Chapter 2.2. General Bacteriology: Laboratory Diagnosis of Bacterial Infections

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CHAPTER PREVIEW

- Specimen Collection
- Direct Detection
- Culture, Identification and AST
- Serology
- Molecular Methods

Laboratory diagnosis of bacterial infections comprises of several steps—as discussed in the highlight box.

Laboratory diagnosis of 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 a clinical specimen (Chapter 9)

    # Molecular diagnosis: Detecting bacterial

    DNA or RNA from a clinical specimen

3. **Culture**

    # Culture media

    # Culture methods

    # Colony morphology, smear, and motility testing

4. **Identification**

    # Biochemical identification

    # Automated identification methods

5. **Antimicrobial susceptibility testing**
6. **Serology**-Antigen and antibody detection (Chapter 9)
7. **Molecular methods**-PCR, real-time PCR

SPECIMEN COLLECTION

Specimen collection depends upon the type of underlying infections **(Table 2.2.1)**. The proper collection of the 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 37 for details)
- **Before the start of antibiotics** specimens for culture should be collected
- **Contamination** with normal 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-mouthed, screw-capped containers **(Fig. 2.2.1A)**

- **Labeling:** All specimens must be appropriately labeled with name, age, gender, treating physician, diagnosis, antibiotic history, type of specimen, and desired investigation name
- **Rejection:** Specimens grossly contaminated or compromised or improperly labeled may be rejected

Table 2.2.1. Types of infections and various specimens collected.

Type of infections	*Specimens collected*
Bloodstream infection, sepsis, endocarditis	Paired blood culture specimens • Collected aseptically by two-step disinfection of skin; first with alcohol followed by chlorhexidine • 8–10 mL of blood (for adults) collected in blood culture bottles **(Fig. 2.2.7B)**
Infectious diseases requiring serology	• Blood (2 mL/investigation) • Collected by minimal asepsis • Collected in vacutainer **(Fig. 2.2.1B)**
Diarrheal diseases	Stool (mucus flakes), rectal swab
Meningitis	Cerebrospinal fluid (CSF)
Infections of other sterile body areas	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 (swabs, sputum not satisfactory)
Upper respiratory tract infections	Throat swab with a 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 • Collected in a well-ventilated area • Gastric aspirate for infants
Urinary tract infections	• Midstream urine • Suprapubic aspirated urine • Catheterized patient—collected from the catheter tube, after clamping distally and disinfecting; not from urobag
Genital infections	• Urethral swab, cervical swab—for urethritis • Exudate from genital ulcers
Eye infections	• Conjunctival swabs • Corneal scrapings

Type of infections	*Specimens collected*
	• Aqueous or vitreous fluid
Ear infections	• Swabs from the outer ear • Aspirate from the inner ear

- If *anaerobic culture* is requested, proper anaerobic collection containers with media should be used
- The specimen should not be sent in a 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. For most of the specimens, transport time should not exceed *two hours*. However, there are some exceptions.

- CSF and body fluids, ocular specimens, tissue specimens, suprapubic aspirate and bone specimen require an *immediate transport* (<15 minutes)
- **Urine (midstream)** added with preservative (boric acid) is acceptable up to 24 hours, otherwise should be transported within 2 hours

Figs. 2.2.1A and B. *A.* Sterile universal container; *B.* Blood collection Vacutainer tube.

- **Stool culture:** Stool specimen should be transported within 1 hour, but with transport medium (Cary-Blair medium) 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** and *lower respiratory* tract specimens can be stored up to 24 hours at 4°C.

For details on specimen collection, refer Chapter 54.

DIRECT DETECTION

Specimens upon receipt to the laboratory are subjected to various direct detection methods, which help in the early institution of antimicrobial therapy and also guide the microbiologist for further culture processing. The most important direct detection method includes the microscopic demonstration of bacteria by various staining techniques. The other methods are the detection of antigen (discussed in Chapter 9) and nucleic acid detection methods (discussed subsequently in this chapter).

STAINING TECHNIQUES

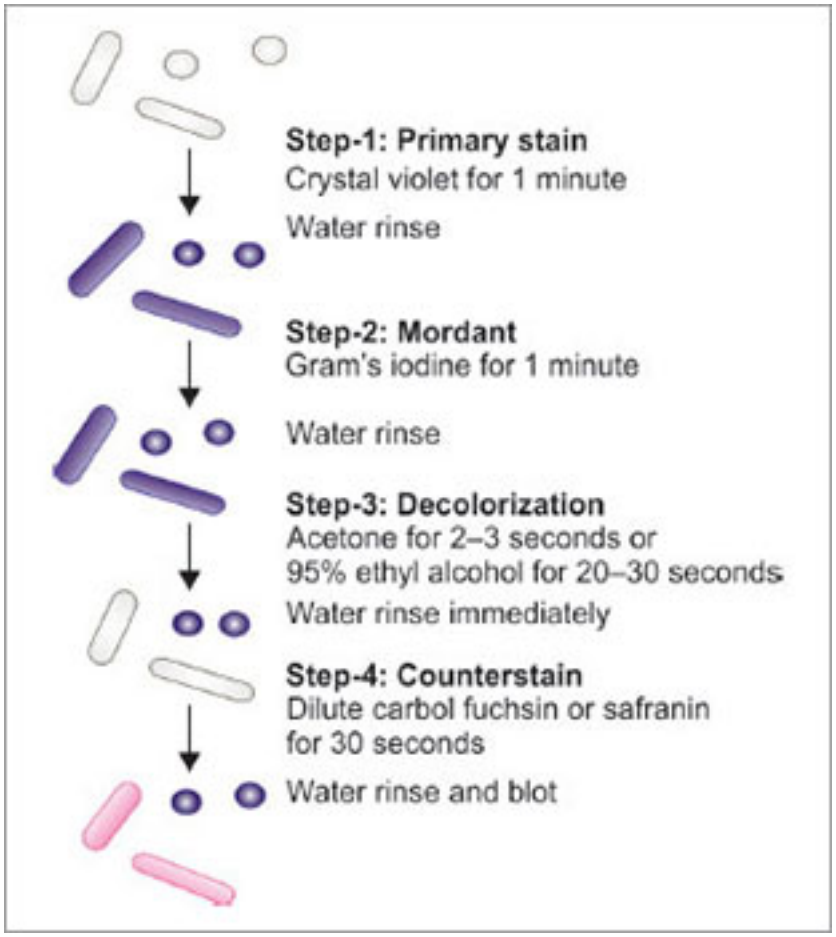
Common staining techniques used in diagnostic bacteriology include:

