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SCHOOL OF BIO & CHEMICAL ENGINEERING

DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT – 1 – Introduction to Microbiology

UNIT I

INTRODUCTION TO MICROBIOLOGY

Microbiology (from Greek μῑκρος, *mīkros*, "small"; βίος, *bios,* "life"; and -λογία, -*logia*) is the study of microscopic organisms, those being unicellular (single cell), multicellular (cell colony), or a cellular (lacking cells).[1] Microbiology encompasses numerous sub-disciplines including virology, mycology, parasitology, and bacteriology.

Eukaryotic micro-organisms possess membrane-bound cell organelles and include fungi and protists, whereas prokaryotic organisms - which all are microorganisms—are conventionally classified as lacking membrane-bound organelles and include eubacteria and archaebacteria. Microbiologists traditionally relied on culture, staining, and microscopy. However, less than 1% of the microorganisms present in common environments can be cultured in isolation using current means.[2]Microbiologists often rely on extraction or detection of nucleic acid, either DNA or RNA sequences.

Viruses have been variably classified as organisms,[3] as they have been considered either as very simple microorganisms or very complex molecules. Prions, never considered microorganisms, have been investigated by virologists, however, as the clinical effects traced to them were originally presumed due to chronic viral infections, and virologists took search—discovering "infectious proteins".

As an application of microbiology, medical microbiology is often introduced with medical principles of immunology as *microbiology and immunology*. Otherwise, microbiology, virology, and immunology as basic sciences have greatly exceeded the medical variants, applied sciences.

Branches

The branches of microbiology can be classified into pure and applied sciences.[7] Microbiology can be also classified based on taxonomy, in the cases of bacteriology, mycology, protozoology, and phycology. There is considerable overlap between the specific branches of microbiology with each other and with other disciplines, and certain aspects of these branches can extend beyond the traditional scope of microbiology.

Pure microbiology Taxonomic arrangement

- Bacteriology: The study of bacteria.
- Mycology: The study of fungi.
- Protozoology: The study of protozoa.
- Phycology/algology: The study of algae.
- Parasitology: The study of parasites.
- Immunology: The study of the immune system.
- Virology: The study of viruses.
- Nematology: The study of nematodes.
- Microbial cytology: The study of microscopic and submicroscopic details of microorganisms.
- Microbial physiology: The study of how the microbial cell functions biochemically. Includes the study of microbial growth, microbial metabolism and microbial cell structure.
- Microbial ecology: The relationship between microorganisms and their environment.
- Microbial genetics: The study of how genes are organized and regulated in microbes in relation to their cellular functions. Closely related to the field of molecular biology.
- Cellular microbiology: A discipline bridging microbiology and cell biology.
- Evolutionary microbiology: The study of the evolution of microbes. This field can be subdivided into:
- Microbial taxonomy: The naming and classification of microorganisms.
- Microbial systematic: The study of the diversity and genetic relationship of microorganisms.
- Generation microbiology: The study of those microorganisms that have the same characters as their parents.
- Systems microbiology: A discipline bridging systems biology and microbiology.
- Molecular microbiology: The study of the molecular principles of the physiological processes in microorganisms.

Other

- Nano microbiology: The study of those organisms on nano level.
- Exo microbiology (or Astro microbiology): The study of microorganisms in outer space (see: List of microorganisms tested in outer space)
- Biological agent: The study of those microorganisms which are being used in weapon industries.

Applied microbiology

- Medical microbiology: The study of the pathogenic microbes and the role of microbes in human illness. Includes the study of microbial pathogenesis andepidemiology and is related to the study of disease pathology and immunology. This area of microbiology also covers the study of human microbiota, cancer, and the tumor microenvironment.
- Pharmaceutical microbiology: The study of microorganisms that are related to the production of antibiotics, enzymes, vitamins,vaccines, and other pharmaceutical products and that cause pharmaceutical contamination and spoil.
- Industrial microbiology: The exploitation of microbes for use in industrial processes. Examples include industrial fermentation and wastewater treatment. Closely linked to the biotechnology industry. This field also includes brewing, an important application of microbiology.
- Microbial biotechnology: The manipulation of microorganisms at the genetic and molecular level to generate useful products.
- Food microbiology: The study of microorganisms causing food spoilage and foodborne illness. Using microorganisms to produce foods, for example by fermentation.
- Agricultural microbiology: The study of agriculturally relevant microorganisms. This field can be further classified into the following:
- Plant microbiology and Plant pathology: The study of the interactions between microorganisms and plants and plant pathogens.
- Soil microbiology: The study of those microorganisms that are found in soil.

- Veterinary microbiology: The study of the role of microbes in veterinary medicine or animal taxonomy.
- Environmental microbiology: The study of the function and diversity of microbes in their natural environments. This involves the characterization of key bacterial habitats such as the rhizosphere and phyllosphere, soil and groundwater ecosystems, open oceans or extreme environments (extremophiles). This field includes other branches of microbiology such as:
- Microbial ecology
- Microbially mediated nutrient cycling
- Geomicrobiology
- Microbial diversity
- Bioremediation
- Water microbiology (or Aquatic microbiology): The study of those microorganisms that are found in water.
- Aeromicrobiology (or Air microbiology): The study of airborne microorganisms. Benefits

Fig. 1. Fermenting tanks with yeast being used to brew beer

While some fear microbes due to the association of some microbes with various human illnesses, many microbes are also responsible for numerous beneficial processes such as industrial fermentation (e.g. the production of alcohol, vinegar and dairy products), antibiotic production and as vehicles for cloning in more complex organisms such as plants. Scientists

have also exploited their knowledge of microbes to produce biotechnologically important enzymes such as Taq polymerase, reporter genes for use in other genetic systems and novel molecular biology techniques such as the yeast two-hybrid system.

Bacteria can be used for the industrial production of amino acids. *Corynebacterium glutamicum* is one of the most important bacterial species with an annual production of more than two million tons of amino acids, mainly L-glutamate and L-lysine.[8]

A variety of biopolymers, such as polysaccharides, polyesters, and polyamides, are produced by microorganisms. Microorganisms are used for the biotechnological production of biopolymers with tailored properties suitable for high-value medical application such as tissue engineering and drug delivery. Microorganisms are used for the biosynthesis of xanthan, alginate, cellulose, cyanophycin, poly(gamma-glutamic acid), levan, hyaluronic acid, organic acids, oligosaccharides and polysaccharide, and polyhydroxyalkanoates.

Microorganisms are beneficial for microbial biodegradation or bioremediation of domestic, agricultural and industrial wastes and subsurface pollution in soils, sediments and marine environments. The ability of each microorganism to degrade toxic waste depends on the nature of each contaminant. Since sites typically have multiple pollutant types, the most effective approach to microbial biodegradation is to use a mixture of bacterial and fungal species and strains, each specific to the biodegradation of one or more types of contaminants.

Symbiotic microbial communities are known to confer various benefits to their human and animal hosts health including aiding digestion, production of beneficial vitamins and amino acids, and suppression of pathogenic microbes. Some benefit may be conferred by consuming fermented foods, probiotics (bacteria potentially beneficial to the digestive system) and/or prebiotics (substances consumed to promote the growth of probiotic microorganisms). The ways the microbiome influences human and animal health, as well as methods to influence the microbiome are active areas of research.

Research has suggested that microorganisms could be useful in the treatment of cancer. Various strains of non-pathogenic clostridia can infiltrate and replicate within solid tumors. Clostridial vectors can be safely administered and their potential to deliver therapeutic proteins has been demonstrated in a variety of preclinical models.

Scope of microbiology

There is vast scope in the field of microbiology due to the advancement in the field of science and technology. The scope in this field is immense due to the involvement of microbiology in many fields like medicine, pharmacy, diary, industry, clinical research, water industry, agriculture, chemical technology and nanotechnology. The study of microbiology contributes greatly to the understanding of life through enhancements and intervention of microorganisms. There is an increase in demand for microbiologists in India and globally. A microbiologist can innovate new diagnostic kits, discover new drugs, teach, research, etc.

Since the microbes are living, it follows that microbiology deals with a group of particular life forms and it comes under the broad domain of biology which includes the study of all aspects of living beings including man.

Where can we fit in microbes in the hierarchy of living beings? Traditionally living beings are divided into plants and animals. But members of microbes can be accommodated in both plants (fungi) and animals (protozoa) and some cannot be accommodated in either plants or animals as they share the characters of both. (For instance *Euglena*was a disputed property till recently between botanists and zoologists.)

In one of the earlier attempts to resolve this problem, Haeckel (1866) a German Zoologist suggested that there should be a third kingdom besides Plantae (plants) and Animalia (animals) to include all the microorganisms. He gave the name *Protista* to this kingdom to include all unicellular microorganisms that are neither plants nor animals.

Haeckel's classification raised some questions like how to distinguish a fungus from a bacterium or from an alga. The discovery in late 1940s of the prokaryotic and eukaryotic nature of the cells rendered the three kingdom classification unsatisfactory.

A recent and comprehensive classification proposed by R.H. Whittaker (1969) has five kingdoms of living beings.

Kingdom Monera Kingdom Protista Kingdom Fungi Kingdom Animalia Kingdom Plantae.

Microorganisms include three of the (Monera, Protista and Fungi) five kingdoms mentioned above. At present it is agreed that within the preview of microbiology, five major groups of microorganisms-viruses, bacteria, fungi, algae and protozoa are dealt with.

As it will be evident from the above discussion, the scope of microbiology extends to both eukaryotic as well as prokaryotic microbes. While discussing the scope of microbiology it should be evident to us that it does not deal with merely the enumeration of structural diversity or classification but extends to all aspects of microbial life. Microbiology is concerned with their form, structure, reproduction, physiology, metabolism classification and most important their economic importance. In other words, what the microbes *can do* and *should not be allowed to do* (some times) as for as human beings are concerned is one of the vital aspects of microbiology on which rests human destiny.

HISTORY

Ancient

The existence of microorganisms was hypothesized for many centuries before their actual discovery. The existence of unseen microbiological life was postulated by Jainism which is based on Mahavira's teachings as early as 6th century BCE. Paul Dundas notes that Mahavira asserted existence of unseen microbiological creatures living in earth, water, air and fire. Jain scriptures also describe nigodas which are sub-microscopic creatures living in large clusters and having a very short life and are said to pervade each and every part of the universe, even in tissues of plants and flesh of animals.

MICROBIOLOGY'S50 MOST SIGNIFICANT EVENTS 1875-1995

1875 – Ferdinand J. Cohn contributes to the founding of the science of bacteriology. He publishes an early classification of bacteria using the genus name Bacillus for the first time.

1876 – Robert Koch publishes a paper on his work with anthrax, pointing explicitly to a bacterium as the cause of this disease. This validates the germ theory of disease. His work on anthrax was presented and his papers on the subject were published under the auspices of Ferdinand Cohn.

1878 – Joseph Lister publishes his study of lactic fermentation of milk, demonstrating the specific cause of milk souring. His research is conducted using the first method developed for isolating a pure culture of a bacterium, which he names *Bacterium lactis.*

1880 – Louis Pasteur develops a method of attenuating a virulent pathogen the agent of chicken cholera, so it would immunize and not cause disease. This is the conceptual breakthrough for establishing protection against disease by the inoculation of a weakened strain of the causative agent. Pasteur uses the word "attenuated" to mean weakened. As Pasteur acknowledged, the concept came from Edward Jenner's earlier success at smallpox vaccination.

1881 – Robert Koch struggles with the disadvantages of using liquid media for certain experiments. He seeks out alternative, and first uses an aseptically cut slice of a potato as a solid culture medium. He also turns to gelatin, which is added to culture media; the resulting mixture is poured onto flat glass plates and allowed to gel. The plate technique is used to isolate pure cultures of bacteria from colonies growing on the surface of the plate.

1882 – Ilya Ilich Metchnikoff demonstrates that certain body cells move to damaged areas of the body where they consume bacteria and other foreign particles. He calls the process phagocytosis. He proposes a theory of cellular immunity. With Paul Ehrlich, Mechnikoff is awarded the Nobel Prize in Medicine or Physiology in 1908.

1884 – Robert Koch puts forth a set of postulates, or standards of proof, involving the tubercle bacillus. Koch's postulates are published in the Etiology of Tuberculosis, in which he demonstrated three major facts: 1) the presence of the tubercule bacillus (as proved by straining) in tubercular lesions of various organs of humans and animals, 2) the cultivation of the organisms in pure culture on blood serum, and 3) the production of tuberculosis at will by its inoculation into guinea pigs. Koch was awarded the Nobel Prize in Medicine ro Physiology in 1905.

1885 – Louis Pasteur oversees injections of the child Joseph Meister with "aged" spinal cord allegedly infected with rabies virus. Pasteur uses the term "virus" meaning poison, but has no idea of the nature of the causative organism. Although the treatment is successful, the experiment itself is an ethical violation of research standards. Pasteur knew he was giving the child successively more dangerous portions.

1889 – Martinus Beijerinck uses enrichment culture, minus nitrogenous compounds, to obtain a pure culture of the root nodule bacterium *Rhizobium,* demonstrating that enrichment culture creates the conditions for optimal growth of a desired bacterium.

1890 – Emil von Behring and Shibasaburo Kitasato working together in Berlin in 1890 announce the discovery of diphtheria antitoxin serum, the first rational approach to therapy of infectious diseases. They inject a sublethal dose of diphtheria filtrate into animals and produce a serum that is specifically capable of neutralizing the toxin. They then inject the antitoxin serum into an uninfected animal to prevent a subsequent infection. Behaving was awarded the Noble Prize in Medicine of Physiology in 1901.

1890 – Sergei Winogradsky succeeds in isolating nitrifying bacteria from soil. During the period 1890-1891, Winogradsky performs the definitive work on the organisms responsible for the process of nitrification in nature.

1891 – Paul Ehrlich proposes that antibodies are responsible for immunity. He shows that antibodies form against the plant toxins ricin and abrin. With Metchnikoff, Ehrlich is jointly awarded the Nobel Prize in Medicine or Physiology in 1908.

1892 – Dmitri Ivanowski publishes the first evidence of the filterability of a pathogenic agent, the virus of tabacco mosaic disease, launching the field of virology. He passes the agent through candle filters that retain bacteria, but he is not sure that the agent is a unique organism.

1899 – Martinus Beijerinck recognizes “soluble” living microbes, a term he applies to the discovery of tobacco mosaic virus. He demonstrate that juice pressed from tobacco leaves that had been filtered free of bacteria retains the ability to cause disease in plants even after repeated dilutions. He calls the disease agent “contagium vivum fluidfium” or contagious living fluid.

1911 – Francis Peyton Rous discovers a virus that can cause cancer in chickens. In 1909, a farmer brought Rous a hen that had a breast tumor. Rous performed an autopsy, extracted tumor cells and injected them into other hens, which that had a breast tumor. Rous performed an autopsy, extracted tumor cells and injected them into other hens which (http://archives. microbeworld.org/microbes/timelines1.aspx) subsequently developed tumors. This is the first

experimental proof of an infectious etiologic agent of cancer. Rous is awarded the Nobel Prized in Medicine or Physiology in 1966.

1912 – Paul Ehrlich announces the discovery of an effective cure (Salvarsan) for syphilis, the first specific chemotherapeutic agent for a bacterial disease. Ehrlich was seeking an arsenic derivative and finally the 606th compound worked. He brought news of the treatment to London, where Alexander Fleming became one of the few physicians to administer it.

1915 – Frederick Twort announces the first discovery of bacteriophages, or bacteria-infecting viruses. Twort's discovery was something of an accident. He had spent several years growing viruses and noticed that the bacteria infecting his plates became transparent, indicating that they had been lysed or broken open and destroyed. Felix d'Herrelle independently describes bacterial viruses and coins the term "bacteriophage".

1926 – Albert Jan Kluyver and Hendrick Jean Louis Donker propose a universal model for metabolic events in cells based on a transfer of hydrogen atoms. The model applies to aerobic and anaerobic organisms.

1928 – Frederick Griffith discovers transformation in bacteria and establishes the foundation of molecular genetics. He shows that injecting mice with a mixture of live, avirulent, rough *Streptococcus pneumonia* Type I and heat-killed, virulent smooth *S. pneumonia* Type II, leads to the death of the mice. Live, virulent, smooth *S. pneumonia* Type II are isolated from the dead mice.

1929 – Alexander Fleming publishes the first paper describing penicillin and its effect on gram-positive microorganisms. This finding is unique since it is a rare example of bacterial lysis and not just microbial antagonism brought on by the mold *Penicillium.* Fleming kept his cultures 2-3 weeks before discarding them. When he looked at one set he noticed that the bacteria seemed to be dissolving and the mold was contaminating the culture. When penicillin is finally produced in major quantities in the 1940s, its power and availability effectively launch the "Antibiotics Era", a major revolution in public health and medicine. With Florey and Chain, Fleming is awarded the Nobel Prize in Medicine or Physiology in 1945.

1931 – C.B. van Niel shows that photosynthetic bacteria use reduced compounds as electron donors without producing oxygen. Sulfur bacteria use H2S as a source of electrons for the fixation of carbon dioxide. He posits that plants use water as a source and release oxygen.

1935 – Gerhard J. Domagk uses a chemically synthesized anti-metabolite, Prontosil, to kill *Streptococcus* in mice. One of the first patients to be treated with Protonsil is Domagk's daughter who has a streptococcal infection that is unresponsive to other treatments. Near death, she is injected with large quantities of Protonsil and makes a dramatic recovery. Domagk is awarded the Nobel Prize in Medicine or Physiology in 1939.

1935 – Wendell Stanley crystallizes tobacco mosaic virus and shows that it remains infectious. However, he does not recognize that the infectious material is nucleic acid and not protein. Together with Northrop and Sumner, Stanley is awarded the Nobel Prize in Chemistry in 1946.

1941 – George Beadle and Edward Tatum jointly publish a paper on their experiments using the fungus *Neurophoramatsa* to establish that particular genes are expressed through the actin of correspondingly specific enzymes. The first gene to be identified controlled the synthesis of an enzyme in a series that led to generation of niacin. This report is the genesis of the "one gene-one enzyme" concept. With Lederberg Beadle and Tatum are awarded the Nobel Prize in Medicine or Physiology in 1958.

1943 – Salvador Lura and Max Delbruck provides a statistical demonstration that inheritance in bacteria follows Darwinian principles. Particular mutants, such as viral resistance, occur randomly in bacterial populations, even in the absence of the virus. More important, they occur in small numbers in some populations and in large number in other cultures. With Hershey, Delbruck and Luria are awarded the Nobel Prize in Medicine or Physiology in 1969.

1944 – Oswald Avery, Colin MacLeod, and Maclyn McCarty show that DNA is the transforming material in cells. They demonstrate that the transformation of *Streptococcus pneumonia* from an avirulent type to a virulent type is the result of the transfer of DNA from dead smooth organisms to live rough ones. They also show that the transforming principle is destroyed by pancreatic deoxyribonuclease – an enzyme that hydrolyzes DNA – but is not affected by pancreatic ribonuclease or enzymes that destroy proteins.

1944 – Albert Schartz, E. Bugie and Selman Waksman discover streptomycin, soon to be used against tuberculosis. Streptomycin has the same specific antibiotic effect against gram-negative microorganisms as penicillin does on gram-positive ones. Waksman is awarded the Noble Prize in Medicine of Physiology in 1952.

1946 – Joshua Lederberg and Edward L. Tatum publish the first paper on a type of bacterial mating called conjugation. The proof is based on the generation of daughter cells able to grow in media that cannot support growth of either of the parent cells. Their experiments showed that this type of gene exchange requires direct contact between bacteria. At the time Lederberg began studying with Tatum, scientists believed that bacteria reproduced asexually, but from the work of Beadle Tatum, Lederberg knew that fungi reproduced sexually and he suspected that bacteria did as well.

1949 – Microbiologist **John Franklin Enders**, virologist **Thomas H. Weller** and physician **Frederick Chapman Robbins** together develop a technique to grow polio virus in test tube cultures of human tissues. This approach gave virologists a practical tool for the isolation and study on viruses. Enders, Weller and Robbins were awarded the Noble Prize in Medicine or Physiology in 1954.

1952 – Joshua Lederberg and Norton Zinder report on transduction, or transfer of genetic information to cells by viruses. They show that a phage of *Salmonella typhimurium* can carry DNA from one bacterium to another

1952 – Alfred Hershey and Martha Chase suggest that only DNA is needed for viral replication. Using radioactive isotopes 35S to track protein and 32P to track DNA, they show that progeny T2 bacteriophage isolated from lysed bacterial cells have the labeled nuclei acid. Further, most of the labeled protein does not enter the cells but remains attached to the bacterial cell membrane.

1953 – Francis Crick and Maurice Wilkins, together with James Watson, describe the double-helix structure of DNA. The chemical structure is based on X-ray crystallography of DNA done by Rosalind Franklin. Crick, Wilkins and Watson are awarded the Noble Prize in Medicine or Physiology in 1962.

1959 – Peter Mitchel proposes the chemiosmotic theory, in which a molecular process is coupled to the transport of protons across a biological membrane. He argues that this principle explains ATP synthesis, solute accumulation or expulsions, and cell movement (flagellar rotation). Mitchell is awarded the Nobel Prize in Chemistry in 1978.

1960 – Francois Jacob, David Perrin, Carmen Sanchez and Jacques Monod propose the operon concept for control of bacteria gene action. Jacob and Monod later propose that a protein repressor blocks RNA synthesis of a specific set of gene, the *lac* operon, unless an inducer, lactose, binds to the repressor. With Lwoff, Jacob and Monod are awarded the Nobel Prize in Medicine or Physiology in 1965.

1961 – Marshall Nirenberg and J.H. Matthaei observe that a synthetic polynucleotide, poly U, directs the synthesis of a polypeptide composed only of phenylalanine. They conclude that the nucleotide base triplet UUU must code for phenylalanine. This is the start of successful efforts to decipher the genetic code. With Robert Holley and Har Gobind Khorana, Nirenberg is awarded the Nobel Prize in Medicine or physiology in 1968.

1961 – Sydney Brener, Francois Jacob and Mathew Meselson use phage-infected bacteria to show that ribosomes are the site of protein synthesis and confirm the existence of messenger RNA. They demonstrate that infection of *Escherichia coli* by phage T4 stops cell synthesis of host RNA and leads to T4 RNA synthesis. The T4 RNA attaches to cellular ribosome and directs protein synthesis.

1964 – Charles Yanofsky and coworkers define the relationship between the order of mutable sites in the gene coding for the Escherichia coli enzymes tryptophan synthetase and the corresponding amino acid replacements in the enzyme. It worked well for tyroptophan synthetase because the enzyme has two subunits, one of which could be mutated. The missense mutants in the alpha subunit could be mapped and related to the genetic fine structure of the gene. The property of correlating a mutation with an amino acid replacement is called colinearity.

1970 – Howard Temin and David Baltimore independently discover the enzyme reverse transcriptase in RNA viruses. Reverse transcriptase uses RNA as a template to synthesize a single-stranded DNA complement. This process establishes a pathway for genetic

information flow from RNA to DNA. With Dulbecco, Baltimore and Temin are awarded the Nobel Prize in Medicine or Physiology in 1975.

1973 – Stanley Cohen, Annie Chang, Robert Helling and Herbert Boyer show that extrachromosomal bits of DNA called plasmids act as vectors for maintaining closed genes in bacteria. They show that if DNA is broken into fragments and combined with plasmid DNA, such recombinant DNA molecules will reproduce if inserted into bacterial cells. The discovery is a major breakthrough for genetic engineering, allowing for such advances as gene cloning and the modification of genes.

1975 – Georg Kohler and Cesar Milstein physically fuse mouse lymphocytes with neoplastic mouse plasma cells to yield hybrid cells called hybridomas that can produce specific antibodies and survive indefinitely in tissue culture. This approach offers a limitless supply of monoclonal antibodies. Monoclonal antibodies permit the generation of diagnostic tests that the highly specific. They also function as probes to study cell function. With Jerme, Kohler and Milstein are awarded the Nobel Prize in Medicine or Physiology in 1984.

1977 – Carl Woese uses ribosomal RNA analysis to recognize a third form of life, the *Archaea* whose genetic makeup is distinct from but related to both *Bactiera* and *Eucarya.*

1977 – Walter Gilbert and Fred Sanger independently develop methods to determine the exact sequence of DNA. Gilbert uses the technique to determine the sequence of the operon of a bacterial genome. Sanger and colleagues use the technique to determine this sequence of all 5,375 nucleotides of the bacteriophage phi-X174, the first complete determination of the genome of an organism. With Paul Berg, Gilbert and Sanger are awarded the Nobel Prize in Chemistry in 1980.

1979-Smallpox (variola) is declared officially eliminated, the last naturally occurring case having been seen in 1977 in Somalia. Small quantities remain held under tightly controlled conditions in the U.S and former U.S.S.R smallpox is the only microbial disease to ever have been deliberately eradicated.

1982 – Stanley Prusiner finds evidence that disease can be caused by a class of infectious proteins he call prions. These abnormal proteins cause scrapie, a fatal neurodegenerative disease of sheep, Prusiner is awarded the Nobel Prize in Medicien or Physiology in 1997.

1983 – Luc Montagnier and Robert Gallo announce their discovery of the immunodeficiency virus (HIV) believed to cause AIDS.

1986 – Kary Mullis uses a heat stable enzyme from *Thermus aquaticus* to establish polymerate chain reaction technology. PCR is used to amplify target DNA many-fold. Mullis is awarded the Nobel Prize in Chemistry in 1993.

1995 – Craig Venter, Hamilton Smith, Claire Fraser and colleagues at TIGR elucidate the first complete genome sequence of a microorganism: *Haemophilius influenza.*

Fig.2. Antonie van Leeuwenhoek

Antonie Philips van Leeuwenhoek - October 24, 1632 – August 26, 1723) was a Dutch tradesman and scientist. He is commonly known as "the Father of Microbiology", and considered to be the first microbiologist. He is best known for his work on the improvement of the microscope and for his contributions towards the establishment of microbiology.

Raised in Delft, Netherlands, Van Leeuwenhoek worked as a draper in his youth, and founded his own shop in 1654. He made a name for himself in municipal politics, and eventually developed an interest in lens making. Using his handcrafted microscopes, he was the first to observe and describe microorganisms, which he originally referred to as *animalcules*. Most of the "animalcules" are now referred to as unicellular organisms though he observed multi cellular organisms in pond water. He was also the first to document microscopic observations of muscle fibers, bacteria, spermatozoa, and blood flow in capillaries (small blood vessels). Van Leeuwenhoek did not author any books; his discoveries came to light through correspondence with the Royal Society, which published his letters.

Early life and career

Antonie van Leeuwenhoek was born in Delft, Dutch Republic, on October 24, 1632. On 4 November he was baptized as *Thonis*. His father, Philips Antonisz van Leeuwenhoek, was a basket maker who died when Antonie was only five years old. His mother, Margaretha (Bel van den Berch), came from a well-to-do brewer's family, and remarried Jacob Jansz Molijn, a painter. Van Leeuwenhoek married Barbara de Mey in July 1654, with whom he would have one surviving daughter, Maria (four other children died in infancy). That same year he returned to Delft, where he would live and study for the rest of his life. He opened a draper's shop, which he ran throughout the 1650s. His wife died in 1666, and in 1671 Van Leeuwenhoek remarried, to Cornelia Swalmius, with whom he had no children.[6] His status in Delft had grown throughout the years. In 1660 he received a lucrative job as chamberlain for the Delft sheriffs' assembly chamber in the City Hall, a position which he would hold for almost 40 years. In 1669 he was appointed as a land surveyor by the Court of Holland; at some time he combined it with another municipal job, being the official "wine-gauger" of Delft and in charge of the city's wine imports[7] and (wine) taxation..

Microscopic study

While running his draper's shop, Van Leeuwenhoek wanted to see the quality of the thread, better than the then-current magnifying lenses available. Therefore, he began to develop an interest in lensmaking, although few records exist of his early activity. Van Leeuwenhoek's interest in microscopes and a familiarity with glass processing led to one of the most significant, and simultaneously well-hidden, technical insights in the history of science. By placing the middle of a small rod of soda lime glass in a hot flame, Van Leeuwenhoek could pull the hot section apart to create two long whiskers of glass. Then, by reinserting the end of one whisker into the flame, he could create a very small, high-quality glass sphere. These spheres became the lenses of his microscopes, with the smallest spheres providing the highest magnifications.

Recognition by the Royal Society

After developing his method for creating powerful lenses and applying them to the study of the microscopic world, Van Leeuwenhoek introduced his work to his friend, the prominent Dutch physician Reinier de Graaf. When the Royal Society in London published the

groundbreaking work of an Italian lensmaker in their journal *Philosophical Transactions of the Royal Society*, De Graaf wrote to the journal's editor Henry Oldenburg with a ringing endorsement of Van Leeuwenhoek's microscopes which, he claimed, "far surpass those which we have hitherto seen". In response the Society published in 1673 a letter from Van Leeuwenhoek, which included his microscopic observations on mold, bees, and lice.[9]

Antonie van Leeuwenhoek was elected to the Royal Society in February 1680 on the nomination of William Croone, a then-prominent physician.[note 3] Van Leeuwenhoek was "taken aback" by the nomination, which he considered a high honor, although he did not attend the induction ceremony in London, nor did he ever attend a Royal Society meeting.[16]

Scientific fame

By the end of the 17th century, Van Leeuwenhoek had a virtual monopoly on microscopic study and discovery. His contemporary Robert Hooke, an early microscope pioneer, bemoaned that the field had come to rest entirely on one man's shoulders.[17] He made about 200 microscopes with different magnification.

On this occasion Van Leeuwenhoek presented the Tsar an "eel-viewer", so Peter could study the blood circulation, whenever he wanted.

Techniques and discoveries

Antonie van Leeuwenhoek made more than 500 optical lenses. He also created at least 25 single-lens microscopes, of differing types, of which only nine survived. These microscopes were made of silver or copper frames, holding hand-made lenses. Those that have survived are capable of magnification up to 275 times. It is suspected that Van Leeuwenhoek possessed some microscopes that could magnify up to 500 times. Although he has been widely regarded as a dilettante or amateur, his scientific research was of remarkably high quality.

The single-lens microscopes of Van Leeuwenhoek were relatively small devices, the biggest being about 5 cm long. They are used by placing the lens very close in front of the eye, while looking in direction of the sun. The other side of the microscope had a pin, where the sample was attached in order to stay close to the lens. There were also three screws that allowed to

move the pin, and the sample, along three axes: one axis to change the focus, and the two other axes to navigate through the sample.

Van Leeuwenhoek maintained throughout his life that there are aspects of microscope construction "*which I only keep for myself*", in particular his most critical secret of how he made the lenses. For many years no-one was able to reconstruct Van Leeuwenhoek's design techniques. However, in 1957 C.L. Stong used thin glass thread fusing instead of polishing, and successfully created some working samples of a Van Leeuwenhoek design microscope.[22] Such a method was also discovered independently by A. Mosolov and A. Belkin at the Russian Novosibirsk State Medical Institute.[23]

Van Leeuwenhoek used samples and measurements to estimate numbers of microorganisms in units of water. He also made good use of the huge lead provided by his method. He studied a broad range of microscopic phenomena, and shared the resulting observations freely with groups such as the British Royal Society.[26] Such work firmly established his place in history as one of the first and most important explorers of the microscopic world. Antonie van Leeuwenhoek was one of the first people to observe cells, much like Robert Hooke.

Van Leeuwenhoek's main discoveries are:

- the infusoria (protists in modern zoological classification), in 1674
- the bacteria, (e.g., large Selenomonads from the human mouth), in 1676
- the vacuole of the cell.
- the spermatozoa in 1677.
- the banded pattern of muscular fibers, in 1682.[note4]

In 1687 Van Leeuwenhoek reported his research on the coffee bean. He roasted the bean, cut it into slices and saw a spongeous interior. The bean was pressed, and an oil appeared. He boiled the coffee with rain water twice, set it aside.[28]

Van Leeuwenhoek's discovery that smaller organisms procreate similarly to larger organisms challenged the contemporary belief, generally held by the 17th century scientific community, that such organisms generated spontaneously. The position of the Church on the exact nature of the spontaneous generation of smaller organisms was ambivalent.

Death and legacy

By the end of his life, Van Leeuwenhoek had written approximately 560 letters to the Royal Society The last few contained a precise description of his own illness. He suffered from a rare disease, an uncontrolled movement of the midriff, which is now named *Van Leeuwenhoek's disease*. He died at the age of 90, on August 26, 1723 and was buried four days later in the Oude Kerk (Delft).

Fig.3 Louis Pasteur

Louis Pasteur (/ˈluːi pæˈstɜːr/, French: [lwi pastœʁ]; December 27, 1822 – September 28, 1895) was a French chemist and microbiologist renowned for his discoveries of the principles of vaccination, microbial fermentation and pasteurization. He is best known to the general public for his invention of the technique of treating milk and wine to stop bacterial contamination, a process now called pasteurization. He is regarded as one of the three main founders of bacteriology, together with Ferdinand Cohn and Robert Koch, and is popularly known as the "father of microbiology".[3][4][5]

Pasteur was responsible for disproving the doctrine of spontaneous generation. He performed experiments that showed that without contamination, microorganisms could not develop.

Although Pasteur was not the first to propose the germ theory, Pasteur also made significant discoveries in chemistry, most notably on the molecular basis for the asymmetry of certain crystals and racemization. Early in his career, his investigation of Tartaric acid resulted in the first resolution of what we now call optical isomers. His work led the way to our current understanding of a fundamental principal in the structure of organic compounds.

He was the director of the Pasteur Institute, established in 1887, till his death, and his body lies beneath the institute in a vault covered in depictions of his accomplishments in Byzantine mosaics.

Education and early life

Louis Pasteur was born on December 27, 1822, in Dole, Jura, France, to a Catholic family of a poor tanner. He was the third child of Jean-Joseph Pasteur and Jeanne- Etiennette Roqui. In 1827, the family moved to Arbois, where he entered primary school in 1831. He was an average student in his early years, and not particularly academic, as his interests were fishing and sketching. His pastels and portraits of his parents and friends, made when he was 15, were later kept in the museum of the Pasteur Institute in Paris. In 1838, he left for Paris to join the Institution Barbet, but became homesick and returned in November. In 1839, he entered the Collège Royal de Besançon and earned his *baccalauréat* (BA) degree in 1840. He was appointed teaching assistant at the Besançon college while continuing a degree science course with special mathematics. He failed his first examination in 1841. He managed to pass the *baccalauréat scientifique* (general science) degree in 1842 from Dijon but with a poor grade in chemistry. After one failed attempt for the entrance test for the *École Normale Supérieure* in Paris in 1842, he succeeded in 1844. In 1845 he received the *licencié ès sciences* (Bachelor of Science) degree.

After serving briefly as professor of physics at the Dijon Lycée in 1848, he became professor of chemistry at the University of Strasbourg, where he met and courted Marie Laurent, daughter of the university's rector in 1849. They were married on May 29, 1849, and together had five children, only two of whom survived to adulthood; the other three died of typhoid. These personal tragedies were his motivations for curing infectious diseases.[3][11]

Career

Pasteur was appointed to the Chair of Chemistry in the faculty of sciences of the University of Strasbourg in 1848. In 1854, he was named dean of the new faculty of sciences at Lille University.

In 1857, he moved to Paris as the director of scientific studies at the *École Normale Supérieure* where he took control from 1858 to 1867 and introduced a series of reforms to

improve the standard of scientific work. In 1862, he was appointed professor of geology, physics, and chemistry at the *École nationale supérieure des Beaux-Arts*, the position which held until his resignation in 1867. In Paris, he established the Pasteur Institute in 1887, in which he was its director for the rest of his life.[4][5][11]

Fermentation and germ theory of diseases

Pasteur demonstrated that fermentation is caused by the growth of micro- organisms, and the emergent growth of bacteria in nutrient broths is due not to spontaneous generation, but rather to biogenesis (*Omne vivum ex vivo* "all life from life"). He was motivated to investigate the matter while working at Lille. In 1856 a local wine manufacturer, M. Bigot, the father of his student, sought for his advice on the problems of making beetroot alcohol and souring after long storage.[21] In 1857 he developed his ideas stating that: "I intend to establish that, just as there is an alcoholic ferment, the yeast of beer, which is found everywhere that sugar is decomposed into alcohol and carbonic acid, so also there is a particular ferment, a lactic yeast, always present when sugar becomes lactic acid."[22] According to his son-in-law, Pasteur presented his experiment on sour milk titled "Latate Fermentation" in August 1857 before the Société des Sciences de Lille. (But according to a memoire subsequently published, it was dated November 30, 1857).[23][24] It was published in full form in 1858.[25][26][27] He demonstrated that yeast was responsible for fermentation to produce alcohol from sugar, and that air (oxygen) was not required. He also demonstrated that fermentation could also produce lactic acid (due to bacterial contamination), which make wines sour. This is regarded as the foundation of Pasteur's fermentation experiment and disprove of spontaneous generation of life.

