MSCZO-604

MICROBIOLOGY & IMMUNOLOGY
M. Sc. III Semester

DEPARTMENT OF ZOOLOGY
SCHOOL OF SCIENCES
UTTARAKHAND OPEN UNIVERSITY

Microbiology & Immunology
(MSCZO-604)

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UNIT 1 STRUCTURE AND CLASSIFICATION OF MICROBIOLOGY

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1.1 Objectives

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1.1 *OBJECTIVES*

The chapter (unit) aims to provide an advanced understanding of Microbiology's core principles and topics and their experimental basis to enable students to acquire specialized knowledge by utilizing dedicated lecture series and subject-oriented research projects. Hence, the main objectives of the program are:

• To communicate the basic knowledge in general microbiology with detailed subdivisions of microbiology.

• For the basic understanding, this chapter includes microbial biochemistry, physiology and molecular Biology to give a basic understanding of microbiology.

• Further bacteriology and virology give individual microbiology sections detailed information on microbiology's economic importance.

• This chapter provides necessary theoretical and practical experience in all divisions of microbiology to become an effective professional.

• This chapter provides broad exposure to various communities' ecological and commercial issues in microbiology.

1.2 *INTRODUCTION*

Microbiology is a branch of science that deals with microorganisms' structure, function, classes and economic importance. Microbiology is one of the exciting, ever-developing fields of science with greater scope as microbes play a major role in our daily lives. This chapter introduces the subject of microbiology to undergraduate beginners who have little knowledge about this subject.

DEFINITION OF MICROBIOLOGY

Microbiology is the study of organisms and agents that are too small to be seen clearly by the unaided eye. More simply, microbiology is the study of microorganisms which are living organisms of microscopic size. Microorganisms are living organisms that are less than 1 millimeter in diameter and cannot be seen by our naked eye. Microorganisms can be viewed through microscopes and can exist as single cells or clusters. Microorganisms include cellular organisms like bacteria, fungi, algae and protozoa. Viruses are also included as one of the microorganisms, but they are acellular.

OCCURRENCE OF MICROORGANISMS

One of the interesting things about microorganisms is that they occur everywhere, even in the atmosphere, water and soil. Microbes colonize almost all natural surfaces. Some microorganisms are even adapted to live comfortably in boiling springs and frozen sea ice. Microbes are the dominant form of life in the universe. More than 50 percent of the biomass on earth consists of microorganisms compared to animals which constitute only 15 percent of the mass of living organisms on earth. The majority of the microorganisms are not dangerous to humans. Microbes help to digest our food and protect our bodies from pathogens. Additionally, they are considered beneficial as they keep the biosphere running by carrying out essential functions such as decomposing dead animals and plants and nutrient cycling, enhancing soil health and crop productivity.

MEMBERS OF THE MICROBIAL WORLD

Based on the nucleus's structure, two types of cells exist. They are

i. Prokaryotes and

ii. Eukaryotes

PROKARYOTIC CELLS

'Prokaryote' is a Greek word, pro - before and karyon - nut or kernel. Prokaryotes are organisms with a primordial nucleus. They have a much simpler morphology than eukaryotic cells and lack a true membrane-bound nucleus and cell organelles like mitochondria, Golgi bodies, endoplasmic reticulum, etc. All bacteria and archaea are prokaryotic.

EUKARYOTIC CELLS

'Eukaryote' is a Greek word, eu - true and karyon - nut or kernel. Eukaryotes possess a membrane-enclosed nucleus and cell organelles. They are more complex morphologically and are usually larger than prokaryotes. Algae, fungi, protozoa, higher plants and animals are eukaryotic.

MICROBIAL GROUPS

Microorganisms are divided into six groups based on their morphological, phylogenetic and physiological characteristics. They are as follows:

1) Bacteria

2) Archaea

3) Fungi

4) Protozoa

5) Algae

6) Viruses

1) BACTERIA are prokaryotes that are usually single-celled organisms. They multiply by binary fission and reproduce asexually. They are the most dominant microorganisms in the soil, water and air. Some bacteria even live in environments with extreme temperatures, pH, or salinity. Many play more beneficial roles in nutrient cycling, decomposition of organic matter, and production of commercial and industrial products like vitamins, antibiotics, etc. Wherein some of them cause diseases and food spoilage. Ex: Bacillus, Pseudomonas.

2) ARCHAEA are phylogenetically related prokaryotes distinguished from bacteria by many features, most notably their unique ribosomal RNA sequences. Many archaea are found in extreme environments. Some have unusual metabolic characteristics, such as methanogens, which generate methane gas. Ex: Methanobacterium.

3) ALGAE are eukaryotes that contain chlorophyll and are capable of performing photosynthesis. Algae are found most commonly in aquatic environments. They reproduce either sexually or asexually. Mostly they are used as food supplements. They are mainly used in the preparation of agar. Ex: Spirulina, Gelidium.

4) FUNGI are eukaryotes. Next to bacteria, they are the most dominant organism in the soil. Fungi range in size and shape from single-celled microscopic yeasts to giant multicellular mushrooms. They possess filamentous mycelium composed of individual hyphae and reproduce sexually or asexually by fission, budding, or through spores borne on fruiting structures. Unicellular fungi like yeast produce alcoholic beverages like wine and beer. Multicellular fungi like molds are useful for the industrial production of antibiotics like penicillin. Ex: Mucor, Rhizopus.

5) PROTOZOA are unicellular eukaryotes that are usually motile and lack cell walls. Many free-living protozoa function as the primary hunters and grazers of the microbial world. They can be

found in many different environments; some are normal inhabitants of the intestinal tracts of animals, where they aid in the digestion of complex materials such as cellulose. Some of them are parasitic and can cause diseases. Ex: Amoeba, Paramecium.

6) VIRUSES are acellular (non-cellular) organisms that are too small and can be visualized only using electron microscopes. All are obligate parasites that require a living cell for reproduction. They are pathogenic to plants, animals and humans. In most cases, they cause human diseases. Ex: Cauliflower mosaic virus, Cucumber mosaic virus.

1.3 *HISTORY AND IMPORTANCE OF MICROBIOLOGY*

1.3.1 IMPORTANCE AND SCOPE OF MICROBIOLOGY

Currently, we are in the era of Microbiology. Microorganisms are the basic research tools as they help understand the chemical and physical basis of life. They are the dominant living organisms in the biosphere and are actively involved in our daily activities. Microbiology primarily paves the way to analyze the biochemical and genetic background of living things. Moreover, as microbes are excellent models for understanding cell functions and play an important role in the field of medicine, agriculture and industry that assures human welfare, microbiology is considered one of the vital branches of science with the utmost promising scopes. Microbiology is not just one small subject to be explored. It has nearly six major branches. They are as follows,

1. **Agricultural Microbiology** deals with soil nutrient cycling by microbes, microbial decomposition of organic wastes, plant-associated microbes that enhance soil fertility, etc.

2. **Food Microbiology** covers the microbes involved in food spoilage, foodborne diseases, commercial food products prepared using microbes, etc.

3. **Industrial Microbiology** explores the utility of microbes in producing antibiotics, enzymes, alcoholic beverages, fermented food products, etc.

4. **Medical Microbiology** deals with the studies related to the microbes that cause diseases, their diagnostic and preventive measures, drug designing, etc.

5. **Aquatic Microbiology** deals with water purification and biological degradation of wastes in aquatic ecosystems by microbes.

6. **Aero Microbiology** talks about the microorganisms prevalent in the air, their abundance and beneficial or harmful issues.

7. **Exomicrobiology** is all about the exploration of life in outer space.

8. **Geochemical Microbiology** analyses the microbial life and their contribution in coal, oil and gas formation areas.

As each branch of microbiology has its specialization that contributes to the development of science and technology, microbiology is always crowned as an innovative, evergreen branch of biology with wider scopes for emerging scientists to explore. We live in a world of microbes without which life will not be trouble-free and comfortable.

1.3.2 HISTORY OF MICROBIOLOGY

The field of microbiology developed further and gained its importance after the fascinating discoveries later in the 1600s by the discovery of microscopes by pioneer scientists. The important discoveries that contributed much to the discipline of microbiology are the conflict over the 'Theory of Spontaneous Generation' followed by 'Koch's Postulates' that completely changed the

view of microorganisms. This chapter gives a vivid outlook on the contributions of many pioneers like Pasteur, Koch, etc.

ROBERT HOOKE (1635 – 1700)

Hooke was the first person to discover the cell (honeycomb-like structures) from the cross sections of a cork. He noticed some microscopic fungi too. He also developed simple microscopes of 30x magnification and observed a few microorganisms.

ANTONY VAN LEEUWENHOEK (1632 – 1723)

Leeuwenhoek is a famous person who is always praised as the Father of Microbiology. He was a Dutch merchant and his hobby was making lenses and microscopes. His microscopes were simple microscopes composed of double convex glass lenses held between two silver plates that could magnify 50 to 300 times. He was the first to describe protozoa and bacteria. He observed some bacteria from plagues of his teeth. He named them animalcules.

THE THEORY OF SPONTANEOUS GENERATION (ABIOGENESIS)

After the discovery of microorganisms by Leeuwenhoek, scientists began investigating the origin of microbes. Since organic matter decomposes quickly outside the living body, it was assumed that microorganisms arise by spontaneous generation. Francesco Redi (1626) supported spontaneous generation theory. He boiled the meat and covered the mouth of the flask with wire gauze. The flies were attracted due to the odour of meat-laid eggs on the wire gauze that later developed into maggots. Thus he established that maggots originated from meat and not from the fly. Additionally, John Needham (1749), an Irish priest, observed the appearance of microorganisms in putrefying meat and interpreted this as spontaneous generation.

LA ZARO SPALLANZANI (1729-1799) - THEORY OF BIOGENESIS

Spallanzani, an Italian priest, boiled beef broth for an hour, sealed the flasks and observed no appearance of microorganisms and disproved the theory of spontaneous generation or abiotic origin of life and proposed the theory of biogenesis. He said every form of life originates from its parents, germ cells, or seeds. This theory of biogenesis was later proved and supported by Louis Pasteur.

LOUIS PASTEUR (1822-1895)

He was a Professor of Chemistry at the University of Lille, France. He is considered a "Pioneer of Microbiology," as his contribution led to the development of Microbiology as a separate scientific discipline. He proved the theory of "Biogenesis" and disproved the "Theory of spontaneous generation" (Abiogenesis) experimentally by using swan-necked flasks. Pasteur passed the untreated and unfiltered air into the boiled nutrient broth, germs settled in the gooseneck and no microbes appeared in the solution. Thus he disproved that living organisms appear from non-living matter. Pasteur also worked on souring wine and beer and found that this alcohol spoilage is due to the growth of undesirable organisms. At the same time, the desirable microorganisms produce alcohol by a chemical process called "Fermentation." He showed that wine did not spoil if heated to 50-60°C for a few minutes. This method is called "Pasteurization," now widely used in dairy units to kill pathogenic microorganisms in milk. He is a founder of the "Germ theory of disease," as he visualized that microorganisms cause diseases. During his research, he discovered the importance of sterilization and steam sterilizers, autoclaves and hot air ovens. He also established the importance of cotton wool plugs to protect culture media from aerial contamination. He differentiated between aerobic and anaerobic bacteria and coined the term "anaerobic" to refer to the organisms that do not require oxygen for growth. He worked on "Pebrine," a silk-worm disease

caused by a protozoan and showed that infection could be controlled by choosing worms free from the parasite for breeding. He developed the process of "attenuation" during his work on "chicken cholera" in fowls. He found that cultures stored in the laboratory for some time would not kill the animals as fresh cultures did. This attenuation is now used in protective vaccination against diseases.

Pasteur showed that a bacterium causes anthrax disease in cattle and sheep. He cultivated anthrax organisms in the sterile yeast water and showed that these cultures could produce disease when inoculated into healthy animals. Pasteur developed a live attenuated anthrax vaccine, incubating at 40-42°C, which proved useful in protecting animals against anthrax. He also worked on swine erysipelas. Pasteur developed a vaccine against rabies (Hydrophobia), which greatly impacted medicine. He obtained the causative agent of rabies by a serial intracerebral passage in rabbits and the vaccine was prepared by drying pieces of the spinal cord. He tested with a boy named Joseph Meister and he saved his life. In 1888, the Pasteur institute was established for mass antirabies treatment. Pasteur gave the general term "Vaccine" (Vacca = cow) in honour of Jenner's cowpox vaccine to various materials used to induce active immunity.

JOHN TYNDALL (1820-1893)

He designed a special chamber to free the dust in the air and kept the sterile broth in the chamber. No microbial growth was observed when a sterilized broth was kept in the chamber. Thus, he proved that dust in the air carried the germs and this is the source of the growth of microorganisms and not the spontaneous generation. He also developed a sterilization method called Tyndallization. Tyndallization is otherwise called intermittent or fractional sterilization. In the case of Tyndallization, heating and cooling by steam for 3 days will remove the germs and their spores.

Heating at 100 degrees Celcius kills the vegetative cells. The spore forms are killed on subsequent heating upon germination of spores.

ROBERT KOCH (1843-1912)/KOCH'S POSTULATES

He was a German country Doctor who later became the Professor of Hygiene and Director of the Institute of Infective Diseases in Berlin. He perfected many bacteriological techniques and known as the "Father of Practical Bacteriology." He discovered rod shaped organisms in the blood of animals that died of anthrax. He experimentally obtained the anthrax organisms in pure culture on a depression slide by inoculating infected blood into the aqueous humour of a bullock's eye. He observed the multiplication of bacteria and spore formation. He injected these spores into mice and reproduced the disease. He found that in certain conditions, the anthrax bacillus forms spores that can survive on the earth for years. He passed anthrax bacilli, from the blood of an infected animal, from one mouse to another through twenty generations and found that they bred true. He worked out its life history. He introduced staining techniques. He prepared dried bacterial films (Smears) on glass slides and stained them with aniline dyes to produce a better contrast under the microscope. He discovered tubercle bacillus (*Mycobacterium tuberculosis*), which is popularly called Koch's bacillus. He injected tubercle bacilli into laboratory animals and reproduced the disease, satisfying all Koch's postulates. He discovered *Vibrio cholerae*, the causative agent of cholera disease. He developed pure culture techniques by introducing solid media. The use of agar-agar from dried seaweeds (*Gelidium* Sp.) in preparing solid bacteriological media was first suggested by Frau Hesse, the wife of Koch's student. This agar-agar is inert with no nutritive value, solidifies at 45°C and melts at 90°C, and was found to be the most suitable solidifying agent in the preparation of culture media. Koch isolated bacteria in pure cultures on these solid media. It

revolutionized bacteriology. He discovered "Old Tuberculin." Koch noted that when tubercle bacilli or its protein extract was injected into a Guinea pig already infected with the bacillus, an exaggerated reaction took place and the reaction remained localized, which is popularly called the "Koch Phenomenon," and it is a demonstration of cell-mediated immunity. The tuberculin test is based on Koch's phenomenon. He erroneously thought that protein extracted from tubercle bacilli, called "Old tuberculin," could be used to treat tuberculosis. Koch did a series of experiments to fulfill the criteria laid by his teacher Henle to establish the causative role between a particular microorganism and a particular disease. They are popularly known as Koch's postulates (Henle-Koch's Postulates). They are:

1. A specific organism should be found constantly in association with the disease.

2. The organism should be isolated and grown in pure culture in the laboratory.

3. The pure culture, when inoculated into a healthy susceptible animal, should produce symptoms/ lesions of the same disease.

4. From the inoculated animal, the microorganism should be isolated in pure culture.

5. An additional criterion is that specific antibodies to the causative organism should be demonstrable in the patient's serum.

EDWARD JENNER (1749-1823)

Jenner was an English country physician who discovered a safe and efficient vaccination against smallpox which ultimately led to the eradication of smallpox (*Variola*). Jenner observed that dairy workers exposed to occupational cowpox infection were immune to smallpox. He proved experimentally that resistance to smallpox could be induced by injecting cowpox material

(Vaccinia) from disease pustules into man (in 1796). He tested his vaccine with a small boy named James Philipp. Pasteur gave the general term "Vaccine" (Vacca = cow) in honor of Jenner's cowpox vaccine to various materials used to induce active immunity. Jenner published his findings in 1798 in a pamphlet, "An inquiry into the cause and effect of a variole vaccine."

JOSEPH LISTER (1827-1912)

He is popularly known as the "Father of Antiseptic Surgery." He was a professor of surgery at

University of Glasgow and Edinburg and later at King's College, London. He was deeply interested in the prevention of post-operative sepsis. He was attracted by Pasteur's germ theory of disease and concluded that sepsis or wound infection might be due to microbial growth derived from the atmosphere. He successfully prevented post-operative sepsis by introducing antiseptic techniques. He chose carbolic acid (Phenol) and used it as a spray on the wound or during surgery. He applied dressings soaked in carbolic acid on wounds. As a result, there was a marked reduction in post-operative sepsis, wound inflammation and suppuration. It saved millions of lives from the jaws of death due to wound infections. Lister's antiseptic surgery later led to the development of aseptic surgery. He suffered much criticism but never lost courage and followed his ideas and revolutionized the science of surgery by introducing the antiseptic system in 1867.

IWANOWSKY (1892)

Dmitri Iwanowsky, a Russian botanist, occupies a pivotal position in the history of virology. In 1866, Adeolf E. Meyer, a Dutch agricultural chemist, described a disease of tobacco called "Mosaic" showed that the disease could be transmitted to healthy plants through the sap of the diseased plant.

Iwanowsky (1892) demonstrated that this disease was caused by an agent that could pass through the filter, which withholds bacteria. He obtained the sap from infected leaves and passed it through a bacterial filter, called Chamberland candle filter, which retained all bacteria and the filtered sap still retained infectivity when applied to healthy leaves. Beijerinck (1898), a Dutch Microbiologist, showed that this infectious agent could diffuse through an agar gel and that it was a non-corpuscular "Contagion vivum fluidum" which he called a "Virus." Stanley (1935), a British Mycologist, was able to obtain the infectious agent of tobacco mosaic in a crystalline form.

METCHNIKOFF (1845-1916)

Elie Metchnikoff, the Russian-French biologist, discovered the phenomenon of phagocytosis, the cellular concept of immunity. In Italy, where he had gone on a research visit, he studied the transparent larvae of starfish and noticed some of their cells could engulf and digest foreign protein particles. These cell eaters are called "Phagocytes." He continued his work on phagocytic action at Pasteur Institute, Paris and found that in human blood, a large proportion of the leucocytes (White blood cells) are phagocytic and attack invading bacteria, which in turn, results in increased numbers of leucocytes in the infected areas, followed by the inflamed area becomes hot, red, swelled and painful due to dead phagocytes forming pus. He spent his last two decades on the study of human aging since he believed that phagocytes eventually begin to digest the host cells, aided by the effects of intestinal bacteria. Moreover, effectively combating them would increase the life span of a human being.

SELMAN A WAKSMAN (1945)

He is an American microbiologist. He isolated *Thiobacillus thiooxidans,* which was an important discovery before he identified the Streptomycin antibiotic from a soil bacterium. In 1939 Waksman

and his colleagues undertook a systematic effort to identify soil organisms producing soluble substances that might be useful in the control of infectious diseases, what is now known as antibiotics. Within a decade, ten antibiotics were isolated and characterized. Three of them with important clinical applications - actinomycin in 1940, streptomycin in 1944, and neomycin in 1949. Eighteen antibiotics were discovered under his general direction.

ALEXANDER FLEMMING (1881-1955)

He was an English scientist who worked at St. Mary's hospital in London. Flemming was associated with two major discoveries - lysozyme and penicillin. In 1922, he discovered lysozyme by demonstrating that nasal secretion has the power of dissolving or lysing certain kinds of bacteria. Subsequently, he showed that lysozyme was present in many tissues of the body.

In 1929, Flemming made an accidental discovery that the fungus *Penicillium notatum* produces an antibacterial substance which he called penicillin. Flemming was culturing Staphylococci in Petridishes and some of his cultures were contaminated with a mold, subsequently identified as *Penicillium notatum*. Around the mold colony, there were clear zones where Staphylococci disappeared. Flemming attributed this to the production of an antibacterial substance by the mold.

Flemming cultured the fungus *Penicillium notatum* in broth cultures, filtered the fungal mat and obtained the penicillin in soluble form in the culture filtrate. In 1940, Howard Florey and Ernst Chain demonstrated its antibacterial action in vivo. Working in the U.S.A., they produced large quantities of penicillin in pure form. In 1945, Flemming, Florey and Chain shared the Nobel prize in physiology and medicine for the discovery of penicillin.

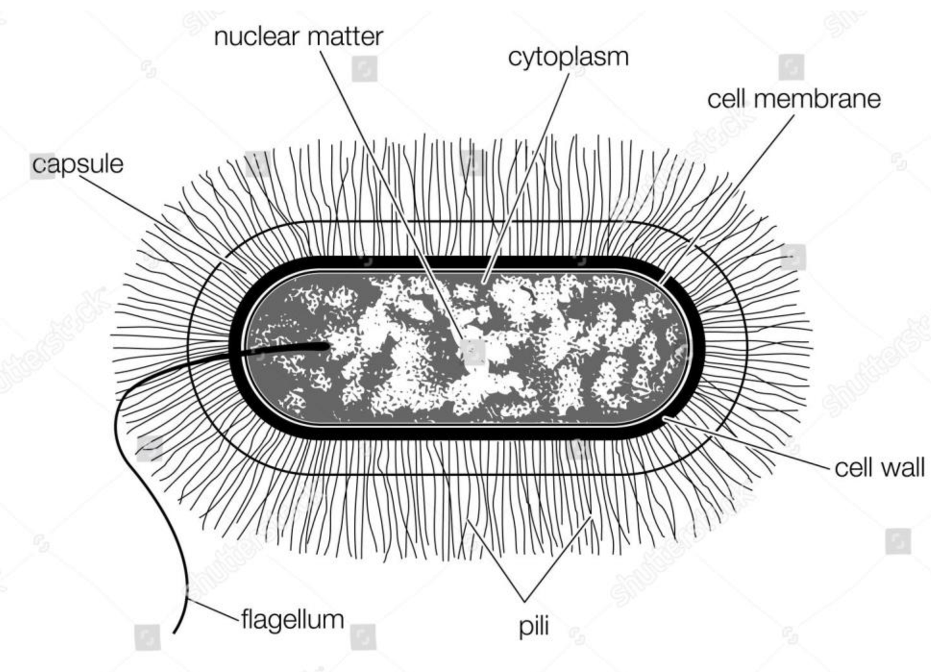
1.4 STRUCTURES AND CLASSIFICATION OF VIRUSES, BACTERIA AND FUNGI

1.4.1 MORPHOLOGY AND PHYSIOLOGY OF BACTERIA AND VIRUS

Bacterial Cell (Morphology And Classification Of Bacteria)

The protoplast is bounded peripherally and has a thin, elastic, semi-permeable cytoplasmic membrane (a conventional phospholipid bilayer). Outside, and closely covering this, lies a rigid, supporting cell wall, which is porous and relatively permeable. The structures associated with the cell wall and the cytoplasmic membrane (collectively the cell envelope) combine to produce the cell morphology and characteristic patterns of cell arrangement. Bacterial cells may have two basic shapes: spherical (coccus) or rod-shaped (bacillus); the rod-shaped bacteria show variants that are common-shaped (vibrio), spiral (spirillium and spirochetes) or filamentous. The cytoplasm, or the main part of the protoplasm, is a predominantly aqueous environment packed with ribosomes and numerous other protein and nucleotide-protein complexes. Some larger structures, such as pores or inclusion granules of storage products, occur in some species under specific growth conditions. A protective gelatinous covering layer outside the cell wall may be called a capsule. Some bacteria bear, protruding outwards from the cell wall, one or more kinds of filamentous appendages:

Flagella are organs of locomotion; **fimbriae**, which appear to be organs of adhesion; and **pili**, which are involved in the transfer of genetic material. Because they are exposed to contact and interaction with the cells and humoral substances of the host's body, the surface structures of bacteria are the structures most likely to have special roles in infection processes.



Classification Of Bacteria

Shape: this can be of 3 main types:

round (cocci)

- regular (staphylococci)

- flattened (meningococci)

- lancet-shaped (pneumococci)

elongated (rods)

- straight (majority of them are like this; e.g., *E. coli*)

- short (coccobacilli; e.g acinetobacters)

- long (fibers) – these are mainly found in OLD cultures

- slender – mycobacterium tuberculosis

- robust – lactobacilli, bacillus

- with split ends - bifidobacteria

- branching - nocardiae, actinomycetes

- curved - campylobacters

- with flat ends – bacillus anthracis

- spindle-shaped - fusobacteria

- club-shaped - corynebacteria

- pleomorphic – haemophili

- spiral – helicobacter, spirilium

Spiral bacteria (spirochetes) are different from the spiral bacteria mentioned above! They are tightly

coiled bacteria.

- Thick: Spirillium

- Uneven: borrelia

- Delicate, regular: Treponema

- Slender with bent ends: Leptospira

Circular	Rod-shaped	Curved Forms	Other Shapes
Diplo- (in pairs)	Coccobacilli (oval)	Vibrio (curved rod)	Helicobacter (helical)
Strepto- (in chains)	Streptobacilli	Spirilla (coil)	Corynebacter (club)
Staphylo- (clusters)	Mycobacteria	Spirochete (spiral)	Streptomyces

Virus (Morphology And Classification Of Viruses)

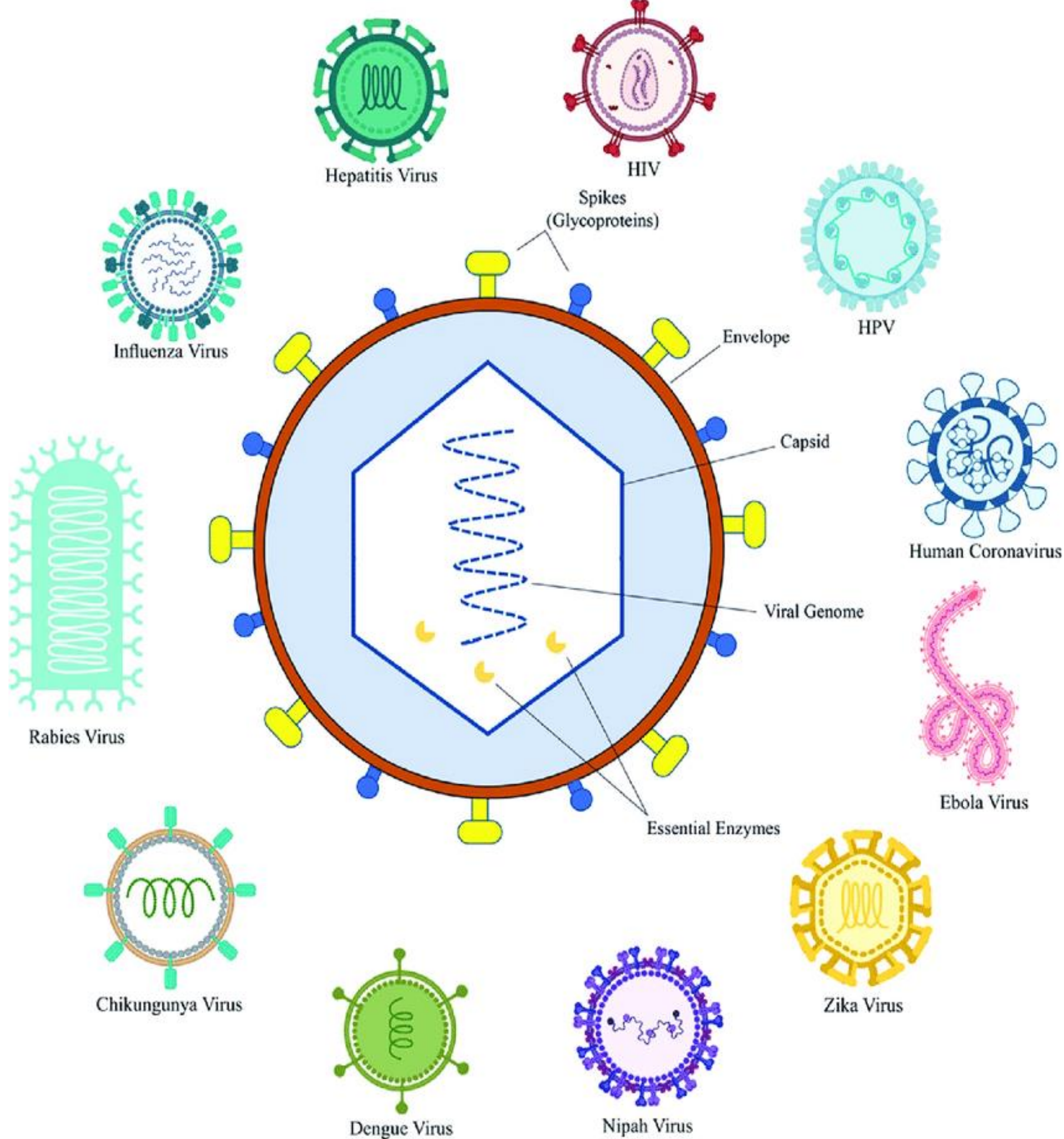
Size: The extracellular infectious virus particle is called a virion. Viruses are much smaller than bacteria. They are too small to be seen under a light microscope. Some large viruses, like the poxviruses, can be seen under the light microscope when suitably stained. The viruses range in size from 20 nm to 300 nm. Poxviruses are one of the largest viruses and parvoviruses are one of the smallest. The earliest method of estimating the size of virus particles was by passing them through collodion membrane filters of graded porosity. The average pore diameter of the finest filter that permitted passage of the virion gave an estimate of its size. With the development of the ultracentrifuge, a second method became available. From the rate of sedimentation of the virus in the ultracentrifuge, the particle size could be calculated using Stoke's law. Electron microscopy is

the third and most direct method of measuring virus size. This method can study both the shape and size of virions.

Structure, shape and symmetry: The virion consists of a nucleic acid surrounded by a protein coat, the capsid. The capsid with the enclosed nucleic acid is called the nucleocapsid. The capsid protects the nucleic acid from harmful agents in the environment. It is composed of many capsomers that form its morphological units. The chemical units of the capsid are polypeptide molecules that are arranged symmetrically. They form a shell around the nucleic acid. The capsid shows two kinds of symmetry – icosahedral (cubical) and helical. An icosahedron is a polygon with 12 vertices and 20 facets or sides. Each facet is in the shape of an equilateral triangle. Two types of capsomers are present in the icosahedral capsid. They are the pentagonal capsomers at the vertices (pentons) and the hexagonal capsomers making up the facets (hexons). There are always 12 pentons, but the number of hexons varies with the virus group. Examples of viruses with icosahedral symmetry of the capsid are Adenovirus and Herpes Simplex Virus. In the nucleocapsids with helical symmetry, the capsomers and nucleic acid are wound together to form a helical or spiral tube, for example, the tobacco mosaic virus. All viruses do not show the typical icosahedral or helical symmetry. Some, like the poxviruses, show a complex symmetry.

Virions may be enveloped or non-enveloped. The envelope of viruses is derived from the host cell membrane, which occurs when the virus is released from the host cell by budding. Protein subunits may be present as projecting spikes on the surface of the envelope. They are called peplomers. The influenza virus carries two kinds of peplomers: haemagglutinin and neuraminidase. Haemagglutinin is a triangular spike and neuraminidase is mushroom-shaped. The envelope is sensitive to the action of lipid solvents. Envelopes confer chemical, antigenic and biological properties on viruses.

The overall shape of the virus particle varies in different groups of viruses. Most animal viruses are roughly spherical. The rabies virus is bullet shaped. Poxviruses are brick-shaped.



Classification Of Viruses

Till about 1950, little was known of the basic properties of viruses. They were named haphazardly based on the diseases they caused or on the place of their isolation. They were grouped according to affinity to different systems or organs of the body (tropism). So, human viruses were classified

as dermotropic, that is, those producing skin lesions (smallpox, chickenpox, measles), neurotropic, that is, those affecting the nervous system (poliomyelitis, rabies), pneumotropic, that is, those affecting the respiratory tract (influenza, common cold) and viscerotropic, that is those affecting visceral organs (hepatitis). Bawden (1941) suggested that viral nomenclature and classification should be based on the properties of viruses and not on host responses. From the early 1950s, viruses began to be classified based on their physiochemical and structural features. Nomenclature and classification are now the official responsibility of the International Committee on Taxonomy of Viruses (ICTV). Viruses are classified into two main divisions based on the type of nucleic acid they possess: riboviruses contain RNA and deoxyriboviruses contain DNA. Further classification is based on other properties like strandedness of nucleic acid, symmetry of nucleic acid, presence of envelope, size and shape of virion and the number of capsomeres. DNA viruses: A few medically important families of DNA viruses are - Herpesviridae, Adenoviridae, Hepadnaviridae, Parvoviridae and Papillomaviridae.

The Herpesviridae family consists of enveloped double-stranded DNA viruses having an icosahedral capsid. Examples of this family are herpes simplex virus and varicella zoster virus. Herpes simplex virus causes skin lesions like herpes labialis. It can also cause viral encephalitis. Parvoviridae consists of non-enveloped single-stranded DNA viruses, for example, Parvovirus B19. The Hepadnaviridae family includes Hepatitis B virus, a partially double-stranded DNA virus. The Papillomaviridae family includes the human papillomavirus, which is responsible for causing skin warts.

RNA viruses: Some medically important families of RNA viruses are – Picornaviridae, Orthomyxoviridae and Paramyxoviridae, Flaviviridae, Rhabdoviridae and Retroviridae. Members of the family Picornaviridae are small (20-30 nm), non-enveloped, icosahedral viruses with a

single-stranded RNA genome. Examples include poliovirus and coxsackievirus. The viruses included in Orthomyxoviridae have enveloped viruses carrying haemagglutinin and neuraminidase peplomers on the envelope. The genome consists of single-stranded RNA in several (eight) pieces. Thus, they have a segmented genome. An example of this family is the influenza virus. Flaviviridae consists of enveloped single-stranded RNA viruses. Examples include yellow fever virus, Japanese encephalitis virus and dengue virus. The members of the Retroviridae family have enveloped RNA viruses with a special enzyme called 'reverse transcriptase.' This enzyme is an RNA-dependent DNA polymerase. It is required in the synthesis of DNA from RNA. An example of the Retroviridae family is Human Immunodeficiency Virus (HIV) which causes AIDS (acquired immunodeficiency syndrome). Baltimore (1970) categorised viruses into seven categories based on the replication mechanism called the Baltimore classification.

1.4.2 CHEMICAL COMPOSITION OF VIRUS & BACTERIA

Chemical Properties Of Virus

Viruses contain only one type of nucleic acid, either DNA or RNA. Viruses are unique because they carry genetic information on RNA. This property is not seen in any other organism in nature. Viruses also contain protein which makes up the capsid. Enveloped viruses contain lipids derived from the host cell membrane. Most viruses do not have enzymes for synthesizing viral components or energy production. Some viruses have enzymes; for example, the influenza virus has neuraminidase.

Resistance: Viruses are destroyed by heat, except for a few. They are stable at low temperatures. For long-term storage, they are kept at -70°C. A better method for prolonged storage is lyophilization or freeze-drying. Viruses are inactivated by sunlight, UV rays and ionizing

radiation. They are, in general, more resistant than bacteria to chemical disinfectants. Phenolic disinfectants have a weak action on viruses.

Chemical Composition Of Bacteria

The cytoplasm, or the main part of the protoplasm, is a predominantly aqueous environment packed with ribosomes and numerous other protein and nucleotide-protein complexes.

Bacterial nucleoid: The genetic information of the bacterial cell is mostly contained in a single, long molecule of double-stranded deoxyribonucleic acid (DNA). The cell solves the problem of packaging this enormous macromolecule by condensing and looping it into a supercoloid state. The bacterial nucleoid lies within the cytoplasm. As DNA-dependent RNA polymerase makes RNA, ribosomes may attach and initiate protein synthesis on the still attached (nascent) messenger RNA. The synthesis of mRNA and protein is therefore seen to be directly coupled in bacteria.

Cytoplasmic membrane: The bacterial protoplasm is limited externally by a thin, elastic cytoplasmic membrane 5-10 nm thick and consists mainly of phospholipids and proteins. Integral, transmembrane and peripheral or anchored proteins occur in abundance and perform similar functions to those described in eukaryotes (e.g., transport and signal transduction). Prokaryotic cell membranes are relatively protein-rich, allowing relatively little space for phospholipids.

1.4.3 REPRODUCTION OF VIRUSES & BACTERIA

Multiplication of Viruses

The multiplication of viruses is called viral replication. Viruses contain the genetic information for replication but lack the enzymes. They depend on host cell machinery for replication. The viral

replication cycle can be divided into six phases – adsorption, penetration, uncoating, biosynthesis, maturation and release.

Adsorption: The virus gets attached to the host cell in this phase. The host cell should have specific receptors on its surface. These receptors recognize viral surface components. This cell-virus interaction helps the virus to attach to the host cell surface.

Penetration: In this phase, the virus enters the host cell. Bacteria have a rigid cell wall. So, viruses that infect bacteria cannot penetrate the bacterial cell. Only the nucleic acid of the virus enters the bacterial cell. Animal and human cells do not have cell walls. Therefore, the whole virus enters the cell. Virus particles may be engulfed by a process called viropexis. In the case of enveloped viruses, the viral envelope may fuse with the cell membrane of the host cell. Then the nucleocapsid is released into the cytoplasm.

Uncoating: This is the process in which the outer layers and capsid of the virus are removed, which mostly occurs by the action of lysosomal enzymes of the host cell, which can also occur by a viral uncoating enzyme. Finally, the viral nucleic acid is released into the cell.

Biosynthesis: In this phase, the viral nucleic acid and capsid are synthesized. The enzymes necessary in the various stages of viral synthesis, assembly and release are also synthesized. Certain 'regulator proteins' are synthesized. They shut down the normal metabolism of the host cell. They direct the production of viral components. Most DNA viruses generally synthesize their nucleic acid in the host cell nucleus. Exceptions are the poxviruses. They are DNA viruses but synthesize all their components in the host cell cytoplasm. Most RNA viruses synthesize all their components in the cytoplasm. Orthomyxoviruses and some paramyxoviruses are exceptions. They

synthesize some components in the host cell nucleus. Biosynthesis consists essentially of the following steps:

1. Transcription of messenger RNA (mRNA) from the viral nucleic acid

2. Translation of mRNA into "early proteins" or "non-structural proteins." They are enzymes responsible for the synthesis of viral components.

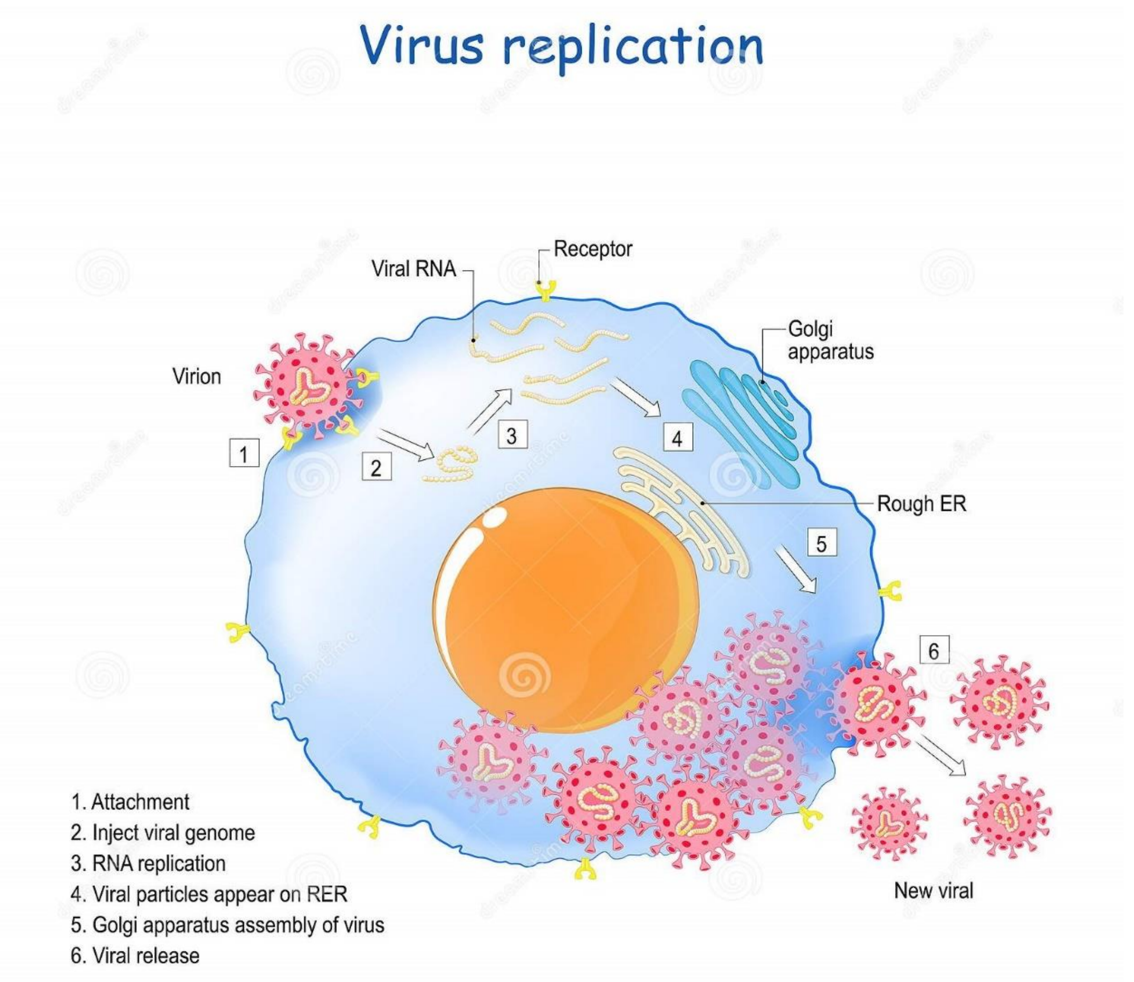
3. Replication of viral nucleic acid

4. Synthesis of "late proteins" or "structural proteins." They are the components

of daughter virion capsids.

Maturation: It is the assembly of daughter virions following the synthesis of viral nucleic acid and proteins. It can take place in the host cell nucleus or cytoplasm. Herpesviruses and adenoviruses are assembled in the nucleus. Picornaviruses and poxviruses are assembled in the nucleus.

Release: Viruses that infect bacteria (bacteriophages) are released by lysis of the infected bacterium. Animal viruses are usually released without cell lysis.



Myxoviruses are released by budding from the cell membrane. The host cell is unaffected. Daughter virions are released into the surrounding medium and may infect other cells. In some viruses (e.g., varicella), transmission occurs directly from cell to cell. In this case, there is a very little free virus in the medium. The poliovirus causes cell damage and may be released by cell lysis. From the stage of penetration till the appearance of mature daughter virions, the virus cannot be demonstrated inside the host cell. During this period, the virus seems to disappear, which is called the "eclipse phase." The time taken for a single cycle of replication is about 15-30 minutes for bacteriophages. It is about 15-30 hours for animal viruses. A single infected cell may release a large number of progeny virions.

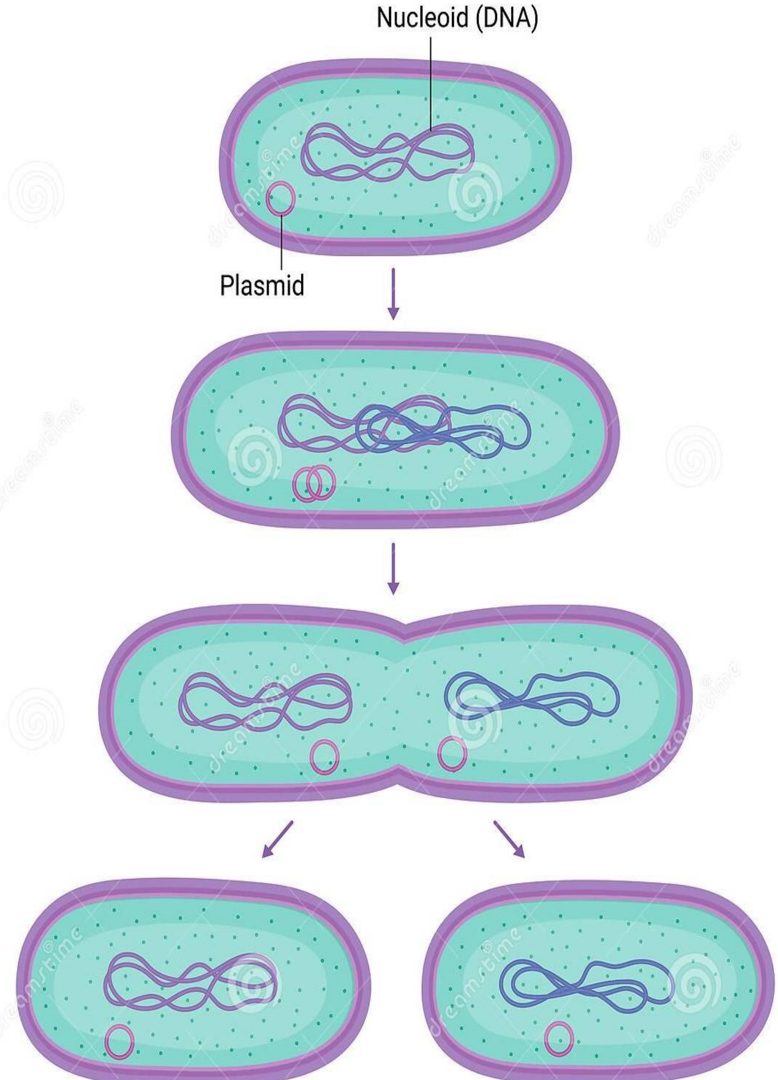
BACTERIAL REPRODUCTION

Bacteria reproduce by binary fission, a form of asexual reproduction and cell division.

- Period I (initiation): the cell grows and proteins that start the next step accumulate inside it.
- Period C (chromosome replication): begins in one spot and diverges out in opposite directions
- Period D (division): a supply of macromolecules is formed. The cytoplasmic membrane inserts between the replicated chromosomes and separate them. The cell wall grows into the cell at a particular spot and forms a septum that divides the maternal cell into two daughter cells.

Division of cocci can occur in one plane, e.g., streptococci or different planes, e.g., staphylococci. Division of rods can occur in the transverse plane, e.g., the majority of chain rods or the vertical plane, e.g. corynebacteria, mycobacteria.

The generation time is the time taken for the number of bacteria to double or the duration of the growth cycle. On average, it is usually about 30 minutes since the number of bacterium doubles generation time, e.g., If the generation time is 30 min, after 24 hours, theoretically, one cell gives origin to 248=2,8 x 1014 cells.



However, the actual amount of cells produced is approximately 5 orders less (i.e., around 109 cells). This amount of cells can be seen by the naked eye and in a liquid broth, it appears cloudy, sedimentation occurs at the bottom, or a pellicle is seen at the top. In a solid medium (agar), a bacterial colony is formed. The result of 109 cells/24 hours applies to stationary cultures, where

nutrients are consumed and metabolites accumulate. The speed of multiplication changes depending on time and the growth of the bacterium can be illustrated by using a growth curve.

1.5 *SUMMARY*

- Bacteria are prokaryotic microorganism that do not contain chlorophyll.

- They are unicellular and do not exhibit true branching.

- The morphological study of bacteria requires the use of microscope like optical or light microscope, phase control microscope, dark/field microscope, electron microscope.

- Staining techniques like simple stain, negative stain, impregnation stain, differential stains are used to exhibit structure of bacteria.

- Bacteria are classified based on the shape as cocci, bacilli, vibrio, Spirilla. And based on arrangements they are classified as diplococci, streptococci, tetrads, sarcina, staphylococci.

- Bacterial cell has cell wall, inner protoplasm and other components.

- Bacterial growth phase has a lag phase, log phase, stationary phase and a decline phase.

- Viruses do not strictly fall into the category of unicellular organisms because they do not have a cellular organization.

- Viruses are obligate intracellular parasites that contain only one type of nucleic acid (DNA or RNA). They are dependent on the synthetic machinery of the host cell for replication.

- The extracellular infectious virus particle is called the virion. The viruses are smaller than bacteria ranging in size from 20-300 nanometers.

- The virion consists of a central nucleic acid core surrounded by a protein coat called the capsid. The nucleocapsid consists of the capsid enclosing the nucleic acid core.

- The capsid protects the nucleic acid from inactivation and is made up of a large number of capsomeres.

- The capsid may have icosahedral, complex or helical symmetry.

- The virions may be enveloped or non-enveloped. The envelope is a lipoprotein. Protein subunits that occur as projecting spikes on the envelope surface are called peplomers, as seen in the influenza virus (haemagglutinin and neuraminidase).

- Enveloped viruses are susceptible to organic solvents. The envelope helps the virus in attachment to the host cell surface.

- Most animal viruses are roughly spherical; some are irregular and pleomorphic. Some have distinctive shapes like bullets (rabies) and bricks (poxviruses).

- Most viruses are inactivated by heat in seconds at 56ºC. Viruses are inactivated if stored for several days at 4 ºC but survive storage at -70ºC. Viruses are inactivated by sunlight, ultraviolet rays and ionizing radiation. Viruses are easily destroyed by chemical disinfectants like chlorine, hydrogen peroxide and hypochlorite.

- Viral multiplication consists of six sequential phases – adsorption, penetration, uncoating, biosynthesis, maturation and release from the host cell.

- Viruses were previously classified on the basis of their affinity towards different systems or organs. Recently, they have been classified based on their physiochemical properties and structure.

The International Committee on Taxonomy of Viruses (ICTV) is responsible for the classification and naming of viruses.

- Viruses are broadly classified into DNA and RNA viruses.

- Baltimore (1970) classified viruses based on their replication mechanisms.

1.6 *TERMINAL QUESTIONS*

1. Describe the morphology of viruses under the following headings – structure, shape and symmetry.

2. Describe the methods of viral cultivation.

3. Enumerate three DNA viruses and three RNA viruses along with the disease caused by each one of these.

4. Give stepwise detailed description of viral replication.

5. Classify bacteria based on shaped and arrangement with examples.

6. Explain the factors affecting the growth of the bacteria.

7. Describe growth curve of bacteria.

8. Describe the structure of cell wall of bacteria.

Objective Questions (Viruses)

1. The genetic material in viruses is:

A. DNA only B. RNA only C. Either DNA or RNA D. Both DNA and RNA

2. Protein subunits presenting as projecting spikes on the surface of the envelope are called:

A. Capsomeres B. Capsid C. Nucleocapsid D. Peplomers

3. Which of the following is the correct sequence of viral replication?

A. Penetration, uncoating, adsorption, biosynthesis, maturation and release

B. Adsorption, penetration, uncoating, biosynthesis, maturation and release

C. Biosynthesis, penetration, uncoating, adsorption, maturation and release

D. Adsorption, biosynthesis, maturation, uncoating, penetration and release

4. Methods used for viral cultivation are:

A. Cell culture B. Animal inoculation C. Embryonated eggs D. All of the above

5. Baltimore classified viruses on the basis of:

A. Diseases caused by them B. Structure C. Replication mechanism D. Physiochemical properties

Objective Questions (Bacteria)

Match the following:

1. Bacilli (a) coma

2. Cocci (b) flexous spiral form

3. Vibrio (c) rigid spiral form

4. Sprillum (d) rod shaped

5. Spirochetes (e) spherical shaped

Match the following:

1. Diplococci (a) groups of four

2. Streptococci (b) groups of eight

3. Tetrads (c) occurs in pairs

4. Sarcina (d) grape like clusters

5. Staphylococci (e) occurs in chains

1.7 *REFERENCE BOOKS*

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Unit 2 TECHNIQUES OF STERILIZATION AND CULTURE MEDIA

CONTENTS

2.1 Objectives

2.2 Introduction

2.3 Sterilization: Principles - dry heat, moist heat, filtration

2.3.1 Tantalization, pasteurization

2.3.2 Radiation - disinfection

2.4 Culture techniques - media preparation

2.4.1 Aerobic and anaerobic culture techniques

2.5 Different Staining methods

2.6 Summary

2.7 Terminal Questions and Answers

2.8 References

2.1 *OBJECTIVES*

After reading this unit you will be able to:

- Define the term " sterilization"
- Explain the various type of sterilization technique and their principles *viz*: dry heat, moist heat, filtration, tyndallization, pasteurization, Radiation - disinfection
- Define the term "Culture media".
- Explain the various techniques for the preparation of culture media.
- Explain the types of anaerobic and aerobic culture media.
- Define the term 'staining".
- Explain preparation of various types of microbiological stains, staining techniques, preparation of stains and their uses.

2.2 *INTRODUCTION*

Sterilization refers to any process that removes, kills, or deactivates all forms of life such as particularly microorganisms such fungi, bacteria, spores,and unicellular eukaryotic organisms and other biological agents such as prions present in or on a specific surface, object, or fluid. Sterilization can be achieved through various means, including heat, chemicals, irradiation, high pressure, filtration, tantalization and pasteurization. After sterilization, an object is referred to as being sterile or aseptic. Some common gaseous sterilants are ethylene oxide, formaldehyde, nitrogen dioxide and ozone while hydrogen peroxide, glutaraldehyde and hypochlorite are liquid sterilants. The most common rays used for sterilization are UV, and gamma rays, in specific cases infrared radiation is also used to make the object sterile.

One of the first attempts at "Sterilization" by heat was said to be used in ancient Rome by a medical practitioner called Galen (130-200 AD). Galen was Greek and cared for wounded Roman Gladiators, in his efforts to save his patients he boiled his instruments prior to use. These basic methods used by the Egyptians and Greeks were relatively effective and likely considered revolutionary at the time, the interesting thing was however, that none of the people implementing these practices had any idea of why heat would reduce infection rate or prevent people from getting

ill from drinking water. The advancements in sterilization and infection prevention reached a stand still during the dark ages due to the general chaos following the black plague where the concept of infection control was lost. So let's fast forward to the second half of the 17th century when a significant discovery was made which paved the way for sterilization as we know it today- The existence of microorganisms. The culture media (growth medium) is a source of nutrients to support the growth of the micro-organisms in-vitro. The media helps in the growth and counting of microbial cells, selection of microorganisms, and survival of microorganisms.

The culture medium can be liquid or gel. The first to have cultured bacteria reproducibly in a liquid culture medium was Louis Pasteur. In 1860,he developed a culture medium containing 'yeast soup' ,ashes, sugar and ammonium salts. His objective was to create a fermentation medium to demonstrate that each fermentation was associated with the development of a particular microorganism [4]. The

Common ingredients of culture media are: **i. Nutrients:** proteins/peptides/amino-acids. **ii. Energy:** carbohydrates. **iii. Essential metals and minerals:** calcium, magnesium, iron, trace metals: phosphates, sulphates etc. **iv. Buffering agents:** phosphates, acetates etc. **v. Indicators for pH change:** phenol red, bromo-cresol purple etc.**vi Selective agents:** chemicals, antimicrobial agents. **vii. Gelling agent:** usually agar (if we want to prepare culture media in jelly form).