- **Simple stain:** Basic dyes, such as methylene blue or basic fuchsin is used as simple stains. They provide color contrast for visualization
- **Differential stain:** Here, two stains are used which impart different colors and help in differentiating bacteria. The most commonly employed differential stains are Gram stain and acid-fast stain
- **Special stains:** These staining techniques are useful to identify various bacterial structures of importance. Examples include—(1) Albert staining (to demonstrate metachromatic granules), (2) Spore staining (e.g. Schaeffer–Fulton stain) and (3) Flagellar staining (e.g. Leifson's method)
- **Negative staining:** A drop of bacterial suspension is mixed with dyes, such as India ink or nigrosin. The background gets stained black whereas the unstained bacterial capsule stands out in contrast.

Gram Stain

This staining technique was originally developed by Hans Christian Gram (1884). Gram stain is the most widely used test in diagnostic bacteriology.

Fig. 2.2.2. Principle and procedure of Gram staining.



Procedure (Fig. 2.2.2)

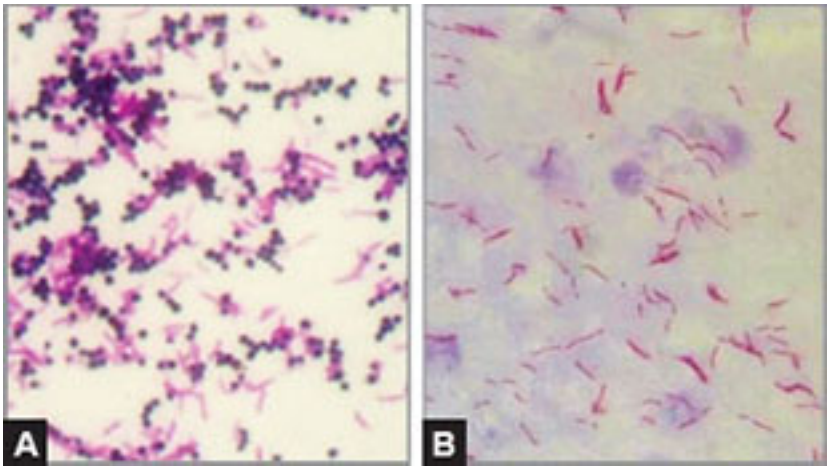
- **Step 1 (Primary stain):** Heat-fixed smear is stained with crystal violet (or gentian violet or methyl violet) for one minute. Then the slide is rinsed with water. Crystal violet stains all the bacteria violet in color
- **Step 2 (Mordant):** Gram's iodine is poured over the slide for one minute. 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 2–3 sec) or ethyl alcohol (20–30 sec). The slide is immediately rinsed with water. Decolorizer removes the primary stain from gram-negative bacteria while the gram-positive bacteria retain the primary stain
- **Step 4 (Counterstain):** 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.

Interpretation of Gram Stain

Smear is examined under oil immersion objective **(Fig. 2.2.3A)**.

- Gram-positive bacteria appear violet colored
- Gram-negative bacteria are decolorized and, therefore, take counterstain and appear pink.

Figs. 2.2.3A and B. *A.* Gram staining demonstrating violet-colored gram-positive cocci in clusters and pink-colored gram-negative bacilli; *B.* Acid-fast stained smear showing long slender slightly curved beaded red acid-fast bacilli.



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Principle of Gram Staining

The gram-positive cell wall has a thick peptidoglycan layer, which acts as a permeability barrier preventing loss of the primary stain. Whereas a gram-negative cell wall is more permeable due to the thin peptidoglycan layer and lipopolysaccharide layer, which gets easily disrupted by the decolorizer; thus allowing the outflow of the primary stain easily.

Uses of Gram Stain

The various uses of Gram stain include:

- To **differentiate bacteria** into gram-positive and gram-negative
- **To start empirical treatment:** Gram stain from the specimen gives a preliminary clue about the bacteria present so that the empirical treatment with broad-spectrum antibiotics can be started early, before the culture report is available
- **Anaerobic organisms** if detected in the Gram stain of the clinical specimen, gives a preliminary clue to perform an anaerobic culture of the specimen
- **Yeasts:** In addition to staining 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.

Acid-fast Stain

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

Ziehl-Neelsen Technique (Hot Method)

Smear is air-dried and heat-fixed before staining.

Procedure

- **Step 1 (primary stain):** Smear is poured with strong carbol fuchsin for 5 minutes. Intermittent heating is done for better penetration of the stain. Rinse the slide with tap water
- **Step 2 (Decolorization):** It is done by pouring 25% sulfuric acid over the slide for 2–4 minutes. The slide is gently rinsed with tap water and tilted to drain off the water
- **Step 3 (Counterstaining):** Methylene blue is poured onto the slide for 30 seconds. Then the slide is rinsed gently with tap water and allowed to dry
- The slide is examined under the bright-field microscope under an oil immersion field (100x).