Pasteur's research also showed that the growth of micro-organisms was responsible for spoiling beverages, such as beer, wine and milk. With this established, he invented a process in which liquids such as milk were heated to a temperature between 60 and 100 °C.[28] This killed most bacteria and moulds already present within them. Pasteur and Claude Bernard completed the first test on April 20, 1862.[7] Pasteur patented the process, to fight the "diseases" of wine, in 1865.[28] The method became known as pasteurization, and was soon applied to beer and milk.[29]

Beverage contamination led Pasteur to the idea that micro-organisms infecting animals and humans cause disease. He proposed preventing the entry of micro- organisms into the human body, leading Joseph Lister to develop antiseptic methods in surgery. Lister's work in turn inspired Joseph Lawrence to develop his own alcohol-based antiseptic, which he named in tribute Listerine.[30]

In 1865, two parasitic diseases called *pébrine* and *flacherie* were killing great numbers of silkworms at Alais (now Alès). Pasteur worked several years proving that these diseases were caused by a microbe attacking silkworm eggs, and that eliminating the microbe in silkworm nurseries would eradicate the disease.[7]

Spontaneous generation

Following his fermentation experiments, Pasteur demonstrated that the skin of grapes was the natural source of yeasts, and that sterilized grapes and grape juice never fermented. He drew grape juice from under the skin with sterilzed needles, and also covered grapes with sterilized cloth. Both experiments could not produce wine in sterilized containers. His findings and ideas were against the prevailing notion of spontaneous generation. He received a particularly stern criticism from Félix Archimède Pouchet, who was director of the Rouen Museum of Natural History. To settle the debate between the eminent scientists, the French Academy of Sciences offered Alhumbert Prize carrying 2,500 francs to whoever could experimentally demonstrate for or against the doctrine.

To prove himself correct, Pasteur exposed boiled broths to air in swan-neck flasks that contained a filter to prevent all particles from passing through to the growth medium, and even in flasks with no filter at all, with air being admitted via a long tortuous tube that would not allow dust particles to pass. Nothing grew in the broths unless the flasks were broken open, showing that the living organisms that grew in such broths came from outside, as spores on dust, rather than spontaneously generated within the broth. This was one of the last and most important experiments disproving the theory of spontaneous generation for which Pasteur won the Alhumbert Prize in 1862.

Never will the doctrine of spontaneous generation recover from the mortal blow of this simple experiment. There is no known circumstance in which it can be confirmed that

microscopic beings came into the world without germs, without parents similar to themselves.

Immunology and vaccination

Pasteur's later work on diseases included work on chicken cholera. During this work, a culture of the responsible bacteria had spoiled and failed to induce the disease in some chickens he was infecting with the disease. Upon reusing these healthy chickens, Pasteur discovered he could not infect them, even with fresh bacteria; the weakened bacteria had caused the chickens to become immune to the disease, though they had caused only mild symptoms.

His assistant, Charles Chamberland (of French origin), had been instructed to inoculate the chickens after Pasteur went on holiday. Chamberland failed to do this, but instead went on holiday himself. On his return, the month-old cultures made the chickens unwell, but instead of the infections being fatal, as they usually were, the chickens recovered completely. Chamberland assumed an error had been made, and wanted to discard the apparently faulty culture when Pasteur stopped him. Pasteur guessed the recovered animals now might be immune to the disease, as were the animals at Eure-et-Loir that had recovered from anthrax.

In the 1870s, he applied this immunization method to anthrax, which affected cattle, and aroused interest in combating other diseases.

Pasteur publicly claimed he had made the anthrax vaccine by exposing the bacilli to oxygen. His laboratory notebooks, now in the Bibliothèque Nationale in Paris, in fact show that he used the method of rival Jean-Joseph-Henri Toussaint, a Toulouse veterinary surgeon, to create the anthrax vaccine. This method used the oxidizing agent potassium dichromate. Pasteur's oxygen method did eventually produce a vaccine but only after he had been awarded a patent on the production of an anthrax vaccine.

The notion of a weak form of a disease causing immunity to the virulent version was not new; this had been known for a long time for smallpox. Inoculation with smallpox (Variolation) was known to result in far less scarring, and greatly reduced mortality, in comparison with the naturally acquired disease. Edward Jenner had also discovered vaccination using cowpox (Vaccinia) to give cross-immunity to smallpox in 1796, and by

Pasteur's time this had generally replaced the use of actual smallpox (Variola) material in inoculation. The difference between smallpox vaccination and anthrax or chicken cholera vaccination was that the weakened form of the latter two disease organisms had been "generated artificially", so a naturally weak form of the disease organism did not need to be found. This discovery revolutionized work in infectious diseases, and Pasteur gave these artificially weakened diseases the generic name of "vaccines", in honour of Jenner's discovery. Pasteur produced the first vaccine for rabies by growing the virus in rabbits, and then weakening it by drying the affected nerve tissue.[38]

The rabies vaccine was initially created by Emile Roux, a French doctor and a colleague of Pasteur who had been working with a killed vaccine produced by desiccating the spinal cords of infected rabbits. The vaccine had been tested in 50 dogs before its first human trial.[39][40] This vaccine was first used on 9-year old Joseph Meister, on July 6, 1885, after the boy was badly mauled by a rabid dog. This was done at some personal risk for Pasteur, since he was not a licensed physician and could have faced prosecution for treating the boy. After consulting with colleagues, he decided to go ahead with the treatment. Three months later he examined Meister and found that he was in good health.[41] Pasteur was hailed as a hero and the legal matter was not pursued. The treatment's success laid the foundations for the manufacture of many other vaccines. The first of the Pasteur Institutes was also built on the basis of this achievement.[20]

Legal risk was not the only kind Pasteur undertook. In *The Story of San Michele*, Axel Munthe writes of the rabies vaccine research:

Pasteur himself was absolutely fearless. Anxious to secure a sample of saliva straight from the jaws of a rabid dog, I once saw him with the glass tube held between his lips draw a few drops of the deadly saliva from the mouth of a rabid bull-dog, held on the table by two assistants, their hands protected by leather gloves.

Because of his study in germs, Pasteur encouraged doctors to sanitize their hands and equipment before surgery. Prior to this, few doctors or their assistants practiced these procedures.

Fermentation

He regarded himself as the first to show the role of microorganisms in fermentation.[48] Pasteur started his experiments only in 1857 and published his findings in 1858 (April issue of *Comptes Rendus Chimie*, Béchamp's paper appeared in January issue), which, as Béchamp noted, did not bring any novel idea or experiments that earlier works had not shown. Particularly on the spontaneous generation because Pasteur in his 1858 paper explicitly stated that the lactic acid bacteria (he named them "lactic yeasts"), which caused wine souring, "takes birth spontaneously, as easily as beer yeast every time that the conditions are favourable." This statement directly implied that Pasteur did believe in spontaneous generation. He condemned the ideas of Pasteur as "'the greatest scientific silliness of the age".[22] However, Béchamp was on the losing side, as the *BMJ* obituary remarked: His name was associated with bygone controversies as to priority which it would be unprofitable to recall.[51] Pasteur and Béchamp believed that fermentation was exclusively cellular activity, that is, it was only due to living cells. But later extraction of enzymes such as invertase by Marcelin Barthelot in 1860 showed that it was simply an enzymatic reaction.[52]

Anthrax vaccine

Pasteur had given a misleading account of the preparation of the anthrax vaccine used in the experiment at Pouilly-le-Fort.[9] The fact is that Pasteur publicly claimed his success in developing anthrax vaccine in 1881.[41] However, his admirer-turned-rival Toussaint was the one who developed the first vaccine. Toussaint isolated the Gram-negative bacteria *cholera des poules* (later named – to add irony – *Pasteurella* in honour of Pasteur) in 1879 and gave samples to Pasteur who used for his own works. In 1880 with his publishing on July 12 at the French Academy of Sciences, Toussaint presented his successful result with an attenuated vaccine against anthrax in dogs and sheep.[53] Pasteur purely on grounds of jealousy contested the discovery by publicly displaying his vaccination method in Pouilly-le-Fort on 5 May 1881. The promotional experiment was a success and helped Pasteur sell his products, getting all the benefits and glory.[54][55][56]

Experimental ethics

Pasteur experiments are often cited as against medical ethics, especially on his vaccination of Meister. Firstly, he did not have any experience in medical practice, and more importantly, a

medical license. This is often cited as a serious threat to his professional and personal reputation.[57][58]

Awards and honours

Pasteur was awarded the prize of 1,500 francs in 1853 by the Pharmaceutical Society for the synthesis of racemic acid. In 1856 the Royal Society of London presented him the Rumford Medal for his discovery of the nature of racemic acid and its relations to polarized light, and the Copley Medal in 1874 for his work on fermentation.

Pasteur Institute

The Pasteur Institute was established by Pasteur to perpetuate his commitment to basic research and its practical applications.

Death

Pasteur was frequently stricken by strokes beginning in 1868, and the one in 1894 severely impaired his health. Failing to fully recover, he died in 1895, near Paris.[20] He was given a state funeral and was buried in the Cathedral of Notre Dame, but his remains were reinterred in a crypt in the Pasteur Institute in Paris, where the crypt is engraved with his life-saving work.

Robert Koch

Robert Heinrich Herman Koch (/ˈkɔːx/;[3] German: [ˈkɔχ]; 11 December 1843 – 27 May 1910) was a celebrated German physician and pioneering microbiologist. As the founder of modern bacteriology, he is known for his role in identifying the specific causative agents of tuberculosis, cholera, and anthrax and for giving experimental support for the concept of infectious disease.[4] In addition to his trail-blazing studies on these diseases, Koch created and improved laboratory technologies and techniques in the field of microbiology, and made key discoveries in public health.[5] His research led to the creation of Koch's postulates, a series of four generalized principles linking specific microorganisms to specific diseases that remain today the "gold standard" in medical microbiology.[5] As a result of his ground

breaking research on tuberculosis, Koch received the Nobel Prize in Physiology or Medicine in 1905.[5]

Personal life

Robert Koch was born in Clausthal, Hanover, Germany, on 11 December 1843, to Hermann Koch and Mathilde Julie Henriette Biewand.[6] Koch excelled in academics from an early age. Before entering school in 1848, he had taught himself how to read and write.[4] He graduated from high school in 1862, having excelled in science and maths.[4] At the age of 19, Koch entered the University of Göttingen, studying natural science.[7] However, after three semesters, Koch decided to change his area of study to medicine, as he aspired to be a physician.[4] During his fifth semester of medical school, Jacob Henle, an anatomist who had published a theory of contagion in 1840, asked him to participate in his research project on uterine nerve structure.[4] In his sixth semester, Koch began to conduct research at the Physiological Institute, where he studied Succinic acid secretion.[4] This would eventually form the basis of his dissertation.[5] In January 1866, Koch graduated from medical school, earning honors of the highest distinction.[4] In July 1867, Koch married Emma Adolfine Josephine Fraatz, and the two had a daughter, Gertrude, in 1868.[5] After his graduation in 1866, he worked as a surgeon in the Franco-Prussian War, and following his service, worked as a physician in Wollstein, Posen.[7] Koch's marriage to Emma Fraatz ended in 1893, and later that same year, he married actress Hedwig Freiberg.[5] From 1885 to 1890, he served as an administrator and professor at Berlin University.[4] Koch suffered a heart attack on 9 April 1910, and never made a complete recovery.[4] On 27 May, only three days after giving a lecture on his tuberculosis research at the Prussian Academy of Sciences, Robert Koch died in Baden-Baden at the age of 66.[7] Following his death, the Institute named its establishment after him in his honour.[4]

Research contributions Anthrax

Robert Koch is widely known for his work with anthrax, discovering the causative agent of the fatal disease to be *Bacillus anthracis*.[8] Koch discovered the formation in anthrax bacteria of spores that could remain dormant under specific conditions.[7] However, under optimal conditions, the spores were activated and caused disease.[7] To determine this causative agent, he dry-fixed bacterial cultures onto glass slides, used dyes to stain the cultures, and observed

them through a microscope.[4] Koch's work with anthrax is notable in that he was the first to link a specific microorganism with a specific disease, rejecting the idea of spontaneous generation and supporting the germ theory of disease.[8]

Koch's four postulates

Koch accepted a position as government advisor with the Imperial Department of Health in 1880.[9] During his time as government advisor, he published a report in which he stated the importance of pure cultures in isolating disease-causing organisms and explained the necessary steps to obtain these cultures, methods which are summarized in Koch's four postulates.[10] Koch's discovery of the causative agent of anthrax led to the formation of a generic set of postulates which can be used in the determination of the cause of most infectious diseases.[8] These postulates, which not only outlined a method for linking cause and effect of an infectious disease but also established the significance of laboratory culture of infectious agents, are listed here:[8]

1. The organism must always be present, in every case of the disease.

2. The organism must be isolated from a host containing the disease and grown in pure culture.

3. Samples of the organism taken from pure culture must cause the same disease when inoculated into a healthy, susceptible animal in the laboratory.

4. The organism must be isolated from the inoculated animal and must be identified as the same original organism first isolated from the originally diseased host.

Isolating pure culture on solid media

Koch began conducting research on microorganisms in a laboratory connected to his patient examination room.[7] Koch's early research in this laboratory proved to yield one of his major contributions to the field of microbiology, as it was there that he developed the technique of growing bacteria. Koch's second postulate calls for the isolation and growth of a selected pathogen in pure laboratory culture.[11] In an attempt to grow bacteria, Koch began to use solid nutrients such as potato slices.[11] Through these initial experiments, Koch observed

individual colonies of identical, pure cells.[11] Coming to the conclusion that potato slices were not suitable media for all organisms, Koch later began to use nutrient solutions with gelatin.[11] However, he soon realized that gelatin, like potato slices, was not the optimal medium for bacterial growth, as it did not remain solid at 37 °C, the ideal temperature for growth of most human pathogens.[11] As suggested to him by Walther and Fanny Hesse, Koch began to utilize agar to grow and isolate pure cultures, as this polysaccharide remains solid at 37 °C, is not degraded by most bacteria, and results in a transparent medium.[11][12]

Cholera

Koch next turned his attention to cholera, and began to conduct research in Egypt in the hopes of isolating the causative agent of the disease.[7] However, he was not able to complete the task before the epidemic in Egypt ended, and subsequently traveled to India to continue with the study.[4] In India, Koch was indeed able to determine the causative agent of cholera, isolating *Vibrio cholerae*.[4][13] The bacterium had originally been isolated in 1854 by Italian anatomist Filippo Pacini,[14] but its exact nature and his results were not widely known.

Tuberculosis

During his time as the government advisor with the Imperial Department of Health in Berlin in the 1880s, Robert Koch became interested in tuberculosis research.[4] At the time, it was widely believed that tuberculosis was an inherited disease.[4] However, Koch was convinced that the disease was caused by a bacterium and was infectious, and tested his four postulates using guinea pigs.[4] Through these experiments, he found that his experiments with tuberculosis satisfied all four of his postulates.[4] In 1882, he published his findings on tuberculosis, in which he reported the causative agent of the disease to be the slow-growing *Mycobacterium tuberculosis*.[11] His work with this disease won Koch the Nobel Prize in Physiology and Medicine in 1905.[4] Additionally, Koch's research on tuberculosis, along with his studies on tropical diseases, won him the Prussian Order Pour le Merite in 1906 and the Robert Koch medal, established to honour the greatest living physicians, in 1908.[4]

Awards and honours

In addition to being awarded a Nobel Prize, Koch was elected a Foreign Member of the Royal Society (ForMemRS) in 1897.[2] His microbial postulates are named in his honour, Koch's postulates.

Edward Jenner

Edward Jenner, FRS (/ˈdʒɛnər/; 17 May 1749 – 26 January 1823) was an English physician and scientist who was the pioneer of smallpox vaccine, the world's first vaccine.[1][2] He is often called "the father of immunology", and his work is said to have "saved more lives than the work of any other human".[3][4][5]

He was also the first person to describe the brood parasitism of the cuckoo.

Early life

Edward Anthony Jenner was born on 17 May 1749[6] (6 May Old Style) in Berkeley, Gloucestershire, as the eighth of nine children. His father, the Reverend Stephen Jenner, was the vicar of Berkeley, so Jenner received a strong basic education.[6]

He went to school in Wotton-under-Edge and Cirencester.[6] During this time, he was inoculated for smallpox, which had a lifelong effect upon his general health.[6] At the age of 14, he was apprenticed for seven years to Mr Daniel Ludlow, a surgeon of Chipping Sodbury, South Gloucestershire, where he gained most of the experience needed to become a surgeon himself.[6]

In 1770, Jenner became apprenticed in surgery and anatomy under surgeon John Hunter and others at St George's Hospital.[7] William Osler records that Hunter gave Jenner William Harvey's advice, very famous in medical circles (and characteristic of the Age of Enlightenment), "Don't think; try."[8] Hunter remained in correspondence with Jenner over natural history and proposed him for the Royal Society. Returning to his native countryside by 1773, Jenner became a successful family doctor and surgeon, practising on dedicated premises at Berkeley.

Jenner and others formed the Fleece Medical Society or Gloucestershire Medical Society, so called because it met in the parlour of the Fleece Inn, Rodborough (in Gloucestershire), meeting to dine together and read papers on medical subjects. Jenner contributed papers on angina pectoris, ophthalmia, and cardiac valvular disease and commented on cowpox. He also belonged to a similar society which met in Alveston, near Bristol.[9]

He became a master mason 30 December 1802, in Lodge of Faith and Friendship #449. From 1812–1813, he served as worshipful master of Royal Berkeley Lodge of Faith and Friendship.[10]

Zoology

Jenner was elected fellow of the Royal Society in 1788, following his publication of a careful study of the previously misunderstood life of the nested cuckoo, a study that combined observation, experiment, and dissection.

Marriage and human medicine

Jenner married Catharine Kingscote (died 1815 from tuberculosis) in March 1788. He might have met her while he and other fellows were experimenting with balloons. Jenner's trial balloon descended into Kingscote Park, Gloucestershire, owned by Anthony Kingscote, one of whose daughters was Catharine.[15]

He earned his MD from the University of St Andrews in 1792. He is credited with advancing the understanding of angina pectoris.[16] In his correspondence with Heberden, he wrote, "How much the heart must suffer from the coronary arteries not being able to perform their functions."

Invention of the vaccine

Inoculation was already a standard practice, but involved serious risks. In 1721, Lady Mary Wortley Montagu had imported variolation to Britain after having observed it in Istanbul, where her husband was the British ambassador. Voltaire, writing of this, estimates that at this time 60% of the population caught smallpox and 20% of the population died of it.[17] Voltaire also states that the Circassians used the inoculation from times immemorial, and the custom

may have been borrowed by the Turks from the Circassians.[18]

Jenner's Hypothesis:

The initial source of infection was a disease of horses, called "the grease", which was transferred to cattle by farm workers, transformed, and then manifested as cowpox.

Noting the common observation that milkmaids were generally immune to smallpox, Jenner postulated that the pus in the blisters that milkmaids received from cowpox (a disease similar to smallpox, but much less virulent) protected them from smallpox.

On 14 May 1796, Jenner tested his hypothesis by inoculating James Phipps, an eight-year-old boy who was the son of Jenner's gardener. He scraped pus from cowpox blisters on the hands of Sarah Nelmes, a milkmaid who had caught cowpox from a cow called Blossom,[23] whose hide now hangs on the wall of the St George's medical school library (now in Tooting). Phipps was the 17th case described in Jenner's first paper[24] on vaccination.

Jenner inoculated Phipps in both arms that day, subsequently producing in Phipps a fever and some uneasiness, but no full-blown infection. Later, he injected Phipps with variolous material, the routine method of immunization at that time. No disease followed. The boy was later challenged with variolous material and again showed no sign of infection.

Donald Hopkins has written, "Jenner's unique contribution was not that he inoculated a few persons with cowpox, but that he then proved [by subsequent challenges] that they were immune to smallpox. Moreover, he demonstrated that the protective cowpox pus could be effectively inoculated from person to person, not just directly from cattle.[25] Jenner successfully tested his hypothesis on 23 additional subjects.

Jenner continued his research and reported it to the Royal Society, which did not publish the initial paper. After revisions and further investigations, he published his findings on the 23 cases. Some of his conclusions were correct, some erroneous; modern microbiological and microscopic methods would make his studies easier to reproduce. The medical establishment,

cautious then as now, deliberated at length over his findings before accepting them. Eventually, vaccination was accepted, and in 1840, the British government banned variolation – the use of smallpox to induce immunity – and provided vaccination using cowpox free of charge. (See Vaccination acts). The success of his discovery soon spread around Europe and, for example, was used *en masse* in the Spanish Balmis Expedition,[26] a three-year- long mission to the Americas, the Philippines, Macao, China, and Saint Helena Island led by Dr. Francisco Javier de Balmis with the aim of giving thousands the smallpox vaccine. The expedition was successful, and Jenner wrote, "I don't imagine the annals of history furnish an example of philanthropy so noble, so extensive as this."

Jenner's continuing work on vaccination prevented him from continuing his ordinary medical practice. He was supported by his colleagues and the King in petitioning Parliament, and was granted £10,000 in 1802 for his work on vaccination. In 1807, he was granted another £20,000 after the Royal College of Physicians had confirmed the widespread efficacy of vaccination.

In 1803 in London, he became president of the **Jennerian Society**, concerned with promoting vaccination to eradicate smallpox. The Jennerian ceased operations in 1809. In 1808, with government aid, the National Vaccine Establishment was founded, but Jenner felt dishonoured by the men selected to run it and resigned his directorship.[27] Jenner became a member of the Medical and Chirurgical Society on its founding in 1805 and presented a number of papers there. The society is now the Royal Society of Medicine. He was elected a foreign honorary member of the American Academy of Arts and Sciences in 1802.[28] In 1806, Jenner was elected a foreign member of the Royal Swedish Academy of Sciences.

Returning to London in 1811, Jenner observed a significant number of cases of smallpox after vaccination. He found that in these cases the severity of the illness was notably diminished by previous vaccination. In 1821, he was appointed physician extraordinary to King George IV, a great national honour, and was also made mayor of Berkeley and justice of the peace. He continued to investigate natural history, and in 1823, the last year of his life, he presented his "Observations on the Migration of Birds" to the Royal Society.

Jenner was found in a state of apoplexy on 25 January 1823, with his right side paralysed. He never fully recovered and eventually died of an apparent stroke, his second, on 26 January

1823, aged 73. He was buried in the Jenner family vault at the Church of St. Mary's, Berkeley, Gloucestershire.[29]

Edward Jenner was survived by one son and one daughter, his elder son having died of tuberculosis at the age of 21.

Legacy

In 1979, the World Health Organization declared smallpox an eradicated disease.[33] This was the result of coordinated public health efforts by many people, but vaccination was an essential component.

Alexander Fleming

Born	6 August 1872 Lochfield, East Ayrshire, Scotland
Died	11 March 1955 (aged 73) London, England
Citizenship	British
Fields	Bacteriology, immunology
Alma mater	• Royal Polytechnic Institution • St Mary's Hospital Medical School • Imperial College London
Known for	Discovery of penicillin

Sir Alexander Fleming FRS FRSE FRCS[1] (6 August 1881 – 11 March 1958) was a Scottish biologist, pharmacologist and botanist. His best-known discoveries are the enzyme lysozyme in 1923 and the antibiotic substance benzylpenicillin (Penicillin G) from the mould *Penicillium notatum* in 1928, for which he shared the Nobel Prize in Physiology or Medicine in 1945 with Howard Florey and Ernst Boris Chain.[2][3][4][5][6][7] He wrote many articles on bacteriology, immunology, and chemotherapy.

Early life and education

Born on 6 August 1881 at Lochfield farm near Darvel, in Ayrshire, Scotland, Alexander was the third of the four children of farmer Hugh Fleming (1816–1888) from his second marriage to Grace Stirling Morton (1848–1928), the daughter of a neighbouring farmer. Hugh Fleming had four surviving children from his first marriage. He was 59 at the time of his second marriage, and died when Alexander (known as Alex) was seven.

Fleming went to Loudoun Moor School and Darvel School, and earned a two-year scholarship to Kilmarnock Academy before moving to London, where he attended the Royal Polytechnic Institution.[8] After working in a shipping office for four years, the twenty-year-old Fleming inherited some money from an uncle, John Fleming. His elder brother, Tom, was already a physician and suggested to him that he should follow the same career, and so in 1903, the younger Alexander enrolled at St Mary's Hospital Medical School in Paddington; he qualified with an MBBS degree from the school with distinction in 1906.

Fleming had been a private in the London Scottish Regiment of the Volunteer Force since 1900,[2] and had been a member of the rifle club at the medical school. The captain of the club, wishing to retain Fleming in the team suggested that he join the research department at St Mary's, where he became assistant bacteriologist to Sir Almroth Wright, a pioneer in vaccine therapy and immunology. In 1908, he gained a BSc degree with Gold Medal in Bacteriology, and became a lecturer at St Mary's until 1914. Fleming served throughout World War I as a captain in the Royal Army Medical Corps, and was Mentioned in Dispatches. He and many of his colleagues worked in battlefield hospitals at the Western Front in France. In 1918 he returned to St Mary's Hospital, where he was elected Professor of Bacteriology of the University of London in 1928. In 1951 he was elected the Rector of the University of Edinburgh for a term of 3 years.

Research

Following World War I, Fleming actively searched for anti-bacterial agents, having witnessed the death of many soldiers from sepsis resulting from infected wounds. Antiseptics killed the patients' immunological defences more effectively than they killed the invading bacteria. In an article he submitted for the medical journal *The Lancet* during World War I, Fleming described an ingenious experiment, which he was able to conduct as a result of his

own glass blowing skills, in which he explained why antiseptics were killing more soldiers than infection itself during World War I. Antiseptics worked well on the surface, but deep wounds tended to shelter anaerobic bacteria from the antiseptic agent, and antiseptics seemed to remove beneficial agents produced that protected the patients At St Mary's Hospital Fleming continued his investigations into antibacterial substances. Testing the nasal secretions from a patient with a heavy cold, he found that nasal mucus had an inhibitory effect on bacterial growth.[10] This was the first recorded discovery of lysozyme, an enzyme present in many secretions including tears, saliva, human milk as well as mucus. Lysozyme degrades the bonds in bacterial peptidoglycan cell walls, particularly in Gram-positive organisms. Unfortunately, lysozyme had little therapeutic potential.

Accidental discovery

"When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionise all medicine by discovering the world's first antibiotic, or bacteria killer," Fleming would later say, "But I suppose that was exactly what I did."[11]

By 1927, Fleming had been investigating the properties of staphylococci. He was already well-known from his earlier work, and had developed a reputation as a brilliant researcher, but his laboratory was often untidy. On 3 September 1928, Fleming returned to his laboratory having spent August on holiday with his family. Before leaving, he had stacked all his cultures of staphylococci on a bench in a corner of his laboratory. On returning, Fleming noticed that one culture was contaminated with a fungus, and that the colonies of staphylococci immediately surrounding the fungus had been destroyed, whereas other staphylococci colonies farther away were normal, famously remarking *"That's funny"*.[12] Fleming showed the contaminated culture to his former assistant Merlin Price, who reminded him, "That's how you discovered lysozyme."[13] Fleming grew the mould in a pure culture and found that it produced a substance that killed a number of disease- causing bacteria. He identified the mould as being from the *Penicillium* genus, and, after some months of calling it *"mould juice"*, named the substance it released *penicillin* on 7 March 1929.[14] The laboratory in which Fleming discovered and tested penicillin is preserved as the Alexander Fleming Laboratory Museum in St. Mary's Hospital, Paddington.

He investigated its positive anti-bacterial effect on many organisms, and noticed that it affected bacteria such as staphylococci and many other Gram-positive pathogens that cause scarlet fever, pneumonia, meningitis and diphtheria, but not typhoid fever or paratyphoid fever, which are caused by Gram-negative bacteria, for which he was seeking a cure at the time. It also affected *Neisseria gonorrhoeae,* which causes gonorrhoea although this bacterium is Gram-negative.

Fleming published his discovery in 1929, in the British *Journal of Experimental Pathology*,[15] but little attention was paid to his article. Fleming continued his investigations, but found that cultivating *penicillium* was quite difficult, and that after having grown the mould, it was even more difficult to isolate the antibiotic agent. Fleming's impression was that because of the problem of producing it in quantity, and because its action appeared to be rather slow, penicillin would not be important in treating infection. Fleming also became convinced that penicillin would not last long enough in the human body (*in vivo*) to kill bacteria effectively. Many clinical tests were inconclusive, probably because it had been used as a surface antiseptic. They started mass production after the bombing of Pearl Harbor.

By D-Day in 1944, enough penicillin had been produced to treat all the wounded with the Allied forces.

Purification and stabilisation

In Oxford, Ernst Boris Chain and Edward Abraham discovered how to isolate and concentrate penicillin. Abraham was the first to propose the correct structure of penicillin.[17][18] Shortly after the team published its first results in 1940, Fleming telephoned Howard Florey, Chain's head of department, to say that he would be visiting within the next few days. When Chain heard that Fleming was coming, he remarked *"Good God! I thought he was dead."*

Norman Heatley suggested transferring the active ingredient of penicillin back into water by changing its acidity. This produced enough of the drug to begin testing on animals. There were many more people involved in the Oxford team, and at one point the entire Dunn School was involved in its production.

After the team had developed a method of purifying penicillin to an effective first stable form in 1940, several clinical trials ensued, and their amazing success inspired the team to develop methods for mass production and mass distribution in 1945.

Fleming was modest about his part in the development of penicillin, describing his fame as the *"Fleming Myth"* and he praised Florey and Chain for transforming the laboratory curiosity into a practical drug. Fleming was the first to discover the properties of the active substance, giving him the privilege of naming it: penicillin. He also kept, grew, and distributed the original mould for twelve years, and continued until 1940 to try to get help from any chemist who had enough skill to make penicillin. But Sir Henry Harris said in 1998: "Without Fleming, no Chain; without Chain, no Florey; without Florey, no Heatley; without Heatley, no penicillin."[19]

Antibiotics

Fleming's accidental discovery and isolation of penicillin in September 1928 marks the start of modern antibiotics. Before that, several scientists had published or pointed out that mould or *penicillium sp.* were able to inhibit bacterial growth, and even to cure bacterial infections in animals. Ernest Duchesne in 1897 in his thesis "Contribution to the study of vital competition in micro-organisms: antagonism between moulds and microbes",[20] or also Clodomiro Picado Twight whose work at Institut Pasteur in 1923 on the inhibiting action of fungi of the "Penicillin sp" genre in the growth of staphylococci drew little interest from the direction of the Institut at the time. Fleming was the first to push these studies further by isolating the penicillin, and by being motivated enough to promote his discovery at a larger scale. Fleming also discovered very early that bacteria developed antibiotic resistance whenever too little penicillin was used or when it was used for too short a period. Almroth Wright had predicted antibiotic resistance even before it was noticed during experiments. Fleming cautioned about the use of penicillin in his many speeches around the world. He cautioned not to use penicillin unless there was a properly diagnosed reason for it to be used, and that if it were used, never to use too little, or for too short a period, since these are the circumstances under which bacterial resistance to antibiotics develops.

Personal life

On 24 December 1915, Fleming married a trained nurse, Sarah Marion McElroy of Killala, County Mayo, Ireland. Their only child, Robert Fleming, (1924 - 2 July 2015) became a general medical practitioner. Robert married Kathleen, a trained radiographer on 10 September 1955 and had two children: Andrew (b. 1956) and Sarah (b. 1959).

Fleming has two great grandsons (James and Christopher) and one great granddaughter (Claire). After Sarah's death in 1949, Fleming married Dr. Amalia Koutsouri-Vourekas, a Greek colleague at St. Mary's, on 9 April 1953; she died in 1986.[*citation needed*]

Death

On 11 March 1955, Fleming died at his home in London of a heart attack. He was buried in St Paul's Cathedral

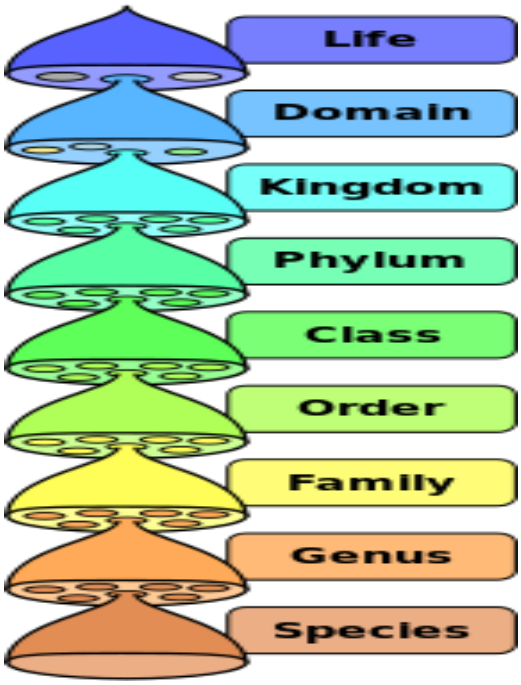

Fig.4 Classification of microorganisms

In biology, **kingdom** (Latin: ***regnum***, pl. ***regna***) is a taxonomic rank, which is either the highest rank or in the more recent three-domain system, the rank below domain. Kingdoms are divided into smaller groups called phyla (in zoology) or divisions in botany.

Definition and associated terms

When Carl Linnaeus introduced the rank-based system of nomenclature into biology, the highest rank was given the name "kingdom" and was followed by four other main or principal ranks.[1] Later two further main ranks were introduced, making the sequence kingdom, phylum or division, class, order, family, genus and species.[2] In the 1960s a rank was introduced above kingdom, namely domain (or empire), so that kingdom is no longer the highest rank.

Systems of classification

Historically, the number of kingdoms in widely accepted classifications has grown from two to six. However, phylogenetic research from about 2000 onwards does not support any of the traditional systems

Classical classification

An initial dichotomy: Two kingdoms

The classification of living things into animals and plants is an ancient one. Aristotle (384–322 BC) classified animal species in his work *The History of Animals*, and his pupil Theophrastus (c. 371–c. 287 BC) wrote a parallel work on plants (*Historia Plantarum* (The History of Plants)).[4]

Carolus Linnaeus (1707–1778) laid the foundations for modern biological nomenclature, now regulated by the Nomenclature Codes. He distinguished two kingdoms of living things: *Regnum Animale* ('animal kingdom') for animals and *Regnum Vegetabile* ('vegetable kingdom') for plants. (Linnaeus also included minerals, placing them in a third kingdom, *Regnum Lapideum*.) Linnaeus divided each kingdom into classes, later grouped into phyla for animals and divisions for plants.

An increasing number of kingdoms Three kingdoms

In 1674, Antonie van Leeuwenhoek, often called the "father of microscopy", sent the Royal Society of London a copy of his first observations of microscopic single-celled organisms. Until then, the existence of such microscopic organisms was entirely unknown. In 1866,

following earlier proposals by Richard Owen and Ernst Haeckel proposed a third kingdom of life. Haeckel revised the content of this kingdom a number of times before settling on a division based on whether organisms were unicellular (Protista) or multicellular (animals and plants).[5]

Four kingdoms

The development of microscopy, and the electron microscope in particular, revealed an important distinction between those unicellular organisms whose cells do not have a distinct nucleus, prokaryotes, and those unicellular and multicellular organisms whose cells do have a distinct nucleus, eukaryotes. In 1938, Herbert F. Copeland proposed a four-kingdom classification, moving the two prokaryotic groups, bacteria and "blue-green algae", into a separate Kingdom Monera.[5]

Five kingdoms (Whittaker system)

The differences between fungi and other organisms regarded as plants had long been recognized. For example, at one point Haeckel moved the fungi out of Plantae into Protista, before changing his mind.[5] Robert Whittaker recognized an additional kingdom for the Fungi. The resulting five- kingdom system, proposed in 1969 by Whittaker, has become a popular standard and with some refinement is still used in many works and forms the basis for new multi-kingdom systems. It is based mainly on differences in nutrition; his Plantae were mostly multicellular autotrophs, his Animalia multicellular heterotrophs, and his Fungi multicellular saprotrophs. The remaining two kingdoms, Protista and Monera, included unicellular and simple cellular colonies.[7] The five kingdom system may be combined with the two empire system.

In the Whittaker system, Plantae included some algae. In other systems (e.g., Margulis system), Plantae included just the land plants (Embryophyta).

RECENT DEVELOPMENTS: SIX KINGDOMS OR MORE?

