# General Bacteriology: Laboratory Diagnosis of Bacterial Infections

CHAPTER 3.3

**CHAPTER PREVIEW**

- Specimen Collection
- Direct Detection
  - Staining Techniques
  - Other Methods of Direct Detection
- Culture, Identification and AST
  - Culture Media
  - Culture Methods
  - Culture Identification
  - Antimicrobial Susceptibility Test
- Serology
- Molecular Methods
- Microbial Typing

## INTRODUCTION

Laboratory diagnosis of bacterial infections is useful for the following purposes:

- **Identification:** To identify the causative bacterial agent responsible for the disease
- **Treatment:** To provide accurate antimicrobial therapy
- **Surveillance purpose:** To assess the disease burden in the community by estimating the prevalence and incidence of the infections
- **For outbreak investigation,** e.g. diphtheria outbreaks in the community, MRSA (methicillin-resistant *S. aureus*) outbreaks in the hospitals
- **To start PEP** (post-exposure prophylaxis): Useful in infectious diseases such as, anthrax and plague
- **To initiate appropriate infection control measures:** For example, contact precaution for MRSA infection, droplet precaution for diphtheria and airborne precaution for tuberculosis (Chapter 21).

Laboratory diagnosis of bacterial infections comprises of several steps—specimen collection, direct detection, culture, identification and antimicrobial susceptibility test, serology and molecular methods (refer box).

**LABORATORY DIAGNOSIS** — Bacterial infections

1. **Specimen collection**
2. **Direct detection**
   - Microscopy: Gram stain, acid-fast stain, Albert stain, histopathological staining, dark ground, phase-contrast and fluorescence microscopy
   - Antigen detection from clinical specimen
   - Molecular diagnosis: Detecting bacterial DNA or RNA from clinical specimen
3. **Culture**
   - Culture media
   - Culture methods
   - Colony morphology, smear and motility testing
4. **Identification**
   - Biochemical identification

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   - Automated identification methods
5. **Antimicrobial susceptibility testing**
6. **Serology**
7. **Molecular methods**
8. **Typing methods**

## SPECIMEN COLLECTION

Specimen collection depends upon the type of underlying infections (Table 3.3.1). The proper collection of specimen is of paramount importance for the isolation of the bacteria in culture.

### General Principles

The following general principles should be followed while collecting the specimen:

- **Standard precautions** should be followed for collecting and handling all specimens (Chapter 21 for details)
- **Before antibiotics start:** Whenever possible, culture specimens should be collected prior to administration of any antimicrobial agents
- **Contamination** with indigenous flora should be avoided, especially when collecting urine and blood culture specimens
- **Swabs** are though convenient but considered inferior to tissue, aspirate and body fluids
- **Container:** Specimens should be collected in sterile, tightly sealed, leak proof, wide-mouth, screw-capped containers
- **Labeling:** All specimens must be appropriately labelled with name, age, gender, treating physician, diagnosis, antibiotic history, type of specimen, and desired investigation name
- **Rejection:** Specimens grossly contaminated or compromised or improperly labelled may be rejected (Annexure 9)
- If **anaerobic culture** is requested, proper anaerobic collection containers with media should be used

**Table 3.3.1: Types of infections and various specimens collected.**

| Type of infections | Specimens collected |
|---|---|
| Bloodstream infection, sepsis, endocarditis | Paired blood culture specimens<br>• Collected aseptically by two-step disinfection of skin; first with alcohol followed by chlorhexidine<br>• 8–10 mL of blood (for adults) collected in blood culture bottles |
| Infectious diseases requiring serology | • Blood (2 mL/investigation)<br>• Collected by minimal asepsis (one-step skin disinfection with alcohol)<br>• Collected in vacutainer |
| Diarrheal diseases | Stool (mucus flakes), rectal swab |
| Meningitis | Cerebrospinal fluid (CSF) |
| Infections of other sterile body area | Sterile body fluids; e.g. pleural fluid, synovial fluid, peritoneal fluid |
| Skin and soft tissue infections | Pus or exudate, wound swabs, aspirates from abscess and tissue bits |
| Anaerobic infections | Aspirates, tissue specimens, blood and sterile body fluids, bone marrow<br>(swabs, sputum not satisfactory) |
| Upper respiratory tract infections | Throat swab with membrane over the tonsil, nasopharyngeal swab, per-nasal swab |
| Lower respiratory tract infections | Sputum, endotracheal aspirate, bronchoalveolar lavage (BAL), protected specimen brush (PSB) and lung biopsy |
| Pulmonary tuberculosis | • Sputum—early morning and spot<br>• Collected in well-ventilated area<br>• Gastric aspirate for infants |
| Urinary tract infections | Midstream urine<br>Suprapubic aspirated urine<br>Catheterized patient—collected from the catheter tube, after clamping distally and disinfecting; not from urobag |
| Genital infections | Urethral swab, cervical swab—for urethritis<br>Exudate from genital ulcers |
| Eye infections | Conjunctival swabs<br>Corneal scrapings<br>Aqueous or vitreous fluid |
| Ear infections | Swabs from outer ear<br>Aspirate from inner ear |

- Specimen should not be sent in container containing **formalin** for microbiological analysis.

### Specimen Transport

The specimens should reach the laboratory for further processing as soon as possible after the collection. If required appropriate transport media should be used (discussed subsequently in this chapter).

For most of the specimens, transport time should not exceed **two hours**. However, there are some exceptions.

- Specimens that require an **immediate transport** (<15 minutes)—such as CSF and body fluids, ocular specimens, tissue specimens, suprapubic aspirate and bone specimen
- **Urine (midstream)** added with preservative (boric acid) is acceptable up to 24 hours, otherwise should be transported within 2 hours
- **Stool culture:** Stool specimen should be transported within 1 hour, but with transport medium (Cary-Blair medium) up to 24 hours is acceptable
- **Rectal swabs**—up to 24 hours is acceptable
- **For anaerobic culture**: Specimens should be put into Robertson's cooked meat broth or any specialized anaerobic transport system and transported immediately to the laboratory.

### Specimen Storage before Processing

Most specimens can be stored **at room temperature** immediately after receipt, for **up to 24 hours**. However, there are some exceptions.

- **Blood cultures—**should be incubated at 37°C immediately upon receipt
- Sterile body fluids, bone, vitreous fluid, suprapubic aspirate—should be immediately plated upon receipt and incubated at 37°C
- **Corneal scraping**—should be immediately plated at bed-side on to blood agar and chocolate agar
- **Stool culture**—can be stored up to 72 hours at 4°C
- **Urine** (mid-stream and from the catheter), **lower respiratory** tract specimen, **gastric biopsy** (for *Helicobacter pylori*)—can be stored up to 24 hours at 4°C.

## DIRECT DETECTION

Direct detection of bacteria in the clinical specimen plays a very important role in early institution of antimicrobial therapy. These methods include microscopic demonstration of bacteria—staining techniques and other methods such as detection of antigen or nucleic acid in the clinical specimen.

## STAINING TECHNIQUES

Structural details of bacteria cannot be seen under a 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 smears are fixed so that they will not be displaced during the staining process. Fixation also protects the internal structures of cells in a fixed position. It is done by two methods.

1. **Heat fixation:** It is done by gently flame heating an air-dried film, used for bacterial smears
2. **Methanol fixation:** Used for blood smears.

**Common staining techniques** used in diagnostic bacteriology include:

- **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. This 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:
  - **Gram stain:** It differentiates bacteria into gram-positive and gram-negative groups
  - **Acid-fast stain:** It differentiates bacteria into acid-fast and non acid-fast groups
  - **Albert stain:** It differentiates bacteria having metachromatic granules from other bacteria that do not have them.

## Gram Stain

This staining technique was originally developed by Hans Christian Gram (1884). Even after more than 130 years of its discovery and even in the presence of newer modern diagnostic facilities, still Gram stain remains the most widely used test in diagnostic bacteriology.

### *Procedure (Fig. 3.3.1)*

- **Fixation:** The smear made on a slide from bacterial culture or specimen is air-dried and then heat-fixed

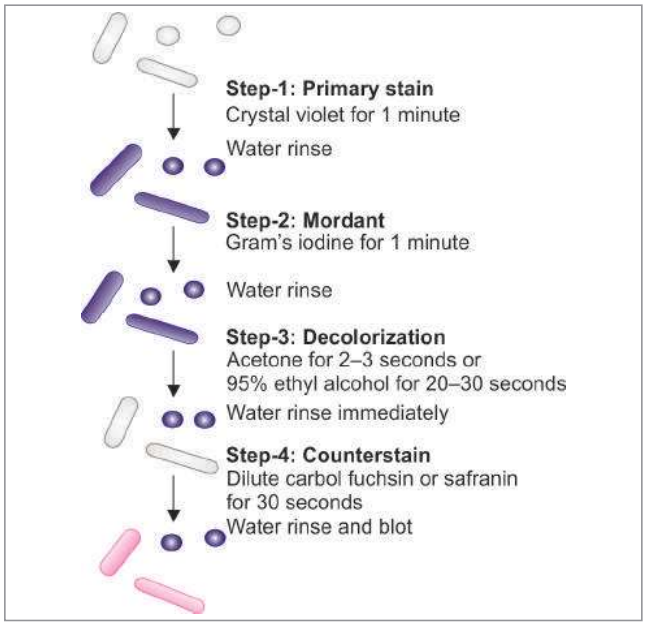



**Fig. 3.3.1:** Principle and procedure of Gram staining.

- **Step 1 (Primary stain):** The smear is stained with pararosaniline dyes such as crystal violet (or gentian violet or methyl violet) for one minute. Then the slide is rinsed with water. Crystal violet stains all the bacteria violet in color (irrespective of whether they are gram-positive or gram-negative)
- **Step 2 (Mordant):** Gram's iodine (dilute solution of iodine) is poured over the slide for one minute. Then the slide is rinsed with water. Gram's iodine acts as a mordant, binds to the dye to form bigger dye-iodine complexes in the cytoplasm
- **Step 3 (Decolorization):** Next step is pouring of few drops of decolorizer to the smear, e.g. acetone (for 1–2 sec) or ethyl alcohol (20–30 sec) or acetone alcohol (for 10 sec). Slide is immediately rinsed with water. Decolorizer removes the primary stain from gram-negative bacteria while the gram-positive bacteria retain the primary stain

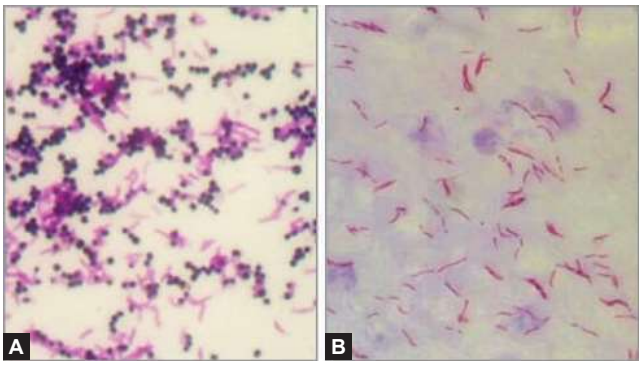
> *Note:* **Decolorization** is the most crucial step of Gram staining. If the decolorizer is poured for more time, even gram-positive bacteria lose color **(over decolorization)** and if poured for less time, the gram-negative bacteria do not lose the color of primary stain properly **(under decolorization)**.