Staining is a technique used to enhance contrast in samples, generally at the microscopic level. Stains and dyes are frequently used in microbiology (microscopic study of microbes), histology (microscopic study of biological tissues), in cytology (microscopic study of cells), and in the medical fields of histopathology, hematology, and cytopathology that focus on the study and diagnoses of diseases at the microscopic level. Stains may be used to define biological tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells), or organelles within individual cells. The staining of bacteria is naturally of later origin than the use of dyes in histological work, as the systematic study of bacteria did not begin until after 18'70. The use of dyes in this followed very promptly after that date, however. The beginnings of bacteriological staining were very adequately summed up by Loeffler (1887).

2.3 *STERILIZATION*

The term sterilization is originated from French word “Sterile” which means “not producing fruit”. Sterilization refers to any process that removes, kills, or deactivates all forms of life such as particularly microorganisms such fungi, bacteria, spores,and unicellular eukaryotic organisms and other biological agents such as prions present in or on a specific surface, object, or fluid. The objects may be culture media and scientific glasswares, equipments, surgical articles and working place.

Sterilization can be achieved by three methods: (i) Physical methods (ii). Chemical methods

(iii) Mechanical methods.

PHYSICAL METHODS: Physical methods of sterilization achieved by dry heat, moist heat, filtration Tandalization, pasteurization, radiation – disinfection

2.3.1 DRY HEAT, MOIST HEAT AND FILTRATION:

(a)DRY HEAT: Dry heat sterilization of an object is one of the earliest forms of sterilization practiced. It uses hot air that is either free from water vapor or has very little of it, no role in the process of sterilization. The dry heat sterilization process is accomplished by conduction; that is where heat is absorbed by the exterior surface of an item and then passed inward to the next layer. Eventually, the entire item reaches the proper temperature needed to achieve sterilization. The proper time and temperature for dry heat sterilization is 160 °C (320 °F) for 2 hours or 170 °C (340 °F) for 1 hour, and in the case of High Velocity Hot Air sterilisers, 190°C (375°F) for 6 to 12 minutes. Dry heat sterilization is used on items that cannot get wet and for glassware, oils, powders, metal instruments, and items wrapped in paper.

There are several different types of dry heat sterilization such as:

- A hot air oven in which there is static air (heating coils on the bottom) or forced air hot air oven (motorized blower) that are used differently.
- Incineration, which burns medical waste that's disposable.
- Flaming, this involves exposing objects to direct fire or flame.

- **(i)Dry heat sterilization by Oven:** A hot air oven is a laboratory instrument that uses dry heat to sterilize laboratory equipment and other materials.
- That equipment cannot be wet or material that will not melt, catch fire, or change form when exposed to high temperatures are sterilized by using the dry heat sterilization method.
- Hot air oven also known as forced air circulating oven.
- Some examples of material which can't be sterilized by employing a hot air oven such as surgical dressings, rubber items, or plastic material.
- We can sterilize Glassware (like petri dishes, flasks, pipettes, and test tubes), Powder (like starch, zinc oxide, and sulfadiazine), Materials that contain oils, Metal equipment (like scalpels, scissors, and blades) by using hot air oven.
- To destroy microorganisms and bacterial spores, a hot air oven provides extremely high temperatures over several hours.
- The widely used temperature-time relationship in hot air ovens to destroy microorganisms are 170 degrees Celsius for 30 minutes, 160 degrees Celsius for 60 minutes, and 150 degrees Celsius for 150 minutes.
- Most of the medical industries use hot air ovens to sterilize laboratory instruments and material due to its simple standard operating procedure and low price. It also provides quick-drying processes.
- The process of dry heat sterilization using a hot air oven originally developed by Louis Pasteur.
- The temperature range of a hot air oven is 50 to 300 ° C. It can be controlled by using a temperature regulator.
- The forced air circulation provided by the oven ensures the temperature uniformity throughout the oven.
- In a hot air oven first, the surface of the material is sterilized then the temperature slowly enters the center of the item.

Working Principle of Hot air oven

Sterilization by dry heat is performed by conduction. The temperature is consumed by the surface of the objects, then moves towards the core of the object, coating by coating. The whole object will ultimately attain the temperature needed for sterilization to take place.

Dry heat causes most of the injury by oxidizing particles. The primary cell components are damaged and the organism dies. The temperature is kept for about an hour to eliminate the most ambitious of the resistant spores.

Hot air oven Parts and Functions (Figure-1 and 2):

External cabinet: 1.The External cabinet is made of stainless sheets. It covers the inner chamber.

2. **Glass wool insulation:** The space between the inner chamber and external cabinet is filled with Glass wool. It provides insulation to the hot air oven.

3**. Inner chamber:** The inner chamber of the hot air oven is made of Stainless steel.

4.**Tubular air heaters:** They help to generate heat within the inner chamber. Two Tubular air heaters are located on both sides of the inner chamber.

5.**Motor-driven blower:** It helps in uniformly circulating the air within the chamber.

6 **.Temperature sensor:** It measures the temperature within the hot air oven and displays it on the controller screen.

7.**Tray slots**: The inner wall of the chamber contains several try slots that hold the trays.

8**. PID temperature controller:** It maintains the accurate temperature during the entire cycle. It also controls the temperature and also displays the temperature values.

9.**Load indicator:** it indicates the hot air oven is overloaded.

10 Mains on/off switch: It helps to turn on/ turn off the hot air oven.

11.Safety thermostat: It is also known as an over-temperature protection device. It keeps your oven and specimen safe in case of controller malfunction.

Types of hot air oven:

There are present different types of hot air oven widely used in hospitals laboratory, education institutions laboratotory and confectionary shop etc.

(i)Gravity Convection

- Gravity Convection Air is distributed by spontaneous convection. As hot air flows up, a gentle flow holds temperatures moderately uniform inside a container and wholly uniform in any distinct position.

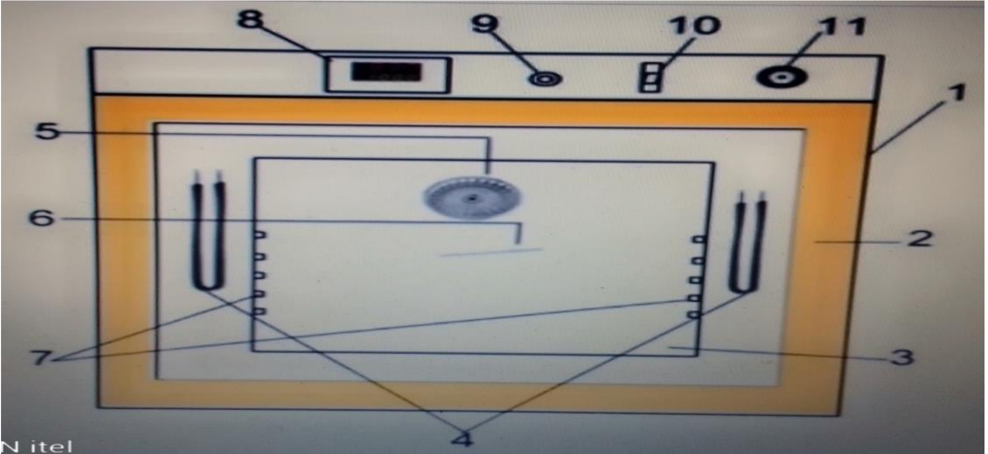

Fig 2.1 Internal Parts of hot air oven

(ii)Forced Convection Ovens

- These ovens carry a fan that gives limited air circulation within the heating container.
- This method provides very fast heat up and restoration times, mixed with especially low-temperature differences inside the working chamber.
- Flexible vents and semi-forced exhaust deliver it a conventional sample-drying oven.

Fig 2.2 Common hot air oven.

(iii) Mechanical Convection

- Mechanical Convection is a gravitation convection oven served with a re-circulating fan in a working container.

(iv) Forced Exhaust Ovens

- In these ovens, air is pushed into the working container by a fan and scattered through an adaptable vent.
- This variety of oven is especially helpful in purposes where the heating process provides vapors or fumes that require to be immediately and continuously discharged from the working container.
- All of the forced air ovens consume at a higher percentage than a convection oven. Though, much larger forced exhaust velocities can be accomplished by adding an air channel and a flexible outlet.
- This adjustment takes an extra $100 and is totally achievable with forced convection ovens.

(v)Side Draught Ovens

- Certain ovens produce airflow from one side to the other i.e. left to right.
- Speedy heat up and restoration time make this type of oven prototype for preheating plastic cloths (hospitals, etc.) or any profession where smooth sheets or plates are used.

Hot air oven Advantages

- No need to water to sterilize the material.
- Not much pressure is created like autoclave which creates it easy to manage and also makes it safer to work with.
- In a laboratory environment, it is more fitting to use as compared to other sterilizers.
- Hot air oven is much smaller in size as compared to autoclaves and also more effective.
- A hot air oven can be more speedy than an autoclave and higher temperature can be achieved as compared to other means.
- The operating procedure is simple as compared to other sterilization methods.
- Its price is low as compared to autoclave.

Hot air oven Disadvantages

- According to the principle of thermal inactivation by oxidation, it can't slaughter some living organisms, such as prions due to the use of dry heat rather than wet heat.
- Most of the materials are not fit with hot air ovens such as surgical dressings, rubber items, or plastic material; they can be a meltdown at low temperatures.

Hot air oven Application/Hot air oven uses

- It is used to dry glassware, sterilize N95 masks, general instruments, and packaging items in life science, microbiology laboratory.
- It is also used in chemical and pharmaceutical industries, food and beverage industries, textile industries.

- It helps in the elimination of moisture from the material thus it is used in curing, drying, baking, and annealing.
- It is also used for the Measurement of mixed liquor suspended solids (MLSS).
- In certain laboratories and hospitals, it is used to store materials at a constant temperature.

(b) MOIST HEAT:

Principle: Moist heat destroys microorganisms by the irreversible denaturation of enzymes and structural proteins. The temperature at which denaturation occurs varies inversely with the amount of water present. Sterilization in saturated steam thus requires precise control of time, temperature, and pressure.Pressure serves as a means to obtain the high temperatures necessary to quickly kill microorganisms. Specific temperatures must be obtained to ensure microbicidal activity. Minimum sterilization time should be measured from the moment when all the materials to be sterilized have reached the required temperature throughout.

Method: The moist heat sterilization achieved by using autoclave. Various types of autoclave are available eg. common laboratory autoclave, horizontal autoclave, vertical autoclave, large automatic hospital autoclave, gravity displacement autoclave. The recommendation for sterilization in an **autoclave is 15 minutes at 121°C** (200 kPa). The temperature should be used to control and monitor the process; the pressure is mainly used to obtain the required steam temperature.

1. Place the material to be sterilized inside the pressure chamber and fill the cylinder with sufficient water.

2. Close the lid and put on the electrical heater. 3.Adjust the safety valve to the required pressure.

4. After the water boils, allow the steam and air mixture to escape through the discharge tap till all the air has been displaced

This can be tested by passing the steam-air mixture liberated from the discharge tap into a pail of water through a connecting rubber tube. When the air bubbles stop coming in the pail, it indicates that all the air has been displaced by steam.

5. Close the discharge tap. The steam pressure rises inside and when it reaches the desired set level (e.g. 15 pounds (lbs) per square inch in most cases), the safety valve opens and excess steam escapes out.

6. Count the holding period from this point of time, which is about 15 minutes in most cases.

7.After the holding period, stop the electrical heater and allow the autoclave to cool until the pressure gauge indicates that the pressure inside is equal to the atmospheric pressure.8.Open the discharge tap slowly and allow the air to enter the autoclave.9.Open the lid of the autoclave and remove the sterilized materials.

Alternative conditions, with different combinations of time and temperature, are given below.

1 atm = 325 Pa

Temperature(°C)	**Approximatecorresponding pressure (kPa=kilo paskal)**	**Minimum sterilization time(min)**
126-129	250 (~2.5 atm)	10
134-138	300 (~3.0 atm)	5

In certain cases (e.g. thermolabile substances), sterilization may be carried out at temperatures below 121 °C, provided that the chosen combination of time and temperature has been validated. The selection of pressure depend upon the item or material or object which we have to sterilized and given in autoclave manual. Object or material may be syringes, cotton, media, surgical gloves etc.

Monitoring of steam sterilization process:

- Like other sterilization systems, the steam cycle is monitored by mechanical, chemical, and biological indicators. Steam sterilizers usually are monitored using a printout (or graphically) by measuring temperature, the time at the temperature, and pressure.

- Chemical indicators are affixed to the outside and incorporated into the pack to monitor the temperature or time and temperature. Autoclave indicator tapes are commercially available and a change in color of the tape suggests proper sterilization.

- Temperature-monitoring probes should be inserted into representative containers, with additional probes placed in the load at the **potentially coolest and least accessible parts** of the loaded chamber. The conditions should be within ±2 °C and ±10 kPa (±0.1 atm) of the required values. Each cycle should be recorded on a time-temperature chart or by other suitable means.

Biological indicator:

The effectiveness of steam sterilization is monitored with a biological indicator using an envelope containing spores of Geo*bacillus stearothermophilus* (formerly *Bacillus stearothermophilus*; e.g. ATCC 7953 or CIP 52.81) for which the D-value (i.e. 90% reduction of the microbial population) is 1.5-2.5 minutes at 121 °C, using about 10^6 spores per indicator *(*this is based on a worst-case scenario that an item may contain a population of 10^6 spores having same resistance as that of *Bacillus stearothermophilus*). After sterilization is over the strip is removed and inoculated into tryptone soy broth and incubated at 56°C for 5 days. No growth of *Geobacillus stearothermophilus* indicates proper sterilization.

Note-The D-value or decimal reduction time (or decimal reduction dose) is the time (or dose) required, at a given condition (e.g. temperature) or set of conditions, to achieve a log reduction, that is, to kill 90% (or 1 log) of relevant microorganisms.

list of commonly used biological indicators (BIs)

Spores of Bacteria	**D Value**
*Bacillus coagulans*0.3	0.3
Clostridium sporogenes	0.8-1.4*Bacillus*
Atropheus 0.5	0.5
Geobacillus stearothermophilus	0.5

Advantage of autoclaving:

1. Nontoxic to patient, staff, environment.
2. Cycle easy to control and monitor.
3. Rapidly microbicidal.

4.Least affected by organic/inorganic soils among sterilization processes listed

5.Rapid cycle time.

6.Penetrates medical packing, device lumens.

Disadvantage of autoclaving:

1. Deleterious for heat-sensitive instruments.
2. Microsurgical instruments damaged by repeated exposure.

3. May leave instruments wet, causing them to rust and Potential for burns.

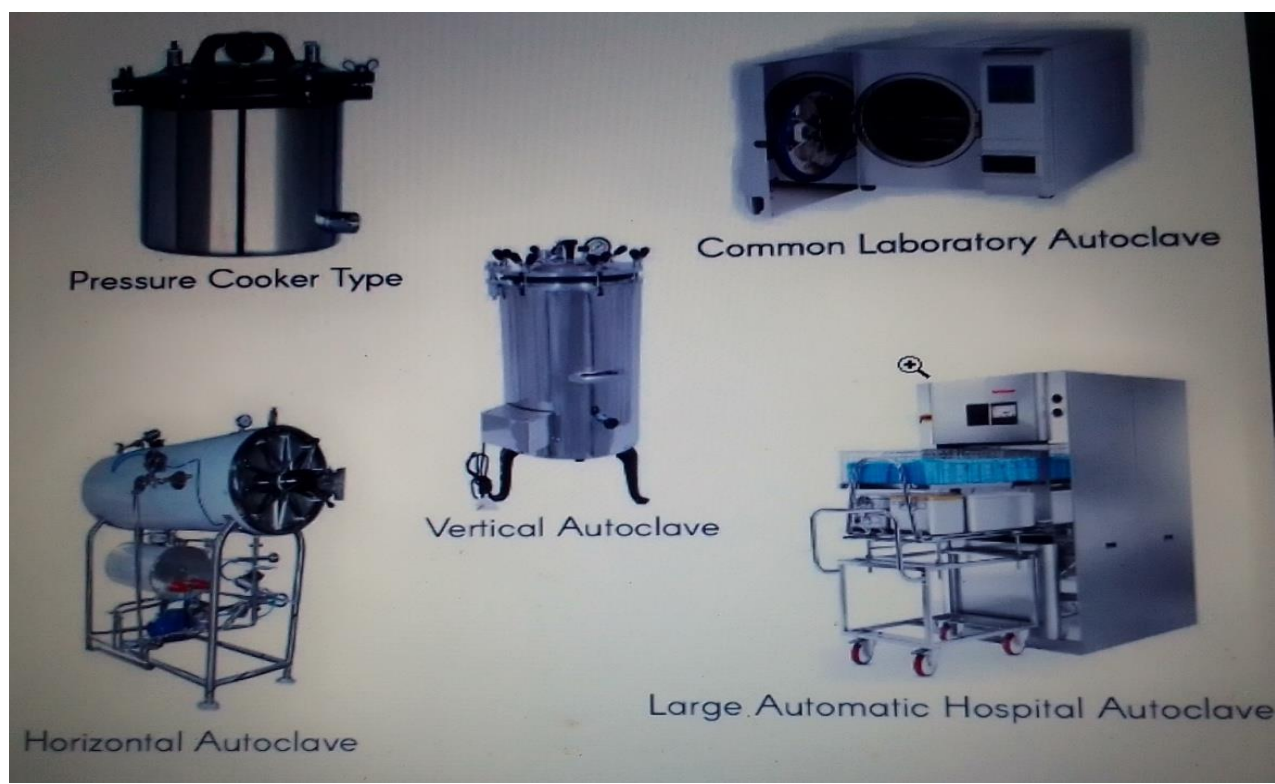

Fig 2.3 Various type of autoclave

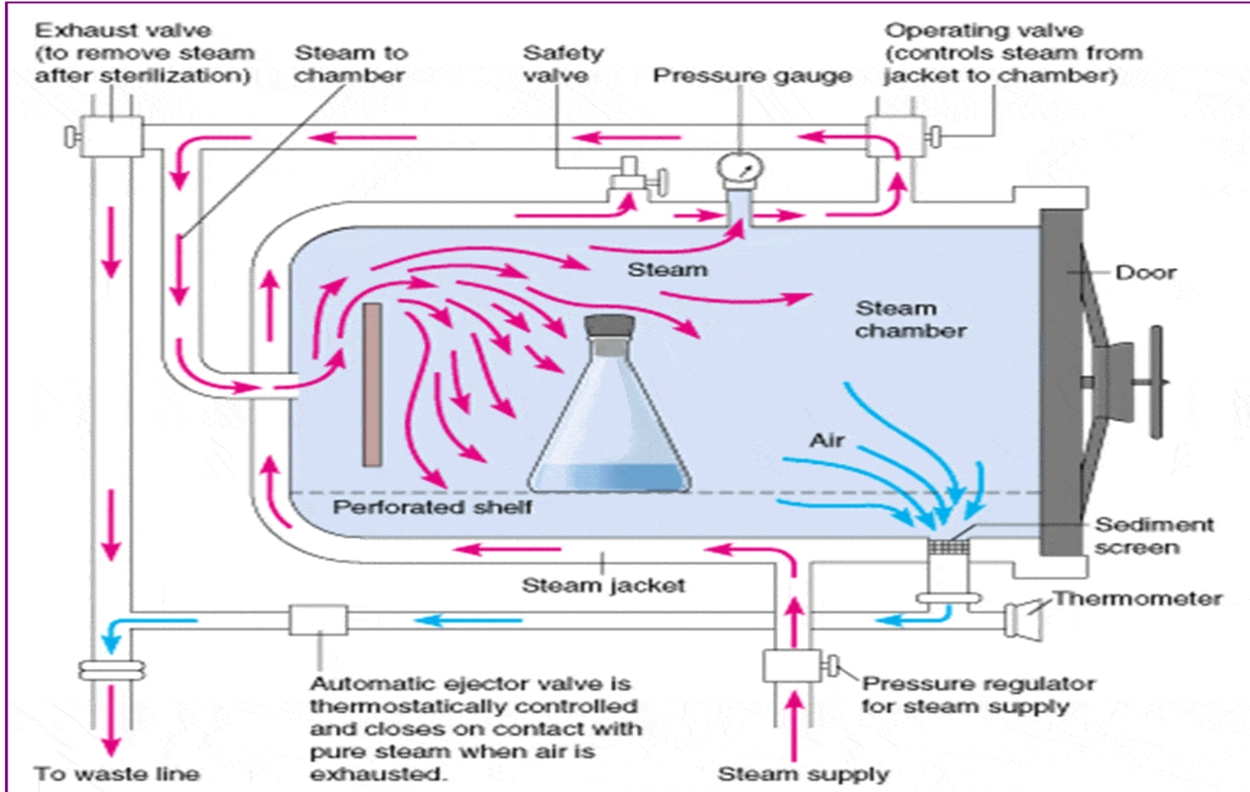

Fig 2.4 working principle of moist hest sterilization and various compartments of autoclave.

(c) Filtration:

Principle: It's the only method that uses force to separate rather than to kill. When you filter a liquid or gas, it passes through a pore, which stops, or filters out, the passage of larger particles. Filtration depends on pore sizes, the smaller the pores the more particles it can screen out, but it also takes more energy to force the liquid through it. Pore sizes can be as small as .01 µm (µm=micrometer), small enough to stop viruses from passing through, but smaller proteins can still get through . There are even really small filters called nano-filters, which stop viruses, proteins and some toxins.

Filtration is different from other sterilization methods due to friction force between two layer of liquid during filtration therefore it is consider as physical method while in different kind of well mechanized filter are also used in filtration process hence it is consider as mechanical method of sterilization .Filtration is the first and only sterilization method that eliminates bacteria by separating the microorganisms from the sterilized medium, but unlike other sterilization methods,

it doesn't kill or stop the bacteria's ability to reproduce. The way it works is actually very simple. We all are probably familiar with water filters found in an office or at home, or a coffee percolator, all of which use the same basic mechanism of filtration.

Types of filters: In laboratories, liquids are filtered through microbial filters to remove any microbes present. It is an effective method of sterilization for heat sensitive liquids. There are four types of filters:

ii Membrane filters: these are thin filters that are made of cellulose. They can be used for sterilization during injection by placing the membrane between the syringe and the needle.

ii Seitz filters: These are usually made of asbestos. They are pad-like and thicker than membrane filters.

iii. Sintered glass filters: These are an alternative type of filter that are made of glass and hence do not absorb liquids during filtration.

iv.Candle filters: these are made of clay-like mud. This special mud has tiny pores made by algae. The microbes get stuck during their travel through the pores now a days modified candle glass filters are widely used in laboratories.

Filtration Techniques:

There is more than one filtration technique. Reverse Osmosis is used in home filtration systems. Other common methods are nano-filtration, ultra-filtration, micro-filtration and particle filtration.

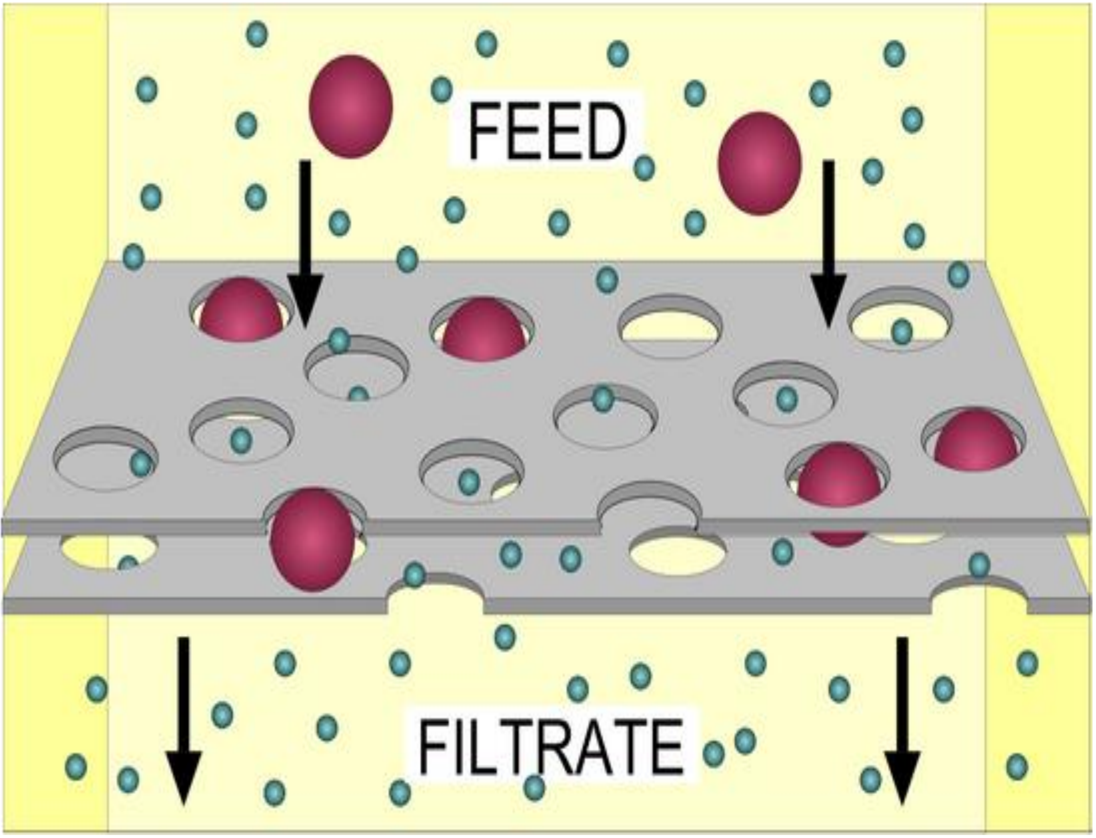

Fig 2.5 The Basics of Filtration: Large particles cannot pass through the smaller holes and get filtered.

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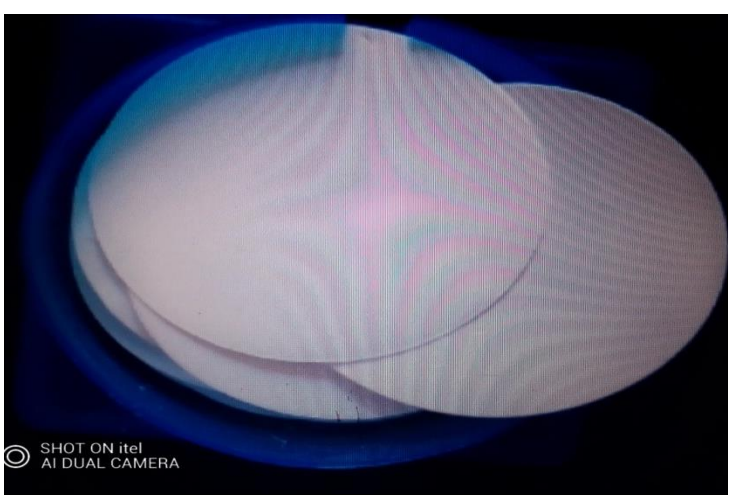

Fig 2.6 Membrane filter

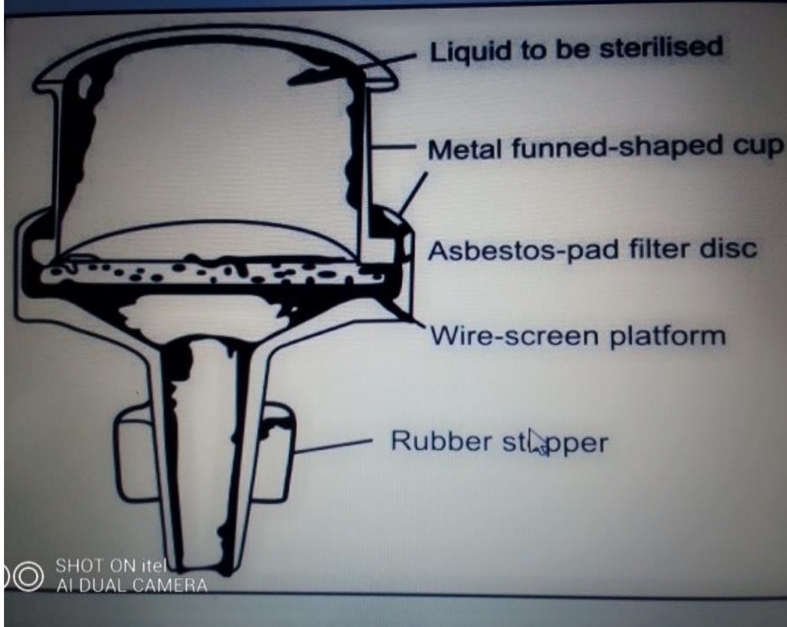

Fig 2.7 Seitz filter

Advantages of Filtration:

- Relatively inexpensive, except for those with the smallest pore sizes.
- Filters do not clog easily.
- Suitable for heat sensitive liquids as filters do not use heat.
- They can filter large volumes of fluid reasonably fast not so much time consuming.

Disadvantages of Filtration:

- Filters can only work on liquids and gasses.
- Autoclaving is usually cheaper than filtration since filters are expensive to replace, especially nano-filters
- Glass filters are very brittle and can break easily.

Fig 2.8 Sintered filter

Fig 2.9 Modified Candle glass filter

2.3.2 TYNDALLIZATION AND PASTEURIZATION:

(a) Tyndallization:

Principle: Tyndallization named after John Tyndall is a lengthy process designed to reduce the level of activity of sporulating bacteria that are left by a simple boiling water method. The process involves boiling for a period (typically 20 minutes) at atmospheric pressure, cooling, incubating for a day, boiling, cooling, incubating for a day, boiling, cooling, incubating for a day, and finally boiling again. The three incubation periods are to allow heat-resistant spores

surviving the previous boiling period to germinate to form the heat-sensitive vegetative (growing) stage, which can be killed by the next boiling step. This is effective because many spores are stimulated to grow by the heat shock. The procedure only works for media that can support bacterial growth - it will not sterilize plain water. Tindalization is ineffective against prions.

Method of sterilization of Media by Tyndallization

i. Tyndallization relies upon the germination of spores to form vegetative cells that can then be killed at 100º C. This germination is accomplished by heating the medium to 100º C for 15-30 min on three consecutive days.
ii. After boiling, the broth medium is incubated at 37º C overnight to allow the heat-shocked spores to germinate into vegetative cells.
iii. Then, when the broth is boiled the next day, the vegetative cells are killed.
iv. The boiling and incubation are repeated three times to ensure that all spores germinate. While this was once considered a means for sterilization, it is not used much today.
The temperature and incubation period depend on object to which we have to sterilized.

Disadvantage of Tyndallization

- One problem is that if there are many spores in the broth, you can get significant growth of bacteria. While those cells are killed by the next boiling step, the dead cells remain in the medium and it is time consuming technique.
- Another problem is that this procedure only works for broth media that support the growth of spore-forming organisms. It is not useful for sterilizing water or buffers.

(b)Pasteurization:

Principle: The process was named after the French microbiologist, Louis Pasteur, whose research in the 1860s demonstrated that thermal processing would deactivate unwanted microorganisms in wine **Pasteurization** or **pasteurisation** is a process in which packaged and non-packaged foods (such as milk and fruit juices) are treated with mild heat, usually to less than 100 °C (212 °F), to eliminate pathogens and extend shelf life. The process is intended to destroy or deactivate organisms and enzymes that contribute to spoilage or risk of disease, including vegetative bacteria, but not bacterial spores.

Types of pasteurization: On the bases of time and temperature the types of Pasteurization are:

Temperature	Time	Pasteurization Type
63ºC (145ºF)*	30 minutes	Vat Pasteurization
72ºC (161ºF)*	15 seconds	High temperature short time Pasteurization (HTST)
89ºC (191ºF)	1.0 second	Higher-Heat Shorter Time (HHST)
90ºC (194ºF)	0.5 seconds	Higher-Heat Shorter Time (HHST)
94ºC (201ºF)	0.1 seconds	Higher-Heat Shorter Time (HHST)
96ºC (204ºF)	0.05 seconds	Higher-Heat Shorter Time (HHST)
100ºC (212ºF)	0.01seconds	Higher-Heat Shorter Time (HHST)
138ºC (280ºF)	2.0 seconds	Ultra Pasteurization (UP)

If the fat content of the milk product is 10percent or more, or if it contains added sweeteners, or if it is concentrated (condensed), the specified temperature shall be increased by 3ºC (5ºF). Egg not shall be heated to at least the following temperature and time specifications:

Temperrature	Time	Pasteurization Type
69ºC (155ºF)	30 minutes	Vat Pasteurization
80ºC (175ºF)	25 seconds	High temperature short time Pasteurization (HTST)
83ºC (180ºF)	15 seconds	High temperature short time Pasteurization (HTST)

The original method of pasteurization was vat pasteurization, which heats milk or other liquid ingredients in a large tank for at least 30 minutes. It is now used primarily in the dairy industry for preparing milk for making starter cultures in the processing of cheese, yogurt, buttermilk and for pasteurizing some ice cream mixes.

The most common method of pasteurization in the United States today is High Temperature Short Time (HTST) pasteurization, which uses metal plates and hot water to raise milk temperatures to at least 161° F for not less than 15 seconds, followed by rapid cooling. Higher Heat Shorter Time

(HHST) is a process similar to HTST pasteurization, but it uses slightly different equipment and higher temperatures for a shorter time. For a product to be considered Ultra Pasteurized (UP), it must be heated to not less than 280° for two seconds. UP pasteurization results in a product with longer shelf life but still requiring refrigeration.

Another method, aseptic processing, which is also known as Ultra High Temperature (UHT), involves heating the milk using commercially sterile equipment and filling it under aseptic conditions into hermetically sealed packaging. The product is termed "shelf stable" and does not need refrigeration until opened. All aseptic operations are required to file their processes with the Food and Drug Administration's "Process Authority." There is no set time or temperature for aseptic processing; the Process Authority establishes and validates the proper time and temperature based on the equipment used and the products being processed.

Advantage of pasteurization:

- **Prolonged shelf life**

Keeping product fresh long enough for it to make it to market and then on to consumers’ pantries is key. Some bacteria and other microorganisms can cause food products to deteriorate faster than it takes for the end consumer to purchase it, so pasteurization is vital to making your food products viable.

- **Preventing disease**

Diseases are found in many food products, and removing the organisms that cause those diseases is critical to ensuring your product is safe for general consumption. For example, eggs are known to spread salmonella and avian flu, and pasteurization kills the organisms that cause those diseases.

- **Quick and safe sanitation**

There are many ways to sanitize food products, but few are as quick or as safe as pasteurization. With pasteurization, the temperature of the product is simply raised enough to destroy any microorganisms that may be present. Other methods may involve chemical treatments or radiation, and may not be the safest to use.

Pasteurization is also faster than most methods that rely on filtration or other means.

- **Consistent product quality**: By eliminating volatile contaminants, the product becomes more stable, therefore the quality of your product is more consistent
- **Potential improvements in flavor and scent:** In some cases removing those bacteria can create a more consistently pleasant experience for the consumer.
- Regulatory compliance: For example, the FDA requires that pasteurized eggs or egg products should be used instead of raw eggs in certain products when serving populations such as school children and nursing home patients.

Disadvantage of pasteurization:

It is coast effective technique and usually uses in food processing and may destroy minerals and useful vitamins (B_6 and B_{12}), proteins which are present inside the food.

2.3.3 RADIATION – DISINFECTION-

There are 2 general types of radiation used for sterilization, **ionizing radiation** and **non-ionizing radiation**.

(A)Ionizing radiation:

Principle: hen ionizing radiation collides with particles, they produce electrons (e^-) and other reactive molecules such as hydroxyl radicals (•OH), and hydride radicals (H•). Each of these reactive molecules is capable of degrading and altering biopolymers such as DNA and protein. Breakage of DNA and degradation of enzymes lead to the death of the irradiated cells.Several sources of ionizing radiation are available, including X-ray machines, cathode ray tubes (electron-beam radiation), and radioactive nuclides (sources of gamma/x-rays).

(i)X-Rays:

X-rays are lethal to microorganisms and higher forms of life but are rarely used in sterilization because their production is expensive and efficient utilization is difficult (since radiations are given off in all directions from the point of origin).

(ii)Gamma Rays:

Gamma radiations are high-energy radiations emitted from certain **radioisotopes such as Caesium-137 (^{137}Cs) and Cobalt-60 (^{60}Co),** both relatively inexpensive bioproducts of nuclear fission. Gamma rays are similar to x-rays but are of shorter wavelength and higher energy. They are capable of great penetration into the matter, and they are lethal to all life, including microorganisms. Gamma rays are attractive for use in commercial sterilization of materials of considerable thickness or volume, eg. packaged food or medical devices.

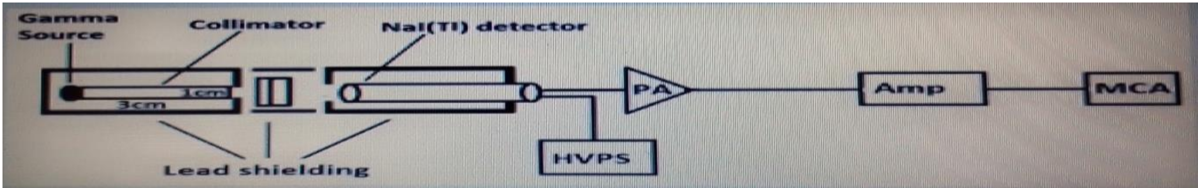

Fig 2.10 Gamma rays pathway

(iii)Cathode rays or electron-beams:

Cathode rays or electron-beams can sterilize materials at room temperature with brief exposure. They have limited penetrating power and are used for the sterilization of surgical supplies, drugs, and other .materials.

Advantages of Ionizing Radiations:

- **High penetrating power:** products can be processed in their fully sealed, final packaging thus limiting the risk of contamination following sterilization.

Fig 2.11 gamma rays torch

Rapidity of action: saves and efforts.

- **Temperature is not raised**: compatible with temperature-sensitive materials, such as pharmaceuticals and biological samples.
- **Flexibility:** can sterilize products of any phase (gaseous, liquid, or solid materials),
- density, size, or thickness.

Disadvantages

- Capital costs are high and specialized facilities are often needed e.g. for gamma irradiation.
- Use of gamma radiation requires handling and disposal of radioactive material.
- Not compatible with all materials and can cause breakdown of the packaging material and/or product. For example, Common plastics such as polyvinyl chloride (PVC), acetal, and polytetrafluoroethylene (PTFE) are sensitive to gamma radiation.

(B)Non-ionizing radiation:

Non-ionizing radiations are quite lethal but do not penetrate glass, dirt, films, water; hence their use is restricted for disinfection of clean surfaces in operation theaters, laminar flow hoods as well as water treatment. The recommended dose is 250-300 nm wavelength, given for 30 minutes. These are spectrum visible and non-visible light. The examples of non-ionizing radiation include **infrared and ultraviolet radiation.** These are following types:

(i)Infra-red rays:

Principle: These are low energy type electromagnetic rays, having wavelengths longer than those of visible light. They kill microorganisms by oxidation of molecules as a result of heat generated. Infra-red rays are used for the rapid mass sterilization of syringes and catheters

(ii)Ultraviolet Light **(UV):**

Sunlight is partly composed of UV light but most shorter wavelengths of light are filtered out by the ozone layer. There are three types of UV radiation; UVA, UVB, and UVC, classified according to their wavelength. Short-wavelength UVC is the most damaging type of UV radiation.

Principle: Many cellular materials including nucleic-acids absorb ultraviolet light. It causes bonding of two adjacent pyrimidines i.e., the formation of pyrimidine dimer, resulting in the inhibition of DNA replication. This leads to mutation and death of exposed organisms. While UV sterilization is ongoing, the area should be closed and UV lamps must be switched off immediately after use.

Fig 2.12 Laboratory UV machine

Uses of UV Sterilization:

UV lights are useful for disinfecting surfaces, air, and water that do not absorb the UV rays. Certain types of UV lights can kill the flu (influenza) virus. Ultraviolet radiation is used for disinfecting enclosed areas such as bacterial laboratory, nurseries, inoculation hood, laminar air flow, and operation theaters. For example, laboratory biological cabinets all come equipped with a "germicidal" UV light to decontaminate the surface after use.

Effects of UV light in SARS-CoV-2 (COVID-19):

UV radiation kills viruses by chemically modifying their genetic material, DNA, and RNA. The most effective wavelength for inactivation, 260 nm, falls in the UVC range. Though we do not have much research regarding the effect of UVC in SARS-CoV-2, concentrated form of UVC is now on the front line in the fight against COVID-19. UVC light is being used to sterilize buses,

UVC-emitting robots to sterilize hospital floors and even banks are using UV light to disinfect money.

Disadvantages:

Damages skin and eyes: Conventional UV light can penetrate and damage skin and also cause cataracts. Does not penetrate, glass, and cloth. microbeonline.com/radiation-sterilization-types-mechanism-applications

2.4 *CULTURE TECHNIQUES-CULTURE MEDIA PREPERATION*

2.4.1 AEROBIC AND ANAEROBIC CULTURE TECHNIQUES

(A)Aerobic bacteria: Aerobic bacteria are the bacteria that survive and grow only in the presence of oxygen. Aerobes grow and live in an ambient environment, i.e. 21% oxygen and 0.03 carbon-dioxide. Aerobic bacteria need molecular oxygen for survival and growth. The reason is aerobic respiration in bacteria is used to derive energy from oxidative phosphorylation and Krebs cycle (also called TCA cycle or citric acid cycle) and some part of the energy from glycolysis. The **Krebs cycle** is the metabolic pathway undertaken by aerobic organisms to generate energy or ATP molecules, which are required to carry out various cellular processes. In aerobes, molecular oxygen serves as a *terminal electron acceptor* during respiration. Hence, aerobic microorganisms exhibit aerobic growth and cannot survive in the absence of oxygen. An example of aerobic bacteria is *Bacillus cereus*.

Aerobic Bacteria can be classified as:

- Obligate aerobes
- Facultative erobes
- Microaerophiles
- Aerotolerent aerobes

Obligate aerobes:

Obligate aerobes are organisms that cannot survive in the absence of oxygen. Therefore, obligatory aerobes *mandatorily* need the presence of oxygen in their environment for survival and

growth. Aerobic bacteria utilize molecular oxygen as a terminal electron acceptor, oxidize sugar and fats, and generate ATP/energy by the process of glycolysis, electron transport chain, and the Krebs TCA cycle. The enzymes involved in the respiratory chain in obligate aerobes are catalase, peroxidase, and superoxide dismutase. These three enzymes are essential for the aerobic biology of the aerobes as they help to counter the toxic effects of the reactive oxygen species generated due to the presence of molecular oxygen. Examples of obligate aerobes are *Bacillus, Mycobacterium,* and *Pseudomonas*.

Facultative aerobes:

Facultative aerobic bacteria do not entirely rely on the availability of oxygen in their environment. Instead, these bacteria utilize anaerobic methods to produce ATP/energy molecules. They can therefore survive even in the absence of oxygen. An example of a facultative aerobe is *Enterobacteriaceae.*

Microaerophiles:

As the name suggests, microaerophiles need only *a small amount of oxygen* for the generation of energy. The presence of oxygen in higher amounts can be lethal to microaerophiles. Microaerophiles lack an electron transport system and are dependent on the fermentation reaction to generate energy. Examples of microaerophiles are *Helicobacter* and *Campylobacter.*

Aerotolerant aerobes:

Aerotolerants do not utilize oxygen for a metabolic activity or for the generation of energy. They are also not adversely affected by the presence of oxygen. Aerotolerant bacteria do not possess enzymes (particularly, catalase, peroxidase, and superoxide dismutase) required for aerobic respiration. Examples of aerotolerant aerobes are *Lactobacilli* and *Streptococci.*

Aerobic Bacteria Examples:

- *Pseudomonas aeruginosa is* a rod-shaped Gram-negative bacterial species that may cause disease in plants and animals, including humans (infections in lungs or blood).

- *Nocardia* is a genus of rod-shaped Gram-positive bacteria. Certain species may cause nocardiosis, a lung disease caused by breathing in dust particles containing infective *Nocardia* species.
- *Mycobacterium tuberculosis* is a causative agent of another lung disease, tuberculosis.
- *E. Coli*
- *Proteus is* a saprophyte found widely in manure soil, sewage, animal feces
- *Salmonella* is a genus of rod-shaped Gram-negative bacteria certain species are commonly associated with food-borne illnesses.
- *Achromobacter* is a genus of rod-shaped Gram-negative bacteria characterized by the presence of peritrichous flagella. They are strictly aerobic, thriving in water and soils.
- *Klebsiella* is a genus of rod-shaped Gram-negative bacteria that are ubiquitous in nature and part of the normal flora of the digestive tract of humans.
- *Citrobacter* is a genus of Gram-negative coliform bacteria. The species are opportunistic pathogens. In humans, certain species can cause urinary tract infections, pneumonia, CNS infections, and neonatal infections.

(B)Anaerobic bacteria: In contrast, to define ***anaerobic bacteria***, that would be bacteria that ***do not*** require oxygen. They are also referred to as *anaerobes*. Anaerobic bacteria do not require oxygen for survival; in fact, oxygen can be toxic and lethal for some anaerobic bacteria. *So how do they derive energy?* Anaerobic bacteria derive their energy from anaerobic respiration and by lactic acid or alcoholic fermentation. In fermentation, they use Nicotinamide Adenine Dinucleotide Hydrogen (NADH) as an *electron carrier*. During glycolysis, NADH traps the energy of the electrons and *converts* them into ATP molecules. The differences between arobes and anaerobes is given in table -1.

Table 1: Aerobic bacteria vs Anaerobic bacteria	
Aerobic bacteria	**Anaerobic bacteria**
Define aerobic bacteria: Aerobic bacteria are bacteria that thrive and grow in an aerobic environment (**with oxygen**). A single bacterium that needs oxygen for survival is referred to as an aerobic bacterium.	Define anaerobic bacteria: Anaerobic bacteria are bacteria that thrive and grow in the **absence** of oxygen. Such bacteria can't tolerate the presence of oxygen in their environment
Bacterial cellular respiration – Aerobically	Bacterial cellular respiration – Anaerobically
Bacterial cellular respiration – Aerobically	Bacterial cellular respiration – anaerobically
Molecular oxygen serves as the terminal electron acceptor	Varies; carbon dioxide, sulfur, ferric, nitrate, or fumarate may serve as an electron acceptor
Survive only in the presence of molecular oxygen	Anaerobes cannot survive in the presence of molecular oxygen. In addition, molecular oxygen is toxic for such bacteria
Such bacteria possess catalase, peroxidase, and superoxide dismutase enzymes to neutralize the reactive oxygen species generated due to aerobic respiration	Nitrate, acetate, methane, and sulfide, like products, are generated.
The amount of energy produced by aerobes is greater than in anaerobes	The amount of energy produced by anaerobes could be less than in aerobes
Aerobes can be found in different areas like water, soil, etc.	Anaerobes are located in regions that are deficient in oxygen or oxygen-depleted areas.
Aerobes get localized at the surface of the liquid growth medium	Aerobes localize themselves at the bottom of the liquid growth medium

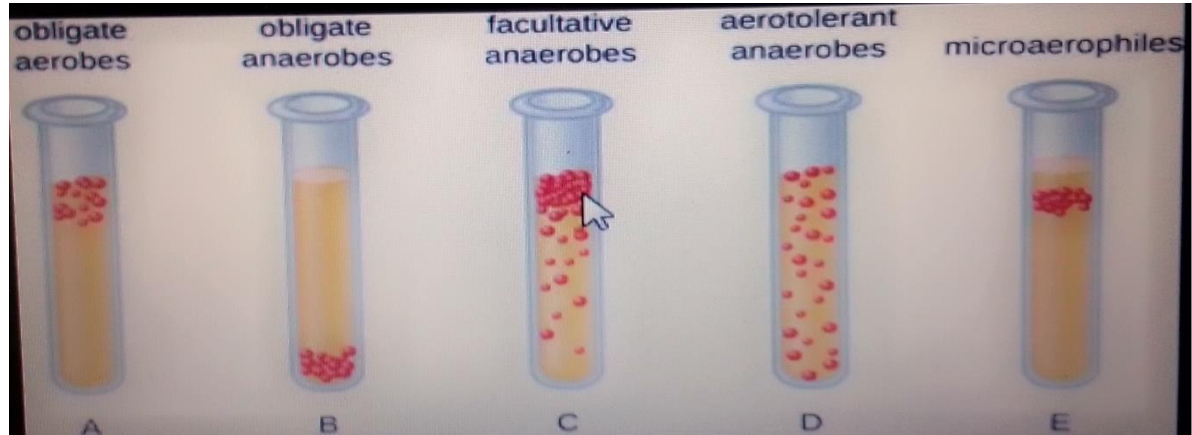

Fig 2.13 Bacteria can be identified based upon their oxygen requirement in a thioglycolate growth medium. Depending upon their oxygen requirement, bacterial populations localize themselves in a different region of the growth medium, ranging from high oxygen requirement or aerobes found near the surface of the growth medium to anaerobes that are located at the bottom of the growth medium.

(C)Types of culture media: Culture media is a gel or liquid that contains nutrients and is used to grow bacteria or microorganisms. They are also termed growth media. Different cell types are grown in various types of medium. Nutrient broths and agar plates are the most typical growth media for microorganisms. Some microorganisms or bacteria need special media for their growth.

Significance – Culture media is used in order to identify the causative agent from infected material.

Types of Culture Media:

The culture media are classified in many different ways:

1. Based on the physical state
    - Liquid media
    - Solid media
    - Semisolid media

Based on the presence or absence of oxygen.

- Anaerobic media

- Aerobic media

Based on nutritional factors

- Simple media
- Synthetic media
- Complex media
- Special media

Nutrient broths or lysogeny broth (LB) medium are the two most popular growth media for bacteria. Agar is frequently added to liquid media before being placed into a petri dish to solidify. These agar plates offer a stable medium for the cultivation of bacteria. It stays solid because relatively few bacteria can break down agar.

Simple Media or non selective media

Simple culture media includes nutrient broth. One percent meat extract and peptone water makes up such broth. Nutrient broth becomes glucose broth when glucose is added to it. Likewise, it becomes nutrient agar when 2-3% agar is added. For purposes of diagnosis, this is the simplest and most common media used in laboratories. A semisolid medium that permits the propagation of motile bacteria can be produced if the concentration of agar is decreased. Simple media: It's a general-purpose media that supports the growth of non-fastidious (non selective) microbes, and it is primarily used for the isolation of microorganisms. Examples are nutrient broth, peptone water, and nutrient agar, glucose broth.

Complex Media

Other than simple media, all forms of media are termed complex media. Complex media contain additional components for bringing out specific qualities or providing the unique nutrients needed for the bacterium's growth. Here, the source of the amino acids contains a variety of chemicals whose precise composition is unknown, thus it is an undefinable media. Examples – MacConkey agar medium and chocolate agar.

Synthetic Media:

A synthetic medium is a defined medium. A defined medium (chemically defined medium) is a medium in which there is no yeast, plant or animal tissue present, and all the chemicals employed are known. These are made from only pure ingredients whose exact composition is known. These are employed in specialised investigations like those on metabolic needs. Example – Dubo's culture medium with tween 80.

Special Media: Special media are of seven different types:

Enriched media – It is created when a basic medium is supplemented with nutrients like eggs, blood or serum. For example, a blood agar medium is used for the growth of bacteria like *Streptococcus* which specifically requires blood for its proliferation.

- **Selective media –** Selective media are used for the growth of only selected microorganisms. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium to prevent other cells, which do not possess the resistance, from growing. Media lacking an amino acid such as proline in conjunction with *E. coli* unable to synthesize it were commonly used by geneticists before the emergence of genomics to map bacterial chromosomes. For example, bile salt acts as a selective agent in BSA or bile salt agar. While preventing the growth of other intestinal organisms, it favours the growth of *Vibrio cholerae*.
- **Differential or indicator media:** It distinguish one microorganism type from another growing on the same medium.This type of media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as neutral red, phenol red, eosiny, or methylene blue) added to the medium to visibly indicate the defining characteristics of a microorganism. These media are used for the detection of microorganisms and by molecular biologists to detect recombinant strains of bacteria. i.e In MacConkey's medium all ingredients such as Peptone, agar, lactose, neutral red and sodium taurocholate are all ingredients are present. In MacConkey agar lactose fermenters produces pink colonies whereas, non-lactose fermenter produces pale or colorless colonies.

  TCBS (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose).

Wilson and Blair media: gives the black colonies of *Salmonella typhi* that develop on sulphite-containing.

- **Enrichment media** – This media contains several ingredients that either stimulate the bacteria being grown or suppress their competitors. Examples – Alkaline peptone water and tetrathionate broth.
- **Transport media** – These are employed when dealing with delicate organisms that might not make it through the transit period or might become covered with non-pathogenic germs. Special media are developed for the transportation of such bacteria to laboratories and these are known as transport media. Example – Stuart's transport medium.
- **Indicator media** – When bacteria multiply in these media containing an indicator, they tend to change their colour. MacConkey's medium is also an example of an indicator medium. Another classic example is the black colonies of *Salmonella typhi* that develop on sulphite-containing Wilson and Blair media.
- **Sugar media** – It contains 1% sugar, which can be any fermentable substance like glucose, mannitol, sucrose and lactose. The generation of acid following the fermentation of sugar transforms the medium into pink due to the presence of an indicator. Also to show that gas is produced, Durham's tube is kept inverted inside the sugar tube and gas bubbles are observed.

(a)Composition of culture media: The formulation of all Oxoid culture media and the components can be divided into different roles or functions:

1. Nutrients: proteins/peptides/amino-acids.

2. Energy: carbohydrates.

3.Essential metals and minerals: calcium, magnesium, iron, trace metals: phosphates, sulphates etc.

4 .Buffering agents: phosphates, acetates etc.

5.Indicators for pH change: phenol red, bromo-cresol purple etc.

6.Selective agents: chemicals, antimicrobial agents.

7.Gelling agent: usually agar.

There is often an overlap of functions of some media components, thus protein hydrolysates will supply amino-nitrogen, energy, some metals/minerals and act as buffering agents. Phosphate buffers are important suppliers of minerals and agar contributes metals.

1. Nutrients: The nutrient components of culture media are carefully selected to recover the required spectrum of organisms in the sample e.g. coliforms or anaerobes. General purpose media such as blood agar in its various forms will often contain mixtures of peptones to ensure that peptides of sufficient variety are available for the great majority of organisms likely to be present. However, more demanding organisms will require supplemental growth factors to be added and examples of such requirements can be seen in media for *Legionella* species.

2. Energy: The most common substance added to culture media as a source of energy to increase the rate of growth of organisms is glucose. Other carbohydrates may be used as: required. Carbohydrates added to media at 5-10 grammes per litre are usually present as organisms. It is usual to add pH indicators to such formulations.

3.Essential Metals and Minerals: The inorganic essential components of culture media are many and can be divided on a semi-quantitative basis: Typical macro-components (gm/litre): Na, K, Cl , P, S, Ca, Mg, Fe. Typical micro-components (mgm-microgm/litre): Zn, Mn, Br, B, Cu, Co, Mo, V, Sr, etc.

As previously mentioned, a formulation may not have specific metals and minerals listed in its formulation. In such cases it is assumed that all the factors required are present in the hydrolysates, buffers and agar components.

4. Buffering Agents: It is important that the pH of a culture medium is poised around the optimum necessary for growth of the desired micro-organisms. The use of buffer compounds at specific pK values is especially necessary when fermentable carbohydrates are added as energy sources. Phosphates, acetates, citrates, zwitterion compounds and specific amino-acids are examples of buffering agents that may be added to culture media.

A side effect of such compounds is their ability to chelate (or bind) divalent cations (Ca ++ and Mg ++). Polyphosphate salts, sometimes present in sodium phosphate, are compounds which can bind essential cations so firmly that they are made inaccessible to the micro-organisms.