The three domains of life

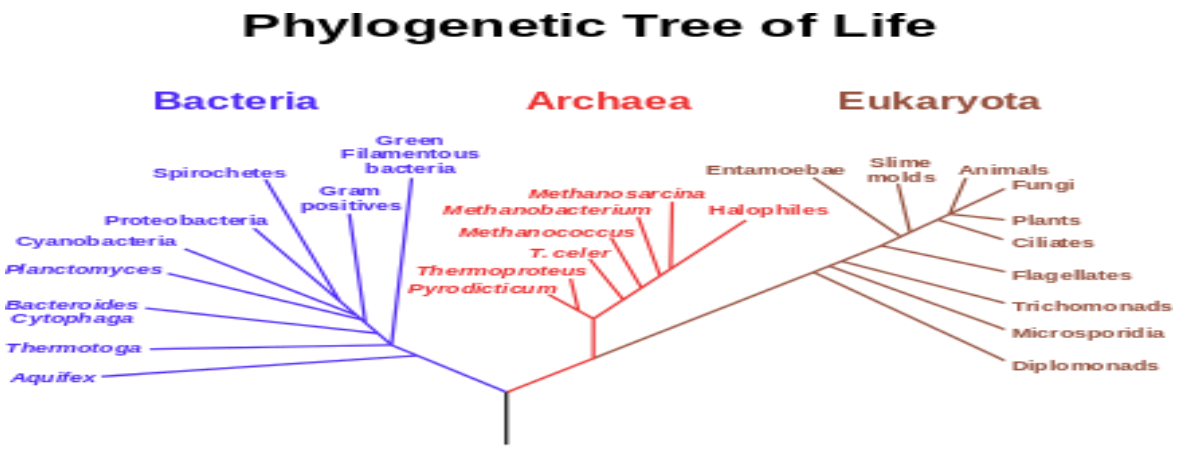

Fig.5 A phylogenetic tree based on rRNA data showing Woese's three-domain system

From around the mid-1970s onwards, there was an increasing emphasis on comparisons of genes on the molecular level (initially ribosomal RNA genes) as the primary factor in classification; genetic similarity was stressed over outward appearances and behavior. Taxonomic ranks, including kingdoms, were to be groups of organisms with a common ancestor, whether monophyletic (*all* descendants of a common ancestor) or paraphyletic (*only some* descendants of a common ancestor).

Based on such RNA studies, Carl Woese divided the prokaryotes (hitherto classified as the Kingdom Monera) into two groups, called Eubacteria and Archaebacteria, stressing that there was as much genetic difference between these two groups as between either of them and all eukaryotes. Similarly, though eukaryote groups such as plants, fungi and animals may look different, they are more closely related to each other from a genetic standpoint than they are to either the Eubacteria or Archaebacteria. It was also found that the eukaryotes are more closely related, genetically, to the Archaebacteria than they are to the Eubacteria.

Although the primacy of the eubacteria-archaebacteria divide has been questioned, it has been upheld by subsequent research.[8]

Woese attempted to establish a "three primary kingdom" or "urkingdom" system.[9] In 1990, the name "domain" was proposed for the highest rank.[10] The six-kingdom system shown

below represents a blending of the classic five-kingdom system and Woese's three-domain system. Such six-kingdom systems have become standard in many works.

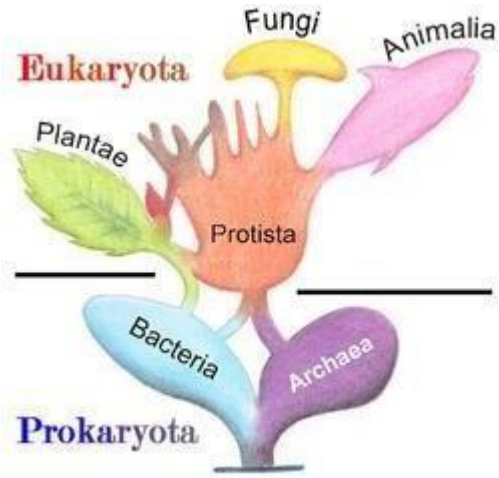

Fig.6.

Phylogenetic and symbiogenetic tree of living organisms, showing the origins of eukaryotes[11] and evolutionary relationships between groups.

Woese also recognized that the kingdom Protista was not a monophyletic group and might be further divided at the level of kingdom.

International Society of Protistologists Classification (2005)

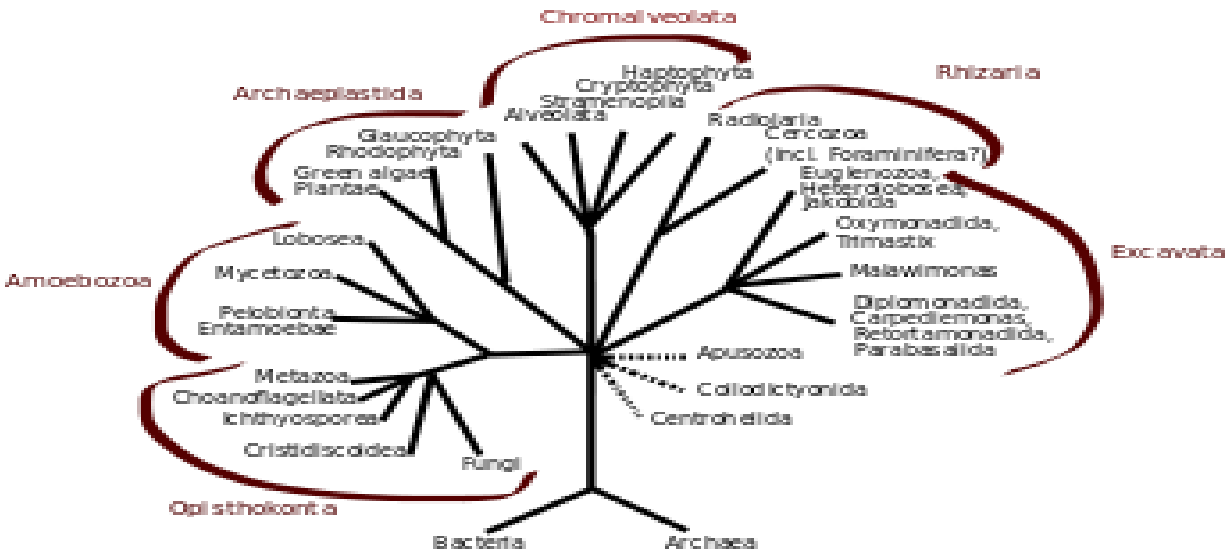

Fig.7

One hypothesis of eukaryotic relationships, modified from Simpson and Roger (2004).

The "classic" six-kingdom system is still recognizably a modification of the original two-kingdom system: Animalia remains; the original category of plants has been split into Plantae and Fungi; and single-celled organisms have been introduced and split into Bacteria, Archaea and Protista.

In this system, the traditional kingdoms have vanished. For example, research shows that the multicellular animals (Metazoa) are descended from the same ancestor as the unicellular choanoflagellates and the fungi. A classification system which places these three groups into different kingdoms (with multicellular animals forming Animalia, choanoflagellates part of Protista and Fungi a separate kingdom) is not monophyletic. The monophyletic group is the Opisthokonta, made up of all those organisms believed to have descended from a common ancestor, some of which are unicellular (choanoflagellates), some of which are multicellular but not closely related to animals (some fungi), and others of which are traditional multicellular animals.[16]

SUMMARY

The sequence from the two-kingdom system up to Cavalier-Smith's six-kingdom system can be summarized in the table below.

<table>
<tr><th>Linnaeus 1735</th><th>Haeckel 1866</th><th>Chatton 1925</th><th>Copeland 1938</th><th>Whittaker 1969</th><th>Woese et al. 1977</th><th>Woese et al. 1990</th><th>Cavalier-Smith 1993</th><th>Cavalier-Smith 1998</th></tr>
<tr><td>2 kingdoms</td><td>3 kingdoms</td><td>2 empires</td><td>4 kingdoms</td><td>5 kingdoms</td><td>6 kingdoms</td><td>3 domains</td><td>8 kingdoms</td><td>6 kingdoms</td></tr>
<tr><td rowspan="6">(not treated)</td><td rowspan="6">Protista</td><td rowspan="2">Prokaryota</td><td rowspan="2">Monera</td><td rowspan="2">Monera</td><td>Eubacteria</td><td>Bacteria</td><td>Eubacteria</td><td rowspan="2">Bacteria</td></tr>
<tr><td>Archae bacteria</td><td>Archaea</td><td>Archae bacteria</td></tr>
<tr><td rowspan="4">Eukaryota</td><td rowspan="3">Protoctista</td><td rowspan="3">Protista</td><td rowspan="3">Protista</td><td rowspan="5">Eucarya</td><td>Archezoa</td><td rowspan="2">Protozoa</td></tr>
<tr><td>Protozoa</td></tr>
<tr><td>Chromista</td><td>Chromista</td></tr>
<tr><td rowspan="2">Vegetabilia</td><td rowspan="2">Plantae</td><td rowspan="2">Plantae</td><td>Plantae</td><td>Plantae</td><td>Plantae</td><td>Plantae</td></tr>
<tr><td>Fungi</td><td>Fungi</td><td>Fungi</td><td>Fungi</td></tr>
<tr><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td></tr>
</table>

- Kingdoms such as Bacteria represent grades rather than clades, and so are rejected by phylogenetic classification systems.

- The most recent research does not support the classification of the eukaryotes into any of the standard systems. As of April 2010, no set of kingdoms is sufficiently supported by research to attain widespread acceptance. In 2009, Andrew Roger and Alastair Simpson emphasized the need for diligence in analyzing new discoveries: "With the current pace of change in our understanding of the eukaryote tree of life, we should proceed with caution."[38]

Viruses

There is ongoing debate as to whether viruses, obligate intracellular parasites that are not capable of replication outside of a host, can be included in the tree of life.[39] [40] A principal reason for inclusion comes from the discovery of unusually large and complex viruses, such as Mimivirus, that possess typical cellular genes.[41]

Current classification

Knowledge of the phenotypic, genotypic and biological characteristics of a microorganism is imperative in differentiating it from its pathogenic and/or toxigenic relatives or other microorganisms that are detrimental to the health of plants, animals, humans and the environment.

Polyphasic approach to microbial identification

The selection of method(s) used for microbial identification depends on the type and nature of the microorganism. The method(s) chosen should be well-described in scientific literature and consistent with those currently used in the field of microbial identification and taxonomic classification and they must enable identification of the organisms to the genus and species and, if possible, strain level. The robustness, precision and validity of the methodologies used to identify the microorganism are critical elements in the assessment of the safety of the product.

The choice of methods for microbial identification is at the discretion of the product proponent. However, the Fertilizer Safety Office recommends that applicants adopt an integrated polyphasic approach that includes classical microbiological and phenotypic analysis along with molecular tools, to accurately identify the active microorganism(s). The strengths and weaknesses of the various identification methods should be taken into consideration, such that the methods chosen complement each other to result in a conclusive and definitive identification of the microorganism, and allow for clear differentiation of the organism from any closely related pathogenic and/or toxigenic species and strains. The methods commonly used in identification and substantiation of taxonomic classification of microorganisms are summarized below.

1. Phenotypic analysis

Preliminary analysis in microbial identification often involves one or more phenotypic methods. Phenotypic methods are suitable for microorganisms that are culturable (i.e., can grow as pure culture on artificial media), have well-established growth parameters, and physiological and biochemical profiles.

a. Analysis of morphological traits

These methods utilize colony and cell morphology to obtain an initial identification of a microorganism. This is accomplished through simple isolation and culturing of the microorganism and subsequent visual observation using microscopy. The morphological properties include:

1. shape,
2. size,
3. surface characteristics and pigmentation,
4. cell wall characteristics (Gram-staining),
5. sporulation characteristics,
6. mechanisms of motility, and
7. other cellular inclusions and ultrastructural characteristics.

b. Analysis of biochemical, physiological and metabolic characteristics

Phenotypic identification methods include the study of the biochemical profile and metabolic properties of a microorganism by testing its growth requirements, enzymatic activities and cellular fatty acid composition.

The biochemical tests use specific growth media, nutrients, chemicals or growth conditions to elicit an observable or measurable biochemical response from the microorganism, thereby enabling its identification and characterization. These tests include: utilization of carbon and nitrogen sources, growth requirements (anaerobic or aerobic; temperature-optimum and range, pH optimum and range), preferred osmotic conditions, generation of fermentation products, production of enzymes, production of antimicrobial compounds, as well as sensitivity to metabolic inhibitors and antibiotics. Examples of recognized tests include: phenol red carbohydrate, catalase and oxidase tests, oxidation-fermentation tests, methyl red tests, Voges- Proskauer tests, nitrate reduction, starch hydrolysis, tryptophan hydrolysis, hydrogen sulfide production, citrate utilization, litmus milk reactions, etc. Several miniaturized and automated commercial systems are currently available with well-defined quality control procedures that allow for rapid identification of microorganisms.

c. Analysis of Fatty Acid Methyl Ester composition (FAME analysis)

Microorganisms can be identified by analysing the fatty acid profiles of whole cells or cell membranes using gas-liquid chromatography or mass spectrometry. The data on the type, content, proportion and variation in the fatty acid profile are used to identify and characterize the genus and species by comparing it against the fatty acid profiles of known organisms.

The expression of microbial phenotypes is highly dependent on environmental variables (e.g., culture pH, temperature, selective vs non-selective media, depletion of nutrients, presence of stressors etc.), and thus, may introduce inconsistencies in the identification process. The phenotypic methods are only acceptable if the response criteria are sufficient to identify the microorganism with a high level of confidence and distinguish it from phylogenetically close relatives that potentially pose safety concerns. Also, the applicability of the method is based on the robustness of information in reference databases. As such, results from phenotypic methods may require supporting data from other methods to accurately identify a microorganism.

2. Molecular Methods

Development of molecular methods has greatly improved the ability to rapidly detect, identify and classify microorganisms and also establish the taxonomic relationship among closely related genera and species. Identification, using molecular methods, relies on the comparison of the nucleic acid sequences (DNA, RNA) or protein profiles of a microorganism with documented data on known organisms. The molecular methods are considered sensitive enough to allow detection of low concentrations of viable or non-viable microorganisms in both pure cultures and complex samples (e.g., soil, peat, wateretc.).

a. Genotypic methods

These include methods such as nucleic acid hybridization (Southern blot analysis or Solution-phase hybridization) and amplification-based or polymerase chain reaction (PCR) technologies. The latter consist of sequence comparisons of conserved genomic regions such as 16S or 18S rRNA, or comparisons of restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) or G+C % content in the genomic DNA with corresponding data on known organisms.

Reliable genotypic identification requires databases with accurate and complete sequence information from a large number of taxa. The commonly used gene sequence databases include:

- GenBank® ;
- Ribosomal Database Project (RDP) ;
- Europe's collection of nucleotide sequence data (EMBL) ; and
- Universal Protein Resource (UniProt) .

Product proponents are not limited to using the reference materials listed above; these are intended as guidance only.

Some of the limitations associated with genotypic methods also include: difficulties in differentiating between species that share identical and/or similar conserved region sequences, limited information on the quality of sequence data available in public databases

and the complexity of taxonomic nomenclature overall. Considering the above, it is important to validate the results of genotypic microbial identification methods with data from other sources (e.g., morphological and/or phenotypic analysis).

b. Protein based methods

Serological methods such as Western blotting, Immuno-precipitation and Enzyme Linked Immuno-sorbant Assay (ELISA) use antibodies to detect specific proteins that are unique and/or characteristic of a microorganism. The applicability of serological methods is dependent on the availability, sensitivity and specificity of the antibodies used. There are commercial kits available for immuno-detection of several microorganisms. Protein based methods also include gel electrophoresis (SDS-PAGE, 2D-gels, etc.) that can separate cellular proteins on a defined matrix and identify microbial proteins of interest by comparing with microorganisms with known protein profiles.

3. Genomics

More recently, complete profiling of the transcriptome, genome, proteome or metabolome have been used to identify and characterise organisms. Several modern technologies such as DNA and protein microarray analyses, mass spectral protein profiling, nuclear magnetic resonance (NMR) spectral analysis, in-silico microbial metabolome platforms are increasingly used in identification and characterization of microorganisms.

The knowledge of the sensitivity and specificity of genomic tools and their application in microbial identification is rapidly evolving. However, challenges related to standardization of genomics methodologies (including optimization of protocols and bioinformatics tools for reliable data annotation, interpretation etc.) continue to hinder their applicability in safety (risk) assessment and regulatory decision making. The Fertilizer Safety Office will consider data generated by genomics-methods on a case-by-case basis. However, validation of genomics data using alternate methods is currently required to substantiate the identification and taxonomic classification of an active microorganism(s) in a supplement product.

II. Taxonomic Classification and Nomenclature:

The taxonomic identification of the microorganism(s) should be based on the currently used and internationally accepted taxonomic classification system. The description of the microorganism(s) in the product and its characteristics must correspond to the characteristics described in standard resources and/or references that are commonly used by the scientific community to validate taxonomic classification. These can include but are not limited to:

- textbooks such as the Bergey's Manual of Systematic Bacteriology ;The Prokaryotes ;
- Applied Microbial Systematic
- Principles of fungal taxonomy
- online resources such as the Catalogue of Life[Footnote 10];
- PubMed Taxonomy[Footnote 11] and UniProt Taxonomy
- peer reviewed journals.

The taxonomic name should follow the nomenclature code officially recognized by the International Committee on Systematics of Prokaryotes (ICSP). Applicants should verify the "Approved List of Bacterial Names" to ensure that the nomenclature is in accordance with the latest Validation List developed and updated by the International Journal of Systematic and Evolutionary Microbiology (IJSEM).

Please note that microbial taxonomic classification and nomenclature, particularly for bacteria, is in a constant state of flux as methodologies evolve to generate more reliable information to identify/classify and/or reclassify the current taxonomic scheme. Cross referencing more than one resource/reference will help in validating the current taxonomic designation and classification of a microorganism.

Methods of Identifying microbes Based on

1. **Morphological characteristics – shape and size – staining techniques**
2. **Chemical characteristics - microscopic observation**
3. **Cultural characteristics - spread , pour and streak plate method**
4. **Metabolic characteristics – biochemical tests**

5. **Antigenic characteristics – Ag –Ab reactions**

6. **Genetic characteristics - DNA sequencing**

7. **Pathogenic characteristics - Infection to human and plants**

8. **Ecological characteristics - climatic conditions**

The Basics of Microscopy.

Brightfield Microscopy

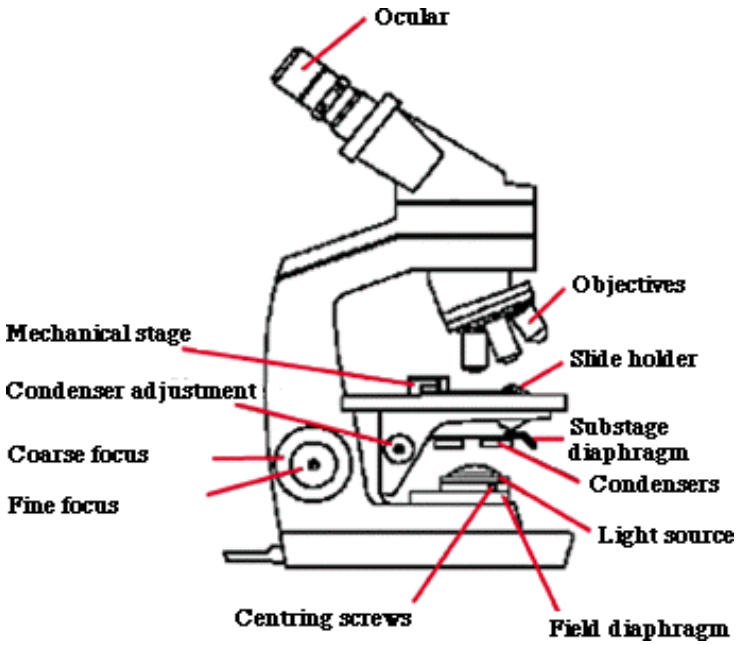

Fig.8

The Basics of Brightfield Microscopy

Bright field microscopy is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample. Bright field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes and its simplicity makes it a popular technique. The typical appearance of a bright field microscopy image is a dark sample on a bright background, hence the name.

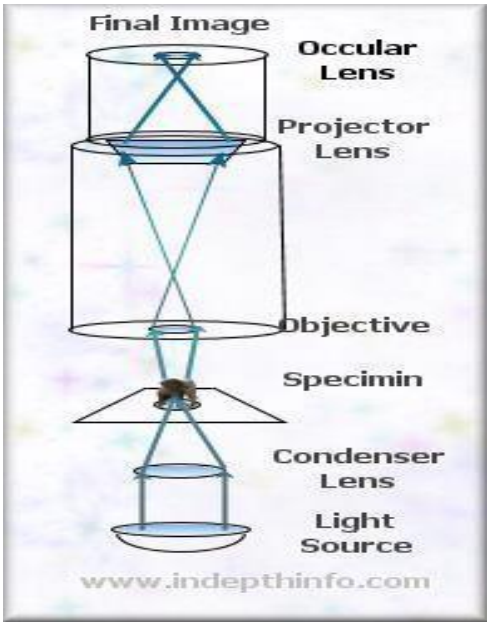

Fig.9

Light path

The light path of a bright field microscope is extremely simple, no additional components are required beyond the normal light microscope setup. The light path therefore consists of:

Light source

1. a transillumination light source, commonly a halogen lamp in the microscope stand; A **halogen lamp**, also known as a **tungsten halogen lamp** or **quartz iodine lamp**, is an incandescent lamp that has a small amount of a halogen such as iodine or bromine added. The combination of the halogen gas and the tungsten filament produces a **halogen cycle** chemical reaction which redeposits evaporated tungsten back onto the filament, increasing its life and maintaining the clarity of the envelope

2. a **condenser lens** which focuses light from the light source onto the sample. A **condenser** is one of the main components of the optical system of many transmitted light compound microscopes. A condenser is a lens that serves to concentrate light from the illumination source that is in turn focused through the object and magnified by the objective lens.

3. **objective lens** : In an optical instrument, the **objective** is the optical element that gathers light from the object being observed and focuses the light rays to produce a real image. Objectives can be single lenses or mirrors, or combinations of several optical elements. Microscope objectives are characterized by two parameters: magnification and numerical aperture. The typically ranges are 4× , 10x , 40x and 100×.

4. **oculars** to view the sample image. An **eyepiece**, or **ocular lens**, is a type of lens that is attached to a variety of optical devices such as microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device. The objective lens or mirror collects light and brings it to focus creating an image. The eyepiece is placed near the focal point of the objective to magnify this image. The amount of magnification depends on the focal length of the eyepiece.

Magnification is the process of enlarging something only in appearance, not in physical size. Typically magnification is related to scaling up visuals or images to be able to see more detail, increasing resolution.

Resolving power is the ability of an imaging device to separate (i.e., to see as distinct) points of an object that are located at a small angular distance..

In optics, the **numerical aperture** (**NA**) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light. In most areas of optics, and especially in microscopy, the numerical aperture of an optical system such as an objective lens is defined by

$$NA = n \sin \theta$$

where n is the index of refraction of the medium in which the lens is working (1.0 for air, 1.33 for pure water, and up to 1.56 for oils; see also list of refractive indices), and θ is the half-angle of the maximum cone of light that can enter or exit the lens. In general, this is the angle of the real marginal ray in the system

Working Performance

Bright field microscopy typically has low contrast with most biological samples as few absorb light to a great extent. Staining is often required to increase contrast, which prevents use on live cells in many situations. Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.

Light is first emitted by the light **source** and is directed by the **condenser lens** on to the specimen, which might be a loose object, a prepared plate or almost anything. A microscope can even be applied to small parts of larger objects, though with a bit more difficulty. (The light does not absolutely need to originate below the specimen.)

The light from the specimen then passes through the objective lens. This lens is often selected from among three or four and is the main determinant for the level of magnification. It bends the light rays and in the case of this example sends them to a **projector lens**, which reverses their direction so that when the image reaches the eye it will not appear "upside-down". Not all microscopes have a projector lens, so the viewer may be seeing a reverse image. In these cases, when the slide is moved, it will appear to be moving in the opposite direction to the viewer.

The light rays then travel to the **oracular lens** or "eye piece". This is often a 10X magnification lens, meaning it magnifies the magnified image an additional ten times. The image is then projected into the eye. It is very seldom that a specimen is in focus the moment it is placed beneath a microscope. This means that some adjustment will have to be made. Unlike in telescopes, the focal length between lenses remains constant when adjusting the focus. The lens apparatus is brought closer to or further from the object. The focus adjustment is often along the neck of the tube containing the lenses, but it might just as well move the slide up and down. The best way to make this adjustment is to make a course adjustment so that it is too close to the object and then back off with the fine adjustment[2]. This helps to ensure that the specimen is not inadvertently smashed by the lens.

Advantages

The name "brightfield" is derived from the fact that the specimen is dark and contrasted by the surrounding bright viewing field. Simple light microscopes are sometimes referred to as bright field microscopes.

Brightfield microscopy is very simple to use with fewer adjustments needed to be made to view specimens.

Some specimens can be viewed without staining and the optics used in the brightfield technique don’t alter the color of the specimen.

It is adaptable with new technology and optional pieces of equipment can be implemented with brightfield illumination to give versatility in the tasks it can perform.

Disadvantages

Certain disadvantages are inherent in any optical imaging technique.

- By using an aperture diaphragm for contrast, past a certain point, greater contrast adds distortion. However, employing an iris diaphragm will help compensate for this problem.

- Brightfield microscopy can’t be used to observe living specimens of bacteria, although when using fixed specimens, bacteria have an optimum viewing magnification of 1000x.

Brightfield microscopy has very low contrast and most cells absolutely have to be stained to be seen; staining may introduce extraneous details into the specimen that should not be present.

Also, the user will need to be knowledgeable in proper staining techniques.

Lastly, this method requires a strong light source for high magnification applications and intense lighting can produce heat that will damage specimens or kill living microorganisms.

The most important feature of a microscope is its **resolution**, the ability to see fine details. Once you can resolve fine details then you can magnify them. Every optical system has a finite resolution, if you magnify objects beyond the resolution the result will be **empty magnification**. So, the actual purpose of a microscope is to see small things clearly.

A second, desirable attribute of a microscope is **depth of field**, which is the range of depth that a specimen is in acceptable focus. A microscope that has a thin depth of field will have to be continuously focused up and down to view a thick specimen.

A third feature that a microscope has its mechanism for **contrast** formation. In order to distinguish a feature from its surrounding background the human eye needs a difference of 2 percent in intensity. Contrast is the ratio between the dark and the light. Typically, most microscopes use absorption contrast, that is the specimen is subjected to stains in order to be seen. This is called **bright field microscopy**. There are other types of microscope that use more exotic means to generate contrast, such as phase contrast, dark field, differential interference contrast.

The fourth desirable feature is a strong **illumination** source. The higher a microscope magnifies the more light will be required. Also, there will be more optical trade off leeway when there is more light present. The illumination source should also be at a wavelength (color) that will facilitate the interaction with the specimen. All microscopes fall into either of two categories based on how the specimen is illuminated. In the typical compound microscope the light passes through the specimen and is collected by the image forming optics. This is called diascopic illumination. Dissecting (stereo) microscopes generally use episcopic illumination for use with opaque specimen. The light is reflected onto the specimen and then into the objective lens.

The four attributes of an optical system trade off with each other. Resolution and brightness is antagonistic towards contrast and depth of field. For example, you can not have maximum resolution and maximum contrast simultaneously. Theoretically speaking, if you had an infinite resolving system there would be no contrast to discern the image. It is up to the microscopist to decide which attribute is needed to view a particular specimen. All of which are controlled be the iris diaphragm, see Koehler illumination.

Electron Microscope

You've probably used a microscope in school -- maybe to observe the wings of an insect or to get a closer look at a leaf. If so, then you know microscopes are used in the classroom to illuminate the surface of your subject of study. These microscopes use transparent glass lenses to magnify the image of whatever you are observing.

However, the size of the smallest features that we can distinguish under the microscope is on the order of the wavelength of the light used. Visible light, which is the one our eyes are sensitive to, ranges between 390 and 700 nanometers (one nanometer is one billionth of a meter). This means that we cannot observe things that are smaller than a few hundred nanometers using our eyes and visible light.

With the advancement of science and technology, we realized there is a whole world of things that we can observe and study at small scales. For example, the size of atoms and molecules is just fractions of a nanometer. An **electron microscope** allows us to see at these small scales.

Electron microscopes work by using an electron beam instead of visible light and an electron detector instead of our eyes. An **electron beam** allows us to see at very small scales because electrons can also behave as light. It has the properties of a wave with a wavelength that is much smaller than visible light (a few trillionths of a meter!). With this wavelength we can distinguish features down to a fraction of a nanometer. Let's explore the different types of electron microscopes, how they work and some of their applications.

Types of Electron Microscopes Scanning Electron Microscope (SEM)

In a **scanning electron microscope** or **SEM**, a beam of electrons scans the surface of a sample (Figure 1). The electrons interact with the material in a way that triggers the emission

of **secondary electrons**. These secondary electrons are captured by a detector, which forms an image of the surface of the sample. The direction of the emission of the secondary electrons depends on the orientation of the features of the surface. There, the image formed will reflect the characteristic feature of the region of the surface that was exposed to the electron beam.

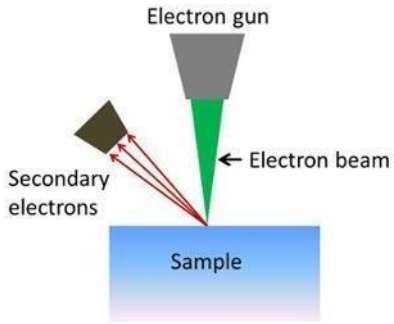

Fig.10. A Scanning Electron Microscope focuses a beam of electrons on the surface of a sample. Secondary electrons are emitted from the sample surface. A detector is used to forman image from the secondary electrons.

Transmission Electron Microscope (TEM)

In a **transmission electron microscope** or **TEM**, a beam of electrons hits a very thin sample (usually no more than 100 nm thick). The electrons are transmitted through the sample (Figure 2). After the sample, the electrons hit a fluorescence screen that forms an image with the electrons that were transmitted. You can better understand this process by imagining how a movie projector works. In a projector, you have a film that has the negative image that will be projected. The projector shines white light on the negative and the light transmitted forms the image contained in the negative.

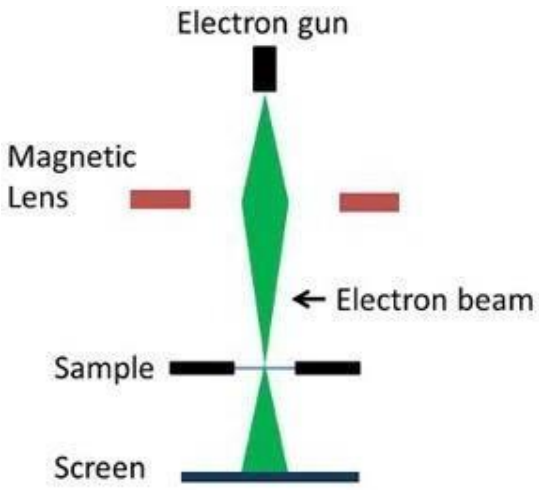

Fig. 11 Transmission Electron Microscope. A beam of electrons is focused on a sample. The electrons pass through the sample to form an image on a fluorescent screen.

Scanning Transmission Electron Microscope (STEM)

A **scanning transmission electron microscope** or **STEM** combines the capabilities of both an SEM and a TEM. The electron beam is transmitted across the sample to create an image (TEM) while it also scans a small region on the sample (SEM). The ability to scan the electron beams allows the user to analyze the sample with various techniques such as **Electron Energy Loss Spectroscopy (EELS)** and **Energy Dispersive X-ray (EDX)** Spectroscopy which are useful tools to understand the nature of the materials in the sample.

Uses of the Electron Microscope

With electron microscopes we can observe the small scale world that makes up most of the things around us. Before the development of the electron microscope we did not know how all these things looked (shape, size, etc.). We were relying on our imagination to picture these objects in our minds.

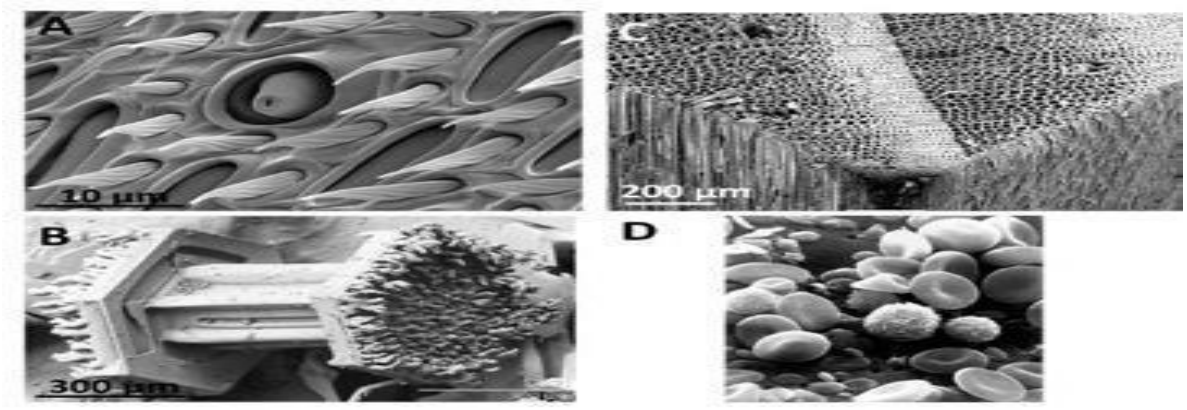

Fig12. SEM Images. A) The surface of the antenna of a wasp. B) A snow flake. C) Wood. D) Blood cells (size of 1-6 micrometers).

Simple Staining Technique

In a **simple staining technique**, a basic, cationic dye is flooded across a sample, adding color to the cells. Before we move on, let's define the word cationic. A **cation** is simply a positively charged ion. The molecules that make up basic dyes have a positive charge. This is important because the cell wall and cytoplasm of bacterial cells have a negative charge. The positively charged dye is attracted to the negatively charged cells, enhancing the ability of the stain to

stick to and color the cells. Now, those nearly colorless cells should pop off the slide in any number of colors.

It is important to note that before a sample can be stained with a simple stain, it must be heat fixed to the slide. During heat fixation, a glass slide is waved over an open flame. This kills the bacteria, attaches the cells to the slide, and enhances the stain uptake. This process makes staining more effective but can damage or distort the cells, changing their appearance from a truly natural, free-living state.

Methylene blue is a classic example of a simple stain. This blue stain will color all cells blue, making them stand out against the bright background of the light microscope. Notice how the background remains generally clear, while the bacterial cells are a deep blue.

Procedure:

1. Clean and dry microscope slides thoroughly.
2. Flame the surface in which the smear is to be spread.
3. Flame the inoculating loop.
4. Transfer a loop full of tap water to the flamed slide surface.
5. Reflame the loop making sure the entire length of the wire that will enter the tube has been heated to redness
6. Remove the tube cap with the fingers of the hand holding the loop.
7. Flame the tube mouth.
8. Touch the inoculating loop to the inside of the tube to make sure it is not so hot that it will distort the bacterial cells; then pick up a pinhead size sample of the bacterial growth without digging into the agar
9. Reflame the tube mouth, replace the can, and put the tube back in the holder.
10. Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the siz of a dime. It should be a thin, even smear.
11. Reflame the inoculating loop to redness including the entire length that entered the tube.

12. Allow the smear to dry thoroughly.

13. Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. Test the temperature of the slide after each pass against the back of the hand. It has been heated sufficiently when it feels hot but can still be held against the skin for several seconds. Overheating will distort the cells

14. Stain the smear by flooding it with one of the staining solutions and allowing it to remain covered with the stain for the time designated below.

    Methylene blue - 1 minute Crystal violet - 30 seconds

    Carbol fuchsin - 20 seconds

    During the staining the slide may be placed on the rack or held in the fingers.

15. At the end of the designated time rinse off the excess stain with gently running tap water. Rinse thoroughly.

16. Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.

17. Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.

18. Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply oil directly to the smear, and focus the smear under oil with the 100X objective.

19. Draw the cells observed.

Gram staining

Gram staining, also called **Gram's method**, is a method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative). The name comes from the Danish bacteriologist Hans Christian Gram, who developed the technique.

Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in a thick layer in Gram-positive bacteria.[1] In a Gram stain test, Gram-positive bacteria retain the crystal violet dye, while a

counterstain (commonly safranin or fuchsine) added after the crystal violet gives all Gram-negative bacteria a red or pink coloring.

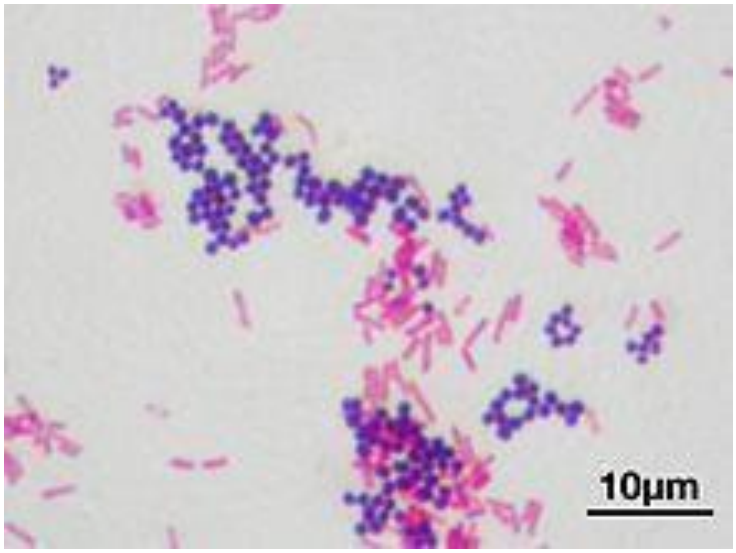

Fig.13 A Gram stain of mixed*Staphylococcus aureus*(Staphylococcus aureus ATCC 25923, Gram-positive cocci, in purple) and*Escherichia coli* (Escherichia coli ATCC 11775, Gram-negative bacilli, in red), the most common Gram stain reference bacteria

The Gram stain is almost always the first step in the identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique. This gives rise to *Gram-variable* and *Gram- indeterminate* groups as well.

