VPM 302: INTRODUCTION TO VETERINARY MICROBIOLOGY

LECTURE NOTES

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VPM 302: INTRODUCTION TO VETERINARY MICROBIOLOGY AND MYCOLOGY

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INTRODUCTORY MICROBIOLOYAND MYCOLOGY
DR. M. A. OYEKUNLE

Microbiology is the study of microorganisms, which are tiny organisms that live around us and inside our body. An organism is a living thing that ingests and breaks down food for energy and nutrients, excretes undigested food as waste, and is capable of reproduction. A microorganism is simply very, very small organism that you cannot see with your naked eye, but with a microscope

TYPES OF MICROORGANISMS

Pathogenic microorganism

An infection is caused by the infiltration of a disease causing microorganism known as pathogenic microorganism. Some pathogenic microorganisms infect humans, other animals and plant.

Example

Yersinia pestis is the microorganism that caused the Black Plague and killed more than 25 million Europeans. You might say that *Yersinia pestis* first infected fleas that were carried into populated areas on the backs of rats. Rodents traveled on ships and then over land in search of food. Fleas jumped from Rodents and bit people, transmitting *Yersinia pestis* into the person's blood stream.

Non-Pathogenic microorganism

Not all microorganisms are pathogens. In fact many microorganisms help to maintain homeostasis in our bodies and are used in the production of food and other commercial products. For example, *flora* are microorganisms found in our intestine that assist in the digestion of food and play critical role in the formation of vitamins such as vitamin B and vitamin K. They help by breaking down large molecules into smaller ones.

What is a microorganism?

Microorganisms are the subject of microbiology, which is the branch of science that studies microorganisms. A microorganism can be one cell or a cluster of cells that can be seen only by using a microscope. Microorganisms are organized into five fields of study: bacteriology, virology, mycology, phycology, protozoology and parasitiology.

Bacteriology

Bacteriology is the study of bacteria. Bacteria are prokaryotic organisms. A prokaryotic organism is a one-celled organism that does not have a true nucleus. Many bacteria absorb nutrients from their environment and some make their own nutrients by photosynthesis or other synthetic processes. Some bacteria can move freely in their

environment while others are stationary. Bacteria occupy space on land and can live in aquatic environment and in decaying matter. They can even cause disease. *Bacillus anthracis* is a good example. It is the bacterium that causes anthrax

Virology

Virology is the study of viruses. A virus is a submicroscopic, parasitic entity composed of nucleic acid core surrounded by a protein coat. Parasitic means that a virus receives food and shelter from another organism and is not divided into cells. An example of a Virus is the *varicella-zoster* virus, which is the virus that causes chickenpox in humans.

Mycology

Mycology is the study of fungi. A fungus is eukaryotic organism, often microscopic, that absorbs nutrients from its external environment. Fungi are not photosynthetic. A *eukaryotic microorganism* is a microorganism whose cells have a nucleus, cytoplasm and organelles. These include yeast and some molds. Tinea pedis, better know as athlete's foot, is caused by a fungus.

Phycology

Phycology is the study of algae. Algae are *eukaryotic photosynthetic* organisms that transform sunlight into nutrients using photosynthesis. A *eukaryotic photosynthetic* microorganism has cells containing a nucleus, nuclear envelope, cytoplasm and organelles and is able to carry out photosynthesis.

Protozoology

Protozoology is the study of *protozoa*, animal-like single-cell microorganisms that can be found in aquatic and terrestrial environment. Many obtain their food by engulfing or ingesting smaller organism. An example is *Amoeba proteus*

NAMING AND CLASSIFYING MICROORGANISMS

Carl Linnaeus developed the system for naming organisms in 1735. This system is referred to as *binominal nomenclature*. Each organism is assigned two Latinized named because Latin or Greek was the traditional language used by scholars. The first name is called the *genus*. The second name is called *specific epithet,* which is the name of the species, and it is not capitalized. The genus and the epithet appear italicized.
.
Sometimes an organismis named after a researcher, as in the case with *Escherichia coli,* better known as (E. *coli.)* The genus is *Escherichia*, which is named after Theodor Escherich, a leading microbiologist. The epithet or species is *coli*, which implies that the bacterium lives in the colon.

Organisms were classified into either the animal kingdom or the plant kingdom before the scientific community discovered microorganisms in the seventeenth century. It was at that time when scientist realized that this classification system was no longer valid.

Carl Woese developed a new classification system that arranged organisms according to their molecular characteristics and then cellular characteristics. However, it wasn't until 1978 when scientists could agree on the new system for classifying organism, and it took 12years after this arrangement before the new system was published.

Woese devised three classification groups called domain. A domain is larger than a kingdom. These are:

Domains

- Eubacteria: Bacteria that have peptidoglycan cell walls. (Peptidoglycan is the molecular structure of the cell walls of eubacteria which consists of N-acetyglucosamine, N-acetylmuramic acid, tetrapeptide, side chain and murein.)

- Archaea: Prokaryotes that do not have peptidoglycan cell walls

- Eucarya: Organisms from the following kingdoms:

Kingdoms

- Protista: Examples- Algae, protozoa, slime, molds.

- Fungi: Examples- one-celled yeasts, multicellular molds and mushrooms.

- Plantae: Examples- moss, conifers, ferns, flowering plant, algae.

- Animalia: Examples- insects, worms, sponges and vertebrates.

Size of microorganism
Microorganisms are measured using the metric system. In order to give you some idea of the size of a microorganism, let's compare a microorganism to things that are familiar to you.

A human red blood cell	100 micrometers (μm)
A typical bacterium cell	10 micrometers (μm)
A virus	10 nanometers (nm)
An atom	0.1 nanometers (nm)

IMMUNOLOGY

Immunology is the study of how an organism defends itself against infection by microorganism. When a microorganism such as bacterium invades your body, white blood cells engulf the bacterial cells and digest it in an immune response called phagocytosis. *Phagocytosis* is the ability of a cell to engulf and digest solid materials by the use of "*pseudopods*" or "false feet."

Phagocytosis was discovered in 1880 by Russian zoologist Elie Metchnikoff, who was one of the first scientists to study immunology. Metchnikoff studied the body's defense against disease-causing agents and invading microorganisms. He discovered that leukocytes (White blood cells) defended the body by engulfing and eating the invading microorganism

DRUGS:

Invading microorganisms activate the body's immune system. It is at this point when you experience a fever and feel sick. In an effort to help your immune system, physicians prescribe drugs called *antibiotics* that contain one or more antimicrobial agents that combat bacteria. An antimicrobial agent is a substance that specifically inhibits and destroys the attacking microorganism.

One of the most commonly used antimicrobial agent is penicillin. *Penicillin* is made from Penicillium, which is a mold that secretes materials that interfere with the synthesis of the cell walls of bacteria causing "lysis" or destruction of the cell wall, and kills the invading microorganism.

HISTORY OF MICROBIOLOGY

The microscope
i.   Zacharias Jansssen

In 1590, Zacharians Janssen developed the first compound microscope in Middleburg, Holland, Janssen's microscope consisted of three tubes. One tube served as the outer casing and contained the other two tubes. At either ends of the inner tubes were lenses used for magnification. Janssen's design enabled scientists to enlarge the image of a specimen three and nine times the specimen's actual size.

ii.   Robert Hooke

In 1665, Robert Hooke, an English scientis, popularized the use of the compound microscope when he placed lenses over slices of cork and viewed little boxes that he called cells. It was his discovery that led to the development of cell theory in the

nineteenth century by Mathias Schleiden. Theoder Schwann, and Rudolf Virchow, Cell theory states that all living things are composed of cells.

iii.   Antoni van Leeuwenhoek

Hooke's experiments with a crude microscope inspired Antoni van Leenwenhoek to further explore the micro world. Van Leeuwenhock, an amateur lens grinder, improved Hooke's microscope by grinding lenses to achieve magnification.   His microscope required one lens. With his improvement, van Leeuwenhock became the first person to view a living microorganisms, which he called Animalcules.

This discovery took place during the 1600s, when scientists believed that organisms generated spontaneously and did not come from another organisms.   This sounds preposterous today; however, back then scientists were just leaning that a cell was the basic component of an organism.

Origin of Organisms

i.   Francesco Redi

In 1668, Italian physician Francesco Redi developed an experiment that demonstrated that an organisms did not spontaneously appear. He filled jars with rotting meat. Some jars he sealed and other he left opened. Those that were open eventually contained maggots, which is the larval stage of the fly. The other jars did not contain maggots because flies could not enter the jar to lay eggs on the rotting meat.

His critics stated that air was the ingredient required for spontaneous generation of an organism. Air was absent from the sealed jar and therefore no spontaneous generation could occur, they said Redi repeated the experiment except this time he placed a screen over the opened jars. This presented flies from entering the jar. There weren't any maggots on the rotting eat.

Until that time scientists did not have a clue about how to fight disease. However, Redi's discovery gave scientists an idea. They used Redi's findings to conclude that killing the microorganisms that caused a disease could prevent the disease from occurring. A new microorganisms could only be generated by another microorganisms when it underwent a reproductive process.   Kill that microorganism and you will not have new microorganisms, the theory went – you could stop the spread of the disease. Scientists called this the Theory of Biogenesis. The Theory of Biogenesis states that a living cell is generated from another living cell.

ii.   Louis Pasteur

Although the Theory of Biogenesis disproved spontaneous generation, spontaneous generation was hotly debated among the scientific community until (1861) when Loius

Pasteur, a French scientific, resolved the issue once and for all. Pasteur showed that microorganisms were in the air. He proved that sterilized medical instruments became contaminated once they were exposed to the air.

Pasteur came to this conclusion by boiling beef broth in several short-necked flasks. Some flasks were left open to cool. Other flasks were sealed after boiling. The opened flasks became contaminated with microorganisms while no microorganisms appeared in the closed flasks. Pasteur concluded that airborne microorganisms had contaminated the opened flaks.

In a follow-up experiment, Pasteur placed beef broth in an open long-necked flask. The neck was bent into an S-shape. Again he boiled the beef broth and let it cool. The S-shaped neck trapped the airborne microorganisms.

The beef broth remained uncontaminated even after months of being exposed to the air. The very same flask containing the original beef broth exists today in Pasteur Institute in Paris and still shows no sign of contamination. Pasteur's experiments validated that microorganisms are not spontaneously generated.

Based on Pasteur's findings, concerned effort was launched to improve sterilization techniques to prevent microorganisms from reproducing. Pasteurization, one of the best-known sterilization techniques, was developed and named for Pasteur. Pasteurization kills harmful microorganisms in milk, alcoholic beverages, and other foods and drinks by heating it enough to kill most bacteria that cause spoilage.

iii.   John Tyndall and Ferdinand Cohn

The work of John Tyndall and Ferdinand Cohn in the late 1800s led to one of the most important discoveries in sterilization. They learned that some microorganisms are resistant to certain sterilization techniques. Until their discovery, scientists had assumed that no microorganisms could survive boiling water, which became a widely accepted method of sterilization. This was wrong. Some thermophiles resisted heat and could survive a bath in boiling water. This means that there was not one magic bullet that killed all harmful microorganisms.

Germ theory

Until the late 1700s, not much was really known about diseases except their impact. It seemed that anyone who came in contact with an infected person contracted the disease. A disease that is spread by being exposed to infection is called a contagious disease. The unknown agent that causes the disease is called a contagion. Today we known that a contagion is a microorganisms, but in the 1700s many found it hard to believe something so small could cause such devastation.

i.   Rober Koch

Koch made some observations on the disease caused *Bacillus anthracci* called anthrax.Based on his findings, Koch developed the Germ Theory. The Germ Theory states that a disease-causing microorganisms should be present in animals infected by the disease and not in healthy animals. The microorganisms can be cultivated away from the animal and used to inoculate a healthy animal. The healthy animal should then come down with the disease. Samples of a microorganism taken from several infected animals are the same as the original microorganism from the first infected animals.

Four steps used by Koch to study microorganisms are referred to as Koch's Postulates. Koch's Possulates state:

1. The microorganism must be present in the diseased animals and not presence in the healthy animal.
2. Cultivate the microorganism away from the animal in a pure culture.
3. Symptoms of the disease should appear in the healthy animal after the healthy animal is inoculated with the culture of the microorganisms.
4. Isolate the microorganism from the newly infected animal and culture it in the laboratory. The new culture should be the same as the microorganism that was cultivated from the original diseased animal.

Koch 's work with anthrax also developed techniques for growing a culture of microorganisms. He eventually used a gelatin surface to cultivate microorganisms. Gelatin inhibited the movement of microorganisms. As microorganisms reproduced, they remained together, forming a colony that made them visible without a microscope. The reproduction of microorganisms is called colonizing. The gelatin was replaced with agar that is derived from seaweed and still used today. Richard Petri improved on Koch's cultivating technique by placing the agar in a specially designed disk that was later called the Petri dish which is still used today.

Vaccination

The *Variola* virus was once of the most feared villains in the late 1700s. The variola virus causes smallpox. If variola didn't kill you, it caused pus-filled blisters that left deep scars that pitted nearly every part of your body. Cows were also susceptible to a variation of variola called cowpox. Milkmaids who tended to infected cows contracted cowpox and exhibited immunity to the smallpox virus.

i. Edward Jenner

Edward Jenner, an English physician, discovered something very interesting about both smallpox and cowpox in 1796. Those who survived smallpox never contracted smallpox again, even when they wee later exposed to someone who was infected with smallpox. Milkmaids who contracted cowpox never caught smallpox even though they were exposed to smallpox.

Jenner had an idea. He took scrapings from a cowpox blister found on a milkmaid and, using a needle scratched the scrapping into the arm of James Phipps, an 8-year-old. Phipps became slightly ill when the scratch turned bumpy. Phipps recovered and was then exposed to smallpox. He did not contract smallpox because his immune system developed antibodies that could fight off variola.

Jenner's experiment discovered how to use our body's own defense mechanism to prevent disease by inoculating a healthy persons with a tiny amount of the disease-causing microorganism. Janner called this a vaccination, which is an extension of the Latin word vacca (cow). The person who received the vaccination became immune to the disease-causing microorganism.

ii.   Elie Metchnikoff

Elie Metchnikoff, a nineteeth-century Russian zoologist, was interested by Jenner's work with vaccinations. Metchnikoff wanted to learn how our bodies react to vaccination by exploring our body's immune system. He discovered that white blood cells (leukocytes) engulf and digest microorganisms that invade the body. He called these cells phagocytes, which means "cell eating". "Metchnikoff was one of the first scientists to study the new area of biology called immunology, the study of the immune system.

Killing the Microorganism

i.   Ignaz Semmelwees

Great studies were made during the late 1800s in the development of antiseptic techniques. It began with a report by Hungarian physician Ignaz Semmelweis on a dramatic decline in childbirth fever when physicians used antiseptic techniques when delivering babies. Infections become preventable through the use of antiseptic techniques.

ii.   Joseph Lister

Joseph Lister, an English surgeon, developed one of the most notable antiseptic techniques. During surgery he sprayed carbolic acid over the patient and then bandged the patient's wound with carbolic acid-soaked bandages. Infection following surgery dramatically dropped when compared with surgery performed without spraying carbolic acid. Carbolic acid, also known as phenol was one of the first surgical antiseptic.

iii.   Paul Ehrlich

Antiseptics prevented microorganisms from infecting a person, but scientists still needed a way to kill microorganisms after they infected the body. Scientists needed a magic bullet that cured diseases. At the turned of the nineteenth century, Paul Ehrlich, a German chemist, discovered the magic bullet. Ehrich blended chemical elements into a convocation that, when inserted into an infected area, killed microorganisms without

affecting the patient.   Today we call Ehrlich's concoction a drug. Ehrlic's innovation has led to chemotherapy using drugs that are produced by chemical synthesis.

iv.   Alexander Fleming

Scientists from all over set out to use Ehrlich's findings to find drugs that could make infected patients well again. One of the most striking breakthroughs came in 1929 when Alexander Fleming discovered *Penicillin snotatum,* the organism that synthesizes penicillin.   Penicillium notatum is a fungus that kills the *Staphyloccus aureus* microorganism and similar microorganisms.

Fleming grew cultures of *Staphyloccus aureus*, a bacterium, in the laboratory. He was also conducting experiments with *Penicillium notatum,* a mold.   By accident the *Staphyloccous aureus* was contaminated with the *Penicillium notatum*, causing   the *Staphyloccocus* to stop reproducing and die. *Penicillium notatum* became one of the first antibiotic. An antibiotic is a substance that kills bacteria.

PREPARING SPECIMEN FOR OBSERVAITON UNDER A   LIGHT COMPOUND MICROSCOPE

There are two ways to prepare a specimen to be observed under a light compound microscope. These are a smear and a wet mount.

Smear

A smear is a preparations process where a specimen that is spread on a slide. You prepare a smear using the heat fixation process.

1. Use a clean glass slide.
2. Take a smear of the culture. (The microorganisms are spread over the   glass slide).
3. Place the live microorganism on the glass slide by smearing it onto the glass
4. The slice is air dried then passed over a Bunsen burner about three times.
5. The heat causes the microorganisms to adhere to the glass slide. This is known as fixing the microorganisms to the glass slide.
6. Stain the microorganisms with an appropriate stain.

Wet Mount

A wet mount is a preparation process where a live specimen in culture fluid is placed on a concave glass side or a plain glass slide. The concave portion of the glass tube forms a cup-like shape that is filled with a thick, sympy substance, such as carboxymethyl cellulose. The microorganism is free to move about within the fluid, although the viscosity of the substance slows its movement. This makes it easier for you to observe the microorganism. The specimen and the substance are protected from spillage and

outside contaminates by a glass cover that is placed over the concave portion of the slide.

STAINING A SPECIMEN

Not all specimens can be clearly seen under a microscope. Sometimes the specimen blends with other objects in the background because they absorb and reflect approximately the same light waves. You can enhance the appearance of a specimen by using a stain. A stain is used to contrast the specimen from the background.

A stain is a chemical that adheres to structures of the microorganisms and in effect dyes the microorganisms so t can be easily seen under a microscope. Stains used in microbiology are either basic or acidic.

Basic stains are cationic and have positive charge. Common basic stains are methylene blue, crystal violet, safranin, and malachite green. These are ideal for staining chromosomes and the cell membranes of many bacteria.

Acid stains are used to identify bacteria that have a waxy material in their cell walls. This form of staining differentiates bacteria.

