior participation of $C_{1,4,2}$ is called alternate pathway and can be brought out by following methods (Fig. 16.2):

a. Zymosan with properdin (a glycene-rich beta-glycoprotein) forms complex in presence of Mg^{++} which activates C_3.
b. Cobra venom with co-factor (glycin-rich beta glycoprotein) present in serum acts directly on C_3.
c. IgA and IgE are capable of activating alternate pathway.

The properdine system can enhance resistance to gram-negative infections; it is involved in the lysis of erythrocytes from patients with paroxysmal nocturnal hematuria. The properdine system can also participate in the mediation of immunologic injury (e.g. nephritis) and may be defective in sickle cell anemia.

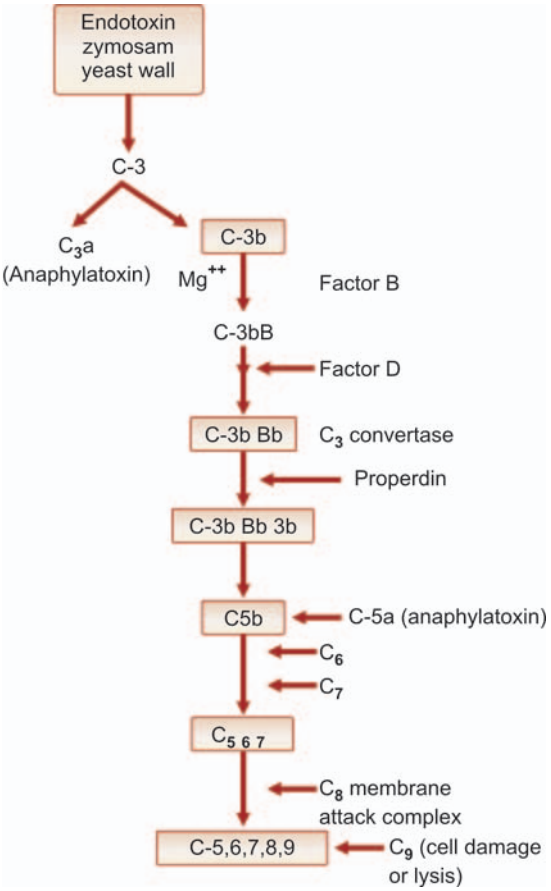


Fig. 16.2: Alternate complement pathway

Low serum complement levels: Low serum complements levels particularly C_3 are found in antigen antibody complex diseases, e.g.

TABLE 16.4: Classical complement pathway (Properties of Proteins of Alternative Pathway)

Protein	Mol. Weight	Serum Conc. (ug/ml)	Sed Coeff.	Property
Properdin	184, 000	25	5.4	• Glycoprotein (γ 2 globulin) • Alternate pathway usually occurs in solution
Factor-B (C_{3b})	93,000	200	5 to 6	• Thermolabile beta globulin a normal serum protein • C_3 proactivator active only when bind to C_{3b} and acted on by a protease—factor D
Factor-D	2,400	2	3	• Globulin-like called C_3 proactivator convertase • It enzymatically convert C_3 proactivator (factor B) and generate Ba and Bb. Bb remains bound to C-3Bb hydrolyzes C_3

TABLE 16.5: Comparative properties of alternative and classic pathways of complement activation

Criteria	*Alternative pathway*	*Classic pathway*
1. Activating agents	Aggregated proteins LPS, inulin, zymosan	IgM, IgG_1, IgG_2, IgG_3
2. Activation site	Unknown	Fc segment of Ig
3. Participation of C1q, C4b and C2a	No	Essential
4. Participation of C_3 and C_5 through C_9	Yes	Yes
5. Divalent cation requirement	Mg^{++}	Ca^{++}, Mg^{++}
6. Non-immunologic initiation	Yes	No
7. Amplification mechanism	Yes	Yes

lupus erythematosus, acute glomerulonephritis and in cryoglobulinemia.

By the way, alternative complement pathway differs from classical complement pathway as shown in Tables 16.4 and 16.5.

Biosynthesis of Complement

C_1 is synthesized in intestinal epithelium.
$C_{2,4}$ is synthesized in macrophages.
$C_{5,8}$ is synthesized in spleen.
$C_{3,6,9}$ is synthesized in liver.
The site of synthesis of C_7 is not known.
Factor B macrophages, lymphocytes and hepatocytes

CONGLUTININ

Conglutinin is a euglobulin of molecular weight 750,000. It is heat stable and is present in bovine serum. It acts as antibody to complement. It is not immunoglobulin and requires Ca^{++} for its activity. It reacts exclusively with bound C_3. Conglutinin causes agglutination of sensitized sheep erythrocytes (conglutination) if they have combined with complement. The titers of serum 1K rise in condition, e.g. infection and autoimmune disease.

Complement Dependent Serological Tests

1. Immune adherence test, i.e. some bacteria react with specific antibody in the presence of complement, particulate material, e.g. erythrocytes, platelets, and bacteria are aggregated and adhere to the cells, e.g. *Vibrio cholerae, Treponema pallidum.*
2. Complement fixation test.
3. Conglutinating complement absorption.
4. Immobilization test, e.g. *Treponema pallidum.*
5. Cytolytic or cytocidal test, e.g. *Vibrio cholerae.*
6. Opsonization.

17 Structure and Functions of Immune System

ORGANS PRODUCING ANTIBODY

These are spleen, lymph nodes and bone marrow. Other tissue like Peyer's patches, appendix, tonsils thymus, etc. are also capable of producing antibodies. These organs contain lymphocytes, macrophages and plasma cells. Lymph nodes and spleen constitute peripheral lymphoid organs while thymus, bone marrow, Peyer's patches, appendix and tonsil form central lymphoid system.

CENTRAL LYMPHOID SYSTEM

Thymus: It develops from the epithelium of 3rd and 4th pharyngeal pouch. In man thymus reaches its maximum size just prior to birth. It continues to grow till puberty. After puberty it undergoes spontaneous progressive involution.

The primary function of thymus is production of thymus lymphocytes. In the thymus, lymphocytes acquire new surface antigen (QTL antigen). Lymphocytes produced in thymus are called T lymphocytes. They are not dependent on antigen stimulation.

T lymphocytes are selectively seeded into certain sites in peripheral lymphatic tissue like thymus dependent regions.

a. White pulp of spleen.
b. Around central arteriole.
c. Paracortical areas of lymph node.

Subpopulations among T cells are suggested using Lyt antigen system in mice and by development of monoclonal anti-T cell antibodies in humans. The most two, widely used groups of anti-T monoclonal antibodies are referred to as OKT and Leu. By utilizing these it has been possible to delineate several functionally distinct populations of human T cell, and to identify distinct stages in T cell differentiation. Antibodies, designated OKT_1 (Leu-1), OKT_3 (Leu-4) and OKT_{11} (Leu-5) react virtually with all T cells in the peripheral blood. Therefore, the antigens corresponding to these antibodies (OKT_1, OKT_3 and OKT_{11}) can be considered common marker to all mature T cells. Among these mature T cells, OKT_4 is expressed in 60 percent whereas 30 percent of the peripheral T cells express OKT_8. The OKT_4 cells have been recognized as helper subset, as they help in antibody synthesis and generation of cytotoxic T cells. On the contrary OKT_8 cells are suppressor T cells. In severe combined immunodeficiency (defective maturation of T cells) OKT_{10} cells, immunologically not competent may be found in the peripheral blood.

NEW CLASSIFICATION OF T CELLS (TABLE 17.1)

T-lymphocytes: Classification of lymphocytes on the basis of surface marker is as under.

Antigen recognition receptor: They have characteristic membrane bound receptors in B cell and T cell (TcR) which they get in the thymus during their maturation from lymphoid stem cell. They react only with antigen present on the surface of another cell. Examples of T-cell receptors are; enzyme receptor (rosettes with sheep RBC), a protein receptor on B-cell and macrophages (processing and immune response of T dependent antigens), T-cell antigen receptor (CD4 act as receptors for HIV) and IL-1 and IL-2 receptors. Thus antigens specificity of mgs in B cells and TCRs in T cells is predetermined.

TABLE 17.1: Functional classification of T cells

Cell type	*Symbol*	*Marker*	*Characters*
Helper lymphocytes	TH	CD 4	1. Provide help signal for C cell (IL-4) 2. Recognize antigen in association with class 2 HLA
Suppressor lymphocytes	TS	CD 8	Recognize antigen in association with class 1 HLA
Effector cytolytic	TC	CD 8	1. Recognize antigen in association with class 1 HLA 2. Present on the surface of nucleated cells
Mediators of delayed hypersensitivity	TDTH	CD 4	Recognize antigen in association with class 2 HLA

Differentiation of T-cells: Thymic antigen is acquired by T-lymphocytes which is detectable using monoclonal antibodies. These antigens or markers or surface molecules of T-cells are now called CD (cluster of differentiation) proteins. As these cells mature, they start expressing their characteristic cell membrane glycoproteins.

T-cell receptor is responsible, for the recognition of specific MHC antigen complexes and is different for every T-cell. So far, over 78 T-cell antigens have been recognized using monoclonal antibodies.

Formerly called T_1, T_3, etc., have been renamed CD-1, CD-3, etc., (CD = cluster of differentiation). Identification of CD and other surface markers on lymphocytes is valuable in immunodeficiency syndromes.

CD testing is usually done with monoclonal antibodies and new determinants and antibodies are identified each year. About 150 CD markers have been identified. Important CD molecules are described as under:

CD-1 It has cortical thymocyte marker having 3 polypeptide chains. It is associated with beta-globulin. It is found only in early stages of thymus maturation.

CD-2 It acts as a receptor for sheep red blood cells and appears in early stages. It persists in all stages of maturation and probably acts as T cell activation and cell adherence.

CD-3 It is associated with T cell receptors on cell membrane. Following binding of antigen, it seems that C-3 transmit signal to the interior of the cell. It is present in all T cells.

CD-4 It has function of a helper. It is present over 60 percent of the circulating. T-lymphocytes. CD-4 acts as a receptor for HIV and recognizes antigen by MHC class II. CD_4 may be found on macrophages and monocytes too. CD-4 has 2 subsets of T helper (Th1 and Th2). Th2 interact with B-lymphocytes resulting in proliferation of B cell and its transformation to plasma cells that produce immunoglobulins. This is mediated by lymphokine like IL-4, IL-5, IL-6 which is secreted by Th-2 cells, Th-1 cells are probably associated with delayed type of hypersensitivity. Th-1 cells secrete lymphokines like interferon responsible for inflammatory response of delayed hypersensitivity. Growth factor are also produced by Th which may be responsible to regulate lymphocyte activity.

CD-5 They are present in many T cells right from the stage of maturation. Their definite function in not known.

CD-8 Present on suppressor and cytotoxic cells. They recognize antigen by MHC class-I molecules. Some CD-8 cells may block response of B cells by producing suppressor agent. They block action immune response due to action on Th or B cells. Cytotoxic T cells may have membrane glycoprotein. CD-8 cytotoxic T cells lyse specific target cells.

Helper T Lymphocytes have following properties:

1. Secrete IL-2
2. Stimulate killer T cells
3. Stimulate B cell functions

Suppressor T Lymphocytes have properties:

1. Suppress killer T Cell
2. Suppress Ig secretion by plasma cells.

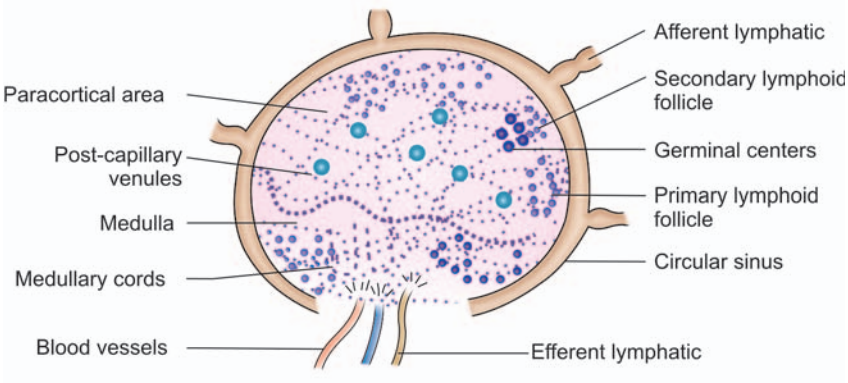


Fig. 17.1: Structure of lymph node

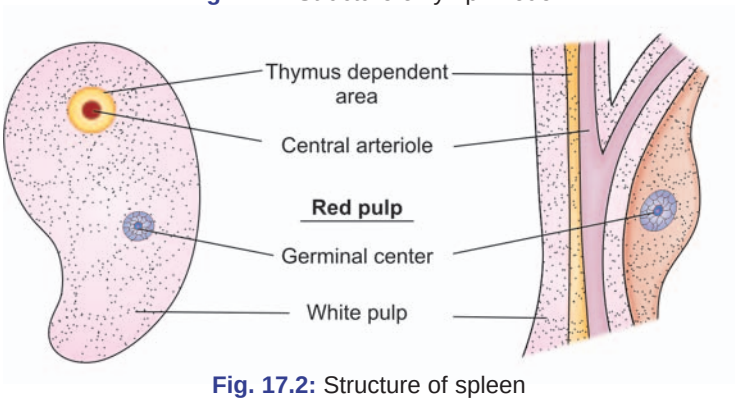


Fig. 17.2: Structure of spleen

Bursa of Fabricius: This is a lympho-epithelial organ arising as a pouch from dorsal part of the cloaca in birds. The bursal equivalent in mammals is not known. It is suggested that tonsils, Peyer's patches, appendix may perform this function. Bone marrow has also been proposed as possible bursal equivalent in mammals.

It is also a site of lymphocytic proliferation. Stem cells from yolk sac, fetal liver and bone marrow enter the bursa and proliferate and develop into bursal "B" lymphocytes. Bursal dependent areas are:

1. Mantle.
2. Germinal follicles.
3. Perifollicular region of spleen and lymph node:
   a. Cortical area.
   b. Medullary cord.

Peripheral Lymphoid System

Lymph Node *(Fig. 17.1)*

Bursa dependent areas containing B lymphocytes are:

1. Cortical follicles.
2. Medullary cords.

Thymus dependent area (containing T cells):
Paracortical area.

Spleen (Fig. 17.2)

Bursa dependent areas:

1. Perifollicular region
2. Germinal center
3. Mantle area.

Thymus dependent areas:

Lymphatic surrounding central arteriole.

Mucosa associated lymphoid tissues: The collection of lymphoid tissue in mucosal lining in alimentary canal, respiratory, genitourinary and other surfaces is known as mucosa associated lymphoid tissue of MALT. Tonsils, adenoids and Peyer's patches of small gut are called gut associated lymphoid tissue. Peyer's patches are small patches of organized B cells and T cells. They play the primary role in defence against microorganisms finding access through digestive tract.

Cells of Lymphoreticular System

1. *Macrophages and microphages:* Particulate antigens, e.g. bacteria are captured by

phagocytic cells, i.e. macrophages of lymphoid system. They are elongated and spindle-shaped cells. They do not synthesize antibody but are important for the immune response.

The blood macrophages (monocytes) are largest of lymphoid cells (12 to 15 μ) found in peripheral blood. Tissue macrophages (histiocytes) are larger (15 to 20 μ). Macrophages are produced in bone marrow. They may be formed locally, e.g. alveolar macrophages of lungs. They have following functions:

i. They are concerned with phagocytosis. They eliminate major portion of ingested antigen through degradation.
ii. They process antigen and convert macromolecular antigen into a soluble form. They bind antigenic determinant to cellular RNA and present them in highly immunogenic form to lymphocytes.
iii. Adhere to glass.
iv. Ingestion of particles by engulfment.
v. Capable of immunologic and non-immunologic phagocytosis.
vi. Macrophages have plasma membrane receptor which can recognize IgG1 and IgG3.
vii. Have independent receptor system which can recognize C'3 component of complement.
viii. They are active in killing bacteria, fungi and tumor cells.
ix. Have the role of activation of 'B' cell and T cells.
x. They secrete interferon and IL-1.

The microphages are leukocytes of blood. Neutrophils are actively phagocytic in acute inflammation. Eosinophils are phagocytic in conditions like allergic inflammation of parasitic infections. Basophils are found in blood and tissue (mast cell). Degranulation of mast cells with release of pharmacologically active agents constitute effector mechanism in anaphylactic and atopic allergy.

2. *Lymphocytes:* They are small round cells found in peripheral blood, lymph, lymphoid organs and many other tissue. According to size they may be large (12 to 15 μ), medium (8 to 12 μ) and small (5 to 8 μ). Small lymphocytes are numerous and consist of spherical nucleus with prominent nuclear chromatin, thin rim of cytoplasm containing scattered ribosomes. They are capable of slow motility.

They may be short-lived or long-lived lymphocytes. Short-lived lymphocytes are effector cells in immune response, while long lived lymphocytes are storehouse of immunological memory. A lymphocyte that has been educated by central lymphoid organ becomes an immunological competent cells. They subserve the functions like:

a. Recognition of antigen.
b. Storage of immunological memory.
c. Immune response to specific antigen.

The nature of immune response depends on whether lymphocyte is a B or T cell (Fig. 17.3). B cells divide and transform into plasma cells which synthesize immunoglobulin. T cells produce activation products (lymphokines) and induce cell mediated response.

T cells activation require antigen and IL-1 resulting in RNA and protein synthesis including production of IL-2. T-activated cell (T blast cell) develops receptor for IL-2. This leads to burst of proliferative activity of blast cells with the secretion of lymphokines.

Activation of B cells occurs in two ways, i.e. (a) stimulation of B cell directly without T cell involvement thus inducing IgM response with little or no memory, (b) stimulation of B cell through T cells with memory. Further B cell activation requires binding of an antigenic determinant to the B cell receptor and stimulating factor IL-4. The newly activated B cell acquires a number of new surface receptors of IL-4. Finally there is differentiation of activated B cell into IgM producing plasma cells, and also switching of the immunoglobulin produced by the stimulated cells of IgM to IgG, IgA or IgE. This is achieved by transfer of a particular gene segment for the variable segment of the heavy chain, to an alternative constant region gene so that antibody produced are of exactly the same specificity but of different class.

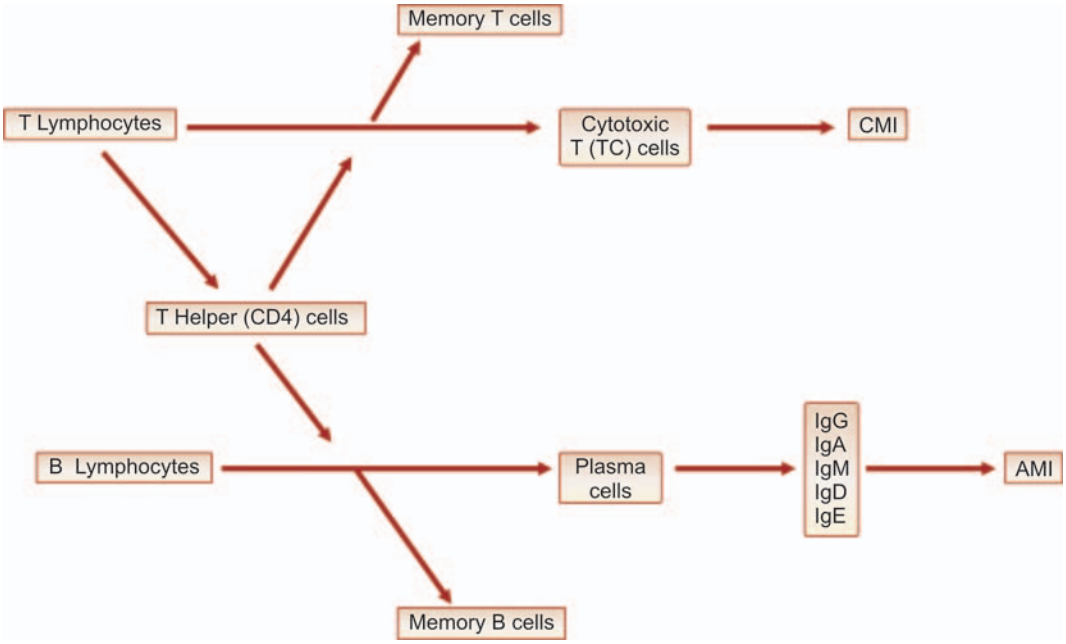


Fig. 17.3: Interaction of T and B lymphocyte

3. *Plasma cells:* Antigenically stimulated B cells undergo blast transformation becoming plasmoblast, intermediate transitional cells and plasma cells. The mature plasma cells is antibody producing cell. It is an oval with eccentric nucleus containing chromatin located peripherally (cartwheel appearance). Cytoplasm is large and contains abundant endoplasmic reticulum and well-developed Golgi apparatus.

   Plasma cell is immunoglobulin producing factory. It has lifespan of 2 to 3 days.

Human "K" cell system: A distinct subpopulation of cytotoxic lymphoid cells is known as K (Killer) cells. They are characterized by the presence of Fc receptor but lack surface Ig surface marker of T cells and are non-phagocytic. They are capable of killing target cell sensitized with IgG antibody. K cells are intermediate lymphocytes. They are non-adhesive to glass or plastic, non-phagocytic, cannot kill the human 'RBC', do not form rosette with sheep RBC and can be sensitized with IgGFc. Additionally K cells secrete lymphokines, intensify cytotoxin action, attract macrophages chemotactic factors, stop migration of MIF and T cell growth. K cell system is independent and they are not recirculating cells.

All T cells do not possess K cell activity still K cells are not in minority. K cell activity is not found in patients with hypogammaglobulinemia. However, X-rays, antimetabolic drugs can destroy K cells. K cells are responsible for delayed hypersensitivity reactions.

Null cells: They do not express B cell or T cell or macrophages markers. Within this population is a group of cells called natural killer cells which spontaneously lyse a number of *in vitro* tumor cell lines. Natural killer cells are activated by interferon and may have an important role in antitumor and antiviral immune mechanism. Natural killer cells are found in the peripheral blood and the lymphoid tissues. They cause direct lysis of cells.

Cellular Basis of Two Components of Immunity

Thymus pathway leads to T cell mediating cell mediated immunity (CMI). Bursal pathway leads to B cell mediating humoral immunity. The following features help to distinguish B and T cells.

1. T cells form rosette with sheep erythrocyte (SRBC) at 37°C while B cells do not form rosette (Table 17.2).
2. B cells bind to sheep erythrocyte coated with antibody and complement (EAC) forming

TABLE 17.2: Characteristics of T cells, B cells and macrophage

	Properties	*T cells*	*B cells*	*Macrophage*
1.	Surface immunoglobulin	–	+	–
2.	Receptor for Fc piece of IgG	–	+	–
3.	EAC rosette (C3 receptor)	–	+	–
4.	SRBC rosette	+	–	–
5.	Thymus specific Ag	+	–	–
6.	Blast transformation with:			
	i. PHA	+	–	–
	ii. Concanavalin	+	–	–
	iii. Endotoxin	–	+	–
7.	Phagocytic action	–	–	+
8.	Adherence to glass surface	–	–	+
9.	Markers of monoclonal antibodies	CD_2 CD_3 CD_4 CD_8	CD_{19} CD_{22}	–

TABLE 17.3: Surface markers on T and B cells

	Function/identity	*CD designation*	*T cells*	*B cells*
1.	Surface Ig	–	–	++
2.	T cells αβ, γδ	–	++	–
3.	TCR signal transducer	CD_3	++	–
4.	Sheep red cell rosettes	CD_2	++	–
5.	MHC class II (T helper cells)	CD_4	++	–
6.	MHC class I (cytotoxic or suppressor cells)	CD_8	++	–
7.	Complement (CR2)	CD_{21}	–	++
8.	Complement (CR1)	CD_{35}	+	++
9.	Il-2 (alpha chain)	CD_{25}	Activated cells	++
10.	Differentiation markers	CD_5	++	Subset
11.	Restricted leukocytes common antigen	CD_{45} R	Memory	+

rosette. EAC rosette is due to presence on B cell membrane of a receptor for C3. T cells do not form such rosette.

3. B cells have immunoglobulin on their surface. They also possess receptor for Fc fragment of IgG. These are absent in T cells.
4. T cells have thymus antigen on their surface. They are absent in B cell.
5. T cells undergo blast transformation with mitogens, e.g. phytohemagglutinin (PHA) or concanavaline A.

   Concanavaline A was first extracted from jack bean in 1919 and was identified as lectin for erythrocytes in 1935. In 1970 it was found to bind to lymphocytes.

   It is potent mitogens of T lymphocytes (optimal dose 3 mg/ml).

   Phytohemagglutinin (PHA) is a protein extracted from red kidney beans. In 1960 it was demonstrated inducing lymphocyte activation (optimum dose in 1 to 5 mg/ml).
6. Viewed under scanning electron microscope, T cells are free from cytoplasmic surface projections and B cells have extensively filamentous surface with numerous microvilli.
7. Monoclonal antibody markers (Table 17.3) for B cells (cluster of differentiation-CD units), e.g. CD-9, CD 10, CD 19, CD 20, CD 21, CD 22, etc.

Monoclonal antibodies markers against T cells are CD 1, CD 2, CD 3, CD 4 (T-suppressors) whereas CD 5, CD 7, CD 8 (T-helpers) and CD 25 (receptor for interleukin-2).

18 The Immune Response

The specific reactivity induced in host by antigenic stimulus is called the immune response. Immune response includes reactions against any antigen living or non-living. Immune response may be beneficial, harmful or non-reactive (tolerance). Immune response is of two types:

a. Humoral immunity.
b. Cell mediated immunity.

Humoral immunity provides primary defence against extracellular pathogens, helps in defences against virus infections, immediate hypersensitivity and certain autoimmune diseases. Cell mediated immunity protects against fungi, viruses, intracellular pathogens, participates in graft rejection, immunity against cells, delayed hypersensitivity and certain autoimmune diseases.

HUMORAL IMMUNE RESPONSE

It consists of 3 steps:

a. Entry and distribution of antigen in tissue and its contact with immunocompetent cells.
b. Processing of antigen and control of antibody forming process.
c. Secretion of antibody, its distribution in tissue, body fluid and manifestation of its effects.

Primary and Secondary Responses

Primary Response (Fig. 18.1)

Primary response to initial antigenic stimulus is slow, sluggish, short lived with a long lag phase and low antibody titer that does not persist for a long time. Antibodies formed are predominantly IgM.

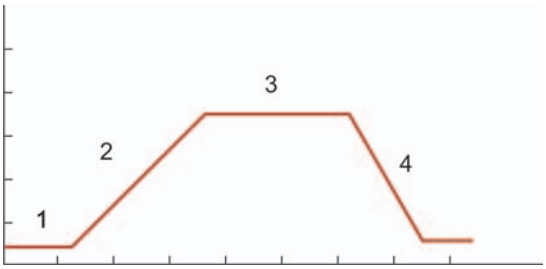


Fig. 18.1: Primary immune response an antigenic stimulus. (1) Latent period, (2) Rise in titer of serum antibody, (3) Steady state of antibody titer, and (4) Decline of antibody titer

Secondary Response (Fig. 18.2)

Secondary response to subsequent stimuli, is prompt, powerful prolonged, and with much higher level of antibody. It lasts for long time. Antibodies predominantly formed are IgG.

When an antigen is injected into an animal carrying specific antibody in circulation, a temporary fall in the level of circulating antibody occurs due to combination of antigen and antibody. This is called negative phase. Then it is followed by increase in titer of the antibody, exceeding initial levels.

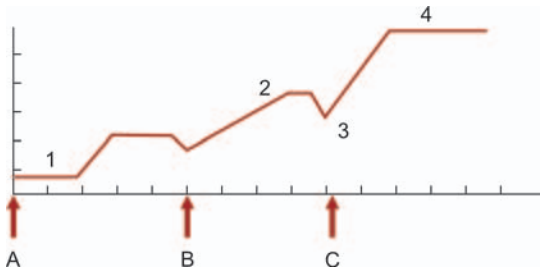


Fig. 18.2: Secondary immune response. Effect of repeated antigenic stimuli A,B,C: (1) Secondary immune response, (2) Negative immune response, (3) Negative phase, (4) High level of antibody after booster dose

FACTORS INFLUENCING ANTIBODY PRODUCTION

1. *Age:* Embryo is immunologically immature, immunocompetence is not complete at birth. The infant has to depend on maternal antibody from 3 to 6 month of age. By this time maternal antibodies disappear. Full competence is acquired only by the age of 4 years. In old age capacity to produce antibody is reduced.
2. *Nutritional status:* Protein calorie malnutrition suppresses both humoral as well as cellular immune response. Deficiency of amino acids and vitamins causes decrease in antibody synthesis.
3. *Route of administration:* The human immune response is better following parenteral administration of antigen than oral or nasal routes. However, for production of IgA antibody, oral or nasal route is suitable.
4. *Size and number of doses:* An antigen is effective only above critical dose. Further increase in dose enhances antibody production. The massive antigen stimulus appears to inhibit antigen antibody system. This is called *immunological paralysis.*

   Secondary antigenic stimulus produces increased antibody response. On the other hand *anamnestic reaction* is the production of heterologous but related antibody that host has earlier produced in response to antigen stimulation, e.g. a person immunized against typhoid bacilli may some time produce antityphoid antibodies in response to infection to some other bacterium. Unlike secondary response anamnestic reaction is transitional.
5. *Multiple antigens:* When two or more antigens are administered effect can be:
   a. Antibody production against different antigens as they had been given separately.
   b. Antibody response to one or other antigen may be enhanced. In triple vaccine (diphtheria, tetanus, *Bordetella pertussis*), the response to diphtheria toxoid is enhanced.
   c. Response may be diminished (antigens competition).
6. *Adjuvant:* The term adjuvant refers to any substance that enhances immunogenecity of an antigen. The most potent adjuvant is Freund's complete adjuvant which is the combination of incomplete adjuvant along with suspension of killed tubercle bacilli. It is not used in human beings. Beside increasing humoral immune response it induces high degree of cellular immunity. The adjuvant commonly used in human vaccine are aluminium hydroxide, phosphate, endotoxin and mineral oils.
7. *Immunosuppressive agent:* X-rays and cortisone if administered before antigenic stimulus, suppress antibody formation. They do not change antibody levels if administered after antibody formation. A number of other agents suppress antibody production if introduced after antigen stimulation, e.g. nitrogen mustard, purine and pyrimidine analogue, actinomycin D, cyclophosphamide, etc. In non-toxic dose they are important in suppressing homograft rejection.

THEORIES OF ANTIBODY PRODUCTION

The following theories are put forward for antibody production.

1. *Instructive theory* suggests that specific patch on the antibody acquires its pattern by being synthesized in contact with antigenic determinants acting as the template. This theory advocates that the template of antigen must be present for the synthesis of antibody. However, it neither explain antibody synthesis long after elimination of antigen nor it explain the phenomenon of memory as expressed in secondary immune response. This theory impresses upon the fact that it has direct instructive role on the gene for antibody protein synthesis. This theory was abandoned when it became apparent that antibody forming cells are devoid of antigens and antibody specificity was a function of amino acid sequence.
2. *Selective theory* explains the view that antibodies are produced in the similar way as other proteins are synthesized just according to genetic codes contained in the

nucleus of the manufacturing cell. However, the antigen selects out appropriate manufacturing cell. *Clonal selection theory* has received universal acceptance as the mechanism of antibody production is based on the fact that natural antibodies are normally present on the surface of lymphocyte. According to it activation of particular clone of lymphocyte is brought about through selection of antigen. Thus selective theory does explain long continuous production of antibodies even in the absence of antigen and also secondary response. In short according to selective theory all facilities are provided to different antigens to select out the particular clone of lymphocyte.

CELLULAR IMMUNE RESPONSE

The term cell mediated immunity refers to the specific immune responses that do not involve antibodies. Induction of CMI consists of specifically sensitizing T lymphocytes against the antigen. When sensitized T lymphocyte comes in contact with antigen determinant, it undergoes blast transformation and clonal proliferation selectively in para cortical areas of lymph node. The activated lymphocytes release biologically active products (lymphokines) responsible for manifestation of CMI. Macrophages under the effect of lymphokines cause destruction of microorganism involved in CMI.

CYTOKINES

They are biological active substances released by cells of immune system. Cytokines are:

A. *Lymphokines:* They are biological active substances released by activated T-lymphocytes. They are basically proteins which regulate immune response, growth and functions of cells of reticulo-endothelial system. They possess several biological activities.

B. *Monokines:* These are soluble proteins of monocytes and macrophages. They are responsible for various biological effects.

Lymphokines, monokines, interferons growth factors, etc have similar effects. Hence they are collectively named as cytokines.

LYMPHOKINES

These are biological active substances released by activated lymphocytes. The different lymphokines are:

Affecting Macrophages

1. Macrophages exhibiting factor (MIF) inhibits migration of normal macrophages.
2. Macrophages aggregating factor (MAF) may result aggregation of macrophages.
3. Macrophages chemotactic factor (MCF) is responsible for migration of macrophages through millipore membranes.
4. Macrophages resistance factor (MRF) makes macrophages resistant to infection.
5. Macrophages anti-spreading factor stops spreading of macrophages on glass surface.
6. Cytophillic factor makes macrophages reactivity specific with antigen.

Affecting Lymphocytes

1. Blastogen factor (BF) may induce blast cell transformation in lymphocytes.
2. Potentiating factor (PF) may result transformation in mixed lymphocyte culture.
3. Cell co-operation factor (CC) is also called helper factor. It increases number and rate of antibody forming cells.

Affecting Granulocytes

1. Inhibition factor (IF) can inhibit migration of lymphocytes.
2. Chemotactic factor (CF) may result migration of granulocytes along a gradient.

Affecting Cultured Cell

1. Lymphotoxins is cytotoxic for some cultured cells.
2. Proliferation inhibiting factor (PIF) may inhibit proliferation of cultured cell without lysing them.
3. Interferon offers protection against virus infection.

Producing Effects *in vivo*

1. Skin reactive factor is capable of inducing skin lesion of delayed hypersensitivity.
2. Inflammatory factor can cause increased vascular permeability.

3. Transfer factor is dialysable, low molecular substance resistant to trypsin, DNA-ase, RNA-ase and freeze thawing. It mediates passive transfer of cell mediated immunity. It is useful in: (i) patients with T cell deficiency, (ii) disseminate infection, (iii) malignant melanoma and other types of cancer, (iv) autoimmune diseases, and (v) diseases of unknown etiology.

Colony stimulating factors: They differentiate pluripotent stem cells in bone marrow and also stimulate growth. They are responsible for adjusting, e.g. the massive granulocyte response encountered in pyogenic infection. Colony stimulating factors are useful to treat hematopoietic dysfunctions in infection and malignancies.

Tumor necrosis factors: It may be alpha or beta. TNF alpha is a serum factor which may induce hemorrhagic necrosis in certain tumor. The same factor may cause wasting disease during chronic infections. It is produced by activated macrophages and monocytes.

TNF beta is lymphotoxin produced by T-cells. Its properties are same as of TNF alpha.

Interferon: This cytokine has 3 classes INFα produced by leukocytes, INFβ produced by fibroblasts and INFγ produced by T-cells activated by antigens or exposure to IL-2. IFNγ is responsible for macrophages activation, augmentation of neutrophils and monocyte functions and antitumor activity.

Other cytokines are:

- The transforming growth factor beta acts as growth factor for fibroblasts and promoting wound healing.
- The leukemia inhibitory factor which is formed by T-cells. It helps stem cell proliferation and eosinophil chemotaxis.

INTERLEUKINS

They are those products of leukocytes which exert regulatory influence on other cells. The term interleukin is used collectively for products secreted by leukocytes.

These substances are produced by leukocytes that regulate other cells. At present we have 13 interleukins (Table 18.1):

1. *Interleukin-1:* It is a stable polypeptide retaining its activity even at 56°C and pH 3 to 11. It is produced by all nucleated cells. Its production is initiated by antigens, toxins, and inflammatory activity. IL-1 activates T cells and promotes the synthesis of lymphokines, B-cell proliferation, antibody synthesis and induces fever. Beside mediat-

TABLE 18.1: Cytokines

Factors	*Source*	*Actions*
IL-1 α/β	Macrophages	Inflammatory
IL-2	T cells	T and B cell proliferation
IL-3	T cells	Pluripotent growth
IL-4	T cells	T and B proliferation and macrophages activity
IL-5	T cells	Eosinophil differentiation, B cell growth
IL-6	T cells	B cell differentiation
IL-7	T cells	B and T cell proliferation
IL-8	T cells	PMN activation
IL-9	T cells	Mast cell growth
IL-10	T cells/B cells	Cytokine inhibition
IFN-α	Multiple	Antiviral
IFN-β	Multiple	Antiviral
IFN-γ	T cells	Antiviral, activation of macrophages
	NK cells	MHC induction
TNF α	Monocytes	Cytotoxicity, cachexia, fever
TNF β	T cells	Cytotoxicity, cachexia, fever
TGF	T cell/macrophages	Inhibits activation of NK and T cells, macrophages, inhibits proliferation of B and T cells
GM-CSF	T cells	Growth of granulocytes and monocytes
G-CSF	Macrophages	Growth of granulocytes
M-CSF	Macrophages	Growth of monocytes

ing inflammatory activity it also resists infection in general.

2. *Interleukin-2:* It is a glycoprotein which acts as a strong modulating agent of immune response. It causes production of T helper cell, T cytotoxic and NK cells. It may convert null cells into lymphokines activated killer cells which are capable of destroying NK resistant tumor cells and hence it may play an important role in the treatment of cancer. On the negative side it is liable to produce side effects like increased permeability and systemic edema.
3. *Interleukin-3:* It is a growth factor for stem cells of bone marrow and seems to stimulate hematopoiesis.
4. *Interleukin-4:* It is a growth factor for T-cells and mast cells. It also activates B cells and act as B cell differentiation factor. It may enhance IgE synthesis and thus play its role in atopic hypersenstivity.
5. *Interleukin-5:* It may induce maturation of eosinophils and proliferation of activated B cells.
6. *Interleukin-6:* It may induce immunoglobulin synthesis by active cells and IL-2 receptors on T-cells. It may play a role in the production of autoimmune diseases like rheumatoid factors. It comes from activated T cells, fibroblast and macrophages.
7. *Interleukin-7:* It was initially known as pre-β cell growth factor made by bone marrow stroma. IL-7 made by thymic stroma acts as on thymocytes and is a T-cell growth and activation factor besides a macrophage activation factor.
8. *Interleukin-8:* They (about 15 members) are produced by macrophages and endothelial cells. They are involved in inflammation and cell migration.
9. *Interleukin-9:* Its main source is T cell and function as T-cell growth and proliferation.
10. *Interleukin-10:* It is also known as cytokine synthesis inhibitory factor. It inhibits the production of IFNγ, inhibits antigen presentation and macrophages production of interleukin-1, interleukin-6 and TNFα. It plays a role in the regulation of IgE.
11. *Interleukin-11:* It is produced by bone marrow stromal cell. It induces acute phase proteins.
12. *Interleukin-12:* Its production is by T-cells and it activates NK cells.
13. *Interleukin-13:* It inhibits mononuclear cell function and is produced by T-cells.

DETECTION OF CMI

1. Skin test for delayed hypersensitivity.
2. Lymphocyte transformation test.
3. Macrophage migration inhibiting factor (MIF).
4. Target cell destruction test.
5. SRBC rosette formation.
6. Enumeration of T and B cells and subpopulation.

SCOPE OF CELL MEDIATED IMMUNITY

Cell mediated immunity plays role in following immunological functions:

1. Delayed hypersensitivity.
2. Immunity as a consequent of infections caused by intracellular microbes, e.g. tuberculosis, leprosy, listeriosis, burcellosis, histoplasmosis, leishmaniasis, measles, etc.
3. Transplantation plus graft and host reaction.
4. Oncogenic immunity.
5. Autoimmune diseases pathogenesis, e.g. thyroiditis, enchephalitis.

19 Hypersensitivity

History: Immune response was thought to be protective. It may be activated by milk protein. Later on Portier and Richet (1902) showed that immune responses possess harmful effects by administering sea anemones to dogs. First dose did not produce any harmful effect but second dose made the dog ill and he died in few minutes. Theobald Smith in 1904 observed same response in guinea pig using non-toxic antigen. With horse antisera and rabbit antisera used in human diseases, harmful effects of immune response became obvious. Von Pirquet (1906) suggested the term allergy which is an altered response of tissue to repeated contacts with antigenic agents. Allergy was thought to include:

a. Hypersensitivity (increased susceptibility).
b. Immunity (increased resistance).

HYPERSENSITIVITY

It is defined as altered state induced by an antigen in which pathological reaction can be subsequently elicited by that antigen or by structurally similar substance.

In hypersensitivity focus of attention is what happens to host as a result of immune reaction. In immunity focus of attention is antigen (killing, neutralization of toxin).

Mechanism: The reactions that appear within minutes are mediated by freely diffusible antibody molecules (immediate type).

The other type is slow evolving responses that are mediated by sensitized "T" lymphocytes. This is cell mediated hypersensitivity (CMI, i.e. delayed type).

Classification

A. On the basis of time required for sensitized host to develop clinical reaction upon re-exposure to the antigen, Chase classified them as:
   i. Immediate reaction.
   ii. Delayed reaction.
   The differences between immediate and delayed reaction are listed in Table 19.1.
B. *Coombs' and Gell classification (1969):* They have classified hypersensitivity reaction into 5 types on the basis of different mecha-

TABLE 19.1: Differences between immediate hypersensitivity reaction and delayed hypersensitivity reaction

	Immediate reaction	*Delayed reaction*
1.	Appears and recedes rapidly	Appears slowly and lasts longer
2.	Induced by antigen or hapten by any route	Induced by infection, injection of antigen with Freund's adjuvant or by skin contact
3.	Circulating antibodies present and responsible for reaction (antibody mediated reaction)	Cell mediated reaction and not antibody mediated
4.	Passive transfer possible with serum	Transfer possible by lymphocytes or transfer factor
5.	Desensitization easy but short lived	It is difficult but long lasting
6.	Lesions are acute exudation and fat necrosis	Mononuclear cell collection around blood vessels
7.	Wheal and flare with maximum diameter in 6 hours	Erythema and induration with maximum diameter in 24 to 48 hours

TABLE 19.2: Hypersensitivity reactions

	Characters	*I*	*II*	*III*	*IV*	*V*
1.	Reacting factor	Ab	Ab	Ab	Lymphocytes	Ab
2.	Ig class and complement	Ig, E, G, C	Ig, G, M, C	Ig G, M, C	—	IgG
3.	Cell	Mast cells		Leukocyte	T cells	T cells
4.	Cutaneous response to Ag:					
	i. Peak reaction	15 to 30 months		3 to 4 hours	24 to 48 hours	—
	ii. Macroscopic	Wheal and flare		Erythema and edema	Erythema and induration	—
	iii. Microscopic	—Mast cells —Eosinophil —Edema		—Inflammation —Polymorph Polymorph	—Lymphocytes —Macrophages	—
5.	Transfer factor	Ab	Ab	Ab	T cell	Ab and T cell
6.	Examples	Anaphylaxis Hay fever Asthma	Thrombocytopenia Agranulocytosis Hemolytic anemia Blood reaction	Arthus reaction Serum sickness Farmer's lung Glomeruler nephritis	—Tuberculosis —Graft rejection	Orchitis (guinea pig) thyrotoxicosis
7.	Detection	P.K. reaction	—	—	1. Lymphocytic transformation 2. MIF	

nisms of pathogenesis (Table 19.2). It is widely used:

Type I: Anaphylactic, reagin dependent, e.g. anaphylaxis, atopy, etc. IgG, IgE and histamine participate in this type of reaction.

Type II: Cytotoxic, e.g. thrombocytopenia, hemolytic anemia, IgG, IgM and complement take part in this reaction.

Type III: Immune complex or toxic complex, e.g. Arthus reaction, serum sickness, etc. In this reaction IgG, IgM and complement take part.

Type IV: It is delayed type of hypersensitivity in which T cells, lymphokines and macrophages take part, e.g. tuberculin type and contact dermatitis. The antigen activates specifically sensitized CD_4 and CD_8. T cells resulting in secretion of lymphokines.

Type V: It is antibody dependent cell mediate and cytotoxic type of reaction, e.g. autoimmune orchitis in guinea pigs.

C. Sell's classification (1972). This classification is on the basis of pathogenesis (Flow chart 19.1).

We shall follow Coomb's and Gell (1969) classification for further discussion:

TYPE I REACTIONS

Here antibodies are fixed on the surface of tissue cells (mast cells and basophils) in sensitized individual. Antigen combines with cell fixed antibodies leading to release of pharmacological substance which produce following clinical reactions:

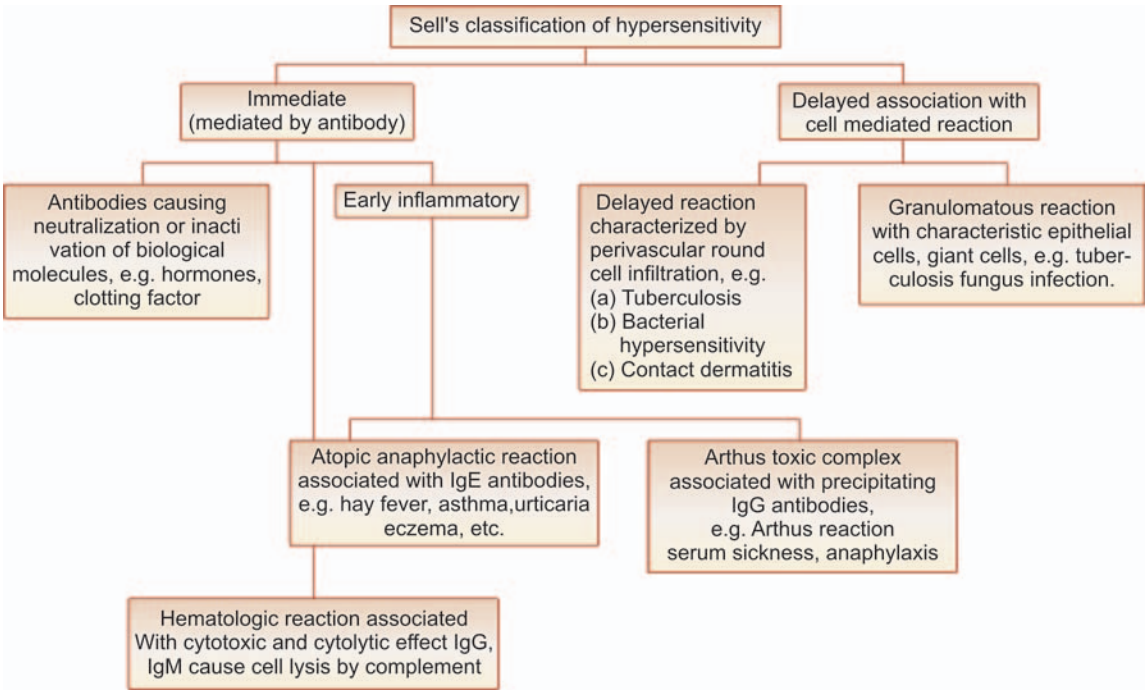
Anaphylaxis

This is a classical immediate hypersensitivity reaction. It is a shock like condition resulting from the action of histamine like substances released as a result of antigen antibody union on a cell.

Antigen as well as hapten can induce anaphylaxis. There should be an interval, of at least 2 to 3 weeks between sensitizing dose and shocking dose. Once sensitized, person remains so for a long time. The shocking antigen should be identical or immunologically closely related to sensitizing antigen. The organs involved in anaphylactic reaction are known as shock organs.

There exist considerable species variation in susceptibility to anaphylaxis. In guinea pig small dose of egg albumin is injected intraperitoneally. After the interval of 2 to 3 weeks a little

Flow chart 19.1: Sell's classification of hypersensitivity



larger dose of same antigen intravenously will bring out manifestation of anaphylaxis, i.e. guinea pig becomes irritable, sneezes, coughs, develops convulsion and dies. At autopsy lungs shows congestion. Here the shock organ is lungs. Death may be due to constriction of smooth muscle of bronchioles.

In rabbit death in anaphylactic shock is due to constriction of pulmonary arteries. Thus causing dilation of right side of heart. In man fatal anaphylaxis is rare. There is itching of scalp and tongue, flushing of skin of body and difficulty in breathing due to bronchial spasm. It is followed by hypotension, loss of consciousness and death. Human anaphylaxis is associated with serum therapy, antibiotic injection and insect stings.

Cutaneous anaphylaxis: It is a local anaphylactic reaction. It can be elicited by scratch or intradermal injection of antigen into the skin in man or guinea pig. At the site of injection of antigen there will be erythema, itching, wheal, pseudopods and spreading flare. The reaction becomes maximum in 10 to 30 minutes and fades away in few hours. Cutaneous anaphylaxis (skin test) is useful for testing hypersensitivity and in identifying the allergen responsible for atopic diseases.

Passive Cutaneous Anaphylaxis

It can be produced by injecting antibody into local area of skin and antigen intravenously. The capillary dilatation takes place rapidly and can be made visible by incorporating Evans blue dye in the serum. The reaction (PCA) is one of the convenient method for detecting small amount of human IgG and IgE antibody.

Schütz-Dale Phenomenon

Isolated tissue, e.g. intestinal or uterine muscle strips from sensitized guinea pigs are kept in Ringer's solution in a bath. On addition of specific antigen to the bath it will contract vigorously. This is called Schutz-Dale phenomenon. It is a specific reaction.

Mechanism of Anaphylaxis

The mast cells and circulating basophils are the key cells in the allergic response. Bridging of cell bound IgE molecules, anaphylotoxins, and non-specific stimuli all precipitate in the release of mediators from some population of these cells. In case of IgE bridging a protease in the cell membrane is activated and cyclic AMP produced. Membrane phospholipids become methylated, forming phosphatidylcholine, leading

to opening of the calcium channel and further conversion to arachidonic acid which is converted to prostaglandin by the lipoxygenase pathway. Mast cells granules that contain preformed histamine, eosinophilic chemotactic factors, neutrophil chemotactic factor, bradykinin and serotonin are broken down and move to periphery of the cell, and the granule and cell membranes fuse, causing a pore to form through which the granule contents are extruded.

In addition to the factors described there are lymphocyte chemotactic factors prostaglandin generating factor, inflammatory factors of anaphylaxis, and the basophil kallikrein, prekallikrein activator and Hageman factor activator involved. However, current research focuses on platelet activating factor as a possible key mediator in anaphylactic and septic shock, and asthma.

By injection of serum, anaphylactic hypersensitivity can be transferred from sensitive person to normal person. IgG and IgE are responsible for anaphylaxis. In man IgE is responsible entirely.

The mediators of anaphylactic response are pharmacological active substances. They are released by degranulation of mast cell (in tissue) and basophil (in circulation). Their release is by two mechanisms.

a. Shocking dose of antigen combines with circulating IgG antibody.
b. Shocking dose of antigen combines with IgE fixed to mast cell. Alternatively antigen combines with circulating IgE forming soluble antigen antibody complex. This gets fixed to mast cell by Fc fragment of IgE. Desensitization can be done either by repeated small injection of antigens or by a single non-fatal shocking dose.

Pharmacological Mediators of Anaphylaxis (Table 19.3)

Histamine: It is most active amine. When released into skin, it stimulates sensory nerves

TABLE 19.3: Mediators of anaphylaxis

Mediator	*Source*	*Function*
Histamine	Preformed in mast cell/ basophil	—Elevates cycle AMP levels —Feedback regulation —Increases vascular permeability —Smooth muscle constriction —Generates prostaglandins —Pulmonary vasoconstriction —Increases gastric secretion —Stimulates suppressor lymphocytes —Cardiac effect
Heparin	-do-	Anticoagulant
Tryptase	-do-	Activates C_3
B-glucosaminidase	-do-	Splits of glucosamine
NCF	-do-	Neutrophil chemotaxis
Serotonin	Performed in mast cell	Increases vascular permeability
Eosinophilic chemotactic factor	Performed in mast cells	—Attract and deactivate eosinophils —Increase eosinophilic complement receptors
Platelets activating factor	Generated in mast cells —Macrophages —Neutrophils —Eosinophils	—Aggregate platelets —Release amines and thromboxane —Increase vascular permeability —Bronchoconstriction —Myocardial depressions —Vasoconstriction —Sequesters platelets
Prostaglandins	Generated in mast cells	—Increase cAMP —Smooth muscle constriction —Increase vascualar permeability
Thromboxane		Aggregation and vasodilation
Leukotrienes	Generated in mast cells —macrophages —neutrophils —eosinophils	—Smooth muscle constriction —Increase vascular permeability —Decrease peripheral blood flow —Generate prostaglandins —Cardiac depression —Coronary vasoconstriction —Decrease lymphocyte response
Neutrophil chemotactic factor	Preformed in mast cells	Attract and deactivate lymphocytes

producing burning and itching sensation. It causes vasodilatation and hyperemia by axon reflex and edema by increasing capillary permeability. It also stimulates smooth muscle contraction and secretions. Histamine is present in platelets, mast cells, basophil, etc.

Serotonin (5-hydroxy tryptamine): It is found in intestinal mucosa, brain tissue and platelets. It causes smooth muscle contraction, increased capillary permeability and vasoconstriction. It is important in anaphylaxis of rat and mice. Its role in man is not known.

Kinins: They cause smooth muscle contraction, increased vascular permeability, vasodilatation and pain. The best known kinin is bradykinin. Its role in human anaphylaxis is not established.

Many other pharmacological active substances are also released during anaphylaxis, e.g. heparin, acetyle choline, prostaglandin E and F and eosinophilic chemotactic factor of anaphylaxis (ECF-A).

Anaphylactoid reactions: It is non-specific reaction involving the activation of complement and release of anaphylotoxins. It has no immunological base. Intravenous injection of peptone, trypsine, etc. provokes clinical reaction resembling anaphylactic shock. This is anaphylactoid reaction.

ATOPY

It is naturally occurring familial hypersensitivity of man manifested by hay fever and asthma. The substances which are more frequently responsible for these manifestations are pollen, feather, animal dander, dust, milk and wheat husk. It is difficult to induce atopy artificially.

Predisposition to atopy is genetically determined and so it runs in families. IgE is known to be an atopic reagin, it is characterized by following properties:

1. IgE can be detected by sensitive technique like passive hemagglutination, etc.
2. Reagin is believed to be synthesized in man. It is now possible to induce atopic sensitivity to guinea pig though with difficulty.
3. Reaginic antibody has an affinity for skin. This is the basis of Prausnitz Kustner (P.K.) reaction. When serum collected from Kustner (having atopic hypersensitivity to certain species of cooked fish) was injected intracutaneously into Prausnitz followed 24 hours later by an intracutaneously injection of small quantity of cooked fish antigen into the same site, within few minutes wheal and flare reaction occurred. Since IgE is homocytotropic, test has to be carried out in man only.
4. They are heat sensitive antibodies and are inactivated at 56°C in one hour. Heating destroys Fc fragment of IgE molecule.
5. It does not pass through placenta.

Atopic sensitivity is due to an over production of IgE. The symptoms are caused by release of pharmacological active substances following combination of antigen and cell fixed reagin. Exposure of antigen to eyes, respiratory tract, intestine and skin may cause conjunctivitis, rhinitis, gastrointestinal symptoms and dermatitis. Sometimes it may cause urticaria even after ingestion of allergen.

Demonstration of atopic antigens:

a. *In vivo:* P.K. reaction.
b. *In vitro:* Schultz-Dale test and degranulation of sensitized mast cells.

TYPE II REACTIONS

Cytotoxic and Cytolytic

This type of reaction is initiated by antibodies that react with either antigenic determinant of cell or tissue element. Cell damage occurs in presence of complement or mononuclear cells.

In sedormid purpura, sedormid (allyl isopropyl acetyluria) is administered. It combines with platelets altering their surface antigenecity. Antibodies are formed against sedormid coated platelet antigen. In subsequent administration of drug, antibodies causes complement dependent lysis of platelet leading to thrombocytopenic purpura.

Cytolytic reactions are responsible for transfusion reaction, hemolytic diseases of newborn and autoimmune anemia.

TYPE III REACTIONS

Immune Complex Diseases

Here the damage is caused by antigen antibody complex. This may precipitate on or around

blood vessels causing damage to cells secondarily or on membrane interfering with their function. In antigen excess, soluble circulating complex may be formed with antibodies. These may be deposited on blood vessel walls or on basement membrane, causing local inflammation and massive complement ($C_{5, 6, 7}$) activation.

Arthus Reaction

When rabbits were injected subcutaneously with normal serum, initial injection were without any local effect but with later injections there occurred local reaction consisting of edema, induration and hemorrhagic necrosis. This is a local manifestation of generalized hypersensitivity and is called Arthus reaction. The tissue damage in this case is due to formation of antigen antibody precipitations which are deposited in blood vessels. This leads to increased vascular permeability and infilteration of site with neutrophils. Leukocyte platelets thrombi reduce blood supply causing tissue necrosis. Leukocytes release lysosomal enzymes causing vasculitis of blood vessels wall. Arthus reaction can be passively transferred with sera containing IgG in high titer.

Serum Sickness

This is a systemic form of type III hypersensitivity. It appears 7 to 12 days following single injection of high concentration of foreign serum like diphtheria antitoxin. Clinical manifestations are fever, lymphadenopathy, splenomegaly, arthritis, glomerulonephritis, endocarditis, vasculitis, urticarial rash, abdominal pain, nausea and vomiting. The pathogenesis is the formation of immune complex (foreign serum and antibodies to it that reaches high titer by 7 to 12 days). It gets deposited on endothelial lining of blood vessels in various parts of body causing inflammatory infiltrations. The plasma concentration of complement falls due to massive activation and fixation by antigen antibody complex. The disease is self-limited. When all foreign antigen is eliminated and free antibody appears, symptoms clear without any sequelae. It differs from other types of hypersensitivity reaction in that single injection can serve both sensitizing and shocking dose.

Other immediate hypersensitivity diseases are:

1. Cot death.
2. Food hypersensitivity.
3. Atopic dermatitis.
4. Goodpasture disease.
5. Myasthenia gravis.

DELAYED HYPERSENSITIVITY (TYPE IV REACTION)

Delayed or cell mediated hypersensitivity is one aspect of cell mediated immunity. The antigen activates specifically macrophages and sensitized T lymphocytes leading to secretion of lymphokines. Locally the reaction is manifested by infiltration with mononuclear cells.

Characters: These reactions have following characters:

1. Antigenic stimulus is necessary.
2. In sensitive subject reaction occurs on exposure to specific antigen, e.g. tuberculin reaction.
3. Induction period is 7 to 10 days.
4. Delayed hypersensitivity is transferred by cells from lymphoid tissue, peritoneal exudate or blood lymphocytes.

Manifestations

1. *General toxemia:* 0.1 ml tuberculin in tuberculous patient produces severe reaction which is manifested as malaise, cough, dyspnea, limb pain, vomiting, rigors and lymphopenia.
2. *Focal reaction:* If antigen in large quantity is introduced in a fresh sensitized tissue, allergic reaction with necrosis of tissue occurs, e.g. tubercular bronchopneumonia.
3. *Local reactions:* It is a typical cutaneous response. If 0.1 ml of 1 : 1000 dilution of old tuberculin is injected intradermally, there will be slowly developing inflammatory response which will be maximum in 48 hours. It subsides in 7 days.

Types: Delayed hypersensitive reactions are of two types:

a. Classical or tuberculin type.
b. Granulomatous reactions.

CLASSICAL TYPE

It plays part in clinical conditions:

1. *Infective disease (bacterial, viral, fungal and parasitic):*
   a. Bacteria hypersensitivity, e.g. Koch's phenomena (tuberculosis), psittacosis, trachoma, lymphogranuloma venerium, leprosy, syphilis, brucellosis, etc.
   b. Viral hypersensitivity, e.g. mumps and revaccination with vaccinia virus.
   c. Fungal hypersensitivity, e.g. coccidiodes, sporotrichosis, blastomycosis and aspergillosis.
   d. Parasitic hypersensitivity, e.g. hydatid cyst, filariasis, schistosomiasis, etc.
2. *Contact sensitivity:* Plant substances like poison oak, poison ivy, poison simac, laundry ink (dhobi's itch on the back or neck), cottonseed, penicillin ointment, nickel, mercuric chloride, quinine, formaldehyde, sulfonamides, etc. may cause skin dermatitis by forming papules, vesicles which ooze and desquamate.

GRANULOMATOUS REACTIONS

It is characterized by granuloma formation consisting of altered mononuclear cells, histiocytes, epitheloid cells and foreign body type of giant cells. It takes longer time than delayed hypersensitivity and need poorly soluble substances. It occurs in tuberculosis, tuberculoid leprosy, etc.

Mechanism of delayed hypersensitivity: Wax D of *Mycobacterium tuberculosis*, complete Freund's adjuvant, etc. induces delayed hypersensitivity. There are two suggestion regarding nature of agent causing delayed hypersensitivity reactions:

1. Cytophilic antibodies in low concentration may bind effectively with macrophages.
2. Sensitized cells with antigen liberate substances altering vascular permeability.

Type V or Stimulation Hypersensitivity

It is an antibody mediated hypersensitivity. In this case antibody reacts with key surface component like hormone receptor. It may switch on or stimulate the cell, e.g. thyroid over activity due to thyroid stimulating antibody which develop into Grave's disease.

Thyroid stimulating hormone from pituitary gland get attached to thyroid cell receptors thus activating adenyl cyclase in the membrane. It converts ATP to AMP. AMP stimulates activity of thyroid cell and hence secretion of thyroxine. The thyroid stimulating antibody in sera of thyrotoxic patient is actually an antibody directed against receptors of TSH. This antibody may bind to these receptors and bring about the same results as that of TSH.

SCHWARTZMAN REACTION

This is not an immune reaction but rather an alteration in factors affecting intravascular coagulation.

Culture filtrate of *Salmonella typhi* if injected intradermally in a rabbit followed 24 hours later by same filtrate intravenously, a hemorrhagic necrotic lesion develops at the site of intradermal injection. The intradermal and intravenous injections need not be of same or even related endotoxin. The absence of specificity and short interval of time between the two doses exclude any immunological basis for reaction.

The initial dose is characteristically of an endotoxin. The intravenous injection can be of variety of substances, e.g. bacterial endotoxin, starch, serum, kaolin, etc. The preparatory injection causes accumulation of leukocytes which condition the site by release of lysosomal enzymes damaging capillary wall. Following the intravenous dose there occurs intravascular clotting. The thrombi leads to necrosis of vessels walls and hemorrhage.

If both the injections are given intravenously animal dies in 12 to 24 hours after second dose. It is suggested that mechanism like Schwartzman reaction may operate in conditions like purpuric rash of meningococcal septicemia and acute hemorrhagic adrenal necrosis found in overwhelming infection.

20 Immunohematology

Blood has been considered in diseases of man right from dawn of time. Blood also was believed to restore youth and vitality of old persons. Blood transfusion became successful only after the discovery of blood groups by Landsteiner. Currently more than 400 red cell antigens are known. Most of them are poorly immunogenic, ABO and Rh blood groups bear significant importance in blood transfusion, medicine, transplantation, etc.

Blood Groups

Various blood group systems available e.g.:

1. ABO
2. Rh
3. MN
4. Lutheran
5. Lewis
6. Duffy
7. Kidd

ABO and Rh systems are the important one and are called major blood group antigens.

ABO Antigens and Isoantibodies

The ABO blood groups system was described first of all by Landsteiner in 1900. It contains 4 major blood groups on the basis of antigen A and antigen B present on the membrane of red cells (Table 20.1). Their presence or absence is under genetic control. Red cells of group A have antigen A, cells of group B antigen B and cells of group AB carry both A and B antigen while group O cells have neither A nor B antigen. Table 20.2 shows ABO distribution in India.

Group A is divided into A-l and A-2 thus increasing blood groups in ABO system to 6, i.e. A-l, A-2, B, A1B, A-2B and O. Other subgroups of group A are, 3, A4 A5. About 80 percent of group A and group AB belong to subgroup A-l and A-1B while the remainders belong to the subgroups A-2 and A-2B, respectively. Since A-2 cells are sensitive to very high titre anti-A serum, confusion may be there in blood grouping. Hence A-2 group can be interpreted as group O and A-2B as B. So it is obligatory to use anti-A serum carefully and selected appropriately that it agglutinates A2 and A-2B cells satisfactorily. Both anti-A and anti-B are natural antibodies of IgM type. These antibodies appear in blood of infant. A and B agglutinogens are not well developed at birth but by the age of one they reach full strength.

H-Substance

Red cells of all ABO groups have a common antigen, which is a precursor for formation of A and B antigens. The amount of the H substance is related to ABO group of the cells. Group O cells have the maximum quantity of H-substance while group A-2B the least

TABLE 20.1: Major ABO groups antigens

Group	*Genotypes*	*Antigen in red blood cells*	*Isoantibodies in plasma*
O	OO	—	Anti-A, Anti-B
A	AA, AO	A	Anti-B
B	BB, BO	B	Anti-A
AB	AB	AB	None

TABLE 20.2: ABO distribution in India

Group	*Frequency*
A	22%
B	33%
AB	5%
O	40%

amount. Since it is universally distributed the H substance is generally not important in blood grouping or transfusion. Sometimes A and B antigens as well as H antigens are absent from red cells. This is called Bombay or OH blood. Such persons carry anti-A, anti-B and anti-H antibodies. So the sera of such persons is incompatible with all red cells except of those with same rare blood group.

A, B and H substances are glycoproteins. In addition to red cells A, B and H substances are present in most of the tissue and body fluids. Blood group antigens are also present in secretions such as saliva, gastric juice and sweat but not CSF of certain individuals and they are called secretors. The secretion of ABH antigen is under control of two allellic genes—Se and se. Individuals inheriting Se are secretors and those with Se-se are not secretors.

Blood group antigens are encountered in animal (stomach of horse and dogs) and plants anti-A agglutinin extracted from Dolichos biflorus and anti-H from Ulex europacus). The blood group agglutinine of plant origin is called lectins.

It is worth to understand that ABO locus is on chromosome number 9. By the way Rh locus is on chromosome-1.

The Rh System

Levine and Steksib (1957) detected an antibody in the serum of woman who recently delivered a baby with Rh refers to a factor present in human red cells which was discovered in 1940 by Landsteiner and Wiener through the use of sera prepared by the injections of red corpuscles of Rhesus monkeys into rabbits and guinea pigs. It was found that the red cells of 80 percent of white persons were agglutinated by anti-Rhesus (anti-Rh) serum and the remainders failed to react this way. The former were referred as Rh positive and the latter Rh-negative.

The chemical nature of Rh substance is by and large unknown. There are different theories and nomenclature for the genes and antigens of Rh system. Fisher's hypothesis was that Rh antigens of Rh system were controlled by 3 pairs of closely linked allelomorphic genes, Cc, Dd and Ee. Every person carries one member of each pair of these genes derived from each parent. Each gene is responsible for production of a specific antigen. The antigen controlled by D locus in the strongest immunogene called Rh-D antigen. The six genes give rise to 8 allelomorphs and 8 antigenic patterns. The 6 sera corresponding to the antigens are denoted by the terms Anti-C, Anti-c, Anti-D, Anti-d and so on. Five of the 6 antisera have been detected in human but no serum containing anti-d has been found. May be the counter-part of D is not antigenic or the gene is amorphic.

As per Wiener view, Rh antigens are controlled by any one of several allelic genes present in a single locus on chromosome. The concerned gene determines the production of appropriate antigen.

The genotype is expressed by 2 letters R, R2 in Wiener's system whereas in Fisher system it is by two sets of letters CDe/cDE. However in practice, Rh positive or Rh negative blood depends on the presence of D antigen on the surface of red cells, which may be detected by testing with anti D (anti Rh serum). About 6 to 7 percent Indians are Rh negative.

Utilization of Blood Groups in Clinical Practice

1. Blood Transfusion

ABO and Rh antigens are considered for routine blood transfusion. The other blood group antigens are quite weak in this regards. In selecting appropriate donors following facts bear some importance.

- Serum or plasma of recipient should be free from antibody capable of damaging donor's erythrocytes.
- Donor should not carry any antibody that may lyse the recipient's erythrocytes.
- Donor's red cells should not possess any antigen which is deficient in the recipient.

Nowadays it is preferred that recipient and donor both have same blood group. O group used to be transfused to the recipient of any group and was called universal donor. Likewise AB group person was designated as universal recipient.

Rh compatibility is important when recipient is Rh negative. If Rh negative person receive

Rh positive blood, antibodies against Rh antigen are formed and subsequent transfusions with Rh positive blood may cause hemolytic reactions. In case of child bearing age women, hemolytic disease of newborn occurs additionally.

2. Blood Group and Diseases

- Blood group antigens may become weak in leukemia.
- *Pseudomonas aeruginosa*, become agglutinable by all blood group sera and also by normal human sera. This is called Thomsen-Friedenreich phenomenon and it may be due unmasking of hidden antigen normally present on all human red cells. This hidden antigen is known as T antigen and anti-T agglutinins are normally present in the sera of all human.
- Duodenal ulcer is found mostly in individuals of blood group O.
- Patients of cancer stomach usually belong to blood group A.

Blood transfusion can be hazardous even after matching blood group and tests from various viral infections are performed. Blood from disaster could be avoided if blood banks irradiates all blood with gamma rays. Bones from cadavers could replace metal implants if they were subjected to gamma rays. These recommendations of irradiation were made known and emphasized by Prof. Mammen Chandy at international conference on the application of radioisotopes and radiation held at Mumbai in Feb., 1988.

21 Miscellaneous

IMMUNE DEFICIENCY SYNDROME

When there lies defect in persons to produce immune response or to express immune response into effective function then there is failure in the development and function of lymphoreticular system (Table 21.1). It is associated with diminished resistance to infection.

Following are types of immune deficiency in man:

1. Reticular dysgenesis means failure of stem cell development.
2. Di George syndrome in which there is defective development of thymus.
3. Agammaglobulinemia where there is deficient production of immunoglobulins, e.g. ataxia telangiectasia.
4. Tumors that affect antibody producing cell and there is excessive production of plasma cells. They form single immunoglobulin. Such tumors are called myelomas.
5. Agranulocytosis means defect in granulocyte production.
6. Chédiak-Higashi disease in which there is neutrophil lysosomal abnormality. The other example is lazy leukocyte syndrome in which there is defective mobilization of leukocytes at sites of infection. In chronic granulomatous disease there is defect in granulocyte function.

TABLE 21.1: Clinical immunodeficiency diseases

Disorder	*Postulated B cell*	*Cell defect T cell*	*Observed immunologic defect*
1. Congenital X linked agammaglobulinemia (Biuton's)	+	–	Absent plasma cells, all classes of immunoglobulins extremely deficient ,cellular immunity normal
2. Transient hypogammaglobulinemia of infancy	+	–	Usually self limited
3. Selective immunoglobulin deficiency (IgA, IgM, IgG subclass)	+	–	IgA, IgM, IgG producing plasma cells absent, respective immunoglobulin absent, cellular immunity normal
4. Thymic hypoplasia (DiGeorge's syndrome)	–	+	Immunoglobulins normal but some antibody responses deficient. Cellular immunity defective
5. Chronic mucocutaneous	–	+	
6. Immunodeficiency with ataxia telangiectasia	+	+	Variable deficiency in immunoglobulins and antibodies. Cellular immunity defective for some antigens
7. Immunodeficiency with thrombocytopenia and eczema (Wiskott-Aldrich syndrome)	+	+	Variable deficiency of enzymes, immunoglobulins and antibodies. Cellular immunity defective for some antigen
8. Immunodeficiency (e.g. thymoma); with short limbed dwarfism, autosomal recessive, combined, X linked	+	+	Extremely deficient antibodies and cellular immunity defective for all antigens

ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

It is characterized by opportunistic infections, defective cellular immunity, autoimmune phenomenon and unusual malignancies, e.g. Kaposi's sarcoma. It may remain symptomless but sometimes certain symptoms may appear, e.g. weight loss, pyrexia and generalized adenopathy which is not responding to therapy, chronic diarrhea, involvement of brain and deep sores around mouth and anus. The four "H" type groups at high risk of contacting AIDS have been identified as: (a) Homosexuals, (b) Heroin addicts, (c) Hemophiliacs, and (d) Haitians.

Dr. Robert C. Gallo discovered HTLV—III (Human T cell lymphotropic virus) in 1983. Now it is called HIV. It has been incriminated for AIDS. Some scientists believe that AIDS may be a result of virus mutation. This is considered to be the first such mutation in medical history to have caused a virulent new disease.

It is suggested that all tissues, body fluids particularly blood, semen, saliva, other secretions and excreta must be assumed infective. So utmost care should be taken to avoid needle injury, or contamination of wound, skin lesions or mucosal surfaces. Clinical and laboratory workers should have six monthly serum samples tested for HIV. They must wear gowns, gloves and plastic aprons. They must protect eyes appropriately. AIDS patients should be nursed properly.

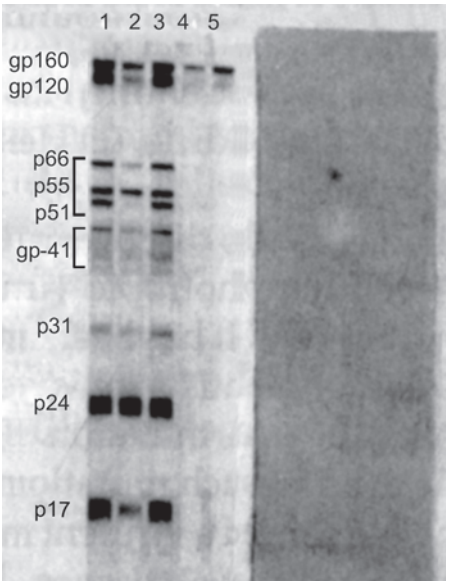


Fig. 21.1: Western blot test number 1, 2 and 3 are showing positive bands

For the diagnosis of AIDS the following procedures may be helpful:

a. ELISA
b. Western Blot Technique (Fig. 21.1) in which viral antigens are separated electrophoretically and transferred onto a nitrocellulose paper. These spots are exposed to the test specimen. If specific antibodies are present they react with corresponding antigen.

AUTOIMMUNE DISEASES

Normally body does not produce antibodies against its own constituents. In certain situations body produces autoantibodies against its own constituents.

Mechanism of Autoimmune Disease

1. Hidden antigen may not be recognized as self antigen and hence production of antibodies against hidden antigen, e.g. thyroid, lens of eye, testis, etc.
2. In some cases new antigen is formed by infection or physical or chemical processes, e.g. drugs. This mechanism may have role in rheumatoid arthritis where there is denaturation of gammaglobulin.
3. Immunization with foreign antigen results in the production of antibodies. These antibodies may cross-react with some host components, e.g. antibodies against *Streptococcus pyogenes* may cross-react with human heart muscle in rheumatic heart disease.
4. There may be break down of general mechanism of immune tolerance through genetic mutation. Breakdown of immunological homeostasis may lead to cessation of tolerance and production of forbidden clones, e.g. systemic lupus erythematosus. Table 21.2 shows classification of autoimmune diseases.

FEATURES OBSERVED IN AUTOIMMUNE DISEASES

1. Immunoglobulin levels are elevated.
2. Autoantibodies demonstration.

TABLE 21.2: Classification of autoimmune diseases

	Disease	*Antigen involved*
I.	**Diseases associated with inaccessible antigens**	
	1. Hashimoto's disease of thyroid	Organ specific antigens are not present normally is circulation
	2. Sjogren's disease	
	3. Sympathetic ophthalmia	
	4. Multiple sclerosis	
	5. Peripheral neuritis	
	6. Male sterility	
II.	**Diseases associated with common body antigen**	
	1. Rheumatoid arthritis	
	2. Acquired hemolytic anemia	Denatured γ globulin, red cell antigen,
	3. Idiopathic thrombocytopenic purpura	platelets antigen,
	4. Systemic lupus erythematosus constituents	nuclear (DNA) and cytoplasmic constituents
	5. Scleroderma	
	6. Polyarteritis nodosa	

3. Deposition of immunoglobulins at the place of lesion, e.g. renal glomeruli.
4. Lymphocytes and plasma cells are collected at lesion site.
5. Coticosteroides and immunosupressive agents are invariably useful.
6. Usually more than one autoimmuno conditions encountered in a patient.
7. Genetic predisposition.
8. It is irreversible.
9. Females are mostly susceptible to autoimmune disorders.

IMMUNOLOGY OF TRANSPLANTATION

Transplantation is one of mankind's old dream. In case of irreparable damage to organ because of disease or injury or when the organ is congenitally defective or absent, grafting or transplantation becomes inevitable for restoration of function. The individual from whom organ is obtained is the donor, the individual on whom it is applied is recipient and organ transplanted is graft or transplant.

Classification of transplant: Transplants are classified in following ways:

1. Based on the organ transplanted they are classified as heart, kidney, skin transplant.
2. On the bases of anatomical site of origin and site of placement of transplant, e.g. orthotopic, when grafts are applied in anatomical normal sites, (e.g. skin graft) whereas heterophic grafts are placed in anatomically abnormal sites, e.g. when thyroid tissue is transplanted in subcutaneous pocket.
3. Transplants could be fresh organs or stored ones.
4. Transplants may be living (kidney, heart) or dead (bone, artery), former is called vital graft whereas latter one is structural graft.
5. Transplant may be classified on the basis of genetic relationship between the donor and recipient (Table 21.3). Transplant may be autograft (taken from individual and placed on himself), isograft (placed on another individual of same genetic constitution, e.g. graft made between identical twins), and allograft (placed in genetically non-identical member of same species). Autograft and isograft are compatible with host tissue genetically and antigenically and hence are successful. On the other hand allograft and xenograft are incompatible genetically and antigenically and hence are rejected.

TABLE 21.3: Terminology of antigens, antibodies and tissue graft

Relationship between donor and recipient	*Genetic terminology*	*Antibody antigen*	*Transplantation terminology*
• Same animal	—	Auto-antibody Auto-antigen	Autograft
• Identical twins and inbred strain	Synergic (isogeneric)	—	Isograft
• Same outbred species or different inbred strains	Allogenic Iso-antigen	Iso-antibody (homograft)	Allograft
• Different species	Heterogenic Xenogenic	Hetero-antibody Hetero-antigen	Xenograft (Heterograft)

Allograft rejection: If skin graft from rabbit is applied on genetically unrelated animal of the same species, initially graft appears to be accepted up to two or three days. By fourth day graft is invaded by lymphocytes and macrophages, blood vessels get occluded with thrombi, vascularity decreases, graft undergoes ischemic necrosis and in ten days' time the graft sloughs off. This sequence of events is called first set response. In this animal which has rejected a graft by the first set response another graft from same donor is applied, it will be rejected in an accelerated way. Initially vascularization does occur but is interrupted by inflammatory response with necrosis and graft sloughs off by sixth day. This is called second set response.

Mechanism of allograft rejection (Fig. 21.2): The first set of response is brought about by T lymphocytes. Hence, it is predominantly cell mediated. Humoral antibodies are also produced during allograft rejection and may be detected by hemagglutination, lymphocyte toxicity, complement fixation and immunofluorescence. Antibodies are formed more rapidly and abundantly during second set response, of course along with cell mediated response.

Humoral antibodies may act in opposition to cell mediated response by inhibiting graft rejection. This phenomenon is called *Immunological enhancement*. If the recipient is pretreated with one or more injections of killed donor tissue and transplant applied subsequently, this transplant may survive quite longer than in control animals. It should be remembered that allograft immunity is a generalized response directed against all the antigens of donor.

Factors favoring allograft survival: (1) Immunosuppression, e.g. neonatal thymectomy, irradiation, corticosteroids and antilymphocytic serum (ALS), (2) Blood cross-matching and tissue typing for various HLA antigens. There are certain privileged sites where allograft are permitted to survive, e.g. intrauterine allograft, cartilage, brain, testis and cornea.

IMMUNOLOGICAL SURVEILLANCE

It means destruction of malignant cell that may arise by somatic mutation. Such malignant mutation does occur frequently in living body and can develop into tumor if constant vigilance of immune system is defective. Increased incidence of cancer is there because of inefficiency of surveillance mechanism on account of ageing, congenital or otherwise immunodeficiency disorders. In other words, cancer should not occur if surveillance is effective enough.

Escape from immunological surveillance may result in development of tumor. The possibilities of escape are: (i) very fast proliferation of malignant cells to an extent which may be beyond the control of immunological attack, (ii) circulating tumor antigen may coat lymphoid cells and thus preventing them from acting on malignant or tumor cells, and (iii) tumor antigen on malignant cells may not be accessible to sensitized cells.

Tumor antigens: Following are tumor surface antigens:

a. Virally controlled antigens may be produced in cell infected with oncogenic virus.

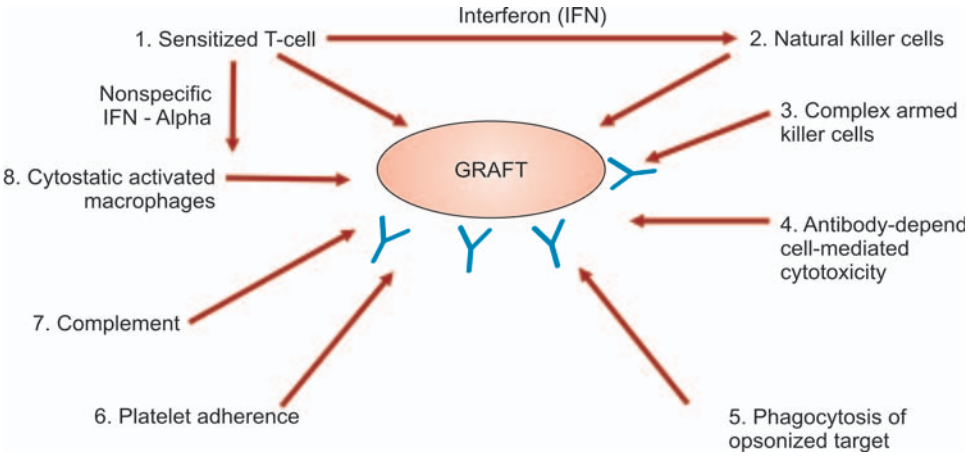


Fig. 21.2: Mechanism of graft rejection

These antigens are of two types, first is 'V' antigen which is identical with an antigen on the isolated virion. Second is 'T' which may be present only on infected cells.

b. Embryonic antigen are L-fetoprotein in hepatic carcinoma and carcinoma embryonic antigen (CEA) in cancer of intestine.
c. Idiotypic antigen: Tumors induced by benzopyrene possess specific transplantation antigen. Even when carcinogen produces two different primary tumors in same animals still each tumor produced by given chemical has its own idiotypic antigen. They may not exhibit the same antigenic specificities and do not confer cross resistance by immunization.

IMMUNOLOGICAL TOLERANCE

It is a kind of immunological unresponsiveness involving antibody production as well as cell mediated response. This non-reactivity may be specific to the particular antigen only whereas immune reaction to other antigens remains unaffected. Tolerance can be induced for any of the substances that induce antibody production. It may be induced by exposure to antigen in fetal life or by exposure to large amount of antigen in adult life.

When antigen comes in contact with lymphocytes they may respond positively (stimulation) or negatively (tolerance). Factors responsible for positive or negative response of lymphocytes are, concentration of antigen, degree to which these recognized antigens fit the combining site of antigen, the way these antigens are presented whether on molecules or cell surfaces and the presence or absence of other lymphocytes which may help to suppress immune response. Tolerance may occur when lymphocytes are confronted with high concentration of antigen. This is called high zone of immunological tolerance. Constant of small concentration of antigen below threshold is required for stimulation results in low zone immunological tolerance.

Tolerance can be overcome spontaneously to injection of cross reacting immunogens. Tolerance to living agent is more lasting than that to non-living substances. Natural occurring tolerance is found in some viral infections, e.g. rubella and cytomegalovirus in which there is constant viremia with decreased ability for production of neutralizing antibodies.

Mechanism of immunological tolerance: The mechanism is not clear. Inactivation of antigen sensitive cells may be the mechanism of tolerance production. Contact of antigen with antigen sensitive cell without being processed through macrophages or T cells may result in inhibition of antigen sensitive cells. Initiation of tolerance by this method may be further maintained by reintroduction of antigen in a suitable manner which cause inhibition of newly emerging antigen sensitive cells.

The other possible mechanism of tolerance is by alteration of regulation mechanism of immune system. Strong activity of suppressor "T" cell can give rise to state of tolerance.

HISTOCOMPATIBILITY ANTIGEN (HLA antigen)

The histocompatibility antigens are glycoprotein substances (molecular weight 59,000) associated with plasma membrane of certain cells of the body. They may act as receptors for various enzymes. Virus induced tumors in animals do contain new histocompatible antigen on the cell surfaces.

The histocompatibility antigens are controlled by a complex linked genes at two regions of chromosomes called subloci. The linked genes are transmitted in a group of alleles (genetic matter) contributed by each of the parents. The precise genetic loci of these genes have not been completely understood. H-2 antigen system was found to be the major histocompatibility antigen for mice and coded for by a closely linked multi-allelic cluster of genes, which was called major histocompatibility complex (MHC). Snell successfully reported the detailed analysis of various loci of this complex by developing the congenie and recombinant strains of mice.

The major antigen determining histocompatibility in man are alloantigen found characteristically on surface of leukocytes. They are HLA and the set of genes coding for them are HLA complex.

TABLE 21.4: Structure of major histocompatibility complex on chromosome-6

Genes	*Centromere*	*DP-DQ-DR-C2-B1-C4-B-C-A*	
Gene Products	Class 2 HLA	1. Complement Components 2. Factor-B	Class I HLA

The HLA complex of genes is located on the short arm of chromosome-6 (Table 21.4). They are:

HLA-A, B, C (Class I).

HLA-D, DR, DQ and DP (Class II) (Fig. 21.3).

Class I antigens (HLA-A and HLA-B) are present on all nucleated cells. Whereas class 2 antigens (HLA-D) have more limited tissue distribution including B lymphocytes, macrophages and activated T cells.

T cytotoxic cells only recognize antigens, e.g. virus, in association with HLA class-I molecules on the surface of a cell thus guiding the T toxic cells to its surface. In case of T helper cells recognize antigen on macrophages and B cells in association with the HLA-D, class 2 antigens (Table 21.5).

HLA loci are multiallelic which means the gene occupying locus can be any one of alternative forms. Each form (allele) determines definite antigen. However, HLA system seems to be pleomorphic with over 23 alleles at HLA locus A and 48 at B. Officially recognized alleles are designated by the locus and a number, e.g. HLA-A_1 HLA-B, etc. (Table 21.6).

HLA specificities are often associated with peculiar diseases, e.g. HLA-B_{27} (ankylosing spondylitis), B_8 (myasthenia gravis), DW, (rheumatoid arthritis) and DW_2 (multiple sclerosis),

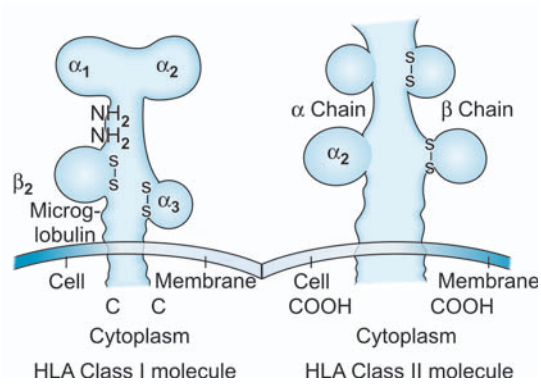


Fig. 21.3: HLA class I molecule and HLA class II molecule

TABLE 21.5: MHC depicting classes, molecules and their functional association with antigen

Class	*II*	*III*	*I*	*IV*
Molecules	DP DO DR	C2, C4, B1	BCA	TLa
Functional Association	Immune associated	Complement	Transplantation antigen	Primitive antigen

TABLE 21.6: The major histocompatibility complex of man

Locus	*Alleles*
HLA — A	1,2,3,9,10,11, AW.
HLA — B	5,7,8,12,13,14,15,17,18,21, BW.
HLA — C	W-1 to 8
HLA — D	W-1 to 11 (Las's)
HLA — DR	DR-1 to DR-6 DRW.
DQ	DQW-1 to DPW-6
DP	DPW-1 to DPW-6
MLC	MLC locus

etc. HLA profile of rheumatic fever patients was studied. DR3 locus antigen is found to be an important immunogenetic marker.

These antigens participate in graft rejection. The major histocompatibility system in man is the human leukocyte antigen (HL—A) system. The HLA complex also bears a complement region (C_2, 4 and properdin factor B) which is mapped between the HLA — B and HLA-DR. It readily evokes the formation of complement fixing cytotoxic antibodies. Mostly HLA—A and HLA—B antigens induce stronger immunological response and these are considered for tissue typing mostly. Recently a new gene is identified. It is RING gene and is located within the class II region of MHC and found to encode molecules which might be involved in the antigen processing, presentation for class-I molecules and recognition T-cells.

An individual inherits one antigen from either of the parents. Thus the individual is typed for two A and two B series antigens. The C and D loci antigens are less important and at the same time, these are weak antigens. This system is useful to do tissue typing and matching to establish compatibility between donor and recipient tissue. Since there are many antigens in major histocompatible system, tissue typing is rather a complicated process. It consists of classifying individuals into tissue types by using antisera specific for various HL—A antigens.

For tissue typing antigen is preferably obtained from lymphocytes. Alternatively other tissue like erythrocytes, reticulocytes, kidney cells and spermatozoa may be procured for the preparation of antigen. Source of antisera are: multiparous women, recipients of multiple blood transfusions, volunteers who are immune to lymphocytes or already on skin graft and lastly patients who have rejected HLA incompatible graft.

In short HLA typing is useful in transplantation, disputed paternity, arthropological studies, susceptibility to diseases.

Tissue typing may be done by three methods:

1. Normal lymphocyte transfer test is performed by injecting intradermally to each donors peripheral blood leukocytes from recipient. Delayed hypersensitivity reaction at the site of injection indicates immune response of recipient leukocytes against donor tissue. The person exhibiting no immune response is the best donor for the recipients.
2. The mixed leukocyte culture (MLC) is based on the principle that T lymphocytes when exposed to HLA incompatible antigen will undergo blast transformation, take up thymidine and divide.
3. Platelets with fixation test.

MHC Restriction

Degradation of antigen is done in lymph node macrophage, fraction of which may appear on its surface. Both macrophage or other cells must possess same MHC antigen for successful immune response and this is called MHC restriction. Cytotoxic T cell (CD-8) responds to antigen with the help of class I antigen whereas helper T cells (CD-4) recognize class MHC antigen.

LIMULUS AMEBOCYTE LYSATE TEST (LAL TEST)

Principle: *Limulus* amebocyte lysate (extract of blood cells from horseshoe crab) forms a firm clot when incubated with endotoxin at 37°C).

This test is less expensive and rapid semi-quantitative which may be useful as under:

1. Detection of lipopolysaccharide (LPS) in toxemia or septicemia.
2. Detection of purity of fluid used for intravenous therapy.
3. Detection of water pollution with Gram negative bacilli.
4. Detection of Gram negative bacilli in food.
5. Diagnosis of meningitis due to Gram negative organisms.

RADIOIMMUNOASSAY METHOD

This is useful for accurate quantitative estimation of polypeptide hormones. It is specific, precise and simple method available.

Principle: Radio iodine labelled hormones compete with non-labelled hormones of a sample under test for antihormone antibodies. The more of the hormone in test sample the less chance the labelled hormone has of combining with the limited number of antibody molecules. Thus by measuring the quantity of labelled hormone combined with antibody a measure in the test sample can be obtained.

This method is useful for an assay of IgE levels in serum and for detection of Australia antigen in human serum.

Immunoblotting

Here mixture of antigen is electrophoretically separated in a polyacrylamide gel. The separated antigens are passed on or blotted electrophoretically from gel to nitrocellulose paper and incubated with antibody No. A (against antigen). Antibody No. A is then revealed by another antibody (antibody No. B). Nitrocellulose sheet with transferred DNA is called Southern blot and when same technique is applied to analyze transferred RNA, it is called Northern blot. Nitrocellulose sheet with transferred proteins (Antibodies to HIV) is called Western blot.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

It is especially useful for detection of rotavirus and Hepatitis-A virus (stool samples). This method is widely accepted as reagents are stable and cheap. Procedure is easy to

understand and perform. Simplicity and sensitivity are the highlights of ELISA.

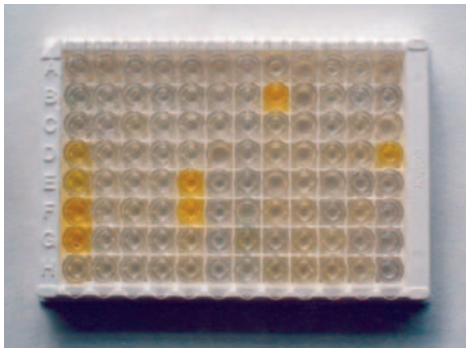
Principle: Antigen is coated on plastic plate or tube. To it is added serum which is supposed to contain antibodies. Addition of antiglobulin linked with enzymes (alkaline phosphatase or peroxidase) is done. This will attach with antigen antibody complex. Now add enzyme substrate (p-nitrophenyl phosphate). There will be color reaction indicating the presence of antibodies.

Procedure: Absorb antigen, (e.g. in malaria schizont may be used as an antigen) to the surface of plastic plates or tubes. Excess of antigen is removed by washing. Test material is then added, say patient serum. Unbound antibody is removed by washing. Now enzyme (alkaline phosphatase) linked antihuman gammaglobulin (known) is added. Again excess is removed by washing. This mixture is incubated at 37°C. Finally corresponding enzyme substrate, (p-nitrophenyl phosphate) is added which may be hydrolized by enzyme yielding yellow product. The optical density of this yellow product is measured in a spectrophotometer (Figs 21.4A to C). The optical density is directly proportional to the amount of enzyme deposited on plates or tubes which in turn is related to the amount of antibodies in a test sample. Likewise we may detect antigen from specimen by coating antibody on the surface of plates or tubes and adding enzyme-linked known antigen. Rest of the procedure is same.

ELISA is useful for assay of hormones (estrogen, progesterone, HCG, T_3, T_4), oncoprotein (alpha 2-hepatoglobulin), snake poisoning, virological infections (Hepatitis B antigen, Hepatitis A antigen, Herpes simplex, Rota, Rubella, EB, CMV, and antibodies to influenza and measle viruses). Beside these it is often useful in bacteriological conditions (enteric infection, antibodies against M protein of streptococci, *E. coli* antibodies in urinary tract infection in urine specimen, *Mycobacterium tuberculosis*, leprosy, etc.), fungal diseases (candidiasis, aspergillosis), surveillance of disease in particular area and histochemical complex reactions.

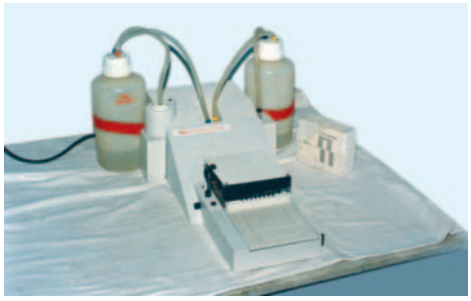
MONOCLONAL ANTIBODIES

Hybridoma technology for the production of antibodies was introduced by Kohler and



A

B



C

Figs 21.4A to C: A. ELISA plates B. ELISA plate reader C. ELISA plate washer

TABLE 21.7: Properties of monoclonal and conventional antibodies

	Property	*Conventional antibody*	*Monoclonal antibody*
1.	Useful antibody contents	Low	High
2.	Composition	— Heterogeneous — Multiple (variable from animal to animal)	— Homogeneous — Defined and consistent
3.	Cross-reaction with other antigen	Partial with antigen bearing common antigenic determinant	Usually absent
4.	Class and subclass of immunoglobulin	Mixture of many classes	Only one
5.	Supply	Limited	Abundant

Milstein for which they have been awarded the Noble prize in medicine in 1984. Now it is possible to successfully generate not only mouse hybridomas but also interspecies (rat-mouse and human-mouse) hybridomas. Even human to human hybridomas have also been produced.

Monoclonal antibodies derived from one clone of cells are homogenous, i.e. composed of same class of immunoglobulin with identical combining site (specificity), affinity and physiochemical properties. The advantages of monoclonal antibodies versus conventional antisera are summarized in the Table 21.7.

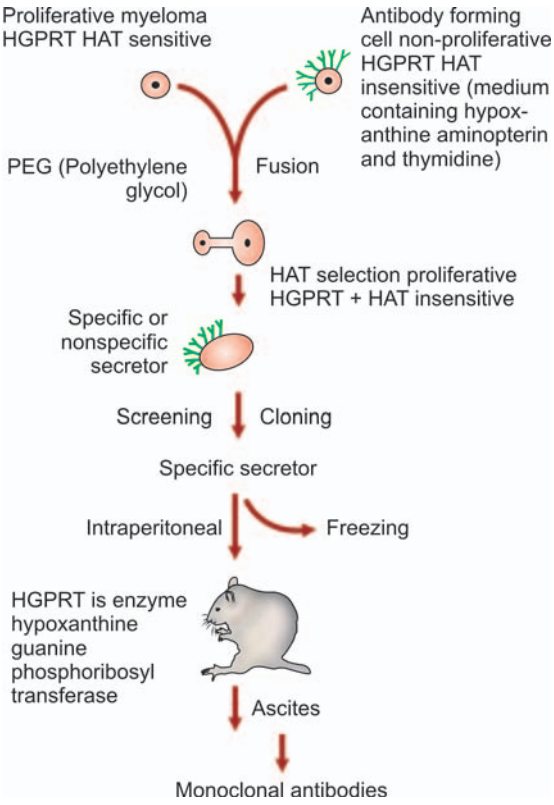


Fig. 21.5: Formation of monoclonal antibodies

Hybrids are made from two types of cells: (a) antibody forming cells, i.e. "B" lymphocyte with limited life span, and (b) myeloma cells with ability to multiply indefinitely (Fig. 21.5). The hybrids secreting the desired antibody are cloned and may be maintained in culture or frozen or grown as solid tumor or as an ascitic tumor in mouse peritoneal cavity. The yield of the antibody is usually up to 100 mg/L tissue culture medium or up to 10 gm/L in serum or ascitic fluid tapped from peritoneal cavity of tumor bearing mice for producing human monoclonal antibodies. Lymphocytes from peripheral blood film, spleen, lymph nodes of immunized volunteers are used as fusion partners (Fig. 21.5).

Hybridoma technique has been widely adapted to generate monoclonal antibodies against a wide variety of antigen as shown in the Table 21.8.

TABLE 21.8: Mouse/human monoclonal antibodies of clinical interest

Normal tissue	Hemopoietic cell antigen, thymocytes, kidney, liver, heart, limbic system neurons and tryphoblast antigen.
Bacteria	Streptococcus group B, *Escherichia coli,* pneumococcus, *Neisseria meningitis, Neisseria gonococci, M. leprae* and mycoplasma.
Viruses	Measles, Hepatitis "B" surface antigen, influenza virus, and Epstein-Barr virus.
Parasites	*Plasmodium falciparum* and *Toxoplasma gondii.*
Hormones	HCG, progesterone, vasopressin, growth hormone, estradiol 17B.
Tumor associated	Leukemia, lymphoma, carcinoma, sarcoma and neuroblastoma.
Miscellaneous	HLA—A/B/C/DR, microglobulin, transferrin receptor, acid phosphatase, digoxin, tetanus toxoid. Clq, H—Y antigen, etc.

The relative eclipse of interest in the *in vivo* use of antibacterial antibodies that followed the introduction of chemotherapy and antibiotics has now been reversed by introduction of monoclonal antibodies and can be produced in unlimited quantities of specific immunoglobulin.

Monoclonal antibodies have numerous application in the diagnosis, treatment and prophylaxis of various clinical disorders (Table 21.8). They provide powerful means for the study of pathogenesis of many diseases especially those related to autoimmune and immune deficiency. Formation of monoclonal antibodies is illustrated below.

NOBEL PRIZE IN MEDICINE 1987

Generation of Immunoglobulin Diversity

The Nobel Assembly of Karolinska Institute in Stockholm awarded the Nobel prize in medicine 1987 to Susumu Tonegawa, a Japanese scientist for his discovery of the genetic principle for the generation of antibody diversity.

We are aware that no immunoglobulin can be produced by a cell unless it has a blueprint for it in its genes. Since the human genome contains only about 100,000 genes it seems unreasonable that they could allow the production of a billion of different antibodies. This was the dilemma unsolved for four to five decades.

Tonegawa, in an important series of experiment reported in 1976, showed for the first time that different genes coding for the proteins that make up an immunoglobulin had physically moved closer on chromosome, thus making it possible to combine at random different proteins that make up these antibodies. He used genetic engineering techniques to compare the arrangement of genes in embryonic and adult mouse B cells. When the embryonic cells mature into adult cells, as they develop, they shuffle the genes, so that some genes end up closer together on the chromosomes.

Tonegawa's experiment showed that during development of a mammal, one each of these different genes (in humans there are 200 different 'V' genes, 20 'D' genes and 4 'J' genes on variable part of heavy chain of immunoglobulin molecule) come together at random producing thousands of different possible variable regions. Since there are four possible variable regions to each immunoglobulin, and on top of this the genes are themselves inherited in a random fashion there are billions of possible variations in the final group of immunoglobulins. It means that the blueprint for different types of proteins are already existent. The foreign protein particle on identification has only to be matched with a corresponding B-cell for immunoglobulin production to start.

This pioneer finding along with genetic engineering which is fast developing today, will, of course, open new avenues for the treatment of many refractory diseases.

Tonegawa performed his valuable experiments during the time he spent working at the Basel Institute for Immunology in Switzerland between 1971 and 1981. In 1981, he moved to Massachusetts Institute of Technology where he has concentrated on the molecular genetics of the T cell receptors.

Part IV

Systematic Bacteriology

22. Staphylococcus
23. Streptococcus
24. Pneumococcus
25. Neisseria
26. Corynebacterium
27. Mycobacteria
28. Bacillus
29. Clostridium
30. Enterobacteriaceae
31. Pseudomonas
32. Vibrio
33. Campylobacter
34. *Helicobacter pyloridis*
35. Brucella
36. Pasteurella, Yersinia and Francisella
37. Hemophilus
38. Bordetella
39. Spirochaetes
40. Miscellaneous Bacteria
41. Rickettsiae
42. Chlamydiae
43. Newer Bacteria
44. Microbiology of Oral Cavity

22 Staphylococcus

MICROCOCCACEAE

The family Micrococcaceae has 3 genera:

a. Micrococci are found on skin or mucous membrane. They are usually not associated with infection.
b. Planococci are motile cocci and are not pathogenic in man. They are arranged in tetrads and produce a yellow brown pigment on nutrient agar, e.g Planococcus citreus and Planococcus halophilus.
c. Staphylococcus genus possesses 30 species. 14 Species are associated with man and animals. Quite a number of CNS infections are caused by *Staphylococcus epidermidis* (70%) followed by *Staphylococcus saprophyticus, Staphylococcus hominis, Staphylococcus simulans, Staphylococcus hemolyticus,* etc.

Staphylococci were first seen in pus by Koch in 1878. Pasteur cultivated them for the first time in 1880. They were named by Sir Alexander Ogston in 1881.

General characters. They are Gram positive cocci, ovoid or spheroidal, non-motile arranged in groups. On nutrient agar they form colonies, white, yellow or golden yellow in color. Their hemolytic capacity is variable. Pathogenic strains produce coagulase, ferment sugar (glucose, lactose, mannitol) with acid production, liquefy gelatin and produce pus in lesion. Under the influence of certain chemicals, (e.g. penicillin) they are lysed or changed into L forms. They are not affected by bile salts or optochin.

Classification

A. On the basis of pigment production 3 types of staphylococci are identified.
   1. *Staphyloccus aureus* produces golden yellow colonies and are pathogenic.
   2. *Staphylococcus albus* produces white colonies and may be non-pathogenic.
   3. *Staphylococcus citreus* produces lemon yellow colonies and are non-pathogenic.

B. On the basis of pathogenecity:
   1. Pathogenic species — *Staphylococcus aureus.*
   2. Non-pathogen species—*Staphylococcus epidermidis.*

C. Baird-Parker classified staphylococcus into subgroups (I-VI). The tests used for classification include coagulase and phosphatase tests, acid production from glucose, arabinose, lactose, mannitol, acetoin and pigment production (Table 22.1).

STAPHYLOCOCCUS AUREUS (Staphylococcus pyogenes)

Morphology: It is ovoid or spherical, (0.8 to 0.9 µ), non-motile, rarely capsulated, non-sporing strain with ordinary aniline dye and is Gram-positive. It is arranged in clusters (grape-like) (Figs 22.1 and 22.2). Cluster formation is due to cell division occurring in three planes with daughter cells tending to remain in close proximity.

Cultural characteristics: *Staphylococcus aureus* is aerobic and grows readily on simple media at optimum temperature 37°C and pH 7.4.

Fluid media: It produces uniform turbidity. No pigment is produced.

Nutrient agar: After 24 hours' incubation colony is pigmented, golden yellow, 2 to 4 mm (pinhead size), circular, convex, smooth, shiny,

TABLE 22.1: Baird-Parker's classification of staphylococci

Tests	Subgroups of staphylococcus						Micrococcus
	I	*II*	*III*	*IV*	*V*	*VI*	
1. Huge and Leifson	Ferments	Ferments	Ferments	Ferments	Ferments	Ferments	Oxidative
2. Coagulase	+	–	–	–	–	–	–
3. Phosphatase	+	+	+	–	–	–	–
4. VP	+	+	–	+	+	+	Variable
5. Arabinose	–	–	–	–	–	–	Variable
6. Lactose	A	A	Variable	–	A	Variable	Variable
7. Maltose	A	A	–	Variable	A	Variable	A
8. Mannitol	A	–	–	–	–	A	Variable
9. Pink pigment	–	–	–	–	–	–	+*

+* *Micrococcus roseus*

opaque, with entire edge and emulsifies easily. Pigment production occurs optimally at 22°C and only in aerobic culture. Pigment production is enhanced when 1 percent glycerol monoacetate or milk is incorporated in medium. Pigment is lipoprotein.

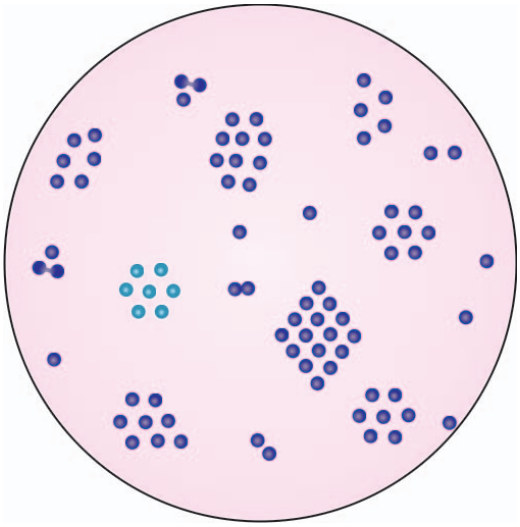


Fig. 22.1: Colonies of *Staphylococcus aureus*

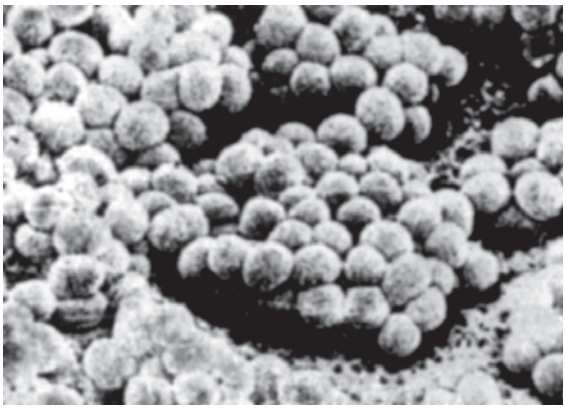


Fig. 22.2: Colonies of *Staphylococcus aureus* (electron microscopic)

Blood agar: A wide zone of beta hemolysis (clear zone) is produced around colonies. Hemolysis is marked on rabbit or sheep blood and weak on horse blood agar.

Egg yolk medium: The organism produces opacity on glucose egg yolk medium through lipolytic enzyme which acts on lipoprotein of yolk (lipovitellin).

MacConkey medium: Colonies are small and pink in color (lactose fermenter).

Selective Media

1. Media containing 8 to 10 percent NaCl (salt milk agar, salt broth).
2. Lithium chloride.
3. Tellurite.
4. Polymyxin.
5. Mannitol salt agar (Fig 22.3).

Fig. 22.3: Mannitol salt agar *Staphylococcus aureus*

Factors Influencing Pigment Production

1. **Temperature:** Maximum pigment production is at room temperature 20° to 25°C.
2. **Oxygen:** Pigment is produced under aerobic condition.
3. **Medium:** Pigment is produced on solid medium. Milk agar and glycerol monoacetate are useful in rapid identification of *Staphylococcus aureus* on the basis of pigment production.
4. **Light:** In presence of light pigmentation of colony is better.

Biochemical Reactions

It ferments a number of sugars producing acid and no gas (glucose, lactose, sucrose, maltose, mannitol). Fermentation of mannitol is important in *Staphylococcus aureus*. It is catalase positive and coagulase positive. It liquefies gelatin. It is lipolytic in media containing egg yolk medium. Phosphatase is produced only by *Staphylococcus aureus.*

Characteristics of Pathogenic Strain

1. Coagulase positive.
2. Mannitol fermentation.
3. Beta hemolysis.
4. Golden yellow pigment.
5. Liquefies gelatin.
6. Phosphatase is produced.
7. Sensitivity to lysostaphin.
8. Hydrolyses urea.
9. Reduces nitrates to nitrites.
10. Tellurite reduction.
11. Deoxyribose nuclease enzyme production.

Resistance: It is among the more resistant of non-sporing organisms. It withstands 60°C for 30 minutes. It resists 1 percent phenol for 15 minutes.

Mercury perchloride (1%) kills it in 10 minutes. Crystal violet (1 in 5,00,000) and brilliant green (one in 10 million) is lethal for it. Staphylococcal resistance to penicillin is due to the production of an enzyme penicillinase (beta lactamase) which inactivate penicillins. Broadly, resistance falls into several classes:

a. Beta lactamase is under plasmid control.
b. Methicillin resistance.
c. Plasmids can carry genes for resistance to tetracycline erythromycin and aminoglycosides.
d. "Tolerance" implies that Staphylococci are inhibited by drug and not killed by it.

Antigenic structure: It has specific antigens in cell wall (Fig. 22.4). It can be demonstrated by agglutination or precipitation. Pathogenic strains are more uniform and 15 types have been recognized.

Staphylococci contain both antigenic polysaccharides and proteins that permit grouping of strains to a limited extent. Peptidoglycan, a polysaccharide plays an important role in the pathogenesis of staphylococcal infection. It elicits production of interleukin-1 and opsonic antibodies by monocytes. It can also be a chemoattractant for polymorphonuclear leukocyte. It produces a localized Schwartzman phenomenon and activates complement. Teichoic acids (polymers of glycerol or ribitol phosphate) linked to cell wall peptidoglycon can be antigenic. Antiteichoic antibodies may be particularly

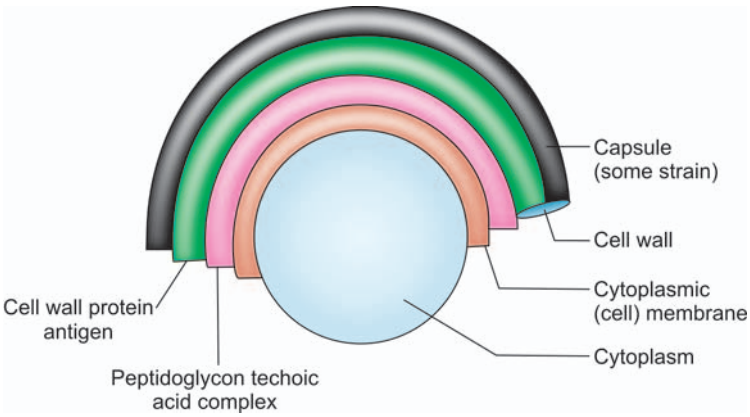


Fig. 22.4: Antigenic structure of staphylococci

associated with active staphylococcal endocarditis. Surface protein may interfere with phagocytosis.

Protein A, a cell wall component of *Staphylococcus aureus* (Cowan 1 strain) happens to bind strongly to Fc portion of any IgG molecule. This makes the Fab portion of an antibody molecule to face outwards, so that it is free to combine with specific antigen. Staphylococcal protein A is involved in coagglutination. Moreover protein A may interfere with phagocytosis and cause damage to platelets.

Antigenic structure is however of little use in the identification of staphylococcus.

BIOTYPES

There are 6 biotypes (A, B, C, D, E, F and G). Biotype A strain is pathogenic to man. Biotype A is characterized by pigment production, fibrinolysis, coagulation test positive, hemolysin (α or β), telberite reduction, etc.

Bacteriophage typing: The phage typing is useful in the investigation of *Staphylococcus aureus* infection. An internationally accepted set of phages is used for typing. Staphylococcal phage typing is by pattern method (Fig. 22.5).

A set of twenty eight phage may be employed. The strains are divided into four groups. Human strains belong to phage group I, II or III. Hospital infections are usually due to strains belonging to group I or III.

Typing set of staphylococcal phages.

Group I.	29, 52, 52A, 79, 80.
Group II.	3A, 3B, 3C, 55, 71.
Group III.	6, 7, 42 E, 47, 53, 54, 75, 77, 83 A, 84, 85.
Not Allocated	42D, 81, 94, 95, 96.

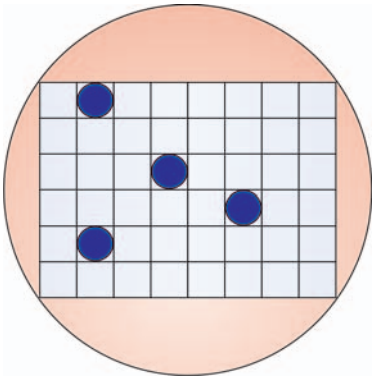


Fig. 22.5: Bacteriophage typing of staphylococci

Enzymes Produced

1. *Coagulase (free coagulase):* It is filterable and heat labile enzyme produced in lag and early log phase of bacterial growth. It is antigenic. About 7 distinct antigenic types have been described. Human strain produces A variety. It has antiphagocytic action.

   Clotting of human or rabbit plasma is brought about by coagulase. Along with coagulase reacting factor (CRF) present in plasma it converts fibrinogen into fibrin.

   Enzyme coagulase can be demonstrated by mixing 0.1 ml of an overnight broth culture of organism and 0.9 ml of 1 in 10 diluted rabbit plasma in a glass tube and incubated at 37°C. Result is noted after every 2 hours. The plasma forms clot if organism is coagulase positive.
2. *Clumping factor* (bound coagulase): It is heat stable protein. Antigenically it is homogenous. It does not require coagulase reacting factor (CRF) and fibrinogen is not converted into fibrin.

   Clumping factor is a component of cell wall. It can be liberated upon autolysis of the cell.

   When a drop of saline suspension of *Staphylococcus aureus* is mixed with rabbit plasma the cocci are clumped or agglutinated. This clumping of staphylococcus is due to combination of fibrinogen with receptor present on the surface of organism. It is not associated with pathogenecity of organisms.

   Table 22.2 depicts difference between clumping factor and coagulase factor.

TABLE 22.2: Differences between clumping factor and coagulase factor

Clumping factor	*Coagulase factor*
1. It remains on the surface of the organism	It is secreted outside the body of the organism.
2. It is heat stable	It is heat labile.
3. One type of clumping factor is present serologically	Seven types of coagulase are differentiated. They are named as A, B, C, D, E, F and G. All human strains of *Staphylococcus aureus* produce "A" type of coagulase

3. *Phosphatase:* It is produced by coagulase positive strain of staphylococci. For its demonstration, organisms are cultured on agar medium containing phenolphthalein diphosphate. When such mixture is exposed to ammonia vapour, colonies assume a bright pink color due to the presence of free phenolphthalein.
4. *Hyaluronidase:* Most strains of *Staphylococcus aureus* form hyaluronidase especially those producing impetigo contagiosa.
5. *Deoxyribonuclease (Fig. 22.6):* Coagulase positive staphylococcus also produces this enzyme.
6. Colony compacting factor.

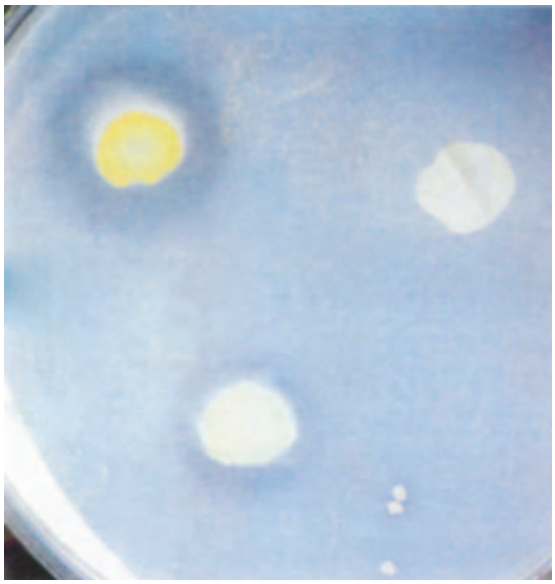


Fig. 22.6: DNase activity of *Staphylococcus aureus* observed on DNase test agar (M482) flooded with 1 N HCl

Toxins

1. *Hemolysin: Staphylococcus aureus* produces at least 4 types of hemolysin known as alpha, beta, gamma and delta. All hemolysins are antigenically distinct. Alpha hemolysin may cause hemolysis of rabbit and sheep red cell rapidly. Alpha hemolysis is produced by coagulase positive strain and is important in the pathogenesis of infection in man. It is leucocidal, cytotoxic, dermonecrotoxic and lethal. It is antigenic and is neutralized by antitoxin.

   Beta hemolysin is produced by staphylococcus isolated from animals. It is hemolytic for sheep red cells. It is produced aerobically as well as anaerobically. It is less toxic to laboratory animals.

   Delta lysin is lytic for sheep RBC. It is less toxic to laboratory animals.

   The gamma lysin, the weakest of other hemolysins, acts on humans, sheep, rabbit and monkey erythrocytes.
2. *Leucocidin:*
   i. The alpha lysin has leucocidal activity.
   ii. Panto valentine is an independent toxin which kills human and rabbit polymorph and macrophages without lysing them. It is heat and oxygen labile and consisting of 2 antigenically distinct protein F and S.
   iii. Leukolysin is closely associated with delta lysin. It is thermostable.
3. *Enterotoxin:* The toxin is responsible for manifestations of staphylococcus poisoning—nausea, vomiting and diarrhea within 6 hours of taking contaminated food. It is heat stable. It is antigenic and is neutralized by antitoxin. It is a trypsin resistant protein. Six antigenic type have been distinguished (A to F). Type A and B are most common. Ingestion of 25 µg of enterotoxin B results in vomiting and diarrhea in humans or monkeys.

   Strain producing this toxin belongs to bacteriophage group III (66/47). Its mode of action is not known. It is believed that it acts peripherally on sensory nerve endings of smooth muscle of intestine. It has emetic effect on cats perhaps by acting centrally.
4. *Fibrinolysin: Staphylococcus aureus* produces staphylokinase during the later stages of growth which causes lysis of fibrin. This fibrinolysin may dislodge the infected intravascular thrombi and then contribute to the development of staphylococcus septicemia.
5. *Exfoliative toxin:* It consists of two proteins which may cause desquamation of staphylococcal scalded skin syndrome. Specific antibodies against it are protective.
6. *Toxic shock syndrome toxin:* Some strains of staphylococci may produce a toxin called toxic shock syndrome toxin-1 which resembles enterotoxin-F and exotoxin-C. In man it may cause fever, shock, skin rash, etc.

It may involve many organs of man. In rabbit it causes effects like toxic shock syndrome but lack skin rash with desquamation.

Other Toxins

a. Nucleases
b. Lipases
c. Proteases
d. Scarlatina toxin.

Numerous staphylococcus strains are found in air, dust, clothing and fomites. They may be pathogenic to newborn babies when they come from fomites. These may be normally present on skin, nose and mouth. They may enter skin through hair follicles, sebaceous glands, sweat glands, cracks, abrasions or injury to the skin.

Virulence Factors

The virulence factors important in *Staphylococcus aureus* are:

1. Cell wall polysaccharide which confer rigidity and integrity to bacterial cells. Besides it activates complement and induces release of inflammatory cytokines.
2. Teichoic acid is an antigenic part of cell wall. It facilitates attachment of cocci to host cell surfaces. It also protects the cocci complement mediated apsonization.
3. Capsule around the bacteria inhibits apsonization.
4. Protein A is chemotactic, antiphagocytic and anticomplementary. It induces platelet damage and hypersensitivity. Protein A is also a 'B' cell mitogen. It has been used as ligands for isolation of IgG.
5. Clumping factor is the bound coagulase.
6. Coagulase.
7. Lipases help the organism in infecting skin and subcutaneous tissues.
8. Hyaluronidase
9. Nuclease is heat stable in *Staphylococcus aureus*.
10. Protein receptors facilitate staphylococcal adhesion to host cell tissue, e.g. fibronectin, fibrinogen, IgG and C1q.

Pathogenecity: Staphylococcus may cause the majority of acute pyogenic lesions in man. Staphylococcal lesions are characteristically localized. Staphylococcal disease may be classified as:

A. *Cutaneous lesions:* Furuncles, styes, boils, abscess, carbuncles, impetigo, pemphigus neonatorum.

The majority of hospital cross infections are of staphylococcal in origin.

B. *Deep infection:* Acute osteomyelitis, tonsillitis, pharyngitis, sinusitis, pneumonia, pulmonary abscess, breast abscess, meningitis, endocarditis and renal abscesses. Staphylococcal septicemia is a rare but serious disease. Staphylococci of phage group II cause bullous exfoliation—the "scalded skin" syndrome. Toxic shock syndrome usually begins within 5 days of onset of menses in young women who use tampons. There is an abrupt onset of high fever, vomiting, diarrhea, myalgias, scarlatiniform rash, hypotension with cardiac and renal failure. This syndrome may recur in successive menstrual periods and is tentatively attributed to staphylococcal toxin.

C. *Staphylococcal food poisoning:* It results when food contaminated with enterotoxin, produced by staphylococcus is consumed, e.g. meat, fish, milk and milk products. Diarrhea and vomiting set in within 6 hours of taking contaminated food.

Laboratory Diagnosis

(a) Hematological Investigations

i. *Total leukocyte counts:* There is leukocytosis and total leukocyte count is above 10,000 cells per cubic mm.
ii. *Differential leukocyte count:* There is increase in neutrophil count. It is usually more than 80 percent.

(b) Bacteriological Methods

Specimen like pus from suppurative lesion and sputum from respiratory infection is taken. In food poisoning, feces and remains of suspected food should be collected. For detection of carrier usual specimen like nasal swab, swabs from perenium and umbilical stump is necessary. It is studied as follows:

i. *Smear examination:* A smear is prepared from the specimen obtained as above. It is stained with Gram method. Stained

smear shows a large number of pus cells and Gram positive cocci arranged in cluster or in small groups.

ii. *Isolation of organism:* The specimen is plated on blood agar media. Staphylococcal colonies appear after overnight incubation at 37°C. They are golden yellow colonies, showing beta hemolysis. The colonies are tested for coagulase and phosphatase production. Antibiotic sensitivity test is done as a guideline to treatment. Bacteriophage typing may be done if the information is required for epidemiological purposes.

Swabs from carrier's feces and from food poisoning are inoculated on selective media like Ludlam's and salt agar media.

iii. *Serological diagnosis:* It is helpful in the diagnosis of hidden deep infections. This includes demonstration of specific antibodies, (e.g. antibodies to teichoic acid in staphylococcal endocarditis), and toxin or leukocidin in patient's blood.

iv. Bacteriophage typing, antibiogram, plasmid, typing, ribotyping and DNA finger printing can be done.

SEROLOGICAL METHODS FOR ASSAY OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN

1. Microdouble diffusion in agar.
2. Double diffuse tube technique.
3. Electroimmunodiffusion.
4. Indirect hemagglutination (IHA) which is quite rapid (results in 4 hours).
5. Solid phase radio-immunoassay which is also a rapid method.
6. ELISA.

Treatment: Benzyl penicillin is a quite effective antibiotic provided strain is sensitive.

Penicillin resistance may be classified into following three types:

1. Production of beta lactamase, e.g. *Staphylococcus aureus* may produce four forms of penicilinase namely A, B, C and D. It may be noted that form A penicilinase is hospital strain. As a matter of fact penicilinase production is controlled by plasmid.
2. It may be by alteration in bacterial receptors. It reduces binding of beta lactam to cells. This may happen at 30 °C and is chromosomal in nature. This resistance includes beta lacamase resistant penicillin, e.g. methicillin and cloxacillin. These strains are also called epidemic methicillin resistant *Staphylococcus aureus* (EMRSA).
3. There may be development of tolerance to penicillin. It means that organism is inhibited and not killed.

Methicillin and cloxacillin are effective against penicillinase producing strains. At present vancomycin is the most effective drug against staphylococci. If topical application is required bacitracin is sufficient. In chronic cases who are resistant to usual therapeutic measures autovaccine may be tried. In some cases autovaccine does show considerable success.

Two types 5 and 8 are found to comprise about 70 percent of isolate from blood of patients. Polyclonal or monoclonal antibody from hybridoma culture (capsular polysaccharides) have been demonstrated to exert opsonophagocytic activity. The protective effects of these antibodies generate hope in the preparation of vaccine.

METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

It was isolated in 1960. They are emerging increasingly because of indiscriminate use of antibiotics. Methicillin resistance is most accurately found by standard agar screen. Classic methicillin resistance is encoded by the methicillin resistance determinant (mec), a 30 to 50 Kb transposon like segment of DNA that is present in methicillin resistant *Staphylococcus aureus* strain. The mec A gene, which resides on mec encodes a variant penicillin binding protein (PBP) called PBP2' or PBP2a. PBP2 has reduced affinity for β-lactam antibiotics and is able to substitute for the essential PBPs if they have been inactivated by β-lactam. They are resistant to most of antibiotics because they carry large conjugative plasmids bearing multiple resistance determinants. They are uniformly sensitive to vancomycin.

Methicillin resistant *Staphylococcus aureus* (MRSA) is responsible for more than 50 percent

nosocomial infections. The prevalence of MRSA is around 10 to 30 percent of all staphylococcus infection. Multidrug resistance is a common feature of MRSA. Ciprofloxacin has been suggested the alternative for the treatment of MRSA infections. The dominant phage pattern of MRSA are 6/47/54/75/77.

Epidemiological markers useful in the identification of methicillin resistant *Staphylococcus aureus* includes antibiotype, phage type, plasmid profile, electrophenotypes and many other genotype typing methods.

Biotyping is simple, quick, reproducible and can be incorporated in bench routine. However strain discrimination is limited.

Phage typing is standardized and reproducible, rapid to perform. It carries drawback as one has to maintain of stocks of check phages. Reverse phage typing may come up as valuable supplement to the routing phage typing. In this case strain differentiation is limited.

Antibiotic susceptibility pattern has been used widely for typing purposes. It is not reproducible.

STAPHYLOCOCCUS SAPROPHYTICUS

1. They are opportunistic microorganisms capable of causing
   a. Urinary tract infection in youg females
   b. In patients undergone cardiac surgery may develop septicemia endocarditis because of *Staphylococcus saprophyticus*.
2. They are resistant to novobiocin.
3. They are coagulase negative.

STAPHYLOCOCCUS ALBUS

It is coagulase negative staphylococcus which is a part of skin flora.

Cultural Characters

Nutrient agar colony is pinhead size and white in color.

Blood agar colony is white and there is no hemolysis around them.

Pathogenecity: It is usually non-pathogenic. It may act as opportunist pathogens causing acne, pustules and stitch abscess. If resistance is poor it may cause serious illness like septicemia. In persons with structural abnormalities of urinary tract it may cause cystitis.