- **Step 4 (Counter stain):** Secondary stains such as safranin or dilute carbol fuchsin is added for 30 seconds. It imparts pink or red color to the gram-negative bacteria. Alternatively, neutral red may also be used as counter stain, especially for gonococci. The slide is rinsed in tap water, dried, and then examined under oil immersion objective.

### *Interpretation of Gram Stain*

Smear is examined under oil immersion objective (Fig. 3.3.2A).

- Gram-positive bacteria resist decolorization and retain the color of primary stain i.e. violet
- Gram-negative bacteria are decolorized and, therefore, take counterstain and appear pink.




**Figs 3.3.2A and B: A.** Gram staining demonstrating violet-colored gram-positive cocci in clusters and pink colored gram-negative bacilli in scattered arrangement; **B.** Acid-fast staining shows long slender straight or slightly curved beaded red acid-fast bacilli.

*Source:* A. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry; B. Department of Microbiology, JIPMER, Puducherry (*with permission*).

### Principle of Gram Staining

Though the exact mechanism is not understood, the following theories have been put forward.

- **pH theory:** Cytoplasm of gram-positive bacteria is more acidic, hence, can retain the basic dye (e.g. crystal violet) for longer time. **Iodine** serves as mordant, i.e. it combines with the primary stain to form a dye-iodine complex which gets retained inside the cell
- **Cell wall theory:** This is believed to be the most important postulate to describe the mechanism of Gram stain
  - Gram-positive cell wall has a thick peptidoglycan layer (50–100 layers thick), with tight cross linkages
  - The peptidoglycan itself is not stained; instead, it seems to act as a permeability barrier preventing loss of crystal violet. More so, alcohol is thought to shrink the pores of the thick peptidoglycan; hence large dye-iodine complexes are not able to penetrate this tightened peptidoglycan layer in a gram-positive bacteria
  - Gram-negative cell wall is more permeable thus allowing the outflow of crystal violet easily. This is attributed to:
    - The thin peptidoglycan layer in gram-negative cell wall which is not tightly cross linked
    - Presence of lipopolysaccharide layer in the cell wall of gram-negative bacteria, which gets disrupted easily by the decolorizer; forming larger pores, that allow the dye-iodine complexes to escape from the cytoplasm.

### Modifications of Gram Staining

There are a few minor modifications of Gram stain which vary slightly from the method described earlier.

- **Kopeloff and Beerman's modification:** Primary stain and counter stain used are methyl violet and basic fuchsin respectively
- **Jensen's modification:** This method involves use of absolute alcohol as decolorizer and neutral red as counter stain. It is useful for meningococci and gonococci
- **Brown and Brenn modification:** This is used for Actinomycetes.

**Uses of Gram Stain**

- **To differentiate bacteria into gram-positive and gram-negative:** It is the first step towards identification of bacteria
- **For identification:** Gram staining from bacterial culture gives an idea to put the corresponding biochemical tests for further identification of bacteria
- **To start empirical treatment:** Gram stain from the specimen gives a preliminary clue about the bacteria present (based on the shape and Gram staining property of the bacteria) so that the empirical treatment with broad-spectrum antibiotics can be started early before the culture report is available
- **For fastidious organisms**, such as *Haemophilus* which takes time to grow in culture; Gram stain helps in early presumptive identification

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- **Anaerobic organisms**, such as *Clostridium* do not grow in routine culture. Therefore, organisms detected in Gram stain, but aerobic culture-negative gives a preliminary clue to perform an anaerobic culture of the specimen
- **Yeasts:** In addition to stain the bacteria, Gram stain is useful for staining certain fungi such as *Candida* and *Cryptococcus* (appear gram-positive)
- **Quality of specimen:** Gram stain helps in screening the quality of the **sputum specimen** before processing it for culture. Presence of more pus cells and less epithelial cells indicates good quality specimen.

## Acid-fast Stain

The acid-fast stain was discovered by Paul Ehrlich and subsequently modified by Ziehl and Neelsen. This staining is done to identify acid-fast organisms, such as *Mycobacterium tuberculosis* and others. Acid-fastness is due to presence of mycolic acid in the cell wall.

### Ziehl-Neelsen Technique (Hot Method)

#### Smear Preparation

Smear measuring 2 × 3 cm in size is prepared in a new clean grease free scratch free slide from the yellow purulent portion of the sputum.

- The smear should neither be too thick nor too thin. When placed over a printed matter, the print should be readable through the smear
- Smear preparation should be done near a flame, as six inches around the flame is considered sterile zone (as heat coagulates the aerosols raised during the smear preparation).

#### Heat Fixation

The smear is air dried for 15–30 minutes and then heat fixed by passing over the flame 3–5 times for 3–4 seconds each time. Coagulation of the proteinaceous material in the sputum will facilitate fixing of the smear.

#### Procedure

***Step 1 (Primary stain)***

Smear is poured with strong carbol fuchsin (1%) for 5 minutes. Intermittent heating is done by flaming the underneath of the slide until the vapor rises. Heating helps in better penetration of the stain.

- Care must be taken to ensure that the smear does not dry out, to counteract drying more solution of stain is added to the slide and the slide reheated
- Rinse the slide with tap water, until all free carbol fuchsin stain is washed away. At this point, the smear on the slide looks red in color.

***Step 2 (Decolorization)***

It is done by pouring 25% sulfuric acid over the slide and allowing it to stand for 2–4 minutes. The slide is gently rinsed with tap water and tilted to drain off the water.

- A properly decolorized slide appears light pink. If the slide is still red, sulfuric acid is reapplied for 1–3 minutes and then rinsed gently with tap water
- The back of the slide is wiped clean with a swab dipped in sulfuric acid.

***Step 3 (Counter staining)***

0.1% methylene blue is poured onto the slide and left for 30 seconds. Then the slide is rinsed gently with tap water and allowed to dry.

- The slide is examined under the binocular microscope using 40× lens to select a suitable area and then examined under oil immersion field (100×)
- Contaminated materials/slide should be discarded in a jar containing 5% phenol.

#### Interpretation

*Mycobacterium tuberculosis* appears as long slender, straight or slightly curved and beaded, red colored acid-fast bacillus. Other non-acid fast organisms present in the smear and the background take up the counter stain and appear blue (Fig. 3.3.2B).

### *Modifications of Acid-Fast Staining*

Hot method (Ziehl–Neelsen technique) is the most commonly done acid-fast staining technique. Other modifications are as follows:

- **Cold method (Kinyoun's method):** It is modification, where the intermittent heating is not required (described in Chapter 63)
- Acid-alcohol can be used as decolorizer alternatively
- Malachite green can be used as counter stain
- The concentration of sulfuric acid may vary depending on the acid-fastness of the structure to be demonstrated. More the content of mycolic acid in the cell wall, more is the acid-fastness, hence more is the percentage of sulfuric acid required for decolorization (Table 3.3.2).

## Albert Stain

Albert stain is used to demonstrate the metachromatic granules of *Corynebacterium diphtheriae*.

### *Procedure*

- Fixation: The smear is heat fixed
- Smear is covered with Albert I stain for 5 minutes, then the excess stain is drained out
- Albert II (iodine solution) is added for 1 minute
- Slide is washed with water, blotted dry and examined under oil immersion field.

**Table 3.3.2:** Acid-fast organisms/structures and percentage of sulfuric acid suitable for staining.

| Acid-fast organisms/structures | Sulfuric acid (%) needed for decolorization |
|---|---|
| *Mycobacterium tuberculosis* | 25% |
| *Mycobacterium leprae* | 5% |
| *Nocardia* | 1% |
| Acid-fast parasites such as *Cryptosporidium, Cyclospora, Cystoisospora,* Microsporidia* | 0.5% |
| Bacterial spore | 0.25–0.5% |

*Microsporidia are now considered to be evolved from fungi.

### *Composition*

Composition of Albert stain includes:

- Albert I: Comprises of toluidine blue, malachite green, glacial acetic acid, alcohol (95% ethanol), and distilled water
- Albert II: Contains iodine in potassium iodide.

### *Interpretation*

*Corynebacterium diphtheriae* appears as green colored bacilli arranged in Chinese letter or cuneiform pattern, with bluish black metachromatic granules at polar ends (Refer Fig. 60.2C, Chapter 60). These can be differentiated from diphtheroids which do not show granules and are arranged in palisade pattern. However, certain bacteria, such as *Corynebacterium xerosis* and *Gardnerella vaginalis* also possess metachromatic granules.

## Other Microscopic Techniques

Other microscopic techniques include:

- **Dark-ground and phase-contrast microscopy**—for demonstration of spirochetes in genital specimens
- **Hanging drop preparation** for stool specimen—for demonstration of darting motility; gives a clue about *V. cholerae*.

# OTHER METHODS OF DIRECT DETECTION

## Antigen Detection

Various immunological methods such as latex agglutination test, immunochromatographic test are available which detect antigens in clinical specimens.

- The classical example includes detection of capsular antigen of pneumococci, meningococci, *H. influenzae* in CSF specimen
- Urinary antigen detection for pneumococci and *Legionella*
- Direct fluorescent antibody test—for detection of *T. pallidum* from tissue sections or exudates.

Details about these antigen detection methods are discussed in Chapter 12.

## Molecular Diagnosis

Bacterial DNA or RNA can be directly detected in the clinical specimens by various molecular methods such as polymerase chain reaction (PCR). It is discussed in detail subsequently in this chapter.

# CULTURE, IDENTIFICATION AND AST

Culture is the most common diagnostic method used for detection of bacterial infections. Specimens are inoculated on to various culture media and incubated. The colonies

grown are subjected to identification and antimicrobial susceptibility test (AST).

## CULTURE MEDIA

A microbiological culture medium is a liquid or solid substance that contains nutrients to support the growth, and survival of microorganisms.

### Constituents of Culture Media

The various constituents of culture media are as follows:

- **Water and electrolytes** (e.g. sodium chloride)
- **Peptone:** It is a complex mixture of partially digested proteins, obtained from various sources such as heart muscle, casein or fibrin, or soya
- **Agar:** It is used for solidifying the culture media, does not add nutritive value to the medium
  - *Source:* It is prepared from the cell wall of seaweeds and available commercially in powder form
  - *Preparation:* Agar powder is dissolved in water and subjected to sterilization by autoclave. When the temperature of the molten agar comes down to 45°C, it is poured into the Petri dishes and then allowed to set for 20 minutes
  - *Concentration:* It is used in concentration of 1–2% for solid medium, 0.5% for semisolid agar and 6% to inhibit *Proteus* swarming.
- **Meat extract:** It is a commercial preparation of highly concentrated meat stock, usually made from beef
- **Yeast extract** (prepared from Baker's yeast) and **malt extract** (contains maltose)
- **Blood and serum:** They are important components of enriched media; provide extra nutrition to fastidious bacteria. Usually 5–10% of sheep blood is used. Alternatively, horse, ox, or human blood can also be used.

### Types of Culture Media

Bacteriological culture media can be classified in two ways.

