Lecture-7, 8 & 9: Microbiological methods for identification of Microorganisms

Microbiological methods for identification of microorganisms

Various methods used to identify organisms cultivated from patient specimens, associated with bacterial identification. **The procedures for diagnosis of infectious diseases is as follows:**

1. Direct examination of patient specimens.

2. Growth and cultivation of the agents from the specimens.

3. Analysis of the cultivated organisms to establish their identification and other pertinent characteristics such as **susceptibility to antimicrobial agents.**

Macroscopic Observation: It provides useful information to both the microbiologist and the physician **the macroscopic observation should include the following:**

- **Swab or aspirate.**
- **Stool consistency (formed or liquid).**
- **Blood or mucus present.**
- **Volume of specimen.**
- **Fluid, clear or cloudy.**

The gross examination also allows the processor to determine the adequacy of the specimen and the need for special processing. Areas of blood and mucus are selected for culture and direct microscopic examination. **Anaerobic cultures may be indicated if gas, foul smell, or sulfur granules are present.**

Microscopic Observation

Microscopy is defined as the use of a microscope to magnify (i.e., visually enlarge) Because most infectious agents cannot be detected with the unaided eye, microscopy plays a pivotal role in the laboratory.

Types of Microscope are:

Bright-field microscopy (also known as **light microscopy**) Many bacteria are difficult to see well because of their lack of contrast with the surrounding medium. Dyes (stains) can be used to stain cells or their organelles and increase their contrast so that they can be more easily seen in the bright-field microscope.

The phase-contrast microscope was developed to improve contrast differences between cells and the surrounding medium, making it possible to see living cells without staining them; with bright-field microscopes, killed and stained preparations must be used.

Dark-field this technique has been particularly useful for observing organisms such as *Treponema pallidum*, a spirochete that is smaller than 0.2 μm in diameter and therefore cannot be observed with a bright-field or phase-contrast microscope.

Fluorescence microscopy is widely used in clinical diagnostic microbiology. For example, the fluorochrome auramine O, which glows yellow when exposed to ultraviolet light, is strongly absorbed by the cell envelope of *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis. When the dye is applied to a specimen suspected of containing *M. tuberculosis* and exposed to ultraviolet light, the bacterium can be detected by the appearance of bright yellow organisms against a dark background.

Differential Interference Contrast Microscope (DIC)

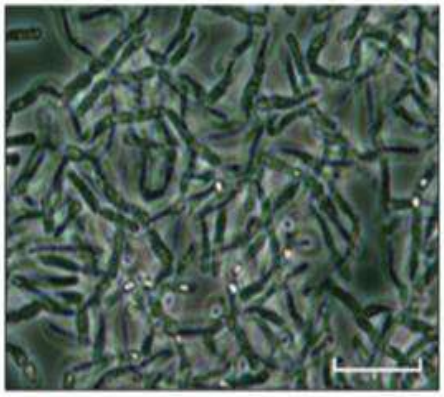
Structures, such as spores, vacuoles, and granules, **appear three dimensional**. DIC microscopy is particularly useful for observing unstained cells because of its ability to generate images that reveal internal cell structures that are less apparent by bright field techniques.

The Electron Microscope

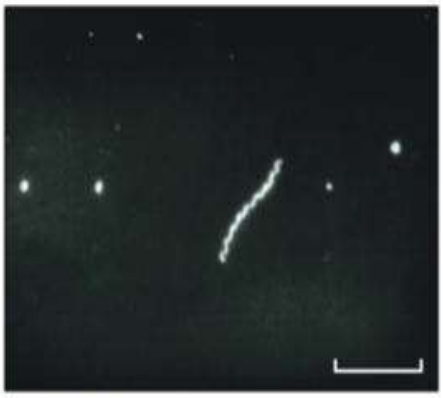
There are two types of electron microscopes in general use: The **transmission electron microscope (TEM)**, which has many features in common with the light microscope; and the **scanning electron microscope (SEM)**. is particularly useful for providing **three dimensional images** of the surface of microscopic objects.

Scanning Probe Microscopes

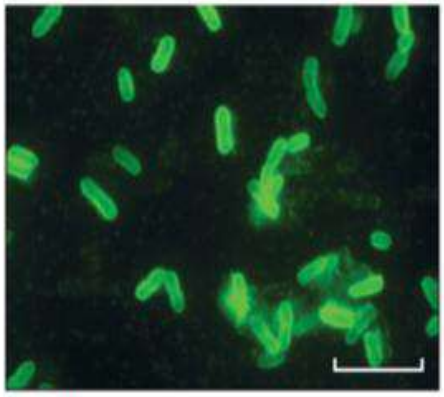
A new class of microscopes, called **scanning probe microscopes**, measures surface features by moving a sharp probe over the object's surface.



(A) Phase Contrast
Cells (dark) are contrasted from the lighter dots (spores).



(B) Dark Field
Unstained cells are seen against a dark background.



(C) Fluorescence
Cells "glow" due to the presence of a fluorescent antibody that binds to the cells.

Direct and Indirect Smears

A direct smear is a preparation of the primary clinical sample received in the laboratory for processing. A direct smear provides a mechanism to identify the number and type of cells present in a specimen**, including white blood cells, epithelial cells, and predominant organism type.**

Indirect smear organisms obtained after purification or growth on artificial media. Indirect smears may include preparation from solid or semisolid media or broth. Care should be taken to ensure the smear is not too thick when preparing the slide from solid media.

Staining:

A specimen must contain at least 105 organisms per milliliter before it is likely that organisms will be seen on a smear. Liquid medium containing 10^5 organisms per

milliliter does not appear turbid to the eye. Specimens containing **10^2–10^3 organisms per milliliter** produce growth on solid media, there are many types of stains, each with specific applications.