Differentiation between *Staphylococcus aureus* and *Staphylococcus saprophyticus*

Properties	*Staphylococcus aureus*	*Staphyloccus saprophyticus*
1. Coagulase test	+	–
2. Mannitol fermentation	+	–
3. DNAase production	+	–
4. Phosphatase production	+	–
5. Alfa lysine production	+	–
6. Presence of Protein A in cell wall	+	–
7. Resistance to Novobiocin	–	+

STAPHYLOCOCCUS CITRUS

It is found as saprophyte and is never pathogenic. On nutrient agar and blood agar it forms lemon yellow pigmented colonies. On blood agar it is never hemolytic. It does not ferment sugar and toxin or coagulase.

Differentiation between Staphylococcus auresu and micrococcus

Properties	*Staphylococcus aureus*	*Micrococus*
1. Gram staining	Gram positive, grape like cluster exhibiting uniform staining	Gram positive in group of four or eight larger than staphylococcus, not uniformly stained and darkly stained
2. Colony character	Golden yellow colonies	White colored colonies
3. Coagulase Test	+	–
4. Carbohydrate fermentation	Fermentatively	Oxidatively

MICROCOCCUS TETRAGEN (GAFFKYA TETRAGENA)

It is a commensal of mucosa of upper respiratory tract. It is Gram positive cocci slightly longer than staphylococci and occurs in tetrads. It is capsulated when in tissue.

Cultural Character

Fluid media: In broth a thick deposit may develop. Supernatant fluid may remain clear.

Nutrient agar: The colony is white. It is emulsified with difficulty.

Blood agar: A zone of hemolysis may appear around colonies.

Biochemical reactions: It ferments lactose, glucose, sucrose and maltose with acid production only. Gelatin is not liquefied.

Pathogenicity: May be responsible for dental abscess, cervical adenitis, pulmonary abscess and rarely endocarditis.

SARCINA

It is similar to micrococcus except that it forms pockets of 8. Some of them may be motile. It is mostly non-pathogenic.

23 Streptococcus

General characters: They are Gram positive cocci arranged in chains, non-motile and non-sporing. They require media enriched with blood, serum or ascitic fluid for their growth. They are important human pathogens causing pyogenic infection with a characteristic tendency to spread. They are also responsible for non-suppurative lesions like acute rheumatic fever and glomerulonephritis.

Classification of streptococcus: Several systems of classification have been employed:

I. **Morphological classification:** Attempt to classify streptococci into long chain (pathogenic strain) and short chain (non-pathogenic) forming cocci (Fig. 23.1) is not a satisfactory method of classification. Length of chain however depends upon the medium in which organism is grown and on several other factors.

II. **Classification based in cultural character:** Streptococci are divided into obligate anaerobe (Peptostreptococci) and aerobes or facultative anaerobes. The aerobic or facultative streptococci are further classified on the base of hemolytic property on blood agar plate.

a. *Alpha hemolytic:* Streptococci produce a zone of greenish discoloration around the colony. This zone of partial lysis of 1.2 mm wide with irregular margin.

b. *Beta hemolytic:* Streptococci produce sharply defined, clear, colorless zone of hemolysis 2 to 4 mm wide.

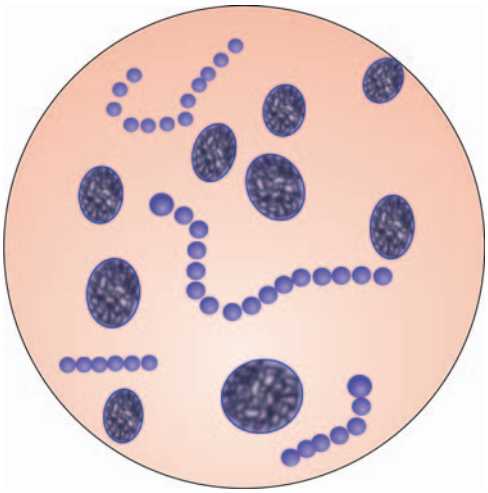


Fig. 23.1: Streptococcus

III. **Classification based on biochemical reactions:** Fermentation is used in differentiating different species of streptococci, e.g. mannitol is fermented by enterococci while *Streptococcus pyogenes* and *Streptococcus viridans* do not ferment mannitol.

IV. **Classification based on antigenic structure:** The aerobic streptococci producing beta hemolysis are divisible into Lancefield group A, B, C, D, E, F, G, H, K, L, M, N, O, P, Q, R, S, T, U, V, on the basis of specificity of polysaccharide-C hapten antigen present on the cell wall. A majority of hemolytic streptococci that produce human infection belongs to group A. Hemolytic streptococcus of group A is known as *Streptococcus pyogenes*. It may be further subdivided into types based on protein (M, T, R) present on cell surface (Griffith typing). More than 80 Griffith types of *Streptococcus pyogenes* have been recognized so far. Table 23.1 shows diseases caused by streptococci of various groups.

TABLE 23.1: Diseases caused by streptococci of various groups

Group	*Disease caused*	*Habitat*
A	Majority of human streptococcal diseases	Man
B	Mastitis in cows, post-natal infections in human and sepsis in newly born	Cow, human genital tract
C	Diseases in various animals. Mild respiratory infections in human.	Various animals and upper respiratory tract of humans.
D	Infections of urogenital tract in humans, endocarditis and wound infections.	Milk products, intestines of humans and animals
E	Diseases in pigs and cows	Pigs and cows
F	Respiratory infections in humans and endocarditis	Upper respiratory tract of humans
G	Mild respiratory infections in humans Genital tract infections in dogs	Upper respiratory tract of humans and dogs
H	Endocarditis	Upper respiratory tract of humans
K	Endocarditis	Upper respiratory tract of humans
L	Genital tract infections in dogs	Dogs
M	Genital tract infections in dogs	Dogs
N	Genital tract infections in dogs	Dogs
O	Endocarditis	Milk products
P	Not known	Chicken, pigs
Q	Not known	Human intestines
R	Not known	Human intestines
S	Not known	Human intestines
U	Not known	Animals
V	Not known	Animals

GROUP A

STREPTOCOCCUS PYOGENES

Morphology: It is 0.5 to 1 µm in diameter and arranged in chain. Chain formation is due to cocci dividing in one plane only and failure of daughter cell to separate completely. The length of a chain depends upon medium in which organism is grown. It is usually encapsulated, non-sporing and non-motile. When capsule is present it is composed of hyaluronic acid (Group A and Group C). Streptococcus Group B and Group C show polysaccharide capsule.

Cultural character: *Streptococcus pyogenes* is aerobic and facultative anaerobes with optimum temperature of growth being 37°C. Enriched media with whole blood, serum, ascitic fluid or glucose favors rapid growth.

a. *Fluid media:* Serum broth, 24 hours after culture shows granular growth with powdery deposits. There is no pellicle formation.
b. *Blood agar:* After 24 hours' incubation colony is small, 0.5 to 1 mm (pin point colonies), circular, transparent, low convex with area of hemolysis. Strains with capsules produce mucoid colonies. Virulent strains produce matted colonies (granular). A virulent strain produces glossy colonies (Fig. 23.2).

Selective media: Blood agar medium having 1:500,000 crystal violet may be used as selective medium (Fig. 23.3).

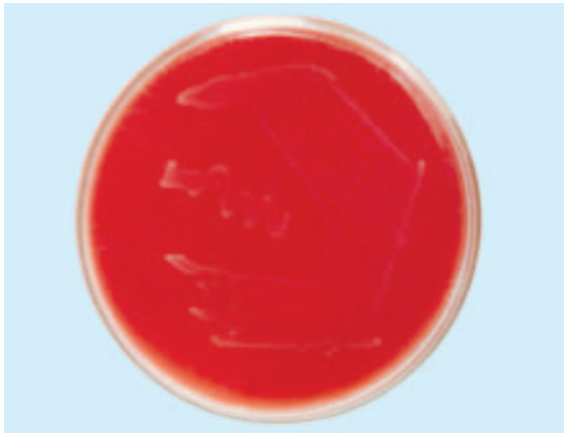


Fig. 23.2: *Streptococcus pyogenes* growth of blood agar medium

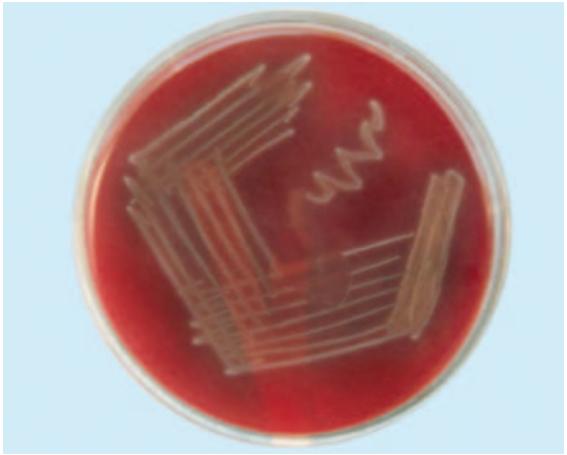


Fig. 23.3: Crystal violet blood agar plate—selective medium for *Streptococcus pyogenes*

Biochemical reactions: It ferments lactose, glucose, salicin, sorbitol, maltose, dextrin, etc. producing acid but no gas. It is catalase negative. It does not liquefy gelatin and is not soluble in 10 percent bile. It hydrolysis pyrrolidonyl naphthylamide (PYR test), producing red colours. It does not ferment ribose.

Resistance: It is easily destroyed by heat at 56°C for 80 minutes. It can survive in dust for several weeks if protected from sunlight. It is resistant to crystal violet. It is susceptible to sulfonamide, etc. *Streptococcus pyogenes* is highly sensitive to bacitracin and this property is used in rapid identification of group A hemolytic streptococci.

Antigenic structure: Hemolytic streptococci possess a group specific polysaccharide C and three type specific protein antigen M, T, R and nucleoproteins (Fig. 23.4).

Polysaccharide C antigen: It confers serological specificity and hence it has 20 Lancefield groups. Human strain belongs to group A and bovine strain belongs to group B.

This antigen is integral part of cell wall and so it has to be extracted for grouping by precipitation with specific antigen. Extraction can be done by:

1. Hydrochloric acid (Lancefield's acid extraction method).
2. Formamide (Fuller's method).
3. An enzyme produced by *Streptomyces albus* (Maxted method).
4. Autoclaving (Rantz and Randall's method).

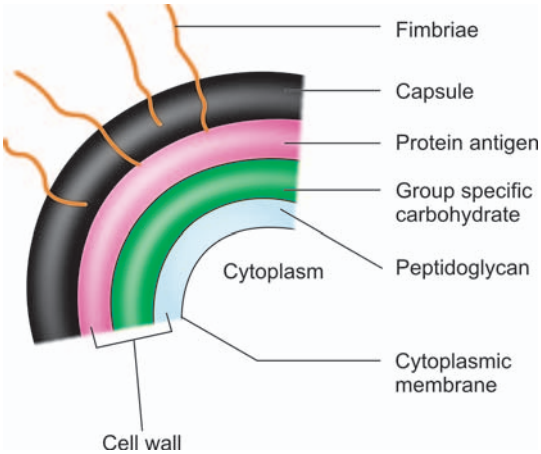


Fig. 23.4: Antigenic structure of *Streptococcus pyogenes*

M antigen: M protein besides determining type specificity also acts as virulence factor by inhibiting phagocytosis. Antibody to M protein is protective. M protein is alcohol soluble and is destroyed by trypsin. Over 65 proteins types have been recognized in group A.

T antigen: It is acid and heat labile but trypsin resistant. It is demonstrated by agglutination with the specific antisera. Antibodies against the antigen are not protective.

It has no relationship to virulence of streptococci. It is obtained from streptococci by proteolytic digestion (otherwise rapidly destroy M proteins). It permits differentiation of certain types. Other types share the same T substance.

R antigen: *Streptococcus pyogenes* (serotypes 2, 3, 28, 48) and in some strain of group B and C, this R protein antigen is found. It has no relation to virulence.

Fimbria are hair like consisting of M protein which are covered by lipotecoic acid and are present in streptococci group A important to attach streptococci to epithelium.

Nucleoproteins: Extraction of nucleoproteins of streptococci by treatment with weak alkali yields mixture of proteins and other substances called P substances which probably make up most of streptococcal cell body.

Other factors: M associated protein (MAP) is identified. Some M types of *Streptococcus pyogenes* produce serum opacity factor.

Toxin Production

1. *Hemolysins:* It is filterable toxic substance. It is of two types:
   a. Streptolysin O demonstrable in deep colonies which is oxygen labile, heat labile, strongly antigenic, important in contributing to virulence (intravenous injection into animal has specific cardiotoxic activity) and it is inhibited by cholesterol.
   b. Streptolysin S is oxygen labile, non-antigenic, inhibited non-specifically by serum lipoprotein and may be nephrotoxic.

2. *Erythrogenic toxin* is filterable and heat stable toxin and is of three types: A, B and C. In small dose if injected intradermally causes erythema in susceptible persons. Administration of larger amount causes generalized rash, fever and malaise. It is erythrogenic toxin and is associated with pathogenesis of scarlet fever.

   The production of erythrogenic toxin A and C is dependent on lysogenization of streptococci with certain temperate bacteriophage. B type is chromosomal.
3. *Streptokinase (fibrinolysin)* is produced mostly by strain of group A, C and G. It is heat stable and antigenic. It promotes lysis of human fibrin clots by activating a plasma precursor. Fibrinolysin appears to play a part in streptococcal infection by breaking down fibrin barrier around lesion and spreading infection.
4. Deoxyribonucleases (streptodornase) causes depolymerization of DNA. It helps to liquefy thick pus. Four distinct streptodornase A, B, C and D have been recognized of which B is more antigenic in man.
5. Diphosphopyricine nucleotidase (DPNase) is antigenic and is specifically neutralized by antibody in convalescent sera. It is believed to be leukotoxic. It acts on the coenzyme DPN and liberates nicotinamide from the molecule.
6. *Hyaluronidase* is a spreading factor present in culture filtrate of *Streptococcus pyogenes*. It breaks down hyaluronic acid of the tissue. This favors spread of infection along intercellular space. Streptococci possess a hyaluronic acid capsule and also elaborate hyaluronidase, self destructive process. But strains that produce hyaluronidase in large amount (M type 4, 22) are non-capsulated and hence no hyaluronic acid.
7. *Protease* is intracellular enzyme produced at acidic pH in cultures grown at 37°C. It destroys M type specific protein and also inhibits production of fibrinolysin and hyaluronidase. It is produced in areas of inflammation. The biological significance of this enzyme is not known.

Amylase, esterase are also produced but it is not known whether they play any role in natural infection or not.

Pathogenecity: *Streptococcus pyogenes* is more invasive and produces septicemia readily. There is a tendency to spread locally along lymphatics and through bloodstream.

1. *Respiratory infection:* Throat is the primary site of invasion causing sore throat. It may be localized in tonsils (tonsillitis) or may involve pharynx (pharyngitis).

   Scarlet fever is caused by a strain producing erythrogenic toxin. This accounts for characteristic erythematous rash. This is uncommon in the tropics and does not occur in India.

   From throat streptococci may spread to the surrounding tissue causing otitis media, mastoiditis, Ludwig angina and suppurative adenitis. It may cause meningitis. Bronchopneumonia may occur when *Streptococcus pyogenes* acts as secondary invader, e.g. influenza. Sometimes empyema may result.
2. *Skin infection:* It may cause suppurative infection of skin, e.g. wound, burns, lymphangitis and cellulitis. Infection of abrasion may lead to fatal septicemia.

   Apart from this it may cause erysepelas and impetigo. Erysipelas is a diffuse infection involving superficial lymphatic. The involved skin becomes red, swollen and indurated. It is found in old patients. In impetigo *Streptococcus pyogenes* of group A type 4, 25 are involved. It is seen in young children. Impetigo may lead to glomerulonephritis in children of tropics but do not often lead to rheumatic fever.
3. *Genital tract: Streptococcus pyogenes* is important cause of puerperal sepsis. The source of infection is nasopharynx of doctors, nurses and attendants, etc.
4. Other infections like abscess of organs (brain, lungs, liver, kidney) may occur. It may cause septicemia and pyemia.
5. *Non-suppurative complications:* They are:
   i. Acute rheumatic fever.
   ii. Acute glomerulonephritis.

The pathogenesis of these conditions is not clearly understood. They require 1 to 3 weeks after acute infection. No organism is detected when sequelae sets in. Comparison of acute rheumatic fever and acute glomerulonephritis is shown in Table 23.2.

Acute rheumatic fever: It is a systemic non-suppurative inflammatory condition characterized by fever, pancarditis, migratory polyarthritis, sometimes chorea and subcutaneous nodules. Usually disease is preceded by an attack of streptococcal sore throat, septic tonsillitis or respiratory tract infection.

The hypersensitivity to streptococci or its products is suggested in pathogenesis of rheumatic fever because of:

a. Absence of organism in the lesion.
b. Latent period between attack of sore throat and onset of rheumatic disease.
c. Similarity of disease produced experimentally in rabbit.

It is suggested that lesion of rheumatic fever may be the result of hypersensitivity to some streptococcal component produced by repeated attacks. Rheumatic fever has marked tendency to be reactivated by recurrent streptococcal infections, whereas nephritis does not have this characteristic. It has also been suggested that there may be an element of autoimmunity involved and antigenic cross reaction between streptococci and heart tissue also has been demonstrated.

Acute glomerulonephritis: It is caused by only few nephritogenic strain of group A (4, 12, 49, 2, 52, 57), type 12 being the most common. The most important antigen is probably in streptococcal protoplast membrane. The immunity in streptococcal infection is type specific and so acute glomerulonephritis is non-recurring condition. Not only sore throat but pyoderma or impetigo may also lead to nephritis. It is self-limited episode that resolves without any permanent damage.

TABLE 23.2: Comparison of rheumatic fever and glomerulonephritis

	Acute rheumatic fever	*Acute glomerulonephritis*
Site of infection	Throat	Throat or skin
Prior sensitization	Essential	Not necessary
Serotype of *Streptococcus pyogenes*	Any	Nephritogenic only (12, 49, 2, 52, 55, 57, 4)
Immune response	Marked	Moderate
Complement levels	Unaffected	Lowered
Hereditary tendency	Present	Not known
Repeated attack	Common	Absent
Penicillin prophylaxis	Essential	Not indicated
Course	Progressive	Spontaneous
Prognosis	Variable	Good

Pathogenesis may be due to antigenic cross reaction, between glomerular antigens and some component of nephritogenic streptococci. More often it may be immune complex disease.

Laboratory Diagnosis

1. *Hematological investigations:*
   a. Total leukocyte count may show considerable increase.
   b. *Differential leukocyte* counts show increase in neutrophil count. Polymorph neutrophil may constitute more than 80 percent.
   c. Erythrocyte sedimentation rate (ESR) is raised especially in rheumatic disease. It is done to estimate the activity of disease.
2. *Bacteriological method:* Most important specimens are throat swab, nasopharyngeal swab, pus swab, sputum, cerebrospinal fluid, blood, etc.
   a. Smear from above material after Gram's straining, show Gram positive cocci arranged in chains.
   b. *Culture:* Specimen is cultured on blood agar or crystal violet blood agar media with loop. After overnight incubation at 37°C, colonies are studied. These are small (pin point); raised colonies showing beta hemolysis. Hemolytic streptococci are grouped further by Lancefield technique. A rapid presumptive identification of group 'A' streptococci can be made by performing bacitracin sensitivity test (Fig. 23.5). For rapid diagnosis swab or pus specimen are cultured in broth and after 2 to 3 hours smears are stained with fluorescein labelled group A antiserum.
   c. *Serological test:* The titer of antistreptolysin O in a patient serum in dilution above 1 : 200 is an indication of strepto-coccal infection. It is also useful for detecting asymptomatic carriers of *Streptococcus pyogenes.* A titer above 286 of antistreptolysin O is suggestive of rheumatic activity. A use in antibody titer to other streptococcal antigens may be estimated, e.g. antiDNAase, antihyaluronidase (especially in skin

infections), antistreptokinase, antiM type specific antibodies, etc. Streptozyme test is useful in detection of antibodies to antigen and enzymes.

3. *Skin test* is known by the name Dick test.
   Dick test: It is done to find out susceptibility of a person to scarlet fever. 0.2 ml erythrogenic toxin is injected intradermally on the forearm and same amount of heated inactivated toxin on the other forearm. A bright red rash appears within 6 hours and becomes maximum in 24 hours and thereafter this fades away. Control forearm does not show any reaction. A positive reaction means no immunity to scarlet fever. A negative reaction means immunity.
4. *Schultz-Charlton reaction:* Erythrogen antitoxin is injected intradermally in a patient with scarlatinal rash. There is local blanching of rash.

Treatment: *Streptococcus pyogenes* is sensitive to penicillin, sulfonamide and several other antibiotics. However, penicillin is the drug of choice. Antitoxic serum was used to be administered effectively in scarlet fever.

GROUP B

Streptococcus agalactiae is responsible for mastitis in cow. It may be present in human throat and vagina as commensal. It can be identified by CAMP (Christie-Atkins-Munch-Petersen) reaction.

On the basis of type specific capsular antigens they are divided into 4 groups, ia, ib, ii and iii. All the strains of type ia and ib are from human source.

It is rarely pathogenic in man. Sometimes they may cause puerperal infection, septicemia, meningitis and ulcerative endocarditis, etc.

DISEASES CAUSED BY GROUP 'B' STREPTOCOCCI

Newborn
- Pneumonia
- Meningitis
- Respiratory diseases
- Osteomyelitis

Adult
- Endocarditis
- Septicemia
- Meningitis
- Arthritis
- Wound sepsis
- Pyoderma

GROUP C

Streptococcus equisimilis is isolated from horses and cows. It may produce streptolysin O and fibrinolysin. The organisms have been isolated from puerperal infection, cellulitis, wound and scarlet fever.

GROUP D

Group D streptococci can be divided into enterococci, e.g. *Enterococcus fecalis* (Fig. 23.6), *Enterococcus fecium, Enterococcus durans, Enterococcus avium* and so on. *Enterococcus fecalis*

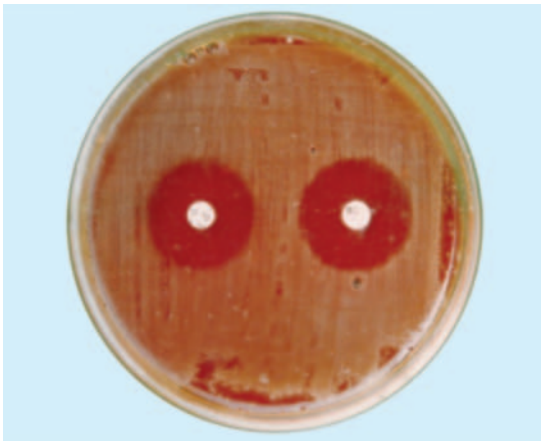


Fig. 23.5: *Streptococcus pyogenes* showing bacitracin and pencillin sensitivity

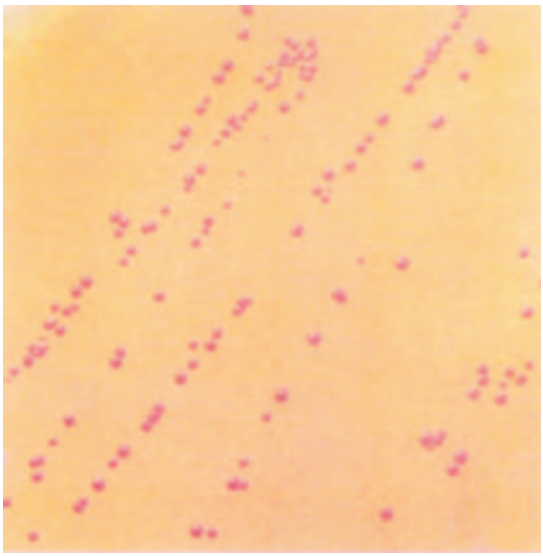


Fig. 23.6: Slanetz and Bartley medium *Enterococcus fecalis*

is the most common of all above. It ferments mannitol with gas production, VP positive with PYR test positive and can be grown on blood tellurite producing black colonies.

Enterococci fecalis may cause urinary tract infection, wound infection, infective endocarditis, biliary tract infection, peritonitis, suppurative abdominal lesions and septicemia. Important characteristics of enterococci are:

- Normal flora of lower intestinal tract and vagina.
- Can grow in 6.5 percent sodium chloride, 40 percent bile and at 45°C.
- On sheep blood agar may produce alpha, beta hemolysis or may be non-hemolytic too.
- Tiny, deep pink colonies appear on MacConkey agar medium.
- Most strains are resistant to penicillin, sulfonamide and also to cephalosporin, gentamycin, streptomycin, etc.
- Survive heat upto 60°C for 30 minutes.
- Non-motile, Gram positive cocci, non-capsulated and may be arranged in pairs or short chain.
- PYR test positive.

Cultural Characters

a. *Fluid media:* It shows uniform turbidity after 24 hour incubation at 37°C.
b. *Blood agar:* Colony is little bigger than *Streptococcus pyogenes*, circular, raised, low convex and emulsifies easily. Most of the strains are non-hemolytic.
c. *MacConkey agar medium:* Colonies are tiny, and deep pink in color.

Biochemical reactions: It ferments mannitol, sucrose, sorbitol, aesculin and grows on tellurite blood agar producing black colonies.

Resistance: It grows at pH 9.6 in presence of 6.5 percent sodium chloride, and also in presence of 40 percent bile. It survives at 60°C temperature for 30 minutes.

Pathogenicity: It invades tissues and may produce pyogenic lesions, e.g. cystitis, pyelitis, vaginitis, cervicitis, puerperal sepsis and subacute bacterial endocarditis.

Non-enterococci, e.g. *Streptococcus bovis, Streptococcus equinus, Streptococcus avium*, etc may cause urinary tract infection, endocarditis, septicemia. They may be non- hemolytic and susceptible to penicillin.

Group F, G, H and O may occur as commensal in the throat.

ALPHA HEMOLYTIC STREPTOCOCCI

Some streptococci do not produce soluble hemolysin. It is characterized by production of alpha hemolytic colonies on blood agar.

It includes:

a. *Streptococcus viridans.*
b. *Streptococcus salivarium.*
c. *Streptococcus mitis.*
d. Streptococcus M.G.

Streptococcus viridans: It is present as a commensal on mucosa of mouth, nasopharynx and saliva of man. On the basis of biochemical reactions it is classified into 5 species *(Streptococcus salivarium, mutans, sanguis, mitior, milleri).* On blood agar it produces alpha hemolysis (Fig. 23.7).

It is normally non-pathogenic. Sometimes it may cause subacute bacterial endocarditis and formation of plaque on teeth leading to dental caries, etc.

Diagnosis of subacute bacterial endocarditis is established by repeated blood culture. It is susceptible to penicillin.

Streptococcus salivarium: It is non-pathogenic found in mouth and intestine of man.

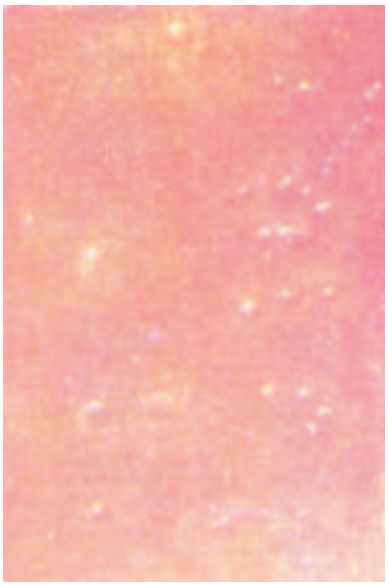


Fig. 23.7: Colonies of *Streptococcus viridans*

Streptococcus salivarium can grow at 45°C and produce large mucoid colonies on agar containing 5 percent raffinose and salicin.

Streptococcus mitis: It does not grow at 45°C. Colonies are non-mucoid, smaller and surrounded by wide zone of alpha hemolysis.

Streptococcus M.G.: It belongs to group E. It is isolated from sputum of patient suffering from primary atypical pneumonia. It is capsulated. The organism is agglutinated by the serum of some patient suffering from atypical pneumonia.

ANAEROBIC STREPTOCOCCUS

Peptostreptococcus putridus: The natural habitat of anaerobic streptococci is female genital tract. The culture on blood agar medium is incubated anaerobically in McIntosh Fildes jar for 48 hours. The colony is 2 to 4 mm, circular, raised and translucent. There is no hemolysis.

It ferments glucose, maltose, fructose with abundant gas production. It produces puerperal sepsis, and puerperal septicemia, brain abscess and other suppurative and gangrenous lesions.

Anaerobic Cocci	
Gram positive	*Gram negative*
1. Peptococcus	1. Veillonella
2. Streptococcus	2. Acidaminococcus
3. Peptostreptococcus	3. Megaphaera
4. Ruminococcus	
5. Caprococcus	

24 Pneumococcus

General characters: They are Gram-positive, lanceolate diplococci and are capsulated. Pneumococci occur primarily in the human throat and are the most common cause of pneumonia. They require enriched medium with blood, serum or ascitic fluid for their growth. On blood agar they produce alpha hemolysis. Of late they are reclassified as *Streptoccocus pneumoniae*.

Morphology: It is typically small (1 µ) slightly elongated with one end broad and other pointed (flame shaped). It occurs in pairs (Fig. 24.1). It is capsulated, capsule enclosing each pair. It is non-motile and non-sporing. It is Gram-positive. In India ink preparation capsule appears as a clear halo (Fig. 24.2).

Cultural characters: It requires serum or whole blood for growth. It grows best at 37°C and at pH 7.6. It is aerobic and facultative anaerobic. Growth is improved by providing them 5 to 10 percent CO_2. Characters of growth on following media are:

a. Serum and glucose broth shows uniform turbidity after 24 hours growth. After 36 hours autolysis occurs. There is no pellicle formation.

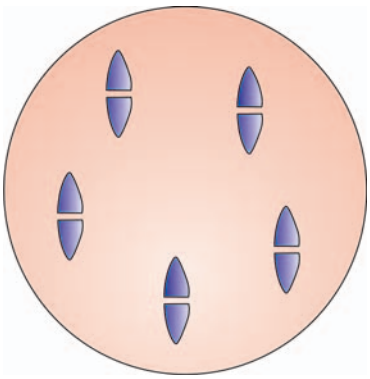


Fig. 24.1: Pneumococcus

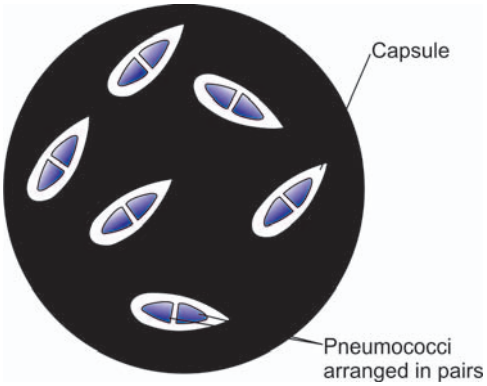


Fig. 24.2: India ink preparation showing capsule of pneumococci

b. *Blood agar:* Colony is small (0.5 to 1 mm), dome shaped with area of greenish discoloration (alpha hemolysis) around them. On further incubation the colonies become flat with raised edges and central umbonation (draughtsman appearance) (Fig. 24.3). Type III pneumococci are longer and have mucoid appearance.

Biochemical reaction: It ferments many sugars, forming acid only. Inulin is fermented by all pneumococci. They are bile soluble (few drops of 10 percent sodium desoxycholate solution are added to 1 ml of overnight broth culture). The culture clears due to lysis of cocci. It is catalase and oxidase negative.

Resistance: It is readily destroyed by heat (52°C for 15 minutes), in one hour by phenol, potassium permanganate and other antiseptics.

It is sensitive to sulphonamide. It is sensitive to optochin (ethyl hydrocuprein) in 1/80,000 and it is useful in differentiating them from streptococci.

Fig. 24.3: Draughtsman colonies of *Pneumococcus*

Strain may be maintained on semisolid blood agar or by lyophilization.

Antigenic structures: Pneumococci possesses a number of antigens.

a. Nucleoprotein: It is neither species specific nor type specific. Antibody to this antigen is not protective.
b. Species specific polysaccharides hapten: It is situated at the cell surface and is not related with capsular antigen.
c. Capsular polysaccharide is found in capsulated form. It determines type specificity of organism and virulence. Pneumococci isolated from lobar pneumonia are classified into 4 types: I, II, III and heterogenous group IV. Members of group IV are further classified into various types. Now about 90 types are known named 1, 2, 3, etc.

Typing may be carried out by:

1. Agglutination of cocci with type specific antiserum.
2. Precipitation of capsular polysaccharides with specific serum.
3. Capsular swelling reaction (Quellung reaction): Here suspension of pneumococci is mixed with type specific antiserum. In presence of homologous antiserum the capsule becomes apparently swollen, delineated and refractile.

Toxin and Other Virulence Factors

1. *Hemolysin:* Pneumococcus produces soluble hemolysin in young culture. It gives characteristic green coloration around colonies. It is oxygen labile and its role in the pathogenesis of pneumococcal infection is not known.
2. *Capsular polysaccharide:* It is specific soluble substance which protects the organism from phagocytosis. It is acidic and has hydrophilic properties. Hence this substance has association with virulence.
3. Pneumococci produce large amount of enzyme resembling receptor destroying enzyme of influenza virus.
4. *Leucocidin:* It kills leukocytes.

Pathogenesis: Pneumococci get attached to nasopharyngeal cell through bacterial surface adhesion (pneumococcal surface antigen-A or choline binding proteins) with epithelial cell receptors. Epithelial binding sites include glycoconjugates containing the disaccharide GlcHAcγβ1-4 Gal or assialo-GM1 glycolipid.

Once nasopharynx is colonized infection may result if pneumococci are carried to eustachian tubes or nasal sinuses, etc.

Pneumococci produce disease through their ability to multiply in the tissue. They produce no toxins of significance. The virulence of organism is a function of its capsule, which prevents or delays ingestion of encapsulated cells by phagocytes.

Pneumococcal infection causes an outpouring of fibrinous edema fluid into alveoli, followed by red cells and leukocytes, which results in consolidation of portion of lung. Many pneumococci are found throughout this exudate. Later mononuclear cells actively phagocytose the debris, and this liquid phase is gradually reabsorbed. The pneumococci are taken up by phagocytes and are digested intracellularly.

In man 80 percent lobar pneumonia and 60 percent bronchopneumonia are caused by pneumococci. It also produces suppurative infection in various parts of body as under:

1. *Lobar pneumonia:* Airborne infection of respiratory tract is a frequent occurrence. The organism is generally eliminated by natural

defence mechanism. If resistance is lowered, organism penetrates bronchial mucosa and spreads through lung along peribronchial tissue and lymphatics. Bacteremia is frequent during early stages. Toxemia is due to diffusion of capsular polysaccharide into blood and tissue. The fall of temperature by crisis and relief coincide with complete neutralization of capsular polysaccharide by anticapsular antibodies. Serotypes 1 to 8, 12, 14, are mostly the cause of pneumonia. Type 3 strain produces particularly severe infection.

2. *Bronchopneumonia:* It is always secondary infection following viral infection of respiratory tract. Bronchopneumonia may be caused by any serotype of pneumococci.
3. Pneumococcal meningitis is most serious pneumococcal infection. It occurs when pneumococcal pneumonia is persistant. Disease is common in children.
4. They may produce suppurative lesions like empyema, pericarditis, otitis media, sinusitis, conjunctivitis and peritonitis.

Laboratory Diagnosis

A. *Hematological investigations:*
   1. Total leukocyte count usually shows leukocytosis. Count may be more than 15,000 per cu mm.
   2. Differential leukocyte count shows usually increase in polymorphonuclear cells.

B. *Bacteriological investigations:*
   1. *Material:* Sputum, cerebrospinal fluid, pleural fluid, pericardial fluid, peritoneal fluid and pus discharge are collected in a sterile container.
   2. *Smear examination:* Gram staining shows flame-shaped cocci arranged in pairs and they are Gram-positive capsulated.
   3. *Culture:* Material is inoculated on blood agar plates and incubated at 37°C under 5 to 10 percent carbon dioxide. Growth occurs after overnight incubation.

      Blood culture: It shows flat, umbonated colonies showing alpha hemolysis. The colonies are treated for inulin fermentation, bile solubility and optochin sensitivity test to differentiate it from *Streptococcus viridans.*
   4. *Immunologic methods:* The detection of pneumococcal capsular polysaccharides in sputum and other body fluids by immunologic methods such as counter-immunoelectrophoresis or latex agglutination provides an alternative to bacteriologic techniques for presumptive diagnosis of pneumococcal infection. Because of crossreactions between the polysaccharides of pneumococci and other bacterial species, immunologic diagnosis is less specific than bacteriologic diagnosis.
   5. *Animal pathogenecity:* Mice are most susceptible to pneumococcal infection (except type 14). It is used for rapid diagnosis. Sputum is emulsified with saline. One ml is inoculated intraperitoneally into 2 mice. Animal usually dies within 24 hours. The encapsulated diplococci can be demonstrated in heart blood and peritoneal fluid.

Treatment: Sulfonamides and penicillins are quite effective drugs. Resistance may develop with antibiotics like sulfonamides and tetracycline. Rarely penicillin resistant strains of pneumococci do occur. It is possible to immune persons with type specific polysaccharides. It provides 90 percent protection. A vaccine has also been developed, (include type 1, 2, 3, 4, 6, 8, 9, 12, 14, 19, 23, 25, 51 and 56) which may be useful for elderly, debilitated or immunosuppressed individuals. This vaccine was licensed in the USA in 1977. In 1983, an expanded polysaccharide vaccine containing 23 types was licensed in the USA. Currently, pneumococcal vaccines of capsular polysaccharides conjugated to proteins such as diphtheria toxoid are being developed.

However pneumococci may be differentiated from *Streptococcus viridans* as shown in Table 24.1.

TABLE 24.1: Differentiation between Pneumococcus and *Streptococcus viridans*

	Pneumococcus	*Streptococcus viridans*
1.	Morphology	
	a. Capsulated	Non-capsulated
	b. Flame shaped diplococci	Oval or round arranged in chain
2.	Quellung test positive:	Negative
3.	*Colonies* Initially dome shaped and later on draughtsman colonies	Dome shaped
4.	Growth in liquid media shows uniform turbidity	Granular turbidity and powdery deposits
5.	Bile solubility is positive	Negative
6.	Inulin fermentation is positive	Negative
7.	Optochin sensitivity is positive	Negative
8.	Intraperitoneal inoculation in mice brings fatal infection	Non-pathogenic

25 Neisseria

General characters: They are Gram-negative, aerobic, non-sporulating, non-motile, oxidase positive cocci arranged in pairs. The genus contains about 30 species. Two important pathogens are:

a. *N. meningitidis.*
b. *N. gonorrhoeae.*

NEISSERIA MENINGITIDIS

Morphology: It is Gram-negative, oval or spherical 0.8 µ to 0.6 µ, arranged in pairs with adjacent sides flattened. Its considerable variation in size and shape and staining property especially in old culture is due to autolysis. In smear from lesion the cocci are more regular and are intracellular. Sometimes microcapsule may be demonstrated by Quellung reaction.

Cultural characters: Growth occurs in media enriched with blood, serum or ascitic fluid. Growth is improved by adding 10 percent carbon dioxide. It is aerobic and grows at optimum temperature 37°C and pH 7.4.

Fluid media: The serum broth shows mild or moderate turbidity after 24 hours incubation at 37°C.

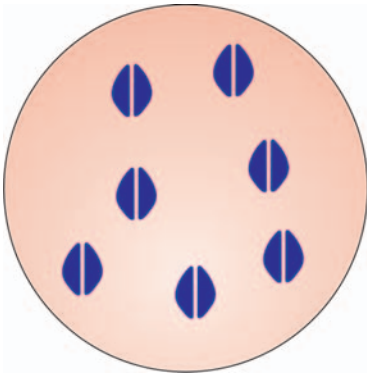


Fig. 25.1: Meningococcus

Blood agar: The colony is moist, elevated, smooth, translucent, round and convex. It is of 1 mm diameter with glistening surface and entire edge. The colony is butyrous in consistency and easily emulsifiable. There is no hemolysis.

Chocolate agar plate: Character of the colony is same as described above. Involution forms appear soon due to active autolysis.

Biochemical reaction: It is catalase and oxidase positive. Glucose and maltose are fermented producing acid and no gas.

Classification: At least 13 serogroups of meningococci have been identified (A, B, C, D, X, Y, Z, W-135, 29E, H, I, K and L) by immunologic specificity of capsular polysaccharides. This grouping is assuming practical importance due to the recent attempts to develop vaccine against protein which is group specific.

The nucleo-proteins meningococci (P substance) have some toxic effect. Certain DNA extracts are capable of inducing streptomycin resistance in meningococci.

Resistance: It is highly susceptible to heat, desiccation, alteration in pH and to disinfectants. It may acquire resistance to streptomycin readily.

Pathogenecity: Meningococci are strict human parasites. The route of infection is usually nasopharynx. They produce cerebrospinal meningitis and meningococcal septicemia. Bacteremia favored by absence of bactericidal antibody (IgG) or its inhibition by blocking IgA antibody or a complement deficiency (C_5, C_6, C_7 or C_8).

From nasopharynx meningococci may spread along perineural sheath of olfactory nerve, through cribriform plate to subarachnoid space. Alternatively spread may be through blood and conjunctiva. On reaching central nervous system a suppurative lesion of meninges is set up involving surface of spinal cord, base and cortex of brain. The cocci are invariably demonstrated from cerebrospinal fluid lying free or intracellular in leukocytes.

Meningococcemia: It presents as acute fever with chills and malaise. Hemorrhagic manifestation is characteristic. In early disease petechial rash may occur which is due to thrombosis of many small blood vessels in many organs, with perivascular infiltration and petechial hemorrhage. Meningococci may be isolated from petechial rash lesion. There may be metastatic involvement of joint, ear, lungs, myocardium and adrenals.

Toxin: Endotoxin is released by the autolysis of organism. There are hemorrhagic manifestations.

Laboratory Diagnosis

a. *Hematological investigations*
   1. Total leukocyte count shows marked leukocytosis. Count may be more than 15,000.
   2. Differential leukocyte count shows increase in polymorphonuclear cells.

b. *Cerebrospinal fluid examination*
   1. Macroscopically fluid is mild to moderately turbid. It is under pressure and contains many pus cells when examined microscopically.
   2. One portion of fluid is centrifuged and studied after Gram staining. Meningococci will be seen mainly inside polymorph and extracellularly also.
   3. The second portion is inoculated on blood agar or chocolate agar under 5 to 10 percent carbon dioxide. After 24 hours' incubation we will identify meningococci on the basis of morphology and biochemical reactions.
   4. Third portion of CSF is incubated overnight and then subcultured on chocolate agar. This method may succeed when direct method fails.

c. *Blood culture*
   In meningococcal and in early cases of meningitis culture is positive.

d. *Nasopharyngeal swab*
   It is useful in carriers.

e. *Petechial lesion*
   Meningococci may be collected from petechial hemorrhage.

f. *Autopsy*
   It is done on meninges, lateral ventricles, surface of brain and spinal cord. After smear and culture, organisms are identified. These must be tested within 12 hours of death of patient.

g. *Retrospective evidence*
   It may be obtained by demonstrating complement fixing antibodies in convalescent sera. However, antibodies to meningopolysaccharides can be measured by latex agglutination or hemagglutination test.

h. *Polymerase chain reaction (PCR)*
   DNA from cerebrospinal fluid or blood may be amplified and detected. Hence PCR may be used as rapid method.

Treatment: Sulfonamides or chloramphenicol are used in the treatment of meningitis. With sulfonamide drug resistant strain may emerge and hence in such situation penicillin G or chloramphenicol is used. Immunization with polysaccharide vaccine has given good protection and so may be used prophylactically. The various preparations of capsular polysaccharides available are:

1. Monvalent, i.e. group A or C.
2. Bivalent, i.e. group A and C.
3. Quadrivalent, i.e. group A, C, Y and W 135. Dose is 50 mg subcutaneously. Children require two doses with interval of 3 months, whereas adults requires single dose. Protective efficacy of this vaccine is claimed as 90 percent.

NEISSERIA GONORRHOEAE

Morphology: It is strictly a parasite of man. The coccus is Gram-negative, oval or spherical 0.8 to 0.6 µ with adjacent side concave (bean shaped) arranged in pair as compared with mieningococci (Table 25.1) (Fig. 25.2). It is found predominantly within the polymorphs.

TABLE 25.1: Difference between gonococcus and meningococcus

	N. meningitidis	*N. gonorrhoeae*
1.	They occur as intracellular diplococci in smear of exudate	Same
2.	Adjacent sides are flat	They are concave
3.	Colonies are butyrous easily emulsified	Viscid colonies difficult to emulsify
4.	Produce acid in glucose and maltose	Acid is produced in glucose only

Cultural characters: It is aerobic. Growth occurs best at 37°C and at pH 7.4. Addition of 5 to 10 percent carbon dioxide is essential. An enriched media is required for its growth. Following media may be used:

Serum broth: Growth is poor at 37°C in 24 hours. Growth is mainly on the surface with no turbidity.

Chocolate agar: After 48 hours of incubation colonies are round, convex, translucent gray white 0.5 to 1 mm in diameter with glistening surface and entire margin. Consistency is viscid and difficult to emulsify.

Thayer-Martin medium: It is a selective medium. It inhibits many contaminants including non-pathogenic neisseria. This medium contains vancomycin, colistin and nystatin.

Chacko Nair egg enriched medium: It supports good growth.

Biochemical reaction: It ferments glucose with formation of acid only. Catalase and oxidase is positive.

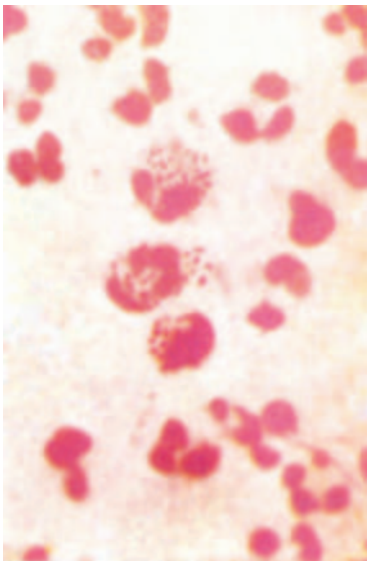


Fig. 25.2: Gonococci from smear of urethral discharge

Classification: On the basis of colony morphology, auto-agglutinability and virulence, there are four biotypes (T_1, T_2, T_3 and T_4). T_1 and T_2 are small, brown, autoagglutinable and virulent. T_3 and T_4 produce large non-pigmented colonies which are avirulent.

Antigenic structure: Gonococci are serologically heterogenous. Gonococci possess polysaccharides and nucleoproteins. They also possess capsule demonstrated by negative staining. H_8 is a surface exposed protein which is heat stable. The outer membrane is a macromolecular complex, an iron regulated protein are other antigenic constant proteins of gonococci and all of them have poorly defined role in pathogenesis.

Resistance: It is killed by heat, drying and antiseptics. It is susceptible to sulfonamides, penicillin and other antibiotics.

Pathogenicity: The gonococcus is a specific human parasite causing a venereal disease called gonorrhea. It produces lesions as follows:

Incubation period is 2 to 8 days. In male it starts as acute urethritis with mucopurulent discharge containing gonococci. Infection may extend along urethra to prostrate, seminal vesicle and epididymis. It may spread to periurethral tissue causing multiple discharging sinuses (watercan perineum).

In females infection involves urethra and cervix uteri. Vagina is spared because of acidic pH. The infection may spread to Bartholin glands, endometrium and fallopian tubes. Salpingitis may lead to sterility.

Proctitis occurs in both sexes. Pharyngitis and conjunctivitis may occur. Blood invasion for primary infection may lead to lesions like arthritis, ulcerative endocarditis and rarely pyelonephritis, meningitis and pyemia. Ophthalmia neonatorum may occur in newborn as a result of infection from genital tract of mother.

Gonococci contain several plasmids. At least one such plasmid carries the gene for beta-lactamase production that makes the gonococcus resistant to penicillin. Gonococci attach to surface epithelial cells with pili. Some gonococci secrete proteases that can break down surface IgA antibodies.

In low concentration of free iron gonococci cannot grow. Gonococci only bind human

transferrin and lactoferrin by producing iron repressible proteins (Fe RPs) that function in the removal of iron from transferrin and lactoferrin. This specificity has been used to explain why gonococci are obligate human pathogens.

Attachment of gonococci to mucosal cells is mediated in part by pili and by protein II. Pili also impede phagocytosis of gonococci by neutrophils and antibody to pili is opsonic. An enzyme IgA protease which inactivates SIg Al interfere with IgA mediated antiadherence activity and hence increased attachment. Other factors which are important in pathogenesis are tumor necrosis factors (damage to fallopian tube), peptidoglycan fragments (toxic for fallopian tube mucosa), several proteases, peptidases and phospholipases.

Laboratory Diagnosis

a. *Hematological investigations:*
   1. Total leukocyte count shows leukocytosis.
   2. Differential leukocyte count shows increase in polymorphonuclear cells.

b. *Bacteriological examination:*
   Smear examination: Gram's staining of smear from purulent discharge (urethra, cervix, etc.) shows Gram-negative diplococci inside polymorphs. The use of fluorescent antibody technique is specific and sensitive method of identification. In male meatus is cleaned with gauze soaked in saline and sample of discharge is collected with platinum loop for culture and smear. In female besides urethral discharge cervical swab should also be studied.

   In chronic infection there may not be any urethral discharge. Here exudate may be obtained after prostatic massage.

Culture: It is done on soft blood agar not chocolate agar incubated in jar containing 10 percent CO_2. Material should be incubated immediately. In case of delay charcoal impregnated swab should be sent to laboratory in Stuart transport medium. In chronic cases, where mixed infection is usual, it is better to use selective medium like Thayer-Martin medium.

Serological test: Serum and genital fluid contain IgA and IgG antibodies against gonococcal pili, outer membrane protein and lipopolysaccharide.

1. *Complement fixation test (CFT):* It is done with polyvalent antigen. It becomes positive 2 weeks after infection and remains positive for long time even after the cure of disease. It becomes positive even after meningococcal infection.
2. *Flocculation test:* It is simpler test than CFT. It has all disadvantages of CFT.

Other serological techniques are: radioimmunoassay, and ELISA. However, these tests lack specificity and reliability as diagnostic aids.

Nucleic acid probe: It may be used to detect gonococci in urethral and cervical specimen.

Treatment: Sulfonamides and penicillin with probenecid is quite effective. Alternatively tetracycline or erythromycin can be used. With the emergence of beta lactamase producing gonococci we require the use of spectinomycin, trimethoprim sulfamethoxazole or cefoxitin.

Following vaccines are being considered:
1. A gonococcal pilus vaccine effective against only homologous strain (prevents attachment of gonococci to mucosal surface).
2. Vaccines containing 3 or 5 serotypes of protein 1 (bacteriocidal effect).
3. Gonococcal lipopolysaccharide vaccine (under study).
4. Finally multicomponents vaccine may be prepared.

Commensal Neisseriae

They may inhabit normal respiratory tract. Their pathogenic significance is uncertain. *N. flavescens* and *N. catarrhalis* have been reported occasionally as having caused meningitis. Characteristic features of some of them are shown in the Table 25.2.

Non-gonococcal Urethritis

Urethritis due to causes other than gonococci is described as non-gonococcal or non-specific urethritis. Here gonococci cannot be demonstrated from urethral lesions.

Etiology

1. Gonococci (L form).
2. Chlamydial infection (TRIC).

TABLE 25.2: Characteristics of species of Neisseriae

Species	*Colonies*	*Growth on nutrient agar*	*Growth at 27°C*	*Fermentation Glucose*	*Maltose*	*Sucrose*	*Serological*
N. meningitidis	Round, smooth, glistening and creamy consistency	—	—	A	A	—	8 antigenic groups
N. gonorrhoeae	Same as above but small and opalescent	—	—	A	—	—	Antigens heterogenous
N. flavescens	Same but pigmented yellow	+	±	—	—	—	Antigenically distinct homogenous group
N. sicca	Small, dry, opaque, wrinkled, and brittle	+	+	A	A	A	Autoagglutinable
N. catarrhalis	Variable, small, translucent, opaque and not emulsifiable	+	+	—	—	—	Autoagglutinable

3. Mycoplasma (*Ureaplasma urealyticum Mycoplasma hominis*).
4. *Hemophilus vaginalis (Gardnerella vaginalis).*
5. *Candida albicans.*
6. *Trichomonas vaginalis.*
7. Mobiluncus (recently classified genus).
8. Acinobacter lwoffi.
9. Chemical and mechanical irritation.

Since etiological diagnosis is difficult to make hence management is also difficult.

Veillonellae

They are small, anaerobic, Gram-negative cocci that are part of normal mouth flora. They ferment few sugars and probably are not pathogenic.

26 Corynebacterium

General character: They are Gram-positive, non-acid fast and non-motile bacilli occurring in palisade. They frequently show club-shaped swelling at the ends and irregular staining. *Corynebacterium diphtheriae* is the pathogenic member and it shows metachromatic granules in Albert stained smear. It produces powerful exotoxin.

CORYNEBACTERIUM DIPHTHERIAE

Morphology: It is thin, slender, rod 3 to 6 µ × 0.6 to 0.8 µ showing clubbing at one or both ends. It is non-sporing, non-capsulated and non-motile. It is Gram-positive and shows pleomorphism. The presence of metachromatic granules (Babes-Ernst granules) serve to distinguish it from diphtheroid. The granules are colored dark purple with methylene blue, Albert or Neisser's differential stain. There may be one or more granule in a simple cell. The granules consist of polymerized metaphosphate.

The bacilli are arranged in characteristic fashion. It is seen in pairs or groups. Bacilli form various angles with each other like V or L. This is called Chinese letter arrangement (Fig. 26.1). This is due to incomplete separation of daughter cells after binary fission.

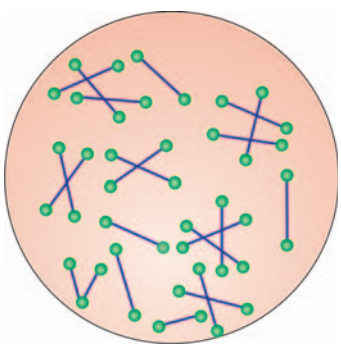


Fig. 26.1: *Corynebacterium diphtheriae*

Cultural character: Enrichment of media with blood, serum or egg is necessary for good growth. The optimum temperature is 37°C and pH 7.2. It is aerobic and facultative anaerobic.

1. *Serum broth:* Turbidity, pellicle formation, amount and nature of deposit is useful in identification of types of *Corynebacterium diphtheriae.*
2. Loeffler's slope culture shows abundant growth after 6 to 8 hours incubation. The colony is small, granular, moist, creamy and glistening with irregular edges.
3. *Blood tellurite medium* (0.04%): It is useful in differentiation of *Corynebacterium diphtheriae* into gravis, mitis and intermedius types (Table 26.1). It acts as selective media inhibiting the growth of other organisms. Diphtheria bacilli reduce tellurite to metallic tellurium giving gray to black color to colonies (Fig. 26.2).

Biochemical Reactions

It ferments glucose and maltose with acid production only. Fermentation of starch, glycogen and dextrin is useful for recognition of gravis, intermedius and mitis. Gravis ferments starch, glycogen and dextrin while intermedius and mitis have no action on starch and glycogen.

It is catalase positive, oxidase negative and do not liquefy gelatin. Urea is not hydrolyzed. It is indole negative and do not form phosphatase.

Resistance

It is readily destroyed by heat (58°C for 10 minutes). It is resistant to drying. It is sensitive

Table 26.1: Differentiation of Corynebacterium diphtheriae into gravis, intermedius and mitis

	Gravis	*Intermedius*	*Mitis*
1.	Morphologically they are short uniformly stained with few granules	Long, irregularly barred, very pleomorphic and poor granulational	Long curved, pleomorphic with prominent granule
2.	Colony is 1 to 2 mm on tellurite blood agar having grayish black center and semi-translucent periphery with commencing crenation of edge (daisy head colony)	Size 1 mm having dull granular center with smooth and glistening periphery carrying lighter ring near the edge (frog egg colony)	Colonies are shiny black, flat with central elevation (poached-egg colony)
3.	Hemolysis is variable	Non-hemolytic	Hemolytic
4.	Consistency is brittle and breaks readily, not emulsifiable	Intermediate between gravis and mitis	Soft, butyrous and emulsifiable easily
5.	Growth in broth is granular deposit, no turbidity and surface pellicle	Turbidity in 24 hour which is cleared in 48 hour having granular deposit	Diffuse turbidity with pellicle later on
6.	Glycogen and starch fermentation positive	Negative	Negative
7.	Antigenic structure—13 types	4 types	40 types

to penicillin, erythromycin and broad spectrum antibiotics.

Antigenic Structure

Antigenically they are heterogenous. Gravis has 13 types, intermedius 4 types and mitis 40 types.

Bacteriophage Typing

About 42 bacteriophages types are known. Type I and III are mitis, IV to VI intermedius, VII is avirulent gravis and remaining virulent gravis. A system of bacteriocin typing has also been described.

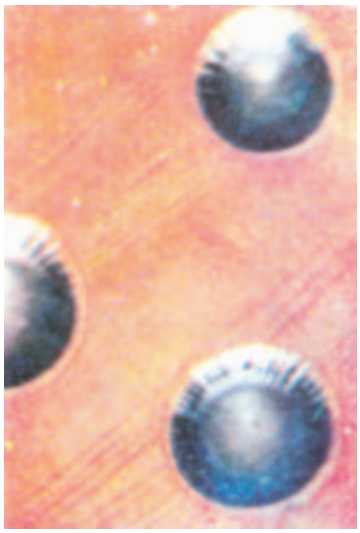


Fig. 26.2: Colonies of *Corynebacterium diphtheriae*

Toxin

Diphtheria bacilli produce very powerful exotoxin. The strain most universally used for toxin production is Park William 8 strain.

Toxin consists of 2 factors A and B. A is a lethal factor and B is a spreading factor. Fragment A inhibits polypeptide chain elongation in the presence of nicotinamide adenine dinucleotide, by inactivating the elongation of EF-2. So toxin fragment-A inactivates EF-2 by catalyzing a reactions that yields free nicotinamide plus an inactive adenosine diphosphate ribose EF-2 complex. It is presumed that abrupt arrest of protein synthesis is responsible for necrotizing and neurotoxic effects of diphtheria toxin. Diphtheria toxin is protein in nature (mol wt. 62,000). It is extremely potent. Lethal dose for 250 gm guinea pig is 0.0001 mg. It is labile.

It can be converted into toxoid (toxin that has lost toxicity but not antigenicity) by:

1. Prolonged storage at 37°C.
2. Incubation at 37°C for 4 to 6 weeks.
3. 0.2 to 4% formalin.

The toxigenicity of diphtheria bacilli depends upon the presence of a beta phage which acts as genetic determinant controlling toxin production. Toxigenicity remains as long as the bacillus is lysogenic. When bacillus is cured of phage it looses toxigenicity.

Toxin production is also influenced by the concentration of iron in the medium. 0.1 mg of iron per ml is the optimum concentration in the medium for toxin production. 0.5 mg per ml of iron in the medium inhibits toxin production. Other factors influencing the toxin production are osmotic pressure, pH, and availability of suitable carbon and nitrogen. The mechanism of action of toxin is not well understood. It inhibits protein synthesis and rapidly kill susceptible cell. It has affinity for myocardium, adrenal tissue and nerve endings.

Mode of Action of Toxin

The bacilli remain confined to site of entry where they multiply and form toxin. This toxin produces area of necrosis. The resulting fibrinous exudate together with epithelial cells, leukocytes, erythrocytes and bacteria form pseudomembrane.

Diphtheria does not occur naturally in animal but infection can be produced experimentally, e.g. cat, dogs, chicks, pigeons, guinea pig, rabbits, etc. Subcutaneous injection in guinea pig, with culture of virulent diphtheria bacilli will cause death of animal in 1 to 4 days. At autopsy of guinea pig, we may find:

1. Gelatinous, hemorrhagic edema and necrosis at the site of inoculation.
2. Swollen and congested lymph nodes.
3. Peritoneal exudate which may be hemorrhagic.
4. Congested abdominal viscera.
5. Enlarged and hemorrhagic adrenals.
6. Blood stained pleural exudate.
7. Pericardial effusion.

Pathogenicity

Incubation period is 3 to 4 days. Pathogenicity is because of toxin production. *Corynebacterium diphtheriae* does not actively invade deep tissues and never enters the bloodstream. The site of infection may be:

1. Faucial.
2. Laryngeal.
3. Nasal.
4. Otitic.
5. Genital, vulval, vaginal, etc.
6. Conjunctival.
7. Cutaneous around mouth and nose, etc.

Anyhow, faucial diphtheria is the most common.

On the basis of clinical severity, diphtheria may be classified:

1. Malignant in which there is severe toxemia. There is cervical lymphadenitis (bull neck). Death is due to circulatory failure.
2. Septic which leads to ulceration, cellulitis and gangrene around the pseudomembrane.
3. Hemorrhagic type is characterized by bleeding from the edge of membrane, epistaxis, conjunctival hemorrhage.

Complications

1. Asphyxia—a mechanical obstruction by pseudomembrane.
2. Acute circulatory failure which may be peripheral or central.
3. Post diphtheritic paralysis, e.g. palatine, ciliary, occurring in 3rd or 4th week of disease with spontaneous recovery.
4. Septic, e.g. pneumonia and otitis media.

Laboratory Diagnosis

Hematological investigations:
It is not significant.

Bacteriological Investigations

Laboratory confirmation of diphtheria is necessary for control measures and epidemiological purposes. Specific treatment should be given immediately without waiting for laboratory test report.

1. *Smear examination:* Gram-staining shows thin, Gram-positive bacilli showing Chinese letter arrangement. Albert staining is done for the demonstration of metachromatic granules (Fig. 26.3).
2. *Culture:* The swab is inoculated on Loeffler's slope, blood tellurite media and blood agar plate. The serum slope shows growth in 6 to 8 hours. Smear is stained with Albert stain and we may find bacilli with metachromatic granules and typical arrangement. Blood tellurite plate may be incubated for at least 2 days before declaring it negative. Individual strain of *C. diphtheriae* within a biotype can be identified by phage typing,

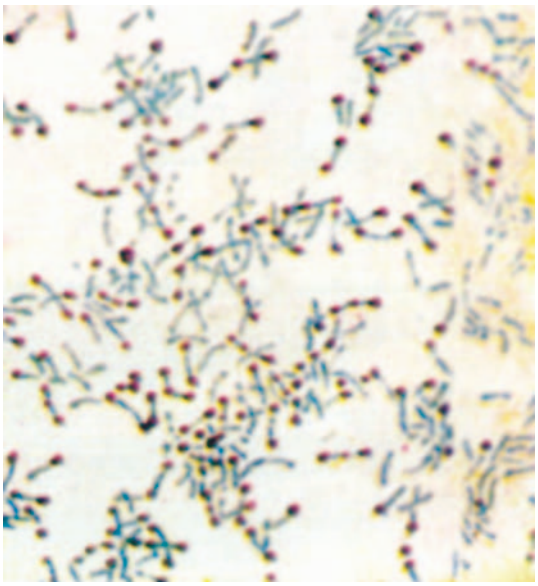


Fig. 26.3: Albert-stained smear of *Corynebacterium diphtheriae* showing metachromatic granules

analytic of bacterial polypeptides or DNA restriction patterns, DNA hybridization tests or PCR, ribotyping, etc.

Virulence Test

It may be done *in vivo* (intradermally or subcutaneously) or *in vitro* (Elek test). Besides this immunoelectrophoresis is also considered reasonably reliable for identification and establishing virulence of *Corynebacterium diphtheriae.*

Subcutaneous test: Overnight growth on Loeffler's slope is mixed in 2 to 5 ml broth and 0.8 ml is injected subcutaneously in 2 guinea pigs. One guinea pig is protected with 500 units of diphtheria antitoxin 8 to 24 hours before. If strain is virulent unprotected animal may die within 4 days. The control animal will remain healthy.

Intracutaneous test: Two guinea pigs are taken. One guinea pig is protected with 500 units of antitoxin the previous day and this animal acts as control. 0.1 ml of emulsion is inoculated at two different sites intracutaneous. To the other animal 50 units of antitoxin is given intraperitoneally 4 hours after skin test in order to prevent death. Toxigenicity is indicated by inflammatory reactions at the site of injection leading to necrosis in 18 to 72 hours while no change in controled animal occurs.

In vitro: The gel precipitation test is called Elek's test. A rectangular strip of filter paper impregnated with diphtheria antitoxin (1000 unit/ml) is placed on the surface of 20 percent normal horse serum agar in petridish while medium is still in fluid form. When it dries, testing strain is inoculated at right angle to filter paper strip. This plate is inoculated at 30°C for 24 to 48 hours. Toxin produced by bacterial growth will diffuse in agar and where it meets optimum concentration will produce line of precipitation. No precipitate will occur in non-toxigenic strain.

Tissue culture test: This may be done by incorporation of *Corynebacterium diphtheriae* into an agar overlay of cell culture monolayers. Toxin produced may diffuse into cells and kills them.

Primary isolates can be rapidly screened for toxigenicity by PCR.

Immunity

In non-immune person (Schick positive) immunity can be produced by active or passive immunization. For active immunization formal toxoid adjuvant such as alum-precipitated toxoid (APT), purified alum-precipitated toxoid (PAPT) and toxoid antitoxin floccules (TAF) are available.

Adjuvant toxoids give rise to severe reaction in older children and hence not suitable especially at the time of polio epidemic. Toxinantitoxin floccules contain horse serum which rarely produce serum hypersensitivity. It is used mainly in older children and adults. Generally children of less than 10 years are immunized with precipitated toxoid.

In endemic areas, active immunization is given at the age of 6 month. A booster dose is given at 18 months and another at school entry (6 years). This gives lasting protection.

Passive immunity: This is an emergency measure when the susceptible are exposed to infection. 500 to 1000 units of antitoxin (anti-diphtheria serum) is given subcutaneously. Being a horse serum one should take precaution against hypersensitivity.

Combined immunity: All cases that receive ADS prophylactically must get combined immunization. An alum-containing preparation is preferred in combined immunization as

the response to plain FT is not satisfactory when given with antitoxin.

Schick test: This is the skin test which is performed to find out susceptibility of a person to diphtheria.

0.2 ml diphtheria toxin is injected intradermally on left forearm and same dose of inactivated toxin (70°C for 30 minutes) is injected in right arm. Reading is taken on 1, 4, 7 days' interval. Four types of reactions are observed.

1. *Positive reaction:* An area of erythema and swelling at the site of injection in 24 to 48 hours reaching maximum in 4 to 7 days measuring 1 to 5 cm. The control area injected with heated toxin will show no reaction. Positive test means person is susceptible to diphtheria and antitoxin level is less than 0.01 Lf unit per ml.
2. Negative reaction means no reaction on either arm. It means person is immune to diphtheria *where antitoxin level exceeds 0.02 Lf unit per ml.*
3. Pseudo reaction is erythema within 6 to 24 hours and disappearing completely in 4 days. This reaction is same on both arms. This indicates person is immune to diphtheria and also hypersensitivity to this component of diphtheria bacilli.
4. *Combined reaction:* Here initial picture is that of pseudo reaction. Erythema disappears in control arm within 4 days. It progresses in test arm to positive reaction. It means person is susceptible to diphtheria and hypersensitive to the bacillus.

Treatment

The diphtheria bacillus is sensitive to most antibiotics. The antibiotics are of little value in treatment as they cannot deal with toxin already present in patient's body. Antitoxin should be given immediately if case is suspected as diphtheria since mortality rate increased with delay in starting antitoxic treatment. The dosage recommended are 20,000 unit intramuscularly (moderate case) and 50,000 to 100,000 unit for severe cases.

However, penicillin, preferably erythromycin or rifampicin or clindamycin, etc. may be used to prevent infection and to stop cases from becoming carriers. They may be used in dealing with established carriers.

DIPHTHEROIDS

Organism which is distinguished morphologically from *Corynebacterium diphtheriae* is called diphtheroid and is non-pathogenic (Table 26.2). It is found on conjunctiva, mucous membrane of nasopharynx, oral cavity and genitalia.

1. *C. hofmanni* is found in human throat.
2. *C. xerosis* has been isolated from xerosis. Its relation with disease is uncertain. It may be found on skin as normal flora.
3. *Propionibacterium acnes* is found in acne pustule and is anaerobic. It produces lipases, which split off free fatty acids from skin lipids. These fatty acids can produce inflammation and contribute to acne. Tetracycline can inhibit lipolytic action.

Morphology: It is short stumpy 1.5 to 2 μ in length with parallel sides and rounded ends. It does not exhibit pleomorphism and has no metachromatic granules. It occurs in palisades or bundles with uniform shape and staining. Actual differentiation can be made out by biochemical reaction and virulence test.

TABLE 26.2: Differences between diphtheria and diphtheroids

Diphtheria	*Diphtheroids*
A. Morphology	
1. Gram-positive and thin	Gram-positive, short and thick
2. Metachromatic granules are more	They are less or absent
3. Pleomorphism present	Very little pleomorphism
4. Chinese letter arrangement	No such arrangement is seen here
B. Culture	
Growth on enriched media	May grow on ordinary media
C. Biochemical reactions	
Fermentation of glucose only	May ferment glucose and sucrose
D. Toxicity	
They are toxic	Usually non-toxic

27 Mycobacteria

General characters: They are acid-fast, slender, rod, aerobic, non-motile, non-capsulated and non-sporing. Growth is generally slow. They do not grow on ordinary media. They require enriched media with egg albumin, e.g. Löwenstein-Jensen's medium.

Classification of Mycobacteria

A. *Tubercle bacilli*
   1. *M. tuberculosis* (human).
   2. *M. bovis* (bovine).
   3. *M. microti* (murine).
   4. *M. avium* (avian).
   5. *M. marinum* (cold blooded).

B. *Lepra bacilli*
   1. Human-*M. leprae.*
   2. Rat-*M. leprae murium.*

C. *Mycobacteria cause skin lesion*
   1. *M. ulcerans.*
   2. *M. balnei.*

D. *Atypical mycobacteria*
   Runyon Group I Photochromogen.
   Runyon Group II Scotochromogen.
   Runyon Group III Non-photochromogen.
   Runyon Group IV Rapid grower.

E. *Johne's bacillus*
   M. paratuberculosis

F. *Saprophytic mycobacteria*
   1. *M. butyricum.*
   2. *M. phlei.*
   3. *M. stercoris.*
   4. *M. smegmatis.*

MYCOBACTERIUM TUBERCULOSIS

Morphology: *M. tuberculosis* is straight or slightly curved rod 1 to 4 µ long and 0.2 to 0.8 µ wide. It may be arranged singly or in groups. It is non-motile, non-sporing and non-capsulated. *Mycobacterium bovis* is usually straighter, stouter and shorter. It is Gram-positive. It is acid fast (acid fastness is due to mycolic acid). Beaded or barred staining is seen in *M. tuberculosis.* It is more uniform without any bead in the *M. bovis* (Table 27.1).

Non-acid fast rods and granules from young culture are also reported and when they are injected into susceptible animal they produce tuberculosis. Perhaps these granules are non-acid fast form of tubercle bacilli. These bacilli are called Much's granules.

TABLE 27.1: Differentiation between *M. tuberculosis* and *M. bovis*

	Test	*M. tuberculosis*	*M. bovis*
1.	Morphology	Long, thin and curved	Shorter, stout and straighter
2.	Staining	Barred and beaded	Uniform
3.	Growth	Eugonic	Dysgonic
4.	Action of glycerol on growth	Enhanced	Inhibited
5.	Colony	Dry, rough, raised and wrinkled	Moist, smooth and flat
6.	Progressive disease in rabbit	—	+
7.	Niacin production	+	—
8.	Nitrate reduction	+	—
9.	Growth in semisolid medium	Grows at surface (aerobic)	Grows 10 to 20 mm below surface (microaerophilic)

Culture Characters

It is aerobic. It grows slowly (generation time 14 to 15 hours). Colonies appear in 2 to 6 weeks. Optimum temperature is 37°C and optimum pH is 6.4 to 7. *M. tuberculosis* grows luxuriantly in culture (eugonic) while *M. bovis* grows sparsely (dysgonic). Addition of glycerol improves the growth of human types. The enriched media is prepared by adding eggs, glycerol, potatoes, meat, bone marrow infusions or aspargine.

Liquid media: In liquid media without dispersing agent growth creeps up the side from the bottom, forming surface pellicles extending along the side of tube. Diffused growth is obtained in Dubo's medium (contain Tween 80). Virulent strains tend to form serpentine cords in liquid media.

Liquid media are generally required for sensitivity test, biochemical tests, and preparation of antigen and vaccine.

Solid media: *M. tuberculosis* forms dry, rough, raised and irregular colony. It is creamy white first and becomes yellowish or buff colored later on. It is not emulsified easily. The colony of bovis is flat, smooth and white, breaking up easily when touched.

Egg based solid media like L.J, Petragnani or American Tradeau Society medium have been used for primary isolation of *Mycobacterium tuberculosis* from clinical samples and have been found more sensitive than agar based media. Otherwise agar based media are more transparent and permit early microscopic detection of colonies. Culture techniques are more sensitive, than staining procedure, but the major limitation are long time taken for their growth.

Solid media used are Dorset egg, Löwenstein-Jensen's medium and Loeffler serum slope. Löwenstein-Jensen's medium is most widely used which contains coagulated hen's eggs, salt solution, malachite green, glycerol and aspargine.

Tubercle bacilli may also be grown on chick embryos and in tissue culture. Recently, it is reported that selenite medium 5 µg of sodium selenite in agar shortens the time period of growth of colonies of *Mycobacterium tuberculosis* (3 to 5 days) otherwise it takes 2 to 6 weeks for the colonies to appear. Disadvantages of selenite include its toxicity and carcinogenic properties. Rapid growth of *M. tuberculosis* may also be obtained by adding blood or hemoglobin to the medium.

Resistance

It is more resistant to drying and chemical disinfectants. Temperature 60°C for 20 minutes can kill it. Moist heat at 100°C kill it readily. In sunlight the culture may be killed in 2 hours. In

MEDIA FOR MYCOBACTERIAL CULTURE

Medium	*Composition*	*Inhibiting agents*
SOLID MEDIA		
1. Löwenstein-Jensen	Coagulated whole eggs with salts, glycerol, potato flour, aspergine, etc.	Malachite green
2. L.J with pyruvic acid	L.J and pyruvic acid	do
3. Petragnani	Coagulated eggs, egg yolks, white milk, potato flour, glycerol, etc.	do
4. Wallenstain	Egg yolks and glycerol	do
AGAR BASED		
1. Middlebrook 7H 10	Salts, vitamins, co-factors, oleic acid, albumin, catalase, glycerol, dextrose, etc.	do
2. Middlebrook 7H 11	Middlebrook 7H 10 and Casium hydrolysate etc.	do
LIQUID		
1. Dubo's medium	Bovine serum albumin, aspargine, caseine hydrolysase, salts and Tween 80	
2. Middlebrook 7H9 Broth	Do	
3. Bactec 12 B medium	Commercial media supplied by firm	

sputum it survives 20 to 30 hours even in sunlight.

It is killed by tincture of iodine in 5 minutes and by 80 percent ethanol in 2 to 10 minutes. Phenol solution (5%) kills it in 24 hours.

Biochemical reactions: The important biochemical tests for its identification are:

1. *Niacin test:* Human tubercle bacilli form niacin when grown on egg medium. Ten percent cyanogen bromide and 40 percent aniline in 96 percent ethanol are added to a suspension of culture. Yellow color indicates positive test.
2. *Aryl sulfatase:* Organism is grown, for 2 weeks in media, containing tripotassium phenolphthalein disulphate. Detection of free phenolphthalein is detected by addition of alkali. Red color indicates positive test. It is positive in atypical mycobacteria.
3. *Neutral red:* The virulent strain of tubercle bacilli may bind the neutral red in alkaline buffer more readily and firmly than bovine types.
4. *Catalase test:* A mixture of equal volume of 30 Vol H_2O_2 and 0.2 percent catechol in distilled water is added to 5 ml of test culture. Effervescence indicates positive catalase test and browning of colonies indicates peroxidase activity.

   Most atypical mycobacteria are strongly catalase positive. Tubercle bacilli is peroxidase positive. Catalase and peroxidase activities are lost when tubercle bacilli develop resistance to INH.
5. *Nitrate reduction:* This is positive in *M. tuberculosis* and negative in *M. bovis.*
6. *Amidase test:* Five amides are used, i.e. acetamide, benzamide, nicotinamide, carbamide and pyrazinamide. A 0.00164 M solution of amide is incubated with bacillary suspension at 37°C. 0.1 ml of $MnSO_4 4H_2O$, 1 ml of phenol and 0.5 ml of hypochlorite solutions are added. These tubes are placed in boiling water for 20 minutes. A blue color means positive test. The ability to split amides has been used to differentiate atypical mycobacteria.

Antigenic structure: Many antigens have been identified. Group specificity is due to polysaccharide. Type specificity is due to protein antigen. Protein antigen is used for tuberculin test. Tuberculin from *M. tuberculosis, M. bovis* and *M. microtic* appear to be indistinguishable.

Bacteriophage: Fresh isolate can be classified into 4 bacteriophage type A, B, C, AB. The predominant types in South India are A and AB.

Pathogenesis: The basis of virulence of bacillus is unknown. It does not produce toxin. May be the various components of bacillus possess different biological activities influencing pathogenesis, allergy and immunity in disease.

Determinants of *Mycobacterium Tuberculosis* Pathology

These determinants are as under:

1. Cording factors are actually glycolipid derivatives of mycolic acid. They are present in outer surface of tubercle bacilli. They are responsible for:
   a. Inhibition of migration of polymorphonuclear leukocytes and ultimately results in the formation of granuloma.
   b. Inducing protective immunity as it is immunogenic.
   c. Growth of tubercle bacilli in serpentine cord.
2. Cell surface glycolipids inhibits phagolysome formation. This allows intracellular survival of tubercle bacilli after ingestion by macrophages.
3. Resistance to antituberculosis antibiotics which is acquired by mutation.

The cell wall of bacillus causes, delayed hypersensitivity. Tubercle protein can elicit tubercular reaction and when bound to lipid can induce delayed hypersensitivity. In tissue it leads to formation of monocytes, macrophages, epithelioid cells and giant cells. The bacterial polysaccharide induces immediate hypersensitivity and causes exudation of neutrophils from blood vessels. Lipid causes accumulation of macrophages and neutrophils. Phosphatids induce the formation of tubercle consisting of epithelioid cells and giant cell. The occurrence of eukaryotic like Adenyl cyclase in *M. tuberculosis*, suggest a role for the enzyme in cell signaling and perhaps in the pathogenesis of *M. tuberculosis.*

There are two types of lesion:

1. Exudative.
2. Productive.

Exudative lesion is an acute inflammatory reaction with accumulation of edema fluid, leukocytes and later on monocytes. It may heal by resolution leading to necrosis or develop into productive type.

The productive lesion is predominantly cellular composed of number of tubercles which may enlarge, coalesce, liquefy and undergo caseation.

Route of infection: Tubercle bacilli enter the body commonly by inhalation, ingestion or by inoculation into skin. When inhaled bacilli are lodged in pulmonary alveoli. They are phagocytosed by alveolar macrophages. Inside phagocyte the bacilli keep on multiplying. Phagocytes with ingested bacilli may act as vehicle transporting the infection to different parts of the body. The multiplication of bacilli is stopped only with the development of specific cellular immunity. It occurs in 6 to 8 weeks after infection.

Primary complex or first infection or childhood type of tuberculosis occurs early in life. It consists of subpleural focus of tuberculous pneumonia in lung parenchyma found in lower lobe or in lower part of upper lobe with enlarged draining lymph nodes. Primary complex is an asymptomatic lesion undergoing spontaneous healing resulting in hypersensitivity to tuberculo-protein and acquired immunity. Rarely it may lead to hematogenous spread and development of miliary tubercle meningitis, etc.

The adult type is due to reactivation of primary infection or exogenic reinfection. Lesion may heal by reabsorption, fibrosis and calcification. It may progress to chronic fibrocaseous tuberculosis with tubercle formation, caseation, and shedding of bacilli in sputum.

Laboratory Diagnosis (Table 27.2)

A. *Hematological investigations:*
   1. *Total leukocyte count:* In early acute cases there is leukocytosis. In miliary infection there may be leucopenia.
   2. *Differential leukocyte count:* In early cases there is increase in neutrophils. Later on there is an increase in monocytes and lymphocytes.
   3. *Erythrocytic sedimentation rate* is usually raised which is an index of progress of the disease.

B. *Bacteriological investigations:*
   Microscopy: Smears may be prepared from specimen like sputum, laryngeal swab,

TABLE 27.2: Current laboratory diagnostic methods of *Mycobacterium tuberculosis*

S.No.	Method	Advantages	Disadvantages
1.	Microscopy (smear for acid-fast bacilli)	1. Low cost 2. Rapid diagnostic	1. Low sensitivity (up to 2/3 of pulmonary tuberculosis cases are negative). 2. Difficult sample collection
2.	Culture	Specific	1. Time consuming (up to 4-8 weeks). 2. Not always possible.
3.	PCR	1. Relatively quick 2. Very specific	1. Relatively expensive 2. High level of training required. 3. Expensive instrumentation 4. Can detect latent disease.
4.	BACTEC	Specific	1. Slow, 2-3 weeks. 2. Expensive
5.	Tuberculosis ELISA test kits	1. Quick (procedure time 110 min). 2. Reproducible with minimal training.	Some equipment required
6.	Rapid tests	1. Very quick (procedure time only 15 min). 2. Minimal training 3. No special equipment required.	1. Lower sensitivity compared to ELISA test kit. 2. No quantitative results

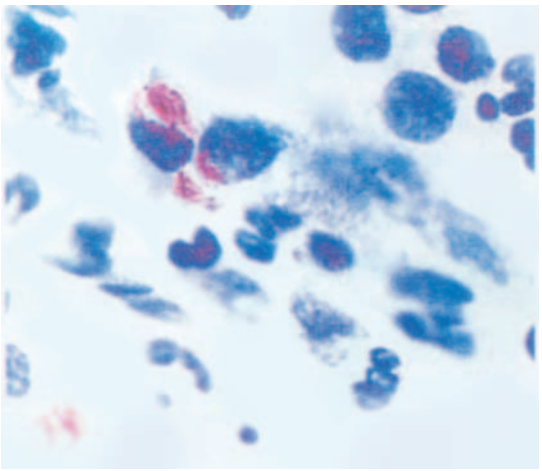


Fig. 27.1: *Mycobacterium leprae* (modified Ziehl-Neelsen staining)

pleural fluid, peritoneal fluid, cerebrospinal fluid, pus, urine, gastric lavage, feces and other infected material.

New glass slides should be used for smear. Smear should be made from thick purulent portion of sputum. The smear is dried, fixed and stained by Ziehl-Neelsen technique. The slide is studied under oil immersion objective. Acid-fast bacilli are seen as pink brightened rods, while background is blue (Fig. 27.1). It has been estimated that at least 50,000 to 1,00,000 acid-fast bacilli should be present per ml of sputum to get positive report. A negative report should not be given till at least 100 fields are examined taking about 10 minutes time.

Where several smears are to be examined daily, it is more convenient to use fluorescent microscopy. Smears are stained with auramine phenol or auramine rhodamine fluorescent stain. They are examined under ultraviolet light and bacilli appear bright rods against dark background.

Concentration Methods

Following are the methods which concentrate the bacilli without inactivating the bacilli and hence can be used for culture and animal inoculation:

1. *Petroff's method:* Sputum is incubated with equal volume of 4 percent solution of sodium hydroxide. Shake till it becomes clear. It takes about 20 minutes. Centrifuge it at 3000 rpm for 30 minutes and sediment is neutralized with N/10 HCl. Sediment is used for smear culture and animal inoculation.
2. Homogenization may be achieved with dilute acid (6% H_2SO_4, 3% HCl or 5% oxalic acid).
3. *Flocculation method:* Specimen is treated with digester containing sodium hydroxide and potash alum. It is neutralized with acid. Floccules start appearing. These floccules are sedimented by centrifugation.
4. *Jungmann's method:* Acidic ferrous sulfate and H_2O_2 is used. A bulky deposit is obtained and used for inoculation.
5. Trisodium phosphate is lethal for many contaminating bacteria but tubercle bacilli are unaffected.
6. N-acetyl-l-cysteine with NaOH is considered rapid and effective method for homogenization of mucopurulent specimen. Pancreatin, desogen, zephiran and cetrimide are other homogenizing agent.
7. Addition of 20 percent clorox to sputum but this method is not suitable for culture.
8. Lauryl sulfate method: Here 1.5 percent sodium hydroxide and 4.5 percent lauryl sulfate is used.

Culture: Culture techniques have been estimated to detect 10 to 1000 viable mycobacteria per ml of specimen. The most common media are based on egg and also contain high concentration of malachite green to overcome contamination with other bacteria. The concentrated material is inoculated into 2 bottles of L.J. media. The tubercle bacilli grow in about 4 to 8 weeks of incubation at 37°C. However, rapid growing mycobacteria may appear in 4 days' time. A negative report is sent if no growth appears by the end of 8th week to 12th week.

Ogawa medium: It is egg based medium. It is most economical because it replaces aspargine by sodium glutamate. Actually it contains mineral salts (potassium dihydrogen phosphate anhydrous, sodium glutamate), distilled water, malachite green dye and homogenized eggs. The modifed Ogawa medium in addition contains magnesium citrate and glycerol. The pH of modified medium is 6, 4. The ingredients are mixed well and dispensed in 6 to 8 ml volume in MacCortneys bottles. Now medium

in MacCortneys bottle is insipissated at 80°C to 85°C for 45 minutes. The inspissation is repeated for 2nd and 3rd time. This medium may be stored in refrigerator for several weeks.

In laboratories where centrifuges are not available, sputum specimens are decontaminated with 4% of sodium hydroxide and inoculated directly on modified Ogawa medium.

Middlebrook 7H 10 and 7H 11 are agar based media. They achieve slightly higher isolation yield than egg based media but are quite expensive.

Blood agar is an alternative culture medium for the isolation of mycobacterium. *Mycobacterium tuberculosis* grows within one or two weeks on blood agar plates. Average number of colonies isolated from clinical specimens on blood agar is significantly higher than the number of colonies on egg based medium. It can also be used as alternative medium for drug sensitivity testing of *Mycobacterium tuberculosis* against antitubercular drugs like isoniazid, rifampicin, streptomycin and ethambutol. Results are obtained much earlier with blood agar. However, they require 5 to 10% carbon dioxide. Desiccation may be prevented by sealing the plates with tapes or by using tubes instead of plates.

Colonies that develop are smeared and stained by Ziehl-Neelsen method and examined. For routine purpose, slow growing, non-pigmented, acid fast and niacin positive is taken as *M. tuberculosis.* Confirmation is done by animal inoculation and biochemical tests.

Sensitivity tests: Drug resistance is a big problem. Drug sensitivity is done by:

1. *Absolute concentrate method:* Media contains serial dilutions of drug is inoculated. Minimum inhibiting concentration is the least drug concentrate that inhibits growth.
2. *Resistance ratio method:* Two sets of media are used containing graded concentration of drug. One set is inoculated with standard strain and other with test strain and resistance ratio is worked out.
3. *Proportion method:* It indicates average sensitivity of strain.
4. *Rapid drug susceptibility test using Resazurin:* Here metabolic activity of *Mycobacterium tuberculosis* is indicated through the use of oxidation reduction indicator Resazurin.
5. A new test, developed at the Albert Einstein College of Medicine in New York involves introducing a gene from firefly into *Mycobacterium tuberculosis* exposed to drugs. Only the drug resistant to *Mycobacterium tuberculosis* will glow. To make the bacteria glow, a gene is introduced for the luciferase enzyme from the firefly into *Mycobacterium tuberculosis,* the genetic engineered microorganism then emits light. When exposed to antituberculosis drugs, the dead *Mycobacterium tuberculosis* is incapable of producing light but drug resistant bacteria will continue to glow. This test is reported to identify drug resistane within two or three days which is likely to be further reduced to few hours.

Newer Drug Screening Methods of *Mycobacterium Tuberculosis*

1. PCR method has special promise for future.
2. E. test is an emerging method for MIC determination anti-microbial agents. All Biodiscs employing various drugs have been found to be promising for *Mycobacterium tuberculosis* and others.
3. FDA staining flurocytometry is based on differentiating the live and dead organisms by fluorescent dyes like fluororescent-diacetate and ethidium bromide. This method is also used in automated method for *Mycobacterium tuberculosis.*
4. Hybridization protection assay based on quantitative assessment of gene hybridization has been reported promising.

Animal inoculation: Two healthy guinea pigs are taken. Tuberculin test is done to ascertain that they are not tuberculous. Concentrated material is injected intramuscularly into the thigh of both animals. Progressive loss of weight is an indication of tuberculosis. One animal is killed at 4 weeks and if no evidence of tuberculosis is noticed at autopsy the other is killed after 8 weeks. At autopsy infected animal shows:

1. Caseous lesion at the site of inoculation.
2. Enlarged caseous draining lymph nodes.

3. Enlarged spleen showing necrotic areas.
4. Tubercles in peritoneum.
5. Tubercles in lungs.
6. Kidney is unaffected.

Diagnosis of tuberculosis is confirmed by demonstration of acid-fast bacilli from lesion. Infection with *M. pseudotuberculosis,* salmonellae, brucellae, pyogenic cocci may simulate the appearance of tuberculous lesion.

IMMUNOLOGICAL INVESTIGATIONS

Immunological investigations may play important role in the diagnosis of tuberculosis especially in following situations:

a. When patient is unable to produce adequate sputum.
b. When sputum smear results are negative.
c. When there is extra pulmonary tuberculosis.

The tuberculin skin test remains one of the few tests that have been in use for the last 100 years for diagnostic purposes. Its limitations includes (1) initial testing can affect results of subsequent tests because of an amnestic recall of immunity (2) error in administration of reading can lead to incorrect results.

Two new T cell based test for the diagnosis of latent tuberculosis have been developed. They are (1) Quanti FERON (QET)—TB Gold, using ELISA to measure antigen specific production of interferon γ (IFN-γ) by circulating T cells in whole blood. (2) T-SPOT. TB in which Elispot technique is used to measure peripheral blood mononuclear cells that produce IFN-γ. In fact IFN-γ release by above mentioned test use more specific *Mycobacterium tuberculosis* antigen (ESAT-6, CFP-10 and TB7-7). The genes encoding these antigens are found in the region of difference I (RDI) of the *Mycobacterium tuberculosis* genome which is deleted from genome of *Mycobacterium bovis* BCG and some non-tuberculous mycobacteria like *Mycobacterium avium*.

Interferon γ release assays mentioned above have following advantages over tuberculin test:

i. Testing needs only one patient visit.
ii. These assays are ex-vivo, which minimize the risk of adverse effect and eliminate potential boosting when testing is repeated.

Adenosine Deaminase Activity (ADA)

It is a simple and cheap, technique helpful in the diagnosis of tuberculous meningitis, tuberculous pleurisy and tuberculous pericarditis. Mycobacteria may be difficult to isolate from these specimens because they are diluted in large fluid volume. Adenosine deaminase activity is determined colorimetrically. The suggested laboratory cut off value of ADA is 40 IU/ liter for pleural fluid, peritoneal fluid and pericardial fluid, where as it is 10IU/liter for cerebral spinal fluid.

Allergy tests: Hypersensitivity to tuberculoprotein by tuberculin test is of limited value as it does not differentiate between clinical disease and sub-clinical disease. It is of some value in indicating active infection in children below 5 years ago. A negative tuberculin test is often helpful to exclude the diagnosis of tuberculosis.

Serology: No serological test is helpful in the diagnosis of tuberculosis. IHA may be positive in cases of established tuberculosis. However, high titers of IgG antibody to PPD, detectable by ELISA test, are considered to exist in many patients with active pulmonary tuberculosis. The soluble antigen fluorescent antibody (SAFA), ELISA and IHA tests are proving of considerable diagnostic significance.

DNA probe for detection of tubercle bacilli is quite rapid and specific.

Rapid techniques for establishing the diagnosis of *Mycobacterium tuberculosis* are polymerase chain reaction and detection of antigen A 60.

Immunity: Infection with tubercle bacillus induces delayed hypersensitivity and resistance to infection. Cell mediated immunity is the only immunity operative in tuberculosis. Humoral immunity has no influence on the course of disease. In non-immune host bacillus is able to multiply inside phagocyte and destroy the cell. In immune host activated T lymphocytes release lymphokines which make the macrophages bactericidal.

The main cell is the activated CD_4 and helper T cell. They may develop along the Th-1 or Th-2 cells. Thus they release cytokines like

interferon gamma, interleukins-1, interleukins-2, toxic necrosis factor alpha-Th-1 dependent cytokines. Above mentioned factors activate macrophages causing protective immunity and containment of the infectivity. Th-2 cytokines induce delayed type hypersensitivity, tissue destruction and progressive disease.

Subcutaneous injection of virulent tubercle bacilli in normal guinea pig produces no immediate response. At the site of injection a nodule appears after 10 to 14 days. The nodule bursts into non-healing ulcer which persists till the animal dies of progressive tuberculosis. Draining lymph nodes are enlarged and caseous. If subcutaneous injection of tubercle bacilli is given to guinea pig infected with *Mycobacterium tuberculosis* 4 to 6 weeks earlier, indurated lesions appear at the site of injection. It undergoes necrosis and shallow ulcer appears which heals rapidly without involving lymph nodes. This is called Koch's phenomenon and is a combination of hypersensitivity and immunity. Koch's phenomenon has 3 components:

1. Local skin reaction.
2. Focal response causing congestion and hemorrhage around tuberculous foci.
3. Systemic response of fever which may be fatal.

Tuberculin

1. Old tuberculin (OT) consists of filtrate of glycerol broth culture of bacilli concentrated to 1/10th of volume by evaporation on water bath.
2. Purified protein derivative (PPD) is prepared by precipitation of tubercle bacilli culture grown in synthetic medium with trichlor acetic acid. PPD is superior to OT because it is stable, and constant in activity. Tuberculin prepared from bovine type is as active as tuberculin prepared from human type.

In Mantoux test, widely used in graded doses of tuberculin which is injected intradermally on forearm. After 48 to 72 hours positive reaction is indicated by erythema, edema and induration of the size measuring 6 to 10 mm in diameter. The graded dose ranges from 1 tuberculin unit (0.01 mg OT or 0.00002 mg PPD) to 100 or 250 TU. Tuberculin injected into hypersensitive host may give rise to severe local reactions and flare up of inflammation and necrosis at main site of injection. So tuberculin is injected in the doses, i.e. 5 TU dose in surveys, 1 TU in person suspected of hypersensitivity and up to 250 TU if reaction is negative. The tuberculin test becomes positive within 4 to 6 weeks after infection or injection of a virulent bacilli.

INTERPRETATION OF MANTOUX TEST

Note the area of inoculation for erythema, edema and palpate the area for any hard nodular feeling. In case there is nodular feeling measure its diameter in both the directions using centimeter scale.

1. No erythema or edema or induration	Negative
2. Slight redness, trace of edema of 5 mm or less	Doubtful 1+ reaction
3. Redness, definite edema, dense hard elevated borders with bullbous or dotted vesicles more than 5 mm diameter but less than 10 mm.	2+ reaction
4. Marked redness, edema, hard elevated borders of 10 mm diameter	3 + reaction
5. Marked redness, edema, necrosis and hard elevated borders of more than 10 mm diameter	4 + reaction

False Negative Tuberculin Test

It is seen in miliary tuberculosis, malnutrition, malignancy, viral infection (measles), sarcoidosis and immunosuppressive therapy or defective CMI.

False Positive

It is seen in *M. avium* or atypical mycobacterium.

Uses of Tuberculin Test

1. Diagnosis of active infection in infants.
2. Measure prevalence of infection in community.
3. Susceptibility of BCG vaccination.
4. Indication of successful vaccination.

Newer Diagnostic Techniques: They are improved techniques and include:

1. **Bactec system:** It is a liquid medium made selective by addition of antibiotics. It contains radiolabelled palmitic acid which is used as substrate by growing mycobacteria leading to production of

labelled carbon dioxide. Frequent measurement of released gas lead to detection of the presence of mycobacteria much earlier than by any other system. *Mycobacterium tuberculosis* can be tentatively identified by subculturing each positive bottle into a bottle containing beta nitro alpha acetylamino beta hydroxy propiophenone (NAP) which inhibits only *Mycobacterium tuberculosis* and allows the growth of mycobacteria other than tubercle bacilli (MOTT).

2. **Septic check acid-fast bacilli system:** These are biphasic systems and consist of an enriched selective broth and a slide with solid media. One side of the slide is covered with non-selective Middlebrook agar. The reverse side of the slide is divided into two sections: one containing beta nitro alpha acetylamino beta hydroxy propiophenone (NAP) incorporated agar/modified egg medium and the other chocolate agar to indicate contamination. Compared to L.J. medium and BACTEC isolation systems best mycobacterial recovery has been observed in septic check followed by BACTEC.
3. **Mycobacteria growth indicator tube:** This system provides easy to use system with high accuracy for early detection of mycobacterial growth (11 to 13 days) from clinical specimens. It is manual detection in which positive results emit a vivid orange fluorescent glow and results compare favorably with BACTEC-460B (8 to 12 days).
4. **Techniques:** Based on immunological relatedness of enzymes like divergencies on the superoxide dismitase (SOD) molecule, an ELISA system has been developed by which pathogenic mycobacteria can be identified.
5. **DNA probes:** In the earlier phase of development, nucleic acid probes were based on total DNA or cloned fragments. Later well-defined oligo-nucleotide probes were developed. For direct clinical confirmation of diagnosis from clinical specimens, these methods are not very sensitive and need more than 1000 organism in the specimen for positivity.
6. **Ribosomal RNA based probes:** RNA based probes may target RNA, ribosomal DNA and spaces sequences. These probes have been reported useful for quick identification of mycobacteria from culture isolates. These probes are developed into chemiluminescent techniques. They are 10 to 100 fold more sensitive than DNA targeting and may be used to confirm the diagnosis directly in the clinical specimen. However, the lowest detection limit is around 100 organisms.
7. **Polymerase chain reaction:** By this 1 to 10 organisms can be detected. PCR assay system for tuberculosis is commercially available and is reported to be reproducible, sensitive as well as specific. Use of immunomagnetic beads and capture resin can significantly increase the sensitivity of the PCR assay.

The other methods found useful are strand displacement amplification (SDA), gene probe amplified *Mycobacterium tuberculosis* direct test, QB replicase based gene amplification. In the list of immunological tests like radioimmune assay, IHA, particle agglutination, etc. Use of ELISA using LAM, 38 kD antigen can be more useful.

Rapid laboratory methods to diagnose tuberculosis: Faster culture methods using radiometric systems such as BACTEC non-radiometric like MGIT are being used increasingly mainly because they reduce the time of culture and drug sensitivity testing to about 2 to 3 weeks. Nucleic acid amplification techniques are used mainly for the cases where there is a chance of infection other than *Mycobacterium tuberculosis*. Undoubtedly many of these techniques are quite expensive.

BCG (*Bacille Calmette-Guérin*)

This is a bovine strain of tubercle bacillus rendered completely avirulent by culturing repeatedly (*M. bovis* maintained for 13 years by 239 subculture on glycerine potato medium). The strain is used in inducing active immunity to tuberculosis. The vaccine contains live avirulent bacilli and is administered to tuberculin negative individual.

The vaccine is given intradermally over deltoid region. It confers 60 percent protection

in India and 80 percent protection in USA and UK. Immunity lasts for 10 to 15 years. After BCG vaccination a positive tuberculin test may last for 3 to 7 years.

BCG VACCINE

One possible explanation for poor efficiency of BCG vaccine is a shortage of antigens that elicit a protective response against *Mycobacterium tuberculosis*. As we know, the attenuated BCG vaccine was originally derived by serial passage of virulent strain of *Mycobacterium bovis*. In the process there was loss of many coding sequences including a group of nine genes known as the region of difference 1 (RD 1). There is a report that immunization with recombinant BCG strain with RD 1 replaced improves protection against *Mycobacterium tuberculosis* in animal models. It appears that the presence of two extra antigens (ESAT 6 and CPF 10) involve more rebust CD 4 T cell response coupled with possible role for these molecules allowing vaccine strain to persist in host might be contributing factors towards improved efficacy and hence enhanced protection.

Complications of BCG

Local

1. Abscess.
2. Ulcer.
3. Keloid.
4. Tuberculoid.
5. Confluent reaction to Heaf's multiple punctures.
6. Lupis vulgaris.
7. Lupoid lesion.

Regional

1. Enlargement of draining lymph node.
2. Abscess formation in lymph node.

General

1. Fever.
2. Mediastinal adenitis.
3. Erythema nodosum.
4. Otitis media.

BCG also confers immunity against leprosy. It is also used in the treatment of leprosy and malignancy.

Mycobacterium habana has been identified as a possible source of vaccine against tuberculosis. It may become alternative to BCG whose efficiency has been questioned of late. It elicits cell mediated response *in vitro* and *in vivo*. Other different BCG strains are:

1. Glaxo freeze dried BCG vaccine 1077.
2. Danish BCG vs vole bacillis.
3. Isoniazid sensitive strains of BCG.
4. 1173-P_2.
5. Tokyo 172.
6. Copenhagon 1331.

Clinical trials of Mycobacterium vaccae vaccine in tuberculosis are in progress.

Treatment: Streptomycin, PAS and INH are first line drugs. The reserve drugs are ethambutol, rifampicin, pyrazinamide, procainamide, cycloserine, capreomycin and thiocetazine.

ATYPICAL MYCOBACTERIA (Table 27.3)

They are also called opportunistic, tuberculoid or mycobacteria other than typical tubercle bacilli (MOTT) or environmental organisms.

They resemble *M. tuberculosis* but exhibit number of atypical characters:

1. They grow much faster than *M. tuberculosis*. Colonies may appear within 7 to 10 days.
2. They may grow on simple media like nutrient agar.
3. They are capable of growing at low temperature (20°C to 25°C) and high temperature 45°C.
4. Most of these bacteria are resistant to antitubercular drugs.
5. Organism is either longer than *M. tuberculosis* or smaller.
6. They are pigmented.
7. They are catalase positive.
8. Aryl sulfatase test is always positive.
9. Cord formation is absent.

Atypical mycobacteria are classified into following 4 groups according to *RUNYON:*

Group I: It is known as photochromogens. It produces no pigment in the dark but when young culture is exposed to light for 1 hour in presence of air and reincubated at 24 to 48 hours yellow orange pigment appears. It is slow growing, e.g. *Mycobacterium kansasii* which produces chronic pulmonary infection in man.

Group II: It is known as scotochromogens. It forms pigmented (orange) colonies even in the dark. It sometimes contaminates culture of

TABLE 27.3: Difference between typical and atypical mycobacterium

Group	*Growth time*	*Colonies*	*Morphology*	*Pigment*	*Catalase*	*Niacin*	*Neutral red*	*Cord test*
HUMAN	6 to 9 weeks	—Dry	—Slender	None	Moderate	+	+	Tight
BOVINE	3 to 6 weeks	—Dry —Smooth —Dysgonic	—Short and thick	None	Moderate	—	+	Tight
GROUP I	3 to 6 weeks	—Dry —Eugonic	—Larger —Beaded	Yellow or light yellow	Violent	—	±	Slight
GROUP II	2 to 3 weeks	—Moist —Smooth —Eugonic	—Large —Coarse —Beaded	Yellow to orange in dark	Violent	—	—	None
GROUP III	3 to 4 weeks	—Dysgonic —Doomed	—Small —Bipolar beads	None rarely light yellow	Moderate	—	—	None
GROUP IV	3 to 5 days	—Usually moist and spreading	—Large —Incompletely acid fast	White to all colors	Variable	—	—	None

tubercle bacilli. It is usually non-pathogenic. However, *M. scrofulaceum* causes cervical adenitis (scrofula) in children.

Group III: It is non-photochromogen. It does not form pigment even on exposure to light. The colonies are buff colored, e.g. *M. intracellulare* (chronic pulmonary infection, renal infection and lymphadenopathy), *M. avium* (cervical adenitis in children and lung infection in adults) and *M. xenopi* (chronic lung infection in man). It can grow even at 45°C. Actually *Mycobacterium avium* and *Mycobacterium intracellulare* are closely similar that they have been considered as one group called *Mycobacterium avium* complex (MAC). In diseases like AIDS, they may cause lymphadenopathy, pulmonary disease, etc.

Group IV: It is also called rapid growers. Colonies appear within 7 days of incubation at 37°C or 25°C. All the chromogenic rapid grower are saprophyte. *M. fortuitum* (pulmonary lesion for which no effective chemotherapy is available) and *M. chelonei* may cause abscesses in man.

SKIN PATHOGENS

Two species, *M. ulcerans* and *M. marinum* are skin pathogens causing chronic ulcer and granulomatous lesion of skin (Table 27.4). Cutaneous localization is because they multiply at skin temperature.

TABLE 27.4: Difference between *M.ulcerans* and *M.marinum*

Characters	*M. ulcerans*	*M. marinum*
Distribution	Tropics	Temperate zone
Clinical course	Chronic progressive ulcer	Self limited ulcer
Bacilli in ulcer	Abundant	Scanty
Rate of growth	Slow, 4 to 8 weeks	Faster, 1 to 2 weeks
Growth at 25°C	—	+
Growth at 37°C	—	+
Culture film	Bacillus in cord	No cord formation
Pigment in light	—	+
Mouse pad lesion	Edema, and rarely ulcer	Marked inflammation and purulent ulcer

M. ulcerans: It forms large clumps of bacilli which are acid fast and alcohol fast. It grows on L.J. media slowly in 4 to 8 weeks. Growth occurs between 30° and 33°C.

It causes ulcer usually on legs and arms following infection through minor injuries. After few weeks area become indurated and breaks down forming indolent ulcer which slowly extends under the skin. It is non-pathogenic to guinea pig and rabbit. In foot pad of mice if injected, forms edema of leg.

M. marinum: It is also called *M. balnei.* It causes tuberculosis in fish and amphibia. It occurs as saprophytes in fresh or salt water. Human infection may occur in epidemic form. The ulcers produced are self limited and undergo spontaneous healing. Growth occurs in 1 week at 30°C. Colonies are non-pigmented in dark and golden yellow on exposure to light.

Foot pad inoculation in mice causes severe and purulent ulcer formation.

MYCOBACTERIUM LEPRAE

India today, has over 40 lakh leprosy cases, or half the world's total. The maximum number are in UP. In some areas of the country, one to three percent of the population has the disease.

About 20,000 people are reported suffering from leprosy in Jammu and Kashmir state.

Morphology: It is long 4 to 5 µ, slender, slightly curved or straight, occurring in bundles of parallel packets. It is weakly acid fast (5% H_2SO_4 is used for decolorization). In smear living bacilli are uniformly stained while dead bacilli are fragmented and irregular.

Bacteriological index is defined as number of viable bacilli in a lesion. Their viability is assessed from stained smear. Internationally agreed bacteriological index is 6^+. Scale commonly used is × 100 oil immersion. It may be expressed as under:

6^+ 1000 or more bacteria in every microscopic field.
5^+ 100 or more bacteria in every microscopic field.
4^+ 10 or more bacteria in every microscopic field.
3^+ 1 or more bacteria in every microscopic field.
2^+ 1 or more in every 10 microscopic field.
1^+ 1 or more in every 100 microscopic field.

Morphological index is more significant and meaningful for assessing the progress of patient on chemotherapy. It is defined as the percentage of uniformly stained bacilli in the tissue.

Cultural characters: It has not been cultured in bacteriological media or tissue culture. However, there are reports of successful cultivation:

1. In Indian Cancer Research Centre Bombay (1962), acid-fast bacilli was isolated from lepromatous patient. They employed human fetal spinal ganglion cell culture. The ICRC bacilli has been adapted to grow on L. J. media.
2. Shepard (1960) found that leprae bacilli can multiply in foot pad of mice.
3. Armadillo has also been found susceptibility to experimental infection. *Mycobacterium leprae* from armadillo or human tissue contains a unique O-diphenoloxidase, perhaps an enzyme characteristic of leprosy bacilli.

   Generation time of leprae bacilli is 20 days.
4. European hedgehog animal model is evaluated and can be kept to be bred in captivity. It is found quite useful for multiplication of leprae bacilli.

Pathogenesis: Leprae bacilli are obligate parasite of man. Portal of entry is most probably skin and nasal mucosa. It needs close and prolonged contact with infective patient for contact. Incubation period is 5 to 8 years.

One gram of lepromatous tissue may contain about 7000 million leprosy bacilli.

The habitat of leprosy bacilli in nerves is the Schwann cell or occasionally the axon which it ensheathes. Under adverse conditions, it may find a retreat in a nerve for which it has great affinity and in which it is not readily detected by immunological means. Sites of multiplication of leprosy bacilli include Schwann cells, smooth and striated muscles. Leprosy bacilli are found in arector pili in skin hair follicles, sweat gland, muscular media of arterioles, endothelial lining of small blood vessels, dartos muscle of scrotum and smooth muscles of iris. Nasal secretions, nasal mucosa, erosions, ulcers and blisters of BL and LL also contain leprosy bacilli. They are found in the sputum, semen, sweat, sebum, tears and breast milk of person with untreated lepromatous leprosy.

The disease occurs in two forms (Table 27.5):

1. Tuberculoid.
2. Lepromatous.

Tuberculoid leprosy is seen in patients with high degree of resistance. The lesions are few, well demarcated consisting of macular anesthetic patches. Neural involvement occurs early and may lead to deformity. Cell mediated immunity is adequate and lepromin test is positive. Prognosis is good.

TABLE 27.5: Difference between lepromatous leprosy and tuberculoid leprosy

	Characters	*Lepromatous leprosy*	*Tuberculoid leprosy*
1.	Cell mediated immunity	Deficient	Adequate
2.	Lepromin test	Negative	Positive
3.	Lepra bacilli in lesion	Numerous	Scanty
4.	Inflammatory cells	Extensive infilteration with mononuclear leucocytes and plasma cells	Infilteration with few inflammatory cell
5.	Lesions	Serious disease with lesion about 1 to 2 cm appearing first on face, ear lobes and situated in skin and subcutaneous tissue	Chronic but benign disease with asymmetrical small patch lesion lesion having raised edges. Patches can also be larger and elevated
6.	Sites involved	May occur on face, extremities, neck (axillae, groin and perineum spread)	Involves face, gluteal region and limbs
7.	Mucous membrane, lymph lymph nodes, eyes and internal organs involvement	Common	Not rare
8.	Nerve involvement	Symmetrical	Thickened peripheral nerve (ulnar, peroneal and greater auricular nerves)
9.	Leonine face	Present	Absent
10.	Mycobacterial antibodies	Present	May be present
11.	Prognosis	Bad	Good

Lepromatous leprosy, where host resistance is very low, the lepromin test is negative and prognosis is poor. Here we find infiltrating skin lesion. Peripheral nerve trunk becomes involved with the progress of diseases. There may be erythematous patch and diffuse infiltrate of nodular lesions.

The borderline type refers to lesions possessing characteristics of both tuberculoid and lepromatous lesion. It may shift to lepromatous or tuberculoid depending upon chemotherapy or change in host resistance.

The indeterminate type is early unstable tissue reaction which is not characteristic of lepromatous or tuberculoid type. In many persons the lesion heals spontaneously. In others, lesion may progress to tuberculoid or lepromatous type.

Ridly and Jopling have introduced leprosy into 5 groups: Tuberculoid (TT), Borderline tuberculoid (BT), Borderline (BB), Borderline lepromatous (BL), and lepromatous (LL) (Table 27.6).

TABLE 27.6: Ridley and Jopling classification

Properties	*TT*	*BT*	*BB*	*BL*	*LL*
1. Lepromin test	++	+	±	—	—
2. CMI	+++	++	+	—	—
3. Mycobacterial antibiotics	±	±	+	++	+++

Lepromin test: The hypersensitivity in a leper may be demonstrated by intradermal inoculation of a material prepared from leprosy nodules. Two types of reactions are known:

1. Fernandes reaction is an early reaction occurring within 24 to 48 hours may last for 3 to 5 days. There is erythema and induration about 10 to 30 mm in diameter.
2. Mitsuda reaction occurring 3 to 4 weeks after the test. It is expression of immunity and is non-specific. It also occurs in tuberculosis and BCG vaccinated person. It is negative in lepromatous leprosy and is positive in tuberculoid leprosy.

0.1 ml of antigen is injected intradermally and then early reaction and delayed reactions are studied.

Lepromin test is done for following purposes:

1. To classify the lesion of leprosy. It is positive in tuberculoid leprosy and negative in lepromatous leprosy.
2. To assess the prognosis and response to treatment. A positive reaction indicates good prognosis. Conversion to lepromin positivity during treatment indicates improvement.

3. To assess the resistance of individual to leprosy. It is desirable to recruit only lepromin positive persons for work.
4. To verify the identity of candidate leprae bacilli. Cultured acid-fast bacilli claimed to be leprae bacilli should give the results similar and parallel to standard lepromin.

Laboratory Diagnosis

1. Smear from lepromatous nodules, skin scraping or nasal mucosa are stained with Ziehl-Neelsen method using 4 to 5 percent H_2SO_4, for decolorization. Smear shows acid-fast bacilli arranged in packed bundles in lepra cells.
2. *Lepromin skin test:* 0.1 ml of lepromin antigen is injected intradermally. The early reaction is seen as induration which increases in size up to 10 mm in diameter till 3 to 5 days. Later it becomes nodules of 2 to 4 mm in diameter in 3 to 4 weeks' time. The center of nodule is necrosed resulting ulcer which heals up in several weeks. This test is useful only in assessing the prognosis of disease. It has no diagnostic value.
3. The immunodiagnostic tests are quite useful for: (a) epidemiological study, (b) identification of individuals at high risk, (c) early detection of disease among patients with symptoms, (d) prognosis of patients, and (e) evaluation of the effect of disease. A number of immunodiagnostic tests are further developed. An ELISA test of comparable sensitivity to the radioimmuno assay test to *M. leprae* has been established. A method has also been developed for early detection of systematic infection in armadillos, and monoclonal antibody are being evaluated for their specificity for *M. leprae.*

Several other new techniques based on different principles are available, e.g. Dot, ELISA, fluorescent leprosy antibody absorption (FLA-ABS) test, passive hemagglutination test, specific carbohydrate epitope found in phenolic glycolipid-1 (PGL-1) of *Mycobacterium leprae.*

At present FLA-ABS is the most sensitive test for detection of presence of *Mycobacterium leprae* in the community and also useful for monitoring the transmission of disease.

Treatment and prophylactic measures: Dapsone is the mainstay of antibacterial treatment of leprosy and is administered for at least 3 to 4 years. However, acedapsone which is administered at 75 days' interval is good in treatment and prophylactically as well. Rifampicin is reported bactericidal to *Mycobacterium leprae*. Recently Clofazimine is also found bactericidal to this organism. A lot of research work is undertaken to prepare vaccine against leprosy. Many quarters claim successful vaccine preparation which is under trial.

MYCOBACTERIUM LEPRAE MURIUM

This is a causative agent of rat leprosy. It is 3 to 5 µ long and curved bacilli. It is Gram-positive, acid fast showing granular staining. It has not been cultured so far. Recently there is claim of its growth on chorioallantoic membrane of fertile hen's egg, tissue culture of rat fibroblast and in mouse macrophages. Growth occurs at 34° to 37°C in 8 to 12 days. Occasionally it may cause lesion in man.

28 Bacillus

General characters: They are rod shaped, sporogenous classified into two groups:

a. Aerobic bacillus.
b. Anaerobic clostridia (Discussed in Chapter 29).

Aerobic bacilli are Gram-positive, non-motile, spore bearing bacilli occurring in chains. They are thick with truncated or convex ends. They include psychrophilic, mesophilic and thermophilic species. The salt tolerance varies from less than 2 to 25 percent of NaCl.

Bacillus anthrax is the only pathogenic species causing anthrax whereas *B. subtilus* is opportunist and *B. cereus* may produce food poisoning.

BACILLUS ANTHRAX

It remains in parasitic form in cattle and sheep. Infection in man is the result of accidental contact with infected animal.

Morphology: It is non-motile, non-acid fast, Gram-positive measuring 1 × 3 to 4 µ. They may be arranged singly or in short chains. The entire chain may be surrounded by capsule. Capsule is polypeptide in nature (D-glutamic acid). Capsule production depends upon a 60 megadalton plasmid, p × O_2.

In culture the bacilli are arranged end to end in chains. The chain of bacilli presents bamboo stick appearance. Spores are formed in soil only in presence of oxygen and not in animal body. Sporulation may be brought about by:

1. Distilled water.
2. 2 percent NaCl.
3. Growth on oxalated agar shows spores which are central, oval and of the same width as the bacillary body.

Cultural characters: It is aerobic growing at optimum temperature of 37°C (range being 12°C to 45°C). The optimum temperature for spore formation is 25°C to 30°C. Growth may occur on ordinary media.

a. *Nutrient broth:* There may be floccular turbidity or no turbidity.
b. *Agar plate:* Colony is irregular, around 2 to 3 mm in diameter, raised, dull opaque, greyish white with a frosted glass appearance and cut glass appearance (in transmitted light). With magnifying glass they look like tangled mass of long hair like curls (barrister wig or medusa head appearance) (Fig. 28.1).

Virulent capsulated strain forms rough colonies whereas avirulent forms smooth colonies.

c. *Blood agar:* The colony is non-hemolytic.
d. *Gelatin stab:* A characteristic "inverted tree"

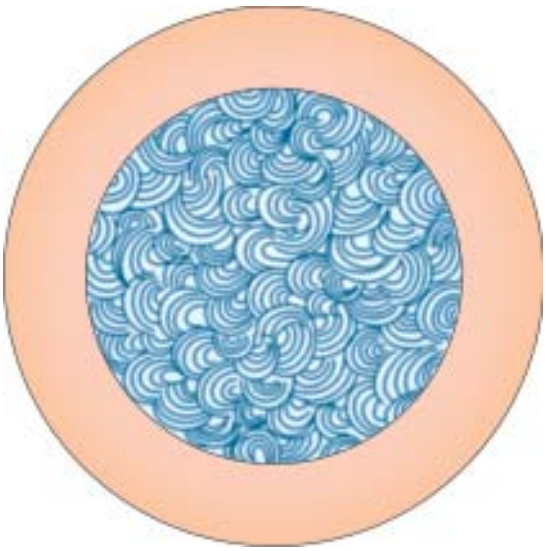


Fig. 28.1: Medusa head appearance of colony on nutrient agar

Fig. 28.2: Gelatin stab showing inverted tree appearance

appearance is seen with slow liquefication starting from the top as shown in Figure 28.2.

e. *Selective medium (PLET):* It consists of polymyxin, lysozyme, ethylene diamine tetra acetic acid (EDTA) and thallous acetate added to heart infusion agar. It is used to isolate anthrax from mixture of spore bearing bacilli.

Biochemical Reactions

Glucose, maltose and sucrose are fermented with acid production only. Nitrates are reduced to nitrite, catalase is positive and gelatin is liquefied.

Resistance: Vegetative form is killed within 30 minutes at 56°C. Anthrax spore remains viable for years in dry state. Dry heat at 40°C requires 3 hours and steam 5 to 20 minutes for sterilization. They survive in 5 percent phenol for weeks. They are killed in 4 percent potassium permanganate and destroyed in 2 percent formaldehyde. They are susceptible to penicillin, sulfonamide, erythromycin, streptomycin, tetracycline and chloramphenicol.

Antigenic structure: 3 antigens have been recognized.

a. Capsular antigen is found in virulent strain. It consists of polypeptide of high molecular weight composed of D-glutamic acid. It is a hapten. Antibodies against this antigen are not protective.
b. Somatic polysaccharides are found as a complex in cell wall. It cross reacts with capsular polysaccharide of type 14 pneumococcus. Antibody to this antigen is not protective.
c. Somatic protein (protective antigen) is present in edema fluid of anthrax lesion. It is heat labile. Its antibody is protective.

Toxin: *Bacillus anthrax* produces substance having exotoxin like properties. They are:

1. Protective antigen or protein.
2. Edema factor.
3. Lethal factor.

The specific toxin is lethal, edema producing and is produced by virulent strain. This toxin seems to have an affinity for cell of reticuloendothelial system.

Pathogenicity: In susceptible animals, the organisms proliferate at the site of entry. The capsules remain intact, and the organisms are surrounded by a large amount of proteinaceous fluid containing few leukocytes from which they rapidly disseminate and reach bloodstream.

In resistant animals, the organisms proliferate for a few hours by which time there appears massive accumulation of leukocytes. The capsule gradually disintegrates and disappears. Thus organisms remain localized.

In nature anthrax is primarily a disease of cattle and sheep, less often of horses and swine. Experimentally fatal infection can be produced in mouse, guinea pig, rabbits, etc. Infection can be produced by cutaneous, subcutaneous, intracutaneous, intramuscular, ingestion and inhalation. Guinea pig dies within 2 to 3 days after subcutaneous injection. Autopsy of dead animal shows hemorrhage, local edema, congested viscera and blood coagulates less firmly. Microscopically bacilli are found in large numbers in the local lesion in blood and viscera. The bacilli are confined to interior of capillaries and tissues and may rarely penetrate.

Route of infection in man: The persons most commonly involved are butchers, shepherds, handlers of hides, hair and laboratory workers. There are three routes of infection:

a. Through skin it results in malignant pustule. The spores enter the skin through cuts, abrasion or hair follicle. This lesion starts as papule in 1 to 3 days after infection. It becomes vesicular containing fluid. The whole area is congested and edematous and several satellite lesions, filled with serous fluid are arranged around central necrotic area covered by a black eschar. Cutaneous anthrax generally resolves spontaneously but may lead to septicemia.
b. Pulmonary anthrax is called wool sorter's disease. It is due to inhalation of dust (containing spores) from infected wool. Hemorrhagic meningitis may occur as a complication.
c. Intestinal anthrax occurs very rarely from ingestion of cooked or partly cooked meat. A violent enteritis with bloody diarrhea occurs with high fatality.

Laboratory Diagnosis

A. *Hematological investigations:*
Leukocytosis occurs when tissues are invaded otherwise total leukocyte count is within normal limit.
B. *Bacteriological investigations:*
1. *Microscopic examination:* Smear prepared from exudate, sputum, etc. on Gram's staining shows Gram-positive non-sporing bacilli occurring in chain.
2. *Culture:* The material is inoculated on nutrient agar plate. Smear shows Gram-positive spore bearing bacilli.
3. *Animal inoculation:* A small amount of exudate or isolated culture from infected man is injected subcutaneously in guinea pig. Guinea pig dies within 36 to 48 hours. Smear from heart blood and spleen shows typical Gram-positive bacilli.
4. *Serological test:* It is a precipitation test. It is used in making rapid diagnosis. The infected tissues are grounded in saline, boiled for 5 minutes and filtered. This tissue extract is layered over anthrax antiserum. Zone of precipitate at the junction of tissue extract and antiserum within 5 minutes at room temperature means test is positive. It is called ASCOLI TEST.
5. *McFadyean reaction:* When blood films containing anthrax bacilli are stained with polychrome methylene blue for a few seconds and examined under the microscope, amorphous purplish material is seen around bacilli. This represents capsular material and is characteristic of anthrax bacilli.
6. *Polymerase chain reaction:* Using this technique *Bacillus anthracis* may be confirmed.

Treatment: Ciprofloxacillin, penicillin, streptomycin and tetracyclines are effective. Scalvo's serum may be used in serious toxic cases. Persons with high occupational risk should be immunized with a cell free vaccine (Sterne strain cell culture) which is available from Centers for Disease Control, Atlanta 30333.

Current strategies for vaccine development include purification of protective antigen, expression of protective antigen in recombinant microbial vaccines and construction of improved live attenuated strains of *Bacillus anthracis*.

Non-living vaccine consisting of alum precipitated or aluminium hydroxide absorbed extracellular components of unencapsulated *B. anthracis* are used in USA for army personnel, agricultural workers, veterinary personnels, etc. The major active component of these vaccines is protective antigen. Live attenuated vaccines having *B. anthracis* (spores) are used. Sterne spore vaccines (loss of plasmid that encodes capsular polypeptide) may be used.

A mutant form of protective antigen that lacks the protease sensitive sequence and that cannot be processed to interact with *E. coli* for LF to mediate toxicity has been produced by genetic engineering as one candidate vaccine against anthrax.

BACILLUS SUBTILUS

It is Gram-positive about 1.5 µ, × 4.5 µ, straight occurring singly or chain, motile and non-capsulated.

TABLE 28.1: Difference between *B. anthrax* and *B. anthracoid*

	B. anthrax	*B. anthracoid*
1.	Non-motile	Generally motile (by swarming, e.g. *B. cereus)*
2.	Capsulated	Non-capsulated
3.	Grows in long chain	Grows in short chain
4.	Medusa head colony	Not present
5.	Hemolysis of sheep RBC absent	Usually well marked
6.	Inverted fir-tree growth in gelatin	Fir-tree growth absent
7.	No turbidity in broth	Turbidity usually present
8.	No growth in penicillin agar (10 unit/ml)	Grows usually
9.	No growth at 45°C	Grows usually
10.	Growth inhibited by chloral hydrate	Not inhibited
11.	Susceptible to gamma phage	Not susceptible
12.	Salicin fermentation negative	Positive
13.	Pathogenic to man and laboratory animals	Not pathogenic
14.	Methylene blue reduced weakly	Methylene blue generally reduced strongly
15.	Liquefaction of gelation slow	Liquefaction of gelatin rapid
16.	Lecithinase reaction weakly positive	Strongly positive with *B. cereus*
17.	Culture filtrates non-toxic to tissue culture cells	Culture filtrates *(B. cereus)* toxic to tissue culture cells
18.	Produces toxin, neutralized by *B. anthrax* antitoxin	Any toxic substance produced not neutralized by *B. anthrax* antitoxin

It grows on blood agar producing wider zone of beta hemolysis. It may grow in broth culture and on nutrient agar, etc.

It does not produce any toxin. Some strains may produce soluble hemolysin. They are opportunist pathogens. They cause egg infection and septicemia. They may contaminate blood transfusion bottle and thus hemolyse the blood.

BACILLUS CEREUS

They are responsible for food poisoning. They can grow in food and produce an enterotoxin that causes diarrhea by a mechanism similar to that of *Escherichia coli* enterotoxin.

Aerobic spore bearer having resemblance with *B. anthracis* are called anthracoid bacillus. They differ from each other as shown in Table 28.1.

29 Clostridium

General characters: The genus clostridium consists of Gram-positive, anaerobic, spore forming, spindle shaped and highly pleomorphic bacilli. Spores are wider than bacillary bodies. The genus contains bacteria causing 3 major diseases of man; tetanus, gas gangrene and food poisoning. Some pathogens, e.g. *Clostridium welchii* now-a-days called *Clostridium perfringens* and *Clostridium tetani* are found normally in human and animal intestine.

Clostridia are motile with peritrichate flagella, except *Clostridium perfringens* and *Clostridium type VI. Clostridium perfringens* and *Clostridium butyricum* are capsulated while others are not so. Pathogenic clostridia forms powerful exotoxins. *Clostridium botulinum* is non-invasive while *Clostridium tetani* has slight invasive properties. Tetanus results from the action of powerful exotoxin of *Clostridium tetani*. The gas gangrene clostridia are toxigenic and invasive causing even septicemia.

CLOSTRIDIUM TETANI

It is widely distributed in soil and in intestine of man and animals.

Morphology: It is slender, long, slightly curved, Gram-positive 4.8 µ × 0.5 µ and occurring singly or in chain. It shows considerable variation in length. Spores are spherical, terminal and bulging, giving the bacilli drumstick appearance (Fig. 29.1). It is non-capsulated and motile.