Interpretation

Mycobacterium tuberculosis appears as long slender, straight, or slightly curved and beaded, red colored acid-fast bacillus and background take up the counterstain and appear blue **(Fig. 2.2.3B)**.

Modifications of Acid-fast Staining

Modifications of acid-fast staining include:

- **Cold method (Kinyoun's method):** In this technique heating is not required, phenol concentration in carbol fuchsin is increased and duration of carbol fuchsin staining is more
- **Decolorization using acid-alcohol** (3 mL of HCl and 97 mL of ethanol) can also be used.

Albert Stain

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

- **Procedure:** Smear is covered with Albert I stain for 5 minutes, then the excess stain is drained out and then Albert II (iodine solution) is added for 1 minute. The slide is washed with water, blotted dry, and examined under oil immersion field
- **Interpretation:** *Corynebacterium diphtheriae* appears as green-colored bacilli arranged in a cuneiform pattern, with bluish-black metachromatic granules at polar ends (Refer to *Fig. 13.1B* of Chapter 13).

Other Microscopic Techniques

Other microscopic techniques include:

- **Dark-ground microscopy**—for demonstration of spirochetes in genital specimens (Refer *Fig. 1.4* of Chapter 1)
- **Hanging drop preparation** for stool specimen—for demonstration of darting motility; gives a clue about *V. cholerae*.

CULTURE, IDENTIFICATION AND AST

Culture is the most common diagnostic method used for the detection of bacterial infections. Specimens are inoculated onto 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 important constituents of culture media are water, electrolytes, peptone, agar (solidifying agent), meat extract, and yeast extract. In addition, blood or serum may be added to provide extra-nutrition to fastidious bacteria
- **Types of culture media:** Based on consistency, culture media are grouped into—liquid (or broth), semisolid and solid media
- **Based on the method of growth detection**, culture media are classified as—conventional or automated culture media
  - *Conventional culture media:* They are of various types such as—simple/basal media, enriched media, enrichment broth, selective media, differential media, transport media, and anaerobic media
  - *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, nutrient broth, and nutrient agar **(Figs. 2.2.4A and B)**
- Basal media support the growth of non-fastidious bacteria, helpful in studying the bacterial growth curve
- They are the preferred media for performing the biochemical tests and to describe colony morphology.

Enriched Media

When a basal medium is added with additional nutrients, such as blood, serum or egg, it is called an enriched medium. They support the growth of fastidious nutritionally exacting bacteria. Examples include:

- **Blood agar:** It is useful to test the hemolytic property of the bacteria, which may be either: Partial or α (green) hemolysis and complete or #-hemolysis **(Fig. 2.2.4C)**
- **Chocolate agar:** It is more nutritious than blood agar, and even supports the growth of *Haemophilus influenzae* that does not grow on blood agar **(Fig. 2.2.4D)**
- **Blood culture media:** They are also enriched media, used for isolating organisms from blood (discussed 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.

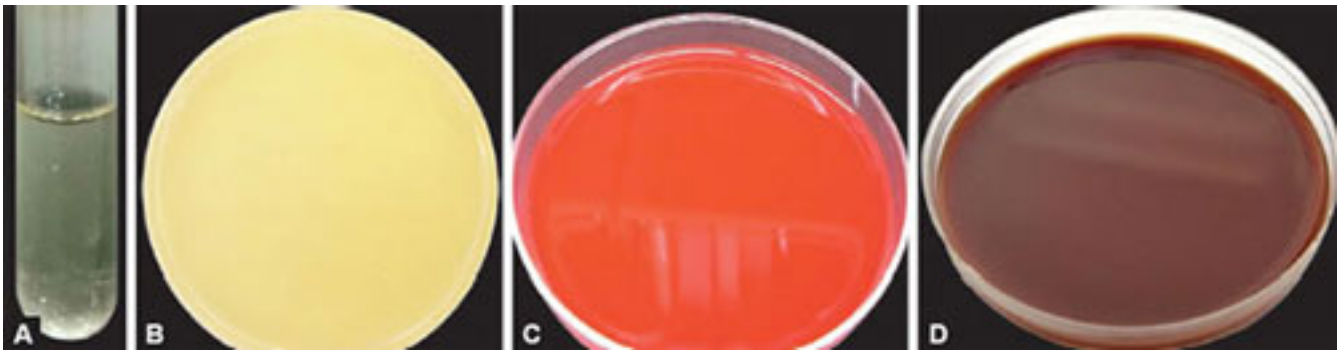
- Useful for isolation of the pathogens from stool specimen, which also contain normal flora
- Examples include—selenite F broth for isolation of *Shigella* and alkaline peptone water (APW) for *Vibrio cholerae*.