History: The method is named after its inventor, the Danish scientist Hans Christian Gram (1853–1938), who developed the technique.

Process

Step 1. Prepare a slide with the culture by transferring the specimen to be examined onto a drop of suspension medium (distilled water) using an inoculation loop. Spread the specimen on the slide to ensure that it is not clumped.

Step 2. Fix the culture by heating the slide over a Bunsen burner to evaporate the water -- make sure not to hold the slide over the flame too long or it will denature the specimen.

Step 3. Drop a few drops of crystal violet stain onto the fixed culture (enough to cover the specimen) and let it stand for 20 seconds. Then pour off the crystal violet stain and gently rinse the excess stain with distilled water.

The objective of this step is:

To allow the crystal violet stain to bind to the peptidoglycan molecules of the Gram + bacteria (if present). Remember, Gram + bacteria have a large peptidoglycan layer located outside the bacterial "inner membrane".

To allow the crystal violet stain to bind to the lipopolysaccharide molecules attached to the "outer membrane" of the Gram - bacteria (if present). Remember, while Gram + bacteria have no "outer membrane", Gram - bacteria have lipopolysaccharide molecules attached to their bacterial "outer membrane".

Step 4. Drop a few drops of iodine solution on the smear and let it stand for 20 seconds. Then pour off the iodine solution and rinse the slide with distilled water.

The objective of this step is to fix the crystal violet to the peptidoglycan molecules on the Gram + bacteria (if present).

Step 5. Drop a few drops of decolorizer (acetone or ethanol) and let the solution trickle down off the slide until the decolorizer has removed enough of the color to drip off clear. Then IMMEDIATELY rinse the slide off with distilled water after 5 seconds. Note that pouring too much decolorier will cause the decolorization of the Gram + bacterial cells (in addition to the Gram - bacteria), and the purpose of staining will be defeated.

The objective of this step is to dissolve the lipopolysaccharide membraine in the Gram - bacteria and expose the thin peptidoglycan layer below.

Step 6. Drop a few drops of basic counterstain (fuchsin or safranin) on the slide and let it sit for 20 seconds, then wash off the solution with distilled water.

The objective of this step is to stain the peptidoglycan layer of the Gram - bacteria a pink/ red color. Remember that the addition of iodine to the crystal violet in Step 4 binds the crystal violet stain

in the Gram + bacteria, so the counterstain is unable to bind to the peptidoglycan wall in the Gram + bacteria in the specimen.

Step 7. Observe the slide under light microscope. Uses Gram staining is a bacteriological laboratory technique[4] used to differentiate bacterial species into two large groups (gram-positive and gram-negative) based on the physical properties of their cell walls.[5] Gram staining is not used to classify archaea, formerly archaeabacteria, since these microorganisms yield widely varying responses that do not follow their phylogenetic groups.[6]

Staining mechanism

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50–90% of cell envelope), and as a result are stained purple by crystal violet, whereas Gram-negative bacteria have a thinner layer (10% of cell envelope), so do not retain the purple stain and are counter- stained pink by the Safranin. There are four basic steps of the Gram stain:

Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture. Heat fixation kills some bacteria but is mostly used to affix the bacteria to the slide so that they don't rinse out during the staining procedure.

The addition of iodide, which binds to crystal violet and traps it in the cell, Rapid decolorization with ethanol or acetone, and *Counterstaining* with safranin.[9] Carbol fuchsin is sometimes substituted for safranin since it more intensely stains anaerobic bacteria, but it is less commonly used as a counterstain.[10]

Crystal violet (CV) dissociates in aqueous solutions into CV+ and chloride (Cl−) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodide (I−or I−3interacts with CV+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. Iodine is often referred to as a mordant, but is a trapping agent that prevents the removal of the CV–I complex and, therefore, color the cell.[11]

When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A gram-negative cell loses its outer lipopolysaccharide membrane, and the inner peptidoglycan layer is left exposed. The CV–I complexes are washed from the gram-negative

cell along with the outer membrane. In contrast, a gram-positive cell becomes dehydrated from an ethanol treatment. The large CV–I complexes become trapped within the gram-positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain is removed from both gram-positive and negative cells if the decolorizing agent is left on too long (a matter of seconds).

After decolorization, the gram-positive cell remains purple and the gram-negative cell loses its purple color. Counterstain, which is usually positively charged safranin or basic fuchsine, is applied last to give decolorized gram-negative bacteria a pink or red color.[12][13]

Some bacteria, after staining with the Gram stain, yield a *gram-variable* pattern: a mix of pink and purple cells are seen. The genera *Actinomyces*, *Arthobacter*,*Corynebacterium*, *Mycobacterium*, and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in gram-negative staining of these gram-positive cells. In cultures of *Bacillus*, *Butyrivibrio*, and *Clostridium*, a decrease in peptidoglycan thickness during growth coincides with an increase in the number of cells that stain gram-negative.[14] In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

Gram-positive bacteria

Historically, the gram-positive forms made up the phylum Firmicutes, a name now used for the largest group. It includes many well-known genera such as *Bacillus*,*Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*. It has also been expanded to include the Mollicutes, bacteria like *Mycoplasma* that lack cell walls and so cannot be stained by Gram, but are derived from such forms.

Gram-negative bacteria

Gram-negative bacteria generally possess a thin layer of peptidoglycan between two membranes (*diderms*). Most bacterial phyla are gram-negative, including thecyanobacteria, spirochaetes, and green sulfur bacteria, and most Proteobacteria and *Escherichia coli*. (exceptions being some members of the *Rickettsiales* and the insect-endosymbionts of the *Enterobacteriales*).[5][15]

Gram-indeterminate bacteria

Gram-indeterminate bacteria do not respond predictably to Gram staining and, therefore, cannot be determined as either gram-positive or gram-negative. They tend to stain unevenly, appearing partially gram positive and partially gram negative, or unstained by either crystal violet or safranin. Staining older cultures (over 48 hours) can lead to false Gram-variable results, probably due to changes in the cell wall with aging. Gram-indeterminate bacteria are best stained using acid-fast staining techniques. Examples include many species of *Mycobacterium*, including *M. tuberculosis* and *M. leprae*.[16][17]

Ziehl–Neelsen stain - Acid-fast stain

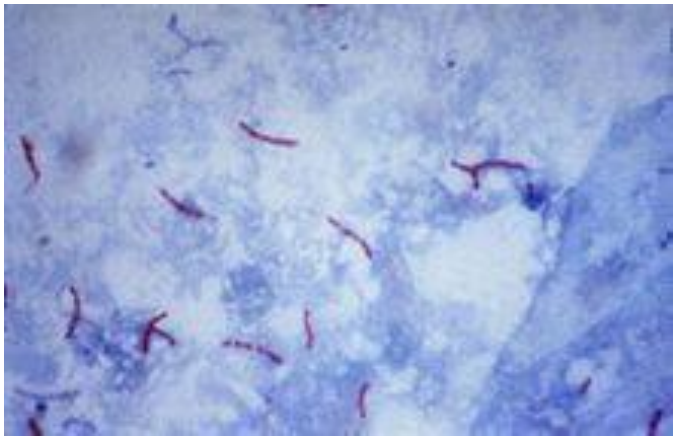

Fig.14.

The **Ziehl–Neelsen stain**, also known as the **acid-fast stain**, was first described by two German doctors: the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Neelsen (1854– 1898). It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria. *Mycobacterium tuberculosis* is the most important of this group because it is responsible for tuberculosis (TB). Other important Mycobacterium species involved in human disease are *Mycobacterium leprae*, *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium bovis*, *Mycobacterium africanum* and members of the *Mycobacterium avium* complex. Acid fast organisms like Mycobacterium contain large amounts of lipid substances within their cell walls called mycolic acids. These acids resist staining by ordinary methods such as a Gram stain.[1] It can also be used to stain a few other bacteria, such as *Nocardia*. The reagents used are Ziehl–Neelsen carbol fuchsin, acid alcohol, andmethylene blue. Acid-fast bacilli will be bright red after staining.

A variation on this staining method is used in mycology to differentially stain acid-fast incrustations in the cuticular hyphae of certain species of fungi in the genus *Russula*.[2][3] It is also useful in the identification of some protozoa, namely Cryptosporidium and Isospora. The Ziehl–Neelsen stain can also hinder diagnosis in the case of paragonimiasis because the eggs in an ovum and parasite sputum sample (OnP) can be dissolved by the stain, and is often used in this clinical setting because signs and symptoms of paragonimiasis closely resemble those of TB.

Procedure

A typical AFB stain procedure involves dropping the cells in suspension onto a slide, then air drying the liquid and heat fixing the cells. The slide is flooded with Carbol Fuchsin, which is then heated to dry and rinsed off in tap water. The slide is then flooded with a 1% solution of hydrochloric acid in isopropyl alcohol (or methanol) to remove the carbol fuchsin, thus removing the stain from cells that are unprotected by a waxy lipid layer. Thereafter, the cells are stained in methylene blue and viewed on a microscope under oil immersion. Studies have shown that an AFB stain without a culture has a poor negative predictive value. An AFB Culture should be performed along with an AFB stain; this has a much higher negative predictive value.

Mechanism Explanation

Initially, Carbol Fuchsin stains every cell. When they are destained with acid-alcohol, only non- acid-fast bacteria get destained since they do not have a thick, waxy lipid layer like acid-fast bacteria. When counter stain is applied, non-acid-fast bacteria pick it up and become blue when viewed under the microscope. Acid-fast bacteria retain Carbol Fuchsin so they appear red.

Modifications

- 1% sulfuric acid alcohol for actinomycetes, nocardia.
- 0.5-1% sulfuric acid alcohol for oocysts of isospora, cyclospora.
- 0.25–0.5% sulfuric acid alcohol for bacterial endospores.
- Brucella differential stain – glacial acetic acid used, no heat applied, secondary stain is loeffler's methylene blue.

- Kinyoun modification (or cold Ziehl–Neelsen technique) is also available.
- A protocol in which a detergent is substituted for the highly toxic phenol in the fuchsin staining solution

Endospore staining

Endospore staining is a technique used in bacteriology to identify the presence of endospores in a bacterial sample, which can be useful for classifying bacteria.[1]Within bacteria, endospores are quite protective structures used to survive extreme conditions, but this protective nature makes them difficult to stain using normal techniques. Special techniques for endospore staining include the Schaeffer–Fulton stain and the Moeller stain. A good stain to use for spore staining is malachite green. It takes a long time for the spores to stain due to their density, so time acts as the mordant when doing this differential stain; the slide with the bacterium should be soaked in malachite green for at least 30 minutes. Water acts as the decolorizer. A counterstain to differentiate the vegetative cells is 0.5% safranin. Types of endospores that could be identified are free endospores, central endospores, central and swollen endospores, and subterminal endospores. One obstacle of this stain is if staining *Mycobacterium* because due to the its thick, wax coats, some cells will stain green, looking positive for spores although this particular bacterium does not produce .

Endospore Staining: Principle, Procedure and Results

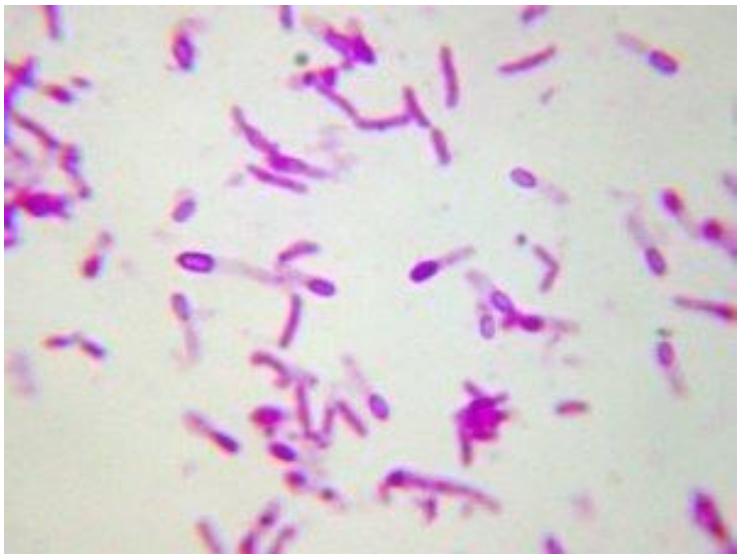

Fig.15

When vegetative cells of certain bacteria such as *Bacillus* spp and *Clostridium* spp. are subjected to environmental stresses such as nutrient deprivation they produces metabolically inactive or dormant form-Endospore. Formation of Endospore circumvent the problems associated with environmental stress and helps them to survive.

Most endospore forming bacteria are found in soil or aquatic environments. However, some species of *Bacillus* and *Clostridium*have **medical significance**. *Clostridium perfringens, C. botulinum* and*C. tetani* are the causative agents of gas gangrene, botulism and tetanus, respectively. *Bacillus anthracis* and *Bacillus cereus* are the causative agents of anthrax and a self limiting food poisoning, respectively.

Principle of Spore Staining:

A **differential staining** technique (the Schaeffer-Fulton method) is used to distinguish between the vegetative cells and the endospores. A **primary stain (malachite green) is used to stain the endospores**. Because endospores resist staining, the malachite green will be **forced** into the endospores by **heating**. In this technique heating acts as a**mordant**.

Water is used to decolorize the cells; as the endospores are resistant to staining, the endospores are equally resistant to de-staining and will retain the primary dye while the vegetative cells will lose the stain. The addition of a counterstain or **secondary stain (safranin) is used to stain the decolorized vegetative cells**.

When visualized under microscopy the cells should have three characteristics:

1. the vegetative cells should appear pink,
2. the vegetative cells that contain endospores should stain pink while the spores should be seen as green ellipses within the cells.
3. Mature, free endospores should not be associated with the vegetative bacteria and should be seen as green ellipses.

Procedure of endospore stain:

1. Prepare smears of organisms to be tested for 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. Heat the slide gently till it starts to evaporate (either by putting the slide on a staining rack that has been placed over a boiling water bath or via bunsen burner).
5. Remove the heat and reheat the slide as needed to keep the slide steaming for about 3-5 minutes. 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 one time that the temperature is appreciably reduced. Do not overheat. The process is steaming and not baking.
6. After 5 minutes carefully 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 side of the slide to remove the secondary stain and blot the slide/ air dry.

Observe the bacteria under 1000X (oil immersion) total magnification. Results: The vegetative cells will appear pink and the spores will appear green.

FLAGELLA STAIN PROCEDURE

Flagellar Staining

A. Preparation of Cultures Background

Solid or liquid media cultures can be used for flagella staining (7). Cultures should incubate between 16 and 20 hours before staining, as older cultures tend to lose flagella. For example, newer cultures are particularly important for *Bacillus* spp. that undergo spore formation and lose flagella during this developmental process. If vortexing is necessary for suspension after centrifugation of liquid cultures, or because cultures clump, do so gently as flagella are easily sheared from the bacterium.

Method

From an agar plate or slant cultures, prepare a suspension by removing a small amount of growth, approximately one-fourth of the colony, with an inoculating loop using proper aseptic technique. Emulsify in 100 ml of distilled water in a microcentrifuge tube by gently vortexing. The emulsion should be only slightly cloudy. Using too much inoculum results in the inability to visualize the flagella.

For staining from liquid cultures, Leifson (7) recommends two rounds of centrifugation and final suspension in distilled water to remove any medium components. Place 100 ml of the liquid culture in a microcentrifuge tube, centrifuge, and remove spent medium. Resuspend in 100 ml of distilled water by gently vortexing, again centrifuge, and remove supernatant. Form a slightly cloudy emulsion by resuspending in ~200 ml of distilled water. Gently vortex. Again, emulsions should be only slightly cloudy prior to proceeding to staining. Optimization of the washing procedure will most likely be necessary to maximize quality of flagella stain.

Preparation of Slides

1. Wipe clean a new microscope slide with 95% ethanol and a Kimwipe. Flame to dry thoroughly. Use slides immediately.
2. When the slide is cool enough to handle, label it using tape with the name of the organism you will be staining.
3. Place 5 to 10 ml of the culture emulsion on one end of the slide using a micropipettor and spread the emulsion using the same pipette tip held parallel to the microscope slide.
4. Allow the sample to dry at room temperature. Do not heat fix as this will destroy the proteinaceous flagella structure.

Flagella Staining

Leifson flagella stain (14)

1. Take a prepared slide and using a wax pencil draw a rectangle around the dried sample. Place slide on staining rack.

2. Flood Leifson dye solution on the slide within the confines of the wax lines. Incubate at room temperature for 7 to 15 minutes. The best time for a particular preparation will require trial and error.

3. As soon as a golden film develops on the dye surface and a precipitate appears throughout the sample, as determined by illumination under the slide, remove the stain by floating off the film with gently flowing tap water. Air dry.

4. View using oil immersion, at 1,000x magnification, by bright-field microscopy. Bacterial bodies and flagella will stain red.

Presque Isle Cultures flagella stain

1. Prepare slide as described above. Place slide on staining rack.

2. Flood slide with Presque Isle Cultures Solution I, the mordant. Incubate at room temperature for 4 minutes.

3. Gently rinse with distilled water. Shake excess water from slide.

4. Flood with Presque Isle Cultures Solution II, the silver stain.

5. Heat over Bunsen burner by moving slide back and forth, just until steam is emitted. If a Bunsen burner is not available then an alternate heat source can be used, but optimization will be necessary. Be careful not to overheat sample, as excess heat will destroy the flagella. Incubate at room temperature for 4 minutes.

6. Rinse with distilled water. Carefully blot dry with bibulous paper.

7. View using oil immersion, at 1,000x magnification, by bright-field microscopy. Bacteria and flagella will appear golden brown. Excess stain is often observed on the slides and illustrates the necessity of beginning the procedure with thoroughly cleaned microscope slides.

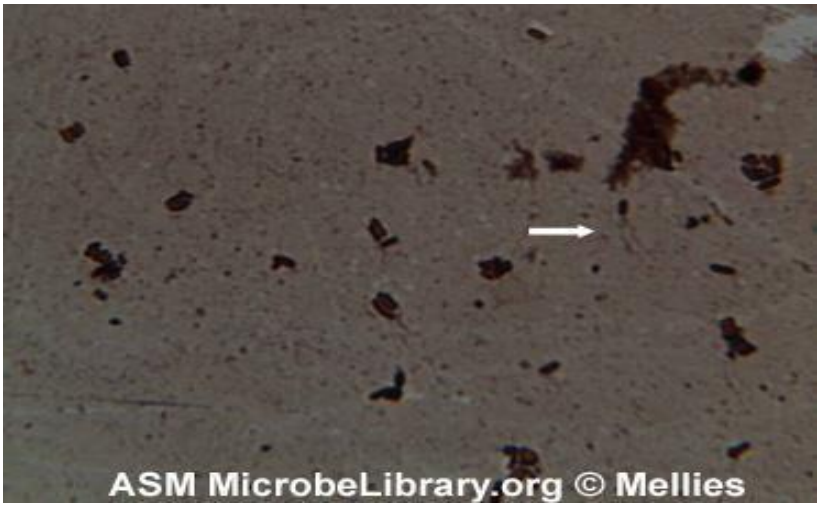

Fig. 16 Arrow points to lophotrichous flagella of *Pseudomonas fluorescens*cultured on nutrient agar, stained using the Presque Isle flagella stain, and visualized under bright-field microscopy using oil immersion at 1,000x magnification.

Capsule Staining

Principle, Reagents, Procedure and Result

The main purpose of capsule stain is to distinguish capsular material from the bacterial cell. A **capsule** is a gelatinous outer layer secreted by bacterial cell and that surrounds and adheres to the cell wall. Most capsules are composed of polysaccharides, but some are composed of polypeptides. The **capsule** differs from the **slime layer** that most bacterial cells produce in that it is a thick, detectable, discrete layer outside the cell wall. The capsule stain employs an acidic stain and a basic stain to detect capsule production.

Principle of Capsule Staining

Capsules stain very poorly with reagents used in simple staining and a capsule stain can be, depending on the method, a misnomer because the capsule may or may not be stained.

Negative staining methods contrast a translucent, darker colored, background with stained cells but an unstained capsule. The background is formed with **India ink or nigrosin or congo red**. India ink is difficult to obtain nowadays; however, nigrosin is easily acquired.

A positive capsule stain requires a mordant that precipitates the capsule. By counterstaining with dyes like **crystal violet or methylene blue**, bacterial cell wall takes up the dye. Capsules appear colourless with stained cells against dark background.

Capsules are fragile and can be diminished, desiccated, distorted, or destroyed by heating. A drop of serum can be used during smearing to enhance the size of the capsule and make it more easily observed with a typical compound light microscope.

Reagents used for Capsule Staining

Crystal Violet (1%) Crystal Violet (85% dye content) = 1 gm Distilled Water = 100 ml

Nigrosin

Nigrosine, water soluble = 10 gm Distilled Water = 100 ml

Procedure of Capsule Staining

1. Place a small drop of a **negative stain** (India Ink, Congo Red, Nigrosin, or Eosin) on the slide. ***Congo Red*** *is easier to see, but it does not work well with some strains.* ***India Ink*** *generally works, but it has tiny particles that display Brownian motion that must be differentiated from your bacteria.****Nigrosin*** *may need to be kept very thin or diluted.*
2. Using sterile technique, add a loopful of bacterial culture to slide, smearing it in the dye.
3. Use the other slide to drag the ink-cell mixture into a thin film along the first slide and let stand for **5-7 minutes.**
4. Allow to air dry (do not heat fix).
5. Flood the smear with **crystal violet stain** (this will stain the cells but not the capsules) for about **1 minutes**. Drain the crystal violet by tilting the slide at a 45 degree angle and let stain run off until it air dries .
6. Examine the smear microscopically (100X) for the presence of encapsulated cells as indicated by clear zones surrounding the cells.

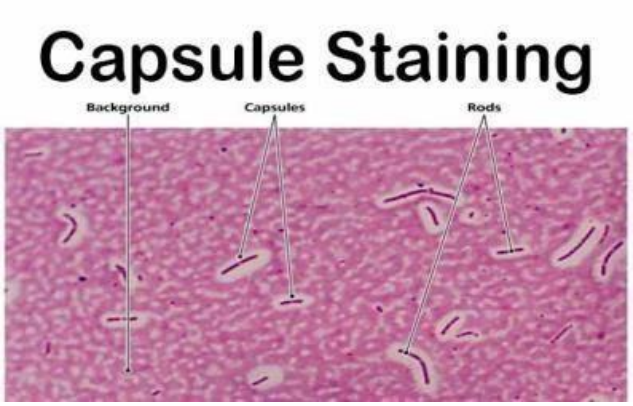

Fig.17

Result of Capsule Staining

Capsule: Clear halos zone against dark background

No Capsule: No Clear halos zone

Examples of Capsule Positive and Negative Positive

*Bacillus anthracis, Klebsiella pneumoniae, Streptococcus pneumonia Neisseria meningitidis Clostridium*spp, *Rhizaobium* spp, etc.

Negative

Neisseria gonorrhoreae, etc.

Mneomonics to remember capsulated bacteria- Some Killers Have Pretty Nice Capsule

S*treptococcus pneumoniae*

K*lebsiella pneumoniae*

H*aemophilus influenzae* ***P****seudomonas aeruginosa* ***N****eisseria meningitidis* ***C****ryptococcus neoformans*

TEXT / REFERENCE BOOKS

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2. Joanne M. Willey, Linda Sherwood, Christopher J. Woolverton, Prescott's Microbiology, 8th Edition, McGraw-Hill Higher Education, 2008

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PART A

S.No.	Questions	outcomes
1	Define microbiology.	**CO1**
2	Emphasis on Koch postulates.	**CO1**
3	What is flagella and pilli.	**CO1**
4	List out few contributions of Louis pasteur.	**CO1**
5	Comment on pure culture	**CO1**
6	Illustrate on types of media.	**CO1**
7	Differentiate lag from log phase.	**CO1**
8	Define decimal dilution method.	**CO1**
9	State on preservation of media.	**CO1**
10	Define growth curve.	**CO1**

PART B

S.No.	Questions	outcomes
1	Give an detailed account on contributions of various scientist in golden period of microbiology.	**CO1**
2	Illustrate with neat diagram on Whittakers five kingdom classification.	**CO1**
3	Discuss with neat diagram on structure of bacteria. Add a note on external structure.	**CO1**
4	Describe with neat diagram about the structure of gram positive and gram negative cell wall of bacteria.	**CO1**
5	Explain with neat diagram about the structure and types of flagella with examples.	**CO1**
6	State on media,its classification and emphazie on its significance with applications.	**CO1**
7	Describe about the bacterial growth curve and add a note on its generation time.	**CO1**
8	Discuss in detail about the enumeration of bacteria with neat diagram.	**CO1**
9	Write a detailed note on sexual and asexual reproduction of bacteria.	**CO1**
10	Discuss the theory of biogenesis vs spontaneous generation citing suitable examples.	**CO1**

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SCHOOL OF BIO & CHEMICAL ENGINEERING

DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT – 2 – Bacteria Culture Media

UNIT II

BACTERIA CULTURE MEDIA

Bacterial Culture Media

One of the most important reasons for culturing bacteria in vitro is its utility in diagnosing infectious diseases. Isolating a bacterium from sites in body normally known to be sterile is are indication of its role in the disease process. Culturing bacteria is also the initial step in studying its morphology and its identification. Bacteria have to be cultured in order to obtain antigens from developing serological assays or vaccines. Certain genetic studies and manipulations of the cells also need that bacteria be cultured in vitro. Culturing bacteria also provide a reliable way estimating their numbers (viable count). Culturing on solid media is another convenient way to separating bacteria in mixture.

History

Louis Pasteur used simple broths made up of urine or meat extracts. Robert Koch realized the importance of solid media and used potato pieces to grow bacteria. It was on the suggestions of Fannie Eilshemius, wife of Walther Hesse (who was an assistant to Robert Koch) that agar was used to solidify culture media. Before the use of agar, attempts were made to use gelatin as solidifying agent. Gelatin had some inherent problems; it existed as liquid at normal incubating temperatures (35 – 37^{o}C) and was digested by certain bacteria.

Composition of Culture Media

Bacteria infecting humans (commensals or pathogens) are chemoorganoheterotrophs. Where culturing bacteria, it is very important to provide similar environmental and nutritional conditions that exist in its natural habitat. Hence, an artificial culture medium must provide all the nutritional components that a bacterium gets in its natural habitat. Most often, a culture medium contains water, a source of carbon and energy, source of nitrogen, trace elements and some growth factors. Besides these, the pH of the medium must be set accordingly. Some of

the ingredients of culture media include water, agar, peptone, casein hydrolysate, meat extract, yeast extract and malt extract.

Classification

Bacterial culture media can be classified in at least three ways; based on consistency, based or nutritional component and based on its functional use.

1. Classification based on consistency.

Culture media are liquid, semi-solid or solid and biphasic.

a. Liquid Media: These are available for use in test – tubes, bottles or flasks. Liquid media are sometimes referred as "broths" (e.g nutrient broth). In liquid medium, bacteria growth uniformly producing general turbidity. Certain aerobic bacteria and those containing fimbriae (Vibrio and Bacillus) are known to grow as a thin film called 'surface pellicle' on the surface of undisturbed broth. *Bacillus anthracis* is known to produce stalactite growth on ghee containing broth. Sometimes the initial turbidity may be followed by clearing due to autolysis, which is seen in pneumococci. Long chains of Streptococci when grown in liquid media tend to entangle and settle to the bottom forming granular deposits. Liquid media tend to be used when a large number of bacteria have to be grown. These are suitable to grow bacteria when the numbers in the inoculum is suspected to be low. Inoculating in the liquid medium also helps to dilute any inhibitors of bacterial growth. This is the practical approach in blood cultures. Culturing in liquid medium can be used to obtain viable count (dilution methods). Properties of bacteria are not visible in liquid media and presence of more than one type of bacteria cannot be detected.

b. Solid media: Any liquid medium can be rendered by the addition of certain solidifying agents. Agar agar (simply called agar) is the most commonly used solidifying agent. It is an unbranched polysaccharide obtained from the cell membranes of some species of red algae such as the general Gelidium. Agar is composed to two long chain polysaccharide (70% agarose and 30% agarapectin). It melts at 95^{o}C (sol) and solidifies at 42^{o}C (gel), does not contribute any nutritive property, it is not hydrolyzed by most bacteria and is usually free from growth promoting or growth retarding substances. However, it may be a source of calcium and organic ions. Most commonly, it is used at concentration of 1 –

3% to make a solid agar medium. New Zealand agar has more gelling capacity than the Japanese agar. Agar is available as fibres (shred) or as powders.

c. Semi-solid agar: Reducing the amount of agar to 0.2 – 0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains (U-tube and Cragie's tube). Certain transport media such as Stuart's and Amies media are semi=solid in consistency. Hugh and Leifson's oxidation fermentation test medium as well as mannitol motility medium are also semi-solid.

d. Biphasic media: Sometimes, a culture system comprises of both liquid and solid medium in the same bottle. This is known as biphasic medium (Castaneda system for blood culture). The inoculum is added to the liquid medium and when subcultures are to be made, the bottle is simply tilted to allow the liquid to flow over the solid medium. This obviates the need for frequent opening of the culture bottle to subculture.

Besides agar, egg yolk and serum too can be used to solidify culture media. While serum and egg yolk are normally liquid, they can be rendered solid by coagulation using heat. Serum containing medium such as Loeffler's serum slope and egg containing media such as Lowenstein Jensen medium and Dorset egg medium are solidified as well as disinfected by a process of inspissation.

2. Classification based on nutritional component

Media can be classified as simple, complex and synthetic (or defined). While most of the nutritional components are constant across various media, some bacteria need extra nutrients. Those bacteria that are able to grow with minimal requirements are said to non-fastidious and those that require extra nutrients are said to be fastidious. Simple media such as peptone water, nutrient agar can support most non-fastidious bacteria. Complex media such as blood agar have ingredients whose exact components are difficult to estimate. Synthetic or defined media such as Davis and Mingioli medium are specially prepared media for research purposes where the composition of every component is well known.

3. Classification based on functional use or application

These include basal media, enriched media, selective / enrichment media, indicator / differential media, transport media and holding media.

a. Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar considered basal medium.

b. Enriched media: Addition of extra nutrients in the form of blood, serum, egg yolk etc. to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few or the enriched media.

c. Selective and enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that do not affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.

d. Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water are used to recovery pathogens from fecal specimens.

e) Differential media or indicator media: certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates, etc. so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Examples: MacConkey's agar, CLED agar, TCBS agar, XLD agar etc.

f) Transport media: Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth

of unwanted bacteria. Some of these media (Stuart's and Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors. Cary Blair medium and Venkataraman Ramakrishnan medium are used to transport feces from suspected cholera patients. Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.

g) Anaerobic media: Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation – reduction potential and extra nutrients. Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1 % thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Robertson cooked meat that is commonly used to growth Clostridium spps medium contain a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Methylene blue or resazurin is an oxidation – reduction potential indicator that is incorporated in the thioglycollate medium. Under reduced condition, methylene blue is colourless.

Preparation and preservation

Care must be taken to adjust the pH of the medium before autoclaving. Various pH indicators that are in use include phenol red, neutral red, bromothymol blue, bromocresol purple etc. Dehydrated media are commercially available and must be reconstituted as per manufacturers recommendation. Most culture media are sterilized by autoclaving. Certain media that contain heat labile components like glucose, antibiotics, urea, serum, blood are not autoclaved. These components are filtered and may be added separately after the medium is autoclaved. Certain highly selective media such as Wilson and Blair's medium and TCBS agar need not be sterilized. It is imperative that a representation from each lot be tested for performance and contamination before use. Once prepared, media may be held at 4 – 5^{o}C in the refrigerator for 1 – 2 weeks. Certain liquid media in screw capped bottles or tubes or cotton plugged can be held at room temperature for weeks.

Types of fungal growth media

For optimal recovery of fungal pathogen, a battery of media should be used, and the followings are recommended:

Media with or without cyclohexamide (Cycloheximide is added to inhibit the growth of rapidly growing contaminating molds.)

Media with or without an antibacterial agent ((Chloramphenicol, Gentamicin and Ciprofloxacin are commonly used antibacterial for this purpose).

Antibacterial agents are used to kill the contaminating bacterial species. If the sample is taken from sterile site, it is not necessary to use media containing antibacterial agents.

Brain-heart infusion (BHI) agar: It is a nonselective fungal culture medium that permits the growth of virtually all clinically relevant fungi. It is used for the primary recovery of saprophytic and dimorphic fungi

Czapek's agar: It is used for the subculture of *Aspergillus* species for their differential diagnosis.

Inhibitory mold agar (IMA): Primary recovery of dimorphic pathogenic fungi. Saprophytic fungi and dermatophytes will not be recovered.

Mycosel/Mycobiotic agar:

Fig.1

It is generally Sabouraud's dextrose agar with cycloheximide and chloramphenicol added.

It is used for the primary recovery of dermatophytes.

Niger Seed Agar: It is used for the identification of *Cryptococcus neoformans*.

Potato Dextrose Agar (PDA): It is a relatively rich medium for growing a wide range of fungi.

Sabouraud's Heart Infusion (SABHI) agar: Primary recovery of saprophytic and dimorphic fungi, particularly fastidious strains.

Penicillium notatum on Sabouraud agar Image source: ASM

Sabouraud's dextrose agar (SDA):

Sabouraud's agar is sufficient for the recovery of dermatophytes from cutaneous samples and yeasts from vaginal cultures.

Not recommended as a primary isolation medium because it is insufficiently rich to recover certain fastidious pathogenic species, particularly most of the dimorphic fungi.

Sabouraud's dextrose agar (2%) is most useful as a medium for the subculture of fungi recovered on enriched medium to enhance typical sporulation and provide the more characteristic colony morphology.

Potato flake agar: Primary recovery of saprophytic and dimorphic fungi, particularly fastidious and slow growing strains.

Bacterial Growth Curve

Objectives:

1. To study the different phases of bacterial growth.

2. To plot standard growth curve of *Staphylococcus aureus.*

3. To determine the generation time of given bacteria.

Principle:

The increase in the cell size and cell mass during the development of an organism is termed as growth. It is the unique characteristics of all organisms. The organism must require certain basic parameters for their energy generation and cellular biosynthesis. The growth of the organism is affected by both physical and Nutritional factors. The physical factors include the pH, temperature, Osmotic pressure, Hydrostatic pressure, and Moisture content of the medium in which the organism is growing. The nutritional factors include the amount of Carbon, nitrogen, Sulphur, phosphorous, and other trace elements provided in the growth medium. Bacteria are unicellular (single cell) organisms. When the bacteria reach a certain size, they divide by binary fission, in which the one cell divides into two, two into four and continue the process in a geometric fashion. The bacterium is then known to be in an actively growing phase. To study the bacterial growth population, the viable cells of the bacterium should be inoculated on to the sterile broth and incubated under optimal growth conditions. The bacterium starts utilising the components of the media and it will increase in its size and cellular mass. The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time or log of cell number versus time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Spectrophotometer. The Spectrophotometer measures the turbidity or Optical density which is the measure of the amount of light absorbed by a bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, and is a convenient and rapid method of measuring cell growth rate of an organism. Thus the increasing the turbidity of the broth medium indicates increase of the microbial cell mass (Fig 1) .The amount of transmitted light through turbid broth decreases with subsequent increase in the absorbance value.

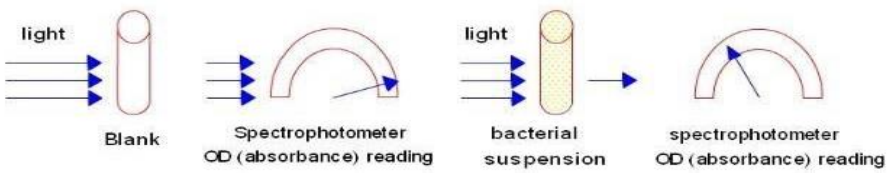


Fig.2 Absorbance reading of bacterial suspension The growth curve has four distinct phases

1. Lag phase

When a microorganism is introduced into the fresh medium, it takes some time to adjust with the new environment. This phase is termed as Lag phase, in which cellular metabolism is accelerated, cells are increasing in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The length of the lag phase depends directly on the previous growth condition of the organism. When the microorganism growing in a rich medium is inoculated into nutritionally poor medium, the organism will take more time to adapt with the new environment. The organism will start synthesizing the necessary proteins, co-enzymes and vitamins needed for their growth and hence there will be a subsequent increase in the lag phase. Similarly when an organism from a nutritionally poor medium is added to a nutritionally rich medium, the organism can easily adapt to the environment, it can start the cell division without any delay, and therefore will have less lag phase it may be absent.