**A. Based on consistency**, culture media are grouped into—liquid (or broth), semisolid and solid media.

**B. Based on the method of growth detection**, culture media are classified as:

1. **Conventional culture media:** They are prepared from nutrients, such as aqueous extract of meat, peptone, etc. The bacterial growth is detected manually by visual inspection of turbidity or colony morphology. They are of various types based on their functional use or application
   - Simple/basal media
   - Enriched media
   - Enrichment broth
   - Selective media
   - Differential media
   - Transport media
   - Anaerobic media.
2. **Automated culture media:** They are mainly available for blood and sterile body fluid culture. The growth is detected automatically by the equipment.

### Conventional Culture Media

#### *Simple/Basal Media*

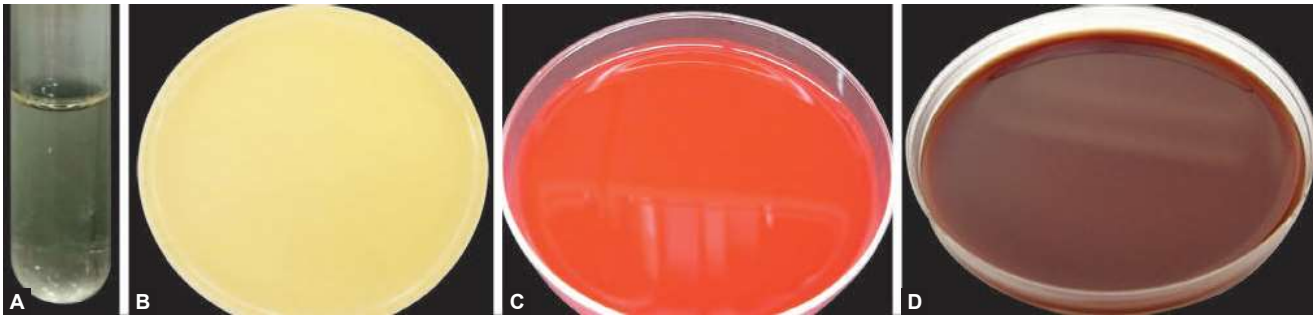
They contain minimum ingredients that support the growth of non-fastidious bacteria. Examples include—

- **Peptone water:** It contains peptone (1%) + NaCl (0.5%) + water (Fig. 3.3.3A)
- **Nutrient broth:** It is made up of peptone water + meat extract (1%). It is available in three forms: (1) meat extract, (2) meat infusion, (3) meat digest broth
- **Nutrient agar:** It is made up of nutrient broth + 2% agar (Fig. 3.3.3B)
- **Semisolid medium:** It is prepared by reducing the concentration of agar to 0.2–0.5 %.

**Uses of Basal Media**

The basal media are used for:

- Testing the non-fastidiousness of bacteria
- They serve as the base for the preparation of many other media
- Nutrient broth is used for studying the bacterial growth curve
- Nutrient agar is the preferred medium for:
  - Performing the biochemical tests, such as oxidase, catalase and slide agglutination test, etc.
  - To study the colony morphology
  - Pigment demonstration.
- Semisolid medium is used for: (1) demonstrating motility of the bacteria; motile bacteria spread throughout the semi-solid medium, making the medium hazy, (2) maintaining stock culture.




**Figs 3.3.3A to D: A.** Peptone water; **B.** Nutrient agar; **C.** Blood agar; **D.** Chocolate agar.

*Source:* A to D. Department of Microbiology, JIPMER, Puducherry (*with permission*).

### Enriched Media

When a basal medium is added with additional nutrients, such as blood, serum or egg, it is called enriched medium. In addition to non-fastidious organisms, they also support the growth of fastidious nutritionally exacting bacteria. Examples include:

- **Blood agar:** It is prepared by adding 5–10% of sheep blood to the molten nutrient agar at 45°C (Fig. 3.3.3C). It is the most widely used medium in diagnostic bacteriology. Blood agar also tests the hemolytic property of the bacteria, which may be either: (1) partial or α (green) hemolysis and (2) complete or β-hemolysis (described subsequently in this chapter)
- **Chocolate agar:** It is the heated blood agar, prepared by adding 5–10% of sheep blood to the molten nutrient agar at 70°C, so that the RBCs will be lysed and the content of RBCs will be released, changing the color of the medium to brown (Fig. 3.3.3D). It is more nutritious than blood agar, and even supports certain highly fastidious bacteria, such as *Haemophilus influenzae* that does not grow on blood agar
- **Loeffler's serum slope:** It contains serum. It is used for isolation of *Corynebacterium diphtheriae*
- **Blood culture media:** They are also enriched media, used for isolating microorganisms from blood. They are available either as conventional or automated blood culture media (described later in this chapter).

### Enrichment Broth

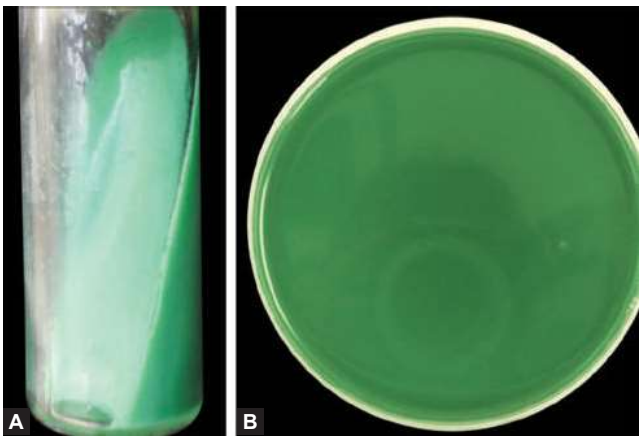
They are the liquid media added with some inhibitory agents which selectively allow certain organism to grow and inhibit others. This is important for isolation of the pathogens from clinical specimens which also contain normal flora (e.g. stool and sputum specimen). Examples for enrichment broth include:

- Tetrathionate broth—Used for *Salmonella* Typhi
- Gram-negative broth—Used for isolation of *Shigella*
- Selenite F broth—Used for isolation of *Shigella*
- Alkaline peptone water (APW)—Used for *Vibrio cholerae*.

### Selective Media

They are solid media containing inhibitory substances that inhibit the normal flora present in the specimen and allow the pathogens to grow.

- **Lowenstein–Jensen (LJ) medium:** It is used for isolation of *Mycobacterium tuberculosis* (Fig. 3.3.4A)
- **Thiosulfate citrate bile salt sucrose (TCBS) agar:** It is used for isolation of *Vibrio* species (Fig. 3.3.4B)
- **DCA (deoxycholate citrate agar and XLD (xylose lysine deoxycholate) agar:** They are used for the isolation of enteric pathogens, such as *Salmonella* and *Shigella* from stool (Figs 3.3.5A and B)
- **Potassium tellurite agar (PTA):** It is used for isolation of *Corynebacterium diphtheriae*.




**Figs 3.3.4A and B: A.** Lowenstein–Jensen medium; **B.** TCBS agar.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

### Transport Media

They are used for the transport of the clinical specimens suspected to contain delicate organism or when delay is expected while transporting the specimens from the site of collection to the laboratory (Table 3.3.3). Bacteria do not multiply in the transport media, they only remain viable.

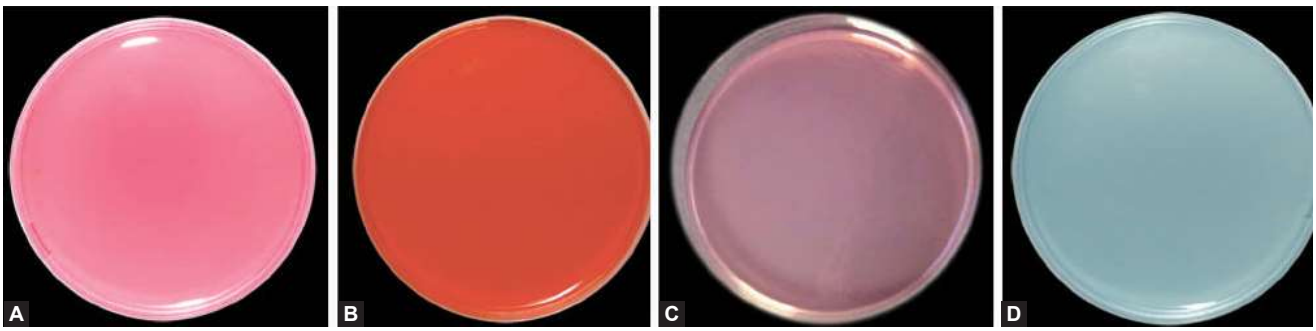
### Differential Media

These media differentiate between two groups of bacteria by using an indicator, which changes the color of the colonies of a particular group of bacteria but not the other group.

- **MacConkey agar:** It is a differential and low selective medium, commonly used for the isolation of enteric gram-negative bacteria (Fig. 3.3.5C)
  - It differentiates organisms into LF or lactose fermenters (produce pink colored colonies, e.g. *Escherichia coli*) and NLF or non-lactose fermenters (produce colorless colonies, e.g. *Shigella*)
  - **Composition:** It contains peptone, lactose, agar, neutral red (indicator) and taurocholate
  - Most laboratories use combination of blood agar and MacConkey agar for routine bacterial culture.
- **CLED agar (cysteine lactose electrolyte-deficient agar):** This is another differential medium similar to

**Table 3.3.3: Transport media used for common bacteria.**

| Organism | Transport media |
|---|---|
| *Neisseria* | Amies medium and Stuart's medium |
| *Vibrio cholerae* | • VR (Venkatraman-Ramakrishnan) medium<br>• Autoclaved sea water<br>• Cary Blair medium |
| *Shigella, Salmonella* | • Buffered glycerol saline<br>• Cary Blair medium |




**Figs 3.3.5A to D: A.** DCA; **B.** XLD agar; **C.** MacConkey agar; **D.** CLED agar.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

MacConkey agar, capable of differentiating between LF and NLF. It is used as an alternative to combination of blood agar and MacConkey agar, for the processing of urine specimens (Fig. 3.3.5D).

### *Anaerobic Culture Media*

Anaerobic media contain reducing substances which take-up oxygen and create lower redox potential and thus permit the growth of obligate anaerobes, such as *Clostridium*. Examples are as follows:

- **Robertson's cooked meat (RCM) broth:** It contains chopped meat particles (beef heart), which provide glutathione (a sulfhydryl group containing reducing substance) and unsaturated fatty acids. It is the most widely used anaerobic culture medium (Fig. 3.3.6A). It is also used for maintenance of stock cultures
- **Other anaerobic media** include:
  - Thioglycollate broth (Fig. 3.3.6B)
  - Anaerobic blood agar
  - BHIS agar (Brain-heart infusion agar) with supplements (vitamin K and hemin)
  - Neomycin blood agar
  - Egg yolk agar
  - Phenyl ethyl agar
  - *Bacteroides* bile esculin agar (BBE agar).