Cultural characters: It is an obligatory anaerobe that grows only in absence of oxygen. The characteristic of anaerobic bacilli is their inability to utilize oxygen as the final hydrogen acceptor. It lacks cytochrome and cytochrome oxidase and is unable to break down hydrogen peroxide because it lacks catalase and peroxidase. Therefore, hydrogen peroxide tends to accumulate to toxic concentration in the presence of oxygen. It also lacks superoxide dismutase and consequently permit the accumulation of toxic free radical superoxide. Hence, it can carry out its metabolism only at negative oxidation reduction potential which means an environment that is strongly reducing. The optimum temperature is 37°C and pH 7.4. It grows fairly well in ordinary media. Cultures have burnt organic smell.

Cooked meat medium: It grows well in this medium with turbidity and gas formation. The meat is not digested but is turned black after prolonged incubation.

Nutrient agar medium: It produces swarming growth forming fine film over the medium. By increasing the concentration of agar in the

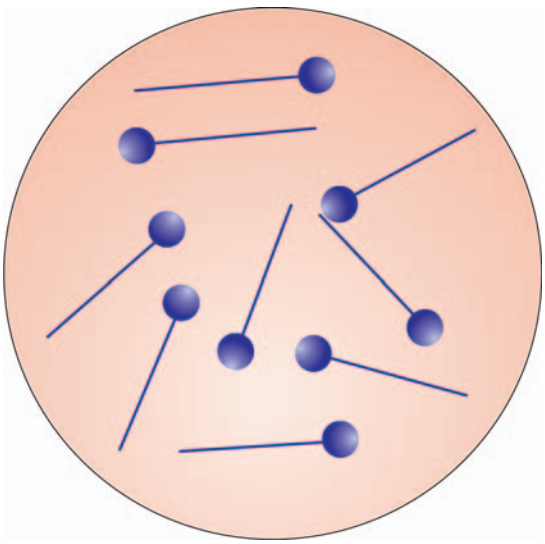


Fig. 29.1: *Clostridium tetani*

medium after 2 to 4 days' incubation, colonies are irregularly round, 2 to 5 mm in diameter, translucent, grayish yellow with granular surface and ill-defined edges.

Blood agar medium: A zone of α hemolysis is produced. It later on develops into beta hemolysis, due to production of hemolysin (tetanolysin).

Lactose egg yolk milk medium: There is no opalescence, pearly layer, proteolysis or lactose fermentation.

Biochemical reactions: It does not ferment any sugar and is slightly proteolytic. It forms indole. Gelatin liquefaction occurs slowly. Coagulated serum is softened. Milk is not coagulated.

Resistance: Spores of *Clostridium tetani* withstand boiling for 15 to 90 minutes. Autoclaving at 121°C for 20 minutes kills spores. Spores otherwise can survive in soil for years. Iodine (1% aqueous solution) and H_2O_2 (10 volumes) kill spores within a few minutes.

Antigen structure: The flagellar antigen differentiate *Clostridium tetani* into 10 types but toxin (neurotoxin) produced is pharmacologically and antigenically identical.

Toxin: *Clostridium tetani* produces three types of toxin:

a. Hemolysin (tetanolysin)
b. Neurotoxin (tetanospasmin)
c. Non-spasmogenic peripherally active neurotoxin.

a. *Tetanolysin:* It is heat labile and oxygen labile and is active against RBC of many animals (rabbit, horse, etc.). Its pathogenic role is not known. May be it acts as a leukotoxin.
b. *Tetanospasmin:* It is oxygen stable and gets inactivated at 66°C in 5 minutes (heat labile). Toxin has been crystallized. Horse is most susceptible. Birds and reptiles are highly resistant. It gets toxoided in presence of low concentration of formaldehyde. It is a good antigen and specifically neutralized by antitoxin. It is protein with 67,000 molecular weight. It acts like strychnine by inhibiting the synthesis and liberation of acetylcholine, thus, interfering with neuromuscular transmission. It can be fixed to cerebral gangliosides. It can cause inhibition of postsynaptic spinal neuron by blocking the release of inhibitory mediator. This results in generalized muscular spasms, hyperreflexia and seizures.
c. A third toxin is a non-spasmogenic peripherally active neurotoxin.

Pathogenesis: Spores implanted in wound multiply only if conditions are favorable. Toxin so produced is absorbed by motor nerve ending. Toxin travels along the axis cylinders of peripheral nerve and reach central nervous system. It is fixed specifically by ganglioside of gray matter of nervous tissue. Its exact mode of action is not known but it may act at synaptic junctions between anterior horn cells and related internuncial neurons leading to abolition of spinal inhibition. As a result, muscle rigidity and spasm occurs.

If given orally it is destroyed by digestive enzyme and so is not effective. Subcutaneous, intramuscularly and intravenous route is equally effective. Intraneural route is more lethal.

If toxin is injected intramuscularly in one of the hind limbs of guinea pig or mice, spasm of inoculated limb appears. This is due to toxin acting on the segment of spinal cord. Subsequently spread of toxin up to the spinal cord causes ascending tetanus and likewise opposite hind limb is involved. If toxin is injected intravenously spasticity develops in the muscles of head and neck first and then spreads downwards (descending tetanus).

Tetanus: Tetanus results from contamination of wound by *Clostridium tetani.* The spores are found in soil. Germination and multiplication occur if certain factors like necrotic tissue, ionisable calcium salts and lactic acid are present. Infection of wound with pyogenic organisms increases the risk of tetanus. Toxin is probably absorbed from the area of infection and through motor nerve endings reach anterior horn cell. Other views are that toxin is absorbed through bloodstream and perineural lymphatics.

The incubation period is 2 days to several weeks and it depends upon site, nature of wound, doses, toxigenicity of organism and immune status of patient. In rural India tetanus is estimated to be the most common cause of death.

There are many clinical types of tetanus:

1. *Tetanus neonatorum:* It occurs from contamination of cut surface of umbilical cord in infants. It has high rate of fatality.
2. *Postabortal and puerperal tetanus:* It results from infection of genital tract with unsterile instrument and dressing. Puerperal tetanus is rare but most dangerous.
3. *Splanchnic tetanus*: There is involvement of muscle of deglutition and respiration with dysphagia.
4. *Cephalic tetanus:* It occurs from the wounds of head. There is unilateral and bilateral contraction of muscles of face.

Laboratory Diagnosis

The diagnosis is always clinical and bacteriological findings confirm the diagnosis.

1. *Microscopic examination:* Smears from wound material after Gram's staining show Gram-positive bacilli with typical drumstick appearance.
2. *Culture:* Diagnosis by culture is more dependable. Excised bits of tissue from necrotic depth of wound is inoculated into cooked meat broth, blood agar and lactose egg yolk medium. The addition of polymyxin B to which clostridia resist, make the medium more selective.

If the material is grossly contaminated with other organisms, heating at 80°C for 10 minutes may be useful for destroying non-sporing organisms.

Animal inoculation: Mouse is a suitable laboratory animal for demonstration of toxigenicity. 2 to 4 days' old cooked meat culture (0.2 ml) is inoculated into the root of tail of a mouse. A second mouse which has received tetanus antitoxin (1000 units) an hour earlier serves as control. Symptoms appear in test animal in 12 to 24 hours with stiffness of tail. Rigidity develops to the inoculated side of the leg, opposite leg, trunk, fore limb in this order. The animal dies within 2 days. However, appearance of ascending tetanus in animal is diagnostic.

Prophylaxis: It is a preventable disease. Immunity to tetanus may result from infection or by immunization (Table 29.1).

Active immunization: Usually two injections 1 ml each of tetanus toxoid is given intramuscularly at the interval of 6 weeks. Third injection is given after 6 to 12 months. A full course of immunization confers immunity for 10 years. Toxin is given either alone or along with diphtheria toxoid and pertussis vaccine (triple vaccine) in which pertussis vaccine acts as adjuvant.

Passive immunization: It is an emergency procedure to be used only once. It is done by giving antitetanus serum (ATS). The recommended dose is 1800 IU subcutaneously or intramuscularly as early as possible after wounding. Unfortunately, it carries the risk of hypersensitivity and immune elimination (half life is 2 days).

Passive immunity without risk of hypersensitivity be obtained by use of human antitetanus immunoglobulin (ATG). This is effective in smaller dose (280 units) and has longer half life (3 to 5 weeks).

TABLE 29.1: Tetanus prophylaxis in wound

Nature of wound	*Immune status*		
	Immune	*Partial immune*	*Non-immune*
Clean (wound toilet performed within 6 hours)	Toxoid × 1	Toxoid × 1	Toxoid × 3
Contaminated (soil, necrotic material present)	Toxoid × 1	Toxoid × 1 ATS Antibiotics	Toxoid × 3 ATS Antibiotics
Infected	Toxoid × 1 Antibiotics	Toxoid × 1 ATS Antibiotics	Toxoid × 3 ATS Antibiotics

NB: Immune patients having full course of 3 injections of toxoid. Partial immune patient has had 2 injections of toxoid. Non-immune patient has had no injection of toxoid or his immune status is not known

Combined immunization: It consists of administering to non-immune person ATS at one site along with a dose of toxoid at other site followed by other doses of toxoid at appropriate intervals. Ideally in emergency combined immunization should be performed instead of passive immunization alone.

CLOSTRIDIUM PERFRINGENS (Clostridium welchii)

It is a normal inhabitant of the large intestine of man and animals. It is found in feces and contaminates the skin of perineum, buttocks and thigh. It also produces food poisoning and necrotic enteritis in man.

Morphology: It is a plump, Gram-positive bacillus with straight, parallel sides, rounded or truncated ends about 4 to 6 µ × 1 µ. It may occur singly or in chains. It is pleomorphic. Filaments and involution forms are common. It is capsulated and non-motile. Spores are central or subterminal.

Cultural characters: It is an anaerobe, growing rapidly at 37°C.

a. *Cooked meat medium:* Fairly good growth occurs at 37°C. The medium becomes turbid within 24 hours with production of gas. The meat is turned pink without digestion (Fig. 29.2). The culture has sour odor.

b. *Nutrient agar:* Two types of colonies appear after 24 hours of incubation; (i) 2 to 4 mm round, smooth, butyrous emulsifiable colonies, (ii) Umbonate colonies with brownish opaque center and lighter radially striated periphery having crenated edges.

Biochemical reactions: Glucose, maltose, lactose and sucrose are fermented with production of acid and gas. In litmus milk it produces acid with gas (Fig. 29.3). Milk is disrupted due to vigorous production of gas. This is called stormy clot. Indole is negative and H_2S is formed abundantly.

Resistance: Autoclaving at 121°C for 18 minutes destroys the spores. Spores are resistant to antiseptics and disinfectants in common use.

Antigenic structures: *Clostridium perfringens* are differentiated into 6 types (A,B, C, D, E, F) on the basis of toxin produced by the strains. Toxins are antigenic and antitoxic sera are used for routine typing of strain.

Toxin: *Clostridium perfringens* produces at least 12 distinct toxins, besides many other enzymes and biological active soluble substances. According to kind and quantity of toxins produced, different strains of *Clostridium perfringens* are divided into 6 types, i.e. A to F. The 4 major toxins, alpha, beta, epsilon and iota are responsible for pathogenicity.

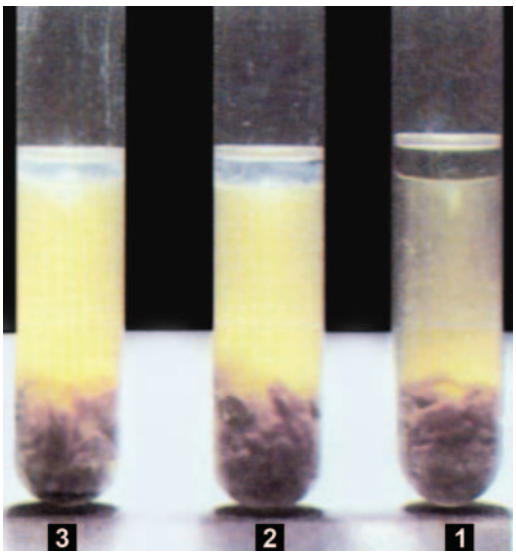


Fig. 29.2: Cooked meat medium
1. Control; 2. *Clostridium sporogenes;*
3. *Clostridium perfringens*

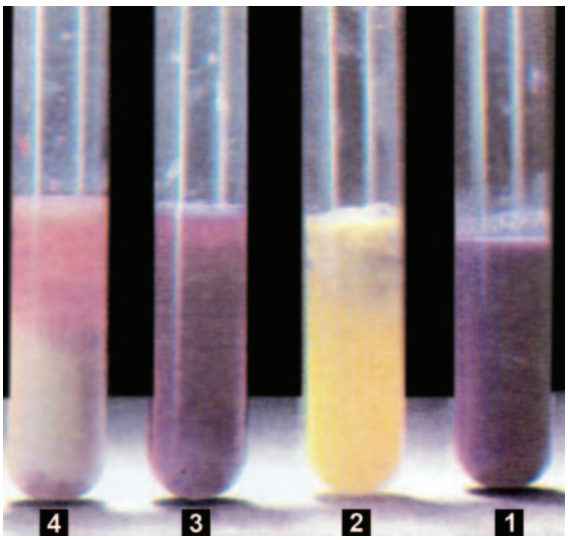


Fig. 29.3: Litmus milk
1. Control; 2. *Clostridium perfringens;*
3. *Pseudomonas aeruginosa*; 4. *Escherichia coli*

Alpha toxin is more important and is produced by all strains of *Clostridium perfringens.* Type A strains produce it more abundantly. It is responsible for toxemia of gas gangrene. It is lethal, dermonecrotic and hemolytic. It also shows lecithinase activity and gives positive Nagler's reaction.

NAGLER'S REACTION

Clostridium perfringens are cultured on plates containing 20 percent of human serum or egg yolk. The organism produces opalescence in media containing human serum and egg yolk. The opalescence is due to lecithinase activity of alpha toxin. Alpha toxin splits lipoproteins and liberates lipids. The lipid deposits around the colony to give opalescence (Fig. 29.4). The reaction is specific and is inhibited by alpha toxin antitoxin sera. It is a useful test for the rapid detection of *Clostridium perfringens* in clinical specimen.

Beta, epsilon and iota toxins have lethal and necrotizing properties.

Besides toxins, *Clostridium perfringens* also produces soluble substances with enzymatic properties, e.g. neuraminidase, hemagglutinin, fibrinolysin, hemolysin, histamine, etc.

Pathogenicity: Only type A and F are pathogenic for man. Type A is responsible for gas gangrene and food poisoning.

A. *Gas gangrene: Clostridium perfringens* type A is the predominant agent causing gas gangrene. Other organisms associated with gas gangrene are *Clostridium septicum, Clostridium edematiciens* and anaerobic streptococci. Organisms enter the wound usually along with foreign particles, e.g. soil, dust, etc. Clostridia may be present on normal skin. Infection may be endogenous.

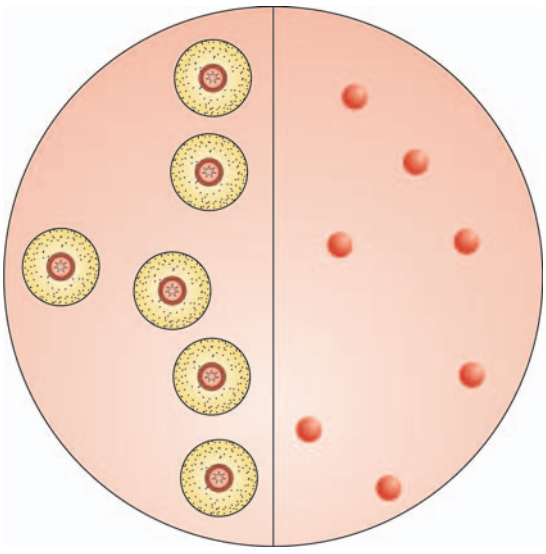


Fig. 29.4: Nagler's reaction

Mere presence of clostridium in wound does not constitute gas gangrene. There are 3 types of anaerobic wound infection:

1. Simple wound contamination with no invasion of underlying tissue. There is usually delay in wound healing.
2. *Anaerobic cellulitis:* In which clostridia invade fascial plane with minimal toxin production and no invasion of muscle tissue. The disease is gradual in onset. It may be limited to gas abscess or extensive involvement of a limb occurs. Infecting clostridia is of low invasive power and poor toxigenicity. Toxemia is absent and prognosis is good.
3. *Anaerobic myositis:* It is most serious and is associated with abundant formation of exotoxin. The clostridia multiply and elaborate toxin which causes further damage. The lecithinase (toxin) damages cell membranes, muscle fibers and increases capillary permeability. Resulting edema may cause increased tension and anoxia in affected muscle. Hemolytic anemia and hemoglobinuria are due to lysis of RBC by a toxin.

   The collagenase destroys collagen barriers in tissue. Hyaluronidases break down intercellular substances. Abundant production of gas reduces blood supply by pressure effect extending the area of anoxic damage. Thus, there is spread of infection and lesion is progressive one.

   The incubation period is 7 hours to 6 weeks. The disease develops with increasing pain, tenderness, edema of affected part with systemic signs of toxemia. Profound toxemia and prostration develops and death occurs due to circulatory failure.

B. *Food poisoning:* Some strains of type A produce food poisoning. They are characterized by marked heat resistance of

spores and production of alpha and beta toxin. They are non-hemolytic strains. Incubation period is 10 to 12 hours. It starts with pain in abdomen, vomiting and diarrhea. Recovery takes place in 24 to 48 hours.

C. *Enteritis necrotican:* A severe and fatal enteritis due to F type strain may occur. The pathogenesis of this disease is suggested to be a low protein diet that predisposes to decreased levels of digestive proteases with subsequent mobility to degrade the clostridia beta toxin. The proteases are further blocked by ingestion of trypsin inhibitors found in sweet potatoes.

Apart from this *Clostridium perfringens* may cause gangrenous appendicitis, biliary tract infection, brain abscess, meningitis, panophthalmitis, urogenital infection, etc. Rarely septicemia and endocarditis may occur.

Laboratory Diagnosis

A. *Hematological investigation:*
   a. Total leukocyte count usually shows no change. Increased count occurs in secondary infection.
   b. Differential leukocyte count shows no change.
   c. Anemia, increased serum, bilirubin and hemoglobinuria may occur due to excessive RBC destruction.

B. *Bacteriological investigation:*
   Specimens: They are collected from:
   1. Muscles at the edge of affected area.
   2. Exudate from area where infection appears more active.
   3. Necrotic tissue and muscle fragment.

Microscopic Examination

Gram-stained smear shows Gram-positive, long and thick bacilli. Gram-positive bacilli without spore are suggestive of *Clostridium perfringens.*

Culture: Material is inoculated on fresh blood agar and cooked meat media. Surface culture is incubated aerobically and anaerobically. Anaerobic culture is studied after 48 to 72 hours of incubation. Further identification is done by:

1. Nagler's reaction.
2. Biochemical reaction.
3. Animal pathogenicity.

Blood collected during bacteremia is cultured in cooked meat medium and glucose broth. It is identified in usual way.

Animal Pathogenicity

On the hind limb of guinea pig 0.1 ml of 24 hour cooked meat broth is injected intramuscularly. Death of animal occurs in 24 to 48 hour. Autopsy shows swelling of injected limb with crepitation due to gas formation. The muscle becomes pink. Organism can be recovered from heart and spleen.

Bacteriological Diagnosis of *Clostridium perfringens* Food Poisoning

From the feces of patient and suspected food, isolation of non-hemolytic, non-motile anaerobic and Gram-positive bacilli is suggestive of *Clostridium perfringens* infection.

Treatment: Antibiotics and surgery in gas gangrene.

CLOSTRIDIUM BOTULINUM

Clostridium botulinum spores are widely distributed in soil, animal manure, sea mud, vegetables, etc. It causes botulism, a severe form of food poisoning.

Morphology: It is Gram-positive about 5 µ × 1 µ, non-capsulated motile by peritrichate flagella producing subterminal, oval and bulging spores. It shows pleomorphism and occurs either singly or in pairs or chains.

Culture characters: It is strict anaerobic. There are 6 different types (A to F). They differ from one another in their culture characters. These types are identified on the base of immunological difference in toxin production. It grows at 20 to 35°C in neutral or slightly alkaline medium.

a. Cooked meat medium: After 2 to 4 days' incubation there is abundant growth. There is blackening of meat particles and gas is also produced.

b. Nutrient agar medium: Single colony is difficult to get because of tendency to spread. Colonies develops after 48 hours.

Colony is irregular, 3 to 8 mm, glistening and with granular surface. Consistency of colony is butyrous and is emulsified easily.

c. *Blood agar:* Colonies on blood agar medium are hemolytic.
d. *Lactose, egg yolk, milk agar medium:* All types of this organism produces opalescence and pearly layer. All are lactose negative.

Resistance: Spore is highly resistant, surviving several hours at 100°C. It withstands 120°C for 20 minutes. However, heat resistance is diminished at acid pH or high salt concentration.

Biochemical reactions: All types ferment glucose, maltose with acid and gas. There are two biochemical types of *Clostridium botulinum:*

a. Proteolytic (types A, B and F).
b. Saccharolytic and non-proteolytic (type C, D and E). H_2S is produced by all types.

Antigenic structure: Six types are distinguished by their toxin production. The toxins are antigenically distinct.

Toxin: It produces powerful exotoxin responsible for pathogenicity. Toxin has been isolated as crystalline protein which is most toxic substance known, i.e. 0.000,000,003 mg is the lethal dose of mice. It is a neurotoxin which acts slowly by inhibiting release of acetylcholine at synapses and neuromuscular junctions. Flaccid paralysis results.

Toxin is stable. It resists digestion in the intestine and is absorbed through intestinal mucosa in an active form. It can be toxoided. The most important and potent exotoxin is that of type A. The other types of toxin are less toxic.

Botulinum toxin is now found useful in strabismus, blepharospasm, hemifacial spasm, tremors (dystonic one), spasmodic torticolis, vocal muscle spasm, stuttering, spasticity and writer's cramp.

Pathogenesis: *Clostridium botulinum* is non-invasive and its pathogenicity is due to toxin, produced in contaminated food. Botulism is because of ingestion of preformed toxin in food. Human botulism is caused by type A, B and E. Source of this toxin is preserved food, meat, canned vegetable and fishes, etc.

Incubation period is 12 to 36 hours. Vomiting, thirst, constipation, difficulty in swallowing, speaking and breathing are the manifestations which may be followed by coma, delerium and death in 1 to 7 days.

Laboratory Diagnosis

Bacteriological Investigations

Specimen: Diagnosis is based on demonstration of bacillus or toxin in food or feces. In early stages toxin may be detected from patient's blood.

Culture: Isolation of organism (toxigenic strain) from vomit, food or feces in absence of toxin is of no significance.

Demonstration of *Clostridium botulinum* toxin: Specimen like food, vomit, etc. are grounded up and soaked overnight in equal volume of isotonic saline solution. It is centrifuged, and supernatant is divided into 3 parts. One portion is heated at 100°C for 10 minutes. Penicillin is added (concentration being 100 units/ml).

One of the guinea pig is protected with polyvalent botulinum antitoxin and 2 ml of unheated material is injected intraperitonially only. Unheated material (2 ml) is injected into second guinea pig. The third animal is injected with 3 ml of heated material.

Second guinea pig develops toxin symptoms like dyspnea, flaccid paralysis and dies within 24 hours. First and third animals show no toxic symptoms.

Typing is done by passive protection with type specific antitoxin. A retrospective diagnosis may be made by detecting of antitoxin in patients serum.

Treatment: Active immunization with toxoid is effective. However, antitoxin is of no use in treatment as toxin already get fixed up to nervous tissue by the time disease becomes apparent. Trivalent antitoxin must be promptly administered intravenously. However, early administration of potent antitoxin (intravenously) with guanidine hydrochloride as an adjunct is recommended. Such measures with artificial respiration have reduced fatality rate from 65 to 25 percent.

CLOSTRIDIUM DIFFICILE

Clostridium difficile is a commensal bacterium of the human intestine found in 2-5% of the population. It was first isolated from the faeces of neonates and was named so due to the difficulties in isolating the organism.

Morphology

It is an anaerobic, spore-forming, long slender, Gram positive bacillus containing oval and terminal spores. It has a tendency to lose its gram reaction.

Toxins

Two types of toxins are produced by clostridium difficile, an enterotoxin (toxin A) and a cytotoxin (toxin B). Toxin A usually results in diarrhea and toxin B produces cytopathogenic effects in several tissue culture cell lines.

Pathogenesis

Prolonged use of antibiotics, especially those with a broad spectrum of activity can result in disruption of normal intestinal flora, leading to an overgrowth of *Clostridium difficile*, which flourishes under these conditions. It thereby results in acute colitis with (Pseudomembranous colitis) or without membrane formation. It is usually a fatal disease if not treated promptly.

Laboratory diagnosis

Toxigenic culture, in which organisms are cultured on selective medium and tested for toxin production, remains the gold standard and is the most sensitive and specific test, although it is slow and requires a considerable effort.

Toxin production can be demonstrated in the faeces by its characteristic effect on human diploid cells and Hep-2. ELSIA is also helpful in detecting the toxin production.

Treatment

Metronidazole is the drug of choice. Other antibiotics that may be effective against *Clostridium difficile* include vancomycin and linezolid. Drugs traditionally used to stop diarrhea should not be used as they frequently worsen the course of *Clostridium difficile* related pseudomembranous colitis.

30 Enterobacteriaceae

General characters: Numerous interrelated bacterial flora of intestine are Gram-negative rods, motile only with peritrichate flagella or non-motile, non-sporing, non-acid fast, ferment glucose with or without formation of gas, reduce nitrates into nitrites, form catalase, oxidase negative and aerobic or anaerobic.

Classification

I. *Based on action on lactose:*
It is an old method. It has practical value in diagnostic bacteriology.
   a. Lactose fermenter
   e.g. *Escherichia coli*
   Klebsiella
   b. Late lactose fermenter
   e.g. *Shigella sonnei*
   Paracolons
   c. Non-lactose fermentation
   e.g. *Salmonella*
   Shigella

II. *Modern taxonomical concept:*
Enterobacteriaceae may be classified into tribes, genera and species by their cultural and biochemical characters. The species are further classified as: biotypes, serotypes, bacteriophage types and colicin types. At present there are five tribes as under:

Enterobacteriaceae

Tribe I	Escherichieae	
	Genus:	*Escherichia*
		Edwardsiella
		Citrobacter
		Salmonella
		Shigella
Tribe II	Klebsielleae	
		Klebsiella
		Enterobacter
		Hafnia
		Serratia
Tribe III	Proteeae	
	Genus: *Proteus*	
Tribe IV	Erwinieae	
	Genus: *Erwinia*	
Tribe V	Yersinae	
		Yersinia pestis
		Yersinia enterolitico
		Yersinia pseudotuberculosis

ESCHERICHIA COLI

It lives only in human or animal intestine. Detection of *E. coli* in drinking water is taken as evidence of recent pollution with human or animal excreta. *Escherichia coli* in contaminated water may be detected using PCR (rapid and sensitive), DNA probes, plating and biochemical tests.

Morphology: It is Gram-negative, non-capsulated, short, plump bacilli 2 to 4 µ × 0.4 to 0.7 µ in diameter and are motile. Spores are not formed.

Cultural characters: It is aerobic and facultative anaerobe growing on simple media. Optimum temperature is 37°C.

1. *Liquid broth:* It shows uniform turbidity after 8 to 24 hours' incubation.
2. *Nutrient agar:* Colonies appear after 12 to 18 hours of incubation. They are circular 1 to 3 mm in diameter, smooth, colorless, having entire edge with butyrous consistency. Colonies are emulsified easily.
3. *MacConkey medium:* The colonies are pink due to lactose fermentation.

4. *Blood agar:* Some strain may show zone of beta hemolysis.

Biochemical reactions: It ferments lactose, glucose, sucrose, maltose and mannitol forming acid and gas. Indole and methyl red (MR) is positive. VP and citrate is negative. Urease is not hydrolyzed. H_2S is not produced.

Resistance: It can survive for months in soil and water. It is killed at 60°C in 20 minutes and chlorine (0.5 to 1 part per million). It is sensitive to streptomycin, tetracycline, chloramphenicol, furadantin and nalidixic acid.

Antigenic structure: There are 4 types of antigens:

1. *Somatic antigen* (O antigen): They are heat stable. They are divided into 175 groups designated as 1, 2, 3 and so on.
2. *Surface antigen* (K antigen). They are heat labile. They interfere with O agglutination unless destroyed by heating at 100 to 121°C (Table 30.1). They are of 3 types.
   a. L antigen is destroyed by heating at 100°C for 1 hour and its capacity to combine with antibody is lost.
   b. A antigen is capsular antigen and is heat stable and is associated with well-marked capsule.
   c. B antigen is destroyed by heating at 100°C for one hour. Still they retain the capacity to combine with antibody.

Only one of L, A and B surface antigen is present in a strain. 100 K antigens are recognized so far. Now K antigen is divided into two types i.e. Type I and Type II.

TABLE 30.1: K antigens of *Escherichia coli*

Characteristics	Type I	Type II
1. Molecular weight	100,000 or more	50,000 or less
2. Heat at 100°C	Stable	Labile
3. pH-6	Stable	Labile
4. O groups	8, 9	Many
5. Acid contents	Hexuronic acid	Glucuronic acid
6. Electrophoretic mobility	Low	High

3. *Flagellar antigen* (H antigen): They are thermolabile and monophasic. About 75 types have been described.
4. *Fimbrial antigen* (F antigen): They are thermolabile and have no significance in antigenic classification of *E. coli.*

The antigenic pattern of a strain is recorded as number of particular antigen it carries, e.g. $O_{111}K_{58}H_2$. The normal colon strain belongs to early O group (1, 2, 8, 4, etc.), while enteropathogenic belongs to later O group (26, 55, 86, 111, etc.).

Toxin production: It produces endotoxin. Besides that, it also produces two types of exotoxins:

a. Enterotoxin which is heat labile, filtrable and causes fluid accumulation in rabbit ileal loop. The mode of action is by activation of adenyl cyclase thereby raising level of cAMP in cell thus causing excretion of fluid and electrolytes in the lumen of intestine.

   Two types of *E. coli* enterotoxins have been recognized, i.e. heat labile toxin (LT) and heat stable toxin (ST). A strain of *E. coli* may produce one or both types of enterotoxins. The production of enterotoxins is under genetic control of transmissible plasmids. Heat labile toxin (LT) is of large molecular weight (80,000) protein which gets inactivated by heating at 60°C for 10 minutes. On the other hand, heat stable toxin (ST) is of low molecular weight (8,000 to 8,500). ST is non-antigenic toxin which seems to stimulate fluid secretion in the gut through mediation of cyclic guanosine monophosphate (cGMP). The demonstration of LT and ST is depicted in Table 30.2.

b. Hemolysin which may be:
   i. Heat labile, filtrable and lethal for animals.
   ii. Associated with bacterial cell and is not filtrable.

Pathogenesis

Endotoxin may cause fever, leukopenia, hypotension, disseminated intravascular coagulation (DIC), etc.

Other lesions produced are:

1. *Gastroenteritis:* Certain serotypes produce fatal type of gastroenteritis in infants, e.g. 4, 26, 46, 55, 86, 111, 112, 119, 127 and 129.

   Sporadic summer diarrhea occurs in children during second or third summer of life in non-epidemic form. Lactic acid

TABLE 30.2: Methods of detection of ETEC enterotoxin

Assay	*Heat labile (LT)*	*Heat stable (ST)*
IN VIVO TESTS		
1. Ligated rabbit ileal loop		
6 hours	±	+
18 hours	+	–
2. Infant rabbit bowel	+	+
3. Infant mouse intragastric	–	+
4. Adult rabbit skin (vascular permeability)	+	–
IN VITRO TESTS		
1. Steroid production in Y1 mouse adrenal cell culture	+	–
2. Morphological changes in Chinese hamster ovary cells (CHO)	+	–
3. Solid phase radioimmuno-assay (RIA)	+	–
4. Enzyme-linked immuno-sorbent assay (ELISA)	+	–

produced from lactose fermentation may cause irritation of the colon. Result is violent nausea, vomiting and diarrhea.

There are six groups of *E. coli* which can cause diarrheal diseases:

a. *Enteropathogenic E. coli also called EPEC:* They are carrying β type of surface (K) antigen. They have caused several serious institutional outbreaks of diarrhea in babies less than 18 months old. Over 25 serotypes are identified, e.g. O_{26} : B_6; O_{55} : B_5; O_{86} : B_7, etc. The pathogenic mechanism of EPEC is their tight adherence to enterocytes resulting in the loss of microvilli and cupping of enterocyte membrane to bacteria.

b. *Enterotoxigenic E. coli also called ETEC:* They produce heat labile and heat stable enterotoxin and thus producing diarrhea. They cause diarrhea in children and also travellers' diarrhea. No biochemical markers are available to identify ETEC strains. Their identification depends on the demonstration of the toxins. They possess surface properties called colonization factors which promote their virulence. Colonization factors may be pili or special type of protein K antigen. Most strains of ETEC belong to O serotypes 6, 8, 15, 25, etc. They are known to cause:
   1. Mild or moderately severe childhood diarrhea in developing countries.
   2. Cholera-like syndrome in adults living in areas where cholera is endemic.
   3. Travellers' diarrhea in persons from developed country who visit developing countries.
   4. Outbreaks of diarrhea in newborn nurseries, in developed countries.
   5. Outbreak of diarrhea due to fecal contamination of food and water in developed countries.

c. *Enteroinvasive E. coli also called EIEC:* They invade intestinal epithelium like other dysentery causing bacilli. They are biochemically atypical as they may be late lactose fermenter or non-lactose fermenter. They cause keratoconjunctivitis when instilled in the eyes of guinea pig (Sereny test). The invasion of HeLa cells in tissue culture also provides another test for its demonstration. They belong to serogroup like O_{28}ac, O_{112}ac, O_{124}, etc.

d. *Enterohemorrhagic E. coli also called EHEC:* They were identified in 1983 following food-borne outbreaks of hemorrhagic colitis caused by *E. coli* O_{157} : H_7. Here there is usually no fever but hemorrhage is marked. EHEC produces a cytotoxin. It is also called "Vero toxin" because of its affects on vero cells in culture.

e. Enteroaggregative *E. coli* (EAEC)

f. Diffusely adherent *E. coli* (DAEC)

2. *Urinary tract infection,* e.g. cystitis, pyelitis and pyelonephritis. Infection may be precipitated by urinary obstruction due to prostatic enlargement, calculi and pregnancy.

Escherichia coli serotype commonly responsible for urinary tract infection are 0, 1, 2, 4, 6 and 7. Strains carrying K antigen are responsible for pyelonephritis while strains from cystitis lack "K" antigen.

Urinary tract infection causing Escherichia coli possess certain characters like binding of *Escherichia coli* to epithelial cell receptors by means of adhesion. Almost all *E. coli* strains that cause pylonephritis in patients with anatomically normal urinary tracts possess a particular pilus (P pilus or gal pilus that mediates attachment to the digalactoside portion of glycosphingolipids present on uroepithelium. The strains that produces pyelonephritis are also hemolysin produce, have aerobactin (a siderophore for scavenging iron) and are resistant to the bactericidal action of human serum. As such, uroepithelial adhesion assay has become one of the important parameters. Six antigenic types F_7 to F_{12} of fimbrial have been identified from uropathogenic *Escherichia coli* strains. Tests used for this purposes are:

i. Electron microscopy to detect the presence of fimbriae.
ii. Uroepithelial adhesion assay.
iii. Hemagglutination tests using human, bovine, chicken erythrocytes with or without mannose.
iv. Salt aggregation test (SAT).
v. Hemolysin production.
vi. Colicinogeny.
vii. Aerobactin production, i.e. siderophore.
viii. Dulcitol fermentation

3. *Pyogenic infection*, e.g. wound infection, abscess, peritonitis, cholecystitis and meningitis.
4. *Septicemia:* It is one of the most common cause of septicemia. The manifestations are fever, hypotension, disseminated intravascular coagulation (endotoxin shock), etc. Mortality is significantly high.

Laboratory Diagnosis

1. *Hematological investigations:*
   a. Total leukocyte count is usually within normal limits. In tissue invasion moderate-leukocytosis may be there.
   b. *Differential leukocyte count:* There may be increase in polymorphonuclear cells in tissue invasion.
2. *Bacteriological investigations:*
   Specimen: In urinary tract infection midstream urine is collected under aseptic conditions. Immediately the urine is examined and in case of delay it should be stored at 4°C.

   In acute diarrhea a sample of feces or a rectal swab is collected. Pus may be collected on sterile cotton swab or sterile container.

Smear Examination

Centrifuged urine deposit is examined for pus cell, RBC and bacteria. Gram-stained smear from centrifuged deposit shows moderate to large number of pus cell and Gram-negative bacilli.

Examination of wet and stained smear of fecal matter is of little use. Treatment of fecal matter with fluorescent labeled O group antisera is useful for an early provisional diagnosis of infantile diarrhea. Here DNA probes for different enteropathogenic forms are quite reliable and useful. ELISA, precipitin test, radioimmunoassay are other useful tests to establish the identity of enteropathogenic strains.

Culture

Material is inoculated on blood agar plate and MacConkey plate. Lactose fermenting, Gram-negative, motile, indole positive, MR positive, VP negative and citrate negative is suggestive of *Escherichia coli.*

The count of organism should be more than 1 lakh per ml (10^5 per ml) in urinary tract infection. It is called significant bacteriuria.

Treatment: It is sensitive to sulfonamides, trimethoprim, tetracyclines, chloramphenicol and aminoglycosides. Resistance to one or more drug is quite common. Nitrofurantin and nalidixic acid may be useful for treating urinary tract infection. In septicemia or serious infection, treatment is required to be started immediately without waiting for even drug sensitivity tests and drug of choice (unlikely to be resistant) is gentamicin.

Towards preparation of vaccine against *E. coli* colonization factor antigen (CFA) is being considered. This prototype vaccine includes CFA I, II and IV and is under evaluation in Sweden.

EDWARDSIELLA

It is non-capsulated, motile bacillus with weak fermentation of sugar (glucose, maltose). It forms indole, H_2S and utilizes citrate.

Edwardsiella tarda is intestinal flora of snake. It has also been isolated from human diarrheaic feces. However, its pathogenic role is not known.

CITROBACTER

It occurs as intestinal commensals in man. It is motile, utilizes citrate, grows in KCN, produces H_2S and may ferment lactose. It has two species:

1. *C. freundii.*
2. *C. intermedius* (H_2S negative).

It has been isolated from enteric fever cases. It may cause urinary tract infection, infection of gallbladder and meninges, etc.

KLEBSIELLA

It is found in the mucosa of upper respiratory tract, intestine and genitourinary tract. It is non-motile, capsulated, growing in ordinary media forming large mucoid colonies of varying degree of stickiness. It has been classified into 3 species on the basis of biochemical reactions.

1. *K. pneumoniae:* It ferments sugar (glucose, lactose, mannitol) with production of acid and gas. Indole and MR are negative, VP and citrate are positive. It hydrolysis urea. It may cause pneumonia, urinary tract infection and pyogenic infections. Serotype 1, 2 or 3 is usually responsible for pneumonia.
2. *K. ozaenae:* It causes foul smelling nasal discharge (ozaena). Biochemical reactions are variable. It belongs to capsular type 3, 4, 5, 6.
3. *K. rhinoscleromatis:* It causes rhinoscleroma. Organisms are seen intracellulary in lesion. It belongs to capsular type 3.

Difference between *Klebsiella* and *Escherichia coli*

	E. coli	*Klebsiella*
1.	Non-capsulated	Capsulated
2.	Motile	Non-motile
3.	Indole and MR positive Citrate and VP negative	Indole and MR negative Citrate and VP positive
4.	Urease negative	Urease positive
5.	Colonies not mucoid and string test negative	Colonies mucoid with string test positive
6.	Slender and long	Short and thick
7.	Gas from glucose fills 1/3rd of Durham's tube	Gas from glucose fills more than 2/3rd of Durham's tube.

ENTEROBACTER

It is motile, non-capsulated, lactose fermenting, indole and MR negative, VP and citrate positive. It liquefies gelatin. There are 2 species.

1. *E. cloacae.*
2. *E. aerogenes* found in human and animal feces, soil, etc.

HAFNIA

It is also intestinal commensal. It is non-capsulated, motile, non-lactose fermenter, indole and MR negative. VP and citrate positive. Only one species is known *H. alvei.*

SERRATIA

It forms pink red or magenta non-diffusible pigment, i.e. prodigiosin. Only one species is recognized, *S. marcescens.* It has been isolated from cases of meningitis, endocarditis, septicemia and respiratory infection. It may be an opportunist pathogen infecting debilited patients of hospital.

PROTEUS

Morphology: It is Gram-negative rods showing great variation in size, 0.5 × 1 to 3 µ. It may be in long filaments or in granular form. It is actively motile and show swarming motility, best seen at 20°C (Fig. 30.1). It is non-sporing and non-capsulated.

Cultural character: It is aerobic and facultative anaerobic. Culture emits characteristic putrefactive (fishy or seminal) odor. *Proteus vulgaris* and *Proteus mirabilis* show swarming type of growth at 37°C while swarming is absent in other species.

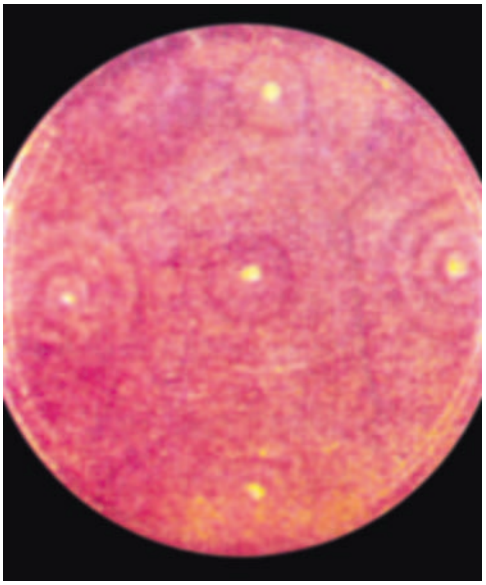


Fig. 30.1: Swarming *Proteus* on blood agar media

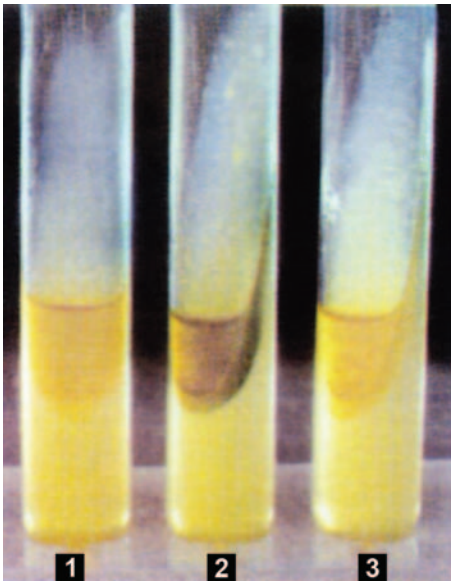


Fig. 30.2: Phenylalanine agar:
1. Control; 2. *Proteus vulgaris;* 3. *Escherichia coli*

Broth: It shows uniform and moderate turbidity after 18 to 24 hours of incubation. There is powdery deposit and ammonical odor.

Nutrient agar: *Proteus vulgaris* and *Proteus mirabilis* swarm on solid media at 37°C after 12 to 18 hour incubation.

Swarming may be due to progressive surface growth spreading from the edge of parent colony.

Swarming can be suppressed by:
1. Six percent agar in media.
2. Chloral hydrate.
3. Sodium azide (1 : 500).
4. Alcohol (5 to 6%).
5. Sulfonamide.
6. Boric acid (1 : 1000), etc.

Swarming does not occur on MacConkey agar medium.

Biochemical reactions: It forms acid and gas from glucose (except *Proteus rettgeri*). It characteristically deaminates phenylalanine to phenylpyruvic acid (PPA) (Fig. 30.2). Hydrolysis of urea is another characteristic property of proteus.

It is MR positive and VP negative. It is non-lactose fermenter. H_2S is produced in *Proteus vulgaris* and *Proteus mirabilis.* Indole is not produced by *Proteus mirabilis. Proteus morganii* and *Proteus rettgeri* are H_2S negative. Citrate is positive in *Proteus rettgeri* and negative in *Proteus morganii.*

Antigenic structure: A number of O and H antigens are produced in proteus. *Proteus vulgaris* is divided into 12 types X_2, X_{19}, XK, XL and A to H. All of these have distinct O antigen (alkali labile polysaccharide). Strain X_{19}, X_2 and XK agglutinate with the sera of typhus patient because of common antigenic factor in O antigen of proteus and antigen of rickettsiae.

Pathogenicity: It is an opportunist pathogen. It may cause urinary tract infection. It may produce pyogenic lesions like abscess, infection of wound, ear or respiratory tract. *Proteus morganii* is reported to cause infantile diarrhea.

Laboratory Diagnosis

Hematological investigations: Leukocytosis with increase in polymorphonuclear cells may occur when tissues are invaded.

Bacteriological investigations: On culture of material (urine, pus, sputum, etc.) we find swarming type of growth. It can be further identified by biochemical tests.

SHIGELLA

It is found exclusively in the intestinal tract of man.

Morphology: It is non-motile, non-capsulated, about 0.5 × 1 to 3 µ in size.

Cultural character: It is aerobic and facultative anaerobic, grows readily in simple media with an optimum temperature of 37°C and pH of 7.4.

Broth: There is uniform growth with mild turbidity after 12 to 24 hours' incubation. There is no pellicle formation.

Nutrient agar: After overnight growth, colonies are small, 2 mm in diameter, circular, convex, smooth and translucent.

MacConkey agar: Colonies are colorless due to the absence of lactose fermentation except *Shigella sonnei* which ferments lactose late and forms pink colonies.

Desoxycholate citrate agar (DCA): It is a useful selective medium for *Shigella*.

Resistance: It is killed at 56°C in 1 hour and 1 percent phenol in 30 minutes. Boiling, pasteurization and chlorination kill the organism. In water and ice it survives and remains viable for 1 to 6 months.

Biochemical Reactions (Fig. 30.3)

It is MR positive and reduces nitrates to nitrites. It does not form H_2S, cannot utilize citrate and is inhibited in KCN. Catalase is positive except *Shigella dysenteriae* type I. Glucose is fermented with production of acid and no gas (except Newcastle and Manchester biotypes of *Shigella flexneri* type 6). Fermentation of mannitol forms the basis of classification as shown above.

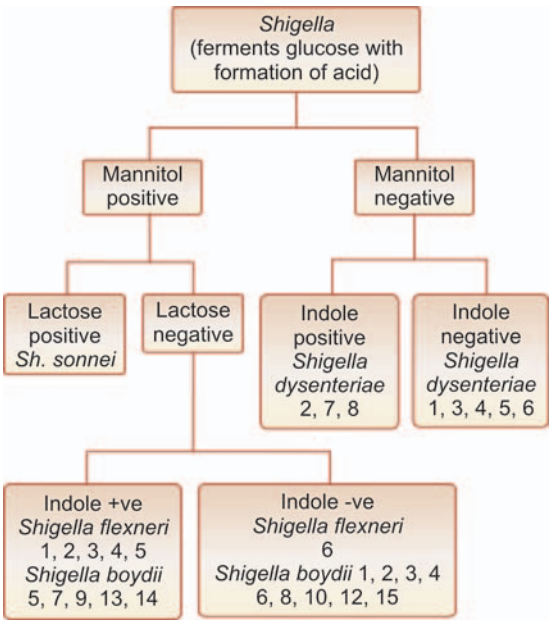


Fig. 30.3: Biochemical reactions

Antigenic structure: It has one or more major antigens and large number of minor somatic antigens. There is antigenic sharing between some members of genus and between *Shigella* and *E. coli.* The somatic O antigen of *Shigella* is lipopolysaccharide. Their serologic specificity depends on the polysaccharides. Common fimbrial antigen may occur. For identification of *Shigella* both antigenic and biochemical properties should be considered.

TOXIN

Following toxins are produced:

a. *Endotoxin:* On autolysis endotoxin is released which is lipopolysaccharide and contributes to the irritation of bowel wall.
b. *Enterotoxin of Shigella dysenteriae* inhibits sugar and amino acid absorption in small intestine of man.

Classification: On the basis of biochemical and serological properties shigella are classified into 4 groups as discussed below:

Shigella dysenteriae: It consists of 10 serotypes. It is unique in forming powerful exotoxin (neurotoxin). It can be toxoided. This shiga exotoxin is found to be immunologically resembling verocytotoxin (VT-1). This VT-1 is also known as Shiga like toxin (SLT1 and SLT2). *Shigella dysenteriae* type I causes most severe bacillary dysentery and also called *Shigella shigae*.

Shigella flexneri: It consists of 6 antigenic types (1 to 6) and several subtypes. Serotype 6 is always indole negative and occurs in three biotypes (Boyd 88, Manchester and Newcastle).

Shigella boydii: Eighteen serotypes are known. This species resemble *Shigella flexneri* biochemically and differs from it serologically.

Shigella sonnei: It is late lactose and sucrose fermenter. Indole is not produced. On the

basis of their capacity to form colicine, it is divided into 17 types. Each type being characterized by the production of specific colicine. It causes mild dysentery.

Pathogenesis: Shigellae cause bacillary dysentery. Ingested bacilli may infect villi of large intestine and multiply inside them. It spreads and ultimately involves lamina propria. Inflammatory reaction results in epithelial necrotic patch which later on become transverse superficial ulcers.

Shigella bacilli are highly infectious. The infecting dose is 10^3 organisms. There is invasion of mucosal epithelium; microabscesses appear in the wall of the large intestine and terminal ileum leading to necrosis of mucous membrane, superficial ulceration, bleeding and formation of a pseudomembrane on ulcerated area. Pseudomembrane consists of fibrin, leukocytes, cell debris, necrotic mucous membrane and bacteria. Then granulation tissue fills the ulcer and scar tissue is formed.

Bacillary dysentery has short incubation period (1 to 7 days). There is frequent passage of loose motion containing blood and mucus with griping pain and tenesmus. *Shigella dysenteriae* type I may cause complications like arthritis, toxic neuritis, conjunctivitis, parotitis, intussusception and myocarditis.

Laboratory Diagnosis

Collection of specimen: Stools are collected under aseptic precaution and examined as under:

Microscopic examination: Wet cover slip preparation shows large number of pus cells with degenerated nuclei, RBC and macrophages. Bacterial flora is considerably diminished.

A loopful of pus or blood tinged mucus from freshly passed fecal sample is cultured on MacConkey and DCA. Selenite broth is used as enrichment medium. After 12 to 18 hours of incubation colorless colonies (non-lactose fermenter) appear on MacConkey medium. These are tested for motility and biochemical reactions. Non-motile organism which is urease, citrate, KCN and H_2S negative, indole and MR positive suggestive of Shigella. Identification is confirmed by slide agglutination with polyvalent and monovalent antisera.

An ECO RI generated 17 Kb fragment of *Shigella flexneri* serotype 5 virulence plasmid may identify specifically all *Shigella* species. This DNA hybridization technique may be useful in the identification of isolates of *Shigella* which fail to agglutinate with reference to *Shigella* antisera.

Serology: The fluorescent antibody technique has been employed for direct identification of shigellae in feces. It is of no value in diagnosis.

Treatment: Drugs like tetracycline or chloramphenicol are effective in *Shigella* infection. Ampicillin is bactericidal for this bacteria. Treatment with antibiotic should be continued for 5 to 7 days. Oral live vaccine using streptomycin dependent strains in polyvalent preparation have given highly significant protection against clinical disease. However, protection conferred was serotype specific and required 3 to 4 doses but immunity lasted for only 6 to 12 months. Single booster dose give prolonged protection for another year. A thymine requiring and temperature sensitive double mutant has been fully characterized and its lack of virulence tested using experimental animals while new attempts are being made to introduce a gene from *Escherichia coli* thus reducing the chances of reverting to pathogenicity and using it as vaccine. Transfer by conjugation of virulent strains of *Shigella sonnei* (produce surface antigen 1) to mutant of *Salmonella typhi*-Ty 21 a is another attempt towards preparation of vaccine. The new strain 1 Gal E protects mice against *Shigella sonnei.*

SALMONELLA

Genus *Salmonella* is found in the intestine of man, animals and birds. Sometimes food (egg and meat) may be contaminated with this organism. It may cause enteric fever, gastroenteritis and septicemia.

Morphology: It is Gram-negative rods, 2 to 4 µ × 0.6 µ in size, motile (except *Salmonella gallinarum* and *Salmonella pullorum*). They are non-capsulated and non-sporing but may have fimbriae.

Cultural character: They are aerobic and facultative anaerobic, optimum temperature for their growth is 37°C and pH is 6 to 8.

Broth: It shows uniform turbidity after overnight culture. There is no pellicle formation.

Blood agar: Colonies are large 2 to 3 mm in diameter, circular low convex, translucent, smooth and non-hemolytic.

MacConkey's media: It is non-lactose fermenter and colorless.

Desoxycholate citrate media: These colonies are non-lactose fermenter (colorless).

Wilson and Blair bismuth sulfite medium: Jet black colonies with metallic sheen due to H_2S formation may appear.

Selenite F and tetrathionate: These broth are commonly used as enrichment media.

Biochemical reactions: It ferments glucose, mannitol and maltose forming acid and gas except *Salmonella typhi* which produces only acid and no gas. It does not produce indole but is MR positive, VP negative and citrate may be positive. Urea is not hydrolyzed and H_2S is produced (Table 30.3).

TABLE 30.3: Biochemical differentiation of *Salmonella*

Salmonella	*Glucose*	*Xylose*	*Taratrate*	*Mucate*
S. typhi	A	d	A	d
S. paratyphi A	AG	—	—	—
S. paratyphi B	AG	AG	—	AG
S. paratyphi C	AG	AG	AG	—

Resistance: It may be killed:

a. By heating at 60°C for 15 to 20 minutes,
b. Pasteurization,
c. Boiling, and
d. Chlorination.

It is sensitive to chloramphenicol. It can survive in ice, snow and water for months together. It is resistant to brilliant green, malachite green, bile salts, tetrathionate and selenium salts.

Antigenic structure: Following antigens are found:

Somatic antigen (O): It is phospholipid protein polysaccharide complex. It can withstand boiling, alcohol and acid treatment. O agglutination takes place more slowly. It is less immunogenic. Hence titer of O antibody after infection or immunization is lower than that of H antigen. About 65 antigenic factors have been identified and each species contains several factors.

Flagellar antigen (H): It is heat labile protein. It is destroyed by boiling, alcohol but not by formaldehyde. It is strongly immunogenic. The flagellar antigen is of dual nature occurring in one of two phases. Phase I is designated as *a,b,c,— z1, z20,* etc. and is species specific. Phase II is non-specific and is shared by several unrelated species of *Salmonella*. It is designated as 1, 2, 3 or complex of *e, n* and *x.*

Vi antigen: It is surface and heat labile antigen. Bacilli inagglutinable with O antiserum becomes agglutinable after boiling or heating at 60°C for 1 hour. It is virulent to mice and Vi antibody may provide protection. The persistence of Vi antibody indicates carrier state. It is present in *S. typhi, S. paratyphi C* and *S. dublin.* It may also be present in citrobacter.

Salmonella classification: As per modern toxonomical techniques all members of genus Salmonella belongs to two species as under:

1. *Salmonella enteric*
2. *Salmonella bongori*

Salmonella enteric

- Corresponds to former subgenus I
- It is further classified into 6 subspecies as under
  - Enterica
  - Salmae
  - Arigonae
  - Diarizonae
  - Houtenae
  - Indica
- *Salmonella enteric* subspecies enteric includes the typhoid, paratyphoid bacilli and many other serotypes responsible for human disease.
- As per this taxonomy the appropriate name suggested for typhoid bacillus is *Sallmonella enteric*, subspecies enteric serotype typhi, serotype name ought to be given in Roman only.

Antigenic classification: On the basis of somatic antigen, Salmonella can be divided into

TABLE 30.4: Kauffman and White scheme

Group serotypes		Antigen		
		O	H	
			Phase I	Phase II
A	*S. paratyphi A*	1, 2, 12	*a*	—
B	*S. paratyphi B*	1, 4, 5, 12	*b*	1, 2
	S. typhimurium	1, 4, 5, 12	*i*	1, 2
	S. chester	4, 5, 12	*eh*	*e, n, x*
C_1	*S. paratyphi C*	6, 7, (Vi)	c	1, 5
	S. cholerae-suis	6, 7	*c*	1, 5
C_2	*S. muenchen*	6, 8	*d*	1, 2
D	*S. typhi*	9, 12 (Vi)	*d*	—
	S. enteritidis	1, 9, 12	*g, m*	—
	S. gullinarum	1, 9, 12	—	—
E	*S. anatum*	3, 10	*e, h*	1, 6

65 serogroups. Each group is designated as A, B, C, D, etc., e.g. members of group A have 1, 2, 12, group B have 1, 4, 5, 12, group C 6, 7 or 6, 8 and group D have 1, 9, 12, 0 factors. Species among each sub-group are recognized by specific flagellar antigen (phase I and phase II).

Kauffman and White have described scheme for antigenic structure and classification of Salmonella (Table 30.4).

Antigenic Variation

1. *S → R variation:* The smooth to rough variation is associated with change in colony morphology and loss of O antigen and virulence. SÄR variation is induced by prolonged incubation of broth culture. It can be prevented by maintaining cultures on Dorset egg media, in cold or lyophilization.
2. *H →O variation:* It is associated with loss of flagellar antigen. This is reversible change. Flagella are inhibited in media containing phenol (1 : 800). Flagella reappear when strain is subcultured in media without phenol.
3. *Phase variation:* The flagellar antigen of *Salmonella* may occur in one of two phases. Phase I antigen is more specific as it is shared by few species only. Phase II antigen is widely shared and is non-specific. Strains that have both phases are called diphasic. *S. typhi* occurs in one phase and called monophasic. A culture in one phase may be converted into other phase by passing through Craigie's tube containing homologous phase antiserum incorporated in agar (Fig. 30.4).

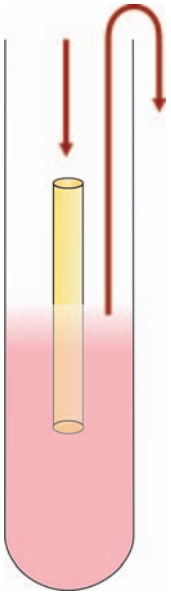


Fig. 30.4: Craigie's tube

4. *V →W variation: S. typhi* carries surface antigen (Vi) which is agglutinable with Vi antiserum (V form). After repeated culture Vi antigen is lost and is inagglutinable with Vi antiserum (W form). Intermediate phase when it is agglutinable with both Vi and O antiserum is called VW forms.

Bacteriophage: Some serotypes of Salmonella strain indistinguishable biochemically and serologically may be subdivided into phage types. It is based on their susceptibility to lysis by different races of bacteriophage. Likewise *S. typhi* having Vi antigen may be divided into 106 phages types designated by letter numbers. Type A is sensitive to all Vi phages. The types predominant all over the world are E-1, A, B-2, C-1, C-2 and F-1. Paratype-B has 53 phage types and *S. typhimurium* has 232 phage types with 30 phages.

Biotyping: On the basis of 15 biochemical properties uptill now 24 primary and 200 full biotypes are recognized of *Salmonella typhimurium*.

Plasmid typing: Quite a large number of plasmids are present in various Salmonella. They can be separated on the basis of molecular weight by electrophoresis and plasmid profile. This technique is comparable with phage typing.

Pathogenesis: It may produce 3 types of lesions:

a. Enteric fever.
b. Food poisoning.
c. Septicemia.

Enteric fever: It is caused by *S. typhi* (70 to 85% in India), *S. paratyphi A* (15 to 21%) and *S. paratyphi B.* Infection is through ingestion. The bacilli may enter the body through lymphoid of pharynx. In the gut organisms attach themselves with epithelial cells of intestinal villi and penetrate lamina propria and submucosa. They are phagocytosed by polymorph or macrophages. They enter mesenteric lymph node to multiply there. Then they enter thoracic duct and subsequently bloodstream.

As a result there is bacteremia and organism are seeded in liver, gallbladder, spleen, bone marrow, lymph node, lung and kidney, etc. In these organs further multiplication occurs. Then there is bacteremia and hence onset of clinical symptoms. The bacilli invade tissue, e.g. Payer's patches and lymphoid follicles of small intestine. The intestinal lesion ulcerates and hemorrhage or perforation may occur (Fig. 30.5).

The organism liberate endotoxin which produces toxic symptoms like headache, anorexia, continuous fever and congestion of mucous membrane. Incubation period is 10 to 14 days. The typical features are step ladder pyrexia, palpable spleen and rose spots that fade on pressure and they appear in 2nd to 3rd week of infection.

S. paratyphi A and *S. paratyphi B* may cause paratyphoid fever resembling enteric fever.

Septicemia: It is frequently caused by *S. cholerae suis.* It produces chills and spiked fever. Local lesions occur in various parts of body producing osteomyelitis, pneumonia, pulmonary abscess and meningitis, etc. The bowel is not invaded and fecal culture is negative.

Food poisoning: It is by ingestion of contaminated food, e.g. meat and egg. Food poisoning is caused by *S. typhimurium, S. enteritides, S. newport,* etc. Incubation period is 12 to 48 hours. There is fever, vomiting, diarrhea (mucus and blood in stool). There may be ulceration of intestinal mucosa. There is no bacteremia.

Laboratory Diagnosis

1. *Hematological investigations:*
   a. Total leukocyte count in typhoid fever shows leucopenia. The count may be 3000 to 8000 per cu mm.
   b. *Differential leukocyte count:* There may be lymphocytosis and monocytosis.
2. *Bacteriological investigations:* Organism may be isolated from blood, urine, feces, persistent discharge and in some cases from cerebrospinal fluids.

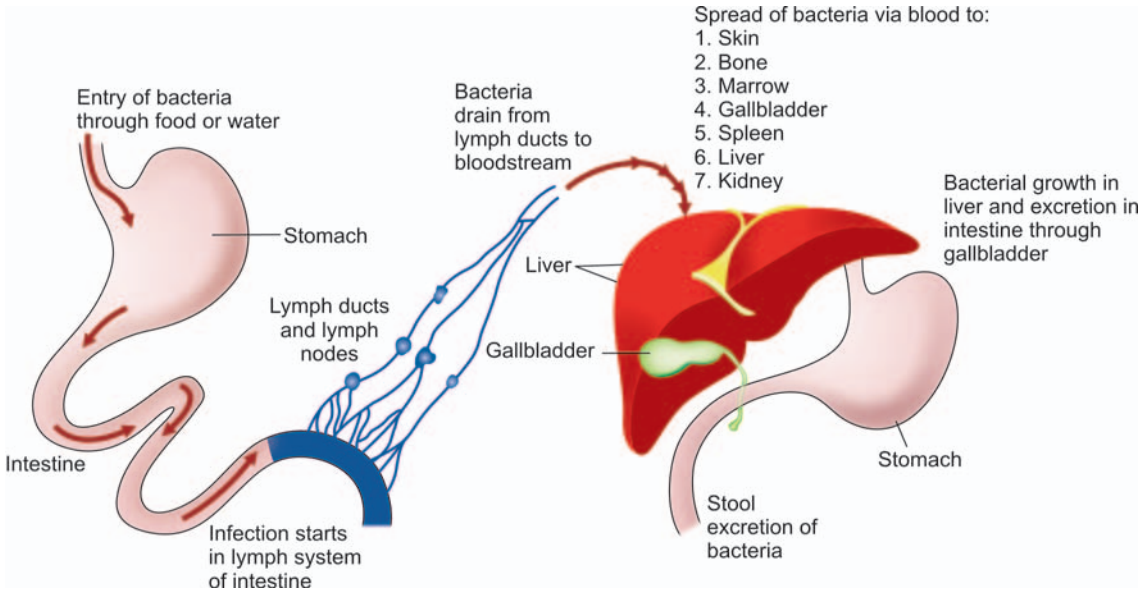


Fig. 30.5: Typhoid fever pathogenesis

a. *Blood culture:* 5 to 10 ml blood of a patient is collected aseptically and is transferred into blood culture bottles containing 100 ml of bile broth. After 24 to 48 hours' incubation of bottle at 37°C, they are subcultured on blood agar and MacConkey, on which bacilli grow as non-lactose fermenting, Gram-negative and motile organism.
b. *Clot culture:* Blood clot is cultured in 15 ml bile broth bottle (0.5% bile salts). It is more frequently positive.
c. *Fecal culture:* It is positive throughout. Repeated cultures are required for successful isolation of organism. Fecal culture is useful more in cases who are on chloramphenicol.

Successful culture depends on use of enrichment and selective media. Fecal samples are plated directly on MacConkey, DCA and Wilson and Blair media. On MacConkey and DCA we find pale colonies. On Wilson and Blair we find black colonies with metallic sheen. *S. paratyphi A* produce green colonies.

For enrichment, specimen are inoculated into one tube each of selenite F and tetrathionate broth. It needs 12 to 18 hours' incubation before subculture.

Bile culture: It is important for detection of carriers and in later stages of disease. Bile aspirated by duodenal tube is processed like fecal specimen.

Urine culture: It is less useful than blood and feces. Culture is positive in 2nd and 3rd week. Clean voided urine is centrifuged and sediments are inoculated into enrichment and selective media.

Other material: Bone marrow culture is positive in most cases. Other specimens like rose spots, pus from lesion, CSF and sputum may be used for culture. At autopsy culture may be obtained from gallbladder, liver, spleen and mesenteric lymph nodes.

Serological test (Widal test): *Salmonella* antibody appears at the end of first week, Widal test is used for this purpose. This is a test to measure H and O antibodies in the sera of patient. Two types of tubes are used:

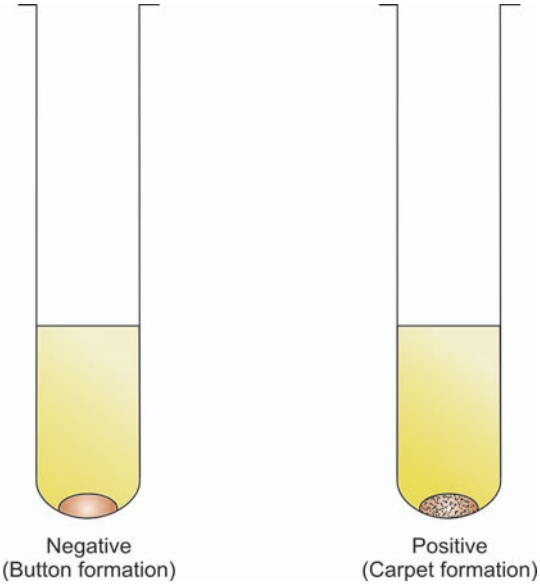


Fig. 30.6: Widal test (Negative and positive tubes)

a. Narrow tube with conical bottom (Dreyer's tube) for H agglutination.
b. Short round bottomed tube (Felix tube) for O agglutination.

Serial two fold dilution of patient serum (1/10, 1/20, 1/40 and so on) are mixed with equal volume of antigen (TO, TH, AO and AH). At 37°C incubation is done for 4 hours and then rack is kept at 4°C overnight. H agglutination leads to formation of cotton wooly clump and O agglutination is seen as matted, granular irregular disc like pattern at the bottom of tube (Fig. 30.6).

Interpretation of Widal Test

1. Agglutination appears by the end of first week. The titer increases steadily till 4th week after which it declines.
2. Demonstration of rising titer of antibody by testing two or more samples are more meaningful.
3. In single test 1/100 titer of O and 1/200 or more titer of H is significant.
4. In immunization, antibody against both *S. typhi* and *S. paratyphi* will be there whereas in infection antibody will be seen only against infecting organism.
5. Immunized person or patients who have had prior infection may develop anamnestic

response during unrelated fever. In anamnestic response there is temporary rise in H titer only, whereas in enteric it is sustained.

6. Bacterial suspension should be free from fimbria otherwise false-positive result occurs.
7. Treated case may show poor agglutination response.

Tracing of typhoid carrier: Sewer swab technique is quite helpful in tracing of carriers. This is done by following the gauze pads, kept in sewers and positive for *Salmonella typhi* cultures, backwards from the main drain, ultimately lead to localization of the house of the carrier. However, typhoid carrier may be detected as follows:

1. Widal test may show raised antibody titers.
2. Vi agglutination test is positive in a titer of 1/10 or more.
3. Several stool cultures may help in the isolation of causative organism.
4. Organism may be cultured from bile obtained after duodenal intubation.

Treatment: Antibiotics like ciprofloxcilin chloramphenicol, furazolidine and ampicillin are effective. Trimethoprim sulfamethaxazole and amoxycillin are also used in the treatment. The treatment of the carrier is cholecystectomy.

Specific prophylaxis consists of TAB vaccine containing *S. typhi* 1000 million and *S. paratyphi A* and *B* 750 million each per ml of heat killed bacteria and preserved in 0.5 percent phenol. It is given in two doses of 0.5 ml subcutaneously at an interval of 4 to 6 weeks. However, encouraging results are noted with live vaccine (streptomycin dependent strain) and killed vaccine given as enteric coated tablets.

Nowadays Vi antigen parenteral is being evaluated. Recently oral vaccine prepared from attenuated strain of *S. typhi* designated as Ty 21a is gaining popularity. It contains capsules of about 1 billion live lyophilized *S. typhi* mutant Ty 21a strain organisms. The Ty 21a vaccine strain is a mutant developed by genetic manipulation lacking UDP galactose-4-epimerase, an enzyme responsible for incorporation of galactose into cell wall lipo-polysaccharide. In the absence of exogenous galactose, the mutant grows as rough non-immunogenic strain devoid of antigen. When the ambient environment as in intestine contains galactose, immunogenic cell wall components could be produced through galactose-1 phosphate but owing to lack of epimerase, intermediate products of galactose metabolism continuously accumulate into bacterial cell wall and cause cell lysis. The restored biosynthesis of cell

TABLE 30.5: Biochemical reactions of the main genera of enterobacteriaceae

Genus	*Motility*	*Glucose (Gas)*	*Lactose (Acid)*	*Sucrose (Acid)*	*Mannitol*	*Indole*	*Methyl Red*	*Voges Proskauer*	*Citrate*	*PPA*	*Urease*	*H_2S*	*KCN*
Escherichia coli	+	+	+	d*	+	+	+	–	–	–	–	–	–
Shigella	–	–	– (except *Sh. sonnei*)	–	– (except *Sh. dysenteriae*)	±	+	–	–	–	–	±	–
Klebsiella	–	+	+	+	+	–	–	+	+	–	+	–	–
Enterobacter	+	+	+	+	+	–	–	+	+	–	–	–	+
Serratia	+	d*	d*	+	+	–	–	+	+	–	–	–	+
Hafnia	+	+	–	d*	+	–	–	d*	+	–	–	+	+
Salmonella	+	+	–	–	d*	+	–	–	+	–	–	d*	–
Citrobacter	+	+	+	d*	+	–	+	–	+	–	d*	+	+
Proteus	+	+	–	+ (except *P.morganii*)	– (except *P.rettgeri*)	d*	d*	–	d*	+	+	d*	+
Providencia	+	+	–	+	d*	+	+	–	+	+	–	–	+
Edwardsiella	+	+	–	–	–	+	=	–	–	–	–	+	–

d* = Different result in different strains

wall polysaccharide accounts for immune effect, while subsequent bacteriolysis accounts for the *in vivo* avirulence of the vaccine. The vaccine strain is no longer isolated from the stools a few days later after giving final (3rd dose) and there is no evidence of spread to contacts. For primary immunization a total of 3 oral doses are given at 2 days' interval. Protection stays for 3 years. It gives humoral (IgA) and cellular response in intestinal tract. Other strains being evaluated are Mutant Ty (Vi^+ and Vi^-) and genetically modified *S. typhimurium* axotropic mutant strain.

Tribe Yersinieae is discussed separately on page 230 to 233 (Chapter 36). On the basis of biochemical reactions main genera of enterobacteriaceae can be differentiated (Table 30.5).

31 Pseudomonas

They are mostly saprophytes being found in water, soil and wherever decomposing matter is found. They are frequently involved as secondary invaders causing suppurative and inflammatory lesions. Pathogenic member is *Pseudomonas aeruginosa.*

PSEUDOMONAS AERUGINOSA (Pseudomonas pyocyanea)

Morphology: It is slender, Gram-negative bacilli of 0.5 µ × 3.5 µ size, actively motile by polar flagellum. It is non-capsulated.

Cultural character: It is aerobic growing on simple nutrient media with optimum temperature of 37°C.

Peptone water: After 18 to 24 hours' incubation it forms dense turbidity and surface pellicle. Bluish green pigment due to water soluble pyocyanin is seen.

Nutrient agar: It produces large, opaque, irregular colonies of butyrous consistency. It gives musty or earthy smell or fruity odor due to production of aminoacetophenone from tryptophane. It produces water soluble pigment pyocyanin which diffuses in medium. Pigments are of following types:

a. Pyocyanin (bluish green).
b. Fluorescein (yellowish green).
c. Proverdin (green).
d. Pyorubin (red).
e. Pyomelanin (black).
   Some strain may be non-pigmented.

Blood agar: It shows beta type hemolysis.

MacConkey: It produces non-lactose fermenting colonies.

Cetrimide agar: It is selective medium.

Antigenic Structure

i. Somatic antigens are 17 designated as 01 to 017. They divide the strains of *Pseudomonas aeruginosa* into 17 serogroups. Serogroup 06 and 011 are responsible for nosocomial infection.
ii. Flagellar antigens are two in number. Flagellar antigen I is uniform while flagellar antigen II is complex one with 5 to 7 factors.
iii. Common fimbreal antigens are 4 in number. They are unstable.
iv. Plasma membrane contains 4 protein antigens of which FI, and H1 and H2 have been characterized.

Typing methods: It is obligatory to type the strain for epidemiological knowledge as under:

A. *Bacteriocin typing:* It is also called pyocin typing. Three types of pyocins are recognized and they are R, F and S. The pyocin producing strains are resistant to their own pyocins but are sensitive to pyocins secreted by other strains. There are 13 indicator strains designated as 1 to 8 and A to E. There are 105 types recognized and identified. It is most popular method used for typing *Pseudomonas aeruginosa*.
B. *Phage typing:* It is tedious and difficult to go for bacteriophage typing.
C. *Sero typing:* Depending upon O to H antigen 17 serotypes of *Pseudomonas aeruginosa* are identified. Although it is reliable but it is done in reference laboratories.

D. *Molecular method:* It is most reliable method practiced for typing. Here restriction endonuclease with pulsed field gel electrophoresis is used for typing.

Biochemical reactions: Glucose is used oxidatively forming acid only. Nitrate are reduced to nitrites and nitrogen. Catalase and oxidase are positive.

Resistance: Heating at 56°C kills the organisms. It is resistant to common antiseptic and disinfectants. It is resistant to most of antibiotics. However, it is sensitive to polymyxin B, colistin, gentamicin and carbencillin, netilmycin, etc.

TOXINS AND ENZYMES PRODUCED BY PSEUDOMONAS

Toxins and enzymes have significant share to increase the virulence of Pseudomonas and they are as under:

1. *Extracellular products:* Pyocyanin inhibits mitochondrial enzymes in tissues. Thus it causes disruption of aviary movements on ciliated nasal epithelium. This is the reason of these organisms being colonized in nasal mucosa.
2. *Extracellular enzymes and hemolysin:* Local lesion may be caused because of the activities of alkaline protease, lactase, hemolysin and lipase.
3. *Exotoxin:* Exotoxin is of two type A and B. Exotoxin A is a polypeptide and it inhibits protein synthesis.
4. *Endotoxin:* It is lipopolysaccharide. It has pyrogenic action and many other activities.

Pathogenesis: *Pseudomonas aeruginosa* is one of the most troublesome agents causing nosocomial infections. It is commonly encountered in secondary infection of wound, burns and chronic ulcers of skin. The bacterium attaches to and colonizes the mucous membranes of skin, invades locally and produces systemic infection. These processes are promoted by pili, enzymes and toxins. Besides these, lipopolysaccharide plays a direct role in causing fever, shock, oliguria, leukocytosis, leukopenia, disseminated intravascular coagulation and adult respiratory distress syndrome.

It has been described as one of the agents responsible for infantile diarrhea. Strains isolated from diarrhea produce heat stable enterotoxin and give positive rabbit ileal loop reaction.

Urinary tract infection may persist for longer time and may give rise to septicemia. Lesions of eye, otitis media, pulmonary empyema, brain abscess and meningitis may occur.

Laboratory Diagnosis

1. *Specimens:* Pus, exudate, sputum and swabs from conjunctiva are examined. Purulent discharge is usually greenish blue in color having sweetish odor.
2. *Culture:* On nutrient agar media characteristic greenish blue colonies appear. It may be confirmed by biochemical tests.

Treatment: Gentamicin, Polymyxin B and Carbencillin or Ticarcillin are effective in *Pseudomonas aeruginosa* infection. Clinical infection with this organism may not be treated with one antibiotic. One of the penicillins like mezlocillin may be used with an aminoglycoside (Gentamicin, amikacin or tobramycin). Ciprofloxacine is also found effective. Lipopolysaccharides vaccine may be administered to high risk patients which may provide some protection against pseudomonas sepsis 10 days later. Such vaccine is instituted in cases of leukemia, burn, cystic fibrosis and immunosuppression.

32 Vibrio

General characters: They are thin, curved, comma shaped Gram-negative, rigid and actively motile (polar flagellum) bacilli. They are non-lactose fermenting, growing at alkaline pH, produce indole and are oxidase positive. They ferment glucose with the production of acid only. Most important *pathogenic* members in man are:

1. *Vibrio cholerae.*
2. Vibrio El Tor.
3. Non-agglutinable vibrio (NAV).
4. *Vibrio parahemolyticus (Fig. 32.1).*

Classification of Vibrio

1. Non halophilic vibrio (Grow in media without sodium salt NaCl)
   a. Vibrio cholera (01 classical and E1 tor biotypes)
   b. Non 01 Vibrio cholera (Non cholera vibrio or no agglutinating vibrio)
2. Halophilic vibrio (Grow in media containing sodium salt NaCl)
   a. *Vibrio parahemolyticus*
   b. *Vibrio alginolyticus*
   c. *Vibrio vulnificus*
   d. *Vibrio mimicus*

VIBRIO CHOLERAE

Morphology: It is short curved, comma shaped about 1.5 µ × 0.2 to 0.4 µ in size and

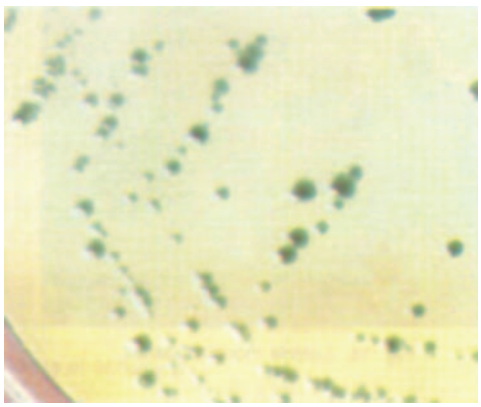


Fig. 32.1: TCBS agar *Vibrio parahemolyticus*

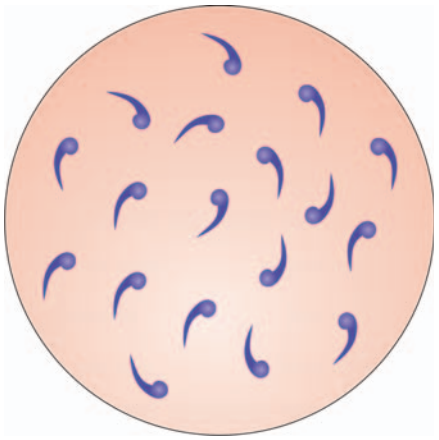


Fig. 32.2: Vibrio cholerae

Gram-negative bacilli. It may occur singly or as "S" shaped semicircular pairs (Fig. 32.2). It is actively motile with a single flagellum. It shows darting type of motility.

Culture characters: It is strongly aerobic. Growth occurs in alkaline pH (7.5 to 9.6) between 22 and 40°C (optimum 37°C). It grows well in ordinary media.

Alkaline peptone water: Rapid growth in about 6 hours is with formation of thick surface pellicle. Turbidity and powdery deposits on prolonged incubation may be present.

Nutrient agar: Colonies are moist, translucent, round 1 to 2 mm in size with bluish tinge in transmitted light. The growth has distinctive odor.

MacConkey's agar: Colonies are colorless.

Blood agar: Colonies show zone of green coloration around them which later become clear due to hemodigestion.

Special Media

For cultivation of *Vibrio cholerae:*

a. *Holding or transport media:*

i. *Venkat Raman (VR) medium:* It contains 5 gm peptone and 20 gm crude sea salt per liter distilled water adjusting pH 8.5 to 8.8. In this medium vibrio remain viable for weeks but do not multiply.
ii. *Cary-Blair medium:* It is a buffered solution of sodium chloride, sodium thioglycolate, disodium phosphate and calcium chloride at pH 8.4.

b. *Enrichment media:*
i. Alkaline peptone water pH 8.6.
ii. Monsur's taurocholate-tellurite peptone water (pH 9.2).

c. *Plating media*
i. Alkaline bile salt agar pH 8.2 (BSA).
ii. Monsur's gelatin-taurocholate trypti-case-tellurite agar (GTTA).
iii. *TCBS medium:* It contains thiosulphate, citrate bromothymol blue and sucrose. It is widely used.

Biochemical tests: Acid without gas is produced from glucose, sucrose, maltose and mannitol. Oxidase is positive, indole may be produced, nitrate reduction positive, liquefies gelatin and urease negative. Cholera red reaction is tested by adding few drops of H_2SO_4 to 24 hour growth in peptone water. With *Vibrio cholerae* red pink color is produced due to formation of nitrosoindole.

Resistance: It is killed by heat at 55°C in 15 minutes. It is destroyed by drying. The acidity of gastric juice at once kills them. It is susceptible to streptomycin, chloramphenicol and tetracycline.

Classification: Heirberg (1934) classified vibrios into 6 groups on the basis of fermentation of mannose, sucrose, arabinose. Subsequently two more groups are added. *Vibrio cholerae* belongs to group I.

Heirberg grouping of vibrios is based on fermentation of:

Vibrio groups	*Mannose*	*Sucrose*	*Arabinose*
I.	A	A	—
II.	—	A	—
III.	A	A	A
IV.	—	A	A
V.	A	—	—
VI.	—	—	—
VII.	A	—	A
VIII.	—	—	A

Antigenic structure: *Vibrio cholerae* contains somatic (O) and flagellar (H) antigen.

a. *O antigen:* It is heat stable and type specific. About 60 groups are identified. *Vibrio cholerae* and El Tor vibrio belong to subgroup I. *Vibrio cholerae* are antigenically divided into Inaba, Ogawa and Hikojima on the basis of O antigenic factors they contain. Inaba contains AC, Hikojima ABC and Ogawa AB.
b. Flagellar antigen (H) is non-specific, heat labile and is common to cholera and cholera like organisms.

Phage types: The strain of cholera is identified as 5 types. All true *Vibrio cholerae* are sensitive.

Toxin: Like other organisms it produces endotoxin. It also produces heat labile entero-toxin called choleragen which is an exotoxin with molecular weight of 80,000. It consists of subunits A (molecular weight 28000) and B (molecular weight 52000). Ganglioside GM_1 serves as mucosal receptor for subunit B which facilitate the entry of subunit A into the cell whereas subunit A activates adenyl cyclase resulting prolonged hypersecretion of water and electrolytes leading to severe dehydration, shock, acidosis and death. The genes for *Vibrio cholerae* enterotoxin are on bacterial chromosomes.

Pathogenesis: Cholera occurs only in humans. Non-agglutinable vibrios are potential patho-gens able to cause symptoms similar to cholera. The disease is transmitted from mild and convalescent cases by contaminated water, milk, fruit, vegetable, etc. Flies may disseminate organisms from feces to food. Cholera is endemic in India, China, Japan and Indonesia. Immunity after infection is short living. Gastric acidity seems an important defense against cholera. Ingestion of at least 10^{10} organisms is necessary to demonstrate some evidence of infection. However, neutra-lizing the acidity of the stomach lowered the number 10^4 organisms.

The action of enterotoxin (exotoxin) seems to be mediated by adenosine—3', 5' cyclic monophosphate. Addition of enterotoxin (cAMP) to the mucosal surface of isolated ileal mucosa stimulates active secretion of chloride and inhibits absorption of sodium. As a matter

of fact, the enterotoxin increases the level of cAMP in mucosal epithelial cells by activating the enzyme adenyl cyclase which converts ATP into cAMP.

Cholera causes an acute gastroenteritis. Incubation period is 24 hours to 5 days. Cholerae vibrio after getting establishment in the intestine, multiplies producing exotoxin get absorbed onto epithelial gangliosides which cause outpouring of fluid into the lumen. It may produce mucinases and endotoxin. Stools are rice water containing mucus flakes, epithelial cells and vibrios. As a result there is tremendous fluid loss, dehydration and hypochloremia.

Laboratory Diagnosis

Hematological Investigations

It is not diagnostically significant in early stages. However, there may be increase of packed cell volume upto 65 to 85 percent, hemoglobulin contents are increased from 15 to 25 gm percent and there may be polycythemia (count may be more than 7 million per cu mm).

Bacteriological Investigations

a. *Specimen:* Feces may be collected with a spoon in a sterile container free from antiseptic. If there are chances of delay, transport media or holding media may be used (VR media). One to three gm stools are emulsified in 10 to 15 ml (VR media). In case of rectal swab, trypticase taurocholate tellurite broth (pH 9.2) or alkaline peptone water is used. If media is not available strips of blotting paper soaked in watery stool may be sent to laboratory.
b. *Smear examination:* Hanging drop preparation shows darting type motility. Gram-staining shows them to be Gram-negative and comma shaped.
c. *Culture:* Specimen is inoculated in Monsur's medium. A tube of alkaline peptone water is inoculated simultaneously with fecal matter. After 6 to 8 hours Gram's stain shows curved Gram-negative bacilli with darting type motility. Sub-inoculation in Monsur's medium is done. The colonies in this medium are tested for biochemical reactions. It shows oxidase positive, nitrate reduction positive, fermentation of glucose, sucrose, mannose and arabinose, cholera red reaction positive, indole positive and slide agglutination with O group polyvalent or mono specific sera differentiates it into Ogawa, Inaba and Hikojima types.

Serology: It is of little use in the diagnosis of cholera. It may be helpful in assessing the incidence of cholera in an area. Indirect hemagglutination, vibriocidal test and antitoxin assay are popular agglutination tests. Among them complement dependent vibriocidal antibody test is most useful.

Treatment: Generally adequate fluid and electrolytes replacement comprises the treatment. However, oral tetracycline is useful in reducing the period of vibrio excretion and need for parenteral fluid.

Vaccine is used for control of cholera infection. Killed cholera vaccine containing killed suspension (12000 million *V. cholerae* per ml) containing equal number of Ogawa and Inaba serotypes is used nowadays which gives 40 to 60 percent protection lasting for about one year. The other vaccine preparations are procholeragenoid with killed Ogawa and Inaba, whole cholera vaccine with adjuvant like aluminium, liposomes or muramyle dipeptide, and live mutant vaccine strain (streptomycin resistant), B subunit toxoid (80 to 85% protection), etc. The candidate live oral cholera vaccine that is currently of greatest interest is *V. cholerae* 01 strain CVD-103-HgR. CVD-103-HgR is reported to elicit significantly higher serum vibriocidal antibody titer in person of blood group O. El Tor type vaccine offers protection about 90 to 100 percent which lasts for 3 years.

Vibrio El Tor

Originally it was isolated from pilgrims at the tor quarantine station on the Sinai peninsula. It was the cause of epidemics of cholera in South East Asia. An association between the risk of cholera and ABO blood groups was

confirmed and appeared to be specific to the El tor biotype. The likelihood of developing severe cholera is related to the ABO blood groups and is least common in people with blood group O. Differentiation between classical cholera and El tor vibrio is as under:

	Test	Classical cholera	El. Tor vibrio
1.	Hemolysis	—	+
2.	Voges Proskauer	—	+
3.	Chick erythrocyte agglutination	—	+
4.	Polymyxin B sensitivity	+	—
5.	Group IV phage susceptibilities	+	—

Vibrio Cholerae 139

The characteristics of *Vibrio cholerae* 139 isolates are as under:

1. Gram-negative curved bacilli.
2. Shows darting motility.
3. Not immobilized by antiserum to *Vibrio cholerae* O1.
4. On TCBS medium yellow colored colonies appear.
5. Oxidase test is positive.
6. Fermentation of glucose, sucrose and mannose without formation of gas.
7. Indole test is positive.
8. Hemolysis of sheep erythrocytes.
9. Resistant to polymyxin B (50 IU).
10. Agglutination of chicken erythrocytes.
11. Agglutination with O139 antiserum.
12. Resistant to furoxone and sensitive to amoxicillin, cotrimoxazole, nalidixic acid, cefotaxime, tetracycline, etc.

Widespread incidence of new strain of *Vibrio cholerae* is reported in 1992 and 1993 in India (Tamil Nadu and Bengal) and Bangladesh. This strain is identified as non-01 type 0139. Toxin produced by this strain can trigger off diarrhea. This strain may lead to another pandemic.

Non-agglutinable Vibrios (NAV)

It may produce cholera-like disease. Morphologically and biochemically it resembles *Vibrio cholerae.* It is non-agglutinable with O antiserum of *Vibrio cholerae.*

Vibrio parahemolyticus: It is enteropathogenic halophilic organism (requires 7 to 8% NaCl) isolated from Japan and India (Calcutta). It causes food poisoning. It can grow in presence of 7 to 8 percent sodium chloride in peptone water (Fig. 32.1).

Vibrio mimicus: This is newly recognized vibrio species that has been implicated in causing gastroenteritis.

Following consumption of raw oysters resulted in cholera like illness in Dacca (Bangladesh) as reported in 1976. Clinical spectrum, epidemiology and pathogenic significance of it is not clear.

Vibrio fluvialis: This is a newly designated species formerly referred to as enteric group EF-6 or group F vibrio organisms.

Aeromonas and Plesiomonas

Aeromonas hydrophilia causes red leg disease in frog. It is reported from cases of diarrhea and from pyogenic lesion of man. *Plesiomonas shigelloides* are reported from diarrheal diseases. Both of them are oxidase positive, polar flagellated and Gram-negative rods. They may be differentiated on the basis of biochemical tests especially utilization of amino acids.

33 Campylobacter

It is a worldwide zoonosis causing diseases communicable to man from animals with reservoirs in animals and fowls. Previously it was known by the name *Vibrio fetus* and used to be discussed with vibrios. But recently different GC ratio (lower than vibrios) led to the emergence of new group called campylobacter.

MORPHOLOGY

They are Gram-negative, bacilli (tapering end) comma shaped, non-sporing and motile. In older culture it assumes coccoid form. GC ratio is 30 to 35 percent (Vibrio 40 to 53%).

They are microaerophilic. They can be cultured on Butzler medium, Skissow medium, etc. Colonies are flat, glossy, effuse with a tendency to spread along the track of inoculation wire.

BIOCHEMICAL REACTIONS

They are oxidase positive, catalase positive/negative and nitrate reduction positive. They do not ferment carbohydrate, non-proteolytic, indole negative and urease negative.

Resistance: May survive for 5 weeks in urine, stools and milk. They are readily killed by HCl concentration of stomach and heat. They resist penicillins, bacitracin.

Toxin: A heat labile enterotoxin with mol. wt. 70,000 affects the Chinese hamster ovary and Y-1 mouse adrenal cells. Fluid is secreted in the lumen of ileal loop of rat and increased production of cAMP by stimulation of adenylate cyclase.

Many strains of *Campylobacter jejuni* and *Campylobacter coli* produce cytotoxin.

PATHOGENESIS

Following ingestion of bacilli along with contaminated edibles or water, there is colonization of organisms in upper small intestine which is rich in bile. *Campylobacter jejuni* multiply actively. Organisms are excreted in stool for 2 to 3 weeks. Tissue biopsy may show nonspecific colitis with an inflammatory infiltration of neutrophils. Lesions include mucosal edema, hyperemia and ulceration of jejunum and colon.

The affinity of cells of intestinal tract mucosa and their invasion is supported by the rapid motility of the organism and its shape. Invasion of colorectum results in its inflammation and hence causing fresh blood in the stools.

CLINICAL FEATURES

The incubation period is 2 to 7 days and illness begins within 12 to 48 hours prodromal period of malaise, fever, headache and myalgia followed by abdominal pain, nausea and diarrhea. Stools are watery sometimes mixed with blood, mucus and leukocytes. There may be dehydration, weight loss, electrolytes imbalance, lassitude and septicemia.

LABORATORY DIAGNOSIS

Specimen required are fresh stools, food, like milk, red meat, poultry and water.

1. *Microscopic Examination of Stools*

   Since large number of organisms are excreted (10^6 to 10^9) in acute phase of disease and hence they are easily demonstrated microscopically exhibiting darting motility. Gram-stained smears are more useful as morphology can be studied better.

2. *Culture*
   They require 3 to 6 percent oxygen, 10 percent carbon dioxide, hydrogen and nitrogen. They also need different media like blood free charcoal medium with cefatoxin and basal medium of sheep blood, salts. They prefer microbial inhibitors like vancomycin, trimethoprim, cycloheximide and polymycin B incorporated in the media. Sometimes vancomycin may be replaced by rifampin. Incubation is at 37°C (*C. jejuni*) to 42°C. The colonies are non-hemolytic, flat, spreading with irregular edge. They may also be discrete, round and convex (1 to 2 mm). Biochemical tests like hypopiruate hydrolysis, H_2S production, DNA hydrolysis can differentiate isolates within species.
3. *Serotyping with H and O Antigens*
   Here H antigen gives better response.
4. *Indirect Hemagglutination Test*
   Here we use soluble thermostable antigens.
5. *Cell Wall Composition*
6. *Phage Typing*
7. *Bacteriocin Typing*.

TREATMENT

It includes electrolyte and fluid replacement orally or intravenously. Erythromycin is the drug of choice. Endovascular infection with *Campylobacter fetus* requires 4 weeks treatment with injection gentamicin. CNS infection may be treated with chloramphenicol (4 weeks).

CAMPYLOBACTER JEJUNI

MORPHOLOGY

It is Gram-negative rod, comma or "S" shaped or gull wing shaped and motile with single flagellum. It does not form spore.

CULTURE

Incubation of inoculated plates is done at 42° to 43°C. Incubation should be done in an atmosphere with 5 percent oxygen and 10 percent carbon dioxide. The selective media are:

TABLE 33.1: Classification of campylobacter

Name of species	*Reservoirs*	*Human disease*
Campylobacter jejuni	Many animals and birds	Diarrhea
Campylobacter fetus	Cattle and sheep	Septicemia in *debilitated* and immunodeficient patients
Campylobacter coli	Pigs	Diarrhea
Campylobacter lardis	Animals and birds	Diarrhea
Campylobacter cinaedi		Infection in homosexual men
Campylobacter hyointestinalis		
Campylobacter fennelliae		

1. Skirrow's medium with vancomycin, polymyxin-B and trimethoprim.
2. BAP medium with cephalothin.

The colonies are colorless or gray, watery, spreading or round and convex.

BIOCHEMICAL CHARACTERS

It is oxidase negative, catalase positive, does not oxidize or ferment sugar. Other tests like nitrate reduction, hydrogen sulfide, hippurate test can be of much use for the identification of species.

PATHOGENESIS

The infection occurs by ingestion of contaminated food, drink, anal-genital sexual activity and contact. Ingestion of 10^4 organisms or more is necessary for infection. The bacilli multiply in small intestine, invade epithelium and produce inflammation leading to appearance of RBC and WBC in the stools. Sometimes bacteremia occurs with emergence of clinical picture resembling typhoid disease. It appears that local tissue damage with the support of toxic activity results in enteritis.

CLINICAL FEATURES

There is an acute onset of abdominal pain, profuse bloody diarrhea, malaise and fever. Mostly this self-limiting illness lasts for about 5 to 8 days.

Fig. 33.1: Smear from culture of *Campylobacter*

LABORATORY DIAGNOSIS

1. Gram-stained smear of stool shows the typical "gull wing" shaped curved Gram-negative bacilli (Fig. 33.1)
2. Wet preparation or dark field phase contrast microscope shows darting motility.
3. Culture and biochemical reactions confirm the diagnosis of *Campylobacter jejuni.*

Treatment

Erythromycin is usually useful.

34 Helicobacter Pyloridis

Previously known by the name *Campylobacter pylori* but quite recently it has been excluded from campylobacter genus and has been renamed *Helicobacter pyloridis* because of its following characters:

1. It bears multiple unipolar flagella with terminal bulbs.
2. It produces large quantity of urease.
3. It has smooth cell surface.
4. Cell wall fatty acid composition and ribosomal RNA sequence differ from campylobacter.
5. DNA base composition is similar to that of other campylobacter species.

MORPHOLOGY

It is small, non-sporulating, curved, Gram-negative rods. It bears polar flagella and is actively motile with corkscrew motion.

CULTURE

It is microaerophilic (10% carbon dioxide). Optimum temperature is 25°C to 37°C and optimum pH is 6.8. Addition of blood or other animal fluid is a must to obtain better growth.

BLOOD AGAR MEDIUM

Within 36 hours incubation, fine colonies appear attaining maximum size in 2 to 4 days. The colonies are circular, cloudy, glistening with entire edge, slightly bluish gray tinge and having butyrous consistency.

The other media used for the growth of *Helicobacter pyloridis* are:

- Brewer's sodium thioglycolate
- Sirrow medium
- Butzler medium
- Smibert medium.

BIOCHEMICAL REACTIONS

It is characteristically urease positive, alkaline phosphatase positive and catalase positive. Hippurate test is negative.

PATHOGENESIS AND CLINICAL FEATURES

Many studies suggest *Helicobacter pylori* as the causative agent of gastritis. After ingestion this organism seek entry within or beneath gastric mucosa. This offers protection against gastric acidity as Helicobacter is sensitive to pH less than 4. This organism produces large amount of urease which metabolizes urea to ammonia and carbon dioxide which in turn offers buffer action against gastric acidity. Hypochlorhydria is associated with this infection which favors colonization, proliferation and persistence of *Helicobacter pylori*. Microscopic studies of involved gastric mucosa reveal loss of microvilli, depletion of intracellular mucin and disruption of intracellular function. Infected gastric mucosal epithelial cells show extrusions of plasma membrane termed as adherent pedestrals that may initiate bacterial adherence. Association of *Helicobacter pyloridis* with gastric mucosal epithelium suggests either the presence of unique receptors in tissue or that unique mucus is produced. There is also persistence antibody response to this organism.

Following facts indicate the involvement of *Helicobacter pylori* in causing gastritis:

1. Treatment with antimicrobial agents (amoxicillin, furazolidone, metronidazole) and bismuth salts eliminate gastritis and eradicate bacilli from the stomach.
2. Demonstration of *Helicobacter pylori* within phagocytic vacuoles of polymorphonuclear neutrophils.

3. Development of antibody response to this organism in infected person.

TOXINS AND ENZYMES PRODUCTION

Toxins and enzymes produced by *Helicobacter pylori* may account for mucosal injury in the absence of actual bacterial invasion.
These are:

1. Extracellular protease with endopeptidase activity causing degradation of gastric mucin.
2. A heat labile protein—cytotoxin factor causing intracellular vacuolation.
3. Urease produced by this organism responsible for metabolism of urea to ammonia and carbon dioxide causing gastric mucosal damage. This ammonia produced may be (i) directly toxic to epithelial cell, (ii) indirectly cause tissue injury by allowing hydrogen ion back diffusion. Apart from this urease is reported to cause vacuolation of tissue culture cells.

LABORATORY DIAGNOSIS

Histology

This is the most reliable test for detecting *H. pylori.* The specimen required is a biopsy of gastric mucosa. A gelatin capsule is reported to be cheap, rapid and effective. The organism can be visualized by direct microscopy using Gram stain, haematoxylin and eosin stain and Warthin- Starry stain.

Culture

The specimen is inoculated on chocolate agar and campylobacter selective medium and is identified on the basis of morphology, staining and biochemical reactions.

Biopsy Urease Test

The gastric biopsy urease test is the most frequently used test for the diagnosis of *H. pylori* infection in routine gastrointestinal endoscopy. The mucosal biopsy taken from the antrum of the stomach is placed into a medium containing urea and an indicator such as phenol red. The urease produced by *H. pylori* hydrolyzes urea to ammonia, which raises the pH of the medium, and changes the color of the specimen.

Urea Breath Test

Patients swallow urea labeled with an uncommon isotope, either radioactive carbon-14 or non-radioactive carbon-13. The detection of isotope-labeled carbon dioxide in exhaled breath done after 10-30 minutes indicates that the urea was split; this indicates that urease is present in the stomach, and hence the presence of *H. pylori*.

Serology

Antibodies against *H. pylori* can be detected by ELISA in patient's serum.

TREATMENT

It includes administration of followings:

1. Antibiotics (tetracycline or amoxycillin or furazolidone).
2. Metronidazole.
3. Bismuth salts.

NOBEL PRIZE IN MEDICINE (2005)

Australian Bary Marshall and Robin Warren are declared recipient of Nobel Prize in Medicine 2005 for their discovery of *Helicobacter pylori* being responsible for gastritis and peptic ulcer. These conditions can be cured by a short course of antibiotics and some other medicines.

This research discovery has really stimulated the search for microbes as possible cause for other inflammatory conditions like Crohn disease, ulcerative colitis, rheumatoid arthritis and atherosclerosis, etc.

Inflammation in the stomach mucosa is also a risk factor for a special type of lymphatic neoplasm in stomach, MALT lymphoma. Since such lymphomas may regress when *Helicobacter pylori* is eradicated by antibiotics, this organism plays an important role in perpetuating this tumor.

Obviously dysfunction in the recognition of microbial products by the immune system can result in disease development. This discovery of Helicobacter has led to a connection between chronic infection, inflammation and cancer.

35 Brucella

They are small non-motile, Gram-negative coccobacilli. They cause brucellosis, which is zoonosis (transmitted from animal to man). The disease in man (undulent, fever, Malta fever) is characterized by an acute septicemia phase followed by chronic stage that may extend over years together and may involve many tissues.

GRAM-NEGATIVE COCCOBACILLI

These may belong to following genera:
Haemophilus: They are Gram-negative, non-motile coccobacilli which require X and or V factor for their growth. Some *Haemophilus* species are capable of causing severe respiratory tract infection, meningitis, arthritis, subacute endocarditis, etc.
Bordetella: These small coccobacilli may be motile or non-motile and may cause whooping cough. They do not require X and V factor for their growth.
Pasturella: These are Gram-negative ovoid bacilli which ferment sugar without gas production. The original genus of *Pasturella* has been subdivided into *Pasturella, Yersinia*, and *Francisella*.
Brucella: These small Gram-negative coccobacilli are non-motile. Three main species, i.e. *Brucella melitensis* (from goat), *Brucella abortus* (from cattles) and *Brucella suis* (from pigs) affect man. *Brucella abortus* has 9 biotypes while *Brucella melitensis* and *Brucella suis* each have 3 biotypes.

Classification: Three species of *Brucella* have been identified as under:

a. *Brucella melitensis* is pathogen of goat and sheep. First of all it was isolated from spleen of patient of Malta fever.
b. *Brucella abortus* is responsible for abortion in cows and buffaloes. Its infection is very common.
c. *Brucella suis* is natural parasite of pigs. Its infection is very infrequent.

Morphology: It is short rod, 0.5 to 0.7 μ × 0.6 to 1.5 μ in size, coccobacilli arranged singly or in chains. It is non-motile, capsulated (mucoid and smooth variants), non-sporing, non-acid fast and Gram-negative bacilli.

Cultural characters: It is strict aerobes. The optimum temperature is 37°C and pH 6.6 to 7.4. Addition of 10 percent CO_2 improves the growth of *Br. abortus* and *Br. melitensis*. Some strains are cultivated on defined media of 18 amino acids, vitamins, salts and glucose. Growth is slow and scanty:

a. In liquid media (broth) growth is uniform. In old culture there may be powdery deposits.
b. *Nutrient agar:* Colonies are small, moist, translucent and glistening with butyrous consistency.
c. *Liver infusion agar:* After 48 to 72 hours, colonies of above description appear.
d. *MacConkey medium:* After 7 days' incubation 0.1 to 1 mm diameter, convex, amorphous, and yellowish colonies appear.

Biochemical reactions: It is catalase positive, oxidase positive and urease positive. Nitrates are reduced to nitrites. No carbohydrate is fermented. Basic fuchsin and thionine are the dyes which can be used to differentiate various species. *B. abortus* and *B. melitensis* show growth in basic fuchsin 1: 50000 whereas *B. suis* shows growth in the presence of thionine 1: 250000.

Resistance: It is killed by pasteurization of milk. It is killed by 1 percent phenol in 15 minutes. It survives in soil and manure for many weeks. It is sensitive to direct sunlight, acid and buttermilk. It is susceptible to chlorotetracycline, oxytetracycline, chloramphenicol, streptomycin, neomycin, etc.

Antigenic structure: Somatic antigen has two components A and M. *Brucella abortus* contains 20 times as much A as M and *Brucella melitensis* about 20 times M as A. *Brucella suis* has intermediate antigenic pattern. The absorbed monospecific sera is used for identification of particular antigen. Antigenic cross reaction occurs between brucella and *Vibrio cholerae*. In addition, a superficial L antigen has been demonstrated that resembles the Vi antigen of salmonellae.

Phage typing: One of the strain phage is Tb. This (Tb) is specific as it lysis strains having character of *Brucella abortus*. Hence, this phage is of great value in identification and classification of brucella.

Pathogenicity: All the three species are pathogenic for man. *Brucella melitensis* is most pathogenic, *Brucella abortus* is least and *Brucella suis* is intermediate. Brucella can produce following types of infections:

a. *Latent infection:* Infection is serological positive but there is no clinical evidence.
b. *Acute brucellosis:* It is also called undulent fever. Incubation period is 4 to 30 days. It is mostly due to *Brucella melitensis.* There is prolonged bacteremia, irregular fever, muscular and articular pain, asthmatic attacks, nocturnal sweating, nervous irritability and chills.
c. *Subacute brucellosis:* It may follow acute brucellosis. Blood culture is less frequently positive. Skin test is positive.
d. *Chronic brucellosis* is usually non-bacteremic. Blood culture is rarely positive. Skin test and agglutination are strongly positive. There is lassitude sweating and joint pain. Illness lasts for years together.

Laboratory Diagnosis

A. *Hematological investigations:*
   a. Total leukocyte counts may show leukocytosis particularly in early acute phase of disease.
   b. Differential leukocyte count may show lymphocytosis.

B. *Bacteriological investigations:*
   a. *Blood culture:* It is most definite method for diagnosis of brucellosis. About 10 ml patient blood is inoculated into a bottle of trypticase-soy broth, tryptose broth or thionine tryptose agar or liver infusion broth, at 37°C in presence of 5 to 10 percent CO_2. Subinoculations are made on solid media every 3 to 5 days till 4 to 8 weeks. Castenada's method of blood culture (Fig. 35.1) is recommended as it minimizes. The chances of contamination of material and risk of infection to laboratory worker. Here both liquid and solid media are available in the same bottle. For subculture, bottle is tilted so that broth flows over the surface of slant. Bottle is incubated in upright position.

   Colonies appear on slant. It is positive in 30 to 50 percent of cases. Culture may be obtained from CSF, lymph node, bone marrows, urine, abscess, etc.

   b *Serological methods:*
      i. *Agglutination test:* It is positive about a week after onset of infection. It is more reliable means of diagnosis. Titer of 1 : 100 or more in presence of clinical symptoms indicates active infection. Individuals immunized with cholera vaccine may develop agglutinin titers to brucellae. Here equal volumes of serial dilution of patient serum and standardized antigen (killed suspension of standard strain of

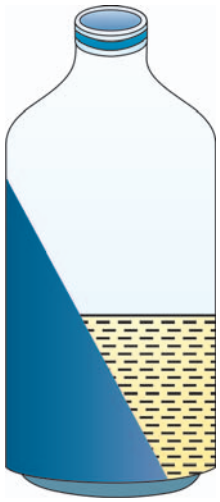


Fig. 35.1: Castenada's blood culture bottle

Brucella abortus) are mixed and incubated at 37°C for 48 hours. Prozone phenomenon is common in brucellosis (1/640 titer). It means agglutination test negative in low serum dilutions although test is positive in higher dilutions. It may be because of presence of blocking antibodies identified as IgA, that interfere with agglutination by IgG and IgM. However, these blocking antibodies appear during subacute stage of infection and tend to persist for many years independently of activity of infection. Blocking antibodies may be detected by the Coombs' antiglobulin method. Radioimmunoassay and ELISA tests can readily distinguish acute from chronic brucellosis and also acute exacerbation of chronic illness. These tests are gradually replacing the standard tube agglutination test. The use of a specific phage is helpful for the identification of *Brucella abortus.*

ii. *2-Mercaptoethanol test:* The addition of 2-mercaptoethanol destroys IgM and leaves IgG for agglutination reaction. The test is not as sensitive as the standard agglutination test but the results correlate better with chronic active disease.

iii. *Complement fixation test:* It is more useful in chronic cases. It detects IgG antibodies.

iv. Indirect immunofluorescence test is specific and sensitive method for detecting antibodies.

v. *Indirect hemagglutination:* It is also very sensitive method.

vi. *Skin test:* It is useful for the diagnosis of chronic cases. It is called brucellin test and also Burn test. A positive reaction with 2 to 6 cm induration means past or present infection. It is non-specific test.

vii. Some rapid methods for the detection of brucellosis are:
   1. Rapid plate agglutination.
   2. Rose Bengal card test.

   Antibodies are detected by:
   1. Milk ring test.
   2. Whey agglutination test.

Treatment: Administration of tetracycline alone or with streptomycin for a period of not less than 3 weeks is quite effective. Response is good in acute cases and not in chronic cases.

A vaccine prepared from *Brucella abortus* strain 19 BA has been employed for human immunization in Russia. However, it has not been used elsewhere.

36 Pasteurella, Yersinia and Francisella

They are Gram-negative, non-motile showing bipolar staining. They are divided into 3 genera on the basis of cultural and biochemical characters, i.e.

a. Yersinia (belongs to Enterobacteriaceae family).
b. Pasteurella.
c. Francisella.

Yersinia contains 3 medically important species: *Yersinia pestis* (plague), *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*.

The genus pasteurella causes hemorrhagic septicemia grouped under common species named *Pasteurella multocida*. The genus francisella consists of single species *Francisella tularensis*.

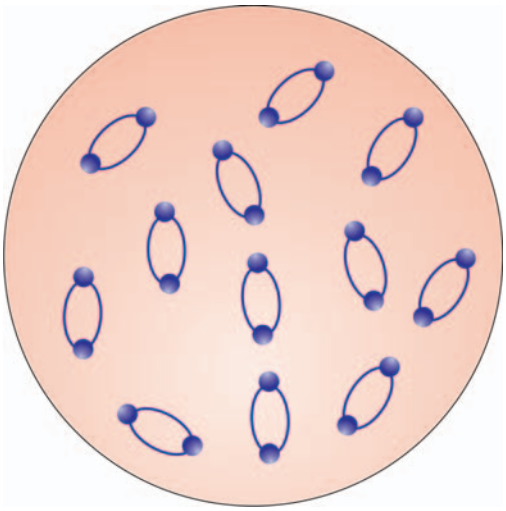


Fig. 36.1: *Yersinia pestis*

Yersinia Pestis (Pasteurella pestis)

It is primarily a parasite of man who is also an accidental host.

Morphology: It is short, plump, Gram-negative, 1.5 µ × 0.5 to 0.7 µ in size with rounded end and convex sides (Fig. 36.1). Pleomorphism is common. It shows bipolar staining (safety pin appearance). It is non-motile (motile at 22°C), non-sporing surrounded by slime layer.

Cultural characters: It is aerobic and facultatively anaerobic. Optimum temperature for growth is 27°C and optimum pH for its growth is 7.2. It can grow in 3 percent sodium chloride.

a. Broth shows flocculant growth occurring at the bottom and along sides of the tube with little or no turbidity.
b. Ghee broth shows growth which hangs down from the surface into the broth resembling stalactites (Fig. 36.2).

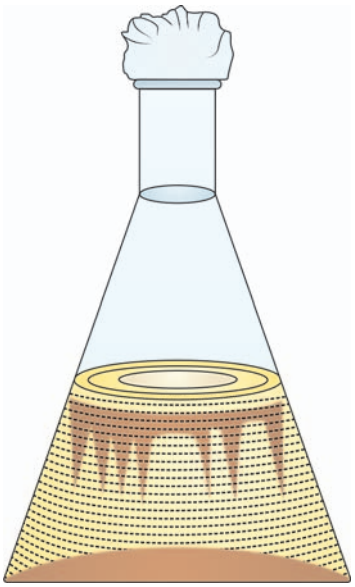


Fig. 36.2: Ghee broth growth

c. *Nutrient agar:* Colonies are small and transparent becoming opaque on continued incubation.
d. *Blood agar:* Colonies are dark brown due to absorption of hemin pigment.
e. *MacConkey medium:* Colonies are colorless.

Biochemical reactions: It is catalase positive, salicin positive and ONPG test positive. It is also coagulase positive.

Resistance: It is destroyed by exposing to heat, sunlight, drying and chemical disinfectants. It is destroyed by 0.5 percent phenol in 15 minutes. It can remain viable in soil or rodent burrows. Many strains are sensitive to penicillin, streptomycin, tetracycline and chloramphenicol.

Antigenic structure: About 18 different antigens are identified. In Surat plague epidemic 1994 new strain belonging to group S is isolated (earlier strains are of group B). Ribotyping and gel analysis of this strain has determined biochemical and genetic make up. There are two types of antigens:

a. Enveloped antigen is heat labile protein. It inhibits phagocytosis and is present in virulent strain. Antibody to this antigen is protective to mice.
b. Somatic antigen.

Toxins: It produces two classes of toxin:

a. Endotoxin which is lipopolysaccharide.
b. Second toxin is protein in nature and possesses properties of both exotoxin and endotoxin.

Virulent strain of plague bacilli produces bacteriocin and it is coagulase positive also showing fibrinolytic activity. Colonies may be pigmented (*hemin media).*

Pathogenesis: Man is infected by blocked flea bites when bacteria are inoculated into the body, or by inhalation of aerosols containing bacilli generated from exhaled breath of pneumonic plague patients. The incubation period is 2 to 6 days for bubonic plague and 1 to 4 days in case of pneumonic plague. *Yersinia pestis* produces disease by virtue of antiphagocytic F1, V, W antigens, exotoxin and endotoxin also add to the pathogenecity. After entering the body, *Y. pestis* multiples in the local lymph nodes (in bubonic plague) and epithelial tissues. Acute inflammatory response is provoked. Characteristic hemorrhagic necrosis occurs initiating bacteremic showers and septicemia. As a result bubonic plague, occurs in about 10 to 20 percent cases. There may be involvement of lungs (pneumonic plague) secondarily. Fibrin thrombi may be extensive in blood vessels of lung, kidney and skin. Infection of serous membranes (pleura, pericardium and meninges) may occur. Endotoxic shock and disseminated intravascular coagulation may also take place.

Plague: Last pandemic started in Hong Kong in 1894 and spread throughout the world. India was worst hit by this pandemic. In sixties, plague reappeared in Vietnam. In man plague occurs in two forms:

a. *Bubonic form:* Incubation period is 2 to 8 days. Lymph nodes draining at the site of entry is infected. It usually enters through flea bite on the legs and inguinal nodes are involved and so the name bubonic (bubon means groin). Lymph nodes become enlarged (1 to 10 cm) with suppuration. Organisms enter blood and can cause septicemia. There may be hemorrhages from skin and mucous membrane.
b. *Pneumonic type:* It spreads through droplet infection. Bacilli spread through lymphatics causing hemorrhagic pneumonia. Cyanosis is prominent.

Chronology of Plague

- 1500-600 BC—recorded in *Bhagvata Purana.*
- 1031-32 AD—Plague reached India from central Asia following invasion of Sultan Mohammad (Arabian chronicles).
- 1325 AD—Plague in Malabar following invasion of Mohammad Tughlaq and again after Timur.
- 1403 AD—Sultan Ahmed's army destroyed by plague epidemic in Malwa.
- 1617 AD—Plague reported during the Mughal emperor Jahangir's reign from Punjab, Ahmedabad, Surat and Deccan and some other parts of India—described by Edward Ferry, ambassador to the Mughal court.

- 1707 AD—Plague in Berhampur.
- 1812-21 AD—Plague hits Kathaiwar, Gujarat and Kutch—supposed to have been imported from Persia.
- 1836 AD—In Mewar and Rajputana—known as Pali plague.
- 1895 AD—In Calcutta—diagnosed bacteriologically on April 17, 1898, by Dr Neild Cook. Imported from Hong Kong.
- 1896 AD—In Bombay, first diagnosed on October 13, 1897. From here plague spread rapidly to most parts of India.
- 1907 AD—Peak year of plague in India with 1,315,892 deaths.
- 1926-27 AD—Severe epidemic in Hyderabad and Deccan.
- 1947-52 AD—A temporary rise in incidence of plague in Calcutta and rise in several old foci in India.
- 1954-58 AD—Plague reappeared in Andhra Pradesh and Mysore and appeared for the first time in Guwahati (Assam).
- 1960-68 AD—Sporadic outbreaks in Mysore, Madras, Himachal Pradesh and Rajasthan.
- Plague cannot be eradicated because of its persistence in wild rodents. Vector flea is xenopsylla.

Laboratory Diagnosis

Hematological investigations: Total leukocyte count may show mild leukocytosis with increase in neutrophils.

Bacteriological Investigations

Specimen: Exudate from involved lymph nodes, sputum, throat swab, CSF, and blood (septicemic cases).

Smear: Giemsa or Gram-staining of smear from sputum or exudate shows Gram-negative coccobacilli with bipolar staining.

Culture: It is done on blood agar plate and then identified by biochemical reactions. On ghee broth we find characteristically stalactite growth.

Animal inoculation: Guinea pig or albino rats are infected subcutaneously with isolated culture of plague bacilli. Animal dies soon. An autopsy shows necrosis, edema with involvement of regional lymph nodes and spleen is enlarged and congested. In septicemia, bacilli may be demonstrated by drawing smear from local lesionized lymph nodes, splenic pulp and heart blood.

- Polymerase chain reaction (rapid and sensitive)
- Bacteriophage lysis.
- Fluorescent antibody binding test.
- Serological methods like IHA using *Yersinia pestis* antigen-F1.

Antigen detection: Using ELISA, F1 glycoprotein antigen may be demonstrated from clinical sample like fluid from bubo or sputum.

Treatment: Tetracycline, doxycycline, chloramphenicol, cotrimoxazole plus gentamycin or kanamycin or streptomycin are effective. Stable, avirulent mutants have been employed for vaccination against plague (e.g. strain EV-76). Heat killed or formalin inactivated suspension of a virulent bacteria and chemical fraction of bacilli may be used as vaccine. A formalin killed vaccine is available. It is mainly indicated for travellers to hyperendemic areas and for persons at special higher risk. Immunity by vaccination remains only for 6 months and requires 7 days for development. The vaccine is given in 2 doses of 0.5 ml subcutaneously at an interval of 7 to 14 days and booster doses every 6 months.

Yersinia pseudotuberculosis: It resembles plague bacilli. It shows poor growth on MacConkey agar and motility at only 22°C. It has 6 serological groups and nine serotypes.

Human infection is very rare. It shows typhoid like illness. Mode of infection in animals is alimentary canal.

Yersinia enterocolitica: It has been isolated from domestic and wild animals. It may cause terminal ileitis, mesenteric adenitis and gastroenteritis. Outbreak of food poisoning is reported in Japan (also see page 265 for more discussion).

Pasteurella multocida *(Pasteurella septica):* It is non-motile, Gram-negative, oxidase positive, and indole positive. It does not grow on MacConkey medium.

In animals like dog, cat, rat, cattle and sheep, organism is carried to upper respiratory tract. Human infection is rare but may follow after trauma or animal bite. It is sensitive to penicillin, tetracycline and streptomycin.

Francisella tularensis: The disease is called tularemia (disease of rabbit). It is transmitted by fleas and ticks. Human infection may occur by direct contact with animals, e.g. bite.

Francisella tularensis is small, capsulated, non-motile and Gram-negative bacilli. It resembles mycoplasma. In infected animal it occurs as intracellular parasite found in liver and spleen.

In man tularemia may present itself as local ulceration with lymph adenitis, glandular enlargement or influenza like respiratory infection. Disease may be water borne. Disease may be diagnosed by culture and inoculation in guinea pig or mice; of organisms and agglutinating antibodies may be demonstrated. It is reported from Japan, Europe and America. A living, avirulent vaccine against tularemia has been used in Russia on a large scale.

37 Hemophilus

They are small (coccobacilli), non-motile, gram-negative bacilli that are parasitic on man or animals. They are characterized by their requirement of one or both of two accessory factors (X and V) present in blood.

Species	Growth-factor X	Growth-factor V	Hemolysis
H. influenzae	+	+	–
H. aegypticus	+	+	–
H. suis	+	+	–
H. hemolyticus	+	+	+
H. ducreyi	+	–	–
H. aphrophilus	+	–	–
H. parainfluenzae	–	+	±
H. vaginalis (*Gardnerella vaginalis*)	±	–	±

Hemophilus Influenzae

Morphology: It is small (1.5 × 0.3 µ), Gram-negative, nonmotile, nonsporing bacillus showing pleomorphism.

Culture characters: They have fastidious growth requirement. X factor present in blood is essential for growth and is heat stable iron prophyrin hematin. X factor is necessary for the synthesis of catalase and other enzymes necessary for aerobic respiration. The other factor is V factor which is a codehydrogenase present in many tissues. It is labile, present in red blood cells and other plant and animal cells. It is synthesized by some fungi and bacteria (*Staphylococcus aureus*). V factor appears to act as hydrogen acceptor in the metabolism of cell.

It is aerobic and grows best at 37°C and pH 7.8.

Blood agar: Growth is scanty as V factor (inside RBC) is not available. So growth is better if source of V factor is provided. *Staphylococcus aureus* is streaked across a blood agar plate on which specimen of *Hemophilus influenzae* has been inoculated. After 37°C overnight incubation we will find colonies of *Hemophilus influenzae* large and well-developed alongside the streak of *Staphylococcus aureus* and smaller colonies farther away. This phenomena is called satellitism (Fig. 37.1).

Other media used are chocolate agar, Leventhal (mixture of blood and nutrient broth), and Fildes agar media (adding peptic digest of blood to nutrient agar).

Biochemical reaction: Fermentation reactions are irregular and nitrates are reduced to nitrites. It is bile soluble. Capsulated strain produces indole. Otherwise biochemical reactions are of least use for identification of organism.

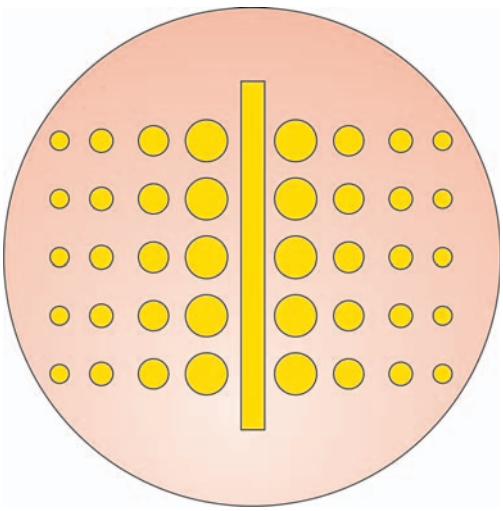


Fig. 37.1: Satellitism

Resistance: They are destroyed by:
1. 55°C for 30 minutes.
2. Refrigeration (0 to 4°C).
3. Drying.
4. Disinfectants.
5. Prolonged incubation due to autolysis.

Preservation is done as under:
a. Chocolate agar slopes.
b. Lyophilization.

Antigenic properties: Capsulated strains possess a polysaccharide antigen. Based on this antigen there are 6 types identified as '*a*' to '*f*'. Type '*b*' strain accounts for most infection.

Pathogenecity: It normally inhabits the nasopharynx and tonsillar region in many normal persons. It may act as:
a. Primary pathogen and may cause meningitis, laryngoepiglottitis, otitis media, pneumonia, arthritis, endocarditis and pericarditis.
b. In second group, the bacillus causes secondary or superadded infection usually respiratory (chronic bronchitis, bronchiectasis, etc.)

Laboratory Diagnosis

a. *Hematological investigations:*
   i. Total leukocyte count shows leukocytosis in pulmonary infection.
   ii. Differential leukocyte count shows increase in neutrophil and in severe infection there may be leukopenia with neutropenia.
b. *Bacteriological investigations:*

Specimen: Sputum, nasopharyngeal swab and cerebrospinal fluid are used for culture.

CSF examination in case of meningitis shows turbidity with increase in cell count 100 to 800 cells/cu mm (polymorphs predominate).

Smear examination: It shows small clumps of fine pleomorphic gram-negative bacilli. Characteristically they do not stain well.

Culture: Material may be inoculated on chocolate agar, blood agar and Levinthal's media. Penicillin may be incorporated in the medium to inhibit the growth of other organisms.

Treatment: *H. influenzae* is susceptible to sulfonamides, chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole and other antibiotics. In resistant cases clarithromycin, amoxicillin-clavulanate and rifampicin (especially carriers) are quite effective. Immunization with capsular polysaccharides is now being considered for mothers who lack antibody. However, available PRP is not adequate vaccine for infants under age 2 years. Hib conjugate vaccine (PRP-T) (Polyribosy iribitol phosphate tetanus) in infants and children is a great success indeed. In addition to eliciting protective antibody, vaccines prevent disease by reducing pharyngeal colonization with Hib. All children should be immunized with Hib vaccine, first dose between 2 and 6 months of age next dose at 12 to 15 months of age.

Hemophilus aegypticus

It is worldwide in distribution and may cause contagious form of conjunctivitis (red eye). It is common in tropics and subtropics.

Hemophilus suis

It is isolated regularly from pigs suffering from influenza. It is not pathogenic for man. Capsulated strain requires X and V factor while non-capsulated strain requires only X factor.

Hemophilus hemolyticus

It is commensal of upper respiratory tract. It requires both X and V factors. It is non-pathogenic.

Hemophilus ducreyi

It is short ovoid bacillus which has tendency to occur in short chains or end to end pairs. It may cause chancroid or soft sore which is venereal disease characterized by tender, non-indurated, irregular ulcer on genitalia.

The culture of *Hemophilus ducreyi* remains the definitive method to diagnose chancroid. Other new techniques are being evaluated, e.g. enzyme immunoassay, dot immunobinding assay, immunofluorescence, DNA probes, etc.

Hemophilus aphrophilus

This requires only X factor. Growth is enhanced by 5 to 10 percent CO_2. It may cause bacterial endocarditis and brain abscesses.

Hemophilus parainfluenzae

It requires only V factor for growth. It is commensal of upper respiratory tract and is reported to cause subacute bacterial endocarditis.

Hemophilus vaginalis (Gardnerella vaginalis)

This is associated with vaginitis and cervicitis (leukorrhea). Since X and V factors are not necessary for growth so it is excluded from genus hemophilus and now named as *Gardnerella vaginalis.* They are small 1 to 3 µ × 0.3 to 0.6 µ in size, Gram-negative, nonsporing, noncapsulated pleomorphic bacilli. They can be cultured on blood agar or chocolate agar media. They may cause non specific vaginitis and cervicitis. Vaginal discharge emits a fishy odor. Gram stain shows clue cells (vaginal epithelial cells covered with many tiny gram negative rods). The fish odor of vaginal discharge is due to presence of amine. Amines get intensified by mixing with a drop of potassium hydroxide and is called amine test. It is treated using metronidazole.

38 Bordetella

They are gram-negative, nonmotile coccobacilli. They do not require X and V factors for growth. The genus *Bordetella* contains three species: *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica.*

BORDETELLA PERTUSSIS

It is found in nasopharynx and respiratory tract mucosa.

Morphology: It is small, ovoid coccobacillus, nonmotile, nonsporing and capsulated. In cultured films the bacilli are arranged in loose clumps with clear space in between (thumb-print appearance).

