جامعة الشرقية
A' SHARQIYAH UNIVERSITY
كلية العلوم التطبيقية والصحية
CAHS

Manual of Basic Practical Microbiology
BIOL 281

Prepared By: Ms. Tasabih M. Saifeldin Ali
Dr. Einas Awad Osman
Reviewed by:
Prof. Emad Hussein

Name : _____
ID Number:_____
Section:_____

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List of Contents

Practical 1: Safety in Microbiology Laboratory

Best Practices for Safely Containing Microorganisms in the Laboratory:

1. **Treat Microorganisms as Potential Pathogens**: Always assume all microorganisms are harmful. Students with compromised immune systems should consult instructors before lab work.

1. **Culture Collection Maintenance**: Keep an organized culture collection, obtaining fresh stock annually to minimize mutations and contamination.

2. **Sterilization**: Sterilize all equipment and materials used in the lab.

3. **Disinfection**: Disinfect work areas before and after use with solutions like 10% bleach or 70% ethanol. Be aware of the location of the nearest eyewash station and sink.

4. **Personal Protection**: Wear safety goggles, lab coats, and gloves when handling cultures. Keep personal belongings separate from the work area and wash hands before handling these items.
5. **Hand Hygiene**: Wash hands with disinfectant before and after working with microorganisms. If unavailable, regular soap is acceptable. Gloves can provide additional protection.

6. **Pipetting Safety**: Always use mechanical pipetting devices—never pipette by mouth. Do not eat, drink, or store food in the lab.

7. **Labeling**: Clearly label all cultures, chemicals, and media, indicating names, dates, and hazard warnings as necessary.

8. **Waste Disposal**: Autoclave or disinfect all waste materials properly. If an autoclave is unavailable, soak materials in a 10% bleach solution for 1-2 hours before disposal.

10. **Spill Cleanup**: Cover spills with a disinfectant solution and clean with paper towels. Notify instructors of any spills and avoid touching broken glass with bare hands.

11. Special Precautions: Be cautious with strong acids, alkalis, and UV lamps. Use disinfectants at proper dilutions and keep equipment organized and tidy. Always read labels before using chemicals and wear gloves.

13. General Lab Practices: Maintain hand hygiene, avoid eating/drinking, use proper pipetting techniques, and manage spills immediately.

14. Additional Considerations: Utilize Biological Safety Cabinets, ensure decontamination of materials, provide training for lab personnel, and comply with institutional safety regulations. [1]

Table 1: PPE (Personal protective equipment) [2]

EQUIPMENT	HAZARD CORRECTED	SAFETY FEATURES
Laboratory coats, gowns, coveralls	Contamination of clothing	• Back opening • Cover street clothing
Plastic aprons	Contamination of clothing	• Waterproof
Footwear	Impact and splash	• Closed-toe
Goggles	Impact and splash	• Impact-resistant lenses (must be optically correct or worn over corrective eye glasses) • Side shields
Safety spectacles	Impact	• Impact-resistant lenses (must be optically correct) • Side shields
Face shields	Impact and splash	• Shield entire face • Easily removable in case of accident
Respirators	Inhalation of aerosols	• Designs available include single-use disposable; full-face or half-face air purifying; full-face or hooded powered air purifying (PAPR); and supplied air respirators
Gloves	Direct contact with microorganisms Cuts	• Disposable microbiologically approved latex, vinyl or nitrile • Hand protection • Mesh

Practical 1: Microbiology Laboratory instruments

1. Item/ Equipment ……. Function:_____ _____ _____ _____	5. Item/ Equipment ……. Function:_____ _____ _____ _____
2. Item/ Equipment ……. Function:_____ _____ _____ _____	6. Item/ Equipment ……. Function:_____ _____ _____ _____
3. Item/ Equipment ……. Function:_____ _____ _____ _____	7. Item/ Equipment ……. Function:_____ _____ _____ _____
4. Item/ Equipment ……. Function:_____ _____ _____ _____	8. Item/ Equipment ……. Function:_____ _____ _____ _____

9. Item/ Equipment …….. Function:_____ _____ _____ _____	14. Item/ Equipment …… Function:_____ _____ _____ _____
10. Item/ Equipment …… Function:_____ _____ _____ _____	15. Item/ Equipment …… Function:_____ _____ _____ _____
11. Item/ Equipment …… Function:_____ _____ _____ _____	16. Item/ Equipment …… Function:_____ _____ _____ _____
12. Item/ Equipment …… Function:_____ _____ _____ _____	17. Item/ Equipment …… Function:_____ _____ _____ _____
13. Item/ Equipment …… Function:_____ _____ _____ _____	18. Item/ Equipment …… Function:_____ _____ _____ _____

19. Item/ Equipment …….. Function:_____ _____ _____ _____	24. Item/ Equipment …… Function:_____ _____ _____ _____
20. Item/ Equipment …… Function:_____ _____ _____ _____	25. Item/ Equipment …… Function:_____ _____ _____ _____
21. Item/ Equipment …… Function:_____ _____ _____ _____	26. Item/ Equipment …… Function:_____ _____ _____ _____
22. Item/ Equipment …… Function:_____ _____ _____ _____	27. Item/ Equipment …… Function:_____ _____ _____ _____
23. Item/ Equipment …… Function:_____ _____ _____ _____	28. Item/ Equipment …… Function:_____ _____ _____ _____

Practical 2: Sterilization & Disinfection techniques

Sterilization is defined as the process where all the living microorganisms, including bacterial spores are killed.