Selective Media

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

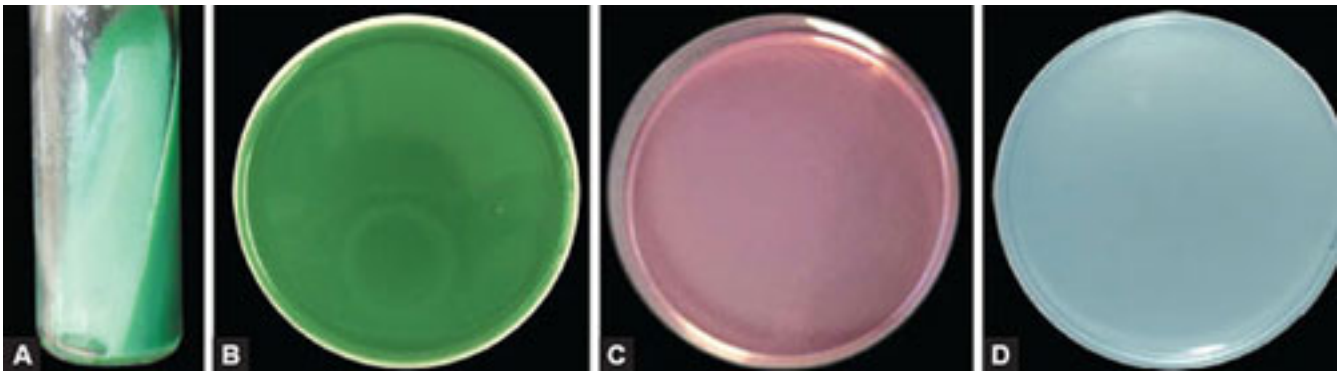
- Lowenstein–Jensen (LJ) medium **(Fig. 2.2.5A)** for isolation of *Mycobacterium tuberculosis*

Figs. 2.2.4A 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)*.

Figs. 2.2.5A to D. *A.* Lowenstein–Jensen medium; *B.* TCBS agar; *C.* MacConkey agar; *D.* CLED agar.



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- Thiosulfate-citrate-bile salt-sucrose (TCBS) agar **(Fig. 2.2.5B)** for isolation of *Vibrio* species.

Transport Media

They are used for the transport of the clinical specimens suspected to contain delicate organisms or if a delay is expected for specimen transport.

- Bacteria only remain viable; do not multiply in this media

- Examples include—(1) Cary-Blair medium for *Salmonella*, and *Shigella*, (2) Venkatraman-Ramakrishnan medium for *Vibrio cholerae* and (3) Amies medium for gonococcus.

Differential Media

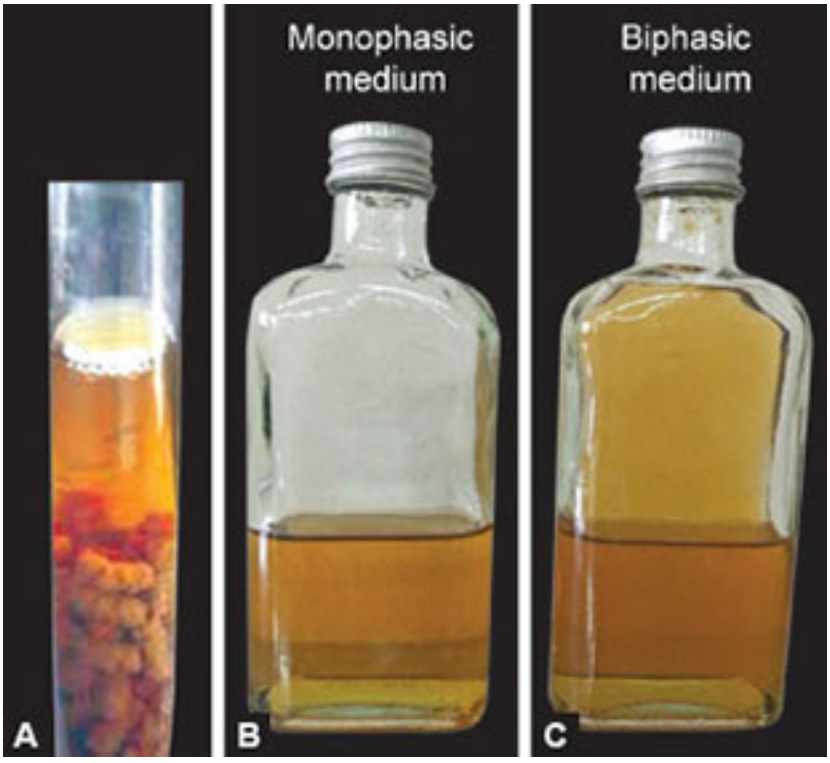
These media differentiate between two groups of bacteria by changes in the color of the colonies produced.

- Examples include— (1) *MacConkey agar* (most commonly used medium for all specimens), and (2) *CLED agar* (cysteine lactose electrolyte-deficient agar, used for urine specimen) **(Figs. 2.2.5C and D)**
- They differentiate 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)*.

Anaerobic Culture Media

Anaerobic media contain reducing substances that take-up oxygen and create lower redox potential and thus permit the growth of obligate anaerobes, such as *Clostridium*. Examples include—*Robertson's cooked meat (RCM) broth* and thioglycollate broth **(Fig. 2.2.6A)**.

Figs. 2.2.6A to C. *A.* Robertson's cooked meat medium; *B.* Brain-heart infusion broth; *C.* Biphasic medium (Brain-heart infusion broth/agar).



