Diagnostic Microbiology

Microbial colonization may result in:

1) Elimination of the microorganism without affecting the host.

2) Infection in which the organisms **multiply and cause** the host to react by making an immune or other type of response.

3) A transient or prolonged carrier state.

- **Infectious disease occurs when the organism causes tissue damage and loss function of body systems.**

Microbiologic diagnosis of an infectious disease

usually involves the following laboratory techniques, which guide the physician along a narrowing path of possible causative pathogens:

1. Morphologic identification of the agent

1. in **stains of specimens** or
2. **sections of tissues** (light and electron microscopy)

2. Cultivation and identification of the organisms.

3. Detection of microbial antigens by immunologic assay (latex agglutination, enzyme immunoassay [EIA].

4. Detection of microbial DNA or RNA.

5. Detection of an inflammatory or host immune response to the pathogenic agents.

Section 4

Cultivation and isolation of viable pathogens

Laboratory media

Culture media are required to isolate the bacteria from the clinical specimens;

following which the appropriate **biochemical tests** can be performed **to identify the causative agent**.

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Constituents of culture media:

The basic constituents of culture media are:

- **Water:** Distilled water
- **Electrolyte:** Sodium chloride or other electrolytes.
- **Peptone:** It is a complex mixture of partially digested proteins.

• **Source:** It is obtained from lean **meat** or other protein material, such as heart muscle, casein or fibrin, or soya flour usually by **digestion with proteolytic enzymes**, such as pepsin

• Constituents: It contains proteoses, aminoacids, inorganic salts (phosphates, potassium and magnesium), accessory growth factors like nicotinic acid and riboflavin

- **Agar:** It is used for **solidifying the culture media**. It is commercially available in powder form; melts in water after boiling and jellifies after cooling also called 'agar-agar' is prepared from the cell wall of variety of seaweeds.
- **Preparation of agar media:**

The appropriate amount of agar powder is added to water and the mixture is dissolved and then sterilized by placing it in an autoclave.

When the temperature of the molten agar comes down to 45°C, it is poured to the Petri dishes and then allowed to set for 20 minutes.

- **Meat extract:** **It is a commercial preparation of highly concentrated meat stock, usually made from beef**. It contains protein degradation products, inorganic salts, carbohydrates and growth factors.

Yeast extract:

Itis prepared commercially from washed cells of Baker's yeast. It contains amino acids, inorganic salts (potassium and phosphates) and carbohydrates. (Figure-5)

A. Peptone water; B. Nutrient agar; C. Blood agar; D. Chocolate agar.

Malt extract: It consists of maltose (about 50%), starch, dextrin, glucose and 5% protein products.

Blood and serum:

They are important components of enriched media and provide extra nutrition to fastidious bacteria. Usually 5- % of sheep blood is used.

Types of culture media:

Bacteriological culture media can be classified in two ways:

A. Based on consistency, culture media are grouped into:

1. Liquid media (or broth)
2. Semisolid media
3. Solid media

B. Based on the growth requirements, culture media are classified as:

1. Routine laboratory media: They are prepared from nutrients, such as aqueous meat, peptone, etc.
They can f be classified into various types based on functional use as
Simple media; Enriched media; Enrichment broth; Selective media; Differential media; Transport media; Anaerobic media.

2. Defined or synthetic media: They are prepared from pure chemical substances the media is known:
Simple synthetic media and
Complex synthetic media.

Simple media

Many bacteria will grow in or on simple media such as **nutrient broth/nutrient agar** that contains ‘peptone’

Enriched media

These contain additional nutrients for the isolation of more fastidious bacteria that require special conditions for growth like agar containing whole **blood (blood agar) or**
agar containing lysed blood (chocolate agar).

Microbiological methods for identification of microorganisms

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

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 stain.

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: Here, 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.

Macroscopic Observation

Processing patient specimens begins with a macroscopic observation.

The **gross appearance of the specimen** may provide useful information to both the microbiologist and the physician.عام أجمالي

Notations from the macroscopic observation should ***include the following:***

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

The gross examination 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 (Optical methods for laboratory diagnosis):

A direct microscopic examination is a useful tool that provides rapid information.

- In critical situations, such as meningitis, the direct microscopic examination can be used to guide therapy choices when therapy must be initiated before culture results are available.

Specimens may be received in many forms.

Preparation of the direct smear depends on the type of material received.

Techniques vary according to whether the specimen is a tissue, swab, or fluid.

Microscopic observation serves several purposes:

1. It can be used to determine the quality of the specimen.
Sputum specimens that represent saliva rather than lower respiratory secretions can be determined by the quantitation of WBCs or epithelial cells.

2. It can give the diagnostic microbiology technologist and the physician an indication of the infectious process involved. Gram stain of a sputum specimen revealing WBCs and gram-positive diplococci is indicative of ***Streptococcus pneumoniae*.**

3. The routine culture workup can be guided by the results of the smear. The technologist can correlate the bacterial isolates with the types detected in the smear; this may alert the technologist to the presence of additional organisms not yet growing, such as anaerobes.

4. It can dictate the need for non-routine or additional testing.
The **presence of fungal elements in a specimen** for bacterial culture would alert the technologist to notify the physician to request a fungus culture.