Culture characters: It is aerobic and facultative anaerobic. It grows best at 35 to 36°C.

Bordet-Gengou glycerine potato blood agar medium is used for its isolation. In this medium, blood is required to neutralize inhibitory agents like toxic acid. Growth is slow and after 48 to 72 hours' incubation on this media refractile, glistening (mercury drop-like appearance), small, smooth, dome-shaped, opaque and grayish white colonies appear. There is hazy area of hemolysis around colonies. Confluent growth gives aluminium paint appearance.

Biochemical reactions: Biochemically, it is almost inactive. It produces oxidase and catalase.

Resistance: It is killed by drying and heating at 55°C for 30 minutes. It remains viable at low temperature (0 to 4°C).

Antigenic structure: Several antigenic factors have been recognized. They are antigenically homogenous and so are agglutinated by common antiserum. Following are antigenic factors:

1. A heat stable lipopolysaccharide endotoxin. Antibodies against it are not protective.
2. A heat labile protein toxin which is dermonecrotic on intracutaneous inoculation in mice.
3. A heat labile agglutinating antigen which is associated with capsule. It has different factor 1 to 14. All strains carry factor 1 and one or more other factor. This is helpful in serotyping.
4. Hemagglutinin occurs in capsular layer and acts at high temperature (42 to 50°C). It is specifically neutralized by the antiserum.
5. Heat labile antigen (histamine sensitizing factor) is present in cell wall.
6. Lymphocytosis prompting factor induces lymphocytosis.

Variation: It undergoes smooth to rough variation. Fresh colonies belong to smooth form (phase I). On subculture, they undergo loss of surface antigen and pass through phases II, III and IV.

Pathogenicity: It is an obligate human parasite. The bacteria liberate toxins and other substances that irritate surface cells, causing coughing and marked lymphocytosis. Before this, organism adheres to and multiplies rapidly, on epithelial surface of trachea and bronchi and interferes with ciliary action. However, blood is not invaded. It causes whooping cough. In man incubation period is 1 to 2 week. It occurs in 3 stages (catarrhal, paroxysmal and convalescent). Each stage lasts for about 2 weeks.

1. *Catarrhal stage:* The onset is insidious. There is low grade fever. It is associated with dry and irritating cough. Diagnosis is difficult at this stage. This is the stage of maximum infectivity.
2. *Paroxysmal stage:* Cough increases in intensity. It comes in bouts. The patient is subjected to violent spasm of continuous cough followed by long in rush of air into empty lungs with characteristic whoop.
3. *Convalescent stage:* Frequency and severity of cough starts decreasing gradually.

Complications

1. Subconjunctival hemorrhage
2. Subcutaneous emphysema
3. Bronchopneumonia
4. Lung collapse
5. Convulsion
6. Coma.

One attack of disease confers immunity for life.

Laboratory Diagnosis

Hematological Investigations

a. Total leukocyte count shows severe degree of leukocytosis in acute stage of disease.
b. Differential leukocyte count shows lymphocytosis in early stage.

Bacteriological Investigations

a. Microscopic examination is done to demonstrate bacilli in respiratory secretion preferable by fluorescent antibody technique.
b. *Culture:*
   i. Cough plate method: Culture plate is held 10 to 15 cm, in front of patient's mouth. Patient is asked to cough in front of plate.
   ii. *Postnasal swab:* Here secretion from postpharyngeal wall is taken with cotton swab or bent wire passed through mouth.
   iii. *Prenasal swab:* Here the swab on flexible nichrome wire is made to pass along the floor of nasal cavity. Material is collected from pharyngeal wall. This method yields highest percentage isolation.

The swabs are plated on glycerine potato blood agar. After 48 to 72 hours' incubation at 35 to 36°C colonies appear. They are confirmed by microscopy and slide agglutination.

Serological Investigations

Rise in titer of antibodies is diagnostic. It is demonstrated by complement fixation test and agglutination.

Treatment: It is generally susceptible to erythromycin, chloramphenicol, tetracycline and ampicillin. Prophylactically, killed vaccine (smooth phase I) of *Bordetella pertussis* detoxified with 0.2 percent merthiolate is recommended. It is quite protective. It is generally combined with diphtheria and tetanus toxoid (triple vaccine) in which *Bordetella pertussis* acts as adjuvant for toxoids producing better results. Three injections are given at the interval of 4 to 6 weeks before the age of 6 months followed by booster dose at the end of one year. Acellular vaccine containing PT, FHA, agglutinogens 1, 2, 3 and *B. pertussis* has been developed. It has fewer complications.

BORDETELLA PARAPERTUSSIS

It is rod-shaped and grows on nutrient agar. Colonies are pigmented. Urease and catalase are positive. It is responsible for 5 percent whooping cough cases.

BORDETELLA BRONCHISEPTICA

This is motile with peritichate flagella. It is antigenically related to *Bordetella pertussis* and *Brucella abortus*. It grows on nutrient agar. Nitrate reduction, urease, oxidase and catalase are positive. It may cause 0.1 percent cases of whooping cough.

39 Spirochaetes

General characters: They are elongated, motile, flexible bacteria which are twisted spirally round the long axis. Characteristically, there are varying number of fine fibrils between cell wall and cytoplasmic membrane of bacterial cell. The spiral shape and serpentine motility of cell depends on the integrity of these filament. Spirochaetes do not possess flagella but are motile. There are three types of motility:

a. Flexion and extension.
b. Corkscrew-like rotatory movement.
c. Translatory.

Spirochaetes belong to the order spirochaetales which is divided into two:

a. Spirochaetaceae (saprophytes).
b. Treponemataceae (human pathogen). It consists of 3 genera (Fig. 39.1):
   i. Borrelia.
   ii. Leptospira.
   iii. Treponema.

BORRELIA

It is large motile, refractive with irregular, wide and open coils. It is 0.3 to 0.7 µ wide and 10 to 30 µ long and is Gram-negative. Borrelia of medical importance are:

1. *Borrelia recurrentis* (relapsing fever).
2. *Borrelia vincentii* (fusospirochaetosis).

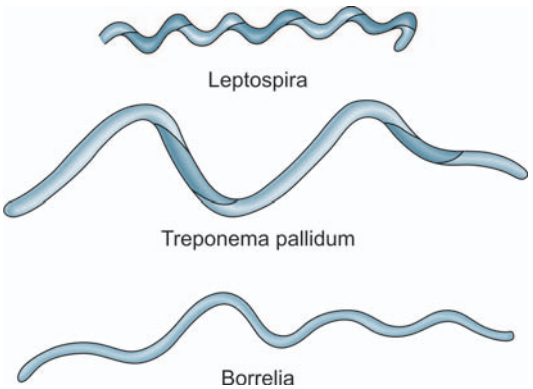


Fig. 39.1: Treponemataceae

Borrelia Recurrentis

Morphology: It is irregular, spiral with one or both ends pointed. It is 8 to 20 µ × 0.2 to 0.4 µ in size. It possesses 5 to 8 loose spiral coils. It is actively motile. It stains best with Giemsa and Leishman's stain.

Cultural characters: Its culture is difficult. Culture is successfully done in ascitic fluid containing rabbit kidney (Noguchi's medium). Growth occurs on chorioallantoic membrane of chick embryo.

Pathogenecity: It causes relapsing fever. Incubation period is 2 to 10 days. It is sudden in onset. Borrelia are abundant in the patient's blood during this period. The fever subsides in 3 to 5 days. After an afebrile period of 9 to 10 days borrelia are not demonstrable in patient blood. The disease subsides after 3 to 10 relapses.

Splenomegaly, jaundice and hemorrhagic lesions in kidney, intestine and meningitis may occur.

Laboratory Diagnosis

Hematological investigations: During fever there is leukocytosis and during afebrile period there is leukopenia. There may be anemia, lymphocytosis and increased serum bilirubin. There may be reduction in platelets.

Bacteriological Investigations

Specimen: Blood is collected during rise of fever.

Smear examination: Thin and thick blood smears are made. They are stained with Leishman's stain or Giemsa stain or diluted carbol fuchsin. Smear is looked for large loosely coiled spirochaetes.

Organisms may be demonstrated by dark field examination of drop of centrifuged blood from buffy coat. However, phase contrast microscopy is considered the method of choice.

Animal inoculation: It is more successful method. About 1 to 2 ml of blood from patient is inoculated into white mice intraperitoneally and within 2 days organisms can be demonstrated from the peripheral blood film of animal. As a routine, smear prepared from blood, collected from tail vein, is examined daily for 2 days.

Serological tests: Cultivation of borrelias and demonstration of antibodies are difficult as well as unreliable to be used for diagnosis. Relapsing fever patient may develop false Wasserman positive reaction.

Treatment: Penicillin, tetracycline and streptomycin are safe and effective as well.

Borrelia Vincentii

It is motile spirochaete about 4 to 20 µ long and 0.2 to 0.6 µ wide having 3 to 8 coils of variable size. It is Gram-negative and is obligatory anaerobic. It is normally a mouth commensal. Under predisposing condition like malnutrition, etc. it may cause gingivostomatitis or oropharyngitis (Vincent angina).

Borrelia vincentii is found generally in symbiotic association with *Fusobacterium fusiform.* This symbiotic infection is called fusospirochaetosis. Fusospirochaetal infection may cause choleraic diarrhea or dysentery but it requires confirmation. Diagnosis may be made by demonstrating spirochaetes and fusiform bacilli in stained smear of exudate from lesion (Fig. 39.2).

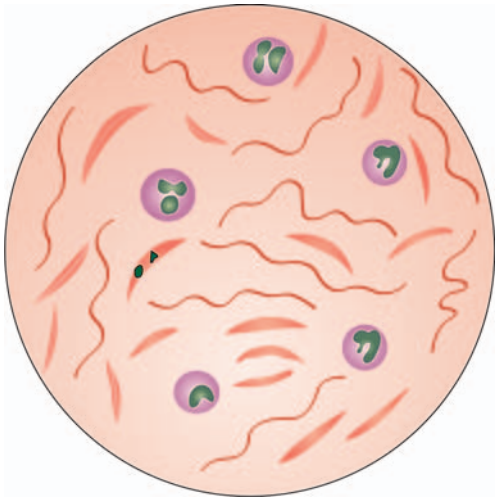


Fig. 39.2: Smear from Vincent's angina

LYME DISEASE

This is named after the town of Lyme, Connecticut. Clinical presentation is annular skin lesion (erythema chronicum migrans). There is associated headache, stiff neck, fever, myalgia, arthralgia or lymphadenopathy. After few weeks to few months, patients may develop neurological symptoms and arthritis which may recur for several years. It is probably the deposition of antigen antibody complexes which is responsible for the recurrent arthritis or neurological manifestations which may occur in few patients. This disease typically occurs in summer. This disease is reported from USA, Europe and Australia.

The disease is transmitted by vector, a small ixodid tick, often *Ixodes dammini,* which carry spirochaetes, i.e. *Borrelia burgdorferi*. The patients develop IgM antibodies to this spirochaete 3 to 6 weeks after the onset of the disease. The serum levels of IgM correlates with disease activity.

Tetracycline, or penicillin if administered initially in the acute stage of disease, prompt recovery occurs with no chance of complication like arthritis, etc.

LEPTOSPIRA

It is actively motile and delicate spirochaete. It possesses number of closely wound spiral and characteristic hooked end. It is very thin and is seen under dark ground illumination. It does not take stain readily.

Classification: Over 100 serotypes and 18 serogroups have been identified which can infect man.

i. *Leptospira icterohemorrhagica* causes Weil's disease (hemorrhagic jaundice). Rats and other rodents are the main reservoirs of infection.
ii. *Leptospira canicola* causes canicola fever in man. In this meningeal symptoms predominate. The reservoirs are dogs, pigs and jackals. Infection in man occurs by invasion through skin abrasion.
iii. *Leptospira hebdomadis* is responsible for seven day fever of east. Organism is natural parasite of field mouse.
iv. *Leptospira autumnatis* causes Akiyami disease in Japan.
v. *Leptospira pyrogen* causes febrile illness among field workers of Indonesia. The reservoirs of infection are certain species of rats.
vi. *Leptospira australis* and *Zanoni* cause cane fever in North Queensland. Certain species of rat are the carrier of organism.
vii. *Leptospira pamona* causes seven day fever among dairy farmers in North Queensland. Pigs act as reservoir.
viii. *Leptospira grippotyphosa* causes swamp fever in agriculturists or workers of Asia, Africa, Israel and USA.
ix. *Lepotospira bataviae* causes leptospirosis in rice field workers of Italy and South East Asia.
x. *Leptospira segroe* has been isolated from human infection in island of Segroe (Denmark). Rodents carry this organism.

Leptospira Icterohemorrhagica

Morphology: It is 7 to 17 µ long and 0.1 µ broad. Coils are many, small and closely set. The ends are characteristically hooked. It is actively motile by rotatory movements. It may be stained with Giemsa's stain or silver impregnation methods of Levaditi and Fontana.

Cultural characteristics: It can be cultured on media enriched with rabbit serum. It is aerobic, microaerophilic with optimum temperature of growth being 25° to 30°C and optimum pH 7.2 to 7.5. It may be grown on chorioallantoic membrane of chick embryo. Culture may be obtained by inoculating the material intraperitoneally in guinea pig and culture the heart blood ten minutes later.

Resistance: It is susceptible to heat (60°C in 10 seconds) and acidic pH (< 6.8). Salt water has deleterious effect. Bile destroys them rapidly. It dies within 3 minutes in water containing 1 ppm chlorine. It is moderately sensitive to penicillin, streptomycin and tetracycline.

Pathogenicity

Weil's disease is very acute epidemic infection characterized by jaundice, hemorrhage from mucous membrane, fever, enlargement of spleen and nephritis. Wild rat is important reservoir. Human infection may result from water or food contamination with leptospirae. Less frequently organism may enter through abrasions in skin. Incubation period is 1 to 8 weeks. It is found in bloodstream and distributed throughout the body. Later they settle in organs like liver and kidney. Central nervous system is frequently involved causing meningitis.

The involvement of kidney in animal is chronic and results in passing large number of organism in urine. Human urine may contain leptospira in 2nd and 3rd week of disease.

Agglutinating, complement fixing and lytic antibodies appear and remain in detectable amount for several years.

Laboratory Diagnosis

Hematological Investigations

i. *TLC* in early cases shows moderate degree of leukocytosis.
ii. *DLC* shows increase in polymorphonuclear cells in early febrile stage.
iii. *ESR* is raised.
iv. *Serum bilirubin* and *blood urea* are raised.

Bacteriological Investigations

Collection of specimen: Blood is collected during first week and urine after second week of illness. Urine specimen should be examined fresh. Serum of patient is examined for

antibodies which are always present from 2nd week onwards.

Examination of blood, urine and CSF: It is done by dark ground microscopy.

Culture: The leptospirae can be cultured in Bijou bottles containing Stuart or Korthof's medium. About 3 to 4 drops of patient's blood are mixed in 3 ml of fluid culture medium. Bottles are incubated at 30°C for two days to two weeks. Samples from culture are examined every third day for presence of leptospira under dark ground illumination.

Animal inoculation: Blood or urine may be inoculated intraperitoneally in about 6 weeks' old guinea pig. After 3 days peritoneal fluid is examined daily for the evidence of leptospira under dark ground illumination and when it is detected cardiac blood is withdrawn for culture purpose.

Serological Test

i. **Microscopic agglutination test:** Dilution of patient serum and culture suspension are incubated at 32°C for 3 hours and then at room temperature for 1 hour before reading the test. Microscopic agglutination test with formalinized antigen is safer and little inferior to live antigen test. A titer of 1/80 is suggestive and 1/100 conclusive of infection.
ii. **Indirect fluorescent antibody test** has been recently used widely.
iii. **Complement fixation test** is neither specific nor sensitive as compared to agglutination test. Complement fixation antibodies disappear soon after recovery and so demonstration of antibody is an indication of recent infection.

Treatment: Penicillin and tetracycline are useful.

TREPONEMA

They are very fine, spiral, slender with pointed or rounded ends. Pathogenic treponemes may cause following diseases in man:

a. *Treponema pallidum* is the causative agent of venereal syphilis.
b. *Treponema pertenue* may cause yaws.
c. *Treponema caratium* may cause pinta.
d. *Treponema vincentii* is found in association with fusiformis in Vincent angina.

Treponema Pallidum

Morphology: It is 6 to 8 µ in length 0.2 µ in diameter with tapering ends. The body is coiled in 8 to 15 regular, rigid, sharp spirals. It is actively motile showing rotation around long axis, backward and forward movements and flexion of whole body. It is feebly refractile and so not stained with ordinary staining techniques. It ordinarily reproduces by transverse fission, and divided organisms may adhere to one another for some time. However, its morphology and motility can be seen under dark ground illumination.

It stains rose red with Giemsa stain. It may be stained with Levaditta, and Fontana method.

Culture: It has not been cultured in artificial media or in tissue culture. Pathogenic strain (Nichol's strain) may be maintained in the testis of rabbit. However, saprophytic strain (Reiter) may be cultured on a defined medium of 11 amino acids, vitamins salts and minerals. Serum albumin also supports its growth.

Resistance: It is inactivated by drying or by heat (41 to 42°C). It is killed in 1 to 3 days at room temperature and in 1 hour at 4°C. It may be stored when frozen at –70°C with 10 percent glycerol or in nitrogen (30°C) for 10 to 15 years.

Antigenic structure: Treponemal infection induces three types of antibodies:

1. The first type of antibody reacts in non-specific serological tests like VDRL, Kahn, Wassermann.
2. It is protein antigen present in *Treponema pallidum* and non-pathogenic strain Reiter treponema. It is group antigen.
3. The third antigen is perhaps polysaccharide in nature and is species specific. It may be demonstrated by *Treponema pallidum* immobilization test.

Pathogenicity

A. *Congenital syphilis:* A pregnant syphilitic woman can transmit *Treponema pallidum* to

the fetus through placenta beginning about the tenth week of gestation. Some of the infected fetuses die and miscarriages result while others are stillborn at term. Some are born live but develop the signs of congenital syphilis in childhood, e.g. interstitial keratitis, Hutchinson's teeth, saddle nose, periostitis and many central nervous system anomalies. However, adequate treatment of mother during pregnancy prevents congenital syphilis.

B. *Acquired syphilis:* Natural infection in man is usually acquired by sexual contact. The organism enters through microabrasions on the skin or mucosa of genitalia. The incubation period is 10 to 90 days. The clinical picture falls into 3 stages: *primary, secondary, tertiary.*

Primary lesion: It is painless, avascular circumscribed, indurated superficially ulcerated lesion. It is called hard chancre. Chancre is covered by thick exudate which is rich in spirochaetes. Regional lymph nodes are swollen, discrete, rubbery and non-tender. Chancre heals up in 10 to 40 days leaving thin scar.

Secondary lesion: It occurs in 2 to 6 months after primary lesion. Then lesion of secondary stage develops with papular skin rashes, mucous patches in oropharynx and condylomata at mucocutaneous junction. Patient is highly infectious during secondary stage. There may be eye and meningeal involvement. Twenty-five percent patients ultimately undergo spontaneous healing in 4 to 5 years' time. Twenty-five percent infection remains latent. In rest 50 percent cases tertiary lesions develop after a latent period of 1 to 33 years.

Tertiary syphilis: It consists of cardiovascular lesions consisting of aneurysm, chronic granulomata and meningovascular manifestations. Only few spirochaetes are present and this stage represents delayed hypersensitivity.

Laboratory Diagnosis

Hematological investigations: There is leukocytosis. In early stage there is increase in polymorphs whereas in chronic cases there is lymphocytosis.

Bacteriological Investigations

Primary stages: The specimen should be collected with care. The lesion is cleaned with a gauze soaked in warm saline. Gentle pressure is applied to the base of lesion and exudate is collected. Wet films are prepared and covered with coverslips. They are examined under dark ground microscope.

Dark ground microscope is very useful. Repeated examination should be done in negative cases. Fluorescent antibody test or smear of exudate gives more positive rate.

Secondary Stage

a. *Dark ground microscopy:* Serous exudate from skin eruption or specimen collected from mucous patches and condylomata are examined as discussed above.
b. *Serological test:*
   1. *Standard Test for Syphilis* (*STS*): Alcoholic extract of ox heart tissue in which added lecithin and cholesterol is used as antigen. The test employed are Wassermann, Kahn, Venereal Diseases Research Laboratory (VDRL).

Wassermann reaction: It is complement fixation test. Patient serum is inactivated at 56°C for ½ hour to destroy complement. It is then incubated with cardiolipin (liquid extract of beef heart) and guinea pig complement. Now sheep erythrocytes and antisheep erythrocyte serum is added to mixture. If hemolysis occurs it means complement was not used up as no antigen antibody reaction occurs. It means test is negative and patient is not suffering from syphilis. If no hemolysis it means antigen antibody reaction occurs and complement is used up. It occurs in positive test when patient is suffering from syphilis.

Kahn's test: Carefully measured 0.15 ml of inactivated serum is taken in 3 tubes containing 0.05, 0.025, 0.0125 ml freshly prepared antigen. They are shaken on Kahn's shaker at 280 oscillation/minute. Normal saline solution is added. Floccules appear in positive test.

VDRL test: It is most widely used as it is simple and rapid test requiring very small quantity of serum. 0.05 ml inactivated serum

is taken in special slide with ring (14 mm diameter). One drop of antigen is added with a syringe delivering 60 drops in 1 ml. Slide is rotated at 120 revolutions per minute for four minutes. It is studied under microscope. Uniformly distributed needles show negative results (non-reactive). Presence of clumps means reactive serum. In reactive serum further dilution is done to obtain reactive titer.

VDRL has one disadvantage as it gives false biological reaction in conditions like leprosy, malaria, relapsing fevers, infectious mononucleosis, hepatitis, tropical eosinophilia, systemic lupus erythematosus, rheumatoid arthritis, bloodless menstruation, vaccination and pregnancy, etc. Positive VDRL tests revert to negative 6 to 24 months after effective treatment of early syphilis.

A positive STS (Wassermann reaction or VDRL) with negative Kahn test is always non-specific.

Rapid plasma reagin test (RPR): In this test finely divided carbon, i.e. charcoal particles and choline may be added to VDRL antigens. There is no need to read the results with the microscope, because results can be seen with naked eyes. The advantages of RPR test are as under:

1. There is no need to heat the test serum.
2. Test may be performed on plastic or paper cards.
3. Blood from patient may be obtained by finger prick only.
4. Test is so easy and simple that it may be used for field study.
5. This test may be performed using either serum or plasma.

Uses of non-treponemal tests: Non-treponemal tests may become positive after pimary lesion. They are positive in over 70 percent primary and around 98 percent in secondary syphilis. Highlights of non-treponemal tests are:

1. A four-fold or more rise in titer occurs during evolution of primary syphilis.
2. A titer of over 32 is observed in secondary syphilis.
3. An increase in reagin titer with time may indicate congenital syphilis.
4. If there is decline in reagin titer after antibiotic treatment of syphilis in early stage indicates effective treatment. Hence, non-treponemal tests may be used to monitor effective antibacterial therapy.

A new serological test: To conduct this test a VDRL antigen may be used to coat the wells of microtiter plate. Antibodies to cardiolipid present in the serum of patient get attached to the antigen in the well. These antibodies may be detected using antihuman IgG labeled with peroxidase enzyme. This test can be sensitive to the extent of over 96 percent in untreated syphilis. This test is based on enzyme immunoassay.

2. *Treponemal test:*
   A. *Reiter's protein complement fixation test* (RPCF): Principle is same as Wassermann reaction but antigen is an extract of *Reiter treponema.* It is least sensitive in early syphilis but is more sensitive in late or latent syphilis. It is more specific than standard tests for syphilis.
   B. Tests using *Treponema pallidum* as antigen:

Tests using Live T. Pallidum

Treponema pallidum immobilization (TPI) in which test serum is incubated with suspension of live treponema and complement. If antibodies are present treponema will be immobilized. Test is positive if more than 50 percent of treponema are immobilized. It is the most specific test available for syphilis.

Test using Killed T. Pallidum

i. *Treponema pallidum agglutination test (TPA):* Nicholas strain inactivated with formaline is used. This test is not specific as false-positive reactions occurs and test is technically difficult.

ii. *Treponema pallidum immune adherence (TPIA):* If a suspension of treponema is mixed with test serum, complement and heparinized whole blood from normal man after incubation treponema will be found to be adhered to erythrocytes in the presence of antibody. In the absence of antibody *Treponema pallidum*

adherence will not occur. It is not widely used.

iii. *Fluorescent treponema antibody (FTA):* This is indirect immunofluorescent test. Nicholas strain of *Treponema pallidum* is smeared on slide (can be stored in deep freeze for months together). Patient's serum is allowed to act on smear. This is treated with antihuman gamma-globulin fluorescence conjugate. Excess of unfixed conjugate is washed off. The slide is examined under ultraviolet microscope. If the test is positive treponema are seen as fluorescent objects.

This test is modified and called FTA-ABS test. Test serum is absorbed with extract of Reiter's treponema which remove group reactive antibody first. This is as specific as TPI test.

Test using T. Pallidum Extract

Treponema pallidum hemagglutination test (TPHA) in which tanned sheep erythrocytes are sensitized with extract of *Treponema pallidum.* This test can be made more sensitive and specific if test serum is absorbed with extract of Reiter's treponema which remove group reactive antibodies. It is reported to be specific like *Treponema pallidum* immobilization test (TPI).

Hemagglutination Treponema test for syphilis (HATTS) and microhemagglutination Treponema pallidum (MHA-TP) are automated version of FTA-ABS test. Main advantages of HATTS and MHA-TP are:

- Simple technique
- Economical
- Have good quality control

However HATTS and MHA-TP lack sensitivity for diagosing primary syphilis.

Tertiary stage: The serological tests are same as in secondary stage.

Treatment: Penicillin, chloramphenicol, erythromycin and tetracyclines are useful.

Diagnosis of congenital syphilis: Prenatal diagnosis can be established by demonstrating maternal antibodies against syphilis organism. Confirmation of diagnosis in infants is made by demonstrating *Treponema pallidum* in skin or CSF by virtue of detection of corresponding antibodies using serological tests like micro-hemagglutination assay for antibodies *Treponema pallidum* (MHA–TP) and FTA–ABS.

Treponema Pertenue

It causes chronic non-venereal disease called Yaws. It is less common in India. The organism is indistinguishable from *Treponema pallidum* morphologically and antigenically. The primary lesion is extragenital papules which form ulcerating granuloma. Like syphilis secondary and tertiary manifestations follow. Destructive lesions of bones are common. Cardiovascular and neurological manifestations are quite rare.

Infection is by direct contact. There appears some cross immunity between syphilis and yaw.

Treponema carateum: It causes *pinta,* a skin disease in which papule appears which does not ulcerate and become lichenoid or psoriform patch. Secondary lesion shows hyperpigmentation and hypopigmentation.

It is not antigenically related to *Treponema pallidum* and hence partial cross immunity between syphilis and pinta exists.

Non-pathogenic treponema: *Treponema microdentum* and *Treponema macrodentum* occur in mouth. The former is shorter with shallow coils and later is large, thicker, regular coil and very actively motile.

Treponema refrigens is found in genital tract of syphilitic and non-syphilitic genital lesion. It has wavy middle and more regular and deeply curved extremities with pointed end.

40 Miscellaneous Bacteria

MYCOPLASMATACEAE

The family Mycoplasmataceae has been divided into 3 genera:

Mycoplasma: This requires sterol for growth.

Acholeplasma: This does not require sterol for growth.

Ureaplasma: This requires sterols for growth, splits urea and is more susceptible to thallium acetate than other mycoplasma.

MYCOPLASMA

It is Gram-negative previously called pleuropneumonia like organism (PPLO), filterables free living pleomorphic organism varying in shape from spherical to branching filaments lacking rigid cell wall. The smallest reproductive units have a size of 125 to 250 nm. It is resistant to penicillin. A characteristic feature of parasitic mycoplasm is the requirement of cholesterol or other sterol as an essential growth factor. They have affinity for cell membrane. A group of parasitic mycoplasma form very tiny colonies on culture and require urea as a growth factor. They are called T strain now called *Ureaplasma urealyticum*. Only few species, e.g. *Mycoplasma pneumoniae* and *Ureaplasma urealyticum* are pathogenic species of mycoplasma encountered in man are shown in Table 40.1.

TABLE 40.1: Mycoplasma species present in human being

Species	*Isolated from*	*Disease in human being*
M. hominis (Type I and II)	Oropharynx and Genital tract	? Pharyngitis ? Urethritis ? Pelvic infection
M. salvarium	Oral cavity	—
M. orale (Type 1, 2, 3)	Oral cavity	—
M. fermentans	Genital tract	—
M. pneumoniae (Eaton's agent)	Respiratory tract	Respiratory infections including primary atypical pneumonia
U. urealyticum (T. strain)	Oropharynx and Genital tract	? Non-gonococcal urethritis ? Sterility

MYCOPLASMA PNEUMONIAE (EATON'S AGENT)

Morphology: It is Gram-negative smallest pleomorphic free living organism which is non-motile. It occurs as granule and filament of various sizes. The granules may be minute elementary bodies 125 to 250 nm in diameter or large from 100 to 1000 nm, balloon, disk, ring or stone shaped. Its method of reproduction is not fully understood but morphologically four types are found:

1. Small coccoid bodies measuring 100-250 nm in diameter. The small bodies are bounded by membrane about 7.5 nm thick. The membrane consists of lipid (phospholipid) with cholesterol interspressed between the phospholipid molecules. It also contains protein mostly on external surface of the membrane.
2. Large cells usually ranging from 500 to 1000 nm which contain a number of particles of subunit size.
3. Very large empty cells about the size of a leukocyte which appear to be degenerative and non-viable.
4. Filamentous forms of various sizes and shapes of 60 to 75 nm in diameter. Filament formation is marked in some species, e.g.

M. mycoides, the cause of contagious bovine pleuropneumonia.

Its method of reproduction is not fully understood but may be:

1. By development within filaments of elementary body and their release.
2. Binary fission.
3. By budding.

It lacks cell wall. It is filtrable. Mycoplasma cell is surrounded by triple layered membrane which is rich in cholesterol and other lipids.

Culture characters: It is facultative anaerobes growing better aerobically. Parasitic strain grows optimally at 35°C to 37°C. Media of its cultivation is heart infusion broth with 2 percent agar (pH 7.8) enriched with 30 percent horse serum and yeast extract. Penicillin and thallium acetate are added to it as selective agents. Colonies appear after 2 to 3 days' incubation and are 10 to 1000 µ in size. The colonies have fried egg appearance, central opaque granular area of growth surrounded by flat translucent peripheral zone (Fig. 40.1).

Colonies cannot be picked up with platinum loop. Subculture is done by cutting out an agar block with colonies and rubbing it on fresh plate. These are mostly hemolytic.

Biochemical reactions: Most species utilize glucose or araginase as main source of energy. Urea is not hydrolyzed except T strain. It is not proteolytic.

Resistance: It is resistant to penicillin, cycloserine and lysozyme that acts on cell wall. It is sensitive to tetracycline. *M. pneumoniae* can grow in presence of 0.002 percent methylene blue in agar while other species are inhibited.

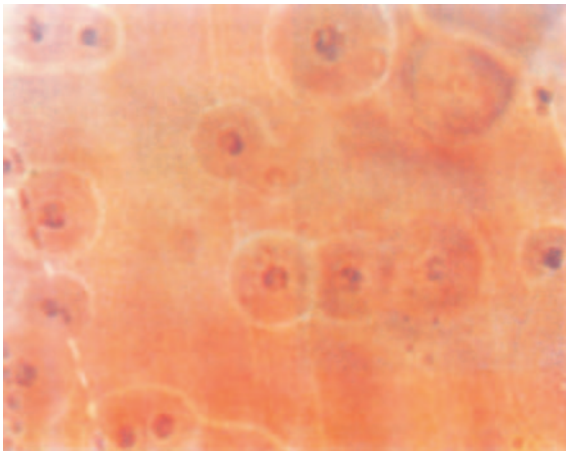


Fig. 40.1: Colonies of *M.hominis*

Antigenic structure: CFT, IHA, IFT are employed for the detection of antibodies in sera for identification by isolates.

Mycoplasma and L forms bacteria: Both lack cell wall but characters of L form bacteria are:

1. L form bacteria resemble parent bacteria biochemically and antigenically.
2. It is not filtrable.
3. Though L form lacks cell walls, still cell wall components may be demonstrated.
4. Sterol is not required for the growth of L form.
5. It is non-pathogenic.
6. It has the same base ratio as parent bacteria.
7. Reversion of L forms to parent bacteria is enhanced by growth in the presence of 15 to 30 percent gelatin or 2.5 percent agar. However, reversion is inhibited by inhibitors of protein synthesis.

Pathogenicity: It may cause primary atypical pneumonia (*M. pneumoniae*). Incubation period is 1 to 3 weeks. Onset is slow with fever, malaise, headache, and sore throat followed by paroxysmal cough with blood tinged sputum and radiological evidence of consolidation lungs (unilateral) involving lower lobe starting at hilum and fanning out to periphery. Rare complications include meningoencephalitis, polyneuritis, monoarticular arthritis, Stevens-Johnson syndrome, pericarditis, myocarditis, hepatitis, diffuse intravascular coagulation, non-cardiogenic pulmonary edema and hemolytic anemia. It is self-limited disease with recovery in 1 to 2 weeks.

The disease is worldwide and transmission is by droplets of nasopharyngeal secretions. It is common in older children and adolescents. The organisms remain for over two months after recovery from diseases.

Laboratory Diagnosis

During acute illness leukocytosis in the range of 10,000 to 15,000 per cu mm occurs in 25 percent cases. Increase in ESR above 40 mm per hour occurs in at least two-third of cases.

Culture: Throat swab or respiratory secretion is inoculated in mycoplasmic medium containing glucose and phenol red. Growth may take 5 to 10 days to appear. It is indicated

by production of acid in the medium with production of beta hemolysis and agglutination of guinea pig erythrocytes. Colonies on agar absorb erythrocycte. Hemadsorption is enzymatic. It is sensitive to erythromycin. It inhibits ciliary motility of hamster trachea organ culture.

Serology: New information about perturbations of host immune response in *M. pneumoniae* infections dictates caution in interpreting serologic data. Due to polyclonal B cell activation "non-specific" antibody rises, coupled with T lymphocyte suppression. This may also explain the appearance of various host tissue antibodies and transient anergy. Since other infectious agents including cytomegalovirus, Epstein-Barr virus and measles produce similar effects, diagnostic confusion may result. *M. pneumoniae* serologic tests never should be included in panels of studies for illness of unknown cause.

Cold agglutinins and agglutinin for MG streptococcus have been detected in atypical pneumonia patients. A titer of 1 : 20 is suggested as positive.

In cold agglutinin test serial dilution of patient serum is mixed with equal volume of 0.2 percent "O" group human erythrocytes. Results are read after leaving the rack containing test tube at 4°C overnight. Titer of 1 : 32 or more is suggestive of this infection. The cold agglutinin reaction occurs with other red cell antigens in infectious mononucleosis, lymphoproliferative diseases and in several respiratory infections, particularly in children younger than 5 years.

Immunofluorescence, hemagglutination inhibition and metabolic growth inhibition are the most sensitive tests. CFT counterimmunoelectrophoresis and IHA tests are less sensitive.

Specific DNA can be detected using hybridization technique and PCR in respiratory secretions.

Treatment: Tetracycline and erythromycin are effective *in vitro* and *in vivo*. Experimental vaccines have been prepared from agar grown *Mycoplasma pneumoniae*.

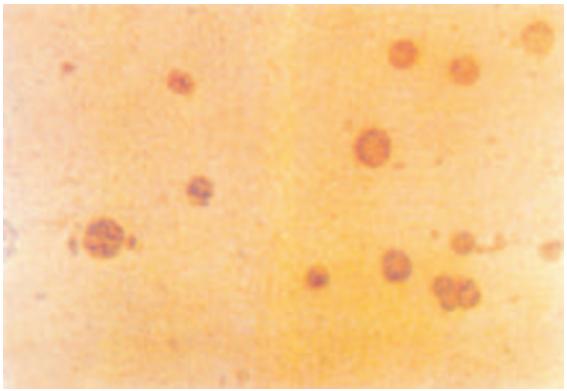


Fig. 40.2: Colonies of *Ureaplasma urealyticum*

***Ureaplasma urealyticum* (T. strain):** It forms colonies and size of colony being 5 to 25 µ (Fig. 40.2). It has been isolated from urogenital tract. It can hydrolyze urea. It also requires cholesterol for growth. It is susceptible to erythromycin and thallium acetate. It may cause non-gonococcal urethritis and Reiter's syndromes.

ACTINOMYCETES

It resembles fungus as it forms mycelial network of branching filaments. It resembles bacteria since it is thin, possesses cell wall containing muramic acid, has prokaryotic nucleus and is susceptible to antibiotics. So they are true bacteria. Actinomycetes are Gram-positive, non-motile, non-sporing, non-capsulated filaments that break up in bacillary and coccoid form. Actinomycetes contain two medically important genera:

a. Actinomyces.
b. Nocardia.

ACTINOMYCES

It causes actinomycosis in man and cattle: *A. bovis* in case of cattle and *A. israelii* and *A. erikonii* in case of human beings. *A. naeslundii* is commensal of mouth but may cause human infection occasionally.

Actinomycosis: It is chronic granulomatous infection. There is indurated swelling of connective tissue with suppuration and discharge of sulfur granules. There are multiple sinuses. In man it is found at following 3 sites:

1. Cervicofacial.
2. Thoracic.
3. Abdominal.

However, actinomyosis may present as mycetoma.

Laboratory Diagnosis

It is made by demonstrating actinomyces in lesion. Specimen collected may be pus or sputum. Sulfur granules may be demonstrated in pus which are white, yellow, varying in size from minute specks to 5 mm. Under microscope, granules seem to be bacterial colonies consisting of Gram-positive filament surrounded by peripheral zone of swollen clubs-shaped structure (sun-ray appearance) (Fig. 40.3). The clubs are Gram-positive and are of host origin.

The granules may be cultured anaerobically on brain heart infusion agar and thioglycollate broth.

NOCARDIA

It is aerobic. All species are Gram-positive. *N. asteroides* and *N. brasiliensis* are acid fast. In sputum it appears as branching filaments which may fragment to form bacillary or coccoid form.

N. asteroides and *N. brasiliensis* may cause pulmonary infection, meningitis and brain abscess. *N. madurae* may cause mycetoma.

Diagnosis: Nocardia may grow on ordinary media forming dry granular, wrinkled colonies which produce pigment (yellow or red).

Table 40.2 shows differentiating features between actinomyces, Nocardia and streptomyces.

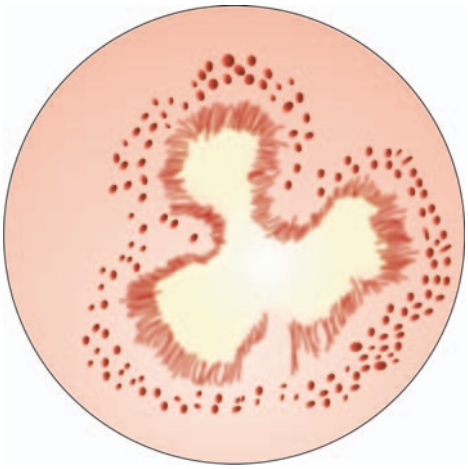


Fig. 40.3: Sun-ray appearance

ACTINOMYCOTIC MYCETOMA

Mycetoma is localized, chronic granulomatous lesion of subcutaneous and deeper tissue involving oftenly foot presenting as tumor with multiple discharging sinus. Mycetoma is usually caused by fungi but may be caused by bacteria like actinomyces, *Nocardia streptomyces*, *Staphylococcus aureus*, etc. In actinomycotic mycetoma color is from white to yellow whereas in fungus mycetoma it is generally black. Isolation of agent in culture establishes the diagnosis.

LISTERIA MONOCYTOGENES

It is small, coccoid, Gram-positive bacillus. It usually occurs in chain. It exhibits slow tumbling motility when grown at 26°C. It is nonmotile at 37°C. It grows on ordinary medium with temperature 4°C to 42°C. Colonies produce beta hemolysis on blood agar (Fig. 40.4). It ferments glucose and salicin with production of acid without gas. Mannitol is not fermented. It is resistant to heat, grows in 0.1 percent potassium tellurite, 13 percent salt at pH 9.6.

It may cause meningitis, granulomatosis infantiseptica. It may cause infertility, abortion and stillbirth. Most human infections are

TABLE 40.2: Distinguishing features of actinomycetaceae

	Actinomyces	*Nocardia*	*Streptomyces*
1. Oxygen requirement	Anaerobes	Aerobes	Aerobes
2. Prevalence	Normal flora of oropharynx	Soil, sputum of healthy man	Soil and water
3. Lesion	Abscess with yellow granules	Subcutaneous and pulmonary	Mycetoma
4. Reproduction	Non-spore former	Non-spore former	Spore former

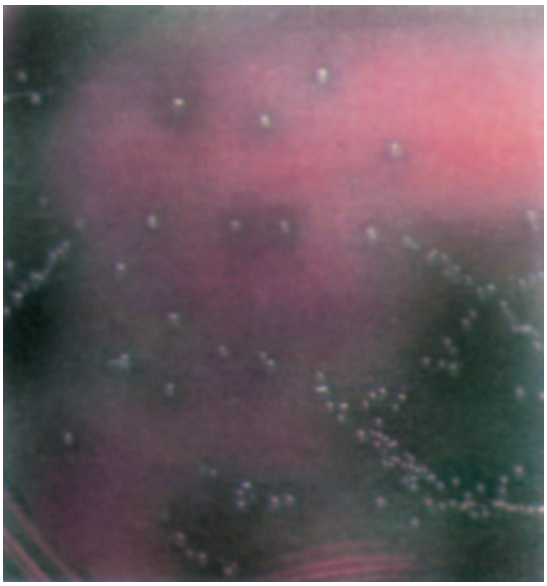


Fig. 40.4: Listeria monocytogenes

caused by serotype 1b and 4b. Monocytosis is a feature of human listerosis. Ampicillin, aminoglycosides or tetracyclines have resulted in clinical cure.

Moraxella Catarrhalis

Moraxella catarrhalis is a Gram-negative, aerobic, oxidase-positive diplococcus which forms a part of normal flora of the upper respiratory tract and genital tract. *M. catarrhalis* was previously placed in a separate genus named *Branhamella* and is now reclassified as *M. catarrhalis.*

It can be grown on ordinary medium as nutrient agar. It is catalase and oxidase positive, does not ferment any carbohydrate but hydrolyses tributyrin, a criteria for identification. Clinically, *M. catarrhalis* results in lower respiratory tract infections, especially in adults with chronic obstructive airway disease. It is also known to cause otitis media, bronchitis, sinusitis, and laryngitis. The majority of this organism produces beta-lactamoses and is resistant to penicillin.

ERYSIPELOTHRIX INSIDIOSA

It resembles listeria in many respects, but is non-motile. In its smooth form it grows as clear minute colonies in which short, non-spore-forming rods are arranged in short rods. However, rough forms have long filament predominantly.

On blood agar only slight hemolysis is produced. Carbohydrates are fermented irregularly and catalase is negative.

Infection in humans follows skin abrasions from contact with fish, shellfish, meat, or poultry. The involved area is painful with edema and purplish erythema with sharp margins that extends peripherally but clears centrally. Sometimes it may cause endocarditis. No permanent immunity is induced following an attack by this organism. The diagnosis rests on isolation of organism from a skin biopsy. The biopsy piece should be incubated in glucose broth for 24 hours and then subcultured on blood agar plates. Penicillin seems to be the antibiotic of choice.

STREPTOBACILLUS MONILIFORMIS

It is microaerophilic, pleomorphic, and Gram-negative bacilli. It grows on artificial medium containing blood and serum, as chains of rods of various length with beaded swellings. It develops into L forms.

It is found in rodent as a natural parasite. In man it causes rat bite fever. Clinically there is fever, polyarthritis and petechial or morbilliform rash.

SPIRILLUM MINUS

It is Gram-negative aerobic bacterium having 2 to 3 spirals per cell. It is motile having amphitrichous flagella. It causes rat bite fever called *SODUKU.* Two to three weeks is the incubation period followed by fever and erythematous rash at the site of bite which becomes chancre-like lesion. There is regional lymphadenitis. Mortality rate is 5 to 10 percent.

Spirillum minus can be cultivated *in vitro.* Diagnosis is made by microscopic examination of exudate from lesion. Exudate, lymph node aspiration and blood may be inoculated in mice.

It is susceptible to penicillin and streptomycin.

DONOVANIA GRANULOMATIS

It is also called *Calymmatobacterium granulomatis.* It is Gram-negative capsulated, short,

plump bacilli with prominent bipolar staining and are responsible for granuloma venereum. It is considered as a sexually transmitted disease which starts as a bubo in the genital region, later spreading to other areas. This organism can be cultivated in the yolk sac of chick embryo and on enriched media like beef heart infusion. It is antigenically closely related to klebsiella species.

LACTOBACILLI

It is Gram-positive rod showing bipolar bodies and barred staining like corynebacteria. Mostly it is non-motile. It produces considerable amount of lactic acid and grows best at pH 5 or less.

It is normally present in mouth. It may produce dental caries. Species like *L. acidophilus* are present in intestine. It may form vitamin B_{12} and vitamin K which may be absorbed by host.

It forms predominant bacterial flora of adult vagina. Lactobacilli of several species are present in vagina called Doderlein bacillus. In prepubertal and post-menopausal vagina, lactobacilli are scanty.

MOBILUNCUS

It is motile, curved, Gram-negative, anaerobic rods isolated from bacterial vaginosis (non-specific vaginitis). However, it is possible that this organism may be a part of normal vaginal anaerobic flora in women.

HELICOBACTER PYLORI

They are motile exhibiting 3 to 5 flagella at one pole, Gram-negative thin spiral rods. Biochemically they are oxidase positive, catalase positive, urease positive, DNAse positive, alkaline phosphatase positive but hippurate negative.

They can grow at temperature 25 to 37°C on Brewer's sodium thioglycollate or Skirrow media or Butzler media or Smibert media. In 2 to 4 days' incubation colonies appear which are circular, domed, cloudy, glistening, entire edge, slightly bluish-gray tinge and butyrous consistency.

Protein profiles of *Campylobacter pylori* strains by polyacrylamide gel electrophoresis differ significantly from the profile of *Campylobacter jejuni.*

They are considered the etiologic agents of peptic ulcer and discussed in details in Chapter 34.

CAT SCRATCH DISEASE

This is benign, self-limiting illness with fever and lymphadenopathy that occurs about 2 weeks after contact with cat. The primary skin lesion appears as a papule or pustule 3 to 10 days after the contact.

The causative organism seems to be a small, pleomorphic, rod-shaped bacilli found mainly in the walls of the capillaries near follicular hyperplasia or within microabscesses. The organism may be demonstrated in tissue with Warthin-Starry silver impregnation stain or by immunofluorescence test using convalescent antisera. Treatment is supportive with little or no affect of antimicrobial drugs. These organisms are Gram-variable and do not grow in culture.

ANAEROBIC GRAM-NEGATIVE BACILLI

Grouped into family Bacteroidaceae and classified as discussed below:

They are thin normal flora of mouth, respiratory tract, intestine and vagina. Genus bacteroides contains the most important anaerobic pathogens as they are frequently isolated from clinical samples and exhibit broad spectrum of resistance to commonly employed antibacterial agents. G + C mol% value of DNA is 26-61.

With bacteroides of great significant is *B. fragilis* which is recovered from most intra-abdominal infections. Enterotoxigenic fragilis (ETBF) has been recently reported to be associated with watery diarrheal disease in young children. Bacteroides contain well-defined species, e.g.

Bacteroides

a. *Bacteroides fragilus:* It is most frequent and non-sporing anaerobe. It may be isolated from blood, pleural and peritoneal fluid, CSF, brain abscess, wound and urogenital infection. It grows most readily on brain-heart infusion agar in anaerobic conditions containing 10 percent CO_2. The capsular polysaccharides of bacteroides appear to

TABLE 40.3: Virulence factors of bacteroides and their role

	Virulence factors	*Role*
1.	Capsular poly-saccharides	Inhibit opsono-phagocytosis, promotes abscess formation and adherence to epithelial cells
2.	Lipopolysaccharide (LPS)	Induces production of specific antibodies and non-specific stimulation of B lymphocytes
3.	Pili and Fimbriae	Promotes adherence to epithelial cells and mucous membrane
4.	Succinic acid	Inhibits phagocytosis and intracellular killing of aerobic bacteria and produce leukotoxic virulence factor.
5.	Enterotoxin	Alter the cytoskeleton and the barrier function of intestinal epithelium
6.	Enzymes like neuraminidase, peroxidase, collagenase, hyluronidase, phospholipase, heparinase, etc.	Contribute to tissue damage and promote spread
7.	Superoxide dismutase	Defends against toxic oxygen radicals and enhances aerotolerance

be important virulence factors (Table 40.3). It lacks the typical endotoxin lipopolysaccharide of Gram-negative organism.

b. *Bacteroides melaninogenicus:* Produces black colonies due to hemin derivative. It is isolated from mixed infection of lung, liver abscess, intestinal lesion, mouth and gums, etc.

Fusobacterium

a. *Fusobacterium fusiformis* is isolated from blood, CSF, bone marrow, abscess, etc.

b. *Fusobacterium girani* is characterized by spinning type of motion. It is isolated from wound infection and peritoneal abscess.

Dialister

Dialister pneumosintes is a minute Gram-negative rod isolated from nasal washing of influenza patients. It has been isolated from brain abscess and meningitis. It is so small that it can pass through Berkefeld V and N filter.

Spherophorous

Spherophorous necrophorous is a very pleomorphic bacillus. On the basis of colony morphology, biochemical reactions and toxin production it is divided into two types. Type A strain is more pathogenic and isolated from man, rabbit and cattle.

Laboratory Diagnosis

1. Conventionally techniques include colony morphology, Gram's staining, pigment production, fluorescence with long wave length ultraviolet light, biochemical tests, etc.
2. Gas liquid chromatography can be of great use to identify various volatile fatty acid profiles and species level diagnosis can accurately be done.
3. Rapid methods are also available some of which are:
   a. Rap 1 DAN A II: It is a 4 hours incubated test system based on the degradation of 18 chromogenic substrate by preformed enzymes. A color chart is provided to aid in interpreting reactions read after the developer is added. A computerized data base is also available.
   b. 4-Methylumbelliferone derivative is a rapid test for presumptive identification of *B. fragilis* group of organisms, using preformed enzyme glucosidase, linked to fluorescent substrate (4-methylumbelliferone). Upon enzymatic hydrolysis of glucoside derivatives, 4–methylumbelliferone produces light blue fluorescence when viewed with wood lamp.
   c. Polymerase chain reaction (PCR) in which PCR amplification of a segment of neuraminidase gene of *B. fragilis* appears to be a sensitive and specific technique for identification of this organism in the clinical specimen.
   d. 165 rDNA PCR restriction fragment length polymorphism (RFLP) is a restriction endonuclease analysis of amplified 165 rDNA. It is considered to be a rapid and reliable method for distinguishing oral isolates of Prevotella nigrescens and intermedia that may be applied confidently to large scale studies.

41 Rickettsiae

They are minute organisms having properties in between bacteria and viruses. The general properties are:

1. It contains both DNA and RNA in a ratio of 1:3.5.
2. Contains muramic acid in the cell wall.
3. Contains enzymes for metabolic functions.
4. Multiplies by binary fission.
5. It is coccobacilli 300 × 600 nm in size visible under light microscope.
6. It is Gram-negative though it stains poorly.
7. Sensitive to many antibiotics.
8. It does not multiply outside the cell. However, it grows readily on yolk of chick sac embryo.
9. It multiplies in cytoplasm of cell except the organisms of spotted fever group which also grow in the nucleus.
10. It produces only endotoxin and no exotoxin.

Rickettsiae family has three genera, i.e. Rickettsia, Orientia and *Ehrlichia*. *Coxiella burnetü* and *Rochalimaea quintana* are ousted from Rickettsiae as former one is not arthropod borne and latter one not being obligatory parasite capable of growing in cell free media. They are maintained in animals and arthropods reservoirs. They are transmitted by arthropod vectors like ticks, fleas, lice, mites, etc. They are obligatory intracellular parasites growing only in the cytoplasm of eukaryotic cell. They require growth factors like acetyl CoA, NAD, ATP. These growth factors of course are provided by host cell.

Morphology: It is pleomorphic cocci bacilli 600 × 300 nm in size, nonmotile, noncapsulated and is Gram-negative.

Culture: It is unable to grow in cell free media except *R. quintana* which grows on blood agar.

It is cultivated in yolk sac of developing chick embryo. Pure preparation of rickettsiae may be obtained by differential centrifugation of yolk sac suspension. It may grow on chorioallantoic membrane. In cell culture, the generation time is 8 to 10 hours at 34°C.

Laboratory animals like mice and guinea pig are helpful for isolation of rickettsiae from patients. It may be propagated in arthropods.

Rickettsial growth is enhanced in the presence of sulfonamides. Obviously these rickettsial diseases are made more severe by sulfonamides.

Resistance: It is destroyed at 56°C and at room temperature when separated from host components. It is susceptible to tetracycline and chloramphenicol.

Antigenic structure: The following types of antigens have been demonstrated:

a. Specific soluble antigen present on the surface of rickettsiae.
b. Species-specific antigen associated with the bodies of rickettsiae. It is strain specific in case of scrub typhus.
c. Alkali-stable polysaccharide may be found in some rickettsiae and some strain of proteus. Weil-Felix test is on the basis of this sharing of antigen. Proteus strains OX_{19} OX_2 and OX_k are used in the test.

Pathology: Rickettsiae multiply in endothelial cells of small blood vessels and produce vasculitis. The endothelial cells become swollen and necrotic. This is followed by thrombosis of the vessel leading to rupture and necrosis of the organs covered by vessel. Vascular

TABLE 41.1: Rickettsiae diseases

Group	*Species*	*Disease*	*Reservoir*	*Arthropod*	*Distribution*
Typhus	*R. prowazekii*	Epidemic typhus	Man	Louse	Worldwide
		Brill-Zinsser disease	Man	—	America Europe Australia
	R. mosseri	Endemic typhus	Rat	Rat flea	Worldwide
Spotted	*Rickettsial*	Rockey mountains Spotted fever	Rabbit, dog, Small rodents	Tick	N. America
	S. sibirica	Siberian tick typhus	Wild animals, cattle, birds	Tick	Erstwhile USSR Mongolia
	R. conorü	South African tick typhus	Dog, rodent	Tick	S. Africa
	R. australis	Queensland tick typhus	Bush rodent	Tick	N. Australia
	N. akari	Rickettsial pox	Mouse	Gamasid mite	USA and USSR
Scrub Typhus	*R. tsutsugamushi*	Scrub typhus	Small rodent and birds	Trabiculid mite	East Asia Australia
Trench Fever	*Rochalimaea quintana*	Trench fever	Man	Louse	Europe
Ehrlichia	*Ehrlichia sennetsu*		Unknown	Unknown	Japan/Malaysia
	Ehrlichia chaffeensis		Amblyomma	Tick bite	USA, Europe Africa
	Ehrlichia phagocyte-philia		Ixodes species	Tick bite	USA, Europe

lesions are common in skin. Vasculitis occurs in many organs and appears to be the basis of homeostatic disturbances. In the brain, aggregation of lymphocytes, polymorphonuclear leukocytes and macrophages are associated with the blood vessels of the gray matter and these are called typhus nodules. The heart shows similar type of lesions of small blood vessels. Other organs may also be involved. Following are rickettsiae diseases (Table 41.1).

TYPHUS FEVER GROUP

a. *Epidemic typhus:* The disease has been reported from all parts of the world. In India it occurs in Kashmir.
The causative agent is *Rickettsiae prowazekii.* Man is the only natural vertebrate host. No extrahuman reservoir has been suggested. Pediculus humanus corporis (human body louse) is the vector. Lice is infected by feeding rickettsiemic patients. Rickettsiae multiply in the gut and appear in feces in 4 to 5 days. Lice die within 4 weeks and remain infective throughout. Lice defecate while feeding and infection is transmitted when contaminated louse feces are rubbed in by scratching through the minute abrasions they cause. However, infection may be transmitted through inhalation of dried louse feces and also through conjunctiva.
About 5 to 21 days is incubation period. It starts with fever and chills followed by rash on 3rd day. The rash is maculopapular, starts on trunk and spreads over limb, sparing face, palm and sole. In 2nd week patients become stuporous and delirious (typhus mean cloudy state of mind). There is 15 to 70 percent fatality.

b. *Brill-Zinsser disease:* It is mild, typhus-like disease in New York among Jewish immigrants from S.E. Europe.

c. *Endemic typhus:* It is milder than epidemic typhus. It is caused by *Rickettsiae moseri.* There is always mild infection of rat transmitted by rat flea *(Xenopsylla cheopis).* Organism multiplies in the gut of flea and is shed in flea feces. Flea remains unaffected but infectious for others throughout.

Man acquires infection through the bite of infected flea or through consumption of food contaminated by rat urine, flea, feces, etc. Man-to-man infection does not occur. Human infection is a dead end. In China and Kashmir, lice is known to transmit murine typhus.

SPOTTED FEVER

Rickettsiae of this group possess common soluble antigen and multiply in nucleus and cytoplasm of host cell. They are all transmitted by tick except *Rickettsiae akari* which is mite borne.

Rickettsiae are transmitted transovarially in ticks. The infection may be transmitted by larval stages or by adult ticks. The ticks are not harmed by rickettsiae. Rickettsiae are shed in feces but transmission to man is by bite.

Rocky Mountain spotted fever is most serious type of spotted fever. It resembles epidemic typhus clinically. Here lesions spread all over the body including palm, sole and buccal mucosa.

Siberian tick typhus is mild rickettsial disease. The disease is transmitted by species of Ixodid tick. Causative agent is *Rickettsiae siberica.* Tick typhus is caused by *Rickettsiae conori* strains. The disease was first observed in India and Megaw (1917) in foothills of Himalayas. The dog tick, *Rhipicephalus sanguineus* is the most important vectors.

Rickettsiae australis is the causative agent of Queensland tick typhus. It is transmitted by Ixodid ticks.

Rickettsial pox is self-limited, not fatal vesicular exanthen. It resembles chickenpox. It is also called vesicular or varicelliform rickettsiosis. It is caused by *Rickettsia akari.* The reservoir of infection is domestic mouse *(Mus musculus)* and vector is mite *(Allodermanyssus sanguineus).* Disease occurs in Russia also.

GENUS ORIENTIA

SCRUB TYPHUS

It is caused by *R. tsutsugamushi* (tsutsuga means dangerous and mushi means insect or mite). It was first observed in Japan. It occurs in area with increased humidity and scrub vegetation. The vector mite is *Trombcula akamushi* in Japan and *T. deliensis* in India.

Man is bitten by mite larvae. The mite feeds on serum of warmblooded animals only once during its cycle and adult mite feeds on plant. Rickettsiae are transmitted transovarially in mites. Rodent acts as reservoir. Incubation period is 1 to 3 weeks. Eschar develops at the site of mite bite in patients. Then there is fever, headache and conjunctival infection. Fatality rate is 0.5 to 60 percent.

EHRLICHIA

They are found in neutrophils, lymphocytes and monocytes. They are subdivided into three groups as under:

1. *Ehrlichia chaffeensis* may cause human monocytic ehrlichiosis. It has affinity for monocytes present in tissues and peripheral blood film. It is transmitted by lone star tick. Patient may develop leukopenia and thrombocytopenia. Rash develop in 20 percent of patients. Masses of ehrlichiae may be seen within cytoplasm.
2. *Ehrlichia phagocytophilia* may cause granulocytic ehrlichiosis. It is not common. Its manifestations to some extent resemble with that of human monocytic ehrlichiosis.
3. *Ehrlichia sennetsu* is the causative agent of sennetsu fever. It resembles glandular fever. Sennetsu in Japanese means mononuclear fever.

GENUS COXIELLA

Q FEVER

Etiology of the disease was not known hence called Query or Q. fever. *Rickettsiae burnetii* or *Coxiella burnetii* is the causative agent. It has been widely distributed and reported from all countries. It is zoonosis. Reservoir is bandicoot and transmitted by Ixodid tick. Transovarial infections have been transmitted in ticks. Ticks transmit disease to cattles, sheep and poultry. The rickettsia are shed in the millions of infected cattle. They are abundant in product of conception of infected animals.

Human infection is acquired by inhaling the dust contaminated by rickettsia, derived from animals and their products. Infection may occur by ingestion of infected milk. It appears that ticks do not play any role in transmitting the infection to man.

Disease is characterized by interstitial pneumonia. No rash occurs in disease. It involves almost all organs frequently, liver (hepatitis) and heart (subacute bacterial endocarditis). Spontaneous recovery occurs. Man-to-man transmission is very rare.

Organism is pleomorphic 0.2 to 0.4 µ × 0.4 to 1 µ in size or as spheres 0.3 µ to 0.4 µ. In flash method of pasteurization, the organisms are killed. It is filtrable, inactivated at 60°C or 1 percent phenol in 1 hour. It grows well in yolk sac of chick embryo, and in various types of cell culture. It also shows phase variation. Fresh isolates are in phase I. They become phase II in repeated passage in yolk sac. Reversion of phase I may occur after passaging in guinea pigs. Phase II cells are autoagglutinable and readily phagocytosed. Phase I is more immunogenic. However, phase II gives better results in CFT in human and animal sera. Q fever sera does not react with rickettsial antigen or with proteus.

TRENCH FEVER

It is exclusively a human disease. No animal reservoir is known. It is transmitted by body louse. Feces of lice become infectious in 5 to 10 days after infectious meals. Lice is unharmed but remain infective throughout.

The causative agent is *Rochalimeae quintana* (Quintana from five day fever). *Rochalimeae quintana* has these characteristics:

a. It occurs extracellularly in arthropod host and is demonstrated in gut lumen of lice.
b. It grows poorly on yolk sac of embryo.
c. It is unable to cause infections in common laboratory animals except monkeys.
d. It is capable of growing on blood agar, etc.
e. Convalescent sera does not react with rickettsial protein antigen.

The disease leads to chronic rickettsiemia.

Laboratory Diagnosis of Rickettsial Diseases

Hematological Investigations

TLC: It shows leukopenia. The count may range from 3000 to 7000 cells/cu mm.

DLC: There is usually normal percentage of cells but sometimes we find neutropenia and lymphocytosis.

Bacteriological Investigations

All strains of rickettsiae can be grown in yolk sac of chick embryo. Tissue culture of mouse sarcomas, rat fibroblast can also support growth. Whole blood or emulsified blood clot is used as inoculum. Usually organisms are isolated from the patient's blood soon after onset of the disease.

Animal inoculation: The most satisfactory method for primary isolation is the inoculation of adult male guinea pig or mice. Blood clot grounded in skimmed milk, etc. inoculated intraperitoneally. In Q fever besides blood and sputum, urine may yield causative organism.

The animals are observed for 3 to 4 weeks and then temperature recorded daily. Their response varies to rickettsial diseases:

a. In Rocky Mountain spotted fever, guinea pigs develop fever, scrotal necrosis and may die.
b. With *Rickettsiae morseri, Rickettsiae conori, Rickettsiae akari* they develop fever and tunica reaction.
c. *Rickettsiae prowazekii* and *Coxiella burnetii* produce fever without any testicular inflammation.

Mice is better than guinea pig in *Rickettsiae tsutsugamushi.* Mice becomes ill and develop ascitis.

Rochialimaea quintana cannot grow in guinea pig and mice. It can be isolated by letting healthy lice feed on patient. The rickettsia may be demonstrated from the gut of such lice. This method is called xenodiagnosis. It may be isolated from patient's blood on blood agar or liquid media containing fetal calf serum. In Rocky Mountain spotted fever skin biopsies taken from the patient between fourth and eighth days of illness reveal rickettsiae by immunofluorescence stain.

Serology

a. *Weil-Felix reaction:* It is an agglutination test in which sera is tested for agglutinins to O antigen of certain nonmotile proteus strains OX_{19}, OX_2 and OX_k. OX_{19}, OX_2 are strains of *Proteus vulgaris* and OX_k of *Proteus mirabilis.* The test is done usually as tube agglutination test although slide agglutination have also been employed.
Sera from endemic typhus agglutinate OX_{19} and OX_2. In tick borne spotted fever OX_{19} and OX_2 are agglutinated. OX_k aggluti-

nation are found in scrub typhus. Test is negative in rickettsial pox, trench fever and Q fever.

Disease	OX_{19}	OX_2	OX_k
Epidemic typhus	+++	±	—
Brill-Zinsser		NEGATIVE	
Endemic typhus	+++	±	—
Tick borne spotted fever	++	++	—
Rickettsiae pox	—	—	—
Scrub typhus	—	—	+++
Trench fever	—	—	—
Q fever	—	—	—

False-positive reaction may occur in urinary or other infections by proteus. The test may be negative in 50 percent scrub typhus.

Procedure: Weil-Felix test is performed by mixing equal volume of serially two-fold diluted patient serum and proteus suspension (OX_{19}, OX_2 and OX_k). Tubes are incubated at 56°C for 6 hours and overnight at 37°C and results are recorded in the morning.

b. *CFT:* Complement fixation test is carried out using soluble antigen obtained from yolk sac. Group specific soluble antigen or type specific washed rickettsia antigen may be used. In Q fever it is important to use phase II antigen for CF test. A satisfactory CF antigen for trench fever has been prepared from *Rickettsiae quintana* cultured on blood agar.

Other serological tests: Agglutination of rickettsial suspension, indirect hemagglutination, toxin neutralization, micro-immunofluorescence, microagglutination and radioisotope precipitation are other useful serological tests.

A skin test for demonstration of delayed hypersensitivity to *Coxiella burnetii* is developed. A positive skin test is considered to be a contraindication for Q fever vaccination.

Treatment

Tetracycline and chloramphenicol are effective, provided treatment started earlier. The antibiotics do not free the body of rickettsiae but they do suppress their growth. However, recovery depends in part upon the immune status of the patient.

Active immunization may be carried out as under:

1. Using formalinized antigens prepared from yolk sac of infected chick embryos or from cell culture. Such vaccines are prepared for; *R. prowazekii* (epidemic typhus), *R. rickettsiae* (Rocky Mountain spotted fever), and *C. burnetii* (Q. fever).
2. A live vaccine (Strain E) for epidemic typhus is effective. It is used experimentally but produces self-limited disease.

BARTONELLA BACILLIFORMIS

It has been classified under family Bartonellaceae in the order Rickettsiales. This is pleomorphic, Gram-negative, motile, rod-shaped organism causing:

a. Fatal febrile anemia called *Oroya fever.*
b. Benign, nodular skin lesion called *Verruga peruana.*

It is seen inside reticuloendothelial cells of infected persons. It can be cultured on semisolid agar with rabbit or human blood. Inoculation of culture in monkey results to Verruga peruana and not Oroya fever. Disease occurs in mountainous areas of American Andes in tropical Peru, Colombia and Ecuador.

42 Chlamydiae

The name chlamydia is derived from the characteristic appearance of inclusion bodies produced by these agents which are seen enclosing the nuclei of infected cells as cloak or mantle (chlamys means mantle). Chlamydiae have following characteristics:

1. It is filtrable and heat labile.
2. It does not grow in cell free media.
3. It possesses ribosomes.
4. It has a variety of metabolically active enzymes, e.g. it can liberate CO_2 from glucose. Some can synthesize folates.
5. It has cell wall like bacteria and rickettsiae.
6. It does not have eclipse phase following cellular infection.
7. It multiplies by binary fission.
8. It has both RNA and DNA.
9. Susceptible to antibacterial agents like ethanol, ether, low concentration of formalin.

Chlamydiae are classified into following groups (Table 42.1):

Group A:

1. It causes:
   a. Lymphogranuloma venereum.
   b. Trachoma.
   c. Inclusion conjunctivitis.
2. It forms compact inclusion with glycogen matrix.
3. Susceptible to sulfonamides and D-cycloserine.

Group B:

1. It causes:
   a. Psittacosis.
   b. Ornithosis.
2. It produces diffuse inclusion without glycogen matrix.
3. It is resistant to sulfonamides and D-cycloserine.

TABLE 42.1: Difference between Group A (*Chlamydia trachomatis*) and Group B (*Chlamydia psittaci*)

Group A	*Group B*
1. Inclusions	
i. Morphology compact and rigid	Vacuoles diffused throughout cytoplasm
ii. Contain glycogen	No glycogen
2. Sensitive to sulfonamides	Not sensitive
3. G + C content of DNA (average) is 45 moles percent	41 moles percent
4. Predominant host is man	Birds and animals
5. Main syndromes include infections of the eye and genital tract, mainly localized	Mainly generalized infections involving lungs, joints, CNS, gut and placenta

Morphology: It exists in 2 forms:

Elementary bodies: They are spherical 200 to 300 nm diameter and extracellular infectious particles.

Reticulate bodies: On entry into cell it enlarges to form reticulate body 200 to 1000 nm in size. This is reproductive form and divides by binary fission to produce large number of elementary bodies (Fig. 42.1).

Differences between Elementary Body and Reticulate Body

Property	*Elementary body*	*Reticulate body*
1. Morphology	Small dense	Large and homogenous
2. RNA:DNA	Centered	3:1
3. Sonication	+	-
4. Resistance to trypsinization	Resistant	Sensitive
5. Infectivity	+	-
6. Toxicity	+	-
7. Location	Extracellular	Intracellular

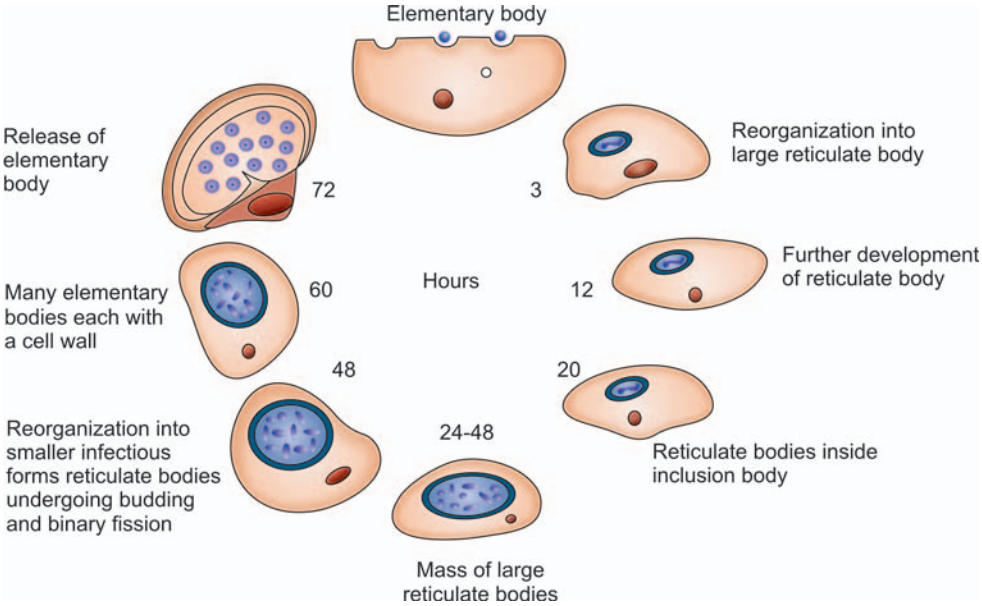


Fig. 42.1: Processing of reticulate bodies

Chlamydiae are Gram-negative. They lack some important mechanisms for the production of metabolic energy. This defect restricts them to an intracellular existence, where host cell furnishes energy-rich intermediates. Its cell wall resembles Gram-negative cell wall. However, its cell wall has high lipid contents, peptidoglycon and perhaps contains muramic acid. The inclusion bodies are basophilic. The inclusion bodies of Group A chlamydia can be stained with Lugol's iodine because of glycogen matrix they possess.

Resistance: It is heat labile (inactivated at 50°C within few minutes). It is susceptible to ethanol, ether, low concentration of phenol and formalin. It can be maintained at 4°C for several days. However, it is preserved at –70°C or lyophilized.

Antigenic structure: It possesses 2 types of antigen:

a. Group antigen is common to all members of chlamydiae. It is polysaccharides resistant to heat, nuclease and proteinase. It is inactivated by periodate and lecithinase. It may be removed by treatment with deoxycholate.
b. Specific antigens are detected by gel diffusion or by immunofluorescence. Specific antigens remain attached with cell wall even after treatment by antitoxin. Toxin neutralization is used for intraspecies typing.

LYMPHOGRANULOMA VENEREUM

It is venereal disease characterized by suppurative inguinal adenitis. The primary lesion is a small papulovesicular lesion appearing on external genitalia. Incubation period is 3 days to 5 weeks. Sometimes lesion may be extragenital. The secondary stage develops 2 weeks later resulting from lymphatic spread to draining lymph nodes.

In male inguinal lymph nodes and in females intrapelvic and pararectal lymph nodes are involved more frequently. Sometimes metastatic complications develop with the involvement of joints, eyes and meninges.

Tertiary stage is chronic and last for years representing scarring and lymphatic block.

Laboratory Diagnosis

a. *Hematological investigations:* It is not informative.
b. *Bacteriological investigations:*
   1. *Microscopic examination:* Smear of material aspirated from inguinal lymph node (bubo) may show elementary bodies.

2. *Animal inoculation:* Material with streptomycin may be injected intracerebrally into mice. It may be inoculated into the yolk sac of developing eggs.
3. *Serological test:* It may be done using group specific complement fixation antigen. False-positive results are common. Type specific antigen may be used but is too complicated for routine use.

Skin test: It is also called Frei's test. Antigen is prepared from infected yolk sac purified by fractional centrifugation and inactivated by phenol or heat. 0.1 ml of this antigen is inoculated intradermally on the forearm. Uninfected yolk sac is inoculated on other arm as a control. A positive reaction is indicated by a nodule appearing at the test site, reaching maximum in 4 to 5 days measuring 7 mm in diameter. Test remains positive for years. This is a group specific antigen and hence false-positive reactions are common. A type specific antigen is there but cannot be used.

TRACHOMA AND INCLUSION CONJUNCTIVITIS

Chlamydiae causing these diseases are essentially similar in nature hence are collectively called TRIC agents.

Trachoma: It is chronic keratoconjunctivitis characterized by follicular hypertrophy, papillary hyperplasia, pannus formation and cicatrization.

Infection is transmitted from eye-to-eye by finger, fomites, flies, dust, etc. The onset is insidious. TRIC agents cannot be demonstrated in early stages as a routine. However, infectivity is maximum in its early stages. Stage IV is non-infectious.

Laboratory Diagnosis

Microscopic examination: In conjunctival scrapings, we can demonstrate characteristic inclusion bodies called *Halberstaedter-Prowazek* or HP bodies (Fig. 42.2) by Giemsa, Castaneda or Machiavello staining. These may be stained with iodine solution because of the glycogen mass.

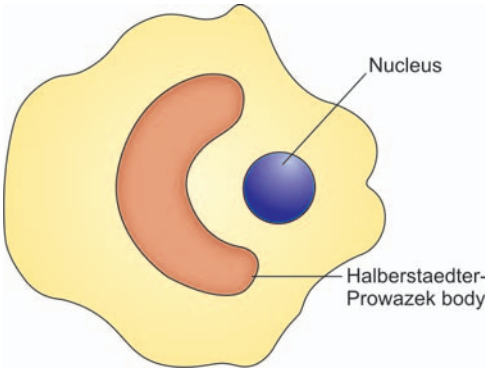


Fig. 42.2: Conjunctival epithelial cell containing reniform body surrounding nucleus

However, fluorescent antibody method is more sensitive.

Culture: It may be grown in yolk sac of 6 to 8-day-old eggs. Material should be treated with streptomycin or polymyxin B before inoculation.

Irradiated McCoy cell culture is more useful for the isolation of chlamydiae.

Serology: CFT may be demonstrated in serum. It is not very useful. Type specific antibodies may be demonstrated by microimmunofluorescence.

Treatment: Sulfonamide and tetracyclines.

Inclusion Conjunctivitis

It is found in genital tract of man. In males it is found in epithelium of urethra causing non-gonococcal or non-specific urethritis. In females it multiplies in cervical epithelium and infection is asymptomatic.

A severe type of conjunctivitis occurs in newborn and adults after infection of eye. It is acute in onset, present for longer time, causes conjunctivitis, produces destructive lesion of cornea and is resistant to treatment.

In newborn, conjunctivitis develops 5 to 9 days after birth. Infecting agents enter the eye during passage through birth canal. It manifests as an acute purulent conjunctivitis affecting usually the lower lid.

Site of infection	*Lesions*	*Chlamydia (Serovars)*
Eye	• Trachoma	Chlamydia trachomatus (CT) A, B, Ba, C
	• Inclusion conjunctivitis	CT (D to K)
	• Ophthalmia neonatorum	CT (D to K)
Genital tract	• Urethritis	
Male	• Epididymitis	CT (D to K)
	• Proctitis	—
Females	• Urethritis	
	• Cervicitis	
	• Proctitis	CT (D to K)
	• Salpangitis	
	• Perihepatitis	
	• Infertility	
	• Abortion	
	• Stillbirth	*Chlamydia psittaci*
	• Periappendicitis	
Male and female	Lymphogranuloma venerium	CT (L1 to L3)
Respiratory Tract	• Infant pneumonia	CT (D to K)
	• Pharyngitis	*Chlamydia pneumonia*
	• Psittacosis	*Chlamydia psittaci* (avian strain)
	• Pneumonia	*Chlamydia psittaci* (oavine strain)

In adult disease is called swimming pool conjunctivitis. Infection is through sexual contacts but spread also can be from eye-to-eye. There is follicular conjunctivitis with sub-epithelial corneal infiltration which resolves spontaneously.

Using specific antisera TRIC agents are classified into 15 types. A, B, Ba, C, D, E, F, G, H, I, K, L_1, L_2 and L_3. Ba and C are associated with trachoma spread being from eye-to-eye whereas, D, E, F, G, H, I, are of genital transmission particularly H and I.

Laboratory Diagnosis

Microscopic examination: Conjunctival scraping after Giemsa's stain will show typical inclusions of epithelial cells.

Culture: It may be cultured on yolk sac of chick embryo.

Chlamydiae pneumoniae: Previously it was known by the name TWAR strain. It has only one serotype and is exclusively human pathogen. There is absolutely no animal host. It is responsible for causing acute respiratory disease in man.

PSITTACOSIS

It is a disease of birds (parrots, pigeons, etc). Man catches infection by inhaling infected bird feces. Infection may spread by sputum of patient. The incubation period is 7 to 10 days.

Manifestation of disease are malaise, fever, severe headache, anorexia and sore throat. Infection spreads from respiratory tract to blood and skin during 2nd week of disease. The lungs show patchy inflammation with sharply demarcated areas of consolidation. Infiltrative cells are predominantly mononuclear. Liver, spleen, heart and kidney may become enlarged and congested. It may lead to meningoencephalitis, endocarditis, pericarditis and arthritis.

Laboratory Diagnosis

Isolation of agent: It may be isolated from sputum, blood and lung tissue. This specimen is treated with streptomycin and is inoculated

into mice intranasally, intraperitoneally, intracerebrally and into the yolk sac 6 to 8-day-old eggs. Inclusion bodies which develop into the tissue of these animals can be demonstrated in smear prepared from peritoneal exudate, lungs, spleen and brain, etc. and also from yolk sac of infected eggs.

Serological Diagnosis

CFT antibodies are demonstrated in paired sera. Antigen used is group specific and may have false-positive results.

A type specific antigen may be used but difficult to use as a routine because of difficult technology.

43 Newer Bacteria

LEGIONELLA PNEUMOPHILA

Legio means army, ella is small, pneumo means lung and philia is love. It was described for the first time in July 1976. Other outbreaks have been diagnosed retrospectively as far as 1965 or earlier. However, sporadic symptomatic or asymptomatic infections occurred all over the world as long ago as 1947. The organism involves multiple system like lungs, kidney, alimentary tract and central nervous system. Outbreak of the disease is in summer and is related to soil or construction sites. Middle aged or elderly male patients are susceptible to this infection. Person-to- person transmission does not take place. It may occur as nosocomial infection. It may involve patients with diseases like malignancy, diabetes mellitus, alcoholism, cardiovascular diseases and cigarette smoking, etc.

The organism is prokaryotic, Gram-negative, aerobic, non-acid fast coccobacilli belonging to new family, i.e. Legionellaeceae. It is pleomorphic rod measuring 2.3 µ × 0.3 to 0.9 µ with blunt or pointed ends (Fig. 43.1). It does not take usual laboratory stain. Paraffin embedded infected lung tissue may be stained with Gumnez stain and Dieterle's silver impregnation stain. Motility by single polar flagella may be responsible for rapid spread of infection in the lung.

It may be cultured on Mueller Hinton chocolate agar medium, Freeley-Gorman agar or charcoal yeast extract agar enriched with ferric-phosphate and L-cysteine hydrochloride. Incubation temperature is 35°C with 2 to 5 percent carbon dioxide. However, pH should be adjusted to the range of 6.85 to 6.95. The organism grows slowly forming tiny cream

Fig. 43.1: Gram stain of laboratory cultured *Legionella*

colored round or flat with entire edges colonies producing brown soluble pigment. Under microscope colonies give cut glass appearance. The growth is evident on charcoal yeast extract agar (Fig. 43.2) in 2 to 3 days' time which is most suitable medium. It may be mentioned here that organism may also be isolated by intraperitoneal inoculation of guinea pigs and by inoculation of the yolk sacs of embryonated hen's eggs.

It is catalase positive, oxidase positive, hydrolyzes hippurate, produces beta lactamase plus gelatinase and emits yellow fluorescence under long wave ultraviolet light. Gas liquid chromatographic study reveals this bacteria to be rich in branched chain fatty acids. It has ten known serotypes and number of isolates, i.e. serotype I (Knoxville-I), serotype II (Togus I), serotype III (Bloomingtone 2), and serotype IV (Los Angeles-I). Recently serotype 5, 6, 7, 8, 9 and 10 have been added. It produces proteases, phosphatase, lipase, DNAse and RNase plus hemolysin and cytolysin.

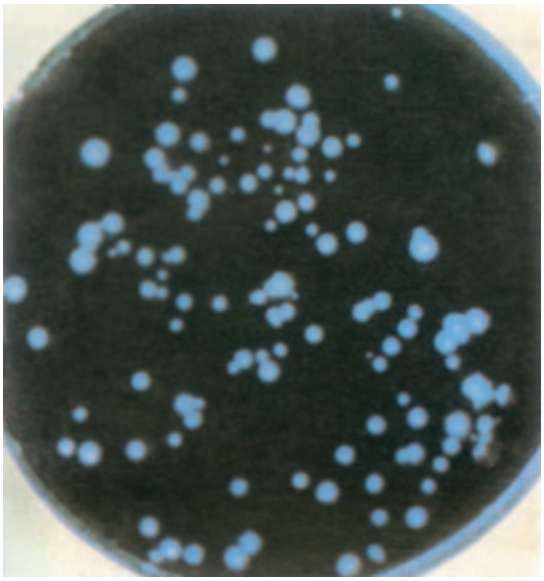


Fig. 43.2: *Legionella* on BCYE (buffered charcoal yeast extract) agar at 35°C

The pathologic changes are mostly confined to lungs. It may cause extensive lysis of inflammatory cells resulting in marked amount of nuclear debris and coagulative necrosis of lung parenchyma. The alveolar septa becomes edematous and are infiltrated with inflammatory cells. Pleura may show evidence of fibrinous pleuritis and serous or seroanguinous pleural effusion. The organism appears to produce exotoxin and endotoxin. However, their role in pathogenesis has not been clearly established. It is worth mentioning that acute tubular necrosis and encephalopathy have been noted in the disease. In a nutshell, the lung changes comprise of acute fibrinopurulent bronchopneumonia around respiratory bronchioles and diffuse alveolar damage. It also produces a disease called Pontiac fever with manifestations like fever, chills, myalgia, headache. Respiratory infection is less prominent.

The causative agent may be demonstrated by direct fluorescent antibody staining of formalin fixed lung aspirate, bronchial washing, lung biopsy, sputum and other organs like liver or spleen. Culture on charcoal yeast extract is also done for rapid diagnosis of the disease. ELISA and radioimmunoassay appears promising. DNA probe may be used.

The treatment is specific as well as supportive. Erythromycin is specific against this bacteria. Tetracycline is less effective. Rifamycin is quite effective and may be administered in patients who are not showing good response to erythromycin. Organism is also susceptible to cefoxitin. The respiratory failure, shock, hypotension and renal failure requires treatment with oxygen, mechanical ventilators with positive expiratory pressure, fluid replacement, vasoactive drugs and dialysis respectively.

CLOSTRIDIUM DIFFICILE

Clostridium difficile is recently accepted widely as the most important cause of severe antibiotic associated diarrhea. Cross contamination is the most likely explanation for spread of this organism in hospitalized infants. The organism may possibly spread among adults who are at risk of developing antibiotic associated diarrhea or colitis. In susceptible individual when exposed to this bacteria, it proliferates, releases toxin and may produce diarrhea, colitis, etc.

For the isolation of this organism stools are cultured immediately on brain heart infusion agar (BHIA) or cycloserine-cefoxitin agar (CCA) media. These plates are incubated at 37°C atmosphere of 10 percent CO_2, 10 percent H_2 and 80 percent N_2 for at least 48 hours in anaerobic incubator. After incubation, plates are examined under long wave (360 nm) ultraviolet light for light green chartreuse fluorescence the characteristic of *C. difficile* colonies. At least 3 colonies from each plate are subcultured into Robertson Cooked Meat media. The culture may be identified after 3 days of incubation by use of volatile fatty acid profile of carbohydrate metabolism generated by gas liquid chromatography and biochemical reaction pattern. The cellular morphology of *Clostridium difficile* is characteristic with apparent bipolar sporing noted in Gram-stain of 3-day-old culture giving *dumb bell* appearance.

However, isolated *Clostridium difficile* are tested for production of toxin *in vitro* using tissue cultures and for production of toxin *in vivo* by inoculation into guinea pig and

clindamycin treated hamsters. This toxin is a necrotizing glycoprotein with molecular weight of 50,000. Animal work has shown that certain antibiotics, like vancomycin are useful and deserve trial on human beings. Trial of antitoxin may also prove useful as *Clostridium difficile* toxin can be neutralized by several clostridial 'toxins' including that of *Clostridium sordellii.*

KINGELLA KINGAE

Previously known as a member of Moraxella. It is oxidase positive, non-motile, hemolytic on blood agar and Gram-negative bacillus. It is a part of normal oral flora and may cause infection of the bone, joints and tendons. It may enter the blood with minor oral trauma, e.g. tooth brushing. It is susceptive to penicillin, ampicillin, erythromycin, etc.

CARDIOBACTERIUM HOMINIS

It is a facultative anaerobe, pleomorphic Gram-negative bacilli which forms the part of normal flora of upper respiratory tract and bowel. It may cause endocarditis. It grows slowly on blood agar medium.

DF-2 BACTERIA

It is Gram-negative bacilli, oxidase positive and catalase positive. It does not show fermentation patterns on routine media. It forms the normal flora of dog. In man it may cause fulminant infection in asplenic patients and alcoholics.

GARDNERELLA VAGINALIS

Formerly called *Corynebacterium vaginalis* or *Haemophilus vaginalis* is Gram-negative bacillus present in the vagina as normal flora. Alone or in combination with anaerobes, *Gardnerella vaginalis* causes vaginitis with a malodorous odor. In wet smear we find "clue cells" which are vaginal epithelial cells covered with many tiny rods and there is an absence of other common cause of vaginitis like trichomonal or yeasts. Vaginal discharge has fish odor. The vaginal discharge is greyish, frothy with pH above 4.6. Metronidazole is used for its treatment.

YERSINIA ENTEROCOLITICA

It is an important cause of enteric infection in man and is found in many parts of the world. In children it causes watery diarrhea of 3 to 14 days' duration with blood in the stool. In adults pain in right lower quadrant of abdomen is most common symptom associated with fever, raised ESR and leukocytosis. Erythema nodusum and arthritis may follow infection with *Yersinia enterocolitica*. Rare cases of myocarditis, subacute hepatitis, hepatic abscess, meningitis, urethritis and glomerulonephritis have also been reported as complication of this infection.

They may be cultured on peptone agar, blood agar, MacConkey desoxycholate agar, etc (Fig. 43.3). It is non-lactose fementer and grows slowly. The colony is small after 24 hours incubation and becoming larger after 48 hours at 22° to 25°C. It is motile and Voges Proskauer positive. It is urease positive and oxidase and phenylalanine negative. O serotype are 1 to 20. The strains causing disease in man are O_3, O_8 and O_9. These strains have been shown to be invasive when tested in HeLa and porcine kidney cells and by Sereny test. Heat stable enterotoxin is produced by strains of serotypes O_3 and O_8. Toxin production is also suggested as plasmid

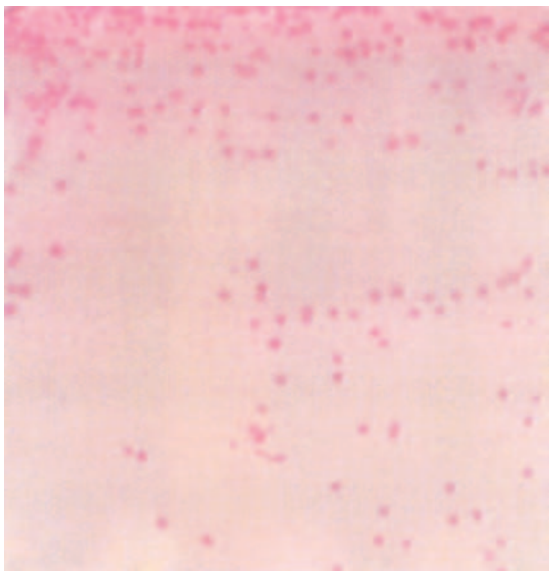


Fig. 43.3: *Yersinia* selective agar *Yersinia enterocolitica*

mediated. Animal models for the disease have been developed in mice, guinea pig, rat and rabbit. Antibiotics effective are tetracycline, trimethoprim-sulfamethoxazole and ampicillin.

FLESH EATING BACTERIA

The virulent form of streptococci are the causative agent identified. They act by liberating toxin that deprives cells of oxygen.

The disease claimed 11 lives in Britain (at least) and fresh cases are reported from New York, California, Florida, Connecticut, Sri Lanka, Singapore and Hong Kong.

BORRELIA BURGDORFERI

It is named following Burgdorfer's discovery of this organism as the etiological agent of Lyme disease. Transmission of this spirochaete is from vertebrate to vertebrate, depending upon blood feeding arthropods. It spreads by tick bite *(Ixodes dammini).*

Laboratory diagnosis includes detection of *Borrelia burgdorferi* directly in tissue, tissue fluid, culture medium, animal inoculation, etc. IFA, ELISA seems to the promising investigation.

Penicillin, erythromycin, ampicillin doxycycline, chloramphenicol, etc. are useful in treating the patient.

44 Microbiology of Oral Cavity

It is beyond doubt that majority of widespread dental diseases are caused by bacteria. The oral cavity has a number of surfaces and crypts for colonization of microorganisms. However, microbial growth is slow. Still large, dense populations of microorganisms can accumulate on tooth surfaces (dental plaques), on dorsum of tongue and in the gingival crevice area and carious lesions. By the way, saliva contains as much as 10^8 bacteria per ml.

Bacterial adhesion would seem to be important in colonization of mucosal surfaces elsewhere in the body by microorganisms like β hemolytic streptococci and enterobacteria. *Streptococcus mutans* and *Streptococcus sanguis* which are mainly found on teeth adhere well to various hard surfaces but not to epithelial surfaces. The protein surface antigen does contribute to the attachment of group A streptococci to epithelial surface. Further, other organisms like spirochaetes *(Treponema denticola)* and Gram-negative anaerobic rods *(Bacteroides Melaninogenicus)* can localize in gingival crevice area or in periodental pockets although they have feeble adherent capacity but are capable of proliferation especially in occlusal fissures of teeth.

Nature of oral microflora: At birth the oral cavity is sterile. With the introduction of food and other contacts microorganisms of all varieties are introduced initially but within days and weeks favored ones become established. One of the earliest settlers is *Streptococcus salivarious. Streptococcus mutans* and *Streptococcus sanguis* appear only after the eruption of teeth whereas spirochaetes and bacteroides appear with the onset of puberty. In adults microflora is quite complex with the predominence of streptococci and filamentous organisms (Gram-positive) in most sites.

Effects of Oral Flora

a. *Beneficial effects:* Man's indigenous flora contributes to his nutrition through synthesis of vitamins and digestion of certain food. Besides, oral flora also contributes to immunity to various organisms as small numbers of oral organisms entering blood stream provide a continual antigenic stimulus resulting low levels of circulating antibodies which may cross react with pathogenic organisms. Oral flora may also compete with pathogens, e.g. *Candida albicans* is restricted in number by indigenous oral bacteria but often grows out and causes disease when indigenous oral bacteria are suppressed by antibiotics.
b. *Harmful effects of oral flora:* Oral organisms seeking entry to tissues in man by various routes may cause not only alveolar abscesses but also abscesses of lungs, brain, extremities and surgical wound infection. These infections are usually mixed with *Bacteroides melaninogenicus* which plays a dominant role. Other unmixed infections initiated by oral microorganisms include candidiases, actinomycosis and subacute bacterial endocarditis.

Organisms may accumulate in the mouth as dental plaques and may start diseases of the teeth and supporting structures. Dental plaques is a mixed colony of bacteria embedded in an intracellular matrix adhering to the tooth surface. Bacterial cells make 60 to 70 percent of the volume and a fully developed plaque may be 300 to 500 cells thick. The matrix

is composed of bacterial and salivary polymer and remnants of epithelial cells and leukocytes.

The plaque changes in composition as it grows. Anaerobic bacteria find a suitable anaerobic environment when sufficient numbers of facultative organisms have accumulated. Moreover, many microorganisms of oral cavity require growth factors produced by neighboring organisms (*Bacteroides melaninogenicus* requires vitamin K derivative and *Treponema macrodentium* requires isobutyric acid and polyamines).

The plaque also changes in metabolism. Organisms in depths are poorly nourished and are exposed to high concentrations of microbial end-products. Hence they develop thickened cell wall, glycogen accumulation and morphological distortion. Beside this, anaerobiasis develops in the depths and promotes fermentation and ultimately production of acid.

Dental caries: Caries means the destruction of enamel, dentin or cementum is clearly caused by bacteria. It is the microbial plaque which represents a large mass of metabolically active bacteria capable of initiating dental caries (cavities), periodontal disease or both. The gnathobiotic rats (rats free of bacteria) fail to develop dental caries even when the animals are genetically susceptible and are fed cariogenic diets. However, infection with specific types of bacteria may cause extensive decay. The experimental caries can be prevented by penicillin.

The mechanism involving tooth decay seems to be basically direct demineralization, due to organic acid produced by the overlying bacterial plaque. Bacteria also contribute proteases. Bacteria like streptococcus in general and lactobacillus have cariogenic potential. These organisms ferment a variety of carbohydrates, producing and tolerating a low pH. *Streptococcus mutans* (cariogenic) has special mechanisms of accumulation thus explaining cariogenecity of sucrose in man. This organism synthesizes extracellular polysaccharides similar to dextran after sucrose is given. This is involved in adhesion of organism to teeth and other hard surfaces.

The second type of decay is occlusal pits and fissures of teeth and third type of decay affects root surface of teeth. Infected teeth may be a major source of subacute bacterial endocarditis (mainly due to *Streptococcus mutans* and *Streptococcus sanguis*).

Periodontal disease: This is the main cause of tooth loss in old age due to infection by microorganisms residing in gingival crevice area. Mainly supporting structures of teeth like gingiva, periodontal membrane supporting alveolar bone are infected. Organisms like Actinomyces, Nocardia, Corynebacterium, streptococci, etc. share the ability to form large quantities of subgingival plaque.

Bacterial hydrolytic enzymes, endotoxins and metabolic end products may cause destruction of the intercellular matrix of connective tissue or may interfere with the anabolism of local tissue cells. Alternatively, other mechanisms involved in the cause of periodontal disease may be inflammatory response by bacterial products directly whereas it is by immediate or delayed hypersensitivity response to plaque antigens indirectly.

Control of bacterial plaques: Dental caries and periodontal disease could be controlled by preventing plaque formation. Mechanical removal of plaque may be achieved by tooth brushing and dental floss. Curtailment of dietary sucrose also reduces decay. Now dietary fluoride is also used prophylactically against caries. Vaccine consisting of *Streptococcus mutans* cells or glycosyl transferase preparations, have reduced *Streptococcus mutans* colonization and dental caries development in experimental animals. Its effectiveness in man has not been evaluated.

Part V

Virology

45 General Characteristics of Viruses

Viruses are unicellular, ultramicroscopic particles containing either RNA or DNA, which reproduce inside living cells, pass through filters that retain bacteria and are covered by a protein coat.

The general properties of viruses are (Table 45.1):

i. Do not possess cellular organization.
ii. Contain one type of nucleic acid, either RNA or DNA but never both.
iii. Lack enzymes necessary for protein and nucleic acid synthesis and so depend upon synthetic machinery of host cells.
iv. They multiply by complex process and not by binary fission.
v. They are unaffected by antibiotics.
vi. They are sensitive to interferon.

Evolutionary Origin of Viruses

The origin of virus is not known. Three hypotheses have been proposed:

1. Viruses became parasites of primitive cells and the two evolved together. This may be the reason that many viruses today cause no host cell damage and remain latent in the host.
2. Viruses evolved from parasitic bacteria. While this possibility exists for other obligatory intracellular organisms, e.g. chlamydiae, there is no evidence that viruses evolved from bacteria.
3. Viruses may be the component of host cells that become autonomous. They resemble genes that escape the regulatory control of host cell. There is evidence that some tumor viruses exist in host cells as unexpressed gene. The likelihood of it is great because some small viruses are evolved in this fashion. However, large viruses (pox and herpes group) show very limited resemblance to host cell DNA.

Rivers' postulates: At the time Koch's postulates were formulated true viral pathogens were unknown. In 1937, TM River created a similar group of rules to establish causative role of viruses in disease Rivers' postulates are as under.

1. The viral agent must be found in the host's body fluid at the time of the disease or in the cells showing lesions.
2. The viral agent obtained from the infected host must produce specific disease in a suitable healthy animals or plant or provide evidence of infection in the form of antibodies against the viral agent. It is important to note all host material used for inoculation must be free of any bacteria or other microorganisms.

TABLE 45.1: Properties of viruses with comparison to prokaryotes

	Cellular organization	*Growth in inanimate media*	*Binary fission*	*Both RNA and DNA*	*Ribosomes*	*Sensitivity to antibiotics*	*Sensitivity to interferon*
Bacteria	+	+	+	+	+	+	–
Mycoplasma	+	+	+	+	+	+	–
Rickettsiae	+	+	+	+	+	+	–
Chlamydiae	+	+	+	+	+	+	–
Virus	–	–	–	–	–	–	+

3. Similar material from such newly infected animals or plants must in turn be capable of transmitting the disease in question to other hosts.

Morphology

Size: Viruses vary widely in size. The largest among them is pox virus measuring about 300 nm. The smallest viruses is foot and mouth disease virus measuring 20 nm.

The methods of estimating the size of virus particles are:

i. Collodion membrane filter of graded porosity.
ii. Ultracentrifugation.
iii. Electron microscope.

Shapes: Some viruses have characteristic shape, e.g. rabies virus has bullet shape, pox viruses are brick-shaped, tobacco mosaic virus is rod-shaped, bacteriophage has head and tail, like sperm, influenza or polio viruses are spheroidal and so on.

Structure and symmetry: Viruses have central core of nucleic acid which is either RNA or DNA but never both. This central core of nucleic acid is covered by protein coat called capsid. The capsid itself is composed of number of subunits called capsomere (Fig. 45.1). The capsomere may be arranged as under:

i. Around coiled nucleic acid which is known as helical arrangement.
ii. As cubes around spheroidal nucleic acid known as icosahedral arrangement.
iii. Some viruses do not fit either helical or icosahedral symmetry due to complexity of these structure, e.g. pox virus, bacteriophage, etc.

Virion may be enveloped or nonenveloped. The envelope is derived from host cell membrane when virus is released by budding. Envelope is lipoprotein in nature.

Protein subunits may be seen as projecting spikes on the surface of the envelope. These are called peplomers. A virus may have more than one type of peplomer, e.g. influenza virus has two peplomers:

i. Triangular spike, i.e. hemagglutinin.
ii. Mushroom-shaped, i.e. neuraminidase.

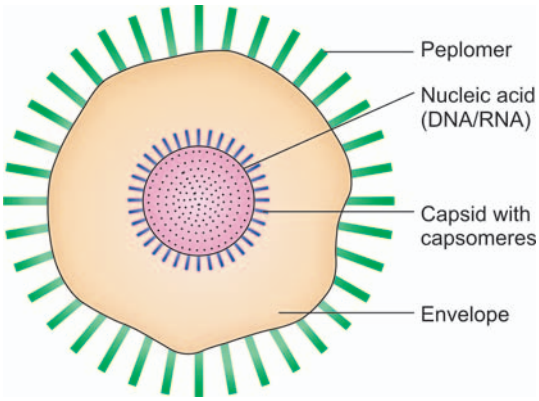


Fig. 45.1: Structure of virus

Reaction to Physical and Chemical Agents

i. *Heat and cold:* Viruses are mostly destroyed by heating at 60°C for 30 minutes except hepatitis virus, adeno-associated virus, scrapie virus. Viruses may be preserved by storage at –20 to –70°C in deep freezer (except poliovirus).
ii. *pH:* They are usually stable at pH 5 to 9.
iii. *Ether susceptibility:* Arbo, myxo and herpes viruses are destroyed by ether whereas entero, reo and adeno are resistant to the action of ether.
iv. *Radiation:* UV light, X-rays and *heavy particles* inactivate viruses, the UV rays dimerizes the pyrimidine bases of nucleic acid strand and gamma rays cause lethal break in the genome.
v. *Vital dyes:* Toluidine blue, neutral red and acridine orange penetrate virus particles. These dyes unite with nucleic acid making viruses susceptible to inactivation by visible light.
vi. *Glycerol:* Viruses remain viable in 50 percent glycerol whereas bacteria die.
vii. *Stabilization by salt:* Magnesium chloride, magnesium sulfate stabilize some of the viruses so that they are not inactivated by heating at 50°C in one hour.
viii. *Disinfectants:* Lysol, dettol, are ineffective against viruses. Higher concentration of chlorine, iodine may kill viruses. Dilute formaldehydes and beta propiolactone, hydrochloric acid, $KMnO_4$, H_2O_2 are most useful disinfectants against virus.

ix. *Antiviral agents:* I-methylisatin β-thiosemicarbazone are used against small pox, amantidine against influenza, rubella and respiratory syncytial virus.

Viral Multiplication (Fig. 45.2)

Virus depends on the synthetic machinery of host cell for replication because it lacks biosynthetic enzymes. The sequence of events are as under:

1. *Adsorption:* The virus is adsorbed at a particular site on the host cell which is called receptor. In case of poliovirus the receptor is lipoprotein present on the surface of primate. The host cell receptor for influenza virus are glycoproteins present on the surface of respiratory epithelium. Adsorption or attachment is specific and is mediated by binding of virion surface structure known as legands, to receptors on cell surface. In HIV surface, glycoprotein (gp120) acts as a legand and it binds to the CD4 60 kD glycoprotein on the surface of mature T lymphocyte.
2. *Penetration:* Virus particles may be engulfed by animal cell by the mechanism called viropexia. Viropexia is like phagocytosis. In case of enveloped virus, viral envelope may fuse with plasma membrane and release nucleocapsid into the cytoplasm.
3. *Uncoating:* This is a process by which the virus lose its outer layer and capsid. In some cases, uncoating is effected by lysozomal enzyme of host cell. For example, in pox virus, uncoating occurs in two steps. Outer coating is removed by lysozyme present in phagocytic vacuole of host cell. This is the first step. In second step, internal core of virus (nucleic acid and internal protein) is released into cytoplasm and is effected by viral uncoating enzyme. Thus DNA is released.
4. *Biosynthesis:* There is synthesis of viral nucleic acid and capsid protein. There is synthesis of regulator protein which shuts down the normal cellular metabolism and direct sequential production of viral component. DNA viruses synthesize their components in host cell nucleus except pox virus which synthesize their components in cytoplasm. Likewise RNA viruses synthesize their components in cytoplasm of host cell except orthomyxoviruses, paramyxovirus and leukoviruses. Biosynthesis consists of following steps:
   i. Transcription of messenger RNA from viral nucleic acid.
   ii. Translation of mRNA into early proteins. They initiate and maintain the synthesis of virus component and shut down the host protein and nucleic acid synthesis.
   iii. Replication of viral nucleic acid.
   iv. Synthesis of late proteins, which are the components of daughter virion capsids.

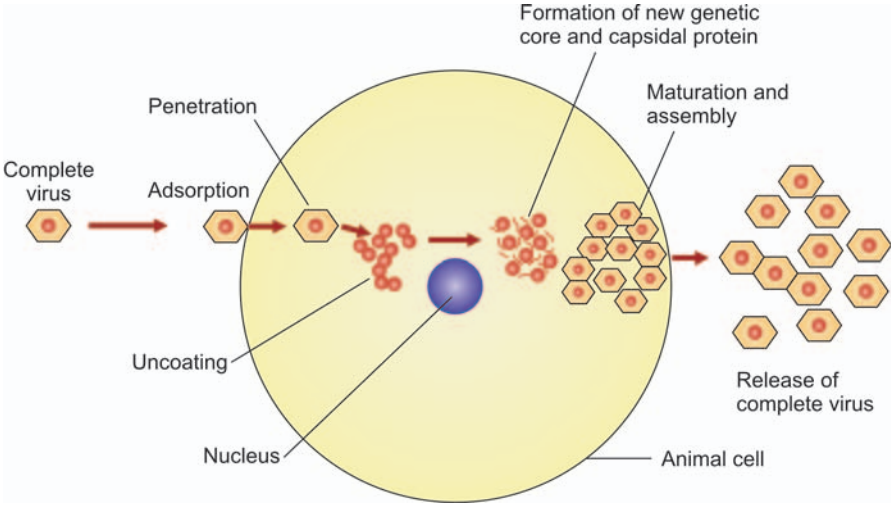


Fig. 45.2: Viral multiplication

Transcription: Mechanism of transcription and nucleic acid synthesis differs in different types of viruses:

a. In single stranded (SS) nucleic acid, complementary strand is first synthesized producing double stranded (DS) replicative forms.
b. Double stranded (DS) viral DNA acts as template and for its replication RNA virus uses various methods for replication:
   i. In poliovirus, SS RNA acts directly on mRNA.
   ii. SS RNA parenteral (positive strand) acts as template for production of complementary strands (negative strand) which act as template for progeny viral RNA.
   iii. In SS RNA (e.g. influenza) parenteral RNA produces complementary negative strands which act both as mRNA and as template for synthesis of progeny viral RNA.
   iv. Oncogenic RNA viruses (leukovirus) exhibit unique replicative cycle. The virus genome is SS RNA which is converted into RNA-DNA hybrid by viral enzyme reverse transcriptase (RNA directed DNA polymerase) from its hybrid DS DNA is synthesized which is integrated into host cell genome (provirus). Provirus acts as template for the synthesis of progeny viral RNA.

This integration of provirus with host cell genome may cause transformation of the cell and development of neoplasm.

5. *Maturation:* Assembly of daughter virion follows synthesis of viral nucleic acid and proteins. It may take place in nucleus (herpes, adeno) or cytoplasm (picorna, pox). Enveloped virus gets envelope from the cell membrane of the host during a process of budding. Nonenveloped viruses are present intracellularly as fully developed virion.
6. *Release:* In bacterial viruses release take place by lysis of infected bacterium. In animal, viruses release occur without lysis (myxo). Some viruses like polio may cause cell lysis during their release.

Cycle of replication:

a. Fifteen to thirty hours in animal virus.
b. Fifteen to thirty minutes in bacterial phage.

Eclipse phase is the time from stage of penetration of virus into host cell till appearance of mature daughter viruses. In this phase, virus cannot be demonstrated in host cell.

Abnormal Replicative Cycles

Magnus phenomenon: When virus yield will have high hemagglutinin titer but low infectivity. They are also called incomplete viruses.

Abortive infection: Sometimes in host cells viral component may be synthesized but maturation or assembly is defective. Either no release occur or the progeny is noninfectious.

Defective viruses: They are viruses which are genetically deficient and so incapable of producing infectious daughter virion.

Virines: They are made up of small amount of RNA complexed with protein of host cell origin.

Prions

They are small proteinaceous infectious particles which resist inactivation by procedures that modify nucleic acid. In short infectious agents which lack nucleic acid genome are called prions. They are about 5 nm in diameter, resistant to heat, ultraviolet rays and nuclease. However, they are sensitive to proteases. Some features of prions are:

1. They cause diseases which are confined to central nervous system.
2. The diseases caused by prions have prolonged incubation period.
3. The diseases due to prions show slow, progressive and fatal course.
4. Prion diseases may show spongiform encephalopathy and vacuolation of neurons.

Immune response: Prions do not cause an inflammatory response. They do not induce the formation of interferons. There is no antibody response against prions. Obviously, it is not possible to screen people for exposure to prions by demonstrating antibodies.

Prion diseases are often called spongiform encephalopathies because of the postmortem

appearance of the brain large vacuoles in the cortex and cerebellum. Specific examples of prion diseases in mammals include:

Scrapie	*Sheep*
TME (Transmissible mink encephalopathy)	Mink
CWD (Chronic wasting disease)	Muldeer, Elk
BSE (Bovine spongiform encephalopathy)	Cows

Humans are susceptible to several prion diseases too some of which are:

CJD	Creutzfeld-Jacob disease
GSS	Gerstmann-Straussler-Scheinker syndrome
Kuru	Alper's syndrome

Humans might be infected by prions by two ways as under:

1. Acquired infection through diet and following medical procedures like surgery, growth hormones injections, corneal transplants, etc.
2. Apparent hereditary mendelian transmission where it is autosomal and dominant trait.

Thus prion diseases are distinct in the sense that they are both infectious as well as hereditary.

Characters of Prions

- Resistant to chemicals, radiation and heat.
- Proteinaceous and filterable
- Do not produce inflammatory reaction in host.
- Do not produce antibody in host
- Abnormal fibers and plaques may be formed in the brain of host by prions.
- Difficult to treat.

Nobel Prize in Medicine 1997

Stanley Prusiner won this prestigious prize through his discovery of prion, a disease causing agent like bacteria or virus. The prion protein can manifest itself as two proteins, i.e. innocent protein and dangerous or disease causing proteins. Stanley Prusiner solved the riddle of prion's properties.

Prions exist normally as innocuous cellular proteins. However, prions possess an innate capacity to convert their structures. That ultimately results in the formation of harmful particles, the causative agents of several deadly brain disease like dementia type in humans and animals.

As a matter of fact this discovery provides key insight into dementia related diseases like Alzheimer's and mad-cow disease. It gives opportunity to understand the biological mechanisms underlying these diseases and also establishes a foundation for drug development and new type of medical treatment strategies.

INTERACTIONS AMONG VIRUSES

When same host is infected by two or more virus particles they may interact as under:

1. *Recombination:* Here the nucleic acid strands break and part of the genome of one parent is joined to part of the genome of the second parent. The recombinant virus is genetically stable and produce progeny like itself on replication. Double stranded DNA genomes recombine efficiently whereas single stranded RNA genomes which are unsegmented do not recombine.
2. *Genetic reactivation:* When an inactive virus particle is rendered active by interaction with other inactive virus particles in the same cell, it is called multiplicity reactivation. Here recombination occurs between the damaged nucleic acids of the parents producing a viable genome that can replicate.
   Sometimes, a portion of genome of the inactivated virus recombines with that of active parent so that certain markers of inactivated parents are rescued and appear in viable progeny. They are genetically stable. This is called marker rescue.
3. *Complementation:* This means the interaction of viral gene products in cells injected with 2 viruses, one or both of which may be defective. The basis for complementation is that one virus provides a gene products in which the second is defective, allowing second virus to grow. However, the genotypes of the two viruses remain unchanged.
4. *Phenotype mixing:* It usually occurs between different members of the same virus family. The intermixed capsid proteins must be able to interact correctly to form a struc-

turally intact capsid. In short, phenotypic mixing is the association of a genotype with heterologous phenotype.

5. *Interference:* It means infection with 2 viruses leading to an inhibition of multiplication of one of these two viruses. Thus it is called interference. The mechanisms involved in interference are:
   i. One virus may inhibit the ability of the second to adsorb to the cell by blocking the receptors (enteroviruses) or by destroying the receptors (orthomyxoviruses).
   ii. One virus may compete with the second virus for components of the replication apparatus (polymerase).
   iii. The first virus may cause the infected cell to produce an inhibitor (interferon) that may prevent replication of the second virus.

Interference has been used as a basis for controlling outbreaks of infection with virulent strains of poliovirus by introducing into the population an attenuated poliovirus that interferes with the spread of the virulent virus.

Cultivation Viruses

Since they are obligate intracellular parasites and cannot grow on inanimate culture medium, 3 methods are used for their cultivation:

a. Animals inoculation.
b. Chick embryo.
c. Tissue culture.

a. *Animal inoculation:* It is one of the oldest methods for the cultivation of viruses. The poliomyelitis virus after intraspinal or intracerebral inoculation in monkeys causes typical paralytic disease and so isolation of viruses. Suckling mice is susceptible to Cox-sackie viruses with manifestation of severe myositis and paralysis. Smallpox virus may be inoculated in the scarfied skin or cornea of rabbit. Brain tissue of rabied dog when inoculated intracerebrally in mice or rabbit develop encephalitis.

Growth of virus in animals may be known by the disease, visible classical lesions or death. Sometimes immunity in experimental animal may interfere with the growth of viruses in that animal. It is not out of place to mention the other utility of animal inoculation, i.e. to study pathogenesis, immune response, and epidemiology.

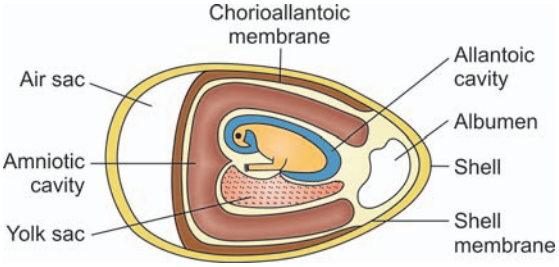


Fig. 45.3: Structure of chick embryo

b. *Chick embryo:* They are better than animal inoculation because of following reasons:
   i. They are clean and bacteriologically sterile.
   ii. They do not have immune mechanism like animals to counteract virus infection.
   iii. They do not need feeding and caging.
   iv. Chick embryo offers several sites for cultivation of viruses, i.e. chorioallantoic membrane (CAM) for variola or vaccinia and herpes viruses, allantoic cavity provides rich yield of influenza and some paramyxoviruses, amniotic sac may be used for the isolation of influenza virus and yolk sac for the cultivation of chlamydiae, rickettsiae and some viruses (Table 45.2). Allantoic inoculation may be used for growing influenza virus for vaccine purposes. Yellow fever (17 D strain) and rabies (flury strain) are other vaccines produced from chick embryo (Fig. 45.3).

TABLE 45.2: Growth of viruses in chick embryo

Route of inoculation	*Virus*	*Lesion on CAM*	*Hem-agglutination*
Chorioallantoic	Poxviruses	+	–
Membrane (CAM)	Herpes simplex	+	–
	Herpes virus B	+	–
Amniotic cavity	Influenza virus	–	+
	Mumps virus	–	+
Allantoic cavity	New castle disease	–	+
	Influenza	–	+
	Mumps	–	+
Yolk sac	JE Virus, West Nile virus	+	–
	Nile virus	+	–

Disadvantages of Egg Inoculation

1. Eggs may be contaminated with mycoplasma and latent fowl viruses which may interfere with the growth of other viruses.
2. The susceptibility of chick embryo is limited to a few viruses only.
3. Even slight amount of bacterial contamination in the inoculum may kill the embryo.

c. **Tissue culture:** Tissue culture of human or animal cells are frequently used for the cultivation of viruses. There are mainly three types of tissue culture:
   1. *Organ culture,* e.g. tracheal ring organ culture is employed for the isolation of coronavirus.
   2. *Explant culture:* Minced tissue may be grown as explant embedded in plasma clots. This is not useful in virology. In the past adenoid tissue explant culture were used for adenovirus.
   3. *Cell culture:* This is very popular and useful technique routinely used for cultivation of viruses. From tissue, fragments cells are dispersed by proteolytic enzymes like trypsin and mechanical shake. After washing the cells, they are suspended in growth medium and distributed in petridishes, test tubes or bottles. The cells adhere to glass surface and grow out to form a monolayer sheet and can be seen *in situ* under low power.

Cell Cultures in Use

Primary cell cultures
- Rhesus monkey kidney cell culture
- Human amnion cell culture
- Chick embryo fibroblast cell culture

Diploid cell strains
- WI-38 (Human embryonic lung cell strain)
- HL-8 (Rhesus embryo cell strain)

Continuous cell lines
- HeLa (Human carcinoma of cervix cell line)
- HEP–2 (Human epitheloma of larynx cell line)
- Vero (Vervet monkey kidney cell line)
- McCoy (Human synovial carcinoma cell line)
- KB (Human carcinoma of nasopharynx cell line)

There are three types of cell cultures (Table 45.3):

a. **Primary cell cultures:** When normal cells freshly taken from body grown for the first time, they are called primary cell culture. They can be maintained in serial culture. They are useful for isolation and cultivation of viruses for vaccine production, e.g. rhesus monkey kidney cell culture, human amnion cell culture, chick embryo fibroblast culture, etc.

b. **Diploid cell strains:** They are capable of 100 divisions in culture. They are useful for the isolation of fastidious pathogens and also for the production of viral vaccines. Examples are human embryonic lung cell strain (WI-38) and rhesus embryo cell strain (HL-8).

c. **Continuous cell lines:** They are single type of cells mainly derived from cancer cells. These also can be grown in successive generation by transferring them from one test tube to another without change in character of cells. These are used only for the isolation of virus. Vaccine preparation on these cells is not safe for human use, e.g. HeLa (human carcinoma of cervix cell line).

TABLE 45.3: Isolation of viruses from cell lines

Cell line	*Virus isolation*
Primary	
• African green monkey	• HSV, RSV, mumps, rubella
• Chick embryo fibroblast	• Rabies, pox-viruses
Diploid Cell	
Human fetal lung (WI-38, MRC-5)	Rabies, adeno, CMV
Continuous	
He La	Polio, pox, reo RSV
HEp-2	Adeno, RSV
MDCK	Influenza
RD	Polio, enteroviruses
Vero	Polio, rabies, measles

Other examples of continuous cell lines are: KB (human carcinoma of nasopharynx cell line), HEP 2 (human epitheloma of larynx cell line), McCoy (human synovial carcinoma cell line), BAK 21 (baby hamster kidney cell line) and Detroit-6 (sternal marrow cell line).

Detection of virus growth on cell cultures: Viruses multiplying in tissue culture manifest their presence by producing:

1. Changes in the cells called cytopathogenic effects (CPE), e.g. measle virus produces syncytium formation and SV_{40} produces prominent cytoplasmic vacuolation.

2. When viruses grow in cell culture, cell metabolism is inhibited and there is no acid production. In normal cell culture because of active metabolism there is active acid production. Phenol red (indicator) can detect the presence of acid formation by changing its color into yellow.
3. *Hemadsorption:* When influenza and para-influenza viruses grow in cell culture their presence may be detected by addition of guinea pig erythrocytes to the culture. If the viruses are multiplying in culture, erythrocytes will adsorb on the surface of cell.
4. *Interference:* Growth of first virus will always check infection by the second virus by interference.
5. *Transformation:* Oncogenic or tumor producing viruses cause cell transformation and loss of contact inhibition.
6. Fluorescent antibody straining is also a method of detecting viral multiplication.
7. Hemagglutination test may be performed by using tissue culture fluid, e.g. orthomyxoviruses and paramyxoviruses.

INCLUSION BODIES

During multiplication of virus in host cells, virus specific structures are produced and they are called inclusion bodies. Sometimes, they may become larger than the individual virus particles. They have distinct size, shape, location and staining properties. The size of inclusion bodies may be from 1 to 30 µ. They are rounded, oval, pyriform or irregular in shape. They can be demonstrated under light microscope. Acidophilic inclusion bodies can be seen as pink structure when stained with Giemsa, or eosin methylene blue stain. Some viruses produce basophilic inclusion bodies. Inclusion bodies are believed to be the site of development of viruses.

Vaccinia infected cells show small multiple intracytoplasmic inclusion (Guarnieri's bodies) (Table 45.4). Large intracytoplasmic inclusions (Bollinger's bodies) are seen in fowlpox. Again Molluscum bodies are intracytoplasmic, quite large about 30 µ and seen in *Molluscum contagiosum*. Negri bodies are intracytoplasmic seen in rabies virus infection.

TABLE 45.4: Cell membrane receptors for viruses

Viruses	*Receptors membrane*
Influenza	Sialic acid on glycoproteins including glycoprotein A molecule
Rabies	Acetylcholine receptors
HIV	CD 4 molecule on T cells
Epstein Barr	C3d receptor on B cells
Vaccinia	Epidermal growth factor receptor
Reovirus type 3	Beta-adrenergic hormone receptor
Rhinovirus	Intercellular adhesion molecule

Intranuclear bodies are Cowdry type A (seen in herpes, yellow fever virus) in granular form and variable in size. On the other hand, Cowdry type B are circumscribed and multiple. They are found in adeno- and poliovirus.

Inclusion bodies which are both intranuclear and intracytoplasmic are encountered in measles virus.

LABORATORY DIAGNOSIS

The appropriate specimen is collected, preserved and transported using proper techniques along with clinical information. The following approach is used for diagnosis of viral disease:

Microscopic Examination

Viruses can be demonstrated and identified by direct microscopic examination of clinical specimens. The various procedures involved include:

Light microscopy: It can reveal characteristics such as inclusion bodies (e.g. Negri bodies) or multinucleated giant cells. Tzanck smear showing herpes virus induced multinucleated giant cells in vesicular skin lesions can be easily observed under light microscopy.

Electron microscopy: It is clinically being used for viruses that are difficult to culture. Rotavirus and hepatitis A virus in feces are increasingly being detected by electron microscopy.

Immunoelectron microscopy: Addition of specific antibody to the specimen enhances the sensitivity of electron microscopy. The added

antibody aggregates with the virus particle thereby making its demonstration easier.

Fluorescent microscopy: Direct or indirect fluorescent antibody technique is useful for detection of viruses or viral antigens in clinical specimens. Some of the common viruses detected by fluorescent microscopy include rabies virus, paramyxovirus, orthomyxovirus, adenovirus and herpes virus.

Identification in Cell Culture

The viruses can be grown by inoculation into animals, eggs or cell cultures. The presence of a virus in the clinical specimen can be detected by observing a "cytopathic effect" in cell culture, hemadsorption etc. A definitive diagnosis of the virus in cell culture is made using known antibody by tests like complement fixation, hemagglutination inhibition and neutralization of the cytopathic effects. Other procedures that can be used are ELISA, fluorescent antibody, radioimmunoassay and immunoelectron microscopy.

Detection of Viral Antigens

ELISA, radioimmunoassay and latex agglutination may be useful for detecting viral antigens.

Detection of Viral Nucleic Acids

The detection of viral DNA or RNA is increasingly becoming the "gold standard" in viral diagnosis. Labeled nucleic acid probes are highly specific with rapid results. Polymerase chain reaction (PCR) technique allows rapid amplification of target DNA sequence so that it can be readily identified using labeled probes in a hybridization assay. The detection of HIV-1, HIV-2, human papillomavirus, hepatitis B virus, hepatitis C virus, enterovirus and Epstein-Barr virus has been simplified using the above technique.

Serology

A rise in antibody titer against the virus during the course of viral infection is a definitive evidence of its etiology. However, an antibody titer in a single specimen does not distinguish between a previous infection and a current one. Paired serum samples, collected during the acute and convalescent phases are required. Presence of IgM specific antibodies is meaningful in certain viral infections. The antibody titer can be determined by the immunological tests mentioned above. Other nonspecific serologic tests include heterophil antibody test (Monospot) used for diagnosis of infectious mononucleosis.

46 Chemotherapy of Viral Diseases

Sometime back close relationship between cellular metabolic process required for viral multiplication and those needed for survival of vertebral cells, was thought to be the genuine reason not to search for antiviral agents.

Now a number of chemicals are available which *inhibit viral replication to greater extent* than cellular metabolism. Reason may be their action on synthesis or functions of viral enzymes or nucleic acids. Very few of them are found useful in practice, e.g.

1. Pyrimidine nucleoside.
2. Methisazone.
3. Amantadine.

All these three have negligible effect on human morbidity or mortality from viral diseases.

Problems of Viral Chemotherapy

Three broad problems to effective antiviral chemotherapy are:

i. Obligatory dependence of viruses upon metabolism of the host cell.
ii. The fact that viral production is well advanced by the time symptoms appear.
iii. Drug-resistant mutant.

All substances which inhibit viral replication also interfere with cellular processes, e.g.

a. *Puromycin or cycloheximide* inhibits both viral and cellular protein synthesis.
b. *Fluorodeoxyuridine or cytosine arabinoside* inhibits the DNA synthesis.
c. *Actinomycin* D inhibits DNA dependent RNA synthesis.

At virostatic concentration, these are toxic for normal cells. These are lethal drugs and hence not useful for human consumption except *iododeoxyuridine* (toxic drug) which is applied topically to treat localized infection, e.g. herpes simplex of the cornea.

Ideal antiviral agent must block viral production without causing lethal damage to uninfected cells. However, there are four key points that should theoretically be susceptible to antiviral attack:

1. Attachment of virus may be dependent on the interaction of specific chemical grouping on the surface of each.
2. Transcription of mRNA from DNA of deoxyribose viruses require virus specified DNA dependent RNA polymerase and some riboviruses utilize a virus specific polymerase.
3. Viral mRNA molecules are apparently different in some fundamental way from RNA of mammalian cells.

The mechanism of action of antiviral chemotherapeutic agent is given in Table 46.1.

TABLE 46.1: Mechanism of action of antiviral chemotherapeutic agent

Drug	*Probable point of action*	*Principal viruses inhibit*
Amantadine	Penetration or uncoating	Influenza A
Iododeoxyuridine Ara—A 1, Arac	DNA replication	Herpetovirus
Rifamycins	Reverse transcriptase assembly	Retroviruses Poxviruses
Thiosemicarbazones	Translation of late viral mRNA	Poxvirus
Interferon	Transcription or translation	All viruses
Poly I, poly C Statolon Pyran copolymer COAM Tilorone Propanediamine	Induction of interferon and stimulation of reticuloendothelial system	All viruses

4. Virus coded enzymes essential to the replication of viral nucleic acid but irrelevant to the cell are synthesized in the infected cell.

Rational Approach to the Search for Antiviral Agents

The approach which led us to discovery of antibacterial antibiotics has produced very poor results for antiviral drugs. Approach should be based upon the search for agents capable of inhibiting biochemical reactions known to be unique to the multiplication of viruses.

Attachment and Penetration

It requires apposition to specific complementary receptor on the surface of virus and cell respectively. Effective antiviral agents should be able to interfere with any one, i.e. receptor or cell and can block infection, e.g. neuraminidase destroys glycoprotein receptors in lungs of mice and render them refractory to influenza infection till new receptors appear after some hours. Amantadine and derivatives do not inhibit attachment but appear to have some effect on the process of penetration.

Transcription

RNA transcriptase viruses Poxviruses	Transcriptase essential for transcription of early mRNA from uncoated viral coat.

All transcription	are viral specific	All except Pox are RNA dependent	May be some specific chemical inhibits the enzymes without effecting DNA dependent cellular RNA polymerases.

Imidazole and interferon block transcription.