2. Exponential or Logarithmic (log) phase

During this phase, the microorganisms are in a rapidly growing and dividing state. Their metabolic activity increases and the organism begin the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially) and finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (That is 2^0, 2^1, 2^2, 2^3 ..... 2^n, n is the number of generations) This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time. The generation time tends to vary with different organisms. *E.coli* divides in every 20 minutes, hence its generation time is 20 minutes, and for *Staphylococcus aureus* it is 30 minutes.

Stationary phase

As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This result in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavourable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally

bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilized. If a cell taken from the stationary phase is introduced into a fresh medium, the cell can easily move on the exponential phase and is able to perform its metabolic activities as usual.

Decline or Death phase

The depletion of nutrients and the subsequent accumulation of metabolic wasteproducts and other toxic materials in the media will facilitates the bacterium to move on to the Death phase. During this, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions and the death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms which can resist this condition can survive in the environment by producing endospores.

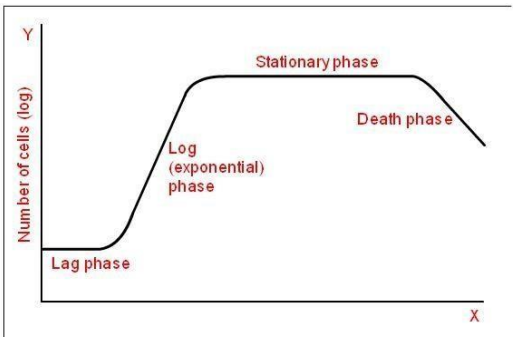


Fig 3 Different phases of growth of a bacteria

CALCULATION:

The generation time can be calculated from the growth curve (Fig 19).

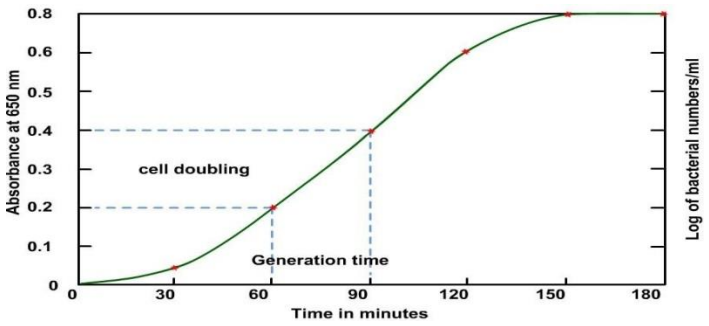


Fig. 4 Calculation of generation time

The exactly doubled points from the absorbance readings were taken and, the points were extrapolated to meet the respective time axis.

Generation Time = (Time in minutes to obtain the absorbance 0.4) – (Time in minutes to obtain the absorbance 0.2)

= 90-60

= 30 minutes

Let No = the initial population number Nt = population at time t

N = the number of generations in time t

$Nt = No \times 2^n$

Therefore,

$$\log Nt = \log No + n\log 2$$

Therefore,

$$n = (\log Nt - \log No) / \log 2$$

$$n = (\log Nt - \log No) / 0.301 \ldots\ldots\ldots (2)$$

The growth rate can be expressed in terms of mean growth rate constant (k), the number of generations per unit time.

$$k = n / t$$

$$k = (\log Nt - \log No) / (0.301 \times t) \ldots\ldots\ldots\ldots (3)$$

Mean generation time or mean doubling time (g), is the time taken to double its size. Therefore,

$$Nt = 2No \ldots\ldots\ldots\ldots (4)$$

Substituting equation 4 in equation 3

$$k = (\log Nt - \log No) / (0.301 \times t)$$

$$= (\log 2N0 - \log No) / (0.301 \times t)$$

Therefore,

Since the population doubles t= g)

$$k = 1 / g$$

$$= \log 2 + (\log No - \log No) / 0.301\ g$$

Mean growth rate constant, Mean generation time, $k = 1 / g$

$g = 1 / k$

Microbial Types based on Nutrition

Introduction

All those events through which necessary and directly usable supplies are delivered to and into individual cells comprise **nutrition**; and any chemical an organism requires as raw material may be called a **nutrient**. Since *a*living body consists partly of inorganic and partly of organic chemicals and since all life functions are directed towards maintaining and perpetuating the body, it is clear that an organism must supply itself with both inorganic and organic metabolites; we recall that organic metabolites are also called **food**.

The physical environment is the ultimate source, which supplies organisms with all required inorganic metabolites. From some of these, the required organic metabolites must then be manufactured and distributed within the living body itself. Thus all organisms build up their bodies at the ultimate expense of inorganic materials withdrawn directly from the physical environment. Excretion products formed within organisms return to the environment largely while the organisms live. And when they die, all other materials of their bodies return to the environment as well. As we shall see, decay caused by saprotrophic bacteria and fungi

gradually retransforms all the returned substances into the same kinds of inorganic materials, which were withdrawn from the environment originally.

The global environment consists of three main subdivisions. The **hydrosphere** includes all liquid components i.e. the water in oceans, lakes, rivers and on land. The **lithosphere** comprises the solid components i.e. the rocky substance of the continents. And the **atmosphere**, which is the gaseous, mantle which envelopes the hydrosphere and lithosphere. Living organisms in general require inorganic metabolites from each of these subdivisions. The hydrosphere supplies liquid water; the lithosphere supplies all other minerals and the atmosphere supplies oxygen, nitrogen and carbon dioxide. Together these inorganic materials provide all the chemical elements needed in the construction and maintenance of living matter. In addition to being sources of supply the three subdivisions of the environment also affect metabolism in various other specific ways.

We shall examine in brief the nutritional aspects of organisms under the following broad heads; nutritional requirements and forms of nutrition

Nutritional Requirements

The microbial cells are extremely complex and in addition to oxygen and hydrogen they contain four other major elements such as carbon, nitrogen, phosphorus and sulphur. About 95 per cent of cellular dry weight in these organisms is accounted for these six elements. The microorganisms in general do not need only these six elements but also others, which are found in lesser -quantity. Such elements are potassium, magnesium, calcium, sodium, iron, manganese, cobalt copper, molybdenum and zinc. These elements, infact, are needed for survival and growth of the organisms. The microorganisms differ greatly with respect to the chemical form in which these elements are utilized as nutrients. This, however, holds good in case of carbon, nitrogen, sulphur and oxygen and therefore, the requirements for these four elements cannot be easily described. Carbon assumes great importance as the main constituent of all organic cell material and represents about 50 per cent of the cells dry weight. Nitrogen is found mostly in proteins, nucleic acids, co-enzymes etc. Phosphorus is a major constituent of nucleic acids while sulphur happens to be a constituent of mainly proteins and coenzymes.

Carbon dioxide is the most oxidized form of carbon and the photosynthetic microorganisms reduce CO2 to organic cell constituents. On the other hand, all the non-photosynthetic microorganisms obtain their carbon requirement mainly from organic nutrients, which contain reduced carbon compounds. These organic compound not only-provide the carbon for synthesis but also meet the energy requirement by entering into energy yielding metabolic pathways and are eventually oxidized to CO2. Some microbes have the ability to synthesize all their cellular components using a single organic carbon source while others in addition to this one major carbon source also need other complex carbon containing components, which they can not synthesize. These are called growth factors, which include vitamins. Some microbes can utilize more than one carbon compound and exhibit a great degree of versatility. The others, however, are specialized in this regard

Sulphur and nitrogen are taken up by most organisms and are subsequently reduced within the cell and utilized in other biosynthetic processes. The sulphur and nitrogen requirements of most organisms can also be met with organic nutrients that contain these two elements in reduced organic combinations such as amino acids. A few microorganisms are capable of reducing elemental nitrogen to ammonia and this process of nitrogen assimilation is known as biological nitrogen fixation.

Most of the microorganisms need molecular oxygen for respiration. In these, the oxygen serves as terminal electron acceptor and, such organisms are referred to as '**obligate aerobes'**. As opposed to this there are a few organisms, which do not use molecular oxygen as terminal electron acceptor. We recall that oxygen is a component of the cellular material of all the microorganisms.

\These microbes are called '**obligate anaerobes'**. In fact, molecular oxygen in toxic to these organisms. Aerobes, which can grow in the absence of oxygen, are called '**facultative anaerobes'** and the anaerobes which can grow in the presence of oxygen are referred to as '**facultative aerobes'**. In addition to these major classes, there are organisms, which grow best at reduced oxygen pressure but are obligate aerobes and these are called '**Microaerophilic'**.

In addition to the four major elements (CNPS) the microorganisms also need a large number of metals in trace quantities. These trace elements are known to be essential for functioning of various enzymes in the microbial cells. Apart from the trace elements the microbes may also need some growth factors (vitamins) which can not be synthesized from single carbon or nitrogen sources and which must be supplied to these organisms to allow their proper growth and development.

The Nutritional Forms

Various nutritional patterns .may be distinguished on the basis of how microorganisms obtain their required organic and inorganic metabolites. In fact, microorganisms do not differ with respect to their procurement of inorganic metabolites; all microorganisms obtain them in finished, prefabricated form from their environment. However, microorganisms do differ with respect to their procurement of organic metabolites (foods). Some microorganisms manufacture their foods from inorganic supplies to them and thus are able to subsist in an exclusively inorganic environment: They are collectively called **autotrophs**. Other micro organic metabolites; they must absorb from the environment certain minimum amounts and kinds of prefabricated organic metabolites (the foods). Such microorganisms are collectively called **heterotrophs**.

Autotrophic microorganisms, which manufacture foods from inorganic sources, require not only externalsource of appropriate nutrient raw materials but also external sources of energy. In some cases, external energy for food manufacture is obtained from light and such microorganisms are collectively called **photosynthesizers**. In other cases, some inorganic nutrients serve as raw materials for food manufacture and other inorganic nutrients, i.e., chemicals, serve as external energy sources. Such microorganisms are collectively called**chemosynthesizers**.

We may, therefore, characterize nutritional forms from the standpoint of nature of food procurement, i.e., its autotrophic (manufacture) or heterotrophic (absorptive) nature, and we may also characterize it from the standpoint of external energy sources, i.e., its photosynthetic or chemosynthetic nature. If we characterize it from both standpoints simultaneously, we may identify following four categories of microorganisms:

Photoautotrophs

Which use light as energy Source and manufacture their food from inorganic raw materials.

Chemoautotrophs

Which use chemicals (inorganic) as energy source and manufacture their food from inorganic raw materials.

Photoheterotrophs

Which use light as energy source and convert reduced organic materials into usable foods.

Chemoheterotrophs

Which use chemical (organic) as energy source as well as absorb some organic chemicals as direct food.

Contrary to the above, if we emphasize raw material sources (i.e., inorganic or organic) and sources of energy (i.e., light or chemicals) simultaneously, we may categorize microorganisms into following four categories:

Photolithotrophs

Which use light as energy source and inorganic materials to obtain their food.

Chemolithotrophs

Which use chemicals as energy source and inorganic materials to obtain their food.

Photoorganotrophs

Which are light as energy source and reduced organic materials to obtain their food.

Chemoorganotrophs

Which use organic chemicals as energy source as well as direct food.

In both type of categories of nutritional forms the first two categories include all the autotrophic microorganisms; the last two, all the heterotrophic microorganisms. The first and third categories include all the photosynthetic micro-organisms; the second and forth, all the chemosynthetic microorganisms (Fig.5.)

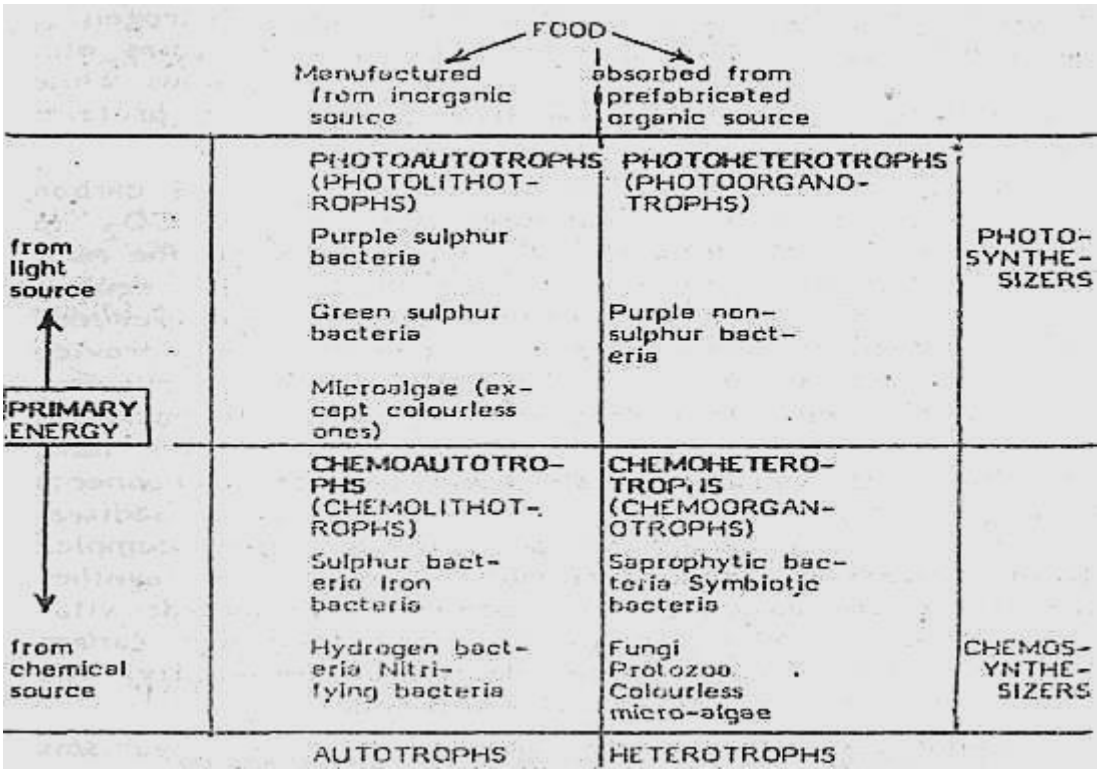


Fig.5. Nutritional classification of microorganims

Autotrophic Microorganisms

In as much as the simplest organic metabolites, i.e., carbohydrates, contain the elements carbon, hydrogen and oxygen, it is clear that every autotrophic microorganism requires an inorganic source of each of these elements. In addition, an external energy source is required to combine the three elements into organic substances.

Autotrophic microorganisms of all kinds use environmental carbon dioxide as their inorganic carbon and oxygen source.

In virtually all cases, the carbohydrate formed is a derivative of glucose, and from this basic food all the other constituents of the microorganism are then produced. But, however, autotrophic microorganisms differ in their energy and hydrogen sources; and taking this aspect into account they can be classified into following two categories:

Photoautotrophs

As in the Fig.1 indicates, this group includes most pigmented bacteria and microalgae (other than colourless ones). In all these photosynthetic microorganisms, the external energy source is **light**, and one or more varieties of **chlorophyll** are present to trap the energy of light. Such microorganisms are therefore largely green, but in many cases other pigments obscure or completely mask the colour of chlorophyll.

The hydrogen source of all photoautotrophic microorganisms except the photoautotrophic bacteria is **environmental water**. Light energy is used to split water and free hydrogen is made available.

The hydrogen source of photoautotrophic bacteria is not water and oxygen is never a byproduct of photo- synthesis. These bacteria are adapted to live in sulphur springs and other sulphurous regions, where hydrogen sulphide (H2S) is normally available. This compound generally serves as the hydrogen source. Two families of bacteria belong to photoautotrophic group, the purple sulphur bacteria and the green sulphur bacteria. The former possess a variety of special chlorophyll known as bacteriochlorophyll. It is green but its colour is masked by the additional yellow carotenoids, which are also present. Green sulphur bacteria possess **chlorobium- chlorophyll**or **bacteriviridin**, which is different from bacteriochlorophyll. Also, its green colour is not masked by the additional yellow carotenoids. For the photoautotrophic bacteria, therefore, the special-pattern of photosynthesis becomes:

Elemental sulphur is the byproduct. It is stored inside the cells in the purple sulphur bacteria, and it is excreted from the cells in the green sulphur bacteria.

Chemoautotrophs

This second group of autotrophic microorganisms consists entirely of bacteria. They can not use light, and their external energy sources in food manufacture are a variety of inorganic metabolites absorbed from the environment. In most cases, these metabolites are combined with oxygen in the cells, resulting in energy and a variety of inorganic byproducts. Water and carbon dioxide are the inorganic raw materials in subsequent food manufacture.

Among the best known chemoautotrophic microorganisms are the **sulphur bacteria**, the **iron bacteria**, the**nitri- fying bacteria** and the **hydrogen bacteria**.

(i) Sulphur bacteria

These bacteria absorb either hydrogen sulphide (H2S) or molecular sulphur (S2) from the environment and combine these metabolites with molecular oxygen. The resulting energy is used toward food manufacture and the byproducts are either S2 (if H2S is original nutrient) or sulphate ions (SO4, if S2 is the original nutrient):

If the byproduct is sulphur, granules of these elements are deposited inside or outside the cell; if the byproduct is sulphate, these ions are either part of the mineral content of the cell or are excreted.

Iron bacteria

These bacteria are stalked ones living in the environment where iron compounds combine them with molecular oxygen, thereby converting them into insoluble substances, energy is gained in the process:

Soluble Compounds Fe Insoluble Fe CompoundsEnergy

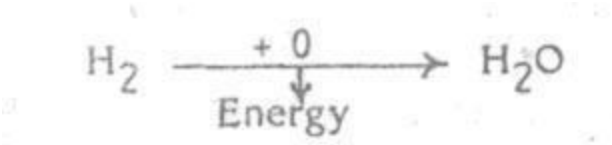
Nitrifying bacteria

These bacteria are of two types, one using ammonia and excreting nitrite ions, the other using nitrite ions and excreting nitrate ions. Both types combine their specific nutrient with molecular oxygen and in each case energy is gained:

For the bacteria themselves, the important product here is energy, which makes food manufacture possible. Forall other living organisms, the important products are the excreted byproducts, which make the global nitrogen cycle possible.

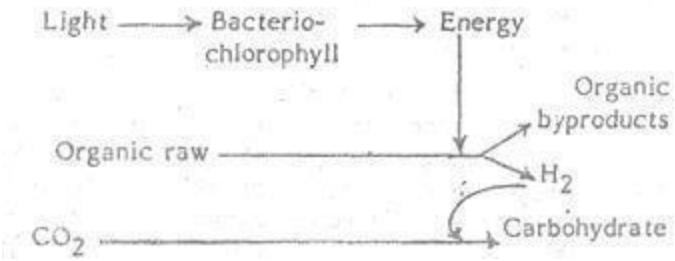
Hydrogen bacteria

These bacteria utilize molecular hydrogen as nutrient. By combining such hydrogen with oxygen, energy isgained and water forms as a byproduct.



Intermediate Forms: Photoheterotrophs

The identifying feature of this interesting nutritional form is that the external energy source is **light** (i.e., the microorganisms are photosynthesizers), but, they require organic raw materials nevertheless. This peculiar form of nutrition, namely, photoheterotrophy, is absolutely unique to one small group of photosynthetic bacteria, the **purple nonsulphur** bacteria. Like the purple sulphur bacteria, the purple nonsulphur bacteria possess **bacteriochlorophyll** as well as red and yellow corotenoids, which mask the colour of the green pigment.



Purple non-sulphur bacteria absorb organic raw materials from the environment, but these metabolites are not or can not be used directly as foods. Instead, the organic materials serve as sources of hydrogen. Extracted hydrogen is then combined with carbon dioxide. CO_2 is usually produced in the oxidation of the organic compound and at the same time used as the hydrogen acceptor. The resulting carbohydrates do serve as usuable foods:

Purple nonsulphur bacteria may manufacture foods in this manner only if light is present and oxygen is **absent**. In some species, a different type of nutrition may also occur in the dark and in the presence of oxygen. Under such conditions, absorbed organic raw materials may be used as foods **directly**. In other words, these microorganisms then are "Chemoheterotrophs". If free $H_2(g)$ is available, the Photoheterotrophs can use it to reduce CO_2 to carbohydrate (CH_2O) at the expense of light energy and they can exist as "Photoautotrophs".

Heterotrophic Microorganisms

Heterotrophic microorganisms can acquire inorganic metabolites such as H2O and CO2 from the environment but can not convert them into foods. They must, therefore, absorb prefabricated organic raw materials from the environment and use them directly as food. It follows that the survival of heterotrophic microorganisms is strictly contingent on the preexistance of autotrophs, for these must be the ultimate sources of the needed organic metabolites.

Chemoheterotrophs:

Majority of heterotrophic microorganisms belongs to this nutritional category, in which organic raw materials represent prefabricated, directly usable foods. Chemoheterotrophs can only use foods, not produce them.

Three kinds of microorganisms are included in the group: Holotrophs, Saprotrophs and Symbionts of various kinds.

Holotrophs

Holotrophs are the free-living bulk-feeders, i.e. most colourless Protista. All have mouth, if not, equivalent ingesting structures, and they digest ingested food and egest any unusable remains.

Saprotrophs

Saprotrophs comprise the slime molds, most colourless algae, and, above all, very many bacteria and fungi. They all subsist on dead organisms or on nonliving derivatives of organisms: dead plants and animals of all kinds; dung, sewage and other elimination and excretion products; and derived materials such as milk, bread and leather. In short, anything and everything nonliving that contains organic components is likely to provide usable food for Saprotrophs. These microorganisms decompose such organic matter chemically and absorb nutrient molecules from the resulting juices. Thus, Saprotrophs bring about **decay**. It is important to note that decay occurs only if and when Saprotrophs are at work.

As a result of their decay-causing nutritional activities, Saprotrophs are vital links in global nutrient cycles. The final decomposition products of decaying organic matter are H_2O, CO_2,

and N_2, and these materials return to the environment from which living matter obtained them originally. Ail the decay-causing microorganisms which participate in the water, oxygen and carbon cycles belong to the saprotrophic Chemoorganotrophs; and those which participate in the nitrogen cycle, three are likewise saprotrophic: the decay causing bacteria and fungi, some of the denitrifying bacteria, and some of the nitrogen-fixing bacteria.

Symbionts

Symbionts include commensalistic, mutualistic Helotic and parasitic types of microorganisms. Most of the mic- roorganisms within each of these categories must absorb inorganic and organic nutrients in pre-fabricated molecular form directly from their hosts. Specific food requirements vary greatly. For example, one bacterial parasite may have to obtain a given vitamin or amino acid in prefabricated form, but another may be able to manufacture such a nutrient from other organic starting materials. Biochemical differences of this sort are exceedingly numerous, and they are one reason why a symbiont can not pick hosts at random. Survival is possibleonly in hosts in which all required types of nutrients are available.

Physical Conditions Required For Growth

To cultivate microorganisms in the laboratory, it is not enough to determine their nutritional requirements, but also the physical environment where the organisms will grow best. Just as microorganisms vary greatly with regard to their nutritional requirements, so also they exhibit diverse response to the physical conditions in their environment. Some important physical factors that affect the growth of microorganisms are discussed.

Temperature

Temperature is the most important factor that determines the rates o£ growth, multiplication, survival, and death of all living organisms. Growth and reproduction of living organisms are dependent on a co-ordinated series of enzyme-catalysed chemical reactions. The rates of enzyme reaction increase with the increase in temperature. Since micro-bial activity and growth are manifestations of enzymatic reactions, their rates of growth are, temperature-dependent. In short.temperature determines the rate of growth, the total amount of growth, the metabolic activity, and the morphology of the organisms. Each microorganism can grow

only within a growth temperature range characteristic of the species. The temperature relationships of a microorganism are usually described by the three cardinal temperatures, the *minimum, optimum,* and the*maximum temperatures* of growth.

The lowest temperature at which organisms grow is the *minimum growth temperature.* Most organisms will survive for a varying length of time below this temperature, but will show negligible growth. Minimum growth temperature is difficult to determine precisely.because of an increase in generation time. Growth is not visible until a population of about 1 x 10^7 cells /ml has been attained. Whether an organism is capable of growth at a particular temperature depends on the visibility of the growth.The *maximum growth temperature* is the highest temperature at which growth occurs. A temperature only slightly above this point frequently kills the microorganisms by inactivating critical enzymes. Maximum growth temperature is relatively easy to establish, because organisms either grow or are destroyed by high temperature. The *optimum temperature* is commonly defined as the temperature at which the most rapid rate of multiplication occurs.For most organisms, optimum growth occurs over a temperature range rather than at a fixed temperature. The optimum temperature is also difficult to agree ,upon, for the optimum temperature of growth may not be optimum for other cellular activities, for example, maximum acid production or pigment production, Sometimes it also changes the nutritional requirement. Generally the upper limit of the optimum growth temperature is only a few degree below the maximum growth temperature. Fig. 22 illustrates the effect of temperature on the rate of two bacterial species.

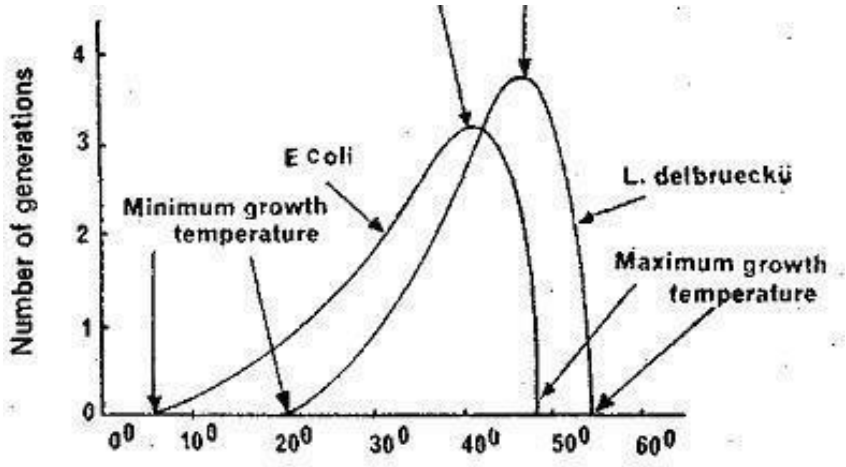


Fig.6 Effect of temperature on the rate of logarithmic multiplication of of Escherichia coli and Lactobacillus delbrueckii

Incubation temperature

Maximum growth temperatures are only 5 to 10 degrees higher than the optimum growth temperatures, whereas minimum growth temperatures are approximately 30 degrees lower.

Classification of bacteria according to growth temperature.

The numerical values of the *cardinal temperatures* (minimum, optimum and maximum), and the range of temperature over which growth is possible, vary widely among bacteria. Some bacteria isolated from hot springs are capable of growth at temperature as high as 95°C; others, isolated, from cold environments, can grow at temperature as low as - 10°C if a high solute concentration prevents the medium from freezing. Bacteria are frequently classified into three groups according to their temperature preferences. These groups are not sharply defined, and the distinctions are arbitrary. However, this sort of classification is useful in describing the collective properties of groups of microorganisms adapted to life in certain environments. Bacteria are normally classified into three broad groups, *psychrophiles, mesophiles,* and *thermophiles.*

Psychrophiles. *Psychrophilic* (Or. *Psychros* = cold) bacteria are the predominant organisms in many uncultivated soils, and in lakes, streams, and oceans. They are commonly defined as micro-organisms capable of growth at 0°C, though they grow best at higher temperatures, between 15° to 30°C. Two groups of psychrophiles have been distinguished : (1) *obligate psychrophiles* cannot grow at temperatures above 19° to 22°C, whereas *(2) facultative psychrophiles* may grow at 30° to 35°C.

Mesophiles. Most of the commonly studied bacteria are m*esophilic* (Gr. *meso* = middle), and these fall into two well defined sub-divisions: (1) those whose optimum growth temperatures are from 20° to 35°C., and (2) those whose optimum temperatures are between 35 to 45°C. The first group consists mainly of saprophytes and plant parasites, whereas the second group consists mainly of animal parasites or commensals. Minimum and maximum growth temperatures, vary correspondingly, but for the most part are within the range of 10 to 52°C.

Thermophiles. *Thermophiles* (Gr. Thermo=heat) have optimum growth temperature of 45°C or higher, and generally grow over a range of 40 to 75°C. Two groups of thermophiles have been observed. *Obligate thermophiles* grow only at high temperatures, usually above 50°C.

Facultative thermophiles grow both at 37°C and 55°C. An organism that is beat resistant, for instance, one that withstands pasteurization, but does riot grow at high temperatures, is termed *thermoduric.*

Table. Classification, of bacteria according to their growth temperature.

Group	Temperature range	Sub-division
1. Psychrophiles	Grow Well at O'C. (a) cannot grow at temp, above 19° to 22'C.	Obligate psychrophiles.
	(b) may grow at 30° to 35'C	Facultative psychrophiles.
2. Mesophiles	Do not grow at 0^8C. Optimum growth temperature is less than 45°C.	
	(a) optimum growth temp. 20° to 35'C.	Saprophytes.
	(b) optimum growth temp. 35*C to 45^8C.	Animal parasites.
3.Thermophiles	Optimum growth temperature is greater than 45°C.	Obligate ther-
	(a) grow above 50''C.	mophiles.
	(b) grow both at 37°C and above	Facultative
	50*C.	thermophiles.

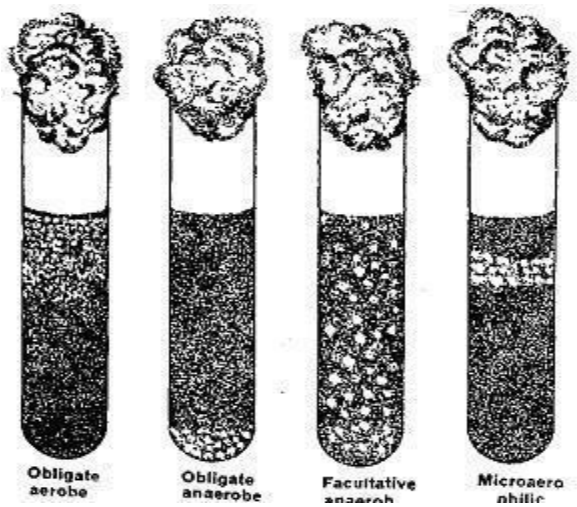


Fig.7. Growth pattern of bacteria in deep agar tubes

Gaseous requirements.

The principal gases that affect microbial growth are oxygen and carbon dioxide. The present atmosphere of the earth 6oniains about 20 percent (V/V) oxygen. Although almost all higher plants and animals are dependent upon a supply of oxygen, this does not hold true for all microorganisms. The responses to oxygen among microorganisms are remarkably variable, and this is an important factor in their cultivation. The organisms are divided into four groups on the basis of their relationship to molecular oxygen :

i. *Strict or obligate aerobex grow only in the presence of free oxygen.*

ii. *Strict or obligate anaerobes grow only in the absence of free oxygen.*

iii. *Facultative anaerobe* can grow both in the presence and the absence of free oxygen

iv. *Microaerophilic* organisms grow best in the presence of a low concentration of molecular oxygen.

Oxygen requirements. Molecular oxygen is relatively insoluble in water, and so must be continuously made available to aerobic microorganisms. Growth of aerobic microorganisms in tubes or small flasks incubated under normal atmospheric conditions is generally satisfactory. However, when aerobic organisms are to be grown in large quantities, it is advantageous to increase the exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers, for which suitable containers are available. Alternatively, shaking and bubbling in sterile air or oxygen is done for increasing the availability of oxygen to microorganisms growing in a liquid medium. However, the amount of oxygen required by various aerobic microorganisms differs considerably. Also, the amount of oxygen required for maximum growth can differ from that required for other metabolic processes. For example, the amount of oxygen required for the growth of *Aspergilus niger* is less than that required for the production of citric acid by *A. niger*.

To cultivate anaerobic microorganism, special techniques are devised to exclude all atmospheric oxygen from the medium. Anaerobic environment can be established by using one of the following methods.

Addition of reducing compounds, e.g. sodium thioglycollate, cysteine hydrochloride, sodium formaldehyde, sulphoxalate, etc. to the medium to absorb oxygen.

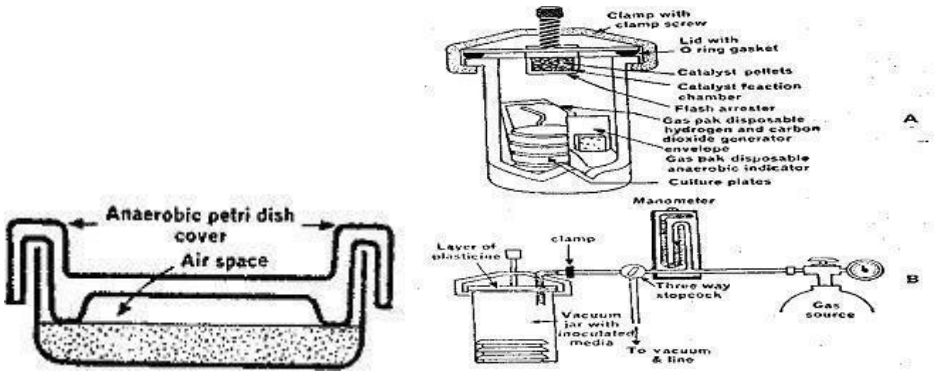


Fig. 8 Anaerobic plate cultivation. Cross section showing Brewer anaerobic petrj dish cover in use. The anaerobic agar contains the reducing agent, sodium thioglycollate. Note that at the periphery of the agar surface the petri dish cover js in contact with the agar, thus sealing the air space.

The thioglycollate absorbs the oxygen from the air space.

Mechanical removal of oxygen from an enclosed vessel containing tubes or plates of inoculated medium. The air is pumped out of the vessel and replaced by nitrogen, helium, or a mixture of nitrogen and carbon dioxide

Chemical reaction within an enclosed vessel containing the incubated medium, to combine the free oxygen into a compound. This can be as simple as the bu rning of a small candle or the combustion of small amount of alcohol to use up some of the free oxygen. A common laboratory method of cultivating an anaerobic micro organism by introducing pyragallol over the cotton plug in the inoculated slant tube is illustrated in Fig.

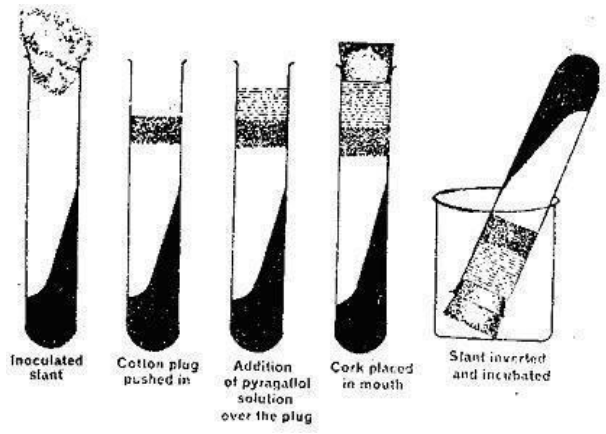


Fig. 9 The Gaspak anaerobic system

Carbon dioxide requirement. All microorganisms utilize carbon dioxide for growth. In some microorganisms the liberation of carbon dioxide from metabolic reactions is adequate to supply this need. However, when cultures are vigorously aerated, particularly when there is a low cell density, the air may sweep the CO2 away as quickly as it is produced. Secondly, a sufficient amount of carbon dioxide is to be provided for the cultivation of *autotrophx.* In case of autotrophs that can be grown under anaerobic condition, the requirement of CO3 can be met by providing buffers such as CaCO3 or NaHCO3 which release C02 when acid is produced by the culture. Carbonates cannot be used in media exposed to air, because the release of CO2 is rapidly swept away, causing the medium to become extremely alkaline.

Hydrogen ion concentration (pH)

Small size and great mobility of hydrogen ions are of supreme importance in many chemical processes, and more so in biological processes, because of the transfer of hydrogen from one molecule to another. The tendency of hydrogen to dissociate from its original combination thus determines the probability of the reaction. The concentration of hydrogen is always low in the natural habitat of micro-organisms, but on the other hand the organisms cannot grow in its complete absence. The effect of hydrogen ions is similar to that of metallic ions, high concentration is toxic, moderately low concentration permits growth, and very low concentration is unfavourable for growth.

The **acidity or alkalinity** of a solution is a function of the relative hydrogen ion (H+) concentration or pH which is expressed as the negative log of the hydrogen ion concentration. Microbial growth and activities are strongly affected by the pH of the medium. However, there are wide differences between the pH requirements of the various species. These differences reflect the normal habits and habitats of the organisms. Microorganisms show the same type of tolerance to acidity or alkalinity that was observed for temperature. Each species usually shows a range of growth responses to varying pH values., and have a pH optimum for maximal balanced growth. Organisms which require pH values of 5 or less for maximal growth rate are termed ***acidopliiles***, and usually have a pH optium of 2 or 3. ***Alkaliphiles*** grow at pH value between 7 and 12, with the optimum around pH 9.5.*Neutro-philes* prefer pH values around neutrality (pH 7).

Bacteria, in general, prefer media of pH values near neutrality, and usually cannot tolerate pH values much below 4-5. There arc some exceptions to this generalization. The classic example is *Thiobacillus thioxidans,* which oxidizes sulphur to sulphuric acid, can grow . at pH 1.0 Acetic acid bacteria and intestinal bacteria which tolerate the acid of the stomach are other exceptions. Animal pathogens are usually favoured by an environment at pH 7.2 to 7.4. At the opposite extreme, bacteria that infect the human urinary tract and hydrolyze urea to give ammonia can grow at pH 11. Yeasts prefer slightly acidic media for growth. Moulds prefer more acidic media (pH 4). Many plant and soil microorganisms, especially *Actinomycetes,* prefer relatively alkaline conditions.