Quick Guide for Staining Techniques

Type	Number of Dyes Used	Observations	Examples
Simple stains	Use a single dye	Size, shape and arrangement of cells	Methylene blue Safranin Crystal violet
Differential stains	Use two or more dyes to distinguish different types or different structures of bacteria	Distinguishes gram-positive or gram-negative. Distinguishes the member of mycobacteria anmd nocardia from other bacteria	Gram stain Zehl-Nielsen acid-faast stain
Special stains	These stains identify specialized structures	Exhibits the presence of flagella. Exhibits endospores	Shaefter-Fultion spore staining

Types of Stains

There are two types of Stains: simple and differential.

Simple Stain

A simple stain has a simple basic dye that is used to show shapes of cells and the structures within a cell. Methylene blue, safranin, carbolfluchsin and crystal violet are common simple stains that are found in most microbiology laboratories.

Differential Stain

A differential stain consists of two or more dyes and is used in the procedure to identify bacteria. Two of the most commonly used differential stains are the Gram stain and the Zehl-Nelson acid-fast stain.

In 1884 Hans Christian Gram, a Danish physician, developed the Gram stain. Gram-stain is a method for the differential staining of bacteria. Gram-positive microorganisms stain purple. *Gram-negative* microorganisms stain pink *Staphylococcus ureus,* a common bacterium that causes food poisoning, is gram-positive, Escherichia coli is gram-negative.

The *Ziehl-Neelsen* acid-fast stain, developed by Franz Ziehl and *Friedrick Neelsen,* a red dye that *attraches* to the waxy material in the cell walls of bacteria such as *Mycobacterim tuberculosis,* which is the bacterim that causes tuberculosis, and *Mycobacterim leprae*, which is the bacterium that causes leprosy. Microorganisms that retain the Zichl-Neelsen acid-fast strain are called acid-fast. Those that do not retain it turn blue because the microorganism doesn't absorb the Ziehl-Neelsen acid-fast stain.

How to Gram-stain a specimen.

Observing Microorganisms

1. Prepare the specimen using the heat fixation process (see "Smear" above).
2. Place a drop of crystal violet stain on the specimen.
3. Apply iodine on the specimen using an eyedropper. The iodine helps the crystal violet stain adhere to the specimen. Iodine is a mordant which is a chemical that fixes the stain to the specimen.
4. Wash the specimen with ethanol or alcohol-acetone solution, then wash with water.
5. Wash the specimen to remove excess iodine. The specimen appears purple in colour.
6. Apply the safranin stain to the specimen using an eyedropper.
7. Wash the specimen.
8. Use a paper towel and blot the specimen until the specimen is dry.
9. the specimen is ready to be viewed under the microscope. Gram-positive bacteria appear purple and gram-negative bacteria appear pink.

Here is how to apply the Zichi-Neelsen acid-fast stain to a specimen.

1. Prepare the specimen (see "Smear" earlier in this chapter).
2. Apply the red dye carbon-fuchsin stain generously using an eyedropper.
3. Let the specimen sit for a few minutes.

4. Warm the specimen over steaming water. The heat will cause the stain to penetrate the cell wall.
5. Wash the specimen with an alcohol-acid or acid-alcohol decolorizing solution consisting of 3 percent hydrochloric acid and 93 percent ethanol. The hydrochloric acid will remove the color from non-acid-fast cells and the background. Acid-fast cells will stay red because the acid cannot penetrate the cell wall.
6. Apply methylene blue stain on the specimen using an eyedropper.

Special Stains

Special stains are paired to dye specific structures of microorganisms such as endospores, flagella and gelatinous capsules. One stain in the pair is used as a negative stain. A negative stain is used to stain the background of the microorganism

Table 3-5. Scientists and Their Contribution

Year	Scientists	Contribution
1854	Hans Christian Gram	Developed the Gram stain used to stain and identify bacteria.
1882	Franz Ziehl and Friedrick Neelsen	Developed the Ziehl-Neelsen acid-fast stain used to stain bacteria

Causing the microorganisms to appear clear. A second stain is used to colorize specific structures within the microorganism. For example, nigrosin and India ink are used as a negative stain and methylene blue is used as a positive stain.

The Schaeffer-Fulton endoscope stain is a special stain that is used to colorize the endospore. The endospore is a dormant part of the bacteria cell that protects the bacteria from the environment outside the cell.

Here is how to apply the Schaeffer-Fulton endospore stain.

1. Prepare the specimen (See "Senear" earlier in the chapter)
2. Heat the malchite gree stain over a Bunsen burner until it becomes fluid.
3. Apply the malachite green to the specimen using an eyedropper.
4. Wash the specimen for 30 seconds.
5. Apply the safranin stain using an eyedropper to the specimen to stain parts of the cell other than the endospore.
6. Observe the specimen under the microscope.

Prokaryotic Cells and Eukaryotic Cells

The life processes of a living thing include

*   Metabolism. Breakdown nutrients for energy or extract from the environment.
*   Responsiveness. React to internal and external environmental changes.
*   Movement. Whether it is the entire organism relocating within its environment, cells within that organism or the organelles inside those cells.
*   Growing. Increase the size or number of cells.
*   Differentiation. The process whereby cells that are unspecialized become specialized. (An example would be a single fertilized human egg, developing into an individual) Prokaryotic cells do not differentiate.
*   Reproduction. Form new cells to create a new individual.

Prokaryotic Cells
A prokaryotic cells is a cell that does not have a true nucleus. The nuclearstructure is called a nuclenid. The nuclenid contains most of the cell's genetic material and is usually a single circular molecule of DNA. Karyo-is Grek for "kernet". A prokaryotic organism, such as a bacterium, is a cell that lacks a membrance-bound nucleus or membrane-bound organetles. The exterior of the cell usually has glycocalyx, flagellum, fimbriae, and pili.

Differences between Prokaryotic and Eukaryotic Cells

Characteristics	Prokaryotic Cells	Eukaryotic Cells
Cells wall	Include peptidoglycan Chemically complex	Chemically simple
Plasma membrane	No carbohydrates No sterols	Contain carbohydrates Contain sterols
Glycocalyx	Contain a capsule or a slime layer	Contained in cells that lack a cell wall
Flagella	Protein building blacks	Multiple micronutrient
Cytoplasm	No cytoplasmic streaming	Contain cytoskeleton Contain cytoplasmic streaming
Membrane-bound Organelles	None	Endoplasmic reticulum Golgi complex Lysosomes Mitochrondria Chloroplasts
Ribosomes	70S	SOS Ribosomes located in Organelles are 70S
Nucleus	No nuclear membranes No nucleui 0.2-2.0 mm un diameter	Have a nucleus Have a nuclear membrane Have a nucleoli 10-100 m in diameter
Chromosomes	Single circular chromosome	Multiple linear chromosomes

	No Bistones	Have histones
Cell division	Binary fussion	Mitosis
Sexual reproduction	No melosis DNA transferred in fragments	Metosis

Parts of Prokaryotic Cells

Glycocalyx

Glycocalyx is a strictly, sugary envelope composed of polysaccharides and/or polypeptides that surround the cell. Glycocalyx is found in one or two states. It can be firmly attached to the cell's surface, called capsule, or loosely attaché, called slime layer. A slime layer is water-soluble and is used by the prokaryotic to adhere to surfaces external to the cell.
Glycicalyx is used by a prokaryotic cell to protect it against attack from the body's immune system.

Flagella

Flagella made of protein and appear "whip-like." They are used by the prokaryotic cell for mobility. Flagella propel the microorganism away from harm and towards food in a movement known as taxis. Movement caused by a light stimulus is referred to as phototaxis and a chemical stimulus causes a chemotaxis movement to occur.

Flagella can exist in the following form

Monotrichous: One Flagellum
Lophotrichus: Two or more Flagella that are at one end of the cell
Amphitrichous: Flagella at two ends of the cell.
Endoflagellum: A type of amphitrichous flagellum that is tightly wrapped around spirochetes. A spirochete is a spiral-shaped bacterium that moves in a corkscrew motion. *Borrelia burgdorferi,* which is the bacterium that causes lyme disease, exhibits an endoflagellum.

Fimbriae

Fimbrae are proteinaceous, sticky, bristle-like projections used by cells to attach to each other and to objects around them. *Neiseseria gonorrhoeae*, the bacterium that causes gonorrhea uses fimbriae to adhere to the body and to cluster cells of bacteria.

Pili

Pili are tubules that are used to transfer DNA from one cell to another cell similar to tubes used to fuels aircraft in flight. Some are also used to attach one cell to another cell. The tubules are made of protein and are shorter in length than flagella and longer than fimbriae

Cell Wall

The prokaryotic cell's wall is located outside the plasma membrane and gives the cell its shape, providing rigid structural support for the cell. The cell wall also protects the cell from its environment.

Pressure within the cells builds as fluid containing nutrients enters the cells. It is the job of the cell wall to resist the pressure the same way that the walls of a balloon resist the build-up pressure whan it is inflated. If pressure inside the cell becomes too great, the cell wall bursts, which is referred to as lysis.

The cell wall of many bacteria is composed of *peptidoglycan*, which covers the entire surface of the cell. *Peptidoglycan* is made up of a combination of peptide bonds and carbohydrates, either N-acetylmuramic acid, commonly referred to as NAM, or N-acetylglycosamine, which is known as NAG

The wall of a bacterium is classified in two ways:

Gram-positive: A gram-positive cell wall has many layers of *Peptidoglycan* that retain crystal violet dye when the cell is stained. This gives the cell a purple color when seen under a microscope

Gram-negative: A gram negative cell wall is thin. The inside is made of *Peptidoglycan.* The outer membrane is composed of phospholipids and lipopolysaccarides. The cell wall does not retain the crystal violet dye when the cell is stained. The cell appears pink when view with a microscope.

Cytoplasmic membrane

The prokaryotic cell has a cell membrane called the cytoplasmic membrane that froms the outer structure of the cell and separates the cell's internal structure from the environment. The cytoplasmic membrane is a membrane that provides a selective barrier between the environment and the cell's internal structures.

The function of the Cytoplasmic Memebrane

The cytoplasmic membrane regulates the flow of molecules (such as nutrients) into the cell and removes waste from the cell by opening and closing passages called channels. In Photosynthesis prokaryotes, the cytoplasmic membrane functions in energy production by collecting energy in the form of light.

The cytoplasmic membrane is selectively permeable because it permits the transport some substances and inhibits the transport of other substances. Two types of transport mechanisms are used to move substances through the cytoplasmic membrane. These are passive transport and active transport.

Cytosol and Cytoplasm

The cytosol is the intracellular fluid of a prokaryotic cell that contains proteins, liquids, enzymes, ions, waste and small molecules dissolved in water, commonly referred to as semifluid. Substances dissolved in cytosol are involved in cell metabolism. The cytosol also contains a region called the nucleoid, which is where the DNA of the cell is located. Unlike human cells, a prokaryotic microorganism has a single chromosome that isn't contained within a nuclear membrane or envelop. Cytosol is located in the cytoplasm of the cell. Cytoplasm also contains the cytoskeleton, ribosomes and inclusions.

Ribosome

A ribosome is an organelle within the cell that synthesizes polypeptide. There are thousands of ribosomes in the cell. A ribosome is comprised of submits consisting of protein and ribosomal RNA, which is referred to as rRNA. Ribosomes and their submits are identified by their sedimentation rate. Sedimentation rate is the rate at which ribosomes are drawn to the bottom of a test tube when spun in a centrifuge. Sedimentation rate is expressed in Sydberg (s) units. The sedimentation rate reflects mass, size and shape of a ribosome and its submits.

Inclusions

An inclusion is a storage area that serves as a reserve for lipids, nitrogen, phosphate, starch and sulphur within the cytoplasm. Scientists use inclusion to identify types of bacteria. Inclusions are usually classified as granules.

MYCOLOGY

Introduction

Mycology is the study of fungi. Fungi have several features that distinguish them from other organisms

1. They have a filametous branching of system of cells with apical growth, lateral branching and heterotrophic nutrition.
2. They are characterized by a life-cycle that begins with germination from spore or a resting structure, followed by a period of growth a the substrate is exploited to produce a biomass
3. Finally, there is a period of sporullation where propapules are formed that can be disseminated from the parent mycelium.
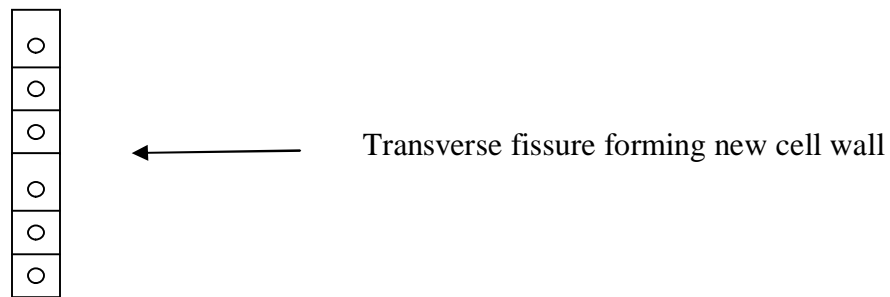
Importance of Fungi

1. They are vital to the biosphere for many reasons not the least, for their decomposing activities on dead substrate that ensure the release of nutrients like carbon, minerals and nitrogen back into the atmosphere(i.e. act as decomposers of complex organic materials in the environment)
2. They are major cause of plant diseases
3. They also cause many diseases of animals and man.

4. Fungi, especially the yeasts are essential to many industrial processes involving fermentation e.g. bread, wine and beer making.
5. They are important in the manufacture many antibiotics.
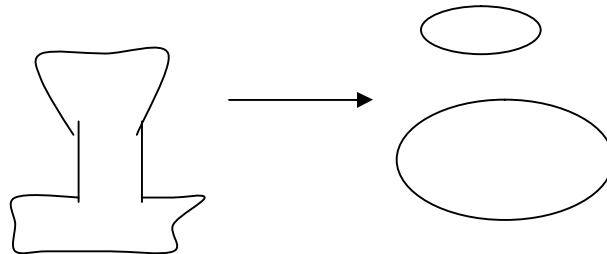6. Fungi are important research tool in the study of fundamentals of biological process.

Reproduction in Fungi

Asexual reproduction: This can be accomplished in several ways

1. A parent cell can divide into two daughter cells by central constriction abd formation of new cell wall- (conidium)

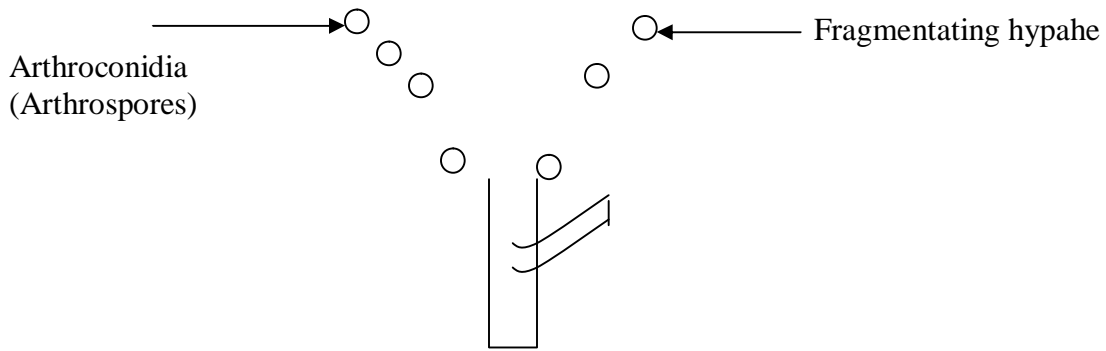Transverse fissure forming new cell wall

2. Somatic vegetative cells may bud to produce new organisms. This is common in yeasts.
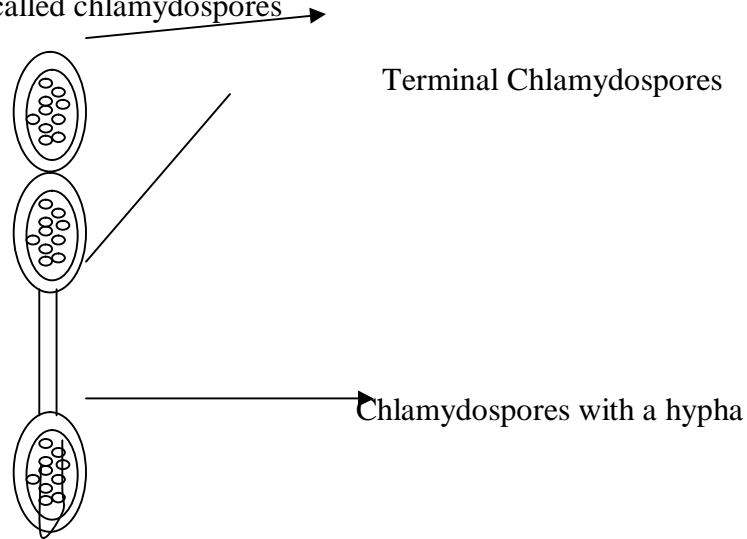

3. The most common method of asexual reproduction is spore production. Asexual spore formation occurs in an individual fungus through mitosis and subsequent cell division. There are several types of asexual spore.
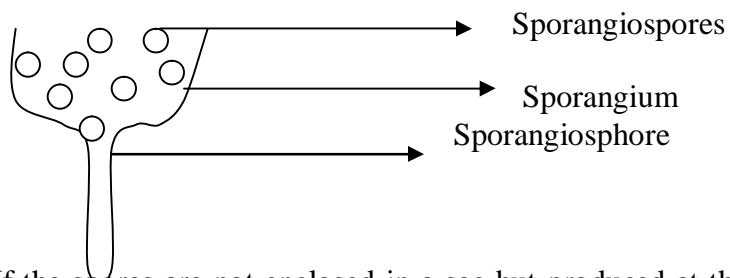
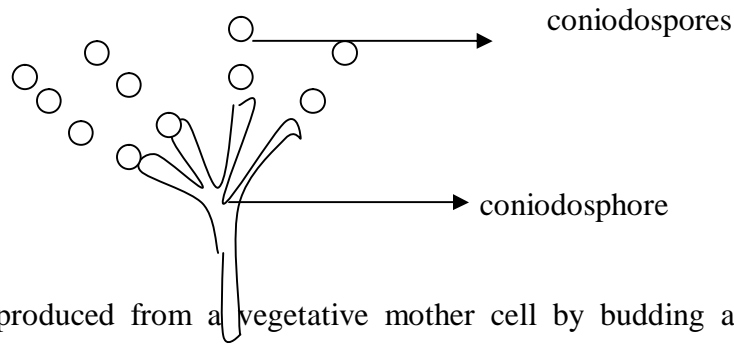   a. Hypha can fragment to form cells that behave as spores. These cells are called arthroconidiao or athrospores.