The effect of these binding or chelating agents will be seen in diminished growth or failure to grow at all, unless care has been taken to supplement the essential cations in the formulation. Opacity forming in a medium, after heating or on standing at 50°C for several hours, is commonly caused by phosphate interaction with metals. Such phosphate precipitates can very effectively bind Fe and lower the available amount of this essential metal in the medium.

5. Indicator Substances: The addition of coloured indicator substances is a very effective way of detecting fermentation of specific carbohydrates in a culture medium. Such compounds should change colour distinctly and rapidly at critical pH values.

Most of the compounds used e.g. phenol red, bromo-cresol purple, fuchsin, etc., are toxic and it is essential to use low concentrations of pre-screened batches/lots. Known sensitive strains of micro-organisms are used in the screening tests.

6. Selective agents: Chemicals or antimicrobials are added to culture media to make them selective for certain micro-organisms. The selective agents are chosen and added at specific concentrations to suppress the growth of unwanted organisms in a polymicrobial sample. It is, of course, essential to have established that the selective agents, at the appropriate concentration, will allow uninhibited growth of the desired organisms.

Common chemical selective agents are: bile salts, dye-stuffs, selenite, tetrathionate, tellurite and azide. Antimicrobial agents are commonly used in mixtures when suppressing polymicrobial contaminating flora. Antimicrobials are more specific in their selective action than the chemical agents shown above. However, the critical weighing and heat-lability of most antimicrobials demand special care and post-sterilisation addition.

The wide variety of organisms and their almost infinite ability to adapt to changing conditions makes a truly selective medium unlikely. Selective media can be said to suppress most of the

unwanted organisms and allow most of the desired organisms to grow. The final formulation is usually a compromise which achieves the best of these criteria.

7. Gelling Agents: Although gelatin is still used for a few specific media and carrageenans, alginates, silica gel and polyacrylamides are sometimes used as gelling agents, the outstanding gel-forming substance used in culture media is agar. Agar is obtained from agarophyte sea-weeds mainly *Gelidium, Gracilaria* and *Pterocladia* species. It is extracted as an aqueous solution at greater than 100°C, decolourised, filtered, dried and milled to a powder.Microbiological agar is specially processed to yield a low toxicity, high clarity, low mineral and high diffusion gel.

Other Components: There are many other substances added to culture media for specific purposes e.g. growth factors for fastidious organisms, eH-reducing compounds for anaerobic organisms (thioglycollate and cysteine), whole blood to detect haemolytic enzymes and encourage the growth of organisms which are vulnerable to oxidation products.

(b)Steps involved culture media preparation: Preparation of culture media formulations, including liquid growth media and culture media based on nutrient agar, is a common procedure in any microbiology laboratory. The culture media formulation process involves many steps and must be carried out with care to avoid cross contamination and ultimately protect the health of consumers.

1. Select culture media protocol from database.

2. Recalculate ingredient quantities according to the required culture media volume.

3. Weigh main ingredients into the container.

4. Weigh trace ingredients on a high accuracy balance and add to the container.

5. Pour in deionized water up to around 80% of the required volume.

6. Mix to dissolve the ingredients, gentle heating may be required.

7. Check the pH using a pH meter and adjust if necessary.

9.Top up the culture media to the required volume.

10.Label the container.

11.Sterilize in autoclave.

(c)Non-selective media used in anaerobic bacteriology:

1. Nutrient broth: Used for the culture of aerobic microorganisms. Beside this peptone water, nutrient agar, glucose broth are also simple media.

2.Cooked meat broth (e.g. Robertson's Cooked Meat Medium): Non-selective for the cultivation of anaerobic organisms; with the addition of glucose, can be used for gas-liquid chromatography.

3.Anaerobic blood agar: It is a non-selective medium for the isolation of anaerobes and facultative anaerobes.

1. 4.Egg-yolk agar (EYA): Non-selective for determination of lecithinase and lipase production by Clostridia and Fusobacteria.

5.Peptone-yeast extract glucose broth (PYG): Non-selective for the cultivation of anaerobic bacteria for gas-liquid chromatography.

(d)Selective and differential media used in anaerobic bacteriology:

1. Bacteroides bile esculin agar (BBE): It is selective and differential for *Bacteroides fragilis* group and good for presumptive identification.
2. Laked Kanamycin-vancomycin blood agar (LKV): It is selective for isolation of *Prevotella* and *Bacteroides* spp.
3. Anaerobic phenylethyl alcohol agar (PEA): Selective for inhibition of gram-negative rods and swarming by some Clostridia.
4. Cycloserine cefoxitin fructose agar (CCFA): Selective for *Clostridium difficile.*

5. Thioglycollate broth: Nonselective for the cultivation of anaerobes; as well as facultative anaerobes and aerobes.

(D)MEDIA PREPERAION AND INOCULATON OF CULTURE MEDIA

(1)MEDIA PREPWERATION FOR AEROBIC BACTERIA:

Nutrient agar medium.

Nutrient agar contains nutrients that suitable to subculture a wide range of microorganisms and makes it an excellent agar media to check on the purity before any biochemical or serological test.Besides, the addition of agar solidifies nutrient agar, which makes it suitable for the cultivation of microorganisms. You can also add up to 10% blood or other biological fluids that meet your experimental purpose.

- Suspend 28.00gm of nutrient agar powder in 1L of distilled water in conical conical flask. Mix and dissolve them completely with the help of glass rod or stirrer.
- Sterilize by autoclaving at 121°C for 15 minutes.
- Maintain the pH if necessary by adding buffer solution.
- Pour the liquid into the petri dish and wait for the medium to solidify. Be sure that you Once the agar solidifies, the agar is ready to use.

Here's a tip for you to ensure that your prepared agar is sterile. you can incubate our freshly prepared agar in an incubator for a few days. If there aren't any microorganisms grow on the agar, we are safe to use it.

We have to store the dehydrated medium at 10-30°C and use before the expiry date on the label. Once we prepare the nutrient agar in the petri dish, store them at 2-8°C.

Composition of dehydrated agar powder: Refindemeat extract-10gm /Ltr, Yeastextract-2.0gm /Ltr, Peptone-5.0gm/Ltr, Sodium-5.0gm/ltr, Agar-15.0gm/Ltr. Maintain the pH pH 7.4 ± 0.2 @ 25°C.

Fig 2.14: Transfer of nutrient agar in Petri dish.

(ii)Inoculation of aerobic culture media:

For the effective detection of the bacterial content of specimens, it is important to achieve growth of individual colonies by using a good technique to inoculate the specimen on culture media. In simple terms, inoculation in microbiology is the process of introducing microbes into a culture media so that it reproduces there in aseptic condition. The specimen of bacteria which have to inoculate is called inoculum.

In Bacteriology, there are several techniques used for inoculating. Some of the most commonly used techniques are as discussed:

(a)Pour Plate Method:

Direct inoculation Method:

- Unscrew the cap or cotton wool plug in the bottle that contains the inoculum.
- Take the clean Pasteur pipette out of its container, then attach the pipette to your left hand.
- Take the bottle or test tube that contains the inoculum using your right hand.
- Take off the cap/cotton wool plug using the small finger on your right hand.
- Flam the bottle/test the tube neck.
- Take 1 mL of the sample by using a sterile pipette lightly (figure-3).

- Dispense 1ml of the dilute sample into the center of the Petri plate using a sterilized micropipette or a calibrated pipette. (Put sample at the dilution you want in the Petri plate that is marked with the specific dilution percentage.)
- Take the pipette off and flame at the top of the bottle or test tube once again. Replace the cap or cotton wool plug.
- Place the test bottle or tube in the laboratory or on its rack.
- Unlock the lid of the bottle, and then flame the mouth. Pour around 15 mL of sterilized, molten media at the right temperature over the sample.
- Cover the plate, then mix the samples thoroughly with a gentle swirling motion on the plate. The plates are usually turned in the “S” or “8” shape.

(All the work is done within the Laminar airflow)

Indirect Inoculation Method:

- Unscrew the cap or cotton wool plug in the bottle that contains the inoculum.
- Take the sterilized Pasteur pipette from the container, and attach the pipette to your right hand.
- Lift the test bottle or tube that contains the inoculum using the left hand.
- The cap or cotton wool plug is removed using the small finger of your right hand.
- Flam the bottle/test tube neck.
- Take 1 mL of your sample using a sterilized pipette to dilute it gently.
- In a tube that has approximately 15mL of the molten medium at a suitable temperature, add 1 milliliters of the sample figure.
- Remove the pipette, and then flame at the top of the bottle or test tube. Replace the cap/ wool plug.
- Place the test tube in the laboratory or on its rack.
- Mix the sample correctly within the media.
- Pour the media onto the sterile Petri plate.

The dish should be moved gently so that the inoculum with the medium is thoroughly mixed, and to make sure that the medium is covering the dish evenly. You can either move the dish across three different directions, first, N-S direction followed by NW-SE and then turn it NE-SW, or move until the inoculum and the medium are well mixed and completely fill the bottom of the dish. (All of this work will be done within the Laminar airflow).

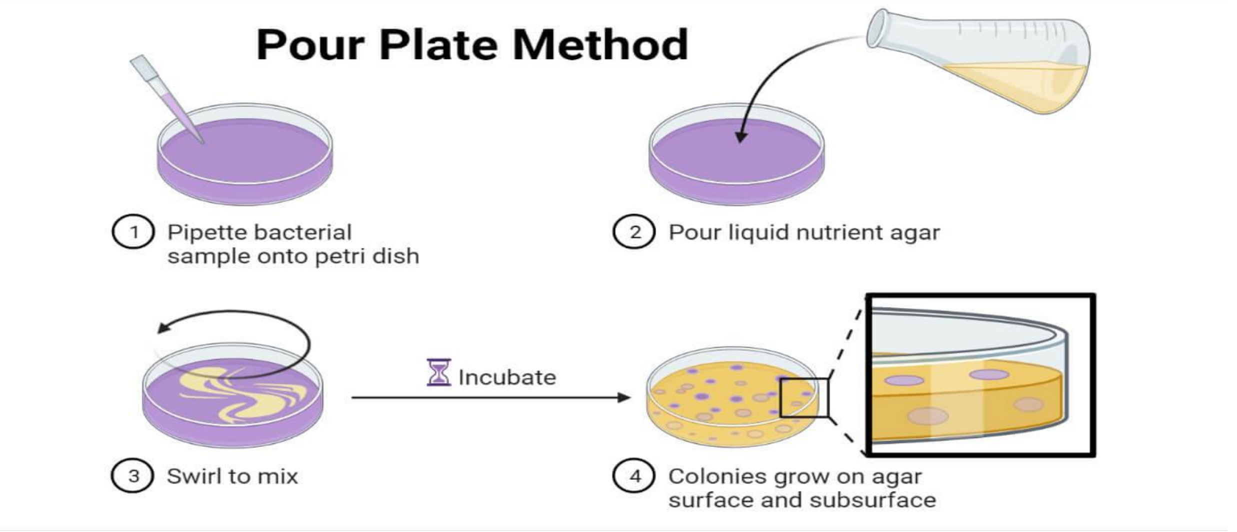

Fig 2.14: Pour plate method of inoculation:

Final Step

- Cover the lid of the Petri plate. Then, let the media fully solidify.
- Place the plate in an inverted position within an incubator, under the appropriate conditions for incubation (mostly for up to 24 hours at 37 degrees Celsius).

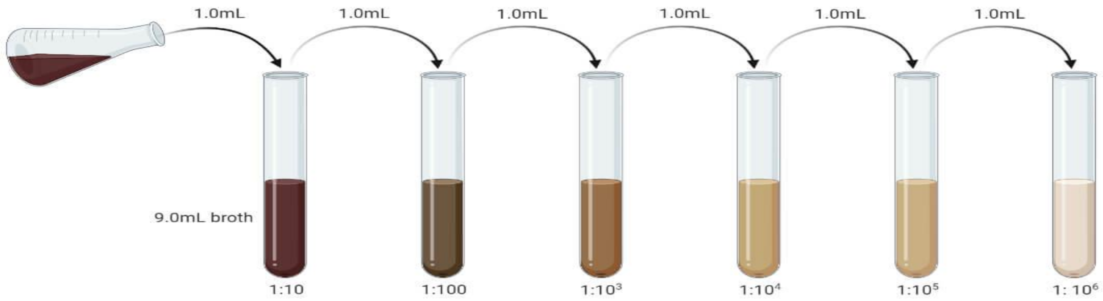

Fig 2.15 Serial dilution of specimen.

Result Interpretation of Pour Plate Method:

After 24 to 48 hours of incubation, count all colonies (note that embedding colonies are smaller than those that are likely to grow in the air). A magnifying colony counter may assist in the counting of tiny embedded colonies. Calculate CFU/mL by using the formula = (number of colonies x dilution factors) /Volume of culture plates*

Or Calculate CFU/mL by using the formula = (number of colonies x concentration of sample)//Volume of sample used*

Imagine that the plate from the 10^5dilution produced 32 colonies. Then, the total amount of colony-forming units per milliliter of the samples is (32) x (104) x1*= 3.2 x 105.

We've used a one-milliliter sample for this pour-plate technique. We can also inoculate more than one culture plate of same specimen in separate petri plate by using serial dilution but we must note the dilution factors off all dilution for the calculation of bacterial colonies. To get the best count or optimum count, the number of colonies needed to be in the range of 20 to 300 CFU/mL. Above this threshold the entire procedure should be repeated. If the total number of colonies is less than 20 it is recommended to utilize the lower dilution sample If the number of colonies is greater than 300, then it's recommended to choose the sample with greater dilution for subsequent repeats.If the colonies have fused or the entire plate is covered by one colony, you should report the result that the colonies are "too numerous to count" (TNTC) and take the sample with a higher concentration.

Applications of Pour Plate Technique:

- It is used to separate pure cultures from mixed cultures.
- It is used to identify and count viable fungi and bacteria (calculate CFU per ml) from liquid samples.
- Utilized to create growth curves to study biochemical and microbial metabolic processes and the effect of environmental conditions on the growth of microbial species.
- It is used to isolate discrete colonies for the purpose of obtaining pure cultures and for studying biochemical characteristics.

- This process is employed in various industrial applications. For instance, it's essential for wastewater treatment plants that are used to test the microbial and chemical contamination of treated water.

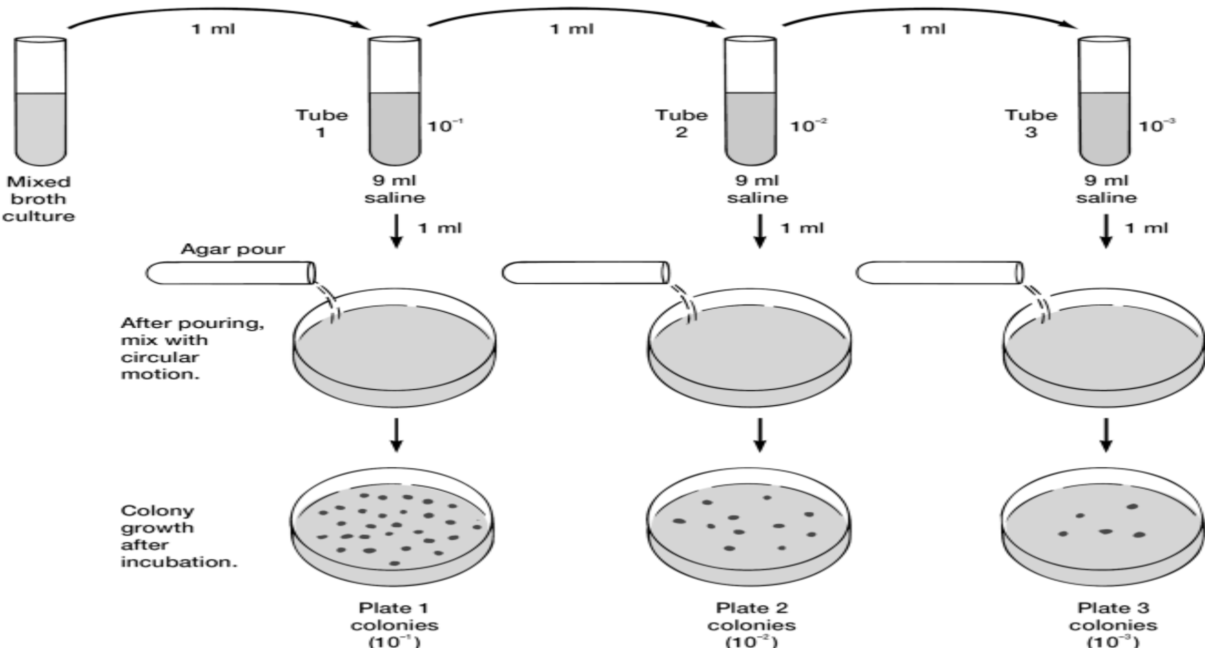

Fig 2.16 Serial dilution and inoculation of three culture plates.

(b)Streak Plate Method:

This method is used to obtain completely isolated colonies from a culture or specimen inoculums through the creation of sections of increasing dilution on a single plate.

Beginning the streak pattern. Label the base of the plate. Then, visualize the plate in four quadrants:

top left (I), top right (II), bottom right (III), bottom left (IV).

Streak the mixed culture back and forth in the first quadrant (top left) of the agar plate. Do not cut the agar, simply scrape the top. Flame the loop to rid of culture residue. Wait for it to cool for the next quadrant.

Streaking again. Proceed to the second quadrant with streaking. Streaks on the medium will overlap. Flame the loop to rid of culture residue. Wait for it to cool for the next quadrant.

Applications of Pour Plate Technique:

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- This process is employed in various industrial applications. For instance, it's essential for wastewater treatment plants that are used to test the microbial and chemical contamination of treated water.

Pharmaceutical companies must evaluate the level of microbial contamination or bioburden of a newly developed medication during its production, storage and

transportation. By sampling the drug through different stages of the process and then,, precautionary measures can be developed to limit or eliminate microbial contamination.

Beginning the streak pattern.

Label the base of the plate. Then, visualize the plate in four quadrants:

top left (I), top right (II), bottom right (III), bottom left (IV).

Streak the mixed culture back and forth in the first quadrant (top left) of the agar plate. Do not cut the agar, simply scrape the top. Flame the loop to rid of culture residue. Wait for it to cool for the next quadrant.

Streaking again. Proceed to the second quadrant with streaking. Streaks on the medium will overlap. Flame the loop to rid of culture residue. Wait for it to cool for the next quadrant.

Streaking yet again. Rotate the plate 180 degrees to get a proper streaking angle in the third quadrant. Be sure to cool the loops before streaking in quadrant four.

Streaking in the center. Streak one last time beginning in quadrant four and into the center of the plate. Flame the loops.

The plate may then be incubated, usually for 24 to 36 hours, to allow the bacteria to reproduce. At the end of incubation there should be enough bacteria to form visible colonies in the areas touched by the inoculation loop. From these mixed colonies, single bacterial or fungal species can be identified based on their morphological (size/shape/colour) differences, and then sub-cultured to a new media plate to yield a pure culture for further analysis.

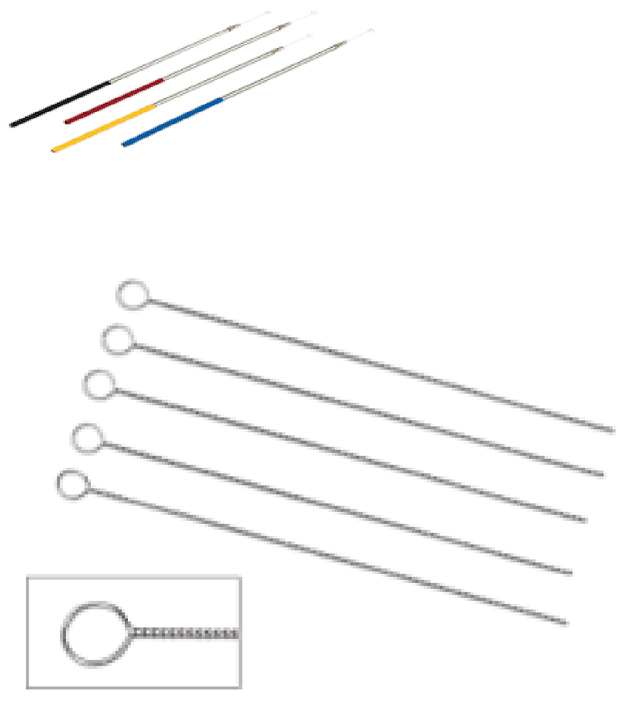


Fig 2.17 inoculation loops

Advantage of streak plate method:

1. From the sample, distinct separate colonies are obtained by the streak plate method.
2. It is a simple method for the isolation of microorganisms.
3. Commonly used for isolation of colonies from pharmaceutical products.

Fig 2.18: Inoculated streak plates.

Disadvantage of streak plate method:

1. Higher risk of contamination prior to isolation.
2. The streak plate method cannot use for quantitative study of enumeration of a number of bacteria in the microbial sample, only qualitatively this method is useful.
3. Only isolation is obtained in the fourth quadrant, so the colony count is not applicable in other quadrants.
4. **(c)Agar stab technique:** It is used in the preparation of stab cultures, from a plate select single colonies. It is used for determination of motile and non motile bacteria. Stab cultures are similar to agar plates, but are formed by solid agar in a test tube. Bacteria is introduced via an inoculation needle or a pipette tip being stabbed into the center of the agar. Bacteria grow in the punctured area. Stab cultures are most commonly used for short-term storage or shipment of cultures.

- Select a well-isolated colony through aseptic technique using an inoculating stab needle (sterile) and stab it a few times via the agar to the base of the tube.
- Substitute the cap and secure loosely during incubation enabling exchange of gases.
- Incubation of this stabbed plate at the suitable temperature is carried out.

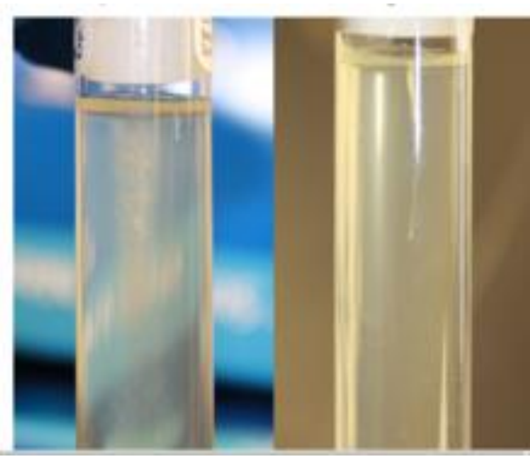


Fig 2.19 Stab culture: Motile and non-motile bacteria can be differentiated along the stab lines. Motile bacteria will grow out from the stab line while non-motile bacteria are present only along the stab line.

(d) Spread Plate Method:

It is used for evenly spreading cells to ensure growth of the isolated separate colonies. Further, it can be used for serial dilutions. The spread plate method is used for enrichment, enumeration and screening and selection of microorganisms.

The spread plate technique is a method of isolation in which a diluted microbial sample containing more than one microorganism is deposited on a solidified agar plate and spread uniformly across the surface with an L-shaped glass rod while the media plate is spun on a turntable. With an accurately diluted sample, cells (CFUs) will be deposited far enough individual on the agar surface to grow into individual colonies.

The main principle behind this Spread Plate technique is that as the Petri dish rotated, at some stage, single cells will be deposited with the bent glass rod on to the agar surface, these cells will be separated from each other by a distance sufficient to allow the colonies that develop to be free from each other.

Requirement:

24 hour nutrient broth cultures , Nutrient agar plates, Lazy Susan turntable,L-shaped bent glass rod,95 per cent alcohol,Beaker (50 mL), Bunsen burner.

Procedure:

1. Prepare different dilutions of the sample.
2. Label the nutrient agar plate with Wax marking pencil. Mention the organism name, type of agar, date, and the plater's name or initials.
3. Lift the plate's lid and use it as a shield to protect it from airborne contamination.
4. Take a clean and sterile Pipette and Pipette out 0.1 ml sample from the appropriate desired dilution series onto the center of the surface of an agar plate.
5. Replace the lid on plate.
6. Properly dispose of the pipetting instrument used to inoculate the medium, because it is contaminated.
7. Sterilize the L-shaped glass spreader by dipping this into 90% alcohol and then flame the glass spreader.
8. Cool the rod for 10–15 seconds
9. After farming the glass rod, lift the lid of the plate and use it as a shield from airborne contamination. Then touch the rod to the agar surface away from the inoculum to cool it.
10. During spreading hold the plate lid with the base of your thumb and index finger and use the tip of your thumb and middle finger to rotate the base. At the same time, move the rod in a back-and-forth motion across the agar surface. After a couple of turns, do one last turn

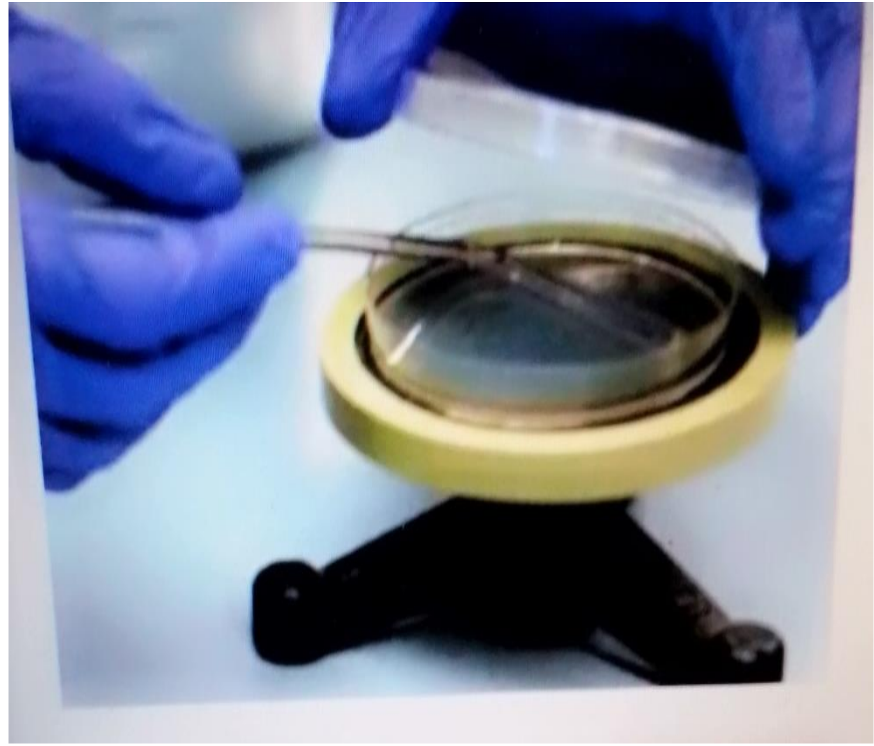


Fig 2.20 Placement of the inoculated plate on the turntable

With the rod next to the plate's edge. Alternatively, place the plate on a rotating platform and spread the inoculum. Or Place the inoculated plate on the turntable. A turntable makes it easier to rotate the plate during the spread plate technique.

11. Remove the rod from the plate and replace the lid.

12. Return the rod to the alcohol in preparation for the next inoculation. There is no need to flame it again.

Incubate the plate in an inverted position at the appropriate temperature for the assigned time.

Note – if you plated a volume of inoculums greater than 0.5ml. Wait a few minutes allow it to soak sample in before inverting the plate.

(2) CULTURE OF ANAEROBIC BACTERIA:

Robertson Cooked Meat medium:

Robertson's Cooked Meat (RCM) medium is used for the cultivation of aerobic, microaerophilic, and anaerobic microorganisms, especially *Clostridium* species. It is also known as cooked meat broth (CMB) as it contains pieces of fat-free minced cooked meat of ox heart and nutrient broth. It supports the growth of both spore-forming and non-spore-forming obligate anaerobes and also differentiates between putrefactive and saccharolytic species. It is a liquid media. Oxygen in culture media can be reduced by various agents such as glucose, thioglycollate, cooked meat pieces, cysteine and ascorbic acid. Thioglycollate broth which contains nutrient broth and 1% thioglycollate is also used to cultivate anaerobes.

Principle:

Before inoculation RCM/CMB medium is boiled to make it oxygen-free. After inoculation, it is covered with a layer of sterile liquid paraffin oil to prevent the entry of oxygen in the medium. The ingredients present in the medium help to maintain the anaerobic (reduced) environment.

1. Unsaturated fatty acids present in meat utilize oxygen for auto-oxidation, this reaction is catalyzed by haematin in the meat.

2. Glutathione and cysteine (both are reducing agents) present in meat also utilize oxygen.

3. Sulphydryl compounds (present in cysteine) also contribute to a reduced oxidation-reduction potential.*(for a detailed explanation of each component and its significance, read the composition and functions of ingredients below)*

Composition: Ingredients per liter of deionized water:

Cooked Meat Medium	250.0 gm
Peptic Digest of Animal Tissue	17.5 gm
Dextrose	5.0 gm
Sodium Chloride	5.0 gm
Yeast Extract	5.0 gm
Iron Filings	10.0 gm
Hemin	10.0 ml
Vitamin K	10.0 ml

Final pH 6.8 +/- 0.3 at 25ºC.

Cooked Meat Medium: Meat particles act as a reducing and detoxifying substance, thereby disabling harmful by-products that may be produced by the replicating organism. Because reducing substances are more available in denatured protein, the meat particles are cooked before use in the medium.

Iron filings: Reducing substance. Iron filings and muscle tissue permit the growth of strict anaerobes.

Nutritional supplements: Nutritional requirements needed by most bacteria are provided by peptic digest of animal tissues, yeast extract, and dextrose. Hemin and vitamin K are added to enhance the growth of anaerobic microorganisms. Amino acids and other nutrients are also supplied by the muscle protein in the heart tissue granules.

(i)Preparation of the medium:

1. Robertson's cooked meat medium is best prepared from ready to use dehydrated granules available from most suppliers of culture media.
2. Using a small tube or scoop pre-marked to hold 1.00g of granules, dispense the medium in 1.00 g amounts in screw-cap bottles or tubes.
3. Add 10.00 ml of distilled water, mix, and allow to soak for 5 minutes.

Sterilize the medium by autoclaving (with caps loosened) at 121°C for 15 minutes. When cool, tighten the bottle caps.

4. Date the medium and give it a batch number.
5. Store the medium in a cool dark place, making sure the bottle cops are tightly screwed.

Shelf-life: 2 years providing there is no change in the volume or appearance of the medium to suggest contamination.

pH of the medium: This should be within the range pH 7.0-7.4 at room temperature.

Before using, the medium must be boiled in a water bath to expel any dissolved oxygen.

(b)Inoculation of Robertson cocked meat culture (RCM):

Freshly prepared medium is preferred in which is inoculated as soon as it has cooled to approximately 35°C. RCM tubes that are not used on the day of preparation should be placed in a boiling water bath or steamer for about 15 minutes to pull out dissolved oxygen. An inoculums of Clostridia is added to the bottom of RCM tubesb containg media by using sterile pipette. For anaerobic organisms use freshly reduced medium and incubate for up to 21 days at 35°C. Examine daily for changes in the medium. Make films and subcultures at intervals.

It is a good practice if the incubation of anaerobic culture media occurs inside a ideal anaerobic incubation system, which provides oxygen- free environment for inoculating media and incubating cultures. It refers to a plastic anaerobic glove box that contains an atmosphere of
H_2, CO_2, and N_2. Glove ports and rubber gloves are used by the operator to perform manipulations within the chamber. There is an air-lock with inner and outer doors.
Culture media are placed within the air-lock with the inner door. Air of the chamber is removed by a vacuum pump connection and replaced with N_2 through outer doors.

The culture media are now transferred from air-lock to the main chamber, which contains an atmosphere of H_2, CO_2, and N_2. A circulator fitted in the main chamber circulates the gas atmosphere through pellets of palladium catalyst causing any residual O_2 present in the culture media to be used up by reaction with H_2.

When the culture media become completely anaerobic they are inoculated with bacterial culture and placed in an incubator fitted within the chamber. The function of CO_2 present in the chamber is that it is required by many anaerobic bacteria for their best growth. A schematic representation of an anaerobic chamber showing its various parts is given in.

When an oxygen-free or anaerobic atmosphere is required for obtaining surface growth of anaerobic bacteria, anaerobic jars are the best suited. The most reliable and widely used anaerobic jar is the Melntosh-Fildes' anaerobic jar. It is a cylindrical vessel made of glass or metal with a metal lid, which is held firmly in place by a clamp.

The lid possesses two tubes with taps, one acting as gas inlet and the other as the outlet. On its under surface it carries a gauze sachet carrying palladium pellets, which act as a room temperature catalyst for the conversion of hydrogen and oxygen into water. Palladium pellets act as catalyst, as long as the sachet is kept dry.

Inoculated culture plates are placed inside the jar and the lid clamped tight. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is then closed and the gas inlet tube connected to a hydrogen supply. Hydrogen is drawn in rapidly. As soon as this inrush of hydrogen gas has ceased the inlet tube is also closed.

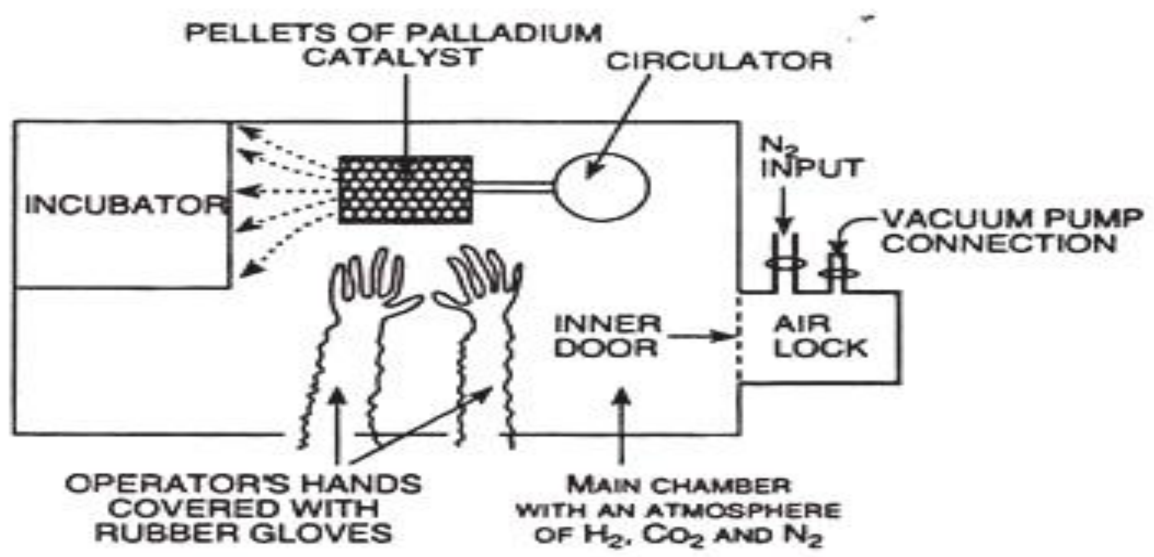


Fig 2.21 Vacuum incubator chamber of anaerobic bacteria culture

When an oxygen-free or anaerobic atmosphere is required for obtaining surface growth of anaerobic bacteria, anaerobic jars are the best suited (figure-13). The most reliable and widely used anaerobic jar is the Melntosh-Fildes' anaerobic jar. It is a cylindrical vessel made of glass or metal with a metal lid, which is held firmly in place by a clamp.

The lid possesses two tubes with taps, one acting as gas inlet and the other as the outlet. On itsunder surface it carries a gauze sachet carrying palladium pellets, which act as a room temperature catalyst for the conversion of hydrogen and oxygen into water. Palladium pellets act as catalyst, as long as the sachet is kept dry.

Inoculated culture plates are placed inside the jar and the lid clamped tight. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is then closed and the gas inlet tube connected to a hydrogen supply. Hydrogen is drawn in rapidly. As soon as this inrush of hydrogen gas has ceased the inlet tube is also closed.

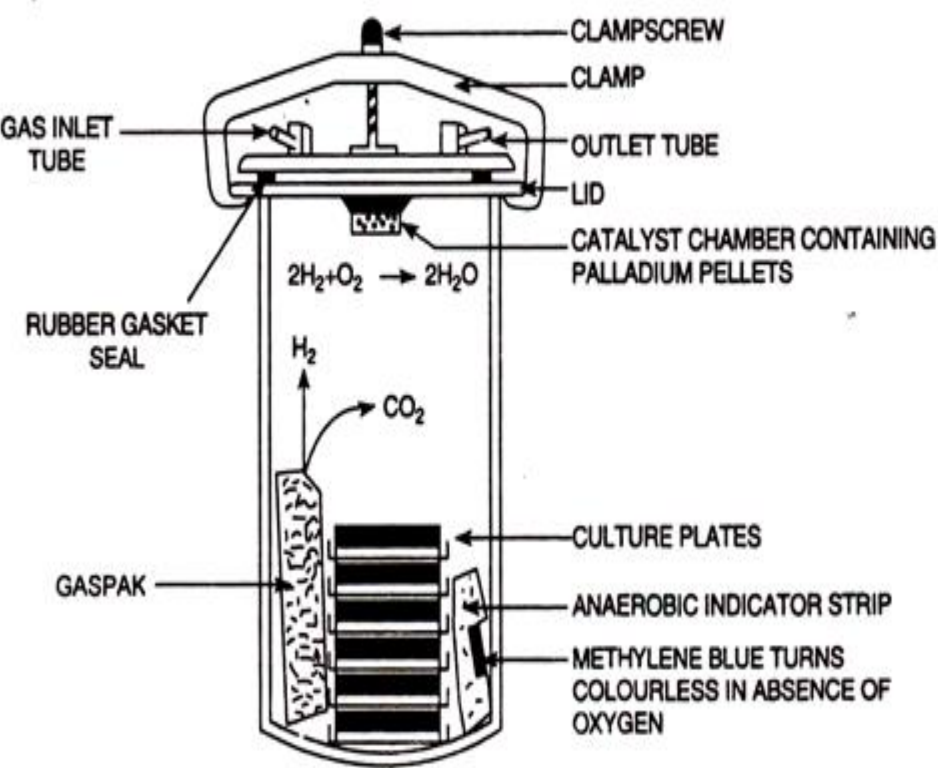


Fig 2.22 Anaerobic jar for inoculation

Result and Interpretation of Robertson's Cooked Meat (RCM) Medium

No turbidity: No growth

Turbidity: Presence of anaerobic bacteria.

Note: To say no growth reporting, there should be blindly subcultured into blood agar and finally incubated aerobically and anaerobically.*Clostridium* species may be divided into two main groups by their action on the medium.

Fig 2.23:Turbidity in media showing the degree of growth of anaerobic bacteria.

(E) SOME IMPORTANT CULTURE MEDIA USED IN MICROBILOGY:

(i) Basic Nutrient Media (Simple media):

Nutrient media contain only basic nutrients eg: Peptone broth. Any selective or enriched ingredients – not added . Certain pathogenic organisms having more exacting nutrition requirement eg: Streptococci.

(a) Nutrient broth:The main example oof nutrient media is nutrient broth. The composition for making the volume 1 Lit is as follows: Peptone 10 gm, Beefextract 10gm,Sodiumchloride 5 gm, distilled water 1 lit., pH 7.6.In market the readymade media is available in plastic box manufactured by several companies eg. Himedia, Ranbaxy, etc

Fig 2.24 Nutrient browth

Fig 2.25 peptone broth

Use: Forpreparing nutrientagarorbloodagar,toprepare liquidinoculums,tostudythegrowthcharacteristicsofbacteria.

(b) Peptone broth: Peptone 10gm**,** Sodiumchloride 5gm,Dist.Water 1lit,pH7.6

Use:Forpreparingnutrientbrothandnutrientagar,forIndoletest,basalmediumforpreparation ofsugarmedia,forinoculum preparationofbacterialculture.

(c)Nutrient agar:

Peptone	10g
Beefextract	10g
Sodiumchloride	5g
Dist.Water	1lit
Agar	10-20gm
pH	7.6

Use: Study thegrowthcharacteristicsofbacteria.

Fig 2.26 Nutrient agar

(ii) Special media

Enrichedmedia: Contains-blood,serum facilitatebettergrowth.
Eg-Bloodagar,Serumagar,Serumglucoseagar,Glucosebroth.

Bloodagar(figure-18):Prepared by adding5-10% steriledefibrinatedbloodinmelted efabrinated blood in melted nutrientagaratatemperatureof55^{0}Ctemperature

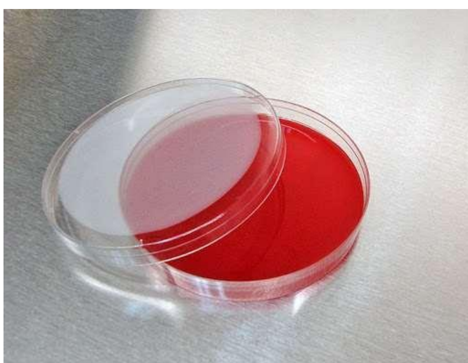


Figure 2.27 Blood agar

EnrichmentMedia: Favoursthegrowthofaparticularorganism.ie. Tetrathionatebroth,SeleniteF broth, Rappaportbroth.

Enrichmentmediaareliquidinconsistencywhileselective mediaare solid.usedforisolationofSalmonella.

Fig 2.28Tetrathionatebroth

Differentialmedia:

MacConkeyagar:

- Peptone-20g
- Sodiumchloride-5g
- Bile salts-5g
- Lactose-10g
- 2%neutralred-5ml
- Agar-20-25g
- Distilledwater-1lit
- pH.-7.4

Fig 2.30 MacConkeyagar dehydrated

Fig 2.31 MacConkeyagar showing bacterial growth.

Brilliantgreenagar(BGA): ForselectiveisolationofSalmonella otherthan*S.typhi*

EosinMethyleneBlueagar(EMB): Forselectiveisolationof*E.coli.*

Characteristic metallic colonies of *E.coli*
On EMB.

Fig 2.32 EMB showing E.coli

Robertson'scookedmeatmedium(RCM):

- Peptone-10g
- Beefextract-10g
- Sodiumchloride-5 g
- Dist.Water-1lit

Add pieces of heart–Nutrientborth

Use: Forcultivationofanaerobicbacteria

e.g.Clostridia

Fig 2.33 RCM showing turbidity growth of Clostridia

Dorset'seggmedium:

Fresheggcontents-75ml ,Nutrientbroth-25ml, 2% malachitegreen-1.25mlUse:ForcultivationofMycobacteria

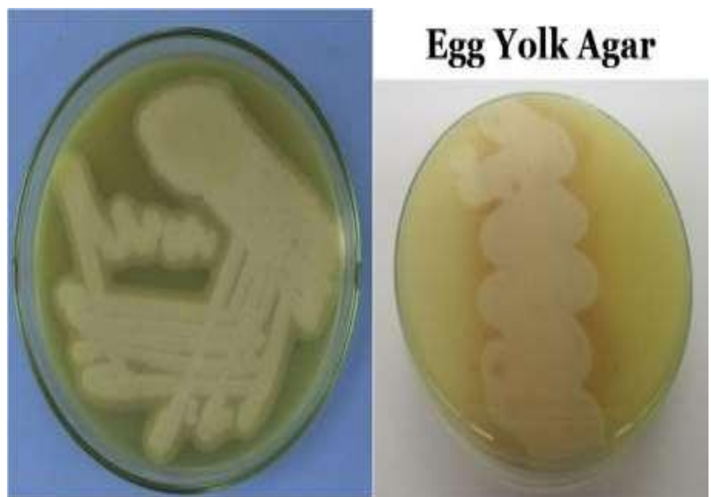


Fig 2.34 Dorset's medium showing growth of Mycobacteria

Sabouraud'sDextroseMaltoseagar: Use:thepH ofthemediumisacidic andissuitablefor cultivationofyeastandfungi

- Peptone-1g
- Dextroseormaltose-4g
- Agar-2g

- Distilledwater-100ml
- pH-5.4

Fig 2.35 Sabouraud's Dextrose Maltose agar medium

2.5 *VARIOUS STAINING METHODS*

Structural details of organisms cannot be seen under a light microscope due to a lack of contrast. Hence, we use dyes to stain cells. Dyes bind with cellular constituents producing color contrast and increasing their visibility. Positively charged (cationic) dyes such as methylene blue, crystal violet, safranin, etc bind with negatively charged cellular constituents such as nucleic acids and acidic polysaccharides and the cell surface of bacteria.

Stains and dyes : A dye is a general-purpose coloring agent, whereas a stain is used for coloring biological material. ➢A stain is an organic compound containing a benzene ring plus a chromophore and an auxochrome group. ➢chromophore is a chemical group that imparts color to benzene. ➢auxochrome group is a chemical compound that conveys the property of ionization of chromogen (ability to form salts) and bind to fibers or tissues.

Composition of stains:

Stain – Majority of the stains used for staining bacteria are of the basic type as nucleic acid of bacterial cells attract the positive ions, e.g. methylene blue, crystal violet.

Acidic stains are used for background staining.

Mordant – It is a chemical that forms an insoluble complex with the stain and fixes it or causes the stain to penetrate more deeply into the cell. These are used in indirect staining. For example, Gram's iodine in Gram staining and phenol in Ziehl Neelson's staining

.Accentuater – It is a chemical which when added to a stain to make the reaction more selective and intense. For example, potassium hydroxide added in Loeffler's methylene blue.

Decolorizer – It is a chemical used to remove the excess stain in indirect regressive staining. For example, ethanol in Gram's staining.

Based on the types and number of dyes used, staining can be categorized into different types

2.5.1SIMPLE STAINS:

Basic dyes, such as methylene blue or basic fuchsin are used as simple stains. They produce color contrast but impart the same color to all the bacteria in the smear. Some other stains commonly used for simple staining include crystal violet, and safranin, Simple stains can be used to determine a bacterial species' morphology and arrangement, but they do not give any additional information

Procedure of simple staining

1. A clean grease free slide is taken .A grease free slide is is made by first washing the slide with detergent wiping the excess water and the slide is passed through flame.
2. On these grease free slide smear is made by using a sterile wireloop and cell suspension.
3. These slide is allowed to air dry.
4. After air drying these slide is rapidly passed through a flame for three to four times for heat fixation.
5. After heat fixation the slide is placed on the staining rack and flooded with a particular stain and these stain is allowed to react for three minutes.
6. Futher the slide is washed under running water.
7. The slide is air dried and washed under oil immersion.

2.5.2 SPECIAL STAINS:

(a)Negative staining: A drop of bacterial suspension is mixed with dyes, such as India ink or Nigrosin. The background gets stained black whereas the unstained bacterial or yeast capsulestands out in contrast. This is very useful in the demonstration of capsules that do not take up simple stains.

India ink preparation

Negative stains are used when a specimen or a part of it, such as the capsule resists taking up the stain. India Ink preparation is recommended for use in the identification of *Cryptococcus neoformans*. **India ink stain** was previously known as the “Nigrosin stain”. **India ink staining** are negative staining techniques used to determine an organism’s cellular morphology. **Negative staining** stains all of the preparation except the items you want to observe. The main purpose of **negative staining** is to study the morphological shape, size and arrangement of bacterial cells that are difficult to stain or too delicate to heat**. Negative staining** is the only staining technique in which bacterial cells are not stained but are made visible against a dark background as colorless bodies.

Principle:

Negative staining is an acid stain contains Nigrosin stain. This means that the dye easily gives up a hydrogen ion (proton) and the chromophore of the dye becomes negatively charged. Since the surface of most bacterial cells is negatively charged, the cell surface repels the dye. The bacteria will appear as bright dots on a dark background.

Method:

1. Place a drop (25µl) of a 7% Nigrosine solution or India ink near one end of a well cleaned slide (depending on the specimen, sometimes India ink needs to be diluted with distilled water)

2. Remove a small amount of culture using an inoculation loop and disperse it into the drop of dye without spreading the drop.

3. Take another microscopic glass slide, place it near the sample-dye mixture at an angle of about 30 ° - 45 °.

4. Move the slide towards the drop of the sample-dye mixture until contact is made with the drop at the specific angle. Then move the slide gently and quickly forward on the sample slide, drawing the dye mixture behind it in a thin film.

5. Allow the smear to air dry, then observe microscopically on high power (45X) and oil immersion (100X) objectives.

(b)Flagella stain*:* Impregnation method:

Bacterial cells and structures that are too thin to be seen under the light microscope are thickened by impregnation of silver salts on their surface to make them visible, e.g., for demonstration of bacterial flagella and spirochetes. Flagellar stains are painstakingly prepared to coat the surface of the flagella with dye or a metal such as silver. The number and arrangements of flagella are critical in identifying species of motile bacteria.

Smear Preparation: 1.With a wax pencil, draw a border around the clear portion of a microscope slide. 2. Approximately one centimeter from the frosted edge, place a drop of deionized (or distilled) water. 3. With a sterile inoculating loop, gently touch a colony of the culture to be examined and then lightly touch the Flagella Stain - for drop of water without touching the slide. Do not mix. 4. Tilt the slide so the drop will flow to the opposite end of the slide. 5. Allow the slide to air dry, at room temperature. Do not heat fix.

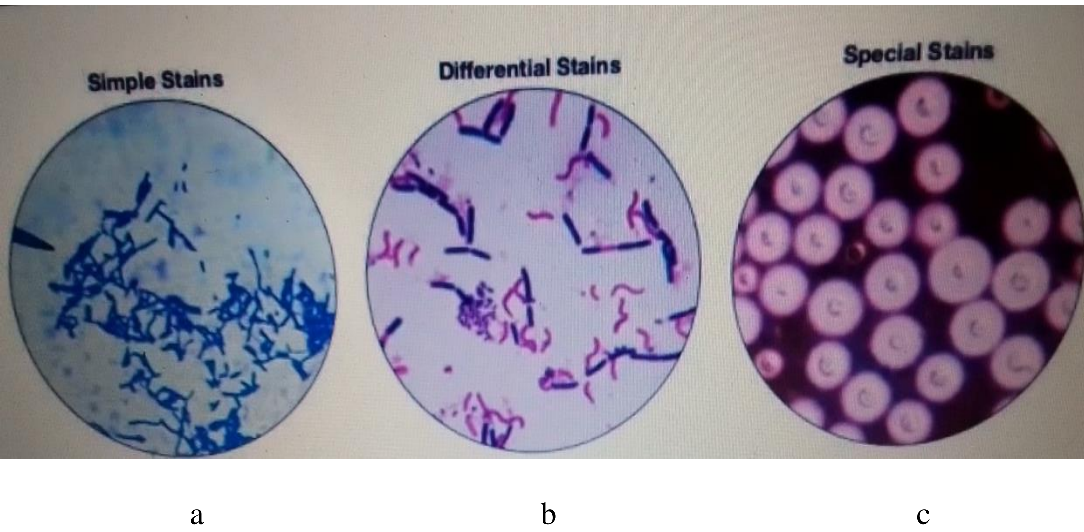
Staining Procedure: 1. Flood the slide with Flagella Stain. Let the slide sit for approximately four minutes. However, staining time may need to be adjusted to between two to eight minutes, depending on the age of the stain, air currents and room temperature, and staining solution depth over the smear.

2. Carefully rinse stain by leaving slide on staining rack and allowing water to flow over the surface of the slide. Do not tilt slide while rinsing.

3. Once the stain is washed off, gently tilt the slide to allow excess water to run off.

4. Air dry slide at room temperature.

5. Examine the slide microscopically using the oil immersion objective. Begin the examination at thinner areas of the smear and work towards the center. Look for fields with several isolated cells rather than clumps of bacteria.



a b c

(a)Crystal violet stain of *E.coli*(b) Gram stain: purple cells are gram positive cells(c) India ink capsule stain *Cryptococcusneoforman*

Fig 2.36 showing various stains with microbes.

2.5.3 Differentaial Stains:

Two stains are used which impart different colors to different bacteria or bacterial structures, which help in differentiating bacteria. The most commonly used differential stains are:

(a)Gram staining

Gram stain is a very important differential staining technique used in the initial characterization and classification of bacteria in microbiology. Gram staining helps to identify bacterial pathogens in specimens and cultures by their Gram reaction (Gram-positive and Gram-negative) and morphology (cocci/rod).

Principle: When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound.

Reagents Used in Gram Staining: Crystal Violet, the primary stain, Iodine, themordantdecolorizer made of acetone and alcohol (95%) ,Safranin, the counterstain.

Procedure of Gram staining:

1. Take a clean, grease free slide.
2. Prepare the smear of suspension on the clean slide with a loopful of sample.
3. Air dry and heat fix
4. Crystal Violet was poured and kept for about 30 seconds to 1 minutes and rinse with water.
5. Flood the gram's iodine for 1 minute and wash with water.
6. Then ,wash with 95% alcohol or acetone for about 10-20 seconds and rinse with water.
7. Add safranin for about 1 minute and wash with water.
8. Air dry, Blot dry and Observe under Microscope.

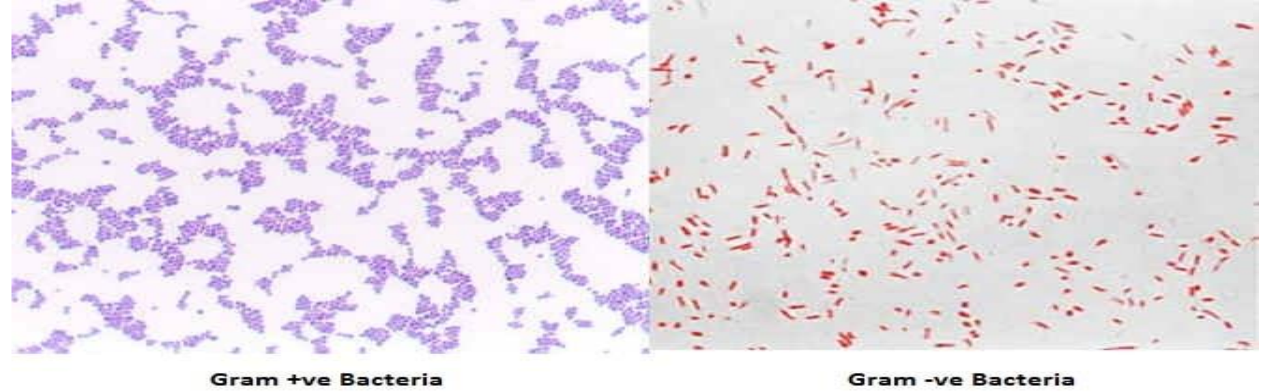


Fig 2.37 Purple or blue color showing gram positive cells while gram negative are red cells

Gram Positive Bacteria: *Actinomyces, Bacillus, Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactobacillus, Listeria, Mycoplasma, Nocardia, Staphylococcus, Streptococcus Streptomyces* ,etc.

Gram Negative Bacteria: *Escherichia coli* (*E. coli*), *Salmonella*, *Shigella*, and other Enterobacteriaceae, *Pseudomonas*,*Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella* etc.

(b) Acid-fast staining (Ziehl-Neelsen technique). It is the differential staining techniques which was first developed by Ziehl and later on modified by Neelsen. So this method is also called ***Ziehl-Neelsen staining*** techniques. Neelsen in 1883 used Ziehl's carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques was developed.

The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups.This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus *Mycobacterium*, are resistant and can only be visualized by acid-faststaining.