When microorganisms are inoculated in a medium originally adjusted to a given pH, it is very likely that this pH will change, depending upon the type of the microbial activity and the composition of the medium. Degradation of proteins and other nitrogenous compounds frequently yields ammonia or other alkaline byproducts; carbohydrate fermentations often produce organic acids. The change in the pH value brought by such reactions continues until the maximum or minimum pH for the organisms is reached, whereupon the culture dies.

The pH of the medium also determines which pathways of metabolism will operate. For example, at an alkaline reaction yeasts ferment glucose to glycerol, whereas at an acid reaction they ferment glucose to ethanol- Organisms such as *Aerobacter aerogenes,* which can form acetylmethyl-carbinol from glucose, will do so only below pH 6.0. The fate of amino acids in the cell is also decided by pH. At an acid reaction they are decarboxylated to the corresponding amines, whereas at alkaline reaction they are deaminated to an acid.

Buffers are often added to prevent the radical shift in the pH of the medium. Most buffers used in media arc mixtures of weakly acidic and weakly alkaline compounds. A combination of KH2PO4 and K2HPO4 is widely employed in bacteriological media. If microorganisms form an acid such as acetic acid in a medium buffered with phosphate, a part of the basic salt (K2HPO4) is converted to the weakly acidic salt.

$$K2HPO4 + HC2H302 \Downarrow KH2PO4 + KC2H3O2$$

The pH of the medium falls only slightly. Conversely, a basic microbia! product reacts with the acidic salt (KH2PO4) to forma dibasic compound that is only weakly alkaline. Many culture media contain amphoteric substances such as peptones. These compounds possess

both amino and carboxyl radicals, which can dissociate as basic and acidic groups- Insoluble carbonates such as CaCO3 aud MgCO3 are also added to media to prevent a drop in pH as acid is produced. Being insoluble, they have no direct effect on pH, but when acid is formed and the reaction falls below pH 7.0, the carbonate decomposes, CO2 is evolved, and the acid is converted to its calcium or magnesium salt- The extent to which a medium should or may be buffered depends on its intended purpose, and is limited by the buffering capacity of the compounds used. Some large fermentation apparatuses are equipped with automatic controls that maintain a constant pH.

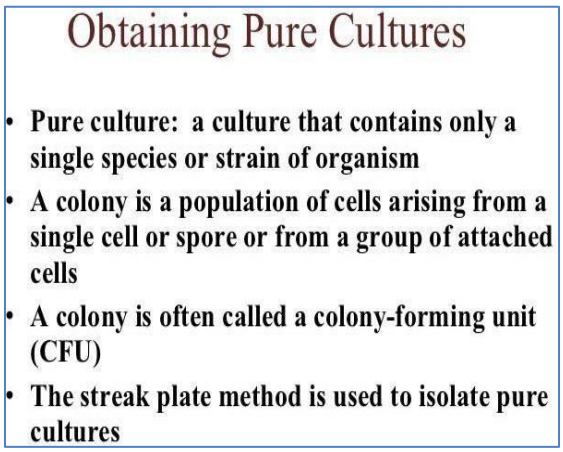
1. Miscellaneous physical requirements. Additional physical factors are to be considered for the growth of certain fastidious organims. For example *photosynthetic* microorganisms(algae, photosynthetic bacteria) must be exposed to a source of illumination, since light is their source of energy. *Halopfules* and *osmophiies* isolated from sea and other natural bodies of water of high salinity can grow only when the medium contains an unusually high concentration of salt.The successful cultivation of microorganism in the laboratory is based upon two basic principles; nutritional requirement to prepare a suitable nutrient medium, and appropriate physical conditions to obtain maximum growth.

<table>
<tr><th colspan="6">Classification of organisms based on their metabolism</th></tr>
<tr><td rowspan="2">Energy source</td><td>sunlight</td><td>photo-</td><td colspan="2" rowspan="2"></td><td rowspan="5">-troph</td></tr>
<tr><td>Preformed molecules</td><td>chemo-</td></tr>
<tr><td rowspan="2">Electron donor</td><td>organic compound</td><td rowspan="2"></td><td>organo-</td><td rowspan="2"></td></tr>
<tr><td>inorganic compound</td><td>litho-</td></tr>
<tr><td rowspan="2">Carbon source</td><td>organic compound</td><td colspan="2" rowspan="2"></td><td>hetero-</td></tr>
<tr><td>carbon dioxide</td><td>auto-</td></tr>
</table>

Factor Class of Organism Minimum Optimum Maximum Example

Temperature (°C)	extreme psychrophile	-2	5	10	*Raphidonema nivale* (snow algae)
	psychrophile	0	15	20	*Vibrio marinus*
	mesophile	10-15	24-40	35-45	*Escherichia* coli
	facultative thermophile	37	45-55	70	*Bacillus stearothermophilus*
	obligate thermophile	45	70-75	85-90	*Thermus aquaticus*
	extreme thermophile	60	75-80	85-110	*Sulfolobus acidocaldarius*
Oxygen Requirements	Aerobes				*Escherichia* coli
	Anaerobes				*Clostridium butulinum*
	Microaerophiles				
pH	acidophile	0.8	2-3	5	*Thiobacillus thiooxidans*
	alkal(in)ophile	ca 7	9-10.5	11-11.5	*Bacillus alcalophilus*
Osmotic pressure	halophile	0.5	1-2	4-4.5	*Vibrio costicola*
(Molar salt conc)	extreme halophile	3	3 5	5.2	*Halobacterium halobium*

Pure culture techniques



Serial dilution method

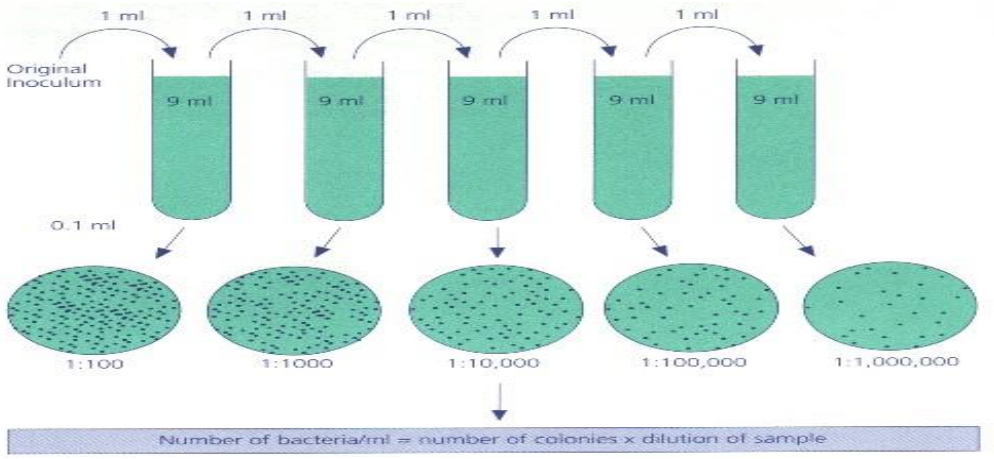


Fig.10

Serial Dilutions

The inoculum is diluted out in a series of dilution tubes which are plated out. The number of colonies on the plate are counted and corrected for the dilution to calculate the number of organisms in the original inoculum.

Spread plate method

Spread plate technique is an additional method of quantifying microorganisms on solid medium. With the spread plate method, a volume of an appropriately diluted culture usually no greater than 0.1 ml is spread over the surface of an agar plate using a sterile glass spreader. The plate is than incubated until the colonies appear, and the number of colonies counted. Instead of embedding microorganisms into agar, as is done with the pour plate method, liquid cultures are spread on the agar surface.

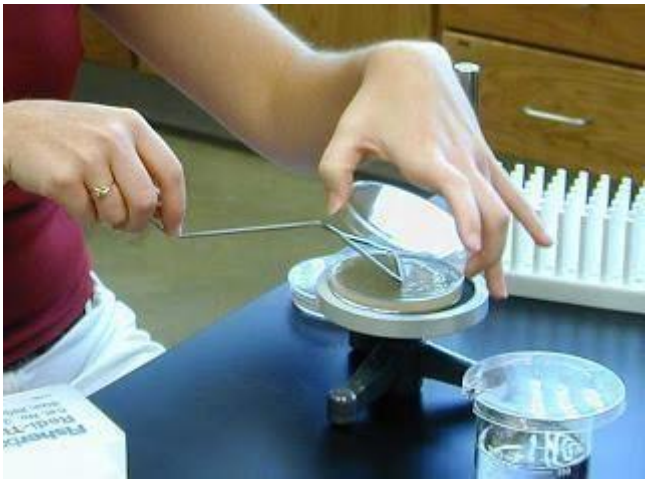


Fig.11

An advantage of spreading a plate over the pour plate method is that cultures are never exposed to 45^{o}C (i.e. melted agar temperatures). Note: Surface of the plate must be dry, so that the liquid that is spread soaks in. volume greater than 0.1ml are rarely used because the excess liquid does not soak in and may cause the colonies to coalesce as they from, making them difficult to count.

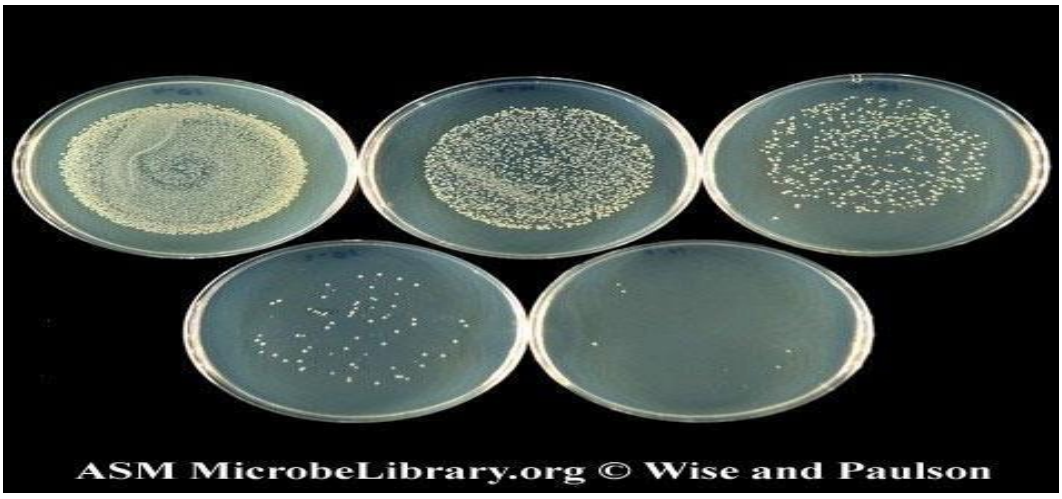


Fig. 12 Picture of spread plates showing bacterial growth (*E. coli*, 40 hours, room temperature) on five plates prepared from a ten-fold dilution series. Care was taken to avoid spreading to the edges of the plates as it is more difficult to count colonies along the edge of the agar.

Duplicate or triplicate plates with 30 to 300 CFUs/plate are used to calculate CFUs/ml.

Pour plate method

Pour plate Technique A pour plate is a method of melted agar inoculation followed by petri dish incubation. A known volume (usually 0.1-1.0 ml) of culture is pipette into a sterile petri plate; melted agar medium is then added and mixed well by gently swirling the plate on the table top. Because the sample is mixed with the molten agar medium, a larger volume can be used than with the spread plate. However, with the pour plate method the organism to be counted must be able to briefly withstand the temperature of melted agar, 45^{o}C.

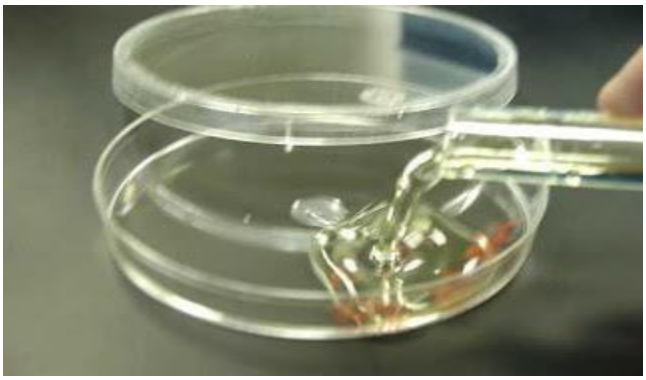


Fig.13.

The cultures are inoculated into melted agar that has been cooled to 45^{o}C. The liquid medium is well mixed then poured into a petri dish (or vice versa*)* Colonies form within the agar matrix rather than on top as they do when streaking a plate. Pour plates are useful for quantifying microorganisms that grow in solid medium. Because the "pour plate" embeds colonies in agar it can supply a sufficiently oxygen deficient environment that it can allow the growth and quantification of microaerophiles.

Streak Plate Technique For organisms that grow well on agar plate, streak plate is the method of choice for obtaining pure culture.

The key principles of this method is that, by streaking, a dilution gradient (number of cells decrease as they move across the agar and away from the point of inoculation) is established across the face of the plate as bacterial cells are deposited on the agar surface. Because of this dilution gradient, confluent growth occurs on part of the plate where the bacterial cells are not

sufficiently separated; in other regions of the plate where few bacteria are deposited separate macroscopic colonies develop that can easily be seen with naked eye. Each well isolated colony is assumed to arise from a single bacterium and therefore to represent a clone of a pure culture.

Purpose of Streak Plate Technique: The purpose of the streak plate is to obtain isolated colonies from an inoculum by creating areas of increasing dilution on a single plate. Isolated colonies represent a clone of cells, being derived from a single precursor cell.

Fig.14.

Many different streaking patterns can be used to separate individual bacterial cells on the agar surface.

Using selective media - Mannitol salt agar for staphylococcus aureus

LAWN CULTURE

- Provides a uniform surface growth of the bacterium.
- Uses
  - For bacteriophage typing.
  - Antibiotic sensitivity testing.
  - In the preparation of bacterial antigens and vaccines.
- Lawn cultures are prepared by flooding the surface of the plate with a liquid suspension of the bacterium.

PART A

S.No.	Questions	Outcomes
1	Define microscope and microscopy.	CO2
2	State on magnification.	CO2
3	What is numerical aperture.	CO2
4	Define staining.	CO2
5	What is sterilization.	CO2
6	List out few disinfectants.	CO2
7	Define tyndallization.	CO2
8	Emphasis on special staining.	CO2
9	Give out the principle of light microscope.	CO2
10	What is monochrome staining.	CO2

PART B

S.No.	Questions	Outcomes
1	Give out the principle and working mechanism of dark field microscope.	CO2
2	Explain the principle and working of phase contrast microscope.	CO2
3	Discuss the various filters used in fluorescent microscope the operation techniques.	CO2
4	Illustrate the principle construction and working of TEM.	CO2
5	Explain the principle and working mechanism of SEM.	CO2
6	Describe the principle and methodology adopted for differential staining.	CO2
7	Write a brief note on capsule and spore staining.	CO2
8	Discuss briefly various sterilization procedures adopted in microbiological laboratory.	CO2
9	Write in detail about the mode of action of various disinfectants routinely used in killing of microbes.	CO2
10	State the importance of moist and dry heat sterilization.	CO2

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DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT – 3 –Microbiology and Pathology

UNIT – III

NORMAL CELL STRUCTURE

Pathology is a branch of medical science primarily concerning the examination of organs, tissues, and bodily fluids in order to make a diagnosis of disease.

Clinical pathology is a medical specialty that is concerned with the diagnosis of disease based on the laboratory analysis of bodily fluids such as blood and urine, as well as tissues, using the tools of chemistry, clinical microbiology, hematology and molecular pathology.

Pathologists are physicians who specialize in the diagnosis and characterization of disease based on the examination of tissues removed from diseased body parts or biopsy samples. They can also diagnose certain diseases and conditions through the laboratory analysis of various bodily fluids such as the blood, semen, saliva, cervical fluid, pleural fluid, pericardial fluid and ascetic fluid.

Rudolf Virchow (1821–1902) is generally recognized to be the father of microscopic pathology. **What is the Disease**? It is the "state in which an individual exhibits an anatomical, physiological, or biochemical deviation from the normal".

Pathology focuses on 3 aspects of disease.

ETIOLOGY: Cause of disease**.** Environmental agents: • Physical • Chemical • Nutritional Multifactorial: • Infections As Diabetes, • Immunological • Genetic Factors: Cancer • Age • Genes Psychological Hypertension

PATHOGENESIS: Mechanisms of development of disease. PathogenesisThe sequence events in the response of thecells or tissues to the etiologic agent, from theinitial stimulus to the ultimate expression of thedisease,"from the time it is initiated to its finalconclusion in recovery or death". **MORPHOLOGY**: structural alterations induced in cell and tissues.

Origins of Pathology

The Greek physician Hippocrates, the founder of scientific medicine, was the first to deal with the anatomy and the pathology of human spine. A student of Virchow's, Julius Cohnheim (1839- 1884) combined histology techniques with experimental manipulations to study inflammation, making him one of the earliest experimental pathologists. Cohnheim also pioneered the use of the frozen section procedure; a version of this technique is widely employed by modern pathologists to render diagnoses and provide other clinical information intraoperatively.

CELL

The **cell** is the basic structural, functional, and biological unit of all known living organisms. Cells are the smallest unit of life that can replicate independently, and are often called the "building blocks of life". The study of cells is called cell biology. Cells consist of cytoplasm enclosed within a membrane, which contains many biomolecules such as proteins and nucleic acids. Organisms can be classified as unicellular or multicellular. While the number of cells in plants and animals varies from species to species, humans contain more than 10 trillion (10^{13}) cells.Most plant and animal cells are visible only under the microscope, with dimensions between 1 and 100 micrometres. The cell was discovered by Robert Hooke in 1665, who named the biological unit for its resemblance to cells inhabited by Christian monks in a monastery.

CELL DEGENERATION

Defined as deterioration of live cells following injury, but with a possibility of the injured cells to reverse to normal when the injury is removed• Injury is defined as any harmful stimulus that induces disturbance in the homeostasis of cells.

CELL REGENERATION

Cell regeneration is the process of renewal, restoration, and growth that makes genomes, cells, organisms to natural fluctuations or events that cause disturbance or damage. Every species is capable of regeneration, from unicellular bacteria to multicellular humans.

NECROSIS

Necrosis refers to a spectrum of morphologic changes that follow cell death in living tissue resulting from the progressive degradative action of enzymes on the lethally injured cells.

Examples of cell injury and necrosis, ischemic and hypoxic injury

Hypoxia refers to any state of reduced oxygen availability it may be caused by reduced amount of hemoglobin.

Ischemia means reduced blood flow usually as a consequence of a mechanical obstruction in the arterial system.

TYPES OF NECROSIS

1. Coagulate necrosis
2. Liquefaction necrosis
3. Caseous necrosis
4. FAT necrosis
5. Fibrinoid necrosis

INFLAMMATION

Inflammation is a localized physical condition in which part of the body becomes reddened, swollen, hot, and often painful, especially as a reaction to injury or infection. Inflammation is a protective response that involves immune cells, blood vessels, and molecular mediators. The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. Inflammation is often characterized by redness, swelling, warmth, and sometimes pain and some immobility.

Inflammation can be classified as either ***acute* or *chronic***.

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A

series of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue.

Prolonged inflammation, known as ***chronic inflammation***, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Inflammation is divided into acute and chronic

Acute is of short duration, primary cell is the neutrophil, characterized by exudation of fluid and plasma proteins.

Chronic is longer, main cells are lymphocytes and macrophages, proliferation of blood vessels, fibrosis and necrosis.

Vascular and cellular responses of both acute and chronic are mediated by chemical factors derived from cells and plasma and triggered by the inflammatory stimulus. Necrotic cells, themselves, release these chemical. These are called **chemical mediators of inflammation.** Inflammation stops when the injurious stimulus is removed.

Acute inflammation has three major components:

Alteration in vessels leading to increased blood flow to the area.

Structural changes in microvessels that allow plasma protein and WBCs to leave the circulation.

Emigration of WBCs from vessels so they can accumulate at the site of injury. Vasodilatation occurs resulting in **increased blood flow to the area**. New capillary beds open up.

This is followed by slowing of the circulation that is brought about by increased permeability. Fluid moves into the extravascular space, the blood becomes thicker and thus moves more slowly. At this point the blood is more viscous; there are higher concentrations of RBCs in the vessels. We call this stasis.

When stasis occurs the WBCs start to move toward the periphery of the vessel. This is call **Margination** or **pavementing**.

The acute inflammation is increased vascular permeability leading to **edema**. How does excess fluid get out of the vessels?

Endothelial gaps. Gaps are do to endothelial contraction mediated by histamine, bradykinin, leukotrienes, substance P and others.

Direct endothelial injury by burns, lytic bacterial infections.

Delayed prolonged leakage beginning after a delay of 2 to 12 hours. Usually caused by thermal injury, radiation and certain bacterial toxins. An example is a late occurring sunburn.

Leukocyte-mediated endothelial injury. Seem mostly in the kidneys and lungs and is related to a hypersensitivity reaction.

Leakage from new blood vessels which have lots of receptors for chemical mediators.

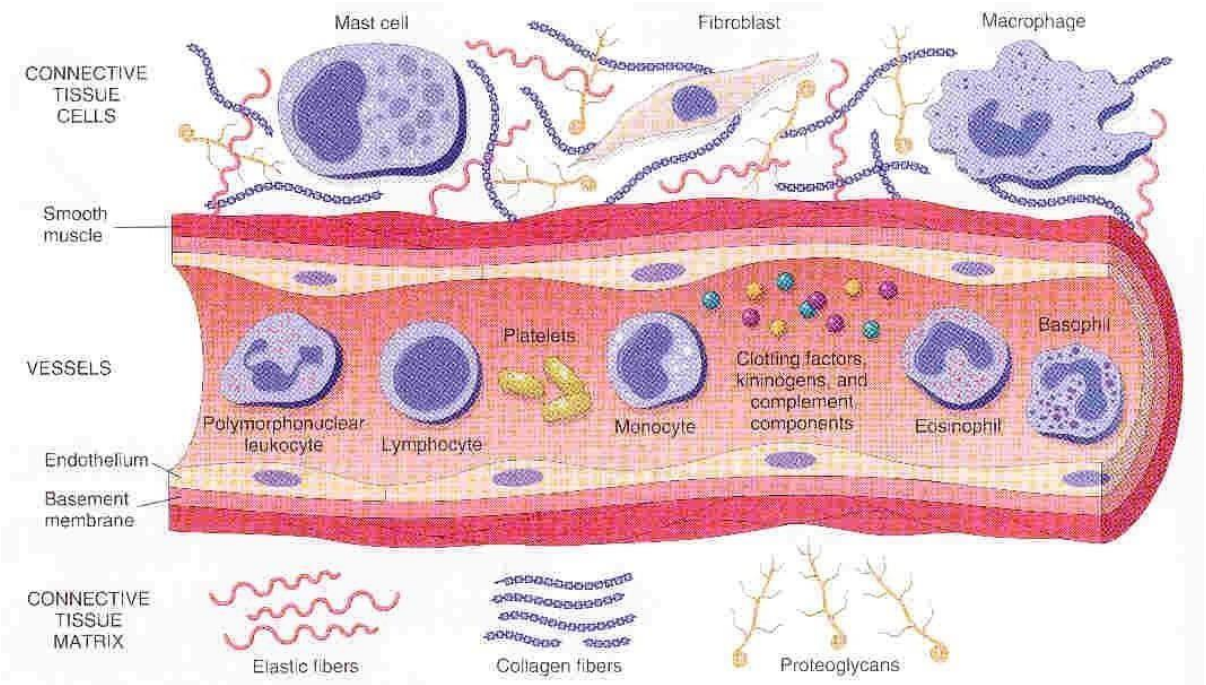

Fig.1

Cellular Events

- Margination, cells have to adhere to vessels. There are adhesion receptors and chemical mediators that allow this to happen. There are genetic disorders where people can not make adequate adhesion factors; these people can not mount a normal inflammatory response.

- Transmigration

- Migration, leukocytes emigrate to the site of injury by a process called chemotaxis. Chemotactic factors can be both endogenous and exogenous. Bacterial products are the most common exogenous factors. Endogenous agents include components of compliment, leukotrienes and cytokines. Chemotactic factors stimulate locomotion.

- Phagocytosis is the ability of the WBC to recognize the pathogen, form an attachment, engulf it and kill it. Various chemical mediators are important in this function. Bacterial killing is accomplished mostly by an oxygen-dependent mechanism. Killing can also occur by the release of granules from the lysosomes.

APOPTOSIS

Apoptosis definition: A form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. Apoptosis plays a crucial role in developing and maintaining the health of the body by eliminating old cells, unnecessary cells, and unhealthy cells. The human body replaces perhaps one million cells per second. When apoptosis does not work correctly, cells that should be eliminated may persist and become immortal.

German scientist Karl Vogt was first to describe the principle of apoptosis in 1842. In 1885, anatomist Walther Flemming delivered a more precise description of the process of programmed cell death. In Greek, apoptosis translates to the "falling off" of leaves from a tree.

Activation mechanisms

The initiation of apoptosis is tightly regulated by activation mechanisms, because once apoptosis has begun, it inevitably leads to the death of the cell. The two best-understood activation mechanisms are of are the intrinsic pathway (also called the mitochondrial pathway) and the extrinsic pathway. The *intrinsic pathway* is activated by intracellular signals generated when cells are stressed and depends on the release of proteins from the intermembrane space of mitochondria. The *extrinsic pathway* is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex (DISC).

A cell initiates intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration, for example, by damage to the membrane, can all trigger the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis.

Before the actual process of cell death is precipitated by enzymes, apoptotic signals must cause regulatory proteins to initiate the apoptosis pathway. This step allows those signals to cause cell death, or the process to be stopped, should the cell no longer need to die. Several proteins are involved, but two main methods of regulation have been identified: the targeting of mitochondria functionality, or directly transducing the signal via adaptor proteins to the apoptotic mechanisms. An extrinsic pathway for initiation identified in several toxin studies is an increase in calcium concentration within a cell caused by drug activity, which also can cause apoptosis via calcium binding protease calpain.

Intrinsic pathway

The mitochondria are essential to multicellular life. Without them, a cell ceases to respire aerobically and quickly dies. This fact forms the basis for some apoptotic pathways. Apoptotic proteins that target mitochondria affect them in different ways. They may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out. they are very closely related to intrinsic pathway, and tumors arise more frequently through

intrinsic pathway than the extrinsic pathway because of sensitivity.There is also a growing body of evidence indicating that nitric oxide is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable.Nitric oxide has been implicated in initiating and inhibiting apoptosis through its possible action as a signal molecule of subsequent pathways that activate apoptosis.

Mitochondrial proteins known as SMACs (second mitochondria-derived activator of caspases) are released into the cell's cytosol following the increase in permeability of the mitochondia membranes. SMAC binds to *proteins that inhibit apoptosis* (IAPs) thereby deactivating them, and preventing the IAPs from arresting the process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of a group of cysteine proteases called caspases,which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability.

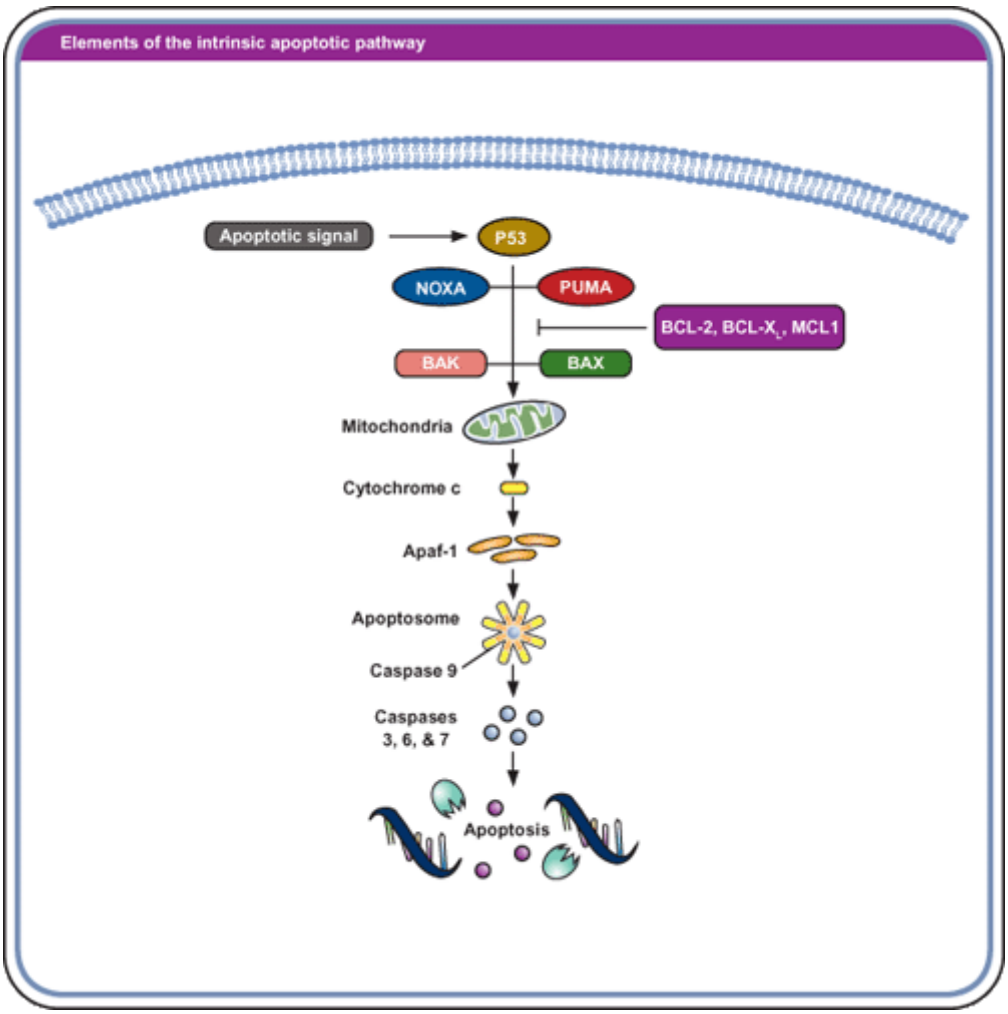

Fig.2

Cytochrome c is also released from mitochondria due to formation of a channel, the mitochondrial apoptosis-induced channel (MAC), in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis.

Extrinsic pathway

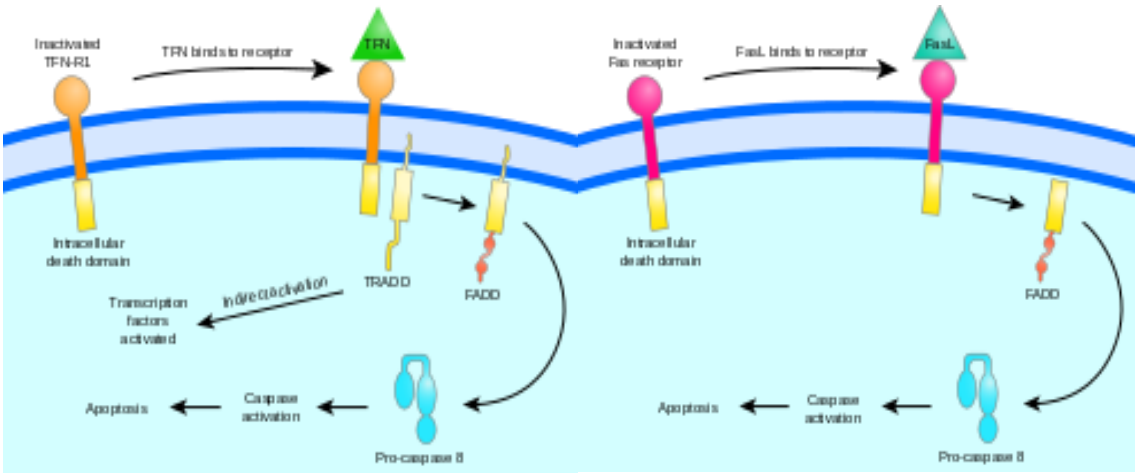

Fig.3.

Overview of TNF and Fas signaling in apoptosis

Two theories of the direct initiation of apoptotic mechanisms in mammals have been suggested: the *TNF-induced* (tumour necrosis factor) model and the *Fas-Fas ligand-mediated* model, both involving receptors of the *TNF receptor* (TNFR) family coupled to extrinsic signals.

TNF path

TNF-alpha is a cytokine produced mainly by activated macrophages, and is the major extrinsic mediator of apoptosis. Most cells in the human body have two receptors for TNF-alpha: TNFR1 and TNFR2. The binding of TNF-alpha to TNFR1 has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD). cIAP1/2 can inhibit TNF-α signaling by binding to TRAF2. FLIP inhibits the activation of caspase-8. Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses.[32] However,

signalling through TNFR1 might also induce apoptosis in a caspase-independent manner.[33] The link between TNF-alpha and apoptosis shows why an abnormal production of TNF-alpha plays a fundamental role in several human diseases, especially in autoimmune diseases.

Fas path

The fas receptor First apoptosis signal (fas) – (also known as *Apo-1* or *CD95*) binds the Fas ligand (FasL), a transmembrane protein part of the TNF family.[30] The interaction between Fas and FasL results in the formation of the *death-inducing signaling complex* (DISC), which contains the FADD, caspase-8 and caspase-10. In some types of cells (type I), processed caspase- 8 directly activates other members of the caspase family, and triggers the execution of apoptosis of the cell. In other types of cells (type II), the *Fas*-DISC starts a feedback loop that spirals into increasing release of proapoptotic factors from mitochondria and the amplified activation of caspase-8.[34]

Common components

Following *TNF-R1* and *Fas* activation in mammalian cells a balance between proapoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (*Bcl-Xl* and *Bcl-2*) members of the *Bcl-2* family is established. This balance is the proportion of proapoptotic homodimers that form in the outer- membrane of the mitochondrion. The proapoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC. Control of proapoptotic proteins under normal cell conditions of nonapoptotic cells is incompletely understood, but in general, Bax or Bak are activated by the activation of BH3- only proteins, part of the Bcl-2 family.

Caspases Caspases play the central role in the transduction of ER apoptotic signals. Caspases are proteins that are highly conserved, cysteine-dependent aspartate-specific proteases. There are two types of caspases: initiator caspases, caspase 2,8,9,10,11,12, and effector caspases, caspase 3,6,7. The activation of initiator caspases requires binding to specific oligomeric activator protein. Effector caspases are then activated by these active initiator caspases through proteolytic cleavage. The active effector caspases then proteolytically degrade a host of intracellular proteins to carry out the cell death program.

Caspase-independent apoptotic pathway also exists a caspase-independent apoptotic pathway that is mediated by AIF (apoptosis-inducing factor)

Once cytochrome C is released it binds with Apoptotic protease activating factor – 1 (*Apaf-1*) and ATP, which then bind to *pro-caspase-9* to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector *caspase-3*.

MAC also called "Mitochondrial Outer Membrane Permeabilization Pore" is regulated by various proteins, such as those encoded by the mammalian Bcl-2 family of anti-apoptopic genes, the homologs of the *ced-9* gene found in *C. elegans*.[28][29] *Bcl-2* proteins are able to promote or inhibit apoptosis by direct action on MAC/MOMPP. Bax and/or Bak form the pore, while Bcl-2, Bcl-xL or Mcl-1 inhibits its formation.

ATROPHY

Atrophy is a decrease in size of a body part, cell, organ, or tissue. The term implies that the atrophied part was of a size normal for the individual, considering age and circumstance, prior to the diminution.

Atrophy also can be defined as gradual loss of muscle or flesh usually because of disease or lack of use of any organ or body parts.

Types of Atrophy & Their Symptoms

Atrophy is a catch-all term used to describe a number of different conditions. For instance, two rare forms of atrophy are:

Glandular atrophy occurs when the glands of the body atrophy due to the extended use of steroids or other drugs, lack of proper nutrition, disease, or hormonal imbalances.

Vaginal atrophy occurs in post-menopausal women when their vaginal walls thin. It's believed to occur because reproduction is no longer biologically necessary.

However, there are three more common types of atrophy - skeletal muscle, spinal muscle, and multiple system.

Skeletal Muscle Atrophy

First, there is **skeletal muscular atrophy**. This type results in the wasting of muscle or a loss of muscle tissue and can be categorized into two types.

Disuse atrophy: This type is caused by a lack of physical activity ('use it or lose it') and can usually be reversed with exercise and proper nutrition.

Neurogenic atrophy: This type is caused by an injury or disease that affects the nerves connected to a muscle. It usually appears more suddenly and is more serious than disuse atrophy. It can be caused by Lou Gehrig's disease, Guillain-Barre syndrome, neuropathy, polio, immobilization, spinal cord injuries, and arthritis, among other things.

Both types of skeletal muscular atrophy cause muscle weakness and a decrease in strength as well as reduced mobility or movement. Some types may occur naturally as a part of aging.