Arthroconidia
(Arthrospores)

Fragmentating hypahe

b. If the cells are surrounded by a thick wall before separation, they are called chlamydospores

Terminal Chlamydospores

Chlamydospores with a hypha
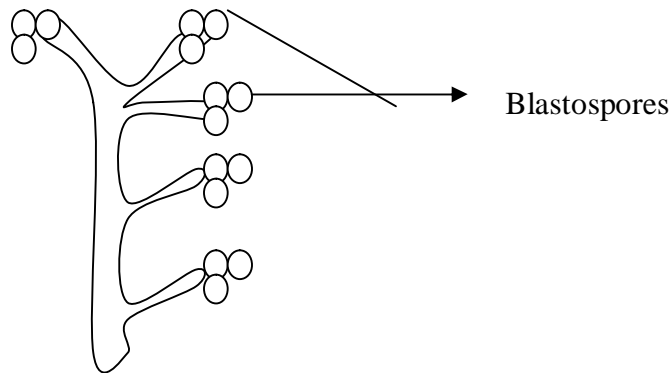
c. If the spores develop within a sac (sporangium/ sporangia) at a hyphal tip, they are called sporangiospores

Sporangiospores

Sporangium
Sporangiosphore

d. If the spores are not enclosed in a sac but produced at the tips or sides of the hypha, they are called comdiospores.

coniodospores

coniodosphore

    e. Spores produced from a vegetative mother cell by budding are called blastospores.

Blastospores

Sexual Reproduction in Fungi

Involves the union of compatible nuclei. Some fungal are self-fertilizing and produce asexually compatible gametes on the same mycelium- homothallic
Other species require out crossing between different but sexually compatible mycelia- heterothallic.

Sexual reproduction yields spores. For example in the zygomycetes the zygote develops a zygospore, in the ascomycetes, an ascospore, in the basiocomycetes a basidiospore.

Fungal spores are important for several reasons:
1. The size, shape colour and number are useful in the identification of fungal species.
2. For fungal dissemination

Fungi metabolism

Fungi prefer moist habitat and they are largely mesophyllic preferring temperature between 15 and 35^0C. The carbon needs of fungi for energy metabolism and biosynthesis has to be met heterotrophically by one of three life styles.
    a. Parasitism of plants and animals- causing diseases.
    b. Saprophytism- growing on dead anima, plant or microbial biomass.
    c. Symbiosis- growing together with algae, plants or insects.

Structure

The body or vegetative structure of fungus is called a thallus (thalli). It varies in complexity and size ranging from a single cell microscopic yeasts to multicellura molds, macroscopic puffballs and mushrooms. The fungal cell is enclosed in a cell wall of chitin.

A. CLASSIFICATION OF FUNGI

Fungi are eukaryotic organisms that do not contain chlorophyll, but have cell walls, filamentous structure, and produce spores. These organisms grow as saprophytes and decompose dead organic matter. There are between 100,000 to 200,000 species depending on how they are classified. About 300 species are presently known to be pathogenic for man and animals

There are five kingdoms of living things. The fungi are in the Kingdom Fungi

KINGDOM	CHARACTERISTICS	EXAMPLE
Monera	Prokaryocyte	Bacteria Actinomycetes
Protista	Eukaryocyte	Protozoa
Fungi	Eukaryocyte*	Fungi
Plantae	Eukaryocyte	Plants Moss
Animalia	Eukaryocyte*	Arthropods Mammals Man

*This common characteristic is responsible for therapeutic dilemma in anti-mycotic therapy

The taxonomy of the Kingdom Fungi is evolving and is controversial. Formerly based on gross and light microscopic morphology, studies of ultra structure, biochemistry and molecular biology provide new evidence on which to base the taxonomy positions. Medically important Fungi are in four phyla.

1. Ascomycota- Sexual reproduction in a sack called an ascus with the production of ascospores
2. Basidiomycota- Sexual reproduction in asack called basidium with the production of basidiospores
3. Zygomycota- Sexual reproduction by gametes and asexual reproduction with formation of zygospores
4. Mitosporic Fungi (Fungi imperfect)- no recognizable form of sexual reproduction, includes most pathogenic Fungi.

B. MORPHOLOGY

Pathogenic fungi can exist as yeasts or as hyphae. A mass of hyphae is called mycelia. Yeasts are unicellular organisms and mycelia are multicellular filamentous structures, constituted by tubular cells with cell wall. The yeasts reproduce by budding. The mycelia forms branch and the pattern of branching is an aid to the morphological identification. If the mycelia do not have SEPTA, they are called coenocytic (nonseptate). The terms "hypha" and "mycelium" are frequently used interchangeably. Some fungi occur in both the yeast and mycelial forms. These are called dimorphic fungi.

Dimorphic Fungi

The dimorphic fungi have two forms

1. YEAST- (Parasitic or Pathogenic form). This sis the form usually seen in tissue, in exudates, or cultured in an incubator at 37^0C .
2. MYCELIUM- (Saprophytic form). The form observed in nature or when cultured at 26^0C. Conversion to the yeast form appears to be essential for pathogenicity. In the dimorphic fungi, fungi are identified by several morphological or biochemical characteristics, including the appearance of their fruiting bodies. The asexual spores may be large (Macroconidia, Chlamydospores), or small (microconidia, blastospores, arthroconidia).

There are four types of mycotic diseases:
1. Hypersensitivity- an allergic reaction to molds and spores
2. Mycotoxicoses- poisoning of man and animals by feeds and food products contaminated by fungi which produce toxin from the grain substrate.
3. Mycetismus- the ingestion of toxin (mushroom poisoning)
4. Infection

We shall be concerned mainly with the last type; pathogenic fungi that cause infections. Most common pathogenic fungi do not produce toxins but they do show physiologic modifications during a parasitic infection (e.g. increased metabolic rate, modified metabolic pathways and modified cell wall structure). The mechanisms that cause these modifications as well as their significance as a pathogenic mechanism are just being described. Most pathogenic fungi are also thermo tolerant, and can resist the effects of the active oxygen radicals released during the respiratory burst of phagocytes. Thus, fungi are able to withstand many host defenses. Fungi are ubiquitous in nature and most people are exposed to them. The establishment of mycotic infection usually depends on the size of the inoculums and on the resistance of the host. The severity of the infection seems to depend mostly on the immunologic status of the host. Thus, the demonstration of fungi, for example, in blood drawn from an intravenous catheter can correspond to colonization of the catheter, to transient fungemia (i.e. dissemination of fungi through the blood stream), or to a true infection. The physician must decide which is the clinical status of the patient based on clinical parameters, general status of the patient, laboratory results, etc. the decision is not trivial, since treatment of systemic fungal infections requires the aggressive use of drugs with considerable toxicity. Most mycotic agents are soil saprophytes and mycotic diseases are generally not communicable from person-to-

person (occasional exceptions: candida and some dematophytes). Outbreaks of disease may occur, but these are due to a common environmental exposure, not communicability. Most of the fungi which cause systemic infections have a peculiar characteristic ecologic niche in nature. This habitat is specific for several fungi which will be discussed later. In this environment, the normally saprophytic organism proliferate and develop. This habitat is also the source of fungal elements and/or spores, where man and animals, incidental hosts, are exposed to the infectious particles. It is important to be aware of these associations to diagnose mycotic diseases. The physicians must be able to elicit a complete history from the patient including occupation, vocation and travel history. This information is frequently required to raise, or confirm, your differential diagnosis. The incidence of mycotic infections is currently increasing (in man) dramatically, due to an immunosuppressive therapy, and the use of more invasive diagnostic and surgical procedures (prosthetic implants). Fungal diseases are non-contagious and non-reportable diseases in the national public health statistics.

C. DIAGNOSIS

1. Skin scrapings suspected to contain dermatophytes or pus from a lesion can be mounted in KOH on a slide and examined directly under a microscope.
2. Skin testing (dermal hypersentivity) used to be popular as a diagnostic tool, but this use is now discouraged because skin test may interfere with serological studies by causing false positive results. It may still be used to evaluate the patient's immunity as well as a population exposure index in epidemiological studies.
3. Serology may be helpful when it is applied to a specific fungal disease: there are no screening antigens for "fungi" in general. Because fungi are poor antigen, the efficacy of serology varies with different fungal infections. The serologic test will be discussed under each mycosis. The most common serological tests for fungi are based on latex agglutination, double immunodiffusion, complement fixation and enzyme immunoassays. While latex agglutination may favor the detection of IgM antibodies, double immunodiffusion and complement fixation usually detect IgG antibodies. Some EIA tests are being developed to detect both IgG and IgM antibodies. There are some tests which can detect specific fungal antigens, but they are just coming into general use.
4. Direct fluorescent microscopy may be used for identification, even on non-viable cultures or on fixed tissue secton. The reagents for this test are difficult to obtain.
5. Biopsy and histopathology. A biopsy may be very useful for the identification and as a source of the tissue-invading fungi. Usually the Gomori methenamine silver (GMS) stain is used to reveal the organism which stain black against a green background. The H&E stain does not always tint the organism, but it will stain the inflammatory cells.
6. Culture. A definite diagnosis requires a culture and identification. Pathogenic fungi are usually grown on Saboouraud dextrose agar. It has a slightly acidic pH (5,6), cyclohezamide, penicillin, streptomycin or other inhibitory antibiotic are often added to prevent bacterial contamination and overgrowth. Two cultures are

inoculated and incubated separately at 25^0C and 37^0C to reveal dimorphism. The cultures are examined macroscopically and microscopically. They are not considered negative for growth until ahter 4 weeks of incubation.

Reference:

Betsy, T and Keogh, J: (2005) Microbiology demystified. Published by the McGraw-Hill Companies, New York, USA.

LECTURE NOTES
VPM 302: INTRODUCTION TO VETERINARY MICROBIOLOGY

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INTRODUCTION TO VETERINARY VIROLOGY

Viruses are obligatory intracellular infectious agents of sizes ranging from 20 to 300 nanometere with an absolute dependence on living cells for their replication.

DISCOVERY OF VIRUSES

The infections caused by viruses such as Rabies, Rinderpest and Smallpox have been known and feared since the dawn of history but the nature of their aetiology remained a myth. The discovery and study of viruses started sometimes in the late nineteenth century much after those of bacteria and fungi have been established.

In 1892, Dmitri Ivanovsky, a Russian scientist, reported that tobacco mosaic disease could be transmitted from a diseased plant to a healthy one using filtered leaf extract from the diseased plant as inoculums. Ivanovsky used Chamberland filter (designed in Pasteur laboratory by Charles Chamberland) to filter leaf extract from tobacco plant infected with tobacco mosaic. The filtrate was used as inoculums to infect a healthy tobacco plant. Ivanosky observed that the inoculated plant developed the disease tobacco mosaic. He then reasoned that the agent of tobacco mosaic couldn't have been a bacterium since Chamberland filter could hold back even the smallest bacterium. The agent must be smaller than bacteria to have passed through the filter. In 1898, Martinus Beijerinck (a Dutch) unaware of the discovery by Ivanovsky also demonstrated the filterability of the agent of tobacco mosaic disease. He also showed that the disease couldn't have been due to a toxin because the filtered sap from infected plant could be used for serial transmission of the disease without loss of potency. The filterable disease-causing agent was termed '*virus*' meaning 'poisonous fluid'.

In 1898, Loeffler and Frosch showed that Foot-and-Mouth-Disease was caused by a filterable agent. In 1901, Walter Reed and his team identified Yellow Fever Virus as a filterable pathogen in humans.

General Characteristics and Properties of Viruses

1. Size: viruses are very small retaining infectivity after passing through filter with pore size small enough to hold back the smallest bacteria. Bacteria are measured in terms of micrometer (μm, 10^{-6} of a metre) whereas viruses are measured in nanometer (nm, 10^{-9} of a metre). Viruses range in size from 20nm to 300nm. The picornaviruses (e.g. Foot-and-Mouth-Disease virus) are the smallest viruses (20nm) while the poxviruses are the largest viruses (300nm). Viruses can not be seen by light microscope because of their small size. They are seen only by the aid of electron microscope. However, poxviruses can be seen by light microscope.

2. Organelles: Viruses do not possess cellular organization and do not have organelles.

3. Genome size: The genomes of viruses are smaller than those of bacteria, ranging from about 2 kilobase pairs (kbp) to 200 kbp.

4. Viruses are completely dependent on living cells, either eukaryotes or prokaryotes for replication and existence. Although some viruses possess their own enzymes such as RNA-dependent RNA polymerase or reverse transcriptase, they cannot reproduce and amplify the information in their genomes without the assistance of the cellular machinery. They do not grow in inanimate/non-living media.

5. Viruses have their genetic information in either DNA or RNA. A virus possesses only one species of nucleic acid either DNA or RNA but never both.

6. Viruses have a receptor-binding protein component for attaching to cells so that they can enter such cell and take over the machinery of the cell to their own advantage in reproducing their progeny.

7. Viruses do not multiply by binary fission but by a complex process involving protein synthesis and nucleic acid production

8. Viruses are unaffected by antibiotics

9. Viruses induce the production of interferon by infected host cell and are sensitive to the interferons.

STRUCTURE AND MORPHOLOGY OF VIRUSES

The knowledge of the structure of viruses is important in their identification and classification into groups. It is also helps us to deduce many important properties of a particular virus. For instance, the processes of attachment and penetration of virus into host cell and subsequent maturation and release vary greatly among viruses and is influenced by the presence or absence of an outer lipid-containing envelope. Enveloped viruses tend to cause fusion of the host cell at some stage during their entry. Non-envelope viruses are more resistant to heat and detergents. These have consequences for control of viral infection especially disinfection of premises following an outbreak.

Basic Components of Viruses

A virus particle or virion is consists of **nucleic acid (DNA or RNA)** that is covered by a protein coat called **capsid**. The combined nucleic acid and capsid is called **nucleocapsid**. The nucleocapsid can either be naked or enclosed by a membrane termed **envelope**. The capsid itself is made up of subunits called **capsomere**.

The proteins that make up the virus particle are termed **structural proteins**. The viral genome also codes for important enzymes called **non-structural proteins** required for viral replication but are not incorporated in the virion.

Viral Nucleic Acid

- This can either be RNA or DNA

- It contains the information necessary for directing the infected cells to synthesis virus-specific proteins

- It may be single stranded or double stranded

- It may be linear or circular

- It may be positive sense or negative sense (a positive sense nucleic acid possesses the same polarity as the mRNA and so can be translated directly into protein without first being transcribed)

- It may be a single piece or segmented

- It is haploid except in retroviruses in which it is diploid

The Capsid

- It is made up of proteins arranged in multiple almost identical units called capsomere

- It offers protection for the nucleic acid against adverse conditions

- It facilitates attachment and entry of the virus into host cell

- It possesses antigens used for virus identification in serological tests

- It determines the symmetry of the virus

The Envelope

- Present only in some viruses

- It is made up of lipids

- It is derived from the plasma membrane of the host cell during the release of the virus from the cell by budding

- In enveloped viruses, capsomeres take the form of projections called spikes or peplomers protruding out through the lipid bilayer of the envelope

- The spikes are glycoprotein in nature

- There may be a stabilizing protein membrane beneath the envelope lipid bilayer. This is referred to as the membrane/matrix protein

- In some viral infections, the envelope is acquired from the endoplasmic reticulum, the Golgi apparatus or the nuclear membrane

- Enveloped viruses are usually susceptible to detergent and are rendered non-infectiuos following damage to the envelope
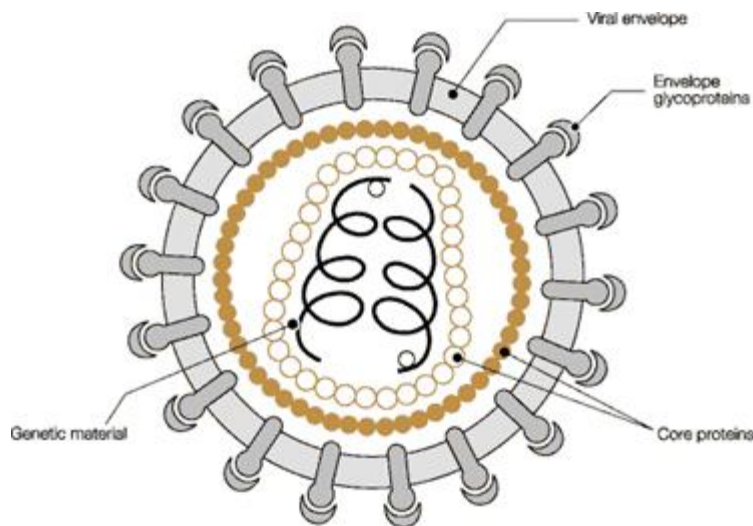

Fig 1 - Structure of a Virus (From: Encyclopedia of Molecular Biology, Blackwell Science Ltd, 1994)
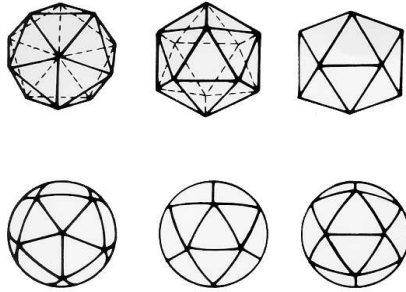
VIRUS SYMMETRY

The orderly structural arrangement of similar protein to protein interface in viruses gives rise to a symmetrical structure. With the help of electron microscopy, it has been found that the morphology of nearly all viruses conforms to one of two basic symmetrical patterns which could be:

1. Cubic/icosahedral

2. Helical

These are the two capsid symmetries described for all viruses
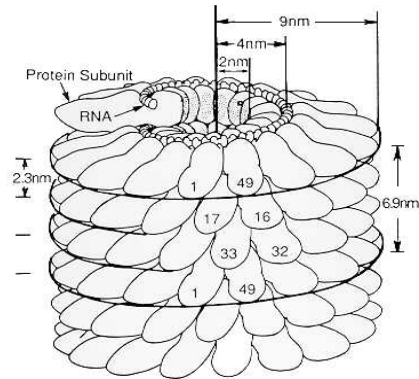
Cubic/Icosahedral symmetry

- Highly structured capsid in which capsomeres are arranged in form of an icosahedrons

- In the icosahedrons, there are 20 triangular faces and 12 apices/corners

- The capsomeres of each face form an equilateral triangle

- The capsomeres contribute to the rigidity of the capsid and help protect the nucleic acid genome

- Each individual capsomere may consist of several polypeptides (in poliovirus, the capsomere is madeup of three proteins)

- All DNA viruses of animals except poxviruses as well as some RNA viruses possess icosahedral symmetry

- Viruses with icosahedral symmetry could be naked (without envelope) or enveloped

- Icosahedral capsids are generally assembled in the host cell prior to incorporation of the viral nucleic acid. Some viral preparations may contain capsids devoid of nucleic acids.