- **Simple stains** are directed toward **coloring the forms and shapes present**,
- **differential stains** are directed toward **coloring specific components of the elements present,**
- **diagnostic antibody or DNA probe–mediated stains** are directed specifically at identification of an organism.

Staining techniques

Structural details of bacteria cannot be seen under light microscope due to lack of contrast. Hence, it is necessary to use staining methods to produce color contrast and thereby increase the **visibility**. Before staining, the fixation of the smear to the slide is done.

Fixation is the process by which the **internal and external structures** of cells are preserved and **fixed in position**. It also **inactivates the enzymes** that might disrupt cell morphology. It toughens (**hardens**) cell structure so that they do not change during staining. It kills and fixes the cells on to the slide.

There are two types of fixation as follows:

1. **Heat fixation**: It is usually done for bacterial smears by gently flame heating an air-dried film of bacteria. This adequately **preserves overall morphology but not structures within the cells**.

2. **Chemical fixation**: It can be done using **ethanol, acetic acid, mercuric chloride, formaldehyde, methanol and glutaraldehyde**. They are used **to protect the fine internal structure of the cells.** This is useful for examination of blood smears. The fixed smear is stained by appropriate staining technique.

Common staining techniques used on microbiology:

- **Simple stain**: Basic dyes, such as **methylene blue or basic fuchsin** are used as simple stains. They provide the color contrast, but impart the same color to all the bacteria in a smear.
- **Negative staining**: A drop of bacterial suspension is mixed with dyes, such as **India ink or nigrosin**. The **background gets stained black** whereas **unstained bacterial/yeast capsule** stand out in contrast. It is very useful in the demonstration of bacterial/yeast **capsules which do not take up simple stains.**
- **Impregnation methods**: Bacterial cells and structures that are too thin to be seen under the light microscope, are **thickened by impregnation of silver salts** on their surface to make them visible, e.g. for demonstration of **bacterial flagella and spirochetes.**
- **Differential stain**: two stains are used which impart different colors to different bacteria or bacterial structures, which **help in differentiating bacteria**. **The most commonly employed differential stains are:**

1. **Gram stain**: It differentiates bacteria into gram positive and gram negative groups (**G+ or G- bacteria**)
2. **Acid-fast stain**: It differentiates bacteria into **acid fast and nonacid fast groups**
3. **Chromatic granules** from other bacteria that do not have them.

Single Enzyme Tests (biochemical tests)

Several tests are commonly used to determine the presence of a single enzyme. These tests usually provide rapid results because they can be performed on organisms already grown in culture.

Catalase Test

The enzyme **catalase** catalyzes the release of water and oxygen from hydrogen peroxide (H2O2 _ catalase H2O _O2); its presence is determined by direct analysis of a bacterial culture. **The rapid production of bubbles when bacterial growth is mixed with a hydrogen peroxide solution is interpreted as a positive test.**

Oxidase Test

Cytochrome oxidase participates in electron transport and in the nitrate metabolic pathways of certain bacteria. Testing for the presence of oxidase can be performed by flooding bacterial colonies on the agar surface with 1% **tetramethyl-p-phenylenediamine dihydrochloride.** Alternatively, a sample of the bacterial colony can be rubbed onto filter paper impregnated with the reagent-A positive reaction is indicated by the development of a purple color.

Indole Test

The enzyme **tryptophanase** are able to degrade the amino acid tryptophan into pyruvic acid, ammonia, and indole. **Indole** is detected by combining with an indicator Kovac's reagent), which results in a pink to red color formation.

Urease Test

Urease hydrolyzes the substrate urea into ammonia, water, and carbon dioxide. The presence of the enzyme is determined by inoculating an organism to broth (**Stuart's urea broth) or agar (Christensen's urea agar)** containing urea as the primary carbon source followed by detecting the production of ammonia. Ammonia increases the pH of the medium so its presence is readily detected using a pH indicator. Change in medium pH is a common indicator of metabolic process and, because pH indicators change color with increases (alkalinity). The urease test helps identify ***Proteus*** spp., and other important bacteria such as ***Corynebacterium urealyticum*** and ***Helicobacter pylori***.

Oxidation and Fermentation Tests

Bacteria utilize of carbohydrates (e.g., sugar or sugar derivatives) and protein substrates. **Oxidation-fermentation** determinations are usually accomplished using a special semisolid medium (oxidative fermentative [O-F] medium) that contains low concentrations of peptone and a single carbohydrate substrate such as glucose.

The glucose fermentative or oxidative capacity is generally used to separate organisms into major groups (e.g., *Enterobacteriaceae* are fermentative; *Pseudomonas* spp. are oxidative).

Amino Acid Degradation

The amino acid substrates most often tested include lysine, tyrosine, ornithine, arginine, and phenylalanine. (The indole test for **tryptophan** cleavage is presented.)

Decarboxylases cleave the carboxyl group from amino acids so that amino acids are converted into amines; lysine is converted to cadaverine, and ornithine is converted to putrescine. Because amines increase medium pH, they are readily detected by color changes in a pH indictor indicative of alkalinity. **Decarboxylation** is an anaerobic process that requires an acid environment for activation, the amino acid substrate of interest (i.e., lysine, ornithine, or arginine), and a pH indicator.

Complementary diagnostic methods are API test and VITEK system.

There some non-traditional methods for identification of pathogens or their products include:

- Molecular testing.
- Saliva based testing.
- Chromogeneic testing.
- Rapid immunochromatographic testing.

All these tests are expensive and not used as ordinary laboratory tests.

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