Treating skeletal muscular atrophy can be done with:

Exercise to help rebuild the muscle

Physical therapy, especially in patients with limited mobility

Ultrasound therapy, which uses sound waves to treat the affected area

Surgery to correct **contracture deformity**, a condition in which the muscles, skin, or connective tissues are too tight, preventing normal movement of the muscle

Spinal Muscle Atrophy

Another type of atrophy is **spinal muscular atrophy**. This affects the muscles in the center of the body first and can hinder moving, walking, and breathing; however, it does not affect cognitive functioning. Spinal muscle atrophy can be broken down into different types.

HYPERTROPHY

Hypertrophy is the increase in the volume of an organ or tissue due to the enlargement of its component cells. OR

Hypertrophy is an increase in the size of an organ caused by an increase in the size of the cells rather than the number of cells. The cells of the heart and kidney are particularly prone to hypertrophy. **Hypertrophy** is also called overgrowth.

Muscle hypertrophy involves an increase in size of skeletal muscle through a growth in size of its component cells. Two factors contribute to hypertrophy: sarcoplasmic hypertrophy, which focuses more on increased muscle glycogen storage; and myofibrillar hypertrophy, which focuses more on increased myofibril size.

A range of stimuli can increase the volume of muscle cells. These changes occur as an adaptive response that serves to increase the ability to generate force or resist fatigue in anaerobic conditions.

Pathogenesis

- The hypertrophied organ has no new cells, just larger cells. The increased size of the cells is due not to cellular swelling but to the synthesis of more structural components.
- Cells capable of division may respond to stress by undergoing both hyperplasia and hypertrophy, whereas in nondividing cells (e.g., myocardial fibers), hypertrophy occurs.
- Nuclei in hypertrophied cells may have a higher DNA content than in normal cells, probably because the cells arrest in the cell cycle without undergoing mitosis.
- Hypertrophy can be physiologic or pathologic and is caused by increased functional demand or by specific hormonal stimulation.

Muscular hypertrophy

The striated muscle cells in both the heart and the skeletal muscles are capable of tremendous hypertrophy.

The most common stimulus for hypertrophy of muscle is increased workload. For example, the bulging muscles of bodybuilders engaged in "pumping iron" result from an increase in size of the individual muscle fibers in response to increased demand.

The workload is thus shared by a greater mass of cellular components, and each muscle fiber is spared excess work and so escapes injury.

The enlarged muscle cell achieves a new equilibrium, permitting it to function at a higher level of activity.

In the heart, the stimulus for hypertrophy is usually chronic hemodynamic overload, resulting from either hypertension or faulty valves. Synthesis of more proteins and filaments occurs, achieving a balance between the demand and the cell's functional capacity.

Endometrial mucosa in pregnancy

The massive physiologic growth of the uterus during pregnancy is a good example of hormone-induced increase in the size of an organ that results from both hypertrophy and hyperplasia.

The cellular hypertrophy is stimulated by estrogenic hormones acting on smooth muscle estrogen receptors, eventually resulting in increased synthesis of smooth muscle proteins and an increase in cell size.

Similarly, prolactin and estrogen cause hypertrophy of the breasts during lactation. These are examples of physiologic hypertrophy induced by hormonal stimulation.

NEOPLASIA

Neoplasia is new, uncontrolled growth of cells that is not under physiologic control. Neoplasm is an abnormal growth of tissue, and when also forming a mass is commonly

referred to as a **tumor** or **tumour**. This abnormal growth (neoplasia) not always forms a mass.

Types

A neoplasm can be benign, potentially malignant (pre-cancer), or malignant (cancer). Benign tumors are localized and do not transform into cancer.

Potentially-malignant neoplasms include carcinoma in situ. They are localised, do not invade and destroy but in time, may transform into a cancer.

Malignant neoplasms are commonly called cancer. They invade and destroy the surrounding tissue, may form metastases and, if untreated or unresponsive to treatment, will prove fatal.

Secondary neoplasm refers to any of a class of cancerous tumor that is either a metastatic offshoot of a primary tumor, or an apparently unrelated tumor that increases in frequency following certain cancer treatments such as chemotherapy or radiotherapy.

Rarely there can be a metastatic neoplasm with no known site of the primary cancer and this is classed as a cancer of unknown primary origin

Causes

A neoplasm can be caused by an abnormal proliferation of tissues, which can be caused by genetic mutations. Not all types of neoplasms cause a tumorous overgrowth of tissue.

Benign conditions that are *not* associated with an abnormal proliferation of tissue (such as sebaceous cysts) can also present as tumors, however, but have no malignant potential. Breast cysts (as occur commonly during pregnancy and at other times) are another example, as are other encapsulated glandular swellings (thyroid, adrenal gland, pancreas).

Encapsulated hematomas, encapsulated necrotic tissue (from an insect bite, foreign body, or other noxious mechanism), keloids (discrete overgrowths of scar tissue) and granulomas may also present as tumors.

Discrete localized enlargements of normal structures (ureters, blood vessels, intrahepatic or extrahepatic biliary ducts, pulmonary inclusions, or gastrointestinal duplications) due to outflow obstructions or narrowings, or abnormal connections, may also present as a tumor. It can be dangerous to biopsy a number of types of tumor in which the leakage of their contents would potentially be catastrophic. The nature of a tumor is determined by imaging, by surgical exploration, and/or by a pathologist after examination of the tissue from a biopsy or a surgical specimen. Cancers may be classified by their primary site of origin or by their histological or tissue types.

Classification by site of origin

By primary site of origin, cancers may be of specific types like breast cancer, lung cancer, prostate cancer, liver cancer renal cell carcinoma (kidney cancer), oral cancer, brain cancer etc.

Classification by tissue types

Based on tissue types cancers may be classified into six major categories:

1. Carcinoma

This type of cancer originates from the epithelial layer of cells that form the lining of external parts of the body or the internal linings of organs within the body.

Carcinomas usually affect organs or glands capable of secretion including breast, lungs, bladder, colon and prostate.

Carcinomas are of two types – adenocarcinoma and squamous cell carcinoma. Adenocarcinoma develops in an organ or gland and squamous cell carcinoma originates in squamous epithelium. Adenocarcinomas may affect mucus membranes and are first seen as a thickened plaque-like white mucosa. These are rapidly spreading cancers.

2. Sarcoma

These cancers originate in connective and supportive tissues including muscles, bones, cartilage and fat. Bone cancer is one of the sarcomas termed osteosarcoma

3. Myeloma

These originate in the plasma cells of bone marrow. Plasma cells are capable of producing various antibodies in response to infections. Myeloma is a type of blood cancer.

4. Leukemia

This group of cancer is blood cancers. These cancers affect the bone marrow which is the site for blood cell production. When cancerous, the bone marrow begins to produce excessive immature white blood cells that fail to perform their usual actions and the patient is often prone to infection.

Types of leukemia include:

Acute myelocytic leukemia (AML) – these are malignancy of the myeloid and granulocytic white blood cell series seen in childhood.

Chronic myelocytic leukemia (CML) – this is seen in adulthood.

Acute Lymphatic, lymphocytic, or lymphoblastic leukemia (ALL) – these are malignancy of the lymphoid and lymphocytic blood cell series seen in childhood and young adults.

Chronic Lymphatic, lymphocytic, or lymphoblastic leukemia (CLL) – this is seen in the elderly. Polycythemia vera or erythremia – this is cancer of various blood cell products with a predominance of red blood cells.

1. Lymphoma

These are cancers of the lymphatic system. Unlike the leukemias, which affect the blood and are called “liquid cancers”, lymphomas are “solid cancers”. These may affect lymph nodes at specific sites like stomach, brain, intestines etc. These lymphomas are referred to as extranodal lymphomas.

Lymphomas may be of two types – Hodgkin’s lymphoma and Non-Hodgkin’s lymphomas. In Hodgkin lymphoma there is characteristic presence of Reed-Sternberg cells in the tissue samples which are not present in Non-Hodgkin lymphoma.

2. Mixed types

These have two or more components of the cancer. Some of the examples include mixed mesodermal tumor, carcinosarcoma, adenosquamous carcinoma and teratocarcinoma. Blastomas are another type that involves embryonic tissues.

Differences between Benign and Malignant Tumors

- ***Rate of growth*** - In general, malignant tumors grow much more rapidly than benign tumors.
- ***Ability to metastasize*** - Benign tumors expand locally, whereas malignant tumors can spread (metastasize) to other parts of the body by way of the bloodstream and lymphatic channels.
- ***Site of*** *recurrence* - While benign tumors may recur locally — that is, near the site of the original tumor — malignant tumors may recur at distant sites, such as the brain, lungs, bones and liver, depending on the type of cancer.
- ***"Stickiness"*** - The cells in benign tumors manufacture chemicals (adhesion molecules) that cause them to stick together. Malignant tumor cells do not produce these molecules and can break off and "float away" to other regions of the body.
- ***Tissue invasion*** - In general, malignant tumors tend to invade nearby tissues, whereas benign tumors do not. In contrast, envision cancer as having "fingers" or "tentacles" that can reach into nearby tissues. In fact, the Latin word cancer derives from the word crab, used to describe the crablike, or fingerlike, projections of cancerous tumors.
- ***Cellular appearance*** - Under a microscope, cells that are benign often look much different from those that are malignant. One of these differences is that the cell nucleus of cancer cells is often larger and appears darker due to an abundance of DNA.
- ***Effective treatments*** - Benign tumors can usually be removed with surgery alone, while cancerous (malignant) tumors will often require chemotherapy and radiation therapy.

- ***Likelihood of recurrence*** - Benign tumors do not recur after surgery, whereas malignant tumors recur much more commonly. Surgery to remove a malignant tumor is more difficult than surgery for a benign tumor. If cells are left over from these, the tumor is more likely to come back.

- ***Systemic effects*** - Malignant tumors are more likely to have systemic or total body, effects than benign tumors. Due to the nature of these tumors, symptoms such as fatigue and weight loss are common. Several types of malignant tumors also secrete substances that cause effects on the body beyond those caused by the original tumor.

- ***Death toll*** - Benign tumors cause around 13,000 deaths per year in the United States. The number of deaths that can be blamed on malignant (cancerous) tumors is over 575,000.

General Etiology and Pathogenesis

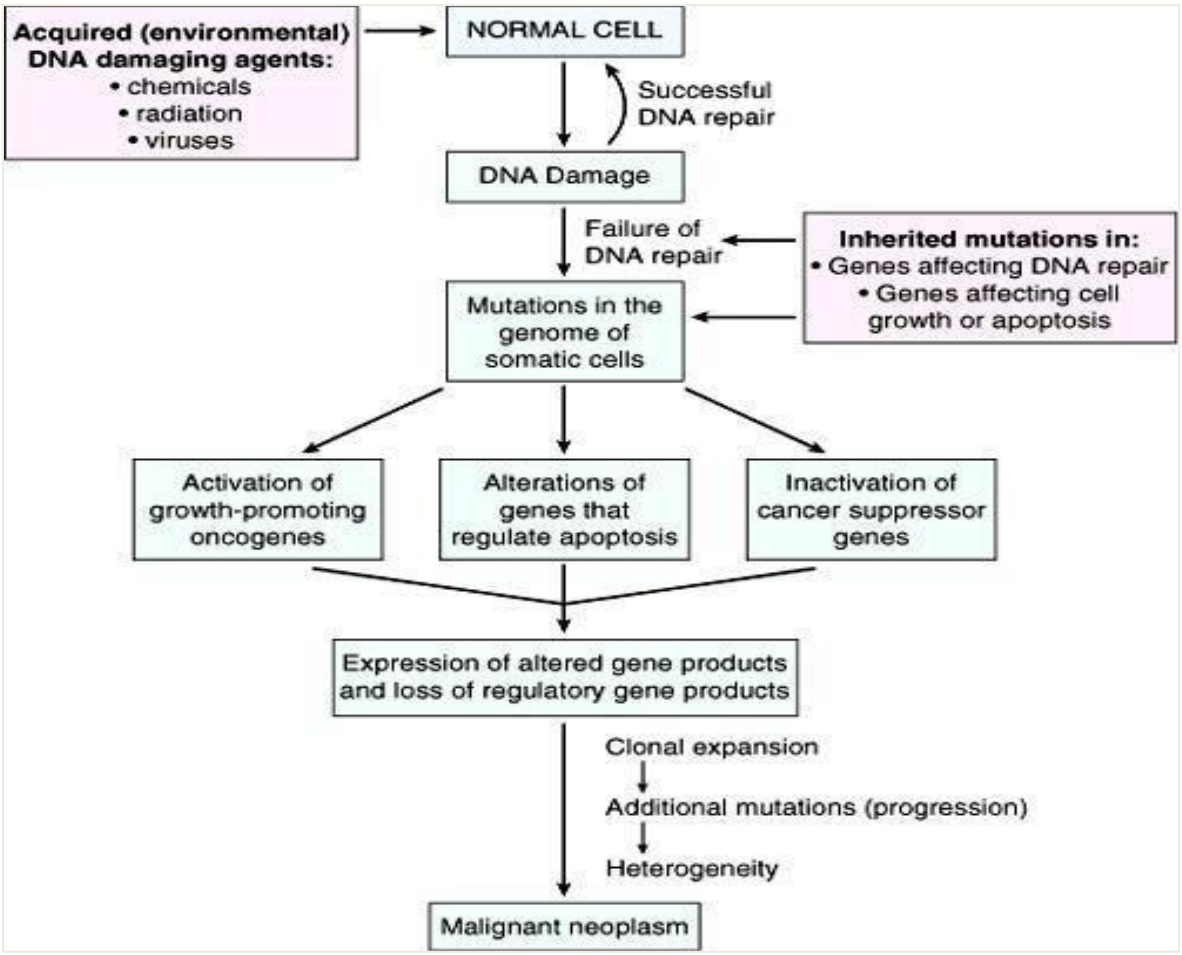

Fig.5

Environmental Carcinogens

- **A cancer-causing agent**
- **Three main types: Chemical**

Direct-acting

Nitrogen mustard, Nitrosomethylurea, Benzyl chloride

Indirect-acting (must be metabolized to activated metabolic forms)

- **Polycyclic aromatic hydrocarbons (PAH)**
- **Produced by incomplete combustion of organic materials**
- **Present in chimney soot, charcoal-grilled meats, auto exhaust, cigarette smoke Physical (radiation)**

Ultraviolet light, Ionizing radiation (X-rays), Asbestos

Biological (viruses and Bacteria)

- **Viral infections account for an estimated one in seven human cancers worldwide**
- **Majority of these are due to infection with two DNA viruses**

– **HBV - linked to hepatocellular carcinoma**

– **HPV - linked to cervical carcinoma**

- **Human papillomaviruses - HPV**
- **Epstein-Barr Virus (EBV)**
- **Human herpesvirus 8 (HHV8)**
- **Hepatitis B virus - HBV**
- **Hepatitis C virus - HCV**

Helicobacter pylori

- **Gastric infection linked to gastric lymphomas and adenocarcinomas**
- **Detection of H pylori in majority of cases of gastric lymphomas**
- **Antibiotic treatment results in gastric lymphoma regression in most cases Spread of tumors Metastasis**, or **metastatic disease**, is the spread of a cancer or other disease from one organ or part to another not directly connected with it. The new occurrences of disease thus generated are referred to as **metastases.**

Metastasis is a Greek word meaning "displacement", from, *meta*, "next", and *stasis*, "placement".

Routes of metastasis

Primary cancers are denoted by "*...cancer*" and their main metastasis sites are denoted by "*...metastases*".

Metastasis occurs by following four routes:

Transcoelomic

The spread of a malignancy into body cavities can occur via penetrating the surface of the peritoneal, pleural, pericardial, or subarachnoid spaces. For example, ovarian tumors can spread transperitoneally to the surface of the liver.

Lymphatic spread

Lymphatic spread allows the transport of tumor cells to lymph nodes and ultimately, to other parts of the body. This is the most common route of metastasis for carcinomas. Lymph node with almost complete replacement by metastatic melanoma.

Hematogenous spread

This is typical route of metastasis for sarcomas, but it is also the favored route for certain types of carcinoma, such as those originating in the kidney (renal cell carcinoma).

Transplantation or implantation

Cancer cells may spread to lymph nodes (regional lymph nodes) near the primary tumor. This is called nodal involvement, positive nodes, or regional disease. Localized spread to regional lymph nodes near the primary tumor is not normally counted as metastasis, although this is a sign of worse prognosis. Transport through lymphatics is the most common pathway for the initial dissemination of carcinomas.

Cellular and Molecular Basis of Cancer Cellular Kinetics

Generation time is the time required for a quiescent cell to complete a cycle in cell division and give rise to 2 daughter cells. Malignant cells, particularly those arising from the bone marrow or lymphatic system, may have a short generation time, and there usually are a smaller percentage of cells in G 0 (resting phase). Initial exponential tumor growth is followed by a plateau phase when cell death nearly equals the rate of formation of daughter cells. The slowing in growth rate may be related to exhaustion of the supply of nutrients and O 2 for the rapidly expanding tumor. Small tumors have a greater percentage of actively dividing cells than do large tumors.

A subpopulation within many tumors, identified by surface proteins, may have the properties of primitive "normal" stem cells, as found in the early embryo. Thus, these cells are capable of entering a proliferative state. They are less susceptible to injury by drugs or irradiation. They are believed to repopulate tumors after surgical, chemical, or radiation treatment.

Cellular kinetics of particular tumors is an important consideration in the design of antineoplastic drug regimens and may influence the dosing schedules and timing intervals of treatment. Many antineoplastic drugs, such as antimetabolites, are most effective if cells are actively dividing, and some drugs work only during a specific phase of the cell cycle and thus require prolonged administration to catch dividing cells during the phase of maximal sensitivity.

Tumor Growth and Metastasis

As a tumor grows, nutrients are provided by direct diffusion from the circulation. Local growth is facilitated by enzymes (eg, proteases) that destroy adjacent tissues. As tumor

volume increases, tumor angiogenesis factors, such as vascular endothelial growth factor (VEGF), are produced by tumors to promote formation of the vascular supply required for further tumor growth.

Almost from inception, a tumor may shed cells into the circulation. From animal models, it is estimated that a 1-cm tumor sheds > 1 million cells/24 h into the venous circulation. Circulating tumor cells are present in many patients with advanced cancer and even in some with localized disease. Although most circulating tumor cells die in the intravascular space, an occasional cell may adhere to the vascular endothelium and penetrate into surrounding tissues, generating independent tumors (metastases) at distant sites. Metastatic tumors grow in much the same manner as primary tumors and may subsequently give rise to other metastases.

Experiments suggest that the ability to invade, migrate, and successfully implant and stimulate new blood vessel growth are all important properties of metastatic cells, which likely represent a subset of cells in the primary tumor.

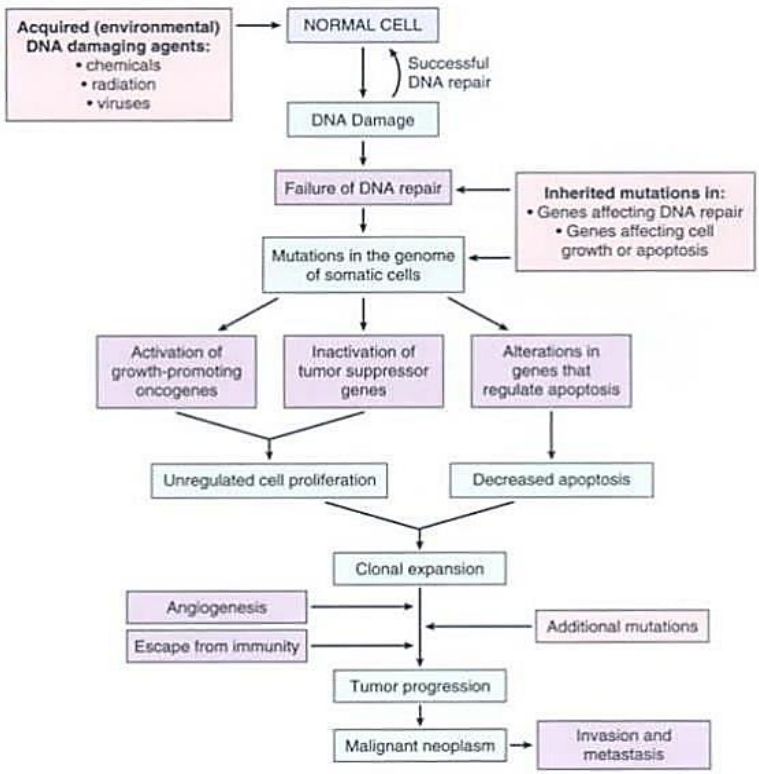

Fig.6 Flow chart depicting a simplified scheme of the molecular basis of cancer

Molecular Abnormalities

Genetic mutations are responsible for the generation of cancer cells and are thus present in all cancers. These mutations alter the quantity or function of protein products that regulate cell growth and division and DNA repair. Two major categories of mutated genes are oncogenes and tumor suppressor genes.

Fig.7

Figure 7-29 Schematic illustration of the roleofcyciins, CDKs, andcyoiin-dependent Hinase inhibitors inregulating the G‹/S cell-cycle transition. External signals activate multiple signal transduction pathways, including those involving the M Y'C and *RAS* gen*es.* which lead to Synthesis and stabilization of cydin D (there are several 0 cyclins, bul, for simplification. we refer tothemas "cyclin D" . Cyclin D binds to CDK4. forming acomplex with enzymatic activity{cyelin D can also bind to CDK6. which appears to have a similar role as COK4 . The eyclin D-CDK4 complex phosphoryiates RB. located in the OF/DP1/RB complex in the nucleus. activating the transcription at activity or mF (E2Fisa family of transcription faciors, which we refer to as "E2F"), which leads to transcription of Cyclin E, cyclin A and other proieins needed for the ceil to go ihrough the late G restriction point. The cell cycle can be blocked by the Cip/Kip inhibitors p21 and p2 7 *(red boxes)* and the IhK4A/ARF inhibitors p18INK4A and p14ARF /green *boxes).* Cell-cycle arrest in res ponse to DNA damage and other oelluiar stresses is mediated through ps3. The levels of ps3 are under negative regulation by MDM2, through a feedback loop that is inhibited by p14ARF.

These are abnormal forms of normal genes (proto-oncogenes) that regulate various aspects of cell growth. Mutation of these genes may result in direct and continuous stimulation of the pathways (eg, cell surface growth factor receptors, intracellular signal transduction pathways, transcription factors, secreted growth factors) that control cellular growth and division, DNA repair, angiogenesis, and other physiologic processes.

There are > 100 known oncogenes that may contribute to human neoplastic transformation. For example, the*RAS* gene encodes the ras protein, which carries signals from membrane bound receptors down the RAS-MAPKinase pathway to the cell nucleus, and thereby regulates cell division. Mutations may result in the inappropriate activation of the ras protein, leading to uncontrolled cell growth. In fact, the ras protein is abnormal in about 25% of human cancers. Other oncogenes have been implicated in specific cancers. These include

- *HER2-NEU* (amplified but not mutated in breast cancer)
- *BCR-ABL* (a translocation of 2 genes that underlies chronic myelocytic leukemia and some B-cell acute lymphocytic leukemias)
- *C-MYC* (Burkitt lymphoma)
- *N-MYC* (small cell lung cancer, neuroblastoma)
- Mutated *EGFR* (adenocarcinoma of the lung)
- *EML4-ALK* (a translocation that activates the ALK tyrosine kinase and causes a unique form of adenocarcinoma of the lung)

Specific oncogenes may have important implications for diagnosis, therapy, and prognosis (see individual discussions under the specific cancer type).

Oncogenes typically result from acquired somatic cell mutations secondary to point mutations (eg, from chemical carcinogens), gene amplification (eg, an increase in the number of copies of a normal gene), or translocations (in which pieces of different genes merge to form a unique sequence). These changes may either increase the activity of the gene product (protein) or change its function. Occasionally, mutation of genes results in inheritance of a cancer predisposition, as in the inherited cancer syndrome associated with mutation and loss of function of *BRCA1* , *BRCA2* , or *p53* .

Genes such as the *p53* gene play a role in normal cell division and DNA repair and are critical for detecting inappropriate growth signals or DNA damage in cells. If these genes, as a result of inherited or acquired mutations, become unable to function, the system for

monitoring DNA integration becomes inefficient, cells with spontaneous genetic mutations persist and proliferate, and tumors result.

As with most genes, 2 alleles are present that encode for each tumor suppressor gene. A defective copy of one gene may be inherited, leaving only one functional allele for the individual tumor suppressor gene. If a mutation is acquired in the other allele, the normal protective mechanism of the 2nd normal tumor suppressor gene is lost. For example, the retinoblastoma (*RB*) gene encodes for the protein Rb, which regulates the cell cycle by stopping DNA replication. Mutations in the *RB* gene family occur in many human cancers, allowing affected cells to divide continuously.

Another important regulatory protein, p53, prevents replication of damaged DNA in normal cells and promotes cell death (apoptosis) in cells with abnormal DNA. Inactive or altered p53 allows cells with abnormal DNA to survive and divide. Mutations are passed to daughter cells, conferring a high probability of replicating error-prone DNA, and neoplastic transformation results. The *p53* gene is defective in many human cancers. As with oncogenes, mutation of tumor suppressor genes such as *p53* or *RB* in germ cell lines may result in vertical transmission and a higher incidence of cancer in offspring.

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PART A

S.No.	Questions	outcomes
1	Define Inflammation	**CO3**
2	What is Apoptosis	**CO3**
3	Emphasis on caspases.	**CO3**
4	List out spread of tumors.	**CO3**
5	What is Neoplasia	**CO3**
6	Differentiate benign from malignant tumor	**CO3**
7	What is Phagocytosis	**CO3**
8	Define Oncogenic virus	**CO3**
9	What is reversible and irreversible cell injury	**CO3**
10	Define Necrosis	**CO3**

PART B

S.No.	Questions	outcomes
1	Describe in detail about the cellular events taking place during inflammation	**CO3**
2	Explain with a neat diagram about phagocytosis	**CO3**
3	Explain in detail about the mitocondrial pathway in apoptosis with neat diagram.	**CO3**
4	Define Neoplasia. classify tumors and discuss about the malignant tumors and benign tumors	**CO3**
5	Explain about the various spread of tumors and add a note on etiology of tumors with its clinical symptoms and their pathology	**CO3**
6	Illustrate in detail about the mechanism of neoplasia.	**CO3**
7	Discuss with neat diagram about the role of role of death receptors in apoptosis.	**CO3**
8	Explain with neat diagram about mechanism of chronic inflammation	**CO3**
9	Differentiate between benign and malignant tumors	**CO3**
10	Discuss about the mechanism of reversible and irreversible cell injury and emphasize on necrosis	**CO3**

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DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT –4 – Fluid and Haemodynamic Dearrangement

UNIT IV

FLUID AND HAEMODYNAMIC DEARRANGEMENT

WHAT IS EDEMA?

The accumulation of fluid in the interstitial spaces located beneath the skin and in cavities of the body. Fluid accumulated body cavities are designated as hydropericardium, hydrothoracic, hydroperitoneum.

It is just a normal response of body to inflammation / injury. For example, a twisted ankle, a bee sting, a skin infection will result in edema in the involved area.

MECHANISM

In the case of congestive heart failure which is associated with reduced cardiac output and renal perfusion, lowers blood pressure(blood volume).

Now, JG cells in the kidney release Renin hormone, which has angiotensinogen converted into Angiotensin-II.

Angiotensin-II stops sweat secretion in the skin, constricts blood vessels and stops urine excretion in the kidney that results in edema.

Increased hydrostatic pressure

Localized increase in hydrostatic pressure results in impact venous outflow that results in edema of lower extremities.

Generalized venous pressure results in systemic edema, that is seen in congestive heart failure.

Reduced plasma osmotic pressure

Results from excessive loss or reduced synthesis of serum protein (albumin) and its loss results in nephrotic syndrome.

Lymphatic Obstruction

Due to impaired lymphatic drainage(waste flow), lymph edema is usually localized. For example – parasitic infection filariasis cause massive lymphatic and lymph node fibrosis of lymph nodes.

Sodium water retention

Sodium water retention may occur with acute glomerular nephritis and renal failure which results in edema.

Renal Edema : Occurs in

- nephrotic syndrome,
- glomerular nephritis,
- renal failure.

Cerebral Edema :

When fluid builds up around the brain, it leads to cerebral edema. It can be resulted from brain trauma or from nontraumatic causes like ischemic stroke, cancer, meningitis etc.,

Pulmonary Edema :

Accumulation of fluid in tissue spaces and also in pulmonary alveoli.

Cardiac Edema :

Results in congestive heart failure.

SHOCK

It is a final common pathway for a number of potentially lethal clinical events including hemorrhage, extensive trauma burns, large myocardial infraction embolism and microbial sepsis (infection).

TYPES OF SHOCK

Cardiogenic shock - result from myocardial pump failure, which is due to intrinsic myocardial damage.

Hypovolemic shock – results in loss of blood or plasma volume, which is caused by hemorrhage, severe burn and trauma

Septic shock – caused by systemic microbial infections. For example endotoxic shock (gram negative bacteria)

Low quartiles

Monocyte, macrophages, neutrophils activation which activates endothelial cells.

Further C activation, C3 α and C5 α activation occurs which leads to local inflammation.

Moderate quartiles

Cytokine, IC 1 activation occurs that induces fever, which increases synthesis of acute phase reactants that is leucocytes that synthesize effects.

High quartiles

Due to vasodilation, that leads to diminished myocardial contractility, blood vessel injury which further leads to thrombosis in that particular area. Pulmonary alveoli damage leading to ARDS(acute respiratory deficiency syndrome) that finally results in septia shock.

- Non Progressive phase
- Progressive phase
- Irreversible phase

CLINICAL SYMPTOMS

- Hypotension
- Weak
- Hemorrhage
- Rapid pulse
- Clammy (unpleasant air)
- Cyanotic skin (bluish skin)
- Vasodialation

HEMORRHAGE

Hemorrhage is an extravasation of blood due to vessel rupture, however rupture of a large artery or vein is almost due to vascular injury, trauma, neoplasmic erosion of vessel wall. It may be external or may be enclosed. Accumulation of blood with a tissue is known as Hematoma.

TYPES OF HEMORRHAGE

Based upon size: ***1-2mm hemorrhage*** – Seen on skin, mucous membrane and denoted as petechiae, which are physically attached with intravascular pressure, thrombocytopenia followed by lysis.

Slightly large (>3mm) – They are called as purpura. Symptoms are vasculitis, amyloidosis (protein deposition).

Large(1-2cm) – It is commonly known as subcutaneous hematoma. This is seen in trauma condition.

SYMPTOMS

- Erythrocytes in local hemorrhage
- Phagocytosis by macrophages
- Hemoglobin enzymaticaly converted in to
- Bilirubin
- Hemosidirin

TYPES OF HEMATOMA

Large accumulation of blood in the body cavities are designated as hemothorax, hemopericardium, hemoperitoneum.

CLINICAL SIGNIFICANCE

- Rapid loss of blood volume upto 20% - Hemorrhage condition
- Iron deficiency anemia / external blood loss – Peptic ulcer
- When RBC are retained in body cavity or tissue during hemorrhage, the hemoglobin synthesis is reduced.
- Bleeding in subcutaneous tissue may result in death if it is located in the brain.

DISSEMINATED INTRAVASCULAR COAGULATION(DIC)

- Acute, subacute or chronic thrombo hemorrhagic disorder.

- Characterized by activation of coagulation sequences which leads to formation of thrombi.
- Clotting can be started either by 2 ways
- Intrinsic pathway – involves the activation of factor-XII by surface contact.
- Extrinsic pathway
- Two major mechanism which trigger DCI
- Release of tissue factor
- Injury of endothelial cells
- Tissue factor
- Gram negative sepsis leads to release of endotoxins
- That activates release of IL, TNF
- Expression of tissue factor on endothelial cells decrease the expression of thrombosis
- Coagulation
- Injury to endothelial cells
- Initiates DIC by release of tissue factor
- That promotes platelet aggregation.

Massive tissue destruction sepsis endothelial injury

- Release of tissue factor
- Thrombosis Platelet aggregation
- Activation of plasma vascular occlusion consumption of clotting factor and platelets
- Fibrolysis proteolysis of clotting factor
- Fibrin split product
- Ischemic tissue damage
- Inhibition of thrombin platelets aggregation and fibrin BLEEDING polymerization

- Respiratory failure
- Hemolytic anemia
- Dyspnea
- Cyanosis
- Acute renal failure
- Sudden circulatory failure
- Shock
- coma

HEMATOLOGICAL DISORDER

Function of RBC – transport oxygen to peripheral tissue.

Reduced O2 carrying capacity of blood usually results from deficiency of RBC and anemia.

TYPES OF HEMATOLOGICAL DISORDERS

1. Anemia of blood loss

Acute blood loss – loss of intravascular volume

- Symptoms are malnutrition , bleeding Chronic blood loss – Reserves are depleted
- Symptoms are shock, cardiovascular collapse and may be death.

2. Hemolytic anemia

- Shorten lifespan of RBC, that is premature destruction of RBC.
- It is of two types acquired disorder and hereditary disorder (intrinsic and extrinsic).
- Clinical symptoms are hemoglobinemia, jaundice, hemoglobinuria.

3. Hereditary sperocystosis

- This genetic disorder is caused by intrinsic defect in RBC membrane that render RBC to become steroid and destructive.

- Symptoms are hyperchromic anemia, erythropoiesis, mild jaundice, hemosiderosis, spleenomegaly.

4. Glucose 6 phosphate dehydrogenase deficiency

- RBCs are vulnerable to injury by exogenesis and endogenesis of oxygen.

- Deficiency of exo monophosphate resulting from impact enzyme function.

- That is the viability of red cells to protect themselves against oxidative injury leading to hemolytic disease.

- Symptoms are hemolytic disease, hemoglobinemia, hemoglobinuria.

5. Sickle cell anemia

- Glutamic acid (HbA) in the normal RBC is converted into valine (HbS) due to point mutation resulting in sickle cell anemia.

- Symptoms are severe anemia, chronic hyperbilirubinemia.

6. Vitamin B12 deficiency

- Vitamin B12 is necessary for DNA synthesis. Autoantibodies are produced against receptor or intrinsic factor on the gastric cell.

- Intrinsic factor does not able to absorb nutrients, which leads to anemic conditions due to decreased level of vitamin B12.

7. Haemostatic disorder

- Antibodies are produced against Rh antigen present on the surface of RBC.

- It leads to antigen antibody complex resulting in lysis of RBC followed by anemia condition.

8. Iron deficiency anemia

Total body iron content, normally it is 2gms in women whereas 6gms in men. Free iron particles are very dangerous, they can tightly bond to ferretin protein found in all tissues but particularly in liver ,spleen and bone marrow.

Example- In liver, ferretin is stored in parenchymal cells.

MECHANISM

- Plasma iron is bound to ferretin, which is transported to marrow precise.
- Where it is transferred to developing red cell and incorporated into hemoglobin.
- Mature RBCs are released into circulation.
- After 120 days, RBCs are ingested by macrophages in the endothelial system.
- Iron is extracted from RBC and returned to plasma.
- Completes its life cycle.

SYMPTOMS

Dietary lack, chronic blood loss, Impaired absorption increased requirement.

BLEEDING DISORDERS

Excessive bleeding can result from

- Increase fragility of vessels
- Platelets deficiency
- De-arrangement of coagulation

Bleeding disorder caused due to vessel wall abnormalities

- Petechiae and purpose hemorrhage
- Drug induced reactions
- Amyloid infiltration of blood vessels (deposition of proteins)

Bleeding related to reduced platelet count

- Thrombocytopenia
- Decreased production of platelet due to leukemia and B12 deficiency.
- Drug induced thrombocytopenia
- HIV induced thrombocytopenia

Bleeding disorder due to related Defective Platelet function

- Defect of adhesion
- Defect of aggregation
- Defect in platelet segregation.

TWO TYPES OF BLEEDING DISORDER

Acquired disorder Hereditary disorder Due to vitamin K deficiency Deficiency of factor XIII and factor IX

Result in impaired synthesis of factor II , VII, IX, XI

Haemophilia A (Factor VIII deficiency)

- Commonly seen in hereditary disorder. This protein serves as a co factor to factor VIII and X in coagulation cascade. X-link trait which is seen in male.

Haemophilia B

- Christmas disease or factor IX deficiency
- This disorder is clinically distinguishable from factor VIII deficiency. It also inherited X-linked recessive trait.

PATHOLOGY OF HAEMOPHILIA

Factor VIII deficiency or haemophilia A

- Sepsis occurs releasing tissue factor which leads to thrombosis.
- Activation of plasma leads to fibrolysis and protolysis of clotting factor.
- Fibrolysis leads to inhibition of thrombin, platelet aggregation and fibrin polymerization that results into bleeding
- Similarly protolysis of clotting factor leads to bleeding.

Deficiency of factor IX or haemophilia B

- Coagulation cascade pathway Factor XII XIIa
- Factor IX leads to inhibition of cascade activation
- Further cascade activation is blocked due to hereditary disease Results in bleeding disorder

TYPES OF LYMPHOMA

- Hodgkin's lymphoma
- Nodular sclerosis
- Mixed cellularity
- Lymphatic depletion
- Cervical and axillary lymphadepathy

Stage 1 - Lymphoma is a cancer occurs in the single lymph nodes.