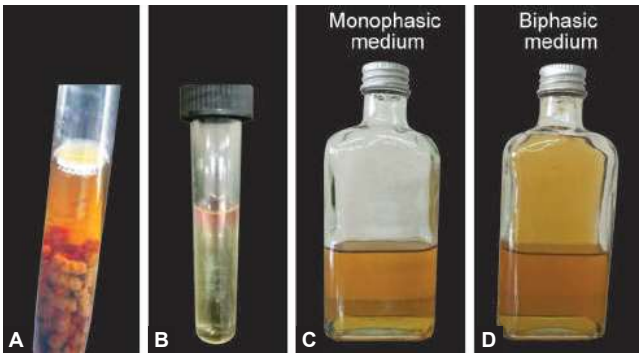
## Blood Culture Media

Recovery of bacteria from blood is difficult as they are usually present in lesser quantity in the blood and many of the blood pathogens are fastidious. Therefore, enriched media are used for isolating microorganisms from blood. Blood culture media are available either as conventional or automated media.

### *Conventional Blood Culture Media*

The conventional blood culture media are of two types.

1. Monophasic medium: It contains brain–heart infusion (BHI) broth (Fig. 3.3.6C)
2. Biphasic medium: It has a liquid phase containing BHI broth and a solid agar slope made up of BHI agar (Fig. 3.3.6D).




**Figs 3.3.6A to D: A.** Robertson's cooked meat medium; **B.** Thioglycollate broth; **C.** Brain-heart infusion broth; **D.** Biphasic medium (Brain-heart infusion broth/agar).

*Source:* A, C and D. Department of Microbiology, JIPMER, Puducherry; B. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

The recovery of organisms in the blood is enhanced by mixing the blood in the broth periodically. If any growth occurs, it can be detected by subcultures.

**Disadvantages**

In conventional blood culture, subcultures are made manually. This process can be performed less-frequently (once a day) as it is cumbersome.

- **From monophasic BHI broth,** subcultures are made onto blood agar and MacConkey agar periodically for 1 week. There is a higher risk of contamination due to opening of the cap of the bottle every time when subcultures are made
- **From biphasic BHI broth,** subcultures can be made just by tilting the bottles so that the broth runs over the agar slope. There is lower risk of contamination as it obviates the opening of the cap of the bottle.

### *Automated Blood Culture Techniques*

Automated blood culture techniques have been in use since last two decades. They are revolutionary, offer several advantages over conventional blood cultures.

- **Continuous automated monitoring:** Following inoculation, the culture bottles are loaded inside the automated culture system

- The incubated bottles are periodically tilted automatically every 10 minutes, which allows mixing of blood with broth which fastens the recovery
- Bottles are periodically monitored for the microbial growth once in every 10 minutes by the instrument. Once positive for microbial growth, the instrument gives a signal (producing beep or color change on the screen).

❖ **Composition:** Automated blood culture bottles contain:
- Tryptic soy broth and/or brain heart infusion broth (as enriched media) added with
- Polymeric resin beads which adsorb and neutralize the antimicrobials present in blood specimen.

❖ **Specimens:** In addition to blood, these bottles can also be used for culture of bone marrow, sterile body fluids such as CSF, peritoneal, pleural and synovial fluid

❖ **More sensitive:** It gives a higher yield of positive cultures from clinical specimens

❖ **Rapid:** It takes less time than conventional methods

❖ **Less labor intensive,** as fully-automated.

### Automated Systems

There are three automated systems commercially available.

1. **BacT/ALERT 3D** (Figs 3.3.7A and B): Its principle is based on colorimetric detection of growth. When bacteria multiply, they produce $CO_2$ that increases the pH, which in turn changes the color of a blue-green sensor present at the bottom of the bottle to yellow, that is detected by colorimetry
2. **BacT/ALERT VIRTUO** (bioMerieux) (Fig. 3.3.8): It is an advanced form of BacT/ALERT which offers several advantages such as (i) automatic loading and unloading of bottles, (ii) faster detection of growth, (iii) can determine the volume of blood present in the bottle


**Figs 3.3.7A and B: A.** BacT/ALERT automated blood culture system; **B.** BacT/ALERT blood culture bottle.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

**Fig. 3.3.8:** BacT/ALERT VIRTUO automated blood culture system.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

3. **BACTEC** (BD Diagnostics): Its principle is based on fluorometric detection of growth; use an oxygen-sensitive fluorescent dye present in the medium
   - In an uninoculated medium, the large amount of dissolved oxygen present in the broth quenches the fluorescent dye
   - Later, actively dividing microorganisms consume the oxygen removing the quenching effect and allowing the fluorescence to be detected.

*Note:* There is an automated culture system available for culture of *Mycobacterium tuberculosis* from various pulmonary and extrapulmonary specimens; called as Mycobacteria Growth Indicator Tube **(MGIT).** This works on fluorometric principle of detection, similar to BACTEC.

### Disadvantages

Automated culture methods do have several disadvantages like (1) high cost of the instrument and culture bottles, (2) inability to observe the colony morphology as liquid medium is used.

## CULTURE METHODS

Culture methods involve inoculating the specimen on to appropriate culture media, followed by incubating the culture plates in appropriate conditions.

### Selection of Media

The first step of a culture investigation is selection of appropriate media, which in turn depends up on the type of specimen to be processed. In general, combination of blood agar and MacConkey agar is commonly used for processing of most specimens. However, there are few specimens for which additional or alternative media are used (Table 3.3.4).

**Table 3.3.4: Selection of media for various specimen types.**

| Specimens | Recommended culture media |
|---|---|
| Exudate specimens* | Blood agar plus MacConkey agar |
| Sterile body fluids | Blood agar, plus MacConkey agar, plus chocolate agar or Automated blood culture bottles |
| Blood | Blood culture bottles (Conventional or automated) |
| Urine | Blood agar plus MacConkey agar CLED agar can be used alternatively |
| Stool | Selenite-F broth plus MacConkey agar plus DCA and/or XLD agar (if cholera is suspected- add TCBS agar) |
| Respiratory specimens | Blood agar, plus MacConkey agar, plus chocolate agar (if diphtheria is suspected - add LSS and PTA) |

*Exudate specimens include pus, wound swab, aspirates, and tissue bits.

*Abbreviations:* CLED, cysteine lactose electrolyte deficient agar; DCA, deoxycholate citrate agar; XLD, xylose lysine deoxycholate; TCBS, thiosulfate-citrate-bile salts-sucrose agar; LSS, Loeffler serum slope; PTA, potassium tellurite agar.

## Inoculation of the Specimens

Inoculation of the specimens onto the culture media is carried out with the help of bacteriological loops made up of platinum or nichrome wire (Fig. 3.3.9A).

- The inoculating loop is first heated in the Bunsen flame by making it red hot (Fig. 3.3.9B) and then made cool waiting for 10 seconds
- The entire process of bacteriological culture method should be carried out in a biological safety cabinet and wearing appropriate personal protective equipment such as gloves, laboratory coat or gown and mask (for respiratory specimens).

**Biosafety Cabinet (BSC)**

It is an enclosed, ventilated laboratory work station, used to protect the laboratory personnel while working with potential infectious clinical specimens.

- They are specially designed in a way that the air is blown into the cabinet away from the worker and then exhausted outside through a duct lined with HEPA filters (Fig. 3.3.10)
- There are various types of BSCs, depending upon air velocity and percentage of air recirculated. Most of the microbiology laboratories require Class 2A BSC. A higher class of BSCs may be required for certain high-risk pathogens.

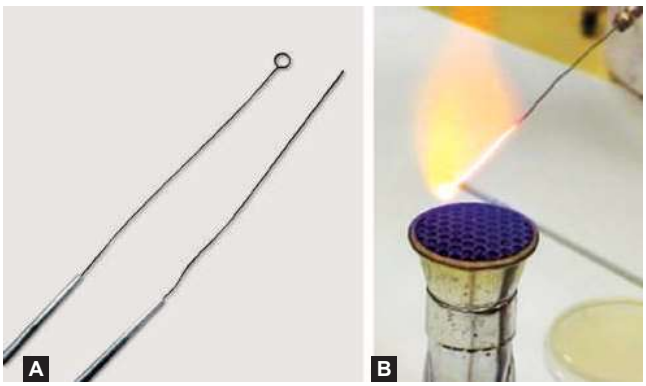
### *Inoculation Methods*

Inoculation methods are of two types.

1. Methods used for inoculating clinical specimens on to the culture media
2. Methods used for inoculating colonies on to various media for further processing.

#### Streak Culture

It is the most common inoculation method; used for the inoculation of the specimens on to the solid media. It is also used for obtaining individual isolated colonies from a mixed culture of bacteria.




**Figs 3.3.9A and B: A.** Bacteriological loop and straight wire; **B.** Flaming the loop (red hot).

*Source:* A. Department of Microbiology, JIPMER, Puducherry (*with permission*).

**Fig. 3.3.10:** Biological safety cabinet.

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

- **Streaking:** A loopful of the specimen is smeared onto the solid media to form round-shaped primary inoculum, which is then spread over the culture plate by streaking parallel lines to form the secondary, tertiary inoculum and finally a feathery tail end (Fig. 3.3.11A)
- **Intermittent heating:** The loop is flamed and cooled in between the different set of streaks to get isolated colonies on the final streaks (Fig. 3.3.11B). Obtaining isolated colonies is the prerequisite to perform tests for identification and AST.

#### Liquid Culture

Liquid culture is used for culture of specimens such as blood or body fluids, which are inoculated by directly adding the specimen in to the liquid medium or with the help of a syringe or pipette.

- **Bacterial growth** is detected by observing the turbidity in the medium. Some aerobic bacteria form surface pellicles (Fig. 3.3.12A)


**Figs 3.3.11A to C: A.** Streak culture (schematic representation); **B.** Isolated colonies grown by following streak culture; **C.** Lawn culture of a bacterial isolate to perform the antimicrobial susceptibility testing.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

- **Uses:** Liquid cultures are useful for—(1) blood, or body fluids culture, (2) automated culture for mycobacteria (MGIT, i.e. mycobacteria growth indicator tube), (3) water analysis
- **Advantages:** Liquid cultures are preferable for culture of—(1) specimens containing small quantity of bacteria, (2) specimens (e.g. blood) containing antibiotics and other antibacterial substances, as they get neutralized by dilution in the medium, (3) It is also preferred when large yields of bacteria are required
- **Disadvantages:** (1) Liquid cultures do not provide a pure culture from a mixed inoculum, (2) there is no visible colonies, therefore unlike solid media, it does not give any preliminary clue about the bacteria.

### Lawn or Carpet Culture

Lawn culture is useful to carry out antimicrobial susceptibility testing (AST) by disk diffusion method (Fig. 3.3.11C). Here, the uniform lawn of bacterial growth is obtained by either swabbing or flooding with a bacterial broth onto the culture plate (discussed in detail subsequently in this chapter).