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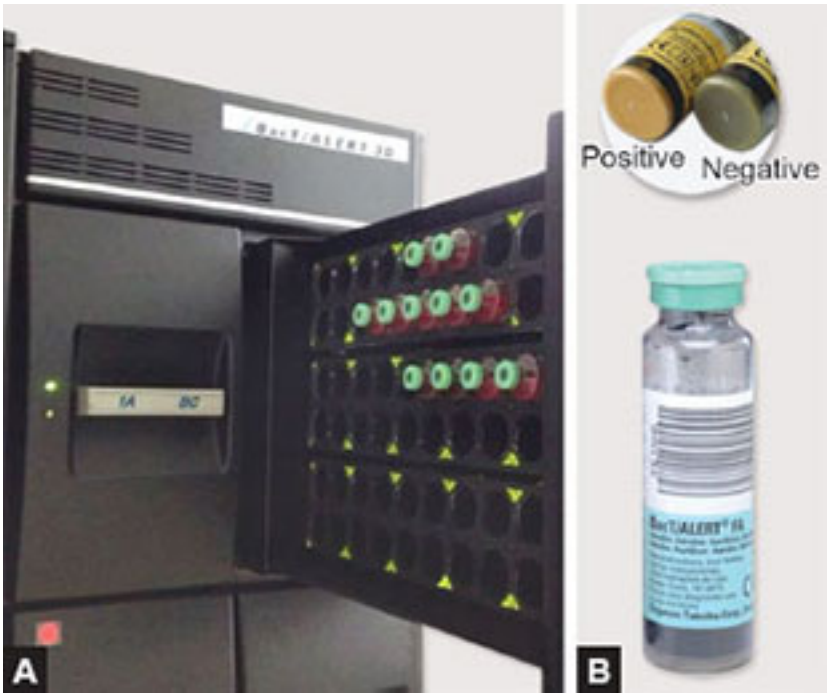
Blood Culture Media

Organisms usually present in lesser quantities in the blood and many of the blood pathogens are fastidious. Therefore, enriched media are used for the recovery of bacteria from the 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.

Figs. 2.2.7A and B. *A.* BacT/ALERT automated blood culture system; *B.* BacT/ALERT blood culture bottle.



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1. **Monophasic medium:** It contains brain–heart infusion (BHI) broth **(Fig. 2.2.6B)**
2. **Biphasic medium:** It has a liquid phase containing BHI broth and a solid agar slope made up of BHI agar **(Fig. 2.2.6C)**.

Automated Blood Culture Systems

Automated blood culture techniques are revolutionary and offer several advantages over conventional blood cultures, such as—(i) continuous automated monitoring, (ii) faster recovery of organisms, and (iii) lesser contamination risk.

- **Medium:** They contain tryptic soy broth and/or brain heart infusion broth
- **Other specimens:** In addition to blood, they can also be used for culture of bone marrow, sterile body fluids such as CSF, peritoneal, pleural fluids, etc.
- **Examples** of automated blood culture systems are BacT/ALERT 3D and BACTEC systems **(Fig. 2.2.7A)**
- **Principle:** When bacteria multiply, they produce CO_2 by metabolism, which decreases the pH of the medium, which in turn triggers the sensor resulting in:
  - Color change at the bottom of the bottle (BacT/ALERT)
  - Production of fluorescence (BACTEC).

- **Growth signal**: The instrument gives a signal (producing beep or color change on the screen) once the growth is detected **(Fig. 2.2.7B)**.

CULTURE METHODS

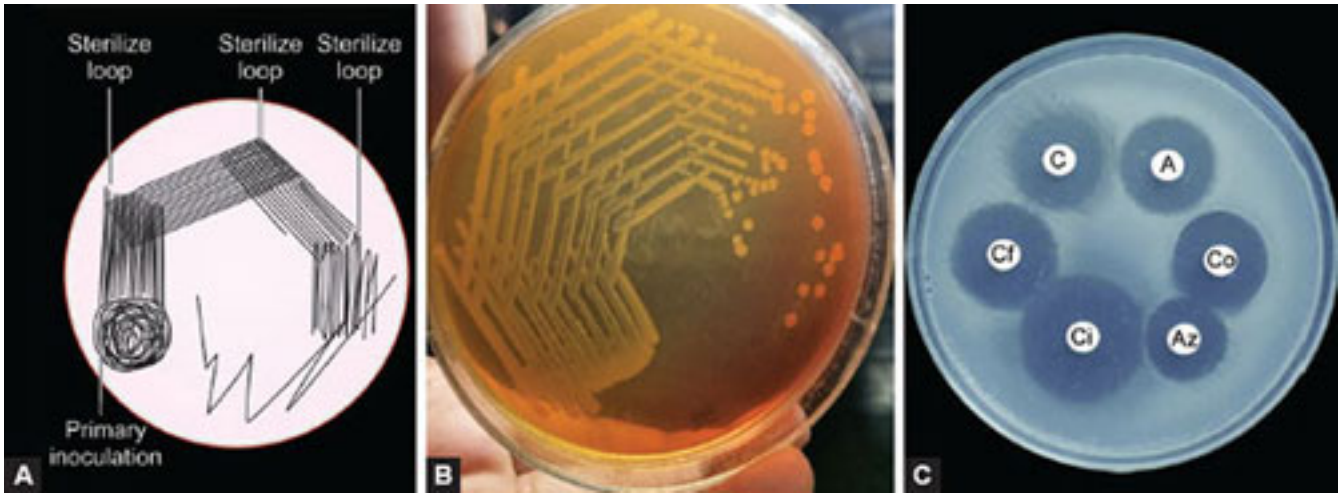
Culture methods involve inoculating the specimen onto appropriate culture media, followed by incubating the culture plates in appropriate conditions.

- **Selection of media:** A combination of blood agar and MacConkey agar is commonly used for the processing of most specimens
- **Inoculation of the specimens** onto the culture media is done with the help of bacteriological loops made up of platinum or nichrome wire
- **Inoculation methods:** Common inoculation methods are streak culture, liquid culture, and lawn culture
  - *Streak culture* method is used for the inoculation of the specimens onto the solid media. Here, 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. This technique is followed to get isolated colonies **(Figs. 2.2.8A and B)**
  - *Lawn* or *carpet culture:* It is useful to carry out antimicrobial susceptibility testing (AST) by disk diffusion method **(Fig. 2.2.8C)**
  - *Liquid culture:* It is used for the culture of blood and body fluids and also for water analysis. Bacterial growth is observed by turbidity in the medium
  - *Pour plate technique:* 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.