Principle:

When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. Then after all cell appears red. Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution. The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. Only decolorized cells absorb the counter stain and take its color and appears blue while acid-fast cells retain the red color.It distinguishes acid-fast bacteria such as *Mycobacterium spp* from non-acid fast bacteria; which do not stain well by the Gram staining. It is used to stain *Mycobacterium* species (*Mycobacterium tuberculosis, M. ulcerans,* and *M. leprae*

Procedure of Acid-Fast Stain:

1. Prepare bacterial smear on clean and grease free slide, using sterile technique.
2. Allow smear to air dry and then heat fix.
   Alcohol-fixation: This is recommended when the smear has not been prepared from sodium hypochlorite (bleach) treated sputum and will not be stained

immediately. M. tuberculosis is killed by bleach and during the staining process. Heat-fixation of untreated sputum will not kill M. tuberculosis whereas alcohol-fixation is bactericidal.

3. Cover the smear with carbol fuchsin stain.
4. Heat the stain until vapour just begins to rise (i.e. about 60 C). Do not overheat. Allow the heated stain to remain on the slide for 5 minutes. Heating the stain: Great care must be taken when heating the carbol fuchsin especially if staining is carried out over a tray or other container in which highly fiammable chemicals have collected from previous staining. Only a small fiame should be applied under the slides using an ignited swab previously dampened with a few drops of acid alcohol or 70% v/v ethanol or methanol. Do not use a large ethanol soaked swab because this is a fire risk.
5. Wash off the stain with clean water.
   Note: When the tap water is not clean, wash the smear with filtered water or clean boiled rainwater.
6. Cover the smear with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolorized, i.e. pale pink.
   Caution: Acid alcohol is fiammable, therefore use it with care well away from an open fiame.
7. Wash well with clean water.
8. Cover the smear with malachite green stain for 1–2 minutes, using the longer time when the smear is thin.
9. Wash off the stain with clean water.
10. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry (do not blot dry).
12. Examine the smear microscopically, using the 100 X oil immersion objective.

Acid-fast: *Mycobacterium tuberculosis, Mycobacterium smegmatis.*

Non-Mycobacterial bacteria: *Nocardia*

Coccidian Parasites: *Cryptosporidium*

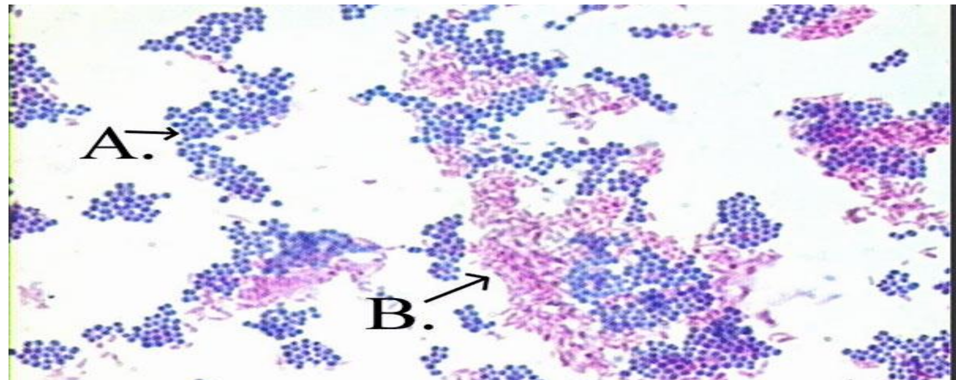


Fig 2.38 Acid fast stains showing ***Bright red to intensive purple (B), Red, straight or slightlycurved rods, occurring singly or in small groups, may appear beaded Non-acid fast: Blue color (A)***

(c) Endospore staining.

Endospore staining is a differential technique that selectively stains the spores and makes them distinguishable from the vegetative part of the cells. Endospores are produced by a few genera of Gram-positive bacilli, such as *Bacillus* and *Clostridium*, in response to adverse environmental conditions. Endospores are highly resistant to environmental conditions such as heat and chemicals (stains and dyes) and therefore require special techniques for staining

Methods for endospore staining:

There are different methods for endospore staining. The most common are

- Schaeffer-Fulton stain technique.
- Dorner's methods
- Modified Zeihl-Nelson's method
- Barthelomew-Mittwar's method
- Abott method
- Moller stain technique

1. Prepare smears of organisms to be tested for the presence of endospores on a clean microscope slide and air dry it.
2. Heat fix the smear.
3. Place a small piece of blotting paper (absorbent paper) over the smear and place the slide (smear side up) on a wire gauze on a ring stand.

4. Saturate the blotting paper with **malachite green** stain solution and steam for 5 minutes, keeping the paper moist and adding more dye as required. Alternatively, the slide may be steamed over a container of boiling water.
5. As the paper begins to dry, add a drop or two of malachite green to keep it moist, but don't add so much at once that the temperature is appreciably reduced.
6. After 5 minutes, remove the slide from the rack using a clothespin.
7. Remove the blotting paper and allow the slide to cool to room temperature for 2 minutes.
8. Rinse the slide thoroughly with tap water (to wash the malachite green from both sides of the microscope slide).
9. Stain the smear with **safranin** for 2 minutes.
10. Rinse both sides of the slide to remove the secondary stain and blot the slide/ air dry.

Result & Interpretation:

Observe the bacteria under 1000X (oil immersion) total magnification. When viewed under 1000X of a light microscope, **vegetative cells appear pink/red, and spores appear green.**

Results:

Vegetative cells are colorless, endospores are red, and the background is black

The difference between various endospore staining methods:

Method	**Primary Stain**	**Decolorizer**	**Counter stain**	**Interpretation**
Modified Zeihl-Nelson's method	Carbol Fuschin	0.25-0.5% sulphuric acid	Leoffler's methylene blue	Spores appear red, bacteria are blue
Dorner method	Carbol Fuschin	Acid-alcohol	Nigrosin	Spores red Bacteria colorless Background Black
Schaeffer-Fulton Stain	Malachite Green	Water	Safranin	Spores appear green vegetative cells appear pink/red

Bartholomew and mittwer method	Malachite Green	Water	Safranin	Spores appear green vegetative cells appear pink/red
Abbott's method	Methylene Blue	Acid alcohol	Aniline fuschin	Spores appear blue bacteria are red
Moeller's stain	Carbol fuschin	Acidified ethanol	Methylene blue	Spores appear red bacteria are Blue
Modified Moller's stain	Kinyoun's Carbol fuschin	2% sulphuric acid and 80% ethanol	Loeffler methylene blue	Spores appear red bacteria are Blue

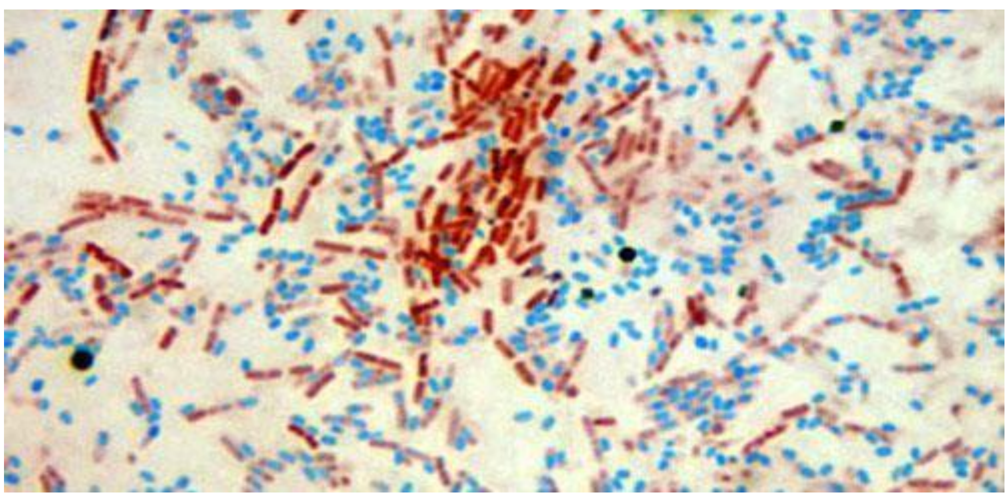


Fig 2.39 bacterial smear showing endospore of L. baccilus.

(d)Capsule staining:

Capsule stain is a type of differential stain which uses acidic and basic dyes to stain background & bacterial cells respectively so that presence of capsule is easily visualized. Capsule is synthesized in the cytoplasm and secreted to the outside of the cell where it surrounds the bacterium. Most of the capsulated bacteria have a capsule made up of a polysaccharide layer but some bacteria have capsule made up of polypeptide, or glycoprotein. Capsules are associated with virulence in several microorganisms, including *Streptococcus pneumoniae* and *Neisseria meningitides*, because capsules resist phagocytosis thus evading the host immune system.

Principle of Capsule Stain:

Bacterial capsules are non-ionic, so neither acidic nor basic stains will adhere to their surfaces. Therefore, the best way to visualize them is to stain the background using an acidic stain (e.g., Nigrosine, congo red) and to stain the cell itself using a basic stain (e.g., crystal violet, safranin, basic fuchsin, and methylene blue).

India ink method:

In this method, two dyes, crystal violet, and India ink are used. The capsule is seen as a clear halo around the microorganism against the black background. This method is used for demonstrating *Cryptococcus.* The background will be dark *(color of India ink).*The bacterial cells will be stained purple *(bacterial cells take crystal violet-basic dyes as they are negatively charged).*The capsule (if present) will appear clear against the dark background *(capsule does not take any stain).*

1. Place a single drop of **India ink** on a clean microscope slide, adjacent to the frosted edge.
2. Using a flamed loop and sterile technique, remove some *Klebsiella pneumoniae* from culture tube or plate and mix it into the drop of India ink. Be sure there are no large clumps of organism, but try to avoid spreading the drop.
3. Place the end of another clean microscope slide at an angle to the end of the slide containing the organism. Spread out the drop out into a film. This is done by contacting the drop of India

ink with the clean microscope slide and using the capillary action of the dye/ slide to spread the India ink across the smear.

4. Allow the film to air dry ***(will take 5-7 minutes).*** *DO NOT heat or blot dry! Heat will melt the capsule!*
5. Saturate the slide with crystal violet for 1 minute and rinse slightly & very gently with water. Be cautious w*ater may remove the capsule from the cell.*
6. Let the slide air dry for a few minutes. *DO NOT blot the slide! Blotting will remove the bacteria from the slide and/or distort the capsule.*
7. Observe the slide under oil immersion.

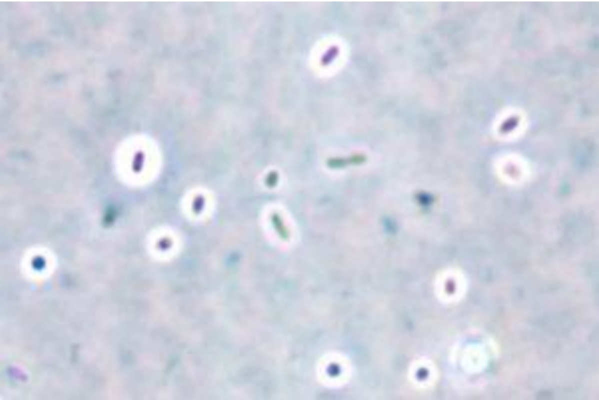


Fig 2.40 Capsule staining by India ink method (at 1000x magnification)

(e)Giemsa stain:

It is a differential stain and contains a mixture of azure, methylene blue, and eosin dye. It is specific for the phosphate groups of DNA and attaches itself to where there are high amounts of adenine-thymine bonding.

Azure and eosin are acidic dye that variably stains the basic components of the cells like the cytoplasm, granules, etc. Methylene blue acts as the basic dye, which stains the acidic components,

especially the nucleus of the cell. Methanol act as a fixative as well as a cellular stain. The fixative does not allow a further change in the cells and makes them adhere to the glass slide.

Preparation of Giemsa Stain

Giemsa is the most commonly used stain for staining blood films for malaria diagnosis. It is available commercially as a ready-to-use product, but the quality varies according to the source. By following simple rules, laboratories can prepare a stock solution of Giemsa stain using Giemsa stain powder, thus ensuring the use of consistent, high-quality stain.

Composition

The essential ingredients of Giemsa stain are the same; however, dilutions can be made depending on their use.

Ingredients	**Gm/L**
Giemsa powder	7.6
Glycerol	500 ml
Methanol	500 ml

Working solution of Giemsa: From giemsa stain take that we prepare above. prepare 10% or 3% Giemsa working solution, depending on your need. About 3 mL of stain is required for each slide with a blood film.working solution of Giemsa stain should be freshly prepared from Giemsa stock solution. Depending upon the method of staining used to stain malaria blood films, the Giemsa working solution is either 10% (for the rapid method) or 3% (for the slow method)

Staining of the slides for thin blood smears:

1. Prepare a thin smear of blood and fix air-dried film in absolute methanol by dipping the film briefly (two dips) in a Coplin jar containing absolute methanol.
2. Remove and let air dry.

3. Stain with a working solution of Giemsa stain
4. Wash by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips). **Note:** Excessive washing will decolorize the film.
5. .Let air dry in a vertical position. Observe under the microscope first at 40X and then using an oil immersion lens.
6. **For Thick blood smears:**

1. Aprepare a thik smear of blood and allow the film to air dry thoroughly for several hours or overnight. Do not dry films in an incubator or by heat, because this will fix the blood and interfere with the lysing of the RBCs.
   Note: If a rapid diagnosis of malaria is needed, thick films can be made slightly thinner than usual, allowed to dry for 1 hour, and then stained.
2. DO NOT FIX.
3. Stain with diluted Giemsa stain
4. Wash by placing the film in buffered water for 3 to 5 min.
5. Let air dry in a vertical position, observe under the microscope at 40X, and then use an oil immersion lens.

Observation:
On microscopic observation, cell organelles, bacteria, and parasites are distinguished based on their morphology and color;

Cell Components	The color observed after staining
Red blood cells	Mauve-pink
Neutrophils	Reddish purple nuclei with pink cytoplasm
Eosinophils	Purple nuclei, faintly pink cytoplasm, and red to orange granules.

Lymphocytes	Dark blue nucleus with light blue cytoplasm.
Platelets	Violet to purple color granules.
Nuclei of host cells	Dark purple
Nuclei of WBCs	Dark purple
The cytoplasm of host cells	Pale blue
The cytoplasm of white cells	Pale blue or grey-blue
Melanin granules	Black green
Bacteria	Pale or dark blue
Chlamydia trachomatis inclusion bodies	Blue-mauve to dark purple depending on the stage of development
Borrelia spirochetes	Mauve-purple
Yersinina pestis coccobacilli	Blue with dark stained ends (bipolar staining)
Malaria parasite	Malaria parasites have a red or pink nucleus and blue cytoplasm. If *P. vivax* is seen, the Schüffner dots are seen as an even carpet of pink dots in the cytoplasm of red blood cells. If *P. falciparum* is observed, Maurer clefts will be seen as unevenly distributed, coarse bodies in the red cell cytoplasm.

Uses of Giemsa Stain:

Wright-Giemsa's stain is commonly used to demonstrate the cellular elements in peripheral blood and bone marrow smears. Giemsa stain is used to obtain differential white blood cell counts. It is also used to differentiate the nuclear and cytoplasmic morphology of the various blood cells like platelets, RBCs, and WBCs.

In Microbiology, Giemsa stain is used for staining inclusion bodies in *Chlamydia trachomatis*, *Borrelia* species, and if Wayson's stain is not available, to stain *Yersinia pestis.* Giemsa stain also is used to stain *Histoplasma capsulatum*, *Pneumocystis jiroveci*, *Klebsiella granulomatis, Talaromyces marneffei* (formerly called *Penicillium marneffei*), and occasionally bacterial capsules. Cytogenetics also uses this stain to stain the chromosomes and identify chromosomal aberrations. It is commonly used for G-banding (Giemsa-Banding)

Parasitology:

In microbiology, this stain is most commonly used in parasitology to detect intraerythrocytic (*plasmodia, babesiae*) and exoerythrocytic (*trypanosomes*, microfilaria) parasites. It is also used for the detection of intracellular amastigotes of *Leishmania* species or *Trypanosoma cruzi.*

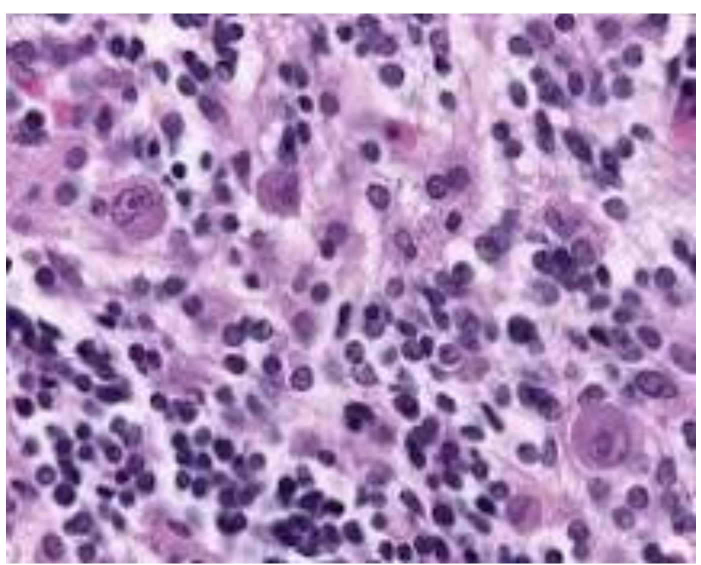


Fig 2.41 WBCs in Giemsa stain showing WBCs.

(f) Acridine orange Stain:

Principle: Acridine orange, a vital stain, will intercalate with nucleic acid, changing the dye's optical characteristics so that it will fluoresce bright orange under ultraviolet light. All nucleic acid–containing cells will fluoresce orange. Acridine orange is a metachromatic stain and under appropriate conditions, RNA will stain orange and DNA will stain green.

Method:

1. Properly prepare and fix the smear prior to staining.
2. Flood slide with acridine orange stain (available from various commercial suppliers). Allow stain to remain on surface of slide for 2 minutes without drying.
3. Rinse with tap water and allow moisture to drain from slide and air-dry.
4. Examine the slide using fluorescent microscopy.

Result interpretation (figure -8):

Bacteria and yeasts will fluoresce bright orange against a green-fluorescing or dark background. The nuclei of host cells may also fluoresce.

Note: RNA is more abundant during cellular growth and may mask the green fluorescence of the DNA within the cell.

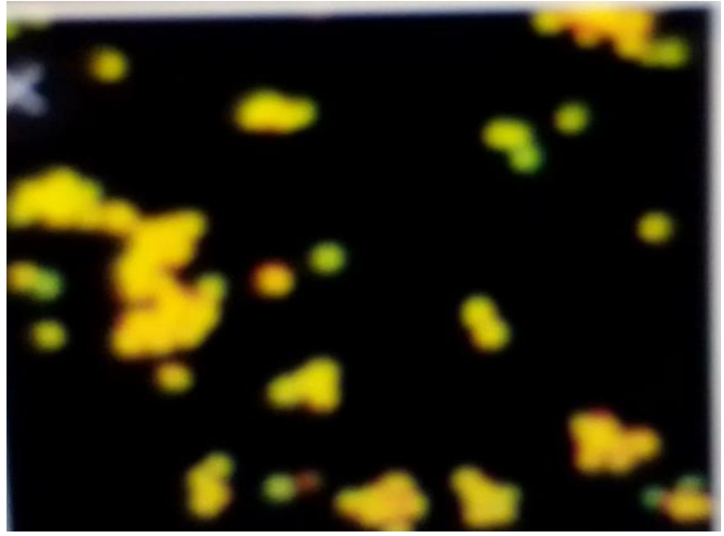


Fig 2.42 Acridine orange stain showing cancer cells.

2.5.3 OTHER STAINS

(a) Auramine-Rhodamine technique:

Principle:

The fluorochrome dye, auramine-rhodamine, forms a complex with mycolic acids found in the acid-fast cell wall of organisms which resist decolorization by acid-alcohol. The counterstain, potassium permanganate, renders tissue and its debris nonfluorescent, thus reducing the possibility of artifacts. The cells visualized under ultraviolet light appear bright yellow or reddish-orange.

Reagents:

- Primary stain: auramine rhodamine solution (caution: possible carcinogen).
- Decolorizer: 0.5% acid alcohol (5 ml HCl in 995 ml 70% alcohol). (caution: flammable, corrosive).
- Counter stain: 0.5% pottassium permanganate (0.25 gm in 50 ml). (caution: corrosive).

(i) Preparation of Smear:

For Sputum: Using a piece of stick, transfer a purulent part of the sputum (containing any yellow caseous material), to a slide and make a thin smear. An area of approximately ½ by 1 inch (or 2-cm square) is recommended. Spread the smear using circular movements. Allow to air dry.

Note: Be sure to prepare smears of suitable thickness. Smears that are too thick may flake during staining and may be difficult to decolorize. Acid-fast organisms that might be present may be obscured. Smears that are too thin may not contain enough sample. Either condition–too thick or too thin–can lead to erroneous results, particularly false negatives. Here (image 1) the smear in the center is of the proper thickness. Hold a smear about 3 to 4 inches over news-print, if you are just able to read the print, the smear is of proper thickness.

For urine: Make a smear of the deposit from three centrifuged early morning urine sediments. Allow to air dry and heat fix the specimen. The use of an electric slide warmer is usually the preferred method for heat-fixing smears. An alternate method of heat-fixing is to pass the dried slide, smear facing upward, 2 to 3 times through the blue cone of a burner flame.

Staining Method:

- Place the fixed smear on a staining rack and flood slide with rhodamine-auramine for 15 minutes. Do not let the surface dry. (Note: Fluorochrome dyes used for acid-fast staining include Auramine O, and Auramine O in combination with another fluorochrome, Rhodamine B).
- Wash off the stain with distilled water.

- Flood slide with fluorescent decolorizer (i.e acid-alcohol) for 2-3 minutes.
- Rinse thoroughly with distilled water.
- Flood slide with potassium permanganate for 3-4 minutes. Do not allow slides to dry.
- Rinse thoroughly with distilled water and air dry.
- Examine microscopically under the same light source as used for fluorescent microscopy (i.e. a K530 excitation filter and a BG 12 barrier or G-365 excitation filter and an LP 420 barrier filter). Slides can be screened on high power (400X) and verified under oil immersion.

Result and Interpretation.

- **Positive Test** – Acid-fast organisms fluoresce reddish-orange against a dark background.
- **Negative Test** – Non-acid-fast organisms will not fluoresce or may appear a pale yellow, quite distinct from the bright acid-fast organisms.

Number of Fields to Examine: The minimum number of fields to examine before reporting a smear as negative for acid-fast organisms.

Final magnification (the objective lens magnification multiplied by the eyepiece magnification) Vs. Number of slides

1. 200x: 30
2. 250x: 30
3. 400x: 55
4. 450x: 70

Reporting of smears

- If Fluorescent AFB are seen, report the smear as **AFB positive,** and give an indication of the number of bacilli present in plus signs (+ to +++)
- If no fluorescent rods are seen, report the smear as NO AFB seen.

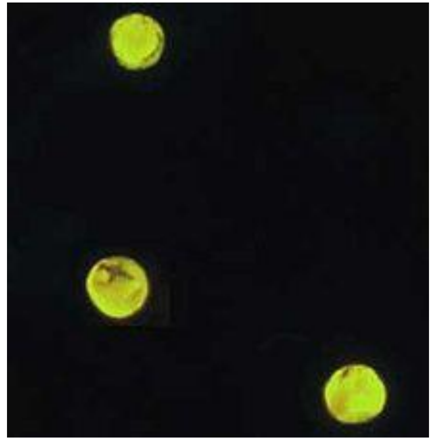


Fig 2.43 Oocysts of Cryptosporidium parvum stained with the fluorescent auramine–rhodamine stain.

(b)Calcofluor white stain (CFW):

It is a chemifluorescent blue dye that is nonspecifically used to bind to the beta linked-polysaccharide polymers of amoebic cysts.It functions by being able to bind to 1-3 beta and 1-4 beta polysaccharides on chitin and cellulose that is present in cell walls on fungi, plants, and algae. Due to the speed in examining the cells, the stain was replaced by Potassium Hydroxide (KOH) wet mount, which is quicker. It used to stain fungal and parasitic elements which are observable under a fluorescent microscope.

Principle:

The chitin containing structures that fluoresce bright white under ultraviolet light in a fluorescent microscope. Calcofluor White stain as a non-specific fluorochrome that binds to chitin and cellulose cell walls, has been described to be the most rapid technique for detection of pathogenic fungi and yeasts such as Pneumocystis carinii, Microsporidium, Acanthamoeba

Naegleria, and Balamuthia species. Calcofluor White Stain visualizes well when mixed with potassium hydroxide for fungal elements. The counterstain used is Evans Stain which forms a dark background that enables the tissues and cells to fluoresce using blue light excitation and not UV rays. Other elements fluoresce reddish-orange while fungal and parasitic elements fluoresce bright apple-green.

Reagents: Calcofluor white stain, Potassium Hydroxide.

Method :

1.Carefully put the specimen on a clean glass slide

2.Add a drop of Calcofluor White Stain to produce an intense fluorescence

3.Add one drop of 10% Potassium Hydroxide

4.Cover the specimen with a coverslip and leave it to absorb the stain for 1 minute

5.Remove excess dye with a dry paper towel by gently pressing on the stain

6.Observe the stain under ultraViolet rays at x100-x400 magnification.

Calcofluor white staining technique was used as a comparative stain specifically for the identification of Onychomycosis fungal infection.

Result: Fungi, Pneumocystis cysts, and parasites appear brilliant apple-green under UV fluorescent microscope in violet and blue light.

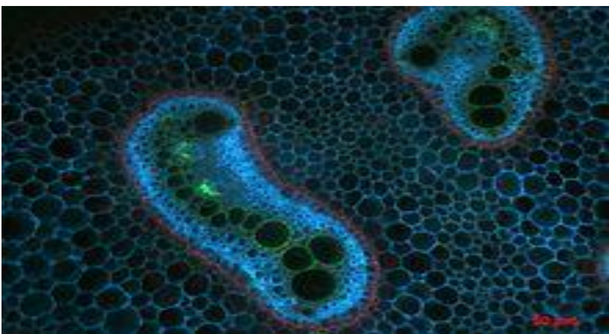


Fig 2.43 The cross section of an eagle fernBright green and bright blue fluorescence with CFW

(c) Lactophenol cotton blue (LPCB) wet mount :

Principle: The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The constituents in LPCB perform different functions i.e Phenol kills fungus, Lactic acid acts as a clearing agent and helps preserve the fungal structures. Cotton blue is an aniline dye that stains the chitin in the fungal cell walls which adds colour to the fungal preparation thereby enhancing and contrasting the structures and Glycerol is a viscous substance that prevents drying of the prepared slide specimen.

Lactophenol cotton blue solution is a **mounting medium** and **staining agent** used in the preparation of slides for microscopic examination of fungi. Fungal elements are stained intensely blue. This operation should always be performed under a biological safety hood and gloves should always be worn.

Preparation of LPCB mount

LPCB can be purchased from commercial suppliers or prepared in-house by mixing the above-mentioned ingredients as mentioned in the steps below.

1. Add 20 mL of lactic acid in a beaker.

2.Add 40 mL of glycerol or glycerine.

3Add 20 mL of distilled water

4.Add 22 g. of phenol crystals (22 ml of melted phenol).

5.Add 0.05 g. aniline blue.

6.Heat and stir the solution to dissolve the stain. Do not boil or go over 100°C.

7.Mix well and let the solution cool. Store the solution at room temperature and dispensed it with a pipette when needed.

Procedure: There are two method used inLPCB method depend upon need of scientist.

Standard Tease Mount

1. Place a drop of 70% alcohol on a clean microscope slide.
   Material from cultures of filamentous fungi should be removed using a stiff inoculating wire, not the loop used for manipulations with bacteria or yeasts.
2. Flame the wire by holding it upright in the hottest part of the Bunsen flame, just above the blue cone, until the whole length of the wire glows red hot.
   You must ensure that the inoculating wire has cooled before placing it in a fungal culture – it should have cooled sufficiently after approximately ten seconds.
3. Remove the cap from the culture tube (but do not put it on the bench). Kill any contaminating microorganisms by flaming the neck of the tube.

4. Remove a small amount of the culture.
   It is often useful to take a little of the agar medium together with the fungus. In any case, the material should be disturbed as little as possible when being transferred to the slide.

5. Flame the neck of the tube once more and replace the cap.

6. Immerse the fungal material in the drop of 70% alcohol.
   This drives out the air trapped between the hyphae.

7. Tease out the material very gently with mounted needles.
   Do not forget to sterilize the inoculating wire and the needles after use by heating to red heat in a Bunsen flame

8. Before the alcohol dries out add one or at most two drops of the stain.
   A common fault is to add too much stain to the preparation.

9. Holding the coverslip between your index finger and thumb, touch one edge of the drop of stain with the edge of the coverslip.

10. Lower the coverslip gently onto the slide, trying to avoid air bubbles. Switch to a higher power 40X objective for a more detailed examination of spores and other structures.

Cellophane Tape Preparation:

The cellophane or transparency tape method for microscopic examination of fungi is simple to perform, inexpensive and, rapid. It preserves the conidial arrangements of the more delicate filamentous molds and allows one to make an accurate identification.

1. Place a drop of lactophenol aniline blue stain on a microscope slide.

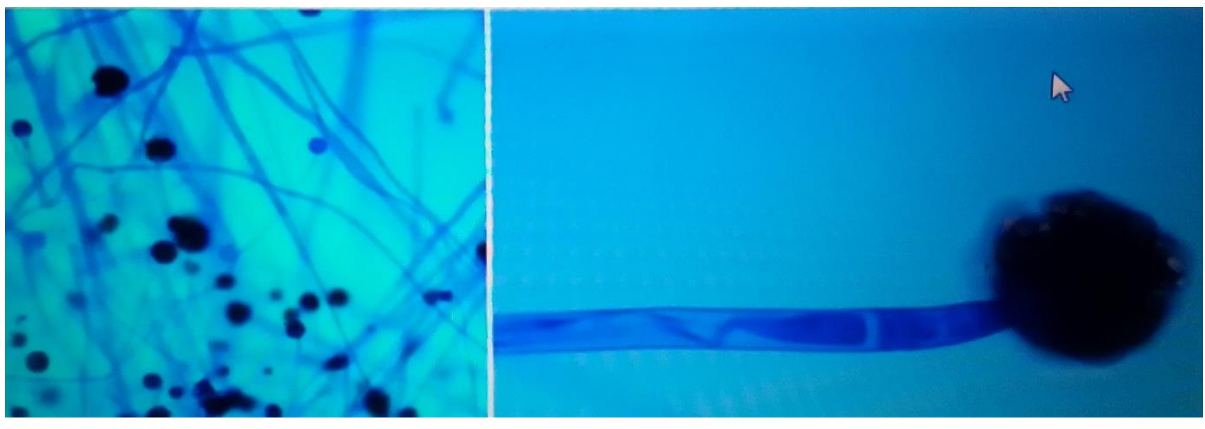
2. Press the sticky side of unfrosted, clear cellophane tape gently but firmly to the surface of the colony, picking up a portion of the aerial mycelium.

3. Stick one end of the tape to the surface of the slide adjacent to the drop of stain.

4. Stretch the tape over the stain, gently lowering it so that the mycelium becomes permeated with stain.

5. Pull the tape taught and stick the opposite end to the glass, avoiding as much as possible the trapping of air bubbles.

6. Your preparation is now ready for examination.Switch to a higher power 40X objective for a more detailed examination of spores and other structures (figure-11).



a b

Fig 2.44 Mucor species: (a) Sparsely septated hyphae sporangiophore bearing sporangia (100X). (b) Mucor sporangiophore bearing sporangia containing sporangiospores (400X)

2.6 *SUMMURY*

There are so many choices available for sterilization of objects. We should select the appropriate sterilization method and the decision will depend on many different parameters including: The material that needs to be sterilized. Is the material heat sensitive? Is it sensitive to moisture, or radiation? The nature of the microorganisms that need to be destroyed or removed. Additional considerations are: Accuracy, Time, safety and budget.

Simple culture media includes nutrient broth. One percent meat extract and peptone water makes up such broth. Nutrient broth becomes glucose broth when glucose is added to it. Likewise, it becomes nutrient agar when 2-3% agar is added. For purposes of diagnosis, this is the simplest and most common media used in laboratories for study purpose.

Selective media contain components that prevent the growth of all undesired bacteria. Selective media are specific for aerobic and anaerobic bacteria. Once we knew that which bacterial species we have to culture than we select the appropriate selective media.

Differential or indicator media distinguish one microorganism type from another growing on the same medium. This type of media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as neutral red, phenol red, eosin y, or methylene blue) added to the medium to visibly indicate the defining characteristics of a microorganism. These media are used for the detection of microorganisms and by molecular biologists to detect recombinant strains of bacteria.

There are various inoculation techniques for bacterial culture some are easier but have some greater disadvantages while some techniques are time consuming and but produces batter result and may be cost effecting with higher accuracy. So we have to select the appropriate inoculation techniques according to our need. Further stain simple stains used to highlight microbes and illustrate cellular shapes and arrangements .Negative stains are used for study of cell morphology while Gram staining is used for the characterization in one of two groups, Gram positive and Gram negative bacteria.

Flagella stain are used for the demonstration of bacterial flagella and spirochetes. The differential stains viz; Gram staining is used for the characterization in one of two groups, Gram positive and Gram negative bacteria while acid fast stain and Auramine-Rhodamine stain are used to differentiate bacteria into acid fast group and non-acid fast groups. The Endospore stain is used for endospore staining produced by a few genera of Gram-positive bacilli, such as *Bacillus* and *Clostridium* etc. However there are various endospore stain available we can use these according to our need. Capsule stain are used the demonstration capsule of bacteria.

In Microbiology, Giemsa stain is used for staining inclusion bodies in *Chlamydia trachomatis*, *Borrelia* species, and if Wayson's stain is not available, to stain *Yersinia pestis.* Giemsa stain also is used to stain *Histoplasma capsulatum*, *Pneumocystis jiroveci*, *Klebsiella granulomatis, Talaromyces marneffei* (formerly called *Penicillium marneffei*), and occasionally bacterial capsules. Cytogenetics also uses this stain to stain the chromosomes and identify chromosomal aberrations. It is commonly used for G-banding (Giemsa-Banding). Calcofluor White Stain is used for the determination of Fungi, Pneumocystis cysts, and parasites.

TERMINANAL QUESTIONS

Q.1. Write the name of Physical methods of sterilizations.

Q.2. Write the short notes on dry heat methods of sterilization.

Q.3. What is temperature and time relationship for hot air ovan.

Q.4. Write the principle and disadvantages of hot air oven .

Q.5. write the principle of autoclave.

Q.6. Illustrate the advantages and disadvantages of autoclave.

Q.7 . Write the principle of filtration.

Q.8. Describe the various type of filters and their uses.

Q.9. Write principle of Tyndallization and pasteurization and

Q.10 What is essential difference between Tyndallization and pasteurization.

Q.11.W rite the principle of radiation sterilization.

Q.12. Write the name various types of rays used in sterilization also write their uses.

Q.13. How do aerobic and anaerobic bacteria derive energy.

Q.14 On the bases nutritional factors write the classification of culture media.

Q.15. What is simple culture media? Give any 3 examples of it.

Q.16. What are special culture media and write their names.

Q.17.What is enriched media. Write the difference between selective and differential media.

Q.18.What is oxoid culture media. Does the term dehydrated and Oxoid are synonyms for each other.

Q.19.Write the formulation or composition of Oxoid culture media

Q.20. Describe the various type of selective culture media for the growth of aerobic and anaerobic bacteria.

Q.21.Write short notes on (a) Inoculation loops (b) *Erlenmeyer flask* (c) Petri dish or plate

(d) Culture vials. (e) Laminar air flow cabinet (f) Anaerobic chamber.

Q.22. Define the term inoculation and inoculums.

Q.23. Describe the various techniques involve in culture inoculation.

Q. 24.illustrate the principle of Robertson'scookedmeatmedium.

Q.25. Write short notes on (a) MacConky Agar (b) Blood Agar (c) Nutrient Agar

Q.26. What is simple staining.

Q.27. Describe the principle of negative staining.

Q.28. Why is a gram staining classified as a differential staining?

Q.29.What is the significant about gram staining?

Q.30. Describe mechanism of action for gram staining

Q.31. Describe the principle of Zeihl Nelson stain.

Q.32. Write the principle of endospore and flagellar staining.

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Note- Student are being advised for theory exam only learn the principle, use advantage and disadvantages of different techniques used in micro biology. No need to learn procedure for theory exam Except gram staining and Zeihl Nelson staining. It is also applicable for sterilization and culture media tecnques (except inoculation techniques).Procedure should be written in practical exam

Unit 3 ENVIRONMENTAL MICROBIOLOGY

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3.1 *OBJECTIVE*

After studying this unit learns will learn:

- What is Microbial Ecology?
- Microorgansims and their ecological relationships
- The Microbiology of Soil and Water
- Basics of Airborne diseases

3.2 *INTRODUCTION*

Microorganisms are a very important component of the ecosystem. They have a role in the various ecological processes. The microorganisms are present in soil, waterbodies, and various other habitats. Microorganisms play two important roles in the ecosystem: firstly, they involve in the production of new organic components and secondly, they help in the decomposition of this accumulated organic matter. Microorganisms also have a great role in nutrient cycling and decomposition processes. Plants are the primary producer in the terrestrial environment. In the aquatic environment the cyanobacteria and algae play similar roles. Likewise, microorganisms also act as produce, decomposers and some also act as consumers.

Environmental microbiology is the study of microorganisms in the environment. The environment here means the air, soil, water and also the animal and plants that inhibit these areas. The modern environmental microbiology includes the study of pathogens and bioremediation etc. The advancement in the field of molecular biology and biotechnology helps in understanding various aspect of environment microbiology.

3.3 *MICROBIAL ECOLOGY*

In the natural habitats the microorganisms live as a population of similar types of organisms like micro-colony growing at a localized site, and as communities comprising different kinds of interactive population. The microbial environment is a dynamic and complex system. The study of microorganisms and their relationship to their specific environment is called microbial ecology.

The microbial environment has overlapping gradients of resource, toxic material and other limiting factors. In a suitable microenvironment, specialized groups of microorganisms can survive with less competition from other microorganisms that have little different functional requirements. The Liebig's law of the minimum stated that the rate of growth of an organism, depends on the amount of the scarcest of its essential nutrients that is available to it. The Shelford's law of tolerance stated that the abundance or distribution of an organism can be controlled by certain factors (e.g. the climatic, topographic, and biological requirements of plants and animals) where levels of these exceed the maximum or minimum limits of tolerance of that organism

In the presence of sufficient nutrients and less competition the microorganisms grow nicely. But in presence of excess nutrients the microorganisms grow rapidly this leads to nutrient depletion and release of toxic substances, which further limit growth. When there is low nutrient and intense competition then many microorganisms become more competitive in nutrient capture and exploitation of available nutrients.

3.3.1 ROLE OF MICROORGANISMS IN THE PRODUCTIVITY OF ECOSYSTEMS

Plants act as a primary producer in terrestrial ecosystems likewise in aquatic and marine environments cyanobacteria and algae play a similar role. Examples of microorganisms acting as a primary producer are deep marine hydrothermal vents. These vents have a large population of tube-shaped worms and giant mussels. These consumers survive on organic matter synthesized by chemolithoautotrophic bacteria e.g. genera *Thiobacillus, Thiomicrospira, Thiothrix* and *Beggiatoa.* Another example of a food chain involving microorganisms as producers is methane fixing microorganisms. Methylotrophs (bacteria) occur as intracellular symbionts with methane-vent mussels (Fleshy gills).

The microorganisms play an important role in the biogeochemical cycling. They help in the transformation of carbon, nitrogen, sulfur and iron. In carbon fixation, methane can be produced from CO_2 and hydrogen (inorganic source) and organic matter. The CO produced by various sources is added to cycle CO-oxidizing bacteria. Cyanobacteria, green algae, photosynthetic bacteria like *Clostridium* and *Chromatium,* and aerobic chemolithoautotrophs all contribute to carbon fixation. Sulfur cycle involves the photosynthetic and chemosynthetic microorganisms. Sulfite is reduced to sulfide by *Alteromonas, Clostridium, Desulfovibrio*and *Desulfotomaculum.*

Nitrogen cycles involve nitrification, denitrification and nitrogen fixation. Nitrification involves oxidation of ammonium ion to nitrite and then nitrite oxidation to nitrate. The first step is carried out by the genera *Nitrosomomas* and *Nitrosococcus*, further oxidation is done by *Nitrobacter* and related chemolithoautotrophic bacteria. Denitrification is done by *Pseudomonas denitrificans*. Nitrite can be transformed to ammonia by *Geobactermetallireducens*, *Desulfovibrio spp.* and *Clostridium.* Nitrogen fixation can be done by both aerobic and anaerobic bacteria. Aerobic microbial nitrogen fixation is done by genera *Azotobacter* and *Azospirillum.* Anaerobic nitrogen fixation occurs with the help of genus *Clostridium*. Cyanobacteria like *Anabaena* and *Oscillatoria* help in nitrogen fixation in fresh and marine water. Some bacteria in association with plants (legumes) fix nitrogen eg. *Rhizobium* and *Bradyrhizobium*. *Frankia* fix nitrogen with many wood shrubs like *Anabaena* with*Azolla*. The nitrogen fixation is catalyzed by nitrogenase enzymes. The microorganisms involved in the iron cycle are *Thiobacillusferrooxidans*, *Gallionella*, *Sulfolobus,Sphaerotilus* and *Leptothrix*. The manganese cycling involves the use of *Leptothrix, Arthrobacter* and *Metallogenium* for Mn^{2+} oxidation and *Shewanella, Geobacter* in manganese reduction.

Therefore, microbes play crucial roles in the decomposition of organic matter (mineralization), the food web (which includes other chemoheterotrophic bacteria), ecological cycles, and the inhibition of growth by producing toxins.

3.3.2 INTERACTIONS BETWEEN MICROORGANISMS AND ANIMALS

Two or more dissimilar organisms live together in close association with each other is known as "Symbiosis". Any microorganism that lives their whole life or portion of life in association with another organism of a different species is called a symbiont. Many microorganisms spend most of their life in special ecological relationships with another organism. There are three types of symbiotic relationships:

1) Commensalism
2) Mutualism
3) Parasitism

These association can be ectosymbiotic or endosymbiotic. In ectosymbiotic association one organism remains outside of the other and in case of endosymbiotic association one organism lives within the other.

Commensalism

Commensalism is an association between two different organisms in which one organism (commensal) is benefited by the relationship and other members is neither harmed nor benefited. An example of commensalism is *Escherichia coli* which lives in the human colon. *E.coli* gets nutrients, warmth and shelter from the human colon and generally does not cause any discomfort or disease.

Mutualism

Mutualism is an association between two different organisms in which both the partners are benefited. There are various examples of mutually beneficial relationships. 1) The flagellated protozoa lives in the gut of termites and wood roaches. The termites are unable to synthesize the enzyme cellulose so, the wood chip ingested by them is digested by the protozoan. The protozoans engulf wood chips and digest the cellulose. The metabolism of cellulose produces acetate and other products. Termites then metabolize the acetate release by their flagellates.

2) Lichens are the association between ascomycetes (the fungus) and specific genera of either green algae or cyanobacteria. In this association the fungal partner is termed as mycobiont and the algal or cyanobacteria partner is termed as phycobiont. The phycobiont synthesizes food via photosynthesis and the fungal takes its food from alga or cyanobacterium. The fungal partner protects the phycobiont from excess light intensities, supplies water and minerals. It also provides a firm substratum within which the phycobiont can grow protected from the environment.

Parasitism

Parasitism is an association in which the symbiont (called parasite) either harms or lives at the expense of another partner (host). There are two types of parasitism 1) Ectoparasite: In this type of association the organism lives on the surface of its host. 2) Endoparasite: In this type of association the parasite lives within the host e.g., *Mycobacterium tuberculosis* causes tuberculosis disease in humans.

3.4 *MICROBIOLOGY OF SOIL*

The soil has a characteristic solid phase, which comprises organic and inorganic components and it is generally aerobic. Plants are the main producer of organic matter. The organic matter is accumulated as leaves and branches over the ground and later become the litter material. Under the ground root growth and decay contribute to the pool of organic matter. In desert environments

algae and cyanobacteria contribute to the accumulation of organic matter. Soil is a dynamic entity and responds to the change in the temperature, moisture and other disturbance (plowing). The microorganisms play an important role in soil chemistry. The soil provides habitat to a number of microorganisms e.g. bacteria and fungi. Microorganisms help in formation and maintenance of soil. The soil consists of different surfaces which influence the nutrient availability and also affect the interaction between different microorganisms.

3.4.1 TYPES OF MICROORGANISMS IN SOIL

The soil is made up of sand, clays, silt and other particles. There are various sizes of pores available in the soil which are used for colonization. The various components form heterogeneous aggregates of various sizes called peds. Most bacteria use particle surfaces and they use nutrients and water from their immediate environment. Bacteria are generally present in small soil pore (2 to 6 μm in diameter) which prevent them from getting eaten up by protozoans. The filamentous fungi are present on the outside of the aggregates. Fungi with the help of their filamentous growth can move nutrients and water from great distances in soil. Various other organisms like protozoa, soil insects, nematodes etc. are also present in the soil. These organisms generally feed on bacteria and fungi.

The soil microorganisms respond to the organic material released near the root of the plant, by increasing their number and changing the characteristics of the microbial community. This region is called the rhizosphere. The genus *Rhizobium* which fixes nitrogen by symbiotic association with the legumes is a prominent member of the rhizosphere community. Other microorganisms which fix nitrogen include genera *Azotobacter* and *Azospirillum* in association with tropical grasses. Stem-nodulating rhizobia found in association with tropical legumes.

Fungi (*Mycorrhizae*) are also associated with the root and were first discovered by Albert Bernhard frank in 1885. There are two types of mycorrhizal association 1) Ectomycorrhizal and 2) Endomycorrhizal. Ectomycorrhizal are generally found in the temperate region associated with certain groups of trees and shrubs e.g. pine trees with fungal components like basidiomycetes, ascomycetes or zygomycetes. Endomycorrhizal association shows penetration of fungal hyphae into the outer cortical cells of the plant root, where they grow intracellularly. They are present in apples, beans, corn, tomatoes and oranges etc. Mycorrhizal association helps in increasing the availability of nutrients especially phosphorus, mycorrhizal helps in water uptake, allowing increased transpiration rates in comparison with non-mycorrhizal plants.

Actinomycetes are also associated with the roots of plants known as actinorhizal. This association is formed by *Frankia* strains with eight plant families. They help in nitrogen fixation.

3.4.2 *FACTORS FOR MICROBIAL GROWTH*

The microorganisms have a diverse habitat and are also able to inhabit those habitats which have extreme physical and chemical environments. The microorganisms are small so they inhibit a small habitat that is why microbial ecologists use the term microenvironment. Microenvironment is a place where a microorganism lives and metabolizes within its habitat. The soil has higher concentration of CO_2, CO and other gasses in comparison with the atmosphere but less concentration of O_2. There is limited diffusion of gasses into and out of these aggregates so there is a possibility that the spaces between aggregates are completely flooded. Which leads to change in the dissolved salts and gases in these smaller pores. The pH also influences the microorganism, like at neutral pH the soil component and microorganism are negatively charged.

3.4.3 *SOIL ENZYMES*

Soil is characterized by the presence of various kinds of enzymes which play an essential role in maintaining soil health by decomposing organic matter, energy availability and NH_4 to plants. Soil enzymes are an integral part of ecosystem processes because they help in catalyzing various reactions in the soil which have biogeochemical significance. Examples of soil enzymes are: β-glucosidase, phenol oxidase, peroxidase enzymes, alkaline phosphatase, acid phosphatase, and fluoresceinhydrolase. Enzymes can exist internally or on surface membranes of viable cells, be excreted into soil solution, or be complexed in the soil matrix or microbial debris. Extracellular enzymes hydrolyze substrates that are too large or insoluble for direct absorption by microorganisms. With the exception of dehydrogenase and perhaps a few other enzymes, which can only exist in live cells, soil enzymes can exist in both viable and complex forms, independent of living cells, stabilised in the soil matrix. The soil enzymes play an important role in the nutrient cycle in the ecosystem. The enzyme's activity varies seasonally because microorganisms respond to change in the environment. Soil type (particularly textural distribution and organic matter) also influence the enzyme activity. The activity of soil enzymes also depends on the pH of the soil. To determine the activity of enzymes various enzyme assays are done, that are used as technological

tools for numerous applications in ecosystem management. Enzyme assays can be used to study the level of degradation and recovery of soils.

Enzyme	**Substrate**	**Product**	**Functions**
Beta glucosidase	Carbon compounds	Glucose	Organic matter decomposition and energy production
Amidase	Carbon and nitrogen compounds	Ammonium	Nutrient cycling and provide NH_4 to plant
Urease	Urea	Ammonia and carbon dioxide	Nutrient cycling and provide NH_4 to plant
Phosphatase	Phosphorus	Phosphate	Nutrient cycling and provide P to plant
Sulfatase	Sulfur	Sulfate	Nutrient cycling and provide S to plant

Table 1 Soil enzymes and their functions

3.5 *MICROBIOLOGY OF WATER*

In the earth 97% water is of marine nature and both marine water and freshwater provide a unique niche for many specialized microorganisms.

In terms of nutrient level both in marine and freshwater, the range varies from microgram to organic matter per liter. The higher amount of nutrients is found in the polluted water bodies or sewage treatment plants. As the amount of nutrients varies there is a shift of microorganisms from oligotrophic (low nutrient responsive) to copiotrophic (high nutrient responsive) occur. The nutrient turnover rate varies in marine and estuarine area. In the marine system the rate is slow but marsh and estuarine areas show high rate of nutrient turnover.

3.5.1 MICROORGANISMS OF WATER

Water has a unique physical environment which provides survival conditions to various types of microorganisms. *Beggiatoa* and *Thiothrix* are bacteria that are present in a waterlogged zone with hydrogen sulfide. Various bacteria which are chemoheterotrophs includes genera *Sphaerotilus, Leucothrix, Caulobacter, Hyphomicrobium, Flexithrix*and and *Flexibacter,* are present in water. Other microorganisms which are present in water are photosynthetic algae (diatoms), aquatic fungi and protozoa (*Foraminiferans and Radiolarian*). In aquatic systems algae are a major source of organic carbon. Protozoan increases nutrient cycling by grazing on other groups of microorganisms. In marine water the major source of organic matter is photosynthesis by phytoplankton (Genus *Synechococcus* and *Picocyanobacteria*). Viruses are also present in the ocean in large populations and lead to major decrease in primary production because they infect cyanobacteria. The ocean also contains large amounts of archaebacteria. They can live in extreme conditions of temperature and salinity.

3.5.2 MICROBIOLOGY OF POTABLE WATER

Potable water (drinking water) is obtained from surface and ground sources and is treated to meet state and federal standards for consumption. Untreated water containing microorganisms, bacteria, toxic chemicals, viruses and fecal matter can cause various water borne diseases such as diarrhea, vomiting or fever. The portable water is obtained from various sources of varying microbiological quality and requires both physical and chemical water treatment. Unwanted microorganisms growth can occur in drinking water distribution systems which causes deterioration of water

quality during storage and transport. Examples of hygienically relevant opportunistic pathogens are *Pseudomonas aeruginosa, Legionella pneumophila, Mycobacteria, Aeromonashydrophila, Klebsiellapneumoniae* and *Campylobacter*. Protozoan present in drinking water also have pathogenic properties (e.g., *Acanthamoeba, Cryptosporidium and Giardia lamblia*), or act as hosts for pathogenic bacteria (*Legionella pneumophila*). Viruses (noroviruses, Hepatitis A virus) were also reported in drinking water which can cause water-born gastrointestinal or other viral illness. Other organisms in drinking water are fungi, algae, invertebrates, worms or snails. Amount of organic and inorganic nutrients in drinking water determine the growth of microorganisms. Heterotrophic organisms like the majority of bacteria in drinking water draw their energy from organic carbon compounds. Inorganic nutrients such as phosphorus, nitrogen or trace elements (iron, magnesium, copper, potassium), are also required for heterotrophic growth but in smaller amounts as compared to organic carbon. Heterotrophic bacteria are the major contributor of bacteria present in drinking water but autotrophic organisms such as nitrifying, sulfate-reducing or iron-oxidizing bacteria have also been reported in drinking water like ammonium oxidizing bacteria (*Nitrosomonas*and *Nitrospira*) are present in treated deep-ground waters rich in ammonium, while sulfate-reducers (*Desulfovibrio* and *Desulfotomaculum*) and iron-oxidizers (*Gallionella, Leptothrix* and *Sphaerotilus*) were associated with microbially induced corrosion processes. Competition, nutrients availability, water temperature or pH and specific kinetic capabilities of individual species are the factors responsible for microorganism's growth. The main water borne parasitic diseases are amoebiasis and giardiasis. Giardiasis is brought on by improperly treated surface waters, and amoebiasis is imposed on by sewage contamination in distribution systems. The cysts of both of these parasites are more resistant to chlorine treatment than are bacteria, but can be removed by the process of flocculation and filtration. Metazoan parasites (helminths, nematodes) found in drinking water can be removed by the process of flocculation, filtration and disinfection.

3.5.3 PURIFICATION OF WATER

Purification of drinking water is essential because use of impure water can cause various water borne diseases. The process of water purification involves a number of steps depending on the type of impurities. The water supplied by the municipality is purified by at least three or four steps. The first step involves collection of raw water (containing suspended material) to a sedimentation

basin and held so that sand and other very large particles can settle out. The partially purified water was then collected in the settling basin and treated with chemicals, such as lime and alum (aluminium sulphate), to aid in the precipitation process. This process helps in removal of microorganisms, organic matter, toxic contaminants and suspended fine particles. This procedure is called coagulation or flocculation. In the next step the water is passed through rapid sand filters which physically trap the fine particle and flocs. This procedure removes up to 99% of the bacteria. At last the water is disinfected by generally chlorination, but ozonation is becoming increasingly popular. The problem with chlorination is that a large dose of chlorine is used which leaves residual free chlorine at a concentration of 0.2 to 2.0 mg/l. So, this leads to formation of Disinfection by-products (DBPs) such as trihalomethanes (THMs), formed when chlorine reacts with organic matter. Some DBPs are carcinogens. This process helps in removing or inactivating disease causing bacteria and indicator organisms (coliforms). Unfortunately, all these processes are not able to remove the *Giardia* intestinalis cysts, *Cryptosporidium* oocysts, *Cyclospora* and viruses**.** *Giardia* cysts can be removed by slowly passing water through a bed of sand in which a microbial layer covers the surface of each sand grain. Coagulation, filtration, chemical oxidants, high pH, and photooxidation are also used to eliminate viruses. Observing and identifying indicators and disease-causing microorganisms are an important part of sanitation. When indicator organisms are not detectable in a specific volume (100 ml) of water, the water is considered potable.