It is of considerable size .

Stage 2 - two or more lymph nodes

Stage 3 - Disseminated involvement from lymph node to liver, lungs and bone marrow.

Hodgkin's lymphoma

- B-cell chronic lymphocytic lymphoma
- Follicular lymphoma
- Peripheral T-cell lymphoma
- Non contagious spread

Follicular lymphoma

- It is painless, generalized lymph adenopathy

Lymphoplasmacytic lymphoma

- B-cell neoplasm of adults (cancer in B-cell)
- Weakness
- Fatigue
- Hepatomoly spleenomegaly
- Lymphadenopathy
- Neurological symptoms
- Bleeding, visual impairment

Mantle cell lymphoma

- Tumor cell closely resembles the normal mantle zone, because that surround germinal cortex(centre)
- Symptoms are painless lymphadenopathy, spleenomegaly.

Marginal zone lymphoma

- B-cell tumor that arises in the lymph nodes and spleen
- It is an interface between the red and white pulp of the spleen.

Peripheral zone lymphoma

- Lymph node consists of pleomorphic, picture of small and large size maligent T-cell

Anaplastic cell lymphoma

- Rearrangement of ALK gene in chromosome.
- The arrangement breaks the ALK locus and lead to lymphoid neoplasm

Adult T-cell lymphoma

- Neoplasm of CD4 cell are observed and affected by human T-cell leukemia
- Symptoms are lymphadenopathy, lymphocytosis, Hepatospleenomegaly.

Burkitt lymphoma

- Recognized as fast growing human tumor and it is the cancer starting in immune cells and B-cells

LEUKEMIA

- Leukemia are neoplasmic polymerization of WBC precursor.
- Diffused replacement of normal bone marrow by leukemic cells.
- Infiltration of leukemic cell is vital organ to Liver, Spleen, Kidney, bone marrow.

Causes:

- Irradiation
- Drugs

- Chemicals(Benzene exposure)
- Viruses
- Genetic factor

Types of Leukemia:

1. Acute leukemia

- Mutation in Haemopoietic.
- Clones of cells proliferate, but loses its ability results in bone marrow failed.

Acute leukemia

It is of two types

i) Acute lymphoblatic leukemia

ii) Acute myleoblastic leukemia

Acute lymphoblastic leukemia

- Seen in 2-4 years old
- Due to genetic mutation
- Derived from B-cell precussor

eg- Burkiff's leukemia

Acute myleoblastic leukemia

- Seen in older age
- Due to genetic mutation
- Bone marrow disorder

Symptoms

- Lymphadenagathy
- Heapatomegaly
- Spleenomegaly
- Thromoocytopenia
- Anaemia
- Septocemia

Treatment

- Frequent bone marrow transplant.

Chronic leukemia

Seen in all age group . The normal bone marrow is replaced by abnormal clone dervied grom pleuropotential stem cells . Leucocytes are abnormal presence of myloblast in bone marrow aspirate.

Symptoms

- Anaemia
- Spleenomegaly
- Hepatomegaly

Treatment

- Stem cell transplant
- Interferons

Chronic lymphocytic leukemia

- It is the chronic lympho proliferative disorder with blood and bone marrow.
- It resists apoptosis.
- This is due to hypermutation in immunoglobulin gene.
- Lympocytes accumulated in RBC's bone marrow, Liver and spleen.

Symptoms

- Anemia, Haepatomegaly, Loss of weight, Thrombocytopenia, Night in sweat(excess).
- Fluderatine

Haircell Leukemia

- Seen in middle age and elderly people characteristics cell in the blood have cytoplasmic hair like structure

Symptoms

- Spleenomegaly

Treatment

- Interfons

Thrombosis

- It is a process of formation of solid mass in circulation from the constieunts of flowing blood the mass itself called thrombosis

Pathophysiology of thrombosis

Role of Blood vessel wall: The integrity of blood vessel wall is important to maintaining normal blood flow. It release few anti thrombotic factor.

eg: Heparin like substance which inactivate the clotting factor by acclerating the factor of antithrombin-III

Role of Platelets: In enothical platelets play of a central role in thrombosis. platelets addition to the injury is de and release cells and aggregate in platelets and result in heamostatic plug.

Role of coagulation system: It is conversion of plasma fibrnogen into solid mass of fibrin

Types of Thrombosis: Thrombosis occur in artery veins and capillary cardiac thrombi. Thrombi can form in any of the chamber of heart/ valve cap which results in infectious entocardiatsis.

Arterial venous Thrombi

- Arthrosclerosis – thickening of arteries
- Renal amyloidosis – deposition of amyloid

Capillary Thrombi

Vasculitis

Fate of Thrombosis

Thrombosis activate fibrolytic system with release of plasma in which may dissolve the thrombosis.

Resolution

- Organisation
- Thromboembolism

If the thrombosis is not removed, Heart get organized plasolytic and appear and begins to phagocyte fibrin and cell debris.

In early stage infected thrombi may dissolve and release stream of emboli which produce ill effect at the site of

EMBOLISM

Embolism are complete obstruction of the cardio vascular system by any mass carried in the circulation transported intravascular mass detect from the site of origin is called embolism.

Various type of emboli

Depending upon the matter in the emboli they can be

- Solid(tumor detach from sight of origin)
- Liquid(amniate fluid)
- Gaseous(acid and other gases)
- Depending upon whether infected/not infected
- Bland-sterile
- Septic-infected

Pathology of embolism

1. Pulmonary embolism

Due to the fatal form of venous thrommbolism- bed patient and

hospitilized patient.

- Pulmonary infraction
- Pulmonary hypertension
- Pulmonary haemorrhage

- Acute or pulmonal
- Sudden death

Systemic embolism

This is due to arterial embolism, it is usually seen in the left ventrical of the heart

Symptoms

- Congential heart disease
- Infectious endocarditis
- Cardiomypathy
- Myocardial infraction

Fat embolism

Obstruction arterous and capillary by globlus consists fat embolism. If the obstruction in the circulation is by fragments of adipose tissue called fat tissue embolism. Fat embolism usually occurs in skeletal injury.

- Anemia
- Thrombocytopenia
- Fatty liver
- Sickle cell anemia

Air embolism

It will usually occur in venous or arterial circulation.

Venous air embolism: Air may be systematic veins during operation on head, neck or trauma.

Jugular vein thromboemboli: These are seen in operation at trauma which occur in time and absorption.

Angiography: Arterial air embolism, cardiao thorasis surgery and trauma.

Angiotoxic fluid embolism .

- Cardio vascular shock
- Unexpected death
- Coma
- Sudden respiratory distress

TEXT / REFERENCE BOOKS

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2. Joanne M. Willey, Linda Sherwood, Christopher J. Woolverton, Prescott's Microbiology, 8th Edition, McGraw-Hill Higher Education, 2008

3. Jawetz, Melnick and Adelberg's Medical Microbiology . McGraw-Hill Medical, 2007

4. University of South Carolina School of Medicine (http://pathmicro.med.sc.edu/book/bact-sta.htm)

PART A

S.No.	Questions	outcomes
1	Define Edema	**CO4**
2	What is shock?	**CO4**
3	Define hemorrhage	**CO4**
4	Define thrombus	**CO4**
5	Define embolism	**CO4**
6	Write a short note on DIC	**CO4**
7	Define Leukemia	**CO4**
8	Define Lymphoma	**CO4**
9	What is iron deficiency anemia?	**CO4**
10	What is Christmas disease.	**CO4**

PART B

S.No.	Questions	outcomes
1	Explain in detail about edema and their pathological role.	**CO4**
2	What is shock? List out the various types of shock and write a short note on their pathology	**CO4**
3	What is Hemorrhage? Discuss types of hemorrhage and add a note on its clinical significance	**CO4**
4	Write a detailed account on Embolism	**CO4**
5	Write any four hematological disorders with a neat diagram.	**CO4**
6	Write in detail about bleeding disorders citing suitable examples.	**CO4**
7	Explain in detail about Lymphoma and its types.	**CO4**
8	Write a detailed account on DIC	**CO4**
9	Explain in detail about the pathological role of Thrombus	**CO4**
10	Discuss in detail about Leukemia and its types	**CO4**

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SCHOOL OF BIO & CHEMICAL ENGINEERING

DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT – 5 – Introduction to Microbiology

Immuno deficiency disease or condition were defence mechanism of body or impact loading to repeated microbial infection of varying severity or sometimes through maligency.

Deficiency of defence mechanism may invole specific function.Classification of immuno deficiency disease:

- **PRIMARY DEFICIENCY:**

- **Results from abnormality in the development of immune system.**

1. X LINKED AGAMMA GLOBULINEMA.

- It is usally seen in male infant.this disease is always associated with pyrogenic bacteria.

- Eg:pnemmoccal infection

- All the immunoglobulin are defected.plasma and germinal cortex are absent.results in decrease in B-cells.CMI is not affected.

- Arthritis and hemolytic anemia are frequently occurred.

2. CELL MEDIATED IMMUNO DEFICIENCY:

- **SCID-severe combined immuno deficiency disease.**

3. DISORDER OF COMPLEMENT SYSTEM

- It is usally seen in autosomal recessive triat.

- Eg:systemic lupus erythematosus.

- Pyrogenic infection is associated with deficiency of complement C3 ,C5 ,C6 ,C7 ,C8

DISORDER OF PHAGOCYTOSIS

There are two types of disorder :

- Extrinsic and Intrinsic
- **Extrinsic**:it is due to the deficiency of complement protein.
- Intrinsic :it is due to deficiency of enzyme l ,glucose 6 phosphate

SECONDARY DEFICIENCY

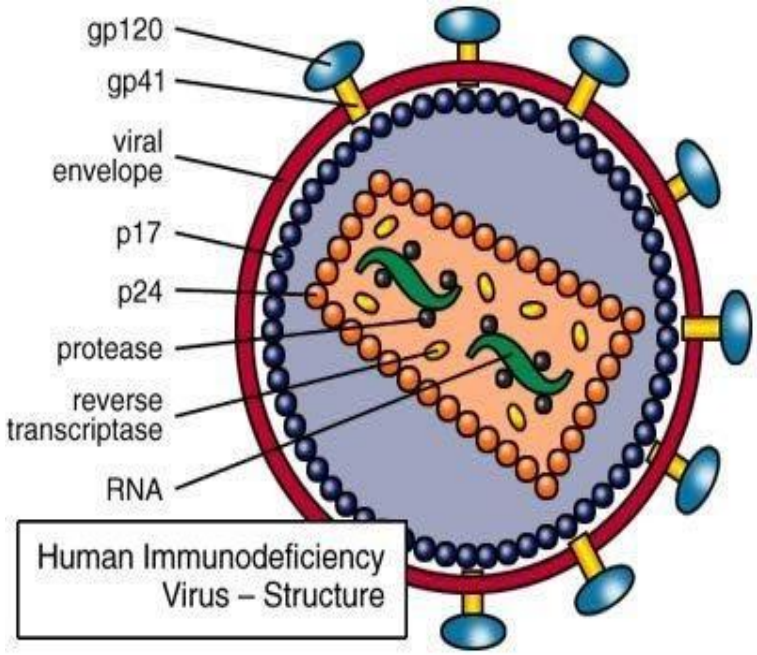

Fig.1

- **Leads to consequence of primary disease.**
- **AIDS: it is caused by human immuno deficiency virus.**
- **MORPHOLOGY :two identical single stranded RNA virus.**
- **It contains reverse transcriptase enzyme.**
- **Icosahedral shape is consists of two spikes on the envelope namely GP120,GP41.**

- **GENETIC PROPERTIES OF VIRUS: It consists of both structural amd non structural genes .**

- **STRUCTURAL GENE: GAG -it hels in synthesis of core and shell of virus .**

- POP:it helps in synthesis of enzyme reverse transcriptase
- END:synthesis of envelope protein GP120,GP40.
- **NONSTRUCTURAL GENE:**
- CAT:It is responsible for viral gene expression.
- REV:it is reponsible for expression of structural protein.
- NPF:Responsible for viral replication.
- VIF:Viral infectivity factor : it is reponsible for infection.
- Mode of transmission: Blood transfusion,drug addict,sexually transmitted.

CLINICAL SYMPTOMS :

It falls into 4 groups:

GROUP1: Fever ,lymphadenopathy . GROUP2: Asymptomatic

GROUP3: Multiple lymphadenopathy.

GROUP 4:Lymph node become atrophy ,night swelling,diarrea,loss of weight,lymphocytosis, opportunistic infection.

- **OTHER DISEASES:**

- Tuberculosis,

- aspergillosis(fungal infection)

- Candiasis (fungal infec)

- Karposis sarcoma(cancer)

SEROLOGICAL TEST:

ELISA

CONFIRMATORY TEST :

Western bloting technique

TREATMENT:

- Zidovudine (antiviral agent) is used to improve life span of HIV patient.

MOLECULAR BASIS OF DIABETICS :

- Regulation of insulin synthesis and secretion:
- The insulin gene is expressed in the B-cells of the pancreatic islets.
- The most important stimulus that triggers insulin synthesis and releases glucose.

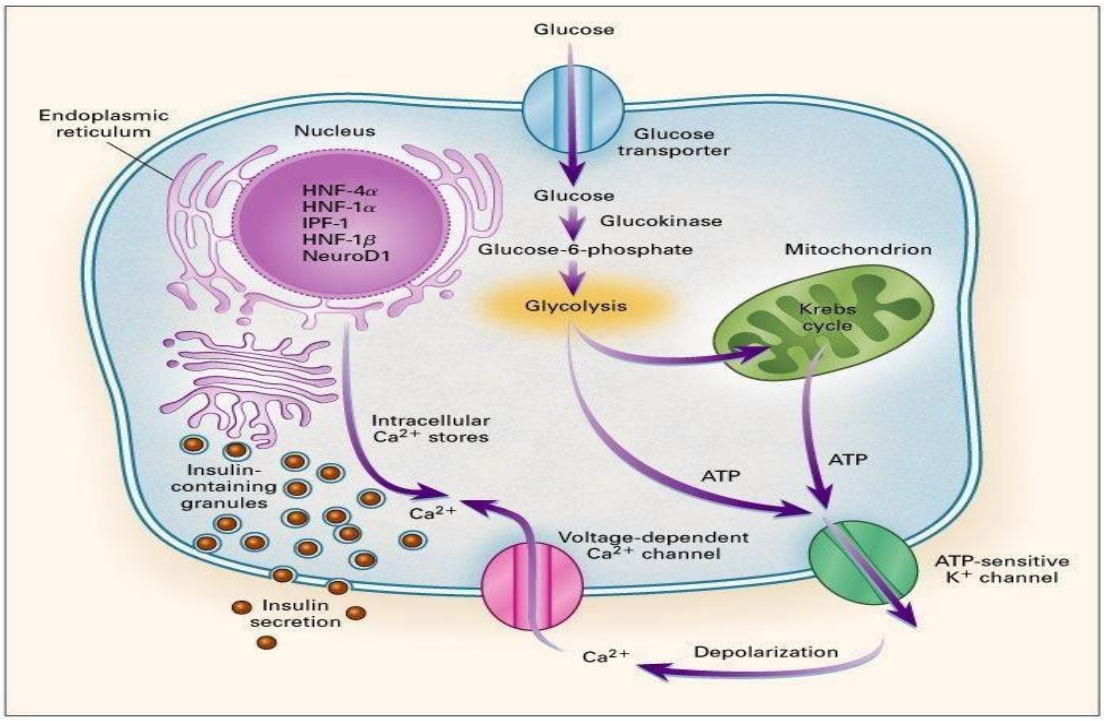

Fig.2

PATHOGENESIS OF TYPE 1 DIABETES:

- Type 1 diabetes is an auto immune disease in which islets destruction is caused by T-lymphocyte reacting against B-cell antigen.
- Genetic susceptibility and environmental factor play a role in pathogenesis.

MECHANISM OF B-CELLS DESTRICTION:

T-lymphocyte react against B-cell antigen and cause cell damage

By release of CD4+ T-CELLS which cause tissue injury.

CD8+ Cytotoxic T –lymphocyte which directly kills Bcells

It also secrete cytokine and activate macrophages.

Islets show cell necrosis and lymphocytic infiltration.

Followed by insulitis.

GENETIC SUSCEPTIBILITY:

MHC locus type 1 diabetics resice in the region that encodes the class-II molecules on chromosome p21

PATHOGENISIS DIABETICS (TYPE II)

- The environmental factors, diatory habit ,obesity and genetic factors are important in diabetics Type -I
- The 2 metabolic defect that characterised .
- Type 2 diabetics are
- Decreased ability of peripheral tissue to respond to insulin.
- Beta-cell disfunction.

- Beta- cell hyperplasia --- normal glycemia
- Beta-cell failure (early) ---- impaired glucose tolerance
- Beta-cell failure (later)---- diabetes type 2

INSULIN RESISTANCE :leads to decrease uptake of glucose in muscles and adipose tissue and inability of an hormone to supress gluconeogenisis.

COMPENSATED B-CELL HYPERPLASIA : It can maintain normal glycemia.

BETA CELL FAILURE(EARLY)-leading to impact glucose tolerance and eventually frank diabetics.

BETA CELL FAILURE (later)-it can directly lead to type II Diabetes.

RESPIRATORY DISORDER

Cystic fibrosis:

- Cystic fibrosis is fundamentally a wide spread disorder in epithelial transport affecting fluid secretion in exocrine gland and epithelial lining of respiratory,gastro intestinal and reproductive gental tract.

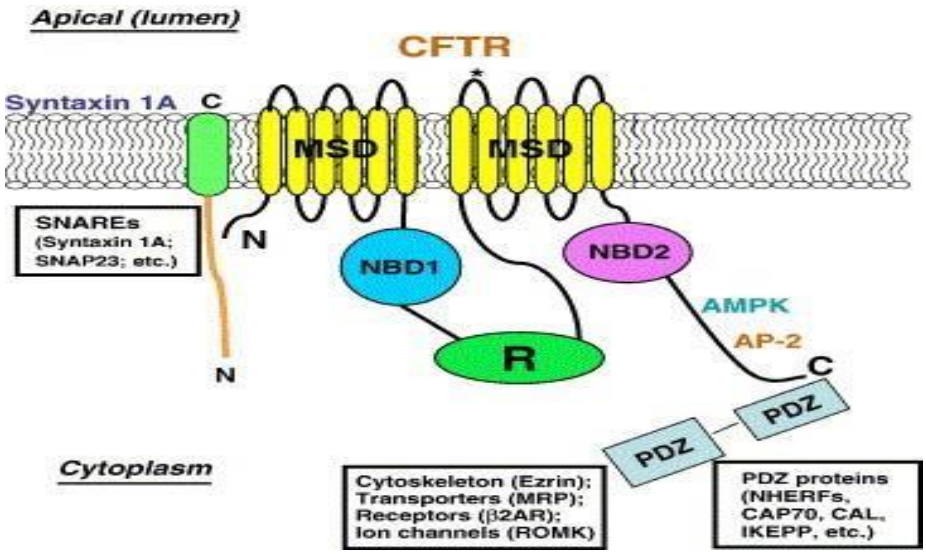

Fig.3

It is a disorder of lethal genetic disease (i.e)autosomal ressive transmission.

The protein encoded by CFTR has two transmembrane domain ,two cytoplasmic nucleotide binding domain. Regulatory domain that contains protein kinase AC phosporylation site CFTR is coded on the chromosome 7.

The two transmembrane domain forms a channel through which chloride passes.

MECHANISM:

Activation of CFTR channel

Mediated by agonist which increases Camp and followed by activation of protein kinase the phosporylate R domain

ATP binding and hydrolysis occurs at nucleotide binding domain Which is essential for opening and closing of channel pore in response to cAMP through which

DISORDER:

- The primary defect in cystic fibrosis results from abnormal function of an epithelial chloride channel protein encoded by CFTR.
- Cystic fibrosis patients have decrease chloride secretion, increase sodium and H2O reabsorption .
- Which leads to dehydration of mucous layer coating epithelial cell followed by defective mucociliary action results in mucus pluging
- Four classes of cystic fibrosis:
- Defective protein synthesis
- Abnormal protein folding
- Defective regulation
- Decrease conductance

CLINICAL SYMPTOMS:

- Chronic lung disease
- Steatorrhea,
- Malnutrition,
- Hepatic cirrhosis,
- Intestinal obstruction,
- Male infertility.

TUBERCULOSIS:

Causitive agent mycobacterium tuberculosis

MORPHOLOGY:

Acid fast bacilli non sporns,non motile,non capsulated,aerobic.

Cultivation: lowenstien jonson medium

MODE OF TRANSMISSION:

Aerosols,

Dropletss

Immuno compossed patients

PATHOGENICITY :

Entry of organism to host by a aerosol .Organism reaches respiratory tract and deposited in lungs.

Alveolar macrophage stimulated in lungs and engulf the tuberculli bacilli

- Macrophage were not able to clean the pathogen since it is
- intracellular paracyte.
- It process and pretent into CDU T Cell
- CD4 T Cell activate interleukein 1
- Interlukin 1 self activate CD4 T cell and release various lymphokine such as macrophage activating ,macrophage chemolactic ,macrophage inhibiting factor
- Macrophage activating fac activates the monocyte toward the infected site
- Macrophage chemolatic fac attract the monocyte towards the injury site
- Macrophage inhibiting fac after the arrival of monocyte to the infected area it inhibits the fuether movement.
- Macrophage fuses to form epitheloid cell , multi nucleated giant cell, T-lymphocyte, fibroblast.

Followed by granuloma formation.

CLINICAL SYMPTOMS:

- Malaise
- Fever
- Loss of appetite
- Fatigue
- Lymphadenopathy
- Dry cough
- Loss of weight
- Haemolysis

LAB DIAGNOSIS

- **The sample is sputum. The staining is acid fast staining .**
- **Next stage is skin test-purified protein derivative (PPD)**

- **Bio chemical test : niacin test and nitrate reductase test**
- **Serological test :ELISA**
- TREATMENT : isoniazid ,ethambutol,para amino salicylic acid.
- Neuropyschiatric disorder/degenerative disorder:
- Alzhimer's disease –dementia:
- The major corticle degenetative disease is alzhimer disease its principle clinical manifestation is dementia . It is common disease in elderly people.
- The disease usually becomes clinically apparent as impartment of higher intellectual function and alteration of behaviour.
- Later progressive disorentiation ,memory loss and aphasia after 5 -10 yrs patient became disabled and immobile.
- Amyloid protein is a transmembrane protein. It consists of cleavage site for 3 distinct enzyme (alpha,beta,gamma secretases)
- AB domain extends from the extra cellular site of protein into the transmembrane domain.
- When ATP is cleaved by gamma secretase subsequent clevage b gamma secretase does not AB.
- Clevage by beta secreatase followed by gamma secretase results in protection of AB which then aggregate and form fibrils.
- Alzheimer's disease have been identified on chromosome 1 and 14.

CLINICAL SYMPTOMS:

- Asymptamatic cores running more than 40 yrs.
- Forget fullness
- Language defect
- Loss of mathematical skill
- Loss of motor skill and mute,unable to walk.

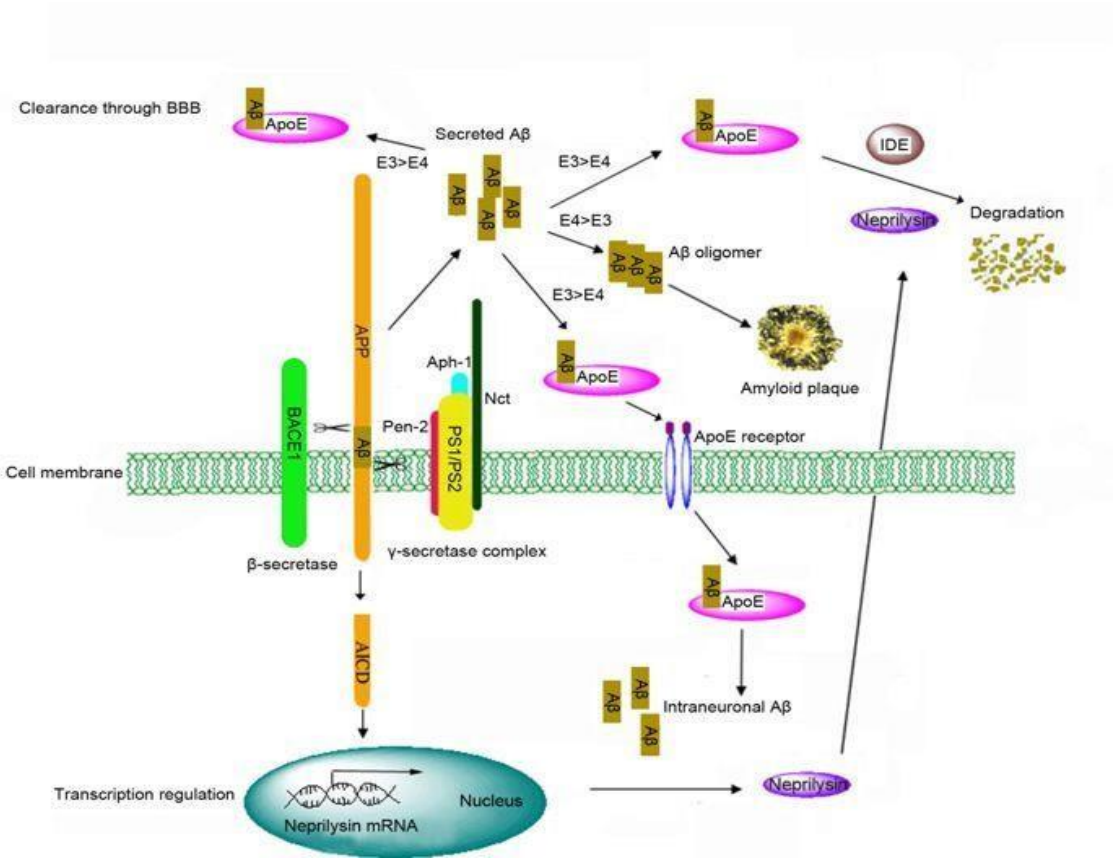

Fig.4

PARKINSON'S DISEASE:

This disease is an example for autosomal dominant or autosomal ressesive inheritant.

PATHOGENESIS AND MOLECULAR GENETICS:

- This disease is associated with the reduction in the striatal dopamine content the severity of the motor syndrome is proportional to the dopamine deficiency.
- The acute parkinsons syndrome and destruction of neuron in the NIGRA follows exposure to one methyl 4-phenyl -1,2,3,6 tetra hydropyridine.
- Mutuation in A53t and A30P have been characterised as gene for parkinson's disease.
- Presence of lewy bodies in a substancia nigra neuron contains alpha synuclein and neuronal protein.
- Lewy body are composed of five fillaments densly paired in the core but lose at the rim .

CLINICAL SYMPTOMS:

- Dementia,
- Hallucinations,
- dementia with lewy bodies.

TREATMENT:

- Autoimmunity
- L-dopa

SKIN DISORDER

It is characterized by red papulo vesicular oozing and lession and crusted lesion. Eczema has been classified into 5 types :

i) allergic contact dermatitis

ii) atopic dermatitis

iii) drug related eczematous dermatitis

iv) Photo eczematous dermatitis

v) Primary irritant dermatitis

- Topically applied
- Unknown
- Haptens
- UV light
- Trauma
- Initially ag at epidermal cell
- Are engulfed by dentritic langerhans cell

- Which then migrate to dermal lymlastics
- Ag are processed by langerhans cells presented to CD4 T cells
- Which are activated and developed into memory T-cells.
- On resposure these memory T cells migrate to affected skin site.
- They release cytokine.
- Neumorous inflamatory cell responsible for the spongeotic dermatitis.

CLINICAL SYMPTOMS:

- Bliaters
- Oozing plaques
- Edematous
- Acanthosis
- Hyperkeratotic

PSORIASIS

- The common chronic dermatitis .it is associated with arthritis. It is a spongiolytic joint disease or acquired immuno deficiency syndrome.
- Psoriatic arthiritis may be mild or may produce some deformitis most frequently affect the skin of elbow ,knee scalp,etc.
- The most typical lesion is demarginated pink colour plaque.
- Nail colour changes yellow or brown colour discolouration

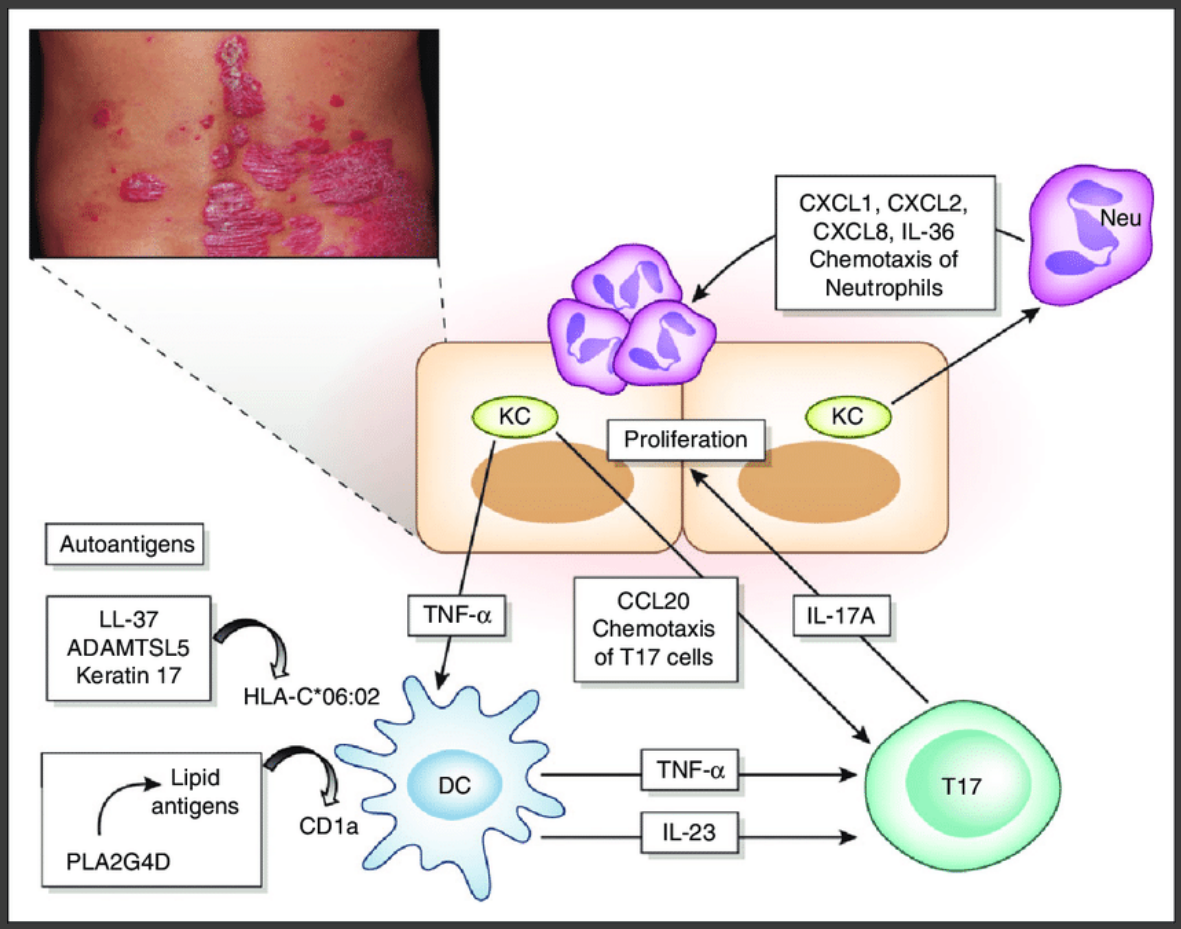

Fig.5

- Definition: The ability of micro-organism to cause disease is known as pathogenicity
- Examples Salmonella typhi causes typhoid.
- The pathogenic effect of one organism differ from other organism.
- Types of bacterial pathogen
- Oppurtunistic pathogen eg. Candida albicans
- Primary pathogen eg. S. aureus
- Virulence a.Vrulent strain.
- b. avirulent strain.
- Eg.smooth and rough strains of S. pneumoniae.
- Invasiveness.
- Toxigenicity.

- Eg. Exotoxin and endotoxin.
- Types of infection
- Natural infection.
- Artificial infection.
- ESTABLISHMENTS OF INFECTIONS.
- Inhalation, 2. ingestion, 3.STD
- Inhalation eg tuberculosis.
- Ingestion eg typhoid. STD eg. Syphillis.
- Adherence factor. 1. host cell factor
- microbial factor.

Penetration . Organism attaches to the epithelial cell of host cell. enters mucosal region submucosal region. spreads to the other parts of the body.

Multiplication org .colonises rapidly by absorbing nutrition from host cell.

Bacteria, virus and fungi as potential pathogens.

BACTERIA

Staphylococcus aureus GRAM POSITIVE COCCI It causes respiratory infection

Skin infection, food poisoning , wound infection,toxic shock syndrome I

FUNGI Candida albicans Budding yeast cell Opportunistic pathogen

Normal flora in human beings

Causes infections in immunocomprimsed host.

Causes respiratory infection, oral thrush, pulmonary infection candida oesophagitis, cutaneous candidiasis,

VIRAL REPLICATION.

Entry of virus Uncoating of virus release of viral genome

Transcription m RNA synthesis Translation viral proteins Synthesis Viral inclusions.

Reduced host cell protein synthesis machinery

Metabolic dearrangements Viral assembly cell lysis Viral antigens new viral progeny.

- Salmonella typhi typhoid
- GRAM NEGATIVE BACILLI
- Motile, non capsulated, nonsporing.
- Produces enterotoxin
- Causes gastro intestinal infections
- Symptoms
- nausea, abdominal pain, vomiting headache fever
- Treatment chloramphenicol.
- FILIARIASIS Wuchereria bancrofti
- morphology adult worm
- microfilaria form.
- 1st 2nd 3rd stage larva
- 3rd larvae is highly infectious.
- LIFE CYCLE OF **W bancrofti.**
- DEFINITIVE HOST. MAN
- INTERMEDIATE HOST ANOPHELES , ADES mosquito

- Infection through bite of infected mosquito to host
- Thirdstage larva are deposited on the skin
- Organism enters peripheral lymphatic vessels
- Larvae quickly migrate to inguinal lymph nodes
- Microfilariae circulate in the blood for 6 months in host
- Microfilariae ingested by mosquito coverts in to L1 L2 L3
- L3 then migrate in to salivary gland of mosquito
- Mosquito bites a man during blood meal
- L3 larvae are rea\leased from the tip of proboscis of mosquito and the life cycle continues.
- Symptoms lymphatic filariasis , hydrocele, elephantiasis, fever, chill
- Lab diagnosis peripheral blood smear GIEMSA Staining
- Treatment Di ethyl carbamazine.
- Mechanism of development of resistance. Virulent to avirulent stage or avrulent to viulent stage by spontaneous or induced mutation Eg transformation.

- Factors responsible to developmnt of resistance.
- Antiphagocytosis
- Intracellular parasite.
- inhibition of oxygen dependent mechanism.
- Antigenic heterogenicity. Sero type variation
- antigenic drift and shift.
- Antimicrobial resistance Production of penicillinase enzyme
- High osmotic pressure.
- Cell membrane permeability defect Alteration in the target site
- Drug inactivation mechanism Decrease drug permeability Transmission of drug resistance.

TEXT / REFERENCE BOOKS

1. Pelczar, Jr E.C.S Chan and noel R.Krieg, Microbiology, 5th edition Tata McGrawHill -2006

2. Joanne M. Willey, Linda Sherwood, Christopher J. Woolverton, Prescott's Microbiology, 8th Edition, McGraw-Hill Higher Education, 2008

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PART A

S.No.	Questions	outcomes
1	Define pathogenicity	**CO5**
2	What are opportunistic pathogen	**CO5**
3	State on virulence and give example.	**CO5**
4	Differentiate Invasiveness from toxigenicity	**CO5**
5	Give out immunodeficiency diseases.	**CO5**
6	What is cystic fibrosis?	**CO5**
7	Emphaize on Parkinson disease?	**CO5**
8	What is Alzheimer disease?	**CO5**
9	List out the clinical symptoms of Mycobacterium tuberculosis	**CO5**
10	What is Eczema?	**CO5**
11	What is Psoriasis?	**Co5**

PART B

S.No.	Questions	outcomes
1	What is microbial pathogenicity? Discuss in detail about the mechanisms of pathogenicity	**CO6**
2	Explain with neat diagram molecular basis of Type I Diabetes.	**CO5**
3	Give a detailed account on the structure of HIV and their pathogenicity add a note on its clinical symptoms	**CO5**
4	Explain in detail about the pathogenicity of candidiasis.	**CO5**
5	Describe in detail about the cystic fibrosis disorder.	**CO5**
6	Discuss the pathological role of Mycobacterium tuberculosis	**CO6**
7	Write in detail about Parkinson disease.	**CO5**
8	Emphasize on Alzheimer disease.	**CO5**
9	Discuss the role of Eczema with neat diagram.	**CO5**
10	Write in detail about Psoriasis and add a note on its symptoms	**CO5**