Retroviruses	Reverse transcriptase	Rifamycin (experimentally)
	may render them to attack at this point	may be useful

Post-transcriptional cleavage (large molecules) of polycistronic mRNA.

Transcribed from DNA and some-RNA virus and subsequent addition of poly (A) may be susceptible to selective chemotherapy.

Translation

Translation of	Affected by:	
Protein from viral RNA	(1) Methisazone (2) Thiosemicarbazone	block the translation of late vaccinia mRNA
Interferon	Blocks translation of all viral mRNA.	

Replication of Viral Nucleic Acid

It occurs rapidly and often in resting cell.

5 iodo, 2 deoxyuridine, adenine arabinoside and cytosine arabinoside	Inhibit DNA synthesis	Systematically toxic but locally effective against DNA viruses, e.g. multiplication in cornea where only few cells divide.

Benzimidazole and derivatives, e.g. 2 α hydroxymethyl and benzimidazole (HBB)	Inhibits RNA replication by picorna viruses in cultured cells.

Post-transcriptional Cleavage of Proteins

Only in PICORNA viruses and Toga (probably)	Protein arisen
Cleavage is important step in assembly of other viruses	Inhibition of cleavage by Rifamycin.

Assembly

Guanidine	Disrupt hydrogen bond and mature virion are not assembled.

Regulation of Gene Expression

Above mentioned stages may be under control of viral protein.

Guanidine may act to throw out the whole of the cycle out of gear.

INTERFERON

Interferons are host-coded glycoproteins with molecular weight 20,000 to 40,000 that inhibit virus replication and are produced by intact animals or cultured cells in response to virus infection or other inducers. They are nontoxic, nonantigenic, inactivated by proteolytic enzymes. They can withstand heating at 37°C for 30 minutes. They are believed to be the body's first line of defence against viral infection. It is ideal antiviral chemotherapeutic agent for the use in man. In man, it is nontoxic, non-allergic and active against broad spectrum of viruses. Interferon differs from antibodies (Table 46.2).

TABLE 46.2: Differences between interferons and antibodies

Interferons	*Antibodies*
1. Are produced by any microbe infected cells	1. Are produced by B lymphocytes
2. Leave the infected cell and enter a nearby healthy cell to dispose of microbes	2. Circulate in blood and lymph to dispose of antigens
3. Induce the healthy cell to synthetize antimicrobial proteins to check microbial multiplication	3. Selectively bind to antigens that are immobilized for easy attack by phagocytes
4. Are quick in action but offer a temporary immunity against microbes	4. Are slow in action but offer long-lasting immunity against antigens
5. Act inside the cells	5. Act outside cells

There are multiple species of interferons that fall into 3 general groups named IFNα, IFNβ and IFNδ. Out of these IFNα family is large being coded by at least 14 genes whereas IFNβ and IFNδ are coded by one or few genes each. These interferons are similar in size but the 3 groups are antigenically distinct. IFNα and IFNβ are resistant to low pH. IFNβ and IFNδ are glycosylated.

Synthesis of interferons: Normal cells do not generally synthesize interferon until they are induced to do so. Interferons are produced by all vertebrate species. RNA viruses are stronger inducers of interferon than DNA viruses. Interferon also can be induced by DS RNA, bacterial endotoxin and small molecules like tilorone. However, IFNδ is not produced in response to most viruses but is induced by mitogen stimulation.

IFNα class of interferons is synthesized by leukocytes, IFNβ by fibroblasts and IFNδ only by lymphocytes.

Diploid strain of human embryonic fibroblast, e.g. WI-38 could provide adequate supply of cells. Precaution for inactivation of viruses used as interferon inducer must be undertaken for purification of interferon. Human skin fibroblast stimulated with poly I, Poly C to produce 5 hours' burst of interferon about once in 24 hours may be used to obtain human interferon.

Synthetic Polynucleotides and Other Inferferon Inducers

Interferon levels in human being following natural infection or artificial stimulation are low as compared to those found in mice but these low levels are protective.

Synthetic chemicals are preferred to live attenuated viruses, e.g. polynucleotide (Poly I and Poly C).

Poly I and Poly C

In human beings they protect against wide variety of viral infection. When administered intramuscularly, one day before infection until 4 days after challenge, with rhino type-13, inoculated by same route, produce less number of virion and less severity of common cold. Topical applications in respiratory tract have some value in human medication without toxic effects.

Drawbacks: Systematic administration is contraindicated except in Grave illness, because of side effect on *hemopoiesis* and *liver function*. Following single dose, animals become temporarily refractory to interferon induction by 2nd dose. This drawback is seen with all varieties of interferon inducers.

Mechanism of action: It is poorly understood. It is clear that interferon induces an antiviral state by prompting the synthesis of other proteins that actually inhibit virus replication.

Interferon molecules bind to cell surface receptors, with IFNα and IFNβ sharing a common receptor and IFNδ choosing a distinct receptor. This binding triggers the synthesis of several enzymes believed to be responsible of antiviral state. These cellular enzymes subsequently block viral replication by inhibiting the translation of viral mRNA into viral protein. Atleast three enzymatic pathways seems to be involved.

1. Protein kinase phosphorylates inactivates a cellular initiation factor and thus prevents

formation of initiation complex required for viral protein synthesis.
2. An oligonucleotide synthetase, 2,5-oligo A, that is required for oligoadenylic acid formation activates a cellular endonuclease RNase, which degrades viral mRNA.
3. Phosphodiesterase that leads to inhibition of peptide elongation. Besides this, interferons may affect viral assembly perhaps as a result of changes at plasma membrane.

Clinical use: Interferon treatment may be helpful in certain severe viral infections like rabies, hemorrhagic fever, herpes encephalitis, persistent virus infections like Hepatitis B, Herpes zoster, etc. Topical interferon in the eye may suppress herpetic keratitis and enhance healing.

All interferons are being tested but interferon IFNδ would be expected to be quite effective due to its higher anticellular activity as anticancer agent.

In short, human interferon can prevent dissemination of Herpes zoster and Herpes simplex in immunosuppressed patients, suppress viremia of chronic active Hepatitis B. If given before trigeminal ganglion surgery for neuralgia, it can temporarily suppress the appearance of lesions of herpes simplex.

Human interferon can:
1. Prevent dissemination of Herpes zoster and Herpes simplex in immunosuppressed patients.
2. Suppress viremia of chronic active Hepatitis B.
3. If given before trigeminal ganglion surgery for neuralgia, it can temporarily suppress the appearance of recurrent lesions of Herpes simplex.

Current Research

Attempts to synthesize new polynucleotides with higher antiviral potency and lower toxicity in man, e.g. N.N. dioctadecyle N, N bis (2 hydroxyethyl) propanediamine administered as oil in water emulsion intranasally induce titer higher of interferon.

Interferon may be used in the treatment of acute life-threatening viral illness. Dosages recommended are 5 million units intramuscularly daily for 14 days (Herpes simplex type I), 5 million units intramuscularly daily for one week (Hepatitis B), 5 million units 3 times a week for 10 months (papillomatosis) and one million units intramuscularly for 3 days in varicella encephalitis. Now recombinant DNA technology has been applied to greatly increase the yields of several interferons for clinical trials. Cloned interferon genes are being expressed in large amount in bacteria and yeasts and availability of genetically engineered interferon is made.

ANTIVIRAL AGENTS

Mechanism	*Anti viruses*	*Viruses killed*
A. Inhibition of viral DNA polymerase	I. Acylovir II. Ganciclovir III. Ribavirin IV. Trisodium phosphonoformate	• Herpes simplex Varicella zoster • Cytomegalovirus • RSV • Lassa virus • Herpes virus • Hepatitis B virus
B. Inhibition of Transcriptase	• Zidovudin • Dideoxycytidine • Dideoxyinosine • Trisolium phosphonoformate	HIV
C. Inhibition of protease	I. Indinavir II. Nelfinavir III. Ritonavir IV. Saqnivir	HIV
D. Blockage in penetration of virus into cells	Amantadine	Influenza virus
E. Protein synthesis inhibition	Interferon	All viruses

Immunomodulators: They are the agents which reverse immunologic effects of the virus and so can be used as adjuvants to antiviral drugs, e.g. interferon, interleukin II, isopronosine thymic humoral factors, etc.

1. *Nucleosides derivatives:*
   a. *Halogenated nucleoside:*
      i. Halogenated pyrimidine is inhibitor of cellular DNA synthesis.
      ii. 5-iodo deoxyuridine is topically used in man in keratoconjunctivitis or dendritis ulcer due to Herpes simplex virus. Five percent iododeoxyuridine in dimethyl sulfoxide solution is used in Herpes labialis increases penetration. Systematically used in Herpes simplex encephalitis cases but value is not confir-

med. Otherwise it is mutagenic and should not be given parenterally.

b. *Thymidine analogue:*
Trifluorethymidine is superior to iododeoxyuridine for human cellular infection with Herpes simplex virus.

Acyclovir (9-[2-hydroxyethoxymethyl] guanine acyloguanosine): It strongly inhibits Herpes simplex virus but has little or no effect on other DNA viruses or on host cells. It has been effective in topical application in the control of herpetic eye lesions in humans and in the healing of only primary lesions. Latent infections in the ganglia are not cured. Parental administration may prevent the reactivation of latent herpes virus infections. However, mutants of herpes virus that lack thymidine kinase fail to phosphorylate the drug and are resistant to it.

Ganciclovir: It is structurally related to acyclovir and is specially useful because of its anticytomegalovirus property in transplant, AIDS patients and immunocompromised patients.

2. *Arabinofuranosyl nucleoside:*
It is more useful than halogenated nucleoside in human medicine. It interferes with both cellular and viral DNA synthesis but their chemotherapeutic index is quite higher.

Cytosine arabinoside (Ara C) has similar antiviral spectrum as iododeoxyuridine, equally effector in herpetic and vaccinal infection especially keratitis in man and animal.

Recently synthesized, adenine arabinoside and 9B-D rabinofuran osyladenine (ara A) have high chemotherapeutic index than Ara C or iododeoxyuridine against herpetic and vaccinal viruses.

Ribavirin: This synthetic nucleoside analogue inhibits both RNA and DNA viruses like influenza, parainfluenza, respiratory syncytial, adeno, arena and lassa viruses.

Vidarabine (Ara-A): It is purine nucleoside analogue with activity against Herpes simplex, varicella, Epstein-Barr viruses, etc. It inhibits viral DNA synthesis. In combination with interferon. It is useful in chronic Hepatitis B infection.

Vidarabine monophosphate (Ara-Amp): It may be used in Herpes simplex encephalitis, genital herpes and varicella zoster infections.

Rimantidine: It is structurally similar to amantidine and is better tolerated. It is more effective and less toxic.

3. *Thiosemicarbazones:*
I-methylisatin β-thiosemicarbazone (methisazone) inhibits multiplication of pox virus. It interacts with perhaps viral or cellular proteins to prevent translation of late viral mRNA. It may have role in treatment of complication of smallpox vaccination such as eczema vaccinatum or vaccina gangrenosa. Drug is not a substitute of smallpox vaccination.

4. *Amantadine:*
I-amantanamine hydrochloride blocks multiplication of influenza A viruses at a stage of penetration or uncoating. Efficiency and safety in man is controversial. Otherwise also it has prophylactic importance only. It is not effective after infection. 1-methyl spiromaleate has been said to show promise in early clinical trial.

5. *Some other compounds of potential value:*
Rifamycin derivative:
Rifamycin inhibits bacterial RNA synthesis by binding to DNA dependent RNA polymerase. No such effect on mammalian polymerases is known.

Studies show that rifamycin inhibits reverse transcriptase of retroviruses at the concentration slightly lower than those blocking cellular DNA dependent DNA polymerase.

Benzimidine:
Enviroxime (2, amino-1-[isopropyl sulfonyl] benzimidazole phenyl-ketone oxime) inhibits rhinovirus.

Derivatives 2 (α-hydroxybenzyl) benzimidazole also called HBB inhibits the multiplication of picorna viruses notable Coxsackie B and ECHO viruses at non-cytocidal concentration in cultured cell but of no value at all *in vivo.*

Guanidine hydrochloride behaves likewise. Drawbacks are:

1. Drug dependence
2. Drug resistance.

AGENTS INHIBITING REVERSE TRANSCRIPTASE

Zydovudine (AZT): Structurally, it resembles thymidine. It is a nucleoside analogue of thymidine having more affinity for reverse transcriptase than natural substrate. Hence binding of natural nucleoside by reverse transcriptase is blocked.

Zydovudine is phosphorylated by cellular kinase and so preferentially bound to the reverse transcriptase during transcription of viral DNA. When zymidine is added instead of thymidine to nascent DNA chain elongation is terminated. Thus, the synthesis of DNA is interrupted immediately after incorporation of zydovudine.

In HIV infection, zydovudine is quite effective therapy. It is more useful in reducing transplacental and perinatal transmission of HIV to neonates. Side effects of this drug are anemia, neutropenia, etc.

Dideoxylnosine (DDC): They act as chain terminators. They are useful in HIV infection that are resistant to zydovudine. Side effects are painful sensorimotor peripheral axonal neuropathy (20 to 30%) and pancreatitis (15%).

Protease inhibitors: HIV protease is essential to the assembly of virion and production of infectious virus particles. The inhibitors HIV protease include saquinavir, ritonavir, indinavir. They inhibit the process polyproteins. They are competitive inhibitors of the HIV. However, use of single drug therapy by protease inhibitors appears to develop drug resistance.

BROAD SPECTRUM NUCLEOSIDE ANALOGUES

1. **Phosphonoformic acid (Foscarnet):** They are classical inhibitors of herpes virus polymerase. It inhibits DNA chain elongation. It is effective in Herpes simplex and cytomegalovirus infections. It also inhibits HIV reverse transcriptase.
2. **Ribavirin:** It has action on cellular enzymes which are important in viral replication by blocking protein synthesis. It is useful in respiratory syncytial virus infection in neonates and infants. It has been used successfully in early stages of Hantavirus infection.

Suramine: This naphthalene derivative is found useful in the treatment of AIDS. It inhibits reverse transcription.

Other upcoming antiviral drugs are AL-721, HPA-23, ddA, ddL, ddC and D4T.

NOBEL PRIZE IN MEDICINE-1988

Professor Gertrude Elion		Discoveries of important principles
Dr George H Hitchings	USA	that have resulted in the development
Sir James Black	UK	of a series of new drugs.

Professor Gertrude Elion and Dr George H Hitchings, both from USA won for their discoveries including trimethoprim, acyclovir (the first successful drug against the Herpes virus), 6 mercaptopurine and thioguanine, two anticancer drugs. Aridothymidine or AZT, a drug used for treating AIDS was developed from their ideas. Thus, their research led to the development of drugs for the treatment of leukemia, malaria, to fight rejection of transplanted organs and AIDS. However, Sir James Black won his share of this Nobel prize for designing the world's first beta blocker propranolol and the first H_2 antagonist.

47 Classification of Viruses

Rapid progress in the field of virology with new information enforces the review and revision of virus nomenclature. Seven or eight schemes of classification for viruses have been produced in the past.

Till 1950 little was known about the viruses. Viruses may affect animals, insects, plants and bacteria. Attempt was made to group or classify the viruses on the basis of their affinity to different systems or organs of the body, e.g.

1 Those producing skin lesion (smallpox, chickenpox, measles).
2. Those affecting nervous system (polio, rabies).
3. Respiratory tract-involving viruses (influenza, common cold).
4. Viruses causing visceral lesions (yellow fever, hepatitis).

It was also suggested that viruses should be classified based on epidemiological criteria. Some of the examples are as under:

1. Enteric virus:
   a. Picornavirus.
   b. Adenovirus.
   c. Reovirus.
   d. Hepatitis virus.
2. Respiratory:
   a. Orthomyxovirus.
   b. Paramyxovirus.
   c. Coronavirus.
   d. Rhinovirus.
   e. Adenovirus.
   f. Reovirus.
3. Arbo (arthropod borne):
   a. Togavirus.
   b. Bunyavirus.
   c. Rhabdovirus.
   d. Orbovirus.

It is not out of place to enumerate the criteria that have been used in forming groups of animal viruses:

1. Type of nucleic acid.
2. Chemical composition (Table 47.1).
3. Susceptibility to physical and chemical changes.
4. Size measurement.
5. Design and construction.
6. Antigenic characters.

Nowadays viruses are classified into two groups depending on the type of nucleic acid they possess; those containing RNA are called ribovirus and those containing DNA are deoxyriboviruses (Fig. 47.1). They may be further classified on the basis of following characters:

i. Strands of nucleic acid.
ii. Symmetry of nucleocapsid.
iii. Presence of envelope.
iv. Number of capsomers.

Further discussion is based on above mentioned characters. Deoxyribose (DNA) viruses are at present placed in five groups and ribovirus (RNA) into nine groups.

Major Groups of DNA Viruses (Fig. 47.1)

1. *Poxvirus:* They are large brick-shaped particle 230 to 300 nm × 200 to 250 nm, visible by light microscope. They may cause smallpox, vaccinia, molluscum contagiosum, cowpox and milker nodes. Examples of pox virus are variola, vaccinia, molluscum contagiosum, avian pox, etc.
2. *Herpes virus:* They are enveloped and icosahedral. They multiply within nucleus. They are covered by ether sensitive envelope. They may cause vesicular skin

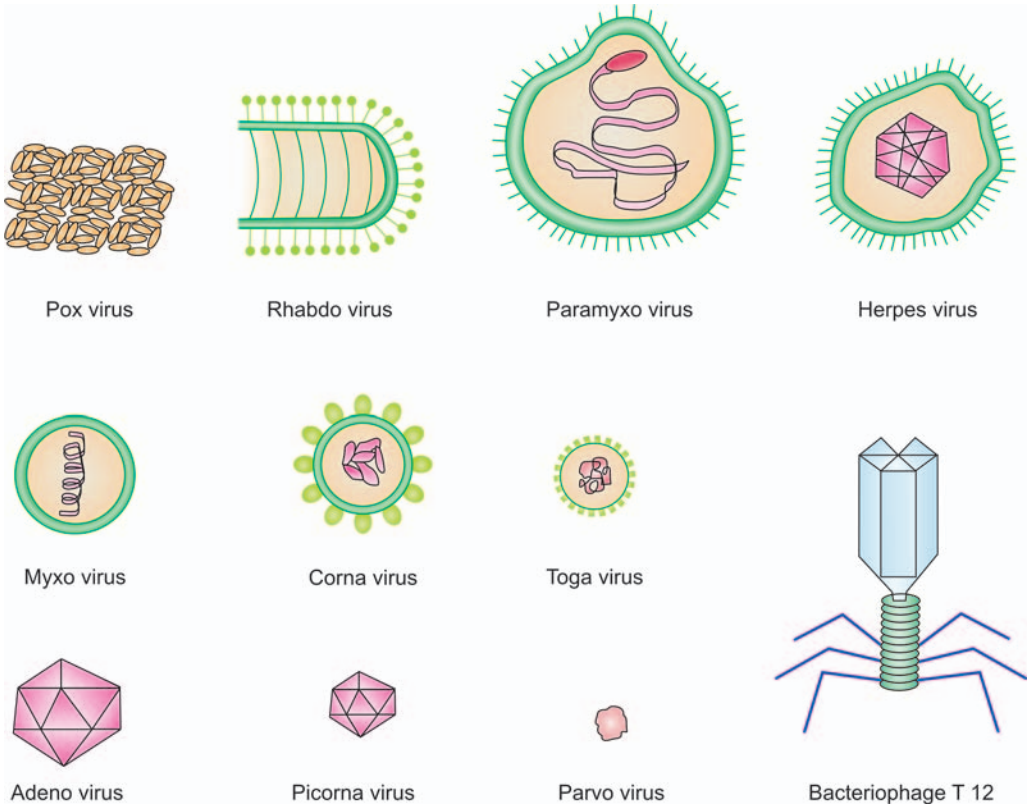


Fig. 47.1: Different types of viruses

TABLE 47.1: Chemical composition of viruses

Family	*Configuration*	*Molecular weight (Dalton)*	*Protein (Polypeptide)*	*Transcription*
DNA:				
Parvo	SS	2	3	–
Papova	DS	3-5	6	–
Adeno	DS	20-25	9	–
Herpetic	DS	100	12-27	–
Pox	DS	160	730	+
RNA:				
Picorna	SS	2-3	4	–
Toga	SS	4	3	–
Bunya	SS	6	3	+
Arena	SS	6	?	?
Corona	SS	9	16	+
Retro	SS	10-12	7-8	+
Ortho	SS	5	7	+
Paramyxo	SS	7	6	+
Rhabdo	SS	4	7	+
Reo	DS	15	7	+

lesions, encephalitis, chickenpox, etc. Examples are Herpes simplex virus, varicella zoster virus, cytomegalovirus, etc.

3. *Adenovirus:* They multiply in nucleus. They are ether resistant. They may cause latent infection of lymphoid tissue, mild respiratory diseases, conjunctivitis, keratitis, etc. Example is adenovirus, etc.
4. *Papova virus:* They are icosahedral, multiply in nucleus and are ether stable. They may cause human warts, papillomata (PA) of rabbits, dogs, etc. polyoma (PO) in mice.

TABLE 47.2: Morphology of virus

Family	Shape	Diameter (nm)	Environment	Symmetry	No. of Capsomers
DNA					
Parvovidae	Spherical	20	–	Icosa	32
Papilloma					
Papova	Spherical	45-55	–	Icosa	72
Polyoma					
Adeno	Spherical	70-80	–	Icosa	252
Herpetic	Spherical	150	+	Icosa	162
Pox	Brick	100 × 240 × 300	–	Icosa	–
RNA					
Picornavidae	Spherical	20-30	–	Icosa	? 60
Toga	Spherical	40-60	+	Icosa	?
Bunya	Spherical	90-100	+	Helical	–
Arena	Spherical	85-120	+	Helical	–
Corona	Spherical	80-120	+	Helical	–
Retro	Spherical	100-120	+	Helical	–
Orth	Spherical of filamentous	80-120	+	Helical	–
Paramyxo	Spherical of filamentous	100-200	+	Helical	–
Rhabdo	Bullet	70-180	+	Helical	–
Reo	Spherical	50-80	–	Icosa	?

Some viruses act as vacuolating agents (VA), e.g. SV_{40}. All are potentially oncogenic. Examples are papilloma virus, polyoma virus, SV_{40}, etc.

5. *Parvovirus:* They are very small 18 to 22 nm in diameter and are ether resistant, e.g. minute virus of mice, Kilhamirat virus (RVM) and adeno satellite virus.

Major Groups of RNA Viruses (Table 47.2)

1. *Orthomyxoviruses:* They are spherical or filamentous, enveloped with lipoprotein, studded with neuraminidase and hemagglutinin subunits. They may cause epidemics and endemics of influenza, etc. Examples are influenza viruses type A, B and C, etc.
2. *Paramyxovirus:* They are similar to myxovirus but are larger and more pleomorphic. They may cause respiratory infections, bad cold, measles, mumps, etc. Examples are parainfluenza virus 1 to 4, measles virus, distemper virus, rinderpest virus, mumps virus, Newcastle virus, etc.
3. *Rhabdovirus:* They are large enveloped, bullet-shaped and ether sensitive. They may cause rabies in mammals and vesicular stomatitis in cattle, etc. Examples are rabies virus, vesicular stomatitis virus, etc.
4. *Togavirus:* They are icosahedral and enveloped by lipid. They require arthropod vectors and may cause meningoencephalitis, lymphadenopathy, bleeding and purpuric rashes, yellow fever, etc. Examples are yellow fever, sindbis and dengue viruses.
5. *Arenavirus:* They are enveloped and ether sensitive causing benign meningitis and encephalitis, e.g. lymphocytic choriomeningitis virus, etc.
6. *Reovirus:* They are ether resistant, naked icosahedral with double stranded RNA causing mild respiratory and enteric diseases.
7. *Picornavirus:* They are small icosahedral, ether and acid resistant causing neuronal damage with paralysis (polio 1 and 3), aseptic meningitis, etc., e.g. polio virus, echo virus, coxsackie virus, rhinovirus, etc.
8. *Leukovirus:* They induce malignant transformation of cells with formation of new antigens and enzymes with loss of contact inhibition, e.g. leukemia, sarcoma in fowls and mice, e.g. Rous sarcoma, murine leukemia, murine mammary tumor virus, etc.
9. *Coronavirus:* They are elliptical or spherical and ether sensitive causing cold and acute respiratory infection, mouse hepatitis, etc. Examples are human, murine and avian virus.

48 Oncogenic Viruses

Viruses that produce tumors in their natural hosts or in experimental animals or induce malignant transformation of cells on culture are known as oncogenic viruses. They may be DNA viruses or RNA viruses (Table 48.1).

DNA Viruses

Papova viridae: They are small double stranded DNA, non-enveloped, 40 to 57 nm in diameter and showing icosahedral symmetry. All members of this family can cause tumor.

Most naturally occurring tumors are benign, i.e. warts in man and animals. *Rabbit papilloma* are initially benign but may become malignant.

Polyoma and *Simian Virus*$_{40}$ (SV_{40}), in nature cause malignant tumors very rarely but when inoculated into newborn rodent they may cause malignant tumor.

Polyoma Virus

Discovery of this virus was made in 1956. It causes a variety of histologically diverse tumors in various parts of the body. Viruses capable of inducing tumors in experimental animals can transform cell *in vitro.* Transformation by polyoma virus is rare event as even input with 1000 pfu per cell, only a minority of cells become transformed.

Though *primary virus* induced change can produce cancerous cell, but more commonly it produces a premalignant cell. Subsequent mutations in the proliferating clone, becoming more malignant or causing abortive transformations in which, transiently *transformed cells resume their normal growth after few generations.*

Neither tumor cells induced by viruses *in vivo,* nor cells transformed in cells culture produced infectious virus, hence infectious DNA cannot be extracted from them. Malignant cell contained 2 types of viral antigen quite distinct from structural proteins of virion (1) T antigen (2) TSTA (tumor specific transformation antigen):

1. T antigen identical with proteins produced in early stages of cytocidal infections.
2. TSTA antigen occurs in cell membrane by transformed cell.

The continued synthesis of these proteins through an indefinite number of cell generation was suggestive of permanent retention of viral genetic material within cell. This was demonstrated by molecular hybridization test. Malignant cell carries several molecules of viral DNA integrated cells chromosomes. The viral DNA is transferred into mRNA specifying at least the viral T and transplantation antigen.

Simian Virus 40 (SV_{40})

Discovered in normal cultures of monkey kidneys cells during production of polio vaccine. Test on baby hamsters showed that it was oncogenic. Complete viral genome is not required for carcinogenesis as happens in polyoma virus. Transformation is increased by the use of UV irradiated SV_{40}, or defective hybrid of SV_{40} and adenovirus. Complete genome of SV_{40} is often present in virus transformed cells and in virus induced tumors as demonstrated by induction of infectious virus production or, as a result of cell diffusion experiments.

TABLE 48.1: Some characteristics of malignant diseases induced by viruses

Virus	*Natural host*	*Malignant diseases tumor*	*Artificial host*	*Malignant cell tumor*	*Transformation of cultured cell*		
					Virus production	*Species*	*Virus product*
Adenovirus	—	—	Baby rodent	Sarcoma	+	Rodent	*
Papova							
Polyoma	—	—	Baby rodent	Sarcoma	+	Rodent	*
SV_{40}	—	—	Baby rodent	Sarcoma	+	Rodent Man	*
Rabbit Papilloma	Rabbit	Papilloma and carcinoma	Rabbit	Papilloma Carcinoma	+	*	*
Herpetovirus							
(i) Marek's virus	Chicken	Lymphomatosis	Chicken	Chicken	*	*	*
(ii) Lucke's virus	Frog	Adenocarcinoma	Tadpole	Adenoma	*	*	*
(iii) Herpes virus samiri	Monkey	Lymphoma	Primates Rabbits	Lymphoma	*	*	*
(iv) EB virus	Man	Lymphoma Nasopharyngeal carcinoma	—		—	Man	*
Herpes simplex 2	Man	Cervical carcinoma	—		*	Man	*
Oncovirus							
Avian	Chicken	Leukosis sarcoma	Chicken Chicken Rodent Monkey	Leukosis sarcoma	+ +	Chicken Chicken	+ +
Murine	Mouse	Leukemia sarcoma	Rodent	Leukemia Sarcoma	+	Mouse Rodent Man	+
Feline	Cat	Leukemia sarcoma	Kitten Dog Rabbit	Leukemia Sarcoma	+ +	Cat Man	+ +
Mammary tumor virus	Mouse	Mammary carcinoma	Mouse	Carcinoma	+		*

(a) Occasional cells spontaneously yield virus.
(b) Virus production can be induced by co-cultivation.
(c) Some sarcoma virus strains are defective and cells of foreign species are non-permissive.
(d) Transformed cells appear natural.
(e) + means viruses are produced.

T and transplantation Ag coded SV_{40} are virus specific. They show no cross reactivity with corresponding antigen of polyoma virus or adenovirus. Temperature sensitive and host cell dependent mutant as well as comprehensive range of adenovirus hybrid have been employed to pin-point the genes responsible for cancer. There is complete map of SV_{40} genome with function of certain genome as well as sites of initiation and termination of DNA replication and transcriptions. Nature of genes that determine cell transformation is not known yet.

Adenoviridae: Adenovirus type 12 produced sarcomas when inoculated in newborn hamster (Trentin 1962). Different adenoviruses of human and animal nature are also oncogenic. Human type, 12, 18, 31 have high oncogenic potential. No evidence of oncogenicity for man has emerged. Cell always contain T and transplantations Ag.

Only 5 percent mRNA recovered from polyribosomes of transformed cell is virus specific. Adenoviral DNA molecules integrated with cell chromosomes are incomplete.

Herpetoviridae: In lower animal evidence of herpetovirus oncogenesis is strong. Oncogenesis seems to be associated with incomplete expression of viral genome.

Herpes virus samiri is closest analogue to human oncogenic herpetovirus isolated from malignant lymphoma in monkey. In Marek disease of fowl etiological agent of malignant lymphoma is herpetovirus so much so that

immunization in flocks of chicken with attenuated live virus vaccine is successful.

Epstein Barr Virus

Half of all malignancies in children (Burkitt's lymphoma) are a result of oncogenic viruses transmitted by insect. In 1964, Epstein recovered EB virus from cultured tumor cells. There are multiple copies of EB virus DNA integrated into the chromosomes of all lines of EB tumor cells. Henle and Henle found EB virus as causative agents of infectious mononucleosis. EB virus causes *Burkitt lymphoma* is supported by the following facts:

1. That EB virus causes infectious mononucleosis resembling early stages of lymphomas.
2. That EB virus causes transformation of lymphocytes *in vitro.*
3. Cases of Burkitt lymphoma show serological evidence of EB virus infection.
4. EB viruses are detectable in Burkitt lymphoma and virion can be recovered after cultivation of cells *in vitro.*

Association of *EB virus and nasopharyngeal carcinoma* is known. EB virus and Hodgkin disease association is also there.

Herpes Simplex Type 2

Association of *Cancer cervix and Herpes simplex type* 2 is there. In malignant cell demonstration of Herpes simplex antigen may be made. Herpes simplex DNA is detected in the chromosomes of malignant cells. UV rays activate Herpes simplex virus type 2 with transformation of cultured cells.

VIRUSES IN HUMAN CANCER

Virus	*Tumor*
DNA viruses	
Epstein Barr virus	• Burkett lymphomas • Nasopharyngeal carcinomas
Herpes simplex	May cause carcinoma cervix
Papilloma virus	• Skin carcinoma • May cause carcinoma cervix
Hepatitis B virus	Carcinoma liver
Hepatitis C virus	Carcinoma liver

Oncogenic RNA Viruses

Only small number of RNA viruses are oncogenic. They are oncogenic under natural conditions and are associated with leukemia in several animals. Oncovirus is the largest subfamily of retroviridae with following 3 groups:

a. *Avian leukosis* viruses.
b. *Murine leukemia* and sarcoma viruses.
c. *Mammary tumor* viruses of mice.

Avian Oncoviruses

Naturally occurring avian leukosis are always transmitted genetically as an integrated DNA provirus transmitted congenitally to produce latent infection. These viruses are related to:

1. *Hemopoietic systems* (lymphomatosis, myeloblastosis, erythroblastosis and osteopetrosis).
2. *Solid tumor.* Best studied variant of avian leukosis virus is Rous sarcoma virus (RSV). RSV produces proliferative foci in the chick fibroblast. All RSV transformed cell are demonstrable malignant cells. Some strains of RSV can transform cultured mammalian cells and produce tumors in newborn.

   In Rous sarcoma virus (RSV) *in vitro* synthesis of DNA provirus occurs.

Murine Oncoviruses

Causative virus of murine leukemia is similar to avian oncovirus in *physical, chemical* and *biological* properties. They produce steady state noncytocidal infection in mouse fibroblast and hence are difficult to detect. Murine sarcoma virus is defective oncovirus capable of multiplication only in presence of murine leukemia virus.

In cultured cell, infection of murine sarcoma virus causes transformation. Cultured cells taken from virus free embryo of high leukemia strain of mice can be *induced to synthesize murine oncovirus by treatment* with certain carcinogens or mutagens.

Mammary Tumor Virus

The mammary tumor virus of mice resembles *leukemia viruses* in several properties but *morphology and morphogenesis* of virus infected cell is different. Mammary tumor virus can be milk transmitted (1936). Genetic transmissions of these viruses in normal mouse can take place.

Feline Oncoviruses

First isolated in 1964, from cat with lymphosarcoma and leukemia. Feline leukemia virus induces leukemia after inoculation into kitten and can be grown in cell culture where it is produced continuously without any cytopathic effect like murine and avian oncovirus.

Oncovirus particle (Sarcoma virus or Fe LV with feline sarcoma virus) agents are causative of solid tumor of cat (fibrosarcoma and liposarcoma). Some preparations of virus induce sarcoma in dogs, rabbits, marmosets, monkeys and also can be transformed to human embryo cells. Fe LV may be more relevant to human cancer virus problem. Cats like humans when subjected to some environmental stress and circumstances develop *leukemia* at same rate.

Evidence points to horizontal transfer in nature of particulate virus that causes leukemia in cats. It suggests endogenous, oncovirus genome copy plays a trivial role supported by *demonstration of excretion of virus into environment by infected cat and transmission by contacts of the infection* and disease in *natural and artificial* closed environments.

Primate Oncovirus

Oncoviruses

There are two distinct classes in non-human primates:

1. *Baboons* carry endogeneous oncoviruses which are vertically transmitted and are otherwise non-oncogenic.
2. *Horizontally transmissible* tumorgenic *oncovirus* shown to induce malignant neoplasm when artificially inoculated into primates. These are oncovirus recovered from sarcoma, lymphomas, leukemias of gibbon, apes or woolly monkeys.

MECHANISM OF VIRAL ONCOGENESIS

The exact mechanism of viral oncogenesis is obscure. However, conclusive evidence exists which suggests particles identified in human neoplasm are causative or contributory agents in these malignancies. Questions to their origin, mode of transmission and their relation to carcinogens, have central issue. However, following hypothesis are suggested:

1. *Provirus hypothesis:* After infection genome of RNA tumor virus is converted into RNA-DNA hybrid by the enzyme reverse transcriptase present in the virus. This RNA-DNA hybrid produces provirus (DNA form of viral RNA) by DNA directed DNA polymerase. This provirus is integrated into host cell. Now this provirus acts as template for viral RNA synthesis and also brings about cell transformation.
2. *Protovirus hypothesis:* Suggests that the regions of DNA in vertebrate cells are transcribed to RNA and then back again to DNA. This process continues and provides mechanism for gene amplification and cellular differentiation. Rarely abnormal events in this mechanism may result in the formation of oncogenic RNA virus genome.

   The hypothesis is supported by the fact that new information (viral sequences) may be detected only in infected and transformed cell which may not be present in uneffected normal cell. The points against the hypothesis are: (i) presence of RNA tumor viral sequences in normal uneffected "C" type particles, (ii) the vertical transmission.
3. DNA oncogenic virus hypothesis suggests integration of viral DNA with host genome. Under its influence the host cell undergoes malignant changes as a result of synthesis of viral coded antigens, i.e. tumor antigen and tumor specific transplant antigen. Virus genome may release the cell from normal regulatory mechanism of morphogenesis such as contact inhibition.

ONCOGENE VIROGENE HYPOTHESIS

Huebner and Todaro (1969) subsequently revised in 1972 and proposed following hypothesis:

Every cell of one or all vertebrates contains DNA copy of genome of oncovirus. Virus specific information is vertically transmitted depending on complex interplay between *host genotype* and *environmental conditions.* Viral

production could be elicited at some stage of life of individuals.

Recent data indicate that most of oncoviruses arising from normal cell are not tumorgenic. Indeed many are xenotropic (incapable of multiplying in the cells of mammalian species from which they were originally induced).

Implication of oncogenic hypothesis, i.e. spontaneous neoplasm is consequence of events within cell shows that naturally occurring tumor of vertebrate does not behave as infectious disease.

This hypothesis is supported by the following facts: (i) presence of viral gene product and genetic information in normal uninfected cell, (ii) by inducibility of C type viruses from many vertebrate cell clones, and (iii) well known vertical transmission of some RNA tumor viruses. This hypothesis does not bear on fact that: (i) some tumors are transmissible by virus, (ii) artificial production of cancer by tumor viruses in laboratory, (iii) oncogenesis by DNA viruses.

Possible Viral Causation of Human Cancer

Human cancer is due to viruses inconsistent with oncogene hypothesis to suggest that all other carcinogens particularly chemical only serve to trigger an endogenesis viral oncogene. Another view is that cancer arises by somatic mutations the incidence of which is known to be increased by hydrocarbons and radiations.

EB is invariably present in Burkitt's lymphoma. Is it an etiological agent or irrelevant passenger? Likewise association of Herpes simplex and cancer cervix is known.

Koch postulates *human tumor cells* are being searched for evidence, viruses coded nonstructural antigen (T Ag), mRNA and *integrated* DNA. All RNA tumor viruses contain a viral RNA-dependent DNA polymerase (reverse transcription). It has stimulated the search for enzymes as well as oncovirus particles in human tumor cells. Oncoviruses recovery from human leukemia is best studied from myeloid cell of patient of acute myelogenous leukemia. Virus grows well in several normal lines of human cells *in vivo.* Resembled oncovirus associated with woolly monkey sarcoma is indicated by nucleic acid hybridization, serological resemblance of reverse transcriptase and other antigen to virion.

Virus is widespread in normal *human beings as all have antibodies against it* and virion can be induced by chemical treatment of various malignant human cell lines. Virus has not been shown to transform human cell to malignant state *in vitro* or *in vivo.*

Prevention of disease by specific viral *vaccine* may offer definitive means whereby *viral etiology cancer will be established.*

NOBEL PRIZE 1989 MEDICINE

Michael Bishop and Harold Varmus University of California San Francisco, USA	Discovery of the cellular origin of viral oncogenes

In their normal form, cellular oncogenes play a role in growth and development by triggering gene expression. Their discoveries suggest that cancer arises from the malfunctioning of normal genes and that damage to genes is likely to be responsible for this.

Present working models in cancer consider tumors as generally due to genetic damage. There are several lines of evidence which support this notion, e.g. hereditary predisposition, occurrence of abnormal chromosomes, deficient DNA repair, the mutagenicity potential of a number of carcinogens, etc. (all showing association with cancers and these all point to defects in the genetic apparatus).

This case is strengthened by Bishop and Varmus' discoveries of cellular genes (protooncogenes) that are involved in normal growth and development which in altered form (oncogenes) in retroviruses can cause tumor growth. The transduction (pick up) of protooncogenes RNA by retroviruses and its incorporation in the latter in an altered form has oncogenic potential. Infection with such virus can set in motion tumorigenic events.

The detection of cellular genes involved in normal growth and development through viral oncogenes is a big step forward in the understanding of cancer. These oncogenes can

now be used as tools and introduced into experimental animals to see their sufficiency individually or in combination in producing cancer. It seems also a good hypothesis that hormones growth factors act as signals for gene expression. Discovery of proto-oncogenes "C"-Jun and "V"-Jun belonging to a multi-gene family which in cooperation with FOS gene and its product through the formation of so called leucine zippers apparently start gene expression at specific sites on DNA.

The work of Bishop and Varmus has provided not only new insights in our understanding of cancer but also opened the way for the study of normal growth and development as it appears that several of these cellular protooncogenes are involved in these normal processes.

49 DNA Viruses

POXVIRUS

They are the largest and most complex viruses of vertebrates. They are DNA viruses. Pox viridae family is divided into following groups on the bases of antigenic reactions and morphological differences:

Group I (Viruses of Mammals)

1. Variola
2. Vaccinia
3. Cowpox
4. Ectromelia
5. Rabbitpox
6. Monkeypox

Group II (Viruses of Birds)

1. Fowlpox
2. Turkeypox

Group III (Tumor Producing)

1. Myxoma
2. Fibroma

Group IV (Miscellaneous)

1. Contagious pustular dermatitis
2. Milker nodule
3. Bovine pustular stomatitis.

Morphology

It is brick-shaped measuring 300 × 200 × 100 nm. It consists of central biconcave DNA core. It is covered by: (a) inner coat adhered to nucleoprotein, and (b) outer irregular layer. On either side of nucleoid is oval structure called lateral body (Fig. 49.1).

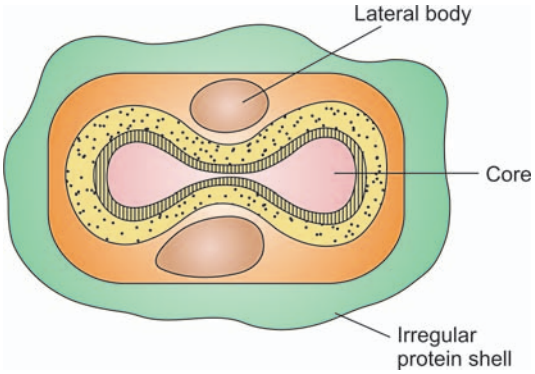


Fig. 49.1: Structure of pox virus

In dry state virus may remain infective at room temperature for one year. In moist state virus can be destroyed at 60°C in 10 minutes. Acid may destroy the virus in hour time (pH 3 to 5). They are susceptible to ultraviolet light, formalin and oxidizing agents.

Antigenic Properties

There are about 8 antigens demonstrated by precipitations in gel. Some of them are:

1. *LS antigen:* It has 2 components: heat labile (L) and heat stable (S). Antibodies to L-S antigen is not protective in any way. They are responsible for flocculation, precipitation and complement fixation reaction.
2. *Agglutinogen:* This is responsible for agglutination with specific antiserum.
3. *Nucleoprotein antigen* (NP): It is responsible for neutralization of infectivity and acquired immunity. However, NP antigen is common to poxviruses.
4. *Hemagglutinin:* It is lipoprotein complex with a molecular weight of 100,000 to 200,000. It

is heat stable and does not exhibit receptor destroying activity. Antibodies to this antigen are not protective.

5. Protective antigen has been isolated during early stage of virus replication. Their role in immunity has not been proved so far.

Cultivation: They can be cultivated on chick embryo (11 to 13 days old), tissue culture (monkey kidney, He La and chick embryo cell), animals (monkey, calves, sheep and rabbit).

SMALLPOX (VARIOLA MAJOR)

It is infectious disease manifested as skin lesion (single crop) which are macular to start with and subsequently may pass through papular, vesicular and pustular stages in 10 to 12 days. There may be systemic involvement.

Pathogenesis

Variola viruses may enter through mucosa of upper respiratory tract. Virus may propagate in regional lymph nodes. They are transported through bloodstream to reticuloendothelial cells. There virus multiplication occurs. From here viruses are again thrown into bloodstream and then they settle at skin and the mucosa with the start of clinical disease. There is formation of macule, papule, vesicle and pustule. Fever at pustular stage may be because of absorption of necrotic cell debris from skin. Since smallpox gives increased degree of protection hence there is no recurrence of disease.

Clinical Features

The incubation period is about 12 days. The pre-eruptive phase is characterized by malaise, fever, vomiting, and headache. After 2 to 3 days skin rashes start appearing. Single crop of eruptions appears on the 3rd to 5th days of onset of illness. The lesion first appears on the buccal mucosa (exanthem) which may develop into macule, papule and vesicle. Vesicle is associated with virus shedding through oropharyngeal secretions (infective stage). There may be conjunctivitis. Corneal exanthems may become the cause of blindness.

Scab starts forming after 12 days and crusts start separating after 3 to 6 week of the onset of disease leaving behind scars. In pre-eruption phase the disease is non-infective but virus can be isolated from blood up to 2nd day of fever. From 6 to 9 days saliva becomes infective, may be because of ulceration of lesions in mouth. In vesicular stage first broken skin lesion and later dried scabs appear.

Laboratory Diagnosis

It is significant especially in non-endemic areas or in areas from where disease has been eliminated. A case identified as smallpox must be informed to health authorities so that proper measures could be undertaken promptly. It becomes still more important when smallpox is declared eradicated from all over the world.

Collection of Specimen

A special kit has been designed by WHO for collection of specimen containing:

1. Hagedorn needle.
2. Clean and sterile slides.
3. Clean Pasteur pipettes.
4. A labeled screw capped container.
5. A double container of metal or wood for dispatching samples to the concerned laboratory.

We can collect specimen like maculopapular, pustular, crusting stage lesions. In special cases blood may be collected in pre-eruptive stage.

Demonstration of Virus

1. By light microscope by demonstrating Guarnieri bodies (Fig. 49.2).
2. By electron microscope.
3. Viral antigen may be demonstrated by serological techniques like complement fixation, hemagglutination, immunofluorescent and convenient and routinely used precipitation in gel (PIG).
4. Isolation of viruses from throat, washing, skin lesions and blood. Specimen may be cultured on chorioallantoic membrane of chick embryo.

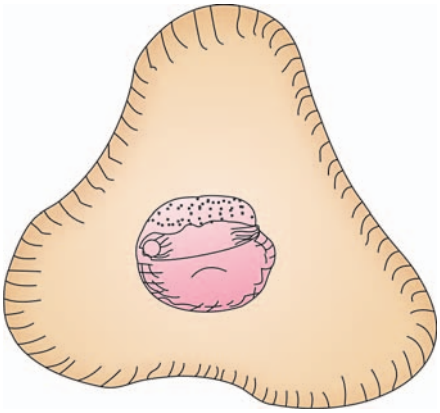


Fig. 49.2: Guarnieri bodies

Demonstration of Antibody

Retrospective diagnosis may be made by demonstration of antibodies rise by testing paired sera. In smallpox convalescent's PIG test is useful for the retrospective diagnosis of smallpox.

Prophylaxis

Some of the preventive measures include immunization and chemoprophylaxis.

Vaccination

There are three strains available for vaccine production:

a. Elstree (Lister Institute)
b. EM_{63} (Moscow)
c. New York Board of Health strain.

Commercial vaccine may be prepared by inoculating vaccinia virus on scarified skin of calves, sheep and buffalo. Alternatively virus may be cultured in bovine cells or CAM of chicken embryo. Vaccine thus prepared is stored and dispensed in liquid form or in freeze-dried form.

Method of Vaccination

It consists of introducing intradermally sufficient live vaccinia viruses. Recommended sites are outer aspect of upper arm at the insertions of deltoid muscles. The methods includes multiple puncture with bifurcated needle and multiple pressure with a sharp needle.

Response of Vaccination

There are 3 types of response:

1. *Primary reaction:* This is manifested 3 to 4 days after inoculation as papule which rapidly becomes vesicle and it enlarges with secondary erythema. On 8th to 9th day center is depressed with turbid contents. It is also associated with axillary lymphadenopathy and fever.

   On 10th day pustule dries up and scab is formed which separates in a week's time. Immunity appears after 10th day and persists for years.
2. *Accelerated reaction (vaccinole):* It occurs in case of limited residual immunity from previous vaccination. It is more rapid than primary reactions. Vesiculation appears with maximum intensity between 3 to 7 days after vaccination. It enhances waning immunity.
3. *Immediate reaction:* It occurs in immune cases. It is most marked on 2nd to 3rd day as papule. It is hypersensitivity response. Immediate response neither indicates level of immunity nor it leads to immunity.

Complication of Primary Vaccination

1. Post-vaccinal encephalitis may occur which may be due to activation of latent infection, neuro-allergy, and interaction of vaccinia virus. Encephalitis may occur 10 to 12 days after vaccination. The mortality is about 50 percent.
2. Vaccinia gangrenosa in which primary lesion fails to heal and extend slowly with loss of tissue. Fresh vesicles may appear with ulceration of nasopharynx. This is associated with abnormality of immune response and is usually fatal.
3. Abortion may occur due to intrauterine infection of fetus. Hence, it is contraindicated in pregnant women.

Since 1977 when last case was reported in Somalia, smallpox has been eradicated worldwide, hence vaccination is unjustified in view of its several complications.

MOLLUSCUM CONTAGIOSUM

It is human disease with multiple discrete nodule 2 mm size, limited to epidermis and

occurs anywhere in the body except palm and sole. Each lesion at its top carries small opening having white core. The incubation period is 19 to 50 days. Transmission is through abrasion and swimming pool. It is very uncommon and involves children and adults. Its transmission in animals is not successful.

Cowpox: It occurs in cattle as ulcer of teats and contiguous part of udder. Lesions appear also in the hands of man. It produces hemorrhage in chorioallantoic membrane of chick embryo and rabbit skin. Vaccinations with vaccinia virus protects human beings.

Milker nodules: It occurs in hands of man from lesions of teats and udder. Warty, non-ulcerating nodules on hand and arm are caused by poxvirus of ORF subgroup. Regional lymph nodes are enlarged. Immunity in man does not last long and second attack may occur after few years.

ORF: Infection of man occurs with virus of contagious pustular dermatitis of sheep. There is single lesion of hand, forearm or face, a slowly developing papule which become flat and vesicular and ultimately heals without scarring. The disease occurs by handling of sheep. There is no infection to man.

Yaba and tanapox: Yaba is benign tumor under natural condition in monkeys of African countries. Laboratory worker handling these animals may develop similar lesions.

Tanapox is isolated from solitary skin lesion in Kenyans. Patient looks quite ill. Perhaps, it is derived from monkey by insect transmission.

ADENOVIRUSES

It is non-enveloped DNA virus with diameter 70 to 90 nm and are spherical. It has icosahedral symmetry. It is relatively stable between 4° and 36°C and can be stored in frozen state. It is heat labile destroyed at 56°C within minutes. It resists ether and bile salts.

It is host specific. Human adenoviruses grow only in tissue cultures of human origin, e.g. human amnion, HeLa or HEp. Cytopathogenic changes include rounding of cell and aggregations into grape-like clusters. Intranuclear inclusion may be demonstrated by staining (Fig. 49.3). Human adenovirus may produce undifferentiated sarcoma in 30 to 90 days when inoculated in newborn hamster.

Adenovirus can be classified into two subgroups based on their ability to agglutinate rat and monkey erythrocytes. Around 33 serotypes are identified of adenovirus infecting man. They cause self-limited infection of respiratory tract, eye and may be intestine. It may cause pharyngitis and tonsillitis (types 1 to 5), pneumonia (types 4 and 7), acute respiratory disease (types 4, 7 and 21), pharyngoconjunctival fever (types 3 and 7), epidemic keratoconjunctivitis (type 8), acute follicular conjunctivitis (types 3 and 7a), intestinal lesions causing gastroenteritis and obesity (type 36). They may cause oncogenesis in hamster (types 12, 18 and 31).

Laboratory diagnosis may be established by isolating virus from throat, eye or feces. The specimen may be inoculated on A 549 cell line (American type culture collection USA) tissue cultures like HeLa, HEP-2, and then noting the cytopathic effects. Serological techniques like complement fixation, hemagglutination, hemagglutination inhibition and neutralization are useful.

a. *Adenovirus* SV_{40}: Monkey kidney tissue culture, used in identification of adenovirus, may be contaminated with SV_{40} virus. They do not produce cytopathic effect on rhesus monkey kidney. Hence, both the viruses replicate and produce mature viral particles. In the process we

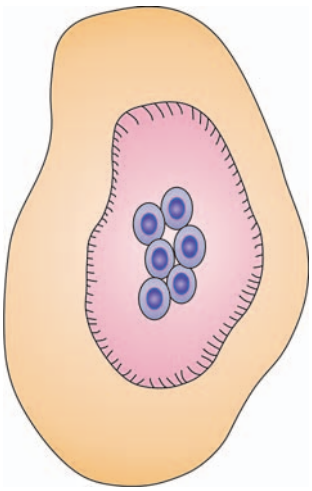


Fig. 49.3: Cowdry bodies (intranuclear)

may get viral particle containing genome of SV_{40} and capsid of adenovirus, which may be called hybrid virus.

b. *Adeno associated virus:* It is defective virus because it does not replicate in the absence of adenovirus. It is non-pathogenic and antigenically different from adenovirus. It is DNA virus about 20 nm. It is also known as adeno-satellite virus.

Herpes Virus (Fig. 49.4)

It is double stranded DNA viruses about 100 to 150 nm in size, lipid enveloped and sensitive to ether and chloroform. It replicates in the nucleus of host cell. It produces intranuclear eosinophilic inclusion bodies. It does not possess common antigen. Examples are:

1. Herpes simplex types 1 and 2.
2. Varicella.
3. Herpes zoster.
4. Cytomegalovirus.
5. EB virus.

1. *Herpes simplex:* It may produce mild vesicular eruption in skin or mucous membrane. It has two types; type 1 strain causes infection of mouth, eyes, central nervous system, etc. and type 2 strain causes infection of genitals. Type 1 and type 2 may be differentiated antigenically also.

   It may be cultured on chorioallantoic membrane producing typical white, shining, non-necrotic pocks. Pocks are less than 0.75 mm in type 1 and more than 1 mm in type 2 strain. They can also be grown on rabbit kidney, HeLa, HEp-2 or human amnion tissue culture. They may produce experimental infection in animals like rabbit, mice, etc.

   It is one of the most common infection of man. Only man is the natural host and may produce illness like herpes labialis, eczema herpeticum, keratoconjunctivitis, and meningoencephalitis. The herpes type 2 is venereal infection and produce genital herpes and neonate herpes. The virus may remain latent for years perhaps in sensory nerve ganglia and get reactivated.

   Laboratory diagnosis is established by isolation of virus from infected material which may be inoculated on chorioallantoic membrane of chick embryo where we get typical pocks. They may also be isolated in rabbit kidney and human amnion tissue culture where typical intranuclear inclusion bodies appear. The isolate may be confirmed by neutralization tests. Serological tests like neutralization and complement fixation tests are also useful for its diagnosis.

   For herpetic keratoconjunctivitis, 5-iodo-2 deoxyuridine is beneficial.

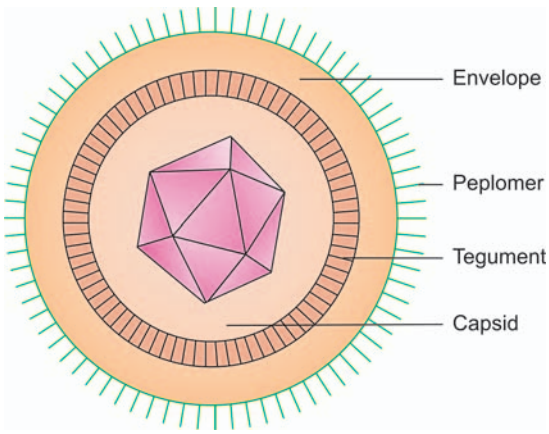


Fig. 49.4: Morphology of herpes virus

2. *Varicella:* Morphology of this virus is identical to that of herpes simplex. It causes chickenpox which chiefly affects children. It is characterized by successive crops of eruptions on skin and mucous membrane. High fever is also there. The source of infection is chickenpox patient or herpes zoster patient. There are no animal reservoirs. Portal of entry is respiratory tract. Incubation period is 7 to 23 days. Chickenpox is usually uneventful disease with complete recovery. Sometimes complications may occur like secondary bacterial infection, e.g. rash, encephalitis and pneumonia. One attack confers life-long immunity.

   The virus does not grow on animal and chick embryo, but may multiply in human embryonic tissue culture producing intranuclear inclusion bodies (Cowdry type A). Laboratory diagnosis may be established by examining smear from vesicle. Giemsa stain shows multinucleated giant cells and

intranuclear eosinophilic inclusion bodies. Virus may be isolated on human tissue culture. If vesicle fluid is examined under electron microscope we may see typical particles of herpes virus. Serological techniques like agar gel precipitation test, complement fixation and neutralization tests may be useful. A fluorescent antibody technique is also helpful for detection of varicella antigen.

Following varicella vaccines (given at 15 months of age) are available:

i. Takahashi live attenuated vaccine (not used because of oncogenicity and possibility of herpes zoster in later life).
ii. Formalin killed vaccine.
iii. Live attenuated OKA (varicella strain) vaccine after prolonged field trial is found quite effective. It is indicated for prevention of chickenpox in immunocompromised children including those with hematological cancers or solid tumor. This vaccine is being evaluated for routine immunization of healthy children.
iv. Development of subunit or recombinant vaccine may eliminate the risk of herpes zoster.

Convalescent sera from herpes zoster patients contain much more levels of antibody than serum from varicella convalescents. Hence, the administration of this sera may confer protection to contacts of chickenpox patients.

3. *Herpes zoster:* It is the disease of old age still it may occur at any age even newborn are not spared. The disease is characterized by appearance of skin eruptions over the distribution of sensory nerves.

The portal of entry is unknown but in some cases varicella virus becomes neurotropic and is localized in nerve cells. From here infection spreads along posterior nerve root fibers with formation of vesicles in the segment of skin supplied by that particular nerve. The rash is usually unilateral. The commonest sites are areas innervated by spinal cord segments T_3 to L_2 and trigeminal nerve mostly ophthalmic branch. The rash disappears in 2 weeks but pain and paresthesia may persist for months together. In some cases lower motor neuron paralysis with meningoencephalitis and generalized zoster may occur.

Laboratory diagnosis is like that of varicella infection. Varicella and zoster viruses appear identical antigenically. It appears that the same virus in children during primary infection produces varicella (generalized infection) and when adults are involved they develop zoster (localized infection).

4. *Cytomegaloviruses:* They are indistinguishable from other viruses of herpes group. They may infect man, monkey, guinea pig, etc. Characteristically they cause enlargement of infected cell with acidophilic or basophilic intranuclear inclusion body. Human strains are antigenically heterogeneous. They can be grown on human fibroblast cultures. Since cytopathic effects are slow in appearance they require prolonged incubation.

It may cause subclinical localized infection of salivary glands, kidney and rarely generalized disease in infants. The infection can be transmitted by urine, saliva droplets via respiratory route and through placenta from infected mother to fetus. The generalized infection is associated with hepatosplenomegaly, jaundice, thrombocytopenic purpura, hemolytic anemia and microcephaly. Apart from this there may be chorioretinitis and cerebral calcification. Sometimes syndrome resembling infectious mononucleosis may occur. It may lead to insidious hepatitis or pneumonia.

Laboratory diagnosis is established by isolating the virus from throat swab, urine and various affected organs. This material is inoculated on human fibroblast tissue culture and after 1 to 6 weeks characteristic cytopathic effects appear with presence of inclusion body. Serological diagnosis like complement fixation and neutralization may be helpful. Histological study of various organs may show large swollen cell with inclusion bodies (intranuclear) suggesting cytomegalovirus infection.

5. *Epstein Barr virus:* It is indistinguishable from other viruses of herpes group. It has the affinity for lymphoblastoid cells. There

is now strong evidence that infectious mononucleosis is caused by Epstein Barr virus (EB virus).

To establish diagnosis blood examination shows leukopenia in early stages followed by leukocytosis. The abnormal mononuclear cell with basophilic vacuolated cytoplasm and kidney shaped nucleus with fenestrated chromatin is seen. By electron microscope examination EB virus may be identified. Serological tests like Paul Bunnel, complement fixation, immunofluorescence and gel diffusion may be useful.

Human Herpes Virus Type 6

- First isolated in 1986 from peripheral blood leukocytes from patients of lymphoproliferative disorders. Reported to be wide spread in UK, Japan, USA, etc.
- Although previously called B-lymphotropic virus, now identified as primarily T-lymphotropic.
- Two genetically distinct variants are recognized and are HHV-6A and HHV-6B).
- HHV-6 frequently develops during infancy.
- HHV-6 can cause exanthem subitum, febrile seizures without rash during infancy, etc.
- In older age HHV-6B has been associated with mononucleosis syndromes, focal encephalitis, pneumonitis, disseminated disease.
- HHV-6A has not been associated with disease.
- Virus is transmitted through saliva and probably by genital secretion.
- There is no established treatment or vaccine available for this virus.

Human Herpes Virus Type 7

- Isolated in 1990 from T-lymphocytes of healthy man from peripheral blood.
- The virus was subsequently isolated from other persons too.
- Virus is acquired during childhood and present in saliva of healthy persons.
- Some cases of exanthem subitum may be associated with this virus.

Human Herpes Virus Type 8

- Unique herpes virus like DNA sequence reported in 1994-95 in tissue derived from Kaposi's sarcoma and body cavity based on lymphoma in AIDS patients.
- HHV-8 isolation in cell culture may define its role in disease.
- They are partially homologous to the DNA of Epstein Barr virus, herpes virus samiri of squirrel monkeys.
- These herpes virus like DNA sequences have also been reported from Kaposi's sarcoma tissues sarcoma from non-AIDS patients, in a subgroup of AIDS related B-cell body cavity based lymphoma, and in brain tumor some proliferative skin lesions of organ transplant recipients.
- These DNA sequences have also been seen in semen of both AIDS and non-AIDS patients.

Initially discovered by molecular biology techniques in Kaposi's sarcoma, this new herpes virus has been isolated in Kaposi's sarcoma using cell cultures. This virus is associated with 3 conditions, i.e. Kaposi's disease, B-cell lymphoma and Castleman's disease.

50 RNA Viruses

PICORNAVIRUSES A

They are very small, 20 to 30 nm in size, non-enveloped and resistant to ether. Picornavirus group of medical importance includes:

A. Enteroviruses
B. Rhinoviruses

Enteroviruses

From the medical point of view important viruses of this group are polio, echo and coxsackie viruses. Enteroviruses are stable, and resistant to bile and ether. They remain unharmed in water and sewage for quite a long time. They are described as under:

a. *Poliovirus:* They are 30 nm diameter with capsomere arranged in icosahedral symmetry and are spherical. They are resistant to ether, chloroform and bile. They survive in low pH and low temperature. They are killed by formaldehyde, cholinination and lyophilization. By neutralization poliovirus strains are classified into types I, II and III. Type I is the commonest and is responsible for epidemic. Natural infection occurs only in man.

   The virus enters body by ingestion or inhalation. The virus multiplies in lymphatic tissue of alimentary canal (from tonsils to Peyer's patches) entering regional lymph nodes and then viruses are carried to bloodstream. From here viruses are taken to spinal cord and brain. They destroy neurons with degeneration of Nissl body. Lesions are mostly in anterior horn of spinal cord. Sometimes we may find extensive lesions like encephalitis. The incubation period is about 10 days with range from 4 days to 4 weeks.

   Laboratory diagnosis is made by isolation of viruses from throat (early stage) and feces (throughout the course of disease). After processing specimen is inoculated into tissue culture and virus growth is indicated by cytopathic effects in 2 to 3 days. Identification of virus should be interpreted along with clinical picture. Serodiagnosis is not of much use, still complement fixation and neutralization test may be valuable.

   Immunization is achieved by using vaccine. Salk killed polio vaccine is formalin inactivated consisting of 3 types of polioviruses. It gives 80 to 90 percent protection against paralytic poliomyelitis. Killed vaccination is given by injection. On the other hand, Sabin live polio vaccine is also available and is prepared by growing the attenuated strain in monkey kidney cells. Live vaccine is easy to administer as it is given orally, much more economical, single dose gives lifelong immunity and gives local immunity in the intestine.

b. *Coxsackieviruses:* They are called coxsackievirus as first of all they were isolated from patients coming from the village of coxsackie in New York. They are classified into group A and B. By neutralization method group A viruses are divided into 24 types. Characteristically the viruses have the ability to infect suckling mice and not the adult mice. All group B viruses grow on monkey kidney tissue culture and some group A viruses grow in HeLa cells.

They may cause vesicular pharyngitis (group A), aseptic meningitis (groups A and B), minor respiratory infections (A_{21}), Bronholm disease manifested as stitch-like

pain in abdomen and chest (group B), myocarditis (group B) and pericarditis (group B).

The laboratory diagnosis may be made by isolating the viruses from lesion or feces by inoculation in suckling mice. Since there are several antigenic types so serodiagnosis is not feasible.

c. *Echoviruses:* Their description designation is enteric cytopathogenic human orphan viruses (ECHO viruses). They are classified into 33 serotypes. They infect man naturally. They are not pathogenic to laboratory animals.

They may produce fever with rash and aseptic meningitis (types 4, 6, 9 and 16). Laboratory diagnosis is by inoculating feces, throat swab or CSF on monkey kidney tissue culture and virus growth is detected by cytopathogenic changes.

Newer Enteroviruses (68 to 72)

Four types of viruses, i.e. 68, 70, 71, 72 associated with diseases of man as under:

68	Pneumonia
70	Acute hemorrhagic conjunctivitis
71	Mumps
72	Hepatitis A.

Rhinoviruses

They differ from enteroviruses in being more acid labile and heat stable. They have been classified into over 100 types and immunity is type specific. Depending upon growth in tissue culture, rhinoviruses are classified as H strains (grow only on human cells) and M strains (grow equally well on human as well as monkey cells). Because of too many serotypes (over 100) it is impossible to make ideal vaccine. However, antiviral chemotherapy may be helpful in bringing specific control.

ORTHOMYXOVIRUSES

It includes the enveloped RNA viruses capable of adsorbing on to mucoprotein receptor on erythrocytes. This results in hemagglutination. They are 80 to 120 nm in size and spherical in shape. Influenza virus represents this group.

INFLUENZA VIRUSES

They are responsible for infectious disease of respiratory tract occurring mostly in epidemic and pandemic forms. The classification of influenza virus into 3 (A, B and C) is based on the antigenic nature of ribonucleoprotein.

Influenza virus is spherical with diameter 80 to 120 nm. The virus has ribonucleoprotein in helical symmetry. Single stranded RNA genome is segmented and nucleocapsid is surrounded by envelope having virus coded protein layer and lipid layer derived from host cell. Attached to lipid layer are hemagglutinin spikes and neuraminidase peplomers (Fig. 50.1). The virus is inactivated at 50°C for 30 minutes, ether, formaldehyde, phenol and salts of heavy metals.

The characteristic feature of influenza virus is its ability to undergo antigenic variation. Depending on degree antigenic variation may be classified as (Table 50.1):

i. Antigenic shift (abrupt, drastic, discontinuous variation in antigenic structure causing major epidemic).
ii. Antigenic drift (gradual changes in antigenic structure regularly, resulting in periodical epidemic).

The virus grows in amniotic cavity and allantoic cavity of chick embryo. It is detected by appearance of hemagglutinin in allantoic and amniotic fluid. They are also grown in monkey kidney cells. Route of entry is respiratory tract. The viral neuraminidase facilitates infection by reducing the viscosity of mucus lining and exposing the cell surface receptor for virus adsorption. These cells are damaged and shed, laying bare the cells in trachea and branchi.

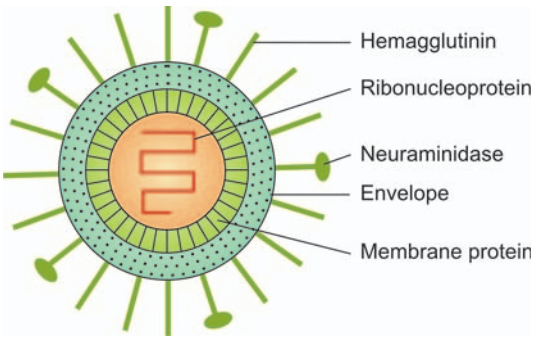


Fig. 50.1: Structure of influenza virus

TABLE 50.1: Emergence of antigen subtypes of influenza A associated with pandemics or epidemic diseases

1889-90	H_2N_8	Severe pandemic
1900-03	H_3N_8	Moderate epidemic
1918-19	H_1 N_1 (formerly Hsw N_1)	Severe pandemic
1933-35	H_1N_1 (formerly H_0N_1)	Mild epidemic
1946-47	H_1N_1	Mild epidemic
1957-58	H_2N_2	Severe pandemic
1968-69	H_3N_2	Moderate pandemic
1977-78	H_1N_1	Mild pandemic
1988-1989	H_1N_1/H_3N_2	Have circulated either in alternating years or concurrently
2009	H_1N_1(Swine flu)	Pandemic April 2009

The incubation period is 1 to 3 days. The onset is abrupt with fever, headache, generalized myalgia and prominent respiratory symptoms. If no complication follows the disease resolves in 2 to 7 days. Complications include pneumonia due to bacterial superinfection, congestive heart failure and encephalitis. Reye's syndrome is associated with influenza B virus.

Diagnosis in the laboratory is established by demonstration of virus antigen (immunofluorescence), isolation of virus (chick embryo or monkey kidney cell culture), serology (complement fixation test, hemagglutination inhibition test) and radial immunodiffusion tests in agarose gel (screening test).

Influenza vaccine is in use. Vaccine may be prepared by growing virus in allantoic cavity and inactivating the virus with formalin. Because of presence of egg protein this vaccine may cause allergic reactions. This difficulty is removed by preparing subunit vaccines (virus treated with ether). The other vaccines in use are: (i) recombinant live vaccines obtained by hybridization between its mutants of established strain, (ii) new antigenic variant, a neuraminidase specific vaccine, and (iii) a live vaccine using temperature sensitive (TS) mutant, etc.

Antiviral drug amantidine hydrochloride which inhibits adsorption of virus to cell is useful in influenza infection. Combined yearly vaccination of persons at high risk, using the best mix of important antigens and administration of amantidine at time of stress, e.g. surgery or hospitalization, etc. is suggested.

PARAMYXOVIRUSES

They are larger and more pleomorphic than orthomyxoviruses. They possesses hemagglutinins, neuraminidases and hemolysin. They are antigenically stable. This group includes viruses like mumps, parainfluenza, respiratory syncytial and measles.

Mumps

It is responsible for acute infectious disease characterized by parotitis. The name mumps is derived from mumbling speech of patients.

The virus is spherical varying from 100 to 250 nm. The envelope has hemagglutinins, a neuraminidase and hemolysin. The virus can be grown on yolk sac or amniotic fluid of chick embryo, and human or monkey kidney cell culture. They are inactivated at room temperature, ultraviolet light or by chemicals like formaldehyde and ether. Two complement fixing antigens have been identified as soluble (S antigen) and viral (V antigen).

Infection may be by inhalation and through conjunctiva. Incubation period is 18 to 21 days. Clinical symptoms start with sudden nonsuppurative enlargement of parotid glands. Skin over the enlarged parotid glands may be stretched, red and hot. Viremia may be responsible for the involvement of other organs. Orchitis and viral meningoencephalitis are important complications of mumps. The pancreas, ovary, thyroid and breast may be involved. However, it is important and most common cause of aseptic meningitis.

Diagnosis is confirmed by isolation of virus from saliva, CSF or urine. For this purpose amniotic cavity of chick embryo or monkey or human kidney cell culture may be used. Serological test like complement fixation, hemagglutination inhibition and neutralization tests may be helpful. Skin test is not very useful but still it can be used to detect susceptible patient.

Mumps infection confers life long immunity. Normal human gamma globulin prepared from mumps convalescent serum appears useful for prophylaxis.

For active immunization killed vaccine (virus grown in allantoic cavity), Jeryl-Lynn strain (live attenuated vaccine) and now live vaccine is available which can be sprayed into mouth without any side effect.

Respiratory Syncytial Virus

Although these viruses resemble paramyxoviruses structurally but they do not have either hemagglutinin or neuraminidase. They are antigenically stable and grow on HeLa cells, HEp-2 and in monkey kidney cells. They are responsible for bronchiolitis and pneumonia. In adults, it may cause afebrile rhinitis and in aged persons it may cause exacerbation of bronchitis.

For diagnosis, nasal and pharyngeal secretion are inoculated in human (He La, HEp2) or monkey kidney cell culture. It takes 5 to 14 days' time. Rapid diagnosis may be made by immunofluorescent technique. Serological techniques like complement fixation and neutralization test may be useful. No vaccine is available at present.

Parainfluenza Viruses

They may produce febrile respiratory infections throughout the year. They possess hemagglutinin, neuraminidase and hemolysin. They grow well in human or monkey kidney cell culture. Growth in chick embryo is poor or absent. They are inactivated by heat and by ether. They are classified into four groups: Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4.

Parainfluenza viruses are responsible for about 10 percent respiratory infection in children. Types 1 and 2 cause croup which is a serious clinical disease. Type 3 causes lower respiratory infections and type 4 causes minor respiratory infections.

Measles

It is highly acute infectious disease characterized for generalized maculopapular rash proceeded by fever, cough, nasal and conjunctival catarrh, etc. (Fig. 50.2).

The viruses possess hemagglutinin and no neuraminidase. They do not grow in eggs but may grow on human embryonic kidney or amnion cell cultures. The virus's core may be inactivated by heat, ultraviolet light, ether and formaldehyde. They are antigenically homogeneous.

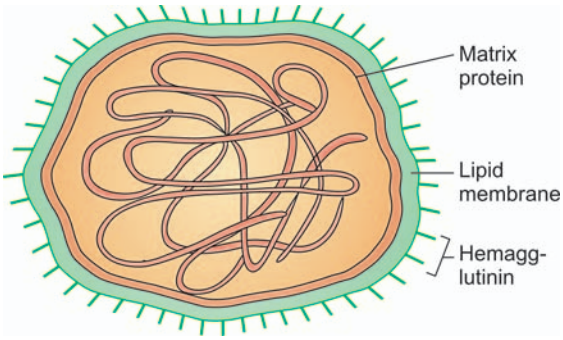


Fig. 50.2: Measles virus

Incubation period is 10 to 12 days. Infection manifests as fever and respiratory tract involvement. At this stage Koplik spots may be seen on buccal mucosa and 2 to 4 days later rash appears. Uneventful recovery occurs in most of the patients. In small number of cases complications like croup or bronchitis, secondary bacterial infection, giant cell pneumonia and meningoencephalitis may occur. Very rarely we may have late complication like subacute sclerosing panencephalitis (SSPE).

The diagnosis may be established by isolating the virus from nose, throat, conjunctiva, blood and urine. Primary human embryonic kidney and amnion cells are quite useful. Rapid diagnosis of virus growth is possible by immunofluorescence. However, smear can be prepared from nasal, pharyngeal and conjunctival secretion and examined microscopically after staining with Giemsa's method for presence of giant cells and inclusion bodies (Cowdry type A). Serological techniques like complement fixation test, neutralization, and hemagglutination inhibition may be useful for establishing diagnosis of measles.

Normal human gamma globulin if given within 6 days of exposure can prevent disease. A formalin inactivated vaccine against measles proved not of much use. Live attenuated vaccine is developed using Edmonston B strain. This vaccine can be given in combina-

tion with mumps and rubella vaccines (MMR). The other live attenuated vaccines are Schwartz and Mortin strain and Backham 31 strain.

RUBELLA VIRUS

It is an enveloped RNA virus causing rash and lymphadenopathy (posterior and suboccipital) in children. In adults, there is involvement of joint and purpura. Infection in early pregnancy may lead to developmental defects in fetus (embryopathy).

The virus is pleomorphic, spherical, 50 to 70 nm in diameter and enveloped. It has one type of antigen (hemagglutinin). It is heat labile and inactivated by ether and chloroform. Rubella virus can be grown in primary African green monkey kidney tissue cell lines (VERO, RK 13), human amnion and thyroid tissue culture. The presence of virus is detected by interference test.

It enters the body by inhalation and replication of virus occurs in cervical lymph nodes. After incubation period (2 to 8 weeks) viremia occurs which lasts till rash, fever and lymphadenopathy appears. Arthritis is common complication especially in females. If rubella occurs in early pregnancy the fetus may die otherwise congenital malformation is common in first trimester. The most common malformation produced by rubella are cardiac defect, cataract and deafness. The other features in babies of congenital rubella are hepatosplenomegaly, thrombocytopenic purpura, myocarditis and bone lesions.

Incidence of defects in rubella infection is closely linked to stage of pregnancy when the infection is acquired:

Stage of pregnancy	*Incidence of defects*
1 month	50%
2 months	25%
3 months	17%
4 months	11%
5 months	6%
6 months onwards	Very low

Rubella virus is found in all excretions of congenitally infected infants. That is the reason of infected babies constituting important source of infection to the staff in nurseries.

Diagnosis can be established by virus isolation from blood (early stage) and throat swabs. Growth on rabbit kidney or vero cell culture is detected by interference with Echo 11 virus. However, serological diagnosis is made by hemagglutination inhibition, neutralization, complement fixation, immunofluorescent of platelets and aggregation tests. Fourfold or more rise in convalescent serums is diagnostic. In congenital rubella diagnosis is made by demonstrating lgM.

Rubella infection gives life-lasting immunity as there is one antigenic type of virus. Prophylaxis is relevant only to women of childbearing age. The vaccines (live attenuated) available are Cendehill and HPV 77 (serial passage in tissue culture). They are administered subcutaneously. Drawback is that arthritis occurs after vaccination. To 336 and HPV 77 DES are other vaccines. New vaccine has come up (RA 27/3) which is administered intranasally conferring local immunity as well. The safe period of giving vaccine in young girls is 11 to 13 years and in women immediately after delivery.

RHABDOVIRUS

They are classified as Rhabdovirus with bullet-like shape. They are enveloped RNA which multiply in cytoplasm of host cell and mature by budding from plasma membrane. The most important virus of this group is rabies virus.

Rabies Virus

They are bullet-shaped with one end blunt and other end pointed, 120 to 200 nm long with cylindrical diameter of 60 to 80 nm. The core contains RNA in helical symmetry. The virion is surrounded by lipoprotein envelop from which project hemagglutinin spikes (Fig. 50.3). They are killed by ultraviolet light, heating at 56°C for one hour and 60°C for 5 minutes, ether, strong acid, strong alkalies and trypsin. All strains are antigenically similar. They induce formation of fixing, neutralizing and hemagglutination inhibition antibodies.

Virus can grow almost in all warm blooded animals, suckling mice being better suited for virus isolation. They can also be grown on chick embryo, duck embryo, tissue cultures prepared from mouse or chick embryo (fibroblast), hamster kidney and human diploid tissue.

Man is infected by the bite of rabid animals, e.g. dog. Saliva of the infective animal contains rabies virus which are deposited in wound conferred by the bite of animal. The viruses travel through nerve fibers to the spinal cord and brain. From central nervous system viruses spread to salivary glands and other tissues. Incubation period is from 1 to 3 months. Prodromal phase usually lasts for 2 to 4 days with manifestations as malaise, anorexia, nausea, vomiting, headache and fever. This stage is followed by sensory phase when patient feels peculiar sensation around wound and attempt to swallow results in painful spasm of muscles of deglutition. This phase is followed by excitory phase in which patient gets generalized convulsions and coma. Many a time death is preceded by paralysis.

It is more important to demonstrate virus in the rabid animal than in patients. However, in patients, demonstration of viral antigen from facial skin biopsy and in corneal smear may be done. Immunofluorescent may be employed for antemortem diagnosis whereas postmortem demonstration of inclusion body or virus antigen in the brain or isolation of virus by mouse inoculation are the standard procedures. For the diagnosis in animals suspected to die of rabies preferably severed head should be sent to laboratory. If possible brain of the animal may be removed and divided into two portion, one in 50 percent glycerol saline (biological test) and the other in Zenker's fixative (microscopic examination). Hippocampus major part of brain should be included as it contains abundant inclusion bodies. Impression smears are stained by Seller technique. The inclusion bodies (Negri bodies) are seen as intracytoplasmic, round and purplish pink structure (Fig. 50.4). By indirect immunofluorescence test using antirabies serum fluorescein conjugate, deposit of virus antigen may be demonstrated in infected cell

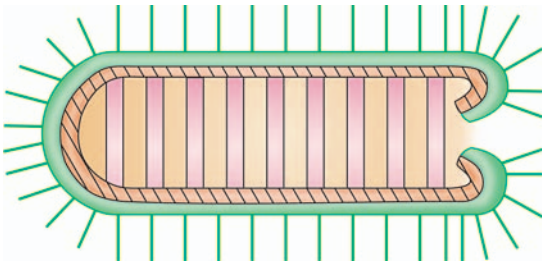


Fig. 50.3: Rabies virus

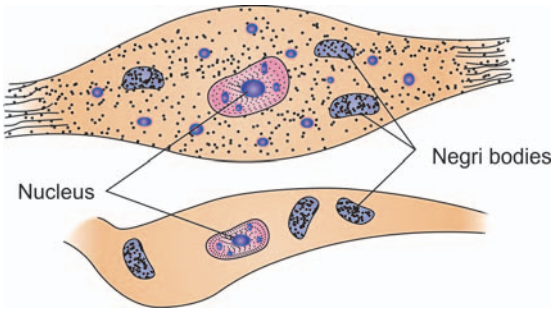


Fig. 50.4: Negri bodies

long before Negri bodies appear. Ten percent suspension of brain is injected intracerebrally into mice. Impression smears of brain show many Negri bodies. Isolation of virus from saliva of man or animal may be tried for this purpose by intramuscular injection to Syrian hamster because of its susceptibility.

Prophylactic measures of rabies consists of washing the wound with water and soap and then cauterizing it with carbolic acid or quartenary ammonium compounds. Currently, following antirabic vaccines are available:

a. Neural vaccines:
   i. Fermi vaccines not in use now.
   ii. Semple vaccine is widely used.
   iii. Sheep brain vaccine.
   iv. Inactivated vaccine is prepared from suckling mouse or rabbit. It carries little risk of neurological complication.
b. Live attenuated chick embryo vaccine is available in two forms:
   i. Low egg passage (LEP)
   ii. High egg passage (HEP)
c. Duck egg vaccine.
d. Tissue culture vaccines have low potency. The recommended dosage schedule of semple vaccine for different classes is as under:

CLASS	SEMPLE VACCINE
CLASS I Licks on fresh cuts with saliva all over the body except head, face and neck, licks on intact mucosa of mouth, nose, and conjunctiva or bites scratches without bleeding and handling raw flesh of rabid animals.	2 ml for 7 days
CLASS II Licks on fresh cut or abrasions on fingers, unlacerated bites or scratches on the fingers (½ cm long) and bites or scratches on all parts of body except head, face, neck or finger which have drawn blood. Number of bites should not be more than five.	5 ml for 14 days
CLASS III Licks and bites or fresh cut or abrasions on head, face and neck, bites, (lacerated) on finger (more than 1 cm), bites causing laceration and drawing blood, more than 5 in number, all jackal and wolf bites and class II patient who has not received treatment within 14 days of injury.	10 ml for 14 days

ARBOVIRUS

They are arthropod borne viruses (transmitted by blood sucking insects vectors). They are RNA viruses, spherical, 20 to 60 nm in diameter having lipid envelope and susceptible to ether, bile salts, etc. Their ability to multiply in arthropods is their special character.

Today 41 arboviruses have been isolated in India. Of these 41 arboviruses, 12 are known to cause human disease in India and an additional 3 have been associated with human disease in other countries but not in India (Sindbis, Wanowrie and Bhanja viruses).

These viruses are:

Family/Genus	*Name*
Togaviridae/Alphavirus	1. Chikungunya 2. Sindbis 3. Japanese encephalitis
Togaviridae/Flavivirus	4. West Nile 5. Dengue types 1, 2, 3 and 4 6. Kyasanur forest disease
Bunyaviridae/ Phlebovirus	7. Sandfly fever Sicilian 8. Sandfly fever Naples
Bunyaviridae/Nairovirus	9. Ganjam
Rhabdoviridae/ Vesiculovirus	10. Chandipura
Ungrouped	11. Wanowrie 12. Bhanja

Five of these viruses, i.e. Kyasanur forest disease, Ganjam, Chandipura, Wanowrie and Bhanja viruses were first discovered in India.

These viruses may be grown by intracerebral inoculation in suckling mice, yolk sac, chorioallantoic membrane of chick embryo, culture of insect tissue and tissue culture primary cell like Vero, HeLa. Three antigens are identified and they are complement fixing, hemagglutinin and neutralizing antigens. Based on antigenic relationship in hemagglutination inhibition and complement fixation test 300 arboviruses are placed into over 20 groups. Medically important groups are A (20 mosquito borne viruses), B (26 mosquito borne and 8 tick borne), C (11 mosquito borne) and so on.

Arbovirus infection in man occurs by the bite of an arthropod and through micropuncture virus is introduced. Viral multiplication occurs in capillaries and lymphatics and from here the virus goes to blood (viremia) of patient. As such virus may be present in macrophages or lymphocytes or adsorbed to the surface of platelets or RBC and thus widespread viral dissemination occurs all over the body. Encephalitis producing virus cause lesions all over the brain. In yellow fever, viral localization may occur in the liver resulting in midzonal necrosis.

Blood collected during acute phase of disease may be used to isolate and identify the viruses for establishing diagnosis. Isolation may also be made from CSF but brain is the best specimen for this purpose. Specimens are

inoculated in suckling mice intracerebrally and animal develops encephalitis after some time. Tissue culture and eggs may be used for culture of virus. Serological techniques like hemagglutination inhibition, complement fixation, gel precipitation and neutralization with antisera are useful for establishing the diagnosis. However, virus isolation from insect vector also helps in identification of arbovirus activity in the area.

Group A viruses:

1. Encephalitis viruses
   a. Western equine encephalitis
   b. Eastern equine encephalitis
   c. Venezuelan equine encephalitis.
2. Febrile illness causing viruses
   a. Chikungunya virus (in India it was demonstrated in 1963)
   b. O'nyong nyong virus
   c. Sindbis virus (also recovered from India).

Group B viruses:

1. Encephalitis virus
   a. St. Louis encephalitis virus
   b. Ilheus virus
   c. West Nile virus
   d. Murry Valley encephalitis
   e. Japanese B encephalitis virus occurs in Korea, Japan, India and Malaysia. They are called Japanese "B" encephalitis to distinguish it from encephalitis A group. Severe epidemics have occurred since its isolation in 1935 in Japan. *Culex tritaeniorhynchus* (mosquito breeding in ricefields) is principal vector. Birds and pigs are reservoir hosts.

      This was recognized in India in 1955 when virus was isolated from *Culex vishnui* mosquito. Epidemics have been reported in West Bengal, Bihar and Assam since 1973 latest being in 1978.

      A formalin inactivated vaccine is used. Vaccination of pig is suggested for checking of epidemics.
2. Yellow fever does not exist in India because of strict vigilance on vaccination and quarantine for travel from endemic area. The other reason may be that stray virus introduced may not be able to get established in vector due to prevalence in local *Aedes aegypti* of dengue fever virus. 17D vaccine is safe for prevention.
3. *Dengue:* This virus is distributed all over tropics and subtropics. It occurs intermittently in large epidemics. It is transmitted from man to man by *Aedes aegypti* mosquito. It is common in India. All four types are identified in India. Control of disease may be achieved by controlling the vector. No vaccine against dengue is available so far.

Four serotypes of dengue viruses are identified abbreviated as DEN-1, DEN-2, DEN-3 and DEN-4.

Laboratory diagnosis includes detection of virus (Immunofluorescence techniques, monoclonal antibodies, radiolabeled RNA probes, etc.), detection of antibodies hemagglutination inhibiton test, IgM capture test, dot enzyme immunoassay) and rapid diagnostic tests (reverse transcriptase PCR, fluorogenic ELISA).

Dengue vaccine is a live tetravalent containing all 4 serotypes. It has been developed in Thailand and in currently undergoing phase II trials in children. Molecular biology techniques used for preparing vaccines either exclude the "enhancing epitopes" in the E components or use other protein like NS-1. More recently, it has been shown that dengue pre-M can also confer protective immunity.

Tick Borne Group

i. Russian spring summer encephalitis virus.
ii. Kyasanur Forest disease viruses cause hemorrhagic fever and occur in India (Karnataka state). Forest birds and animals are believed to be the reservoir hosts.
iii. Omsk hemorrhagic fever virus.
iv. *Louping ill:* This is only arthropod borne togavirus infection occurring in Britain. In sheep it causes cerebellar damage and characteristic ataxic movements. It may be transmitted by tick *(Isodex ricinus)* to man who may develop mild encephalitis.

Bunyaviridae

The term Bunyaviridae is derived from a locality in Africa Bunyamwera. This group of viruses is the largest arboviruses.

Morphology

They are enveloped, single stranded, RNA viruses about 60 to 100 nm in diameter, having helical symmetry. They multiply in the cytoplasm of host cell and attain maturation by budding into vacuoles.

Classification

There are about 100 species in this group. Recent classification is as under:

1. Bunyavirus.
2. Phlebovirus.
3. Nairovirus.
4. Unkovirus.

These are further divided into strains and serological types based on reservoir animals/vectors, geographical distribution and serology.

Pathogenesis

They produce infection in man rarely.

The vector (mosquito, etc.) infects the virus directly into the bloodstream through saliva. Multiplication of virus occurs in vascular endothelium and in the reticuloendothelial cells of lymph nodes, spleen, liver, etc. After 4 to 7 days viremia occurs with systemic symptoms.

Clinical Picture

It may start with fever, chills, aches, arthritis, myositis, hemorrhagic skin rash, nephritis or encephalitis.

Laboratory Diagnosis

Culture on cell lines like, BHK-21, Vero may be used. With repeated passage there are very little cytopathogenic effects. However, granular cytoplasmic inclusion may be demonstrated.

Serological methods like complement fixation test, hemagglutination inhibition test and neutralization test are useful.

51 Severe Acute Respiratory Syndrome (SARS)

Towards the tail end of 2002 a new syndrome emerged in southern China. It was named as Severe Acute Respiratory Syndrome (SARS). The initial outbreak was in peak in April 2003. By June 2003 there had been 8,000 cases worldwide and 775 deaths.

SARS is caused by a novel coronavirus (CoV). It does not appear to be related with 3 known classes of coronavirus. It is hypothesized on the basis of available data that animal virus recently mutated and developed the ability to productively infect man. Groups 1 and 2 contain mammalian virus while Group 3 contains avian virus. SARS CoV defines 4th class of coronavirus and it exhibits following features:

1. It has 29,727 nucleotides in length.
2. It has 9 open reading frames that are not found in other coronavirus and may code for proteins that are unique to SARS virus.
3. It is large, enveloped having positive stranded (27 to 30 kb) and may cause respiratory and enteric diseases in man and animal.
4. Its genome is largest found in any RNA virus.
5. Human coronaviruses are found both in Group I (H Cov -229 E) and Group II (H CoV-OC 43) and are responsible for 30 percent mild respiratory tract infection (Figs 51.1 and 51.2).

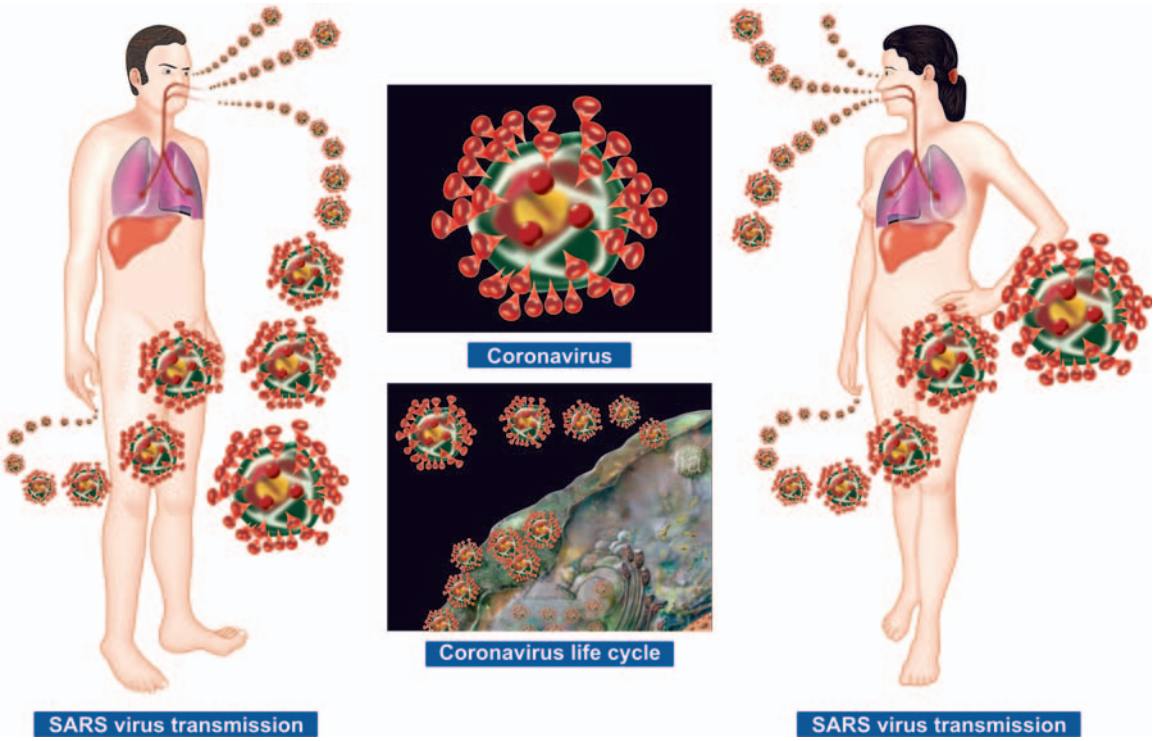


Fig. 51.1: Severe acute respiratory syndrome (SARS)

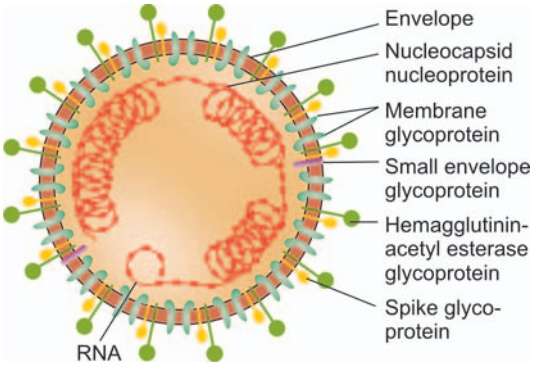


Fig. 51.2: SARS-Associated coronavirus

CLINICAL PICTURE

SARS is transmitted by inhalation because the virus may be present in droplets aerosol of respiratory tract secretions of the patients. Incubation period of SARS is 5 to 7 days. Manifestations of SARS are as under:

1. Fever 38°C or more.
2. Dry non-productive cough.
3. Myalgia.
4. Sore throat.
5. Shortness of breath.
6. Atypical pneumonia.
7. Mortality and morbidity resembles 1918 influenza epidemic.
8. Death may occur from progressive respiratory failure in 3 to 30 percent of cases.

LABORATORY DIAGNOSIS

1. Isolation of SARS CoV in monkey Vero E 6 cells in tissue culture.
2. Reduction in lymphocyte count.
3. Rise in aminotransferase activity which indicates damage to the liver.
4. Reverse transcription PCR using respiratory secretions.
5. Indirect immunofluorescent to demonstrate rising antibodies.
6. ELISA to detect rising titer of antibodies.
7. Chest radiograph.

MANAGEMENT

There is no consensus so far treatment for SARS is concerned. Only symptomatic treatment and quarantine of the patient seems to be feasible for the time being. The drug of particular interest and promising is the one which blocks the protease function. No vaccine has been developed. A major hurdle towards development of vaccine is antigenic shift.

52 Avian Influenza (Bird Flu)

Birds are especially important species because all known subtypes of influenza A viruses circulate among wild birds, which are considered the natural hosts for influenza A viruses. Influenza viruses that infect birds are called avian influenza viruses.

The causative agent is H5 N1 a subtype of influenza A virus. It generally affects bird population all over Asia. Outbreaks of avian influenza A (H5 N1) have been confirmed among poultry in Cambodia, China, Hong Kong, Indonesia, Japan, Laos, South Korea, Thailand and Vietnam. The virus H5 N1 was first isolated from birds in South Africa in 1961.

Influenza A viruses may be divided into subtypes on the basis of their surface proteins, i.e. hemagglutinin (HA) and neuraminidase (NA). There are 15 known H subtypes. Whereas all subtypes are found in birds, only 3 subtypes of HA (H1, H2 and H3) and two subtypes of NA (N1 and N2) are known to have circulated widely in man. This virus makes wild birds sick, but may make domesticated birds very sick and may kill them. Several instances are on records where human infections and outbreaks have occurred in last 8 years. There is possibility of different variations of H5 N1. Following information has been gathered about H5 N1:

1. All genes are of bird origin. Since virus has not acquired genes from human influenza virus so possibility of person to person spread is more likely.
2. There is possibility of different variations of H5 N1 virus which is in circulation at this time. Genetic sequencing of virus samples from South Korea and Vietnam suggest that viruses of these countries are different.

SPREAD OF INFECTION

Infected birds shed viruses in saliva, nasal secretions and feces. Bird to birds transmission is by contact with contaminated excretions. H5 N1 infection in man may be because of contact with infected poultry or contaminated surfaces. Of late human to human transmission has been reported.

SYMPTOMS

The symptoms in man include fever, cough, sore throat, muscle aches. Additionally there may be eye infection, pneumonia, acute respiratory distress and other severe life threatening conditions.

PREVENTION AND TREATMENT

Prevention measures include killing of sick and exposed birds, isolation and treating the patients. Travellers to countries in Asia with H5 N1 outbreaks must avoid poultry farms and any surface contaminated with feces from poultry. Other preventive measures include wearing of mask and gloves, cleaning kitchen surfaces, cooking chicken till boiling temperature, controlling human traffic into poultries and reporting to authorities any unusual death or illness of chicken or other birds as well as illness of workers in poultry farms.

Antivirals drugs like oscmltamvir and zanamavir are quite effective. Some strains however do show resistance to amantadine and rimantadine.

53 Acquired Immune Deficiency Syndrome (AIDS)

IMPORTANT EVENTS IN AIDS

1981 The first case of AIDS reported in USA.
1983 LAV, i.e. HIV isolated.
1984 Serological tests developed to identify infected persons.
1985 Report of anti-HIV activity of suramin, ribavirus published.
1985 Report of *in vitro* anti-HIV activity of HPA 23, interferon alpha, foscamet and zidovudine published.
1986 Clinical trials of zidovudine show efficacy in AIDS and advanced AIDS-related complex.
1987 Zidovudine (AZT)—licensed for clinical use in many countries. Large scale clinical trials of zidovudine and other agents began.
1991 Didanosine is licensed for use by USFDA in selected AIDS patients.

AIDS is a immunoregulatory disorder that is often fatal because it predisposes the person to severe opportunistic infections or possibly to neoplasms. It happens so because of depletion of helper T cells owing to infection by HIV (human immunodeficiency virus).

History

AIDS was first recognized in USA in July 1981. In August 1981, AIDS was reported in intravenous users. In June 1982, clusters of AIDS patients appeared among homosexuals and later in hemophiliacs and blood transfusion associated patients. In January 1983, it was reported in heterosexual cases in female.

Isolation of etiological agent of AIDS was first reported in May 1983 by Luc Montagnier from Pasteur Institute, Paris. They isolated a retrovirus from a West African patient with generalized lymphadenopathy and they named it lymphadenopathy associated virus (LAV). In March 1984, Robert Gallo from National Institute of Health, Bethesda (USA) reported isolation of retrovirus and named it HTLV-3. In March 1985, ELISA test kit was approved by FDA. In May 1985, blood bank screening for HIV was introduced. In 1986, the virus was named HIV.

India started a serosurveillance among high risk groups in 1985 to know the magnitude of HIV infection. First case of HIV infection in India was reported in 1986 and that of HIV-2 in 1991.

Structure and Properties of HIV

It belongs to the Lentivirus subgroup of Retroviridae family. HIV is an RNA retrovirus (see also page no. 328). The unique morphologic feature of HIV is its cylindrical nucleoid in the mature virion. The diagnostic bar-shaped nucleoid may be seen in electron micrographs. Under electron microscope it exhibits the characteristic exotic flower appearance (Fig. 53.1). Dr. Rober C. Gallo discovered HIV in 1984.

The virus contains the 3 genes required for a replicating retrovirus—gag, pol and env (Fig. 53.2). The virus has outermost envelope rich in glycoproteins (gp^{41}, gp^{120}, gp^{160}) and inner core with two component proteins (p^{18}, p^{24}) while the enzyme reverse transcriptase capable of the retrograde transcription of viral RNA to viral DNA marks its special feature. The core proteins, the surface proteins and the regulatory proteins (p^{31}, p^{66})

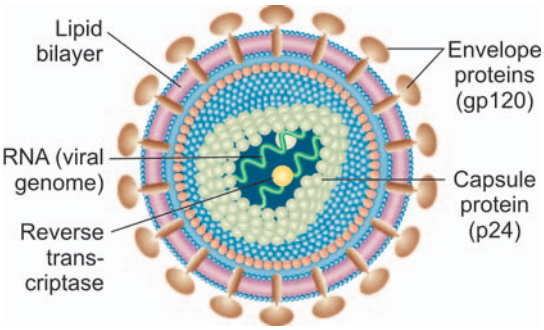


Fig. 53.1: Face of AIDS virus

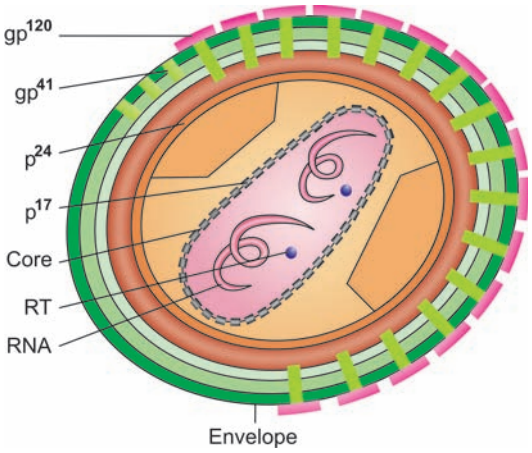


Fig. 53.2: Structure of HIV

are under the genetic control by the gag, env and the pol loci respectively. Amongst the three inbuilt control mechanisms the env locus is under frequent genetic alteration, leading to modification in the antigenic structure of the virus.

In addition following 5 genes code for polypeptides are identified as under:

TAT (transactivation gene)	
REF (regulator of virus gene)	May be involved in regulation of HIV expression.
NEF (negative factor gene)	
VIF (viral infectivity factor gene)	

VPU in HIV-I—May weaken transcriptional activator.

VPX in HIV-II—May be required for efficient budding.

HIV is T lymphotropic especially for helper T cells identified by monoclonal antibody OK T_4 (Leu-3). Virus infection causes cytopathic effect including the formation of multinucleated giant cells followed by cell death. This explains the quantitative and functional depletion of T_4 lymphocyte subset that is the hallmark of AIDS lymphodepletive terminal event. In the end stage, there is reduced T_4 count (less than 60%), nonspecific proliferation of B lymphocytes producing functionally incompetent wasteful serum immunoglobulins (IG and IgA) and nonreactivity (energy) to recall antigens. The final outcome is total depletion of lymph node, impairment of the CMI making the patient susceptible to vast spectrum of opportunistic infection (bacterial, viral, fungal and parasitic).

Etiopathogenesis: HIV effects T4 lymphocytes through infected semen, the contaminated blood and blood products and rarely through saliva, urine and the fecal material.

Once the virus binds with CD4 receptor, the outermost cover is lost at the site of entry. The inner protein coat is being subsequently cast off and the bare enzyme RT transcribes viral RNA and DNA (provirus). Subsequent to its entry into host nucleus the viral DNA genome (provirus) integrates with host DNA genome. In the absence of immunological activation the T4 lymphocyte continues to survive with provirus and the subsequent integration in host DNA is halted leading to the latent HIV infection. What influences the viral replication or dormancy is unpredictable at this stage. The viral messenger RNA (mRNA) subsequent to its transcription redirects the host cytoplasmic machinery to synthesize the newer viral particles. The cell eventually dies and newly generated viruses bud out from dying cell, leading to lymphodepletive terminal events.

The end stage is characterized by reduced T4 count (60%), nonspecific unrestrained proliferation of B lymphocytes producing excessive functional incompetent wasteful immunoglobulins (IgG, IgA) and nonreactivity to recall antigens. The lymph node in the terminal stage may show a total depletion with the characteristic "burnt-out" picture. The final outcome is long-standing impairment of CMI making the affected person unusually susceptible to a vast spectrum of life-threatening opportunistic infection and malignancies of varying types.

Clinical picture: The incubation period seems to be long, ranging from 6 months to more than 2 years. AIDS occurs in homosexuals (75%) but bisexual males, heterosexuals, intravenous drug users and hemo-philiacs treated with blood products or factor VIII are also at risk to get infected.

TABLE 53.1: Break-up of HIV infected persons in India

Category	*% of total*
• Heterosexually promiscuous	45.30
• Homosexuals	0.12
• Blood donors	19.33
• Patients on dialysis	0.38
• Antenatal mothers	0.42
• Recipient of blood/blood products	1.98
• Relatives of HIV patients	0.90
• Suspected AIDS related cases or AIDS cases	3.68
• Drug addicts (I/V)	18.03
• Miscellaneous	9.86

AIDS is characterized by pronounced suppression of immune system, the development of unusual neoplasms especially Kaposi's sarcoma or wide variety of severe opportunistic infections. Other symptoms include fatigue, malaise, unexplained weight loss, fever, shortness of breath, chronic diarrhea, white patches on tongue (hairy leukoplakia or oral candidiasis) and lymphadenopathy.

The most common complication of AIDS may be (*protozoal Toxoplasma gondii,* etc.), fungal, (*Pneumocystis carinii, Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum,* etc.), bacterial (*Mycobacterium avium, Mycobacterium tuberculosis, Listeria monocytogenes,* salmonella, *Nocardia asteroides,* etc.) and viruses (cytomegalovirus, Herpes simplex, adenovirus, Hepatitis-B, etc.).

HIV can infect other cell types including B lymphocytes and monocytes *in vivo* and a variety of human cell lines *in vitro*. Viruses can spread throughout the body, and are demonstrated in lymphoid cells, brain, thymus, spleen and testes. No animal models have been developed for AIDS.

Inactivation of HIV: 10 minutes' treatment at 37°C can inactivate this virus, with:

i. 10% household bleach
ii. 50% ethanol
iii. 35% isopropanol
iv. 1% NP_{40}
v. 0.5% lysol
vi. 0.5% paraformaldehyde
vii. 0.3% H_2O_2

Besides extreme pH (pH 1.0 and 13.0), heating at 56°C for 10 minutes can inactivate HIV.

Global situation of AIDS: 23 million people all over the globe are now infected with HIV. 8500 people are infected with HIV each day and as per calculation it is ample clear that by 2000 AD every 6th or 7th person in world will be a victim of this infection. WHO data suggested 1.3 million adult AIDS cases in 193 countries with epicentre of epidemic shifting from Africa to Asian subcontinent. India has emerged quickly as the country with large number of people infected with AIDS virus. More than 3 million of India's 950 million people are estimated to be infected with HIV. Trailing India in the number of people infected are South Africa 1.8 million, Uganda 1.4 million, Nigeria 1.2 million, Kenya 1.1 million.

The first case of AIDS in India was registered in 1986. Since then, HIV prevalence has been reported in all states and union territories of India. By October 31, 1997, of a total of 3.20 million individual practising risk behaviors and suspected AIDS case who were screened for HIV infection, 67,311 were found to be seropositive. Up to May 31, 1998, the number of AIDS cases in India is reported as 6052, the largest number of cases are from Maharashtra (2,955) followed by Tamil Nadu (1,424) and Manipur (301). The break-up of HIV cases in India is depicted in Table 53.1.

Laboratory Diagnosis

Direct Methods

i. *Cultivation of T lymphocytes:* T lymphocytes with normal lymphocytes are cultured on special cell lines (Hg, HUT_{78} U_{937}) with interleukin II. Stimulation of T cell and demonstration of multinucleated giant cells is possible by 2 to 20 weeks. It is the classical method to confirm the HIV infection.
ii. *Detection of reverse transcriptase:* It is possible on virus grown cells by radiolabeling.

iii. *Solid phase ELISA:* It is useful to detect HIV antigen.
iv. *Phase contrast microscopy:* It is helpful in assaying the prevention of reclustering of MT-4 cells.
v. *Electron microscopy:* Demonstration of fuzzy envelope of infected T_4.
vi. *Animal study:* Reproduction of human lesions in nonhuman primates have a limited diagnostic importance.

Indirect Methods

Antibodies to HIV usually appear in 6 to 8 weeks after the exposure to virus. Following methods are simple, easy and quite useful to diagnose AIDS patients.

i. ELISA
ii. *Western Blot Assay:* The antigene fractions specific for HIV, initially resolved by SDS polyacrylamide gel are transferred on nitrosocellulose membrane by electroblotting. The antigenic profile on membrane contains P^{17}, P^{24}, P^{31}, gp^{41}, P^{55}, P^{56}, gp^{160} in the increasing order of molecular size. The later steps involve reaction with HRP labelled anti-IgG antibody and chromogen substrate reaction. The development of pink colored bands indicate the site of specific antigen antibody complex on the membrane.

However, IgM-Western Blot Assay test is slightly different from Western Blot Assay. In this test basic technique is identical, diaminobenzedine as a substrate and P^{24} as a positive control are two deviations. Besides resolution of brown components indicate the site of immune complex. Appearance of all the three gene products (env, gag and pol) are consistent with a classical HIV infection. Otherwise two env bands (gp^{41}, gp^{120}) show a positive pattern too.

The ELISA at times, particularly so in multiparous women gives rise to false-positive results because of antibodies crossreacting with T_4 lymphocytes. This is rarely seen in Western Blot technique (Fig. 53.3).

iii. *Radio-immunoprecipitation assay:* This technique involves radioactive antigen.
iv. *Polymerase chain reaction:* The polymerase chain reaction (PCR) is a new and exciting technology. It is gaining widespread use in the diagnosis and management of genetic, oncologic, hematologic and infectious diseases. In this method, there is direct detection of disease specific sequences of either RNA or DNA from the tissue or body fluid of patient. It is quite sensitive.

This technique is widely used in HIV infection. In addition to quantitative PCR technique is useful in monitoring the efficacy of antiviral chemotherapy.

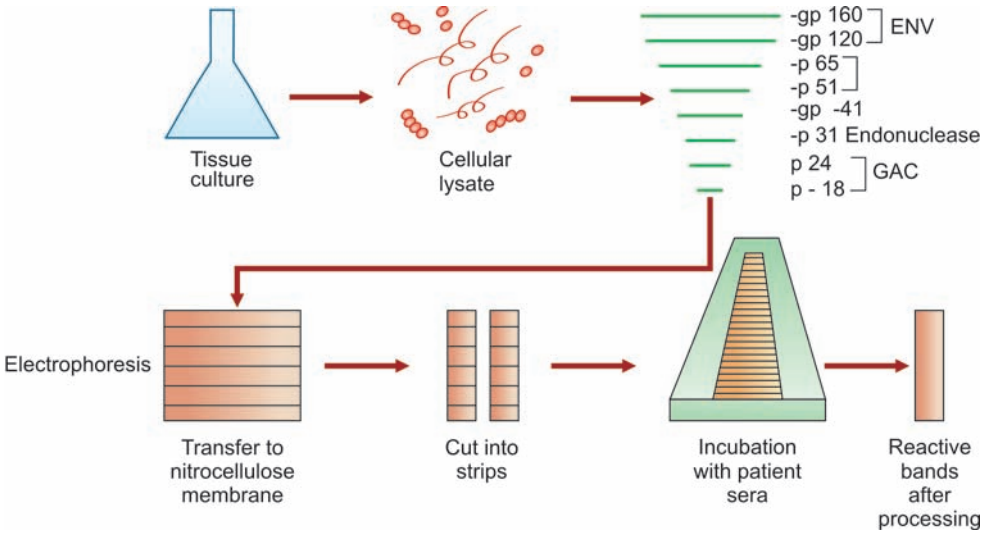


Fig. 53.3: Western blot technique

v. Dot blot hybridization.
vi. Agglutination tests using RBC/latex gelatin, colloidal gold, immuno dot/strip and PAT (particle agglutination tests).
vii. Detection of antibody to nef gene product (27 KD protein) along with p^{20} and gp^{120}.
viii. *Fujerbio agglutination test:* In this test, antigen coated gelatin particles are agglutinated by antibody present in the serum of patient. It is quite simple and convenient test. However, false-positive reactions do occur.
ix. *Karpas test:* In this test, HIV infected cells are fixed on teflon coated slide wells to which serum of the patient is added. After some time horse radish peroxidase labeled antihuman immunoglobulin is added. Later appropriate and corresponding substrate is added. Color develops if test is positive. No color develop if test is negative. It is easy to do and not expensive as slide immunoperoxidase test.

Surrogative markers: They are direct predictors of HIV infection and are:
1. Low CD 4 cells
2. Low B_2 microglobulin
3. Increased neopterin

Nobel Prize for Medicine 2002

Sydney Brenner and John Sulston of Britain and H. Harvitz of US were awarded Nobel Prize for Medicine 2002. Their pioneer work on gene research shed new light on killer diseases, e.g. AIDS and cancer fetched them Nobel Prize. They discovered about how gene regulate organ growth and a process of progammed cell suicide.

Working with tiny worms, these workers identified key genes regulating organ development and programmed cell death, a necessary process for printing excel cell.

Brenner broke new ground by linking specific mutation to particular effects on organ development. Sulston discovered that certain cells in the developing worm are destined to die, through programmed cell death. He demonstrated the first mutation of genes that participate in that process. Harvitz identified the first two "death genes" in the worm and showed that humans have a gene similar to one of them. Now scientists know that most genes controlling cell death in worms have counterparts in humans. Information about programmed cell death has helped scientists understand how some viruses and bacteria invade human cells.

Other Investigations

Reduced T_4 lymphocytes, reversal T_4: T_8 ratio, defective T cell plus NK cell cytotoxicity and anergy to tuberculosis indicate a basic T cell defect which possesses a definite role in the diagnosis of HIV infection.

WHO and national HIV testing policy recommends HIV testing for following purposes:
- Screening blood, organ and tissue for transplantation.
- Epidemiological surveillance.
- Diagnosis of symptomatic infection (AIDS).
- Early diagnosis of HIV infection among asymptomatic persons with only informed consent.

Laboratory tests for diagnosis of HIV infection are:
1. Screening test
   a. ELISA
   b. Rapid tests like
      - Latex agglutination
      - Dot blot test
   c. Simple tests, i.e.
      - Particle agglutination test.
2. Supplement tests: These tests are required for validation of the positive results of screening tests. They include:
   - Western blot tests
   - Immunofluorescence test
3. Confirmatory tests
   - Virus isolation
   - Detection of p^{24} antigen by ELISA
   - Detection of viral nucleic acid may be detected by *in situ* hybridization and polymerase chain reaction.

Treatment: In a nutshell, thymus and bone marrow transplantation, interferon and interleukin therapy is useful in reconstituting basic T cell dysfunction. The antibiotics have a key role in taking care of opportunistic infection. Many HIV drugs are found useful like AZI and suramine (block the action of reverse transcriptase). Inactivation of the virus with monoclonal antibodies is another approach to combat against HIV infection. Phosphonoformate, 'Posearnet', are other anti-HIV drugs reported recently.

Now it is beyond doubt that zidovudin benefits disappear within a year because HIV mutates into new forms that are resistant to this drug. Hence, a new AIDS therapy devised by Yung Kang Chaw is advocated. It consists of administration of 3 drugs, i.e. zidovudine (AZT), dideoxyinosine (DDI) and either nevirapine or pyridinone.

Vaccine against HIV infection: DNA recombinant vaccine with yeast and vaccinia virus, the synthetic peptides are few candidates. Unfortunately, due to genetic alterations vaccine trials are ineffective.

Recent report on the trial of fusion inhibitor *Enfuvirtide* is encouraging. This new drug prevents the entry of HIV 1 into the target cells of the host. It also prevents fusion of HIV transmembrane (gp^{41}) glycoprotein with the CD 4 receptor of the host cell.

Currently treatment for HIV comprised of HAART (Highly active antiretroviral therapy). Here two nucleoside analogue reverse transcriptase inhibitors or NRTI plus either protease inhibitor or a nonnucleoside reverse transcriptase inhibitor (NNRTI).

STRATEGIES OF HIV TESTING IN INDIA

Strategy I: (Blood Donation)

Serum is subjected once to ELISA, Rapid, Simple tests for HIV. If negative, serum is considered free of HIV. In case it is positive, sample is taken as HIV infected.

Strategy II: (Surveillance and Diagnosis)

Test serum sample is taken as negative for HIV if first ELISA test report is negative, and subjected to a second ELISA which utilizes a system different from first one.

Strategy III: (Diagnosis)

Here 3 ELISA kits are used. First ELISA is done with highest sensitivity, and second and third ELISA with highest specificity.

Preventive Measure in Biosafety

Steps	*Preventive Measures*
1. Pre-use screening	• Avoid avoidable risky procedures like mixing, grinding etc. • Avoid the use of sharp objects • Keep away person with ulcerating or weeping skin lesions
2. Barrier precautions	• Use of gloves contact with blood, body fluids, mucous membrane, broken skin etc, • Use of mask protective eye wear face shield to prevent droplet infections • Use of gowns, aprons, long shoes to prevent splash of blood, body fluids
3. In use precaution	• Prevention of injuries by sharp objects, i.e. needles, scalpel etc. • Needles need not to be recapped.
4. After use precaution	• Drop all used instrument in disinfectant jar. • Placing the jar with disinfectant as close as possible to the working place.

54 Miscellaneous Viruses

Hepatitis Viruses

Viruses responsible for hepatitis are hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, Epstein Barr (E B viruses), yellow fever, cytomegalovirus, rubella, Herpes simplex and coxsackievirus. Recently, some more viruses are included in this list, i.e. Marburg virus, Lassa virus and Ebola virus.

The term viral hepatitis refers to infection with hepatitis type A, hepatitis type B and non-A non-B virus like hepatitis-C, hepatitis-D and hepatitis-E virus (Table 54.1). Difference between Hepatitis A and Hepatitis B type is tabulated in Table 54.2.

a. Hepatitis A Virus

Type A hepatitis occurs endemically in all parts of the world with frequent minor and major outbreaks. The older the patient more are the chances of this infection. Lower socioeconomic status class and females have high prevalence. Infection shows peak in the month of March and April. Incubation period is 2 to 6 weeks. Routes of spread may be stools, urine and nasopharyngeal secretions. Modes of transmission can be fecal-oral. The virus enters the body by ingestion, starts multiplying in intestinal epithelium and then through blood it reaches liver.

Hepatitis A virus (Figs 54.1 and 54.2) is a spherical RNA virus which belongs to picornavirus group (26 to 30 nm in diameter). Lipid is not an integral component and so this virus is stable to treatment with ether, acid and survives heating at 56°C for 30 minutes, and 1 ppm chlorine for 30 minutes.

It is inactivated by ultraviolet irradiation heating at 100°C for 5 minutes, autoclaving 121°C for 20 minutes, 1:4000 formalin at 37°C for 3 days and by RNase. This virus was first identified in 1937 by Feinstone, Kapikian and Purcell using immune electron microscope. Only one serotype is known and there is no antigenic cross-reactivity with hepatitis B virus.

Culture of this virus in the laboratory has been possible and so provision of vaccine is now there. Provost and Hillermann in 1979 claimed cultivation of this virus in liver cell cultures of marmosets and in fetal rhesus kidney cell lines.

Laboratory diagnosis is possible by the demonstration of virus by immunoelectron microscopy. The other tests are complement fixation, immune adherence, hemagglutination, radioimmunoassay and ELISA (enzyme

TABLE 54.1: Properties of viral hepatitis (various types)

Hepatitis type virus	*Route of transmission*	*Mortality*	*Risk of chronic liver diseases*	*Incubation period*	*Virus classification*
A. Unenveloped SS RNA	Feco oral	Low	None	2 to 6 weeks	Picornavirus
B. Enveloped DS DNA	Parenteral	High	High	1 to 6 weeks	Hepadnavirus
C. Enveloped SS RNA	Parenteral	Moderate	High	1 to 5 months	Flavivirus like
D. Enveloped circular SS RNA	With hepatitis B	High	High	2 to 6 weeks	Animal satellite virus
E. Unenveloped SS RNA	Feco oral	High in pregnancy	None	3 to 6 weeks	Calcivirus like

TABLE 54.2: Differences between hepatitis-A type and hepatitis-B type

Characters	*Hepatitis type A*	*Hepatitis type B*
1. Diameter of virus	27 nm	43 nm
2. Symmetry of virus	Icosahedral	Icosahedral
3. Nucleic acid	RNA	DNA (double stranded)
4. Primary route	Feco-oral	Parenteral and also feces, saliva, transplacental
5. Incubation period	2 to 6 weeks	2 to 6 months
6. Mortality	Low	High
7. Chronic active hepatitis	Rare	Common
8. Onset	Acute	Insidious
9. Fever	Present	Rare
10. Age incidence	Children	All age group
11. Extrahepatic lesion	Absent	Common
12. Australia antigen in serum	Absent	Present
13. Viremia	Brief	Prolonged
14. Viruses in stool	Present	Mostly absent
15. Human gamma globulin prophylaxis	Effective	Not effective

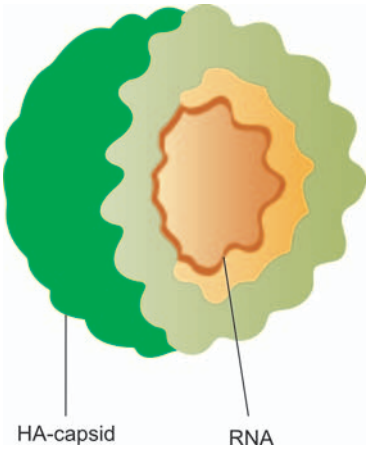


Fig. 54.1: Hepatitis A virus (27 nm)

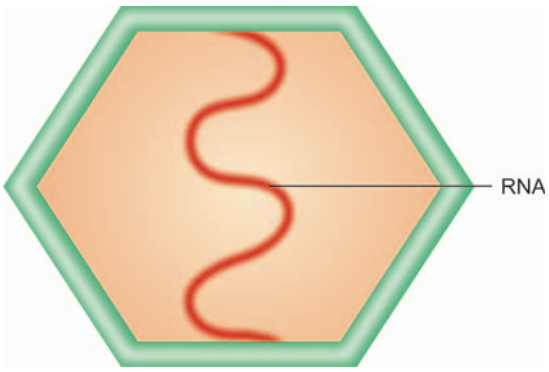


Fig. 54.2: Hepatitis A virus

linked immunosorbent assay). More sensitive serologic assay, e.g. the microtiter solid phase immunoradiometric assay and immune adherence have made it possible to detect this virus in stool, liver homogenates, bile and also to measure specific antigen in serum.

Hepatitis A vaccine which is live attenuated procured from H-2 strain is reported safe and highly immunogenic at a subcutaneous dose of 10^6 $TCID_{50}$ (1 ml).

Another hepatitis A vaccine comprises of formalin inactivated virions grown in human fibroblasts or monkey kidney cell line for active vaccination. Two doses are injected one month apart. Booster dose may be given after 6 months giving adequate immune response in 99 percent cases lasting for years together. It is expensive because of low yield of viruses from cultured cell. It is required in sewage workers, homosexuals and intravenous drug users and for visitors visiting HAV endemic regions.

b. Hepatitis B Virus

23.6 percent cases of endemic hepatitis in India are HBs Ag positive with majority of patients beyond third decade. Incidence of this infection is more in males. Incubation period is 28 to 160 days. The common subtypes reported from India are adr, adw. In Tamil Nadu, adr is reported in 64 percent of cases studied by Thyagarajan *et al.* In north India, the peak incidence occurs in the month of July and August.

Routes of spread are serum, saliva, tears, nasopharyngeal secretion, urine, feces, seminal fluid, breast milk, gastric fluid, synovial fluid and intestinal juice. Mode of transmission may be blood transfusion, intravenous drug abuse, tattooing, ear piercing, sharing of razors, sexual transmission, insect vectors and human bites, etc.

They are spherical DNA viruses. Electronic microscope shows three types of particles in sera from type B hepatitis patients (Fig. 54.3).

The most abundant form is spherical (20 nm in diameter). The second type of particles are tubular with diameter of 20 nm and varying length. These two types of particles represent Australia antigen. The third type of particles are double shelled spherical (42 nm diameter) and also called Dane's particles. Dane's particles are considered complete type B hepatitis virus (Fig. 54.4).

The terms in current use for virus, viral antigen and antibodies are:

HBs Ag: It is hepatitis B surface antigen found on 20 nm spheres tubules and outer envelope of Dane particle also called Australia antigen.

HBc Ag: It is hepatitis B core antigen present in the core of Dane's particles.

HBV is hepatitis B virus identical with Dane's particles.

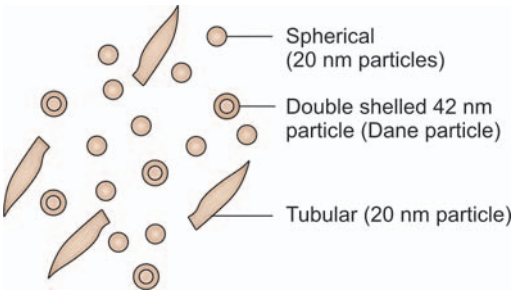


Fig. 54.3: Hepatitis B virus

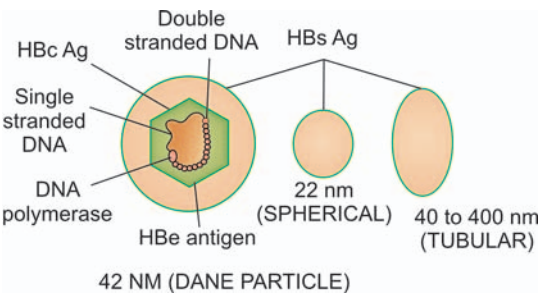


Fig. 54.4: Structure of hepatitis B (3 types)

Hepatitis B—"e" antigen was described in 1972 by Magnius *et al*. It is smaller than HBs Ag and HBah Ag. Takashi, 1979 demonstrated that HBe Ag exists in the core of Dane's particles in cryptic form.

Delta antigen is the latest to the addition in list of antigens. It was reported for the first time by Rizzetto in 1977. The antigen is distinct from known antigenic determinants of hepatitis B virus. It is localized in hepatocyte nuclei that do not contain HBc Ag.

Subtypes of HBs Ag: In addition to the common antigen "a" HBs Ag contain 2 pairs of subdeterminants b/y and w/r. These four antigen types are: a d w, a b r, a y w, and a y r. Subtypes are useful as epidemiological markers.

A variety of serological techniques are available for detection of hepatitis B antigens and antibodies. HBs Ag becomes detectable about a month after exposure to infection with peak levels in preicteric phase of disease. It disappears with recovery of disease. Antibodies to HBs Ag appear within week after disappearance of HBs Ag and they may persist for years. These antibodies are rarely detectable in chronic cases. HBs Ab is not detectable in the serum of patient. Antibodies of HBc Ag are detectable in preicteric phase. However, HBc Ag may be demonstrated in infected liver cells by immunofluorescence. Viral DNA-polymerase may be detectable in preicteric phase of illness.

Serological tests are immunodiffusion, counter immune electrophoresis, complement fixation, indirect hemagglutination inhibition, immuneadherence, hemagglutination, reverse passive latex agglutination, radioimmunoassay and ELISA. Nowadays, new monoclonal Enzygnost kit is available which is more specific, simpler to perform and needs a shorter total incubation period than polyclonal kit.

Hepatitis B carriers: Carrier form may be defined when there are persistence of HBs antigen in circulation for more than 6 months. Carriers may be of following types:

1. Super carriers: Here HBe antigen, high titre HBs antigen and DNA polymerase remain present in blood. Needless to say that very minute amount of serum or blood from carriers may transmit this infection.