3.5.4 MICROBIOLOGY OF SEWAGE & BIOREMEDIATION

The sewage water contains various microorganisms e.g. bacteria, viruses, protozoa, fungi, flatworms or roundworms. Different bacterial species were found in the sewage water e.g. *Actinomyces, Bifidobacterium, Clostridium, Propionibacterium* and *Peptostreptococcus* genera. Sewage water contains mainly anaerobic bacteria because sewer environments have conditions, which are favorable for the growth of anaerobic bacteria. These bacteria carry out a number of fermentation processes leading to production of hydrogen sulfide, methane and volatile organic compounds. Examples of sulfate-reducing bacteria are *Desulfovibrio, Desulfotomaculum, Desulfobacter, Desulfuromonas* and *Desulfococcus* genera. Bacterial stains from *Simplicispira, Comamonas, Azonexus, Thauera*and other genera are responsible for forming biofilm on sewer walls. The pathogenic microorganisms are responsible for a number of diseases. Number of

opportunistic pathogens e.g. *Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Klebsiellapneumoniae*, *Proteus vulgaris* or *Pseudomonas aeruginosa* are present in wastewater and can cause different systemic infections. Various species of *Longilinea, Georgenia, Desulforhabdus, Thauera, Desulfuromonas* and *Arcobacter* genera were found in the sewerage system. Microorganisms help in biological digestion of sewage that results in conversion of contaminated, infectious liquid into an inert sludge and a harmless effluent. This effluent is then chlorinated and discharged to the receiving stream, leaching bed, or other disposal area. Sewage treatment involves two fundamental biological processes 1) Aerobic digestion and 2) Anaerobic digestion

1) Aerobic digestion involves aeration of water received from the primary settling tanks until active masses of microorganisms settle out as sludge and leave a clear effluent of low organic content. A part of the sludge is again mixed with the incoming raw sewage and the remaining is pumped into the digester tanks. The raw sewage contains high amounts of organic matter and the activated sludge contains various microorganisms e.g. bacteria, fungi, protozoa, rotifers, and sometimes nematodes. The growth of bacteria is logarithmic and their predators (e.g. protozoans) also grow fast. When the food becomes a limiting factor the microorganism starts to die off leading to formation of floc. Rotifers can eat small particles of floc. This floc is later removed in a sedimentation tank.
2) Anaerobic digestion involves use of large digestion tanks, septic tanks and cesspools. It is a slow process and also sensitive to an imbalance in the biological population. When the sludge in a digestion tank or septic tank settles to the bottom the aerobic organisms die off due to lack of oxygen forming spores or cysts. Anaerobic digestion involves two phase 1) Acid phase: facultative anaerobes metabolize the organic matter and convert it into organic acids, aldehydes, and alcohols (Acid-forming bacteria). This leads to lower pH and retardation of bacterial action. 2) Methane phase: It is the second phase that starts with the increase in the group of obligate anaerobes. They metabolize the organic acids, producing carbon dioxide and methane.

Bioremediation

Various human activities like Agriculture, Urbanization and Industrialization results in accumulation of waste products causing environment degradation. The accumulation of toxic

waste like heavy metals, nuclear wastes, pesticides, greenhouse gasses and hydrocarbons can also cause health problems. The removal of these toxins is necessary and bioremediation is one of the methods used for the removal of these toxins. The use of microorganisms to transform toxic compounds to nontoxic degradation products is called bioremediation. This technique involves the use of some bacteria and other microorganisms (like archaebacteria, yeasts, fungi, algae), to extract or degrade inorganic and organic contaminants. Both aerobic and anaerobic microorganisms are involved in the process of bioremediation. Environmental factors such as: presence of nutrients, temperature, oxygen addition or diffusion, pH, heavy metals, presence of toxic compounds, physiology of biodegradative microorganisms, metabolic activity, effectiveness of contaminants removal mechanisms and type of microorganisms influence the success of bioremediation. The success of bioremediation depends on the interaction between biological, metabolic and environmental factors. Bioremediation can be done either Ex situ or In situ depending on cost, site characteristics, type and concentration of pollutants. The selection of appropriate bioremediation techniques is crucial for the success of the processor. The selection criteria used for bioremediation technique are nature of pollutant (agrochemicals, chlorinated compounds, dyes, greenhouse gasses, heavy metals, hydrocarbons, nuclear waste, plastics, and sewage), depth and degree of pollution, type of environment, location, cost, and environmental policies. Ex situ bioremediation is generally more expensive compared to in situ techniques as a result of additional cost attributable to excavation.

Ex situ bioremediation techniques

Ex situ bioremediation techniques involves the excavation of the pollutants from polluted sites and then they are transported to the other site for treatment. Ex situ bioremediation techniques depend upon the cost of treatment, depth of pollution, type of pollutant, degree of pollution, geographical location and geology of the polluted site.

A) Biopile is an ex situ bioremediation which involves above-ground piling of excavated polluted soil, followed by nutrient amendment, and sometimes aeration resulting in increased microbial activities to enhance bioremediation. The components of this

technique are: aeration, irrigation, nutrient and leachate collection systems, and a treatment bed.

B) Windrows depend on periodic turning of piled polluted soil to enhance bioremediation by increasing degradation activities of indigenous and/or transient hydrocarbonoclastic bacteria present in polluted soil. The periodic turning of polluted soil, together with addition of water result in increased aeration, uniform distribution of pollutants, nutrients causing increase in microbial degradative activities.

C) Bioreactor is a vessel in which raw materials are converted to specific products by a series of biological reactions. Different operating modes of bioreactor are batch, fed-batch, sequencing batch, continuous and multistage.

D) Land farming bioremediation technique is a less expensive process because it requires less equipment. Due to site of treatment sometimes this technique is also considered as in situ bioremediation technique. Pollutant depth determines whether the land farming will be carried out ex situ or in situ. In this process the polluted soils are generally excavated and/or tilled. On-site treatment of excavated polluted soil is an in situ bioremediation.

In situ Bioremediation techniques

These techniques involve treating polluted substances at the site of pollution. This technique is less expensive compared to ex situ bioremediation techniques because no extra cost is required for excavation processes but sometimes on-site installation of some sophisticated equipment is expensive.

A) Bioventing involves controlled stimulation of airflow by delivering oxygen to an unsaturated zone, which results in increased activity of indigenous microorganisms to enhance bioremediation. The success of bioventing-based bioremediation depends upon the number of air injection points resulting in uniform distribution of air.

B) Bioslurping techniques involve the use of vacuum-enhanced pumping, soil vapor extraction and bioventing to achieve soil and groundwater remediation by indirect provision of oxygen and stimulation of contaminant biodegradation.

C) In biosparging technique air is injected into the soil subsurface to stimulate microbial activities in order to enhance pollutant removal. Unlike bioventing, air is injected at the saturated zone that results in upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation.

D) Phytoremediation technique involves the use of interactions (physical, biochemical, biological, chemical and microbiological) in polluted sites to reduce the toxic effects of pollutants. Toxic heavy metals and radionuclides are generally removed by extraction, transformation and sequestration. Organic pollutants are generally removed by degradation, rhizoremediation, stabilization and volatilization. *Brachiariamutica and Zea mays* are phytoremediators of heavy metal-contaminated sites.

E) Permeable reactive barrier (PRB) technique is generally used as a physical method for remediating contaminated groundwater, due to its design and mechanism of pollutant removal.

F) Intrinsic bioremediation (natural attenuation) is an in situ bioremediation technique, which is characterized by the passive remediation of polluted sites with no human involvement. This technique is less expensive but takes longer time to achieve the target.

Bioremediation is an effective process and in this technique microorganisms play an important role. So, the microbial abundance, diversity and community structure along with environmental factors are very crucial in determining the fate of any bioremediation technique. Two techniques are used to speed up microbial activities during the bioremediation process.

1) Biostimulation involves the addition of nutrients or substrates to a polluted sample in order to stimulate the activities of autochthonous microbes. The activity of microorganisms

increases and decreases according to the amount of pollutant concentration. So, addition of agro-industrial wastes with appropriate nutrient content (nitrogen, phosphorus and potassium) will help in solving the problem of nutrient limitation in most polluted sites.

2) Bioaugmentation is a process of introducing or increasing microbial population with degradative capabilities. The use of diverse microorganisms is useful as compared to pure isolate because metabolic diversities may lead to complete and rapid degradation of pollutants. This process is effective but has few disadvantages like competition among endogenous and exogenous microbial populations, the risk of introducing pathogenic microbes into an environment, and the possibility that the inoculated microorganisms may not survive in the new environment.

If multiple bioremediation techniques are used side by side then this will lead to an increase in remediation efficacy (by decreasing the shortcoming of individual technique), and also reduce the overall cost. Efficacy of the technique can also be increased by using genetically engineered microorganisms (GEM). Advantage of Bioremediation technique is it is cost-efficient, environment-friendly and sustainable method for waste treatment. Disadvantages of bioremediation technologies are

(a) Some highly chlorinated contaminants and high molecular weight PAHs are not easily degraded by microorganisms.

(b) Degradation of some chemical results in the production of more toxic and mobile intermediates than the parent compound e.g. reductive dehalogenation of TCE can result in accumulation of vinyl chloride, a toxic product and a carcinogen.

3.6 *AIRBORNE DISEASES*

Microorganisms like bacteria, viruses and fungi if transmitted through air results in airborne disorder or disease. These organisms are transmitted via sneezing, coughing, spraying of liquids, and the spread of dust, talking, or any activity that results in the generation of aerosolized particles.

The Airborne transmission is of two types obligate or preferential depending on whether it is only transmitted through droplet nuclei or if it has multiple other ways of transmission. Airborne microorganisms are transmitted via fine mist, dust, aerosols or liquids. The source of infection (infected organism) generates the aerosolized particles. These aerosols are sometimes also generated by biological waste products that accumulate in garbage cans, caves, and dry arid containers. During aerosolization, the microbes which are less than 100 micrometers in size float in the air. These microorganisms in the droplets are then dispersed through air currents to various distances and may be inhaled by susceptible hosts. While a majority of the particles will drop off within the surrounding area, the infected aerosolized particles usually remain suspended in the air and may even travel considerable distances. The rate of transmission from the source to susceptible individuals decreases as the distance between them increases. The airborne microorganisms cause an inflammation of the upper airways affecting the nose, sinuses, throat, and lungs resulting in sinus congestion, sore throat, and lower respiratory tract symptoms. The control of airborne transmission from patients to susceptible hosts is necessary. To control or to prevent airborne transmission various measures required like 1) The control of airflow with the use of specially designed ventilation systems 2) The practice of antiseptic techniques 3) Wearing personal protective equipment (PPE) and 4) Performing basic infection prevention measures like hand washing. Airborne diseases affect both humans as well as animals. However, it is not necessary that exposure to infected particles may result in disease. Because the infection depends on the host's immunity, the amount of exposure, and the duration of exposure to the infected patient. There are various factors that influence airborne transmission

1) Temperature: Different microorganisms are active at different temperatures. Some are active at low temperature but some are resistant at low temperature.
2) Sun exposure: The strength and duration of exposure is an essential for the survival of microbes in the air.
3) Humidity: The humidity also affects the rate at which airborne droplet nuclei spread from person to person. High humidity levels protect against UV light destruction as water vapor forms a protective barrier around the droplet nuclei.
4) Wind: Air currents increase the distance traveled by infectious airborne particles but wind decreases the concentration of droplet nuclei resulting in decrease in airborne infectivity.
5) Tropical Storms: They decrease the quantity of fungal spores for a few days in the air.

6) Socioeconomic and living conditions: Conditions of living also determine the rate of transmission e.g. Number of people per room, Room ventilation and aeration. Air Conditioning also increased airborne infection spread in closed environments.
7) *Place of living:* Because of high population density in urban areas as compared to rural areas there is increased transmission of bacterial and viral pathogens in urban areas.
8) Poor sewage and drainage systems: Accumulated Biowaste increases the risk of formation and spread of airborne infection.

Organism	**Disease**
Bacillus anthracis (Bacteria)	Anthrax
Aspergillus fumigatus (Fungus)	Aspergillosis
Blastomycesdermatitidis(Fungus)	Blastomycosis
Varicella zoster virus	Chickenpox
Rotavirus	Diarrhea
Influenza viruses	Influenza
Rhinovirus	Common cold
Neisseria meningitidis (Bacteria)	Meningococcemia and life-threatening sepsis
Streptococcus pneumoniae (Bacteria)	Pneumonia
Legionella pneumophila	Legionellosis
Measles virus	Measles
Mumps virus	Mumps
Variola virus	Smallpox
*Cryptococcus neoformans*and *Cryptococcus gattii* (Fungus)	Cryptococcosis
Mycobacterium tuberculosis	Tuberculosis
SARS-associated coronavirus (SARS-CoV)	Severe acute respiratory syndrome (SARS)
Middle East respiratory syndrome coronavirus (MERS-CoV)	Middle East Respiratory Syndrome (MERS)

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	Coronavirus Disease 2019 (COVID-19)

Table 2: Airborne diseases and causative organisms

3.7 *SUMMARY*

Microorganisms are a very important component of the ecosystem. They have a role in the various ecological processes. The microorganisms are present in soil, waterbodies, and various other habitats. In the natural habitats the microorganisms live as a population of similar types of organisms like micro-colony growing at a localized site, and as communities comprising different kinds of interactive population. Plants act as a primary producer in terrestrial ecosystems likewise in aquatic and marine environments cyanobacteria and algae play a similar role. Examples of microorganisms acting as a primary producer are deep marine hydrothermal vents. These vents have a large population of tube-shaped worms and giant mussels. The microorganisms play an important role in the biogeochemical cycling. They help in the transformation of carbon, nitrogen, sulfur and iron. When two or more dissimilar organisms live together in close association with each other is known as symbiosis. Any microorganism that lives their whole life or portion of life with association with another organism of a different species is called a symbiont. Many microorganisms spend most of their life in special ecological relationships with another organism. There are three types of symbiotic relationships 1) commensalism 2) mutualism and 3) parasitism. The microorganisms play an important role in soil chemistry. The soil provides habitat to a number of microorganisms e.g. bacteria and fungi. Microorganisms help in formation and maintenance of soil. Water has a unique physical environment which provides survival conditions to various types of microorganisms. *Beggiatoa*and *Thiothrix* are bacteria that are present in a waterlogged zone with hydrogen sulfide. Various bacteria which are chemoheterotrophs including genera *Sphaerotilus, Leucothrix, Caulobacter, Hyphomicrobium, Flexithrix* and *Flexibacter* are also present in water. Other microorganisms which are present in water are photosynthetic algae (diatoms), aquatic fungi and protozoa (foraminiferans and radiolarian). Purification of drinking water is essential because use of impure water can cause various water borne diseases. The use of microorganisms to transform toxic compounds to nontoxic degradation products is called bioremediation. Microorganisms like bacteria, viruses and fungi if transmitted through air results in airborne disorder or disease. These organisms are transmitted via sneezing, coughing, spraying

of liquids, spread of dust, talking, or any activity that results in the generation of aerosolized particles.

3.8 *TERMINAL QUESTIONS AND ANSWERS*

Q1 Which of the following comes under the category of positive association?

A) Neutralism C) Parasitism

B) Commensalism D) Ammensalism

Q2 Which of the following CANNOT be transmitted via infectious droplets?

A) Rubella C) Common cold

B) Tuberculosis D) None of the above

Q3Bioaugmentation involves

A) Eliminating sludge C) Plants usage for bioremediation

B) Addition of microbes to a cleanup site D) Bioventing

Q4 The most commonly used disinfectant for drinking water throughout the world is

A) Alum C) Nitrogen

B) Lime D) Chlorine

Q5 Activated sludge process is an example of ………… growth process.

A) Anaerobic suspended C) Anaerobic attached

B) Aerobic suspended D) Aerobic attached

Answers: 1 (B), 2(D), 3(B), 4(D), 5(B)

Q6 Describe the process of bioremediation.

Q7 Explain the process of water purification.

Q8 Write a short note on

A) Soil Enzymes
B) Microbiology of sewage water
C) Interaction between microorganism and animals
D) Microbiology of potable water
E) Airborne disease

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UNIT-4 CLINICAL MICROBIOLOGY

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4.1 *OBJECTIVES*

After studying this unit learns will learn about:

- Pathogenic microbes of bacterial, viral, fungal and protozoan diseases
- Preventive measures of cure and control of microbial pathogens
- Classifications, source and mode of action of Antibiotics
- *Escherichia coli* and *Staphylococcus aureus* case studies, collection, identification and causative Agents
- AIDS Virus

4.2 *INTRODUCTION*

Clinical microbiology deals with isolation and identification of microorganisms from the clinical specimens. It utilized the information obtained from various research to study the various pathogenic microorganisms. The clinical microbiology can be defined as the branch of science which deals with the interrelation of disease causing organisms under normal and pathological conditions and also study the pathological process with an account of the treatment till the clinical and/or complete recovery is presented. Number of microorganisms are reported to cause various diseases to human and other living beings. Infections are caused by bacteria, fungi, viruses, and other parasites. The first step of infection diagnosis is to collect the patients sample at a body site where the detection of a pathogen or its associated biomarkers is likely to signify disease. The sample is tested in the laboratory and preserved for further examination if any. After complete diagnosis proper treatment is prescribed.
Clinical microbiology utilizes the culture-based methods and phenotypic methods for identification of culture organisms. Technological advancement in the microbiology laboratories helps to provide rapid and accurate diagnostic for diseases. Rapid, accurate diagnosis helps in preventing spread of diseases.

4.3 *PATHOGENIC MICROBES OF BACTERIAL, VIRAL, FUNGAL AND PROTOZOAN DISEASES*

Organisms which invade other living beings and result in a disease are known as pathogens. They utilize the other organisms (host) for their own needs and cause harm to the host. The microorganisms evolved themselves in a way to survive and reproduce in a particular host. The human is the ideal host due to its nutrient-rich habitat, warm, wet environment, steady temperature, and capacity for self-renewal. The human body contains microbes (bacterial, fungal, and

protozoan) known as the normal flora. The natural flora is only present in certain areas of the body (skin, mouth, large intestine, and vagina). These natural florae are harmless and can only cause harm when the host is immunocompromised or if they reach a normally sterile part of the body. Pathogens are different from the natural flora as they have specialized mechanisms to break the cellular and biochemical barrier of the host. The host immune system recognizes and tries to eliminate the pathogens. So, to survive and multiply in a host pathogen must be able to (1) Colonize a nutritionally compatible niche in the host body; (3) Avoid, subvert, or circumvent the host innate and adaptive immune responses; (4) Able to reproduce using host machinery and (5) Exit and spread to a new host. The symptoms caused by any disease are manifestations of immune responses.

Bacterial diseases

Bacteria are prokaryotic organisms which are small and structurally simple (rods, spheres, or spirals). Some of the bacterial species are known to cause disease to humans. Obligate pathogenic bacteria can replicate only inside the cells of the human body whereas, facultative pathogens can replicate in an environmental reservoir (water or soil) and only cause disease if encounter a susceptible host. Some bacteria can only cause disease when the host is immunocompromised; they are known as opportunistic pathogens. Furthermore, some bacteria are host specific and can only infect a single species or a group of related species as compared to others which are generalists e.g. *Shigellaflexneri* can cause epidemic dysentery only in humans and other primates but *Salmonella enterica*, can cause food poisoning in humans as well as other vertebrates (chickens and turtles). *Pseudomonas aeruginosa* can cause disease in both plants and animals. The pathogenicity of bacteria is due to genes (virulence genes) which code for proteins known as virulence factors. Bacteria secrete toxic proteins that directly interact with host structural or signaling proteins to induce host response that is beneficial to pathogen colonization or replication or bacteria produce proteins that are required for delivery of such toxins to their host cell targets e.g. *Vibrio cholerae* cause Cholera and *Bacillus anthracis cause* Anthrax.

Bacteria belong to prokaryotes which differ from the eukaryotes in various biological processes (DNA replication, transcription, translation, and fundamental metabolism) therefore, on the basis

of these differences antibacterial drugs are designs which specifically inhibit bacterial processes without disrupting the host. Antibiotics used to treat bacterial infections are small molecules that inhibit bacterial enzymes involved in a particular biological process specific for bacteria e.g. cell wall synthesis that is only present in bacteria not in humans. Few examples of human diseases caused by bacteria are as follows:

Tuberculosis caused by *Mycobacterium tuberculosis;* Pertussis caused by *Bordetella pertussis;* Diphtheria caused by *Corynebacterium diphtheria*; Anthrax is caused by *Bacillus anthracis*; Leprosy caused by *Mycobacterium leprae*and; Syphilis is caused by *Treponema pallidum.*

Viral diseases

Viruses are particles made up of a small genome (either DNA or RNA which may be single stranded or double stranded). The viral genome is packed in a coat made up of protein, which in some viruses is further enclosed by a lipid envelope. Viruses are obligate parasites, that utilize the host molecular machinery for their own replication and assembly. A virus first infects a cell and produce thousands of progeny, then it kills the host cell by lysis to release the progeny viruses that in turn infect new cells. The clinical manifestation of viral infection is the cytolytic *effect* of the virus e.g. cold sores formed by *herpes simplex* virus and the lesions caused by the *smallpox* virus are due to the damage of the epidermal cells in a local area of infected skin. Viruses are able to cause chronic infections and some of the viruses are responsible for causing cancer. Designing drugs for viral infection is hard because viruses use host cell machinery. So, the best way for controlling viral diseases is to prevent them by vaccination of the potential hosts e.g. smallpox, poliomyelitis. Few examples of human diseases caused by viruses are as follows: Influenza or flu caused by Orthomyxoviruses; Chickenpox by Varicella-zoster virus; Measles by Rubeola virus; Mumps by Paramyxovirus; Poliomyelitis by Polio virus and Hepatitis B is caused by Hepatitis virus

Fungal and Protozoan diseases

The fungi are eukaryotic organisms they may include both unicellular *yeasts* and filamentous, multicellular *molds*. They show *dimorphism,* which is the ability to grow in either yeast or mold form and this transition is frequently associated with infection e.g. *Histoplasmacapsulatum* at low temperature grows as a mold, but if inhaled into the lung, it can cause disease histoplasmosis. *Candida albicans* is a normal flora of the human and causes disease in immunocompromised persons. Few examples of human diseases caused by fungus are as follows: Aspergillosis caused by *Aspergillus fumigatus, A. flavus*; Candidiasis caused by *Candida albicans;* Pneumocystis pneumonia is caused by *Pneumocystis carinii*; Blastomycosis caused by *Blastomycesdermatitidis*; Sporotrichosis caused by *Sporothrixschenckii*; Tinea versicolor caused by *Malassezia furfur.*

Protozoan parasite life cycle includes more than one host. First example is Malaria, which is caused by four species of *Plasmodium*, which are transmitted to humans by the bite of the female *Anopheles* mosquito. Second example is *Sickle cell anemia* which is caused by *Plasmodium falciparum.* Both the pathogens are eukaryotes therefore designing drugs is hard as it will also harm their eukaryotic host. Therefore, antifungal and antiparasitic drugs are more toxic and less effective as compared to antibiotics. Treating these organisms is difficult because they switch forms during their life cycles. So, drugs effective in killing one form are often ineffective in killing other forms. Few examples of human disease caused by protozoans are as follows: Giardiasis caused by *Giardia lamblia*; Trichomoniasis caused by *Trichomonas vaginalis*; Malaria is caused by *Plasmodium falciparum, P. malariae, P.ovale, P.vivax*; Kala-azar is caused by *Leishmaniadonovani*; Ameobiasis is caused by *Entamoebahistolytica* and African sleeping sickness is caused by *Trypanosomabruceigambiense, T. bruceirhodesiense.*

4.4 PREVENTIVE MEASURES OF CURE AND CONTROL OF MICROBIAL PATHOGENS

The microbial Infections are unavoidable as the environment (air, water and soil) is the habitat of enormous amounts of microorganisms. A few of them cause disease and some infections even

remain asymptomatic. There are various ways by which we can protect ourselves from the disease and treat a disease once it has developed. The step can be taken at individual level, national level and global level for detection, prevention, and treatment of a disease.

Preventive measures of cure of microbial pathogens

1) Vaccination is the key method to develop immunity against a particular disease. Vaccine comprise of attenuated microorganism or its toxins which lose the ability to cause disease but are able to evoke an immune response. The immune system recognizes the vaccine as foreign, eliminates it and generates the memory cell for future recognition. When a large number of people get vaccinated against a disease, the chance of its spreading becomes less because of the development of herd immunity.
2) Antibiotics are used for the treatment of bacterial infection. The problem with antibiotics is that the bacteria develop resistance against it. But they cannot be used for the treatment of the viral infection. Antiviral drugs are used for the treatment of viruses by inhibiting virus reproduction or by boosting the immune system.

   Preventive measures of control of microbial pathogens

   Various measures can be taken by individual, nation, and global communities to control the microbial pathogens.

   1) **Measures taken by Individuals**

      Awareness about microorganisms and daily habits like a) immunizations, b) Proper hygiene like washing hands, cleaning drinking water and clean cooking practices c) Use of proper medicines and medical facilities d) Protection from infected animals and insects e) Safe intercourse (for the protection of HIV) f) Traveling safety during international and national travel g) Follow healthy habits like eating well, getting enough sleep, exercising, avoiding tobacco and illegal drug use.

   2) **Measures taken by Governments**

      Government should make policies for the protection of the nation from disease outbreaks by effective and well-coordinated programs that monitor public health.

An effective response against an infectious disease is required to safeguard public health. The improvement in medical facilities is the must for the safeguard of public health. The public should be provided with safe and nutritional food. The food should not be contaminated by microorganisms and animal husbandry should take proper care of animals to prevent any animal borne disease. During the time of an endemic or pandemic the national and international rules should be followed by every country. The developed nations should provide vaccines and medicines to poor countries of the world.

4.5 CLASSIFICATIONS, SOURCE AND MODE OF ACTION OF ANTIBIOTICS

Classification based on Nature of the Source

(a) Natural compounds obtained from microorganisms e.g. benzylpenicillin, cephalosporins and gentamicin. They have high toxicity.

(b) Semi-synthetic members are structurally modified natural products: e.g. ampicillin and amikacin.

(c) Synthetic products e.g. moxifloxacin and norfloxacin. They are therapeutically effective and have lower toxicity as compared to natural antibiotics.

Classification on the basis of Chemical Structure

Different classes:

i) β-lactams antibiotics are characterized by the presence of β-lactam ring e.g. penicillins, cephalosporinsetc.

ii) Macrolides antibiotics are characterized by presence of macrocyclic lactone ring usually 14-, 15- or 16-membered-ring compounds to which various amino sugars are attached. They are isolated from *Streptomyces* species.

iii) Tetracyclines antibiotics are characterized by the presence of a linearly fused tetracyclic nucleus to which a variety of chemical groups are attached. They can be obtained from the *Streptomyces aureofaciens* and *Streptomyces rimosus* or they are semisynthetic.

iv) Aminoglycosides

v) Sulfonamides antibiotics are synthetic compounds which contain the sulfonamide chemical group (R-SO-NR R).

vi) Quinolones are synthetic antibiotics which are obtained from the heterobicyclic aromatic compound quinoline.

Classification on the basis of Mechanism of Action

1) Inhibition of cell wall synthesis: Antibiotics inhibit the synthesis of bacterial cell wall by arresting the formation of the peptidoglycan layer. The bacterial cell wall is made up of peptidoglycan. The peptidoglycan undergoes cross-linking of the glycan strands by the enzyme transglycosidases, and the peptide chains stretch from the sugars in the polymers and cross links, one peptide to another. The D-alanyl-alanine part of the peptide chain is cross-linked by glycine residues in the presence of penicillin binding proteins. The β-lactam class of antibiotics binds to the bacterial membrane receptors penicillin-binding proteins (PBPs); as they are similar to the natural substrate of PBP which is D-alanyl-D-alanine. The PBP is associated with β-lactam rings and is not able to synthesize new peptidoglycan. This disruption in the bacterial peptidoglycan layer causes lysis of bacteria.

2) Inhibition of protein synthesis: Antibiotics can inhibit the protein synthesis by binding with the ribosomal subunits (50S and 30S). Examples of classes of antibiotics that inhibit bacterial protein synthesis are aminoglycosides, macrolides, tetracyclines, etc.

3) Inhibition of nucleic acid synthesis: Antibiotics can inhibit the synthesis of DNA (replication) and RNA (transcription) in bacteria. DNA synthesis can be inhibited by affecting the activity of type II topoisomerase enzymes: DNA gyrase and DNA topoisomerase IV e.g. quinolones and metronidazole. Antibiotics can inhibit the RNA synthesis by disrupting the bacterial transcription process e.g.Rifamycin class and fidaxomicin/lipiarmycin antibiotics inhibit RNA polymerase in bacteria.

4) Key metabolic pathways inhibition: Antibiotics can also inhibit the folate metabolism in bacteria by affecting the activity of enzyme dihydrofolate reductase (DHFR), which is involved in

thymidylate synthesis, DNA replication and cell survival. Trimethoprim is the inhibitor of DHFR by binding to the active site of DHFR. Sulfonamides inhibit the enzyme dihydropteroate synthase in a competitive manner as it has higher affinity for the enzyme as compared to the p-amino benzoic acid which is a natural substrate.

Classification based on Type of Pharmacological Effects

1) Bactericidal antibiotic leads to death of the bacterial cell by inhibiting cell wall synthesis, cell membrane function or protein synthesis e.g. β-lactams, aminoglycosides, glycopeptides, ansamycins, quinolones, streptogramins, lipopeptides and macrolides
2) Bacteriostatic antibiotics lead to slowing of bacterial cellular activity and growth without causing cell death e.g sulfonamides, tetracyclines, chloramphenicol, oxazolidinones and macrolides.

Classification by the Spectrum of Activity

1) Broad spectrum antibiotics: They are effective against a wide variety of pathogenic bacteria (both Gram-positive and -negative bacteria) e.g. Ampicillin, kanamycin A etc.
2) Narrow-spectrum antibiotics: They are effective against only one type of pathogenic bacteria (Gram-positive or Gram-negative bacteria) e.g. Penicillin G, Cephalosporinsetc.

4.6 ESCHERICHIA COLI AND STAPHYLOCOCCUS AUREUS CASE STUDIES, COLLECTION, IDENTIFICATION AND CAUSATIVE AGENTS

Escherichia coli (E. coli)

Escherichia coli (E. coli) is a gram-negative bacillus. *E. coli* is a part of normal flora of the human body. As a part of normal intestinal flora it does not cause any disease but when found outside of the intestinal tract, *E. coli* may cause urinary tract infections (UTI), pneumonia, bacteremia, and peritonitis etc. Numerous stains of E. coli are reported to cause diseases ranging from mild self-limited gastroenteritis to renal failure and septic shock. *E. coli* also causes nosocomial infections

e.g. catheter-associated UTIs and ventilator-associated pneumonia. Intestinal illnesses will be described by the causative *E. coli* subtypes, including enterotoxigenic *Escherichia coli* (ETEC), enterohemorrhagic *Escherichia coli* (EHEC), enteroinvasive *Escherichia coli* (EIEC), enteropathogenic *Escherichia coli* (EPEC), and enteroaggregative *Escherichia coli* (EAEC). Extra intestinal illness is a result of translocation of gut *E. coli* into other parts of the body or the environmental spread in hospitals and long term care facilities. *E. coli* can be found in floors of hospitals and long-term care facilities, soil, on vegetables, in water, and in undercooked meats. Pathogenic strains result in intestinal illness in humans when ingested. ETEC is the cause of watery diarrhea in the region of poor sanitation and it is responsible for causing traveler's diarrhea. ETEC is also the leading cause of dehydrating diarrheal illness in infants and children.

The diagnosis of *E. coli* infection should be done for all patients who suffer from diarrhea, (bloody diarrhea or hemolytic uremic syndrome). The Stool specimens of the patients should be collected. The stool sample should be examined as soon as they are received. If the whole stool sample will not be processed immediately then it should be either refrigerated or frozen at -70°C and examined within 1-2 hours after refrigeration. If stool samples are not examined within this time then it should be placed in a transport medium (commercially available e.g. Cary-Blair, Stuart's, Amie's, buffered glycerol saline) and should be examined within 2-3 days. If the sample will not be examined within 3 days then it should be frozen immediately (-70°C) and stored for days. *E. coli* isolation is done by plating the samples onto MacConkey agar and incubated for 24 h at 37°C and characteristic lactose-fermenting colonies were identified using the identification test.

Staphylococcus aureus

Staphylococcus aureus is cocci-shaped, Gram-positive bacteria that is responsible for a wide variety of clinical diseases. These bacteria can grow aerobically or anaerobically at temperatures 18° C to 40° C. Human body is the major reservoir for these bacteria as they are found on the skin and mucous membranes. Health care workers, patients who use needles on a regular basis (*i.e.,* diabetics and intravenous (IV) drug users), hospitalized patients, and immunocompromised

individuals show higher rates of *S. aureus* colonization. The transmission of *S. aureus* can occur directly from person-to-person by contact or by fomites. *S. aureus* generally does not cause infection on healthy skin but when they enter the internal tissues or bloodstream. These bacteria result in numbers of serious infections. *S. aureus* cause multiple human infections, including bacteremia, infective endocarditis, skin and soft tissue infections (e.g. impetigo, folliculitis, furuncles, carbuncles, cellulitis, scalded skin syndrome, and others), osteomyelitis, septic arthritis, prosthetic device infections, pulmonary infections (e.g. pneumonia and empyema), gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections. *S. aureus* evaluation is done by studying clinical signs, history and physical findings. The blood and sputum sample is used for the diagnosis. The staphylococci presence in a lesion might first be suspected after examination of a direct Gram stain. But, small numbers of bacteria in blood prevent microscopic examination and require culturing first. Biochemical identification tests are done e.g. catalase positive (all pathogenic *Staphylococcus* species), coagulase positive (to distinguish *Staphylococcus aureus* from other *Staphylococcus* species), novobiocin sensitive (to distinguish from *Staphylococcus saprophyticus*), and mannitol fermentation positive (to distinguish from *Staphylococcus epidermidis*). Drug susceptibility testing is done for correct treatment. The catalase test is done by flooding an agar slant or broth culture with several drops of 3% hydrogen peroxide. Catalase-positive cultures bubble at once. The isolation of bacteria is done by streaking material from the clinical specimen onto a solid media (blood agar, tryptic soy agar or heart infusion agar) .Treatment of infection remains a chance due to the presence of multi-drug resistant strains such as MRSA (Methicillin-Resistant Staphylococcus aureus). The antimicrobial treatment involves the use of penicillin (MSSA, or methicillin sensitive *S. aureus* strains) and vancomycin for MRSA strains.

4.7 AIDS VIRUS

The HIV virus (Human immunodeficiency virus) responsible for causing AIDS (Acquired immune deficiency syndrome) is a retrovirus. It is classified as: Genus- Lentivirus; Family- Retroviridae; Subfamily- Orthoretrovirinae. The virus is of two types HIV type 1 and type 2 (on the basis of genetic characteristics and differences in the viral antigens). The HIV type 1 virus is developed

from non-human primate immunodeficiency viruses (simian immunodeficiency virus, SIV) from Central African chimpanzees whereas the type 2 HIV is developed from West African sooty mangabeys. HIV like other viruses consist of a genetic material enclosed by a protein coat. The Genetic material of HIV consists of two identical single-stranded RNA molecules. The mature HIV virus is round and 100 nm in diameter. The outer envelope is made up of lipid membrane and contain gp120 surface protein (SU) which is anchored to the membrane by transmembrane protein gp41 (TM). It covers the symmetrical outer capsid membrane which is made by the matrix protein (MA). The conical capsid is made up of inner capsid protein p24 (CA). The tapered pole of the capsid is attached to the outer capsid membrane. Inside the capsid two identical molecules of viral genomic RNA is present with several molecules of the viral enzymes RT/RNase H and IN bound to the nucleic acid.

Number of steps are involved during the infectious cycle of HIV virus

1) **Attachment:** The first step of infection is the attachment of virus particles to the cell. The attachment occurs viaprotein-protein interactions. The surface protein (glycoprotein gp120) of HIV virus binds with CD4 receptors (e.g. T helper cells, macrophages, dendritic cells and astrocytes) of the host cell.

2) **Absorption:** Attachment of the receptor leads to conformational change causing the fusion of viral and cellular membrane. The fusion process causes translocation of the viral capsid into the cytoplasm. The capsid is then taken up by an endosome, and change in the pH of the phagosome triggers the release of the capsid contents into the cytoplasm. The enzyme reverse transcriptase (RT) activation occurs in the cytoplasm.

3) **Integration of RNA genome into the cellular genome**: After its activation in the cytoplasm the enzyme HIV reverse transcriptase, transcribes the single-strand HIV RNA genome into cDNA (complementary DNA). In parallel to DNA synthesis, the enzyme RNase H degrades the RNA. Then the single-stranded cDNA is converted

into double-stranded DNA (proviral DNA) by the DNA-dependent DNA polymerase activity of RT. This proviral DNA is transported to the cell nucleus via nucleopores in association with integrase (IN). The enzyme integrase inserts the proviral genome into the human host cell genome.

4) **Replication of viral genome and its component synthesis:** After the integration of the proviral genome into the cellular genome, the LTR promotor of the proviral genome act as attachment site for cellular DNA-dependent RNA polymerases and a various transcription factors leads to the synthesis of viral mRNA and genomic RNA.

5) **Release of viral progeny:** The first virus particle is detectable after 12hr of integration and the first progeny is released after 24hr of infection.

The genome of the HIV provirus (proviral DNA) is produced by the reverse transcription. The DNA genome is flanked at both ends by LTR (long terminal repeat) sequences. The 5′ LTR region codes act as a promotor for transcription of the viral genes. The genome codes for proteins of the outer core membrane, the capsid protein, the nucleocapsid, a smaller nucleic acid-stabilizing protein, enzymes protease, reverse transcriptase, RNase H (p15) or RT plus RNase H, integrase, glycoproteins gp120 and gp41. It also codes for regulatory proteins: Tat (transactivator protein) and Rev (RNA splicing-regulator) which are involved in initiation of HIV replication. While the other regulatory proteins Nef (negative regulating factor), Vif (viral infectivity factor), Vpr (virus protein r) and Vpu (virus protein unique) code by the genome help in virus replication, budding and pathogenesis. The HIV infection can be detected into the HIV patients by either detection of Antibodies or by virus detection. Viral RNA can be detected in the blood with the help of a nucleic acid test (NAT) about 11 days after infection. For the diagnosis of antibodies screening tests are used and if reactions occur then confirmation tests like ELISA (enzyme linked immunosorbent assay) or variants of this test system, particle agglutination tests are done. HIV can be transmitted by number of ways like via body fluid (blood, plasma or serum, breast milk, genetic secretion) and transmitted from a mother to her child during pregnancy and delivery. Other ways include

unprotected sexual intercourse, blood transfusions, organ transplantation, use of contaminated needles, syringes and other injecting equipment. The treatment of AIDS utilized the use of antiviral drugs. These drugs are either protease inhibitors, integrase inhibitors, reverse transcriptase inhibitors or viral replication inhibitors. The combination of two or more drugs are found effective but have side effects that can reduce the quality of life. The stability of the HIV virus is due to the lipid envelope. HIV virus can remain stable at pH 3-10 for several hours. HIV is sensitive to disinfectants as treatment with 70% ethanol, 50% isopropanol, 4% formaldehyde or peracetic acid as well as strong detergents like sodium dodecyl sulphate (SDS), NP-40 or Triton X-100 can inactivate it within minutes. HIV remains stable for several hours when subjected to physical conditions e.g. ultraviolet light, gamma irradiation or ultrasonic waves. At lower temperatures HIV is relatively stable but high temperature can lead to degradation of HIV RNA.

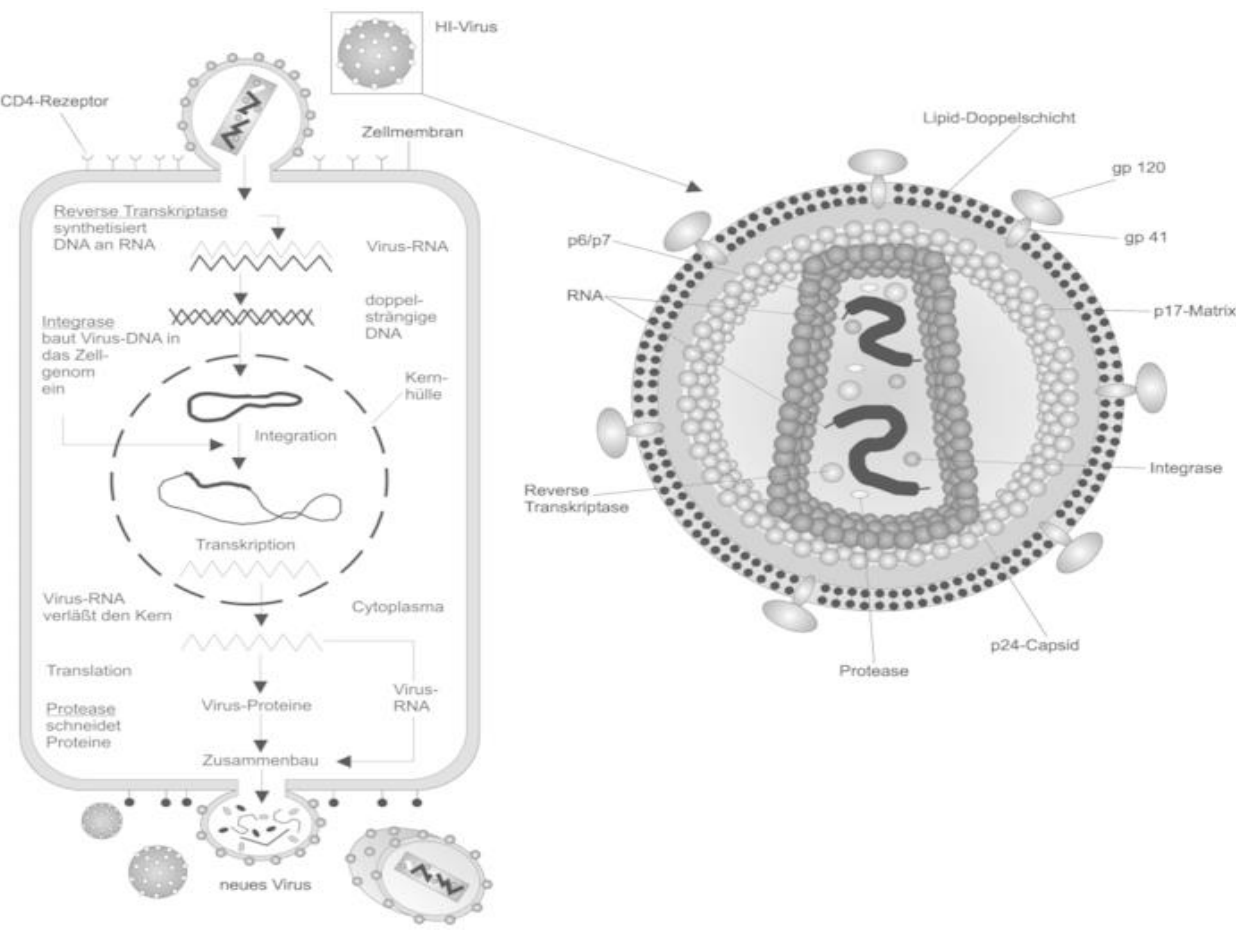

Fig 4.1 HIV Virus infection cycle and structure (credit: https://commons.wikimedia.org/wiki/File:Hiv_gross_german.png)

4.8 SUMMARY

Clinical microbiology deals with isolation and identification of microorganisms from the clinical specimens. It utilized the information obtained from various research to study the various pathogenic microorganisms. Organisms which invade other living beings and result in a disease are known as pathogens. They utilize the other organisms (host) for their own needs and cause harm to the host. Bacteria are prokaryotic organisms which are small and structurally simple (rods, spheres, or spirals). Some of the bacterial species are known to cause disease to humans. A virus first infect a cell and produce thousands of progeny, then it kills the host cell by lysis to release the progeny viruses to infect new cells. Fungal and Protozoan are known to cause number of diseases. The microbial Infections are unavoidable as the environment (air, water and soil) is habitat of enormous amounts of microorganisms. A few of them cause disease and some infections even remain asymptomatic. There are various ways by which we can protect ourselves from the disease and treat a disease once it has developed. Antibiotics can be classified based on source and mode of action. *Escherichia coli (E. coli)*, a gram-negative bacillus, is a part of normal flora of the human body. As a part of normal intestinal flora it does not cause any disease but when found outside of the intestinal tract, *Staphylococcus aureus* is cocci-shaped, gram-positive bacteria that is responsible for a wide variety of clinical diseases. These bacteria can grow aerobically or anaerobically at temperatures 18° C to 40° C. Human body is the major reservoir for these bacteria as they are found on the skin and mucous membranes. The HIV virus (Human immunodeficiency virus) responsible for causing AIDS (Acquired immune deficiency syndrome) is a retrovirus. It is classified as: Genus- Lentivirus; Family- Retroviridae; Subfamily- Orthoretrovirinae. The virus is of two types HIV type 1 and type 2 (on the basis of genetic characteristics and differences in the viral antigens). The HIV type 1 virus is developed from non-human primate immunodeficiency viruses (simian immunodeficiency virus, SIV) from Central African chimpanzees. Whereas the type 2 HIV is developed from West African sooty mangabeys.

4.9 TERMINAL QUESTIONS AND ANSWERS

1) Which of the following bacteria is predominantly present as normal flora in urine?

a) *Escherichia coli*

b) Staphylococcus epidermidis

c) *Staphylococcus aureus*

d) *Streptococcus pyogenes*

2) Name the bacteria that is a common cause of toxic shock syndrome

a) *Staphylococcus epidermidis*

b) Staphylococcus aureus

c) Staphylococcus intermedius

d) None of the above

3) Which of the following metabolic characteristic is a distinguishing characteristic and identification of colonies of *E. coli*?

a) Hydrogen sulfide formation

b) Indole Formation

c) Lactose fermentation

d) Urea Degradation

4) HIV belongs to which of the following genus member of the virus?

a) Ortho myxovirus

b) Retrovirus

c) Parvovirus

d) Reovirus

5) All of the following are common pathogens that are naturally found in soil, Except?

a) *Candida albicans*

b) Coccidiodeimmitis

c) Sporothrixschenckii

d) *Cryptococcus neoformans*

ANSWER: (1) a , (2) b , (3) c , (4) b , (5) a

6) Describe various preventive measures of cure and control of microbial pathogens.

7) Give a systematic detail of classification of Antibiotics.

8) Write a short note on

A) HIV Virus
B) Staphylococcus Aureus
C) Escherichia Coli
D) Bacterial diseases
E) Viral disease
F) Fungal and Protozoan diseases

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Block II: Immunology

UNIT 5 INTRODUCTION TO IMMUNE SYSTEM

CONTENTS

5.1 Objectives

5.2 Introduction

5.3 Resistance and immunity

5.3.1 Cellular immunity v/s humoral immunity

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5.4 Summary

5.5 Terminal Questions and Answers

5.6 References

5.1 OBJECTIVES

After reading this unit you will be able to understand

- What is immunity?
- Basics of Cellular immunity v/s humoral immunity
- Phenomenon active and passive immunity

5.2 INTRODUCTION

Immunology is the study of the immune system. The term is derived from the Latin word immune, which means 'to exempt'. Immunity is defined as a defense mechanism of organisms against pathogens, so that these animals are normally capable of resisting the infection by most pathogens. Immune system involves both specific and nonspecific components. The nonspecific components act as barriers for a broad range of pathogens irrespective of their antigenic make-up. The specific parts of immune system generate antigen specific immune response. The molecular and biochemical mechanism involve in immunity generation has been understood only during the last few decades.

The antigens are recognized by the host cells through chemical interactions, which elicit the production of a specific antibody by host. The antibody or immunoglobulins are proteins. The antigen may be protein, polysaccharide, or nucleic acid or any other substance. It should also be recognized that the entire surface of an antigen molecule is not necessary for its antigenicity, instead specific group of atoms called antigenic determinant or epitope, consisting of 5-8 amino acids, is needed for immune response and antibody production.

The cellular immunity involves T- cells and these cells are of two types (T helper cells and T cytotoxic cells). In case of virus infection the viral components are digested by the immune cells and represented on the surface of the cells via receptors and then this receptor-antigen complex is recognize by the T-cell receptor.

5.3 RESISTANCE AND IMMUNITY

The first line of defense is provided by the less specific component known as **innate immunity.** Generally most of the components of innate immunity are present before the encounter with the infection and constitute a set of disease-resistance mechanisms. These mechanisms are non-

specific to a particular pathogen and include both cellular and molecular components. Innate immunity can be seen to comprise four types of defensive barriers:

1) Anatomic: Skin act as a mechanical barrier which retards the entry of microbes. The Acidic environment (pH 3–5) of an organ retards the growth of microbes. The Mucous membranes is inhibited by normal flora which competes with microbes for attachment sites and nutrients. Mucus secreted by different organ can entrap foreign microorganisms and Cilia propel microorganisms out of body.

2) Physiologic: physiological barriers include normal body temperature which inhibits the growth of some pathogens. Increase in temperature known as Fever response also inhibits the growth of some pathogens. The Acidity of stomach contents kills most ingested microorganisms. Lysozymes secreted in various body secretions kills bacteria by cleaving their cell wall.

3) Phagocytic: Various cells internalize (endocytose) and break down foreign macromolecules e.g blood monocytes, neutrophils, tissue macrophages

4) Inflammatory: inflammatory response induces leakage of vascular fluid, containing serum proteins which have antibacterial activity and influx of phagocytic cells into the affected area.

Immunity

An antigen is a foreign substance capable of stimulating an immunological response by the production of an antibody or cell-mediated immunity. Antigens may be bacteria, viruses, pollen, blood cell, surface molecules of transplanted tissue and organs.

Commonly, antibodies recognize and interact with specific regions on antigens called antigenic determinants or epitopes (Fig.5.1). The nature of this interaction depends on the size, shape and chemical nature of the organic determinant as well as the chemical structure of the binding site on the antibody molecule. The molecular weight of most antigens is 10,000 or more. A foreign substance that has a low molecular weight is often not organic, unless it is attached to a carrier molecule. The small compounds are called haptens. Haptens are usually small organic molecules, they become antigenic if they are coupled to a suitable macromolecule called carrier. Penicillin is a good example of a hapten. Haptens such as the dinitrophenyl (DNP) group are important tools in experimental immunology.

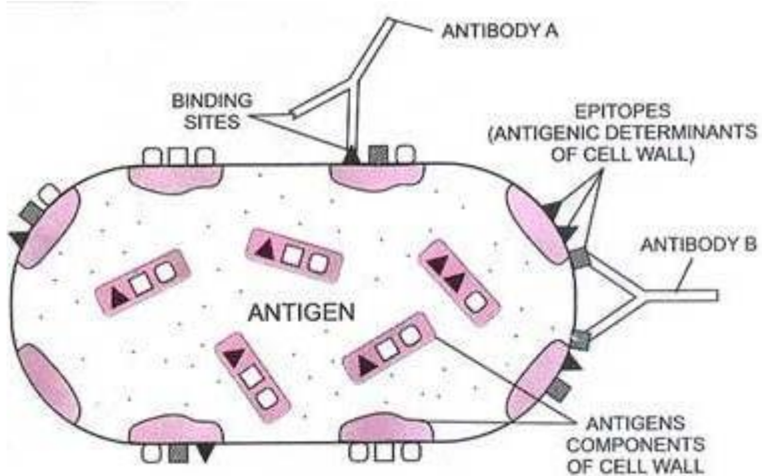

Fig. 5.1 Antigenic determinants (epitopes) (Source: https://microbiologyinfo.com)

The immune system of the body is monitored with the help of white blood cells called lymphocytes, which circulate between blood and lymph system (fig.5.2). Lymph is a colourless fluid and is circulated like blood with the help of vessels that make a lymphatic system just like blood circulation. The lymphocytes are two types: B cells and T cells. The B cells synthesize antibodies causing agglutination of unwanted antigens, while T cells carryout cellular immune responses where cells having unwanted antigens on their surface are attacked without any mediation by antibodies. The former response is called humoral immunity and the latter the cellular immunity. The T cells interact with antigen through cell surface proteins called T cell receptors, which are specially attached to the cell membrane. B cells and T cells, both originate in bone marrow, which is the primary source of all lymphocytes.

In mammals, B cells mature within bone marrow, but T cells migrate out and mature in special organs like thymus. Once they mature, B cells leave bone marrow and T cells leave the organ like thymus and travel to secondary lymphoid organs like lymph nodes, tonsils and spleen. In these organs they can be activated to produce antibodies or T cell receptors. These cells then pass to blood system and back again to the lymph.

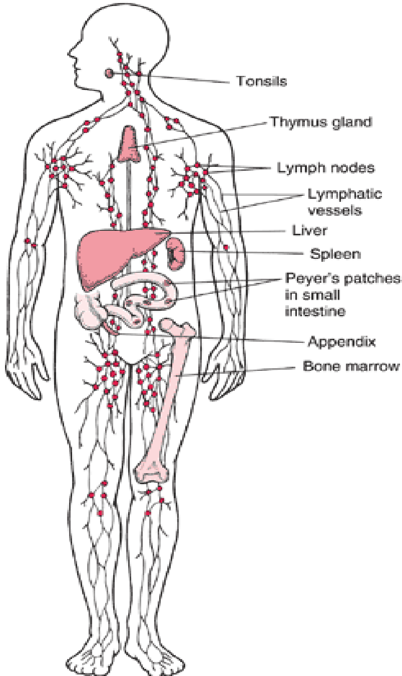

Fig.5.2 An outline of lymphoid system in a human being. (Source https://www.lgdalliance.org/the-lymphatic-system)

5.3.1 Cellular immunity v/s humoral immunity

Cell-mediated immunity

Cell-mediated immunity is primarily driven by mature T cells, macrophages, and the release of cytokines in response to an antigen. Cell –mediated immunity involves specialized lymphocytes called T cells (or T lymphocytes) that act against foreign organisms or self altered cells. It is very effective against bacteria and viruses located within the infected host cells and against fungi, protozoa and helminthes. It is an important factor in our defense against cancer too.

T cells involved in cell-mediated immunity rely on antigen-presenting cells that contain membrane-bound MHC class I proteins in order to recognize intracellular target antigens. The binding specificity between MHC proteins and foreign antigens is essential for the maturation and differentiation of naive T cells into helper or T cytotoxic cells.

Cell-mediated immunity typically comes into play at body sites where cells are infected by a virus, bacteria, or fungi (intracellular invaders). With the assistance of MHC class I proteins, T cells can also recognize cancerous cells.

Naive T cells, which have not yet become activated, circulate in the bloodstream and the lymphatic system. When naïve T cells encounter antigen-presenting cell, they get activated and rapidly proliferating into different T-cell subsets. These subsets perform different functions –1) CD4+ helper T cells releases a set of signaling proteins called cytokines. These cytokines can damage the target cell directly or help activate cytotoxic T cells and macrophages. 2) CD8+ cytotoxic T cells cause direct lysis of target cells 3) whereas macrophages, which are a type of antigen-presenting cell, also play an important role in T-cell creation.

T lymphocytes Mainly two types i) T cytotoxic (Tc) and ii) helper T cells (T_H) that distinguished by the presence of either CD_4 or CD_8 membrane glycoproteins on their surface. T cell displaying CD4 generally function as T_H cells and recognize antigen in complex with MHC class II, whereas those displaying CD_8 generally function as Tc cells and recognize antigen in complex with MHC class I.

Navie CD8+ T cells browse the surface of antigen presenting cell with their T cell receptors. When these cells bind to an MHC-peptide complex, they become activated, proliferate and differentiate into an effector cell called a cytotoxic T lymphocyte (CTL). The CTL has a vital function in monitoring the cells of the body and eliminating any cells that display foreign antigen complexed with class I MHC, such as virus- infeted cells, tumor cells and cells of a foreign tissue graft. To proliferate and differentiate optimally, naïve CD8+ T cells also need help from mature CD4 + T cells.