Incubatory Conditions

Most of the pathogenic bacteria are aerobes or facultative anaerobes; grow best when incubated at 37°C in the bacteriological incubator.

Figs. 2.2.8A 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.



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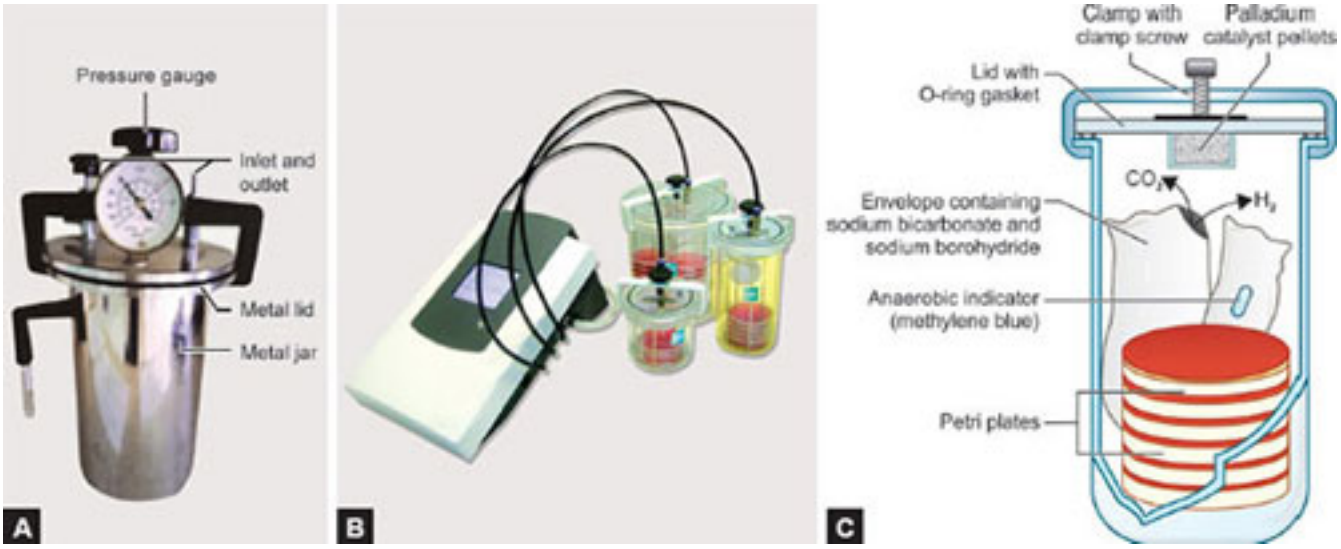
For capnophilic bacteria (e.g. *Brucella*, *Streptococcus*, pneumococcus) candle jar is used to provide 5% CO_2. For obligate anaerobes, anaerobic culture methods are used (see below).

Anaerobic Culture Methods

Obligate anaerobic bacteria can grow only in the absence of oxygen. The following are the methods used to create anaerobiosis.

- **Evacuation and replacement method** by using:
  - *McIntosh and Filde's anaerobic jar* ***(Fig. 2.2.9A)***: It was the most popular method in the past
  - *Anoxomat:* It automatically evacuates air and replaces it with hydrogen gas from a cylinder. It is easier to use and highly effective for creating anaerobiosis **(Fig. 2.2.9B)**.
- **Absorption of oxygen by chemical methods**: *GasPak system* works on this principle. It is the most commonly used method for anaerobiosis. Here, the oxygen is removed by chemical reactions **(Fig. 2.2.9C)**
- **Anaerobic glove box** and *anaerobic work station* for easy processing, incubation and examination of the specimens
- **Reducing agents** such as glucose, thioglycollate, and cooked meat pieces can be used to reduce oxygen in culture media.

Figs. 2.2.9A to C. *A.* McIntosh and Filde's anaerobic jar; *B.* Anoxomat anaerobic system; *C.* GasPak anaerobic system.



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Robertson cooked meat broth **(RCM)** is commonly used; which uses chopped meat particles as reducing agent **(Fig. 2.2.6A)**.

Colony Morphology

After overnight incubation, the culture media are removed from the incubator and examined under bright illumination.

- The appearance of the bacterial colony on the culture medium helps in their preliminary identification
- The colony characteristics that help in the preliminary identification are—
  - Size, shape, consistency (e.g. dry, moist or mucoid)
  - Color (pink or pale on MacConkey agar)
  - Pigment production (e.g. blue-green pigments by *Pseudomonas)*
  - Hemolysis on blood agar: e.g. (i) α or partial hemolysis, (ii) β or complete hemolysis.

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
- 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 automated identification systems.

Conventional 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)*
   - Inulin fermentation and bile solubility test (for pneumococcus).

Automated Systems for Bacterial Identification

Automated identification systems are revolutionary in diagnostic microbiology.

- They have several advantages—(i) produce faster results, (ii) can identify a wide range of organisms with accuracy, which are otherwise difficult to identify (e.g. anaerobes) through conventional biochemical tests
- Examples of automated systems used for bacterial identification are—
  - MALDI–TOF: Matrix-assisted laser desorption ionization time-of-flight
  - VITEK 2 automated ID and AST system.

ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility test (AST) is the most important investigation carried out by a microbiology laboratory. Bacteria exhibit great variations in susceptibility to antimicrobial agents. Therefore, AST plays a vital role to guide the clinician in tailoring the empirical antibiotic therapy to pathogen-directed therapy.