Disinfection is the process of elimination of most pathogenic microorganisms (excluding bacterial spores) on inanimate objects. Chemicals used in disinfection are called disinfectants. disinfectants can kill all microorganisms.

*When dealing with Microorganisms, it is necessary that aseptic techniques are applied; **Aseptic Techniques to Prevent Contamination:**

- Work in a sterile environment (e.g., near a Bunsen burner or in a laminar flow hood).
- Sterilize inoculating instruments before and after use.
- Minimize the time Petri dish lids or culture containers are open.
- Wear gloves and clean your workspace with disinfectant before and after working.

Types of Sterilization and disinfection

A. Physical

1. Heat (Dry, Moist)
2. Radiation (Non- Ionizing UV rays lie in the range of 200-280 nm)
3. Filtration (HEPA (High Efficiency Particle Air))

B. Chemical

1. Alcohols
2. Aldehydes
3. Phenol
4. Halogens
5. Heavy Metals
6. Surface Active Agents
7. Dyes

DRY HEAT:

Red heat: bacteriological loops, straight wires, tips of forceps and searing spatulas are sterilized by holding them in Bunsen flame till they become red hot. This is a simple method for effective sterilization of such articles but is limited to those articles that can be heated to redness in flame.

Flaming: This is a method of passing the article over a Bunsen flame, but not heating it to redness. Articles such as scalpels, mouth of test tubes, flasks, glass slides and cover slips are passed through the flame a few times.

Incineration: This is a method of destroying contaminated material by burning them in incinerator. Articles such as soiled dressings; animal carcasses, pathological material and bedding etc.

This technique results in the loss of the article, hence, is suitable only for those articles that have to be disposed (Execution).

Hot air oven: Articles to be sterilized are exposed to high temperature (160o C) for duration of one hour in an electrically heated oven. The oven should be fitted with a thermostat control, temperature indicator, meshed shelves and must have adequate insulation. Articles sterilized: Metallic instruments (like forceps, scalpels, scissors), glassware (such as petri-dishes, pipettes, flasks, all-glass syringes), etc…

Sterilization process: The sterilizing temperature for a defined period (holding time) and the time taken for the articles to cool down. Different temperature-time relations for holding time are 60 minutes at 160o C, 40 minutes at 170o C and 20 minutes at 180o C. Increasing temperature by 10 degrees shortens the sterilizing time by 50 percent[3].

MOIST HEAT:

A. At temperature below 100o C:

1. Pasteurization: This process employed in food and dairy industry. There are 3 method:

H**older method** TEMP: heated at 63o C for 30 minutes, and

Flash method TEMP: heated at 72o C for 15 seconds, followed by quickly cooling to 13o C.

Ultra-High Temperature (UHT) TEMP: 140o C for 15 sec and 149o C for 0.5 sec.

2. Inspissation: This is a technique to disinfect egg and serum containing media. The medium are placed in the slopes of an inspissator.

TEMP: heated at 80-85o C for 30 minutes on three successive days.

B. At temperature 100o C:

1. Boiling: Boiling water TEMP: 100o C, certain metal articles and glassware can be disinfected by placing them in boiling water for 10-20 minutes. The articles are subjected to free steam at 100o C. Traditionally Arnold's and Koch's steamers were used or Autoclave.

2. Tyndallisation : at **100o C** Media such as TCBS, DCA and selenite broth are sterilized by steaming. Sugar and gelatin in medium may get decomposed on autoclaving, hence they are exposed to free steaming for 20 minutes for three successive days.

C. At temperature above 100o C:

Autoclave: an autoclave the water is boiled in a closed chamber. As the pressure rises, the boiling point of water also raises. At a pressure of 15 lbs inside the autoclave, TEMP: 121o C. Exposure of articles to this temperature for 15 minutes.

*For Prions; Higher temperatures or longer times are used; 135o C or 121o C for at least one hour are recommended. Advantages of steam: It has more penetrative power than dry air, it moistens the spores (moisture is essential for coagulation of proteins), condensation of steam on cooler surface releases latent heat, condensation of steam draws in fresh steam.

Chemical Disinfectants:

1. ALCOHOLS: Alcohols dehydrate cells, disrupt membranes and cause coagulation of protein.

Examples: Ethyl alcohol, isopropyl alcohol and methyl alcohol Application: A 70% aqueous solution (spirit) is more effective at killing microbes than absolute alcohols. Used as antiseptic on skin. It can also be used to disinfect surfaces. It is used to disinfect clinical thermometers.