Navie CD4+ T cells also browse the surfaces of antigen- presenting cells with their T-cell receptors. If and when they recognize an MHC-peptide complex, they can become activated and proliferate and differentiate into one of a variety of effector T-cell subsets. T helper type I (T_HI) cells regulate the immune response to intracellular pathogens and T helper type 2 (T_H2) cells regulate the response to many extracellular pathogens. Two additional T_H cell subsets have been recently identified. T helper type 17 cells ((T_H17),so named because they secrete IL-17, play an important role in cell-mediated immunity and may help the defense against fungi. T follicular helper cells (T_{FH}) play an important role in humoral immunity and regulate B-cell development in germinal centers. Which helper subtype dominate a response depends largely on what type of pathogen (intracellular versus extracellular, viral, bacterial, fungal, helminth) has infected an animal.

The cytotoxic cells kill the cells that are infected with a virus or any other microorganism and helper T cells send chemical signals to help stimulate the responses of other cells like macrophages, B cells and $T_{H.}$

T cells posses antibody like receptor called T cell receptors, found on the surface of these cells to allow response through direct contact with target cells. This receptor are enclosed by genes that are assembled from many gene segments during T cell development in the thymus but are not secreted like antibodies, as in case of B cells. Each receptor (both in T_H and helper T cells) consists two polypeptide chains (called α and β) linked with disulphide bonds.

MHC molecule

There are two types of MHC molecules i) class I MHC molecules: it found on all nucleated cells is involved in binding peptide fragments from intracellular antigens (e.g. virus) and presenting the cells to cytotoxic T cells. It consists of a single polymorphic transmembrane polypeptide chain called α (consisting of three domins (α_1, α_2, α_3) and an extracellular invariant protein β_2 microglobulin is not encoded within MHC, it is not glycosylated and non- covalently associated with three domins of α. and ii) class II MHC molecules: it found on specialized cells (e.g. B cells, thymus cells) is involved in binding extracellular or endocytosed peptide fragments and presenting them to helper T cells. It consists of two transmembrane polymorphic polypeptide α and β each consisting of two helper T cells. It consists of two transmembrane polymorphic polypeptides α and β each consisting of two extracellular domins (α_1 and α_2, β_1 and β_2). Both chains (α and β) are incoded within MCH, both are glcosylated and remain non- covalently connected.

Humoral immunity

Humoral immune system involves the production of antibodies that act against foreign organism and substances. These antibodies are found in extra cellular fluids such as blood plasma, lymph and mucus secretions. B cells are responsible for the production of antibodies. The humoral immune response is defensive primarily against bacteria, bacterial toxins and viruses that are circulating freely in the body fluids. Immature B cells are lymphocytes that circulate all through the body in the lymphatic system. These lymphocytes communicate a variety of antigen-specific molecules that are necessary for the detection of infectious agents in the human body. When

immature B cells meet an antigen in the lymphatic system, they undergo a separation process that leads to the formation of memory B cells and effector B cells.

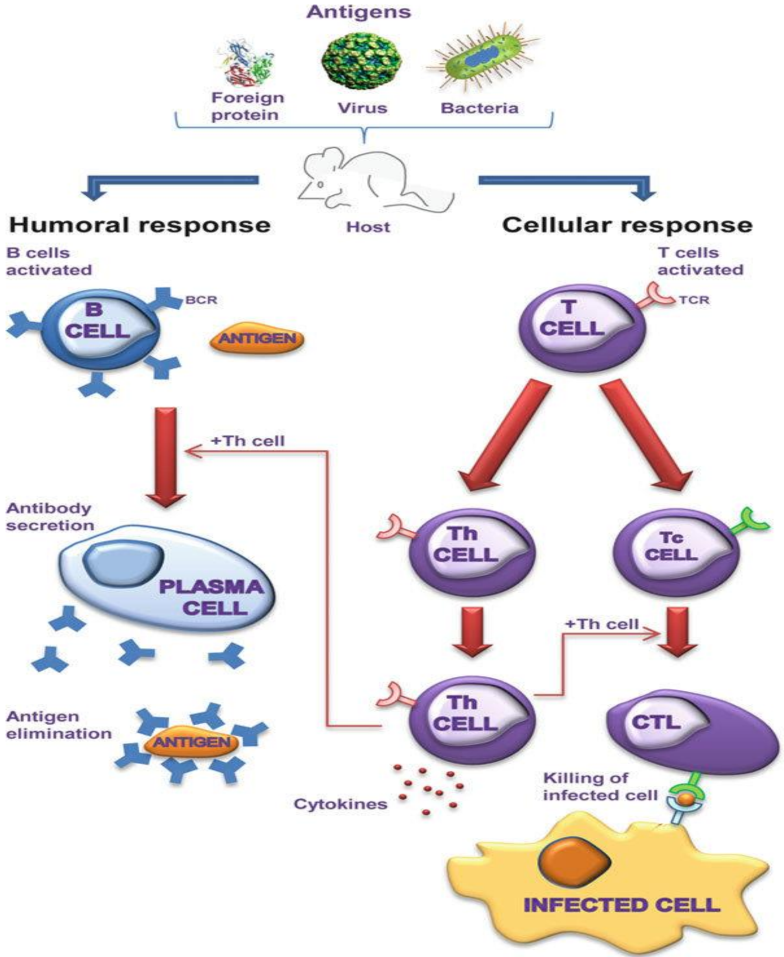

Fig. 5.3 The humoral and cell-mediated branches of the immune response (Source: ResearchGate)

During this separation, memory B cells and effector B cells produce the same antigen-specific molecules as their parent immature B cell. With the help of T cell lymphocytes, in turn activated by MHC class II receptors that identify microbial-associated antigens, the activated memory B cells convey these antigen-specific molecules on their surface while the effector B cells secrete these molecules in the blood to bind the antigen of interest.

Antibodies

Antibody is a protein formed in response to an antigen. Antibody specifically combines with the antigen, to inactivated, neutralized, and destroyed it. The B-cells synthesize and secrete the antibodies. They are present in the blood serum. Most of the antibodies are gamma globulins. Hence they are called immunoglobulins (Ig).

The antibodies are also the surface receptor of the B-cells and when the particular antigen bind with this specifically which activate the B cell to produce and secrete more antigen-specific antibodies. Once a B cell fully matures, it is known as a plasma cell and will continue to produce and secrete antigen-specific antibodies for the remainder of its life cycle.

Structure of antibodies

The antibody structure consists of two identical heavy (H) chains, each chain having a molecular weight of 50,000 daltons. The smaller pair is composed of identical light (L) chains, each with a molecular weight of 25,000 daltons. The chains are joined by disulphide links and other bonds to form a Y-shaped molecule. This molecule is flexible and can assume a T shape. The end portions of the Y's arm are called variable (V) region and the lower parts of the Y's arms are called constant (C) region. This region is also called Fc (the trunk of the Y shape) region. The Fc region is composed of constant domains from the heavy chains. It role is in modulating immune cell activity.

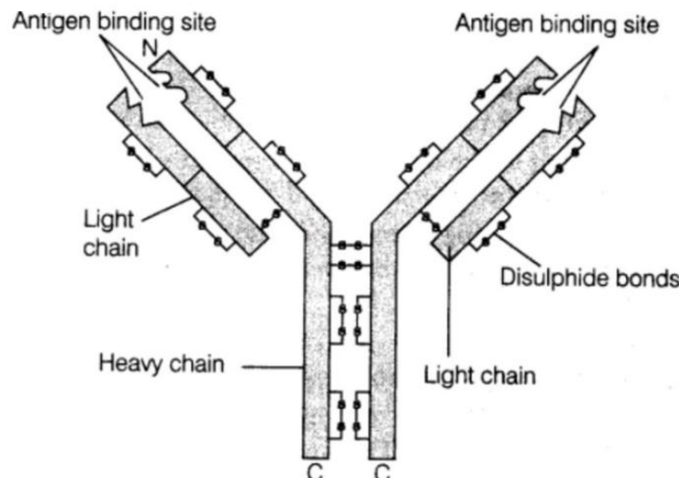

Fig. 5.4 Structure of an antibody molecule (Source: learnatnoon.com)

These Y-shaped proteins contain antigen-binding sites that specially bind to their target antigens.

Previously, antibodies capably bind to their target antigen, they can either neutralize their target antigen directly by blocking normal antigen binding or they can induce the recruitment of other immune cells or molecules that promote the antigens removal or destruction. In mammals, such antibodies come in a variety of forms commonly known as isotypes.

Previously antibodies are in the blood stream; these free-floating proteins are ready to function as defensive molecules with direct and indirect immune functions. These functions include:

- neutralization of infectious agents – via blocking or antibody-dependent cellular cytotoxicity
- activation of the complement system – compliment dependent cytotoxicity
- binding of foreign substances to be destroyed - opsonization and phagocytosis

Antibodies neutralize antigens generally during mechanisms of addition and accumulation. For example, the aggregation of neutralizing antibodies upon antigen-matching viral particles would block this virus's capacity to infect other cells.

Antibodies can also take part in processes that lead to the lysis or killing of infected or antigen-presenting cells throughout the activation of the complement cascade or communication with effector cells and release of cytokines. The complement system is a part of innate immunity that enhances the capability of antibodies and lymphocytes to clear the body of pathogens and infected cells. Finally, antibodies that coat pathogens or infected cells can attract (opsonize) and be converted into internalized by macrophages during phagocytosis.

5.3.2 Active/s passive immunity

The Acquired immunity based on antibodies is the most efficient type of acquired immunity divided into two types: active and passive immunity.

Active Immunity

Active immunity responds directly to a foreign antigen inside the body. In acquired or adaptive immune system, the body remembers the pathogens it has encountered in the past. In active immunity, there are two types of immunity: natural and artificial. Natural immunity of an animal is also known an innate immunity, or inherited immunity. This relates to a general or non- specific type of resistance which prevents infection by different kinds of pathogens. The extents of this

natural immunity differ in different organism. For example, man can easily create antibodies in the natural world in response to harmful infections like measles. Furthermore, antibodies are produced artificially when they are produced in response to controlled exposure to an attenuated pathogen, which is vaccination.

Because the virus induces the immune system to build up antibodies that specially identify and kill the pathogen the next time it is encountered, an individual who recovers from a first event of measles is opposed to further infection by the measles-causing virus.

Example of active immunity

The virus induces the immune system to develop antibodies that specifically recognize and kill the pathogen the next time it is encountered, an individual who recovers from a first episode of measles is resistant to further infection by the measles-causing virus.

Passive Immunity

Passive immunity involves the immune response by the antibodies attained from outside the body. The primary response by the body to a pathogen it encounters for the first time is rather weak, so the first encounter is always a little harsh on the body. Passive immunity is protection from a disease provided by antibodies created outside of the individual’s body.

The passive immunity is two types:

- Natural Passive Immunity- natural means, involves the transfer of antibodies from to mother to unborn child, though the placenta during the later part of pregnancy and also via breastfeeding.
- Artificial Passive Immunity- artificial type refers to the original production of antibodies in some other individual (human or lower mammal) followed by injection of these antibodies with the help of a needle or syringe.

Examples of Passive Immunity

The most common examples of passive immunity are mothers and their children. Passive immunity is provided to babies by their mothers earlier than they are born and for a length of time later. The maternal antibodies found in their mother's placenta and breast milk help them stay healthy.

Pregnant women use their placentas and blood circulation to provide nutrition and protection for their newborns. Maternal antibodies and other immune defenses pass through the bloodstream to

the unborn baby. Although the newborn is generally immune to bacteria and sickness before delivery, it becomes exposed to them once it leaves its mother's body.

5.4 SUMMARY

The immune system is forever working to defend the body from infection, injury, and disease. It relies on a sufficient supply of nutrients for its baseline functions as well as for ramping up its movement when necessary. Immunity is a defense mechanism of animals against parasites, so that these animals are normally capable of resisting the infection by most pathogens.

In biology, **immunity** is the ability of multicellular organisms to oppose harmful microorganisms. Immunity involves both specific and nonspecific components. The nonspecific components act as barriers or eliminators of a broad range of pathogens irrespective of their antigenic make-up. Other components of the immune system adapt themselves to each new disease encountered and can generate pathogen-specific immunity.

Distinct humoral immunity**, cell-mediated immunity** does not depend on antibodies for its adaptive immune functions. Cell-mediated immunity is primarily driven by mature T cells, macrophages, and the release of cytokines in response to an antigen.

Cell –mediated immunity involves specialized lymphocytes called T cells (or T lymphocytes) that act against foreign organisms or tissue. It is very effective against bacteria and viruses located within the infected host cells and against fungi, protozoa and helminthes. It is an important factor in our defense against cancer too. The major types of lymphocytes involved in cell-mediated immunity include naive T cells, helper T cells, killer T cells and macrophages.

Humoral immunity and cell-mediated immunity are two types of an adaptive immune response that allow the human body to protect itself in an embattled way beside harmful agents such as bacteria, viruses and toxins. Even as there is some overlap between these arms of the immune response - both rely on the functions of lymphoid cells - there are also some important differences.

Humoral immune system involves the production of antibodies that act against foreign organism and substances. These antibodies are found in extra cellular fluids such as blood plasma, lymph and mucus secretions. B cells are responsible for the production of antibodies. The humoral immune response is defensive primarily against bacteria, bacterial toxins and viruses that are

circulating freely in the body fluids. Acquired immunity based on antibodies is the most efficient type of acquired immunity divided into two types: active and passive immunity.

Active immunity responds directly to a foreign antigen inside the body. In acquired or adaptive immune system, the body remembers the pathogens it has encountered in the past.
Passive immunity involves the immune response by the antibodies attained from outside the body. The primary response by the body to a pathogen it encounters for the first time is rather weak, so the first encounter is always a little harsh on the body. Passive immunity is protection from a disease provided by antibodies created outside of the individual's body.

5.5 TERMINAL QUESTIONS AND ANSWERS

1. Multiple Choice Questions:

1. Humoral immunity is mediated by

A. B- cells

B. Macrophages

C. both A and B

D. Phagocytes

2. Humoral immunity is also called as

A. Antigen mediated immunity

B. Antibody mediated immunity

C. Non-specific immune response

D. All of these

3. The function of memory B cell is

A. Antibody production

B. immunologic memory

C. Regulated antibody production

D. None of these

4. Which of the following immunity is obtained during a lifetime?

A. Acquired immunity

B. Active immunity

C. Passive immunity

D. None of the above

5. Which of the following cells is involved in cell-mediated immunity?

A. Leukemia

B. T cells

C. Mast cells

D. Thrombocytes

Answers: 1. A, 2. B, 3. B, 4. A, 5. B

2. Short Answer Question:

1. What is the active immunity?

2. What is the passive immunity?

3. What do you understand by Humoral immunity?

3. Long Answer Question:

1. What is the difference between active and passive immunity? Describe the suitable example?

2. Explain humoral immunity.

3. Describe Cell-mediated immunity.

5.6 REFERENCES

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Unit 6 APPLICATION OF IMMUNOLOGICAL PRINCIPLES

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6.1 *OBJECTIVE*

After studying this unit learns will learn about:

- Diagnostics
- Widal test and ELISA test
- Basics of Vaccines
- Active vs. passive immunity

6.2 *INTRODUCTION*

The multicellular organisms have a defense mechanism against the number of pathogens and this defense mechanism is known as the immune system. Immune system recognizes a variety of pathogens and utilizes a number of different destructive mechanisms to safeguard the organisms. This system is made up of various organs, cells and biochemical pathways which are generally interconnected. The advancement in the field of immunology leads to use of various immunological knowledge for improvement in health care services. Various test like widal test, ELISA etc helps in early and accurate detection of a disease possible. Centuries ago observers noticed that the individual who once suffered from a disease became immune to that particular disease and on later encounter with the same disease they will not suffer from the disease. This observation leads to the development of the science of immunology. Immune system protects an individual from any foreign particle (called antigen) which is potentially harmful for the organism. It provides immunity to the individual. The immunity involves both humoral and cellular components. The humoral part involves the use of Immunoglobulin (Antibodies) and the cell-mediated immunity involves the various cellular components. Nowadays with the help of enormous knowledge of various immunological principles, various tests are done to recognize the number of pathogens (diseases). And to prevent various diseases vaccination is done in advance. The concept of vaccination is given by Louis Pasteur.

6.3 *DIAGNOSTICS*

Immunoassays have been developed to assist in the diagnosis of disease brought on by infectious microorganisms. The detection and quantification of antibodies produced against an infectious agent, a microbe, or a non-microbial antigen are the foundation of these biochemical and serological procedures. The basic concept behind these techniques are the specificity of an antibody to a specific antigen.

6.3.1 WIDAL TEST

The causative organism of *typhoid fever* is *Salmonella typhi* and the characteristic inflammation are Peyer patches, intestinal ulceration, and mesenteric adenitis. In 1896 Widal developed the first serologic test for typhoid fever, an agglutination assay that detects the presence of antibodies against the O and H antigens of *Salmonella Typhi*. In case of acute infection, first O antibodies appear (6–8 days), which rise progressively and then later fall leading to their disappearance within a few months. H antibody appears later (10–12 days) but persists for longer. High O antibody titer usually indicates acute infection; High H antibody titer helps to identify the type of enteric fever. *Salmonella* antibody appear in serum at the end of the first week of endemic fever and rise sharply during the 3rd week. It is recommended to test two samples of sera at an interval of 7 to 10 days to demonstrate a rising antibody titer. *Salmonella* antigen suspensions can be used as slide and tube techniques.

PRINCIPLE

Patient samples contain antibodies which react with colored *Salmonella* antigens. Bacterial suspension which contains antigen will agglutinate on exposure to antibodies to *Salmonella* organisms.

Widal Antigens preparation

1) Bacterial H suspension is prepared by adding 0.1 per cent formalin to a 24 hours broth culture or saline suspension of an agar culture.
2) Bacterial O suspensions is prepared by culturing the organisms on phenol agar (1:800) to inhibit flagella.

3) Standard smooth strains of the organism are used; *S. Typhi* 901, (O and H strains)
4) The growth is then emulsified in saline by mixing 20 times its volume with alcohol, heated for 30 minutes at 40° C to 50° C and then centrifuged.
5) The antigens are mixed with chloroform (preservative) and then dyed with appropriate dye for easy identification.

Procedure of Slide Widal Test

1.Put one drop of positive control on one reaction circles of the slide.
2.Put one drop of Isotonic saline on the next reaction circle (-ve Control).
3.Put one drop of the sample on the remaining four reaction circles.
4.Add one drop of WidalTEST antigen suspension 'H' to the first two reaction circles.
5.Add one drop each of 'O', 'H', 'AH' and 'BH' antigens to the remaining four reaction circles.
6.Uniformly mix the contents of each circle with a mixing stick (different stick for different circle).
7. Gently rock the slide back and forth and check for agglutination in a microscope within one minute.
8. Agglutination is a positive test result

Limitations of Widal Test

- This test can give false positive result in patients with previous vaccination or infection with *S.Typhi*.
- This test shows cross-reactivity with other *Salmonella* species,
- This test cannot distinguish between a current infection and a previous infection or vaccination against typhoid.
- False positive Widal test results are reported to occur in case of typhus, acute falciparum malaria (particularly in children), chronic liver disease associated with

raised globulin levels and disorders like rheumatoid arthritis, myelomatosis and nephrotic syndrome.

- False negative results may come in early stages of infection.

6.3.2 ELISA TEST

ELISA stands for Enzyme-Linked Immunosorbent Assay. The principal of this technique is: specific antibodies recognize a specific antigens and these antibodies are attached with an colorless substrate (known as chromogenic substrate), which in the presence of an enzyme (e.g. alkaline phosphatase, horseradish peroxidase, and -galactosidase) produce a colored product.

Types of ELISA

Various forms of ELISA techniques are used for qualitative detection or quantitative measurement of either antigen or antibody.

A) INDIRECT ELISA

This technique is used for the detection or quantitative determination of antibody. Sample containing primary antibody (Ab1) is added to an antigen-coated microtiter well to react with the attached antigen. Washing is done to remove any free Ab1 and to determine the presence of antibody-antigen complex, enzyme-conjugated secondary anti-isotype antibody (Ab2) are attached, which binds to the primary antibody. Then once again washing is done to remove any free Ab2. The next step is addition of a substrate for the enzyme. The specialized spectrophotometric plate readers are used to measure the amount of colored reaction product. It can measure the absorbance of all of the wells of a 96-well plate in seconds. This technique is used for the detection of human immunodeficiency virus (HIV) that causes AIDS.

B) SANDWICH ELISA

This technique is used to detect antigen. In Sandwich ELISA the antibody instead of antigen is immobilized on a microtiter well. Then the sample containing antigen is added, which reacts with the immobilized antibody. Washing is done to remove free antigen and after washing a second enzyme-linked antibody specific for a different epitope on the antigen is added, which reacts with

the bound antigen. Again, washing is done to remove the free second antibody. Then the substrate is added to produce a colored reaction. After that the colored reaction product is measured.

C) COMPETITIVE ELISA

This technique is used to measure the amount of antigen. Firstly, the antibody is incubated in solution with a sample containing antigen. The antigen-antibody complex is then added to an antigen coated microtiter well. If a large amount of antigen is present in the sample, then the less free antibody will be available to bind to the antigen-coated well. An enzyme-conjugated secondary antibody (Ab2) which is specific for the isotype of the primary antibody is added to determine the amount of primary antibody bound to the well. So, here higher the concentration of antigen, the lower will be the absorbance.

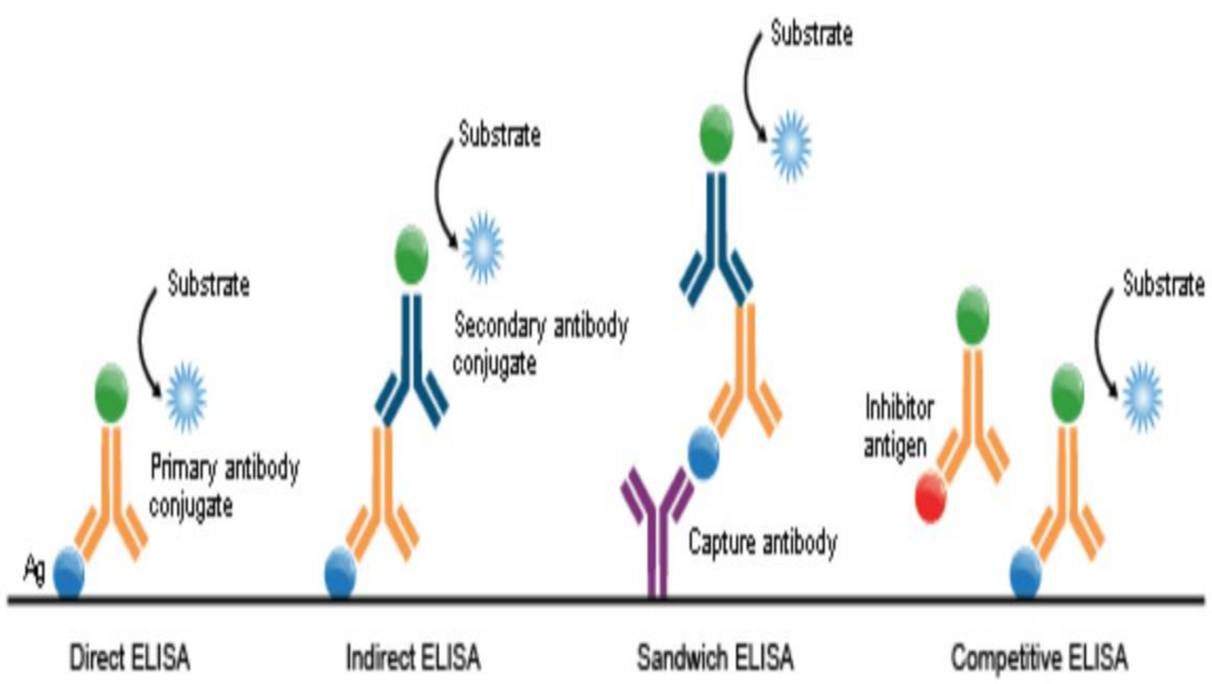

Fig 6.1 Types of ELISA (Credits:

https://upload.wikimedia.org/wikipedia/commons/c/c9/ELISA_types.png)

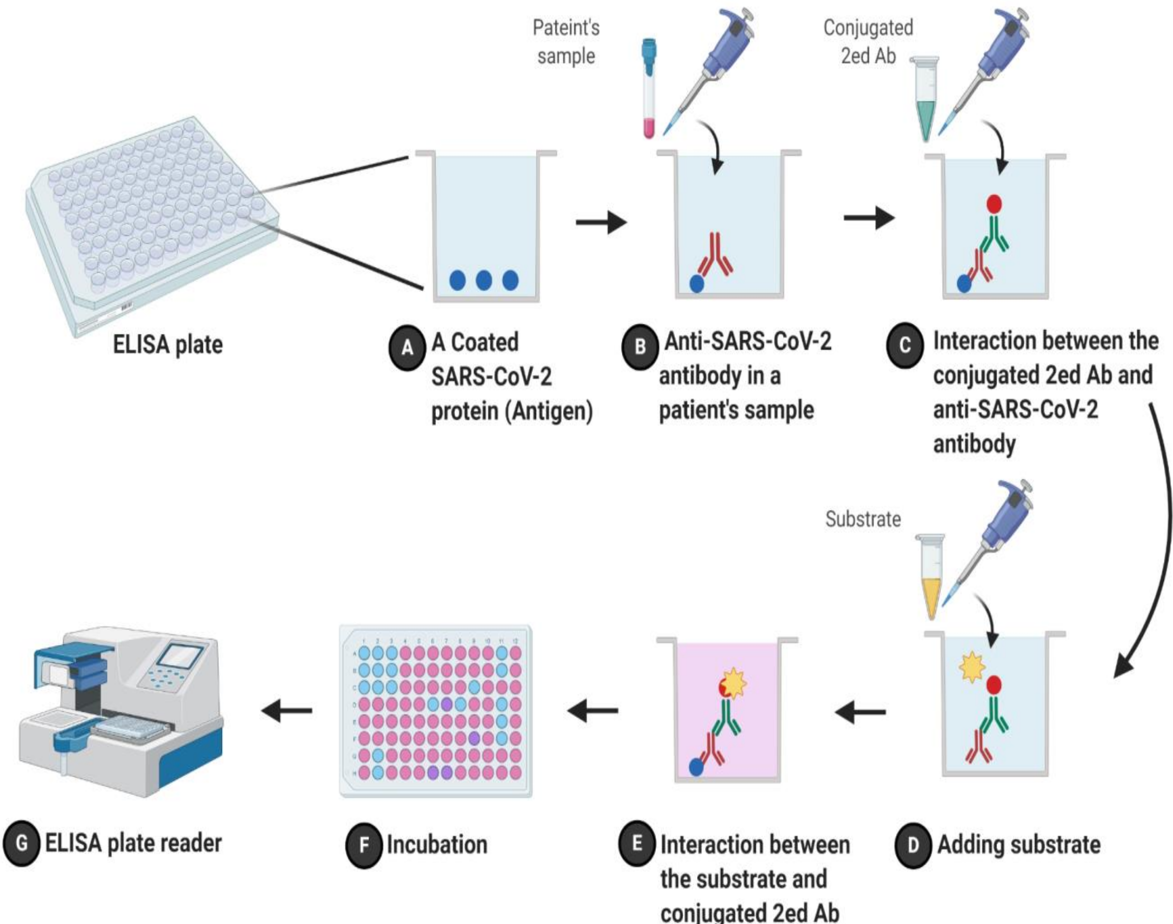

Fig 6.2 Basic steps of ELISA (credit: https://upload.wikimedia.org/wikipedia/commons/8/8e/Fig._E._COV.jpg)

6.4 *VACCINES*

Edward Jenner and Louis Pasteur initial work on vaccination help in understanding the process of immunity generation from previous infection. As a result of the invention of numerous vaccines, the prevalence of diseases including Diphtheria, Measles, Mumps, Pertussis, Rubella, Poliomyelitis, and Tetanus has significantly decreased. The following requirements must be met for a vaccination to be effective:1) Approved for usage by humans 2) Affordably priced; 3) Effectively delivered to at-risk populations. The manufacturing of vaccines is a very tedious job. After clinical trials, human trials are also done to ensure the safety of the vaccine. Even those

candidate vaccines that pass the initial study and are approved for trial in humans are not necessarily being passed as vaccines for common usage. It is not necessary that every vaccine candidate that was successful in laboratory and animal trials prevents disease in humans. Some potential vaccines might result in unacceptable side effects, and some may even worsen the disease they were meant to prevent. Vaccines which contain live virus vaccines may be a threat to those with primary or acquired immunodeficiency. For a successful vaccine even, the low frequency adverse side effects should be addressed. The advancement in the knowledge of molecule biology and immunology enables scientists to develop vaccines effectively e.g. with the knowledge of the differences in epitopes recognized by T cells and B cells, immunologists can design vaccine candidates to maximize activation of both types of the immune system. Proper vaccination helps to reduce death among children due to various diseases. Some vaccines have severe side effects but advancement in the field of immunology helps in synthesis of vaccines which have less side effects and are effective in generation of immunity against the disease. In some cases, multiple immunization is required e.g. Sabin polio vaccine is given multiple times so that appropriate immune response is generated to each of the three strains of poliovirus. Some vaccines are given to adults depending on the risk group e.g. Vaccines for meningitis, pneumonia, and influenza are given to individuals with low immunity or groups living in close quarters. Anthrax vaccine is given to workers, who come into close contact with infected animals or products from them. Anthrax spores can be used as bioweapons, so sometimes military personnel and civilians of risk areas are also immunized against it. Travelers are also given vaccines according to their destination. Vaccination is not 100% successful as some recipient's respond poorly. But this is not a serious issue because a large percentage of the population is immune to an infectious agent, the possibility of a vulnerable individual contacting an infected individual is very low that the vulnerable one is not likely to become infected. This phenomenon is known as herd immunity.

VACCINES DESIGNING

Various points must be kept in mind during vaccines designing

1) Immune system has two components: humoral and cell mediated immunity and it is important which branch is activated by vaccine. Therefore, vaccine designers must know the differences between activation of the humoral and the cell mediated branches.

2) The second point is the generation of immunologic memory, if a vaccine that induces a protective primary response but does not induce the formation of memory cells, will leave the host unprotected after the primary response to the vaccine weakens. Some diseases have a short incubation period and the disease symptoms are already underway by the time memory cells are activated e.g. influenza virus. Therefore, effective protection against influenza can only be provided by maintaining high levels of neutralizing antibodies by repeated immunizations to those at highest risk are immunized each year.

ATTENUATED VIRUSES AND BACTERIA

The attenuated microorganisms lose their ability to cause disease but retain their capacity for transient growth within an inoculated host. The microorganism can be attenuated by growing them (pathogenic bacterium or virus) for prolonged periods under abnormal culture conditions. Those mutants are selected which grow successfully in the abnormal culture conditions and are therefore less capable of growth in the natural host.

Advantages of attenuated vaccine

1) These vaccines are capable of transient growth, resulting in prolonged exposure of immune-system to the individual epitopes on the attenuated organisms, causing increased immunogenicity and production of memory cells. Therefore, these vaccines are given only once, no repeated booster's doses are required.

2) The attenuated vaccine can replicate within host cells due to which they are able to induce cell mediated response e.g. Sabin polio vaccine, the attenuated viruses colonize the intestine and produce protective immunity against all three strains of virulent poliovirus. Although most of the attenuated vaccines require a single immunizing dose, but Sabin polio vaccine is given multiple times so that appropriate immune response is generated to each of the three strains of poliovirus.

Disadvantages of attenuated vaccines

1) The attenuated vaccines can revert to a virulent form e.g. the rate of reversion of the Sabin polio vaccine is about one case in 2.4 million doses of vaccine. This is the reason some countries use exclusively inactivated polio vaccines.

2) Attenuated vaccines may show complications similar to those that occur in case of the natural disease e.g. few recipients of the measles vaccine have been reported to develop post-vaccine encephalitis or other complications.

Nowadays with the help of genetic engineering techniques the virus can be attenuated irreversibly by selectively removing genes that are necessary for virulence e.g. herpesvirus vaccine for pigs.

INACTIVATION OF ORGANISM BY HEAT OR CHEMICAL TREATMENT

Other methods of vaccine production include inactivation of the pathogen by heat or by chemical method so that it is not able to replicate in the host. It is important not to damage the structure of epitopes on surface antigens during inactivation. Heat inactivation can cause extensive denaturation of proteins thus, any epitopes which depend on three-dimensional structure are possibly to be changed significantly. Formaldehyde or various alkylating agents is used for chemical inactivation e.g. Salk polio vaccine. Killed vaccines are given multiple times to maintain the immune status of the host. Killed vaccines only induce a predominantly humoral antibody response and they are less effective in inducing cell-mediated immunity and in eliciting a secretory IgA response as compared to attenuated vaccines. Risk associated with inactivated whole-organism vaccines is: complications occur from first Salk vaccines because formaldehyde failed to kill all the virus in two vaccine lots, which caused paralytic polio in a high percentage of recipients. The disadvantage associated with attenuated or killed whole organism vaccines can be avoided by using vaccines that are made up of specific, purified macromolecules derived from pathogens e.g. 1) inactivated exotoxins 2) capsular polysaccharides, and 3) recombinant microbial antigens

1) Bacterial Toxins

Some bacterial pathogens (diphtheria and tetanus causing bacteria) produce exotoxins and these exotoxins result in many symptoms of disease that are due to infection. Vaccines for Diphtheria and tetanus are produced from purified bacterial exotoxin (by inactivation by formaldehyde to form a toxoid). Immunization with these toxoids produced antibodies against the toxoid and these antibodies are able to bind with toxin and neutralize its effects. It is important to do the production of toxoid carefully without disturbing the structure of the epitope. Now large amounts of purified toxin can be produced by cloning the exotoxin genes and then expressing them in host cells.

2) Capsules polysaccharides

In the case of some bacteria the pathogenesis is due to antiphagocytic properties of their hydrophilic polysaccharide capsule. The phagocytosis of bacteria by macrophages and neutrophils occurs when the coat is covered by antibodies and/ or complement e.g. the vaccine for *Streptococcus pneumoniae* and *Neisseria meningitidis*. These vaccines are not able to activate T_H cells.

3) Pathogenic protein synthesized by Recombinant Techniques

The surface antigen of a pathogen is encoded by genes and these genes can be cloned and expressed in bacterial, yeast, or mammalian cells by recombinant DNA technology. These surface antigens can be used as vaccine e.g. vaccine for hepatitis B virus (HBsAg)

4) Synthetic Peptides as Vaccines

Synthetic peptides can be used as vaccines but they are not as immunogenic as proteins, and it is hard to generate both humoral and cellular immunity to them. The use of conjugates and adjuvants can assist in raising protective immunity to peptides, but barriers to the widespread use of peptide vaccines remain and pose an interesting problem for immunologists.

5) Recombinant-Vector Vaccines

In this technique attenuated viruses or bacteria can be used as vectors by introducing gene that encode major antigens into them. The attenuated organism will replicate inside the host and express the antigenic protein. example of organisms used for vector vaccines are vaccinia virus, the canarypox virus, attenuated poliovirus, adenoviruses, attenuated strains of *Salmonella*, the BCG strain of *Mycobacterium bovis*, and certain strains of *Streptococcus*.

6) DNA Vaccines

Plasmid DNA encodes antigenic proteins and if it is directly injected into the muscle of the recipient. Then the muscle cells take up this DNA and synthesize the protein antigen that is expressed, causing both a humoral and a cell-mediated response. The DNA taken up by the muscle cells either get integrated into the chromosomal DNA or region in episomal form for a long time. The advantages of DNA vaccine are: 1) The encoded protein is expressed in the host in its natural

form (no denaturation or modification) so, natural immune response occurs. 2) It induces both humoral and cell-mediated immunity 3) It causes prolonged expression of the antigen, which produces significant immunological memory. 4) No refrigeration is not required for storage of the plasmid DNA 5) The same plasmid vector can be modified to make a variety of proteins 6) For rapid delivery of these vaccine to large population, plasmid DNA is coded with microscopic gold beads and then delivered to muscle with an air gun (called a gene gun).

7) Multivalent Subunit Vaccines

The synthetic peptide vaccines and recombinant protein vaccines are less immunogenic and they do not induce cell-mediated response therefore, different methods are used to produce synthetic peptide vaccines that contain both immunodominant B-cell and T-cell epitopes. The first method is preparation of solid matrix–antibody antigen (SMAA) complexes by attaching monoclonal antibodies to particulate solid matrices and then saturating the antibody with the desired antigen. The second method utilizes the use of detergent to produce multivalent vaccines. Detergent is used to incorporate protein antigens into protein micelles, lipid vesicles or immunostimulating complexes. The protein is initially mixed with the detergent and then the detergent is removed which leads to the formation of micelles (proteins orient themselves with their hydrophilic residue towards the aqueous region and the hydrophobic residues at the center to avoid the aqueous region). Liposomes (lipid vesicles) with protein antigens are synthesized by mixing the proteins with a suspension of phospholipids to form vesicles bounded by a bilayer. Immunostimulating complexes (ISCOMs) are lipid carriers synthesized by mixing protein with detergent and a glycoside called Quil A.

6.5 *ACTIVE VS. PASSIVE IMMUNITY*

There are various ways by which the immunity can be acquired e.g.

1) By natural processes such as transmission from mother to fetus, or through an earlier infection with the organism

2) Artificial means like by injection of antibodies or vaccines.

Emil von Behring and Hidesaburo Kitasato showed that immunity generated in one animal can be transferred to another organism by injecting it with serum from the first. This process where

preformed antibodies are transferred to a recipient is known as passive immunity. Naturally produced antibodies (in preganant women) can pass the placenta and be transferred to unborn fetuses. Passive immunity is provided to the developing foetus by maternal antibodies against poliovirus, rubella, rubeola, mumps, and streptococci. Maternal Antibodies present in colostrum and milk also provide passive immunity to the Breastfeeding infants. Artificially the passive immunity can be generated by injecting a recipient with preformed antibodies. In the past when vaccines and antibodies were not available, passive immunity was the major defense against fatal diseases (by injecting animal sera e.g. horse serum). The following events can benefited from passive immunization: 1) Antibody deficiency brought on by inherited or congenital B-cell abnormalities, together with other immunodeficiencies. 2) The risk of getting a disease that will lead to complications (such as a leukemia patient exposed to varicella or measles); and 3) the absence of sufficient time for active immunization to provide appropriate protection. Antiserum administered passively protects against insect and snake venom. Additionally, visitors or healthcare professionals who may soon come into contact with an infectious agent but lack active immunity are immediately protected by passive immunization. Because the immune system is not activated during passive immunization, no memory will form against the diseases. Passive immunization provides short time protection or alleviation of an existing condition, but active immunization provides protective immunity and immunologic memory. After a successful active immunization, a subsequent exposure to that specific pathogen will elicit an intensified immune response that successfully destroys the pathogen. Active immunization can occur 1) naturally by microorganism infection 2) Artificially by administration of a vaccine. In active immunization, the immune system gets activated and proliferation of antigen-reactive T and B cells cause formation of memory cells.

6.6 SUMMARY

The multicellular organisms have a defense mechanism against the number of pathogens and this defense mechanism is known as the immune system. Immune system recognizes a variety of pathogens and utilizes a number of different destructive mechanisms to safeguard the organisms. This system is made up of various organs, cells and biochemical pathways which are generally interconnected. The advancement in the field of immunology leads to use of various immunological knowledge for improvement in health care services. The branch of immunology

contributes to the biological technique by antibody based and fluorescence-based techniques. These techniques are used for the diagnosis of various diseases. The basic concept behind these techniques are the specificity of an antibody to a specific antigen. The causative organism of *typhoid fever* is *Salmonella typhi* and the characteristic inflammation are Peyer patches, intestinal ulceration, and mesenteric adenitis. In 1896 Widal developed the first serologic test for typhoid fever, an agglutination assay that detects the presence of antibodies against the O and H antigens of *Salmonella typhi*. ELISA stands for Enzyme-Linked Immunosorbent Assay. The principal of this technique is: specific antibodies recognize a specific antigen and these antibodies are attached with a colorless substrate (known as chromogenic substrate), which in the presence of an enzyme (e.g. alkaline phosphatase, horseradish peroxidase, and -galactosidase) produce a colored product. Edward Jenner and Louis Pasteur initial work on vaccination help in understanding the process of immunity generation from previous infection. Now a number of vaccines are developed for various diseases e.g. diphtheria, measles, mumps, pertussis, rubella, poliomyelitis, and tetanus and the incidence of these diseases has decreased dramatically. Emil von Behring and Hidesaburo Kitasato show that immunity generated in one animal can be transferred to another organism by injecting it with serum from the first. This process where preformed antibodies are transferred to a recipient is known as passive immunity. Passive immunization provides short time protection or alleviation of an existing condition, but active immunization provides protective immunity and immunologic memory

6.7 *Terminal Questions and Answers*

Q.1 Vaccination was invented by

a) Jenner
b) Pasteur
c) Koch
d) Salk

Q.2 Vaccines against viruses are usually

a) Given at birth
b) Expensive
c) Either live-attenuated or killed

d) Mainly polysaccharide

Q.3 ELISA (enzyme-linked immunosorbent assay) allows for rapid screening and quantification of the presence of _______ in a sample.

a) amino acid

b) DNA

c) antigen

d) RNA

Q.4 Abbreviation of ELISA is __________________

a) Ion Selective Field Effect Transistors

b) Enzyme Linked Field Effect Transistors

c) Adenosine triphophosphate

d) Enzyme Linked Immunosorbent Assay

Q.5 The causative agent of of Typhoid is

a) Salmonella typhii

b) E. coli

c) None of the above

d) Both

.Answer: Q.1 (a), Q.2 (c), Q.3 (c), Q.4 (d), Q.5 (a)

Q.6 Write a short note on

A) Vaccine

B) ELISA

C) Widal Test

Q.7 Differentiate between Active and Passive immunity

Q.8 Describe various types of vaccines

6.8 *REFERENCES*

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Unit 7 ADJUVANT

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7.1 *OBJECTIVES*

This unit will provide you a comprehensive knowledge about immunologic adjuvants. Besides the history and introductory information about an adjuvant, this section covers the characteristics, uses, types, examples and mechanisms of action of adjuvants. This chapter also describes the role of adjuvants in improving efficacy of vaccines and immunomodulation.

7.2 *INTRODUCTION*

In Immunology, vaccines are the preparations that are used to stimulate the body's immune response against diseases. In the early days of vaccine designing, efficacy of different batches of same vaccine was found to be variable in significant manner assumed to be caused by contamination of the chemicals and apparatus used during the process. However, it was soon found that removing those contaminants actually seemed to reduce the effectiveness of the vaccines. These contaminants are generally considered as adjuvants, also called immune potentiators or immunomodulators, and have been used for decades to improve the immune response to vaccine antigens.

An adjuvant, comes from the Latin word ***adiuvare*** meaning to help or aid, ***is an ingredient or substance used in some vaccines that enhances, accelerates or modulates the specific immune response in people receiving the vaccine. In other words, they help poorly immunogenic vaccines to work better***. Adjuvants were originally described by Gaston Ramon, a French veterinarian, in 1924 as ***'substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone'***. While working at the Pasteur Institute, Ramon observed higher antibody levels in those horses, immunized with tetanus or diphtheria toxoids, which had developed abscesses at injection site. Ramon then induced sterile abscesses by injecting chemicals like starch, breadcrumbs together with the toxoids and a further enhancement was seen in the antibody production. He concluded that substances which were capable of inducing inflammation at the injection site promoted antibody formation. In 1926, Alexander Glenny performed a similar experiment by injecting a foreign antigen together with alum (aluminium potassium sulfate). As a result of Glenny's work, addition of aluminium salts as an adjuvant became a common practice to enhance vaccine efficacy.

Vaccines that are formulated from weakened highly purified antigens or killed antigens contain naturally occurring adjuvants that help the body to produce a strong immune response.

However, most of the vaccines developed in modern times include only small components of antigens, such as their proteins, rather than the entire microorganism (virus or bacteria). In both types of vaccines especially those containing killed organisms or highly purified antigens, adjuvants help the body to produce a strong protective immune response in the person from the disease for which he or she is being vaccinated.

With the increasing threat to various infectious, allergic and autoimmune diseases and also to cancer and infertility problems, interest of vaccine manufacturers and public health authorities, e.g., WHO, towards developing vaccine adjuvants has been growing rapidly. They have established goals for improving present vaccines, for developing new vaccines and for developing appropriate adjuvants for vaccines having low immunogenicity. Use of new technologies related to analytical biochemistry, macromolecular purification, recombinant technology, and better understanding of immunological mechanisms and disease pathogenesis has improved the development and application of adjuvants.

7.3 *CHARACTERISTICS AND USES OF IDEAL ADJUVANT*

Adjuvants help in providing increased immunity to a particular disease by modifying or augmenting the effects of a vaccine by stimulating the immune system for its more vigorous response to the vaccine. They increase the amount and quality of the antibody produced. They generally mimic PAMPs (Pathogen-associated molecular patterns), specific sets of evolutionarily conserved molecules which function as antigenic moieties. These include liposomes, lipopolysaccharide, coat proteins of antigens, components of bacterial cell walls, and endocytosed nucleic acids such as RNA, double-stranded RNA, single-stranded DNA, and unmethylated CpG dinucleotide-containing DNA. Similar to a natural infection, presence of PAMPs as adjuvant in a vaccine enhances the innate immune response to the antigen by increasing the activities of dendritic cells, lymphocytes, and macrophages. This triggering of innate immune responses ultimately promotes the adaptive responses by shaping the consequences of T-cell activation (induction of cytotoxic or helper T lymphocyte responses) to vaccines and so provide long-term protection.

State of inflammation is a prerequisite for an effective vaccination. This can be seen in the form of more local reactions (such as redness, swelling, and pain at the injection site) and more systemic reactions (such as fever, chills and body aches). In general, immunization with purified proteins leads to a poor immune response due to their inability to create a state of inflammation.

In contrast to this, in adjuvanted vaccines, substances used as adjuvants enhance immune responses by inducing inflammation by antigen-independent mechanisms at the injection site.

Adjuvants help in aggregation and precipitation of soluble protein antigens in the form of particles, which not only facilitates their efficient uptake by antigen presenting cells (APCs) but also reduces the rate at which antigen is cleared from the system.

Adjuvants are also used to increase the immunogenicity of weak antigens, to enhance speed and duration of immune response, to stimulate and modulate humoral responses (including antibody isotype), to improve induction of mucosal immunity and to decrease the dose of antigen required by reducing costs and eliminating inconvenient needs for booster doses. Adjuvants play a very important role in enhancing immune responses in immunologically immature patients, particularly infants.

7.4 *NATURAL AND CHEMICALLY DEFINED ADJUVANT AND THEIR ROLE ON IMMUNOMODULATION*

In immunology, several kinds of adjuvants are used in vaccination program. These are selected on the basis of their ability to promote the highest immune response and cause least toxicity.

Aluminium salts, such as aluminium hydroxide, aluminium phosphate, and aluminium potassium sulfate, were the earliest, most widely employed and the only approved adjuvants for human vaccination. These have been used since the 1920s with diphtheria and tetanus vaccines. These were safe and cost-effective and found to strengthened the body's immune response to these vaccines. Among aluminium salts, alum (aluminium potassium sulfate), aluminium oxyhydroxide, aluminium hydroxyphosphate and aluminium phosphate are the adjuvants globally licensed for human use. Aluminium adjuvants induce humoral responses by increasing half-life of vaccine antigens, by improving processing and presentation of antigens by the antigen presenting cells (APCs) and by inducing production of immunomodulatory cytokines. However, aluminium adjuvants are less effective in stimulating cell mediated immunity mediated by CD4 Th1 cells and CD8 T cells.

With the use of advanced technologies in the field of immunology, newer adjuvants have been designed in such a way that they target specific components of the body's immune system and provide strong and long-lasting protection against a disease.

7.4.1 TYPES OF ADJUVANTS

Adjuvants can be classified into different types on the basis of their source (natural, synthetic or endogenous), mechanism of action, or physical or chemical properties as follows –

(A) Damage-Associated Molecular Patterns (DAMPs)-Type Adjuvants

I. **Mineral salts / Inorganic compounds:**

Examples- Aluminium hydroxide, aluminium phosphate, aluminium potassium sulfate, aluminium oxyhydroxide, aluminium hydroxyphosphate, calcium phosphate hydroxide and calcium phosphate gels.

II. **Oil emulsions and Saponin-based formulations:**

Examples- MF59 (microfluidised detergent stabilized oil-in-water emulsion), QS21 (purified plant saponin from *Quillaja saponaria*), Montanide ISA-51 and ISA-720 (stabilized water-in-oil emulsion).

(B) Pathogen-Associated Molecular Patterns (PAMPs)-Type Adjuvants

I. **Microbial derivatives (natural and synthetic) function as TLR ligands (Toll-like receptor ligands):**

Examples- Lipopolysaccharide, monophosphoryl lipid A (MPLA), Detox (MPL + *M. Phlei* cell wall skeleton), AGP [RC-529] (synthetic acylated monosaccharide), OM-174 (lipid A derivative), CpG motifs (synthetic oligonucleotides containing immunostimulatory CpG motifs), modified LT and CT (genetically modified bacterial toxins to provide non-toxic adjuvant effects).

II. **Synthetic TLR ligands**

Examples- Imidazoquinolines, and some guanosine & adenosine analogs.

III. **Bacterial products:**

Examples- Killed bacteria of the species *Bordetella pertussis*, *Mycobacterium bovis*, toxoids.

IV. **Synthetic polymer adjuvants (Large biocompatible polymers)**:

Examples- Polylactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA). Chitosan (linear polymer formed by the deacetylation of chitin. It consists of randomly arranged chains of b-(1-4)-linked-D-glucosamine and N-acetyl-D-glucosamine monomers)

(C) Particulate Adjuvants:

Examples- Liposomes, virosomes (uniflagellar liposomal vehicles incorporating influenza haemagglutinin), AS04 (Al salt with MPL), ISCOMS **(Immune stimulatory complexes,** structured complex of saponins and lipids), polylactide co-glycolide (PLG), inert vehicles (e.g., gold particles), nanoparticles (made from many different compounds such as poly amino acids, polysaccharides, polystyrene, biodegradable polymers in addition to nondegradable elements such as gold, silver, iron, and silica)

(D) Combined Adjuvants (constructed by combining PAMP and DAMP- type adjuvants in a single vaccine formulation): most commonly used adjuvants for both polyclonal and monoclonal antibody production.

Examples- Freund's Adjuvant (**Freund's complete adjuvant, Freund's incomplete adjuvant), Titermax (mixtures linear, blocks or chains of non-ionic copolymers polyoxypropylene and polyoxyethylene), RiBi (oil-in-water emulsions). The antigen is mixed with squalene [a metabolizable oil] and emulsified in a saline solution containing Tween 80)**

i) Freund's Complete Adjuvant (FCA)

FCA is a water-in-oil emulsion that contains mineral oil, the surfactant mannide monoleate and heat-killed *Mycobacterium tuberculosis*, *Mycobacterium butyricum* or their extracts, used for creating a state of inflammation. FCA has the ability to localize the antigen for slow release within the immunized host for a certain period of time. FCA also stimulates both cell mediated and humoral immunity. However, the FCA is mainly used for pre-clinical studies due to its toxic side effects due to the non-metabolizable mineral oil.

ii) Freund's Incomplete Adjuvant (FIA)

FIA is different from FCA as it does not contain *Mycobacterium* or its components. The FIA is much less toxic than the FCA, but due to the lack of microbial components, it is less effective than the FCA in inducing immune responses.

(E) Cytokines (Endogenous human immunomodulators):

Examples- hGM-CSF or hIL-1, IL-2, IL-4, IL-12 (cytokines that can be administered either as protein or plasmid encoded), Immudaptin.

7.4.2 ROLE OF ADJUVANTS ON IMMUNOMODULATION

Adjuvants perform their action by stimulating components of innate immune responses needed to initiate protective adaptive responses. Most of the modern adjuvants are able to generate sufficient B cells but not enough CD-8 T cells. However, some of them can stimulate adequate cell mediated responses rely on signaling through antigen processing cells.

Generally, vaccines containing highly purified antigens are considered poor vaccines because they lack signals that trigger innate immune responses and hence cannot generate the signaling pathway required to enhance adaptive responses. Conversely, modified vaccines with adjuvants, when mimicking natural infections, cause cell damage and generate strong mandatory innate responses needed to optimize the adaptive responses which promote the uptake of vaccine antigens by dendritic cells (antigen-presenting cells). They perform this either by directly triggering innate immune responses that provide a stimulus for dendritic cell function and antigen presentation or by delivering the antigen in a form optimized for dendritic cell processing and antigen presentation.

Adjuvants which cause cell and tissue damage provide the body with DAMPs and are called DAMP-type adjuvants whereas adjuvants which contain microbial products are called PAMP-type adjuvants. These molecules trigger innate responses through pattern-recognition receptors (PRRs) including Toll-like receptors (TLRs) or Nod *(nucleotide oligomerization domains)* - like Receptors (NLRs) on dendritic cells (Fig. 7.1). The activation of PRRs on dendritic cells triggers cytokine release. These cytokines promote helper Th1 and Th2 cell responses which in turn activate B and T cells and so promote protective adaptive immunity.