Icosahedral models (left to right) on fivefold, threefold, and twofold axes of rotational symmetry (Copyright © 1996 <u>The University of Texas Medical Branch at Galveston</u>)

Helical Symmetry

- Single stranded RNA viruses such as paramyxoviruses, orthomyxoviruses and rhabdoviruses have helical symmetry

- The capsid is in form of a helix

- Helical viruses resemble long rods that may be rigid or flexible

- The flexuous helical nucleocapsid is always contained within a lipoprotein envelope

- The envelope is lined internally by a matrix protein (M-protein)

- The M-protein may be rigid as in the case of bullet shaped rhabdoviruses or readily distorted as in orthomyxoviruses and paramyxoviruses

- The capsid of helical viruses is formed by the insertion of protein units between each turn of the nucleic acid helix.

- The capsid protein helix thus coincides with that of the nucleic acid and the length of the helix is determined by the length of the RNA molecule

- Helical capsid devoid of nucleic acid cannot be formed.

- In RNA viruses, each capsomere consists of a single polypeptide molecule

The helical structure of the rigid tobacco mosaic virus rod (Copyright © 1996 <u>The University of Texas Medical Branch at Galveston</u>)

Complex Symmetry

- Large viruses with large genome have complicated symmetry

- For example, poxviruses have complex symmetry which is neither icosahedral nor helical

CLASSIFICATION OF VIRUSES

Following the discovery of viruses, earliest studies on them were based on their filterability, and observations on the diseases which they caused. Early classification systems were premised on pathogenic effects of the viruses and their transmission patterns. However, with the invention of electron microscope and sophisticated molecular techniques that permitted ultra-structural studies, details of the structures and compositions of viruses began to emerge. Thereafter, it became possible to group viruses on the basis of shared features of the virions.

Consequently, the following general parameters are have been used for classification of viruses:

1. Pathogenicity

2. Ecological characteristics

3. Physico-chemical characteristics

Pathogenicity:

In this classification, viruses affecting same tissues producing similar syndrome and pathological manifestations are grouped together.

- Viruses affecting the respiratory tract: Influenza, rhinoviruses, parainfluenza, adenovirus

- Vesicular viruses: Foot-and-Mouth-Disease, vesicular stomatitis

- Central nervous system viruses: rabies, equine encephalitis, entroviruses (polio), mumps

- Mucous membrane viruses: Myxomaviruses

- Enteric viruses: rotaviruses,

- Limitations of this classification system: some viruses affect more than one system of the body and they will belong to several groups. Pantropic viruses affecting multiple systems such as canine distemper, Newcastle disease, rinderpest, pestes des petits ruminants will belong to respiratory, enteric and CNS groups.

Ecological characteristics:

Ecological features of viruses such as the involvement of vectors or vertebrate reservoirs in their transmission cycles and maintenance in nature can be used for classification. Viruses are classified into arboviruses and non-arboviruses or roboviruses and non-roboviruses.

Arboviruses: these are viruses that are transmitted biologically between blood sucking arthropods (such as ticks, culicoides, mosquitoes) and vertebrate hosts. They cause disease in the vertebrate host but not in the arthropods. Examples include African swine

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fever virus (soft ticks), Yellow fever virus (mosquitoes), Equine encephalitis virus (mosquitoes), African horse sickness virus (culicoides).

Roboviruses: These are viruses with rodent reservoirs. Infected rodents are asymptomatic. They shed the virus in their urine and contaminate the human surroundings, food, drinks and formites. Example include Lassa fever virus with rat as reservoir.

Physico-chemical characteristics:

These are the most reliable, verifiable and satisfactory parameters for classifying viruses.

Viruses are classified based on the following criteria:

- Type of nucleic acid (RNA or DNA)

- Number of strands of the nucleic acid (single or double stranded)

- Physical construction of the nucleic acid (linear, circular, circular with break, segmented, non-segmented)

- Polarity of the viral genome: positive polarity (viral genome can be used directly as mRNA) and negative polarity(viral genome must be transcribed into mRNA)

- Symmetry of the nucleocapsid

- Presence or absence of envelope

- Size of the virus

- Antigenic and chemical compositions

- Susceptibility to physical and chemical changes

Based on these criteria, viruses are grouped into families, subfamilies and genera. Further subdivision is based on the degree of antigenic similarity and serological tests.

Antigenically identical viruses can be further grouped by differences in biological characteristics including virulence, cellular receptors and nucleotide sequences.

VIRUS NOMENCLATURE

The nomenclature of viruses has been in a constant state of flux for many years. This is may not be unconnected with emerging facts about the composition and morphology of viruses with advanced studies made possible by new technologies.

- Some viruses are named according to the type of disease they cause. Examples include poxviruses, herpesviruses (creeping lesions)

- Other are named based on acronyms of disease (papovavirus   -papilloma, -polyoma – vacuolating) or acronym of observable characteristics (picornavirus   -pico/small – rna –virus)

- Viruse are also named based on morphology as revealed by electron microscopy. Coronaviruses (halo or corona/crown of spikes), Togavirus (Toga/cloak), Rhabdovirus (Rhabdo/Rod-shaped), Calicivirus (Calix/cup-shaped depression)

- Some viruses are named after geographical regions where they were first isolated (E. g. Coxsackie-, Marburg-, Gumboro-, Mokola- virus)

- Occasionally, viruses are named after individual discoverer (Epstein-Barr virus)

The International Committee on Taxonomy of Viruses (ICTV) was established in 1973 to develop and expand the universal scheme in which characteristics of virions are used to assign them to five hierarchical levels (order, family, subfamily, genus and species). The hierarchical levels are denoted with the following suffixes:

Order: -virale

Family: -viridae

Subfamily: -virinae

Genus: -virus

Species: -virus

The primary considerations for virus taxonomy are the type and nature of the genome, the mode and site of replication and the structure and morphology of the virion.

Virus orders are designated by the suffix –virale. Phylogenetically-related families are grouped together. Many virus families are yet to be assigned into orders. Only two orders containing viruses of animals are so far recognized. The virus order Mononegavirale is made up the families Paramyxoviridae, Rhabdoviridae, Bornaviridae and Filoviridae. Members of the order Mononegavirale have common attributes including a single stranded, non-segmented, negative sense RNA genome. Their replication strategies are also similar. The second viral order is the Nidovirale comprising of the families Coronaviridae and Arteriviridae.

REPLICATION OF VIRUSES

Viruses rely completely on living host cells for their replication. The small genome size put them at disadvantage. Also, they lack organelles and other machineries required for protein synthesis. Although some viruses enter the host cell with few virus-encoded enzymes, others do not possess any protein of their own and therefore depend completely on those produced by the host cell. Virus replication is facilitated by the host cell which provides the required energy and synthetic machinery and sometimes essential enzymes for replication and also by the viral nucleic acid which carries the genetic information required for the synthesis of viral components.

The replicative cycle of a virus can be divided into a number of stages:

1. Attachment to surface receptors on a susceptible host cell

2. Entry into the cell

3. Uncoating of the viral nucleic acid

4. Transcription of the viral nucleic acid and translation of mRNA for synthesis of virus-encoded proteins

5. Replication of the viral nucleic acid into progeny/daughter nucleic acid

6. Assembly of newly formed virus particles

7. Release of the daughter virions from the host cell

The duration of the replicative cycle ranges from 6 to 40 hours. Infection of a susceptible host c ell is usually followed by an eclipse phase.

Eclipse phase: this is the initial stage of virus replication whereby the infecting virus loses its physical identity and most or all of its infectivity. At this time, no virus is detectable in the infected host. The eclipse phase is followed by the productive stage as new virus particles are formed and released from the cell.

Steps in virus replication

Attachment: viruses have evolved to the point where they can utilize a wide range of essential host cell surface protein as receptors. They bind with their own receptor-binding proteins (ligands) to the receptors on the host cell plasma membrane. Virus receptors on cells could be glycoproteins or glycolipids. The interaction between the virus receptor-binding proteins and the corresponding receptor on the host cell contributes to host specificity.

Entry: following attachment, the virus gains access to the host cell internal environment where replication takes place. Viruses employ three different mechanisms for this internalization:

I. Receptor-mediated endocytosis (viropexis): The site of virus attachment to the plasma membrane is coated internally with the protein clathrin and the virus-receptor complex is taken into the cell in a manner similar to phagocytosis. A cage-like lattice, in form of endosome (vesicle) is then formed after internalization. Fusion of the endosome with lysome degrades the membrane and the nucleocapsid of the virus is released (seen in rhabdoviruses, orthomyxoviruses and flaviviruses). Acidification of the clathrin coated cage-like structure within the host cell cytoplasm also leads to breakdown of the viral structures in certain viruses.

II. Fusion of viral envelope with the plasma membrane of the host cell (seen in retroviruses, herpesviruses and paramyxoviruses)

III. Direct introduction of viral genome into the cytoplasm (injection) through channels in the plasma membrane. This is seen in some non-envelope viruses such as picornaviruses.

Uncoating: this implies the release of viral genome from the nucleocapsid for transcription to take place. However, in certain viruses, transcription may proceed without complete release of the viral genome. The genome of reoviruses may be fully expressed without being fully uncoated. The mechanism involved in the process of uncoating is not fully understood. Uncoating may occur on the cell membrane, cytoplasm, or nucleus and is facilitated by celluar enzymes in some viruses. In poxviruses, uncoating takes place in two stages. The initial stage is facilitated by host cell

enzymes while the later stage is mediated by virus-specified proteins. In non-envelope viruses, uncoating may be due to proteolytic activity of lysosomal enzymes. Uncoating leads to loss of virus infectivity.

Replication of viral nucleic acid and synthesis of viral proteins: there is synthesis of viral nucleic acid using the viral genome as template. The nucleic acid may be messenger RNA or replicative intermediates. Proteins including enzymes (non-structural proteins) and capsids (structural proteins) are also produced. Regulatory proteins which shut down the normal cellular metabolic processes and direct sequential production of viral molecules are also synthesized. Replication of DNA viruses takes place in the nucleus of the host cell except poxviruses in which case replication takes place in the cytoplasm. The RNA viruses replicate in the cytoplasm except orthomyxoviruses which require host hoost DNA transcription, paramyxoviruses which have a non-obligatory nuclear pahse of replication and retroviruses which replicate via a DNA intermediate (provirus).

Major activities occurring at this stage include:

- Transcription of mRNA from viral genome
- Translation of mRNA into early protein which initiate and maintain the synthesis of virus components and shut down the host protein and nucleic acid sunthesis
- Replication of viral nucleic acid destined for encapsidation
- Synthesis of late proteins which are the components of daughter virion capsid

Transcription: the mechanisms of transcription and nucleic acid synthesis differ in different types of viruses. In single stranded (ss) nucleic acid, complimentary strand is first synthesized producing double stranded (ds) replicative intermediates. In picornaviruses, ssRNA acts directly as mRNA because of their positive polarity. The

parental positive-sense strand acts as template for production of complimentary strand with negative sense. The negative sense complimentary strand then acts as the template for the synthesis of the positive sense progeny viral nucleic acid. In single stranded negative sense RNA viruses (rhabdoviruses), a complimentary positive sense RNA is produced from the parental negative sense RNA. The complimentary positive sense RNA then acts both as mRNA for protein synthesis and as the template for the synthesis of negative sense progeny viral RNA destined for encapsidation. Retroviruses exhibit a unique replicative process. The virus ssRNA genome is first converted into a RNA-DNA hybrid by the action of the viral enzyme reverse transcriptase (an RNA-dependent DNA polymerase). The RNA strand of the RNA-DNA hybrid is removed after the synthesis of a DNA strand complimentary to the DNA strand of the RNA-DNA hybrid. This results in the formation of a dsDNA. The dsDNA (provirus) is then integrated into the host cell genome where it acts as the template for the synthesis of progeny viral RNA. The integration of the provirus into the host cell genome may cause transformation of the cell and development of neoplasm.

Translation: this is the synthesis of viral proteins. It involves the attachment of the mRNA to ribosomes in the cytoplasm of the host cell. The genetic information contained in the mRNA is used to order specific amino acid for protein synthesis including capsid protein and enzymes.

Assembly/maturation: Assembly of daughter virion may take place in the nucleus (DNA viruses) or in the cytoplasm (RNA viruses). The progeny nucleic acid is incorporated into the capsids which are preformed (spontaneously produced capsid referred to as procasids). Non-envelope viruses are afterwards present in the host cell as

fully developed virion while envelope viruses acquire their envelope from plasma membrane during their release.

Release: envelope viruses are usually released by budding from the plasma membrane of the host cell. In non-envelope viruses, release is by cytolysis (disintegration) of the host cell.

DIAGNOSIS OF VIRAL DISEASES

There are well over 1000 known viruses of vertebrate and it is impossible for a single laboratory to have all the resources required for the diagnosis of all these viruses. Laboratories tend to specialize on particular viruses and serve as reference laboratories.

Stages of viral disease diagnosis:

1. Clinic: presumptive diagnosis based on clinical signs and history

2. Pathology: Observed lesions and pathological changes at gross and histopathological levels. History may provide clues.

3. Microbiological diagnosis: confirmatory diagnosis

Principles of microbiological diagnosis:

i. Isolation of viruses

ii. Detection of viral nucleic acid/specific genes

iii. Detection of viral antigen

iv. Detection of specific virus-induced antibody

Sample collection:

a. Samples must be collected at from the right site and at the right time

b. Samples to be collected must relate to the clinical signs and pathological changes observed

c. Samples must be collected as soon as clinical signs are observed

d. Knowledge of the pathogenesis of the disease may dictate the type of sample to be collected

e. Proper labeling of samples for identification and to avoid confusion

f. Samples must be sent to the laboratory with history and tentative diagnosis

g. Transport samples with ice packs (4 ^{O}C) if transit time is less than 24 hours

h. For transit above 24 hours, use dry ice at -70 ^{O}C.

i. Long term storage is achieved with liquid nitrogen at -196 ^{O}C.

j. Use transport medium containing buffer (isotonic saline) with bovine albumin/foetal calf serum (protein to prolong virus survival), antibiotic and antifungal agent (to prevent contaminants)

Samples for diagnosis of viral diseases:

- Respiratory tract infection: nasal swab, tracheal swab, nasopharyngeal aspirate, lung tissue.

- Enteric infection: Faeces, rectal swab.

- Genital tract infection: prepucial washing, semen, genital swab.

- Eye infection: Conjunctival swab.

- Skin infection: Vesicular fluid, epithelial scrapings, biopsy of solid lesions.

- Central nervous system: cerebrospinal fluid, faeces, nasal swab, brain tissue.

- Generalized infection: Nasal swab, faeces, blood leukocytes.

- Post mortem examination: relevant organ.

- Every case: Blood for serum to be used in serology.

Virus isolation:

Advantages:

- It allows for further studies on the virus isolate

- It is required for preparation of vaccines

- It is required for preparation of antigens for rapid diagnostic kits

- It is highly sensitive and reliable

Disadvantages:

- It is slow and time consuming

- It is labour intensive

- It is expensive to acquire and maintain required facilities

- Some viruses may not grow

- Selection of appropriate media may require critical consideration

Methods of virus isolation

1. Virus isolation in cell culture

2. Virus isolation in embryonated egg

3. Virus isolation in laboratory animals

4. Virus isolation in susceptible host

5. Virus isolation in arthropods

Virus isolation in cell culture

➢ This is the most commonly used method of virus isolation.

➢ It involves inoculation of sample onto confluent tissue culture monolayer.

➢ The inoculated tissue culture monolayer is incubated at 35-37 ^{O}C.

- The culture is examined daily for evidence of virus growth (cytopathic effect, interferon, haemadsorption and antigen detection).

- Virus is harvested from the culture by freeze-thawing and low-speed centrifugation

- The supernatant is used as antigen for virus identification

- The effect of the growing virus in cell culture can be detected by light microscopy

Types of tissue culture

Primary tissue culture: this is made directly from tissue. It may involve one or two passage. In primary tissue culture, there are mixed cell types. It is good for isolation of influenza virus, parainfluenza virus and enteroviruses. Three types are recognized: monolayer, suspension and organ culture.

a. Monolayer: derived from tissue taken directly from the susceptible host (monkey kidney, mouse embryo). The tissue is cut into very small pieces and digested into cells by proteolytic enzymes such as trypsin or collagenase. The cells are grown on glass or plastic surface as monolayer in as artificial medium containing growth factors supplemented with foetal calf serum and antibiotics.

Type of artificial medium:

- Growth medium: for cell cultivation. Its constituents include salts at physiological concentration, glucose, amino acids, vitamins, antibiotics and antifungal agents and 10-20% foetal calf serum. It is maintained at pH 7.2-7.4. After formation of confluent monolayer, the growth medium is changed to maintenance medium.

- Maintenance medium: contains similar constituents as growth medium except that the foetal calf serum is 2 -5% for survival of cells and no further division.

b. <u>Suspension culture</u>: cells are grown in form of suspensions in growth medium and not monolayer. Lymphocyte cultures are cultivated in suspension.

c. <u>Organ culture:</u> organ culture are not trypsinized into cell but grown as whole. Examples include tracheal ring organ culture.

<u>Secondary culture:</u> they are obtained from primary culture by passages or subcultivation. Repeated passages of primary culture will produce cell lines with almost homogenous cell type. Types of secondary culture include semi continuous cell line and continuous/established cell line.

a. <u>Semi continuous cell line</u>: this is derived from fibroblastic cell of animal or human foetal tissue. They have been subcultured through about 50 passages (generations of repeated subcultivation). They have diploid number of chromosome. They are used for vaccine production. They have limited life span. Examples include HDCS, MRC-9, WI-38. This type of cell culture is good for herpes simplex and rhinovirus.

b. <u>Continuous cell line</u>: this is also called established cell line. Hey are widely used fro diagnostic purposes. They are derived from neoplastic cells or from normal cells that have been transformed by repeated subcultivation to behave like tumour cells. Continuous cell line can grow indefinitely. They have heteroploid (aneuploid) chromosome (variable/abnormal number of chromosome). It is good for vaccine production and research purposes. Examples include Mardin Darby bovine kidney (MDBK), MDCK, Vero cell (African green monkey kidney cells), Hep, HeLa, Crandell feline kidney (CRFK).