### Pour Plate Technique

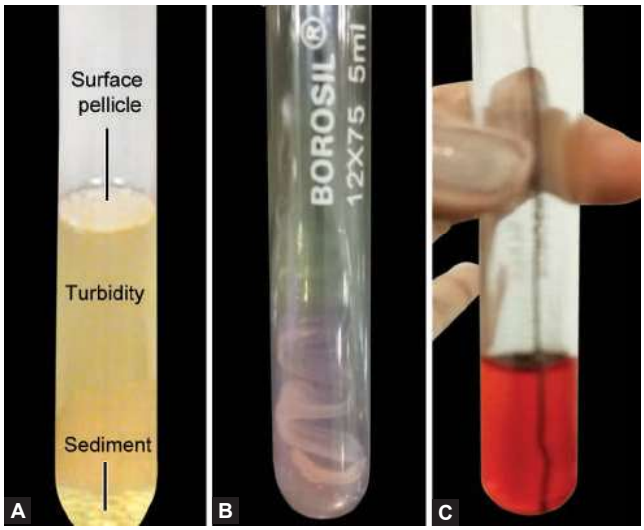
Seldom used for quantifying the bacterial load present in the specimens such as urine or blood. Here, serial dilutions of the specimen are added on to the molten agar. After being cooled and solidified, the Petri dishes are incubated and then the colony count is estimated.

### Stroke Culture

This is carried out on agar slopes or slants by streaking the straight wire in a zigzag fashion (Fig. 3.3.12B). It is used for biochemical test such as urease test.

### Stab Culture

It is made by stabbing the semisolid agar butt by a straight wire. It is used for motility testing using mannitol motility medium (Fig. 3.3.12C), and triple sugar iron agar test (here, both stroke and stab cultures are made).




**Figs 3.3.12A to C: A.** Liquid culture in test tube (turbidity indicates growth); **B.** Stroke culture; **C.** Stabbing with inoculation wire (stab culture).

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

## Incubatory Conditions

Most of the pathogenic bacteria are aerobes or facultative anaerobes; grow best at 37°C, i.e. body temperature of human beings. Therefore, the inoculated culture plates are incubated at 37°C aerobically overnight in an incubator.

**Bacteriological Incubator**

It is an equipment used to incubate the culture plates, biochemical tests and AST plates (Fig. 3.3.13). The incubator maintains optimal temperature. Some incubators are specially designed to maintain other conditions, such as humidity and $CO_2$.

**Fig. 3.3.13:** Bacteriological incubator.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

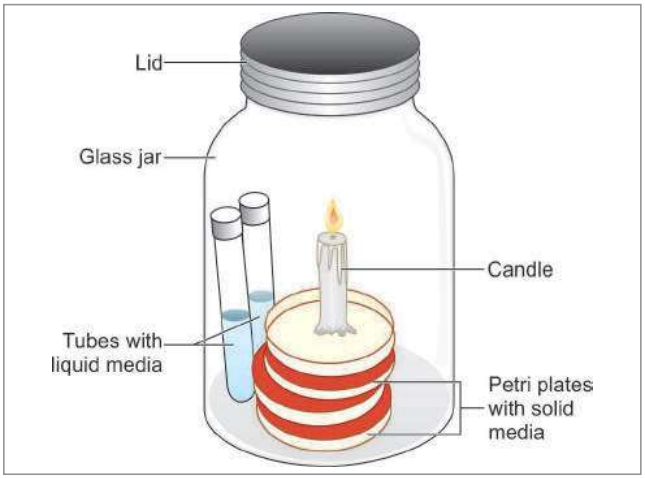



**Fig. 3.3.14:** Candle jar.

### *Other Incubatory Conditions*

The incubatory conditions may vary depending upon the bacteria to be isolated.

- **For capnophilic bacteria:** Candle jar is used. Here, inoculated media are placed inside a jar, along with a lighted candle and then jar is sealed
  - The burning candle reduces oxygen to a point where the flame goes off (Fig. 3.3.14). This provides an atmosphere of approximately 3–5% $CO_2$
  - This is useful for capnophilic bacteria, such as *Brucella*, *Streptococcus*, pneumococcus and gonococcus.
- **For microaerophilic bacteria,** such as *Campylobacter* and *Helicobacter* require 5% oxygen for optimum growth
- **For obligate anaerobes,** anaerobic culture methods are used (see below).

## Anaerobic Culture Methods

Obligate anaerobic bacteria can grow only in the absence of oxygen, hence for the growth of such bacteria, anaerobic environment is needed. The following are the methods used to create anaerobiosis.

### *Evacuation and Replacement*

This involves evacuation of the air from jar and replacement with inert gas like hydrogen followed by removal of the residual oxygen by use of a catalyst. It is carried out either by:

- **Manual method** by using McIntosh and Filde's anaerobic jar (Fig. 3.3.15A): It was the most popular method for creating anaerobiosis in the past, now not in use
- **Automated system (Anoxomat)**: It automatically evacuates air and replaces by hydrogen gas from a cylinder (Fig. 3.3.15B)
  - The catalyst used to combust residual oxygen is a sachet containing aluminum pellets coated with palladium


**Figs 3.3.15A to C: A.** McIntosh and Filde's anaerobic jar; **B.** Anoxomat anaerobic system; **C.** Anaerobic work station (Whitley pvt. Ltd.).

*Source:* A. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry; B. Department of Microbiology, JIPMER, Puducherry; C. Dr Padmaja A Shenoy, Department of Microbiology, Kasturba Medical College, Manipal, Karnataka (*with permission*).

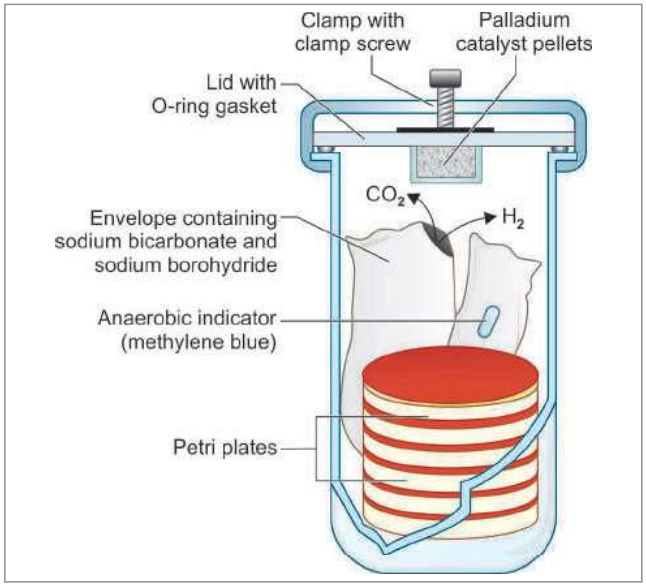



**Fig. 3.3.16:** GasPak anaerobic system.

- It is easier to operate than McIntosh jar method and claims to be highly effective for creating anaerobiosis.

### *Absorption of Oxygen by Chemical Methods*

**GasPak system** (BD diagnostics) works on this principle. It is the the most commonly used method for anaerobiosis, especially for laboratories with less sample load.

- Here, the oxygen is removed by chemical reactions, instead of evacuation and replacement technique used in Anoxomat
- It uses a sachet containing sodium bicarbonate and sodium borohydride which react chemically in presence of water, to produce hydrogen and $CO_2$ gas
- The traces of oxygen is removed by using the same catalyst used for Anoxomat (aluminium pellets coated with palladium) placed below the jar lid (Fig. 3.3.16)
- **Indicator of anaerobiosis:** The effectiveness of anaerobiosis can be checked by:
  - **Chemical indicator:** Reduced methylene blue remains colorless in anaerobic conditions, but turns blue on exposure to oxygen
  - **Biological indicator** using obligate aerobe such as *Pseudomonas:* Absence of its growth indicates that complete anaerobiosis has been achieved.

**GENbag (bioMérieux):** It consists of an airtight transparent bag with a generator sachet, which rapidly produces carbon dioxide and creates an anaerobic environment. Its application is similar to that of GasPak system.

### *Anaerobic Glove Box and Anaerobic Work Station*

These systems provide facility for easy processing, incubation and examination of the specimens without exposure to oxygen (Fig. 3.3.15C).

### *Reducing Agents*

Oxygen in culture media can be reduced by various reducing agents, such as glucose, thioglycollate, cooked meat pieces, cysteine and ascorbic acid. **Robertson cooked meat broth** is the most widely employed anaerobic culture medium which uses chopped meat particles (beef heart) as reducing agent (Fig. 3.3.6A).

### *Pre-reduced Anaerobically Sterilized (PRAS)*

PRAS media are prepared entirely under oxygen-free conditions from initial sterilization to packaging in sealed foil packets.

## Colony Morphology

After overnight incubation, the culture media are removed from the incubator and are examined under bright illumination. The appearance of bacterial colony on culture medium is characteristic for many organisms; which helps in their preliminary identification. The following features of the colony are studied.

- Size—in millimeters; e.g. pinhead size is characteristic of staphylococcal colony, whereas pinpoint size is characteristic of streptococcal colony
- Shape—circular or irregular
- Consistency—dry, moist or mucoid
- Density—opaque, translucent or transparent
- Hemolysis on blood agar (see below)
- **Color of the colony:** Colonies may be colored due to certain properties of the media or organisms. For example, pink colonies produced by lactose fermenters on MacConkey agar and black colonies by *Corynebacterium diphtheriae* on potassium tellurite agar due to the reduction of tellurite. Color of the colonies may also be due to pigment production by the bacteria
- **Pigment production:** Bacteria may produce two types of pigments
  1. **Diffusible pigments**, e.g. blue-green pigments produced by *Pseudomonas aeruginosa*
  2. **Non-diffusible pigments:** They do not diffuse into surrounding media, hence only the colonies are colored, not the surrounding media; e.g. *S. aureus* producing golden-yellow colonies.

**Hemolysis on Blood Agar**

Certain bacteria produce hemolysin enzymes that lyse the red blood cells surrounding the colonies on blood agar, forming a zone of hemolysis (Fig. 3.3.17). Hemolysis may be:

- **Partial or α hemolysis:** Partial clearing of blood around the colonies occurs with green discoloration of the surrounding medium; outline of the RBCs is intact (e.g. pneumococci, viridans streptococci)
- **Complete or β hemolysis:** Zone of complete clearing of blood around the colonies due to complete lysis of the RBCs (e.g. *Staphylococcus aureus* and *Streptococcus pyogenes*)
- **No hemolysis (γ hemolysis, a misnomer):** There is no color change surrounding the colony (e.g. *Enterococcus*)

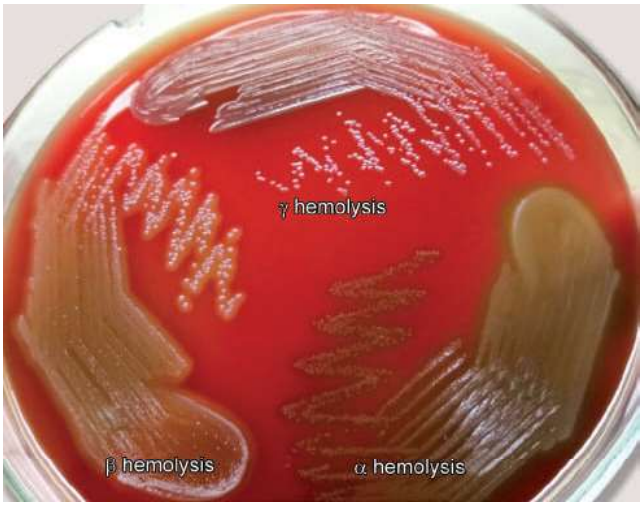



**Fig. 3.3.17:** Hemolysis on blood agar.

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

### Culture Smear and Motility Testing

The colonies grown on the culture media are subjected to Gram staining and motility testing by hanging drop method.