2. Simple carriers: Here HBs antigen is found in blood in low titre. However, HBe antigen and DNA polymerase are absent in blood. Transmission of infection is possible when large amount of blood is transferred, e.g. blood transfusion. By the way simple carriers are more common.

Prophylaxis of hepatitis B infections: Hepatitis B virus infection may be checked by observing the followings:

1. Screening of blood donors.
2. Use of sterile disposable syringes or needles.
3. Reduction in the number of sexual partners.
4. Use of condoms.
5. Blood spills should be cleaned with 2% glutaraldehyde or 0.5% sodium hypochlorite.

Passive immunization: Hepatitis B globulin (HBIG) should be given as early as possible within 48 hours of exposure. A second dose should be given after 30 days in conjunction with active immunization. HBIG is prepared from donors with high titre of anti-HBs. It may be administered in the doses of 300 to 500 IU intramuscularly.

Indications of passive immunization include needle stick injury, splash of blood from HBs antigen positive patient, to newborne infants born to HBs positive mothers.

Active immunization: Indications of active immunization are:

1. Healthy personnels in direct contact with blood and sharp instruments.
2. Patients in need of repeated blood transfusion and blood products.
3. Patients on dialysis.
4. Parenteral drug users.
5. Prostitutes and sexually promiscuous.
6. Spouses of known HIV positive.

The vaccines available against Hepatitis B are:

1. Plasma derived vaccine containing 22 nm particles of HBs antigen. It is cheap, immunogenic and safe. It is administered intramuscularly in deltoid region in three doses at 0, 1 and 6 months.
2. Recombinant yeast Hepatitis B vaccine is safe, immunogenic and free from side effects. It is administered intramuscularly into deltoid region in three doses 0, 1 and 6 months.
3. Synthetic vaccine are yet under evaluation.
4. Hybrid virus vaccines are prepared by incorporating HBs antigen into vaccinia virus DNA. This vaccine is cheap having long self life.

The virus shows considerable genetic and antigenic diversity. There are different genotypes and many subtypes thus, indicating high mutability. Hence, there is little heterologous or even homologous postinfection immunity in infection due to this virus.

It is noteworthy that hepatitis virus has not been grown in culture. However, it has been cloned in Escherichia.

Patient may be treated by administering interferon in large doses. It has reduced the level of HBV and related antigens in the blood with improvement of health in some patients. Vidarabine (arc-A) and acyclovir have been successful in reducing HBV levels.

Hepatitis C Virus

It belongs to family Flavivirdae. It is 50 to 60 nm virus with linear single stranded RNA of positive polarity enclosed within a core and surrounded by an envelope carrying glycoprotein spikes. It has multiple genotypes which has made vaccine preparation difficult. It is difficult to culture this virus. Recently, it has been included in a separate genus called Hepacivirus under family flavivirdae.

Hepatitis D Virus

Morphology

It is an RNA virus which has the outer shell of HBs antigen and the core is the delta antigen. It is smaller than HBV and measures around 37 nm (Fig. 54.5). The RNA is small chained and codes for 2 to 3 proteins. Animal model useful for its study is chimpanzee. It requires the helper function of HBV to produce disease. It is an incomplete virus. This virus is unique amongst the satellite viruses as it can also infect a second animal species Marmota Monax in association with woodchuck hepatitis virus.

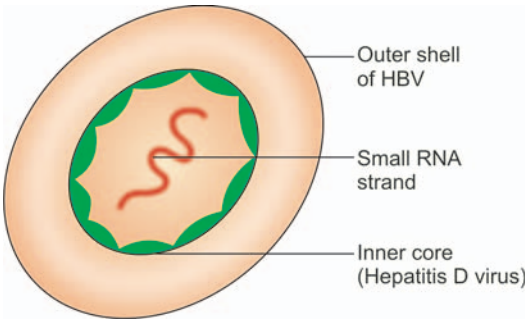


Fig. 54.5: Hepatitis D virus

Patients having repeated blood transfusions and drug abusers are likely to be infected with this virus.

Pathogenesis: Coinfection of HBV and HDV will usually cause an acute disease. In case a person becomes a chronic HBs Ag carrier, chronic delta hepatitis occurs.

HBV replication rate is evidenced by HBe Ag. When HDV infection occurs it will compete with HBV for replication resulting in low rate of HBV replication and hence, HBe Ag is absent. Thus, in patients with high HBs Ag and absent HBe Ag, HDV infection will cause chronic delta hepatitis.

Clinical features: Both acute and chronic HDV infection is more severe than acute and chronic (uncomplicated) HBV infection.

About 39 percent cases of fulminating hepatitis are HDV positive. About 16 percent of all HDV infections are fulminating.

Laboratory diagnosis: Radioimmunoassay, at present, carries some diagnostic importance. Even electron microscope is not of much use.

Treatment: Alfa steroid therapy is considered useful in both acute and chronic hepatitis D infection.

Hepatitis E Virus

This virus causes enterically transmitted non-A non-B hepatitis. This virus is now named hepatitis E virus. Hepatitis caused by E virus is found in epidemic form as well as sporadic form. Large epidemics of this infection has occurred in many countries including India with very high mortality in pregnant ladies. The higher mortality in pregnancy has been attributed to reversal host factors like heightened immune response, malnutrition and altered harmonal status. Disseminated intravascular coagulation, which is the most common cause of mortality in hepatitis E associated pregnancy, seems to be a severe manifestation of a Schwartzman like reaction.

Morphology: The virus is spherical, unenveloped measuring 34 to 37 nm with spikes and indentations seen on the surface of virus particle. These features resemble with calcivirus (Norwalk virus).

Resistance: This virus is heat labile, sensitive to freezing-thawing, cesium chloride and pelleting by ultracentrifugation.

Successful cloning of viral genome of this virus is now possible. Development of diagnostic tests and preparation of vaccine, now seems to be feasible. The diagnosis of acute hepatitis E can be conveniently established by demonstrating virus specify IgM/IgG antibodies against recombinant proteins from open reading frame (ORF-2,3) by enzyme immunoassay. In this assay, polystyrene beads coated with HEV proteins representing sequence from ORF-2 (SF-3 antigen ORF) and ORF-3 (8.5 antigen) of Burmese strain of HEV.

Hepatitis F Virus

- It is putative virus causing non-B, non-C, non-D hepatitis.
- May cause hepatitis in persons having blood or blood product transfusion (negative of hepatitis B virus and hepatitis C virus).
- These viruses have not been studied and characterized.

Hepatitis G Virus

It was discovered independently by 2 different groups, one naming it hepatitis G, other calling it GB virus. Like hepatitis C, hepatitis G virus is a blood borne RNA virus sequence analysis of 10 kilobase genome shows that Hepatitis G and three recently identified viruses Hepatitis GBV-A, GBV-B and GBV-C have genome structure characteristic of flaviviridae family. It is transmitted by transfusion. Infection may

occur in patients coinfected with hepatitis C, and that hepatitis G virus does not change severity of hepatitis C virus. Hepatitis G virus may be the major infection associated with bone marrow failure. Hepatitis producing bone marrow failure (aplastic anemia) is not usually severe.

Reoviruses

It is 70 nm in diameter enclosing second protein shell containing genome of double stranded RNA. The virion is icosahedron. It may be cultured on monkey kidney, human amnion and HeLa cells. Type 3 reovirus has been isolated from Burkitt lymphoma showing oncogenic potential. This role in disease is not proved. They have been isolated from patients of respiratory infection and gastroenteritis.

Coronavirus

It is pleomorphic, 80 to 160 nm in diameters, enveloped with wedge-shaped projection on the surface. The core contains RNA. These viruses have been isolated from cases of common cold. When inoculated in human embryonic trachea it may cause loss of ciliary movements in ciliated epithelial cells. It has many serotypes.

Lymphocytic Choriomeningitis

Man may acquire this infection from excreta of rodents. It may cause influenza like illness or meningitis. It is enveloped RNA virus. Particle inside virus shows electron dense granules resembling sand. It is a natural parasite of mice.

Persistent Virus Infections

Persistent viral infection are kept into following three categories:

1. Latent infection is intermittent acute episodes of disease between which viruses are not demonstrable, e.g. smallpox, measles, varicella, etc.
2. Chronic infection is persistent infection in which virus is always demonstrable but disease is either absent or is associated with immunopathological disturbance, e.g. leukemia, Aleution disease, etc.
3. Slow infection is a persistent infection with a long incubation period followed by slowly progressive disease that is lethal, e.g. scrapie. Discussion on slow virus infection follows:

Slow virus diseases: These diseases are characterized by very long incubation, slow course, fatal termination, predilection for involvement of central nervous tissue, absence of immune response and genetic predisposition.

Slow virus infection are grouped as under:

I. Group A consists of slowly progressive infection of sheep, e.g. leukoviruses causing Visna and Maedi.
   a. Visna is a demyelinating disease of sheep with incubation period of 2 years. Onset of disease is insidious with pareses, total paralysis and death. Virus can be isolated and grown in sheep choroid plexus tissue culture from CSF, saliva or blood of infected sheep.
   b. Maedi is slow progressive fatal hemorrhagic pneumonia of sheep. Incubation period is 2 to 3 years.

II. Group B consists of four infections (1) scrapie (2) kuru (3) mink encephalopathy (4) Creutzfeldt-Jakob disease.

These four conditions are collectively called subacute spongiform viral encephalopathy.

1. Scrapie is a slow virus disease of sheep. Incubation period is about 2 years. The animal becomes irritable, develops pruritus, and later on emaciation and paralysis occur leading to death.
2. Kuru is an endemic disease of New Guinea. Incubation period is 5 to 10 years. Symptoms are cerebellar ataxia and tremors. The disease ends fatally in 3 to 6 months. It occurs in man. The disease is transmitted by tribal practice of eating the dead bodies of their relatives.
3. Mink encephalopathy is a disease of mink and resembles scrapie of sheep.

4. Creutzfeldt-Jakob disease is subacute encephalopathy of man with progressive incoordination and dementia because of degeneration of brain. Death occurs about a year after onset.

III. Group C consists of two unrelated CNS diseases of man (1) subacute sclerosing panencephalitis, and (2) progressive leukoencephalopathy.

1. *Subacute sclerosing panencephalitis (SSPE)*: The disease sets in many years after initial infection characterized by progressive deterioration of mental and motor functions. Brain cells do show serological and microscopic evidence of measles virus infection but virus cannot be cultured in routine cultural techniques. Patient shows high levels of measles virus antibodies in serum. Subacute sclerosing panencephalitis may develop as a very late but rare complication of live measles virus vaccination. Rubella infection may present similar picture as a rare complication.
2. *Progressive multifocal leukoencephalopathy:* It is a rare subacute demyelinating disease of old persons whose immune status is quite low because of malignancy or immunosuppression. Motor functions, speech and vision deterioration do occur resulting in death after 3 to 4 months. Papova virus has been demonstrated from the brain of these patients.

Other possible examples of slow virus disease of man are: Echo virus in Guillian-Barré syndrome and paramyxovirus in multiple sclerosis.

Mechanism of Slow Virus Infection

Following possibilities are proposed so far as mechanism of slow virus infection is concerned:

a. Spread of virus *in vivo* is restricted.
b. Infected cells spontaneously produce virus at low rate *in vivo.* Hence, destructive effect is slow.
c. Virus acts for a series of host mediated pathologic processes that destroy tissue. It is the evolution of tissue damage that is slow.

Marburg Virus

It is an RNA virus resembling rhabdovirus structure. It is larger and more pleomorphic. Natural asymptomatic infection does persist in monkeys in Uganda and Kenya. A fatal natural death was reported in 1975 in man. Originally the virus was isolated from hemorrhagic fever that occur simultaneously in laboratory workers in Marburg, Frankfurt (Germany) and Belgrade (Yugoslavia) in 1967. The mortality rate is over 20 percent. It causes fever, headache, muscle pain, diarrhea, vomiting and maculopapular rash.

Ebola Virus

They are 80 nm in diameter and 800 to 1400 nm in length. They are RNA virus. The envelope comprises of lipid bilayer covered with peplomers. It is SS RNA having helically wound tubular nucleocapsid.

Culture: Vero cells may be used for the culture of Ebola virus. Virus multiplies in the cytoplasm forming large inclusion bodies. BGM cell line is also useful to study cytopathogenesis.

Pathogenesis: It is thought that Ebola virus enters the host through broken skin and mucous membrane. Incubation period is 4 to 10 days.

Norwalk Agent

They are small (27 nm in diameter) RNA virus resembling picornaviruses. They are capable of inducing antibody formation. They have been found to cause gastroenteritis. They have not been successfully cultured so far.

Rotavirus

They are 62 to 66 nm in diameter characterized by well-defined outline like rim of a wheel (Fig. 54.6). They are RNA double stranded virus. They can be grown on monolayer and organ culture of human fetal intestine. Etiological role is established and may have pathogenic role in infantile diarrhea.

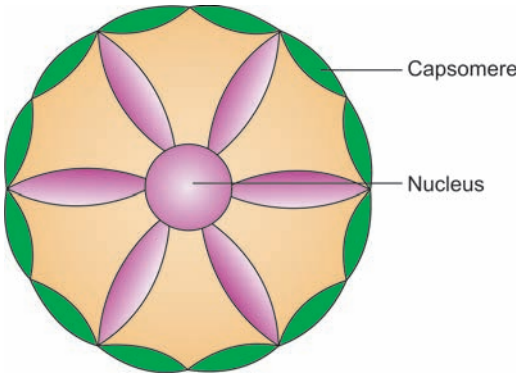


Fig. 54.6: Rotavirus

Rotaviruses infect cells in the villi of small intestine. They multiply in the cytoplasm of these cells and damage their transport mechanisms. Damaged cells may slough into the lumen of the intestine and release large quantities of virus which appear in the stool. Diarrhea may be due to impaired sodium and glucose absorption.

Laboratory Diagnosis

Laboratory diagnosis of rotavirus is based on detection of virus and viral antigen. Following laboratory procedures are useful for the purpose:

1. Electron microscopy is quite helpful but it does not specify serogroup.
2. ELISA.
3. Latex agglutination test.
4. PAGE (Polyacrylamide gel electrophoresis). It is more useful for epidemiological purposes as serogrouping is possible with it.

Rotavirus Vaccines

Bovine Rotavirus Vaccine

i. RIT 4237 vaccine.
   a. It is safe and provides partial protection against rotavirus illness.
   b. This is greater for severe illness than for all rotavirus diarrhea.
   c. Protection appears to be induced more readily in infants over 6 months, for disease due to serotype 1 rather than serotype 2 rotavirus.

Because of low efficacy in young infants and variable protection observed in developing countries, RIT 4237 vaccine has been withdrawn by its manufacturers.

ii. WC-3 vaccine.
   A single efficacy treatment has been carried out in USA with bovine vaccine WC-3. Marked protection against rotavirus illness was observed among infants 3 to 12 months given a single dose. Use of vaccine in developing countries is being planned.
   Dose: 10^7–10^8 PFU with little or no excretion of virus and there were no side effects.

Rhesus Rotavirus Vaccine

- Dose in 10^4 PFU
- Mild and brief fever reaction were observed in 20 to 30 percent in 2 to 4-month-old infants who received 10^4 PFU dose.
- Potential advantage over bovine strain is that its VP_7 neutralizing specific surface antigen is identical to that of rotavirus serotype 3.
- Protection by RRV-1 (formerly MMU-18006) is largely serotype specific (i.e. for serotype 3).
- Protection is substantial against severe disease and can be achieved with single dose, even in very young infants.

Rhesus Human Reassortment Vaccine

This vaccine is developed which incorporates in RRV-1 strain, the gene segments of human rotavirus that encode for the synthesis of surface protein (VP_7) specific for each human rotavirus serotype 1, 2 and 4. Combining these reassortment strains with the parent RRV-1 (serotype 3) would yield tetravalent vaccine that might be useful to induce immunity against each of the 4 major human rotavirus serotypes. Its evaluation is being conducted in Burma, Brazil, India, Israel and Thailand.

Other Rotavirus Vaccine

i. Cloned rotavirus gene segments in prokaryotic and eukaryotic cells.
   a. To produce large quantity of rotavirus antigen for use in non-living vaccine.

b. When cloned, *E. coli* is used to develop a potential live vaccine based upon *E. coli* that elaborate protective antigens of rotavirus.
c. Expression of VP_7 is achieved in eukaryotic cells using recombinant vaccines virus as vector.
d. In USA, expression of rotavirus antigen is achieved successfully in cultured insect cells using baculovirus vector.

ii. When cold adapted mutant of human rotavirus is used several such mutants are developed.

Lassa Virus

It was described for the first time in Lassa in 1969 and subsequently seen as outbreaks in other corners of Africa. Rodents may be the source of virus. The virus may be demonstrated in sera by complement fixation test. Their isolation can be done by tissue culture. It has caused mortality up to 45 percent in some outbreaks. The disease manifests with fever, sore throat, ulceration of mouth and pharynx, vomiting, abdominal and chest pain and diarrhea. It does cause leukopenia and proteinuria.

The virus is a member of Arenaviruses and contain RNA with projection form surface. It is 70 to 150 nm in size.

Retrovirus (Table 54.3)

It is single stranded RNA virus which is transcribed from RNA to DNA by reverse transcriptase. The name retro is derived from Reverse transcriptase. It is 100 nm, round, enveloped and covered by surface projection. The nucleoprotein is helical and surrounded by icosahedral capsid protein to form nucleoid.

The replication of the virus is unique and is initiated with the transcription of positive SS RNA of virus to negative SS DNA by viral reverse transcriptase (enzyme released during uncoating from capsid). The negative DNA becomes double stranded circular DNA which gets integrated into host cell chromosome by viral enzyme DNA ligase. Now viral DNA genes code for virus component.

TABLE 54.3: Classification of retrovirus (3 groups)

Group	*Human pathogens*
I. Oncovirinae	1. Human tumor leukemia virus 2. HIV
II. Lentivirinae	1. Human tumor leukemia virus 2. HIV
III. Spumavirinae	1. Human foamy virus (HFV)

Parvoviruses

They belong to family parvoviridae and are small about 20 nm, nonenveloped, icosahedral viruses having single stranded DNA. It has three genera: Parvovirus, Dependovirus (defective virus always requiring helper virus for growth and replication) and Erythrovirus. Parvovirus B 19 has been included in Erythrovirus.

Parvovirus B 19: They may be associated with aplastic crisis in sickle cell disease and other hemolytic anemias as they have affinity for immature red blood cell precursors. In immunodeficient cases it may cause persistent anemia, erythema infectiosum of cheek (slapped cheek appearance) further spreading to trunk and limbs followed by lymphadenopathy and arthralgia. It may involve children fever with rash sometimes referred as fifth disease as this condition was placed 5th in the old list of six exanthematous fever of children. If pregnant woman happen to develop primary infection, the result may be erythroblastosis fetalis with hydrops.

Nipah virus: It was discovered in 1999 and is recognized zoonotic virus. The virus is named after the location where it was first detected in Malaysia. Nipah is closely related to another zoonotic virus (1994) called Hendra virus. Nipah and Hendra are members of Paramyxoviridae.

Certain species of fruit bats are natural hosts of both Nipah and Hendra viruses. The bats appear to be susceptible to infection with these viruses. Bats do not themselves become ill. The role of species other than pigs in transmitting infection to other animals has not been determined. Human to human transmission of Nipah virus has not been reported.

The incubation period is 4 to 18 days. Patient develops high fever muscle pains, inflammation of brain with drowsiness,

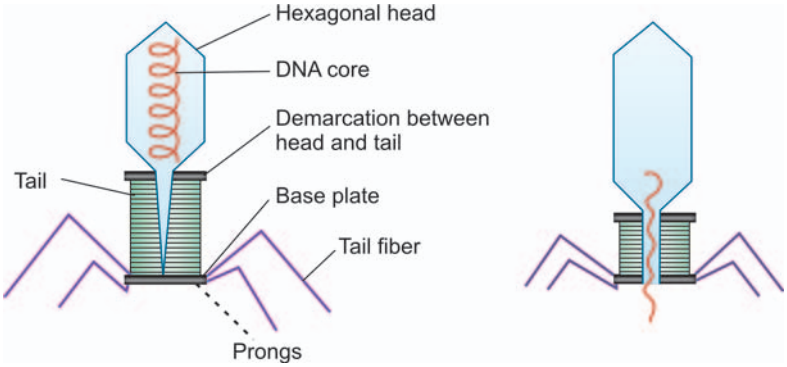


Fig. 54.7: Bacteriophage

disorientation, convulsions and coma. Fifty percent cases die.

Early treatment with ribavirin can reduce both duration of feverish illness and severity of disease.

Bacteriophage

They are viruses that infect bacteria. They are also called phage and are 10 to 100 µ (Fig. 54.7). Recently, more concentration is being given to phages of *E. coli* especially T even phage. T phages are easy to purify because of their large size and high frequency of recombination which is quite useful for genetic studies.

Bacteriophage each exists in structurally different states, e.g. extracellular form, vegetative form or prophage strain of bacteria which are themselves not susceptible to lysis but carry phage (prophage) are known as lysogenic strains.

Even numbered T phages made up of head and tail are tadpole in shape. Head contains DNA and has the shape of two halves of icosahedron connected by short hexagonal prism. Tail varies in dimension and structure. It is composed of tube through which DNA passes during cell infection. Tube is surrounded by parallel rows of protein manomer, are connected to this disc at head end and to the plate at tip end. This sheath is capable of contraction. The plate tip end is hexagonal, has pin at every corner and is connected with 6 very long thin tail fibers.

Lifecycle of phage starts with adsorption and penetration. Receptors of different phages are located in different layers of cell wall of say *E. coli.* Nucleic acid (DNA) is injected into the cells.

NONBACTERIAL GASTROENTERITIS (VIRAL GASTROENTERITIS)

This is the second most common viral disease. Sign and symptoms last only for 24 to 48 hours. Viruses responsible are:

1. Rotavirus
2. Norwalk agent — Proved etiological agents
3. Hawaii virus
4. Coronavirus
5. Adenovirus
6. Echovirus
7. Coxsackie virus
8. Reovirus
9. Asteroid virus
10. Poliovirus
11. Hepatitis virus

Diagnosis is made by isolating the virus from fecal samples. Viruses like adeno and echo are excreted for a week after onset of disease. Rectal swab (fecal sample) in TC 199 with albumin, streptomycin, penicillin and polymyxin is taken. It may be inoculated in chick embryo, tissue culture and laboratory animals. Diagnosis may be confirmed by electron microscopy, immunofluorescence, hemadsorption, hemagglutination, inhibition and neutralization with specific antisera.

ACUTE ASEPTIC MENINGITIS (VIRAL MENINGITIS)

The term acute aseptic meningitis syndrome includes number of disorders which have in common an acute onset usually self-limiting

course with meningeal manifestations of varying degree, an increase in cells of spinal cord and absence of organisms on direct smear.

Viruses responsible are:

1. Mumps
2. Echo 4, 6, 8, 11, 14, 18, 30
3. Coxsackie B_{16}
4. Polio
5. Measles
6. Herpes simplex
7. Lymphocytomegalovirus
8. Arboviruses
9. E.B. virus

Laboratory diagnosis may be established by examining cerebrospinal fluid and inoculating an egg proceeded by animal pathogenecity. In case of Echo and Coxsackie, mice is the animal of choice and route of injection is intracerebrally. Serological procedure like complement fixation test, neutralization and hemagglutination test may be undertaken for the diagnosis.

PERINATALLY ACQUIRED VIRAL INFECTION

Infants may come in contact with vaginal secretion and get viral infection. Viruses responsible are:

1. Herpes virus
2. Cytomegalo virus
3. Varicella zoster virus
4. Coxsackie B virus
5. Hepatitis B virus.

INTRAUTERINE INFECTION

1. Rubella virus
2. Cytomegalo virus
3. Herpes simplex (cerebral abnormality)
4. Coxsackie B (congenital heart defect)
5. Influenza (teratogenic effect?).

POSTNATAL VIRAL INFECTION

1. Respiratory syncytial virus
2. Influenza virus
3. Varicella virus
4. Adenovirus
5. Measles virus.

Part VI

Mycology

55 Mycology

Fungi and yeasts constitute eumycetes. These eukaryotes lack chlorophyl pigments. They possess differentiated nuclei surrounded by nuclear membrane and reproduce either by budding or by forming spores. They have rigid chitinous cell walls. They lack differentiation of root, stem and leaves. Morphologically the fungi may be either simple, oval cells or long, tubular, septate hyphae showing true lateral branching.

The yeasts and fungi need organic compounds as nutrients. Their role in nature appears to be as scavanger, i.e. breaking down the complex carbohydrates and proteins of dead bodies of other organisms. Needless to mention that only a few of them are pathogenic. In many ways, fungi have been of service to man, as in the making of bread, fermented drinks, cheese, antibiotics, etc.

MORPHOLOGICAL CLASSIFICATION OF FUNGI

Fungi can be divided into four groups each of which have some human pathogenic species.

1. **Moulds:** They are filamentous and mycelial fungi. They grow as long filaments or hyphae which branch and interlace to form a meshwork or mycelium. They reproduce by forming various kinds of spores. The part of the mycelium which grows on and penetrates into the substrate, absorbing nutrients for growth is called vegetative mycelium. The part of mycelium which protrudes into the air is called aerial mycelium. On artificial medium they are seen as filamentous mould colony which may be dry and powdery. The pathogenic members are trychophyton, microsporum and epidermophyton.
2. **Yeasts:** They are unicellular occurring as spherical or ellipsoidal cells. They reproduce by budding. On solid media they form moist, compact, creamy, mucoid colonies resembling those of staphylococci. *Cryptococcus neoformans* is the only important pathogens.
3. **Yeast like fungi:** They grow partly as yeasts and partly as long filamentous cells joined, end to end forming a pseudomycelium. On solid media moist creamy colored colonies are produced. *Candida albicans* is the example.
4. **Dimorphic fungi:** They grow in mycelial form at low temperature, i.e. 22°C or in soil whereas growth at 37°C or in animal body occurs in yeast form. The pathogenic members are *Histoplasma capsulatum,* sporotrichum, blastomyces and *Coccidioides immitis.*

SYSTEMATIC CLASSIFICATION

Based on sexual spore formation fungi are kept in 4 classes as described below:

1. **Phycomycetes:** They are fungi having nonseptate hyphae. They form endogenous asexual spore (sporangiospore) contained within sac-like structure called sporangia. Sexual spores are also found and are of two varieties—oospore and zygospore.
2. **Ascomycetes:** They form sexual spores (ascospores) within a sac. This sac is called ascus. They include both yeasts and filamentous fungi. They form septate hyphae.
3. **Basidiomycetes:** They reproduce by means of sexual reproduction. Basidiospores are borne at the tip of basidium. These basidia are sometimes quite large leaf-like structure as in mushroom. They form septate hyphae.

4. **Fungi imperfecti:** They consist of group of fungi whose sexual phases have not been identified. Fungi of medical importance belong to this group, e.g. *Sporothrix schenkii.*

LABORATORY DIAGNOSIS

A presumptive clinical diagnosis of mycosis must be confirmed by a laboratory diagnosis methods. This includes the following:

Direct Microscopy

Potassium Hydroxide (KOH) Preparation

The specimen placed in a drop of 10% KOH on a slide covered with a coverslip can be observed under a microscope. Fungal chains of arthrospores and free arthrospores may be seen and their recognition permits a diagnosis of fungal infection. Budding yeast cells mixed with long filaments are indicative of a yeast-like fungus.

In case of doubt as to the nature of any fungus-like elements in the specimen, KOH is replaced by lactophenol blue, or clacflour stain.

Gram Stain

Gram positive yeasts such as those of Candida species can be observed by Gram stain.

India Ink Preparation

India Ink is helpful in demonstrating the capsule e.g. *Cryptococcus neoformans*.

Culture

Sabouarud's Dextrose Agar (SDA) is the most suitable medium as fungal growth is favored by a high sugar concentration and is relatively tolerant to acidity (pH 5.4). The agar is prepared as slopes in test tubes stoppered with cotton-wool as most of the fungi are aerobic. Chloramphenicol is incorporated in the culture medium to prevent contamination by bacteria. Similarly addition of cycloheximide (actidone) can suppress the contaminating fungi. The fungal growth can be identified by its color and morphology on visual examination and pigmentation on the reverse.

Microscopic examination is done to evaluate the morphology of hyphae, spores and other structures. Teased mounts are made in lactophenol blue and examined under a microscope. The morphology of various spores is characteristic of different fungi. Slide culture is useful for studying the exact morphology of the fungus.

Tissue Sections

Although many fungi can be seen with Haematoxylin and Eosin stain, special stains also used for fungi include Periodic acid Schiff stain (PAS), Gomori methenamine Silver stain (GMS), Mayer's mucicarmine stain and Gridley fungal stain.

Serology

Serological diagnosis of fungal infections usually lacks complete specificity because some of the pathogenic fungi have common antigens. Fractional separation of the active antigenic components of a fungus has not been achieved with complete success. However, serology may be useful in reaching a presumptive diagnosis.

Mycology is discussed in this section in following scheme: Superficial mycosis, subcutaneous mycosis, systemic mycosis, opportunistic fungus and miscellaneous others.

Superficial Mycosis

Pityriasis Versicolor

Etiology: Pityrosporum orbiculare (Malassezia furfur)

Specimen: Scraping from skin lesion

Direct microscope examination: KOH preparation shows clusters of yeast-like cells and short branched hyphae.

Culture: Not cultured so far.

Other diagnostic tests: Examination under Wood's lamp shows fungus giving yellow fluorescence.

Clinical picture: Pityriasis versicolor is a superficial chronic fungus infection of the horny layer of the epidermis involving the trunk of the body.

The normal skin pigmentation is altered resulting in a blotchy appearance. The infected areas are usually brownish. The lesions fluoresce a pale yellow under a Wood's light.

Treatment: Application of soap and water followed by application of sodium thiosulphate solution or 3 percent salicylic acid in 7 percent alcohol. Miconazole and clotrimazole are also effective.

Tinea Nigra

Etiology: Cladosporium mansonii (in Asia, Africa) *Cladosporium werneckii*.

Specimen: Scrapings from skin lesions.

Direct microscopic examination: KOH preparation shows brownish branched septate hyphae and budding cells.

Culture: On Sabouraud's dextrose agar the colonies are moist, shiny, black yeast like after 3 weeks' incubation at 35°C.

Microscopic examination of above-mentioned colonies shows dark hyphae and budding cells which are single or two celled.

Clinical picture: Lesions are largely confined to the palms where they appear as irregular, flat darkly discolored areas (dark brown to black). This lesion is flat and not scaly.

Treatment: Three percent sulfur and 2 percent salicylic acid, tincture of iodine or weak Whitfield ointment may be beneficial.

Black Piedra

Etiology: Piedra hortai

Specimen: Infected hair with nodule

Direct microscopic examination: KOH preparation shows on hairshaft, dark brown, dichotomous branched hyphae. Septation in thick-walled hyphae give appearance of arthospores. Broken nodules show asci containing 2 to 8 ascospores.

Culture: On Sabouraud's dextrose agar after incubation at 25°C for 3 weeks, colonies grow as greenish black, elevated in center or flat, glaborous or smooth to cribriform.

Microscopic examination of these colonies shows dark, thick-walled hyphae, multiseptate with many chlamydospores. Rarely asci and ascospores like those seen in hair are spotted.

Clinical picture: Piedra hortai forms hard, dark nodules on the shaft of infected scalp hair.

Treatment: Clip or shave the infected area and apply antifungal agents like 1 : 2000 solution of bichloride of mercury, 3 percent sulfur ointment or benzoic and salicylic acid combination.

White Piedra

Etiology: Trichosporon cutaneum.

Specimen: Infected hair with nodule.

Direct microscopic examination: KOH preparation shows on hair shaft, transparent greenish brown mycelial mass. Hyphae are at right angle to shaft and segmented into oval cells. Rarely there is budding but no asci.

Culture: On Sabourand's, dextrose agar after incubation at 25°C for 3 weeks there appear rapidly growing, shiny colonies which are first cream colored and later on center heaped and colony wrinkled.

Microscopic examination of the colonies show transparent greenish brown mycelial mass which form round to rectangular cells. No asci are observed.

Clinical picture: There is involvement of scalp or beard hair. Soft, pale nodules appear on the shaft of the hair.

Treatment: Use of 1 : 2000 solution of bichloride of mercury, 3 percent sulfur ointment or benzoic acid and salicylic acid combination is quite beneficial.

Dermatophytoses: Ringworms, athlete's foot, jock itch and dermatomycosis are other names given to dermatophytoses.

Dermatophytoses refer to infection of skin, nails or hair that are caused by fungi classified as dermatophytes. It does not cause systemic disease and with only one minor exception *(Trichophyton verrucosum),* none of this group of fungi can grow at 37°C.

All fungi of dermatophytoses are discussed in three genera, i.e. Microsporon, Trichophyton and Epidermophyton.

MICROSPORUM

Microsporum gypseum: This fungus resides in soil (geophilic).

Direct microscopic examination: KOH preparation (Figs 55.1 A and B) of infected hair shows spores surrounding the hair (ectothrix infection).

Culture: On Sabouraud's dextrose agar the colonies are flat, light brown and very powdery in appearance at 25°C.

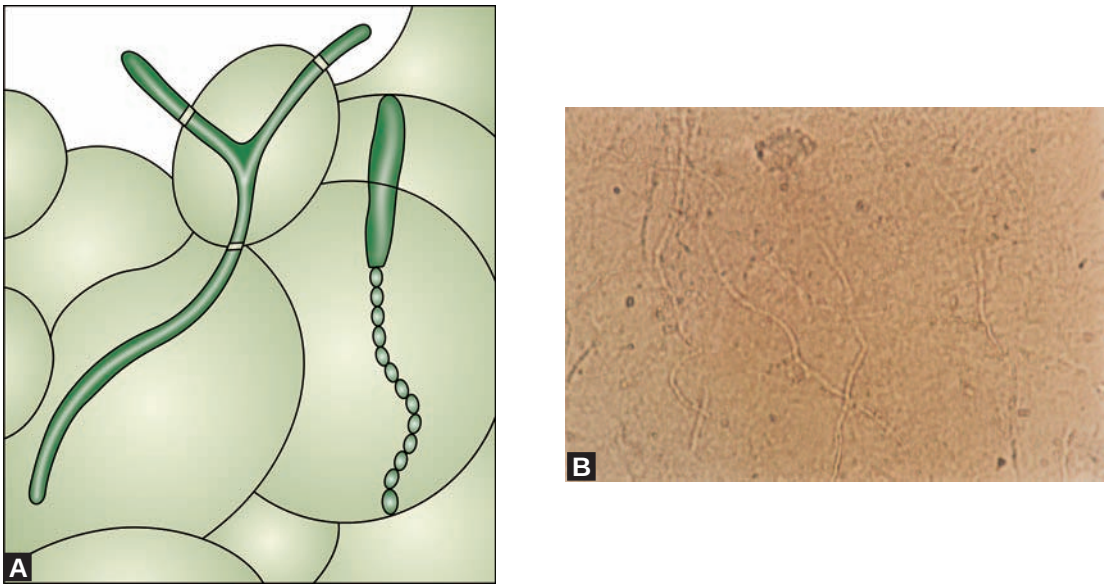
Microscopically it forms thin-walled, spindle-shaped spores which contain 4 to 6 septa (Figs 55.2 A and B).

Microsporum canis: This fungus is zoophilic and causes sporadic outbreaks of hair and skin infection. Erythema is common in lesions caused by this fungus.

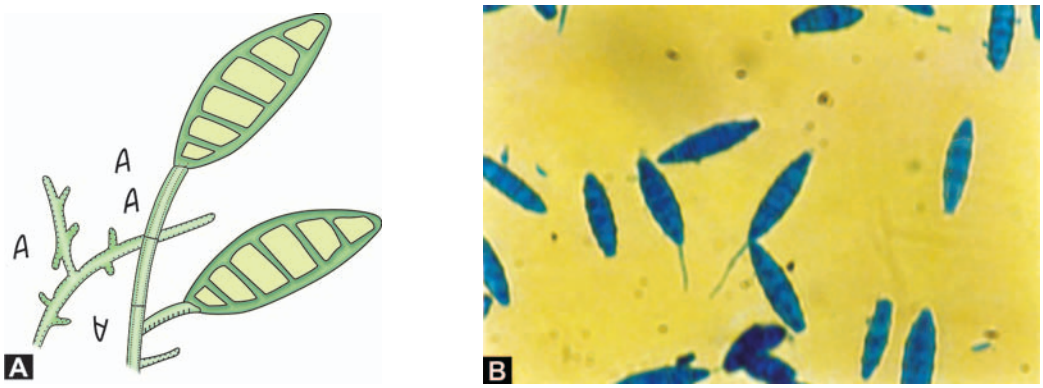
Direct microscopic examination: KOH preparation of hair and skin shows spores.

Culture: On Sabouraud's dextrose agar the colonies are white and fluffy. Reverse side of the colony is pigmented as bright brownish yellow at 25°C.

Microscopically this fungus is characterized by the formation of large, thick-walled, spindle-shaped macroconidia which contain 8 to 12 septa (Fig. 55.3).



Figs 55.1 A and B: (A) Dermatophyte in KOH preparation of skin or nail showing branching hyphae and arthrospores (B) KOH wet mount showing branched septate hyphae with arthroconidia (40 x)



Figs 55.2A and B: (A) *Microsporum gypseum* (B) LCB Mount of *M. gypseum* showing macronidia with rat tail filaments (40 x)

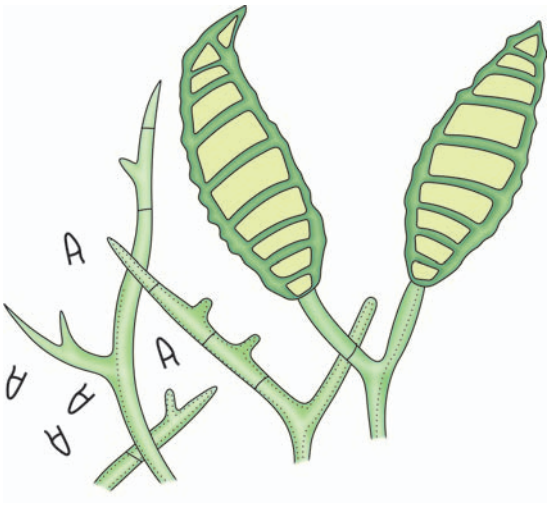


Fig. 55.3: *Microsporum canis*

Microsporum audouini: It is anthropophilic and is the etiologic agent in most epidemics of ringworm of the scalp in children and rarely attacks animals.

Direct microscopic examination: KOH preparation shows spores.

Culture: On Sabouraud's dextrose agar forms white fluffy colonies after 1 to 2 weeks incubation at 25°C. The underside of the colony is pale yellow to light orange in color.

Microscopically there are thick-walled chlamydospores.

Trichophyton

Trichophyton Rubrum

Direct microscopic examination: KOH preparation of tissue (skin and nails) shows branching septate hyphae or chain of arthrospores.
Culture: On Sabouraud's dextrose agar at 25°C for 1 to 4 weeks the growth is velvety with red pigment on reverse side of medium (Figs 55.4 A and B) and (Fig. 55.7 A).

Microscopic examination of growth shows few long pencil shaped macroconidia. There are rarely cigar shaped macroconidia or spiral hyphae. The microconidia show bird on fence arrangement (Fig. 55.5)

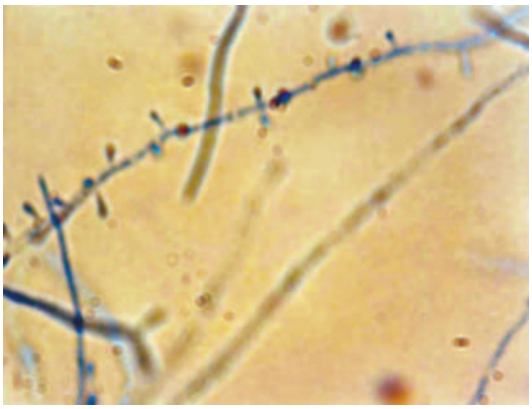
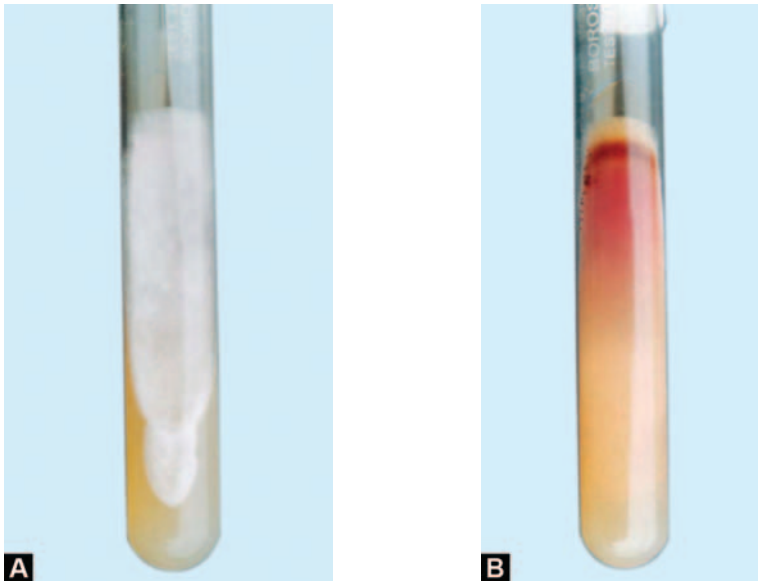


Fig. 55.5: LCB Mount of *T. rubrum* showing bird on fence arrangement of the microconidia (40x)



Figs 55.4 A and B: (A) Fluffy white growth of *Trichophyton rubrum* on Sabouraud's dextrose agar medium; (B) Deep red color on reverse of culture medium (*T. rubrum*)

Diseases Produced

1. *Tinea corporis*
2. *Tinea pedis*
3. *Tinea cruris*
4. *Tinea barbae*
5. *Tinea unguium*

Trichophyton Mentagrophytes

Direct microscopic examination: KOH preparation of hair shows spores surrounding the hair (ectothrix).

Culture: On Sabouraud's dextrose agar at 25°C for 1 to 4 weeks there appears white to tan colored, cottony or powdery colonies. Pigment is variable (Fig. 55.7 B).

Microscopic examination of growth shows grape like clusters of microconidia. There are rarely cigar shaped macroconidia or spiral hyphae seen (Figs 55.5, 55.6 and 55.8).

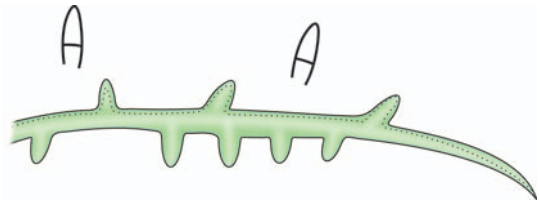
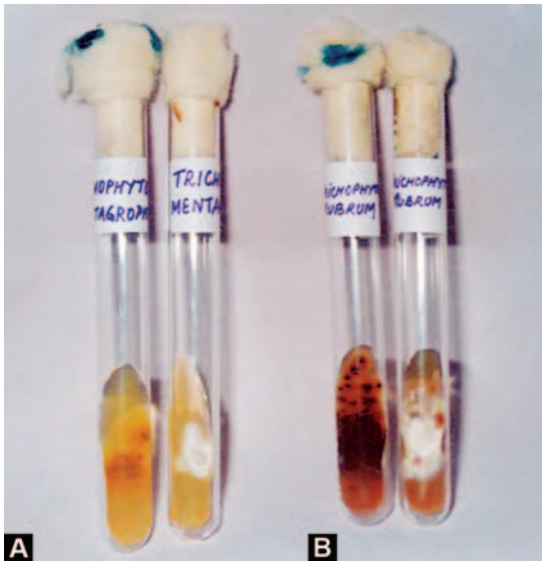


Fig. 55.6: *T. rubrum*



Figs 55.7 A and B: (A) Colony of *T. rubrum* (a) Obverse showing floccose pink white colonies (b) reverse showing red to tan pigment. (B) Colony of *T. mentagrophytes* (a) obverse showing powdery white colonies, (b) reverse showing yellow to brown pigment

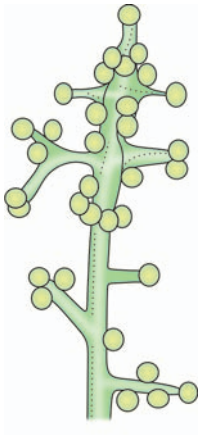


Fig. 55.8: Trichophyton mentagrophytes

Diseases Produced

1. *Tinea corporis*
2. *Tinea pedis*
3. *Tinea cruris*
4. *Tinea barbae*
5. *Tinea unguium*

Trichophyton Tonsurans

Direct microscopic examination: KOH preparation of hair shows spores inside hair shaft (endothrix).

Culture: On Sabouraud's dextrose agar medium at 25°C for 1 to 4 weeks colonies (Fig. 55.9) appear which are cream or yellow colored with central furrows (Fig. 55.9(4)).

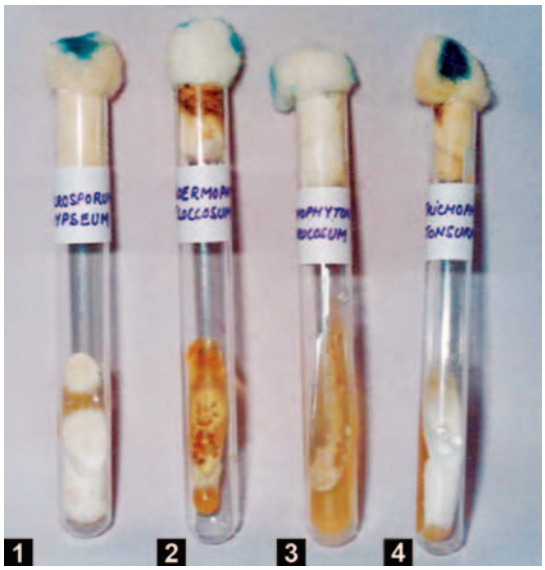


Fig. 55.9: Left to right: Culture tubes showing colonies of: (1) *M. gypseum* (2) *E. floccosum* (3) *T. verrucosum* (4) *T. tonsurans*

Microscopic examination of these colonies shows numerous microconidia (Figs 55.10A and B). Rarely there is thick-walled irregular macroconidea.

Diseases produced: Tinea cruris.

Trichophyton Schoenleinii

Direct microscopic examination: KOH preparation of the hair shows endothrix invasion with hyphae and air spaces in the hair shafts.

Culture: On Sabouraud's dextrose agar, colonies are slow growing, waxy or suede-like with a deeply folded honey-comb-like thallus and some sub-surface growth. No macroconidia and microconidia are seen in routine cultures; however numerous chlamydoconidia may be present in older cultures. However, characteristic "nail head" hyphae also known as "favic chandeliers" may be observed.

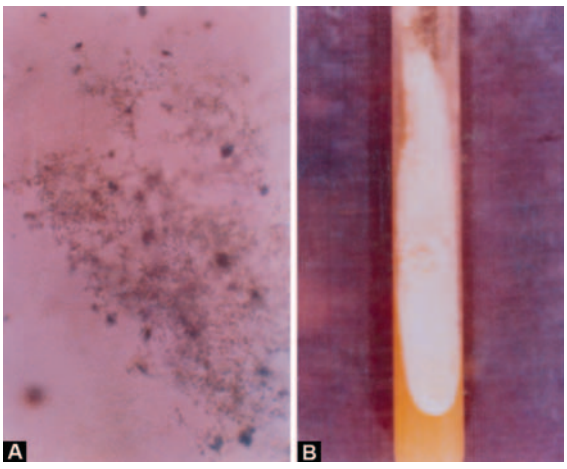
Disease produced: Favus.

EPIDERMOPHYTON

Epidermophyton floccosum: This fungus contains only one species and infects only man. It attacks skin and nails only.

Direct microscopic picture: KOH preparation shows macrospores which are smooth-walled and contain 2 to 3 septa having blunt or rounded ends (Fig. 55.11).

Culture: On Sabouraud's dextrose agar colonies are yellow to greenish and are quite



Figs 55.10A and B: (A) Grape-like arrangement of microconidia of trichophyton. (B) Powdery colonies of trichophyton mentagrophyte on SDA medium

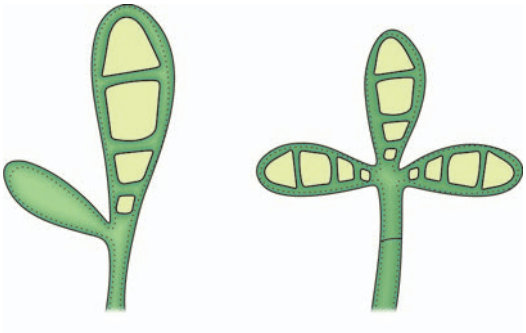


Fig. 55.11: *Epidermophyton floccosum*

wrinkled and folded at 25°C. The colonies have very fine fuzzy texture almost like suede leather (Fig. 55.9(2)).

Microscopically one can find club-shaped macrospore usually formed singly or in clumps of 2 to 3. No microspores are formed.

Pathogenesis: The pathogenecity of dermatophytes is not understood. The fungus grows in the keratinized layer of the skin, throughout the thickness of nails and inside hair shaft, the keratin being attacked by extracellular enzymes. Usually hyphae do not penetrate into living tissue and mechanism by which these superficial infections stimulates inflammatory reactions is not clear. It could be a reaction to products of fungal metabolism or to fungal constituents.

As far as a variability of pathogenecity for different hosts, the dermatophytes vary in their ability to attack particular structures or areas of the body. *Microsporum audouini* usually confines its attack to the hair of children under the age of puberty and no microsporum species attacks nails. Trichophyton is a common cause of skin and nail infections but does not attack hair. Epidermophyton attacks skin and never the hair.

Immunology: Persons with dermatophytoses sometimes have skin lesions believed to represent an allergic reaction to fungal antigens (Id's reaction), that spread from the site of infection. These lesions, dermatophytids, are sterile and appear as vesicles, symmetrically distributed on the hands.

'Id's reaction: It is in fact secondary eruption encountered in sensitized tinea patients. It may be due to circulation of allergenic fungal

products from the primary site of dermatophytic infection. Id reaction is also called dermatophytid. Actually fungal products are absorbed from the skin resulting in itching. The lesions are sterile. There are two types of Id reaction:

1. Lichen scrofulosorum like reaction which is seen in tinea capitis in children. There are small grouped follicular lesions on the body. These lesions are symmetrical and central in distribution. They are seen mostly on limbs and face.
2. Pompholyx like lesion seen in patients having tinea pedis infection. Lesions appear on the sides and flexor aspects of fingers and palms. The lesion may be popular or vesicular, pompholyx like mostly bullous. It is because of type III type of hypersensitivity.

Treatment: Griseofulven, clotrimazole and miconazole are effective drugs.

SUBCUTANEOUS MYCOSIS

Rhinosporodiasis

Etiology: Rhinosporidium seeberi

Specimen: Polyp material.

Direct microscopic examination: KOH preparation of polyp material crushed between two slides shows fungal spherules (10 to 200 µ) containing endospores.

Culture: Culture is not possible.

Histological examination: It shows number of fungal spherules (100 to 200 µ) containing endospores.

Clinical picture: The infection causes the development of polyp in the submucosa of the nose, mouth and other areas, like conjunctiva and occasionally the skin. Reports of this infection are mostly from India and Sri Lanka. Affected persons give a history of repeated bathing in polluted river and pond water. Natural infection with this fungus occurs in horses, mules and cows. There is no tendency of dissemination.

Treatment: Excision of polyp.

Chromoblastomycosis

Etiology: *Fonsecaea pedrosoi.*
Fonsecaea compactum.
Fonsecaea dermatitides.
Phialophora verrucosa.
Cladosporium carrioni (Fig. 55.12).

Specimen: Scraping from skin lesions and skin biopsy tissue.

Direct microscopic examination: KOH preparation shows dark brown, round, thick-walled, fungal bodies with septae.

Culture: On Sabouraud's dextrose agar at 25°C for 1 to 3 weeks' incubation, black colored velvety growth appears. Microscopic examination is done for species identification.

Histological examination: It shows fungus which is seen as round or irregular dark brown bodies with septae (Fig. 55.13).

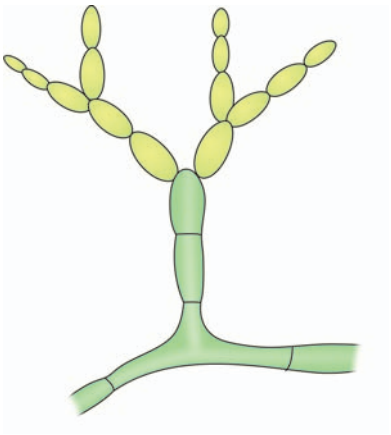


Fig. 55.12: Cladosporium

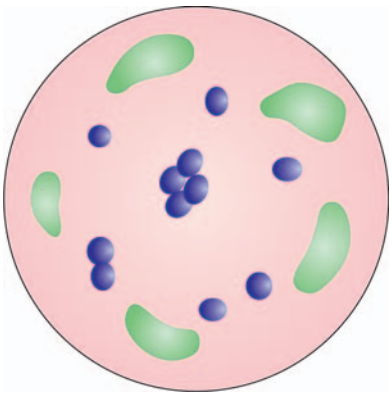


Fig. 55.13: Chromomycosis (pigmented fungal cells in giant cell)

Clinical picture: Chromoblastomycosis is a chronic warty dermatitis, usually of legs and feet caused by traumatic inoculation of one of the five closely related pigmented fungi which normally grow on wood. The lesions appear as warty, ulcerating and cauliflower-like growth.

Treatment: Early lesions may be excised. However, neither surgery nor medical treatment has been successful with late lesions.

Mycetoma

Etiology

A. Maduromycotic mycetoma (True fungi).
   1. *Madurella mycetomi*
   2. *Madurella grisea*
   3. *Phialophora jeanselmei*
   4. *Alescheria boydii*
   5. *Cephalosporium*

B. Actinomycotic mycetoma (Higher bacteria)
   1. *Nocardia asteroides*
   2. *Nocardia brasiliensis*
   3. *Actinomadurae madurae*
   4. *A. pelletere*
   5. *Streptomyces somaliensis*

Specimen: Pus curretage material, biopsy from skin, and grains or granules of pus.

Gross and microscopic examination of granules: Granules are washed, separated and examined grossly first. White to yellow granules are seen in *A. boydii, Nocardia asteroids, Nocardia brasiliensis, A. madusae, Streptomyces somaliensis,* etc. Brown to black granules are seen in *M. mycetomi, M. grisea, P. jeanselmoi, A. pelleteri,* etc.

Microscopic examination of maduromycotic mycetoma shows grains composing of broad hyphae which are often septate with chlamydospore. However, actinomycotic mycetoma grains are composed of very small less than 1 µ, Gram-positive bacteria-like filaments.

Culture: It is done on Sabouraud's dextrose agar with chloramphenicol and cyclohexamide at 25°C for 1 to 2 weeks incubation for maduromycotic mycetoma and only Sabouraud's dextrose agar without antibiotics for actinomycotic mycetoma. Each fungus is identified by its colonial characters and microscopic morphology.

Histology examination: H and E stained section shows granules or grain surrounded by eosinophilic material.

Clinical picture: The fully developed mycetoma is a chronic suppurative, granulomatous lesion with progressive destruction of contagious tissue and vascular dissemination (lymphatics and blood vessels). Skin and subcutaneous tissues are involved originally, but as the disease progresses fascia and bone become infected. The foot is the most infected part of the body. With the progress of disease, the foot becomes grossly deformed with multiple sinus formation and fistula tract which communicate with each other and with deep abscesses including ulcerated areas of the skin. Within suppurative foci the fungi or bacteria form characteristic grain which are composed of colonies embedded in and surrounded by an eosinophilic and homogenous material.

Treatment: It is very important to differentiate between actinomycotic (bacterial) and eumycotic (fungal) mycetoma. Actinomycotic mycetoma usually responds to antibiotics used for Gram-positive bacterial infections. For eumycotic mycetoma amputation of the infected extremity is done.

Sporotrichosis

Etiology: Sporotrichum schenckii

Specimen: Pus and skin biopsy

Direct microscopic examination: KOH preparation shows cigar shaped yeast cells. However, fungus is not demonstrable (Fig. 55.14A).

Culture: It is done on Sabouraud's dextrose agar at 25°C and 37°C incubation for 1 to 3 weeks. First cream white and later brown to black colonies which are rough and yeast like with wrinkled surface appear. At 37°C yeast colonies appear.

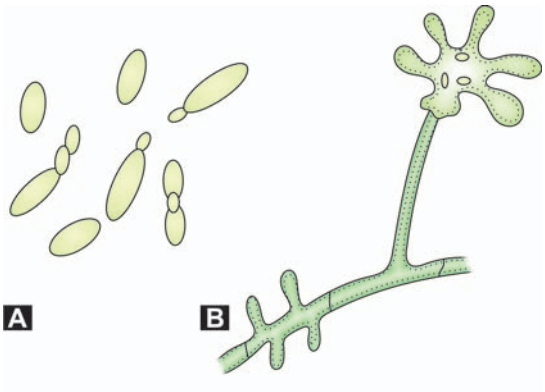
Microscopic examination of these colonies show delicate branching septate hyphae. On lateral branches of these hyphae there appear microconidia (2 to 5 µ) pyriform in shape and in clusters. However, microscopic examination

of yeast colonies (37°C) shows flower like cluster of oval to fusiform budding cells (1 to 3 µ × 3 to 10 µ) (Fig. 55.14 B).

Histological examination: H and E stained section shows asteroid bodies composed of central fungus cell with eosinophilic material radiating from it.

Methanamine silver stained section shows fungus as cigar shaped yeast cells.

Other laboratory tests: Mice inoculated intratesticular or intraperitoneal and then tissue examined for cigar shaped bodies.



Figs 55.14A and B: Sporothrix schenckil. (A) Blastospores in tissue or at 37°C culture, (B) Conidia formation in 20°C culture

SYSTEMATIC MYCOSIS

Cryptococcosis

Etiology: Cryptococcus neoformans.

Specimens useful:

1. CSF
2. Sputum
3. Scraping from skin lesions
4. Exudates
5. Urine
6. Tissue (autopsy).

Direct microscopic examination: KOH preparation shows budding yeast cell whereas India ink preparation reveals capsulated yeast cells with budding.

Culture and identification: On Sabouraud's dextrose agar with chloramphenicol at 37°C and 25°C for 1 week, there appears smooth mucoid cream colored colonies. Microscopic examination of these colonies show encapsulated budding yeast cells.

Other identifying characters are hydrolysis of urea, brown colored colonies, and pathogenicity to mice.

Other Laboratory Tests

1. Precipitation.
2. Complement fixation test.
3. Latex agglutination test.
4. Countercurrent immunoelectrophoresis.
5. Histological examination of H and E stained section shows budding yeast cells with little tissue reactions.

Pathogenesis: Inhalation of *Cryptococcus neoformans* is assumed to initiate pulmonary infection, with subsequent hematogenous spread to other viscera and the central nervous system. In the severe, chronic and disseminated form, brain, meninges, lungs, skin, bones, etc. are involved.

Treatment: Amphotericin B is particularly effective.

Candidiasis

Etiology: Candida albicans

Specimens:

1. Swabs or scraping from skin
2. Sputum
3. Exudates
4. Feces

Direct microscopic examinations: KOH preparation shows yeast cells with budding and pseudohyphae.

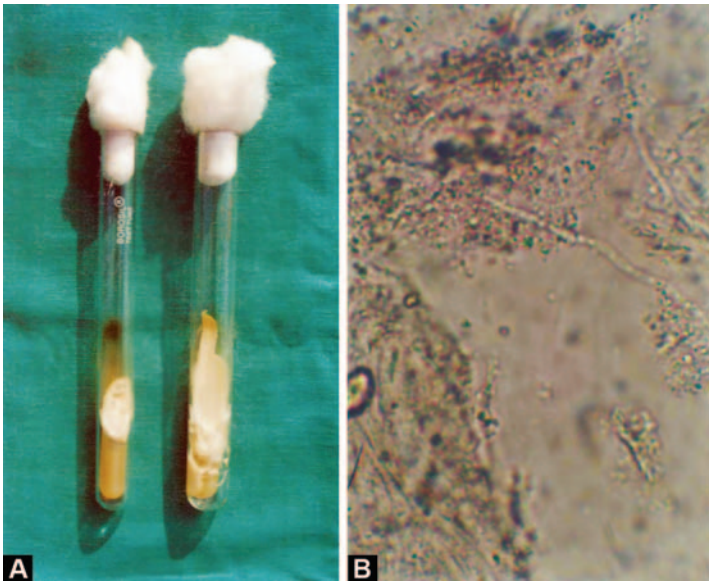
Gram-stained smear shows Gram-positive yeast cells with budding and pseudohyphae.

Culture and identification: On Sabouraud's dextrose agar with chloramphenicol after 1 to 7 days incubation at 37°C and 25°C shows creamy white smooth colonies. Microscopically they are Gram-positive yeast cells with budding and pseudohyphae (Figs 55.15 A and B).

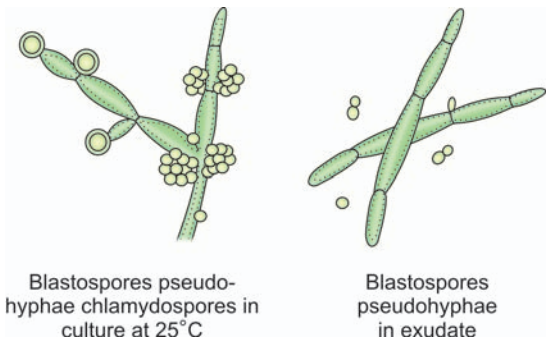
Additional characteristic features are chlamydospores production in corneal agar (Fig. 55.16) and germ tube formation in human serum at 37°C within two hours.

Other laboratory tests:

1. Precipitation test
2. Agglutination test
3. Indirect fluorescent test.



Figs 55.15 A and B: Waxy colonies of *Candida albicans* on SDA medium (A), Microscopic view of lactophenol cotton blue mount of *Candida albicans (B)*



Figs 55.16: *Candida albicans*

Clinical picture: It is an acute or chronic, superficial or disseminated mycosis causing thrush (white, creamy patches on the tongue), paronychia (inflammation of subcutaneous tissues at the base of fingernails or toe-nails), vulvovaginitis, bronchocandidiasis (chronic bronchitis), pulmonary candidiasis, endocarditis, meningitis and septicemia.

Treatment: Nystatin and miconazole are useful for candidiasis without the involvement of internal organs. For systemic candidiasis amphotericin B and 5 fluorocytosine are of some value.

Histoplasmosis

Etiology: Histoplasma capsulatum

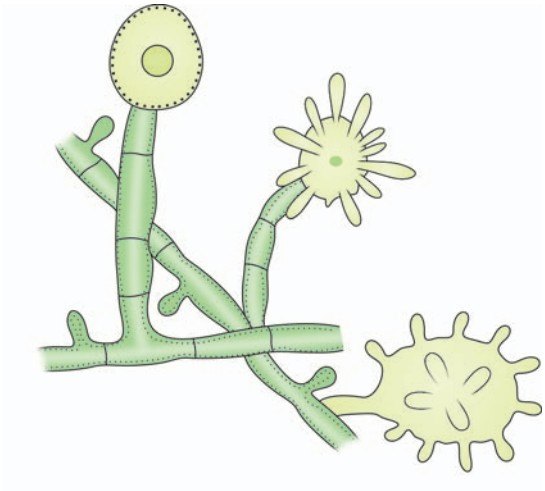
Specimen:

1. Sprutum
2. Scrapings from skin lesions
3. Buffy coat of blood cells
4. Urine
5. Bone marrow
6. Lymph node and skin biopsy.

Direct microscopic examination: KOH preparation, preferably Giemsa stained smear shows round to oval yeast cells (2 to 4 µ) present within macrophages or free in tissue.

Culture and identification: On Sabouraud's dextrose agar with chloramphenicol and cyclohexamide at 25°C for 1 week there is slow growth raised, cottony first and buff brown colored later on. Microscopic examination of this growth shows septate hyphae, delicate and branching with smooth, round or pyriform microconidia which are sessile or short stock. Macroconidia are 7 to 15 µ in size, thick-walled, round and tuberculate (Fig. 55.17).

At 37°C on glucose cysteine blood agar or, after 4 to 6 weeks there grow yeast form, small mucoid, cream colored colonies which on



Figs 55.17: *Histoplasma capsulatum*

microscopic examination are seen as oval budding cells about 2 µ in size.

Other laboratory tests:

1. Precipitation
2. Complement fixation
3. Latex agglutination
4. H and E stain or methanamine silver stain
5. Skin test is positive in infected patient exhibiting delayed hypersensitivity.

Pathogenesis: Histoplasma capsulatum is present in soil and inhalation of conidia leads to pulmonary infection. Miliary lesions appear throughout the lung parenchyma and hilar lymph nodes become enlarged. With healing the pulmonary lesions become fibrotic and calcified. Sometimes the initial infection may pass unnoticed.

In small number of infected cases the infection becomes progressive and widely disseminated with lesions in practically all tissues and organs. In fact, disseminated form of histoplasmosis often coexists in patients who have tuberculosis, leukemia or Hodgkin's disease.

The tissue lesions are characterized by granulomatosis inflammation with epithelioid cells, giant cells, even caseation necrosis. The characteristic features are swollen, fixed and wandering macrophages containing small, oval yeast cells (Fig. 55.18).

Treatment: Disseminated histoplasmosis can be treated with amphotericin B.

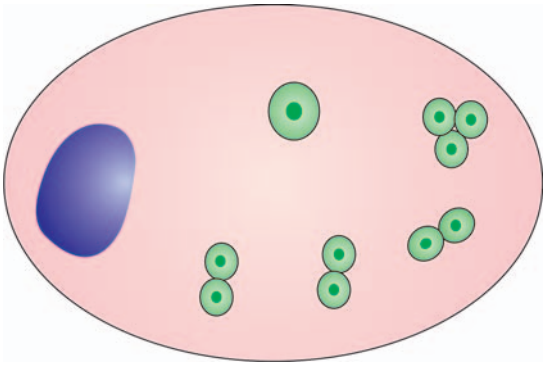


Fig. 55.18: Macrophage containing blastospores of *Histoplasma capsulatum*

Coccidioidomycosis

Etiology: Coccidioides immitis

Specimen:

1. Sputum
2. Pus
3. CSF
4. Tissue biopsy.

Direct microscopic examination: KOH preparation shows spherules 30 to 60 µ in size with endospores.

Culture: On Sabouraud's dextrose agar with chloramphenicol and cycloheximide at 25°C for 1 to 3 weeks' incubation there appear white and moist thin cottony colonies. Later on they become matted of buff colored. Microscopically this growth is seen as septate hyphae which break up into endospores.

On blood agar without antibiotics at 37°C for 1 to 3 weeks' incubation there appears yeast-like moist colonies which microscopically is seen as spherules 30 to 60 µ in size with thick wall containing endospores (Fig. 55.19).

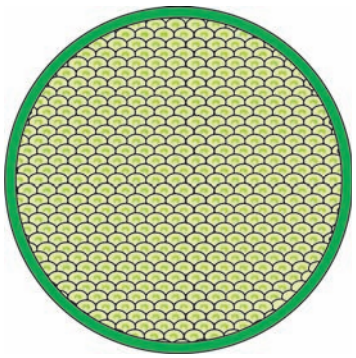


Fig. 55.19: *Coccidiodies immitis* (spherule with endospores)

Other laboratory tests:

1. Precipitation test.
2. Complement fixation test.
3. Histological examination of H and E stained section shows spherules 30 to 60 µ in size with endospores.
4. Skin test is positive in infected patients exhibiting delayed type of hypersensitivity.

Pathogenesis: Coccidioides immitis infection is established by inhalation of airborne spores. The infection may remain asymptomatic or mild symptoms like pneumonia may appear. The lesions of lung may heal up by calcification or thin-walled cavity may appear. In blood borne dissemination the lesions may spread to skin, bones and central nervous system.

The inflammatory lesions of acute pneumonitis due to this fungus are histologically like those caused by pyogenic bacteria. However, in chronic pulmonary disease and in disseminated lesions the inflammation is granulomatous characterized by abundant histiocytes, giant cells and caseation necrosis. Small spherules are found within macrophages or giant cells and larger more mature spherules lie freely in tissue spaces.

Treatment: Treatment with amphotericin B is highly encouraging. Failures occur because of prolonged therapy and severe intoxication.

Paracoccidioidomycosis

Etiology: Paracoccidioides brasiliensis

Specimen: Sputum
Pus
Exudates
Skin biopsy.

Direct microscopic examination: KOH preparation shows thick-walled cells 10 to 30 µ with multiple buds.

Culture: On Sabouraud's dextrose agar with cycloheximide and chloramphenicol after 1 to 4 weeks' incubation at 25°C, there grows colonies which are covered with white, velvety hyphae. The underside of the colony is light tan. Microscopic examination of the colony shows septate hyphae, puriform conidia 3 to 4 µ, sessile or with short sterigmata. Occasionally chlamydospores are also seen.

At 37°C the colonies are waxy, wrinkled and cream colored. Microscopically there may be single or multiple thick-walled budding cells.

Other laboratory tests:

1. Precipitation test.
2. Complement fixation.
3. Histological examination reveals large 10 to 30 µ round or oval cells with multiple budding.
4. Skin test is positive (delayed hypersensitivity) in infected patient.

Clinical picture: Coccidioidomycosis is an inapparent, benign, severe or fatal mycosis. There are 3 clinical forms as under:

a. Primary pulmonary form occurs 7 to 28 days after inhalation of a single spore of the infectious agent. Symptoms are fever, malaise and cough. Skin test becomes positive. There may be development of rash called erythema nodosum or erythema multiforme.
b. Benign form coccidioidomycosis exhibits precipitin and complement fixation titer positive apart from positive skin test. There is development of lung cavity. Usually this form exists for years and it may go unnoticed, usually causing no problem to the patient.
c. Disseminated form occurs in very few patients. Here disease may involve brain and other organs. Precipitin titer disappears but complement fixation titer continues to rise and there is reversion from skin test positive to skin test negative.

Treatment: Coccidioidomycosis is a difficult disease to manage and carries a grave prognosis. Amphotericin B may be useful.

Blastomycosis

Etiology: Blastomyces dermatitidis.

Specimens:

1. Sputum
2. Pus
3. Exudate
4. Urine
5. Skin biopsy.

Direct microscopic examination: KOH preparation shows broadly attached bud, large (7 to 20 µ), spherical, thick-walled yeast cell (Fig. 55.20).

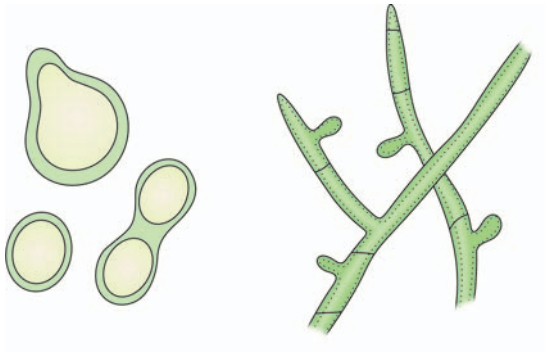


Fig. 55.20: *Blastomyces dermatitidis*

Culture: On Sabouraud's dextrose agar at 25°C for 1 to 4 weeks' incubation there appears white or brownish cottony growth. Microscopic examination of them shows, septate hyphae with round or pyriform microconidia borne on lateral conidiophores.

At 37°C incubation there appears wrinkled waxy and soft colonies. Microscopically there are round, thick-walled budding cells 8 to 15 µ in diameter.

Other laboratory tests:

1. Precipitation test
2. Complement fixation tests
3. Histological section when studied microscopically shows budding yeast cells which are thick-walled. Each yeast cell carries a single broad based bud.

Pathogenesis: Infection usually begins in the lungs and spreads hematogenously to establish focal destructive lesions in bones, skin, prostate, and other viscera. The gastrointestinal tract is spared.

The lesions are characterized by granulomatous inflammation, microabscesses, and extensive tissue destruction. The yeast cells are visible within the abscesses and granulomata.

Treatment: Amphotericin B or hydroxystibamidine may be used.

Pneumocystis Carinii

Analysis of gene sequences of ribosomal RNA, mitochondrial proteins and major enzymes (thymidylate synthase, dehydrofolate reductase) has demonstrated that *Pneumocystis carinii* is more closely related to fungi than to protozoa. Biochemical studies have suggested that cell wall of *P. carinii* contains glucans. Drugs that inhibit glucans synthesis in fungi are highly active against *P. carinii* in animals.

P. carinii contains two prominent antigen groups. A major surface glycoprotein complex of 95 to 140 kDa represents a family of proteins encoded by multiple genes. This complex plays a central role in the host-parasite relationship in this infection. This antigen facilitates the adherence of *P. carinii* to lung cells, contains protective epitopes and is capable of antigenic variations. The other antigen which migrates as a band of 35 to 55 kDa is most common antigen recognized by host. This may serve as a marker of infection.

OPPORTUNISTIC MYCOTIC INFECTIONS

Aspergillus

Etiology: Aspergillus fumigatus

Specimen:

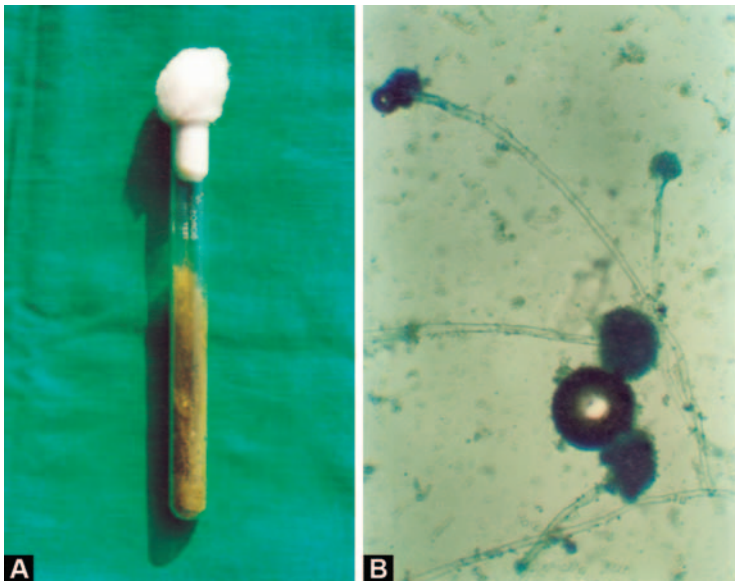
1. Exudate
2. Sputum
3. Lung biopsy.

Direct microscopic examination: KOH preparation of the specimen shows septate filamentous hyphae.

Culture: On Subouraud's dextrose agar with chloramphenicol after 25°C incubation for 1 to 4 days, there appear gray green colored colonies. Microscopic examination of these colonies shows septate hyphae bearing conidia in chain like fashion on sterigmata of conidiophore (Fig. 55.21 A and B).

Other laboratory tests: Histological examination of H and E stained and methenamine silver stain section shows septate hyphae branching dichotomously.

Aspergillus fumigatus (Fig. 55.22) is uniquely positioned for diagnosis by PCR by virtue of the presence of a gene coding for 16 kDa virulent factor. Primers were designed for the 5 and 3 ends of the gene that are virtually specific for the amplification of a 444 bp frag-



Figs 55.21 A and B: (A) Growth of *Aspergillus niger* on SDA medium (B) Microscopic view of lactophenol cotton blue mount of *Aspergillus niger*

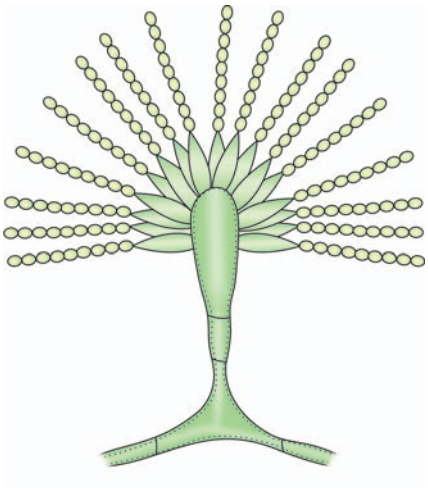


Fig. 55.22: Aspergillus

ment. A procedure that uses chitinase and microwave treatment is described for the extraction of genomic DNA of aspergillus species from the sputum and bronchial aspirate of patient with established aspergillosis. A colorimeteric method for detection of PCR product is developed based on immunoaffinity reaction. It is established that at least 1 pg of DNA is extractable from the clinical samples to produce enough quantity of PCR product for detection on agarose gel or by immunoaffinity based color reaction. An absorbance value of 0.9 to 1.5 against 0.2 for negative control at 405 nm for colorimeteric PCR is observed. This method is useful for screening a large number of clinical samples from immunocompromized as well as from suspected cases of aspergillosis. Other methods include agarose gel electrophoresis of PCR products and colorimeteric immuno PCR under discussion.

Clinical picture: The disseminated form of aspergillus is granulomatous, necrotizing disease of the lungs which often disseminates hematogenously to various organs. Most often this form of the disease is fatal and diagnosis is usually made at autopsy. Hypersensitive reaction to inhalation of fungal spores may cause asthmatic attack and mucifibrinous sputum containing eosinophils.

Another form of aspergillus is called fungal ball. Here fungus settles in old lung cavity. Usually these lung cavities are the result of old tuberculous lesions. If the organism remains in these cavities it grows into huge mass of mycelium in the form of fungus ball. Radiographically these fungus balls may appear to move about.

Treatment: Amphotericin B may be used. Fungus ball may be removed surgically.

Penicillosis

Etiology: Penicillium

Specimen: Skin lesion scrapings

Direct microscopic examination: KOH preparation shows small, round spores and hyphae.
Culture: On Sabouraud's dextrose agar with chloramphenicol at 25°C for 1 to 4 days the colonies appear which are white first and later on become blue green. Surface of these colonies is usually velvety or powdery.

Microscopic examination of these colonies show brush like arrangement of conidia, sterigmata and conidiophores (Fig. 55.23).

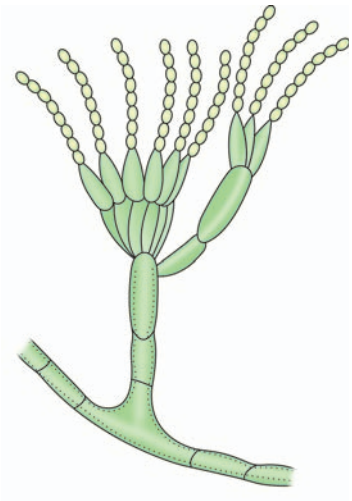


Fig. 55.23: *Penicillium conidia* firm within phialide

Clinical picture: Penicillium species are implicated in otomycosis and mycotic keratitis. In otomycosis there is inflammation, pruritis, exfoliation of epithelium and often, deafness when ear canal is occluded by a plug of hyphae.

In mycotic keratitis there may be corneal ulcer or hypopyon or both. However, corneal trauma, corneal disease and glaucoma predispose to mycotic keratitis.

There may be pulmonary and cerebral penicillosis.

Treatment: In otomycosis 5 percent aluminum acetate solution may be used to reduce edema and to remove epithelial debris. Aqueous solution of 0.02 to 0.1 percent phenyl mercuric acetate, thymol (1%) in metacresyl acetate and iodochlorohydroxyquin are more effective drugs.

In mycotic keratitis, topical application of amphotericin B is quite useful.

PENICILLOSIS MARNEFFEI

It is the only dimorphic fungus of genus penicillium predominantly found in Southeast Asia spreading continuously in the surrounding areas like Southeastern China, Hong Kong, etc. So far 4 cases are reported from India too.

It is a dimorphic facultative intracellular fungus. It exists as mycelial form at 25°C and yeast like form at 37°C. Unlike other yeasts, it divides by fission with formation of transverse septum.

The yeast form can multiply in macrophages of host. The primary site of infection is reticuloendothelial system. Ultimately infection may result into granulomatous suppurative reaction to immunity. In immunocompromising patients necrotizing reaction may occur.

This infection is chronic entity with incubation period of 4 weeks. Probably infection occurs by inhalation of airborne conidia of this fungus. It presents with low grade fever, chills, malaise, weight loss, cough, generalized popular skin rash with central umbilication, lymphadenopathy and hepatosplenomegaly. There may be umbilicated popular molluscum contagiosum like skin lesion on face, upper trunk, pinna, arms, etc. There may be diarrhea fungemia, necrotic papules, nodules, pustules etc. This fungus infects bamboo rats and man.

Penicillosis marneffei is included in the list of AIDS defining disease in endemic areas. It has also been reported in immunocompetent conditions.

The laboratory diagnosis needs demonstration and culture of fungus from body fluids and tissue biopsy specimens as discussed as under:

A. *Indirect diagnosis:* Microscopic examination of Wright's stained impression smear of skin, lymph node, bone marrow aspirate may be done. This fungus can be stained with H and E, Gomeri's methanamine silver stain, etc. Calcoflur white staining is useful for the demonstration of yeast. In tissue yeast cells may be round or oval 2.5 to 4 µm

especially in macrophages. However, they are curved when found extracellularly.

B. *Culture:* They can be cultured from blood, bone marrow, sputum, lymph nodes, pleural fluid, urine, etc. On Sabouraud's dextrose agar at 25°C grayish white colonies appear within 2 days and slowly become wooly growing to granular in texture and yellowish or gray in the center. The periphery of colony is white and has radical folds. The reverse is bright rose color. The hyaline short hyphae are septate and branched. The conidiophores are arranged laterally and terminally.

C. *Immunological methods:* They are IFAT, immunoblot assay, latex agglutination, immunodiffusion, PCR, etc.

Effective drugs are amphotericin B, itraconazole, ketoconazole, etc.

Zygomycosis

Etiology:

1. Mucor
2. Rhizopus.

Specimen:

1. Skin lesion scraping
2. Tissue biopsy.

Direct microscopic examination: KOH preparation may show mycelial fragments and spores.

Culture: On Sabouraud's dextrose agar after 25°C incubation for 1 to 4 days there may be the growth of cottony colonies. For identification whether mucor or rhizopus, microscopic examination of this growth is helpful. Mucor colonies, microscopically are seen as non-septate hyphae having branched sporangiophores with sporangium at terminal end whereas in case of rhizopus there are non-septate hyphae and sporangiophores arise in groups exactly above rhizoids (Fig. 55.24).

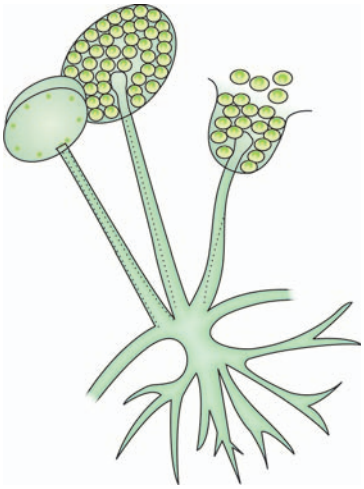


Fig. 55.24: Rhizopus

Histological sections: Microscopic examination shows broad, non-septate, irregular hyphae in thrombosed vessels or sinuses surrounded with leukocytes and giant cells.

Clinical picture: Zygomycosis and phycomycosis (mucormycosis) is a systemic disease which may involve internal organs with predilection for blood vessels. Sometimes phycomycosis may be seen as a chronic infection of subcutaneous tissue.

Treatment: In some cases amphotericin B may be useful.

MISCELLANEOUS MYCOLOGY TOPICS

I. Mycotoxins: Some fungi produce mycotoxins (poisonous substance) that are capable of causing acute or chronic intoxication and damage. Severe damage to liver, kidney and bone marrow may be caused as a result of eating of poisonous mushrooms, e.g. *Amantia phalloides.* Ingestion of small quantities of toxin if contaminated food (aflatoxin from *Aspergillus flavus*) may cause chronic damage or neoplasm in animals or man. Also derivatives of fungal products (LSD) may result profound mental derangement.

II. Antifungal drugs: Followings are major antifungal drugs:

1. *Iodides:* It is most commonly used for the treatment of sporotrichosis (cutaneous lymphatic form). Disseminated sporotrichosis is frequently resistant to iodide therapy. It is customary to begin treatment with potassium iodide with a dose of 1 ml of saturated solution three times a day which may be gradually increased to 12 to 15 ml/day. Treatment should be continued for at least 6 weeks.

2. *Hydroxystilbamidine Isethionate:* It is an aromatic diamidine. Only *B. dermatitides* is susceptible. Contents of vial containing 225 mg should be added to 200 ml of glucose or saline solution and should be administered intravenously in 45 to 70 minutes.
3. *Amphotericin B:* It is an antibiotic derived from *Streptomyces nodosus.* When suspended in liquid it is unstable at 37°C. Its mode of action is by binding to sterol present in the cell membrane of some fungi. Such binding increases permeability of the cell with leak of essential component like glucose and potassium. Susceptible fungi to amphotericin B are *Blastomyces dermititidis, Histoplasma capsulatum, Cryptococcus neoformans,* Candida, *Sporthrix schenkili,* etc. Aspergillus are most frequently resistant. To the 50 mg vial, 10 ml of sterile water is added and vial is shaken for 3 minutes. This suspension is added to 5 percent glucose solution in a final concentration no greater than 10 mg/100 ml. The contents are given intravenously over a 2 to 6-hour period.
4. *Griseofulvin:* It is active against dermatophytes only. It causes stunting and shrinking of hyphae. Drug is administered orally 10 mg/kg daily. Although symptomatic improvement is noticed after 2 to 3 days of administration, treatment must continue for at least 4 weeks.
5. *Flucytosine:* It is a fluorinated pyrimidine. Fungi like *Cryptococcus neoformans,* Candida, Cladosporium are susceptible to it. It is administered orally 150 mg/kg/day in equally divided 4 doses at 6 hour intervals.
6. *Imidazoles: In vitro* it is effective in yeasts and filamentous fungi including dermatophytes. Little is known about its mode of action. However, its relatively high concentration preferentially damages the fungal cell wall and plasma membrane making them permeable to intracellular amino acids, phosphates and potassium, thus inhibiting intracellular macromolecular synthesis. Doses recommended are 100 mg/kg/day in children whereas in adults 1.5 gm every 6 hourly through oral route.

Other antifungal drugs are *saramycetin* (effective against histoplasmosis and dermatophytosis), hamycin *(B. dermatitidis),* tolnaftate (dermatophytes), nystatin (candida infection of skin and mucous membrane), pimaricin (keratitis due to fusarium), etc.

Part VII

Parasitology

56 Introduction

Medical parasitology is the study of the parasites which cause disease in man. Here, as a matter of fact we study host/parasite relationship, geographical distribution, habitat, morphology, lifecycle, mode of infection, disease manifestations, host response, laboratory diagnosis, treatment and prophylactic-cum-preventive measures.

Poor hygiene, warm climate, poor sanitation and nonavailability of safe water encourage the parasitic infection and provide predisposing factors.

A parasite is an organism which takes its nourishment from another host without giving anything in return. The parasites include protozoa and helminths. When a parasite lives in its host like tapeworm, malarial parasite, it is known as endoparasite and is said to cause an infection. When a parasite lives on its host like a body louse, it is known as ectoparasite and is said to cause an infestation. Ectoparasites are important as vectors transmitting pathogenic microbes.

The host is the organism which harbours the parasite. The parasite does take nourishment and other benefits from host. The host is one in which the adult stage of parasite lives, or where sexual reproduction takes place, this is known as definitive host, e.g. man is the definitive host of *Taenia solium,* but the mosquito is the definitive host of the malarial parasite. The host in which the larval stages of the parasite are found is known as intermediate host, e.g. pig is the intermediate host of *Taenia solium* and man is the intermediate host of malarial parasite. A vertebrate host in which a parasite may remain viable without development or multiplication is called paratenic host. This type of host may serve to pass on the infection to another host and so may be called transport host.

Host parasite relationship may be there in the form of strict parasitism, commensalism, symbiosis, mutualism, saprophytism.

The discipline of medical parasitology deals only with parasites belonging to human beings and animals.

TAXONOMY AND CLASSIFICATION

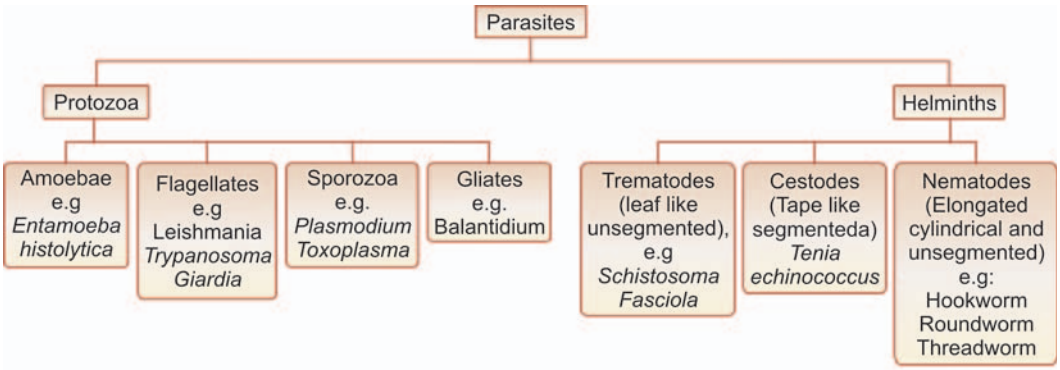
The science of classification of living forms is called taxonomy. It provides an orderly and agreed upon system of naming organisms.

Danish in eighteenth century orginated Binomial nomenclature in the system that is still used to name all living things. In this system the first word designates the genus of the organism and the second word is the specific epithet, e.g. *Entamoeba histolytica*. Together the genus and the specific epithet identify the species to which the organism belong.

Until the end of the nineteenth century organism were divided initially into 2 kingdoms (a) plant (b) animals and in 1866 Haeckel proposed another, i.e. (c) prostista for all microorganisms.

Of late in 1969 five kingdom system of classification was suggested by Whittakar. It remains the widely accepted system of classification which comprises of following five kingdom.

a. Monera (prokaryote; unicellular)
b. Protista (eukaryote; unicellular)
c. Fungi (eukaryote; uni or multicellular)
d. Plantae (eukaryote; multicellular)
e. Animalia (eukaryote; multicellular).



Protozoa:
- Unicellular
- Consist of true membrane bound-nucleus and cytoplasm

Classes of Protozoa

Class	*Examples*
1. Rhizopoda 2. Ciliates 3. Flagellates 4. Sporozoa	Amoebae Balantidium • Leishmania • Plasmodium

Helminths:
- Multicellular
- Elongated, bilaterally symmetrical covered with thick cuticle and vary in length

Classes of Helminth

Class	*Examples*
1. Nematodes	Roundworm
2. Cestodes	Tapeworm
3. Trematodes	Schistosome

Parasites of medical importance fall into 2 kingdom, i.e. protista and animalia. Protista includes the microscopic single called, eukaryotes known as protozoe. In contrast helminthis are macroscopic, multicellular worms possessing well-differentiated tissues and complex organs belong to kingdom animalia.

For centuries, parasitic diseases continue to cause high morbidity especially in the developing countries. Infection may be low among persons in temperate regions with good hygiene than unhygienic population of tropics. AIDS has given opportunity to parasitic diseases which were, as a matter of fact rare and less pathogenic.

HISTORY IN BRIEF

Protozoa were referred to as animacules in 1764, the term infusoria was suggested by Wrisberg in 1836, Alfred Donne suggested that flagellate was responsible for vaginal discharge in women. This flagellate was named trichamonas.

The first discovery of parasite in the blood of a patient suffering from malaria was made in 1880 by Alphonso Laveran. Stains prepared and used by Romanowsky revolutionized the study of parasites.

History of Protozoa

1836	Trichomonas	Alfred Donne
1843	Trypanosoma	Gruby
1857	Balantidium	Malmsten
1859	Giardia	Lambl
1875	Entamoeba	Losch
1880	Plasmodium	Laveran
1882	Sarcocystis	Lankester
1888	Babesia	Babes
1903	Leishmania	Leishman
1907	Cryptosporidium	Tyzzer
1908	Toxoplasma	• Nicolle and Manceaux (from rat) • Darling (from man)
1912	Pneumocystis	Delanoe and Delanoe

In 1758, Linnaeus identified Fasciola without the help of microscope and later suggested that roundworm and threadworm can cause diseases in man. Helminths were discovered in various organs of body, e.g. Trichinella larval form from muscles of infected man, paragonimus from Bengal tiger in 1878 in a Zoo of Amsterdam, wuchereria in the form of larva (microfilaria) in hydrocele fluid by Lewis in Calcutta in 1872.

In the light of new information parasites like *Pneumocystis carinii, Toxoplasma gondii,* isospora and cryptosporidium, have been proved to cause extensive damage.

History of Helminths

1685	Echinococcus	Hartmann
1758	Ascaris	Linnaeus
	Fasciola	Linnaeus
	Entrobius	Linnaeus
1782	Taenia	Goezy
1821	Trichinella	Tidemann
1843	Fasciolopsis	Busk
1851	Schistosoma	Bilharz
	Hymenlepsis	Bilharz
1853	Dracunculus	Bastion
1863	Wuchereria	Demarquay
1875	Clonorchis	McConnell
1876	Gastrodiscoides	Lewis and McConnell
1876	Strongyloides	Normand
1878	Paragonimus	Kerbert
1911	Ancylostoma	Looss
1952	Toxocara	Beaver
1960	Anisakis	Van Thiel
1964	Angiostrongylus	Beaver and Rosen

PARASITE

A living organism which gets nourishment from another living organism where it lives is called parasite. They may be:

a. Ectoparasite (living on the surface of other organisms lice, ticks, mites, etc.)
b. Endoparasites (lives inside the body of other organism, e.g. *Entamoeba histolytica, Ascaris lumbricoides*, etc.)
c. Obligate parasite (who must spend some part of their life cycle in or on host, e.g. plasmodium).
d. Facultative parasite (may be free living but can obtain the nutrition from hosts too).
e. Accidental parasite (when parasite attacks unnatural host).
f. Aberrant parasite (when the parasite comes to a site, during migration where it cannot live or develop further).

HOST

An organism which harbors the parasite. Host may be of following types:

a. Definitive host (when it harbors parasite in adult form or where parasite utilizes sexual method of reproduction).
b. Intermediate host (harbor's larval stages of parasite).
c. Paratenic host (when host harbors parasite exhibiting no development but this parasite remains viable).
d. Reservoir host (which make parasites available for transmission to other hosts).
e. Natural host (which is naturally infected with certain species of parasite).
f. Accidental host (which is by and large under normal circumstances not infected with parasite).

Association of Parasite and Host

This association may be as follows:

i. Symbiosis is an association in which both are so dependent upon each other that one cannot live without the help of other and none of the partners suffers any harm from the association.
ii. Commensalism is an association in which parasite is deriving benefit without causing injury to its host, e.g. *Entamoeba gingivalis, Trichomonas tenax,* etc. It is a form of symbiosis.
iii. Mutalism is an association where parasite and host both are benefitted. It is another form of symbiosis.
iv. Parasitism is an association where parasite gets benefit and the host gets nothing in return but always suffers some injury, however, minimal the injury may be.

MECHANISMS OF DISEASE PRODUCTION BY PARASITES

The Effect of Parasites on their Host

The damage which pathogenic parasites produce in the tissue of the host may be effected in any of the following ways:

Mechanical Injury

It may be inflicted by a parasite by means of pressure as it grows larger, e.g. hydatid cyst or by blockage of ducts such as blood vessels producing infarction, e.g. strongyloides or of lymphatic vessels to produce edema and elephantiasis, e.g. filariasis or the intestinal tract to produce obstruction, perforation and necrosis, e.g. *Ascaris lumbricoides* and destruction of architecture of the human red blood cells by *Plasmodium falciparum.*

Deleterious Action of Toxic Substances

In *Plasmodium falciparum,* production of toxic substances may cause rigors and other symptoms characteristic of clinical malaria. In *Entamoeba histolytica* lesions in human gut may be due to the production of histolytic enzyme (tissue dissolving).

Deprivation of Nutrients, Fluids and Metabolites

Parasites may produce disease by competing with the host for food, e.g. *Diphylobothrium latum* (fish tapeworm) is known to deprive its host of vitamin B_{12} thus causing megaloblastic anemia. Likewise certain nematodes, e.g. *Ancylostoma duodenale* and some other blood sucking arthropod parasites quickly lead to severe iron deficiency anemia in patients infested by large numbers of these parasites.

Introduction of Pathogenic Microorganisms

Bacterial infection of lesions produced initially by a parasite may occur and in some cases may prove lethal, e.g. tetanus is a well-known complication of *Dracunculus medinensis* infection. This is the result of the spores of *Clostridium tetani* contaminating the ulcers of the foot and digit, produced by *Dracunculus medinensis.* Similarly, the very high urinary carrier rate of *Salmonella typhi* in parts of Egypt is due to equally high incidence of urinary Schistosomiasis caused by human bloodflukes, *Schistosoma hematobium.*

REACTION OF THE HOST TO PARASITE

The reaction of host to the presence of the parasite, its developmental stages or the products of its metabolism may produce two types of host reaction, i.e. (a) tissue reaction, (b) immunological response.

Tissue Reactions

Excessive proliferation of certain tissues following invasion by some parasites is known to occur in man, e.g. fibrosis (cirrhosis) of liver following deposition of the ova of *Schistosoma mansoni,* hepatic fibrosis and hyperplasia of biliary tract due to the infection of *Clonorchis sinensis* and *Fasciola hepatica,* etc. Hyperplasia of reticuloendothelial tissue (especially spleen and liver) is a well-known feature of leishmaniasis.

Immunological Response

Tissue damage may be caused by immunological response of the host, e.g. nephrotic syndrome *(Plasmodium malariae)* and cercarial dermatitis due to penetration of human skin by schistosome (cercarie).

57 Protozoa

Protozoa are subdivided into four groups (Table 57.1).

RHIZOPODA

ENTAMOEBA HISTOLYTICA

Geographical Distribution

Entamoeba histolytica has been found in all populations throughout the world where search has been conducted. It is more prevalent in the tropics and subtropics than the cooler climates. In the Western Hemisphere, *Entamoeba histolytica* has been found from Anchorage, Alaska (60°N) to the strait of Magellan (52°S), and in the Eastern Hemisphere from Finland (60°N) to Natal, South Africa (30°S).

Habitats

Trophozoites of *Entamoeba histolytica* live in the mucous and submucous layers of large intestine.

Morphology

Table 57.2 shows morphological features of *Entamoeba histolytica* and their distinction from macrophages and PMN.

Three stages are encountered (a) active ameba trophozoite (b) inactive cyst and (c) intermediate precyst.

TABLE 57.1: Classification of the pathogenic protozoa

Class	*Organ of locomotion*	*Important human pathogens*	*Graphical prevalence*
I. Rhizopoda	Pseudopodia	*Entamoeba histolytica* *Hartmonella* and *Naeglaria*	Worldwide May be worldwide but reported so far in South Australia, USA, New Zealand, Britain and Czechoslovakia
II. Mastigophora	Flagella	1. Flagella of blood and tissues: *Trypanosoma brucei* *Trypanosoma cruzi* *Leishmania donovani* 2. Flagellates of the genital and alimentary tract *Trichomonas vaginalis* *Giardia lamblia*	Africa South Africa Asia, Africa, Southern Europe, South and Central America Worldwide Worldwide
III. Sporozoa	None, exhibit a slight amoeboid movement	*Plasmodium falciparum* *P. vivax* *P. malariae* *P. ovale*	Tropical and subtropical areas of Africa, South America, Near East India, Pakistan, Bangladesh, Sri Lanka, Southern China, Malaysia, South Pacific, Islands and North Australia
IV. Ciliata	Cilia	*Balantidium coli*	Worldwide

TABLE 57.2: Comparison of some host cell in feces confused with *E. histolytica*

S. no.	*Character*	*Macrophages*	*PMNs*	*E. histolytica*
1.	Size (μ)	30 to 60	mean 14	12 to 60
2.	Ratio of nuclear material to cytoplasm	1:4 to 1:6	1 to 1	1:10 to 1:12
3.	Nuclear morphology	Large, may be irregular	2 to 4 segments connected by chromatin strands	Round, vesicular with a central karyosome and peripheral chromatin
4.	Ingested material	PMNs, RBC, tissue debris	—	RBC
5.	Cytoplasm	May contain red staining bodies	Granular	Uniform, finely granular
6.	Trichrome staining characteristics	Green cytoplasm dark red nucleus	Green cytoplasm, dark red nucleus	Green cytoplasm dark red nucleus

Trophozoite

Under the microscope the living parasite on warm stage exhibit remarkable locomotion. Movement results from long finger-like pseudopodial extension of ectoplasm into which the endoplasm flows. It is 15 to 30 μ in size.

Cytoplasm is divisible into a clear translucent ectoplasm and granular endoplasm. Cytoplasm may contain erythrocytes, white blood cells and tissue debris.

Nucleus is 4 μ spherical in shape and placed eccentrically. It has well-defined nuclear membrane, inner line of which is lined with uniform and closely packed fine granules of chromatin. Karyosome is centrally placed.

Precystic Stage

It is colorless, round or oval, smaller than trophozoite but larger than cyst about 10 μ to 20 μ, endoplasm free from RBC, etc. with sluggish pseudopodial activity. Characters of nucleus remain intact.

Cystic stage: Cysts are encountered only in the lumen of intestine under unsuitable conditions. Cyst begins as a uninucleated body but divides by binary fission and develops into binucleated and quadrinucleated structure. The cyst is 6 to 15 μ with clear and hyaline cytoplasm containing oblong bars with rounded ends called chromatoid bars (1 to 4). A distinct glycogen mass is found in the early stages of cyst formation. In quadrinucleated cyst chromadial bars and glycogen mass disappear. It is the mature and infective form.

Life cycle: Cysts of Entamoeba are formed in bowel of man and are passed with stools. Cysts are swallowed with contaminated food and drinks by man. They pass through stomach and reach intestine. Cyst wall is weakened because of alkaline pH and cytoplasmic mass containing 4 nuclei (metacyst) comes out. The nuclei divide by binary fission giving rise to 8 daughter trophozoites. Trophozoites which are actively motile moves towards ileocaecal region (Fig. 57.1).

Strain differentiation: Strains of *Entamoeba histolytica* can be differentiated from the pathogenic strains by certain physiological differences such as ability to grow at reduced temperatures, genome size, DNA base ratio contents and DNA homology. Strain differentiation can also be done on the basis of phenotypic isoenzyme patterns called zymodemes. These zymodemes are identified using phenotypic isoenzymes, e.g. glucose phosphoisomerase (GPI), phosphaglucomutase (PGM), malate NADP oxidoreductase (ME), hexokinase (HK), etc. On the basis of different isoenzyme patterns *Entamoeba histolytica* can be divided into 22 zymodemes. Out of these 22 zymodemes only 7 (II, VI, VII, IX, XII, XIII and XIV) are potentially pathogenic.

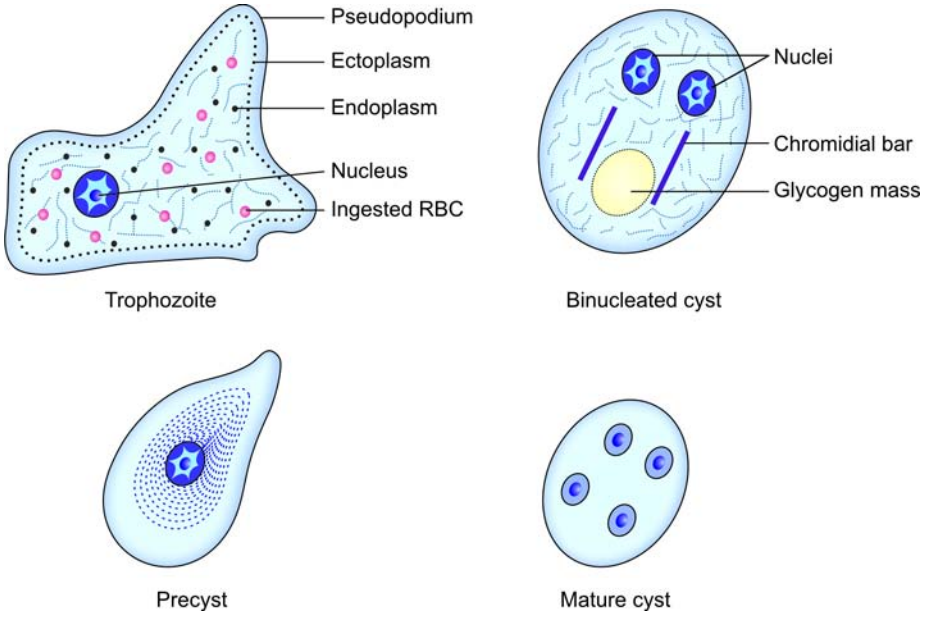


Fig. 57.1: Life cycle of *Entamoeba histolytica*

Pathogenesis

- Invasiveness depends with particular zymodemes.
- Invasiveness also correlates with phagocytic process, collagenase, immunologic cytotoxic proteins, host's inflammatory response and capacity to induce histolysis.
- Bacteria may enhance pathogenecity.

Pathology

- Bacteria may enhance pathogenecity.
- Man is the reservoir of infection. Infections occur by 4 nucleated cysts.
- *Entamoeba histolytica* produces dysentery with frequent passing of stools mixed with mucus and blood.
- Intestinal lesions are acute amebic dysentery and chronic intestinal amebiasis.
- Extraintestinal lesions (metastatic) include: liver (amebic hepatitis and amebic liver abscess), lungs (primary small abscess or multiple abscess in one or both lungs or secondary single abscess in lower lobe of right lung which is contacted from amebic liver abscess), brain (a small cerebral abscess), spleen (splenic abscess), skin (granulomatous lesion (ameboma) near visceral lesion, e.g. liver.
  - Amebic vaginitis
  - Amebic ulcer of penis.

TABLE 57.3: Differences between bacillary and amebic dysentery

Bacillary	*Amebic*
• Frequency 10 per day	6 to 8 per day
• Amount of stool small	More
• Consists of blood, mucus but hardly fecal matter	Feces mixed with blood and mucus
• Bright red colored	Dark red
• Viscid, mucus adherent to container	Liquid or formed mucus not adherent to container
• Odorless	Offensive
• Alkaline	Acidic
• Microscopic findings	
i. Pus cells numerous	Less in number
ii. RBC lies discretely	RBC in clumps
iii. Macrophages with ingested RBC	Absent
iv. Charcot Leydon crystals absent	Present
v. *Entamoeba histolytica* absent	Present
vi. Bacteria scanty	Numerous

The differences between bacillary and amebic dysentery are given in Table 57.3.

Characters of Amebic Intestinal Ulcers

Size varies from pinhead to one inch. Other features of lesions include shape which is round or oval with narrow neck and broad

base (flask-like). Margins are ragged or undermined. Superficial ulcers do not extend beyond muscularis mucosa but deep ulcer may extend to submucous coat. Superficial ulcer heals without any scar but deep ulcer healing invites scar formation. If ulcer is studied microscopically it consists of central area of necrosis (no ameba seen) but towards periphery, trophozoites of *Entamoeba histolytica* are seen in large numbers. The ulcers might be distributed along the whole length of intestine more at caecum, hepatic flexure and rectum.

Chronic ulcers are small, superficial, causing scarring, thinning, dilatation, succulation of intestinal wall, extensive adhesion with neighboring areas and formation of tumor-like mass.

Laboratory Diagnosis

- Macroscopic examination of stool (dark red stool mixed with blood and mucus).
- Microscopic examination of stool for demonstration of trophozoite or cyst of *Entamoeba histolytica*, cellular exudate, Charcot Leydon crystals, etc. *Entamoeba coli* has characters resembling *Entamoeba histolytica*.
- Proctosigmoidoscopy, scraping and biopsy samples collected under direct vision by endoscopy may reveal trophozoites of *Entamoeba histolytica*, if otherwise not demonstrable.
- Culture can be done using lock egg serum medium, locks egg albumin medium, Craig's medium, Balamuth's medium, etc.
- Serological techniques are not useful for the patient of acute intestinal lesions. However, in chronic intestinal cases and in extraintestinal cases, they are useful. Serological tests include:
  - i. Indirect hemagglutination (Significant titer 1:256 and more)
  - ii. ELISA (Using monoclonal antibody)
  - iii. Counter current immunoelectrophoresis
  - iv. Latex agglutination
  - v. Gel diffusion precipitation
  - vi. Indirect immunofluorescence.
- DNA probes makes the diagnosis rapid and specific.

SEROLOGICAL TESTS IN INVASIVE AMEBIASIS

1. Latex agglutination slide test to detect antibodies to *Entamoeba hislolytica*.
2. Cellulose acetate precipitin test is simple specified and inexpensive. It becomes positive early during early invasive amebiases. It becomes negative in 3 months after successful treatment.

The principle is based on the fact that specified antibodies and soluble antigen diffuse on cellulose acetate paper towards each other. It forms a line of precipitation where they meet.

Treatment

Metronidazole, chloroquine, tinidazole, diloxanide furoate, emetine and secnidazole are effective drugs.

Non-pathogenic Amebae

Entamoeba coli (Table 57.4)

- It habitates in the lumen of large gut but does not invade tissues.
- Trophozoite is 10 to 40 µ, blunt granular slow pseudopodia.
- Cyst is 10 to 30 µ thread-like pointed chromotoid bodies (1 to 8).
- It is commensal of large intestine.

TABLE 57.4: Differences between *Entamoeba histolytica* and *Entamoeba coli*

Entamoeba histolytica	*Entamoeba coli*
Cyst	
i. 5 to 20 µ	10 to 40 µ
ii. Nuclei 4 or less	Up to 8
iii. Chromotoidal body cigar like	Thread like
iv. Karyosome smaller and central	Large and eccentric
Trophozoite	
i. 10 to 60 µ in size	10 to 50 µ
ii. Single pseudopodium	Multiple
iii. Cytoplasm finely granular	Coarsely granular
iv. Cytoplasm encloses RBC	Cytoplasm encloses bacteria debris as inclusion bodies
v. Actively motile	Sluggishly motile
vi. Nucleus invisible	Visible

Entamoeba gingivalis

- It is found only in trophozoitic form and cystic forms are absent.
- Trophozoite is 5 to 35 µ, having hyaline, blunt but rapid pseudopodia and actively motile.
- Present in pyorrhea alveolaris and may cause pyorrhea.

Endolimax nana

- Trophozoite is 6 to 15 µ, pseudopodia blunt, hyaline, slow, motility is sluggish.
- Cyst is 5 to 14 µ, glycogen mass absent, chromoidal bodies are spherical and nuclei 1 to 4.
- Present as commensal in human intestine.

Iodameba butschlii

- Trophozoite is 6 to 20 µ, pseudopodia blunt, hyaline and slow showing sluggish motility.
- Cyst is 5 to 18 µ with large mass of glycogen (dark brown) and nucleus is one.
- It is found in the lumen of colon.
- It is non-pathogenic.

FREE LIVING SOIL AMEBA

Primary Amebic Meningoencephalitis (PAM)

Etiologic Agent

Naegleria fowleri: It occurs in trophozoitic form and cystic form (Fig. 57.2). Trophozoite may be in ameboid form (10 to 30 µ exhibiting eruptive locomotion) or flagellated form (pear shaped with two flagella at anterior end and

TABLE 57.5: Differentiation between free living amebae and *Entamoeba histolytica*

Free living ameba	*Entamoeba histolytica*
1. Nucleolus large and distinct	Nucleolus small and indistinct
2. Contractile vacuoles present	Absent
3. One nucleus in cyst	1 to 4 nuclei
4. Glycogen and chromatoid bodies in the cyst are absent	Present
5. Mitochondria present	Absent
6. Cell wall has pores	Pores absent in cell wall

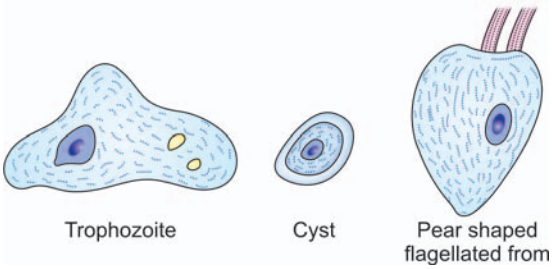


Fig. 57.2: *Naegleria fowleri*

is non-replicating). Both forms are uninucleated with large karyosome.

Cystic form measures 7 to 10 µ having smooth double cyst wall. Cysts are not formed within tissue.

Table 57.5 lists the differences between free living ameba and *Entamoeba histolytica.*

GRANULOMATOUS AMEBIC ENCEPHALITIS

Etiologic Agent

Acanthamoeba culbertsoni: Trophozoites are larger than Naegleria organisms (15 to 40 µ in size, produce fine hyaline projection called acanthopodia without any flagella). Cystic form is 10 to 25 µ with double wall. The outer wall is wrinkled one (ectocyst) and polygonal or round inner wall (endocyst). Nucleus is single, large, dense with centrally located nucleolus (Fig. 57.3). It differs from Naegleria in not having flagellated form and in forming cyst in tissue (Table 57.6).

Incubation period is 1 to 7 days in case of Naegleria and several weeks to several months in case of acanthamoeba. Swimming in lakes, ponds and swimming pool causes infection in Naegleria. In Acanthamoeba inhalation, ingestion or through skin (traumatic) and eyes are the modes of infection.

Life cycle: In case of *Naegleria amebae* invade the nasal mucosa, pass through olfactory plate into the meninges and start an acute purulent meningitis.

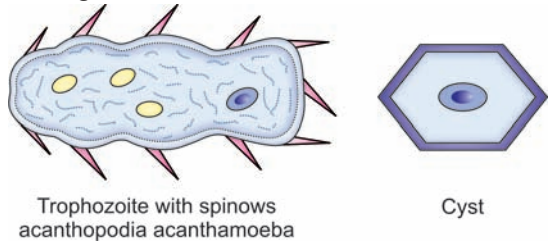


Fig. 57.3: Acanthamoeba

TABLE 57.6: Differentiation between *Naegleria* and *Acanthamoeba*

	Naegleria	*Acanthamoeba*
1. Trophozoite	10 to 30 µ with single pseudopodium	15 to 40 µ with thorn like pseudopodia
2. Cyst	7 to 10 µ with smooth surface	10 to 25 µ with wrinkled surface
3. Flagellate form	Present	Absent
4. Mitosis and nuclear membrane persists	Nucleolus divides	Nuclear membrane dissolves
5. Culture	Positive	Positive
6. Tissue form	Trophozoite	Cyst and trophozoite
7. Lesion	Acute suppurative inflammation	Granulomatous inflammation
8. Disease	Primary amebic meningoencephalitis	Granulomatous amebic encephalitis
9. Clinical course	Acute	Subacute or chronic
10. Portal of entry	Nose	Upper respiratory tract
11. Predisposing factor	Swimming in contaminated water	Immune incompetence
12. Leukocytes in CSF	Predominantly neutrophils	Predominantly lymphocytes

In Acanthamoeba, it reaches brain by way of bloodstream from lower respiratory tract and ulcers of skin and mucosa.

Clinical Picture

In Naegleria disease is acute in onset resulting in rapid worst condition of the patient. To start with there is upper respiratory tract infection, low fever, and headache. Within 1 to 2 days meningitis develops with symptoms of frontal headache, high fever, nausea, vomiting and nuchal rigidity. Finally there is cerebral edema and patient becomes comatosed and dies within few hours. It causes primary amebic meningoencephalitis (PAM).

In Acanthamoeba course of infection is subacute or chronic. There is focal granulomatous lesion of the brain called granulomatous amebic encephalitis (GAE).

Laboratory Diagnosis

It is established by demonstration of amebae, culture on nutrient agar plate with a suspension of *Escherichia coli, Klebsiella*, etc. serology (immunofluorescent test and immunoperoxidase in Acanthamoeba only).

Treatment

Amphotericin B may be used for *Naegleria*. Ketoconazole with topical miconazole is useful for *Acanthamoeba keratitis.*

Differences between Naegleria and Acanthamoeba is depicted in Table 57.6.

MASTIGOPHORA

GIARDIA LAMBLIA

Geographical Distribution

It occurs all over the world. It is prevalent in 2 to 25 percent population.

Habitat

Duodenum and upper part of intestine.

Morphology

It is found in the following two forms (Fig. 57.4):

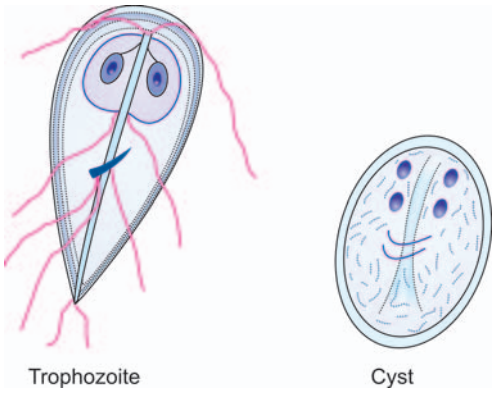


Fig. 57.4: *Giardia lamblia*

Trophozoite: It resembles longitudinally-cut pears. It is 10 to 20 μ in length and 6-15 μ in width and 1 to 3 μ in thickness. The dorsal surface is convex and ventral surface is concave. There is an oval shaped adhesive area in the anterior ventral surface. The dorsal surface area is meant for diffusion of nutrients. The anterior end is rounded and posterior end is pointed. It is bilateral symmetrical. There are a pair of axostyles, two nuclei and 4 pairs of flagellae. It multiplies by binary fission.

Cyst: Trophozoites are transformed into cysts under unfavorable conditions. The cyst is:

- Oval
- 8 to 14 μ × 6 to 10 μ.
- Contains 4 nuclei usually lying at one end or lie in pairs at opposite poles.
- Ramnant flagellae and margins of sucking disk lie inside cytoplasm.
- They are passed in stools.

Life cycle: Cyst is the infective form. Cysts are ingested through water and edibles. Acidic pH of stomach initiates excystation which is completed in duodenum thus releasing trophozoites (2 trophozoites from each cyst). The trophozoites establish themselves in the intestinal villi and start multiplying by binary fission. It can also localize itself in biliary tract.

Symptomatology

The role played by this parasite in pathogenesis is not fully understood. Acute manifestations of giardiasis are nausea, anorexia, explosive watery stools, steatorrhea, malabsorption, severe flatulence with abdominal distention and mid epigastric cramps. The foul smelling stool, flatus, marked abdominal distention along with the absence of pus and the blood are more suggestive of giardiasis. The most common chronic complaints are periodic bouts of soft, mushy, foul smelling stools, flatulence and abdominal distention. Biliary disease is simulated in adults, whereas children show predominantly a diarrheal syndrome often steatorrheal in nature.

Pathology

Mucosal suction biopsies from duodenum and adjacent to duodenum have shown invasion of the mucosa but there are no signs of host cell injury. Electron microscope studies suggest that the organism may be attacking the fuzzy coat of the microvilli (Fig. 57.5). Trophozoites have been demonstrated inside host cells by some investigators. Autopsy findings, in some instances, have shown extensive ulceration and sloughing of mucosa in the presence of heavy infection. Passage of *Giardia lamblia* trophozoites up to the bile duct to the gallbladder may occur with signs and symptoms of biliary tract disease.

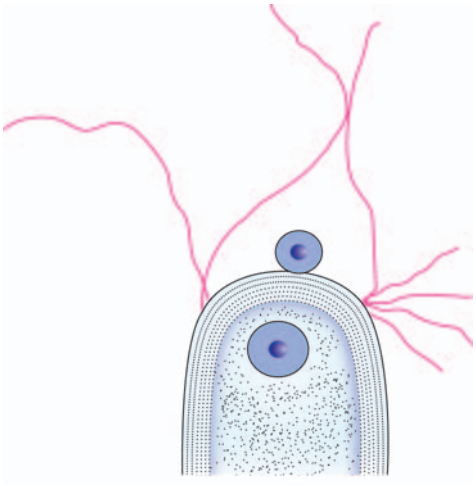


Fig. 57.5: *Giardia lamblia* trophozoite perched on top of an epithelial cell

Secondary vitamin A deficiency has been suggested by the lower carotene levels in children infected with giardia.

Two predisposing factors to symptomatology appear to be achlorhydria and hypogammaglobulinemia. Giardiasis appears to cause general disaccharidase deficiency, resulting in lactose intolerance which is usually restored after chemotherapy. Immunoglobulin deficiency has also suggested that lack of secretory IgA in patients may lead to colonization of bacteria in the jejunum and increased susceptibility to giardia. By deconjugating bile acids the bacteria may cause steatorrhea, but opportunist *Giardia lamblia* may aggravate the condition.

Laboratory Diagnosis

- Demonstration of cysts in the stool microscopically.

- Demonstration of trophozoites in duodenal aspirate.
- Intestinal biopsy.
- Immunological techniques like ELISA, indirect immunofluorescence, counter-current immunoelectrophoresis.
- *Giardia lamblia* specific antigen can be detected using enzyme immunoassay (EIA) technique performed on microplate or micromembrane. It gives result in 10 minutes.

Treatment

- Metronidazole, quinacrine, furazolidone and tinidazole may be effective.

Prevention

- Safe water supply (boiled treatment with hypochlorite or iodine).
- By checking fecal contamination.
- Treating properly giardiasis patients.

TRICHOMONAS VAGINALIS

Geographical Distribution

It is encountered in all climates and all social groups.

Habitat

- In female it is found mainly in vagina and in male it is in urethia.

Morphology

It is found only in trophozoitic form which bears following characters (Fig. 57.6):

- Pear shaped measuring 10 to 30 µ × 5 to 10 µ.
- It has short undulating membrane which comes up to the middle of the body.
- Possesses 4 anterior flagellae, a prominent axostyle which bifurcates the body into two and projects posteriorly.
- There is a costa, parabasal body, rounded nucleus (anteriorly).
- Chromatin granules are present all over, more densely near costa and axostyle.
- Flagellae give characteristic webbing or rotatory motility.

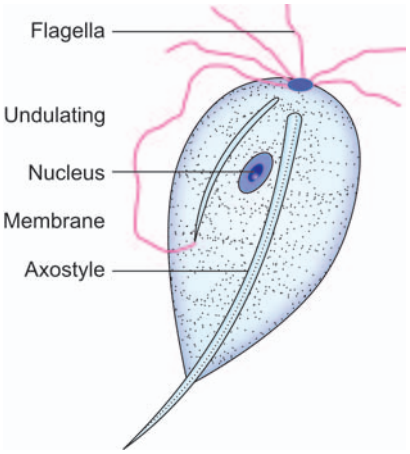


Fig. 57.6: *Trichomonas vaginalis*

- It multiplies by binary fission in longitudinal axis.

Mode of Transmission

It is primarily a venereal disease in which transmission can also be from person-to-person contact. However, newborns may get infected during birth. Fomities also form another way of transmission of infection.

Incubation Time

It varies from 4 to 30 days.

Pathogenesis and Pathology

Within few days following the introduction of viable *Trichomonas vaginalis* into the vagina, the proliferating colonies of this flagellate cause degeneration and desquamation of the vaginal epithelium. It is followed by leukocytic inflammation of the tissue layer. Very large numbers of trichomonads and leukocytes are now present in the vaginal secretion, which is liquid, greenish or yellow, and covers the mucosa down to the urethral orifice, vestibular glands and clitoris. As the acute condition changes to the chronic stage the secretion loses its purulent appearance due to decrease in number of trichomonads and leukocytes, increase in epithelial cells and establishment of a mixed bacterial flora.

Trichomonas vaginalis in male genitalia may be symptomless or may be responsible for an irritating, persistent or recurring urethritis.

Clinical Picture

The vaginal secretion is extremely irritating, almost unbearable and is constantly flowing. These symptoms may continue from a few days to many months. After each menstruation there is a tendency for acute stage to recur. Eventually, the chronic condition transforms into latent one and secretions become normal with no manifestation although trichomonas are still present. Difference in the intensity of symptoms may be due to differences in virulence of strains of this organism.

In male patients it may be symptomless or may cause urethritis and prostatitis.

Laboratory Diagnosis

1. In female patient, *Trichomonas vaginalis* may be demonstrated in sedimented urine, vaginal secretion or from vaginal scraping.

   In male patient *Trichomonas vaginalis* may be found in the centrifuged urine and prostatic secretions following massage of the prostatic gland.

   However, care should be taken to prevent contamination of the specimen with feces, since *Trichomonas hominis* may be seen and thus misdiagnosed as *Trichomonas vaginalis*. The smear is stained using Giemsa, PAS, Papanicolaou, Leishman, Diff Quick and acridine orange.
2. *Culture:* It is quite sensitive technique. Media used for culture include, CPLM (cysteine, peptone, liver maltose), Bushley's Feinberg Whittington, etc.
3. Indirect hemagglutination test (IHA) employing glycoprotein obtained from *Trichomonas vaginalis* as an antigen. It is considered to be highly specific.

Treatment

1. Administration of metronidazole 250 mg thrice daily orally for five days.
2. Suppositories of diiodohydroxyquin are also useful.
3. Douching with lactic acid and vinegar to promote acid pH is also advocated.

TRICHOMONAS TENAX

- Harmless commensal of oral cavity, periodontal area, carious cavities of tooth, tonsillary crypts, etc.
- Measures 5 to 10 µ, i.e. smaller in size as compared to *Trichomonas vaginalis.*
- Transmission is through fomites, salivary droplets and kissing.

TRICHOMONAS HOMINIS

- Measures 8 to 12 µ.
- Carries 5 flagellae, undulating membrane going over the full length of protozoal body.
- It is a common commensal of caecum.

TRYPANOSOMA BRUCEI

Geographical Distribution

Western and central parts of Africa.

Habitat

It is a parasite of connective tissues. It attacks the regional lymph nodes through lymphatics and then goes to blood and finally may involve central nervous system.

Morphology

It occurs in trypomastigote form in the vertebrate host as spindle-shaped elongated organism with pointed anterior and blunted posterior ends. The nucleus is central in position and the kinetoplast is situated at the posterior end. The flagellum starts just adjacent to the kinetoplast, curves around the body to form an undulating membrane and continues beyond the anterior end as free flagellum. The trypomastigotes of *T. brucei* are highly pleomorphic, varying in shape and size in different stages. The size may vary from 10 µ × 3 µ to 20 µ × 3 µ. Many antigenic variants of the organism occur.

Life Cycle

The definitive hosts are man, domestic animals and wild game. The intermediate hosts are several species of Tsetse fly (Golossina), e.g. *Glossina palpalis*. The infective metacyclic form of the organism is introduced into man by the bite of the insect (tsetse fly). After multiplication at the site of inoculation, the parasite attacks the blood of the definite host. The

trypomastigote forms are picked up by the tsetse fly during its blood meal from the infective definitive host to continue cycle.

Clinical Picture

A typical trypanosomal chancre appears at the site of insect bite. There is an enlargement of lymph node and involvement of central nervous system. There may be fever, headache, loss of sleep at night. Later there is meningoencephalitis manifested as sleeping sickness. If not treated the disease is fatal one.

Laboratory Diagnosis

It comprises:

1. *Demonstration of trypanosomes form:*
   i. Peripheral blood
   ii. Bone marrow
   iii. Lymph node aspirate
   iv. Cerebrospinal fluid.

   Demonstration of trypanomastigote may be done as under:
   a. Unstained preparation by direct microscopy.
   b. Leishman's stained smears.
   c. Culture on NNN medium.
   d. Animal inoculation (white rat, white mice, guinea pig).
2. *Serological diagnosis:*
   i. Indirect immunofluorescence (IIF).
   ii. Indirect hemagglutination (IHA).
   iii. ELISA.
   iv. Complement fixation test (CFT).
   v. Card agglutination trypanosomiasis test (CATT).

Treatment

Suramin and pentamidine are the drugs of choice. Nitrofurazone, arsenicals and malaresopal are also useful.

TRYPANOSOMA CRUZI

Geographical Distribution

Central and South Africa.

Habitat

A parasite of the muscular and nervous tissues and also of reticuloendothelial system.

Morphology

It is same as *Trypanosoma brucei* except that it is C or U shaped in stained films of blood measuring 3 μ × 3 μ. It does not multiply in peripheral blood. Amastigote forms are seen in cells of striated muscle (heart and skeletal muscle) neurological and reticuloendothelial cells as round or oval bodies 2 to 4 μ with nucleus and kinetoplast. Multiplication occurs only at this stage.