Detection of viral growth in cell culture

- Observation for cytopathic effect: evidence of growth of virus in cell culture is by the detection of cytopathic effect (CPE). Cytopathic effect is defined as degenerative changes caused by the growth of viruses in cell culture or virus-induced damage in cell culture. The following are examples of cytopathic effect which are usually observed by microscopy:

1. Cell lysis or cell disintegration

2. Syncythial formation: formation of multinucleated giant cell due to cell fusion. This is found with lentivirus, herpesviruses, paramyxoviruses

3. Rounding up of cell or cellular transformation (change from spindle shape to spherical shape)

4. Formation of intranuclear (adenovirus, herpesvirus, parvovirus) or intracytoplasmic (poxvirus, rhabdovirus, reovirus) inclusion bodies.

Viruses can be categorized into *burster* or *creeper* viruses based on the type of CPE produced.

Burster (lytic) viruses: these induce cell lysis and cellular transformation in cell culture

Creeper viruses: these induce formation of multinucleated giant cells.

- Plaques: Macroscopic/gross observation of CPE on monolayer cell by overlaying with molten agar. Plaques are foci of dead virus-infected cells which do not take up the stain (acridine dye) when stained and so appear as clear spots in a stained monolayer.

- Haemadsorption: adherence of erythrocytes to monolayer cells because of the growth of haemagglutinating virus in the cells. Haemagglutinin is expressed on the cell membrane of cells in monolayer and this attracts and binds erythrocytes. Feline panleukopaenia virus haemagglutinate porcine erythrocyte while porcine parvovirus haemagglutinate chick, guinea pig, monkey, human and cat erythrocytes.

- Immunofluorescence

- Interference

- Virus neutralization test

- Haemagglutination inhibition test

Virus isolation in embryonated egg

➢ This is no longer widely used

➢ However, it remains the most preferred for isolation of influenza A virus and avian viruses

➢ 10 to 12 day old embryonated eggs are used

➢ Eggs must be from specific pathogen-free flocks

Route of administration into embryonated egg: this is determined by tissue affinity of the particular virus and includes

- Allantoic cavity

- Amniotic cavity

- Yolk sac

- Chorioallantoic membrane (CAM)

- Intravascular inoculation in well-developed chick embryo

Evidence of virus growth in embryonated egg:

- Death of the embryo

- Dwarfing/stunting of embryo

- Formation of pock on the CAM

- Virus isolation in laboratory animals

Virus isolation in laboratory animals

Suckling mice: suckling mice is used for cultivation of arthropod-borne viruses. Suckling mice is inoculated intracerebrally and observed for signs of disease and death. Virus is identified from specimen collected from infected animal by complement fixation test, virus neutralization test or by other rapid diagnostic method.

Virus isolation in susceptible host

This involves inoculation of natural host species of the virus. This is used for evaluation of vaccine, production of polyclonal antibody and for pathogenicity testing/verification of Koch's postulate.

Virus isolation in arthropods

This is not a common method and is obsolete. Example includes isolation of Dengue virus in adult *Toxorhynchites*, male *Aedes aegypti* and *Aedes albopictus* mosquitoes by intrathoracic inoculation.

RAPID DIAGNOSTIC TECHNIQUES

These include:

1. Electron microscopy
2. Viral nucleic acid detection
3. Serology/detection of viral antigen and antibody

1. Electron microscopy

This is used to demonstrate the presence of virus in clinical specimens and to study the morphology or symmetry of the virus. With electron microscopy, it is possible to recognized mixed viral infection. It is also used for detection of non-viable viruses and those that cannot be grown *in vitro*. However, equipment required for the procedure is

rather expensive. Besides, large number of viral particles in excess of 10^6/ml must be present in the sample before they can be detected. It is difficult to differentiate viruses with similar morphology especially those from the same family with electron microscopy.

Method:

- Homogenize sample.

- Centrifuge at low speed to remove large particulate debris.

- Ultracentrifugation to sediment available virus particles.

- Negative staining with heavy metal compound such as phosphotungstic acid or uranyl acetate. (negative staining stains the background to increase contrast so that bright virion stand out against a dark background).

- Addition of immune serum (immunoelectron microscopy) to increase sensitivity by clumping/agglutinating virus particles and enhance recovery following centrifugation).

- Observe at X13,000-100,000 magnification.

2. **Detection of viral nucleic acid:**

Oligonucleotide probes are used for the detection of viral DNA or RNA. Insufficient viral nucleic acid in sample is increased by amplification using polymerase chain reaction. Probes anneal to the targeted viral nucleic acid sequence which can be the whole genome, specific gene or nucleic acid segment. Variable or conserved sequence can be targeted. Double stranded genomes are first separated by heating. Oligonucleotide probes are labeled with radioactive isotopes such as ^{32}P or ^{35}S to allow viewing. Non-radioactive

labels such as alkaline phosphatase fluorescein and horse radish peroxidase can be used for direct viewing whilebiotin and digoxigenin are used for indirect viewing.

I. Dot-blot hybridization

- Nucleic acid, usually DNA is extracted from sample

- Extracted nucleic acid is spotted directly onto charged nylon or nitrocellulose membrane

- Nucleic acid binds firmly onto membrane after baking

- Fluorescent dye- or radioisotope- labeled probe is added

- The membrane is washed to remove unbound materials

- Binding of probe to targeted nucleic acid is detected by autoradiography or by colour precipitation

II. In-situ hybridization

Viral nucleic as is detected in frozen section of infected cells with the aid of labeled oligonucleotide probes. Intracellular location of viral nucleic acid is revealed by autoradiography or immunoperoxidase cytochemistry

III. Southern blot hybridization

- Restriction enzymes are used to cleave DNA into short oligonucleotides

- Oligonucleotides are separated by agarose electrophoresis or acrylamide gel electrophoresis

- Separated oligonucleotides are transferred by blotting onto nitrocellulose membrane or nylon

- Probes are added and reaction detected by autoradiography or by colour development

IV. Northern blot hybridization

RNA hybridization similar to southern blot

V. **Western blot**

Application to protein identification

VI. **Polymerase chain reaction**

- Extraction of DNA or RNA nucleic acid from sample

- Amplification of the extracted nucleic acid in a series of repeated cycles of denaturation, primer annealing and polymerization using heat-resistant Taq (*Thermus aquaticus*) polymerase. Specific primers recognize and bind to the targeted gene to initiate the amplification reaction.

- Amplified sequences are stained with ethidium bromide and separated by electrophoresis

- The separated sequence is viewed under ultraviolet transillumination.

- This procedure is very specific and highly sensitive

3. **Serology**

For the detection of viral antigen or virus-induced antibody in the serum of the host

I. **Immunodiffusion**

Immunodiffusion is the simplest and most direct method for detecting antigen-antibody reaction. It is cheap and easy to perform but of low sensitivity. It is used for the detection of reaction of an antigen with its antibody by formation of precipitation. This reaction is influenced by the relative concentrations of antigens and antibody. Optimal precipitation is seen in the region of equivalence and decreases in the zones of antigen excess or antibody excess. Immunodiffusion can be categorized as single or double.

i. Single radial diffusion

This was introduced by Mancini in 1965. In single immunodiffusion, either antigen or antibody is static and the other reactant remains free to migrate and complex with the static component. Single radial immunodiffusion can be used to quantify the amount of serum protein or immunoglobulin in a sample.

Method:

- Molten agar mixed with a specific antibody to a particular antigen is poured into a Petri dish

- After the agar solidifies, a well is made at the centre

- A precisely measured amount of sample containing the antigen is poured into the well

- The antigen is allowed to diffuse radially from the well for 24 – 48 hours.

- Where the antigen meets its antibody, a precipitin ring is formed.

- The diameter of the ring is measured by a precision viewer (a calibrated microscope)

- The diameter of the ring correspond to the concentration of the antigen in the sample

- A 'standard' is prepared for comparison

- To prepare the standard, a serial 1 in 2 dilution of known quantity of standard antigen is prepared.

- About four wells are created in the agar and each dilution of the standard placed in different wells

- This is allowed to react and form precipitin rings at the same time interval as the test sample

- The amount of antigen in the test sample is calculated and compared with those observed in the different dilutions of the standard.

ii. Double diffusion in agar (Ouchterlony test)

In this technique, both the antigen and the antibody are free to move towards each other through a semisolid medium (agar) and form detectable precipitate (immune complex). This method permits the comparison of the relatedness of antigens in different samples.

Method

- Molten agar is poured into a Petri dish or layered on a glass slide.

- The agar is allowed to solidify and small wells of equal diameter punched in the agar

- One central well and 6-8 peripheral wells a few millimeter apart are thus made

- Known antibody is put in the central well

- Different test samples containing antigens or different serial dilutions of a particular sample are placed in the peripheral wells

- These are allowed to diffuse towards one another and react to for precipitin line in a moist chamber for 18 – 24 hours

- Observe for precipitin lines

- The concentrations on the antigen and the antibody determine the thickness of the precipitin line and its distance between the wells.

- The types of patterns of precipitin lines shows the relatedness (complete identity, partial identity and non-identity) of the antigens in the peripheral wells

II. Immunofluorescence (IF) (Direct method)

- Smear of clinical sample is made of microscope slide

- The sample is reacted with specific antiserum coupled with fluorescein isothiocyanate (FICT) fluorescent dye

- Excess antiserum is washed away

- View under ultraviolet microscope

- FICT-labelled antigen-antibody complex is visible as a green fluorescence in positive sample

 Immunofluorescence (indirect method)

- Similar to the direct IF except that the specific antiserum is not labeled

- Fluorescein-labelled antiglobulin is added after the addition of the unlabelled specific antiserum to detect the presence of antigen-antibody complex

- This method is called the 'sandwich' method because there are three layers instead of two as seen in the direct method. The three layers are: the specimen to be tested which is the source of antigen, unlabelled virus specific antiserum (prepared in rabbit) and fluorescein isothiocyanate-labelled antirabbit antiserum

- The advantage of this method is that only one labeled serum (antispecies antiserum) is required to test for many viruses.

Note: Immunoperoxidase (enzyme) can be used as the label. It will react with its substrate to produce a precipitate that can be detected by light microscope without the need for ultraviolet microscope.

III. Enzyme Linked Immunosorbent Assay (ELISA) and Radioimmuno Assay (RIA)

- Polystyrene wells of a microtitre plate are coated with specific capture antibody

- Specimen from which antigen is to be detected is added

- Excess is washed away

- Enzyme-labelled specific antibody (detector antibody) is added. Enzyme can be horse radish peroxidase or alkaline phosphatase

- Excess is washed away

- Substrate that will react with the bound enzyme on the labeled specific antibody is added

- Reaction of the substrate with the enzyme produces a visible colour change which is detectable by spectrophotometry

- RIA is similar to ELISA but the label is radioactive iodine (^{125}I). Bound antibody is measured by a gamma counter. This technique is now obsolete because of health hazard associated with radioactive label used and has been superseded by ELISA.

IV. Virus neutralization test

- In microtitre plate wells, make 1:2 serial dilution of serum from which antibody is to be detected

- Add constant amount of stock virus of interest to each of the wells

- Add equal amount of susceptible cell to the wells

- Observe for CPE

- There will be no CPE in positive serum sample with high antibody titre because of the neutralizing effect of the antibody on the stock virus

- End-point is where virus neutralization stops (that is the well just before the onset of CPE)

V. Haemagglutination Inhibition (HAI) Test

- This assay is specific, easy and reliable

- Make twofold dilution of test serum

- Add known concentration of stock virus of interest (usually 4 haemagglutinating/HA unit)

- Add 0.5% erythrocyte

- Observe for haemagglutination

- There will be no haemagglutination in positive serum sample with high antibody titre because of the inhibitory effect of the antibody on the stock virus

- The highest dilution that inhibit erythrocyte agglutination is the HAI titre

- Non-specific inhibition of haemagglutination in sera can be inactivated by heating at 56 ^{o}C for 30 minutes or by treatment with kaolin, trypsin periodate or bacterial neuraminidase

VI. Complement fixation test

This method is no longer widely used. It is based on the principle that the reaction of an antigen with its antibody results in the formation of immune complex which activates and fixes complement. Free (unfixed) complement causes haemolysis while fixed complement does not cause haemolysis. The technique is not very sensitive and large amount of virus/antigen is required in the sample.

Method

- A known antiserum is heat-treated to destroy complement

- The test sample is added to the known antiserum

- The mixture is incubated and allowed to react

- A precise amount of guinea pig complement is added

- If the test sample is positive (presence of virus/antige), antigen-antibody complex is formed with the known antiserum. This will fix the guinea pig complement

- Sheep red blood cells treated with specific rabbit antibody are added to the reaction as indicator to detect complement fixation

- Unfixed guinea pig complement will cause lysis of the sheep red blood cells indicating a negative reaction

- Fixed complement will not cause lysis of the sheep red blood cells indicating a positive reaction.

INTERFERENCE

Interference occurs when infection of a host cell with one virus prevents superinfection of the same cell with another virus. This is a situation that plays out when the multiplication of a particular virus in a host cell population leads to resistance against superinfection and multiplication of another virus in the same host cell population.

Types of Interference

Heterologous: Interference: Interference between two completely different viruses.

Homologous: Interference: Interference between two related viruses or two strains of viruses.

Autointerference: Interference between a virus and its defective particles**.**

Mechanism of Interference

1. Change on receptor sites thereby preventing the attachment of the second virus

2. Competition for cellular sites and enzymes

3. Altering the metabolic pathway for the replication of the second virus

4. Inducing the production of interferon

INTERFERONS

Interferons were first discovered by Lindenmann and Isaacs in 1957. Interferons are non-viral proteins with molecular weight of about 20 Kilo Dalton (KDa) produced by cells (especially leukocytes and fibroblasts) in response to virus infection and stimulation by natural or synthetic double stranded RNA and Chlamydiae.

Classes of Interferons

1. Type I interferons: These are alpha and beta interferons (IFN-α and IFN-β). Alpha IFN is sunthesized by virus-infected monocytes, macrophages, epithelial cells and fibroblasts while IFN-β is produced by epithelial cells and fibroblasts. They have common receptor and are stable at pH 2.0. Type I interferons are activated by natural killer cells and they enhance antigen processing and presentation to T- and B- lymphocytes.

2. Type II interferon: Gamma interferon (IFN-ɣ). This is produced by activated T-cells in response to previously encountered antigen. It is unstable at pH 2.0.

Properties of Interferons

i.   They are produced very early in the course of virus infection (usually within 48 hours)

ii. They are not produced by virus but by virus-infected cells

iii. They are not virus specific: Interferon produced against a virus is equally effective against another virus

iv. They are species specific: Interferon produced in one species of animals is not effective in another species

v.   They do not directly kill viruses

vi. They do not act as antibody

vii. Double stranded RNA are the principal inducers of interferons

Major functions of Interferons

i.   They inhibit viral replication

ii. They inhibit cell division

iii. They modulate immune response to infection

-    They enhance the display of histocompatibility antigens on cell surfaces which helps in antigen-driven activation of T-cells

- They modulate B- and T- cells activities

- They enhance the cytotoxicity of natural killer (NK) and cytotoxic T cell (T_C)

Interferons have been used as adjuncts to immune-therapy and chemotherapy of cancer because of their immunomodulatory action and inhibitory effect on cell division.

MYCOLOGY

DR. O. E. OJO

DIAGNOSIS OF FUNGAL DISEASES

- Diagnostic cultural procedures for fungi should be carried out in the biosafety cabinet.

- Fungi produce spores which are easily get carried by air and can be dispersed in the environment.

- Dispersal of pathogenic fungi in air is of public health concern.

- Clinical signs and history are essential for making a tentative diagnosis.

- Specimen collection: samples are collected based on observed clinical sings and pathological lesions.

- Superficial nycosis (e.g. dermatophytosis): hair sample and skin scrapings

- Sub-cutaneous and systemic mycoses: biopsy, exudates, aspirates and tissue at postmortem

- Swab samples are inadequate for fungal diagnosis

- Stringent transportation conditions are not required for samples for fungal identification. Do not freeze. Most fungal agents can be recovered from samples in transit for about 14 days

- Penicillin-streptomycin or chloramphenicol is added to combat bacterial contaminants

Diagnostic procedures:

Microscopy:

Direct microscopic examination of wet preparation: skin scrapings and hair are mounted on microscope slide, treated with few drops of 10% KOH for about 2-3 hours to clear the specimen of debris and cover with cover slip. This is examined under the microscope at X40 magnification to view arthropores or hyphae. Phase contrast microscopy helps in

direct microscopy. Direct microscopy is fast, does not need staining and provides clear visualization.

Stained preparations:

- Methylene blue and Gram's methods can be used to demonstrate Yeast cells in samples.

- Moulds are demonstrable by lactophenol cotton blue.

- Wood's lamp: spores of *Microsporum canis* fluoresce when infected hairs are examined by Wood's lamp

- Silver nitrate/methenamine silver impregnation: for demonstration of fungal elements in tissue by impregnation

- Periodic acid Schiff: preferred for the demonstration of fungal elements in histological section. Fungi stain red

- Giemsa and Wright's stain: for biopsy and tissue from post mortem

- Gomori: for biopsy and tissue from post mortem. Fungi stain deep black while the tissue stains green

- Nigrosin and Indian ink: for demonstration of the capsule of *Cryptococcus neoformans*. When stained, budding cells with wide capsule is revealed.

Fungal culture:

Culture media:

- Sabouraud dextrose agar: primarily for isolation of dermatophytes from cutaneous samples or yeasts (*Candida spp*) from clinical samples. This medium has acidic pH (5.5) which inhibits the growth of most bacteria.

- Czepak's agar: for colonial study of *Aspergillus spp*

- Sabhi agar/inhibitory mould agar: recommended for improved recovery of fastidious, slow-growing fungi.

- Potato dextrose agar: for isolation of mould and yeast

- Selective culture: addition of chloramphenicol (16µg/ml), penicillin (20 I.U/ml), streptomycin (40 U/ml), gentamicin (5 µg/ml) and cycloheximide/actidione (0.5mg/ml) increases selectivity by inhibiting contaminations by bacteria and fast growing fungi (e.g. Zygomycetes)

- Enriched media: Brain-heart infusion agar is supplemented with 5% blood. This is used to stimulate the growth of yeast phase of dimorphic fungi. Incubation is at 37 ^{O}C.

Culture procedures:

Fungi are cultured in tubes or in plates (Petri dishes). Clinical samples are inoculated directly onto prepared medium and incubated aerobically.

Tube: agar media are poured and allowed to solidify in slanting position to prevent dehydration during prolonged incubation. The tubes are plugged with cotton wool or covered with screw cap. The cap should not be screwed tightly after inoculation to allow access to oxygen.

Plate: this provides large surface area and good hydration for fungal growth. It allows easy examination and subculture from the plate. The plate is taped round to prevent escape and dispersal of fungal spores.