AST Methods

Antimicrobial susceptibility testing methods are:

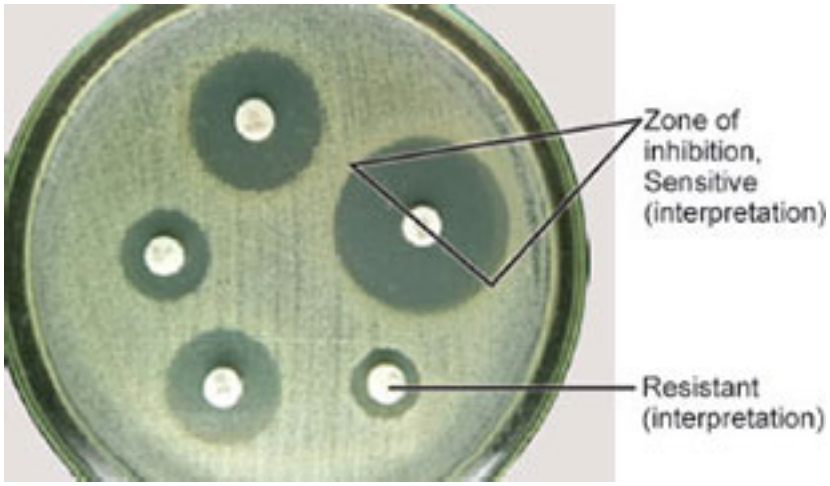
- Disk diffusion method, e.g. Kirby–Bauer's disk diffusion (DD) test
- Dilution tests: Broth dilution and agar dilution methods
- Epsilometer or E-test
- Automated AST: e.g. VITEK and Phoenix systems.

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.

- **Procedure:** Mueller–Hinton agar (MHA) is the medium used for DD test
  - Bacterial suspension is inoculated onto this medium by spreading (lawn culture) with sterile swabs
  - Antimicrobial disks are then placed on the surface of MHA plate. Then the plates are incubated at 37°C for 16–18 hours and then interpreted.
- **Interpretation:** Susceptibility to the drug is determined by the zone of inhibition of bacterial growth around the disk. The interpretation of zone size into sensitive, intermediate or resistant is based on the standard zone size interpretation chart, provided by standard guidelines (e.g. CLSI) **(Fig. 2.2.10)**.

Fig. 2.2.10. Kirby–Bauer disk diffusion method.



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

Dilution Tests

Here, the inoculum of the test organism is added to serial dilutions of antimicrobial agent in presence of a suitable medium. After overnight incubation, it is examined to determine the MIC.

- **MIC** (minimum inhibitory concentration): It is the lowest concentration of the drug that will inhibit the visible growth of an organism
- **Types:** Based on the platform where the test is performed, there are various types of dilution methods
  - *Broth dilution:* It uses Mueller-Hinton broth. It can be performed using tube (broth macrodilution) or microtiter plate (broth microdilution)
  - *Agar dilution:* It is performed on Mueller-Hinton agar.

Epsilometer or E-test

E-test is an absorbent strip that contains predefined gradient of antibiotic concentrations along its length. It is a MIC-based method, that uses the principles of both dilution and diffusion of the drug into the medium.

Automated Antimicrobial Susceptibility Tests

Several automated systems are available now, such as: VITEK 2 and Phoenix system for bacterial identification and AST. They are MIC-based methods, that work on the principle of broth microdilution. They provide more rapid results compared with traditional methods.

Interpretation of AST

The result of AST is always expressed in the following interpretative categories.

- **Susceptible (S):** Indicates that the antimicrobial agent is clinically effective when used in a standard therapeutic dose
- **Intermediate (I):** Indicates that the antimicrobial agent is not clinically effective when used in standard dose, but may be active when used in increased dose

- **Resistant (R):** Indicates that the antimicrobial agent is NOT clinically effective when used in either standard dose or increased dose, and therefore should not be included in the treatment regimen.

SEROLOGY

The serological tests play an important role in the diagnosis of various bacterial infections. These include detection of either antigen or antibody in the serum of the patient, by various immunological assays—precipitation, agglutination, ELISA, rapid tests, etc. The detail of these methods is discussed in Chapter 9.

MOLECULAR METHODS

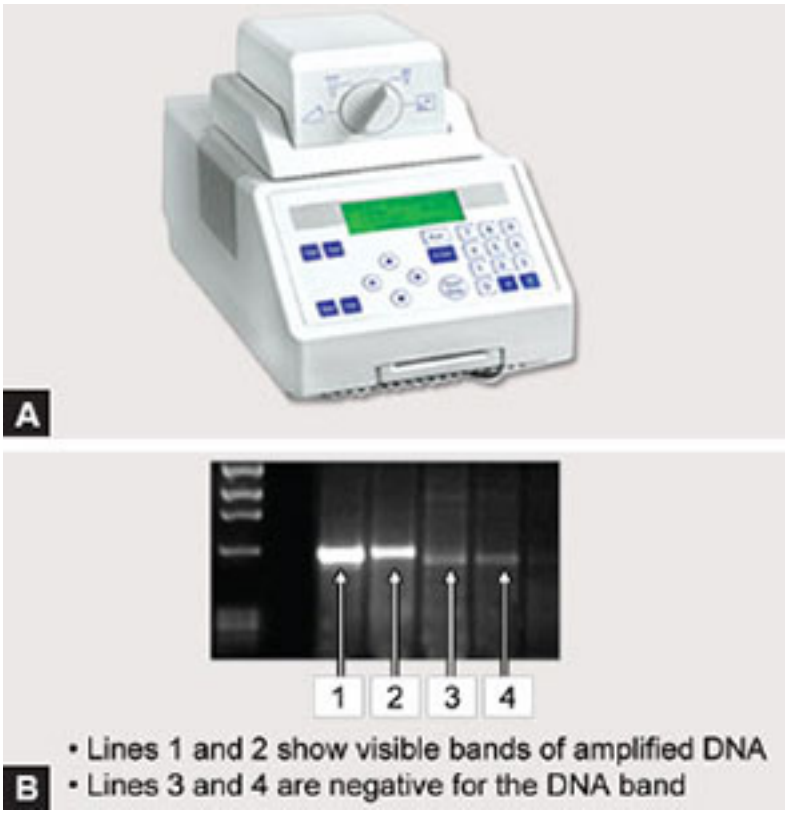
Molecular methods are broadly grouped into amplification-based and non-amplification-based methods.