In certain specimen types, the direct microscopic examination does not provide useful information and is not appropriate.

Throat and nasopharyngeal specimens are examples.

Gram stains for ***N. gonorrhoeae*** on specimens from the vagina, cervix, and anal crypts are **not recommended** because these sites contain other bacteria that can have the same morphology, although Gram stain direct smears are recommended to diagnose bacterial **vaginosis.**

Gram stains on stool specimens are not usually routine, although they can be useful to determine whether the patient has an inflammatory diarrhea based on the **presence of WBCs**.

Bacterial cultivation:

The process of bacterial cultivation involves the use of **optimal artificial media** and incubation conditions to isolate and identify the bacterial etiologies of an infection as rapidly and accurately as possible.

Media used for bacterial incubation conditions are selected for their **ability to support the growth** of the bacteria most likely to be involved in the **infectious of etiologic agents**, specimen inoculum is usually spread over the surface of plates in a standard pattern.

Typically, a calibrated loop of 1µL is used for urine cultures.
However, in situations where a lower count of bacteria may be present such as a suprapubic aspiration, a 10 µL loop may be needed to identify the lower count of organisms.

There are some criteria frequently used to characterize bacterial growth include the followings:

1. Colony size (usually measured in millimeters or described in relative terms such as pinpoint, small, medium, large).

2. Colony pigmentation.

3. Colony shape (includes form, elevation (raise), and margin (border) of the colony).

4. Colony surface appearance (e.g., glistening (shining), opaque, cloudy, dry, transparent).

5. Changes in agar media resulting from bacterial growth (e.g., hemolytic pattern on blood agar, changes in color of pH indicators, pitting of the agar surface).

6. Odor (certain bacteria produce distinct odors `smells` that can be helpful in preliminary identification)

Biochemical tests:

In diagnostic bacteriology, enzyme-based tests are designed to measure the presence of one specific enzyme or a complete metabolic pathway that may contain several different enzymes.

Single Enzyme Tests;

which include several tests are commonly used to determine the presence of a single enzyme. These tests (**chemical tests**) usually provide **rapid results** because they can be performed on organisms already grown in culture.

these tests are easy to perform and interpret and often play a key role in the identification scheme

Examples of these biochemical tests include:

1. Catalase Test: The enzyme catalase catalyzes the release of water and oxygen from hydrogen peroxide (H_2O_2 + catalase => H_2O + O_2); its presence is determined by direct analysis of a bacterial culture.

The catalase test is key to the identification scheme of **many gram-positive organisms,** and the interpretation must be done carefully to **differentiate** different bacterial types within G+ bacteria.

2. 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% tetra methyl-p-phenylenediamine dihydrochloride.

The test is initially used for differentiating between groups of gram-negative bacteria.

Among the commonly encountered gram-negative bacilli, Enterobacteriaceae, Stenotrophomonas maltophilia, and Acinetobacter spp. are **oxidase-negative,** whereas many other bacilli, such as ***Pseudomonas*** spp. and ***Aeromonas*** spp., are **positive**.

3. Indole Test: Bacteria that produce 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, aldehyde ([4-dimethylamino] benzaldehyde, hydrochloric acid, and penta-1-01, also referred to as Kovac's), which results in a blue color formation.

This test is used in numerous identification schemes, especially to presumptively identify ***Escherichia coli,*** the gram-negative bacillus most commonly encountered (done) in diagnostic bacteriology.

4. 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 or 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) or decreases (acidity) in the medium's pH, they are commonly used in many identification test schemes.

The urease test helps identify certain species of ***Enterobacteriaceae***, such as ***Proteus spp***., and other important bacteria such as ***Corynebacterium urealyticum*** and ***Helicobacter pylori***.

5. Oxidation and Fermentation Tests:

Bacteria use various metabolic pathways to produce biochemical building blocks and energy.

Determining whether substrate utilization is an oxidative or fermentative process is important for the identification of several different bacteria.

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

However, the **utilization pattern for several other carbohydrates** (e.g., lactose, sucrose, xylose, maltose) is often needed to help identify an organism's genus and species.

6. Amino Acid Degradation tests:

Determining the ability of bacteria to produce enzymes that either deaminate, dihydrolyze, or decarboxylate certain amino acids is often used in identification schemes.

The amino acid substrates most often tested include lysine, tyrosine, ornithine, arginine, and phenylalanine.

Establishing Inhibitor Profiles:

The ability of a **bacterial isolate to grow in the presence of one or more inhibitory substances can provide valuable identification information**.

In addition to the information gained from **using inhibitory media or antimicrobial susceptibility testing, other more specific tests** may be incorporated into bacterial identification schemes. Because most of these tests are used to identify a particular group of bacteria.

A few examples of such tests include the followings:

1. Growth in the presence of various NaCl concentrations (identification of **Enterococci** and **Vibrio spp**.).

2. Susceptibility to Optochin and **solubility in bile** (identification of *Streptococcus pneumoniae*).

3. Ability to hydrolyze esculin in the presence of bile (identification of **Enterococci** spp. in combination with NaCl).

4. Ethanol survival (identification of *Bacillus* spp.)