Incubation condition:

Fungi are cultured aerobically. A flat pan containing water is placed under the incubator to provide humidity.

Conditions for Fungal incubation

Fungi	Temperature (^{O}C)	Duration of Incubation
Dermatophytes	25	2 to 4 weeks
Aspergillus spp	37	1 to 4 days
Pathogenic yeast	37	1 to 4 days
Dimorphic fungi (mould)	25	1 to 4 weeks
Dimorphic fungi (yeast)	37	1 to 4 weeks
Zygomycetes (fast growing fungi)	37	1 to 4 days

Fungal growth identification:

Criteria for identification:

- Condition of growth (temperature and duration of incubation)

- Colonial characteristics: Size and appearance, colour of obverse and reverse sides, surface elevation or depression

- Microscopy: examination of sporing head for conidia, presence or absence of septa, colour =of hyphae (hyaline/colourless, dematiaceous/pigmented), presence of special hyphal structure such as being racquet-shaped and spiral hyphae

- Biochemical reaction of yeasts

- Immunological test/serology: gel diffusion test, complement fixation test, agglutination reactions, delayed hypersensitivity test (purified protein derivatives)

- Molecular identification: using polymerase chain reaction, dot blot hybridization, specific nucleic acid probes and other molecular characterization

CANDIDA SPECIES

Candida spp occur worldwide and can be found as commensals on plant as well as in the digestive and urogenital tracts of humans and animals. There are more than 200 species in the genus *Candida.* However, *Candida albicans* is the species most often implicated in human and animal disease. Other species are *C. tropicalis, C. krusei, C, stellatoides, C. paratropicalis*. *Candida albicans* does not have a sexual stage. It grows aerobically at 37 ^{O}C on a wide range of media including Sabouraud dextrose agar (SDA). Colonies are composed of budding oval cells about 5 by 8µm. *Candida albicans* may exhibit polymorphism (pseudohyphae and true hyphae) in animal tissues. On some culture media under certain conditions, it may also form thick-walled resting cells referred to as chlamysospores (chlamydoconidia).

Clinical conditions

Candida spp are associated with opportunistic infections. These diseases occur sporadically and are associated with immunosupression resulting from malnutrition, metabolic disorders (diabetes mellitus), pregnancy, corticosteroid therapy, stress and elimination of competing bacterial flora subsequent to prolonged antimicrobial use.

Clinical conditions associated with *Candida albicans*

Mycotic stomatitis in puppies, kittens and foals; gastro-oesophageal ulcers in pigs, foals and calves; rumenitis in calves; enteritis and dermatitis in dogs; thrush of the oesophagus and crop in chickens; cloacal and vent infections in geese and turkeys; reduced fertility, abortion and mastitis in cattle; pyometra in horses; urocystitis and pyothorax in cats; ocular lesions in cats and horses and disseminated/generalized infection in dogs, cats, swine and cattle.

Diagnosis:

- Clinical samples: biopsy specimen, tissues from postmortem, sputum, exudates and discharges, skin scrapings, milk, faeces.

- Tissue section stained with methenamine silver impregnation or Periodic acid Schiff to demonstrate budding yeast cells and hyphae

- Isolation of *C. albicans* following aerobic culture of specimen of SDA with or without cyclohexide supplement. Culture is incubated at 37 ^{O}C for 2 to 5 days.

Identification criteria

- Colonial appearance: colonies of most species of *Candida* are similar. The colonies are whitish, slimy and convex. They are about 4 to 5 mm in diameter after 3 days of incubation.

- Only *C. albicans* grows in the presence of cycloheximide, other species are inhibited with cycloheximide.

- *Candida albicans* produces germ tubes within 2 hours when incubated in serum at 37 ^{O}C. Germ tubes are round yeast cell with slender cylindrical protrusions produced by *Candida albicans* within 2 hours when incubated in serum at 37 ^{O}C.

- *Candida albicans* produces chlamydospores in submerged culture on corn meal agar. Chlamydospores are thick-walled resting cells formed from pseudohyphae in submerged colonies growing in cornemeal agar.

- Microscopy: Gram staining reveals Gram positive organism. Colonies of *Candida* yield characteristic budding yeast cells.

- Biochemical profiles: carbohydrate assimilation and fermentation tests for the definitive identification of *Candida species.*

Treatment:

Nystatin and Miconazole are used for the treatment of localized infection without internal involvement. Amphotericin B and 5-fluorocytosine used for systemic treatment.

CRYPTOCOCCUS SPECIES

Although the genus *Cryptococcus* contains about 37 species, only *C. neoformans* produces clinical infection. *Cryptococcus neoformans* is a round to oval yeast of about 3.5 to 8.0 μm in diameter. *Cryptococcus species* exist only in the yeast form (there is no mould/mycelia form). A budding daughter cell is usually seen on a narrow neck joined to the mother cell. They are aerobic, non-fermentative organisms which form mucoid colonies on a variety of media including SDA. When freshly recovered directly from infected animal host, the yeast possesses thick mucopolysaccharide capsule demonstrable by Indian ink. Four serotypes (A, B, C and D) of *C. neoformans* are recognized. Classification into serotypes is based on the type of capsular antigen possessed. Serotypes A and D are designated as *C. neoformans var neoformans* while serotypes B and C are termed *C. neoformans* var *gatti*.

Cryptococcus neoformans can be recovered from the droppings of pigeons and other birds. Soil enriched by such the droppings is also a good source of the organism. *Cryptococcus neoformans* has affinity for creatinine present in the droppings. Pigeons can excrete *C. neoformans* for several months without showing signs of clinical infection.

Clinical infection: *Cryptococcus neoformans* produces opportunistic infection in animal host. Infection is transmitted by inhalation of *C. neoformans* in dust. The organism is associated with the following clinical conditions:

➤ Cattle: mastitis and nasal groanulomas

➤ Horses: nasal granulomas, sinusitis, pneumonia, meningoencephalitis, abortion and cutaneous lesions

➤ Dogs: generalized infection with prominent neural and ocular signs

➤ Cats: respiratory, neural, ocular and cutaneous diseaases

> ➤ Humans: respiratory (tuberculosis-like) lesions and meningoencephalitis

Microbiological diagnosis:

Note: exercise caution in handling samples from suspected cases because of the risk of zoonotic transmission of *Cryptococcus neoformans* to humans.

- Clinical samples for diagnosis: exudates, cerebrospinal fluid, biopsy specimens and tissues from postmortem.

- India ink preparation of sample will reveal budding yeast with prominent capsule

- In tissue section, capsule can be demonstrated by Mayer's mucicarmine method.

- Melanin can be detected in cell wall of *C. neoformans* by the Fontana-Masson technique

- Isolation of the organism from clinical sample is achieved by inoculation onto SDA with incorporation of chloramphenicol. Cycloheximide should not be added. Inoculated medium is incubated at 37^OC for up to 2 weeks. Ability to grow at 37^OC distinguishes *C. neoformans* from other *Cryptococcus* species. In addition, *C. neoformans* produces brown colonies on birdseed agar.

- Identification criteria: Mucoid colonies, presence of capsule and urease activity

- Latex agglutination test for the detection of soluble capsular material of *C. neoformans* within 3 weeks of infection in CSF, urine and serum

Treatment: Amphotericin B and Flucytosine

SPOROTHRIX SCHENCKII

Sporothrix schenckii is widely distributed in the environment where it grows as a mould producing conidiophores and slender hyphae of about 1 to 2 μm in diameter. The organism causes sporadic infection in horses, cats, dogs and humans. *Sporothrix schenckii* is present in many parts of the world especially in the tropical and subtropical regions. It grows as a saprophyte on dead and senescent vegetations such as rose thorns, timbers, hays, straws and mosses.

Clinical infections: *Sporothrix schenckii causes* chronic cutaneous and lymphocutaneous sporothricosis. The disease may become generalized and disseminated all over the body. Disseminated infection is common in immunocompromised patients. Sporadic cases have been reported in horses, cats, dogs, cattle, goats, pigs and humans. The organism enters the body through abrasion on the skin especially at the extremities (limbs). Nodular lesions develop on the lymph nodes and along the lymphatics. The nodules rupture leaving ulcers and exudates.

Diagnosis:

- Clinical samples: exudates, scrapings from ulcers, aspirates and tissues from affected lymph nodes

- Direct microscopy: examination of methylene blue-stained smear of exudates from lesions in affected cats reveals large number of yeast cells. These are sparse in exudates from other animals.

- Gram staining: smear made from pus reveals Gram positive budding yeast cells

- Histopathological examination of tissue section stained by PAS or methenamine silver impregnation may reveal yeast cells.

- Fluorescent antibody or immunoperoxidase technique applied to tissue section permit specific identification of yeast cells

- Fungal isolation:

Mould form: Samples are inoculated on SDA at 25 $^\circ$C to produce mould colonies. The colonies which develop rapidly are white becoming black or brown, wrinkled and leathery. Microscopic examination of the mould colonies reveals pear-shaped conidia borne in a rosette pattern on slender conidiophores. In old cultures, conidia form singly on hyphae.

Yeast form: to demonstrate the yeast form of the organism, samples are inoculated onto brain heart infusion agar containing 5% blood and incubated at 35-37 $^\circ$C. Cream colour to tan yeast colonies develop within 3 weeks. At microscopy, cigar-shaped yeast cells of about 2-3 µm by 3-5 µm in size are observed.

Treatment: administration of inorganic iodide (potassium iodide or sodium iodide) in feed. Treatment should continue for 30 days after complete recovery. Ketoconazole may be given along with inorganic iodide in intractable cases. Intravenous administration of amphotericin B could also be considered.

ASPERGILLUS SPECIES

Aspergillus species are saprophytic mould widely distributed in the environment. They are present in the soil and in decomposing organic matter. *Aspergillus fumigatus* can be found in overheated, poor quality hay and in compost heaps. Spores are dispersed in dust and in the air. The genus *Aspergillus* comprises of over 190 species but only a few of them are of clinical relevance causing opportunistic infections in humans and animals. *Aspergillus fumigatus* is the species most often implicated in tissue invasion. Other species commonly associated with aspergillosis are: *A. niger, A. flavus, A. terreus, A. deflectus, A. nidulans* and *A. flavipes.*

Most *Aspergillus spp* are grouped as fungi imperfecti while some are classified as ascomycetes. They possess septate hyphae whichare hyaline in nature and about 8.0µm in diameter. Non-branching conidiophores can be seen to have emerged at right angle from a specialized hyphal foot cells. The tip of the conidiophores enlarged to form a vesicle bearing numerous flask-shaped phialides. The phialides produce chains of spherical pigmented conidia (phialoconidia) of about 5.0 µm in diameter and may be smooth or rough. In some species (*A. fumigatus*), the phialides are borne directly on the vesicle (uniseriate) while in others (*A. niger*), the phialides are borne on metulae attached to the vesicle.

Aspergillus species are aerobic and grow rapidly to form visible colonies after 2 to 3 days of incubation. The colour of the obverse side of the colonies varies with species and condition of culture. The different colours which may be may be blue-green, black, brown, yellow, or reddish can be used for presumptive identification. *Aspergillus fumigatus* can resist high temperature and grows at temperature ranging from 20 – 50 ^{O}C.

Clinical infections: respiratory infection develops following inhalation of *Aspergillus* spores. Occasionally, infection may occur following ingestion of spores in feed or following tissues invasion through trauma. Systemic infection is associated with immunosuppression. Mycotoxicosis occurs following expression of toxins by *Aspergillus spp* such as *A. flavus* growing in cereals and other foods.

Forms of aspergillosis:

- Allergic aspergillosis
- Colonizing aspergillosis: invasive aspergillosis, disseminated aspergillosis (other organs and the central nervous system), bronchopneumonia, naso-orbital aspergillosis,
- Aspergilloma: tumour in lungs and other body organ resulting from inhalation of *A. niger*

Poultry:

i. Brooder pneumonia: Occurs in newly-hatched chickens in incubators

ii. Pneumonia and air sacculitis: Most common in chickens and poults of up to 6 weeks of age. Older birds are occasionally affected.

iii. Generalized aspergillosis: Dissemination of infection usually from the respiratory tract

Horses:

i. Guttural pouch mycosis: confined to the guttural pouch and often unilateral

ii. Nasal granulomas: Produces a nasal discharge and interferes with breathing. Fungi other than *Aspergillus spp* may initiate this condition

iii. Keratitis: Localized infection following ocular trauma

iv. Intestinal aspergillosis: enteric infection resulting in diarrhea in foals

Cattle:

i. Mycotic abortion: occurs sporadically, produces thickened placenta and plaques on skin of aborted foetus

ii. Mycotic pneumonia: uncommon condition of housed calves

iii. Mycotic mastitis: may result from the use of contaminated intramammary antibiotic tubes

iv. Intestinal aspergillosis: may cause acute or chronic diarrhea in calves

Dogs:

i. Nasal aspergillosis: invasion of nasal mucosa and turbinate bones occurring sporadically

ii. Otitis externa: *Aspergillus spp* may constitute part of a mixed infection

iii. Dissemination aspergillosis: uncommon but may result in osteomyelitis or discospondylitis

Cats:

i. Systemic aspergillosis: rarely encountered usually following immunosuppression

Diagnosis:

i. Presumptive diagnosis is based on clinical signs.

ii. Sample collection: biopsy samples, tissues from postmortem lesions.

iii. Staining of tissue sections by methenamine silver impregnation or by PAS methods to reveal hyphal invasion.

iv. For isolation, small tissue specimens are applied to the scarified surface of SDA and incubated aerobically at 37 ^{O}C for 2 to 5 days. Hyphae grow from specimen to form

colonies. Colonies can be up to 5 cm in diameter after incubation for 5 days. The colour of the reverse side is pale yellow to light tan. The colour of the obverse side is determined by the pigmentation of the conidia.

Colonia identification criteria:

- Temperature of growth (45-50$^{\text{o}}$C) for identification of thermotolerant species (*A. fumigatus*).
- Presumptive identification can be made on the basis of colonial appearance and conidial arrangement on sporing heads (size, shape of vesicle, position of phialides; size, shape and colour of conidia).

Colonial appearance:

Only a small number of the species are responsible for the majority of infections in animals.

- *A. fumigatus* colonies rapidly become velvety or granular and bluish green with narrow white peripheries. Older colonies are slate-gray
- *A. niger* colonies are black and granular because of the large pigmentation of the sporing heads
- *A. flavus* colonies are yellowish green with a fluffy texture
- *A. terreus* colonies are cinnamon-brown with a granular texture

Because colonies of *Aspergillus fumigatus* are similar to those of *Penicillium spp* in appearance, microscopic differentiation of smear from colonies may be necessary. The

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conidiophores of *Penicillium spp* often possess secondary branches (metulae) bearing several phialides.

    v.   Serological tests: procedure such as ELISA is reliable for identification in dogs

   vi.   Molecular procedures such as PCR

MUCORMYCOSIS

Mucormycosis refers to the mycosis caused by any of the fungal species belonging to the genera classified under the order 'Mucorales' in the class Zygomycetes. These genera include: *Rhizopus*, *Mucor*, Rhizomucor and *Absidia*. The term 'mucorales' originated from the latin word '*mucere'* meaning 'to be mouldy or musty'. Members of the order 'Mucorales' include those Zygomycetes that reproduce asexually by formation of spores within sac-like sporangia and also sexually by forming zygospores.

Members of the order mucorales are commonly referred to as 'pin' mould because their dark sporangia resembles pin head, they are also called bread mould because they often grow on stale bread. They are widely distributed in nature as saprophytes in soil, dung, vegetation and other organic matters. Their spores are dispersed in air. They are transmitted through inhalation of spore to produce disease of the respiratory system or by ingestion leading to gastrointestinal disorder and associate lymph nodes. Disseminated infection may occur where the organisms invade the circulatory system and form foci of infection in different organs. Infection may be associated with immunosuppression.

Identification of Mucorales

Rhizopus, *Mucor*, Rhizomuclor and *Absidia* grow well on SDA at 37 ^{O}C (Rhizomucor are thermophiles which will survive and grow at temperature up to 60 ^{O}C). Their growth is inhibited by cycloheximide. They grow rapidly and cover the entire surface of the agar plate. The colonies could be fluffy, wooly or cottony with gray-white, brown, brownish-gray colour. The fungi grow from border to border in the Petri dish without producing distinct colony margin. As colonies mature, gray-brown colour with black pepperlike stipples are produced indicating the production of sporangia. The genera are differentiated on the basis of microscopic morphologic features.

Generally, at microscopic they possess ribbon-like aseptate hyphae (there may be septa in older culture), sporangia that is borne on top of a sporangiophore (branched or unbranched) terminating in a bulb-like structure termed columella. Within the sporangia are numerous sporangiospores. The columella may rest

within another structure referred to as apophysis. They also have root-like structures called rhizoids for anchoring into the medium on which they are growing.

Differentiating morphological features of members of mucorales

Feature	*Rhizopus*	*Mucor*	*Rhizomucor*	*Absidia*
Sporangia	Spherical	Large and spherical	Spherical	Pear-shaped
Sporangiophore	Simple but occasional branched	Simple but tend branch	Simple but may branch	Branch freely
Apophysis	Not prominent	Absent	Not prominent	Prominent
Rhizoids	Coarse, spiked and located under sporangiophores	Absent	Poorly developed found between sporangiophores	Delicate, located between sporangiophores

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Introductory microbiology lecture notes (VPM 302)

CULTURING MICROBES

Definition of medium (plural: media)

A medium (plural: media) is any solid or liquid preparation made specifically for the growth, storage or transport of bacteria. A medium must be sterile, i.e. it must contain no living organisms.

There is need for us to adequately understand certain terminologies used by bacteriologists in communicating about media; I believe this will be best achieved by following the outline of a simple laboratory procedure. To grow an organism such as *Staphylococcus aureus*, the bacteriologist takes an appropriate sterile medium and adds to it a simple amount of material which consists of, or contains, living cells of that species; the 'small amount of material is called an inoculum , and the process of adding the inoculum to the medium is inoculation. The inoculated medium is then incubated i.e. kept under appropriate conditions of temperature, humidity etc. for a suitable period of time. The incubation is usually carried out in thermostatically controlled equipment called incubator. During incubation, the bacteria grow and divide –giving rise to a culture; thus, a culture is a medium containing organisms which have grown (or still growing) on or within that medium.

A liquid medium may be used in a test tube (which is stoppered by a plug of sterile cotton wool, or which has a simple metal cap or in a glass, screw-cap bottle; a *universal bottle* is a cylindrical bottle of about 25ml capacity, while a *bijou* is smaller (about 5-7ml).