- *Nucleic acid amplification techniques (NAATs)* have been increasingly used in diagnostic microbiology. Various NAATs used are:
  - Polymerase chain reaction (PCR)
  - Real-time polymerase chain reaction (rt-PCR)
  - Automated real-time PCR such as cartridge-based nucleic acid amplification test (CBNAAT).
- *Non-amplification molecular methods* include DNA hybridization method, e.g. line probe assay.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single or few copies of a piece of DNA to generate millions of copies of DNA. It was developed by Kary B Mullis (1983) for which he and Michael Smith were awarded the Nobel Prize in Chemistry in 1993.

Figs. 2.2.11A and B. *A.* Thermocycler machine; *B.* Visualization of amplified DNA under UV light.



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

Principle of PCR

PCR involves three basic steps:

1. **DNA extraction from the organism:** This involves lysis of the organisms and release of the DNA by-boiling or adding enzymes (e.g. proteinase K).

2. **Amplification of extracted DNA:** This is carried out in a special PCR machine called thermocycler **(Fig. 2.2.11A).** The extracted DNA is subjected to repeated cycles (30-35 numbers) of amplification which takes about 3-4 hours. Each amplification cycle has three steps:

   # Denaturation at 95°C: This involves separation of the dsDNA into two separate single strands

   # Primer annealing (55°C): Primer is a short oligonucleotide complementary to a small sequence of the target DNA.

   It anneals to the complementary site on the target ssDNA

   # Extension of the primer (72°C): This step is catalyzed by Taq Polymerase enzyme which keeps on adding the free nucleotides to the growing end of the primer.

3. **Gel electrophoresis of amplified product:** The amplified DNA is electrophoretically migrated according to its molecular size by performing agarose gel electrophoresis. The amplified DNA forms clear band, which can be visualized under ultraviolet (UV) light **(Fig. 2.2.11B).**

Applications of PCR

Polymerase chain reaction is now a common and often indispensable technique used in medical diagnostics for a variety of applications. It has the following advantages compared to the conventional culture methods:

- **More sensitive:** It can amplify very few copies of a specific DNA
- **More specific:** The use of primers targeting specific DNA sequence of the organism makes the PCR assays highly specific
- PCR can also detect the organisms that are highly fastidious or non-cultivable by conventional culture methods
- PCR can be used to detect the genes in the organism responsible for drug resistance (e.g. *mec* A gene detection in *Staphylococcus aureus)*.

Modifications of PCR

1. **Reverse transcriptase PCR (RT-PCR):** Conventional PCR amplifies only the DNA. For detection of RNA, reverse transcription step is done prior to PCR which converts RNA into DNA
2. **Nested PCR:** It uses two sets of primers that are targeted against two different DNA sequences of same organism. It is more sensitive and specific. Nested PCR is used for detection of *Mycobacterium tuberculosis* (targeting *IS6110* gene) in samples
3. **Multiplex PCR:** It uses more than one primer that is targeted against DNA sequences of several organisms in one reaction
    - It is useful for the syndromic diagnosis of infectious diseases that are caused by more than one organism
    - For example, for the diagnosis of pyogenic meningitis, PCR can be performed using different primers targeting common agents of meningitis such as pneumococcus, meningococcus and *H. influenzae*.

Real-time PCR (rt-PCR)

It is an advanced PCR technology, which is used to amplify and simultaneously detect or quantify a DNA sequence on a real-time basis.

- **Advantages:** It is quantitative, takes lesser time, higher rate of sensitivity and specificity as compared to conventional PCR
- **RT rt-PCR:** Reverse transcriptase rt-PCR formats can detect and quantify RNA of the test organism in the sample on a real-time basis—e.g. for COVID-19.

EXPECTED QUESTIONS

1. I. Write short notes on:

1. Gram staining.
2. Selective media.
3. Transport media.

4. Anaerobic culture methods.

5. Polymerase chain reaction.

2. II. Multiple Choice Questions (MCQs):

1. **Recommended transport medium for stool specimen suspected to contain** *Vibrio cholerae is:*

   a. Buffered glycerol saline medium

   b. Venkatraman-Ramakrishnan medium

   c. Nutrient broth

   d. Blood agar

2. **Which is an enriched medium?**

   a. Selenite F broth

   b. Peptone water

   c. MacConkey agar

   d. Chocolate agar

3. **Robertson cooked meat broth is an example of:**

   a. Enriched media

   b. Enrichment media

   c. Anaerobic media

   d. Nutrient media

4. **The blood culture bottle contains:**

   a. Enriched media

   b. Enrichment media

   c. Anaerobic media

   d. Nutrient media

5. **The three components of PCR involve all,** *except:*

   a. DNA extraction

   b. Amplification

   c. Gel documentation

   d. Blotting

1. b	**2.** d	**3.** c	**4.** a	**5.** d