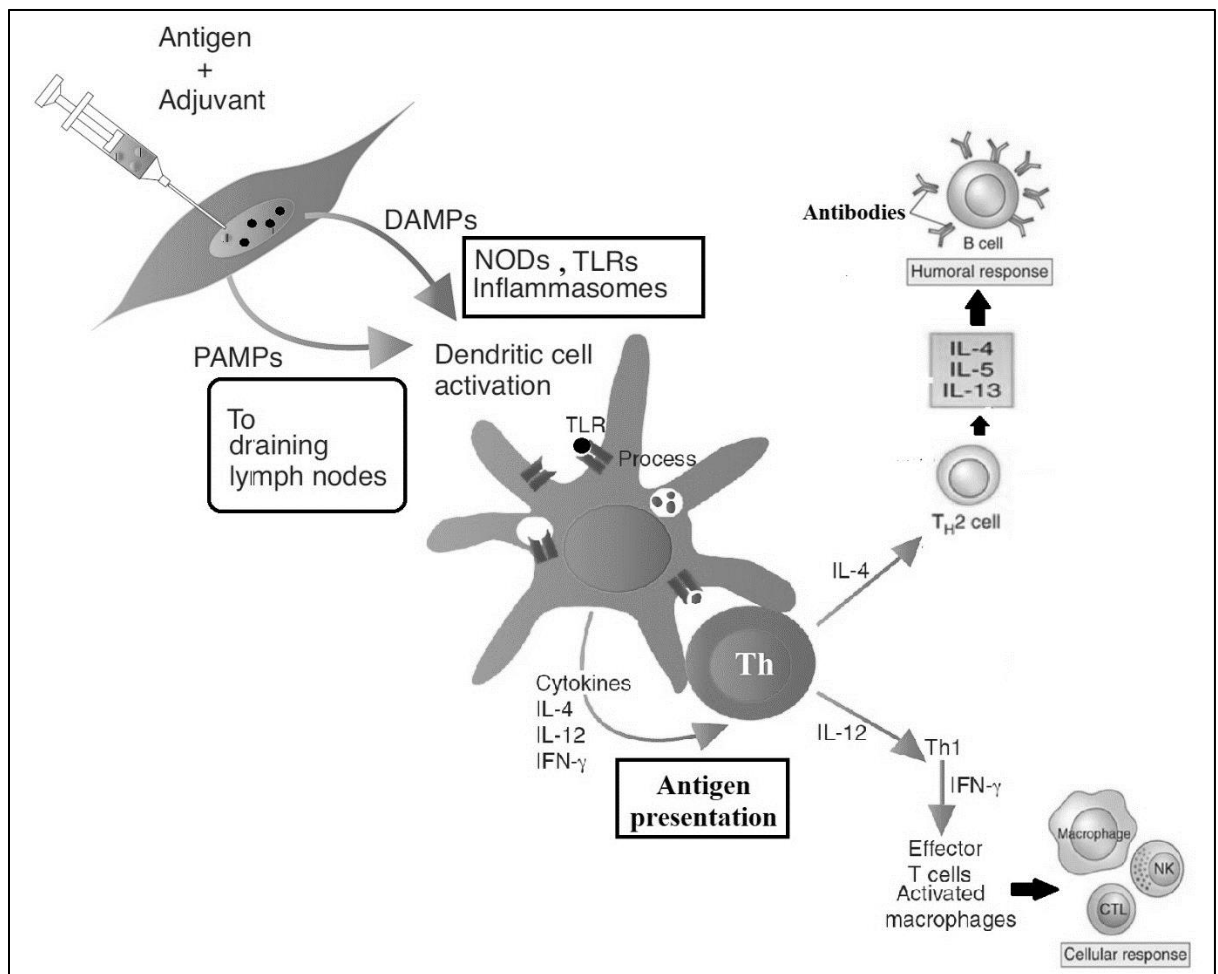

Fig. 7.1 The basic mechanisms of action of adjuvants. DAMPs, damage-associated molecular complexes; NODs, nucleotide oligomerization domains; PAMPs, Pathogen-associated molecular patterns; TLRs, Toll-like receptors; Th- T helper cell; CTL- Cytotoxic T Lymphocyte; NK- Natural Killer cell (Photo credit to Tizard I.R., 2019).

DAMPs released by adjuvants bind to receptors on antigen presenting cells and activate their inflammasome pathway. In this pathway, inflammasomes (multiprotein complexes) generate cytokines such as interleukin (IL)-1 and IL-18 leading to activation of innate immunity and helper T cell. PAMP-type adjuvants directly activate PRRs on dendritic cells and so cause the release of proinflammatory cytokines such as IL-1, IL-6, and tumour necrosis factor-α (TNF-α). They also stimulate the production of neutrophil-attracting chemokines such as CCL-3, CCL-4, CCL-8, and CCL-20.

Antigen-processing cells are playing a major role during the effective adjuvant action. After activation, these cells engulf antigen based on its size, charge, and hydrophobicity. They then effectively present the antigen to helper T cells with the help of major histocompatibility

complex (MHC) molecules. These cells can be directly influenced by adjuvants and are playing a critical role in determining the nature of the immune response. Particulate adjuvants are generally optimized for ingestion and processing by antigen-presenting cells.

7.5 SUMMARY

An adjuvant, comes from the Latin word ***adiuvare*** meaning to help or aid. It can be defined as "***a substance used in some vaccines for enhancing and modulating the specific immune response in patients receiving the vaccine".*** They increase the amount and quality of the antibody produced.

Similar to a natural infection, presence of adjuvants in a vaccine enhances the innate immune response to the antigen by increasing the activities of dendritic cells, lymphocytes, and macrophages which ultimately promotes the adaptive responses by activating the cytotoxic or helper T lymphocyte and hence provide long-term protection. Major characteristic of most of the adjuvanted vaccines is that they generally enhance immune responses by inducing inflammation by antigen-independent mechanisms at the injection site.

Other important roles of adjuvants include:

- Enhancement of the immunogenicity of weak antigens
- Stimulation and modulation of the humoral responses (including antibody isotype)
- Induction of mucosal immunity
- Reduction of amount and dose of antigen required and elimination of inconvenient needs for booster doses.

Among adjuvants, aluminium salts i.e., alum (aluminium potassium sulfate), aluminium oxyhydroxide, aluminium hydroxyphosphate and aluminium phosphate are the most common adjuvants globally approved for human use. Adjuvants can be classified into different types as DAMPs, PAMPs, particulate and combined type on the basis of their source, mechanism of action and properties.

Vaccines with adjuvants, when mimicking natural infections, cause cell damage and generate strong mandatory innate responses needed to optimize the adaptive responses which promote the uptake of vaccine antigens by dendritic cells (antigen-presenting cells). They perform this either by directly triggering innate immune responses that provide a stimulus for dendritic cell

function and antigen presentation or by delivering the antigen in a form optimized for dendritic cell processing and antigen presentation.

7.6 *TERMINAL QUESTIONS AND ANSWERS*

- **i.** *Full form of DAMP is……………………. and PAMP is……………………………*
- **ii.** *Examples of aluminium salts used as adjuvants are …………, ……….. and ……….*
- **iii.** *Adjuvants enhance innate immune responses towards vaccine mainly by activating ……cell.*
- **iv.** *……and ….. are the major cytokines function as adjuvants.*
- **v.** Adjuvants generally trigger innate responses through pattern-recognition receptors (PRRs) including………….. or……… on dendritic cells.
- **vi.** Example of adjuvant with a water-in-oil emulsion is …………

Answers

- **i.** *Damage-associated molecular patterns, Pathogen-associated molecular patterns*
- **ii.** Aluminium hydroxide, aluminium phosphate and aluminium potassium sulfate
- **iii.** Dendritic Cell
- **iv.** IL-4 and IL-12
- **v.** Toll-like receptors (TLRs), Nod *(nucleotide oligomerization domains)* (NLRs)
- **vi.** Freund's Complete Adjuvant (FCA)

1. MODEL EXAMINATION QUESTIONS:

- **i.** What do you mean by adjuvants? Make a note on various types of adjuvants used in vaccination.
- **ii.** Differentiate between DAMPs and PAMPs with examples.
- **iii.** Write a note on various classes of adjuvants with example.
- **iv.** Describe various characteristics of adjuvants.
- **v.** What do you mean by combined adjuvants? Describe composition and function of Freund's Adjuvant.
- **vi.** Describe immunomodulating mechanism of action of various kinds of adjuvants.

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UNIT 8 ANTIGEN V/S ANTIBODY

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8.3 Antigenic determination

8.4 Hapten s and antigenicity

8.5 Theories of antibody formation

8.6 Structure and classification of immunoglobulins and their properties

8.7 Immunoglobulin's synthesis at molecular level

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8.1 *OBJECTIVES*

After studying this unit learns will learn about:

- Concepts of Antigenic determination
- Haptens and antigenicity
- Theories of antibody formation
- Structure and classification of immunoglobulins and their properties
- Immunoglobulin's synthesis at molecular level

8.2 *INTRODUCTION*

The disease-causing organisms are called pathogens and the process by which they cause the disease is called pathogenesis. The immune system fights with the infection and protects the infected organism. It has two components: cellular immunity and humoral immunity. The T- cells are responsible for cellular immunity and Antibody producing B-cells are involved in Humoral immunity. An antigen is any substance that elicits a specific immune response. The antibodies are produced in the response of antigen. Antibodies have a wide range of reactivity and can distinguish between closely related molecules. The membrane bound receptor (antibody) determines the specificity of each B cell and this specificity is generated by random rearrangements of a series of gene segments that encode the antibody molecule. T cell specificity is also generated by random gene rearrangement. Details on immunoglobulin classification, structure, and antigenic determination will be included in this unit.

8.3 *ANTIGENIC DETERMINATION*

Antigens are typically large, complex substances that B and T cells cannot fully identify. Instead, lymphocytes (B and T cells) recognize discrete sites on the antigen called antigenic determinants, or epitopes. In a complex antigen, epitopes represent the immunologically active regions. B and T cells bind with the epitope although both the cells recognize antigens differently. B cells identify

an epitope alone, whereas T cells recognize an antigen (epitope) in conjunction with an MHC molecule on the surface of either an antigen-presenting cell or an altered self-cell. B cells are responsible for the humoral immunity and they recognize a large variety of epitopes (surfaces of bacteria or viral particles, and released soluble proteins, glycoproteins, polysaccharides, or lipopolysaccharides by pathogens). The binding between antibody and epitope of antigen is facilitated by weak noncovalent interactions. In order to have a strong bonding, the antibody binding site and the epitope of an antigen must be complementary to each other so that interacting groups come near to each other. Epitope size should not be larger than antibody binding site. The shape of the epitope is determined by the structure formed by sequence of amino acids at the binding site and their chemical nature. Most antibodies generated in response to native protein do not interact with denatured protein in the case of globular protein antigens because the tertiary conformation of the native protein determines the structure of the epitope (in case of B-cell). Only protein epitopes shown along with MHC molecules on self-cells are recognized by T cells, which are responsible of the cell-mediated immunity branch. The complex antigens are first processed and then with the association with the MHC molecules (present on cell surface) they are presented. In order to be recognized by the T-cell the foreign protein (antigen) must be degraded into small antigenic peptides and these antigenic peptides are displayed by the MHC molecules (class I or class II) on the cell surface. This process of conversion of antigen into MHC-associated peptide fragments is called antigen processing and presentation. The exogenous antigens are present outside of the host cell and they enter inside the host cell by the process of endocytosis or phagocytosis. Antigen presenting cells (e.g. macrophages, dendritic cells, and B cells) contain class II MHC molecules. These cells degrade the ingested exogenous antigen into peptide fragments during the endocytic processing. This complex (MHC-peptide association) is then exported to the surface of the cell. T cells with CD4 receptors recognize the antigen presented by class II MHC molecules and these are called T helper cells.

The endogenous antigens are generated in two ways 1) viral protein synthesized by the cells infected with the virus and 2) unique proteins synthesized by cancerous cells. These endogenous antigens are degraded into peptide fragments and then associated with class I MHC molecules within the endoplasmic reticulum. This complex (peptide–class I MHC complex) is then transported to the cell membrane. All the nucleated cells contain class I MHC molecules and use this pathway to process endogenous antigen. T cells with CD8 receptors recognize the antigen

associated with class I MHC molecules and these cytotoxic T cells attack and kill cells displaying the antigen–MHC class I complexes hence called T Cytotoxic cells. So, T-cell recognize the denatured antigen presented together with MHC molecule.

8.4 *HAPTENS AND THE STUDY OF ANTIGENICITY*

During the 1920s and 1930s Karl Landsteiner designed a chemically defined system for studying interaction between antibodies and epitope on an antigen. He used small organic molecules called Haptens which are antigenic but not immunogen. The haptens are chemical coupled with a large protein called a carrier. This hapten-carrier conjugate is now immunogenic and produces antibodies in immunized animals. These antibodies are specific for the hapten determinant, the carrier protein epitope, and new epitopes produced by the combination of parts of both the hapten and carrier. Alone hapten cannot function as an immunogenic epitope but many single hapten molecules when coupled to a carrier protein (or non-immunogenic homopolymer) act as immunogen. The hapten-carrier system provides immunologists an opportunity to study the chemically defined determinant which can be chemically modified to study the effect of various chemical structures on immune specificity. Landsteiner first immunized the rabbits with a hapten carrier conjugate. He then tested the reactivity of the rabbit's immune sera for hapten and closely related haptens-carrier protein complex. So, he tested whether an anti hapten antibody was able to bind with other hapten which have slightly different chemical structure. If a reaction takes place it is known as cross-reaction and the specificity of the interaction can be studied by observing which modifications prevented or permitted cross-reactions. He concluded that the overall structure of a hapten plays a major role in determining whether it can react with a given antibody. His work shows the specificity of the immune system and also demonstrated the wide range of diversity of epitopes that the immune system is capable of recognizing. Various drugs, peptide hormones, and steroid hormones, are examples of haptens.

8.5 *THEORIES OF ANTIBODY FORMATION*

Initially the greatest mystery for the immunologists was the specificity of the antibody molecule for antigen. To explain the specificity of the immune system two major theories were proposed: The selective theory and The instructional theory.

In 1900 Paul Ehrlich gave first insight into selective theory. He proposed that cells of the blood have a variety of receptors (side chain receptors) that react with the infectious agents and inactivate them. He also proposed the binding between receptors and infectious agents occur in lock and key fashion (influence with Emil Fischer lock and key hypothesis for enzyme-substrate complex). Ehrlich suggested that receptors and infectious agent's association would induce the cell to generate more receptors with the same specificity. According to Ehrlich's theory, the receptor specificity was determined before its exposure to the receptor and antigen specifically binds with the appropriate receptor. Ehrlich's theory was proven correct except that one cell made many receptors. One cell does not make many receptors but each cell produces many copies of just one membrane bound receptor (one specificity). After an antigen binds with the B cell membrane receptor specifically, it triggers the B cell to proliferate and produce many copies of these receptor in soluble form (now known as antibody)

During the 1930s and 1940s, the selective theory was challenged by various instructional theories. The concept of instructional theories revolves around antigen, which determines the specificity of the antibody molecule. Friedrich Breinl and Felix Haurowitz instructional theory suggested that, particular antigen would act as a template around which an antibody folds and then antibody assume a configuration complementary to that of the antigen. The instructional theories were rejected in the 1960s.

During the 1950s Niels Jerne, David Talmadge, and F. Macfarlane Burnet gave a selective theory known as the clonal selection theory. This theory stated that an individual lymphocyte (B and T cells) has antigen specific receptors (specific for a single antigen) on their membrane. The receptor specificity is determined before it is exposed to an antigen. When an antigen binds with its specific receptors, it activates the cells to proliferate into a clone of cells that have the same immunologic specificity as the parent cell.

8.6 STRUCTURE AND CLASSIFICATION OF IMMUNOGLOBULINS AND THEIR PROPERTIES

The antibodies are the membrane receptor of B cell (B-cell receptor) and on encountering antigen they secrete the soluble form of these specific receptors known as Immunoglobulins (Antibodies).

These antibodies are made up of proteins. The three-dimensional structure of antibodies is achieved by folding of polypeptide chains into an organized series of antiparallel β-pleated strands which form different domains. To form a tertiary structure the β- strands are arranged into a pair of β- sheets in each domain. The individual proteins have different numbers of strands per sheet but generally most antibody domains have 110 amino acids and 3 to 5 strands per β-sheet. The intrachain disulfide bond stabilized the β-sheet in each domain. Adjacent domains are connected with each other with the help of polypeptide chains. The structure is also stabilized by the hydrophobic interactions. All types of antibodies contain a common structure of four polypeptide chains (Heterodimers) i.e. two identical light chains (L) and two identical heavy (H) chains (larger polypeptides). Each heavy chain and light chain have an amino-terminal variable (V) region that is made up of 100 to 110 amino acids and differs from one antibody to the next. The rest part of both heavy and light chains is known as the constant (C) regions. The variable region of the light chain is known as V_L and the constant region is known as the C_L. The variable region of the heavy chain is known as V_H and the constant region is known as the C_H. The light chain is connected to its partner heavy chain by disulfide bond and by non-covalent interaction between the V_H and V_L and C_{H1} and C_L domain. The two heavy chains are linked by variable disulfide bonds.. The C terminal region of the two heavy chains is also involved in noncovalent interaction between corresponding domains. The structure of the antibody is Y shaped with two identical antigen binding regions at the tip of the Y shaped structure. Both light and heavy chain Amino acid contribute to the formation of antigen binding regions. The base portion of Y shaped structure is made by the C-terminal domains of the heavy chain. So, basically the antibody consists of three relatively compact regions which are joined together by a hinge region. This region is susceptible to cleavage by papain (proteolytic enzyme). The two antigen binding regions are known as Fab regions. The non-antigen binding region is known as Fc region (identical in all antibodies of class). This region easily crystallizes (Fc for fragment crystallizable). The Fab region binds with antigen and the Fc region helps in binding antibodies to the Fc receptor on phagocytic cell or effector molecules.

The amino terminal of the light chain is variable but the carboxyl terminal is constant, the two major light chain constant sequence regions are known as the kappa (κ) and lambda (λ) chain. The lambda (λ) region is further divided into λ1, λ2, λ3 and λ4 due to few variations in amino acids among them. In the variable part of the light chain there are regions of hypervariability which

interact with antigen and known as complementarity determining regions, or CDRs. The heavy chain is divided into five major classes μ (mu), δ (delta), γ (gamma), ε (epsilon) and α (alpha). Each different heavy-chain constant part is known as isotype and the isotype of a given antibody molecule determines its class.

1) IgM class: contain heavy chain of μ(mu) isotype
2) IgD class: contain heavy chain of δ(delta) isotype
3) IgG class: contain heavy chain of γ(gamma) isotype
4) IgE class: contain heavy chain of ε(epsilon) isotype
5) IgA class: contain heavy chain of α (alpha) isotype

Due to minor differences in the amino acid sequences of α and γ heavy chain it is subdivided into sub-isotypes and antibodies are therefore divided into subclasses:

1) γ (gamma) have four sub-isotypes (γ1, γ2, γ3 and γ4) therefore for classes of IgG (IgG1, IgG2, IgG3 and IgG4)

2) α (alpha) have two sub-isotypes (α1 and α2) therefore two sub classes of IgA (IgA1 and IgA2)

IgG, IgA and IgD antibodies heavy chains have four domains and a hinge region, but IgM and IgA have five domains but no hinge region. the polymeric form of antibody IgM and IgA are liking by a polypeptide known as J chain, which is linked by two disulfide bonds to the Fc region in the two different monomeric unit, IgM antibody in serum always found as pentamer but most Serum IgA exist as monomer and sometime di, tri and tetrameric forms are also seen.

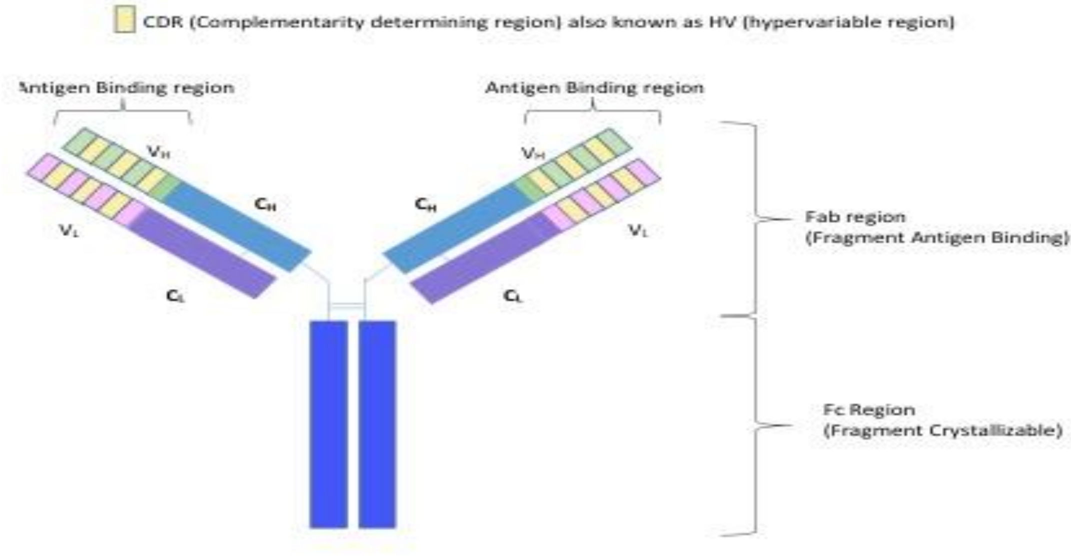

Fig 8.1 Antibody Structure

(Credit:https://commons.wikimedia.org/wiki/File:Antibody_Structure_and_Antigen_Binding_Regions.jpg)

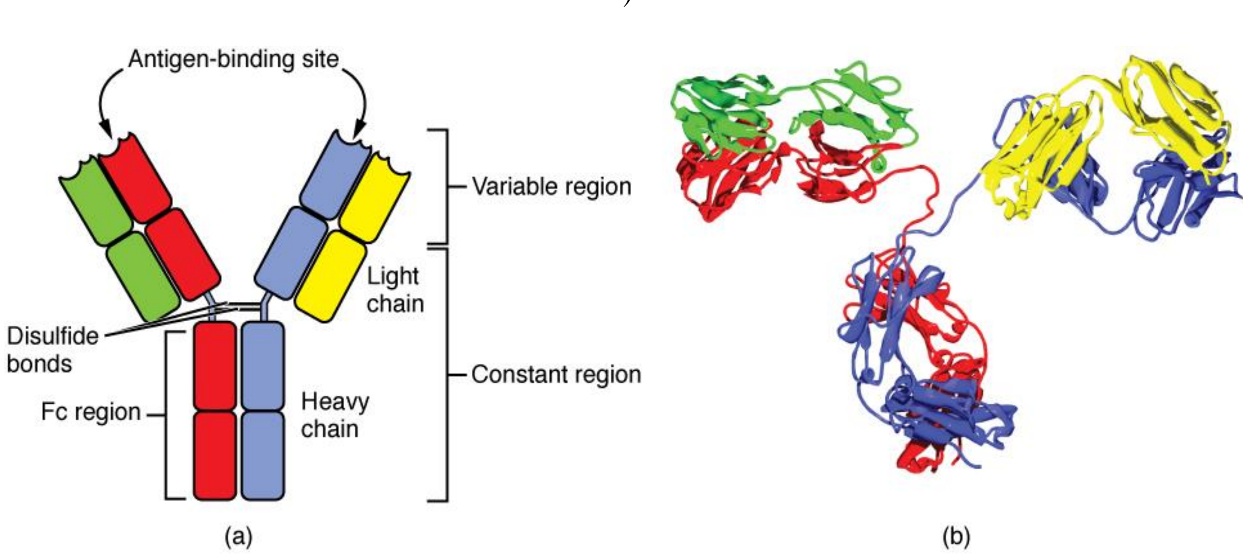

Fig 8.2 Antibody Molecular Structure

(Credit:https://commons.wikimedia.org/wiki/File:2220_Four_Chain_Structure_of_a_Generic_Antibody-IgG2_Structures.jpg)

Function of IgM

1) The first antibody class that is produce during primary immune response
2) Low affinity antibody because they are produced by B cells which do not undergo affinity maturation
3) They are pentavalent, so they can very effective in binding pathogens.
4) It protects us from common pathogen specially pathogens from gut and other mucosal areas
5) It is good in fixing complement
6) It effectively induces lysis of the pathogen it binds with
7) They form dense antibody pathogen complex which is easily engulfed by macrophages

Function of IgG

1) It is the most common antibody isotype in the serum
2) It is most diverse type of antibody and contain several subclasses
3) They bind to Fc receptor and increase phagocytosis by macrophages
4) IgG1 and IgG3 of human is good in fixing complement
5) IgG1 (human) can mediate ADCC by NK cells

Function of IgA

1) It is the major isotype which is found in the secretion (mucus in gut, milk from mammary gland, tears and saliva
2) In the body secretion IgA continually neutralize the toxins and pathogen and also interact with body natural flora present on mucosal surface and prevent them from entering the circulatory system
3) It does not fix complement so; no inflation is induced that's why it continually acts on pathogens that pose no threat.
4) It has long half-life because Fc region is resistant to many proteases
5) In circulation it occurs in monomeric form but in mucosal tissue di and polymeric forms are also seen
6) The di and polymeric form binds with the receptor of epithelial cells causing endocytosis and transport of molecules from basolateral to apical side of epithelial cells and into the lumen of tissue

7) IgA1 (serum) and IgA2 (secretions) mediate ADCC by NK cells and cause degranulation of granulocytes

Function of IgE

1) It plays role in allergy and asthma
2) It provides protection against helminths
3) It is produced in small quantities
4) It has very potent effect
5) It causes degranulation of eosinophils and basophils thereafter release histamine which damages the pathogens.

8.7 *IMMUNOGLOBULIN'S SYNTHESIS AT MOLECULAR LEVEL*

The immune system produces a number of receptors for the recognition of various pathogens and it also minimizes the expression of receptors that recognize the self-antigens. The receptor can differentiate between the small structural differences in the structure of the antigen. Susumu Tonegawa won Noble prize for physiology or medicine in 1987 for his work on "genetic principle for generation of antibody diversity. His discovery challenged the concept of one gene encoded one polypeptide. Tonegawa with his colleagues showed that the light chain of antibody is encoded by three families of gene segments present in the germ line. they demonstrated that two segments of DNA, one from each family, are conjoined, only in B lymphocytes to form mature light chain variable regions of the immunoglobulin (Ig) gene, the third segment encodes for the constant region of the gene. Different combinations of these segments result in formation of diverse repertoire of light chain receptor genes. All the receptors genes of B and T cells are assembled from multiple gene segment rearrangements.

As we already discussed, the first 110 amino acids (amino terminal) of light and heavy chains are very variable among different antibody molecules. This variable part is known as variable (V) region and the remaining part is known as constant region (C). Light chains have only four sequence for constant region and heavy chain have only eight sequences for constant region. So, it is seemed that the expressions of both (variable and constant) region are independently

controlled. Also, it is seen that the same antibody variable region is attached with more than one heavy chain constant region.

Two theories are proposed for explaining the diversity of antibodies.

1) Germ-line theories: This theory proposed that the genetic information of each antibody is encoded, in its entirety, within the germ-line genome. This is not possible because to form so many diverse antibodies large amount of genetic information is required which is more than the total genome of the organism.

   During 1965, William Dreyer and J. Claude Bennett suggested that each heavy and light chains of antibodies are encoded in two separate segments in the germline genome and segments encoding for V and C region are brought together in B- cell DNA to form antibody gene.

2) Somatic hypermutation theory: This theory suggested that, a restricted number of genes for antibody are acted upon by unknown mutational mechanisms in somatic cells to produce a diverse receptor repertoire in mature B-lymphocytes. Despite the fact that somatic cell gene recombination has never been observed, this explanation supports the diversity of antibodies.

Now we know that each antibody is encoded by multiple, germ-line, variable-region gene segments and these segments are differently rearranged in each naïve immune cell to generate diverse primary receptor repertoire. On encountering an antigen, these rearranged genes undergo somatic hypermutation and antigenic selection causing expansion and exquisitely tuned repertoire of antigen specific B cells.

The light chain V region gene segments have a leader sequence and a V segment which encode for 1-97 amino acids. Other segment knows as J segment of the gene encode for 98-110 amino acids. The heavy-chain variable region gene sequence: A leader sequence is separated by heavy chain variable region (V_H) gene fragment, which encode for 1-101 amino acid. Other sequence is heavy chain joining gene sequence (J_H) that determines the sequence of 107-123 amino acids. Additionally, 102-106 amino acid sequence present between V and J sequence is determine by D segment region.

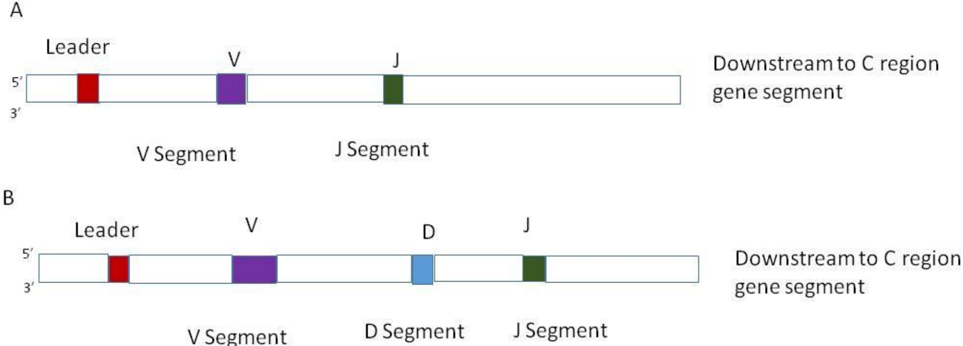

Fig 8.3 A. Light chain V region gene segment

B. Heavy chain V region gene segment

During the development of B cells one V segment and one J segment attached together with the Constant (C) region to form antibody light chain genes. The rearrangement of genes occurs in DNA before transcription.

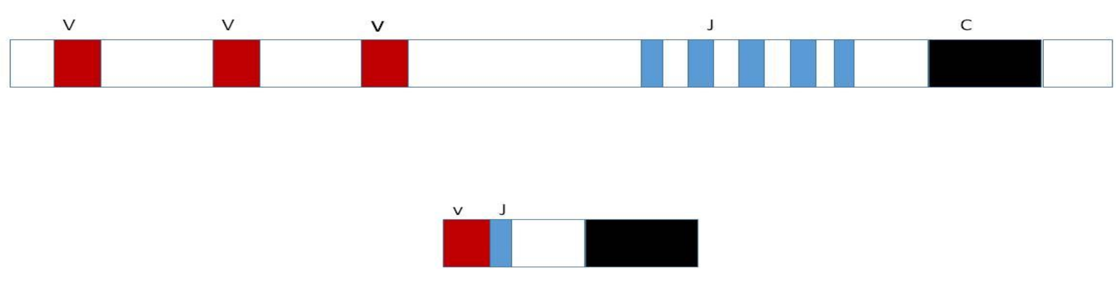

Fig 8.4 Antibody Gene rearrangement

8.8 *SUMMARY*

The disease-causing organisms are called pathogens and the process by which they cause the disease is called pathogenesis. The immune system fights with the infection and protects the infected organism. It has two components: cellular immunity and humoral immunity. Antigens are usually large and complex molecules and not recognized in their entirety by B and T lymphocytes. Instead, lymphocytes (B and T cells) recognize discrete sites on the antigen called antigenic determinants, or epitopes. In a complex antigen, epitopes represent the immunologically active regions. B and T cells bind with the epitope although both the cells recognize antigen differently. T cells recognize antigen (epitope) with the association with an MHC molecule on the surface either an antigen-presenting cell or an altered self-cell, while B cells recognize an epitope alone. B cells are responsible for the humoral immunity and they recognize a large variety of epitopes (surfaces of bacteria or viral particles, and released soluble proteins, glycoproteins, polysaccharides, or lipopolysaccharides by pathogens). To explain the specificity of the immune system two major theories were proposed: the selective theory and the instructional theory. The antibodies are the membrane receptor of B cell (B-cell receptor) and on encounter with the antigen they secrete the soluble form of these specific receptors known as Immunoglobulins (Antibodies). Each antibody is encoded by multiple, germ-line, variable-region gene segments and these segments are differently rearranged in each naïve immune cell to generate diverse primary receptor repertoire. On antigen encounter these rearranged genes undergo somatic hypermutation and antigenic selection causing expansion and exquisitely honed repertoire of antigen specific B cells.

8.9 *TERMINAL QUESTIONS*

Q1. **How many types of antibodies are there?**

A. Five.

B. Three.

C. four

D. two

Q2. The basic Ig unit is made up of:

A. 2 identical heavy and 2 identical light chains.

B. 2 identical heavy and 2 different light chains.

C. 3 different heavy and 3 identical light chains.

D. 2 different heavy and 2 different light chains

Q3. The specificity of an antibody is because of

A. Its valence

B. The light chains

C. The Fc portion of the molecule

D. The variable portion of the heavy and light chain

Q4. Which of the following statements is true about hapten?

A. It induces low immune response

B. It needs carrier to induce immune response

C. It is a T-independent Antigen

D. It has no association with MHC

Q5. For specific antigen recognition by T cells,

A. antigen is bound by a T cell membrane antibody

B. denaturation of antigen does not reduce epitope recognition

C. antigen exposure during T cell maturation is required

D. MHC molecules are not required

ANSWERS 1 (A), 2(A), 3(D), 4(B), 5(B)

Q6. Write a Short note on

A) Haptens
B) Antigenic determinants, or epitopes.

C) Antigens

D) Theories of antibody formation

Q7. Describe the structure and function of various immunoglobulin.

Q8. Describe immunoglobulin's synthesis at molecular level.

8.10 *REFERENCES*

1) Kuby Immunology by Judith A. Owen, Jenni Punt, Sharon A. Stanford (Seventh Edition) W.H. Freeman and Company New York
2) Lydyard, P.M., Whelan, A. and Fanger, M.W., (2004). Immunology. Garland Science/BIOS Scientific Publishers Limited

Unit 9 *IN VITRO* AND *IN VIVO* REACTIONS

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9.1 Objectives

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9.1 *OBJECTIVES*

After studying this unit learns will learn about:

- *In vitro* and *In vivo* reactions involved in various immunological functions.
- Reactions related to antigen-antibody interaction, i.e., precipitation and agglutination
- Biological activity of complement fixation and its role in cytolysis
- mechanisms of different *In vivo* reactions involved in triggering hypersensitivity

9.2 *INTRODUCTION*

In Latin, *In vivo* describes something "within a living organism" while *In vitro* describes something "in glass" that occurs outside the body of living organism in a test tube or petri dish. In biology, *in vivo* reactions are those reactions which generally take place within the living organisms, like performing experiments in an animal model, or in a human being during clinical trial. These reactions involve all naturally occurring mechanisms in a living organism. In the present chapter, processes like phagocytosis, hypersensitivity, complement fixation and cytolysis are described as *in vivo* reactions to understand their role in immunomodulation.

In vitro reactions refer to interactions of cells, tissues or other biological components that have been removed from the living organism(s) of interest. These are very important in studying different biological mechanisms under controlled and isolated environment. For example, before an experimental drug is studied *in vivo*, it is important to evaluate its mechanism of action and complexity thoroughly by means of *in vitro* reactions using *in vitro* models. In this chapter, precipitation and agglutination, *in vitro* interactions between antigen and antibodies are also described. In precipitation, antibody and soluble antigen interacting in aqueous solution forms a lattice that eventually develops into a visible precipitate whereas agglutination is a visible clumping resulting from the interaction between antibody and a particulate antigen**.**

9.3 *PHAGOCYTOSIS*

Despite the strong non-specific innate defenses of our protective epithelial layers, many pathogenic microbes enter inside the body through wounds and animal bite. After they enter, a set of specialised membrane receptors and proteins begin identifying the pathogen's microbial components and activate effective defense against it As a part of the innate immune system,

phagocytes such as macrophages, neutrophils, and dendritic cells in the tissues as well as monocytes in the blood carry out the cellular eating or phagocytosis process to get rid of invasive pathogens.As a component of innate immune system, phagocytes such as macrophages, neutrophils and dendritic cells in tissues and monocytes in the blood carry out the process of cellular eating, or phagocytosis, of intruder pathogen to eliminate it. The process of phagocytosis is seen in both invertebrates and vertebrates. In 1880s, Elie Metchnikoff first described the process of phagocytosis similar to leucocytes using cells from star fish (an echinoderm) and concluded that phagocytosis plays a major role in immunity. It was found that defects in phagocytosis lead to severe immunodeficiency.

Among phagocytes, macrophages are the most common types in tissues which recognize microbes and engulf them as endosome called phagosome by extending their plasma membrane. Microbes are then killed and degraded by hydrolytic enzymes of lysosome. Other common types of phagocytes of vertebrate body are neutrophils and dendritic cells (major component for initiating adaptive immune responses).

9.3.1 RECEPTORS OF PHAGOCYTES INVOLVED IN RECOGNITION OF MICROBES AND PHAGOCYTOSIS:

Phagocytes recognize microbes through variety of receptors found on their surfaces, these are-

(A) **Pattern Recognition Receptors (PRRs)-** Receptors which directly recognize specific conserved molecular components called **pathogen associated molecular patterns (PAMPs)** or **microbe associated molecular patterns (MAMPs) are** present on the microbial surfaces. Examples of PAMPs include: cell wall components of bacteria and fungi, complex carbohydrates such as mannans and beta glucans, LPS, other lipid containing molecules, peptidoglycans, viral proteins and surface proteins.

Examples of PRRs: C-type lectin receptors (CLRs) for example, mannose receptor, Dectin-1 and scavenger receptors for example, SR-A and SR-B.

(B) **Toll-like receptors-** These are named after a similar receptor found in fruit flies encoded by the Toll gene. Toll-like receptors of phagocytes recognize specific bacterial molecules or PAMPs and activate the immune response.

(C) **Antibodies**- Some antibody producing immune cells recognize specific parts of microbial components as antigens and generate phagocytosis.

(D) Opsonin Receptors- By identifying soluble phagocytosis-enhancing proteins called opsonins that have attached to microbe surfaces through a process known as opsonization (from the Greek for "to make tasty"), some receptors can indirectly trigger phagocytosis. Opsonins also activate phagocytosis by their binding to PAMPs, hence sometimes referred to as soluble pattern recognition proteins.

Examples of Opsonins and Opsonin receptors –

- **Opsonins-** Surfactant collectin proteins, SP-A and SP-D, Mannose binding lectin (MBL), L-ficolin associated with microbial acetylated sugars and the complement component C1q as opsonins (found in blood as well as in mucosal secretions of lungs and other parts of body).
- **CD91-Opsonin receptors-** These are found on surfaces of alveolar and other macrophage populations. Due to structural similarities in different opsonins, CD91 opsonin receptors recognize SP-A and SP-D, MBL, L-ficolin and the complement component C1q and promote phagocytosis.

Phagocytic receptors of macrophages also contribute towards cell cleaning that is to take up and clear cellular debris, dead cells (either due to necrotic cell death or from apoptosis i.e. programmed cell death).

9.3.2 MECHANISMS INVOLVED IN KILLING AND DEGRADATION OF PHAGOCYTOSED MICROBES

Binding of microbes to the surface receptors of phagocytes initiate polymerization of actin through signaling. This causes formation of pseudopodia by the evagination of membrane of phagocytes around the microbe particles and their engulfing by forming phagosomes. In macrophages, the phagosomes fuse with lysosomes whereas in neutrophils, with preformed primary and secondary granules and form phagolysosomes. Antimicrobial agents of phagolysosomes then kill and degrade the engulfed microbes. After degradation, undigested microbe particles are either released from cell surface of phagocytes by exocytosis or presented on cell surface through MHC molecules for activating adaptive responses (Fig. 9.1).

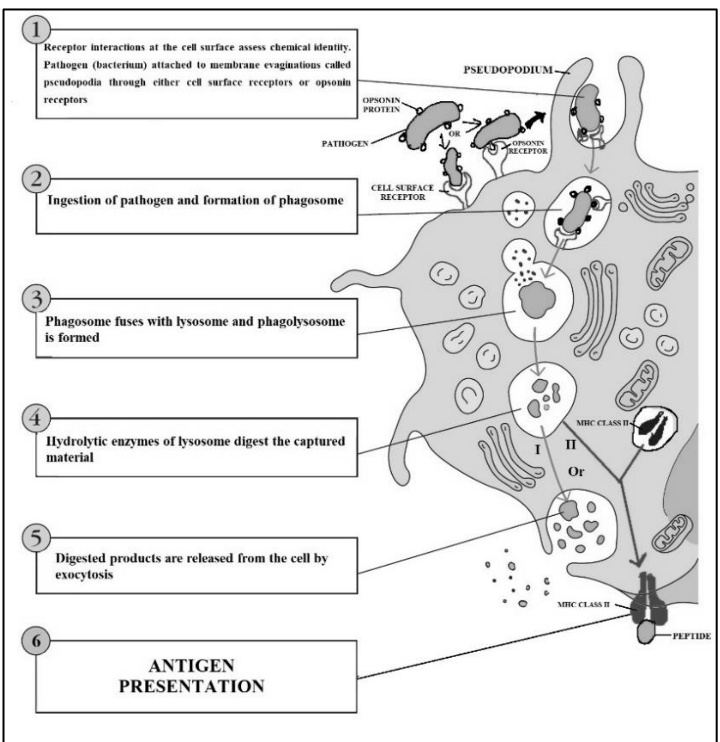

Fig. 9.1 Schematic diagram of the steps in phagocytosis of a pathogen showing different fates of degraded foreign peptides (I) Exocytosis (II) Antigen presentation (Image source: Owen et. al. 2015, Book- Kuby Immunology).

9.3.2.1 Major antimicrobial agents of phagolysosomes involved in degradation of microbes are:

a. Antimicrobial proteins and peptides (defensins and cathelicidins)

b. Low pH

c. Acid activated hydrolytic enzymes (lysozyme and proteases)

d. Specialized molecules related to Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) that mediate oxidative attack

9.3.2.2 Mechanism of oxidative attack:

Degradation of intracellular components of phagocytosed microbes occurs in various phagocytes such as neutrophils, macrophages and dendritic cells by Oxidative attack. This process is mediated by specialized highly toxic molecules of ROS and RNS.

- **Examples of Reactive Oxygen Species (ROS):** Superoxide ion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO).
- **Examples of Reactive Nitrogen Species (RNS):** Nitric acid (NO), peroxynitrite ($ONOO^-$) and S-nitrosothiols (RSNO).

A unique enzyme found in phagocytes is NADPH oxidase, also called phagosome oxidase, which generates ROS during the process of phagocytosis. Generation of ROS by NADPH oxidase is supported by several fold increase in oxygen uptake by phagocytes through a metabolic process known as the **respiratory burst**. NADPH oxidase reacts with oxygen to form superoxide ions ($^{\cdot}O_2^-$) whereas other ROS such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO) are generated by the action of additional enzymes.

The generation of RNS occurs by the action of enzyme nitric oxide synthase (iNOS, or NOS2). iNOS oxidizes L-arginine to produce L-citrulline and nitric acid (NO), a potent antimicrobial agent. Nitric acid (NO) combines with superoxide ions, generated by NADPH oxidase, to form additional molecules as RNS, peroxynitrite ($ONOO^-$) and toxic S-nitrosothiols. ROS and RNS produces toxic effects to phagocytosed microbes by degrading their molecules through various chemical reactions such as oxidation, hydroxylation, chlorination, nitration, S-nitrosylation and destruction of iron-sulfur clusters in proteins.

9.4 *PRECIPITATION AND AGGLUTINATION*

9.4.1 PRECIPITATION REACTIONS

In vitro reactions, are commonly utilized in many immunological assays, in which soluble antigens and antibodies interacts in an aqueous solution and forms a crosslinked lattice structure, that can be visualized as precipitate known as **precipitation reaction**. Interacting antibodies are termed as **precipitins**. In precipitation, formation of antigen-antibody (Ag-Ab) complex occurs immediately after adding these two but conversion of this complex into a visible precipitate is very slow and generally takes one or two days.

Requirements for proper Ag-Ab precipitation are:

(a) Precipitation depends on valency of both antibody and antigen.

i. The antibody must be **bivalent**, Ab with monovalent Fab fragments will not form precipitate.

ii. The antigen must be either **bivalent** or **polyvalent**; that is, antigen must have at least two copies of the same epitope, or have different epitopes that react with different antibodies present in polyclonal antisera.

(b) Antigen contains multiple, distinct epitopes but only a single copy of each epitope, therefore, it precipitates well with specific polyclonal antibodies but fails to precipitate with specific monoclonal antibodies as it cannot form a crosslinked lattice structure with monoclonal antibodies (Fig. 9.2).

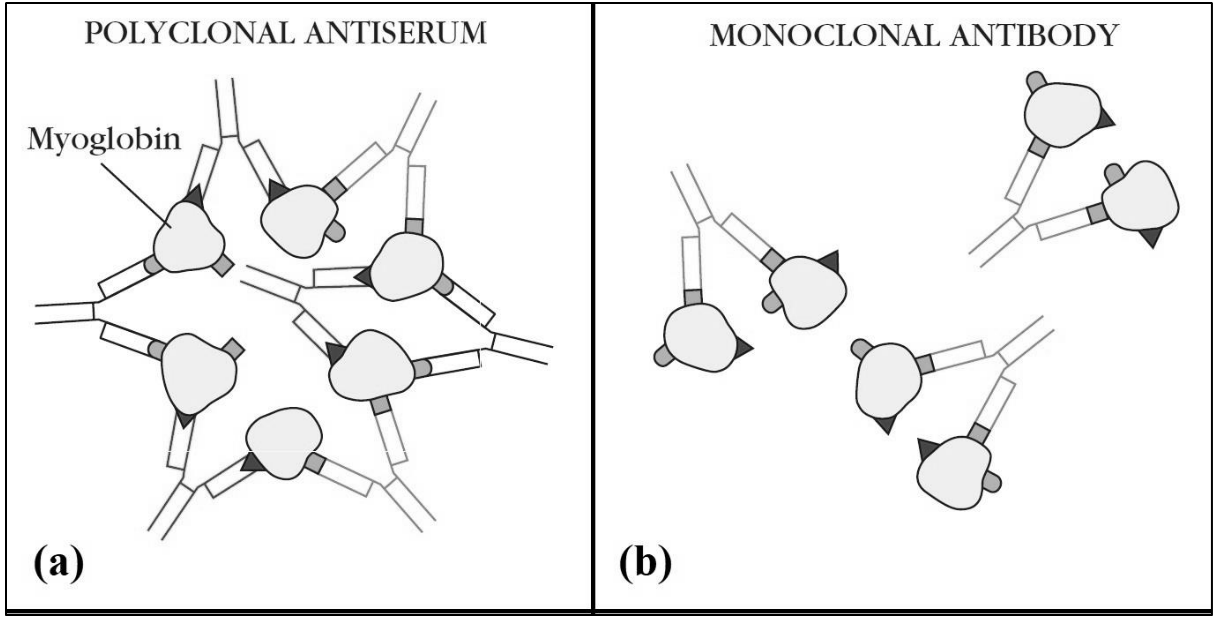

Fig. 9.2 Precipitation reactions. (a) Polyclonal antibodies form lattice or large aggregates with antigens (b) Monoclonal antibodies recognize only a single epitope of antigens can link only with two molecules of antigens and resist precipitation (Image source: Goldsby et. al. 2002, Book- Immunology).

9.4.1.1 Precipitation Reactions in Fluids

For quantitative measurement of the amount of antigen or antibody present in a sample of interest, precipitation reaction can be performed by placing a constant amount of antibody in a series of tubes and adding increasing amounts of antigen to the tubes. After precipitation, each tube is centrifuged, the supernatant is poured off and the amount of precipitate of each tube is measured. Plotting the amount of precipitate against increasing antigen concentrations yields a **precipitin curve**. With the help of this curve, antigen concentrations of unknown samples can be estimated by measuring the amount of precipitates. In precipitin curve, there is an **equivalence zone** which represents the optimal precipitation. Excess of either antibody or antigen do not form extensive lattices and ultimately inhibited precipitation.

9.4.1.2 Precipitation Reactions in Gels

Immune precipitation reactions can also be performed in an agar gel in which antigen diffuses into the antibody-containing matrix and forms a visible line of precipitation. It is also termed as **Immunodiffusion**. In this reaction, precipitation line is generally formed in the region of equivalence having optimal antigen-antibody ratio. Similar to precipitation reaction in fluids, precipitation is inhibited in regions with antibody excess or antigen excess. Immunodiffusion process helps in determining the relative concentrations of antibodies or antigens, the relative purity of an antigen preparation and also in comparing antigens.

There are two types of immunodiffusion reactions:

(a) **Radial immunodiffusion** (the Mancini method)- In this type, antigens of an antigen sample placed in a well are allowed to diffuse into agar matrix containing a suitable dilution of an antiserum. Due to this reaction, a **precipitin ring** is formed around the well at the region of equivalence (Fig. 9.3a). The area of the precipitin ring is proportional to the concentration of antigen. A standard curve obtained between area of precipitin and known concentrations of antigen helps in estimation of the concentration of any antigen sample.

(b) **Double immunodiffusion** (the Ouchterlony method)- In this method, both antigen and antibody are kept in their respective wells in an agar plate. From wells, both antigen and antibody diffuse radially towards each other and precipitate as a **precipitin line** at the region of equivalence (Fig. 9.3b).

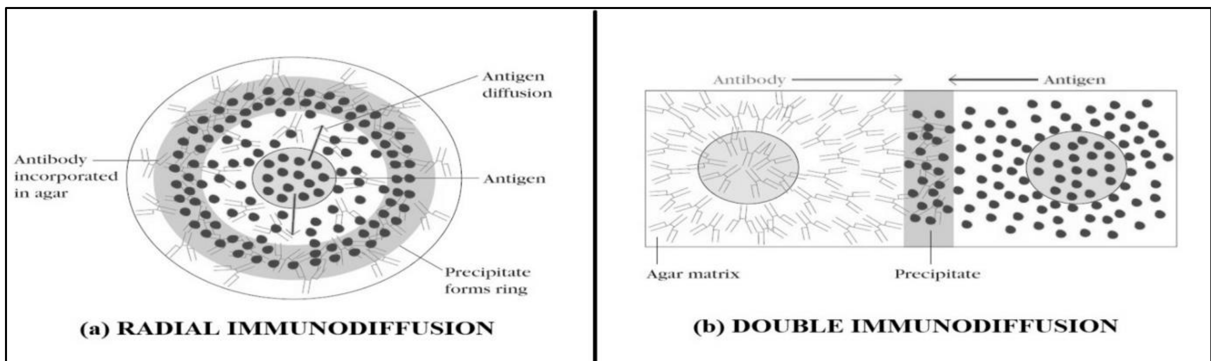

Fig. 9.3 Diagram showing process of (a) radial immunodiffusion (Mancini method) and (b) double immunodiffusion (Ouchterlony method) (Image source: Goldsby et. al. 2002, Book- Immunology).

Specialized double immunodiffusion technique, is coupled with electrophoresis technique, is called **immunoelectrophoresis.** In this technique, components of an antigen mixture are first electrophoresed to separate by their charge. An antiserum is then added to the troughs, made by cutting the agar gel parallel to the direction of the electric field. Antibody and antigen then diffuse toward each other and form lines of precipitation at the region where they bind in appropriate

proportions (Fig. 9.4). It is a qualitative technique employed in clinical studies to detect the presence or absence of proteins and also their production rates in the serum.

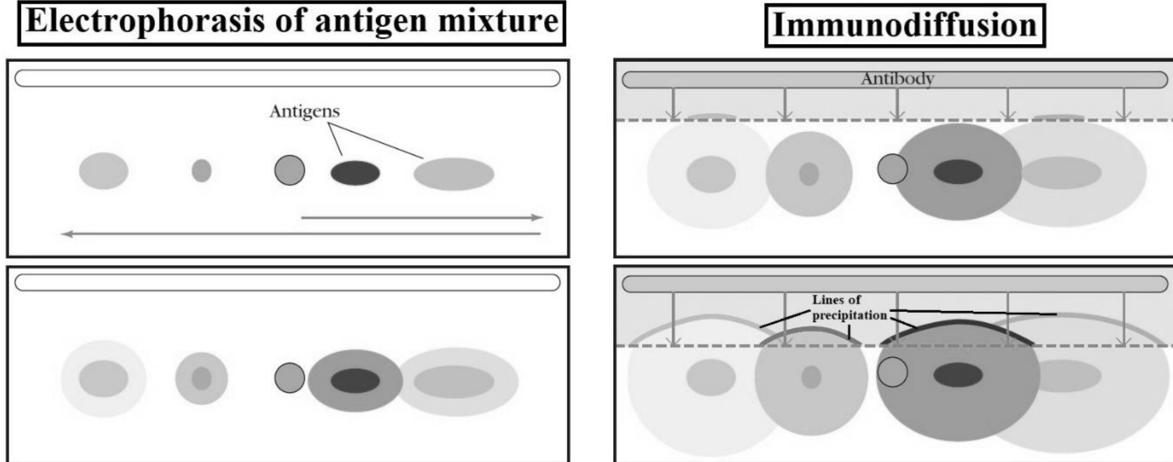

Fig. 9.4 Diagram showing two step Immunoelectrophoresis of an antigen mixture (a) Electrophoresis of antigen mixture (b) Immunodiffusion and formation of Ag-Ab complex at lines of precipitation.

9.4.2 Agglutination Reactions

Another type of *in vitro* immune reactions in which antibody interacts with a **particulate antigen** (not with soluble antigens as in precipitation) and form a visible clumping is called **agglutination.** In Latin, the word ***agglutination*** means ***agglutinare*** (gluing to) therefore in other words **agglutination** is the clumping or aggregation of particles. Antibodies that are involved in such reactions are called **agglutinins.**

Similar to the principles of precipitation reactions, agglutination reactions also depend on the crosslinking of polyvalent antigens. Like precipitation reaction, excess of antibody also inhibits agglutination reactions by a phenomenon called **prozone effect**. It is a common effect seen in many types of immunoassays. Mechanisms which can cause the prozone effect are:

(a) At high concentrations of antibody, number of epitopes may increase by the number of antibody binding sites. Due to this, most antibodies bind antigen only univalently instead of multivalently. Univalent binding of antibodies inhibits agglutination as it prevents the crosslinking of one antigen to another and hence cause prozone effect.

(b) A polyclonal antiserum may contain high concentrations of **incomplete antibodies** (mainly of the IgG class) which can bind to antigens but are unable to induce agglutination. They also

inhibit agglutination by the complete antibodies or IgM (a good agglutinin), added subsequently. Such antibodies are therefore also called **blocking antibodies**. Examples of these types of antibodies include Anti-Rh antibodies and anti-brucella antibodies. At high concentrations, these incomplete antibodies instead of binding to complete antibodies, most of the antigenic sites are occupied by incomplete IgG, which prevent agglutination. Incomplete antibodies lack agglutinating activity due to having restricted flexibility in their hinge region which make difficult for them to cross-link epitopes on two or more particulate antigens.

9.4.2.1 Methods and applications of Agglutination

Agglutination tests are considered the most sensitive tests available for clinical diagnosis of wide range of non-infectious immune disorders and infectious diseases. Additionally, agglutination reactions are easy to perform as a test in a variety of applications in which serum or other body fluids are used for the detection of both antigens and antibodies. Various factors which are responsible for the quality of result in these tests are the time of incubation with the antibody; amount and type of the antigen and conditions of the test environment (e.g., pH and protein concentration). Common methods of agglutination employed in diagnostic immunology are:

(a) **Latex agglutination**- It is used for detecting the presence of a desired antigen or antibody molecules with the help of latex beads (particles) immobilized with antibodies or antigens respectively. For example, to detect an antigen in a test specimen, latex beads conjugated with respective antibodies are added. If antigen is present in test sample, it will bind to the antibodies of latex beads and form a visible, cross-linked aggregate. Similar test can be performed for antibody detection by using latex beads bound with antigens.

(b) **Direct bacterial agglutination-**This test uses whole pathogens, e.g., Bacteria, as a source of antigen. It estimates the antibody level produced in a host infected with that disease-causing pathogen on the basis of visible, cross-linked clumps formed by the binding of antibodies to surface antigens on the bacteria.

For example, the Widal test is a reaction that involves agglutination of typhoid causing bacteria (*Salmonella typhi*) when they are mixed with serum of person infected with typhoid.

(c) **Hemagglutination**- Agglutination reactions resulted from clumping of antibodies with cross lining red blood cells (RBCs) are called **hemagglutination.** In this reaction, erythrocytes function as the biological carriers of bacterial antigens, and purified polysaccharides or proteins for detecting the presence of corresponding antibodies in a specimen sample. The most

common application of hemagglutination is the **blood typing** i.e. the detection and matching of blood group types in donor and recipient during blood transfusions. In addition to this presence of antibodies in virus infected persons has also been detected by a process called **viral hemagglutination**. For example, if a person is infected by a viral disease, such as measles, his serum will contain antibodies to neutralize the activities of the virus. In normal conditions, hemagglutination occurs when virus particles and RBCs are mixed (Fig. 9.5a). However, absence of hemagglutination reaction on mixing serum of diseased person with RBCs represents the presence of antibodies in the serum (Fig. 9.5b). Inhibition of hemagglutination occurs due to the neutralization of the viruses by specific antibodies. It is considered a positive test result for the presence of virus-specific antibodies. Diagnostic tests for influenza, mumps and other viral infections are also based on hemagglutination inhibition tests.