Culture: NNN medium

Life cycle: It is similar to that of *T. brucei* except that it is transmitted by the reduviid bug. Within bug the trypomastigote forms taken up during the bite, are transformed to epimastigote form and finally trypomastigote forms which are excreted into the feces of insect. Man is infected by fecal matter rubbed into the site of the insect bite or by infection of conjunctiva or other exposed membranous surface with finger.

Clinical Picture

After 7 to 14 days of incubation period patient suffers from acute or chronic symptoms of Chaga's disease. It is characterized by fever, conjunctival congestion, edema of one side of face, enlargement of spleen and lymph nodes, anemia and lymphocytosis. These manifestations last for 30 days and often end fatally because of myocardial failure and meningoencephalitis.

The chronic form is found in adults and characterized by cardiac arrhythmia and neurological problems like psychic change and spastic paralysis. The disease lasts for 10 to 12 years. Degeneration of the intramural autonomic nervous system may cause megaesophagus or megacolon. Cardiomyopathy is the other complication.

Laboratory Diagnosis

- Direct microscopic examination of peripheral blood smear (Leishman's stained) shows trypomastigote form.
- Inoculation in animals (Guinea pig).
- Xenodiagnosis by allowing laboratory bug to feed on patient blood and examining its intestinal contents after 2 weeks.

- Biopsy of lymph node or muscle.
- Serological tests like:
  a. Complement fixation test
  b. Immunofluorescence test
  c. ELISA
  d. Intradermal test (delayed hypersensitivity) using *T. cruzi* antigen.

Treatment: Nitrofurazone is effective.

LEISHMANIA DONOVANI

Geographical Distribution

Visceral leishmaniasis is widely distributed but local endemicity is usually rather sharply delimited. It is endemic in many places in America, Africa, China, South Europe, Europe and India. In India, it is common in Assam, Bengal, Bihar, Orissa, Tamil Nadu and eastern parts of Uttar Pradesh up to Lucknow.

Habitat

The natural habitat of *Leishmania donovani* in man is reticuloendothelial system especially spleen, liver, bone marrow, intestinal mucosa. It may be found in endothelial cells of kidneys, suprarenal capsules, lungs, meninges, cerebrospinal fluid and also in the macrophages of intestinal wall.

Morphology

Leishmania donovani exists in two forms: (a) amastigote form also called aflagellar form and (b) Promastigote form also called flagellar form or leptomonad form (Fig. 57.7).

Amastigote Form

In this form this parasite is found in the cells of reticuloendothelial system of vertebrate hosts like man, dog, hamster, etc.

Promastigote Form

They are found in sandfly and in cultures.

a. Shape: Pear-shaped bodies (early stage) to long slender spindle-shaped bodies.
b. Size: 5 to 10 µ × 2 to 3 µ (early stage). 15 to 20 µ × 1 to 2 µ (fully developed).
c. Nucleus: Centrally placed.
d. Kinetoplast: It lies transversely near the anterior end and consists of parabasal body and blepharoplast.
e. Eosinophilic vacuole: It is a light staining area lying in front of kinetoplast over which axoneme runs.
f. Flagellum: It projects from the front and may be of the same length as the body of parasite. Undulating membrane is absent.

Culture

In vitro it can be cultured in NNN (Novy, MacNeal and Nicolle), bacto agar biphasic medium, Schneider's medium with 20 percent calf serum (proved to be excellent), etc. Apart from this tissue culture media and insect culture are also found useful.

The incubation in NNN medium is undertaken in water of condensation and incubation is done at 22° to 24°C. To prevent contaminants, antibiotics and antifungal drugs may be added to above medium.

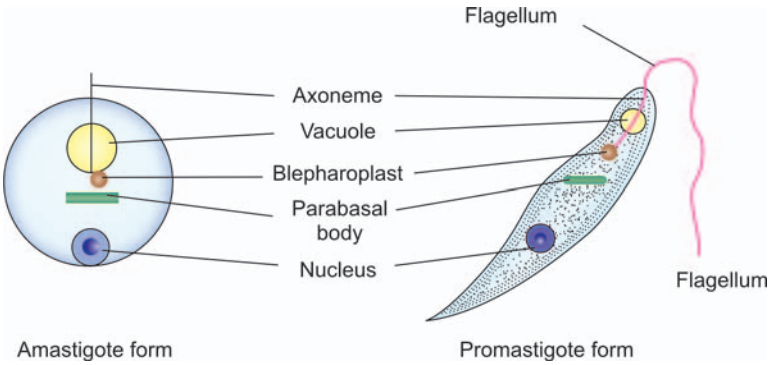


Fig. 57.7: *Leishmania donovani*

The salient features of amastigote form are as under:

a. Shape: Rounded or ovoidal.
b. Size: 2 to 4 µ along the longitudinal axis and living intracellularly in monocytes, polymorphonuclear leukocytes or endothelial cells.
c. Cell membrane: It is very delicate.
d. Nucleus: Lying within the cytoplasm is a relatively large nucleus measuring about 1 µ in diameter and it is oval or round lying in the middle or along the side of cell wall.
e. Kinetoplast: It lies tangentially at right angles to the nucleus. It consists of:
   i. Parabasal body which is a rod-like structure.
   ii. Blepharoplast is a dot-like structure lying near parabasal body.
f. Axoneme: It is a delicate filament which arises from blepharoplast and extends to the margin of the body.
g. Vacuole: It is a clear, unstained space lying alongside the axoneme.

Animal Models

Hamster is the most commonly used animal in routine.

Mode of Transmission

- Natural transmission by the bite of sandfly *(Phlebotomus)*.
- Mother to fetus (vertical transmission).
- Blood transfusion.
- Accidental inoculation of culture in the laboratory.

Life Cycle

The female sandfly (Phlebotomus) after sucking leishmania along with blood of the patient, invariably require fruit juice feed before it becomes infective. Phlebotomus is small hairy fly (1.5 to 3.5 mm). Its usual biting time is at dusk or night.

Leishmania donovani undergoes development inside the body of female sandfly. The promastigote forms after multiplication ascend to pharynx and reach the proboscis. It takes 9 days to complete the cycle in sandfly. Ultimately the buccal cavity of sandfly is blocked by promastigote form. For taking second meal the sandfly has to release the promastigote form from its mouth into the bite wound caused by its proboscis.

The promastigotes thus enter the circulation are mainly destroyed by vertebrate host (man). Still some promastigotes take shelter inside cells of reticuloendothelial system where promastigote form is transformed into amastigote one. They undergo multiplication there at a slow rate. When the infected cells of reticuloendothelial system rupture, the free amastigote forms attack other cells. Sometimes they may be phagocytosed.

Pathology and Clinical Picture

Spleen

Macroscopic Picture

- Enlarged grossly
- Capsule thickened
- Soft in consistency
- Cut surface shows congestion.

Microscopic Picture

- Vascular space engorged with blood and widely dilated.
- Reticular cells of Billroth cords are increased and packed with amastigote form of parasite (*Leishmania donovani* bodies, i.e. L.D. bodies).
- Trabeculae are thin and atrophied.
- Malpighian corpuscles disappear.
- No fibrosis occurs.

Liver

Macroscopic Picture

- Enlarged
- Congested
- Cut surface shows nutmeg appearance.

Microscopic Picture

- Kupffer cell hyperplasia and loaded with LD bodies.
- Engorgement with blood of sinusoidal capillaries. They are dilated too.

Bone Marrow

- Macrophages (loaded with LD bodies) and plasma cells abundantly replace hematogenous tissue.

Miscellaneous:

- Cloudy swelling of kidney.
- Degenerative myocarditis.
- Anemia
- Intestine may present with ulcer because of secondary infection.
- Lymph nodes are not involved in Indian infection.

The clinical picture is as under (Kala-azar):

- Irregular fever
- Hepatosplenomegaly
- Marked emaciation and anemia
- Loss of weight
- Dry skin and brittle hair
- Pigmentation of the skin
- Perverted appetite
- Epistaxis, cancrum oris, lung infection, edema, etc. are some of the complications. The incubation period is 3 to 6 months.

AIDS and Visceral Leishmaniasis

- Sera of visceral leishmaniasis patients co-infected with HIV may be non reactive in serodiagnostic tests.
- Visceral leishmaniasis rapidly accelerates the onset of AIDS and shortens the life span of HIV infected patients.
- HIV spurs the spread of visceral leishmaniasis. AIDS increases the risk of 100 to 1000 times in endemic regions.
- Both visceral leishmaniasis and HIV produce cumulative deficiency of immune responses thus increasing disease severity complications and net consequences.

Laboratory Diagnosis

1 Non-specific tests
   a. TLC indicates leukopenia.
   b. DLC shows neutropenia with relative lymphocytosis and monocytoses.
   c. WBC: RBC ratio 1:2000 (Normal: 1:750).
   d. Reverse albumin globulin ratio detected by following tests.
      i. Naplier Aldehyde test where 1 ml of patient's serum is mixed with 1 drop of formalin (40%) and incubated at room temperature. Gellification means test is positive.
      ii. In 2 ml of patient serum few drops of 4 percent stibamine solution is added. Positive test means appearance of profuse flocculant precipitate. However, this test is less sensitive as compared to aldehyde test.
      iii. Electrophoresis.

2. Demonstration of LD bodies (amastigote) from specimens (Blood, spleen, bone marrow, liver, etc.)
   a. Biopsy or aspirate from these specimens is smeared on clean glass slide fixed with methyl alcohol and stained with Giemsa stain. LD bodies are demonstrated. LD bodies should be differentiated from *Histoplasma capsulatum, Toxoplasma gondii.* Konetoplast is characteristically present in LD bodies which is absent in *Histoplasma capsulatum* and *Toxoplasma gondii.*
   b. Culture may be done on following media.
      i. Grace, insect medium.
      ii. Schneider's drosophila medium.
      iii. Brain-Heart infusion agar medium.
      iv. Tobie's medium.
      v. NNN (Novy, MacNeal, Nicolle) is the popular medium used all over. Basically it is rabbit blood agar slope to which antibiotics are added to check contamination. The specimen is inoculated in the water of condensation and incubated at 24°C for 7 days. Growth in the form of PROMASTIGOTE ONLY is demonstrated. The specimen is declared negative only after 5 to 6 weeks of incubation.
3. Animal inoculation is performed by injecting intraperitoneally the specimen in either hamster or mice. The inoculated animals are kept at 26°C. Amastigote form of parasite is recovered from injected animal.
4. Leishmanin skin test is also called Montenegro test. It is based on the principle of delayed hypersensitivity. About 0.1 ml of antigen suspension (10%) is injected intradermally on dorsoventral part of forearm. Induration of 5 mm or more after 48 to 72 hours means test is positive. Skin test is negative in Indian Kala-azar.

5. Immunological tests include tests to detect antigen (e.g. ELISA and Indirect fluorescence test) and tests to detect antibodies (e.g. complement fixation test—CFT) using Witebsky, Klingenstein and Kuhn antigen also called WKK antigen (obtained from human tubercle bacille) and CFT using Kedrowsky's acid fast bacilli. Other tests used to demonstrate antibodies are using antigen procured from culture are:
   i. CFT
   ii. Countercurrent immunoelectrophoresis
   iii. Indirect immunofluorescence (1:32)
   iv. ELISA
   v. Indirect hemagglutination.
6. Some newer test are:
   i. DOT ELISA
   ii. IIF using monoclonal antibodies
   iii. Nodular lesions may appear on depigmented macules or erythematous patches. Characteristic feature of the nodule is that they never ulcerate.

Direct Agglutination Test (DAT)

- It is a rapid and reliable screening test for visceral leishmaniasis. The antigen used is a suspension of trypsin treated amastigotes. A titer of 3200 or more indicates positive result.
- A rapid latex agglutination test detects leishmania antigen in urine of patients suffering from visceral leishmaniasis.

Treatment

- Pentavalent antimonial drugs, e.g. meglumine antimonate, sodium stiboglucomate.
- Other drugs like pentamidine, amphotericin B and allopurinol are used as an alternative to pentavalent antimonial drugs.

Preventive Measures

- Treatment should be continued with adequate dose for recommended duration and control over the cases of leishmaniasis.
- Use of insecticidal spray like DDT.
- Use of mosquito net, mesh doors and repellents.

Preliminary work in Brasil suggests vaccination with multiple recombinant leishmania antigen may be useful
- A killed promastigote plus BCG vaccine preparation is being investigated as prevention measure.

POST KALA-AZAR DERMAL LEISHMANIASIS (DERMAL LEISHMANIASIS)

Geographical Distribution

Mainly it is seen in Indian regions like Bihar, West Bengal, etc.

Pathogenesis and Clinical Picture

It occurs in patients residing in endemic areas who take drugs for a short while or drugs not in recommended doses. It usually occurs in 2 to 8 percent cases of visceral leishmaniasis about 2 years after recovery from visceral lesions.

It is manifested as:
   i. Depigmented macules distributed over trunk and extremities.
   ii. Erythematous patches on face resembling distribution like butterfly.

Laboratory Diagnosis

Biopsy from lesions shows amastigote form in a stained smear/section. The biopsy material may be cultured on NNN medium or inoculated into animals for establishing diagnosis.

Treatment

Such patients require treatment with pentavalent antimonial drugs in double doses and this treatment should be continued for long time.

LEISHMANIA TROPICA

Along Mediterranean Sea coast.

Geographical Distribution

Central and Western India. The infection does not coexist with kala-azar.

Habitat

Amastigote in reticuloendothelial cells of skin (clasmatocyte). Promastigote form in sandfly, i.e. *Phlebotomus sergenti* in India.

Morphology

Resembles *Leishmania donovani.*
Culture: NNN medium.

Clinical Features

Incubation period is 6 months. It causes disease called oriental sore, Delhi boil, Bhagdad boil, etc. Lesions are cutaneous (exposed parts). Lesion starts as a granulomatous nodule surrounded by red margin. The lesion ulcerates in the center. Later satellite lesions appears around the ulcer.

Pathology

Deposition of promastigotes as a result of bite of infected sandfly on the surface of skin. Promastigote enters through punctured wound and transform into amastigote form inside histiocytes and endothelial cells. Inflammatory granulomatous reaction occurs with infiltration of lymphocytes and plasma cells. As a result of disturbance of blood supply, necrosis and then ulcer formation occurs.

Laboratory Diagnosis

Smear from specimen collected by puncturing the edge of lesion is prepared and stained with leishman stain to demonstrate amastigote form. Material may also be cultured on NNN medium to demonstrate promastigote form.

Treatment: Same as in kala-azar.

LEISHMANIA BRASILIENSIS

Geographical Distribution

Central and South America.

Habitat

Amastigote form occurs in the macrophages of skin and mucous membrane of the nose and buccal cavity.

Morphology

Resemble *Leishmania donovani.*

Life Cycle

Same as *Leishmania donovani* but the vector is forest sandfly (Lutzomyia).

Reservoir: Small forest rodent.

Culture: NNN medium.

Clinical Features

A specific ulcerative granuloma of the skin occurs after incubation period (few days). Later the lesion involves mucosa of mouth, nose, pharynx and larynx. The lesions may appear as papules, nodules or ulcers.

Pathology

The initial lesion has tendency to enlarge radially forming an ulcer with clearcut margin and oozing surface. Amastigotes are there in large numbers towards the periphery of lesion.

Laboratory Diagnosis

Direct smear, culture and animal inoculation and serological techniques (IFA, ELISA and Leishmanin skin test) are of immense value in establishing the diagnosis.

Treatment: Antimonial preparation.

SPOROZOA

MALARIAL PARASITE

GEOGRAPHICAL DISTRIBUTION

It occurs in all countries in the tropics and subtropics (40°S to 60°N). *Plasmodium ovale* is not reported in India whereas *Plasmodium vivax, Plasmodium falciparum* and *Plasmodium malariae* exist in India.

Habitat

It is found in parenchymal cells of liver, erythrocytes and other organs.

Life cycle: All species complete life cycle in man and female anopheles mosquito.

Life cycle in Man (Schizogony)

Female anopheles mosquito bite to man results in the injection of sporozoites which circulate in blood (about 1 hour) when some sporozoites attack liver cells and start pre-erythrocytic cycle. In 6-15 days' time release of thousands of merozoites occurs after completion of pre-

erythrocytic cycle. Some of these merozoites are phagocytosed whereas others start erythrocytic schizogony.

In *Plasmodium vivax* and *Plasmodium ovale* hepatic form known as hypnozoites persist and remain active in hepatocytes for considerable time before they grow and undergo pre-erythrocytic schizogony with liberation of merozoites in bloodstream causing lapse of the infection.

During erythrocytic schizogony (Table 57.7) parasite assumes a form of ring and then of trophozoite. In trophozoitic form malarial pigments start appearing. Trophozoites are transformed to schizont with division of chromatin surrounded by pieces of cytoplasms. On attaining maturity schizont ruptures with the liberation of merozoites. They in turn invade fresh erythrocytes.

After undergoing erythrocytic schizogony some macrozoites are transformed into gametocytes. Gametogony occurs in the erythrocytes of capillaries of internal organs. Mature gametocytes seek entry to peripheral blood from where they are carried to vector (mosquito) when it bites the patient.

Life cycle in Mosquito (Sporogony): The female anopheles mosquito during blood meals from malarial patient sucks blood containing plasmodia in various forms and stages. The presence of male and female mature gametocytes should be there for continuing sexual cycle (sporogony) in the mosquito. At least 12 gametocytes per cu mm of blood and macrogametocytes must be in excess. The other forms of plasmodia, i.e. asexual forms are destroyed in the stomach of mosquito.

First of all gametocytes become rounded and exflagellation occurs with the formation of 4 to 8 filamentous structure (microgametocyte). These filaments are detached and form microgametes. One of these microgametes penetrates the macrogamete. This follows the transformation of fertilized macrogamete into zygote. It happens from 20 minutes to 2 hours after ingestion of blood by mosquito.

Zygote becomes very active and now called ookinete which penetrates the muscle wall of stomach and comes to lie below the outer limiting membrane of stomach wall in the form of oocyst (6 to 12 µ). Oocyst further increases in size to 60 µ in diameter, containing many sickle-shaped structures called sporozoites. Oocyst containing sporozoites is called sporocyst.

Sporocyst ruptures with the liberation of sporozoites in body cavity of mosquito. Sporozoites are disseminated in all parts of body except ovaries. Sporozoite has special affinity for salivary glands. They pass through the glands and reach the lumen of salivary duct with maximum concentration of sporozoites. The mosquito is now infective.

Pathogenesis of Plasmodium

1. The plasmodia that invade the red blood cells grow and segment at the expense of these host cells, which rupture when schizogony is complete. The debris of ruptured cells, the released merozoites, and their metabolic products, stimulate chemoreceptor of the temperature regulating mechanism of the host to conserve heat. As the number of the invaded red cells increases and the asexual cycle of parasite becomes more synchrosed, the quantity of pyrogen released at one line becomes sufficient to produce the characteristic chills and fever of a malaria attack.
2. The species of plasmodia differ greatly in their ability to multiply in the blood *Plasmodium vivax* prefers to invade the youngest erythrocyte, whereas *Plasmodium malariae* prefers the older red cells. Hence these species parasitize around 2 percent of host red blood cells. *Plasmodium falciparum*, on the other hand, invade erythrocytes of all ages and, thus, is capable of parasitizing a very high percentage of erythrocytes.
3. Due to the varying number of merozoites produced in schizogony by the species of plasmodium, *Plasmodium falciparum* multiplies more rapidly than *Plasmodium vivax* which, in turn multiplies more rapidly than *Plasmodium malariae* and *Plasmodium ovale*. The parasitemia of *Plasmodium falciparum* also tends to be higher because more than one parasite frequently develop in a single erythrocyte.

TABLE 57.7: Morphological features in different species

	P. falciparum	*P. vivax*	*P. malariae*	*P. ovale*
RBC				
Size	Normal	Enlarged	Normal	Enlarged
Shape	Round and crenated	Round	Round	Oval or round
Color	Normal	Pale	Normal	Normal
Stippling	Maurer's dots (large red)	Schuffner's dots (small red)	Ziemann's dots	James dots (many small red)
Pigment	Black or dark brown	• Fine golden brown in cytoplasm	Black or brown large	Black or brown
Parasite	Small, dark multiple infection of one RBC	• Large	Tendency to form band across the RBC otherwise of moderate size	Regular shape
Stages in PBF	Only rings and Gametocytes	Trophozoites Schizonts Gametocytes	Trophozoites Schizonts Gametocytes	Trophozoites Schizonts Gametocytes
Ring stage	Small, 1.5 µ, double chromatin multiple rings and vacuole formation	Large 2.5 µ single Thicker chromatin	Characters of *P. vivax*	Characters of *P. vivax*
Trophozoites	Compact, small, no vacuole seen	Large irregular ameboid, vacuole prominent Thick chromatin	Band formation	Compact chromatin is large irregular clumps
Schizont	Small Compact Not seen in PBF	Large, filling RBC Yellow brown pigment	Fills RBC Segmented, Daisy head pigment (dark brown)	Fill 3/4th RBC Segmented Dark yellow brown pigment
Micro-gametocyte	• Larger than RBC • Kidney shaped • Cytoplasm blue • Fine granules scattered • In smear many in number	• Fills enlarged RBC • Round • Cytoplasm pale blue Many brown granules	• Smaller than RBC • Round • Cytoplasm pale blue • Pigment and chromatin like *P. vivax*	• Of the size of RBC • Round • Cytoplasm pale blue • Pigment and chro-matin as in *P. vivax*
Macro-gametocyte	• Crescent • Deep blue • Cytoplasm • Ends sharply rounded • In abundant in PBF	• Round • Fills enlarged • RBC • Pigment as small masses • Many in PBF	• Round • Smaller than RBC • Cytoplasm dark blue • Scanty in PBF	• Round • Of RBC size • Cytoplasm dark blue • Rarely in PBF

Contd...

Contd...

	P. falciparum	*P. vivax*	*P. malariae*	*P. ovale*
Number of merozoites in a schizont	20-24	16	8	8
Erythrocytes infected	Any age	Young	Old	Young
Maximum parasitemia	1,000,000/µl	25000/µl	10,000/µl	25000/µl
Relapse	No	Yes	No	Yes
Drug resistance	Yes	No	No	No
CNS involvement	++++	+	+	±
Nephrotic syndrome	+	±	++++	+
Anemia	++++	++	++	–

4. With each schizogony, the parasitized cells are destroyed, but there also in considerable destruction of unparasitized cells due to lysis and phagocytosis, phagocytosis especially occurs in the spleen and liver. In falciparum malaria with a very high parasitemia, hemolytic jaundice may be evident and anemia may be severe.
5. Malignant character of falciparum malaria is not so much related to its rapid its rapid multiplication and invasiveness as to the manner in which it causes lesions in the human host. Characteristic lesions are due to blockade of small vessels by sticky parasitized erythrocytes. The blockade causes stasis, then local anoxia, then increased vascular permeability, which allows plasma and unparasitized cells to leak into the perivascular space. This incidently results in additional loss of erythrocytes.
6. These factors combine to cause a decrease in circulating erythrocytes in the circulating blood volume, local tissue anoxia and edema.

Clinical Picture

- Fever peaks (exhibits cold, hot and sweating stages)
- Anemia
- Splenomegaly and hepatomegaly.

Complications of *P. falciparum*

- Cerebral malaria occurs when non-immune person remains untreated for 7 to 10 days after plasmodial infection. It is associated with fever, confusion, convulsion, coma and death.
- Black water fever is characterized by sudden massive hemolysis followed by fever and hemoglobinuria. It is always associated with consumption of small doses of quinine. There is intravascular hemolysis with the manifestations like methemalbuminemia, hyperbilirubinemia, hemoglobinuria. Kidneys and liver are particularly involved. RBC and hemoglobin fall considerably. Sequelae of black water fever includes uremia, renal failure, circulatory failure, liver failure, anemia and pigment calculi. Renal failure is the cause of death.
- Pernicious malaria results from anoxia due to obstruction of capillaries in various organs followed by necrosis of tissues. It occurs in recently infected persons *(P. falciparum)* without immunity to plasmodium. It may involve (i) Nervous system (cerebral malaria) cerebrospinal involvement and peripheral nerves plus cord nerves rarely, (ii) Gastrointestinal having gastric, choleric and dysenteric forms, (iii) May involve cardio-

vascular, respiratory and genitourinary system. Hence pernicious malaria may be of following types:

a. Septicemic form may eventually cause cardiac failure and death.
b. Acute hemolytic form may cause anemia.
c. Hemorrhagic form may manifest like purpura.
d. Pneumonic form may result in pulmonary edema.
e. Nephrotic form with nephritis, nephrosis, etc.

Transmission Modes

- Mosquito bite (Table 57.8).
- Transfusion malaria.
- Congenital malaria.
- Use of contaminated syringe as in drug addicts.

Immunity

- Glucose 6 phosphate dehydrogenase deficients are protected from malaria as this enzyme is necessary for respiration of plasmodium.
- Absence of Duffy blood group protects against *Plasmodium vivax* as this blood group antigen seems to be receptor for *Plasmodium vivax.*
- *Plasmodium falciparum* does not multiply in sickle red cells as they contain abnormal hemoglobin S.
- Severe malnutrition and iron deficiency appear to offer some protection against malaria.

TABLE 57.8: Differences between sporozoite induced and trophozoite induced malaria

	Sporozoite induced	*Trophozoite induced*
1. Transmission	Mosquito bite	Blood, e.g. blood transfusion
2. Pre-erythrocytic schizogony	+	
3. Incubation time	Prolonged	Short
4. Relapses	May occur	Absent
5. Severity	Less	More severe
6. Radical drug treatment	Indicated	Not required

- Infants are immune to malaria because of presence of fetal hemoglobin (Hb-F), diet deficient in amino benzoic acid and passive antibodies, i.e. maternal antibodies.
- Premunition is a state of resistance in an infected person which harbors parasites but remains asymptomatic. This immunity disappears soon after eradication of plasmodial infection. Hence low levels of parasitemia must be there to maintain immunity.

Pathology: Changes in various organs are mentioned as under:

Liver

Macroscopic (Gross)

- Liver enlarged.
- Color differs from dark red to slate gray.

Microscopic

- Dilatation of capillaries and sinusoids.
- Hypertrophy and hyperplasia of Kupfer's cells lining sinusoids.

Spleen

Macroscopic

- Enlarged
- Congested
- Dark red
- Soft
- Capsule tense.

Microscopic

- Hyperemia.
- Hyperplasia of reticuloendothelial and lymphoid elements.
- Macrophages loaded with red cells and parasitic debris plus pigments.

Kidney

Macroscopic

- Congested.

Microscopic

- Degenerative changes in the epithelium of tubules.
- Acute tubular necrosis.
- Immune complex deposition especially in *Plasmodium malariae.*

Bone Marrow

Macroscopic

- Dark red.

Microscopic

- Hyperplasia of reticuloendothelial cells containing pigments.
- Parasitized erythrocytes.

Brain

Macroscopic

- Congested and edematous.
- Petechial common in white matter.

Microscopic

- Capillaries congested and loaded with parasitized erythrocytes.
- Ring hemorrhages.
- Necrosis.

Heart

- Petechial hemorrhage in epicardium.
- Congested capillaries engorged with parasitized erythrocytes.
- Degenerative changes in heart muscles.

Lungs

- Congested.
- Capillaries contain parasitized erythrocytes.
- Pulmonary edema may be there.

Gastrointestinal Tract

- Edematous.
- Congested.
- Petechial hemorrhage, but necrosis and ulceration only in *Plasmodium falciparum.*

Laboratory Diagnosis

- Peripheral blood film for parasites (thick and thin smear) is studied microscopically after staining often with Leishman technique. Morphological features are noted and plasmodium is identified in thin film.
- Serological techniques like IHA, ELISA, etc. These tests are of very limited importance in establishing diagnosis. However, it does carry epidemiological importance.

Newer diagnostic techniques are:

i. Use of fluorescent dyes.
ii. Radioimmunoassay.
iii. Agar gel diffusion.
iv. Dot-blot assay.
v. Use of automated equipment.
vi. Visualization of parasite by quick buffy coat method.
vii. ELISA using polyclonal or monoclonal antibodies.
viii. DNA probe.
ix. RNA probe.
x. PCR.
xi. Immunochromatographic test kit which is a credit card size manual kit based on capture of a circulating *Plasmodium falciparum* histidine rich protein-2 (Pf HRP-2) in whole blood.
xii. A rapid immunodiagnostic: Strep test for detection of *Plasmodium falciparum* specific parasite lactate dehydrogenase (PLDH) may be used.

Treatment

- Quinine.
- Chloroquin.
- Primaquin.
- Pyrimethamine + salfadoxine.

Prevention and Control

- Mosquito repellants and bed nets
- Spray of chemicals
- Biological control measures like use of special fishes (Gambiens) in water, destruction of reproductive system using radiations, etc.
- Proper treatment of malarial patients
- Use of malarial vaccine which are under trial, e.g. sporozoites vaccines, vaccine with exo-erythrocytic merozoites, merozoites of erythrocytic stage vaccine, vaccine prepared from purified gametocytes and SPF-66.

Now PCR and genetic engineering techniques have brought new hopes in the preparation of ideal vaccine against malarial infection.

TOXOPLASMA GONDII

Geographical Distribution

It is worldwide in distribution.

Habitat

Endothelial cells, leukocytes, body fluid and tissue cells.

Morphology

It occurs in following three forms:

Trophozoites (Fig. 57.8)

- Oval-shaped or crescent-shaped with one end pointed and the other rounded.
- Measure 7 × 7 µ.
- Lack flagella, cilia and pseudopodia.
- Staining by Giemsa's or Wright's technique.
- Nucleus lies near blunt end.
- Multiplication by endogeny which means internal budding.
- Rapid proliferation of trophozoites in acute infection (invade mammalian cells) is called tachyzoites (Fig. 57.9).
- Trophozoites may be found extracellular too.
- Exhibit asexual multiplication, i.e. schizogony.

Tissue Cysts

- Measure 10 to 200 µ.
- Contain thousands of organisms.
- Demonstration by periodic acid Schiff stain.
- Slowly multiplying parasites within cyst are named bradyzoites.

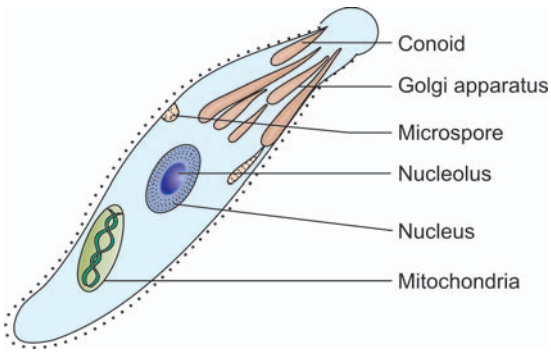


Fig. 57.8: Trophozoite of *Toxoplasma gondii*

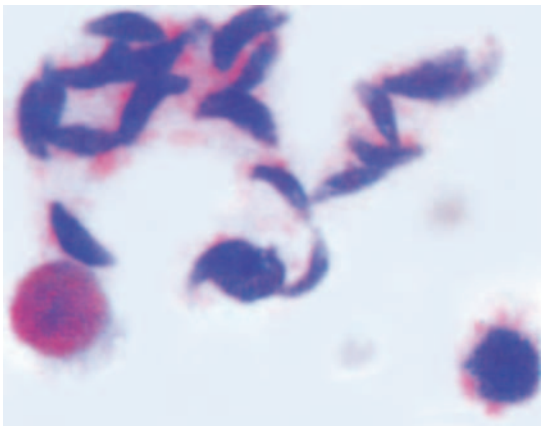


Fig. 57.9: Toxoplasma gondii

- Act as source of infection and thus responsible for transmission of infection.
- Tissue cysts are formed during the chronic stage of infection.
- May involve any organ of the body predominantly skeletal muscles, heart muscles and brain.
- Exhibit asexual multiplication, i.e. schizogony.

Oocysts

- Spherical or oval 10 to 12 µ.
- Contain sporoblast.
- Freshly passed oocyst are not infective.
- They attain infectiveness only after development in soil or water. Infective form contains 8 sporozoites.
- Oocysts are formed as a result of sexual reproduction, i.e. gametogony or sporogony.
- Oocysts develop only in the definitive host, i.e. cat and members of feline family.

Life Cycle

Life cycle is completed as under:

Enteric Cycle in Cat

Ingested oocyst releases sporozoites. These sporozoites penetrate the epithelial cells of the intestine. They become rounded and grow within host cells. Thereafter asexual division occurs resulting in the formation of merozoites. Some merozoites manage to seek entry in extraintestinal tissues and thus tissue

cysts formation occur in other organs of body. Other merozoites are changed into sexual forms and thus start sexual reproduction named gametogony. A motile microgamete fertilizes a macrogamete which results in the formation of oocyst which undergo development through various stages before it becomes infective.

Exoenteric Cycle in Man

Ingestion of oocyst contaminated improperly cooked meat results in infection. Alternatively, oocyst from cat may be ingested. Only asexual form of reproduction occurs in man with the formation of merozoites only. Merozoites enter lymphatics and blood. They develop into tissue cysts in different organs of the body. Human-to-human infection is possible through placenta giving rise to congenital toxoplasmosis. It is worthwhile to point out that oocysts are not formed in the intestine of man.

Mode of Transmission

It is by ingestion of oocyst or tissue cyst containing edibles, through placenta to the fetus, occupational (laboratory or slaughter house workers), etc.

Pathogenesis

Many organs are involved once parasites gain access to bloodstream. Initial invasion of cell occurs followed by their multiplication and finally disruption of cell. It results in focal area of necrosis with lymphocytes, and monocytes around it. By and large the infection is asymptomatic and its reason may be effective protection immunity (extracellular antibody and intracellular T cell factors).

Clinical Picture

- Congenital toxoplasmosis with manifestation in newborn like chorioretinitis, blindness, epilepsy, mental retardation. It may give rise to abortion or stillbirth. Other less important lesions are of eyes, liver and spleen.
- Infection of mother during pregnancy.
- Ocular toxoplasmosis with lesions like chorioretinitis.
- Widespread infection in immunocompromised persons, e.g. AIDS, organ transplantation recipients, malignancies, etc. Other lesions are encephalitis, meningitis, chorioretinitis, myositis, etc.
- However, acquired toxoplasmosis has presentation like lymphadenitis, maculopapular rash, myocarditis, etc.

Laboratory Diagnosis

1. **Isolation of *Toxoplasma gondii:*** It is possible by injecting body fluid or infected tissue into peritoneal cavity of mice or tissue culture. Mice should be examined for demonstration of *T. gondii* in the peritoneal fluid, 6 to 10 days after injection of material.
2. **Demonstration of trophozoites or cysts:** Their demonstration in placenta or tissues of the newborn confirms the diagnosis of congenital toxoplasmosis. They may also be demonstrated in brain biopsy, bone marrow biopsy, CSF, amniotic fluid, etc. thus diagnosing the infection as an acute one.
3. **Lymph node biopsy** shows reactive follicular hyperplasia and irregular clusters of epithelioid histiocytes.
4. **Serological Tests:**
   - Latex agglutination.
   - Toxoreagent agglutination test is simple, rapid test exhibiting over 94% agreement with dye test. Here latex particles are coated with soluble antigen.
   - IHA
   - ELISA
   - Complement fixation test
   - Indirect immunofluorescence test
   - Methylene blue dye test of Sabin and Feldman in which equal amount of diluted patient serum, *Toxoplasma gondii*, normal human serum are mixed and incubated at 37°C for one hour. To each tube 1 drop of methylene blue (pH 11) is added and a drop of mixture is examined microscopically. If the patient serum contains specific antibodies, less than 50 percent of free toxoplasma does not take up stain and the cytoplasm remains colorless. The highest dilution of test serum which inhibits the staining is the titer.

The test becomes positive in 1 to 2 weeks after infection and remains positive for years altogether.

Presence of specific IgM/IgA antibodies in serum, a high dye titer value (>300 IU/ml) are the tests indicative of recent toxoplasma infection. However, all these tests are unreliable because sometime IgA antibodies persists for months while IgM may persist for years following primary infection. Sabin and Feldman dye test titers also remain very high for several months. Avidity ELISA test is found to be a useful complimentary test to differentiate recent infection from chronic infection.

Treatment

- Pyrimethamine and sulfadiazine combination is quite useful.
- Spiramycin as such or in combination with sulfadiazone may be tried.

ISOSPORA BELLI

It is the protozoa of intestine of man. It is frequently found in Asia, Africa and South America.

Morphology

- It is oval in shape.
- Measures 20 to 30 × 10 to 20 micron.
- Oocyst develops to 2 sporoblasts which transform to sporocysts.
- Each sporocyst measures 9 to 15 × 8 to 14 micron and contains 4 crescent-shaped sporozoites.
- Oocyst is surrounded by thin, smooth and two layered cyst wall.

Life Cycle (Fig. 57.10)

- Ingestion of contaminated food or water is the source of infection in man.
- Eight sporozoites are released from each oocyst in the upper part of small intestine where they invade epithelial cells.
- In the cytoplasm asexually multiplication of parasite occurs to produce trophozoites.
- Some trophozoites undergo sexual reproduction (gametogony) and produce oocysts.
- Sporulation of oocyst occurs in 5 days both within the host and external environment.
- Sporulated and unsporulated oocysts are passed through stools.

Pathogenicity

- May cause mild and self-limiting diarrhea.
- In AIDS cases, it may be associated with more severe infection.
- Patient may present with:
  - — Fever
  - — Headache
  - — Malaise
  - — Cholecystitis
  - — Persistent diarrhea
  - — Weight loss
  - — Steatorrhea
  - — Death.

Laboratory Diagnosis

- Demonstration of characteristic oocysts in stool examination in unstained and iodine stained direct smear preparation as well as by zinc sulfate and formalin ether concentration methods.

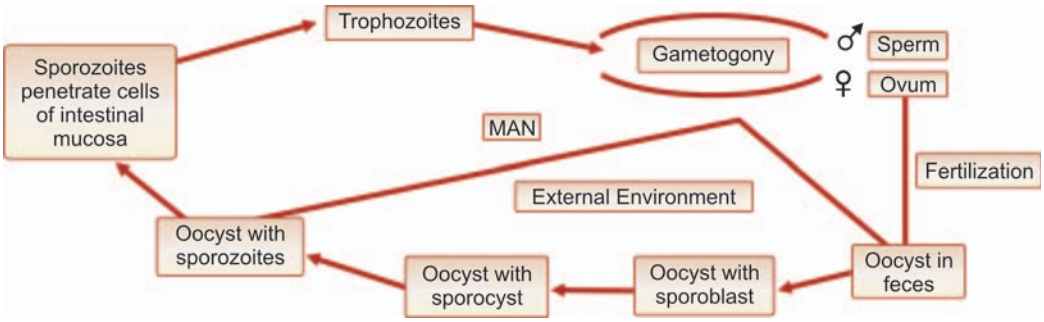


Fig. 57.10: Life cycle of *Isospora belli*

- Intestinal biopsy to demonstrate oocysts.
- Trophozoites may be demonstrated in tracheobronchial, mediastinal and mesenteric lymph nodes, gallbladder, liver and spleen of especially AIDS patients.

Treatment

Co-trimoxazole is the drug of choice in case antimicrobial treatment is indicated.

CRYPTOSPORIDIUM PARVUM

Geographical Distribution

It is cosmopolitan reported from USA, Russia, Australia, Denmark, Brazil, UK, Bangladesh, etc.

Morphology

Oocyst is demonstrable in stool and it bears following characters:

- It is 4 to 5 μ in diameter.
- Contains 1 to 6 large dark granules and many small granules.
- Mature oocyst (postsporulation) in which 2 to 4 sausage-shaped sporozoites are seen.
- Oocyst exists in 2 forms.
  i. Thin wall oocyst
  ii. Thick wall oocyst.

Life Cycle

Life cycle is completed in one host. Infected form is a mature oocyst containing 4 sporozoites which is ingested. Excystation occurs in small intestine where sporozoites are released which attack the epithelial cells. In the epithelial cell sporozoite is transformed into trophozoite and then to first generation schizont (eight merozoites). These merozoites attack other epithelial cells and form second generation schizont (4 merozoites). These merozoites may attack other epithelial cells and later on develop into microgametocytes or macrogametocytes. A microgametocyte may give rise to 12 to 16 microgametes. On the contrary one microgametocyte gives rise to only one microgamete. A zygote is formed as a result of fertilization which develops into oocyst. Thus life cycle is completed.

Pathogenesis and Clinical Picture

This protozoa can be seen on brush borders of intestinal epithelial cells and in the crypts of Liberkuhn. The lesions may be located in small intestine and also in colon, cecum or rectum.

Cryptosporodiasis clinically resembles giardiasis. It appears as short-term cholera-like diarrheal disease. Diarrheal stools are foul smelling. It has high incidence in AIDS patients.

Laboratory Diagnosis

- Microscopic examination of stool for the demonstration of oocyst.
- Concentration method of stool examination is sugar floatation or Sheather's method.
- Cryptosporidium takes red stain when stained with modified acid fast method.
- Intestinal biopsy for microgametes and macrogametes. We can also demonstrate schizont.
- ELISA.
- Enzyme immunoassay (EIA) is useful to detect cryptosporidium antigen. It is available in microplae format. It is sensitive, specific but expensive. Specific antigen may be detected in fresh or preserved stool specimens.
- Indirect immunofluorescence test.

Treatment

It is self-limiting infection. No drug treatment is effective till now.

BABESIA

Geographical Distribution

Northeast USA, France, Ireland, Mexico, Russia and Scotland.

Habitat

Erythrocytes.

Morphology

Babesia are small plasmodiae-like protozoan which are pear-shaped. Human infections are reported by *Babesia bovis*, *Babesia divergens* and *Babesia microti* species.

Transmission of Infection

It is by ticks of genera Ixodes and Dermacentor. Like malaria it may be transmitted by blood transfusion too.

Incubation Period

1 to 2 weeks.

Life cycle: Like malaria, sexual cycle occurs in mammals whereas asexual cycle occurs in ticks. Ring-shaped and ameboid-shaped parasites appear in peripheral blood film. The parasite multiplies by binary fission or budding, dividing upto 4 buds at a time. A cluster of 4 small merozoites (tetrad) is a primary diagnostic finding of babesiosis.

Pathogenesis and Clinical Features

There is invasion and destruction of erythrocytes. Manifestations include splenomegaly, swelling, cellular degeneration and necrosis of hepatic sinusoids.

Laboratory Diagnosis

- Peripheral blood smear examination after staining with Giemsa or Wright stain.
- Animal inoculation in which blood of patient is inoculated in hamster or gerbil. It is sensitive technique for recovery of babesia.
- Serological techniques like indirect immunofluorescent antibody, complement fixation, ELISA, IHA and rapid agglutination are useful.

Treatment

Pentamidine.

PARASITES ASSOCIATED WITH AIDS

1. Cryptosporidium
2. Isospora belli
3. Cryptospora
4. Microsporidia
5. Pneumocystis carinii
6. Blastocystis hominis
7. Leishmania species
8. Toxoplasma gondii
9. Acanthameba species
10. Trypanosoma cruzi

CILIATA

BALANTIDIUM COLI

Geographical Distribution

Worldwide.

Habitat

Largest protozoal parasite inhabiting large intestine of man. Also found in pigs and monkeys.

Morphology

Trophozoites (Fig. 57.11):

- Oval
- 50 to 200 µ × 40 to 70 µ.
- Surface is pointed with delicate cilia.
- Anterior endpointed and has cytostome.
- Posterior end is round.
- Cytoplasm contains kidney-shaped large macronucleus and small micronucleus.

Cyst (Fig. 57.11)

- Oval
- Thick outer wall
- Cilia absent
- Protozoa is enclosed in double-layered wall.

Life cycle: No intermediate host is required. The cysts are passed in stool. Infection occurs by ingestion of cyst with contaminated food or drinks. In the wall of intestine excystation occurs and trophozoites develop which live and subsequently multiply by binary fission on the mucosa of large intestine.

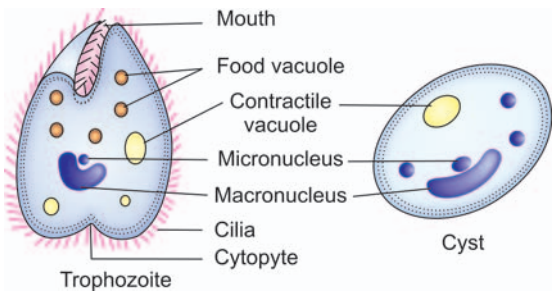


Fig. 57.11: *Balantidium coli*

Pathology

Rarely, there is mucosal damage caused by trophozoites. Because of enzyme hyaluronidase produced by parasite may cause mucosal damage resulting in small superficial ulcer which may penetrate up to submucosa. Thus there is diarrhea and later frank dysentery develops. Abdominal colic, nausea and vomiting may occur.

Laboratory Diagnosis

The trophozoites and cysts may be demonstrated in the stool of patient.

Prophylaxis

- Improvement of personal hygiene.
- Prevention of food contamination with stools of man as well as pigs or monkeys.

58 Helminths

NEMATODES

TRICHINELLA SPIRALIS

Geographical Distribution

Common in Europe and USA.

Habitat

It is a parasite buried in duodenum or jejunal mucosa, fertilization of female occurs which discharges ova in circulating blood which encyst in striated muscle of man, pig or rat.

Morphology

Adult worm (male) measures 1.4 to 1.6 mm (smallest nematode) and female is 3 to 4 mm long. Larvae measure 100 × 6 µ. They remain encysted in striated muscle of host and keep growing till maximum size is attained in 35 days. Lifespan of male worm is 1 week and female dies in 16 weeks. Most of larvae die within 6 months while others survive for 10 to 30 years.

Life cycle: It is passed in one host (man, pig, etc.). Male and female worms lie in the folds of intestinal villi. Fertilized female bores its way into intestinal wall. Larvae discharged into blood stream are localized in the striated muscle where encystation of larvae occurs. Infection of a new host is by ingestion of raw flesh containing encysted larvae.

Pathogenicity and Clinical Picture

Mode of infection is by ingesting infected pig's flesh. During intestinal invasion gastrointestinal symptoms appear. During invasion of muscle patient complains of remittent fever, urticarial rash, respiratory, myocardial and neurological symptoms. Stage of encystation occurs in striated muscle.

Laboratory Diagnosis

- Stool examination for adult worm or larvae demonstration.
- Blood examination to detect eosinophilia.
- Muscle biopsy.
- X-ray examination to detect calcified cyst.
- Serological tests like complement fixation test, latex agglutination test, etc.

Treatment

Thiabendazole is quite effective.

TRICHURIS TRICHIURA (WHIPWORM)

Geographical Distribution

Worldwide.

Habitat

Adult worm lives in large intestine of man.

Morphology

Adult worm in large, small, whip-shaped parasite having thin anterior end. Female worms measure 3 to 5 cm while male is slightly smaller. Cephalic end of parasite has a stylet which helps in the penetration into intestinal mucosa. The worm is oviparous.

Eggs are barrel-shaped, 25 × 50 µ containing mucous plug at both the ends. The outer shell is bile pigmented which encloses unsegmented embryo.

Life cycle: No intermediate host is required. Eggs are passed in stools of infected patient. A rhabditiform larva develops from egg and infection to healthy person occurs by ingestion of embryonated eggs in food and water. The egg shell is dissolved in the stomach and larvae liberated pass down the cecum which grow into adult worms and embed their anterior parts in the intestinal mucosa. They grow in adult form. The life-cycle is completed in one host, i.e. man.

Pathogenicity and Clinical Picture

In severe infection, abdominal pain, anorexia, vomiting, flatulence and in chronic cases anemia may develop.

Laboratory Diagnosis

It is established by detecting characteristic eggs in stool. Sometimes adult worm may be detected in stools but rarely.

Treatment

Thiabendazole, mebendazole are effective drugs.

ASCARIS LUMBRICOIDES (ROUNDWORM)

Geographical Distribution

It is cosmopolitan.

Habitat

Adult worm lives in the lumen of the small intestine (jejunum) of man.

Morphology

Adult Worm

- Elongated cylindrical measuring 15 to 50 cm × 2 to 6 mm.
- Oral cavity has 2 lateral lips and one median lip.
- Body cavity contains toxin (ascaron) in which digestive and reproductive organs float. Ascaron is allergic.
- Posterior end of male is curved ventrally.
- Male worm is smaller than female worm.

Egg Fertilized

- 45 to 75 µ × 35 to 50 µ.
- The shell has innermost very thin vitelline membrane, a thick glycogenous middle layer and coarsely laminate outermost layer.
- Embryo is unsegmented and made up of coarse lecithin granules.
- Floats in saturated salt solution.

Unfertilized

- Larger in size 88 to 94 µ × 45 µ.
- The shell is relatively thin.
- The innermost layer vitelline is absent.
- Embryo contains disorganized mass containing refractile granules.
- It does not develop into larvae.
- It does not float in saturated salt solution.

Life Cycle

- The female adult worm liberates fertilized eggs in small intestine which are passed with stools. The freshly passed fertilized eggs contain unsegmented ovum. At this stage they are not infective to man.
- Rhabditiform larvae develop inside in 9 to 15 days in moist soil (22° to 33°C). A moulting occurs within the egg and then the egg becomes infective.
- Infective eggs are swallowed with raw food material which pass through stomach into small intestine.
- Outer shell of egg is dissolved and larva comes out in the intestinal lumen measuring 250 µ × 14 µ.
- The larvae penetrate the mucosa and reach portal circulation thereafter to liver (live for 3 to 4 days), heart and finally to the lung alveoli.
- Larvae grow upto 2 mm and moult twice (5th and 6th days).
- The larvae from alveoli migrate to bronchioles, trachea and are swallowed back in the stomach. During this period two more moulting occur (in 25th to 29th days). In the intestine larvae develop into adult worm.

Pathogenicity and Clinical Picture

The larvae in lung alveoli cause migrating pneumonitis (allergic reaction) and this allergic reaction may be due to ascaron.

Adult worm may cause malnutrition, vague abdominal pain, colic pain, poor digestion, diarrhea, etc.

- Perforation of bowel, appendicitis, diverticulitis may occur.

Laboratory Diagnosis

- Detection of adult worms in stool.
- Microscopic detection of eggs in feces or bile obtained by duodenal intubation.
- Eosinophilia.
- Dermal reaction (allergic), scratch test with powdered ascaris antigen.

Treatment

Piperazine, decaris, albendazole, mebendazole are effective.

ANCYLOSTOMA DUODENALE (HOOKWORM)

Geographical Distribution

It is widely found in tropical and subtropical countries.

Habitat

The adult worm resides in small intestine of man particularly in jejunum.

Morphology

Adult Worm

- It is small, pinkish, fusiform in shape.
- The anterior end is curved.
- Female worm is 13 mm × 0.6 mm with pointed posterior end.
- Male worm is smaller 11 mm × 0.4 mm having copulatory bursa with tripartite dorsal rays and a pair of long copulatory spicules.
- The oral cavity has single pair of dorsal teeth and 2 pairs of ventral teeth which help the worm to get attached to the mucosa.

Egg

- Oval, measuring 75 µ × 40 µ.
- It has thin outer shell enclosing a segmented ovum with 4 segments.
- There is a clear space between egg shell and segmented ovum.
- It floats in saturated saline.
- It is non-bile stained.

Life Cycle

The adult worm lives in small intestine of man attached to the mucosa. After fertilization female lays eggs which are passed out with stool. The larvae develop from eggs outside the body of host. Freshly passed egg contains segmented embryo and in 24 hours free living rhabditiform larva is liberated. After 7 to 8 days rhabditiform larva moults twice (once on 3rd day and second on 5th day) and transforms into filariform larva which is infective. On coming in contact with skin of foot, it penetrates into hair of follicle or bores through intact skin to reach right side of heart (through lymphatics and venous blood). Thereafter larvae reach lung and after piercing capillary wall reach alveolar space. Third moulting occurs at this stage. From alveolar space larvae reach bronchioles, trachea, larynx and are swallowed back to stomach. On reaching duodenum and jejunum another moulting occurs. Here, in 5 to 7 weeks, larvae grow to mature adult form which are sexually mature.

Pathogenicity and Clinical Picture

Ancylostomiasis or hookworm disease is characterized by anemia. At the site of filariform larvae penetration, dermatitis may occur. The larvae then migrate to subcutaneous vessels and cause creeping eruption. When larvae reach lung multiple hemorrhage and transient bronchopneumonia occur. Adult worms cause irritation and petechial hemorrhage in the intestinal mucosa.

Laboratory Diagnosis

- Stool examination for adult worm (naked eye).
- Microscopic examination of stool for ova.
- Study of duodenal contents for adult worm and ova.

Indirect Evidences

- Blood examination for anemia and eosinophilia.
- Stool examination for occult blood and Charcot-Leydon crystals.

Treatment

- Thiabendazole, mebendazole, etc.
- Anemia is treated with iron, folic acid and vitamin B_{12}.

NECATOR AMERICANUS (TABLE 58.1)

Geographical Distribution

It is the most common species in Sri Lanka and India (except Punjab and UP). It was first discovered in America. It occurs in tropics and South Africa, Far East and Australia.

STRONGYLOIDES STERCORALIS

Geographical Distribution

It is worldwide in distribution, more predominant in Brazil, Far-East and Africa.

Habitat

The female adult worm lives in the mucous membrane of small intestine.

TABLE 58.1: Differences between *Ancylostoma duodenale* and *Necator americanus*

Ancylostoma duodenale	*Necator americanus*
• Adult worm larger and thicker	Smaller and more slender
• Anterior end bends in the same direction as body curvature	Bends in opposite direction to body curvature
• A spine is present at posterior end of female	Absent
• Buccal capsule has 6 teeth	4 chitinous plates
• Vulval opening behind the middle of the body	It is in front of the middle of the body
• More pathogenic because of — large size — armed with teeth — more migratory leaving more bleeding points	Less pathogenic

Morphology

Females are 2.5 mm × 40 to 50 µ (hardly visible to naked eye). Males are shorter and broader than female.

Eggs measure 30 µ × 5.5 µ, thin shelled, transparent and oval, containing larvae ready to hatch. Rhabditiform larvae come out of eggs into the lumen of intestine from where they are passed with stool. Hence larvae are detected in man's stools.

Larvae are of two types—rhabditiform and filariform larvae.

Life Cycle

No intermediate host is required. Life cycle is completed in one host only. Man is the optimum host.

Pathogenesis and Clinical Picture

The skin lesions include urticarial rash at the site of entry and a linear, erythematous urticarial wheal around anus caused by migrating filariform larvae. Pulmonary lesions include hemorrhages in the lung alveoli and bronchopneumonia.

Laboratory Diagnosis

- Detection of rhabditiform larvae in freshly passed stools.
- Eosinophilia.
- Microscopic examination of duodenal biopsy for larvae.
- Sputum examination for larvae.
- Complement fixation test using filarial larvae as an antigen.

Treatment

- Thiabendazole.

ENTEROBIUS VERMICULARIS (THREADWORM)

Geographical Distribution

It is cosmopolitan.

Habitat

Adult worm (female resides in cecum and appendix of man).

Morphology

The male adult worm is 5 mm × 0.5 mm in diameter while female measures 8 to 12 mm × 0.3 to 0.5 mm. The posterior end of female is sharp and pointed while it is curved in males. Male dies after fertilization while gravid female dies after oviposition within 2 to 3 weeks.

Eggs are colorless, planoconvex, 50 to 60 µ × 30 µ, surrounded by transparent shell and contains coiled tadpole-like larvae.

Life Cycle

The female worm when fully gravid passes down to migrate several inches outside the anus to deposit eggs. These eggs are transferred by fingers (autoinfection) and by contaminated food or fomites to the mouth and they are swallowed. On reaching the intestine, outer shell is dissolved by digestive enzyme thus liberating the larvae. In the presence of oxygen, larvae become infective.

Pathogenesis and Clinical Picture

The movement of adult worm (female) at the time of laying eggs causes intense itching inducing the patient to scratch the affected area (anal canal and perianal skin). The scratched area may become eczematoid.

Laboratory Diagnosis

- Detection of adult worm in the stools.
- Demonstration of eggs in stool and finger nails, perianal skin scraping and washings from underwear.

Treatment

- Piperazine.

DRACUNCULUS MEDINENSIS (GUINEA WORM)

In 1984, there were around 40,000 guinea worm cases in 12,840 villages in 89 districts of seven endemic states. However, only 371 cases of guinea worms were reported during the year 1994 which is a reduction of over 99% as compared to year 1984. Majority of these cases were from the states of Rajasthan (93%) followed by Madhya Pradesh 4 percent and Karnataka 3 percent. This is because of successful strategy adopted by Guinea Worm Eradication Programme including continuous detection and surveillance of guinea worm cases, prompt and free treatment, vector control through temephos application to drinking water sources, supply to fine nylon meshy stainers, etc.

The last case of guinea worm infection, Banwari Lal, 25 years old was reported in Jodhpur district of Rajasthan in 1996. The International Commission for the certification of Dracunculiasis Eradication has declared India to be free from guinea worm in the year 2000.

Geographical Distribution

India (especially Rajasthan), Burma, Arabia, Africa, West Indies, Russia and America.

Habitat

The adult female is usually found in subcutaneous tissue (legs, arms, back).

Morphology

Female adult worm is long, cord-like, measuring 60 to 100 cm × 1 to 1.5 mm. At blunt end there lies triangular mouth lined with thick cuticle. Male worm measures 2 to 4 cm in length. The body is cylindrical, smooth and milk white in color. The worm in viviparous and discharges embryo in successive batches. Body fluid is toxic. The life-span of female is one year and that of male is 6 months.

Embryos are coiled bodies with rounded head and long slender tapering tails. They measure 650 to 750 µ × 17 to 20 µ.

Life Cycle

Two hosts are required, man being definitive host and cyclops serve as intermediate host. Cyclops with larvae are swallowed in drinking water. The cyclops are digested in the stomach of man and larvae are liberated. They penetrate gut wall and enter retroperitoneal connective tissue where they mature into adult

males and females. Male dies after fertilization of female. The gravid female reaches the skin of those parts of body which usually come in contact with water (feet, legs, shoulder, back). On reaching skin, toxin in secreted and blisters are produced. When blisters burst on coming in contact with water, embryos are discharged in water. Embryos reach cyclops and the cycle is repeated.

Pathogenesis and Clinical Picture

The worm produces a reddish papule (2 to 7 mm) which later on becomes vesicle. The central portion is necrosed to form ulcer with burning itch.

Laboratory Diagnosis

- Detection of adult worm.
- Detection of embryo as milky fluid.
- Intradermal test.
- X-ray (calcified worm).

Treatment

- Extraction of worm.
- Ambithus to kill worm.

WUCHERERIA BANCROFTI

Geographical Distribution

It is confined to the tropics and subtropical regions. In India, endemic areas are along the sea coast and banks of large rivers.

Habitat

Adult worms are found in lymphatic vessels and lymph nodes of man only. Microfilaria are found in the blood.

Morphology

Adult Worm

- Male and female worms remain coiled together.
- Adult female worm is about 8 to 10 cm long and 0.24 to 0.3 mm wide.
- Adult male is 4 cm long and 0.1 mm wide.
- Anterior end is ventrally curved in case of male.
- Life-span of adult worm is 10 to 15 years.

Microfilaria

- 230 to 296 μ in length covered by delicate sheath.
- Double row of nuclei are present all along the length except posterior end.
- Life-span in human blood is 70 days.

Life Cycle

The man is the definitive host and mosquito (culex, aedes and anopheles) acts as intermediate host.

Adult worms live in the lymph nodes, lymphatics and body cavities of man. After fertilization the gravid female produces larvae (microfilaria) which are active and reach general blood circulation periodically.

When female mosquito bites the infected man microfilaria are sucked with blood meal and reach mosquito gut. There microfilaria shed their sheath and penetrate the gut wall to reach the thoracic muscles of mosquito where microfilariae develop into infective filariform larvae. After 2 moultings (4th and 6th days) the infective forms migrate from thoracic muscles to the mouth parts.

When such mosquito bites a healthy person the infective larvae migrate by process of chemotaxis to the wound produced by mosquito. They penetrate the skin through wound and reach subcutaneous lymphatics and then gradually migrate to various tissue. They undergo 2 moultings and mature to develop in adult worm in about one year.

Clinical Picture and Pathogenesis

Site of localization is according to the site of bite. It may be in lymphatic system of superior or inferior extremities.

The microfilaria in peripheral blood stream are harmless. However, it may cause eosinophilia, lymphangitis, choroid degeneration and granulomatous lesions in the tissues.

The adult worm may cause inflammatory lesion in the body tissues. Recurrent attacks of inguinal lymphadenitis, orchitis, funiculitis, epididymitis, etc. are caused due to mechanical irritation by parasite or by the action of

TABLE 58.2: Differences between classical and occult filariasis

Classical	*Occult*
• Due to adult worms	Microfilaria
• Lymphatics and lymph nodes are involved	Lymphatic vessels lungs, liver and spleen
• Microfilaria present in peripheral blood	Absent in blood but present in tissues
• Lesions produced are lymphangitis and lymphadenitis	Eosinophilic granuloma
• No therapeutic response	Responds to microfilaria killing drugs like DEC

toxins secreted by worms. Secondary bacterial infection may precipitate acute exacerbation.

Mechanical obstruction of the lymphatics by adult worms and inflammatory reaction in surrounding tissue causes localized edema, lymph, varices, hydrocele, chyluria, chylous ascitis and chylous diarrhea. Recurrent attacks of lymphangitis leads to elephantiasis.

In occult filariasis microfilaria are absent in peripheral blood but they are present in lymph nodes and other internal organs like lungs, liver and spleen (Table 58.2). Occult filaria is also called tropical pulmonary eosinophilia. It is believed to be due to immune response. It is characterized by high eosinophil counts, high antibody titer to filariae, high IgE levels, nocturnal wheezing, cough and dyspnea, etc.

Laboratory Diagnosis

- Demonstration of microfilaria
  i. Direct unstained smear.
  ii. Stained smear (Leishman stain).
  iii. Concentration techniques like membrane concentration.
  iv. DEC provocation test.
  v. Microfilaria may be demonstrated in lymph, chylous, urine, hydrocele fluid, etc.
- Demonstration of adult worm in biopsied lymph node and calcified worm in X-rays.
- Serodiagnosis is done using IHA, ELISA, IFA and RIA tests. Other test like intradermal, CFT using *Dirofilaria immitis* antigens, are neither specific nor sensitive.
- Xenodiagnosis.

Treatment

Diethylcarbamazine (DEC).

BRUGIA MALAYI

Geographical Distribution

It occurs in India, Indonesia, Malaysia, Thailand, Vietnam, China, Burma, Korea, etc.

Habitat

Adult worm is found in the lymphatic system.

Morphology

Adult worms are slightly smaller otherwise resemble *Wuchereria bancrofti*. Microfilaria shows following peculiarities.

- Smaller in size and lies folded with head close to tail.
- Possesses secondary kinks.
- The nuclei are curved hence difficult to count.
- Two distinct nuclei one at the tip of tail and another subterminal one.
- Cephalic space twice as long as broad.

Life Cycle

It resembles *Wuchereria bancrofti*. Intermediate host is mansonia (Indian species *Mansonia annulifera*). Larval development is completed in 6 to 8 days. The microfilaria is nocturnal periodic.

Pathogenicity and Clinical Picture

Lymphangitis and elephantiasis are produced.

Treatment

Diethylcarbamazine (DEC).

ONCHOCERCA VOLVULUS

Geographical Distribution

Africa, Central America and South Arabia.

Habitat

Adult worms reside in the subcutaneous connective tissue of man.

Morphology

Male adult worm is 3 × 0.13 mm with coiled tail. The female measures up to 50 × 0.4 mm. The gravid female may live up to 15 years. Cuticular oblique and annular thickening is more prominent in females.

Microfilariae are unsheathed and non-periodic found in skin. They measure 300 µ × 6 to 8 µ. The column of nuclei does not extend up to the tail.

Life Cycle

Man is the definitive host while day biting female black fly (simulium) is intermediate host. Development in black fly is completed in 6 days.

Pathogenicity and Clinical Picture

The incubation period is one year. The adult worm resides in subcutaneous connective tissues only. Following lesions are produced.

- Subcutaneous nodule formation due to adult worm.
- Dermatitis, pruritis may be caused by toxin from larva or adult worm. Development of hydrocele, elephantiasis of leg and scrotum may occur.
- Occular lesions are caused by microfilariae.

Laboratory Diagnosis

- Demonstration of microfilaria in shaved pieces of skin and adult worm inside excised nodules.
- In ocular lesion, microfilariae are detected by means of slit lamp.
- Eosinophilia.

Treatment

Enucleation of nodules and use of drugs like DEC or Surmic.

LOA LOA

Geographical Distribution

Occurs in Central and West Africa.

Habitat

Adult worms live in subcutaneous connective tissues of man often in subconjunctival tissues of eye.

Morphology

Male adult worm measures 3 mm × 0.35 mm. Female measures 6 cm × 0.5 mm. The life-span of adult worms may be 15 years or more.

Embryo measures 300 µ × 7 µ and is enveloped in a sheath. They are found in peripheral blood during daytime.

Life Cycle

It passes its Life cycle in man and chrysops. Larval form follows the same course as in other microfilariae. Loa loa is maintained in nature by interhuman transmission.

Pathogenicity and Clinical Picture

The incubation period is 3 to 4 years. With the bite of chrysops, embryo is introduced in man. It gets migrated rapidly to various parts of the body and in subdermal connective tissue. It shows predilection for creeping in and around eyes. The disease is named as loiasis.

Treatment

Diethylcarbamazine DEC.

MANSONELLA OZZARDI

Geographical Distribution

West Indies, Central America and South America.

Habitat

Adult worm lives in the mesentery of man.

Morphology

Adult worm (female) is 7 cm × 0.25 mm. The cuticula is smooth and the tail end possesses a pair of flaps like papillae.

Microfilariae are found in blood. They are small unsheathed and non-periodic. The tail end is sharply pointed.

Pathogenicity

Non-pathogenic.

Laboratory Diagnosis

By demonstrating microfilaria in blood.

CESTODES

It means girdles or ribbon. They are segmented tapeworm whose size vary from few mm to several meters. They do not have body cavity or alimentary canal. Nervous system and excretory system is rudimentary. They are hermaphrodite.

Medically important tapeworms are:

A. Pseudophyllidean tapeworms
   - *Diphyllobothrium latum* (fish tapeworm)
   - *Sparganum mansoni* and *sparganum proliferum*

B. Cyclophyllidean tapeworms
   1. Genus taenia
      - *Taenia saginata* (beef tapeworm)
      - *Taenia solium* (pork tapeworm).
   2. Genus echinococcus
      - *Echinococcus granulosus* (dog tapeworm)
      - *Echinococcus multilocularis.*
   3. Genus hymenolepis
      - *Hymenolepis nana* (dwarf tapeworm)
      - *Hymenolepis diminuta* (rat tapeworm).

DIPHYLLOBOTHRIUM LATUM (FISH TAPEWORM)

Geographical Distribution

Central and Southern Europe especially in Scandinavian countries. Also occurs in Siberia, Japan, North America and South Africa. It has not been reported from India so far.

Habitat

Small intestine of man, dog, cat, fox, etc.

Morphology

The adult worm measures 3 to 10 meters in length and the life-span is 5 to 15 years. The head (scolex) is elongated and spoon shaped with 2 slit-like grooves (bothria) measuring 2 × 3 × 1 mm^3. It lacks rostellum or hooklets. The neck is thin and unsegmented. The body segments are 3000 to 4000 and are greater in breadth than length. Each segment measures 2 mm × 10 to 20 mm.

Life Cycle (Fig. 58.1)

There are two intermediate hosts, i.e. (i) fresh water cyclops and (ii) fresh water fish. Man is the definitive host.

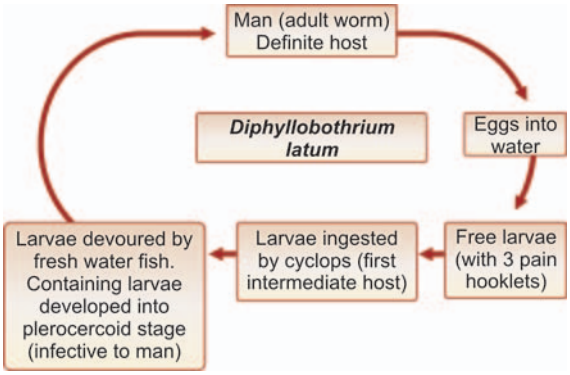


Fig. 58.1: Life cycle of Diphyllobothrium latum

Clinical Picture

- Gastrointestinal problem
- Macrocytic anemia
- Eosinophilia.

Laboratory Diagnosis

- Microscopic examination of stool for the demonstration of operculated eggs.
- Examination of segments passed in feces.

Treatment

Niclosamides, quinacrine, praziquantel are useful drugs.

Prophylaxis Prevention

Proper cooking of fish, checking of fecal contamination of water and deworming of pet dogs and cats.

TAENIA SAGINATA (BEEF TAPEWORM)

Geographical Distribution

Worldwide in distribution. In India it is prevalent in Mohammadens who are beef eaters.

Habitat

The scolex of grown-up worm embedded in the mucosa of the wall of ileum and rest of worm extends through the lumen.

Morphology (Fig. 58.2)

Adult Worm

- It is white transparent tape-like.
- It measures 5 to 12 meters (up to 24 meters).
- Scolex is pear-shaped 1 to 2 mm in diameter with 4 round suckers without rostellum and hooks.
- Neck is long and narrow.
- Proglottids may be up to 2000 in number, terminal gravid segment 2 cm × 0.5 cm, genital pore is situated near the margin at the posterior end of each segment alternating irregularly in right and left margin, gravid uterus having 15 to 30 lateral branches.
- Gravid segments are expelled singly.
- Adult worm is usually single in a host.
- Life-span of adult worm is nearly 10 years.

Larvae

- It is called *cysticercus bovis.*
- It is found only in cattle.
- It is eliptical in shape (7.5 mm × 5.5 mm).

Egg

- Spherical and measures 30 to 40 µ in diameter.
- Bile stained.
- Thin outer transparent shell.
- Embryophore is brown in color, thick-walled radially striated.
- Contains an oncosphere with 3 pairs of hooklets.
- Does not float in saturated saline solution.
- About 80,000 eggs in a proglottid which are liberated by rupture of mature proglottid.
- Eggs remain viable up to 8 weeks.

Life Cycle

Man is the definitive host whereas cow or buffalo acts as intermediate hosts.

Eggs are passed out with stools on the ground and cow or bufallo swallow these eggs while grazing in the field. The eggs are not infective to man. In the intestine eggs rupture with liberation of oncospheres which penetrate gut wall with the help of hooks, enter blood stream and are filtered into muscular tissues (tongue, neck, shoulder and cardiac muscles) where they settle and grow. In 8 days oncospheres are transformed into *cysticercus bovis.*

Man is infected by eating uncooked beef containing cysticerci (measly beef). In the intestine scolex exvaginates (stimulation by bile) and anchor to the gut wall by its suckers and slowly grows into adult worm. It attains sexual maturity in 2 to 3 months and starts producing eggs.

Clinical Picture

It is usually asymptomatic. It may cause abdominal discomfort, hunger pain, indigestion, diarrhea alternating with constipation, loss of appetite, pruritus ani, intestinal obstruction and appendicitis.

Laboratory Diagnosis

- Demonstration of proglottids or eggs.
- Serodiagnosis is done with the help of tests like indirect hemagglutination, IFA and ELISA.

Treatment

Niclosamide, mebendazole, praziquantel and bithional are the drugs used.

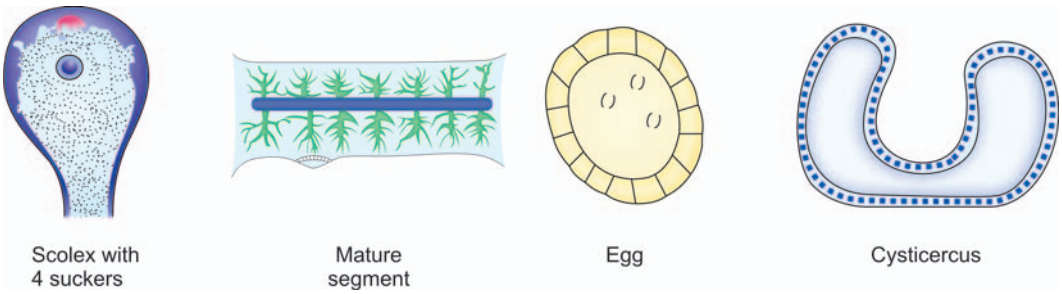


Fig. 58.2: *Taenia saginata*

TAENIA SOLIUM (PORK TAPEWORM)

Geographical Distribution

It is worldwide especially in pork eating persons.

Habitat

Adult worm lives in the small intestine.

Morphology (Table 58.3)

Adult worm (Fig. 58.2)

- It measures 2 to 3 meters long.
- Scolex is pinhead size, globular in outline with 4 suckers and head is provided with rostellum with two rows (small and large) of hooklets.
- Neck is short 5 to 10 mm.
- Proglottids 1000, gravid segment longer than broader while immature segment broader than longer, genital pores lie laterally at middle of each segment (alternating left and right), testis with 150 to 200 follicles, ovary with two symmetrical lobes and an accessory lobe and gravid segments are passed passively in the stool.

Larva (Fig. 58.3)

- It is called *cysticercus cellulosae.*
- Occurs in pig and man.
- It is small, oval, milky white bladder measuring 8 to 10 mm in breadth and 5 mm in length.
- Contains milky fluid rich in albumin and salts.
- It lies parallel to muscle fibers as white spot which represents future head invaginated into bladder.

TABLE 58.3: Differences between *Taenia solium* and *Taenia saginata*

Taenia solium	*Taenia saginata*
• 2 to 3 meters	5 to 10 meters
• Below 1000 proglottids	Above 1000
• Nonpigmented suckers	Pigmented
• Hooklets present	Absent
• Gravid proglottid 1.2 cm × 0.6 cm	2 × 0.6 cm
• Proglottids expelled in the chain of 5 to 6	Single proglottid crawls out of anus
• Uterine branches 5 to 10 dendritic	15 to 30 dichotomus
• 150 to 200 testicular follicles	300 to 400
• Accessory ovarian lobe	Absent
• Vaginal sphincter absent	Present
• Neck of worm short	Long
• Life-span up to 25 years	10 years
• Scolex globular	Quadrate
• Rostellum present	Absent

- The pork containing cysticercus is usually named as measly pork.

Eggs (Fig. 58.3)

Morphological features resemble that of eggs of *Taenia saginata.*

Life Cycle

Similar to the Life cycle of *Taenia saginata.* However, larval stage (cysticercus cellulosae) occurs in man too. Man acquires infection by eating inadequately cooked pork (containing cysticercus cellulosae) or by ingesting eggs of *Taenia solium* by consuming contaminated food and drinks.

Clinical Picture

As in *Taenia solium.*

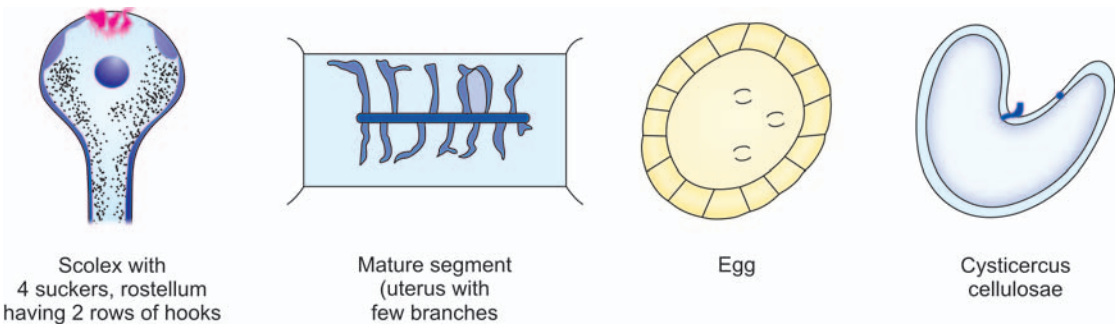


Fig. 58.3: *Taenia solium*

Diagnosis

- Demonstration of proglottids or eggs.

Treatment

- Same as of *Taenia saginata.*

CYSTICERCUS CELLULOSAE

Morphology

- Measures 10 mm by 5 mm.
- It has opaque invaginated scolex with 4 suckers, hooks and bladder filled with fluid.
- Development to the infective form usually takes 9 to 10 weeks.

Transmission

- By ingestion of eggs of *Taenia solium.*
- By autoinfection (reverse peristalsis).
- External re-infection from anus to finger to mouth.

Clinical Features

- It may be asymptomatic.
- Symptomatic picture includes involvement of eye, skin, viscera and muscles. Neurocysticercosis involve CNS and spinal cord and presents as epilepsy, hydrocephalus, encephalitis, diplopia, aphasia, amnesia, etc.

Laboratory Diagnosis

- Biopsy of nodule and study of its histological picture.
- Radiology.
- Serological tests like complement fixation test, indirect hemagglutination, ELISA and immunofluorescence using crude antigen (extracted from pig cysticerci) and purified antigen.

Treatment

Surgical excision or drugs like praziquantel is quite effective.

ECHINOCOCCUS GRANULOSUS (DOG TAPEWORM)

Geographical Distribution

Worldwide more prevalent in temperate climate than tropical areas. It is quite common in cattle and sheep predominating places. From India, West Asia and Mediterranean countries, high incidences of hydatid cyst are reported.

Habitat

Adult worm is found in the small intestine of canines like dog. Larval form is found most commonly in liver, lungs, etc. of man.

Morphology

Adult Worm

- Attached to the wall of intestine of canines like dogs.
- It has scolex, neck and 3 to 5 segments.
- It measures about 3 to 9 mm in length.
- Scolex is spherical provided with rostellum carrying 30 to 40 hooks in 2 rows. There are four suckers.
- First one or two segments are immature followed by segment sexually mature.
- The last segment is gravid one containing about 400 to 500 eggs. The uterus bursts open before evacuation of gravid proglottides into the intestine. Thus the process of release of eggs occurs.
- Life-span is about 6 months.

Eggs

- Spherical
- 31 to 40 µ
- The outer shell surrounds the inner embryophore.
- Oncosphere has 3 pairs of hooklets within embryophore.
- Eggs resemble other taeniid species of dog.
- Eggs survive in the soil for 6 to 12 months.
- Eggs are infective to man, cattle and sheep.

Larval Form

- It is found within hydatid cyst growing in the organs of sheep, cattle and man.
- Future scolex of adult worm remains invaginated within vesicular body.
- Larval form remains live and continues to develop for many years.

Life Cycle

Dogs, pigs, fox and jackal are definitive hosts. Intermediate hosts are sheep, goat, cattle, horse, pig and man.

As a result of disintegration of gravid proglottids in the intestine eggs are discharged through stools of definite host. Ingestion of contaminated food (with eggs of *Echinococcus granulosus*) by intermediate host results in hatching out of hexacanth embryo out of eggs (8 hours after ingestion). This liberated oncosphere penetrates the mesenteric blood vessels and gets distributed to various organs of the body like liver, lungs, etc. Wherever embryo settles it grows into hydatid cyst containing thousands of scolices.

The hydatids are ingested by definite host, e.g. dog and grow into adult worms in 6 to 7 weeks in the intestine and start laying eggs which are passed through stools. Thus Life cycle is repeated.

Hydatid Cyst (Fig. 58.4)

It grows very slowly. At the end of a year it is about 5 cm in diameter. Hydatid cyst consists of:

Ectocyst

- It is 1 mm thick.
- It is outer cuticular layer which is as matter of fact laminated hyaline membrane.
- It is elastic in nature and resembles the white of a hard boiled egg.

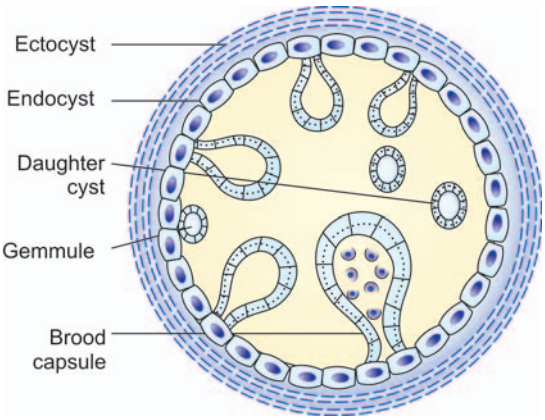


Fig. 58.4: Structure of hydatid cyst

Endocyst

- It is an inner germinal layer.
- It is cellular and consists of a number of nuclei dispersed in a protoplasmic mass.
- It is about 1/4th of mm.
- It has a role in the formation of outer layer, secretion of hydatid fluid and to form brood capsule with scolices.

Hydatid Fluid

- It is secreted by endocyst.
- It is clear, colorless or pale yellow fluid, weakly acidic, of low specific gravity, containing sodium chloride, sodium sulfate, sodium phosphate, and calcium salts of succinic acid. It is antigenic, highly toxic and provides nutrition for developing scolices.

Hydatid Sand

- It is granular deposit.
- It consists of liberated brood capsules, free scolices and loose hooklets.

Formation of Daughter Cysts

The endogenous daughter cyst develops inside the mother cyst and can also develop from detached fragment of germinal layer. On the other hand exogenous daughter cyst development results owing to increased intracystic pressure which may cause either herniation or rupture of germinal as well as laminated layers through weakened part of the adventia, e.g. bone hydatid disease.

Animal Model

They are golden hamsters and guinea pigs.

Immunology

Both humoral (IgG or IgE) and cell-mediated immune responses are involved. Immune unresponsiveness is reported in about 10 percent cases.

Clinical Features

Location of cyst and its stage of development give rise to particular signs and symptoms. It may remain asymptomatics for years altoget-

her and is found accidentally. Rupture of cyst gives rise to allergic symptoms and signs, e.g. rupture in lungs causes chest pain, cough, dyspnea and hemoptysis. Cyst in cerebrum may give rise to epilepsy. A cyst in kidney may cause hematuria.

Laboratory Diagnosis

- DLC shows eosinophilia in many cases.
- X-rays, CT scan, angiography, ultrasound and magnetic resonance imaging (MRI).
- Exploratory cyst puncture is dangerous because of accidental spilling of contents resulting to secondary spread or anaphylaxis.
- Detection of scolex in stool, sputum or vomiting in cases where cyst ruptures into bile duct, intestine or bronchus.
- Casoni test where 0.2 ml of hydatid fluid (filtered by Seitz or membrane) is injected intradermally in one ventral aspect of forearm and an equal volume of saline as a control in the other forearm. In positive case a large wheel about 5 cm in diameter with multiple pseudopodia-like projections appears in 30 minutes only at test side which fades away in 60 minutes. It is sensitive but not specific test.
  - Serodiagnostic tests are as under:
  - Complement fixation test (CFT).
  - Indirect hemagglutination (IHA).
  - Counter current immunoelectrophoresis.
  - ELISA.
  - Radio-immunoassay (RIA).
  - Dot ELISA.
  - Indirect immunofluorescence test (IFT).

Treatment

- Surgical extraction of hydatid cyst.
- Albendazole and mebendazole are also reported as effected in treating metacestodes of *E. granulosus.*

ECHINOCOCCUS MULTILOCULARES

The salient features are:

- It is prevalent in Germany, Switzerland, North Vietnam, China, Russia, etc.
- Causes multilocular hydatid disease.
- Adult worm is relatively smaller than *E. granulosus.*
- Eggs are more resistant to cold.
- Life cycle involves foxes and oriental rodent.
- Major source of human infection is fruits and vegetable contaminated with feces of fox, etc.
- Liver is more commonly involved.
- Cyst lacks fluid and there is no hyaline membrane and capsule. Cyst is sterile. Germinal layer is hyperplastic.
- It has features resembling malignant neoplasm.
- Metastasis occurs in blood and secondary lesions usually in the brain and lungs.

HYMENOLEPIS NANA (DWARF TAPEWORM)

Geographical Distribution

It is cosmopolitan in distribution more predominantly seen in warm climate.

Habitat

It lives in the upper two-third of the ileum.

Morphology

- It is one of the small intestinal cestodes infecting man.
- It is 1 to 4 cm long and 1 mm thick.
- It has short lifespan (2 weeks).
- Scolex has 4 suckers and a single row of hooklets.
- There are 200 segment and each mature segment measures 0.3 mm × 0.9 mm.

Eggs

- Spherical or ovoid measuring 30 to 45 µ.
- There is thin colorless outer membrane and an inner embryophore.
- Embryophore encloses hexacanth oncosphere.
- The space between two membranes contains yolk granules and 4 to 8 polar filaments arising from two knobs of embryophore. Eggs are non-bile stained and float in saturated saline solution.

Life Cycle (Fig. 58.5)

The Life cycle takes place in man. There is no intermediate host. *H. nana* undergoes multiplication in the body of host unlike other helminths.

A different strain of *H. nana* infects rat and mice. The eggs passed in rodent stools are ingested by rat flea *(Xenopsylla cheopis)* which act as intermediate host. The eggs develop into cysticercoid larvae in the hemocele of these insects. Rodents get infected when they eat these insects. Human strain may infect rodent which constitutes a reservoir of infection for the human parasite.

Clinical Picture

Generally it does not produce any problem. However, sometimes symptoms occur due to allergic response like abdominal discomfort, diarrhea, pruritus, weight loss, weakness, irritability and keratoconjunctivitis.

Laboratory Diagnosis

- Demonstration of eggs from the feces either in direct smear or formal ether concentration is useful in establishing the diagnosis.

Treatment

Niclosamide.

TREMATODES

SCHISTOSOMA

They are also called blood flukes. *Schistosoma hematobium* (bilharzia) involves predominantly genitourinary system, *S. mansoni* the gastrointestinal tract and *S. japonicum* the small and large intestine, liver and central nervous system.

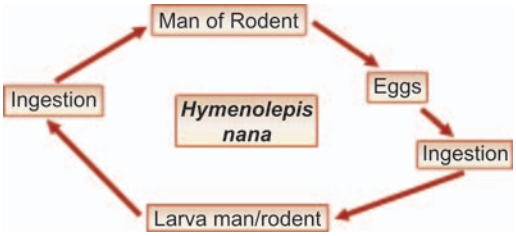


Fig. 58.5: Life cycle of *Hymenolepis nana*

General Clinical Symptoms

S. hematobium causes urinary schistosomiasis characterized by painless terminal hematuria. In *S. mansoni* infection is manifested as dysenteric attacks, hepatomegaly, periportal cirrhosis, portal hypertension, cor pulmonale and myelitis. *S. japonicum* infection is manifested as dysenteric attack, periportal fibrosis and pigmentation of liver, enlarged spleen because of portal hypertension and hematemesis (esophageal varices).

SCHISTOSOMA HEMATOBIUM (BILHARZIA HEMATOBIUM)

Geographical Distribution

Nile valley, most parts of Africa and West Asia. Some endemic areas in Ratnagiri, south of Bombay were reported by Gadgil and Shah in 1952.

Habitat

The adult worm lives in urinary bladder and pelvic plexuses of veins.

Morphology

The male is 10 to 15 mm long and 1 mm thick. It is covered by finely tuberculated cuticle. There are two muscular suckers the oral sucker is small while the ventral sucker is large and prominent. There is gynecophoric canal in which the female worm is held. It begins behind the ventral sucker and extends to the caudal end. The adult female is 20 mm by 0.25 mm with cuticular tubercles confined to the two ends (Fig. 58.6).

The eggs are ovoid 150 μ by 50 μ with a brownish transparent shell having a terminal spine at one pole.

Life Cycle (Fig. 58.7)

Man is a definitive host. The infection is acquired from fresh water containing infective cercaria larvae. The intermediate host is snail. In India the species of snail is limpet *Ferrisia tenuis.*

Cercariae swim about in water after being released from infected snail. If within 1 to 3

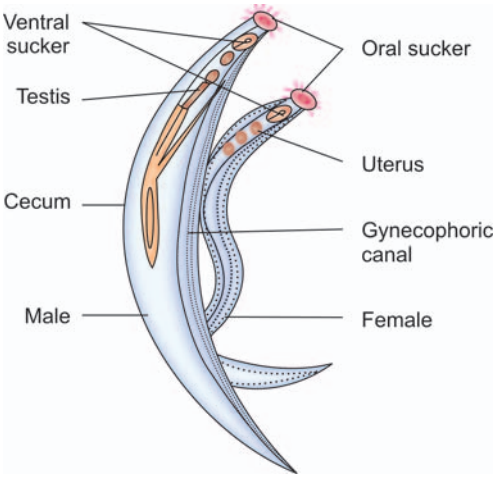


Fig. 58.6: Morphology of *Schistosoma haematobium*

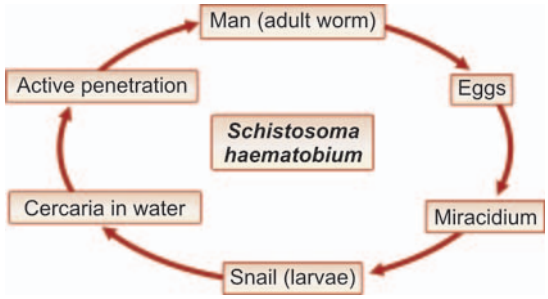


Fig. 58.7: Life cycle of *Schistosoma haematobium*

days they come in contact with host (bathing or wading in water), they penetrate through the intact skin. Skin penetration is facilitated by lytic substances secreted by penetration glands present in cercaria.

On entering the skin, the cercaria shed their tails and become schistosomules which enter the peripheral venules. Through vena cava they enter right heart, lungs, left heart and ultimately go to systemic circulation finally reaching liver. About 20 days after skin penetration they become sexually mature and differentiated inside intrahepatic portal veins. Then they reach vesiate and peloic venous plexus via inferior mesenteric veins. They further gain maturity and start laying eggs. Eggs start appearing in urine 10 to 12 weeks after cercarial penetration. The lifespan of adult worm is 20 to 30 years.

Pathogenicity and Clinical Features

The clinical features during penetration are cercarial dermatitis (itching) and anaphylactic or toxic symptoms (fever, headache, malaise and urticaria).

The typical manifestation because of egg laying and extrusion is painless hematuria. Cystoscopy shows hyperplasia and inflammation of bladder mucosa with minute papular or vesicular lesion.

In chronic stage there may be generalized hyperplasia and fibrosis of the vesical mucosa with a granular appearance known as sandy patch. There is pseudoabscesses formation at the sites of deposition of the eggs with the infiltration of lymphocytes, plasma cells and eosinophils. To start with trigone is involved but later on inflammation of entire mucosa with thickening and ulceration occurs followed by secondary bacterial infection which ultimately leads to chronic cystitis. There may be deposition of uric acid and oxalate crystals around the eggs and obstructive hyperplasia of ureter and urethra. Schistosomiasis favors urinary carriage of typhoid bacilli and chronic schistosomiasis is associated with bladder cancer tentatively.

Laboratory Diagnosis

- Blood examination for eosinophilia and aldehyde test.
- Eggs with terminal spine may be demonstrated by microscopic examination of centrifuged deposits of urine of the patient (urine especially at the end of micturition). Eggs are also seen in seminal fluid, feces, vesical or rectal biopsies, etc.
- Intradermal allergic test (Fairley's test).
- Complement fixation test.
- Bentonite flocculation.
- Indirect hemagglutination test.
- Immunofluorescence.
- Gell diffusion.
- ELISA
- Circumoval precipitation where there is globular or segmented precipitation around schistosome eggs incubated in positive sera.
- Cercarian Hullen reaction where there is development of pericercarial membrane around cercariae incubated in positive sera.

Treatment

Metrifonater or praziquantel are useful.

59 Medical Entomology

Insects form the largest group of species in the animal kingdom and most important source of human diseases. Their medical importance lies to their capacity to cause morbidity and mortality and their extensive distribution over the face of the earth. In most instances, the microorganism were first of all the parasites of insects, and majority of microorganism are so well-adopted to the insects host that they produce no tissue damage.

Arthropods as transmitters of pathogens: As transmitters of various pathogens to man, insects vary in the intimacy of their association with the disease producing microorganisms. They may only be mechanical vectors of etiologic agent. Most important insect transmitted diseases employ the insect as biological vector, requiring a period of incubation or development in this host. For example, trypanosome or malarial parasite develop inside tsetse fly and female anopheles mosquito respectively before they become infective to man. Rocky Mountain spotted fever, scrub typhus, filaria infection are other examples of biological vectors.

Non-blood sucking flies may deposit a vomit drop containing pathogens on human food or drink, e.g. typhoid infection of man. Some non-blood sucking flies may ingest filth during their larval (i.e. maggot) stage and their associated pathogens may be retained in their intestine during the period of pupation, later to be deposited by the adult fly on human food or human tissues.

Blood sucking insects may introduce microorganisms into the human skin. They obtain parasite organisms in a blood meal from patient and later deposit them a vomit drop in the puncture wound (plague), or in fecal pellets near the puncture wound (typhus) made in the skin of uninfected person. Still others discharge, the organisms (malaria sporozoites) through hypopharynx in minute droplets of salivary secretion at the time they puncture, a blood meal or (filaria larvae) from proboscis sheath, thus, enabling the pathogens to migrate.

Bugs have thwarted human efforts to establish stable and safe environments throughout the past centuries. During sixth century BC, malaria and plague flourished in newly established cities.

Although various insects were known to live on the outer surfaces of mammals, untill 19th century little was known of the relationship between insects and disease agents. Towards, the end of the 19th century, scientists launched an intensive, systematic study of infectious diseases. One product of this work was the demonstration in 1893 by T smith *et al* that ticks transmit the protozoa *Babesia bigemina*, which causes Texas cattle, or red water fever.

The discovery of this insect microbial association provided a model that investigators could use to show the significance of other insects to disease epidemiology. Sir Ronald Ross applied the model in his way, demonstrating the importance of *Anopheles* spp. mosquitoes to malaria.

In recent years, knowledge of insects borne diseases has increased immeasurably. Along with several areas of related research this knowledge forms the specialization referred to as medical entomology. This field of study is concerned with the recognition and description of insects, the distribution of insect

associated diseases, the effect of disease agents on the insect vector, the effect of disease agent on the host, and the control of insects.

Sources of Pathogens Transmitted by Insects

Man himself is the only source from which anopheles mosquito obtains the infection. Filaria worms which infect man are almost invariably derived from human sources. However, *Brugia malayi* may be derived from resevoir host strains. Bubonic plague is obtained by flea from the rat. Epidemic typhus passes from man to man, with the body louse as the transmitting insect.

Insect as Etiologic Agents of Disease

In addition to their role in transmission of pathogenic organisms to man, insects themselves play a significant role as disease producing organisms. Larval stage of the myiasis producing flies, the chigoe and the sarcoptic mite, invade the tissue of man and produce serious lesions. Trauma results when tick introduces its hypostome and chelicerate into the skin preparatory to obtaining a blood meal from man. Certain ticks produce paralysis in some persons, presumably due to toxins in their secretions. Venoms introduced into the skin by the bite of scorpion or black widow spider or bee at times produce both local reactions and profound systemic shock. Blood sucking flies, in depositing droplets of saliva in the skin, may provoke serious allergic reactions.

Physiology of Insects

The growth hormone of all insects is ecdysone, secreted by the prothoracic gland. It stimulates growth and differentiation of all tissue except muscle which requires a neurosecretory hormone.

Biology of Insects

The insects were originally aquatic which always breathe by means of gills. Many insects forms have to remain on land or to the air and, as a result, the gills have been sunk into the body to form back lungs, or have completely atrophied and have been replaced by a tracheal system of respiration. In aerial species wings have developed. Conquest of several of the most deadly human diseases has been or will be made possible only by control of insect transmitters.

Life Cycle of Insects

Life cycle of an insect starts when egg produced by female is inseminated by a spermatozoon from male. In some cases the egg proceeds with its development even without fertilization. Among the less highly developed groups of insect, the stages in development consist of egg followed by one or more larval stages (resembling the adults) and finally adult stage. However, in several groups of insects, there is a appreciable morphologic transformation between the last larval stage and the adult with a pupa or "resting stage" inbetween. Still between the simplest and most complex types of development, there are groups with partial metamorphosis.

Bed Bugs (Cimicidae)

- 13 mm long.
- Broad flat reddish brown insects.
- In males the abdomen is pointed at the tip.
- In females abdomen is evenly rounded.
- The greater part of the body is covered with bristles (Fig. 59.1).
- Eggs are pearly white and oval with lid.
- Complete Life cycle in 15 to 50 weeks.
- Lifespan varies from many months to around one year.
- Bed bugs, e.g. *Cimex hemipterus* and *Cimex lectularius* may be naturally infected with hepatitis-B virus which can transmit this virus mechanically or via feces.
- Bed bugs may cause dermatitis or asthma, etc.
- Control includes
  - Pouring of boiling water to kill egg and nymph
  - Use of kerosene, turpentine, benzene and petroleum, etc.

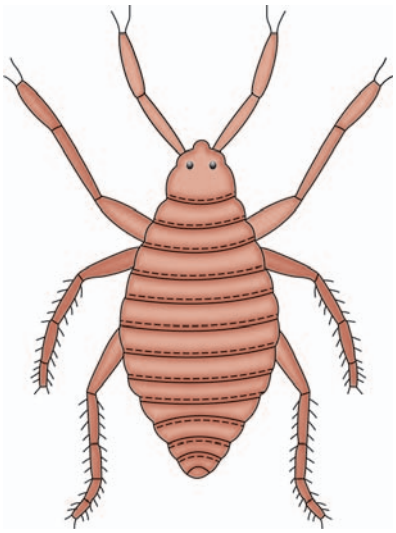


Fig. 59.1: Bed bug (female)

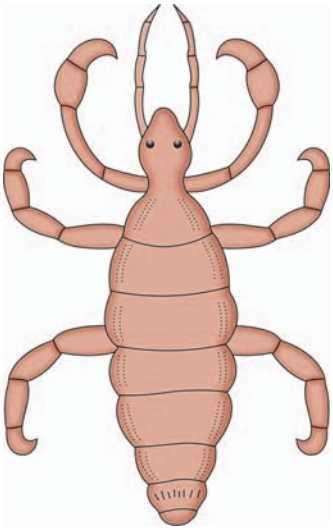


Fig. 59.2: *Pediculus humanus* (male)

- Use of 5 percent DDT.

Lice *(Pediculus humanus corporis, and Pediculus humanus capitis)* Pubic or Crab louse

- Have dorsoventral flattened body
- Two to four mm in size; elongated and grayish white with piercing and sucking mouth parts.
- Lack wings but have clawed legs.
- Eggs are 0.8 mm long (Fig. 59.2).
- Principal habitats are body or hair.
- Lifespan of adults up to 1 month.
- May transmit typhus fever and cause irritating dermatitis.
- May be responsible for transmission of *Salmonella* species.
- Control includes
  - Use of benzyl benzoate, DDT, benzocaine.
  - Mixture of kerosene oil and olive oil.
  - Kerosene oil having 0.12 percent pyrethrins.

Housefly (*Musca domestica*)

- Male measures 5 to 6 mm and female 6 to 7 mm.
- Dusky gray in color.
- The head is broad, frons straw and dark brown, antennae brown.
- Possess squarish ovoid thorax having 4 dark stripes.

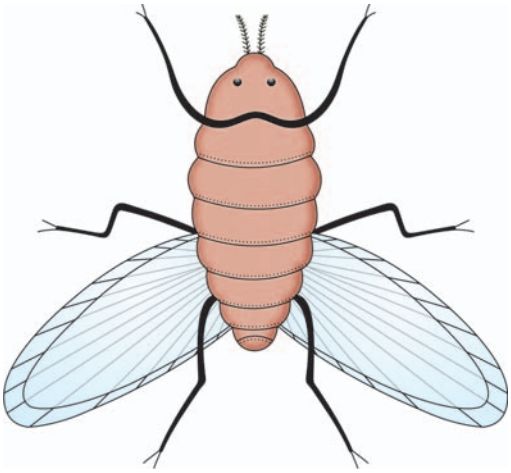


Fig. 59.3: Housefly

- Wings are transparent having straw colored base.
- Foot or terminal segment at the end of each leg is provided with a pair of horny claws, pair of ventral cushions, each with many glandular hair (Fig. 59.3).
- Eggs are pearly white each one is 1 mm long.
- Completes life cycle in about 2 weeks.
- Responsible for transmitting pathogenic microorganisms mechanically, e.g. *Salmonella* tubercle bacilli *Yersinia pestis*, *Bacillus anthracis*, *Brucella abortus, Chlamydia,* etc.
- There is a close association between house flies and myiasis.

- Control includes
  a. Use of flypaper and flytraps
  b. Garbage to be kept in close fly proof containers which is incinerated at frequent interval.
  c. Insecticidal spray, e.g. chlorinated hydrocarbon (1% water emulsion) to be sprayed twice a week over breeding area for fly ova.

Mosquitoes (Fig. 59.4)

- Slender, delicate forms with 3071 species all over the world.
- Have elongated piercing sucking mouth parts adopted for sucking blood, especially in female mosquito.
- Have long antennae feathery in male and hairy in females.
- Scales on wings look spotted in anopheles mosquitoes and uniformly black in Culex (Table 59.1).
- Life-span of male mosquito is about 7 days and 1 month in case of female.
- Responsible for transmission of diseases, i.e. malaria (*Anopheles*), yellow fever (*Aedes*), Eastern equine encephalomyelitis (*Aedes*), Western equine encephalomyelitis (*Aedes*), Venezuelan equine encephalomyelitis (*Aedes, Anopheles, Culex*), chikungunya fever (*Aedes*), dengue fever (*Aedes*), Japanese encephalitis (*Culex*), filariasis (*Aedes, Culex* and *Anopheles*), etc.
- Prevention and control includes:
  a. Reduction of breeding places of mosquitoes like drainage, clearing or, filling of ditches and elimination of water containers.
  b Use of insecticides.
  c. Use of mosquito repellant, e.g. residual sprays or lotion, gases or vapors.

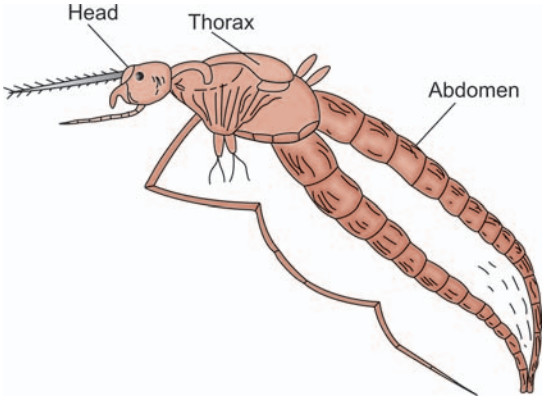


Fig. 59.4: Mosquito

TABLE 59.1: Differentiating features of *Culex*, *Aedes* and *Anopheles*

Anopheles	*Aedes*	*Culex*
1. Eggs	Elliptical	Cigar like
• Boat shaped		
• Laid in flowing, fresh water	• Cavities of trees filled with water, domestic containers	• Stagnant dirty water
2. Larva		
• Lies parallel to water surface	• Hangs at an angle	• Hangs from water surface at an angle
• Surface feeder	• Bottom feeder	• Bottom feeder
3. Pupa		
• Green	• Colorless	• Colorless
4. Adults		
• Grayish, wings carry dark spots	Blackish brown with white silvery stripes on thorax and abdomen	Uniformly grayish body
• Resting posture inclined, head down at 45° angle.	• Horizontal to ground	Horizontal to ground
• Body slender with delicate legs	• Well built with stout legs	• Well built with stout legs
• Abdomen with few or no scales	• Covered with scales	• Covered with scales
• Maxillary palp equal to proboscis (terminally pointed—females)	Equal to proboscis in male but shorter in females	Maxillary palp longer than proboscis in male but shorter in females

d. Diversion of mosquitoes from man to animals (cattles). This is called zooprophylaxis, a method hardly practised deliberately but often naturally effective.
e. Genetic control is successful by introducing sterile male mosquitoes (by radiation or exposure to UV light) into natural population.
f. Introduction of larvicidal fishes like gambusia and gold fish to breeding places of mosquitoes.
g. Many birds eat adult mosquitoes, e.g. swifts, night hawks, etc.
h. Wall lizards, frogs and spiders destroy mosquitoes.
i. Many aquatic plants e.g. chara, utriculatria, etc.
j. Certain bacteria help of destroy larvae.

Fleas (Fig. 59.5)

- Adults are small, oblong, compressed, hard skinned, bristly, wingless and dark brown in color.
- They have concealed antennae and long jumping legs.
- The head is broadly joined to thorax.
- The abdomen consists of 10 segments; the last 3 are modified for sexual purposes.
- All parts of body are furnished with backward projecting bristles and spines which prevent it from slipping backwards and moving ahead between dense hair.
- Male fleas are easily identified by rakish upward tilt of abdomen. In cases of female it is rounded.
- The eggs are deposited in dark and dry places in the haunts of its host that takes about 70 to 75 days to mature.
- Fleas may transmit plague (*Yersinia pestis*) endemic typhus fever, etc.
- Flea bite may cause flea dermatitis.
- For control DDT, rotenone (1%), malathion (4%), pyrethrin (1%) may be used. In case of infested floor, rugs, carpet, pillows, etc. spray of kerosene solutions or emulsions of 3 percent malathion or 1 percent diazinon are useful. Rodent control can also check fleas.

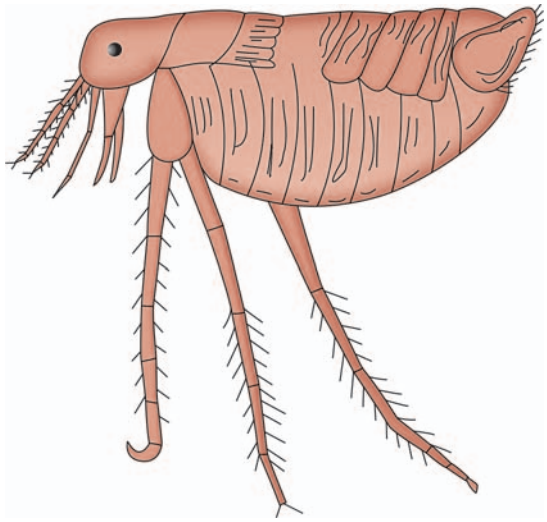


Fig. 59.5: Flea

Sandfly *(Phlebotomus)*

- Small about 2 mm long
- Humpbacked, fawny in color with prominent black eyes.
- Body, wings and legs are hairy.
- Wings veins do not cross with each other.
- Oval lanceolate wings are carried errect.
- Only the females feed on blood.
- Feeding usually occurs at night and flies hide in dark, often damp places in day time.
- Have long, slender antennae, long maxillary palpi and a proboscis longer than head (Fig. 59.6).
- The egg (long, ovoid) laying starts after 30 to 36 hours of blood meals and life cycle is completed in 60 to 65 days.
- May be responsible for transmitting cuta-

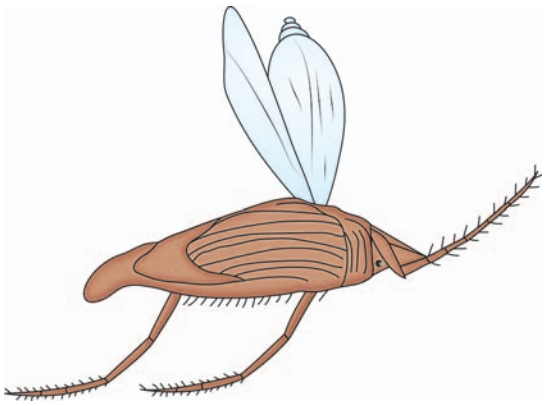


Fig. 59.6: Sandfly

neous leishmaniasis, visceral leishmaniasis, viral 3 days fever, tularemia, loasis, etc.
- Control includes
  a. Elimination of breeding grounds, cracks, crevices from walls and floors, etc.
  b. Use of insecticides like DDT, etc.
  c. Oiling pools of water can kill adult females who dive to lay eggs.

Fly Maggots (Fig. 59.7)

- The adult fly is dirty yellowish brown with the tip of abdomen rusty black.
- The posterior respiratory openings of maggots consists of stigmatal plates. These are hardened, dark colored eye like spots surrounded by sclerotized ring and button like mark.
- The position and shape of plates, the development of ring plus button and the forms of slit (straight, bent or looped) are of great importance in the identification.
- The first stage maggots are recognizable by the absence of anterior as well as posterior spiracular plates.
- The life cycle is completed in about 10 weeks. Eggs are transformed into larvae in 2 weeks. Larva take several weeks to become pupa and finally to adult.
- May cause myiasis (infestation of living organs or tissue by maggots).
- Myiasis may be atrial (involving oral, nasal, aural, vagina, urethra, etc.), cutaneous (involving skin), intestinal, etc.
- There are numerous species of fly maggots but the only larva that sucks blood by puncturing skin of man is congo floor maggot *Auchmeromyia luteola*, found all over tropical Africa, etc. where people sleep on the floor. The larvae lie buried under the floor mat during day time and come forth during night to pierce skin with their mouth hook and suck blood.
- Control includes:
  a. Proper disposal of carcases to prevent laying their eggs.
  b If feasible maggots are removed using forceps after spray of 5 percent chloroform in light vegetable oil and lesion dressed with antiseptics.

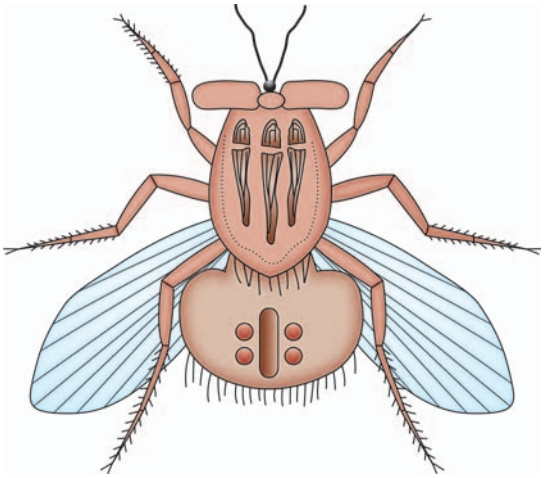


Fig. 59.7: Maggot fly (adult)

  c. Adult fly of both sexes are attracted by swormlure-2, chemically defined bait and they are intoxicated by 2 percent dichlorovos.
  d. A genetic control measure using mass reared, sterile male has also been tried successfully for species like *Cochiomyia hominivorax*.

Cockroaches

- Body is narrow elongated, symmetrical, smooth and flattened dorsoventrally.
- Adult measures 2.5 to 5 cm in length and 2 cm in width.
- The color is reddish brown.
- The body is segmented into head, thorax and abdomen.
- Body bears 2 pairs of wings (outer pair leathery and inner pair membranous).
- Development from egg through nymph to adult requires 32 to 90 days depending on species and temperature.
- Domestic cockroaches are intermediate host of *Hymenolepis diminuta*, etc. They may transmit enteric viruses and many bacteria. About 40 species of pathogenic bacteria have been isolated from contaminated cockroaches. *Aspergillus fumigatus* and *Aspergillus niger* have also been isolated from cockroaches. Cysts of *Giardia* and *Entamoeba histolytica* have also been found. An interesting report proposes the transport of *Toxoplasma gondii* from feces of the domestic cat by cockroaches. They also serve as a potentially important source

of contactant, inhalant, infectant, and ingestant allergens. Hence, they may cause itching, dermatitis, localized necrosis, asthma and hey fever.

- Control measures are:
  i. Kerosene oil spray on cockroach hiding place. Care must be taken to protect food.
  ii. Use of suitable insecticides, e.g. fenitrothion, propoxur or dioxacarb used at 1 percent.

Ticks (Fig. 59.8)

- It may be soft bodied ticks (Argasidae) or hard bodied ticks (Ixodidae).
- In the soft bodied ticks, there is no hard dorsal plate, the mouth parts are situated ventral to the anterior extremity, and the spiracles are usually located directly behind the third pair of coxal segments, e.g. *otobius, antricola,* etc.
- In Ixodidae body is ovoid, dorsoventrally flattened and unsegmented. Capitulum projects from anterior end of body. Dorsal surface of body is covered by shield-like scutum bearing eyes. Walking legs are 4 pairs with adhesive pads and claws. Female is larger than male. They produce ixodin which acts as anesthetic agent and host does not feel pain.
- Ticks are responsible for causing human diseases. They also act as vectors of many human pathogens including rickettsiae, viruses and protozoa. Thus, responsible for tick-borne rikettsial infection (Rocky mountain fever, Queensland tick typhus, etc.), viral (encephalitis, hemorrhagic fever, severe myalgia with fever, etc.), protozoa (babesiosis), other diseases like Lyme's disease erythema chronicum migrans, etc.

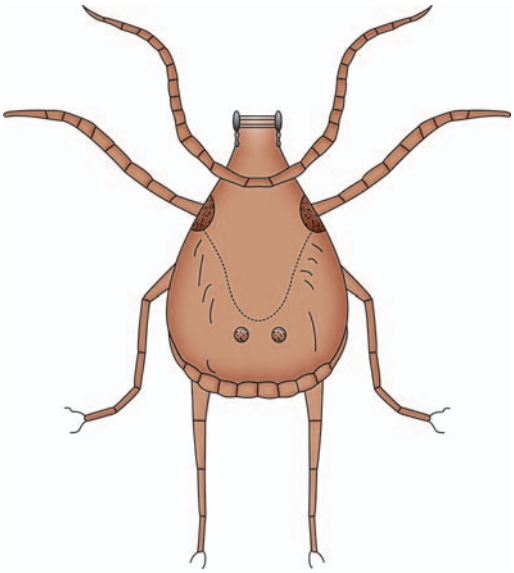


Fig. 59.8: Ticks (Ixodidae)

Mites

- Usually a millimeter in greater length or breadth than ticks.
- They have hypostome unarmed with tooth-like anchoring processes.
- Parasitic form feeds on blood, lymph, digested tissues, or sebaceous secretions on near the surface of the skin.
- Some tunnels subcutaneously causing an intense pruritus.
- Certain mites may serve as both reservoir and vector of Rickettsia tsutsugamushi.
- House dust mites either produce or concentrate potent allergens commonly found within the home.

Part VIII

Clinical Microbiology

60 The Normal Flora

The collection of species found in the normal, healthy individual and which usually co-exist peacefully in a balanced relationship with their host are known as indigenous or normal flora of body.

It is not possible to eliminate the normal flora of the skin and intestine. Antibiotics are capable of drastically reducing their number to a minimum. So the pathogenic organism gets the opportunity to overgrow and cause lesions or diseases.

The normal flora is acquired during and shortly at birth and changes continuously throughout life. It is basically environmentally determined. Breastfed infants have lactic acid streptococci and lactobacilli in their gastro-intestinal tract on the other hand bottlefed children show a much greater variety of organisms.

The term flora is used because the majority of the organisms are bacteria. Man has about 10^{14} bacteria associated with him, most of them in the large intestine.

Location of Normal Flora

Normal flora are found in those parts of the body that are exposed to or communicate with the external environment, e.g. skin, nose, mouth, intestine and urogenital tracts. Internal organs and tissues are normally sterile.

Skin

Staphylococcus epidermidis is one of the most common species (90%) of aerobes occurring in densities of 10^3 to 10^4 per square centimeters. *Staphylococcus aureus* may be present in the moist regions of the body (axillae, perineum, between toes, scalp).

Anaerobic diphtheroids are found below the skin surface in hair follicles, sweat and sebaceous glands, e.g. *Propionibacterium acnes*. *Candida* may occur on scalp and around nails. They are infrequent on exposed skin, but can cause infection in moist skin folds (intertigo).

Nose and Mouth

They are heavily colonized by streptococci, staphylococci, diphtheroids and Gram-negative cocci. Some of the species found as a part of the flora in healthy persons are potentially pathogenic (*Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Neisseria meningitidis, Lactobaccilus, Candida* etc.). The microbial density of normal flora here is 10^{11} per gram wet weight of tissue. The surface of teeth and the gingival cervices carry large number of anaerobic bacteria. Plaque is a thin film of bacteria attached with polysaccharide matrix which the bacteria secrete. When teeth are not cleaned regularly there is increased activities of certain bacteria like *Streptococcus mutans*, which may lead dental caries. Acid fermented by these organisms from carbohydrates, can invade dental enamel.

The pharynx and trachea carry their own normal flora, e.g. alfa and beta streptococci, staphylococci, neisseria and diphtheroids.

Gastrointestinal Tract

The stomach contents give shelter to transient organisms, the acidic pH providing unfavorable environment. Gastric mucosa may be colonized by acid tolerant lactobacilli and streptococci.

The upper intestine is colonized (10^4 organisms per gram) but density increases in the ileum. Here streptococci, lactobacilli, enterobacteria and bacteroids may all be present.

In the large intestine, bacterial numbers are quite high (10^{11} per gram) and many different species are found. The majority (95 to 99%) are anaerobes, bacteroides being common and major component of stool matter. A number of harmless protozoans occur in the intestine, e.g. *Entamoeba coli.*

Urogenital Tract

Urethra in males and females is lightly colonized with *Staphylococcus epidermides*, *E. fecalis* and diphtheroids. In females before, puberty, the predominant organisms are staphylococci, streptococci, diphtheroids and *Escherichia coli.* Subsequently lactobacilli predominates, its fermentation of glycogen being responsible for maintenance of an acid pH, which prevents overgrowth by other vaginal organisms. In case vaginal pH rises, *Candida* may overgrow causing a condition called thrush.

Advantages of Normal Flora

1. They prevent colonization of potential pathogen, e.g. skin bacteria produce fatty acids, gut bacteria release bacteriocin, colicin plus metabolic wastes and lack of oxygen, vaginal lactobacilli maintain acid pH, etc.
2. Gut bacteria release vitamin B and K.
3. Antigenic stimulation provided by intestinal flora is considered important in ensuring the normal development of the immune system.
4. Antibodies produced in response to normal flora cross react with pathogens thus raising immune status of the host. The endotoxin liberated by normal flora trigger alternative complement pathogen.

Disadvantages

1. The disadvantage of the normal flora lie primarily in the potential hazard for spread into previously sterile parts of the body, e.g. when intestine is perforated, skin is broken extraction of teeth, *E. coli*, from perianal skin ascend the urethra to cause urinary tract infection.
2. Overgrowth by pathogenic members of normal flora may occur in conditions like after administration of antibiotics, increase in stomach or vaginal pH or when immune system becomes ineffective.
3. Isolation of normal flora may cause confusion in the diagnosis.

61 Collection and Transport of Clinical Specimens

By and large report from bacteriological laboratory can indicate on what has been found by microscopic and cultural examination. An etiological diagnosis is thus confirmed or denied. Failure to isolate the causative organism, however, is not necessarily the fault of inadequate technical method but it is frequently the result of faulty collecting technique. The following points should be remembered in collecting the material for microbial examination:

1. Preferably specimen should be obtained before antibiotic or other antimicrobial agents have been administered. If the culture has been taken after initiation of antibacterial therapy, laboratory should be informed so that specific counteractive measures, such as adding penicillinase or merely diluting the sample may be carried out.
2. Material should be collected where the suspected organism is most likely to be found and with as little external contamination as possible. This is particularly true of draining lesion containing coagulase positive staphylococci.
3. The other important factor for successful isolation of causative agent is the stage of disease at which the specimen is collected for culture. Enteric pathogens are present in much greater number during acute or diarrheal stage of intestinal infections and they are most likely to be isolated at that time. Viruses responsible for meningoencephalitis are isolated from cerebrospinal fluid with greater frequency when fluid is taken during the onset of disease rather than at a time when the symptoms of acute illness have subsided.
4. Specimen should be of a quantity sufficient enough to permit complete examination and should be kept in sterile containers. A serious danger to the laboratory workers as well as to all others involved is the soiled outer surface of sputum container or a leaking stool sample.
5. If morning sputum sample collection is required then patient or attendant of patient should have full instructions.
6. Arrangement should be made for prompt delivery of specimens to the laboratory. It is often difficult to isolate *Shigella* from stool specimen that has remained in the hospital ward too long as it results in overgrowth of commensals and an increasing death rate of Shigella.
7. The laboratory should be provided with sufficient clinical information to guide the microbiologist in the selection of suitable media and appropriate techniques. It is essential that close cooperation and frequent consultation among clinician, nurse and microbiologist be the rule rather than the exception.
8. The collection of specimens for anaerobic culture, the use of double-stoppered collection tube gassed out with oxygen-free carbon dioxide and nitrogen is recommended. The specimen (pus, body fluid or other liquid material) is injected through the sterilized rubber stopper while avoiding the introduction of air.

The following points must be kept in mind while collection of samples from a patient suspected to have anaerobic infection:

1. Avoid contamination with normal flora.
2. Avoid contact with oxygen.

3. Do not refrigerate the samples.
4. Process immediately after collection.
5. Transport media should be used only when delay in culturing specimen is inevitable.

Specimen Containers and their Transport

A sterile container should be used and specimen be plated as soon as possible. Apart from usual containers the most useful piece of collecting equipment is cotton, calcium alginate or polyester tipped applicator stick. Collection of material from throat, nose, eye, ear, wound at operative site, urogenital orifices and rectum is done in a Pyrex test tube (20 × 150 mm) which is cotton plugged having applicator stick and small test tube containing thioglycollate broth. The swab inoculated with material from the patient is placed in inner broth tube to prevent drying out and promptly sent to the laboratory.

In case significant delay is unavoidable between collection of specimen and culturing, transport media are required. Transport media prolong the survival of microorganisms, e.g. Stuart medium maintains a favorable pH and prevents both dehydration of secretions during transport as well as oxidation and enzymatic self destruction of pathogen present in the specimen. Cary and Blair transport medium is used for fecal culture to isolate *Salmonellae, Shigellae, Vibrio*, etc. For anaerobic culture double stoppered collection tube gassed out with oxygen-free carbon dioxide or nitrogen are recommended for collection of specimen and transporting it to laboratory.

Some bacteria such as meningococcus in cerebrospinal fluid are quite sensitive to low temperature and require immediate culturing. On the contrary clinical material likely to contain abundant microbial flora may be held at 4°C in a refrigerator for several hours before culturing if it cannot be processed immediately, e.g. urine, feces, etc. Refrigeration will preserve the viability of most pathogens and also prevent the overgrowth of commensals.

If specimen is to be delivered by post it must be sealed in such a way as to prevent the leakage of material and outer envelope must be marked "Microbial Specimen and Fragile—Handle with Care".

Handling of Specimen in the Laboratory

It is not always practical for many specimens to be inoculated as soon as they arrive in the laboratory. Refrigeration at 4°C to 6°C offers a safe and reliable method of storing many clinical samples until they can be conveniently handled. However, some may require immediate plating.

Urine specimens for culture may be refrigerated for 24 to 48 hours without affecting the bacterial flora. Swabs from wound, urogenital tract, throat, rectum and samples of feces and sputum may be refrigerated for 24 to 48 hours without appreciable loss of pathogens. Gastric washings and resected lung tissue for the culture of *Mycobacterium tuberculosis* should be processed immediately after delivery as tubercle bacilli may die rapidly in these specimens.

Sputum, bronchial secretions, bone marrow and purulent material from patients suspected of having systemic fungal infection should be inoculated as soon as possible, particularly when diagnosis of histoplasmosis is considered. However, pieces of hair or scraping from skin and nail submitted for fungal culture may be kept at room temperature for several days before inoculation. Specimen submitted for isolation of virus should be frozen immediately. Specimen of clotted blood for virus serology may be refrigerated but never frozen.

Selection of Laboratory Investigations

Diagnostic tests in infectious diseases fall into following 4 groups:

1. The demonstration of an infectious agent (bacterial, viral, protozoal, helminthic or mycotic) in specimens obtained from the patient.
2. The demonstration of a meaningful antibody response in the patient. It requires two serum specimens usually obtained at an interval of 10 to 20 days or longer.
3. The demonstration of meaningful, cell mediated responses or skin tests to antigens associated with a particular infectious disease.
4. The demonstration of deviation in a variety of clinical laboratory determinations that non-specifically suggest or support a suspicion of infectious diseases.

62 Collection and Preliminary Processing of Specimens

Specimen collection from different parts of the body: It has two-fold objective: (a) to indicate the commensal organisms commonly found and pathogens of utmost importance in material from different parts of the body, (b) to indicate basic routine for collection and initial investigations of material. One should know that bacteriological examination is usually completed in 2 to 3 days' time whereas culture of *Mycobacterium tuberculosis* takes 3 to 8 weeks. Collection of specimen from particular parts of the body is as follows:

1. **Skin:** The commensals are *Staphylococcus albus* and diphtheroid bacilli. Many more bacteria and some fungi are also present on skin but only temporarily. The important pathogens are *Staphylococcus pyogenes* (boils), *Streptococcus pyogenes* (impetigo, cellulitis), *E. coli*, *Proteus*, *Pseudomonas*, *Candida albicans* (intertrigo), dermatophytes, pox and herpes virus.

   Swab moistened with sterile broth or saline may be used for collection of specimen. Crust of scab in a sterile bottle is more useful especially in viral lesions. In fungal lesions scraping from skin, hair and nail may be used. For bacteria Gram staining, culture on blood agar and MacConkey's media may be made, for *Candida albicans* culture on Sabouraud's medium and for dermatophytes wet preparation of scraping in KOH and culture on Sabouraud's medium is made.
2. **Conjunctiva:** The common commensals are staphylococci, diphtheroid, *Streptococcus viridans*, non-pathogenic *neisseriae*, etc. The important pathogens are *Neisseria gonorrheae*, *Staphylococcus aureus*, pneumococcus, *Haemophilus influenzae*, chlamydiae, adenovirus and herpes virus.

   It is desirable to have microscopic slides and culture plates in clinics and wards. Alternatively patient may be sent to laboratory. Smears are made and culture inoculated with material taken from conjunctival surface by sterile platinum loop. Otherwise conjunctival swab is never taken as adequate specimen for bacteriological investigations like Gram stained smear and inoculation on blood agar, chocolate agar, aerobically and in 5 to 10 percent CO_2.
3. **Ear:** Commensals of external ear are same as of skin. However, middle ear is sterile. The important pathogens are *Staphylococcus pyogenes*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, pneumococcus, *Haemophilus influenzae*, etc. A swab may be used to collect specimen from external ear or from middle ear that is discharging through eardrum. Gram's staining and culture on blood agar, chocolate agar, MacConkey media may be done.
4. **Throat swab:** The commensals are *Staphylococcus albus*, *Streptococcus viridans*, non-hemolytic streptococcus, diphtheroids, lactobacilli, non-pathogenic neisseriae, etc. The important pathogens are *Streptococcus pyogenes*, *Staphylococcus pyogenes*, diphtheria bacilli, *Haemophilus influenzae*, Vincent's organisms and *Bordetella pertussis*, etc.

   Throat swab is taken with patient's mouth wide open and tongue depressed. Care is taken to touch only pharyngeal mucosa. Blood culture, although it does not provide information at once, is usually positive for *Haemophilus influenzae* and should be carried out as the best means of confirmation of diagnosis. In the presence of membrane especially when diphtheria

is suspected part of the membrane must be removed.

Initial investigation is done by doing Gram's staining, smears stained with dilute carbol fuchsin for Vincent bacteria and Albert staining for diphtheria bacilli. Media used are blood agar, Löffler's serum and tellurite (diphtheria), chocolate agar (*Haemophilus influenzae* and *Neisseria meningitidis*), Bordet-Gongou medium for *Bordetella pertussis.*

5. **Alimentary tract:** Commensals include lactobacilli, anaerobes of the bacteroides and clostridia, streptococci and certain protozoa. The pathogens are *Escherichia coli,* shigellae, salmonellae, *Vibrio cholerae, Staphylococcus aureus, Candida*, rotaviruses, etc. Stool specimens are collected in a screw capped bottle which may be obtained from laboratory. If it is difficult to collect stool specimen then rectal swab may be taken. Naked eye inspection of the specimen is done for consistency, presence of blood or mucus. For culture inoculation is done on blood agar, MacConkey and DCA plates. Enrichment media like selenite broth may be used. Gram's staining should be done if staphylococcal or candida enteritis is suspected.
6. **Blood culture:** Essentially blood is sterile and hence no commensals are present. Detection of organism in blood is always abnormal finding. The important pathogens are *Staphylococcus aureus* (acute septicemia), *Neisseria meningitidis* (chronic septicemia), *Streptococcus viridans,* enterococci (subacute bacterial endocarditis), salmonellae (enteric fever), brucellae (undulent fever) and plasmodium (malaria).