**Hanging drop preparation** is one of the most common and easiest method to demonstrate bacterial motility.

- A drop of bacterial broth is prepared on a coverslip and kept over a cavity slide
- Then the edge of the drop is focused under the microscope for demonstration of motile bacteria, as they usually migrate towards the edge to get more oxygen
- Hanging drop may give some clue about the identification, especially for gram-negative bacilli.

## CULTURE IDENTIFICATION

Identification of bacteria from culture is made either by conventional biochemical tests or by automated identification systems.

### Biochemical Identification

Based on the type of colony morphology and Gram staining appearance observed in culture smear, the appropriate biochemical tests are employed.

1. **Initially,** catalase and oxidase tests are done on all types of colonies grown on the media
2. **For gram-negative bacilli:** The following are the common biochemical tests done routinely, abbreviated as 'ICUT':
   - Indole test
   - Citrate utilization test
   - Urea hydrolysis test
   - Triple sugar iron test (TSI).
3. **For gram-positive cocci:** The useful biochemical tests are as follows:
   - Coagulase test (for *Staphylococcus aureus*)
   - CAMP (Christie-Atkins-Munch-Petersen) test for group B *Streptococcus*
   - Bile esculin hydrolysis test (for *Enterococcus*)
   - Heat tolerance test (for *Enterococcus*)
   - Inulin fermentation (for pneumococcus) and
   - Bile solubility test (for pneumococcus)
   - Antimicrobial susceptibility tests done for bacterial identification are as follows:
     - Optochin susceptibility test—done to differentiate pneumococcus (sensitive) from viridans streptococci (resistant)
     - Bacitracin susceptibility test—done to differentiate group A (sensitive) from group B (resistant) *Streptococcus*.

Some of the important biochemical tests are described below. Coagulase test and other biochemical reactions for gram-positive cocci are described in the respective chapters.

### Catalase Test

When a colony of any catalase producing bacteria is mixed with a drop of hydrogen peroxide (3% $H_2O_2$) placed on a slide, effervescence or bubbles appear due to breakdown of $H_2O_2$ by catalase to produce oxygen (Fig. 3.3.18).

- Catalase test is primarily used to differentiate between *Staphylococcus* (catalase positive) from *Streptococcus* (catalase negative)
- It is also positive for members of the families Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, etc.

### Oxidase Test

It detects the presence of cytochrome oxidase enzyme in bacteria, which catalyzes the oxidation of reduced cytochrome by atmospheric oxygen.

- When a filter paper strip or disk, soaked in oxidase reagent is smeared with a bacterial colony producing cytochrome oxidase enzyme, the smeared area turns deep purple within 10 seconds due to oxidation of the dye to form a purple colored compound indophenol blue
- Interpretation (Fig. 3.3.19A) and examples:
  - **Oxidase positive (deep purple):** Examples include *Pseudomonas*, *Vibrio*, *Neisseria*, *Bacillus*, *Haemophilus*, etc.
  - **Oxidase negative (no color change):** Examples include; members of family Enterobacteriaceae, *Acinetobacter*, etc.

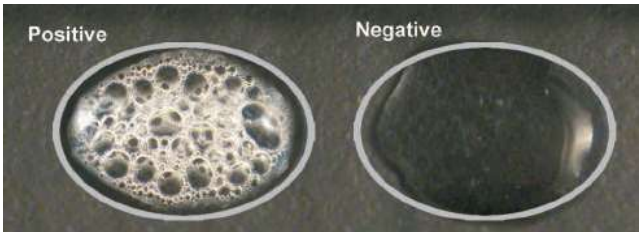
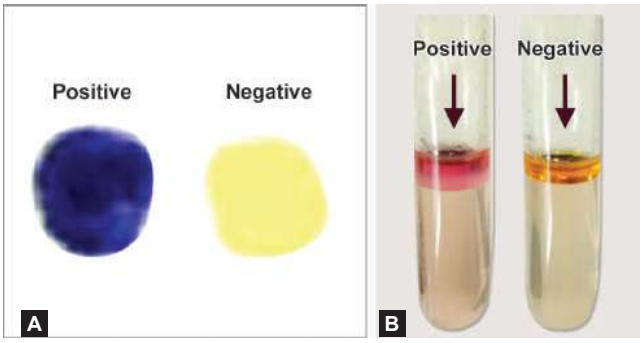


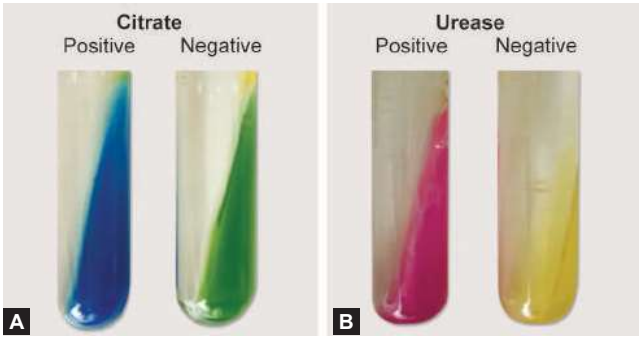

**Fig. 3.3.18:** Catalase test.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).




**Figs 3.3.19A and B: A.** Oxidase test; **B.** Indole test.

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).




**Figs 3.3.20A and B: A.** Citrate utilization test; **B.** Urea hydrolysis test.

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

### Indole Test

It detects the ability of certain bacteria to produce an enzyme tryptophanase that breaks down amino acid tryptophan present in the medium into indole.

- When Kovac's reagent is added to an overnight incubated broth of a bacterial colony, it complexes with indole to produce a cherry red color ring near the surface of the medium
- **Indole positive** (Fig. 3.3.19B): A red colored ring is formed near the surface of the broth. Examples include *Escherichia coli, Proteus vulgaris, Vibrio cholerae*, etc.
- **Indole negative** (Fig. 3.3.19B): Yellow colored ring is formed near the surface of the broth, e.g. *Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas, Salmonella*, etc.

### Citrate Utilization Test

It detects the ability of a few bacteria to utilize citrate as the sole source of carbon for their growth, with production of alkaline metabolic products. Test is performed on Simmon's citrate medium. Citrate utilizing bacteria produce growth and a color change, i.e. original green color changes to blue (Fig. 3.3.20A)

- Citrate test is positive for *Klebsiella pneumoniae, Citrobacter, Enterobacter*, etc.
- The test is negative for *Escherichia coli, Shigella*, etc.

### Urea Hydrolysis Test

Urease producing bacteria can split urea present in the medium to produce ammonia that makes the medium alkaline.

- Test is done on Christensen's urea medium, which contains phenol red indicator that changes to pink color in alkaline medium (Fig. 3.3.20B)
- **Urease test is positive for:** *Klebsiella pneumoniae, Proteus* species, *Helicobacter pylori, Brucella*, etc.
- **Urease test is negative for:** *Escherichia coli, Shigella, Salmonella*, etc.

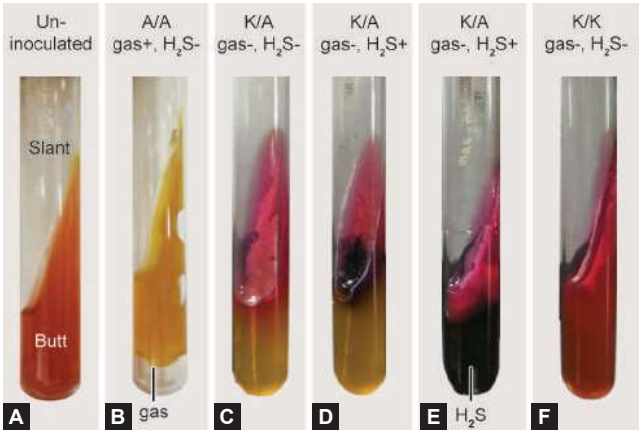
### Triple Sugar Iron (TSI) Agar Test

TSI is a very important medium employed widely for identification of gram-negative bacteria. TSI medium contains three sugars—glucose, sucrose and lactose in the ratio of 1:10:10 parts. Uninoculated TSI medium is red in color; has a slant and a butt (Fig. 3.3.21A). After inoculation, the medium is incubated at 37°C for 18–24 hours.

#### Interpretation

TSI detects three properties of bacteria, which includes fermentation of sugars to produce acid and/or gas and production of $H_2S$ (Figs 3.3.21A to F and Table 3.3.5).

- **Acid production:** If acid is produced, the medium is turned yellow from red. Accordingly the organisms are categorized into three groups
  1. **Nonfermenters:** They do not ferment any sugars, hence the medium (both slant and butt) remain red, producing Alkaline slant/Alkaline butt (K/K) reaction (Fig. 3.3.21F); e.g. *Pseudomonas* and *Acinetobacter*
  2. **Glucose only fermenters:** They ferment only glucose and produce little acid only at the butt, whereas the slant remains alkaline giving rise to Alkaline slant/Acidic butt (K/A) reaction (Fig. 3.3.21C); e.g. *Salmonella* and *Shigella*
  3. **≥ 2 sugars fermenters:** They ferment glucose and also ferment lactose and/or sucrose to produce large




**Figs 3.3.21A to F:** Triple sugar iron test.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

| Table 3.3.5: Various reactions in TSI with examples. | |
|---|---|
| **Reactions in TSI** | **Examples** |
| **Acidic slant/acidic butt** | **≥2 sugars fermented** (1) glucose, (2) lactose or/and sucrose |
| A/A, gas produced, no $H_2S$ (Fig. 3.3.21B) | *Escherichia coli* *Klebsiella pneumoniae* |
| **Alkaline slant/acidic butt** | **Only glucose-fermenter group** |
| K/A, no gas, no $H_2S$ (Fig. 3.3.21C) | *Shigella* |
| K/A, no gas, $H_2S$ produced (small amount) (Fig. 3.3.21D) | *Salmonella* Typhi |
| K/A, no gas, $H_2S$ produced (abundant) (Fig. 3.3.21E) | *Proteus vulgaris* |
| K/A, gas produced, $H_2S$ produced (abundant) | *Salmonella* Paratyphi B |
| K/A, gas produced, no $H_2S$ | *Salmonella* Paratyphi A |
| **Alkaline slant/alkaline butt** | **Non-fermenters group** |
| K/K, no gas, no $H_2S$ (Fig. 3.3.21F) | *Pseudomonas, Acinetobacter* |

amount of acid so that the medium (both slant and butt) change to yellow giving rise to Acidic slant/Acidic butt (A/A) reaction (Fig. 3.3.21B); examples, *E. coli* and *Klebsiella*.

- **Gas production:** If gas is produced, the medium is lifted up or broken with cracks (Fig. 3.3.21B); examples, *E. coli* and *Klebsiella*
- **$H_2S$ production:** If $H_2S$ is produced, the medium changes color to black (Figs. 3.3.21D and E); examples, *Salmonella* Typhi and *Proteus vulgaris*.