Most solid media are jelly-like materials which consist of a solution of nutrients etc. 'solidified' by agar (a complex polysaccharide gelling agent obtained from certain sea weeds other gelling agents include silica gel, gelatin etc.). A solid medium is commonly used in a plastic *petri dish* (usually about 9cm although varied sizes are available). The medium in molten (liquid) state is poured into the petri dish and allowed to set; a petri dish containing the solid medium is called a *plate*.

note

a. *Culture media* are solutions containing all of the nutrients and necessary physical growth parameters necessary for microbial growth.
b. Note that not all microorganisms can grow in any given culture medium and, in fact, many can't grow in any known culture medium.

Types of media

Media can be classified on three bases

i. physical form (solid vs. broth),
ii. chemical composition, and

iii. functional type

i. Physical states of media

Liquid media are defined as water-based solutions that do not solidify at temperature above freezing and that tend to flow freely when the container is tilted. These media termed, termed **broths, milks, or infusions**, are made by dissolving various solutes in distilled water. Growth occurs throughout the container and can then present a dispersed, cloudy or particulate appearance. Examples include nutrient broth (which contains beef extract and peptone dissolved in water), methylene blue milk and litmus milk (opaque liquids containing whole milk and dyes) and brain heart infusion (find out its composition).

At ordinary room temperature, **Semisolid media** exhibit a clot-like consistency because they contain an amount of solidifying agent (agar or gelatin) that thickens them but does not produce a firm substrate. Semisolid media are used to determine the motility of bacteria and to localize a reaction at a specific site. They contain a small amount of agar (0.3-0.5 %).

Solid media provide a firm surface on which cells can form discrete colonies (mounts of daughter cells originating from a mother single cell) and are advantageous for isolating and culturing bacteria and fungi. The come in two forms: liquefiable and nonliquefiable. Liquefiable solid media, sometimes called

reversible solid media, contain a solidifying agent that changes its physical properties in response to temperature. The most widely used of these agents is **agar** (a complex polysaccharide isolated from the red alga *Gelidium*. The benefits of agar include;

a. Agar is solid at room temperature, and it melts (liquefies) at the boiling temperature of water of water (100^oC).Once liquefied, agar does not resolidify until it cools to 42^oC, so that it can be inoculated and poured in liquid form at temperature (45°-50^oC) that will neither harm the microbe nor the handler.

b. Agar is flexible and mouldable, and it provides a basic framework to hold moisture and nutrients and not attacked by a vast majority of microorganisms.

c. An agar gel does not melt @ 37^oC- a temperature used for the incubation of most bacteria.(by contrast, a gelatin can be liquefied by some bacteria , and is molten @ 37^oC .

Any medium containing 1% to 5% agar usually has the word *agar* in its name. Although gelatin is not nearly as satisfactory as agar, it will create a reasonably solid surface in concentrations of 10% to 15%.

Nonliquefiable solid media solid media have less versatile applications than agar media because they do not melt. They include materials such as rice grains (used to grow fungi), cooked meat media (good for anaerobes), and potato slices; all of these media start out solid and remain solid after heat sterilization. Other

solid media containing egg and serum start out liquid and are permanently coagulated or hardened by moist heat.

ii. **Chemical composition (non-synthetic/complex vs. synthetic/chemically defined)**

Media whose chemical composition is chemically defined are termed synthetic. Such media contain pure organic and inorganic compounds that vary little from one source to another and have a molecular content specified by means of an exact formular. Defined media are used widely in research, as it is often desirable to know what the experimental microorganism is metabolizing.

Complex, or non-synthetic, media contain at least one ingredient that is not chemically definable; not a simple, pure compound and not representable by an exact chemical formula. Most of these substances are extracts of animals, plants, or yeasts, including such materials as ground-up cells, tissues, and secretions. Examples are blood, serum, and meat extracts or infusions.

iii. Functional type

General-purpose media are designed to grow as broad a spectrum of microbes as possible. As a rule, they are non-synthetic and contain a mixture of nutrients that could support the growth of pathogens and nonpathogens alike. Examples include nutrient agar and broth, brain-heart infusion, and trypticase soy agar (TSA).

An Enriched medium contains complex organic substances such as blood, serum, haemoglobin, or special **growth factors** (specific vitamins, amino acids) that certain species must have in order to grow. Bacteria requiring such growth factors and complex nutrients are termed **fastidious** .examples of enriched media include blood agar(an agar based medium enriched with 5-10% blood), chocolate agar(an agar made by heating blood agar to 70-80□C until it becomes chocolate brown in colour).

Enrichment medium allows certain species to outgrow others by encouraging the growth of wanted organism(s) and/or by inhibiting the growth of unwanted species. Hence, if an inoculum contains only a few cells of the required species (among a large population of unwanted organisms), growth in a suitable enrichment medium can increase ('enrich') the proportion of required organisms. Examples include slenite and Rappaport vassiliadis broth used for enrichment of *Salmonella* spp. **Note**-enrichment media occur more often in liquid form.

A selective medium contains one or more agents that inhibit the growth of a certain microbe or microbes (A, B, C) but not others (D) and thereby encourages, or selects, microbe D and allows it grow.

Medium	Selective Agent	Used for
Mannitol salt agar	Nacl 97.5%	*Staphylococcus* spp.
MacConkey agar	Bile, crystal violet	Isolation of gram

		negative enterics
Salmonella/Shigella agar	Bile, citrate, brilliant green	Isolation of *Salmonella* and *Shigella* spp.
Enterococcus faecalis broth	Sodium azide	Isolation of faecal enterococci
Lowenstein-Jensen	Malachite green dye	
Sabouraud's agar	PH of 5.6 (acid)	Isolation and maintenance of Mycobacteria
		Isolation of fungi-inhibits bacteria

Differential medium grows several types of microorganisms and are designed to display visible differences among those microorganisms. Differentiation shows up as variations in colony size or colour, in media colour changes, or in the formation of gas bubbles and precipitates. These variations come from the type of chemicals these media contain and the ways that microbes react to them. For example,

when microbe X metabolizes a certain substance not used by organism Y, then X will cause a visible change in the medium and Y will not. The simplest differential media show two reaction types such as the use or non-use of a particular nutrient or colour change in some colonies but not in others. Some media are sufficiently complex to show three or four different reactions.

Dyes can be used as differential agents because many of them are PH indicators that change colour in response to the production of an acid or a base. For example, MacConkey agar contains neutral red, a dye that is yellow when neutral and pink or red when acidic. A common intestinal bacterium such as *Escherichia coli* that gives off acid when it metabolizes the lactose in the medium develops red to pink colonies, and one like *Salmonella* that does not give off acid remains its neutral colour(off-white).

Examples of Differential Media			
S/N	Medium	Substances that facilitate differentiation	Differentiates Between
1.	Blood agar	Intact red blood cells	Types of haemolysis
2.	Mannitol salt agar	Mannitol, Phenol red, and 7.5% NaCl	Species of *Staphylococcus* ;NaCl also inhibits the salt sensitive

			species
3.	Hektoen (HE) enteric	Brom thymol blue, acid fuchsin, sucrose, salicin, thiosulfate, ferric ammonium citrate and bile	*Salmonella, shigella*, other lactose fermenters from nonfermenters;Dyes and bile also inhibit gram positive bacteria
4.	Urea broth	Urea, phenol red	Bacteria that hydrolize urea to ammonia
5.	Sulphur Indole Motility (SIM)	Thiosulphate, iron	H_2S gas producers from non producers
6.	Tripple-sugar iron agar (TSA)	Triple sugars, iron, and phenol red dye	Fermentation of sugars, H_2S production
7.	Xylose-Lysine deoxycholate (XLD) agar	Lysine, xylose, Iron, thiosulphate, phenol red	*Enterobacter, Escherichia, Proteus, Providencia*, Salmonella and *Shigella*

Miscellaneous Media

A reducing medium contains a substance (Thioglycollic acid or cystine) that absorbs oxygen or slows the penetration of oxygen in a medium, thus reducing its availability. Reducing media are important for growing anaerobic bacteria or determining oxygen requirements.

Transport media are used to maintain and preserve specimens that have to be held for a period of time before clinical analysis or to sustain delicate species that die rapidly if not held under stable conditions. Stuart's and Amies transport media contain salts, buffers, and absorbants to prevent cell destruction by enzymes, PH changes, and toxic substances, but will not support growth. **Assay media** (e.g Mueller Hington) are used to test the effectiveness of antimicrobial drugs and by drug manufacturers to assess the effect of disinfectants, antiseptics, cosmetics, and preservatives on the growth of microorganisms. Enumeration media are used by industrial and environmental microbiologists to count the numbers of organisms in milk, water, food, soil, and other samples.

Pure culture technique

    a. Clonal populations**:**
        i. *Pure culture technique* is a method of culturing microorganisms in which all of the individuals in a culture have descended from a single individual.
        ii. This is done so as to:
            1. inhibit evolutionary change within cultures

        2. allow the characterization of types microorganisms without the confounding presence of other, different types of microorganisms

b. Colony isolation:

    i. The basis of *pure culture technique* is the isolation, in colonies, of individual cells, and their descendants, from other colonies of individuals.

    ii. This is usually done by culturing methods employing petri dishes such as:

        1. streaking

        2. pouring

        3. spreading

c. Isolation from the wild**:**

    i. When isolating microorganisms from complex mixtures it is always a good idea to repeat the isolation procedure at least once (e.g., restreak an isolated colony) to make sure that an isolated colony is truly derived from only a single cell (i.e., closely overlapping colonies can be indistinguishable from colonies founded from single cells).

    ii. Following their isolation from the wild, microorganisms may be characterized by inoculation into differential medium to determine what type of nutrients they require or can use, and what types of by-products they produce. This aids in identification.

d. Pouring a plate

a. A *pour plate* is a method of melted agar inoculation followed by petri   dish incubation.

b. Steps include:

   i. cultures are inoculated into melted agar that has been cooled to 45□C

   ii. The liquid medium is well mixed then poured into a petri dish (or *vice versa*)

   iii. colonies form within the agar matrix rather than on top as they   do when streaking a plate

c. Pour plates are useful for quantifying microorganisms that grow in solid   medium.

d. 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.

e. Spreading a plate

a. Quantification technique:

   -*Spreading a plate* is an additional method of quantifying   microorganisms on solid medium.

   -Instead of embedding microorganisms into agar, as is done with   the pour plate method, liquid cultures are *spread* on the agar   surface using a devise that looks more or less like a hockey   stick.

b. An advantage of spreading a plate over the pour plate method is that cultures are never exposed to 45□C melted agar temperatures.

Preservation of cultures

Culture Preservation is important for the following reasons:

i. Scientific reasons

ii. Identification

iii. Vaccine production

 iv. Industrial use

Methods of preserving cultures include:

I. Refrigeration

ii. Stabs

iii. Slants

iv. Lyophilisation

v. Freezing

i. Refrigeration (a.k.a., 4□C)

This is effective short term preservation. All of the following can be refrigerated

➤ broth cultures
➤ stabs
➤ slants

➢ streaks

ii. Stabbing

In this method, Cultures are usually stabbed deeply into agar using an inoculating needle in bijou bottles, test tubes and other holding wares. The stabs are incubated until visible cultures are formed; they are then sealed and stored at room or lower temperature.

iii. Slant method

Slant method involves streaking the organism of interest onto the surface of a solid medium in a slant tube. The slant is then incubated until visible culture formation. The slant is then sealed and stored at room or lower temperature.

iv. Lyophilisation

Lyophilisation is the freeze-drying of cultures. Cultures are first frozen and then dried under high vacuum. To revive cultures they are rehydrated by broth. Lyophilisation can be an effective long term method of storage.

v. Freezing

Broth cultures are mixed with various ingredients (e.g. glycerol) to limit damage upon freezing and then frozen to temperatures ranging from -50☐C to -95☐C. To revive cultures, they are thawed, pelletted, and resuspended into broth. Freezing can be an effective long term method of storage.

Colony morphology

Differentiating colonies

I. Colony morphology gives important clues as to the identity of their constituent microorganisms.

Important classes of characteristics include:

1. size
2. type of margin
3. colony elevation
4. colony texture
5. light transmission
6. colony pigmentation

II. Colony size

a. *Colony size* is dependent not just on the type of organism but also on the growth medium and the number of colonies present on a plate (that is, colonies tend to be smaller when greater than ascertain amount are present) and on culture medium characteristics.

b. Usually stabilizes after few days:

➢ Colony size usually stabilizes after a day or two of incubation.
➢ Exceptions include:

-slow growing microorganisms

-during growth under conditions that promote slow growth

> With slow growth [colonies](#) may continue to experience growth past this time,   especially if an effort is made to prevent solid medium from drying out.

III. Type of margin

    d. Colonies can vary in the shape of their margins.

    e. See illustration below

IV. Colony elevation

Colonies can vary in their elevations both between microorganisms and growth conditions, and within individual colonies themselves.

V. Colony texture

Surface appearance:

    i. Colonies can vary in their texture.

    ii. Possible textures include:

        1. shiny to dull

        2. smooth to wrinkled

        3. rough

        4. granular

        5. mucoid

NOTE: A shiny, smooth, and/or mucoid appearance tends to be associated with the presence of capsular material.

VI. Colony light transmission

The *light transmission* through colonies can range from:

- ➢ complete (transparent)
- ➢ through intermediate (translucent)
- ➢ through completely lacking (opaque)

VII. Colony pigmentation

Colonies can come in a rainbow of colors.

SOME DEFINITIONS

- ➢ **Petri dish -***Petri dishes are circular*, vertical sided plates used to contain agar and with tops for aseptic purposes.
- ➢ **Loop -**A platinum wire formed into a *loop* is heated to an orange glow to sterilize it then is used to transfer a culture from one physical location to another. Platinum wire is used as inoculation needle because when heated, it cools fast.
- ➢ **Streaking -***Streaking* is a method of applying cultures to solid medium:

  i. a sterile loop is cooled and brought into contact with a culture

ii. the loop is then brought into contact with the surface of solid medium whereupon it is streaked (i.e., dragged) along the surface of the solid medium

iii. colonies grow along the points of the streak

Streaking a plate

Colony isolation: A petri dish is streaked in manner such that individual colonies may be isolated.

Control of Microbes by Physical and Chemical Agents

I. Definitions

A. Antimicrobial agent = general terms for an agent that kills microbes or inhibits their growth
1. prefix designates organism type (bacteri-, fungi-)
2. suffix designates whether it kills (-cidal) or inhibits growth (-static)

B. Sterilization = destruction or removal of ALL living cells, spores, or viruses

C. Disinfection = killing, inhibition, or removal of all microbes that may cause
   disease by disinfectant; usually on inanimate objects.

D. Sanitation = reduction of microbial populations to levels that are considered
   safe for public health by cleaning (partial disinfection) by sanitizer; usually
   on inanimate objects.
E. Antisepsis = prevention of infection of living tissue by application of
   antiseptic.

F. Antibiotic = a microbial product or derivative that kills or inhibits growth of
  a susceptible microbe.

II. Conditions influencing effectiveness of antimicrobial agent

A. Population size: larger population size = longer time to kill all organisms
   because death is logarithmic.
B. Population composition
1. Some organisms are more resistant to killing than others
2. Some growth stages are more resistant than other (i.e. endospores, stationary
   phase cells)
C. Concentration/intensity of agent

  Generally and up to a point, the more concentrated the agent, the more
  effective it is at killing. There are exceptions to this. 70% ethanol is more
  effective than 100%.
D. Duration of exposure (contact time): longer exposure = more killing

E. Temperature: many times higher temperature = greater killing

F. Local environment

1. lower pH enhances killing

2. Organic material protects antimicrobial agents

  Use on inanimate objects Use on living tissue

  sterilant disinfectant sanitizer antiseptic antibiotic

III. Use of physical methods in control

A. Heat

1. Mechanism of action: denaturation of proteins and DNA, disruption of
   membranes, oxidation of proteins (for dry).

2. Quantitative descriptions

a) thermal death point = lowest temperature in which a microbial
   population is killed in 10 minutes;
b) thermal death time = shortest time needed to kill all organisms in a

microbial suspension at a particular temperature; depends on original number

   of microbes.

c) decimal reduction time (D) = time required to kill 90% of microbes in
   a sample at a particular temperature

d) z value = increase in temperature required to drop D 10-fold (larger Z
   value = more heat resistant)

3. Types of heat

(a) Moist heat

(i) Boiling

   Ten minutes of heating destroys most vegetative cells but not bacterial
   endospores. Hence, we can regard this as a form of disinfection since not all
   population are completely destroyed.

(ii) Autoclaving

   This involves the use of autoclave @ a pressure of 15 psi , high temperature
   of 121°C for 15 minutes. Bacterial endospores and vegetative forms are
   destroyed. It is a form of sterilization. Examples include bact. Media, rubber
   appliances, surgical instruments, cotton wool and discarded cultures.

(iii) Pasteurizing

   This involves heating (usually below boiling) to kill all pathogens and to
   reduce the number of microbes that may cause spoilage(disinfection).e.g
   milk is pasteurized at 63°C for 30 mins.

(iv) Tyndallization (fractional stream sterilization)

   This involves alternating treatments (x3) of heat at 100° for 30 min
   followed by incubation at 37°C for 1 day which allows germination of
   spores and then subsequent killing (sterilization).

(b) Dry heat

 (i) Hot air

This is the use of hot air through hot air oven (160-170°C for 2-3 hours) to

sterilize items that cannot be in contact with water and that can withstand very high temperature. Examples include powders, glass wares, liquid paraffin etc. This method cannot be used for items that cannot stand exposure to heat for long times and a lot of time is needed in this method.

(ii) Direct flaming

This involves exposing the appliances to direct flames(sterilization).Examples include inoculation loop, spatula, mouth of test tubes and flasks.

B. Filtration

This is the process of separating microbial contaminants from liquids using filters. Sterilization here depends on pore size of filters used.