   Blood may be collected strictly aseptically using autoclaved syringe. It is always best to obtain blood at a time when patient's temperature is rising as at this time patient is having highest number of bacteria in the blood. About 5 to 10 ml blood is transferred in 50 ml culture media in blood culture bottles. For Salmonellae organisms bile glucose broth and for other organisms glucose broth is used. Bile broth is used in enteric fever since bile lyses WBCs and releases the intracellular organism.

   In case of infective endcoarditis, multiple cultures are required because of intermittent nature of bacteremia.

   Sometimes it may be necessary to use special medium such as broth with 10 percent sucrose to recover the bacteria from blood.

   In some laboratory sodium polyanethol sulphonate is used in broth culture media which prevents blood from clotting and inhibits bactericidal activity of blood. All cultures are incubated at 37°C. Subculture is done after 24 hours, 96 hours and 14 days' incubation at 37°C.

   Some laboratories use blood culture media containing radiolabelled metabolic substrates. The gases in the culture bottles are monitored by automation to detect $^{14}CO_2$, a metabolic by-product that indicates growth.
7. **Urinary tract:** Apart from skin commensals, urinary tract is sterile. The important pathogens are *Escherichia coli,* Proteus, Citrobacter, Pseudomonas, Moraxella, Acinetobacter, Staphylococcus, *Streptococcus fecalis,* salmonellae, *Mycobacterium tuberculosis*, etc. For non-tuberculosis purposes a midstream fresh urine is collected in an autoclaved cotton swabbed test tube. In tuberculosis patient first morning urine sample or 24-hour urine collection is required in clean and dry container. Specimen must be processed immediately after collection. In case 3 to 4 hours' delay is inevitable then urine sample may be kept in refrigerator at 4°C. Urine samples are streaked on blood agar and MacConkey agar plates. Microscopic examination of urine should also be done especially for pus cell, RBC and bacteria.

63 Diagnostic Microbiology—An Approach to Laboratory Diagnosis

Microbiology is perhaps the least easy to codify in fixed routines. Every patient with the suspected infection is a new biological problem that both clinician and bacteriologist can solve. Only by following their noses wherever the investigations lead them, and any attempted identification of infecting microbe in the laboratory may lead the microbiologist along unexpected paths. Clinical microbiology is an explanatory art that demands flexibility of mind and technique. The latest methods are less important to the (would-be) practitioner than a set of uniformly good procedures with which to explore the common and not-too-rare infection of man with which he stands a reasonable chance of discovering new ones.

Etiology of microbial diseases may be established mainly by three methods: (1) Microscopy, (2) Culture and (3) Serology/skin tests. Unstained microscopic examination is useful to demonstrate trophozoites of protozoa, eggs of helminths, pus cells/red blood corpuscles in body fluids and motility of bacteria. Unstained wet preparations using dark ground illumination are useful for the demonstration of *Treponema pallidum*. Ziehl-Neelsen staining is useful for the demonstration of *Mycobacterium tuberculosis* and leprae bacilli. Gram-staining is useful in gonorrhea, Vincent's angina and infections caused by other organisms. Microscopy is also useful for diagnosis of fungal diseases and demonstration of inclusion bodies. However, fluorescent antibody technique is gaining momentum in establishing its place in rapid diagnosis.

Cultural examination includes isolation and identification of organism. For identification we need colony morphology, biochemical tests, pathogenicity or toxigenicity test and differentiation of types of bacteriocin, phage typing, etc.

Serology is useful in demonstrating antibodies in the serum of patients. Examination of single specimen of serum is not diagnostic. Four-fold rise in the levels of specific antibodies in patient serum during course of illness is usually diagnostic. The most common serological tests are: (i) agglutination, e.g., Widal's test for enteric fever, (ii) precipitation test, e.g. VDRL used for syphilis and (iii) complement fixation test, e.g. Wassermann test used for syphilis. New serological test like indirect hemagglutination (IHA), radioimmunoassay, and ELISA are currently in use.

Skin tests are not very reliable diagnostic procedures. The important examples of skin test are: Casoni test for the diagnosis of hydatid cyst (immediate hypersensitivity), tuberculin test for the diagnosis of tuberculosis (delayed type hypersensitivity) and Frei's test for the diagnosis of lymphogranuloma venereum (delayed type of hypersensitivity).

The other nonspecific tests like red blood cell count, total and differential leukocyte count, erythrocyte sedimentation rate (ESR), C-reactive proteins, etc. are also useful for establishing the diagnosis and determining the prognosis of disease.

Before proceeding for the laboratory diagnosis of a disease we must know the etiology (causative) agents, specimen required, collection of specimen, its transport to laboratory and processing of specimen, i.e. microscopy for morphological study, culture identification, serology to demonstrate four-fold rise in titer in paired sera and skin tests.

Some common problems concerning the laboratory diagnosis establishment are discussed as under:

PYREXIA OF UNKNOWN ORIGIN (PUO)

Peterson and Beeson defined PUO as illness of 3 weeks' duration temperature exceeding 38.3°C on several occasions. Invariably diagnosis is not established even after one week's stay in the hospital.

The causes of PUO are:

Acute (short duration)

1. Enteric fever
2. Amebic hepatitis
3. Urinary tract infections
4. Malaria
5. Subacute bacterial endocarditis
6. Brucellosis
7. Typhus fever

Chronic (long duration)

1. Brucellosis
2. Pulmonary tuberculosis
3. Typhus
4. Subacute bacterial endocarditis
5. Kala-azar.

Investigations are done as under:

I. Urine for microscopic examination especially for pus cells. In case of presence of pus cells culture and drug sensitivity is done.

II. Blood examination:
   a. **TLC** and **DLC** shows leukocytosis in urinary tract infection, subacute bacterial endocarditis and amebic hepatitis. Leukopenia is a feature of enteric fever and malaria. Lymphocytosis may be observed in brucellosis.
   b. **RBC** count and hemoglobulin estimation indicates anemia.
   c. Peripheral blood film may show malarial parasite.
   d. Bone marrow may show **LD** bodies.

III. Sputum examination is done for the demonstration of acid-fast bacilli by direct, concentrated and culture methods.

IV. Stool examination for trophozoites and cysts of *Entamoeba histolytica* is done. One may also look for Charcot-Leyden cystals.

V. Blood culture is done by collecting 5 to 10 ml of blood from the patient aseptically using autoclaved syringe. Blood culture bottles are used containing 50 ml of broth (Fig. 63.1). Glucose bile broth is used for enteric fever, glucose broth for subacute bacterial endocarditis or other pyogenic organisms and liver infusion broth (Castaneda's media) for brucellosis. These bottles are incubated at 37°C and subcultured on solid media (blood agar and MacConkey agar plates) after 24 hours, 48 hours, 7 days and 14 days. For enteric look for non-lactose fermenter colonies (NLF) which are Gram-negative bacilli, oxidase negative, ferment glucose and mannitol, Indole and VP negative, and MR plus citrate positive. Final confirmation for enteric bacilli is done by slide agglutination with specific antisera. For subacute bacterial endocarditis subculture is done on blood agar plate after 2, 7 and 30 days and study the colonies of *Streptococcus viridans, Staphylococcus albus*, and *Streptococcus fecalis.* For brucellosis incubation is done in 5 to 10 percent CO_2 and look for Gram-negative coccobacilli, non-lactose

Fig. 63.1: Blood culture bottle

fermenter, non-motile and oxidase negative bacilli. Confirmation is done by agglutination with specific antisera.

VI. **Serology:** Widal's test is done for enteric fever. Agglutination test for brucellosis may be done. Weil-Felix test using OX_2, OX_{19} and OXk antigen is done for typhus fever. Formal gel test can be done for kala-azar.

VII. Pus may be examined microscopically for trophozoites of *Entamoeba histolytica* and necrotic debris.

SORE THROAT

Etiology

a. *Membranous*
   1. *Corynebacterium diphtheriae*
   2. Candida
   3. Vincent's angina

b. *Non-membranous*
   1. *Streptococcus pyogenes*
   2. *Staphylococcus aureus*
   3. Pneumococcus
   4. Pertussis
   5. *Corynebacterium diphtheriae*
   6. Adenovirus
   7. Rhinovirus

Collection of Specimen

Throat swab (Fig. 63.2) is taken aseptically using tongue depressor. It is necessary that collection of throat swab should be undertaken under proper light. It is advisable to take two throat swabs. If membrane is present one must make it a point to remove part of the membrane because chances of recovery of causative organism are more from membrane.

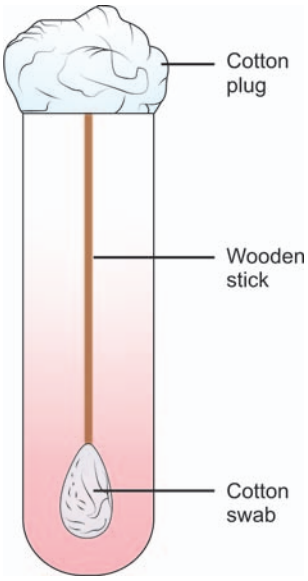


Fig. 63.2: Throat swab

Culture

Immediately after the collection of specimen, inoculate it on blood agar, blood tellurite and Löffler's serum slope. Incubate these plates at 37°C for 12 to 48 hours. In the meantime smears from throat swabs are prepared, then Gram's and Albert staining is done. In stained smear, look for Gram-positive bacilli, which are thin, slender, pleomorphic with metachromatic granules showing Chinese latter arrangement. These characters are suggestive of *Corynebacterium diphtheriae*, Gram-positive cocci in chains (streptococcus), in clumps (staphylococcus) and if in pairs (pneumococcus). Budding yeast cells are found in candida. In Vincent's angina we find curved and spiralled organisms, i.e. fusiform bacilli.

In diphtheria cases we may find small, convex colonies on blood agar plates. On Löffler serum slope there is abundant growth which is moist, cream colored or pigmented. Blood tellurite agar is selective and indicator medium (black colonies) showing differentiation among gravis, intermedius and mitis. Sugar sets are used. Sugar media is prepared in Hiss serum. In case of gravis, starch and glycogen are fermented without formation of gas. Pathogenicity tests are useful like Elek's test, animals inoculation test, e.g. rabbit and guinea pig in which material may be injected intradermally or subcutaneously.

In *Staphylococcus aureus* cases we find colonies 2 to 3 mm in diameter, smooth, glistening butyrous, opaque and golden yellow showing beta hemolysis on blood agar plate. They are catalase positive and Gram-positive cocci arranged in clusters. They exhibit pathogenicity tests like coagulase production, mannitol fermentation, lipase, DNase and phosphatase production.

The *Streptococcus pyogenes* is Gram-positive cocci arranged in chains. They are catalase

negative. On blood agar plate they are small, low convex, semitransparent, discrete colonies showing beta hemolysis. They are matt to mucoid when freshly isolated. Biochemically they ferment lactose, glucose, sucrose and mannitol with only acid production. Most common Lancefield group responsible for throat infection is A.

Pneumococcus shows alpha hemolysis. The colonies are small, flat and transparent. They show positive bile solubility test, ferment insulin and optichin sensitivity test is positive. They may kill mice when injected intraperitoneally.

URINARY TRACT INFECTION

It is a common urological condition. Sometimes it is impossible to eradicate it because of development of drug resistant bacteria. So the wrong therapy is likely to make sensitive organism resistant to drugs. Hence prior isolation of causative organisms and their sensitivity to antimicrobial drugs should be done before any rational treatment is given to the patient.

Causes

1. *Escherichia coli*
2. Klebsiella
3. Enterobacter
4. Serratia
5. Proteus
6. Providentia
7. *Pseudomonas aeruginosa*
8. Alcaligenes
9. Acinetobacter
10. Moraxella
11. *Streptococcus fecalis*
12. *Staphylococcus pyogenes*
13. *Streptococcus pyogenes*
14. Salmonella
15. *Neisseria gonorrheae*
16. *Mycobacterium tuberculosis*
17. *Candida albicans.*

Collection of Specimens

Midstream urine is collected aseptically in a sterilized container. Two successive clean voided midstream urine specimens may be collected in order to have 95 percent confidence level when using a bacterial count of 10^5 per milliliter as an index of significant bacteriuria. It is necessary to process the urine in the laboratory within one hour of collection. In case of inevitable delay urine may be stored in refrigerator at 4°C. The technique of dividing voided urine from male patients into urethral, midstream and post-prostatic massage may help to increase the accuracy of localizing urinary tract infection. Since specimens of urine either clean voided or catheterized are frequently contaminated so the recovery of even pathogens does not necessarily establish urinary tract infection. Bacterial counts on fresh, voided urine from infected patient show more than 100,000 (10^5) organism per millilitre significant bacteriuria. However, bacterial counts of less than 10^5 per milliliter may occur in patient on antibacterial therapy or in patients who are excessively hydrated causing dilution of urine (Fig. 63.3).

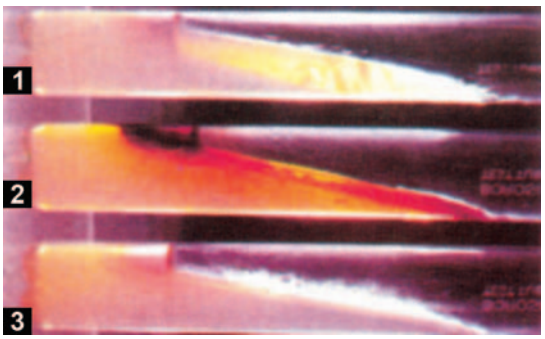


Fig. 63.3: Nitrate agar; 1. Control, 2. *Escherichia coli*, 3. *Acinetobacter calcoaceticus*

Cultural Procedure

Flame sterilized and cooled, about 4 mm, platinum loop (standardized) charged with uncentrifuged urine is streaked on blood agar and MacConkey agar plate. A bacteriological loop of 4 mm diameter holds 0.01 ml of urine which should be inoculated on to the medium, growth of more than 100 colonies is considered significant. Incubate both plates at 37°C and study the plates next morning. Estimate the total count from blood plate and Gram-negative bacterial count from MacConkey agar plate. After determining the plate bacterial count, proceed with the identification of the organisms present and then find out their susceptibility to antibiotics.

If no growth occurs after 24 hours, hold the plates at 37°C for another day and if negative, report it as "no growth after 48 hours."

The other quantitative methods of estimation of bacteriuria in urine are:

1. Blotting paper strip method where two blotting papers (12 × 6 mm) are dipped in urine and impressed—one on blood agar and the other on MacConkey plate. After 37°C incubation colonies are counted. Twenty to thirty colonies represent significant bacteriuria (10^5/ml).
2. *Uricult:* It is dip slide with nutrient agar on one side and MacConkey's medium on other side. Viable count is determined by com- paring density growth on each medium and matching with manufacturer's chart.
3. *Microstik:* These are strip-like uricult and used as per instructions of manufacturer.
4. *Tri-phenyl tetrazolium chloride test:* 2 ml urine with 0.5 ml of reagent is incubated at 37°C for 4 hours. Appearance of red precipitate in solution means 10^5/ml bacterial count.
5. *Nitrite test:* Nitrites are not found in normal urine. Its presence means coliform bacteria in urine (Fig. 63.3).
6. *Catalase test:* The presence of catalase as evidenced by frothing on addition of hydrogen peroxide indicates bacteriuria, although positive result is obtained also in hematuria.

MENINGITIS

Causes

a. *Bacterial:*
   1. Meningococci
   2. Pneumococci
   3. *Hemophilus influenzae-b*
   4. Streptococci
   5. Staphylococci
   6. Coliform organism
   7. *Mycobacterium tuberculosis*
   8. *Treponema pallidum*
   9. Leptospira

b. *Viral:*
   1. Mumps
   2. Coxsackie
   3. Echo
   4. Herpes simplex
   5. Lymphocytic choriomeningitis
   6. Poliomyelitis
   7. Arbovirus

c. *Fungal:*
   1. Cryptococcus
   2. Coccidioides
   3. *Histoplasma capsulatum*

Collection of Specimen

Specimen required for investigation of meningitis is cerebrospinal fluid. It is collected aseptically in an autoclaved container by performing lumbar puncture and specimen should be arranged to be transported to laboratory quickly. If delay is inevitable then specimen may be kept at 37°C for not more than 4 hours. It should never be kept in refrigeration or icebox.

Processing and Culture of Specimen

In the laboratory gross examination of cerebrospinal fluid is done to note color, turbidity and any deposit or clot. Microscopic examination is also done for white cell count. Some fluid is immediately transferred into glucose broth. Fluid left behind is centrifuged. Supernatant may be used for biochemical test like protein, sugar and chloride. Smear is prepared from deposit and stained by Gram's method. Deposit is also used for culture on blood agar plate. Similarly, subculture from glucose broth may be done on blood agar plate. After 37°C incubation for 48 hours plates are studied and colonies are identified by various biochemical reactions. Drug sensitivity is also determined.

In tuberculosis cases cerebrospinal fluid frequently contains clot (cobweb or spiderweb). Smear and then Ziehl Neelsen stain from this clot may show acid-fast bacilli. Lowenstein Jensen medium may be used for the culture of *Mycobacterium tuberculosis* and for animal inoculation. Cerebrospinal fluid deposit may be injected into guinea pig.

For demonstration of fungus microscopic examination is done. Subsequently for cultural purposes Sabouraud's agar media may be used. Some serological tests may be helpful in diagnosing fungal etiology.

For the demonstration of virus as etiological agent electron microscope, tissue culture and serological techniques may be undertaken.

INFECTIVE DIARRHEA

Causes

a. Bacterial:
   i. *Escherichia coli* (O_4, O_{29}, O_{55}, O_{80}, O_{111}, O_{124}, O_{130}, etc.)
   ii. Shigella
   iii. Salmonella
   iv. *Vibrio cholerae*
   v. *Vibrio parahemolyticus*
   vi. Staphylococcal infection
   vii. Campylobacter
   viii. *Clostridium difficile*
   ix. *Clostridium perfringens*
   x. *Bacillus cereus*
   xi. Pseudomonas
   xii. Proteus
   xiii. Klebsiella.

b. *Viral:*
   i. Rotavirus
   ii. Norwalk agent
   iii. Adenovirus
   iv. Asteroid virus
   v. Echo virus (11, 14, 18 types)
   vi. Coxsackie virus

c. *Parasitic:*
   i.
      i. *Entamoeba histolytica*
      ii. *Giardia lamblia*
      iii. *Ascaris lumbricoides*
      iv. Whipworm
      v. Tapeworm
      vi. Alkylostoma (hookworm)
      vii. Plasmodium (rare cause)

d. *Fungal:*
   Candida albicans.

Collection of specimen: Stool specimen is preferably collected aseptically in screw-capped water tight container provided with small plastic spoon (Fig. 63.4). This spoon may be used to transfer safely into the container small amount of stool specimen. From young children where collection of specimen is difficult rectal swab may be obtained. If culture of stool is required and delay in transporting the stool specimen to microbiology laboratory is inevitable, transport media (glycerol saline) may be used. If *Vibrio cholerae* is expected it is advisable to use at least alkaline peptone water.

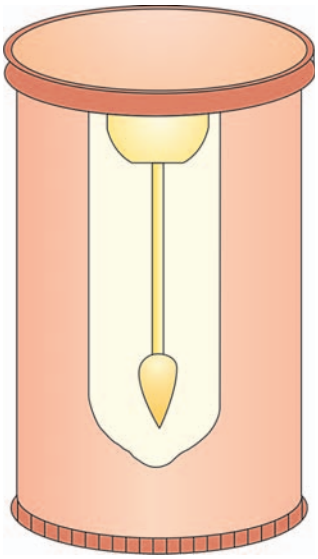


Fig. 63.4: Container used for collection of stool specimen

Further Processing and Culture Procedure

Naked eye examination for consistency, presence of blood, pus and mucus is done. Wet microscopic (both saline and iodine) preparations are used for detection of ova and cysts. Plasmodium may cause diarrhea and so peripheral blood film should be scanned for plasmodium.

Aerobic culture on blood agar for pathogenic strain of *Escherichia coli* and *Staphylococcus aureus* is done. MacConkey or DCA medium may be used for Shigellae and Salmonellae. Selenite F broth may be used if salmonellae is suspected which may be subcultured subsequently on MacConkey or DCA media. Just reporting of *E. coli* without serotyping is not sufficient. Pseudomonas, proteus and klebsiella are not always normal flora of intestine and their presence in stool may be abnormal especially when associated with diarrhea.

Virus culture is tedious and difficult job in ordinary laboratories and is difficult to obtain. However, where electron microscope

facilities are available, rotavirus may be identified by virtue of its characteristic cartwheel appearance. Of course serological techniques are also helpful in establishing viral etiology.

Further we may proceed blood culture for septicemia, Widal test of salmonellae infection, Chinese hamster cell culture or rabbit ileal loop assay for enterotoxigenic strains of *Escherichia coli* and mycological culture for *Candida albicans* can be done. If feasible, detection of parenteral infection and immunological deficiency may be made out.

WOUND INFECTION

Etiology

Aerobes:
1. *Staphylococcus aureus*
2. *Streptococcus pyogenes*
3. *B. proteus*
4. Pseudomonas
5. Coliform bacilli.

Anaerobes:
1. Streptococci
2. Bacteroides—Nonspore former
3. *Clostridium perfringens*
4. *Clostridium tetani*—Spore former
5. *Clostridium septicum*
6. *Clostridium edematiens*

Fungi:
1. Candida.
2. Aspergillus.

Source of infection may be exogenous (from environment) or endogenous (from commensal of body). Open wound may be infected with multiple organisms whereas closed undrained wound is usually infected with single organism like *Staphylococcus aureus, Streptococcus pyogenes* and so on.

Collection of specimen: Specimen should be collected aseptically in an autoclaved container. Pus specimen is comparatively better than swabs. If swab is present over the wound peel it off and then swab is well-soaked in wound material. If abscess is there then aspirate it with sterilized needle. It is always advisable to obtain two swabs, one for smear and second for culture.

Chemicals like liquid (sodium polyanethole sulfonate) can be used to neutralize the toxic substance present in blood.

Processing of a tissue in broth is more useful than taking a swab. The swab should be moist. Specimen should be taken from margin of the ulcer.

Direct anaerobic culture can be performed on exudates which do not show squamous cells under microscope and also on fluids showing bacteria in smears where organisms morphologically resemble anaerobic bacteria

The material collected is sent to laboratory. First of all material is examined grossly for especially bluish green color (pseudomonas), and presence of gas (clostridium, klebsiella, *Escherichia coli*).

Smear stained with Gram's stain shows whether organism is Gram-positive or Gram-negative. Gram-staining also provides the information if the organism is cocci or bacilli. If Gram-positive cocci are arranged in cluster (staphylococcus), or in chains they are streptococcus. Gram-positive bacilli (spore bearer) may be *Clostridium tetani* or *Clostridium perfringens.* Gram-negative bacilli may be pseudomonas, proteus, klebsiella, *Escherichia coli*, etc.

Specimen may be cultured on blood agar plate (aerobically and anaerobically) and Robertson cooked meat medium (anaerobic culture). Aerobically specimen may be cultured on blood agar plate and MacConkey medium (Fig. 63.5). Now colonies or growth on these media may be studied and smears also be prepared:

1. *Staphylococcus aureus* shows golden colored, beta hemolytic colonies which are catalase and coagulase positive.
2. *Streptococcus pyogenes* shows pin-point, beta hemolytic colonies which are catalase negative. Ninety-nine percent belong to Lancefield group A.
3. Pseudomonas colonies are bluish green in color. They are oxidase positive and use glucose oxidatively (Hugh Leifson media).
4. Proteus colonies show characteristic swarming character. PPA and urease tests are positive. Species identification depends upon H_2S production, indole and citrate.

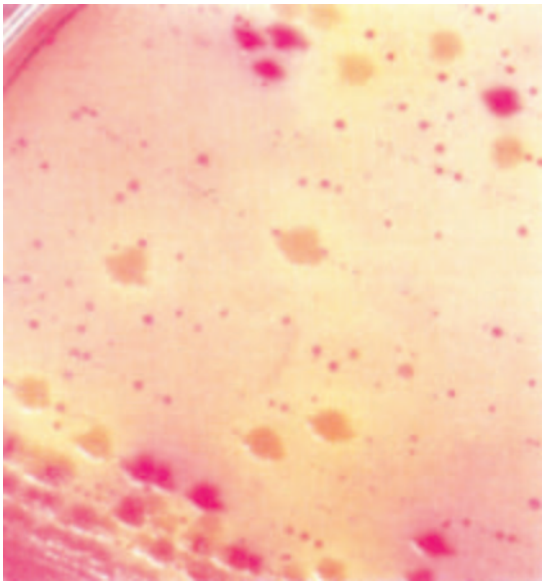


Fig. 63.5: MacConkey Agar; *Escherichia coli* (large pink colonies), *Salmonella typhi* (colorless colonies), *Staphylococcus aureus* (Small pink colonies), *Streptococcus faecalis* (pinpoint colonies)

5. Coliform colonies are pink in color on MacConkey (lactose fermenter). Identification of species is based on biochemical tests (IMViC).

Clues to the development of anaerobic infections are as follows:

1. Foul smell
2. Gas formation
3. Black exudate.
4. Presence of granules (sulfur)
5. History of aminoglycoside therapy without any improvement.
6. Treatment with cytotoxic and immunosuppressive drugs.

For anaerobic culture, inoculation is done immediately in Robertson cooked meat medium or thioglycollate medium and blood agar plates. If delay is inevitable we may use Stuart transport medium.

1. *Clostridium perfringens* is identified on the basis of morphological characters, demonstration of capsule and biochemical reactions (litmus milk stormy clot, Nagler's reaction positive and strongly sacchrolytic activity).
2. *Clostridium tetani* shows characteristic drumstick appearance. Demonstration of production of toxin and its neutralization by specific antitoxin is essential. Only demonstration of *Clostridium tetani* is not of much importance as they may be present as saprophytic wound contaminants.

FOOD POISONING

Etiology

1. *Staphylococcus aureus*
2. *Clostridium botulinum*
3. *Salmonella typhimurium*
4. *Salmonella enteritidis*
5. *Bacillus cereus*
6. *Clostridium perfringens*
7. *Vibrio parahemolyticus.*

Collection of specimen: Stools are collected aseptically in an autoclaved container. In case of any delay in sending the stools to laboratory stools may be collected in glycerol saline.

If isolation of causative organism is to be made from food then macerate food in Ringer's solution and then proceed for culture.

It is worthwhile to discuss collection and cultural method of each individual organism.

1. *Staphylococcus aureus* may be cultured from food or even from feces. Food is macerated in sterile Ringer's solution and inoculated on blood agar and 10 percent salt agar plates. Incubate at 37°C for 24 hours and look for golden colored colonies showing beta hemolysis with coagulase test positive.
2. *Clostridium botulinum* may be demonstrated in food by preparing smear and doing Gram-staining. Presence of spore bearing Gram-positive bacilli is suggestive of proceeding further. Food is macerated, centrifuged and filtered through filter paper. Now it may be injected into mice and guinea pig. The mice may show paresis of hind gut and difficulty in breathing. Similarly guinea pig shows difficulty in breathing, flaccid abdominal muscles, salivation and congestion of internal organs. Further extensive thrombosis and hemorrhages may be noted at necropsy.

For isolation of *Clostridium botulinum,* food is macerated in sterile saline which is heated at 65 to 80°C for ½ hour to get rid of non-sporing bacteria. However,

unheated samples should also be processed as spores of type E strains may be inactivated by heat. Inoculate the material on Willis and Hobb's medium containing neomycin and incubate at 32°C for 24 hours anaerobically. *Clostridium botulinum* is identified on the base of biochemical tests like fermentation of glucose, fructose and maltose with production of acid and gas.

Fluorescent antibody test is of great diagnostic aid. Smear of food sample is prepared and stain it with fluorescent tagged antibody and study it under fluorescent microscope.

3. *Salmonella:* Fecal matter is streaked on MacConkey, DCA, bismuth sulfite agar, selenite F, and tetrathionate broth. Incubate them at 37°C for 24 hours. Now study the colonies and do biochemical and serological tests for confirmation of salmonella.

   In case isolation from food is desired then 25 gm of food may be taken in two sterile screw capped bottles. Now add 25 ml of 25 percent of Ringer's solution in each bottle. Incubate them at 37°C for 2 hours and add 50 ml double strength tetrathionate broth to the other. Now incubate at 37°C for 24 hours and subculture from each on MacConkey, DCA, and bismuth sulfite agar. If culture is negative repeat subculture after three days and then after 24 hours. Colonies are picked up and processed for biochemical and serological tests.
4. *Clostridium perfringens* may produce non-hemolytic colonies on horse blood agar plate. They are markedly heat resistant spores which are characteristically associated with mild form of food poisoning. However, intermediate heat resistant spore may also produce food poisoning. Heat resistant spores strain may be isolated by inoculation of feces in Robertson cooked meat medium. Heat it at 80 to 100°C for 1 hour, cool it and incubate at 37°C for overnight. Subculture it on Willis and Hobbs medium and incubate it anaerobically at 37°C for 24 hours. They show positive Nagler's reaction (if done) and are Gram-positive bacilli with square-cut ends.

   Isolation of typical food poisoning strain of *Clostridium perfringens* from food is difficult as spores of it are rarely abundant in food. Food is macerated in broth, subcultured on Willis and Hobbs's medium containing neomycin and horse blood agar plate. Incubate them at 37°C for 24 hours anaerobically. Identify Nagler positive, non-hemolytic (horse blood), Gram-positive bacilli with square-cut ends. Heat resistance of spore is demonstrated by first growing spore bearing bacteria in Ellner medium. Final identification may be done by slide agglutination test with series of agglutinating antisera prepared against different food poisoning strain of *Clostridium perfringens.* For heat sensitive and moderate heat resistant spores, the food and stool may be processed like food as described above.
5. *Bacillus cereus* may be isolated from food by macerating food in saline and inoculating it on blood agar and nutrient agar media. Identification is done by colony characters. They are large Gram-positive bacilli, motile, non-capsulated and show low pathogenicity for mice. They are usually associated with fried rice. Their spores withstand cooking and then germinate later with production of toxin. This toxin is responsible for food poisoning.
6. *Vibrio parahemolyticus* is associated with food. Feces may be collected and inoculated on bile salt agar medium. After 37°C incubation for 24 hours, the colonies are identified by virtue of colony characters and biochemical reactions like fermentation of glucose, oxidase positive, catalase positive, indole positive, V.P. negative, urease negative, lysine, orinithine, decarboxylase positive and arganine dehydrolase negative.

PULMONARY TUBERCULOSIS

Sputum

Collection of specimen: Patient should be advised to brush his teeth, rinse his mouth

with water and then cough up the specimen in a wide-mouthed clean container. It is usually desirable to collect 24 hours' collection of sputum. Nowadays first morning sputum sample is preferred. The most purulent or necrotic or hemorrhagic portion is removed by sterile forceps, platinum loop or Pasteur pipette for further processing.

Smear preparation and stainings: It is prepared by squash method, by keeping small portion of selected material on the slide and keeping the other slide over it. Now draw the slide apart. Two smears are ready. Dry them in the air and fix them by passing over the flame two or three times. Now stain them by Ziehl-Neelsen method and look for acid-fast bacilli. If negative repeat the specimen for two more occasions.

Concentration method (Petroff's method): Mix equal portions of sputum and 4 percent sodium hydroxide. Incubate at 37°C for 15 minutes to half-an-hour. Centrifuge it at 3000 revolutions per minute for half-an-hour. Now decant the supernatant and neutralize the deposit by adding N/10 HCl drop by drop. Again centrifuge it and discard the supernatant. From deposit prepare smear; do Ziehl-Neelsen stain and look for acid-fast bacilli. For quicker demonstration of acid-fast bacilli auramine O stain may be done and study under fluorescent microscope. Löwenstein-Jensen media may be used for culture by rubbing the deposit over it.

Laryngeal Swab

It is obtained by using sterilized nichrome or brass wire. Patient is asked to sit comfortably and with one hand pass laryngeal mirror while with the other hand pass the swab wire and collect the specimen in duplicate. Put the swab into the test tube containing 10 percent sulphuric acid for 5 minutes. Now transfer it to another tube containing 4 percent sodium hydroxide for 5 minutes. Drain each swab of excess of fluid and inoculate two bottles of Löwenstein-Jensen media.

Gastric Lavage

It is usually done in children who have the habit of swallowing sputum. Gastric juice is having harmful effect on acid-fast bacilli. So it becomes necessary to process it as soon as possible. If delay is inevitable then neutralize it with N/10 sodium hydroxide and keep it in refrigerator till processed.

The specimen is collected before breakfast with Ryle's tube. Specimen is allowed to stand in a refrigerator. Supernatant is discarded and sediment centrifuged at 3000 revolutions per minute for 15 minutes. Smears are made and stained by Ziehl-Neelsen or auramine O. The remaining deposit is treated with equal amount of 4 percent sodium hydroxide. Incubate, centrifuge and discard the supernatant. Now neutralize the deposits with N/10 HCl and culture on Löwenstein-Jensen medium. Material is also used for inoculation in guinea pig, rabbit or mice.

Culture on Löwenstein-Jensen medium appears in 10 to 14 days at the earliest. It may take more than 8 weeks. Human strains grow luxuriantly than bovine strains. The addition of glycerol in the medium enhances the growth of human strain. However, two tubes of Löwenstein-Jensen media are used for each specimen. Every week media is inspected for any evidence of growth, aeriation and removal of water of condensation. Growth of typical human strain is raised, irregular, wrinkled and creamy white in color. On liquid medium (Dubos') growth appears on the top as wrinkled pellicles. However, diffused growth may be obtained by adding Tween-80.

Animal inoculation: Guinea pig may be used for human as well as bovine strain. However, it is resistant to atypical mycobacteria. Rabbit is more susceptible to bovine than human strain whereas mice is susceptible to atypical mycobacteria.

About 0.5 ml each of concentrated material is injected intramuscularly into two guinea pigs. Local swelling appears in 10 days' time involving neighboring lymph nodes. Tuberculous nodules appear subsequently in other organs like spleen, liver, lungs and kidney. Death of animal may occur in 8 weeks' time.

Animals are examined weekly for the appearance and progress of lesion. One animal is killed as soon as lesion appears. If no lesion

appears then the other animal is killed after 8 weeks and autopsy is performed to record the lesions. Smears are prepared from lesions and Ziehl-Neelsen stain is done to demonstrate acid-fast bacilli.

Infective Endocarditis

Causes

- *Streptococcus viridans*
- *Enterococcus fecalis*
- *Staphylococcus epidermids*
- *Staphylococcus aureus*
- *Haemophilus influenzae*
- *Coxiella burnetti*
- *Chlamydia psittaci*
- Bacteroides
- Members of Enterobacteriaceae
- *Candida albicans*

Collection of Specimen

About 5 ml of blood is drawn by venepuncture aseptically and into blood culture bottle containing 50 ml of 1 percent glucose citrate broth. The ratio of blood to broth should be about 1:10. The dilution of blood in broth helps in inhibiting the action of antibodies, antibiotics and leukocytes on bacterial growth. It is recommended that 3 to 6 samples of blood (10 ml each) may be collected over 24 hours. Samples of blood preferably should be collected at the height of fever.

Culture Procedure

Culture bottles are incubated at 37°C and after 48 hours subcultures are made on blood agar, MacConkey agar or any other suitable media. If this subculture is negative further subculture on 7th, 14th and 21st day are necessary, before declaring the culture as negative. Castaneda's bottle can also be used.

Enzymes like penicillinase may be added to inhibit the action of chemotherapeutic agents on bacteria and p-amino benzoic acid may be used for sulfonamides.

Infection with *C. burnetti* is diagnosed by animal inoculation and demonstration of antibodies in serum.

For anaerobic organisms thiol broth with added CO_2 under marked vacuum may be used. A dilution 1:17 of blood in broth is used.

Thus the organisms grown are identified by usual methods.

SEPTICEMIA/BACTEREMIA

It is a condition where there is actively multiplying micro-organism in the blood stream and formation of toxic products in the blood with manifestations (sign and symptoms).

Laboratory diagnosis

Etiology of septicemia

1. *Salmonella typhi*
2. *Salmonella paratyphi A*
3. *Salmonella paratyphi B*
4. *Salmonella paratyphi C*
5. Brucella
6. *Hemophilus influenzae*
7. *Escherichia coli.*
8. *Klebsiella pneumonia*
9. Enterobacter
10. Proteus
11. Bacterioides
12. Pseudomonas
13. *Streptococcus mitis.*
14. *Staphylococcus aureus*
15. *Staphylococcus epidermidis.*
16. *Streptococcus pyogenes*
17. Pneumococcus.
18. *Neisseria meningitides*
19. *Listeria monocytogenes*

1. Blood culture (1:10) is done with glucose broth. For anaerobic culture thioglycollate borth is used. Alternatively RCM may be used. Sodium polyanethol sulfonate (SPS) may be added which acts as anticoagulative, anti-phagocytic, anticomplementory etc. It interferes with certain antibiotics e.g. aminoglycosides and thus enhances growth rate and increases the rate of isolation.
2. Castaneda method
3. Automated blood culture system, e.g. bacteria (detection of CO_2 by radiometric or optical methods)

4. Culture of other specimen:
   a. I/v catheter tips.
   b. Swab from infected burn.
   c. Swab from wound or abscess
   d. Urine in urinary tract infection
   e. Sputum in respiratory tract infection
5. Non culture method to detect circulating antigens and other microbial products.
   a. Latex agglutination test e.g. H. influenzae-b antigen, *N. meningitides*, *Streptococcus penumoniae* and yeasts etc.
   b. Counter current immunoelectrophrosis (CIEP) used to quickly identify pneumococcus, Klebsiella, *H. Influenzae* etc.
   c. Limulus ambocyte lystate assay tests may be used to detect circulating lipopolysaccharide (endotoxin) of Gram negative bacteria in blood.
6. Gas liquid chromatography detects metabolic end products of anaerobic microbacterial activity in serum.

64 Rapid and Automation Methods in Diagnostic Microbiology

The last few years have seen exciting advances in finding out new ways of identifying microorganisms within a few minutes of arrival of a specimen in the laboratory. Specific microbial components may be detected by countercurrent immunoelectrophoresis also designated as CIE. Another nice method for identification of microorganism is gas liquid chromatography (GLC) in which specific metabolic products are detected.

Microcalorimetry may be used to find out rapidly the number of bacteria in liquid specimen like urine, by detecting their heat output.

Luminescent biometry is again a useful method in which amount of bacterial adenine triphosphate is detected and thus the number of bacteria in a given amount of liquid can be known by measuring the brightness of flash of light when that amount is mixed with standard preparation of luciferase (lighting system of fire of flies). Radiorespirometry using C^{14} glucose in basal nutrient medium with different antibiotic is used for antibiotic sensitivity and thus recommending appropriate antibiotic for an infection in only 3 hours' time. Additionally following methods are discussed:

1. *Gas Chromatography:* The primary use in microbiology is to detect metabolic by-products of bacterial growth. Gas chromatography is useful in the identification of anaerobic bacteria and analysis of body fluids for the compounds that indicate the presence of microorganisms (sensitive and rapid procedure). Methods such as frequency modulated electron capture gas chromatography and pyrolysis gas chromatography are under investigation as a means to identify various bacteria and fungi (or their metabolic end products) from specimen source. Cell wall fatty acid analysis and whole cell carbohydrate study by gas liquid chromatography offer much promise. High pressure liquid chromatography is extremely useful for assay of antimicrobial agents in serum and body fluids.
2. *Bactometer:* It is an instrument capable of detecting changes in electric impedance as a result of growth and metabolic activity of bacteria in a suitable growth medium. This has been primarily evaluated for use in detecting clinically significant number of bacteria in urine and blood. Bottles of appropriate broth containing specimen is used. Wire electrodes are kept in this bottle. These wire electrodes are inserted through rubber stopper of bottles for connection to bactometer. Impedence changes are continuously monitored permitting detection within hours.
3. *Measurement of Radiolabelled Compounds:* The instrument in wide use in clinical microbiology is the Bactec system. This instrument measures the release of CO_2^{14} labelled substrates that result from bacterial metabolism. It employs sampling device and an ionization chamber. Specimens are inoculated into the bottles of broth medium containing C^{14} labelled substrate. Depending on the instrument model, bottles are monitored either automatically or manually for measurement of CO_2. The instrument converts this into growth index. This unit of measurement is recorded

through light or on meter of instrument as well as on a paper tape. Bactec has a number of other capabilities like identification of *Mycobacterium, Neisseria,* etc.

4. *Autobac Multi-Test System:* This instrument consists of an incubator shaker, photometer system, curvette information device and remote printer. Each chamber in a disposable curvette contains a different substrate (or inhibitor) or antibiotic. Identification is based primarily on the pattern of growth and or substrate utilization in the various chambers plus reaction patterns for various organisms are contained in the computer data base. This instrument is designed to perform antibiotic sensitivity results, identification of both fermentative and nonfermentative organisms after relatively short period of time.
5. *MS-2:* Abbot MS2 is the instrument with multifunction capabilities. It consists of control module and an analysis module. Besides susceptibility testing, the system can also identify the Enterobacteriaceae. The major advantage of the MS-2 system is its level of automation and results are provided within 5 hours. The disadvantage is its limitation to the identification of only the Enterobacteriaceae.
6. *Dynateh MIC-2000:* It is a semiautomated system for preparing and performing microbroth antibiotic susceptibility and identification tests. The system consists of 96 channel dispenser for accurately delivering a volume of liquid such as antibiotic dilution from test tubes to a 96-microtiter plate (the MIC plate) and an inoculator with 96 pins for accurately transferring liquid inoculum from disposable inoculum tray to the 96 wells of MIC plate. An illuminated magnifying viewer to assist in discriminating between wells with and without visible growth is also included.
7. *Replican System:* Replican system is a commercially available replica plating method for the identification of Enterobacteriaceae. This system consists of a variety of biochemical and antibiotics containing device, a viewing table allowing visual inspection and electronic recording of individual reactions, and a computer terminal complete with hard-copy printout. This device allows the simultaneous inoculation of up to 36 organisms on a single agar plate. It is considered to be efficient, economical and effective laboratory tool for identification of organisms.
8. *Automicrobic System:* It is designed for the detection, enumeration identification and drug susceptibility testing of microorganisms in clinical specimens, with all its functions to be performed simultaneously or consecutively by the same instrument. This system consists of a diluent dispenser, injector or filling module incubator reader, computer and terminal printer. It uses a novel array of highly selective media in which a mixture of substrates and inhibitors is used to permit the growth of one or group of closely related microorganisms, to concurrently inhibit growth of any other organisms present, and to enumerate the total number of organisms in the sample. The advantages of automicrobic system are its versatility, its rapidity of results with urine samples, its identification potential and its degree of automation. This system can handle up to 240 specimens simultaneously and all of the operations are automatic.
9. *Genetic Probes:* A labelled (usually P^{32}) sequence of DNA or RNA is used to detect specific DNA sequence. The nucleotide sequence of the test organism is taken out by enzyme (endonuclease), fragmented transferred to a nitrocellulose sheet and the fragments separated by electrophoresis. The specific DNA (labelled) sequence is added to the paper, where it hybridises with a fragment which has the same sequence, and detected by autoradiography.

65 Molecular Techniques in Microbiology

INTRODUCTION

It is a new area in applied medical microbiology. This in fact offers immense help in the form of molecular tests in establishing laboratory diagnosis of enormous infectious diseases. Apart from this, it provides information about prognosis of infectious diseases and response of treatment. Surprisingly molecular tests have replaced conventional culture, etc. Molecular test are convincingly accepted because conventional tests are slow, insensitive, expensive, time consuming or not always available at all and so on.

AMPLIFIED TESTS

1. **Polymerase chain reaction (PCR):** This technique offers synthesis of unlimited quantity of the target nucleic acid sequence. This technique is susceptible to contamination causing false positivity. Basic PCR is described on page 317 apart from basic PCR, other PCR are discussed briefly as under:
   a. *RT-PCR:* This technique is used to amplify RNA. Here complimentary DNA (cDNA) is formed from RNA with the help of an enzyme reverse transcriptase. Subsequently, cDNA is amplified by PCR.
   b. *Nested PCR:* By this technique both sensitivity and specificity of PCR is increased considerably. In this technique two pairs of amplification primers are required. One of the primers is used in the first round of PCR to amplify needed sequence. The amplified material so obtained is subjected to second round of PCR with the second set of primers. Actually, it anneals to the sequences found in the first of material.
   c. *Multiplex PCR:* In this case two or more sets designed for amplification of different targets are used. It may help in amplification of more than one target sequence in clinical specimens. As a matter of fact multiplex PCR is less sensitive than PCRs with single set of primers.
2. **Transcription mediated amplification (TMA):** It is in fact an isothermal RNA amplification technique. In this technique RNA target is reversely transcribed into cDNA. Now RNA copies are formed with RNA polymerase. In about 2 hours time 109 fold amplification of the target RNA can be synthesized. Advantages of TMA include: (1) there is no need of thermal cycle, (2) there are very less possibility of contamination. TMA uses a reverse transcriptase enzyme with endogenous RNase H activity and T7 RNA polymerase.
3. **Nucleic acid sequence based amplification (NASB):** It is a again isothermal RNA amplification based technique. Here RNA target is reverse transcribed into cDNA and then RNA copies are produced with the help of RNA polymerase. In a way this technique resembles with TMA. It does not require thermal cycles. Here AMVRT, RNase H and T7 bacteriophage, RNA polymerase are used in NASB. Needles to add that NASB based kits are available for detection and quantitation of HIV-1RNA and CMV RNA.

4. **Ligase chain reaction (LCR):** Ligase chain reaction is in fact probe amplification technique. Here amplified products have only a sequence present in initial probes.

In ligase chain reaction a nick is formed when two oligonucleotides probe hybridize adjacent to one another in each side of denatured DNA strands. Now enzyme DNA ligase seals the nick. At this point each ligated product and original target act as template in subsequent steps of denaturation, annealing and ligation resulting in exponential increase of liagted products. Ligase chain reaction does require thermal cycler. LCR is being used in *Mycobacterium tuberculosis, Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

NONAMPLIFIED TESTS

Nucleic Acid Probe

Nucleic acid probes are segments of DNA or RNA labeled with radioisotopes or enzymes that may hybridize to complementary nucleic acid with high degree of specificity. However, the utility of DNA/RNA probe is as under:

1. Finding of microbes directly in clinical samples.
2. To determine microbes which cannot be cultured .
3. Cultured microorganisms may be identified.
4. Identification of even strain.
5. Identification of toxins and other virulent substances.
6. Identification of resistant markers.

Molecular Techniques in Clinical Laboratory

1. Successful detection of unculturable or slow growing microorganisms, e.g. HCV, bartonella, human herpes virus 8, etc.
2. Molecular technique in virology are quite rapid, sensitive and cost effective. As a matter of fact nucleic acid-based tests are cost effective and ideal especially in immunocompromised patients for viral meningitis, HSV encephalitis and CMV infections.
3. Prognosis in microbial infection using molecular techniques is possible, e.g. HIV-1 viral load predictor of progression as AIDS. Severity of viral disease can also be detected using molecular techniques by subtyping some viruses, e.g. HPV is he cause of carcinoma cervix. HPV types 16,18 are connected with high-risk progression whereas HPV types 6, 11 are of low-risk progression.
4. Molecular techniques are of immense use to find out genes connected with drug resistance, e.g. methicillin resistant *Staphylococcus aureus*, rifampicin resistant *Mycobacterium tuberculosis*, vancomycin resistant enterococci.

Viral load assay is useful in monitoring antiviral therapy in HIV, HBV and HCV infections. Apart from this molecular techniques are applied to monitor HIV patient response to antiviral therapy. Drug resistance mutation in RT protease genes of HIV-1 can also be detected by molecular techniques.

66 Serological and Skin Tests

Various serological techniques are used to demonstrate antibodies in the serum of patients. The formation of these antibodies in the serum of patient is the result of provocation by microbial infection. Their demonstration is of great diagnostic value especially in conditions where isolation of causative organism takes quite a long-time. In the interpretation of these tests following points should be kept in mind:

1. The duration of antibody responses to various organisms differs. Evidence in support of fresh infection is rising titer of antibodies. It can be demonstrated by collecting two serum samples at a week's interval. Four-fold rise of antibody titer is considered significant.
2. It is important to know basal titer of normal healthy individuals of the same age, habitat and social habitat of the patient. This is important before attaching any significance to antibody titer measured by particular test.
3. Antibody responses are not detectable for a week's time after onset of infection. So the serum sample collected within a week of a disease is not expected to give appreciable or significant antibody titer.
4. Anamnestic reactions is defined as rise in the level of previously formed antibody in response to nonspecific stimulus of quite different infection. It is recognized with Salmonella H agglutinins showing increase in titer following unrelated febrile illness.
5. Detectable antibodies may not be formed in a patient suspected of suffering from illness in which antibodies are mostly formed. Hence, antibodies are not detected in patients' blood even weeks after the onset of disease. This may be explained on the basis of derangement of antibody forming mechanism.
6. Antibodies are not necessarily protective in nature and so not related to person's immune status.

Here we will discuss important serological tests like widal test, serological test for brucellosis, antistreptolysin O (ASO) antibody test, cold hemagglutination test, Paul-Bunnel test, Weil-Felix test and streptococcus-MG agglutination test.

1. **Widal test:** This investigation is done for the diagnosis of enteric fever or other Salmonella infection. Conventionally, H and O suspensions of *Salmonella typhi* and paratyphi are used. For the test serial dilutions of patient's serum (1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320....) are pipetted in small amounts (0.3 ml) into special small tubes. One complete set of dilutions is prepared for each bacterial suspension used in the test. An equal volume (0.3 ml) of appropriate bacterial suspension is added to each tube of particular row. Control tubes are also set up containing saline and bacterial suspension but no patient serum. Control is set up to ensure that the bacteria do not agglutinate spontaneously. Now these tubes are incubated at 37°C in water bath for overnight. Floccular agglutination of H suspension and granular agglutination of O suspension are noted after 12 to 24 hours' incubation. However, in H suspension after 55°C incubation for 2 hours,

floccules become apparent. The results of the test are expressed as antibody titer.

Low levels of antibodies that agglutinate Salmonella suspension may be detected in the blood of patient without history or relevant illness or of immunization. Hence, it may be taken as normal. Anamnestic reaction is more common in H agglutinin than O agglutinin. The most reliable serological evidence of Salmonella infection is a 4-fold or more increase in O agglutinin levels between first and second or later weeks of illness. However, absence of antibodies response does not exclude the possibility of typhoid as patient may fail to produce detectable levels of antibodies.

2. **Serological test for brucellosis:** Brucellosis may cause persistent pyrexia. For brucellosis agglutination test may be done using suspension of *Brucella abortus*. Demonstration of rising titer of 1 : 100 or more indicates active brucellosis. In chronic cases we may find low agglutination titer or prozone phenomena (failure of serum to cause agglutination until serum is further diluted). Prozone phenomena may be because of blocking action of nonagglutinating IgG antibodies. Coomb's test or complement fixation test with a titer of 1 : 32 or more in either test points towards active brucellosis.

   Patient's serum is inactivated at 56°C for 30 minutes. Serial dilution of serum, i.e. 1 : 10, 1 : 20, 1 : 30, 1 : 40, 1 : 80 and so on are prepared. Now equal quantity of bacterial suspension is added in each tube. After 37°C incubation for 48 hours agglutination of each tube is noted.

3. **Antistreptolysin O (ASO) antibody titer:** ASO titer is expressed in Todd unit. One todd unit is the amount of serum (antibodies) neutralizing 2½ MHD of standard hemolysin. A titer of 400 units or higher is considered highly significant and diagnostic, i.e. streptococcal infection.

   Patient's serum is inactivated and double dilution is prepared. 0.5 ml of diluted antigen (streptolysin O) is added to each serum dilution. Mixture is incubated in water bath at 37°C for 15 minutes. To each tube 0.5 ml of rabbit blood cells are added. Now incubate the mixture at 37°C for another 45 minutes. ASO titer is represented by the last tube in which hemolysis has not occurred.

4. **Cold hemagglutination test:** This test is used for the diagnosis of primary atypical pneumonia caused by mycoplasma. Prepare serial dilution of the serum of the patient. In the tube add 0.5 ml of human RBC of blood group O (1%). Shake the rack and keep in refrigerator at 4°C overnight. Reading is taken immediately after taking out the rack from refrigerator. Clumping of RBC is labeled as positive reaction.

5. **Paul-Bunnel test:** It is used for the diagnosis of infectious mononucleosis. Serum of the patient is inactivated at 56°C for half an hour. Serial dilution of this inactivated serum is prepared. Now 1 ml of sheep red blood cells (1%) are added to each tube. After incubation at 37°C for 4 hours, tubes are examined for any evidence of clumping of red blood cells. Titer of 1 : 100 is suggestive of infectious mononucleosis. If positive then the test is further done using guinea pig kidney suspension and ox red cell for removal of Forssman antibody.

6. **Weil-Felix test:** This test is used for the diagnosis of typhus fever. In this test sera of the patient is tested for agglutinins to O antigens of certain nonmotile proteus strains: OX_{19}, OX_2 and OXk. The basis of test is the sharing of an alkali stable carbohydrate antigen by some rickettsiae and by certain strain of proteus, i.e. *P. vulgaris* (OX_{19} and OX_2) and *P. mirabilis*, i.e. (OXk). The test is mostly done as tube agglutination test. However, rapid slide agglutination methods have also been employed for rapid screening. The antibody appears rapidly during the course of disease. Peak titer is 1 : 1000 to 1 : 1500 in second week and decline rapidly during convalescence. The test is of no value in the diagnosis of rickettsial pox, trench fever and Q fever. False-positive

reaction may occur in cases of urinary or other infection caused by proteus, typhoid fever and liver disorders. Hence, rising titer should be demonstrated.

7. **Streptococcus MG agglutination test:** This test is used for the diagnosis of primary atypical pneumonia caused by mycoplasma. Here unheated patient serum is used. Serial dilutions of patient serum are prepared. 0.5 ml of diluted heat killed suspension of MG streptococcus antigen is added to each dilution. After 2 hours' incubation of mixture at 37°C, it may be refrigerated overnight. Reading is taken as in Widal's test. A titer of 1 : 20 or more is suggestive of this infection.

8. **Skin tests:** The following skin tests are commonly used as diagnostic aid for detecting immune status and for detecting hypersensitivity:
   a. Schick test is used to find out immune status against diphtheria. Small amount of diphtheria toxin injected into skin of nonimmune person causes erythema which begins to appear one to two days after injection, reaches maximum in 4 days and persists for about a week or two. There may be small swelling at the site of injection. In persons whose level of diphtheria antitoxin is sufficient to protect him against an attack of diphtheria the toxin is neutralized and hence no reaction.
   b. Dick test may be used to determine the need of immunization against scarlet fever. Reaction is more rapid and is transitory.
   c. Tuberculin test is based on type IV hypersensitivity. The test material used may be old tuberculin or purified protein derivative. Positive tuberculin test indicates past tuberculous infection and also probable immunity. In positive reaction we find palpable induration (10 mm or more) which is maximum in two to three days after injection. Erythema may be there but is in part nonspecific and so difficult to interpret. The purpose of tuberculin test is: (i) diagnostic for individual patient, (ii) to find out foci of infection, i.e. in the absence of vaccination high incidence of active tuberculosis is encountered meaning active dissemination of disease, (iii) survey of population to find out the frequency of tuberculosis in the community, (iv) selection of subject for BCG vaccination, i.e. negative tuberculin test indicates the requirement of BCG vaccination, (v) assessment of the efficacy of BCG vaccination.
   d. Brucellin test may be used for the diagnosis of brucellosis but this test is of little diagnostic importance.
   e. Lepromin test is used in leprosy patients.
   f. Frei's test is used for the diagnosis of lymphogranuloma venerium.
   g. Skin test may be used in cat scratch fever. Pus from active cases diluted 1:5 and heated 60°C for 10 hours can be used as skin antigen. It gives positive reaction in some individual with a clinical picture.

The other skin tests are for the diagnosis of chanceroid *(H. ducreyi),* tularemia and Schultz-Charlton reaction (streptococci Group A), herpes simplex, mumps, etc.

67 Microbiology in the Service of Human Being

Microbes have positive contribution in day-to-day activities of human being. Some microbes are useful in souring milk, ripening of cheese, production of antibiotics, fermentation, acetic acid, butanol and so on. Some organisms are capable of killing mosquitoes and can be useful in the control of disease like malaria, commensals micro-organisms are useful in maintaining health and normal functions of the body. Normal flora of intestine is also responsible for production of vitamins. They also maintain balanced and favorable atmosphere for other forms of life by releasing carbon, nitrogen, oxygen in the environment, etc. They are used in research and study and understanding of genetics. Microorganisms are also used in genetic engineering for the production of hormones, vaccines, etc. On the other extreme organisms exhibit affinity for ores and amay be used to locate ores like gold, sulfur, etc. Microorganisms are at the same time harmful too especially in causing infectious diseases and their proposed use in bioterrorism.

Micrbiology is the branch of science dealing with study of microbes like bacteria, viruses, fungi and parasites. There are other branches of microbiology like medical microbiology, industrial microbiology, food microbiology, soil microbiology, plant microbiology, etc.

MEDICAL MICROBIOLOGY

Medical microbiology is closely associated with diagnosis, epidemiology, control of infections and treatment. One needs to understand that disease producing capacity of microorganism may be enhanced by environmental and host factors, e.g. contaminated food, drinks, air malnutrition, tissue damage and over crowding. Medical microbiology has the constitution as a model in structure of genetics, molecular-biology, diagnostic, prevention and control of various microbial diseases.

Medical microbiologist may plan out various steps likely to be effective to control infections as under:

1. Microorganisms can be eliminated form its source or breeding places. Thus, transmission to new host is checked.
2. Securing clean hand and edibles, drinks with control of house files and cockroaches. This checks intestinal infections like typhoid, cholera, dysentery, food poisoning, etc.
3. Seeking proper disposal of hospital wastage and sewage which is a health hazards.
4. Use of insecticides killing vectors and hence checking malaria, dengue, louse borne disease.
5. Prophylactic vaccination.
6. Surveillance of communicable diseases by keeping track of source and spread of infecting agent and study of conditions that may favor the spread of infection in the community. Overall it gives prognostic predictions about future trends and assessment of deliberate control measures.

ROLE OF MICROBIOLOGY

Role of medical microbiologist may include as follows:

1. Diagnosis is made by isolation the causative agent from pathological lesions. Serological

methods and latest techniques like PCR, probes, etc. are of great help.
2. Prognosis of disease may be reviewed by microbiologist, e.g. Widal test. Rising titer is interpreted as active disease. Declining titer means curing and that treatment is effective.
3. Guidance in treatment is provided by antibiotic sensitivity test report.
4. Medical microbiologist can also help in finding out source of infection, e.g. in a sudden outbreak of typhoid disease in a hospital ward, one can find out carrier of Salmonella (kitchen workers) by subjecting their stool samples for bacteriological culture.
5. Microbiologist is engaged in the preparation of vaccine against outbreak of infection around.

IMPORTANCE IN DIAGNOSIS OF INFECTIOUS DISEASE

Medical microbiologist are enthusiastic to confirm diagnosis by making smears, preparing cultures from lesions (boil, cellulites) to demonstrate microorganisms and suggesting effective antibiotics, Each investigation of a patient is as a matter of fact a challenge and thrill. It is dealt as a research project in miniature. In a way a clinician by requesting an investigation is asking a questions and microbiology laboratory attempts to reply in a realiable and rapid way.

Adequate clinical management of microbial diseases depends on accurate identification of involved organism and antimicrobial sensitivity. Molecular biological techniques have increased the speed and sensitivity of detection methods as well allowing laboratories to identify organisms that do not grow or grow slowly in culture. These techniques also allow microbiologists to identify genes that result in resistance in antibiotics and to fingerprint individual isolates for epidemiological tracking.

Infections diseases still remain a major cause of morbidity despite decades of progress in diagnostic front, preventive measures and treatment. Every now and then new antimicrobial agents ate introduced with the hope that infectious diseases would be eliminated. Emergence of resistance of enterococci to vancomycin, pneumococci to penicillin and so on are clinical problems and matter of concern. In a way that is how microorganisms are developing the ability to elude activities of antibiotics. Result is ever increasing antibiotic resistance. Further, the emergence of unique infectious disease or novel mechanisms of resistance will always cause us to haul the old tools out of closet until these agents have been identified and characterized.

Smallpox is completely eradicated from the world since 1977. We have progressed towards conquering over many infectious disease like anthrax, plague, etc. Some infectious diseases are re-emerging, e.g. malaria, influenza, dengue cholera, tuberculosis. Many infectious agents have been discovered recently, i.e. HIV, Marburg virus, Ebola virus, Hunta virus, Nipah virus, Hendra virus, etc. Of course campaign is in progress with vaccines and other effort on war footing to deal with these infectious challenges.

68 Community Microbiology

Role of Microbiology in Community

Microbiology is closely concerned with the epidemiology and control of infection in any community where the transmission and disease producing capacity of the infecting microorganisms may be enhanced by environmental or host factors like contaminated food, drink or air, malnutrition, tissue damage and overcrowding.

Epidemiology is concerned with etiological factors and it has had conspicuous successes in their elucidation among both infectious and noninfectious diseases, e.g. cholera, typhoid fever, pellagra, lung cancer, etc. Improved public health measures like safe water supply, proper disposal of sewage, good housing and nutrition of citizen are major factors in the decline of epidemic infectious diseases.

Certain infections are due to opportunistic invasion of tissues by commensal microorganisms previously resident elsewhere in the body. These are called endogenous infection (originating from within). They are not ordinarily transmissible from one person to another, e.g. urinary tract infection, subacute bacterial endocarditis, etc. On the other hand measles, whooping cough, chickenpox, etc. are infectious and communicable. They are transmissible from one person to another and are called exogenous (originating from without) infections. Yet there is another group of infections that are transmissible from vertebrate animals, animal hosts to man and not communicable ordinarily from man-to-man. They are called zoonosis, e.g. bubonic plague, brucellosis, rabies, etc.

No one can deny the importance of carriers in the study of sources and modes of spread of infections. Both bacteriological and epidemiological studies brought to light the convalescents and more persistent stool excreter of typhoid fever thus confirming the hypothesis of Koch that in typhoid fever, the acute or convalescent patient was a common source for subsequent infection. After some time it was shown that carriers (individual with no clinical disease but carry and disperse a pathogenic microorganism) were important links in the chain of dissemination of various infections in the community. Antibodies titer to specific pathogens or their toxin has shown that inapparent infections may have a role in raising the resistance of a community to epidemic outburst of disease.

Microbiologists and health officers with sound knowledge of the epidemiology of communicable disease can plan out various steps likely to be quite effective for its control. Even in the field of prophylactic immunization microbiologist can be a valuable partner.

The main causes of death, among the infections a century ago were tuberculosis, pneumonia, bronchitis, typhoid fever, diarrheal diseases and scarlet fever. Only recently AIDS, pneumonia and bronchitis took a heavy toll of life. The death rate from tuberculosis, and diarrheal disease have started declining. Rheumatic heart disease still has high death rate. New infections are emerging all over the world like HIV, campylobacter, *Helicobacter pyloridis,* etc.

The extraordinary reduction in deaths from infections is due to improvement in nutrition, environmental sanitation and living conditions and use of antimicrobial drugs. Still infection remains the major cause of sickness especially

children, e.g. respiratory infections (cold, sore throat, catarrh bronchitis, etc.), specific fevers (measles, whooping cough, chickenpox, mumps, etc.), diarrheal diseases and staphylococcal infections. There is no doubt to the fact that infections if not treated promptly and effectively may lead to impairment of physical or mental development or death. Laboratory services for general practitioners are therefore quite essential and so collaboration between the practitioner and microbiologist will definitely help to improve the quality of patient care in the community.

A community infection of special kind occurs among patients admitted in hospitals (nosocomial infection). However, many infectious diseases are being controlled by good environmental sanitation, by the destruction of insect vector like louse, flea, mosquitoes or by prophylactic vaccination, e.g. typhus, plague, etc. Smallpox is completely eradicated from the world since 1977. At the same time many infections like AIDS, malaria, tuberculosis, leprosy, diarrheal diseases, pneumonia, viral hepatitis, etc. take a heavy toll of life and health in most of the developing countries.

Control of community infections: It may be achieved as under:

1. Pathogen can be eliminated from its source or breeding ground and hence transmission to a new host is checked, e.g. syphilis, gonorrhea, mycobacterial infections.
2. Securing clean hands, water, milk and food with control of flies. Thus, intestinal infections like typhoid, dysentery, cholera and food poisoning can be controlled.
3. Proper disposal of sewage.
4. Prophylactic vaccination.
5. Use of insecticides thus killing vectors and hence control of malaria, yellow fever, dengue, louse-borne typhus, etc.

Surveillance: Broadly it means natural history of disease associated with continuous observation of its occurrence. Surveillance of communicable disease connotes the active follow-up of specific infections in terms of morbidity and mortality in time and place, keeping track of the source and spread of infecting agent and the study of conditions that may favor or inhibit the spread of infection in the community. Of course it requires teamwork with the obvious involvement of the microbiologist. It permits prognostic predictions about future trends and objective assessment of deliberate control measures.

Surveillance in the control of infections is quite effective, e.g. campaign against poliomyelitis, first with killed and later with live vaccine resulted in the virtual disappearance of paralytic polio. It has been followed by an intense epidemiological surveillance at both laboratory and clinical levels in order to monitor the effectiveness and safety of vaccine and to detect viruses other than polio virus which may cause infantile paralysis.

It may be concluded that use of antimicrobial drugs in acute infections, isolation of patients, blocking of channels of spread and protection of susceptible host allows the microbiologist and workers of other disciplines, to reduce the load of infection and control its spread whether locally within the family, hospital or within the general community.

Microbiology influences the community infection as under:

- Collection of exact data
- Laboratory investigation
- Spread of infection
- Zoonosis

69 Emerging and Re-emerging Microbial diseases

Continuously we are facing emerging new microbial disease all over the world causing heavy morbidity and mortality. This threatening situation may even intensify in coming years. Now we have HIV-AIDS which is in a way every bit venomous as the influenza, plague and cholera epidemics of past centuries. AIDS was unknown till 1980 but is now responsible for over 20 million deaths. In India alone over 4 million persons are living with AIDS. Irony is that there is no proper treatment for AIDS patients. Chance of development of vaccine is also bleak for the time being.

Newly emerging microbial diseases other than AIDS include Legionnaires disease which is a form of pneumonia caused by *Legionella pneumophila*, ebola hemorrhagic fever, SARS, Rift valley fever, monkey pox, Vibrio cholera 0139. Nipah viral fever, etc. Bioterrorism has itself added new dimension to this problem. All of them are challenging keeping all of those associated with them always on their toes.

The emergence of new strain enable the microorganisms to spread rapidly even in persons protected by antibodies. Hence development of effective vaccines is becoming difficult e.g. avian influenza (bird flue), swine flue (H_1N_1).

The expanding area of human inhabitation has put numerous persons at risk of microbes previously rare or unknown as a cause of disease. Microbes because of climatic changes may adapt to their environment adding antimicrobial resistance to evolutionary pathway. It also makes the microorganisms to spread to new geographical regions.

New types of personal behavior and new food processing methods may enhance transmission of microbes. New diseases and modern treatment may result immune suppression and this increases susceptibility to pathogenic microorganisms.

EMERGING MICROBIAL DISEASES

According to WHO, emerging microbial diseases are diseases the incidence of which in man has either increased during the last two decades or threatens to increase in near future. According to this definition one can include newly emerging microbial disease or those spreading to new geographical regions. Also included in this category the infectious disease controlled by antibiotics but now of late acquired resistance to these drugs.

In the past 20 years as many as 30 new microbial diseases have emerged. In most of cases proper treatment is not available, prevention and control is also difficult as no vaccine has been developed against them. Some of the newly emerged microbial diseases are as under:

Bacteria

Year	*Microbe*	*Disease*
1977	*Legionella pneumophila*	Legionnaires
1981	Toxin producing *Staph. aureus*	Toxic shock syndrome
1982	*Borrelia burgdorferi*	Lyme disease
1983	*Helicobacter pylori*	Gastritis and dyspepsia
1992	*Vibrio cholera* 0139	Epidemic cholera
1992	Bartonella henselae	Cat scratch fever
	Mobiluncus Spp.	Vaginosis
	Clostridium difficile	Pseudo membranous colitis

Viruses		
1973	Rota virus	Infantile diarrhea
1977	Ebola virus	Ebola hemorrhagic fever
1978	Hantian virus	Hemorrhagic fever and renal syndrome
1980	HTLV-1	T cell lymphoma/leukemia
1982	HTLV-2	Prevalent in intravenous drug uses no associated with disease
1983	HIV-1	AIDS
1985	HIV-2	AIDS
1986	Bovine spongiform encephalitis (BSE) agent	BSE in cattle
1988	HHV-6	Exanthema subitum
1988	Hepatitis E	Hepatits
1989	Hepatitis C	Transfusion Hepatitis
1992	H_5N_1	Avian flu
1995	HHV-8	Kapsoi sarcoma
1996	Hepatitis G	Hepatitis
1999	Nipah Virus	Flu
2003	Novel Coronavirus	SARS
2003	H_5N_1	Bird flu
2009	H_1N_1	Swine flu

Parasites		
1976	*Cryptosporidium parvum*	Acute and chronic diarrhea
1985	*Enterocytozoon bieneusi*	Persistent diarrhea
1986	*Cyclospora cayetanensis*	Persistent diarrhea
1992	*Encephalitozoon hellim*	Conjunctivitis
1993	*Encephalitozoon cuniculi*	Disseminated disease

PANDEMIC OF SWINE FLUE (H_1N_1) OUTBREAK OF APRIL 2009

Novel H_1N_1 causative organism has in fact, two genes from an avian virus (Eurasian swine 1979), three from old fashioned H_1N_1 in North American swine two genes from the triple reassortants in North American swine and final one from human transmitted from birds in 1968. In fact swine influenza viruses 2009 are influenza virus and the subtypes of influenza. A virus known as H_1N_1, H_1N_2, H_3N_1, H_3N_2 and H_2N_3.

The 2009 flue outbreak is due to new strain of subtype H_1N_1. In late April 2009, Margaret Chan (WHO Director General) declared a "Public Health Emergency of International concern when first case of H_1N_1 virus was reported in the USA. Following the outbreak on 02 May, 2009, it was reported in pigs at a garm in Alberta, Canada with a link to outbreak in Mexico. The pigs are suspected to have caught this new strain of virus from a farm worker who recently returned from Mexico, then showed symptoms of influenza like illness.

Due to reassortment there has been transfer of genes among strains crossing swine avian and human species boundaries. The new strain was described as reassortment of at least four strains of influenza "A" virus subtype H_1N_1 including one strain endemic in humans, one endemic in birds and two endemic in swine. Subsequent analysis revealed it was a reassortment of just two strains, both found in swine.

The intensity of problems can be imagined with the available data of over 254206 cases and 2837 deaths due to swine flue all over the world till 31st August, 2009.

Signs and symptoms: In humans symptoms include fever, cough, sore throat, body aches, headache, chills and fatigue, etc. There is increased percentage of patients reporting diarrhea and vomiting. Severe illness and death has occurred as a result of illness associated with this virus.

Usually people younger than 25 years of age are the victim of H_1N_1 2009. Adults older than 64 years do not appear to be at higher risk of H_1N_1 related complications. Children younger than 5 years, old pregnant women are at high risk of serious complications. Apart from this diabetes, heart disease, asthma, kidney, etc. patients are at high risk of serious complications.

Vaccines: CDC has isolated the new H_1N_1 virus and modified it to make doses of vaccine. There are efforts to produce good vaccine with H_1N_1 second virus. There is a suggestion by WHO strategic advisory immunization to fomulate vaccine with oil in water adjuvant and live attenuated influenza (H_1N_1) virus.

Other preventive measures:

1. Cover nose and mouth with tissue paper, while coughing or sneezing. Throw the tissue paper in trash after use.
2. Wash hands with soap and water especially after cough or sneeze, alcohol based hands cleaners are quite effective.

3. Avoid touching eyes, nose or mouth.
4. It is advisable to stay at home when sick (at least 24 hours after fever is gone).

Treatment: Antiviral effective are oseltamivir or zanamvir for treatment. Now there are reports of H_1N_1 strain resistant to oseltamivir from Denmark, Japan, etc.

RE-EMERGING MICROBIAL DISEASES

Re-emerging microbial diseases are those that have reappeared after a noteable decline in their incidence. Plague appeared in a big way in India in 1994 after a gap of around 27 years. Other important examples of re-emerging infection are cholera in 1995, dengue in 1996, etc. Other examples are as under:

Relaxation in immunization practices can fast result in resurgence of disease like spread of diphtheria in Russia and around. Spread of Hepatitis-B is related to techniques like kidney dialysis and multiple blood transfusions.

FACTORS RESPONSIBLE FOR EMERGING AND RE-EMERGING OF MICROBIAL DISEASES

1. Defective public health system
2. Unhygienic living environment.
3. Rapid urbanization
4. Industrial and other developmental activities.
5. Ecological changes because of human occupation of inhabited area.
6. Rapid means of transportation to and from any part of the world.
7. Natural disasters.
8. Migration of population.
9. Alterations of microorganisms.
10. Antimicrobial resistance
11. Development of insecticide resistance in vectors e.g. DDT resistance anopheles, vector of plasmodium.

Control and Prevention

1. Global surveillance
2. Improved diagnostic facilities
3. Immunization
4. Continuous research on war footing

By the end of the 20th century, vaccine really conquered many of man's most dreaded plague, eliminating smallpox and disease wiping out mumps, measles, rubella, whooping cough, diphtheria and poliomyelitis at least in developed countries.

Causative microorganisms	*Disease*
Myco. tuberculosis multi-drug resistant	Tuberculosis
Multi-drug resistant *Salmonella typhi*	Typhoid fever
Leptospira interrgans	Leptospirosi
Pseudomonas pseudomallei	Meliodosis
Bacillus anthracis	Anthrax
Yersinia pestis	Plague
Drug resistant *Plasmodium falciparum*	Malaria

70 Nosocomial Infections

Infections which are acquired from hospitals are called nosocomial infections. If the organisms come from another patient it is called cross infections and if the patient himself carries the infection to some other site then it is autoinfection. Infection may become apparent during the stay of the patient in the hospital or after his discharge from the hospital. There is actual increase in the frequency and severity of infection especially due to antibiotic resistant enterobacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Thus prolonged stay of the patient in the hospital is undesired and may be a serious matter for the patient and his family. Moreover, his maintenance in hospital and treatment is quite expensive. At the same time prolonged undesired stay of the patient in the hospital means occupation of bed which might otherwise be used for another needy patient.

PATIENTS REQUIRING ISOLATION

Some patients really need isolation. Patients of tuberculosis, typhoid, diphtheria, Lassa fever or smallpox should not be treated or nursed in open ward as these diseases are serious and easily transmissible. Similarly, infants with measles or whooping cough should not be nursed in general ward but may be treated at home. *Staphylococcus aureus* infection cases especially resistant to many antibiotics belonging to phage types (80/81 or 75/77) capable of causing serious epidemic of hospital sepsis, certainly require isolation. Isolation is also desirable for babies with *Escherichia coli* gastroenteritis and for many patients with *Pseudomonas aeruginosa* or diarrheal diseases infections. Isolation cubicles are suggested for these purposes which should be so designed, equipped and managed that no microorganism can pass from them to a ward. Attendant should use gown on entering the cubicle and remove on leaving. Washing facilities for the patient and attendant of the patient must be provided in the cubicle. Dressings should be discarded into paper bags which may be removed to incinerator. Beddings and clothings should be kept in disinfectant solution before sending to laundry. Excreta should also be treated with disinfectant and crockery should not be permitted to return to kitchen without sterilization. When the patient finally leaves the cubicle, it must be thoroughly washed with disinfectant and all equipment must be sterilized as far as possible.

HOSPITAL INFECTION AND PREVENTION

We should be aware of some important hospital infections and about their prevention:

1. *Wounds and burns:* It is important to remove all tissue debris from accidental wounds and burns as bacteria can establish more easily in damaged tissue. A careful and aseptic technique for dressing of wound preferably in dressing room reduces chances of cross infection.
2. *Urinary tract infection:* Catheter or other instruments into the bladder may cause urinary tract infection. Used catheters are difficult to sterilize and may be the cause of cross infection also, hence disposable sterilized catheter should be used aseptically. Continuous bladder drainage with indwelling catheter becomes necessary and

so receptacles of the catheter should not be open to ward air (ascending infection) but should preferably be kept in disposable plastic bag.

3. *Alimentary tract infections:* Outbreak of *E. coli* gastroenteritis in children and of *Shigella sonnei* dysentery do occur quite oftenly in hospital. Isolation, general hygiene and exclusion of carriers are important preventive measures.
4. *Baths as means of cross infections:* It is commonly seen that series of babies are made to have bath in a same sink thus resulting in dispersal of pathogenic organisms especially *Staphylococcus aureus* through nursery. Hence, it is emphasized that if newborn babies need to be bathed, this should be done in stainless steel bowls which can easily be autoclaved after the bathing of each baby.

EPIDEMIOLOGICAL MARKERS USEFUL IN INVESTIGATING HOSPITAL INFECTION

- Antibiogram and resistogram.
- Biotyping.
- Phage typing.
- Bacteriocin typing.
- Serotyping.
- Serum opacity factor (analysis of marker proteins, analysis of enzyme production, e.g. *Staphylococcus aureus*).
- RNA electrophoresis as is done in rotavirus.
- Cytotoxicity assay, e.g. *Proteus mirabilis.*
- Reverse phage typing, e.g. *Staphylococcus aureus.*
- Plasmid profile.

PREVENTION OF NOSOCOMIAL INFECTIONS

- Proper washing of hands.
- Isolation of patients, e.g. plague, influenza, measles, etc.
- Careful and appropriate use of instruments.
- Use of antibiotics only if required. It may be given to carrier staff or patient.
- Use of blood transfusion only if must. Disinfectant of excreta and infected material.
- Surveillance of infection properly and regularly.
- Use of vaccine, e.g. tetany gas gangrene, hepatitis B, etc.

FACTORS RESPONSIBLE FOR HOSPITAL INFECTIONS

- Neonates and aged patients have risk of getting hospital infection because of long stay and decreased immunity.
- Impaired defence mechanisms of patients due to disease or treatment.
- Hospital environment contains relatively heavy load of microorganisms.
- Major invasive diagnostic or therapy procedures.
- Advance treatment of cancer, organ transplantation, etc.
- Presence of multidrug resistant bacteria, etc.

The incident of hospital infection in developed countries is 2 to 12 percent. These are the hospitals where people suffering from various health problems are cured. Surprisingly the hospitals are also responsible for transmitting diseases (especially infectious) to the patients being treated in the hospitals. One cannot forget what Florence Nightingale once said that hospitals should do no harm to the patients. Unfortunately, in spite of best efforts microorganism free environment in hospitals has never been achieved.

SOURCES OF HOSPITAL INFECTION

- Infecting microorganisms from fellow patients which may be multidrug resistant.
- Infecting organisms from hospital staff.
- Infecting organisms from instruments, blood products, intravenous fluid, etc.
- From patient's normal flora, etc.
- Insects are also source of multidrug infection
- Organism may be present in air, dust, water, antiseptic solution, food, etc.
- Surfaces contaminated by patient's secretions, blood fluid, etc.

MODE OF INFECTION

- Airborne.
- Contact, e.g. hands, clothing, etc.

- Food and water.
- Hospital equipment and instruments.
- By parenteral route.

OUTLINE OF INVESTIGATIVE APPROACH OF HOSPITAL INFECTION OUTBREAK

It includes number of patients involved with their distribution in wards, times of onset, their symptoms, whether all or majority of cases followed operation if so whether they were operated in same operation theater and other clues as to the way in which they became infected. If their infection is by identical bacteria effort should be made to trace human carrier or other sources of infection. Outbreak of infection in the hospital is generally because of defective ventilation in the ward or theater, in aseptic technique or in sterilization of dressings or instruments and improper cleanliness of hospital kitchen plus its workers.

71 Hospital and Laboratory Waste

Hospital and laboratory waste includes all waste biological as well as nonbiological which is discarded and unlikely to reuse it in the hospital in future. Mainly hospital waste is of two following types:

Infectious waste: This type of waste of the hospital is harmful and may cause infectious diseases. In fact this type of waste may be microbiological waste, blood, body fluids, contaminated laboratory waste, sharps, pathological waste, soiled dressing, cotton plasters contaminated with blood or body fluids, cotton plugs, etc. Infectious waste is about 10 to 15% of total hospital waste.

Noninfectious waste: It is the waste of the hospital that is noninfectious. It comprises of 80 to 85% of total hospital waste. This category of hospital waste does not cause any harm.

Since we are facing diseases like AIDS, Hepatitis B, etc. It is necessary to follow universal precautions which are as under:

1. Consider all patients and clinical specimens potentially infectious especially for AIDS, Hepatitis B, etc.
2. All specimens especially blood must be dispensed in leak proof impervious bags for transportation.
3. Use of gloves, face mask with glasses while handling blood or fluid specimens.
4. Wear proper laboratory coats while working in the laboratory.
5. Pipetting by mouth must not be done.
6. Decontaminate the laboratory working surface with decontaminant after completion of laboratory procedures.
7. In case of spillage of blood or other specimen treat the surface with appropriate disinfectant.
8. Biological safetyhood must be used for laboratory procedures.
9. All potentially contaminated material should be decontaminated before disposal or reuse.
10. Remove all protective wearing/clothing before leaving the laboratory or attending the patients in operation theater or OPD.

INFECTIOUS WASTE OF HOSPITAL AND LABORATORY

1. Microbiology waste
2. Blood and body fluids
3. Used sharps
4. Pathological waste, i.e. samples and tissues
5. Bandages and cotton swabs
6. Animal carcasses
7. Beddings.

INFECTIOUS MICROORGANISMS ASSOCIATED WITH HOSPITAL OR LABORATORY

1. HIV
2. Hepatitis B
3. *Brucella*
4. *Mycobacterium tuberculosis*
5 *Bacillus anthracis*
6. *Francisella tularensis*
7. *Shigella*
8. *Salmonella*
9. *Coccidiodes immitis*

SEGREGATION OF HOSPITAL WASTE

Hospital waste is segregated and disposed of as under:

1. Yellow plastic bags and containers with human anatomical and pathological waste

are directly dispatched for deep burial or incineration.
2. Red plastic bags and containers having infectious waste material are quickly sent for sterilization by autoclavation. Thereafter they are disposed of by land filling.
3. Blue plastic bags and containers with plastic and rubber disposable material are first of all cut into small pieces to prevent reuse followed by treatment with sodium hypochlorite solution. Now they are autoclaved and disposed of by land filling or burial. If feasible treated material as mentioned above may be transported to reputed plastic industry for further reuse. By no means should it be allowed to incinerate or burn as they contain halogenated polyvinyl chloride. It emits dioxin gas into the air which is highly carcinogenic.
4. Blue or white transparent puncture proof containers need a little different treatment before disposal. Sharps must be destroyed, disinfected with 1% sodium hypochlorite solution and then finally disposed of in sharp pit that is covered and protected and not accessed to rag pieces for reutilization.

However, noninfectious waste can be collected in any appropriate container and dispatched to municipal garbage bins.

TECHNIQUES FOR WASTE TREATMENT

Chemical Method to Dispose of Hospital Waste

Formaldehyde 6 to 8%, glutaraldehyde, hydrogen peroxide 6 to 30% or sodium hypochlorite 0.1% are in common use.

Incinerator

Advantages

- Ensures complete and safe disposal of waste.
- Volume of waste is reduced considerably.

Disadvantages

1. Sharps cannot be disposed of.
2. Not suitable at all for PVC plastic material because of possible emission of harmful gases.
3. It is quite expensive to operate and maintain.

Autoclave

Uses

1. Microbiological and pathological waste
2. Blood and its products
3. Sharps
4. Plastic waste
5. Body fluids.

Advantages

1. Quite economical
2. Easy to operate
3. Waste is transformed into noninfectious form
4. Waste is disinfected without hazardous emission.

Disadvantage

In fact there is no reduction of volume of waste at all.

Microwaving

Uses

1. In real sense it involves the use of radiations produced by microwave to break down molecular chemical bonds and as a result disinfect waste.
2. This process may require preshredding the waste and injecting it with steam treatment chamber. Thus, they evenly heat it for 25 minutes at 97°C to 100°C under series of microwave units (radiation spectrum between frequencies of 300 to 300,000 MHz).

Advantage

It disinfects waste without any harmful emission.

Disadvantages

As it requires pretreatment of shredding of the waste, it is full of risks for infectious waste. Further, it cannot be used for cytotoxic hazardous and radioactive waste.

Hydroclaving

Advantages

1. It is used to dispose of microbiological and pathological waste, sharps, etc.
2. No pretreatment is needed.
3. Perfect fragmentation and dehydration is done.
4. It is ideal for infectious waste.
5. Volume as well as weight reduction makes its transportation and land filling easy and less expensive.
6. There is no emission of toxic gases etc.

It is quite simple, cheap to operate and maintain.

There is hardly any disadvantage of hydroclaving.

Plasma Technology

It allow a complete and satisfactory destruction of waste.

Advantages

1. It does transformation of all hydrocarbonated products into combustible gases without leaving behind any solid remnant.
2. Here there is no need of segregation of waste.

DISPOSAL OF HOSPITAL AND LABORATORY WASTE

1. Land filling
2. Burial
3. Sewage draining

Infectious hospital and laboratory material after treatment may be disposed of by land filling or deep burial. Waste in fluid form can be discarded in sewage drains. Apart of this treatment incineration is also a vital method of disposal.

72 Diagnostic Virology

Laboratory Diagnosis of Viral Disease

With rapid techniques for the diagnosis of many virus infections and availability of antiviral drugs, diagnostic virology is becoming a routine procedure. The information regarding viral etiological agent may lead to precise diagnosis, more accurate prognosis, possibility of anticipating need for special treatment and it may spare the patient from unnecessary hazard of antibiotic treatment for an imagined bacterial infection. Importance of etiological diagnosis is emphasized in many viral infections like, rubella in pregnant women, herpetic lesion of eye (early treatment suggested), upper respiratory tract infection and aseptic meningitis.

In majority of virus infections the organisms are easily demonstrated in early phase of illness before the symptoms appear. Best time of collection, nature and site of collection of specimen depends upon individual virus diseases. Rapid diagnosis is made by microscopic or serological demonstration of virus particles or antigens present in the specimen of the patient. However, isolation by growing it in embryonated eggs, tissue culture or in animal is time consuming but more sensitive and conclusive method.

Following are the indications for the diagnosis of viral infections.

1. Viruses like herpes have antiviral drugs. Drugs against HIV are also becoming available.
2. For proper management of diseases caused by rubella (abortion in first trimester). Post-exposure immunization in early establishment of rabies prevents the development of rabies. Cesarean section is required if the patient has primary genital herpes. If a baby is borne of an HBsAg positive mother, immunization birth is a must.
3. Prevention of spread of viruses hepatitis HIV B and C from carriers to others is possible by screening of blood donors.
4. Dangerous epidemics like dengue, yellow fever, encephalitis influenza, poliomyelitis is of importance in alerting authorities to start antimosquito control measures, immunization, etc.
5. Reporting of new viruses.

Collection of Specimen

The appropriate specimen should be collected from patient, preserved and transported to laboratory in proper manner with clinical information. There should be least delay in sending samples to the laboratory. Some viruses are relatively robust, e.g. pox viruses and they do not require precaution during transport. Of course precautions are taken to prevent infection to those who transport the specimen. There are viruses which cannot survive at room temperature and so material containing them should be kept at 4°C. Drying is another hazard to virus survival and so specimen should be placed in 0.2 percent bovine albumin mixed in buffered salt solution. Further, bacteria present in specimen may interfere with isolation of virus and they must be killed by using appropriate antibiotics.

Processing of Specimen and Procedure

In the laboratory following methods are used for the demonstration of etiological agent or detection of specific antibodies.

1. *Microscopic examination*: Ordinary microscope for detection of inclusion body for rabies in dog or elementary bodies in pox virus infection is useful. Ordinary microscope is also useful for examination of stained smear or tissue sections for characteristic histological changes. Electron microscope although expensive yet is of immense use as wider range of viruses are seen and recognized. Electron microscope provides one of the diagnostic methods for the demonstration of rotavirus. Immunofluorescent techniques are being used for the rapid identification of viruses.
2. *Demonstration of virus antigen*: In smallpox, antigen is abundant in lesions and so used to be demonstrated rapidly by precipitation in gel (PIG) or immunofluorescence techniques.
3. *Isolation of virus*: The methods of isolation to different viruses are different. If animal inoculation is applied viruses are identified by diseases and particular lesions they produce. In case embryonated eggs are used identification of virus may be made by lesions they produce on the chorioallantoic membrane or by the presence of their antigen in fluid from cavities. Tissue culture is becoming more popular as it is convenient and cheap.
4. *Serological diagnosis*: Rising titer of antibodies to virus during disease is supporting and almost confirmatory evidence about the etiological agent. For it, always paired sera (collected during early course of disease and convalescent sample about 10 to 14 days later) is used. The serological methods widely used are, complement fixation, hemagglutination inhibition, neutralization, immunofluorescence, radioimmunoassay (RIA), immunoelectrophoresis, ELISA, etc.

73 Emergency Microbiology

Almost daily clinicians and microbiologists come across clinical situations where rapid detection and characterization of an infection are essential for the diagnosis and effective treatment of infectious diseases. Only a few techniques in clinical microbiology laboratory can be performed within 1 to 2 hours. Hence they can be of much use in patients with overwhelming sepsis, e.g. meningococcemia. Other cases with meningitis or other life-threatening acute infections may benefit greatly by emergency laboratory procedures. In general, emergency procedures rely upon:

1. The visualization of the agent causing disease.
2. The detection of specific antigens of the offending organisms.
3. The detection of substances produced by the organisms while infecting the patient.
4. The detection of substances produced by the host to the infectious process.

While each of these approaches is valuable, results obtained by any of these techniques do not eliminate the need to submit specimens for conventional studies culture and antibiotic sensitivity test.

VISUALIZATION OF THE AGENT OF DISEASE

Microscopic detection of stained or unstained microorganisms is of great value for the detection of infection. Microscopic methods like phase contrast, Darkfield, light and electron microscopy have been employed to detect agents of infection. Phase contrast microscope may be employed for visualizing fungal elements in clinical specimen, Darkfield microscope is quite helpful in the diagnosis of early syphilis and electron microscope for visualizing viral particles from clinical specimen like stool. Light and ultraviolet microscopes are widely employed especially for Gram and acid-fast stain. Acid fast bacteria are detected in clinical specimens by Ziehl-Neelsen, Kinyoun and auramine/rhodamine stain.

When appropriately applied to clinical situations, Gram stain is of immense use. Table 73.1 presents a list of diseases and specimens that may assist in the rapid diagnosis of infections.

Recently, application of fluorescent acridine orange stain to clinical specimens such as urethral exudate is recommended as it is more sensitive than visible microscopy. An additional benefit is that the smears stained with acridine orange can later be Gram stained without previous decolorization.

ANTIGEN DETECTION

The detection of antigens and substances associated with the intact infecting agent or of antigens that are disassociated from the agent is an alternate means of rapidly detecting the presence of an infecting agent.

Its application is in the use of specific antiserum to serologically identification of pneumococci in clinical specimen by Quelling reaction. Use of fluorescein conjugated antibody reagents is helpful in rapid detection of organisms like *Bordetella pertussis, Corynebacterium diphtheriae, Legionella pneumophila*, viral, and rickettsial infections.

Increased sensitivity in detecting microbial antigens has been achieved by the use of more sensitive serologic tests like indirect particle agglutination, counter immunoelectropho-

TABLE 73.1: Relation of Gram's smear from clinical specimens and diseases

	Diseases	*Specimens*	*Results*
1.	Septicemia	Buffy coat	Intracellular/extracellular bacteria
2.	Endocarditis	Skin lesion	Intracellular/extracellular bacteria
3.	Meningitis	Sediments of spinal fluid	Intracellular/extracellular bacteria
4.	Wound infection	Biopsy aspirate	Presence of predominant type(s) of organisms present
5.	Urinary tract infection	Midstream urine	Presence of > 1 organism/oil immersion
6.	Pneumonia	Expectorated sputum	Predominance of sputum polymorphonuclear leukocytes and general absence of squamous epithelial cells. Predominant organisms present

resis, radioimmunoassay and ELISA. The later test for cryptococcal antigen has proven to be of significant value in detecting cryptococcal infections in man. Counterimmunoelectrophoresis has been successfully used for rapid detection of antigens like *Neisseria meningitidis*, *Pneumococcus*, *Haemophilus influenzae* type b, streptococci, *Escherichia coli* with K or capsular antigen in CSF and other clinical specimens. This method is not very sensitive but is quite useful in identifying the causative agent in meningitis quickly. Radioimmunoassay and ELISA are more sensitive and used for the detection of hepatitis virus, rotavirus and many other microbial antigens.

An exciting development is the serological detection of microbial antigens using monoclonal antibodies. Antibodies produced this way are more sensitive, specific and more active and so no false-positive results. The Limulus Amebocyte Lysate test (LAL test) is shown to be very useful for rapid diagnosis of gram-negative meningitis, gram-negative bacteriuria and gonococcal urethritis.

Detection of Substances Produced by the Infecting Organisms in Specimens

A classic example is the detection of *Clostridium botulinum* toxin in serum and stool specimens obtained from patients with botulism. A more recent example is the diagnosis of antibiotic associated colitis by detecting *Clostridium difficile* toxin in stool specimen.

Infectious process may be detected by identifying the presence of microbial enzymes in clinical specimen, e.g. aminopeptidases, propanediol oxidorductase, and B lactamase.

One method of detecting microbial activity is by the detection of microbial metabolites in clinical specimens. Rapid diagnosis of invasive candidiasis is by detection of D-arabinitol, a fungal metabolite.

Detection of Substances Produced by the Host in Response to Infection

Classic examples are serodiagnostic tests employed to diagnose syphilis which measure treponemal and reaginic antibodies. However, these tests are of little value in first week of disease and more useful in late primary, secondary and tertiary disease. Detection of IgM antibody is of proven value in the rapid diagnosis of toxoplasmosis, rubella and many viral infections.

Concentration of lactic acid in cerebrospinal fluid and joint fluid may be of value in diagnosing infectious meningitis and infectious arthritis. Lactic acid concentrations in excess of 30 to 50 mg/dl have proven helpful in identifying the patient with bacterial meningitis.

74 Bacteriology of Milk, Air and Water

BACTERIOLOGY OF MILK

Milk is an excellent medium for the growth of even pathogenic organisms.
It may contain following types of bacteria:

- Acid forming bacteria, e.g. *Streptococcus lactis*, *Streptococcus fecalis*, lactobacilli, etc.
- Alkali forming bacteria, e.g. alkalegenes aerobic spore bearers, achromobacter, etc.
- Gas forming bacteria, e.g. coliform bacteria, *Clostridium perfringens, Clostridium butulinum,* etc.
- Proteolytic bacteria, e.g. *Bacillus subtilis, Bacillus cereus*, *Proteus vulgaris*, staphylococci, micrococci, etc.
- Inert bacteria (produce no change in milk) achrobacters and pathogenic bacteria in milk are examples of this group of bacteria.
- Human milk may contain organisms like *Staphylococcus epidermidis, Streptococcus mits*, Gaffkya tetragena, *Staphylococcus aureus*, etc.

Sources of Bacteria in Milk

- Milk ducts of udder.
- Milk equipment like unsterilized collecting vessels of milk.
- The milker's hand also contribute to bacterial contamination of milk. It can be avoided by washing hands properly before milking.
- Unclean udders and dust in milking shed.
- Contaminated water used for cleaning the udder and adulteration of milk with contaminated water contribute to contamination of milk.
- Milk from diseased animal, e.g. brucellosis, tuberculosis, etc.
- Carriers of typhoid, paratyphoid, dysentery, staphylococci, streptococci, etc. involved in milking may act as sources of pathogenic bacteria of milk.

Milk-borne Diseases

1. Infection of animals transmitted to man
   - Tuberculosis
   - Brucellosis
   - Enterotoxigenic staphylococcal
   - Streptococcal
   - Q-fever
   - Anthrax
   - Campylobacter fetus
   - *Yersinia enterocolytica*
   - Leptospira.
2. Milk contaminated with excreta of small mammals, etc.
   - Tick-borne encephalitis
   - Streptobacillus moneliformis.
3. Infections primarily of man transmitted through milk
   - Typhoid fever
   - Paratyphoid fever
   - Cholera
   - Shigellosis
   - Tuberculosis
   - Hepatitis
   - Staphylococcal food poisoning
   - Enteropathogenic *Escherichia coli* causing diarrhea or dysentery.

Pasteurization of Milk

It eliminates the risk of most of the infections like tuberculosis, brucellosis, Q-fever, salmonellosis, etc. Pasteurization can be done.

- Holder method (heating at 62°C for 30 minutes followed by rapid cooling to 5°C).

- Flash method (heating at 72°C for 15 seconds followed by rapid cooling to 4°C and large quantity milk can be pasteurized by this method.
- Ultra high temperature (UHT) method where milk is heated 125° to 132°C for a few seconds two to three times and then rapidly cooling it. It bottled as quickly as possible.

Biological Standards of Milk

- Untreated milk may contain 500 bacteria per ml or more. It is also called raw milk.
- Pasteurized milk should not have coliform bacteria in 0.1 ml milk. On submission to phosphatase test it must give reading not more than 10 gm of p-nitrophenol per ml of milk.
- Sterilized milk must satisfy turbidity test.
- Ultraheated milk should contain bacteria less than 10 bacteria per 0.01 ml.

Bacteriologic Examination

1. Collection of milk sample is done by mixing thoroughly bulk milk contained in a container. Sample to be tested is collected well below the surface with a sterile dipper and poured into a sterilized screw capped or stoppered bottle of 125 ml or so capacity. The sample is kept in an insulated container and dispatched to the laboratory without delay as there it is put for test as early as possible. Unopened bottles or polythene bag of milk may be sent to the laboratory similarly.
2. Bacteriologic examination of milk comprises of following tests:
   - Viable count of raw milk may be about 500 to several million per ml. The test is done by doing plate count by serial dilution of the milk samples (1:10, 1:100, 1:1000 with sterile ringer solutions). After thorough mixing 1 ml of appropriate dilution is mixed with 10 ml of preheated and cooled milk extract milk agar medium which is poured in plates (Petri dishes). They are incubated at 30° to 31°C for 72 hours. Count the number of colonies after incubation and multiply by dilution factor. Now report the results as number of viable bacteria per ml of milk.
   - Coliform count is tested by inoculating different dilutions of milk into MacConkey's liquid medium (3 tubes) and record the production of acid and gas after incubation. The absence of acid and gas from at least two tubes means that milk has passed the test. The coliform contamination occurs from dirty utensils, dust and dairy workers. The presence of coliforms in properly pasteurized milk (phosphatase test negative) indicates that contamination has taken place after pasteurization. The coliform should be absent in 1 ml milk.
   - Methylene blue reduction test is a simple substitute of viable count. Viable bacteria reduce the dye in milk when kept in cool and dark place. Solution of methylene blue used gives a final concentration of 1/300,000. The milk is decolorized after reduction of methylene blue. The rate of reduction of methylene blue is related to the degree of bacterial contamination of milk. Raw or untreated milk is taken as satisfactory in case it fails to decolorize methylene blue in 30 minutes under standard conditions.
   - Resazurine test is also dye reduction test. Reduction of resazurine by bacteria passes through a series of color changes, i.e. blue, lilac, pink to finally colorless if no reduction occurs. Generally, 10 minutes test is done, in which shade of color is recorded after incubation with milk for 10 minutes.
   - Phosphatase test is based on the principle that phosphatase enzyme normally present in milk is inactivated after proper pasteurization of milk. The standard technique of detecting phosphatase is based on its ability to break down disodium p-nitrophenyl phosphate and liberate p-nitrophenyl. This test is done by taking 1 ml of milk to

be tested and adding it to 5 ml of buffer substrate solution (buffer + disodium phenyl phosphate) in a test tube. After incubating the tube in water bath at 37°C for 2 hours the color of the solution mixture is recorded. Yellow color solution of p-nitrophenol means milk contains phosphatase. In properly pasteurized milk the concentration of p-netrophenol is less than 10 µg per ml of milk.

- Turbidity test distinguishes between pasteurized and sterilized milk. This is a definitive test for sterilized milk. Absence of turbidity indicates that milk has been heated to at least at 100°C for 5 minutes. The soluble proteins in milk get denaturated and so cannot be precipitated by ammonium sulfate.
- Test for tubercle bacillis is done by centrifugation of milk at 3000 rpm for 30 minutes and sediments inoculated into two guinea pigs. Animals are observed for a period of 3 months for tuberculosis (after killing the animals one after 4 weeks and second at 8 weeks after inoculation). Tubercle bacilli must be isolated only in culture.
- Test for brucellosis is done by inoculating milk on serum dextrose agar. The milk is centrifuged and deposit of milk may be injected intramuscularly into guinea pigs. The animals are killed after 6 weeks and serum is examined by *Brucella abortus* suspension and spleen of animal is used for culture of brucellae.
- Milk ring test is very sensitive test for the presence of brucella antibodies in the milk of infected cows, which is detectable in the bulked supply from a dairy herd. To confirm a positive milk ring test, Whey's agglutination test can be done to detect the antibodies.

In case of suspected food poisoning centrifuged milk should be examined for *Staphylococcus aureus, Salmonella, Campylobacter* and *Yersinia enterocolitica.*

BACTERIOLOGY OF AIR

There has never been pure air. Foreign substances including microbes, pollens and many more of them are present in the air in all the places at all times. The levels of bacterial contamination of air is expressed as the number of bacteria carrying particles cubic meter or per cubic per feet.

Since a man breath in about 500 cubic feet of air in a day. The contents of air is quite important especially when it contains pathogenic organisms. Naturally, a person becoming infected will be greatest if he inhales air containing large number of pathogenic microbes. Pathogenic bacteria usually do not multiply in the air.

The bacterial contents in outdoor air depends on:

- Density of human and animal population
- Nature of soil
- Amount of vegetation
- Humidity of air
- Temperature of air
- Wind conditions
- Rainfall
- Sunlight.

The characters of outdoor air are:

- The air in ocean is almost free from bacteria.
- Outdoor air contains usually nonpathogenic microorganism like micrococcus, *Bacillus subtilis*, sarcina, achromobacter, fungi and spores.
- Number of organisms is less indoor air.
- Air at higher altitude contains much less microbes.

Characters of indoor air are:

- Contains droplets of organisms disseminated by man and animals usually through nose and mouth.
- Droplet nuclei are a type of particles implicated in spread of airborne infection.
- Droplet nuclei are 1 to 10 µ in size and are formed by evaporation of droplet coughed, sneezed or by aerosols and disseminated into air. These can be formed in the laboratories and autopsy rooms. Particles are drawn into the alveoli of the lungs and are retained there.
- Diseases by droplet nuclei include, tuberculosis, influenza, chickenpox, measles, Q-fever, other respiratory diseases, etc.
- Some larger droplet expelled during coughing or sneezing settle down on floor,

carpet, furniture, bedding, etc. Many pathogenic organisms can survive there for quite a long time and these dust particles can be blown by air. Diseases transmitted by dust particles are streptococcal, staphylococcal, psittacosis and coccidioidomycosis, etc.

Indications of Bacteriological Examination of Air

- Surgical theaters.
- Kitchen or premises where food articles are prepared and packed.
- Premises of pharmaceutical preparation.
- Hospital wards where hospital infections have occurred.

Methods of Bacteriological Examination of Air

They are as follows:

Settle Plate Method

- Also called sedimentation method.
- It is based on the principle of measuring the rate at which bacteria carrying particles settle down.
- Open plates of culture media are exposed to air for ½ to 1 hour and incubated at 37°C for 24 hours.
- The number of colonies are counted.
- This method gives an impression about the number of microorganisms and species present in air.
- This method is especially used for testing the air in surgical theaters and hospital wards.
- Blood agar plates can be used to detect both pathogenic and nonpathogenic organisms.
- This method is simple but capable of measuirng only the rate of deposition of large particles from air.

Slit Sampler Method

- It gives count bacteria carrying particles containing in a given volume of air.
- This method is better than settle plate method. Efficiency of detection even small particles containing organism is quite high.
- In this method a known volume of air is directed onto a plate of culture medium through a slit of 0.25 mm width, the plate being mechanically rotated in order to ensure uniform distribution of organisms all over the plate containing medium.
- One cubic foot of air is allowed to pass through the slit and likewise 10 cubic feet of air is tested. The culture media are incubated and colonies counted which gives the bacterial number present in the air.

Bacteriological Examination of Environment Dust

Air Centrifugation

- This involves the principle of centrifuging particles on a culture medium borne on a plastic strip.
- After sampling the strip is removed from the instrument and incubated at 37°C for 48 hours and colonies are counted.
- However, this method in less efficient for particles less than 5 µ in size.
- This method is inferior to slit sampler method.

Sweep Plate Method

- Useful for detection of organisms from personal clothing, bed clothes, curtains having bacteria laden dust.
- An open culture plate is swept 8 to 10 times over the dusty surface with swab soaked in nutrient broth and culture plate incubated and colonies counted after overnight incubation.

Dust Sampling

- Moistened cottonwool swabs can be used for collecting dust from floor, wall, furniture, etc.
- The swabs are placed in broth as well as in Robertson cooked meat media and incubated.
- After subculturing on plates, the organisms can be identified.
- This is used as a routine for reviewing the level of asepsis in surgical theater especially for demonstration of spores of *Clostridium tetani* and other spore bearers

in theater dust (in positive cases fumigation of theater is advised and theater is closed till bacteriological report shows negative results regarding *Clostridium tetani*).

Interpretation

- Most bacteria found in the air are harmless saprophytes or commensals.
- About 1 percent of airborne organisms in the wards or closed room are harmful.
- Streptococcus may be found in large number, i.e. 10 per cubic feet in a room occupied by the streptcoccal infected patients like streptococcal tonsillitis or scarlet fever.
- *Staphylococcus aureus* is predominant airborne organisms (0.1 to 10 organism per 1 cubic feet air).
- Man may be infected even when he inhales only one *Mycobacterium tuberculosis* in 500 cubic feet air that he respires during 24 hours.
- Air contamination standards are as follows.
  a. Operation theater, etc. should have not more than 10 per cubic feet.
  b. Dressing room, operation theaters for neurosurgery and theaters catering burn patients the bacterial count should not exceed 1 per cubic feet.
  c. Factories, offices, homes should have count below 50 per cubic feet.

BACTERIOLOGY OF WATER

Water is said to be contaminated when it contains bacterial, parasitic, viral agents, poisonous chemical substances, industrial or other wastes or sewage. The term polluted water is synonymous with contaminated water. A safe water is one that cannot harm the consumer even when ingested over prolonged period.

Bacterial Flora of Water

Natural Flora of Water

- Micrococcus
- Pseudomonas
- Serratia
- Flavobacterium
- Alkaligenes
- Acinetobacter.

Mixed with Soil

- *Bacillus subtilis*
- *Bacillus mycoides*
- *Enterobacter aerogenes.*

Mixed with Sewage

- *Escherichia coli*
- *Streptococcus faecalis*
- *Clostridium perfringens.*
- *Salmonella typhi*
- *Vibrio cholerae.*

Sewage Proper Bacteria

- *Bacillus vulgaris*
- *Clostridium sporogenes*
- Nocardia.

Water-borne Diseases

Bacterial

- Cholera
- Typhoid
- Paratyphoid
- Diarrhea
- Bacillary dysentery
- *Yersinia enterocolitica*
- *Campylobacter jejuni*
- *Campylobacter coli.*

Viral

- Hepatitis-A
- Hepatitis-E
- Poliomyelitis
- Rotavirus diarrhea.

Protozoal

- Amoebiasis
- Giardiasis
- *Balantidium coli* diarrhea.

Helminths

- Roundworm
- Whipworm
- Threadworm
- Hydatid disease.

Aquatic Host

- Cyclope (Guinea worm, fish tapeworm)
- Snail (Schistosomiasis).

Indicators Bacteria of Human Pollution (Intestinal/Feces) of Water

- *Escherichia coli* in particular and coliform bacteria as a whole.
- *Streptococcal faecalis.*
- *Clostridium perfringens* which can reduce sulfite.

Coliforms

- *Escherichia coli* is a typical example of fecal group whereas *Klebsiella* is an example of non-fecal group of organisms.
- The presence of *Escherichia coli* in water is an indication of recent excretal pollution of water as *Escherichia coli* survive in water for few days.
- Presence of *Escherichia coli* in water is an indicator of fecal contamination as they are only discharged from human or animal intestine. About 200 to 400 billion of these organisms are circulated by man per day. *Escherichia coli* are always foreign contents in drinking water.
- *Escherichia coli* and other coliform bacteria can be easily detected in culture even one bacteria in 100 ml. This is not so with pathogenic organisms because pathogenic organisms are few in number and tend to die out more rapidly.
- In short presence of *Escherichia coli* in a sample of water indicates the probable presence of intestinal pathogens in water supply but definitely fecal contamination is there.
  a. Streptococcal faecalis are regularly found in excreta although their number is less than *Escherichia coli.* Their presence in water along with *E. coli* is a confirmatory test of recent fecal contamination of water.
  b. *Clostridium perfringens* is also present in stools in small number. Presence of spores suggest that fecal contamination is there. In absence of coliform bacteria their presence indicates that fecal pollution of water occurred quite sometimes ago.

Sampling of Water

- Sample of water should be regularly and frequently collected to find out seasonal variations regarding microbes.
- Collection of water should be done in sterilized bottles which bear important information like data, time, locality and source, etc. These bottles containing samples are dispatched to laboratory as soon as possible.
- Volume of water collected should be 200 to 300 ml to be sufficient enough for laboratory tests.
- Due care should be taken in the collection of water to avoid contamination during collection procedure.
- Each 100 ml of water should contain 0.1 ml of fresh 1.8 percent (w/v) aqueous solution of sodium thiosulfate to neutralize the bacteriocidal effects of chlorine.
- The emphasis should be to examine water bacteriological daily if the water supply is serving more than 100,000 population.

Types of Water to be Sampled

Water Sample from Tap

- Remove the dirt from tap with clean cloth.
- Open the tap and let it flow for about 3 to 5 minutes.
- Close the tap and sterilize it with gas burner, spirit lamp for 1 to 2 minutes.
- Open the tap now and allow the water to run for about 1 minute.
- Sterilized container is opened and allow the required quantity of water from tap to flow in. Always leave airspace in the water container to facilitate shaking at the time prior to subjecting the water to bacteriology tests.
- The cap of container or stopper and brown paper is fixed in place and properly tightened with thread or string and transported as required.

Sample from Reservoir

- Open the bottle and fill it by hold the lower part of bottle and submerging it to a depth of 20 cm, with its mouth facing sideward and then upwards.
- All sterilized precaution are to be observed in this collection process.
- Bottle is closed with lid or cap and packed. Ideally, it should be sealed and bear on label attached all vital information.

- After collection it should be dispatched to laboratory for processing as early as possible.

Sample from Well

- Attach a stone or lead of suitable size weight to the sampling bottle with a piece of thread or string.
- About 20 meter length of clean thread or string is tied on the bottle.
- Bottle is opened by removing the cap and lowered into the well right to the bottom of well.
- When water get filled up with water it is pulled out.
- A little water is thrown out to create air space in the bottle.
- The water bottle is closed with cap, properly packed and sealed.
- A lable bearing important information is sticked on the bottle.
- Sample should be preferably tested in the laboratory within 1 hour of collection and in no case testing should be delayed beyond 6 hours of collection of sample (better bottle kept in ice).
- Where delay is inevitable, water can be filtered using a membrane filter and then it can be transported on an absorbent transport medium.

The following tests are important for bacteriological examination of water.

Presumptive Coliform Test (Fig. 74.1 and Table 74.1)

Two following methods can be used for the estimation of probable number of coliform bacilli in water.

i. *Multiple tube method:* With this method most probable number (MPN) of coliform bacilli are reported in 100 ml of water.

TABLE 74.1: Classification of water samples based on bacteriological tests

Class	*Presumptive coliform count per 100 ml of water*	*Escherichia coli count per 100 ml of water*
I. Excellent	0	0
II. Satisfactory	1 to 3	0
III. Suspicious	4 to 10	0
IV. Unsatisfactory	10 or more	0.1 or more

Double strength and single strength liquid MacConkey medium sterilized in tubes having Durham's tubes for detection of gas production is required medium contains promocresol purple as an indicator.

For the test proceed as under:

- One 50 ml of water to 50 ml of double strength medium.
- Ten ml of water to each of 5 tubes containing 10 ml double strength medium.
- One ml of water to each of 5 tubes containing 5 ml of single strength of medium.
- 0.1 ml of water to each of 5 tubes containing single strength medium.
- The above set of tubes are incubated at 37°C and examined after 18 to 24 hours. The probable number of coliform per 100 ml are read off from probable table of McCrady. Tube showing formation of acid (change of color) and gas bubbles (trapped in Durham's tube) is taken as positive.

Differential coliform test is also called Eijkman test and is done to confirm whether the coliform bacilli demonstrated in presumptive test are *Escherichia coli* or not. After the presumptive test, subcultures are made from all tubes showing acid and gas to fresh tubes of single strength MacConkey's medium which is brought to 37°C. Now these tubes are incubated at 44°C in water bath and examined after 24 hours. Tubes showing formation of acid and gas are having growth of *Escherichia coli* (number of bacilli estimated from McCrady table). Further confirmation of *Escherichia coli* can be obtained by biochemical tests, i.e. indole production and citrate utilization.

ii. *Membrane filter*

- In some countries used as standard technique for presence of coliform bacilli in water.
- A measured volume of water is filtered through cellulose ester membrane.
- Bacteria are retained on the surface of membrane. The membrane is inoculated (face upwards) in an appropriate medium for 15 to 20 hours.
- Number of colonies are counted directly.
- This test is not suitable for turbid water.
- Advantage of this test is that bacterial colonies are counted in 20 hours.

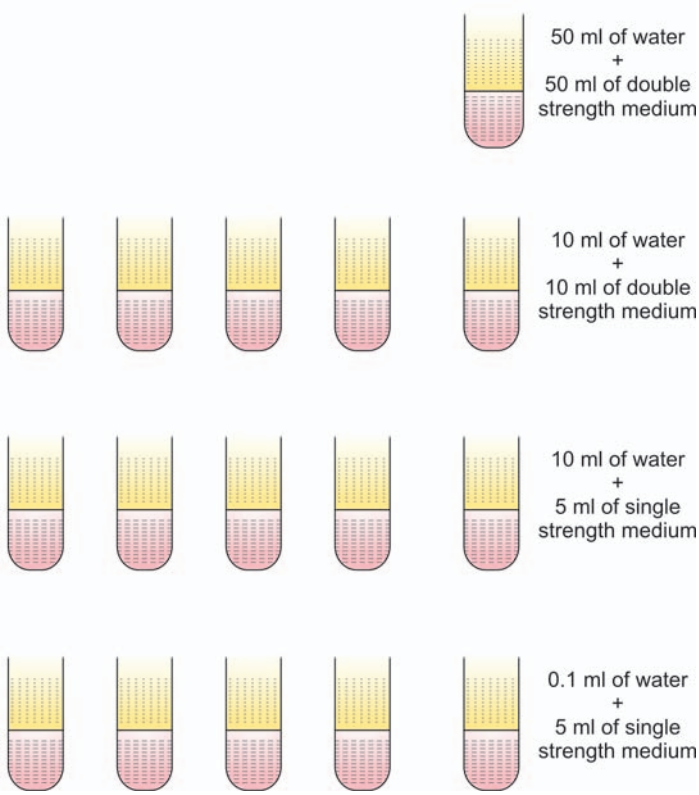


Fig. 74.1: Presumptive coliform test

Detection of Fecal Streptococci and *Clostridium perfringens*

- Their presence in water shows fecal contamination of water in doubtful cases.
- Subculture from presumptive positive tubes in coliform test are made into tubes containing 5 ml of glucose aside broth and incubated at 45°C. Production of acid in medium indicates the presence of *Streptococcus faecalis*.
- The water sample is inoculated in litmus milk and incubated anaerobically at 37°C for 5 days. Stromy fermentation (clot reaction) with acidity confirms *Clostridium perfringens*.

Colony Count

- One ml of water is placed in Petri dish (10 cm diameter).
- Ten ml melted yeast (45° to 50°C) agar is added on water, mixed them thoroughly and allow to solidity.
- Another plate is prepared as discussed above.
- One plate is incubated at 22°C and other at 37°C for 18 to 24 hours.
- Colonies are counted after incubation with the help of hand lens.
- Each colony represents one bacterium and so number of colonies counted mean number of bacteria per ml of water.
- Organisms growing at 22°C shows the amount of decomposed organic matter present in water. These organisms are no pathogenic.
- Growth of organisms at 37°C is more important index of contamination of water.

Viruses in Water

- Although methods are available for isolation of enteroviruses and other cytomegaloviruses from water, they are the part of routine water tests.
- Viruses in water are killed by chlorination, with free residual chlorine more than 0.5 mg per liter of water. These should be minimum contact period of 30 minutes at pH below 8. There should be 1 nephalometric turbidity unity or less.

Part IX
Appendices

Appendices

Appendices

APPENDIX 1
Disease candidates for worldwide eradication—International task force for disease eradication (1990-1991)

Disease	*Current annual toll worldwide*	*Chief obstacles to eradication*	*Conclusion*
Mumps	Unknown	Lack of data on impact in developing countries; difficult diagnosis	Potentially eradicable
Rubella	Unknonw	Lack of data on impact in developing countries; difficult diagnosis	Potentially eradicable
Hepatitis B	250,000 deaths	Carrier states; *in utero* infection not preventable; need routine infant vaccination	Not now eradicable but could eliminate transmission over several decades
Neonatal tetanus	770,000 deaths	Inexhaustible environmental reservoir	Not now eradicable but could prevent transmission
Diphtheria	Unknown	Difficult diagnosis; multiple dose vaccine; carrier state	Not now eradicable
Whooping cough	60 million cases 700,000 deaths	High infectiousness; early infections; multiple dose vaccine	Not now eradicable
Yellow fever (Not in India)	710,000 deaths	Sylvatic reservoir, heat labile vaccine	Not now eradicable

APPENDIX 2
Potential virulence factors of anaerobic gram-negative bacilli

	Virulence factor or property	*Possible significance*
1.	Adherence to peritoneal mesothelium	Factor in development of peritonitis
2.	Adherence to gingival cervicular epithelium	Factor in development of periodontal disease
3.	Capsule	Inhibits macrophage migration: antiphagocytic for aerobes and anaerobes: promotes abscess formation
4.	Superoxide dimutase and catalase	Confer oxygen tolerances
5.	Immunoglobulins proteases	Resists host defence
6.	Hyaluronidase collagenase chondroitin sulfatase neuraminidase	Tissue digestion or dissolution (Spreading factor)
7.	Heparinase and other coagulation promoting factors	Impairment of blood supply to infected area
8.	Lipopolysaccharide	Inflammation and bone resorption in periodontal disease
9.	Leukotoxin	Cytopathic for a variety of mammalian cell types
10.	Butyrate	Cytotoxic substance
11.	Soluble inhibitors of chemotoxis	Blunting of inflammatory response

APPENDIX 3
Clinical clues of anaerobic infection

1. Foul odor of lesion or discharge
2. Location of infection in proximity to mucosal surface
3. Tissue necrosis, abscess formation
4. Infection secondary to human or animal bite
5. Gas in tissue or discharge
6. Gas gangrene
7. Previous therapy with aminoglycoside antibiotics (e.g. neomycin)
8. Black discolor or red fluorescence under UV light of blood containing exudate (Pigmented bacteroides)
9. Septic thrombophlebitis
10. Presence of sulfur granules in discharge (actinomycosis)
11. Failure of culture to grow aerobically although organisms are seen on Gram stain of original specimens

APPENDIX 4
Sexually transmitted diseases

1. AIDS
2. Gonorrhea
3. Syphilis
4. Genital herpes (type 2 virus)
5. Urethritis (mycoplasma)
6. Urethritis (*Trichomonas vaginalis*)
7. Urethritis (Chlamydiae)
8. Soft chancre *(H. ducreyi)*
9. Lymphogranuloma venerium
10. Granuloma inguinale
11. Condyloma acuminatum
12. *Corynebacterium vaginalis (Gardnerella vaginalis)*
13. Urethritis (*Candida albicans*)
14. Group B streptococcal infection
15. Reiter's disease
16. Scabies
17. Viral hepatitis (B)
18. Pediculosis pubis

APPENDIX 5
Microbial representative of zoonosis

Disease	*Associated animal*	*Major mode of transmission*
Anthrax	Domestic livestock	Direct contact with infected and contaminated soil
Brucellosis	Domestic livestock	Direct contact with infected tissues, ingestion of milk from diseased animal
Bubonic plague	Rodents	Fleas
Relapsing fever	Rodents	Lice and ticks
Rocky mountain spoted fever	Dogs and rodents	Ticks
Tularemia	Wild rabbits	Direct contact with infected tissues, deer flies, ticks
Leptospirosis	Dogs, rodents and wild animals	Direct contact with infected tissues and urine
Trypanosomiasis	Humans, wild game animals	Tsetse flies
Chaga's disease	Human, wild animals	Kissing bugs
Kala-azar	Cats, dogs, rodents	Sandflies
Toxoplasmosis	Cats, birds, wild rodent	Unknown, may be contamination of food and water
Yellow fever	Monkeys	Mosquitoes
Rabies	Bats, cats, dogs, men, wolves	Bites, contamination of wounds with infectious saliva
Eastern equine encephalitis	Birds, horses, etc.	Mosquitoes

APPENDIX 6
Arthropod-borne diseases

Vector	*Diseases*
Mosquito	Malaria Yellow fever Dengue Encephalitis Filariasis
Tick	Spotted fever Tularemia Relapsing fever Kyasanur Forest disease
Mite	Scrub typhus Rickettsial pox
Louse	Epidemic typhus Relapsing fever
Flea	Plague Murine typhus
Tsetse fly	Sleeping sickness
Blackfly	River blindness
Sandfly	Kala-azar Bartonellosis
Housefly	Salmonellosis Bacillary dysentery Cholera Amoebiasis Poliomyelitis Trachoma
Triatomid bug	Chaga's disease

APPENDIX 7
Differences between *Mycobacterium tuberculosis* and saprophytic mycobacteria

Properties	*Mycobacterium tuberculosis*	*Saprophytic mycobacteria*
1. Acid and alcohol fast	Acid and alcohol fast	Only acid fast
2. Growth	Slow	Rapid
3. Media	Special like LJ medium	Any ordinary media medium
4. Animal pathogenicity	Pathogenic	Non-pathogenic

APPENDIX 8
Collection specimen in salmonella infection

Time	*Specimen*	*Medium required*
1 to 7 days	Blood or clot for culture	Bile broth
7 to 21 days	Widal's test (serum)	In a clean glass tube
10 to 13 days	Bone marrow for culture	Bile broth
7 to 21 days	Stool for culture	Enrichment media like tetrathionate broth, selenite F and then selective media like DCA, Salmonella, Shigella, Wilson and Blair medium
7 to 21 days	Urine for culture	DCA, Salmonella, Shigella, Wilson and Blair medium

APPENDIX 9
Mechanism of action of chemotherapeutic agents

	Mechanisms	*Chemotherapeutic agents*
1.	Acting on bacterial cell wall	Penicillin Cephalosporin Bacitracin Novobiocin Vancomycin Cycloserine
2.	Interfering with protein synthesis	Streptomycin Lincomycin Tetracycline Chloramphenicol Manamycin Neomycin Erythromycin
3.	Enzymatic blockade	Sulfonamide Trimethroprim
4.	Interfering with cell membrane	Polymyxin Colistin Nystatin Amphotericin-B
5.	Interfering with nucleic acid metabolism	Griseofulvin colchin

APPENDIX 10
Representative antibacterial substances in animal tissues or fluid

Substance	*Common sources*	*General chemical composition*	*Types of micro-organism affected*
Complement	Serum	Believed to be a protein, carbohydrate lipoprotein complex	Gram-negative
Histone	Components the lymphatic system	Protein	Gram-positive
Interferon	Virus infected cells	Protein	Various viruses and certain protozoa
Leukin	Leukocytes	Basic peptides (protein like)	Gram-positive
Lysozyme	Include leukocytes, saliva, perspiration, tears, egg whites	Protein	Mainly Gram-positive
Phagocytin	Leukocytes	Protein	Gram-negative
Properdin	Serum	Protein	Gram-negative and certain viruses
Protanime	Spermatozoa	Protein	Gram-positive
Spermidine	Prostate and pancreas	Basic polymines	Gram-positive
Spermine	-do-	-do-	-do-
Tissue polypeptides	Component of the tissue lymphatic system	Basic peptidase	Gram-positive

APPENDIX 11
Genetic deficiencies in complement components and inhibitors

Deficient component	*Species: Man abnormalities*
C_1r	Lupus erythematosus
C_4	Lupus erythematosus
C_2	Lupus erythematosus
C_3	Recurrent severe bacterial infection
C_5	Lupus erythematosus
C_6	Disseminated gonococcal infection and recurrent meningococcal meningitis
C_7	Disseminated gonococcal infection
C_8	Disseminated gonococcal infection
C_3b INA	Marked C_3 deficiency with recurrent bacterial infection
C_1 INH	Hereditary angioneurotic edema

APPENDIX 12
Examples of surface antigens detected by selected monoclonal antibodies on T and B cells

T cells		*B cells*
Antigen	*Subset*	*Surface antigen*
Leu 4, OK T_3	All T cells	Ig heavy and light chains
Leu 5, OK T_{11}	All T cells with sheep RBC receptor	DR antigen
Leu 3, OK T_4	Helper inducer T cells	B cell specific antigen (B_1, B_2, Leu 12)
Leu 2, OK T_8	Suppressor or cytotoxic T cells	Fc receptors

APPENDIX 13
Transmission of diseases spread by arthropodes

Diseases transmitted by rat flea
1. Bubonic plague
2. Endemic typhus
3. Chigerosis
4. H. nana

Diseases transmitted by hard ticks
1. Tick typhus
2. Kyasanur Forest disease
3. Tularemia
4. Human babesiosis

Diseases transmitted by soft ticks
1. Q fever
2. Endemic relapsing fever

Diseases transmitted by trambiculid mite
1. Scrub typhus
2. Rickettsial pore

Diseases transmitted by cyclops
1. Guinea worm diseases
2. Fish tapeworm

Diseases transmitted by louse
1. Epidemic typhus
2. Epidemic relapsing fever
3. Trench fever

APPENDIX 14
Transmission of infectious diseases

Diseases transmitted by water and edibles

1. Diarrheas
2. Cholera
3. Typhoid fever
4. Poliomyelitis
5. Hepatitis A infection
6. Various intestinal parasitic infections

Diseases transmitted by blood

1. Hepatitis-B
2. Hepatitis-C
3. HIV
4. HTLV
5. Cytomegaloviruses
6. Plasmodia
7. *Trypanosoma cruzi*
8. *Treponema pallidum*

APPENDIX 15
Vector-borne diseases

Diseases transmitted by biological transmission of vectors

1. Plague bacilli in rat fleas
2. Malarial parasite in mosquitoes
3. Microfilaria in mosquitoes

Diseases transmitted by mosquitoes

1. Malaria
2. Filaria
3. Japanese encephalitis
4. Dengue
5. West Nile
6. Yellow fever

Diseases transmitted by sandfly

1. Kala azar
2. Oriental sore
3. Sandfly fever
4. Oroya fever
5. Chandipura

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