## Automated Systems for Bacterial Identification

Automated identification systems are revolutionary in diagnostic microbiology. They have several advantages—(i) produce faster result, (ii) can identify a wide range of organisms with accuracy, which are otherwise difficult to identify (e.g. anaerobes) through conventional biochemical tests.

- **MALDI–TOF** (Matrix-assisted laser desorption/ionization time-of-flight), e.g. VITEK MS (bioMérieux): Refer the highlight box and Fig. 3.3.22 for details
- **VITEK 2** (bioMérieux) for automated identification and antimicrobial susceptibility test: Refer the highlight box and Figure 3.3.23 for details
- **Phoenix** (BD Diagnostics) for automated identification and antimicrobial susceptibility test
- **MicroScanWalkAway system** (Beckman Coulter) for automated identification and antimicrobial susceptibility test.

**MALDI-TOF**

MALDI-TOF technology (Matrix Assisted Laser Desorption Ionization Time-of-Flight) has revolutionized the identification of organisms in clinical microbiology laboratories.

*Contd...*

**Fig. 3.3.23:** VITEK 2 system with its panels (reagent cards) for identification and antimicrobial susceptibility test.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

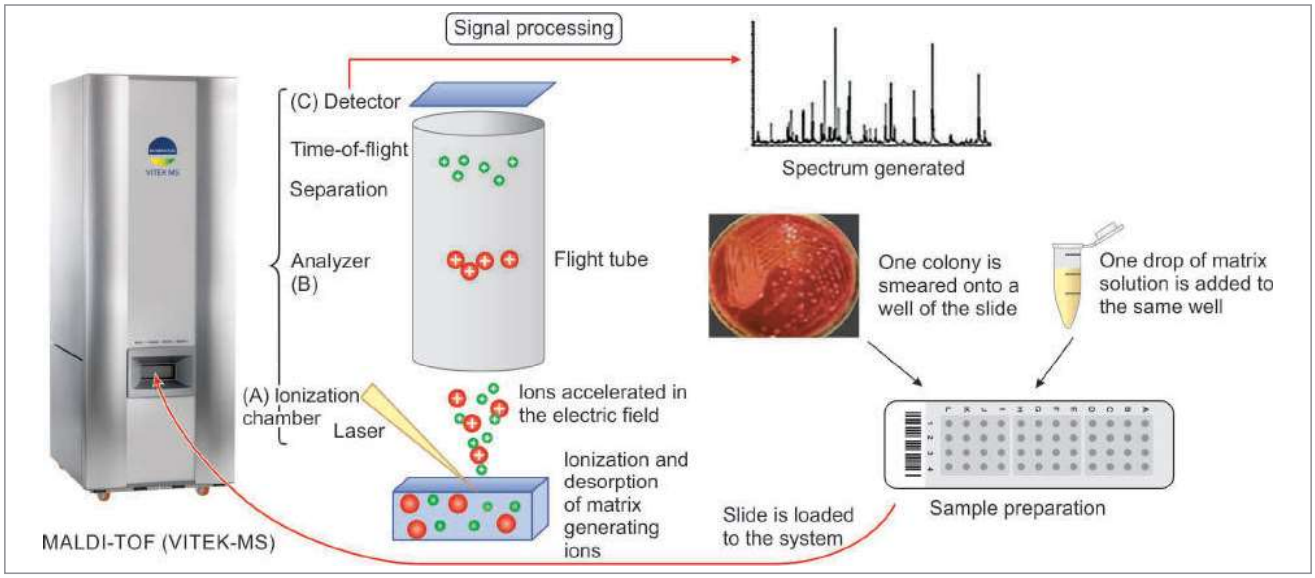



**Fig. 3.3.22:** MALDI-TOF and its working principle.

*Source:* Department of Microbiology, JIPMER, Puducherry (*with permission*).

*Contd...*

- It can identify bacteria, fungi, and mycobacteria with a turnaround time of few minutes and with absolute accuracy
- Two systems are commercially available: VITEK MS (bioMérieux) and Biotyper system (Bruker).

**Principle (Fig. 3.3.22)**

MALDI-TOF examines the pattern of ribosomal proteins present in the organism.

**Sample preparation:** The colony of an organism is smeared onto a well of the slide and one drop of matrix solution (composed of cyano-hydroxy-cinnamic acid) is added to the same well and mixed; then the slide is loaded in the system.

**Steps after loading:** Overall, mass spectrometry can be divided into three steps occurring in three chambers of the system.

1. **Ionization chamber:** Here, the wells are irradiated with the laser beam. The matrix absorbs the laser light causing desorption and ionization of bacterial ribosomal proteins, generating singly protonated ions
2. **Analyzer:** These ions are then accelerated into an electric field which directs them to the analyzer chamber. The analyzer (mass spectrometer) separates them according to their time-of-flight (TOF) in the flight tube. The smaller molecules travel faster, followed by the bigger, according to the mass to charge (m/z) ratio
3. **Detector:** It converts the received ion into an electrical current which is then amplified and digitized to generate a characteristic spectrum that is unique to a species due to its conserved ribosomal proteins. The test isolate is identified by comparing its spectrum with a known database.

**VITEK 2 Automated System**

VITEK 2 is the most widely used automated system in India; can perform both identification and antimicrobial susceptibility testing (AST) of bacteria and yeast. Principle of VITEK for identification is discussed below, VITEK for AST is discussed later in this chapter.

- It uses colorimetric reagent card containing 64 wells; each well contains an individual test substrate. Separate cards are available for gram-negative, gram-positive bacteria, fastidious bacteria and yeasts (Fig. 3.3.23)
- Substrates in the well measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, etc. which helps in identification of the organism
- The reaction pattern obtained from the test organism is compared with the database and the identification is reported with a confidence level of matching (excellent matching to the unidentified organism)
- **Incubation:** The cards are incubated in the system at 35.5 ± 1°C. The reading is taken once every 15 minutes by the optical system of the equipment, which measures the presence of any colored products of substrate metabolism (by advanced colorimetry method)
- The result of identification is usually available within 4–6 hours.

## ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility test (AST) is the most important investigation carried out by a Microbiology laboratory.

- Bacteria exhibit great strain variations in susceptibility to antimicrobial agents. Therefore, AST plays a vital role to guide the clinician for tailoring the empirical antibiotic therapy to pathogen-directed therapy
- AST is performed only for pathogenic bacteria isolated from the specimen, and not for the commensal bacteria. For example, *E. coli* isolated from urine specimen should be subjected to AST, whereas *E. coli* isolated from stool is a commensal; hence, AST is not performed.

### Classification of AST Methods

AST methods are classified into phenotypic and genotypic methods.

- The phenotypic methods are further grouped into-
  - Disk Diffusion Method, e.g. Kirby–Bauer's disk diffusion (DD) test
  - Dilution tests: Broth dilution and agar dilution methods
  - Epsilometer or E-test
  - Automated AST, e.g. Vitek, Phoenix and Microscan systems.
- Genotypic methods such as PCR detecting drug-resistant genes.

### Disk Diffusion Method

Kirby–Bauer's disk diffusion (DD) test is the most widely used AST method. They are suitable for rapidly growing pathogenic bacteria; however, they are not suitable for slow growing bacteria. It is mostly performed from colony (called colony-DD), or performed directly from the specimens (called direct DD).

#### *Procedure (Colony Disk Diffusion)*

Antibiotic disks are impregnated on to a suitable medium lawn cultured with the test isolate.

- **Antibiotic disks:** Antibiotic disks are available commercially or prepared in-house. Sterile filter paper disks of 6 mm diameter are impregnated with standard quantity of antibiotic solution
- **Medium:** Mueller–Hinton agar (MHA) is the standard medium used for AST. For certain fastidious organisms such as *S. pyogenes* and *S. pneumoniae*, Mueller–Hinton blood agar (MHBA) containing 5% of sheep blood is used
- **Inoculum:** The inoculum is prepared by — (1) directly suspending the colony in the normal saline or (2) by inoculating into a suitable broth and incubating at 37°C for 2 hours
- **Turbidity:** The turbidity of the inoculum is adjusted to 0.5 McFarland opacity standard, which is equivalent to approximately $1.5 \times 10^8$ CFU/mL of bacteria
- **Lawn culture:** The broth is then inoculated on to the medium by spreading with sterile swabs
- **Disks impregnation:** After MHA plate is dried (3–5 min), the antibiotic disks are placed and gently pressed on its surface. Disks should be placed atleast 24 mm (center to center) apart on the MHA plate. Ordinarily, maximum

up to 6 disks can be applied on a 100 mm plate (Fig. 3.3.24)

- **Incubation:** The plates are then incubated at 37°C for 16–18 hours and then interpreted.

### Interpretation

The antibiotic in the disk diffuses through the solid medium, so that the concentration is highest near the site of application of the antibiotic disk and decreases gradually away from it

- Susceptibility to the drug is determined by the zone of inhibition of bacterial growth around the disk, which can be measured by using Vernier caliper (Fig. 3.3.25)
- The interpretation of zone size into sensitive, intermediate or resistant is based on the standard zone size interpretation chart, provided by CLSI or EUCAST guidelines (Table 3.3.6).

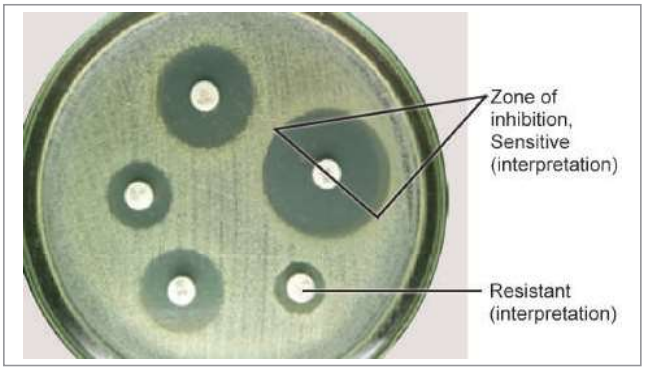



**Fig. 3.3.24:** Kirby–Bauer disk diffusion method.

*Source*: Department of Microbiology, Pondicherry Institute of Medical sciences, Puducherry (*with permission*).

*Note:* CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) are international agencies, which provide guidelines for zone size interpretation, and are updated annually.

### Direct Disk Diffusion Test

The direct DD (or direct susceptibility test, i.e. DST) test can be performed when results are required urgently and single pathogenic bacterium is suspected in the specimen (for positively-flagged blood culture bottle, sterile body fluids or urine).