 Types of filters

a) depth filters

b) membrane filters

c) HEPA filters – High efficiency particulate air filter

Advantages: easy, can filter heat sensitive materials

Disadvantages: filters are expensive, some things do not filter well

C. Radiation

1. 260 nm ultraviolet (UV) light

(a) Mechanism of action: causes the formation of thymine dimers in DNA

which inhibits DNA replication

(b) Pros: Very effective sterilant

(c) Cons: Cannot penetrate solid or opaque objects

2. Ionizing radiation – very short wavelength radiation that causes atoms to loose electrons or ionize
(a) Mechanism of action

  (1) breaks hydrogen bonds

  (2) oxidizes double bonds

  (3) polymerizes some molecules

  (4) produces free radicals

(b) Types

  (1) X rays – artificially produced

  (2) gamma rays – emitted during radioisotope decay

  (c) Pros: Can penetrate better than UV

  (d) Cons: Concern about radioactive contamination, production of toxic or carcinogenic byproducts, alteration of nutritional value; takes a long time

D. Cold

1. Mechanism of action: prevents growth by decreasing rate of chemical reactions
2. Microbiostatic for most organisms - does not disinfect

3. Important in food storage

E. Desiccation

1. Mechanism of action: disruption of metabolism

2. Microbiostatic for most organisms

3. Important in food storage

F. Osmotic pressure

1. Mechanism of action: plasmolysis

2. Important in food preservation

IV. Use of chemical agents in control

A. Ideal properties

1. Effective against broad range of microbes

2. Effective at high dilutions and in the presence of organic material

3. Nontoxic to people and objects

4. Stable

5. Non-offensive odour

6. Soluble

7. Inexpensive

8. Sporocidal

B. Types

1. phenolics

a) phenols and related compounds such as Lysol

b) mechanism of action: denature proteins; disrupt membranes

c) pros: 1, 2, 4

d) cons: 3, 5

2. alcohol

a) 70% ethanol; isopropanol

b) mechanism of action: denature proteins; dissolve lipids

c) pros: 1 ,3 (antiseptic), 7

d) cons: 8; 4 (contact time)

3. halogens – iodine based

a) iodine (I2) or more commonly organic carrier: iodine complex
   (iodophor) – Betadine
b) mechanism of action: oxidized cell constituents, especially proteins at
   –SH groups; iodinates proteins and inactivates them frequently at tyrosine
   residues
c) pros: 1, 3, 4, 6, 8 (at high concentrations)

d) cons: some pseudomonads can survive in iodophors

4. halogens – chlorine based

a) chlorine gas; sodium hypochlorite (bleach); calcium hypochloride

b) mechanism of action: conversion to hypochlorous acid (HOCl) and
   then atomic oxygen which oxidizes cell components
c) pros: 1, 3, 6

d) cons: 2, 4, 8

5. heavy metals

a) Hg; Ag; arsinic; Zn; Cu; silver nitrate; silver sulfadiazine; copper
   sulfate
b) mechanism of action: combine with proteins (usually via sulfhydryl
   groups) to inactive them; can precipitate proteins
c) pros:

d) cons: for many (3), bacteriostatic,

6. Surface acting agents

a) Soaps and detergents

(1) mechanism of action: help in removal of organisms from
    surfaces

(2) not very effective disinfectants

b) quaternary ammonium compounds

(1) positively charged quaternary nitrogen and long hydrophobic
chain
(2) mechanism of action: disrupt membranes, may inactivate some
proteins
(3) pros: 3, 4

(4) cons: 8

7. aldehydes

a) formaldehyde; glutaraldehyde

b) mechanism of action: inactivates proteins by crosslinking to numerous
functional groups on proteins including (-NH2, -OH, -COOH, -SH)
c) pro: 1, 8 (sterilant)

d) con: 3, 5

8. sterilizing gases

a) ethylene oxide

b) mechanism of action: inactivates proteins by alkylating them

c) pros: penetrates plastics, 1, 8 (sterilant)

d) cons: 3

V. Evaluation of antimicrobial agent effectiveness

A. Regulated by EPA (disinfectants) and FDA (antiseptics)

B. Phenol coefficient test

Comparison of particular agent with phenol using *Salmonella Typhi* and
Staphlococcus aereus as testers
C. Use dilution test

Controlled contamination of a steel cylinder with a microbe exposure to

different concentration of test disinfectant for 10 min transfer to culture media

Determine # of organism

Determine dilution that resulted in killing of all organisms at a 95% confidence level

SUPERFICIAL MYCOSES

Dermatophytoses are a category of cutaneous fungal infections caused by more than 30 species. The superficial (cutaneous) mycoses are usually confined to the dead outer layers of skin, hair, feathers, horn, hooves, claws , nails and do not invade living tissues. The fungi are called dermatophytes. Dermatophytes, or more properly, keratinophilic fungi, produce extracellular enzymes (keratinases) which are capable of digesting keratin. Keratin is the primary structural protein of skin, nails, feather, claw, horn hooves and hair, the digestion of keratin manifests as scaling of the skin, loss of hair, and crumbling of the skin. Dermatophyte infections are called ringworm (tinea).

Whether a fungus is categorized as mold or yeast is based upon the microscopic appearance in the tissue or on routine culture media (the asexual stage). If the hyphal structures are observed , the fungi is termed a mold; if a single-celled, budding structure is observed, the fungus is termed a yeast. On routine culture media , molds will have a ''fuzzy'' or wooly appearance , and a yeast will be bacteria-like in its colonial morphology and consistency. Some pathogenic fungi will produce either hyphal-like structures or yeast like structures, depending upon the conditions in which they are growing. Such fungi are called DIMORPHIC fungi.

The common dermatophytes include Microsporum, Trichophyton, and Epidermophyton.

Morphology

In their nonparasitic state, including culture, dermatophytes produce septate, branching hyphae collectively called mycelium. The asexual reproductive units (conidia) are found in the aerial mycelium. These units may be either macroconidia : pluricellular, podlike structures up to 100 µm long ; or microconidia: unicellular spheres or rods less than 10 µm in any dimension. Shape, size, structure, arrangement, and abundance of conidia are diagnostic criteria.

In the parasitic state , only hyphae and arthroconidia (arthrospores), another asexual reproductive unit, are seen.

Growth Characteristics

The traditional medium for propagating dermatophytes (and other pathogenic fungi) is Sabouraud's dextrose agar, a 2% agar containing 1% peptone and 4% glucose. The selectivity is enhanced by the addition cycloheximide (500 µg/ml), which inhibits other fungi, and gentamicin and tetracycline (100 µg/ml of each) or Chloramphenicol (50 µg/ml), which inhibit bacteria. Dermatophytes are aerobes and nonfermenters. Some attack proteins and deaminate amino acids. They grow optimally at $25^{o}C$ to $30^{o}C$ and require several days to weeks of incubation.

Some dermatophytes in skin and hair (but not in culture) produce a green fluorescence due to a tryptophan metabolite that is visible under ultraviolet light (λ = 366nm, sometimes referred to as wood's light. Of animal dermatophytes, only *Microsporum canis* produces this reaction.

Ecology

The dermatophytes (which means *skin plants*) causing animal infections may have different natural sources and modes of transmission:

anthropophilic - These are usually associated with humans only; transmission from man to man is by close contact or through contaminated objects.

zoophilic - These are usually associated with animals; transmission to man is by close contact with animals (cats, dogs, cows) or with contaminated products.

geophilic - These are usually found in the soil and are transmitted to man by direct exposure.

Diseases caused by dermatophytes

Group	species	Disease
a. Epidermophyton	E. fluccosum	Infection of skin and nails of fingers and toes
b.Microsporum	M.audouinii	Ringworm of scalp in children
	M. canis	Infection of skin and hair on dogs, cats and other animals. Causes tinea capitis of children
	M. gypseum	
c. Tricophyton	T. mentagrophtyes	A saprophyte in soil and parasite of lower animals

	T. rubrum	Primarily a parasite of hair
	T. verrucosum	Causes ringworm and infects hair
	T. gallinae	Causes a ringworm in cattle
		Infection in chicken

There are three genera of dermatophytes:

1.Trichophyton species

These infect skin, hair and nails. They rarely cause subcutaneous infections, in immuno-compromised individuals. *Trichophyton* species take 2 to 3 weeks to grow in culture. The conidia are large (macroconidia), smooth, thin-wall, septate (0-10 septa), and pencil-shaped; colonies are a loose aerial mycelium that grow in a variety of colors. Identification requires special biochemical and morphological techniques

2. Microsporum species

These may infect skin and hair, rarely nails. This organism could be easily identified on the scalp because infected hairs fluoresce a bright green color when illuminated with a UV-emitting Wood's light. The loose, cottony mycelia produce macroconidia which are thick-walled, spindle-shaped, multicellular, and echinulate (spiny). *Microsporum canis* is one of the most common dermatophyte species infecting humans.

3. Epidermophyton floccosum

These infect skin and nails and rarely hair. They form yellow-colored, cottony cultures and are usually readily identified by the thick, bifurcated hyphae with multiple smooth, club-shaped macroconidia

Laboratory diagnosis

Direct Examination

In 50% to 70% of cases, hairs and skin scales infected with *M.canis or M. Audouinii* may emit a bright greenish-yello fluorescence under ultraviolet light e.g woods light.

Microscopic Examination

Skin scrapings and hair are examined microscopically for the presence of hyphae and arthroconidia. The scraping should include materials from the margins of any lesion. The sample is placed on a slide , flooded with 10% to 20% potassium hydroxide, covered with a cover slip, and heated gently.

Microscopic examination should begin under low power (100X) and subdued light. Infected hairs are encased in an irregular sheath of arthrospores that may double their normal thickness. At higher magnification (400X) of such hairs, individual, spherical arthroconidia are recognizable.

Stains and penetrating and wetting agents (permanent ink, lactophenol cotton blue, dimethylsulfoxide) improve visualization. Calcoflour white reagent imparts fluorescence to fungal structures and facilitates diagnosis where a fluorescent microscope is available.

Culture

Scrapings are planted onto and into the surface of selective media (Sabouraud's agar with chloramphenicol and cycloheximide, Dermatophyte Test Medium [DTM],Rapid Sporulation Medium [RPM], which are incubated at 25°C (room temperature) for up to 3-weeks. An alkaline reaction suggests presence of dermatophyte.

THERAPY

Skin infections can be treated (more or less successfully) with a variety of drugs, such as:

Tolfnatate (Tinactin) available over the counter - Topical

Ketoconazole seems to be most effective for tinea versicolor and other dermatophytes.

Itraconazole - oral

Terbinifine (Lamisil) - oral, topical.

Echinocandins (caspofungin)

For infections involving the scalp and particularly the nails, griseofulvin is commonly used. This antimycotic must be incorporated into the newly produced keratin layer to form a barrier against further invasion by the fungus. This is a very slow process requiring oral administration of the drug for long periods - up to 6 to 9 months for fingernail infections and 12 to18 months for toenail infections.

Itraconazole and terbinafine are the drugs of choice for onychomycoses.

DIMORPHIC FUNGI

A.BLASTOMYCOSIS (*Blastomyces dermatitidis*)

Most of the systemic fungi have a specific niche in nature where they are commonly found. *Blastomyces dermatitidis* survives in soil that contains organic debris (rotting wood, animal droppings, plant material) and infects people collecting firewood, tearing down old buildings or engaged in other outdoor activities which disrupt the soil. In addition to an ecological niche, most fungi that cause systemic infections have a limited geographic distribution where they occur most frequently. Blastomycosis occurs in eastern North America and Africa.

HISTOPLASMOSIS (*Histoplasma capsulatum*) Histoplasmosis is a systemic disease, mostly of the reticuloendothelial system, manifesting itself in the bone marrow, lungs , liver, and the spleen. In fact, hepatosplenomegaly is the primary sign in the young, while in adults, histoplasmosis more commonly appears as pulmonary disease. This is one of the most common fungal infections, occurring frequently in South Carolina, particularly the northwestern portion of the state. The ecological niche of *H. capsulatum* is in blackbird roosts, chicken houses and bat guano. Typically, a patient will have spread chicken manure around his garden and 3 weeks later will develop pulmonary infection. There have been several outbreaks in South Carolina where workers have cleared canebrakes which served as blackbird roosts with bulldozers. All who were exposed, workers and bystanders, contracted histoplasmosis. Histoplasmosis is a significant occupational disease in bat caves in Mexico when workers harvest the guano for fertilizer. In the endemic area the majority of patients who develop histoplasmosis (95%) are asymptomatic. The diagnosis is made from their history, serologic testing or skin test. In the patients who are clinically ill, histoplasmosis generally occurs in one of three forms: acute pulmonary, chronic pulmonary or disseminated. There is generally complete recovery from the acute pulmonary form (another "flu-like" illness). However, if untreated, the disseminated form of disease is usually fatal. Patients will first notice shortness of breath and a cough which becomes productive. The sputum may be purulent or bloody. Patients will become anorexic and lose weight. They have night sweats. This again sounds like tuberculosis, and the lung x- ray also looks like tuberculosis, but today radiologists can distinguish between these diseases on the chest film

(histoplasmosis usually appears as bilateral interstitial infiltrates). Histoplasmosis is prevalent primarily in the eastern U.S. In S.C., a histoplasmin skin test survey of lifetime, one county residents, white males, 17 to 21 years old, was performed on Navy recruits. The greatest number of positive skin tests appeared in the northwestern part of the state. A similar study of medical students conducted at Medical University of South Carolina, about 25 years ago, bore the same distribution.The skin test is NOT used for diagnostic purposes, because it interferes with serological tests. Skin tests are used for epidemiological surveys.

Clinical specimens sent to the lab depend on the presentation of the disease: Sputum or Bronchial alveolar lavage, if it is pulmonary disease, or biopsy material from the diseased organ. Bone marrow is an excellent source of the fungus, which tends to grow in the reticulo-endothelial system. Peripheral blood is also a source of visualizing the organism histologically. The yeast is usually found in monocytes or in PMN's. Many times an astute medical technologist performing a white blood cell count will be the first one to make the diagnosis of histoplasmosis. In peripheral blood, *H. capsulatum* appears as a small yeast about 5-6 microns in diameter. (Blastomyces is 12 to 15 microns). Gastric washings are also a source of *H. capsulatum* as people with pulmonary disease produce sputum and frequently swallow their sputum.

Mycology

When it is grown on Sabouraud dextrose agar at 25 degrees C, it appears as a white, cottony mycelium after 2 to 3 weeks. As the colony ages, it becomes tan. In the mold form, *Histoplasma* has a very distinct spore called

a tuberculate macroconidium . The tubercles are diagnostic, however there are some non-pathogens which appear similar. A medical mycologist will be able to distinguish them. Grown at 37 degrees, C the yeast form appears. It is a white to tan colony. The yeast cell is 5-6 microns in diameter and slightly oval in shape. This is not diagnostic. To confirm the diagnosis, one must convert the organism from yeast to mycelium or vice-versa or use the DNA probe.

Serology

Serology for histoplasmosis is a little more complicated than for other mycoses, but it provides more information than blastomycosis serology.

There are 4 tests:

- Complement Fixation
- Immunodiffusion
- EIA (antibody)
- EIA (antigen)

Each of these serological tests has different characteristics that make them useful.

The complement fixation test is like the one for blastomycosis, except there are 2 antigens, one to the yeast form of the organism and the other to the mycelial form. Some patients react to one form and not the other, while some individuals react to both. The reason for the different responses is not clear. One disadvantage is that complement fixing antibody develops late in the disease, about 2 to 3 months after onset. A second disadvantage is that it

cross reacts with other mycotic infections. An advantage of the C-F test is that it is quantitative, so the physician can follow the course of the disease by observing the titer of several samples. The interpretation of the immunodiffusion test is a little more complicated than with blastomycosis because there are two bands which may appear. An H band indicates active disease and will appear in 2 to 3 weeks. An M band can indicate past or present disease, or result from a skin test. This is one reason why skin tests are not used for diagnosis because they can interfere with other tests. Skin tests will also affect the complement fixation test.

Recently, a radioimmunoassay for histoplasma polysaccharide antigen has been developed. This is a proprietary test so the evaluation of the results have been questioned. The drug of choice (DOC) is amphotericin B, with all its side effects. Itraconazole is now also being used for mild cases.

C.COCCIDIOIDOMYCOSIS (*Coccidioides immitis*)

Coccidioidomycosis is primarily a pulmonary disease. About 60 % of the infections in the endemic area are asymptomatic. About 25 % suffer a "flu-like" illness and recover without therapy. This disease exhibits the typical symptoms of a pulmonary fungal disease: anorexia, weight loss, cough, hemoptysis, and resembles TB. CNS infection with *C. immitis* is more common while it is less frequent with the other fungal diseases. The ecological niche of *C. immitis* is the Sonoran desert, which includes the deserts of the Southwest (California, Arizona, New Mexico, Nevada, Utah

and Texas) and northern Mexico. It is also found in small foci in Central and South America.

Desert soil, pottery, archaeological middens, cotton, and rodent burrows all harbor *C. immitis*. *C. immitis* is a dimorphic fungus with 2 life cycles. The organism follows the SAPROPHYTIC cycle in the soil and the PARASITIC cycle in man or animals. The saprophytic cycle starts in the soil with spores (arthroconidia) that develop into mycelium. The mycelium then matures and forms alternating spores within itself. The arthroconidia are then released, and germinate back into mycelia. The parasitic cycle involves the inhalation of the arthroconidia by animals which then form spherules filled with endospores. The ambient temperature and availability of oxygen appear to govern the pathway The organism can be carried by the wind and therefore spread hundreds of miles in storms so the distribution is quite wide. In 1978, cases were seen in Sacramento 500 miles north of the endemic area, from a dust storm in Southern California. The spores of the organism are readily airborne. The cases that occur in South Carolina are usually in patients who have visited an endemic area and brought back pottery, or blankets purchase from a dusty roadside stand, or in Navy and Air Force personnel who were exposed when they were stationed in the endemic area. The disease manifests itself after they are transferred to a base in South Carolina. A few interesting cases occurred in cotton mills in Burlington and Charlotte, N.C. The cotton, grown in the desert of the Southwest, was contaminated with the fungus spores and the mill workers inhaled the spores while handling the raw cotton and developed coccidioidomycosis.

Histopathology

The inflammatory reaction is both purulent and granulomatous. Recently released endospores incite a polymorphonuclear response. As the endospores   mature into spherules, the acute reaction is replaced by lymphocytes, plasma cells, epithelioid cells and giant cells.

Serology

There are four tests for diagnosis:

- Complement-Fixation
- Slide agglutination
- Immunodiffusion
- EIA C-F antibody is slow to rise and develop in about 1 month. This test is excellent for coccidioidomycosis because it is quantitative. However, these antibodies cross-react with some other fungi (Blastomyces and Histoplasma). The C-F test is also a PROGNOSTIC test. If the titer keeps rising, then the patient is responding poorly and the course may be fatal. If the C-F titer is dropping then the prognosis for that patient is favorable. A titer of greater than 1:128 usually indicates extensive dissemination. Life-long immunity usually follows infection with *C. immitis.* There is a much greater mortality rate in dark-skinned people (Mexicans, Filipinos, and Blacks). They are 25 times more likely to develop progressive disease and death. The reason for this is obscure.

Amphotericin B, fluconazole and itraconazole are the drugs of choice.