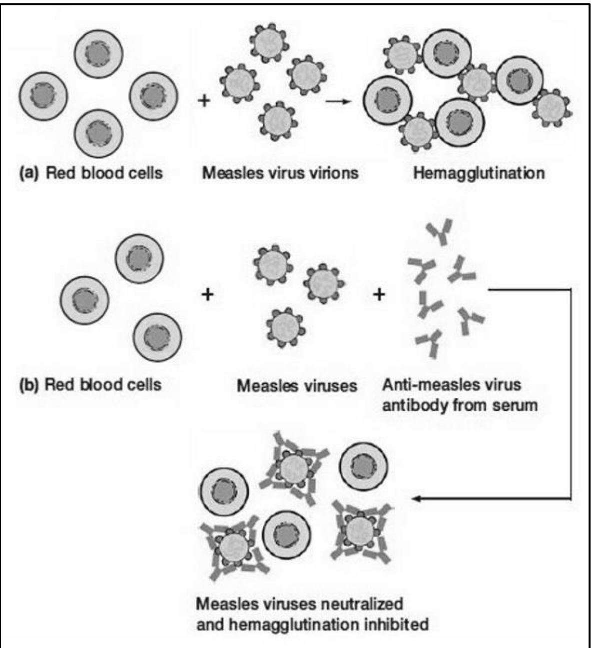

Fig. 9.5 Viral hemagglutination (Image source: Kumar V., 2021)

(d) **Agglutination inhibition**- A modification of the agglutination reaction, provides a highly sensitive assay for small quantities of an antigen. For example, one of the early types of ELISA based home pregnancy test kits included latex particles coated with human chorionic gonadotropin (HCG) as hapten carrier conjugate and antibodies to HCG (Fig.9.6). During pregnancy test, absence of agglutination in kit shows the neutralization of HCG present in the urine taken from women by anti-HCG antibodies of kit, considered as positive result for pregnancy. However, if the urine of women does not contain HCG, a clumping is seen in latex

as a result of HCG conjugate and anti-HCG antibodies of kit. Similar agglutination inhibition tests are also performed for detection of illegal drugs in the blood of a person.

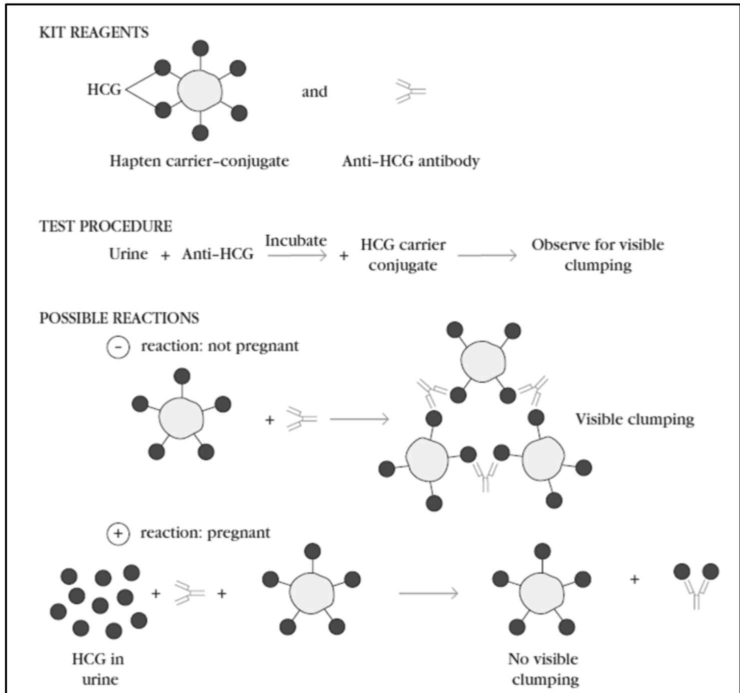

Fig. 9.6 Mechanism of action of traditional home pregnancy test kit utilizing principle of hapten inhibition to determine the presence and absence of human chorionic gonadotropin (HCG) (Image source: Goldsby et. al. 2002, Book- Immunology).

9.5BIOLOGICAL ACTIVITY OF COMPLEMENT FIXATION AND CYTOLYSIS

9.5.1 DEFINITION OF COMPLEMENT SYSTEM:

The complement system, an essential part of body defense system, comprises of a set of heat-labile serum proteins, called **complements**, that interact with one another in catalytic cascades (chain reactions similar to the components of the blood clotting system) to eliminate pathogenic antigens by cooperating components of both the innate and the adaptive immune systems. Once the cascade reactions are triggered, all the complements which generally exist as inactive precursors become active and act as enzymes of these chain reactions. These catalytic cascades of complements ultimately cause the disruption of cell membranes and promote cytolysis.

9.5.2 HISTORY OF COMPLEMENT SYSTEM:

The term "complement system" is derived after experiments performed by **Jules Bordet** in the 1890s at the Institut Pasteur in Paris. He observed that sheep antiserum to the bacterium *Vibrio cholerae* caused lysis of the bacteria and that heating the antiserum destroyed its

bacteriolytic activity (Fig. 9.7a). Surprisingly, the bacteriolytic ability was restored to the heated serum by adding fresh serum that was devoid of antibacterial antibodies (Fig. 9.7b).

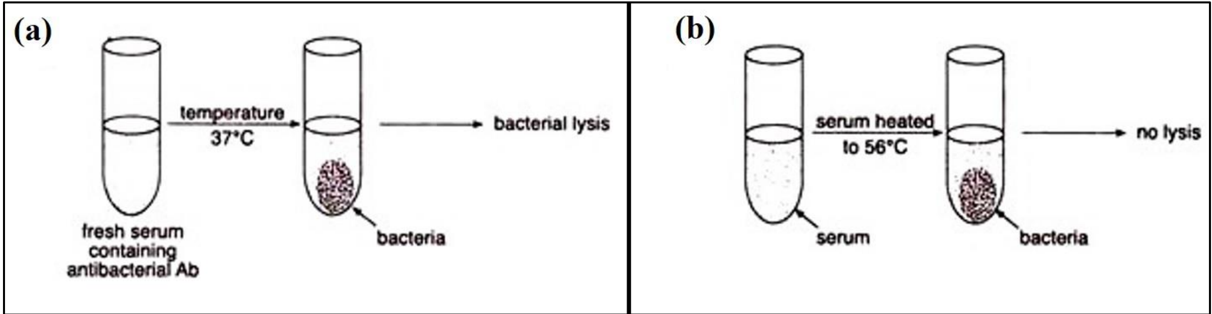

Fig. 9.7 Bacterial lysis experiments performed by Jules Bordet (Image source: Chonkar S., 2016)

On the basis of this finding, Bordet concluded that bacteriolysis needed two different substances:

1. The heat-stable specific antibodies that are bound to the bacterial surface
2. Heat-labile (sensitive) non-specific component responsible for the lytic activity.

Bordet had made efforts to purify the second, non-specific component and used these, along with purified serum fractions, to identify those fractions that cooperated with the antibodies to induce lysis of the red blood cells (hemolysis). The famous immunologist **Paul Ehrlich**, also carried out similar experiments independently in Berlin and coined the term “complement”, defining it as "the activity of blood serum that completes the action of antibody."

Complement system is generally considered as a non-specific innate defense mechanism which involves components of adaptive immune system, i.e., antibodies for its activation. In most of the cases, complement provides the actual protection from the response whereas antigen-antibody interaction only determines the specificity of the response. In other words, antibodies specify the target and complement kills it.

9.5.3 COMPONENTS OF COMPLEMENT:

It was discovered that the action of complement involves the interactions among more than 30 glycoproteins of a complex group, distributed among the blood plasma and cell membranes, considered as complement components. Some of these components are enzymes, some are regulatory molecules, whereas some are structural proteins without any enzymatic activity. Out of these, about 16 are most common with high biological significance. These proteins are designated by the letter “C” and are numbered C1, C2…….C9. Serum also contains factors designated as B,

D, P, H, and I. These components are synthesized in various sites throughout the body such as in liver, monocytes, macrophages, fibroblasts and epithelial cells of the gastrointestinal and genitourinary tracts. Most of the complement components (eg., C3, C6, C8 and B) are produced in the liver by hepatocytes, although some are also produced by other cell types; C1 by epithelial cells of the gastrointestinal and genitourinary tracts, C2, C3, C4, C5, P, D and I by macrophages. Molecular weight of these complements varies from 80,000 Daltons for C9 to 4,00,000 Daltons for C1q. Complement components constitute approximately 15% of the globulin protein fraction in plasma, and their combined concentration can be as high as 3 mg/ml.

Complement products generally assist the humoral immune system of body, they amplify the initial antigen-antibody reaction for generating more effective defense mechanism. Due to continuous cleavage and activation of successive complement proteins, complement fragments bind on the surface of pathogen covalently. Proteolytic cleavage of each precursor of complement protein resulted into two major fragments (Fig. 9.8) i.e., the larger fragment (designated as ‘b’) and the smaller fragment (designated as ‘a’). The larger ‘b’ fragment consists of two biologically active sites, first one is an attachment site for binding cell membranes to the target cell towards the site of activation and the other one is enzyme site for enzymatic cleavage of the next complement component. The smaller ‘a’ fragment are diffusible in nature, these move away from the site and play a role in initiating a localized inflammatory response (chemotactic activity).

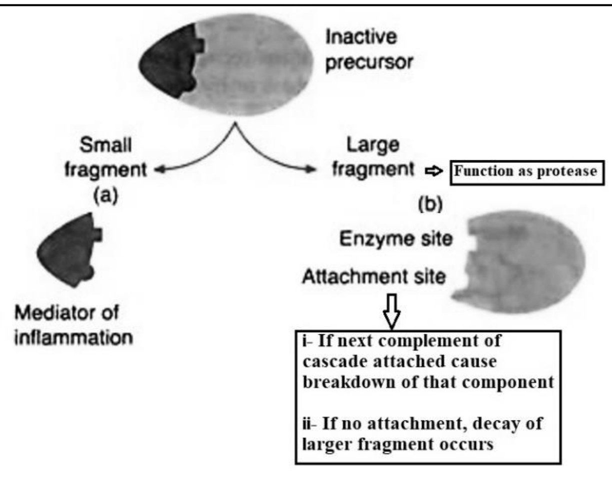

Fig. 9.8 Basic principle of underlying the cleavage of complement components (Image source: Chonkar S., 2016)

9.5.3.1 Complement components can be classified into seven functional categories-

1. Initiator complement components- These proteins bind to their activating ligand (membrane bound molecules), undergo conformational alterations resulting in changes in their biological activity and initiate their respective complement cascades. For example- the Clq complex, Mannose Binding Lectin (MBL), and the ficolins.

2. Enzymatic mediators- It includes several complement components (e.g., Clr, Cls, MASP2, and factor B) which function as proteolytic enzymes that cleave and activate other members of the complement cascade. These proteases are activated either by creating conformational changes through their binding to other macromolecules or by cleavage via another protease enzyme. The two most important enzyme complexes that cleave complement components C3 and C5, respectively, are called the C3 and C5 convertases.

3. Membrane-binding components or opsonins- Upon activation of the complement cascade, several proteins are cleaved into two unequal fragments, the larger fragments serve as opsonins, enhancing phagocytosis by binding to microbial cells and serving as binding tags for phagocytic cells bearing receptors for these fragments. For example, cleavage of C3 and C4 components, the larger fragments, C3b and C4b, serve as opsonins (Generally larger fragments are denoted by letter "b" and smaller fragment by "a").

4. Inflammatory mediators- Some small complement fragments produced during activation of complement activation by proteolytic cleavage act as inflammatory mediators. These fragments enhance the blood supply to the area in which they are released by vasodilation through their binding to receptors on endothelial cells lining the blood capillary. They also attract other cells to the site of tissue damage. Examples include C3a, C5a, and C4a.

5. Membrane attack proteins- Proteins associated with the membrane attack complex (MAC) insert into the cell membranes of invading microorganisms and punch holes that result in lysis of the pathogen. The complement components of the MAC include C5b, C6, C7, C8, and multiple copies of C9.

6. Complement receptor proteins- Membrane-bound receptor molecules are found on the cell surfaces of phagocytes specific for complement components. These are named with "R," such as

CR1, CR2, and C5aR. These bind with complement proteins and initiate signal specific cell functions. For example, complement receptors CR1 bind to complement components such as C3b on the surface of pathogens, triggering phagocytosis of the C3-bound pathogen whereas binding of the complement component C5a to C5aR receptors on neutrophils stimulates neutrophil degranulation and inflammation.

7. Regulatory complement components- These are membrane-bound soluble regulatory proteins which protect host cells from unintended complement-mediated lysis. Examples of these regulatory proteins include factor I, which degrades C3b and Protectin, which inhibits the formation of the MAC on host cells.

9.5.4 BIOLOGICAL FUNCTIONS OF COMPLEMENT SYSTEM:

Different biological functions of Complements are as follows:

1. **Cytolysis:** A Membrane Attack Complex (MAC) assembled from complement proteins directly kills some pathogens by creating pores in microbial membranes. In a sequential manner firstly complement components are activated and polymerized on cell surface. This resulted in the formation of pores on phospholipid membrane of pathogen cells and eventually cause cytolysis by disrupting bilayer (Fig. 9.9).

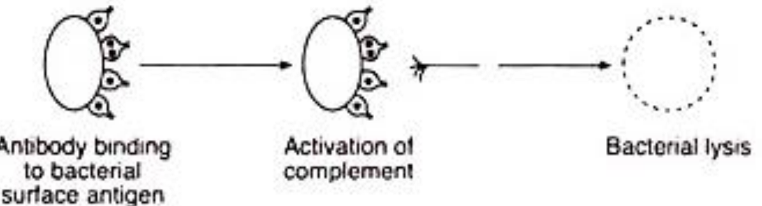

Fig. 9.9 Cytolysis of bacteria (Image source: Chonkar S., 2016)

2. **Opsonization:** Membrane bound complement components also called opsonins may bind and opsonize foreign organisms such as bacteria. Recognition of these opsonins by phagocytic leukocytes (macrophages) through specific receptors leads to receptor-mediated phagocytosis of foreign organisms (Fig. 9.10).

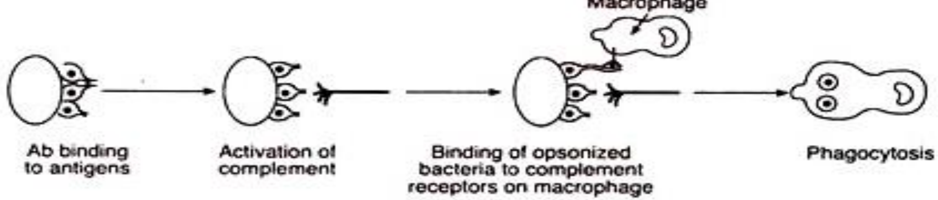

Fig. 9.10 Process of opsonization (Image source: Chonkar S., 2016)

3. **Activation of inflammation:** Complement proteins can also elicit inflammatory responses, interlink components of the acquired immune system, clear immune complexes from the serum, and/or eliminate dying cells. Inflammation is caused by mast cell activation and neutrophil recruitment due to action of certain proteolytic fragments of complement protein.

9.5.5 THE MAJOR PATHWAYS OF COMPLEMENT ACTIVATION

As a most evolutionarily ancient participants in the immune system, complement components in vertebrates have evolved majorly three pathways, that are **classical**, **lectin** and **alternate pathways** for their activation to combat various microbial attack. Although each of these three pathways of complement activation are initiated with different events, they all involved in the generation of an enzyme complex capable of cleaving the C3 molecule into two fragments, C3a and C3b. The enzymes that catalyze this biochemical transformation are referred to as **C3 convertases**. The classical and lectin pathways use the dimer C4b2a for their C3 convertase activity, while the alternative pathway uses C3bBb to cleave the C3 molecule. The final result in all of the pathways is an increase in the concentration of C3b which is a centrally located and multifunctional complement protein. Addition of C3b components help in the formation of **C5 convertases**, the second set of convertase enzymes of the cascade.

A. The Classical Pathway of Complement:

The classical pathway of complement is initiated through the formation of soluble antigen-antibody complexes by direct interaction of antibody with antigen. The classical pathway of complement activation proceeds through following successive stages:

(i) Activation of C1 component

(ii) Production of C3 convertase

(iii) Production of C5 convertase and

(iv) Action of membrane attack complex (MAC)

(i) Activation of C1 component:

At initial stage, formation of soluble antigen-antibody complex induces a conformational change in the Fc (fragment crystalized) region of the antibody molecule that exposes a binding site for the C1 component of the complement system.

Structure of C1 component- C1 is a complex multidomain protein found in serum in inactive condition. Three subunits which constitute this complex protein are C1q, C1r and C1s, out of

which C1q has ability to recognize and bind to the Fc region of the antibody whereas C1r and C1s exists as inactive proteases with their two subunits each. C1q and dimers of each C1r and C1s held together to form a complex called **C1qr$_2$s$_2$** which is stabilized by Ca^{2+} ions (Fig. 9.11).

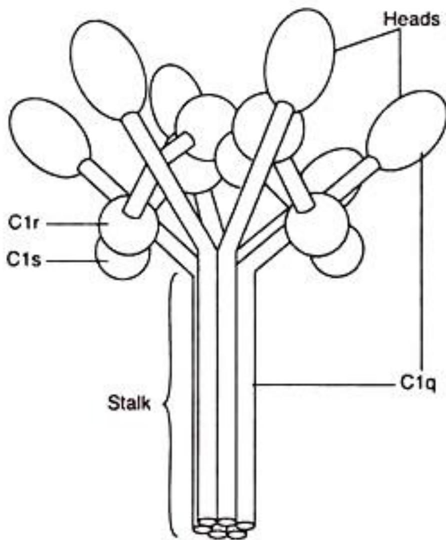

Fig. 9.11 Structure of C1qr$_2$s$_2$

Major subunit in the structure of C1 component is C1q, a large molecule consisting of 18 polypeptide chains associated together to form a structure having six collagen-like triple helical arms. Two third portion of this structure at amino-terminus forms the stalk and remaining one third of polypeptides at carboxy-terminus form the globular flower like structure, which has binding sites for antibody. In normal condition, C1r$_2$s$_2$ complex remains in inactive form with configuration 'S' and avoid binding with C1q. Each C1r and C1s includes two domains named **catalytic domain** and **interaction domain**. Due to action of interaction domain in presence of antigen- antibody complex in the serum C1r$_2$s$_2$ complex binds with C1q.

C1q binds to Fc region of an antibody of antigen-antibody complex by its globular heads. This binding activates serine proteases C1r and C1s, the proteolytic enzymes which release serine residues at the active site after being activated.On binding to antibody, one molecule of C1r becomes enzymatically active byself-cleavage. This activated C1r molecule then cleaves and activates the second C1r and both C1s molecules. On activation C1r$_2$s$_2$ complex shows "8" configuration (Fig. 9.12) and binds with C1q globular flower and active C1 component or C1qr$_2$s$_2$is formed. The activated serine protease C1s binds, cleaves and activates the next two components of the classical pathway, i.e., serine protease C4 and C2.

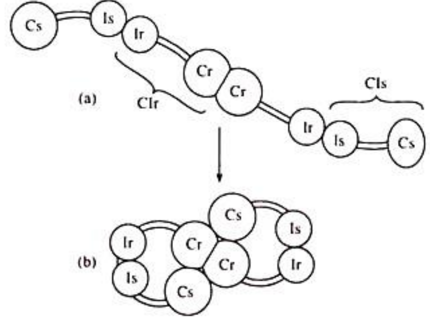

Fig. 9.12 Relaxed and tensed configuration of $C1r_2s_2$ complex (Image source: Chonkar S., 2016)

Activation of classical pathway via IgM and IgG:

Binding of antibodies, either IgM or IgG, to antigenic determinants on pathogen cell surface generally initiates the cascade reaction of complement system. Due to the structural differences between IgM and IgG, activation level of these antibodies varies greatly. When pentameric IgM is bound to antigen on a target surface, it requires at least three binding sites for C1q attachment. However, IgG molecule contains a single C1q binding site in the CH_2 domain of its Fc region. As globular head of C1q requires at least two Fc sites for a stable C1-antibody reaction, two IgG are required to be present on a target surface. Therefore, at the activation of Clq binding, IgG requires less amount of time but a good number of IgG molecules whereas IgM activation is time consuming one but more efficient, even a single IgM molecule can initiate the process (Fig. 9.13).

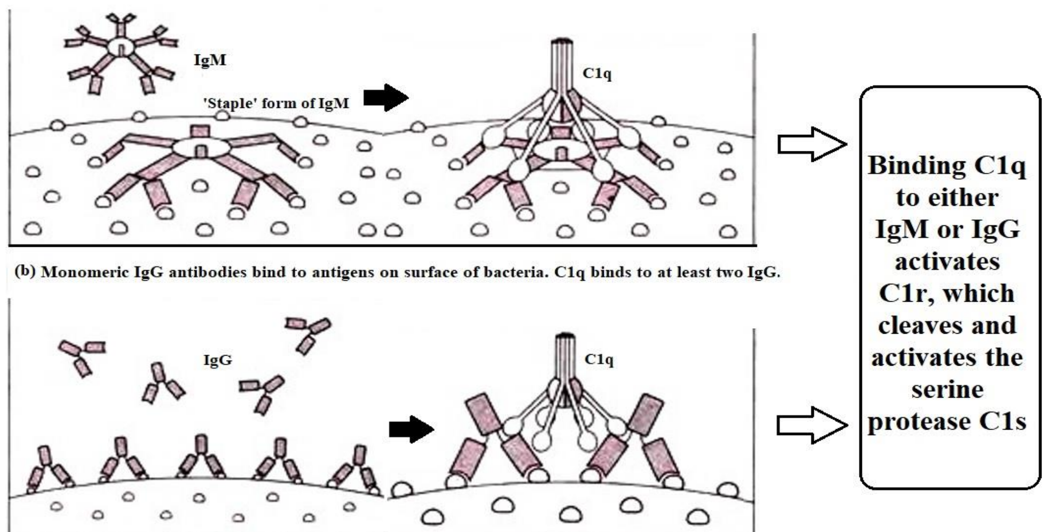

Fig. 9.13 The classical pathway of complement activation is initiated by binding of C1q to antibody (IgM and IgG) on a bacterial surface (Image source: Chonkar S., 2016)

(ii) Production of C3 convertase:

Active C1 component also known as **serine protease enzyme,** $C1qr_{2}s_{2}$, has two distinct substrates, C4 and C2. C4 component is a large globular glycoprotein containing three polypeptide chains named α, β and γ. Activation of C4 is done when C1s hydrolyses a small fragment C4a from the amino terminus of the chain, exposes a binding site on the larger fragment C4b. The C4b fragment attaches to the target surface of the C1 component bound to antibody on the pathogen surface. Additionally, the activated C1s protease acts on C2 serine protease, which cleaves C2 into larger C2a and smaller C2b fragments. The smaller fragment C2b will be cleaved away from the site of action and C2a larger fragment will remain active at the active site. By the above two reactions, C4b2a active complex is formed which in turn acts on the substrate C3 component. C4b2a is called C3 convertase of the classical pathway.

(iii) Production of C5 convertase:

Complement component C3 (very similar to C4) has two types of polypeptide chains, i.e., α and β. C3 convertase (C4b2a) helps to cleave the smaller fragment C3a from the amino terminus of the α chain of C3 component leaving active C3b at the site of action. C3 convertase is highly efficient enzyme in amplifying the amount of C3b. Even a single C3 convertase molecule can accelerate the formation of more than 200 molecules of C3b which eventually bind with C4b2a to form a tri-molecular complex called C4b2a3b, also called C5 convertase.

(iv) Action of membrane attack complex (MAC):

C4b2a3b or C5 convertase acts on C5 complement component, cleaves C5a from the action site and C5b attaches to the antigenic surface. This bound C5b initiates formation of membrane-attack complex (MAC) by associating with C6, C7, C8 and C9 components gradually and ultimately forms C5b6789 (MAC) which accelerates lysis of cell membrane of antigen by making a large pore in its lipid bilayer (Fig. 9.14).

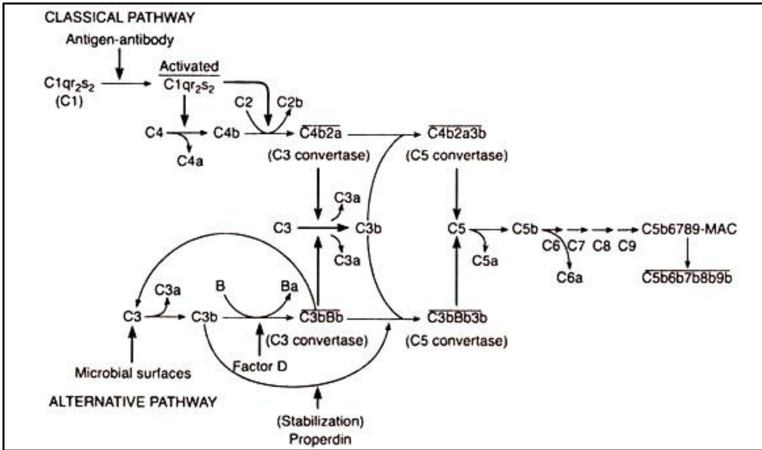

Fig. 9.14 The overview of complement activation pathways (Classical and Alternate) (Image source: Chonkar S., 2016)

B. The Alternative Pathway:

In addition to the classical pathway, complement system can also be initiated by another method called alternative pathway. In contrast to classical pathway, the alternative pathway is initiated by the cell-wall constituents of both gram-positive and gram- negative bacteria as antigens. Microbial surfaces directly affect the serine protease C3 and gradually cleave C3 into C3a and C3b. This conformational change extends its effect on another factor i.e. factor B which is cleaved into Ba and Bb. Fragment Ba is removed from active site whereas Bb associates with the C3b in presence of Mg^{++} and forms C3bBb. This C3bBb is considered as the C3 convertase of alternative pathway.

Binding of C3b exposes a site on factor B that again serves as the substrate for an enzymatically active serum protein called factor D. actually, factor D cleaves the C3b bound factor B, and helps to form C3bBb. In the alternate pathway, the action of unstable C3bBb becomes stabilized with increased convertase activity period by the presence of another exclusive serum protein **properdin**. Formation of C3bBb accelerates auto-catalysis of more C3 component and forms C3bBb3b as C5 convertase. Even after having different structural constituents of C3 and C5 convertase in these two pathways of complement system, their mode of action is same.

In alternate pathway, C3bBb3b, subsequently associated with C5, C6, C7, C8 and C9 respectively, results in Membrane Attack Complex (MAC) formation which binds to the antigenic surfaces of microbes (antigen) (Fig. 9.14). MAC gradually displaces the membrane phospholipids, forms a large trans-membrane channel and gradually destroys the membrane and lysis of the antigen occurs.

C. The Lectin mediated pathway:

The third pathway of complement system is lectin-mediated pathway. This pathway is activated by the binding of mannose-binding protein (MBP) present in blood plasma to mannose containing proteoglycans on the surfaces of the bacteria and yeast, it forms MBP-MASP (Mannose-binding protein-mannose-associated serum protease). In lectin pathway, MBP-MASP acts on the substrate C_4 and C_2 component protein. Later cascade reactions are similar to the classical pathway of complement activation.

9.5.6 Deficiencies related to complement

Generally, deficiency of complement components results in lack of resistance to various infection and low susceptibility to infection. Most severe deficiency related complications occur in individuals deficient in C3. Deficiency in C1 inhibition causes **Hereditary angioneurotic edema**, deficiency of C2 and C4 results in **Systemic lupus erythematosus (SLE)**, whereas, persons who lack reactions related to C3 and C4 activation show symptoms of **Recurrent polycoccal infections.**

9.6 *MECHANISM OF IN VIVO REACTIONS AND HYPERSENSITIVITY*

Immune system generally protects the host organism from invading foreign particles, called antigens, through triggering different responses without causing any harm to the host. The effector molecules eliminate antigens through mediating immune responses by inducing local inflammatory reactions. However, under some circumstances, the inflammatory responses induce adverse *in vivo* reactions which cause tissue damage or even death. This over reactivity by the immune system to some antigens is referred to as **hypersensitivity**. In other words, ***hypersensitivity*** *is increased reactivity or increased sensitivity by the animal body to an antigen to which it has been exposed previously*. Hypersensitivity is either antibody mediated or cell mediated. Hypersensitivity reactions are highly variable in their timing of occurrence after coming into contact with the antigens. On this basis, these are divided into two types:

(a) **The immediate type hypersensitivity responses**: These appear very fast within few minutes after contact with antigens. These are humoral immune responses involving B cells and antibodies.

(b) Delayed type hypersensitivity responses: These appear slowly after several hours of contact with antigen. Generally, these type of hypersensitivity reactions are mediated by T cells.

The term **“Allergy”** is often used as a synonym for hypersensitivity (especially for type-1 hypersensitivity), which describes a state of altered reactivity to an antigen. Hypersensitivity has been categorized into several types on the basis of differences in the effector molecules generated during the course of the reaction. This classification also considers the cause of hypersensitivity whether it can be passively transferred by antibodies or by specifically immune lymphoid cells. The most widely adopted current classification is that of **R.R.A. Coombs** and **P.G. Gell** who classified all hypersensitivity reactions into following categories (Fig. 9.15):

(i) Immunoglobulin-mediated (immediate) hypersensitivity reactions- types I, II, and III hypersensitivity

(ii) Lymphoid cell-mediated (delayed-type) hypersensitivity-type IV hypersensitivity

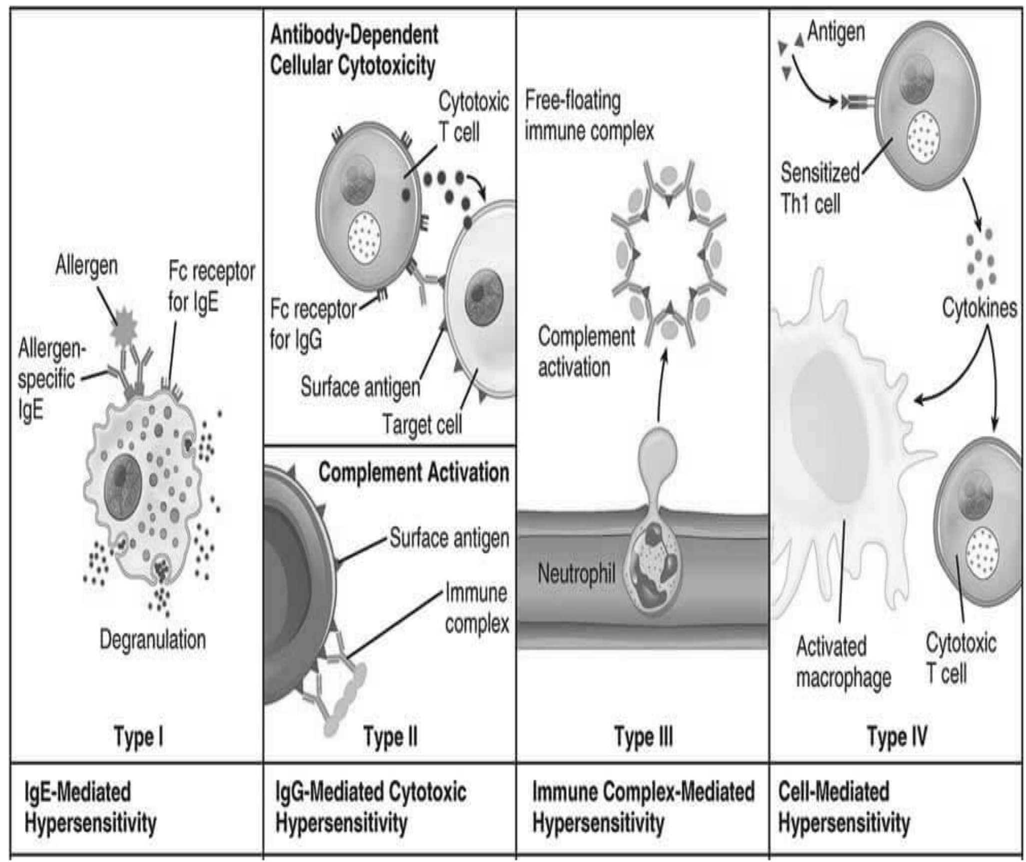

Fig. 9.15 Types of reactions involved in hypersensitivity *(Image Source: Betts J.G. et. al., 2013)*

9.6.1 MECHANISM OF HYPERSENSITIVITY REACTIONS

Hypersensitivity reactions are commonly classified according to the type of immune response and the effector mechanism responsible for cell and tissue injury. These mechanisms include some that are predominantly antibody dependent and others predominantly T cell dependent, however all hypersensitivity mechanisms include components and *in vivo* reactions of both humoral and cell-mediated immunity.

9.6.1.1 IMMEDIATE (TYPE I) HYPERSENSITIVITY

It is caused by IgE antibodies specific for environmental antigens and is the most prevalent type of immediate hypersensitivity, commonly grouped under allergy or atopy. These are often caused by activation of interleukin-4 (IL-4), IL-5, and IL-13 producing Th2 (T helper-2) cells and the production of IgE antibodies. Crosslinking of the bound IgE with specific antigen (allergen) triggers the mast cells and basophils to induce inflammation and release pharmacologically active agents that lead to occurrence of many clinical manifestations such as rhinitis, asthma and in severe cases anaphylaxis.

Some antigens (allergens), such as insect venom, sea foods, beans, milk, pollen, and dust mite, can induce the formation of IgE antibodies in individuals with a corresponding predisposition (Fig. 9.16). In sensitization phase, the IgE antibodies bind to mast cells via Fc receptors. During effector phase, if the individual is re-exposed to the allergen, multivalent antigenic molecules bind with the membrane-bound IgE and crosslink adjacently placed IgE molecules. The bridging of IgE molecules triggers two signal mediated processes, one leading to the discharge of primary mediators (e.g., histamine, kininogen) by degranulation of mast cells (Fig. 9.16) and the other involves de novo synthesis and release of secondary mediators such as arachidonic acid. This immediate release of different types of mediators ultimately induces vasodilation, smooth muscle contraction, mucus secretion, edema, and skin blisters. Most allergens are small proteins and act as active proteases. Due to their small size, they can easily diffuse through the skin or mucosa. Cytokine IL-4 favors the differentiation of Th2 cells. The allergen stimulates the induction of CD4 containing Th2 cells which secrete cytokines that cause IgE production by plasma cells.

Types of primary mediators:

(a) **Biogenic amines-** Histamine and adenosine
(b) **Chemotactic mediators-** Eosinophil chemotactic factor, neutrophil chemotactic factor
(c) **Enzymes-** Proteases and hydrolases (generate kinins)

(d) **Proteoglycans-** Heparin (natural anticoagulant) and chondroitin sulphate. These serve to store the other mediators in the granules.

Types of secondary mediators:

(a) **Lipid mediators-** Leukotrienes and Prostaglandins. Both are derivatives of arachidonic acid.

(b) **Platelet activating factor (PAF)-** It helps in secretion of histamine and have important proinflammatory function.

(c) **Cytokines-** Mast cells secrete IL4, IL5, IL6 and TNF-alpha. IL4 increases IgE production by B cells.

Binding of IgE molecule to the Fc receptor on mast cells and basophils causes vasodilation which increase vascular permeability. This type of hypersensitivity may occur as a systemic or local reaction:

- **Systemic reactions:** skin erythema, followed by respiratory difficulty due to bronchial constriction.
- **Local reactions:** generally seen on the skin or mucosal surface at the site of Antigen exposure. For example, allergy to penicillin, *Aspergillus* spores, rupture of *Echinococcus* cyst etc.

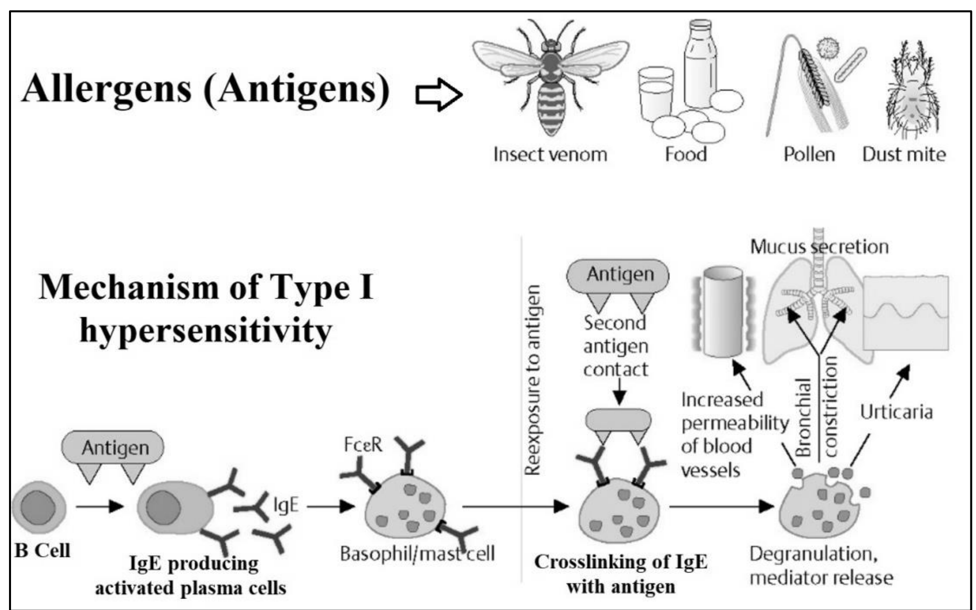

Fig. 9.16 Type I hypersensitivity (Image source: Aryal S., 2022)

9.6.1.2 ANTIBODY-MEDIATED (TYPE II) HYPERSENSITIVITY

Type II reactions are mediated by IgG and IgM antibodies specific for antigens present on the surface of the cells or other tissue components. These reactions cause cell death or tissue injury through antibody activated complement system. The antigens may be intrinsically produced on the cell membrane or may be an exogenous antigen absorbed on the cell surface. In this type, antibodies are formed against target antigens that are cell membrane components therefore instead of hypersensitivity, these are more like cytotoxic reactions.

Different mechanisms are involved in this type of reactions are:

(a) Complement-dependent Mechanisms: In these mechanisms, complement components and antibody direct the lysis and opsonization. It may occur by two ways, in the first pathway, antibody reacts with an antigen present on the surface of the cell causing **activation of the complement system** resulting in the **assembly of the membrane attack complex that causes cell lysis** by drilling holes through the lipid bilayer (Fig. 9.17c).In the second pathway, the **cells become susceptible to phagocytosis by opsonization** that is the **fixation of either antibody or C3b fragment** to the cell surface (Fig. 9.17a and b). Opsonization induced phagocytosis and destruction of the cells is done by macrophages and neutrophils expressing receptors for Fc or C3b fragment.

(b) Antibody dependent cell mediated cytotoxicity (ADCC): In this mechanism, antibody mediated cytolysis does not involve fixation of complement, but instead requires the cooperation of leucocytes. Antigen coated target cells are killed by a variety of leucocytes having receptors for Fc fragments of IgG antibodies bound to antigens without phagocytosis (Fig. 9.17d). ADCC may be mediated by monocytes, neutrophils, eosinophils and NK cells. ADCC is a mechanism of type II hypersensitivity reaction for parasites or tumor cells which are too large to be phagocytosed.

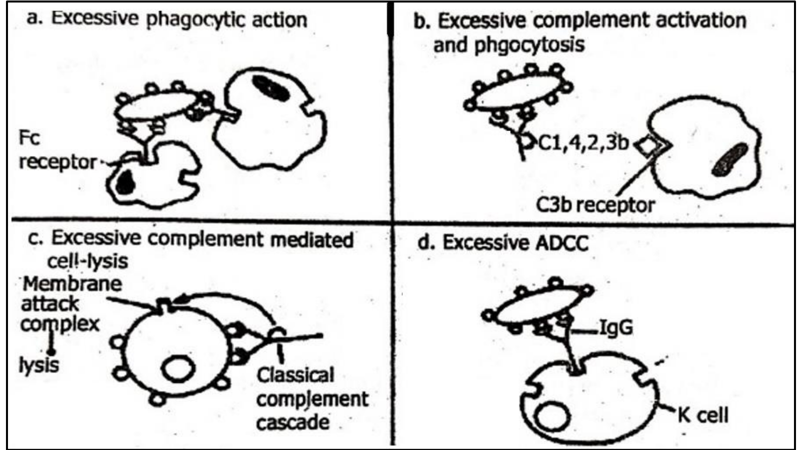

Fig. 9.17 Type II hypersensitivity reactions (Image source: Aryal S., 2022)

Type II reactions are observed in following conditions:

i. **Erythroblastosis fetalis-**The immunization of individuals to erythrocyte antigens during pregnancy is a typical example of a type II reaction, in which there is an antigenic difference between the mother and foetus. Antibodies from the mother cross the placenta and cause destruction of foetal blood cells. Children who inherit the RhD erythrocyte antigen from their father can induce immunization against the RhD+ antigen in their RhD- mother. Sensitization usually occurs at birth when foetal blood cells come into contact with the maternal immune system. In any subsequent pregnancies, maternal anti-RhD antibodies of the IgG type can pass into the placenta and cause severe hemolysis of foetal RhD+ erythrocytes.

ii. **Autoimmune haemolytic anaemia**, **agranulocytosis** or **thrombocytopenia-**In these conditions' individuals produce antibodies to their own blood vessels.

iii. Other examples: Drugs (e.g., penicillin) can passively bind to erythrocytes. Antibodies directed against penicillin then lead to lysis of the erythrocytes.

9.6.1.3 IMMUNE COMPLEX-MEDIATED (TYPE III) HYPERSENSITIVITY

Reactions which are induced by immune complexes i.e., antigen-antibody complexes, are called type III hypersensitivity reactions. During an immune response, IgM and IgG antibodies specific for soluble antigens in the blood form **antibody-antigen complexes (immune complexes**). These immune complexes may deposit in the walls of blood vessels and basement membrane of various tissues such as lungs, kidneys and in the joints (synovia), causing inflammation, thrombosis and tissue injury (Fig. 9.18). Immune complexes can

induce inflammatory processes in these structures by binding complement factors C3a and C5a (anaphylatoxins).

A specific type III reaction is the **Arthus reaction** which includes an acute, localized inflammatory response. In this reaction, when an antigen has made its entry inside the skin of an individual who has pre-synthesized IgG antibodies, the immune complexes are formed. These complexes can bind to Fc receptors of most cells due to which degranulation inflammatory cells are recruited and complement is activated, leading to the release of C5a and local inflammation, platelet accumulation, and eventually to blood vessel occlusion (disruption) with necrosis (Fig. 9.18).

This type shows **two** patterns of immune-complex mediated injury:

A) SYSTEMIC DISEASE (serum sickness type, Systemic Lupus Erythematosus, SLE)

- This is because of abundant number of antibodies, a large number of immune complexes are deposited at the site of injury especially within the wall of blood vessel, the subsequent events will result in **necrotizing vasculitis** (inflammation of blood vessels) and accumulation of neutrophils causing **Systemic Lupus Erythematosus (SLE).**

B) LOCAL DISEASE (Arthus reaction)

- **Arthus reaction:** Intraductal injection of antigens to a personalized person may lead to local intradermal Ab – Ag complex formation and local vasculitis, redness, swelling.
- **Example:** Repeated (booster) vaccination with diphtheria or tetanus rarely leads to local vasculitis.

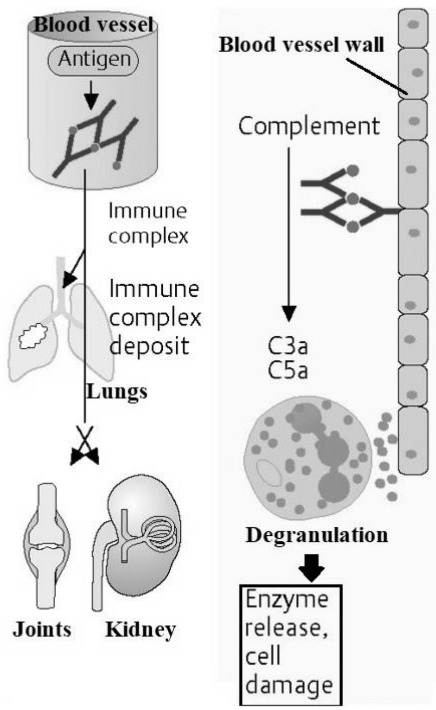

Fig. 9.18 Type III hypersensitivity reactions (Image source: Aryal S., 2022)

9.6.1.4 T CELL-MEDIATED (TYPE IV) HYPERSENSITIVITY

In this type of hypersensitivity reactions, instead of components of humoral immunity, T cells induce immune responses and tissue injury. This T cell induced tissue injury occurs may be due to the T cell mediated inflammation or directly killing of target cells by T cells. In most of these diseases, the major mechanism involves the activation of CD4+ helper T cells, which secrete cytokines that promote inflammation and activate leukocytes, mainly neutrophils and macrophages (Fig. 9.19). Tissue injury promoted by Cytotoxic T cells is less frequent.

Haptens are small molecules with very small molecular weight (often < 1 kDa). Because of their low molecular weight, they are generally non-antigenic, but they can penetrate the epidermis and bind to certain proteins in the skin called carrier proteins. Hapten-carrier complexes are bound by antigen-presenting cells of the skin (Langerhans cells), which then migrate to regional lymph nodes where activation of T-cells takes place. This is called sensitization phase which generally lasts for 10-14 days. Second exposure of the individual to the hapten, stimulate antigen-specific T cells for their migration to the skin where they accumulate and proliferate (Fig. 9.19). They also cause edema formation and local inflammation with the help of cytokines. Generally, compounds containing nickel or chrome and chemicals found in rubber latex are typical triggers of type IV hypersensitivity reactions.

There are **two** patterns of type IV reactions that involve either CD4 or CD8T Cells.

A) Acute (within 2-3 days)

- **Tuberculin test**, **contact dermatitis**: mediated by CD4+ T helper cells.CD4+ T cells recognize antigen (tuberculin) which leads to the formation of sensitized CD4+ T cells.
- Upon cutaneous injection into previously sensitized individual sensitized CD4+ T cells become activated and secrete cytokines.
- **Tuberculin/Mantoux test:** intradermal injection of tuberculin (purified tuberculoprotein) leads to swelling after 48-72 h if the patient has been exposed to *Mycobacterium tuberculosis* previously.

B) Chronic (> 1 week)

- Chronic activation of T cells leads to **Granuloma formation**, graft rejection: mediated by CD8+ cytotoxic T cell.
- lymphocytes surrounding epithelioid cells lead to the formation of granuloma.
-

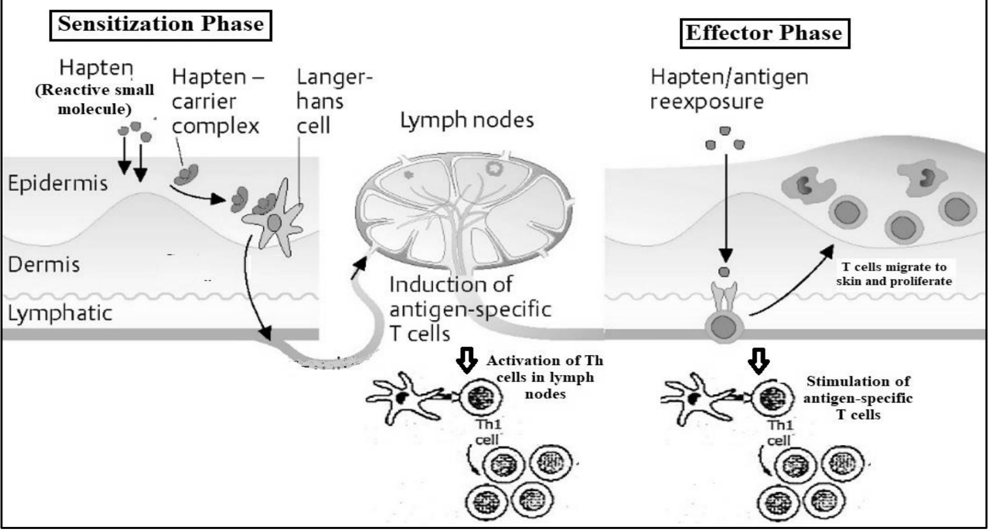

Fig. 9.19 Type IV hypersensitivity reactions (Image source: Aryal S., 2022)

9.7 *SUMMARY*

In biology, *in vivo* reactions are those reactions which generally take place naturally within the living organisms, like phagocytosis, hypersensitivity, complement fixation and cytolysis, whereas, *in vitro* reactions refer to interactions of cells, tissues or other biological components that have been removed from the living organism(s) of interest under controlled, isolated environment. Example of *in vitro* reactions include precipitation and agglutination.

As a component of innate immune system, phagocytosis or cell eating is a process performed by phagocytes such as macrophages, neutrophils and dendritic cells in tissues which engulf intruder pathogen as endosome called phagosome by extending their plasma membrane. Microbes are then degraded by hydrolytic enzymes of lysosome. Degraded microbial peptides are then either excreted out of the cell or being presented on the cell surface of phagocytes for initiating immune response (antigen presentation). Phagocytes recognize microbes through variety of receptors found on their surfaces such as **Pattern Recognition Receptors, Toll-like receptors, Antibodies** and **Opsonin Receptors.** During phagocytosis, microbes are degraded by antimicrobial proteins and peptides (defensins and cathelicidins), Low pH, acid activated hydrolytic enzymes (lysozyme and proteases) and specialized molecules related to Reactive oxygen species (ROS) and reactive nitrogen species (RNS) that mediate oxidative attack.

Commonly utilized *In vitro* reactions, based on antigen and antibody interactions are precipitation and agglutination. **Precipitation** occurs when antigen-antibody interaction forms a crosslinked lattice structure which slowly converts into a visible precipitate. Generally, antigens precipitate properly with crosslinking polyclonal antibodies which are termed as **precipitins**. Precipitation is done for quantitative analysis of antigen or antibody by techniques called **Immunodiffusion (Radial and double immunodiffusion)** and **immunoelectrophoresis**.

Another type of *in vitro* immune reactions in which antibody interacts with a **particulate antigen** and form a visible clumping is called **agglutination.** Agglutination tests are considered the most sensitive tests available for clinical diagnosis of wide range of non-infectious immune disorders and infectious diseases, by detecting the amount of either antigens or antibodies.

As a part of immune system, the complement system, comprises of a set of heat-labile serum proteins, called **complements**, they interact with one another in catalytic cascades to eliminate pathogenic antigens by disrupting their cell membranes and promoting cytolysis. Complements are activated by proteolytic cleavage through either of the three pathways, i.e.,

classical, **lectin** and **alternate pathways.** Although each of these three pathways of complement activation are initiated with different events, they all involved in the generation of an enzyme complex capable of cleaving the C3 molecule into two fragments, C3a and C3b. The enzymes that catalyze this biochemical transformation are referred to as **C3 convertases**.

Immune system generally protects the host organism from antigens through triggering different responses especially local inflammatory responses without causing any harm to the host. Sometimes, these inflammatory responses induce adverse *in vivo* reactions which cause tissue damage as **hypersensitivity**. It is an increased sensitivity by the animal body to an antigen to which it has been exposed previously. Hypersensitivity is either antibody mediated or cell mediated. Hypersensitivity reactions are highly variable may be divided into two types, i.e., Immunoglobulin-mediated (immediate) hypersensitivity reactions include **types I, II, and III hypersensitivity** and Lymphoid cell (T cell)-mediated (delayed-type) hypersensitivity include **type IV hypersensitivity**.

9.8 *TERMINAL QUESTIONS AND ANSWERS*

- **i.** *In vivo* reaction means………” while *In vitro* reactions means……….
- **ii.** The theory of phagocytosis was developed by…………
- **iii.** Immunoelectrophoresis process involves………..and…………………..
- **iv.** Examples of pattern recognition receptors are…………,…………and………..
- **v.** …………reaction is involved in diagnosing the presence of antibodies to typhoid bacteria and.
- **vi.** Examples of ROS are …………, …………..and………………….
- **vii.** Generation of ROS by NADPH oxidase by excessive oxygen uptake by phagocytes is called……………
- **viii.** In precipitation, antibodies bind with ……….and in agglutination, antibodies interacting with……….forming a crosslinked lattice structure.
- **ix.** *Complement was first observed by…….and termed by………….*
- **x.** *The component of the complement that binds with IgM or IgG is……….*
- **xi.** *Another name of C5 convertase is…………*
- **xii.** *Major initiator of classical complement pathway is………., whereas, of alternate pathway is……………*

xiii. Another name of C1 component is……………..

xiv. The component of the membrane attack complex are……………..

xv. Systemic Lupus erythematosus (SLE) is caused by deficiency of complement components……and……

xvi. Component B and D are found in the cascade reactions of ………… pathway.

xvii. The delayed hypersensitivity reaction is mediated by……cells.

xviii. The antibody involved in type I hypersensitivity or allergic reaction is……..

xix. Examples of primary mediators in type I hypersensitivity are ………and………., whereas, of secondary mediators are……………..and……………

xx. Erythroblastosis fetalis is an example for type……..hypersensitivity.

xxi. The cells that play major role in the type III hypersensitivity reaction are……..

Answers

i. Reactions within a living organism, reactions outside the body

ii. Elie **Metchnikoff**

iii. **Electrophoresis, double immunodiffusion**

iv. Mannose receptor, Dectin-1 etc. and scavenger receptors

v. **Agglutination reaction**

vi. Superoxide ion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO)

vii. Respiratory burst

viii. **Soluble antigens, particulate antigens**

ix. **J. Bordet,** Paul Ehrlich

x. C1 component or $C1qr_2s_2$

xi. C4b2a3b

xii. **Antigen-antibody binding, component binding to microbial surface**

xiii. $C1qr_2s_2$

xiv. C5bC6789

xv. C2 and C4

xvi. Alternate pathway

xvii. T dependent T helper (TDTH) cell

xviii. IgE

xix. Histamine and adenosine, leukotrienes and prostaglandins

xx. Type II

xxi. Neutrophils

1.MODEL EXAMINATION QUESTIONS:

vii. What is phagocytosis? Write the basic mechanism of phagocytosis.

viii. Write a note on various types of receptors which mediate the process of phagocytosis.

ix. What is opsonization? Write a note on opsonin and opsonin receptors.

x. Write an essay on complement system describing its pathways and components.

xi. Describe various components of complement and their functions.

xii. Describe cascade reactions take place during a classical pathway of complement activation.

xiii. What is oxidative attack? Write a note on various ROS and RNS and their interaction during oxidative attack on pathogens.

xiv. What is hypersensitivity? Describe various types of hypersensitivity reactions with examples.

xv. Write a note on diseases related to type II hypersensitivity.

xvi. Write a short note on (a) Arthus reaction (b) ADCC (c) Delayed type hypersensitivity (d) Tuberculin reaction (e) Disease related to complement deficiency

xvii. Differentiate the process of precipitation and agglutination with examples, also write their application.

xviii. Write a note on double immunodiffusion with example.

xix. What is viral hemagglutination? Describe its mechanism in diagnosing viral diseases.

9.9 *REFERENCES*

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