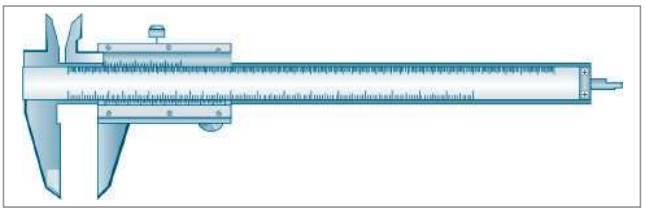


**Fig. 3.3.25:** Vernier caliper.

**Table 3.3.6:** Commonly used disk concentrations and interpretation of disk diffusion test (as per CLSI 2020 guideline).

| Breakpoints for Enterobacteriaceae (CLSI 2020) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Antimicrobial agents** | **Disk strength (µg)** | **Zone diameter break points (mm)** | | | **MIC Breakpoints (µg/mL)** | | |
| | | **Sensitive** | **Intermediate** | **Resistant** | **Sensitive** | **Intermediate** | **Resistant** |
| Ceftazidime | 30 | ≥ 21 | 18-20 | ≤ 17 | ≤ 4 | 8 | ≥ 16 |
| Ceftriaxone | 30 | ≥ 23 | 20-22 | ≤ 19 | ≤ 1 | 2 | ≥ 4 |
| Ciprofloxacin | 5 | ≥ 26 | 22-25 | ≤ 21 | ≤ 0.25 | 0.5 | ≥ 1 |
| Piperacillin-tazobactam | 100/10 | ≥ 21 | 18-20 | ≤ 17 | ≤ 16 | 32-64 | ≥ 128 |
| Amikacin | 30 | ≥ 17 | 15-16 | ≤ 14 | ≤ 16 | 32 | ≥ 64 |
| Meropenem | 10 | ≥ 23 | 20-22 | ≤ 19 | ≤ 1 | 2 | ≥ 4 |
| Colistin | - | - | - | - | - | ≤ 2 | ≥ 4 |
| **Breakpoints for gram-positive organisms (CLSI 2020)** | | | | | | | |
| **Antimicrobial agents** | **Disk strength (µg)** | **Zone diameter break points (mm)** | | | **MIC Breakpoints (µg/mL)** | | |
| | | **Sensitive** | **Intermediate** | **Resistant** | **Sensitive** | **Intermediate** | **Resistant** |
| Cefoxitin (*S.aureus*) | 30 | ≥ 22 | - | ≤ 21 | ≤ 4 | - | ≥ 8 |
| Levofloxacin (*S.aureus*) | 5 | ≥ 19 | 16-18 | ≤ 15 | ≤ 1 | 2 | ≥ 4 |
| Cotrimoxazole (*S.aureus*) | 1.25/23.75 | ≥ 16 | 11-15 | ≤ 10 | ≤ 2/38 | - | ≥ 4/76 |
| Tetracycline (*S.aureus*) | 30 | ≥ 19 | 15-18 | ≤ 14 | ≤ 4 | 8 | ≥ 16 |
| Linezolid (*S.aureus*) | 30 | ≥ 21 | - | ≤ 20 | ≤ 4 | - | ≥ 8 |
| Vancomycin (*S.aureus*) | - | - | - | - | ≤ 2 | 4-8 | ≥ 16 |
| Ampicillin (*Enterococcus*) | 10 | ≥ 17 | - | ≤ 16 | ≤ 8 | - | ≥ 16 |
| Linezolid (*Enterococcus*) | 30 | ≥ 23 | 21-22 | ≤ 20 | ≤ 2 | 4 | ≥ 8 |
| Vancomycin (*Enterococcus*) | 30 | ≥ 17 | 15-16 | ≤ 14 | ≤ 4 | 8-16 | ≥ 32 |

*Abbreviation*: CLSI, Clinical and Laboratory Standards Institute.

- Here, the specimen is directly inoculated uniformly on to the surface of an agar plate and the antibiotic disks are applied
- The results of the direct-DD test should always be verified by performing AST from the colony subsequently
- This test is of no use when mixed growth is suspected in the specimen, e.g. pus, stool, sputum, etc.

## Dilution Tests

Here, the antimicrobial agent is serially diluted, each dilution is tested with the test organism for antimicrobial susceptibility test and the MIC is calculated.

- MIC (minimum inhibitory concentration) is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation
- Depending upon whether the dilutions of the antimicrobial agent are made in agar or broth, there are two types of dilution tests.

### *Broth Dilution Method*

It is of two types: macro broth dilution (performed in tubes) and micro broth dilution (performed in microtiter plate). The procedure of macro broth dilution is explained below.

- Serial dilutions of the antimicrobial agent in Mueller-Hinton broth are taken in tubes and each tube is inoculated with a fixed amount of suspension of the test organism. A control organism of known sensitivity should also be tested. Tubes are incubated at 37°C for 18 hours
- The MIC is determined by noting the lowest concentration of the drug at which there is no visible growth, i.e. broth appears clear (Fig. 3.3.26)
- The minimum bactericidal concentration (MBC) can be obtained by subculturing from each tube (showing no growth) onto a nutrient agar plate without any antimicrobial agent. The tube containing the lowest concentration of the drug that fails to show growth, on subculture, is the MBC of the drug for that test strain (Fig. 3.3.26).

### *Agar Dilution Method*

Here, the serial dilutions of the drug are prepared in molten agar and poured into Petri dishes. The test strain is spot inoculated. This method is more convenient than broth dilution and has the added advantage of:

- Several strains can be tested at the same time by using the same plate
- It directly measures the MBC; there is no need of subculturing as it is done with broth dilution method.

## Epsilometer or E-test

This is a quantitative method of detecting MIC by using the principles of both dilution and diffusion of antibiotic into the medium.

- It uses an absorbent strip containing predefined gradient (serial dilution) of antibiotic concentration immobilized along its length

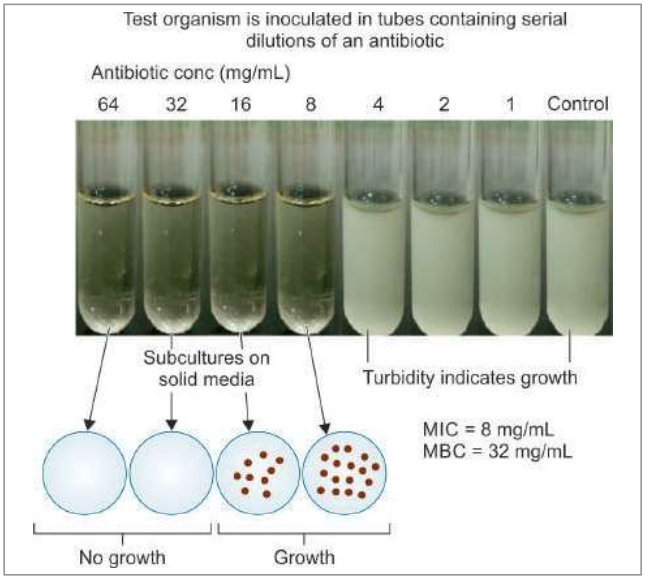



**Fig. 3.3.26:** Macro broth dilution method.

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

- It is applied to a lawn inoculum of a bacterium. Following incubation of the test organism, an elliptical zone of inhibition is produced surrounding the strip
- The antibiotic concentration at which the ellipse edge intersects the strip, is taken as MIC value (Fig. 3.3.27).

## Automated Antimicrobial Susceptibility Tests

Several automated systems are available now, such as:

- VITEK 2 identification and antimicrobial sensitivity system (bioMerieux)
- Phoenix System (Becton Dickinson)
- Micro Scan Walk Away system.

Most systems are computer assisted and have sophisticated softwares to analyze the growth rates and determine the antibiotic susceptibility report. They work by the principle of micro broth dilution. They use commercially available panels that contain antibiotic solution in serial dilutions. They provide more rapid results compared with traditional methods.

### *VITEK 2 Automated System for AST*

VITEK 2 is the most widely used automated AST system in India; can perform AST of bacteria and yeasts; whereas other automated AST systems can perform AST of bacteria only, not for yeasts.

- It works on the principle of microbroth dilution
- It uses a reagent card containing 64 wells, which contain doubling dilution of antimicrobial agents. The organism suspension (of 0.5 McFarland turbidity) is added to the wells (Figure 3.3.23 and Table 3.3.7)
- The cards are incubated in the system at 35.5 ±1°C. The reading is taken once in every 15 minutes by the optical system of the equipment. It measures the presence of


**Fig. 3.3.27:** Epsilometer or E-test.

*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

any turbidity (by nephelometry) which indicates the organism has grown in that antibiotic well

- The MIC is determined as the highest dilution of the antimicrobial agent which inhibits the growth of organism and there is no turbidity in the well
- The results are available within 8-10 hours for gram-negative bacilli and 16-18 hours for gram-positive cocci.

### *Role of MIC-based Methods*

The clinical microbiology laboratory should perform a MIC-based method whenever possible. This is because the MIC-based methods are much superior to disk diffusion test for a number of reasons.

- **For confirming the AST results** obtained by disk diffusion tests, as they are more reliable and accurate than the latter
- AST for bacteria for which disk diffusion test is not standardized should only be performed by MIC testing
- For performing AST for slow growing bacteria, such as tubercle bacilli
- **To select the most appropriate antibiotic:** Lower is the MIC, better is the therapeutic efficacy. If >1 antimicrobial agents are found susceptible, then the antibiotic having the lowest MIC (when compared with the susceptibility breakpoint) should be chosen for therapy. This is better guided by calculating the **therapeutic index**; which is the ratio of susceptibility breakpoint divided by the MIC of the test isolate. It is discussed in detail in Chapter 26
- **MIC-guided therapy:** There are certain situations, where the antibiotic treatment is MIC-guided
  - Clinical conditions such as endocarditis, pneumococcal meningitis/pneumonia, etc.
  - Vancomycin for *S. aureus*: vancomycin should be avoided if MIC is >1µg/mL.

**Table 3.3.7:** Antibiotic panel used in VITEK AST card for Enterobacteriaceae.

| Enterobacteriaceae | Antimicrobial agent used in VITEK |
|---|---|
| First line | Ampicillin |
| First line | Amoxicillin- clavulanic acid |
| First line | Ciprofloxacin |
| First line | Ceftriaxone |
| First line | Ceftazidime |
| First line | Gentamicin |
| Second line | Cefoperazone-sulbactam |
| Second line | Piperacillin- tazobactam |
| Second line | Cefepime |
| Second line | Amikacin |
| Restricted | Meropenem |
| Restricted | Doripenem |
| Restricted | Ertapenem |
| Restricted | Imipenem |
| Restricted | Colistin |
| Restricted | Tigecycline |

## Molecular Methods (Detecting Drug-resistant Genes)

Molecular methods are available targeting specific drug resistant genes; for example:

- *mecA* gene for MRSA detection by PCR
- *van* gene detection for vancomycin resistant *S. aureus* (VRSA) and vancomycin resistant *Enterococcus* (VRE) by PCR
- GeneXpert for detection of rifampicin resistance (in *M. tuberculosis*) and line probe assay for detection of resistance to many anti-tubercular drugs.

## Interpretation of AST

The result of AST (whether disk diffusion or MIC based methods) is always expressed in four interpretative categories.

- **Susceptible (S):** Indicates that the antibiotic is clinically effective when used in standard therapeutic dose
- **Intermediate (I):** Indicates that the antibiotic is not clinically effective when used in standard dose; but may be active when used in increased dose. Antibiotics reported as 'I' should be avoided for treatment if alternative agents are available
- **Susceptible dose dependent (SDD):** Indicates that the antibiotic will be clinically active only if given in increased dose. This category is available only for few agents such as cefepime for Enterobacteriaceae
- **Resistant (R):** Indicates that the antibiotic is NOT clinically effective when used in either standard dose or increased dose; and therefore should not be included in the treatment regimen.