2. ALDEHYDES: Damages nucleic acids. It kills all microorganisms, including spores. Examples: Formaldehyde, Gluteraldehyde Application: 40% Formaldehyde (formalin) is used for surface disinfection and fumigation of rooms, chambers, operation theatres, biological safety cabinets, wards, sick rooms etc.

3. **PHENOL**: Act by disruption of membranes, precipitation of proteins and inactivation of enzymes. Examples: 5% phenol, 1-5% Cresol, chloroxylenol (Dettol) Applications: used ito prevent infection of surgical wounds. Phenols are coal-tar derivatives. They act as disinfectants at high concentration and as antiseptics at low concentrations. They are bactericidal, fungicidal, mycobactericidal but are inactive against spores and most viruses.

4. HALOGENS: They are oxidizing agents and cause damage by oxidation of essential sulfydryl groups of enzymes. Chlorine reacts with water to form hypochlorous acid, which is microbicidal. Examples: Chlorine compounds (chlorine, bleach, hypochlorite) and iodine compounds (tincture iodine, iodophores) Applications: Tincture of iodine (2% iodine in 70% alcohol) is an antiseptic.

5. HEAVY METALS: Act by precipitation of proteins and oxidation of sulfydryl groups. They are bacteriostatic. Examples: Copper sulfate, organic mercury salts (e.g. merthiolate) Applications:. Silver sulphadiazine is used topically to help to prevent colonization and infection of burn tissues. Mercurials are active against viruses at dilution of 1:500 to 1:1000. Merthiolate at a concentration of 1:10000 is used in preservation of serum. Copper salts are used as a fungicide.

6. SURFACE ACTIVE AGENTS These compounds have long chain hydrocarbons that are fat soluble and charged ions that are water-soluble, they concentrate on the surface of membranes. They disrupt membrane resulting in leakage of cell constituents. Examples: Cetrimide and benzalkonium chloride act as cationic detergents. Application: They are active against vegetative cells, Mycobacteria and enveloped viruses. They are widely used as disinfectants at dilution of 1-2% for domestic use and in hospitals.

7. DYES: Acridine dyes are bactericidal because of their interaction with bacterial nucleic acids. Examples: Aniline dyes such as crystal violet, malachite green and brilliant green.. A related dye, ethidium bromide, is also germicidal. It intercalates between base pairs in DNA. They are more effective against gram positive bacteria than gram negative bacteria and are more bacteriostatic in action. Applications: They may be used topically as antiseptics to treat mild burns. They are Also used as selective agents in certain selective media.

HYDROGEN PEROXIDE: It acts on the microorganisms through its release of nascent oxygen. Hydrogen peroxide produces hydroxyl-free radical that damages proteins and DNA.

Application: It is used at 6% concentration to decontaminate the instruments, equipment such as ventilators. 3% Hydrogen Peroxide Solution is used for skin disinfection and deodorizing wounds and ulcers. Strong solutions are sporicidal[3].

Practical 3: Preparation of Nutrient agar plates, Slopes and Nutrient Broth medium

Bacterial culture media are nutrient-rich environments used in laboratories to grow and maintain bacterial cultures. These media provide the essential nutrients, water, and conditions (like pH and oxygen levels) necessary for bacterial growth. Culture media can be classified into different types based on their composition, & consistency:

- **Based on Composition:**
  - **Simple Media:** Basic nutrient supply for bacterial growth, e.g., Nutrient Agar.
  - **Enriched Media:** Contains extra nutrients like blood, serum, or egg to grow fastidious organisms, e.g., Blood Agar.
  - **Selective Media:** Contains agents that inhibit the growth of some bacteria while allowing others to grow, e.g., MacConkey Agar.
  - **Differential Media:** Helps distinguish between different types of bacteria based on biochemical reactions, e.g., Mannitol Salt Agar.
  - **Transport Media:** Used to transport samples without bacterial growth or death, e.g., Stuart's medium.

- **Based on Consistency:**
  - **Solid Media:** Contains agar, providing a solid surface for bacterial colonies, e.g., Tryptic Soy Agar.
  - **Liquid Media (Broth):** No agar, used for growing bacteria in a liquid environment, e.g., Nutrient Broth.
  - **Semi-solid Media:** Lower agar concentration, used for motility tests, e.g., SIM medium.

Steps of Culture Media preparation:

1. After following instruction on the dehydrated media, place the weighed powder to a proper flask, pour Distilled water required volume to the flask gradually till the powder dissolves.

2. Heat the dissolved media on a hot plate till it is clear then transfer the Foil covered flask to the Autoclave.

3. **Autoclaving:** Sterilize the media using an autoclave. Most media are sterilized at **121°C at 15 psi pressure for 15-20 minutes**. This kills all microorganisms, spores, and contaminants.
   *Some Medium contain heat labile substances, accordingly it will be Noted that dissolve till heating, then it will be poured into the proper tubes, petri dishes or bottles

4. **Cooling (for solid media):** If preparing agar plates, allow the sterilized agar media to cool down to about 45-50°C before pouring into Petri dishes to avoid condensation.

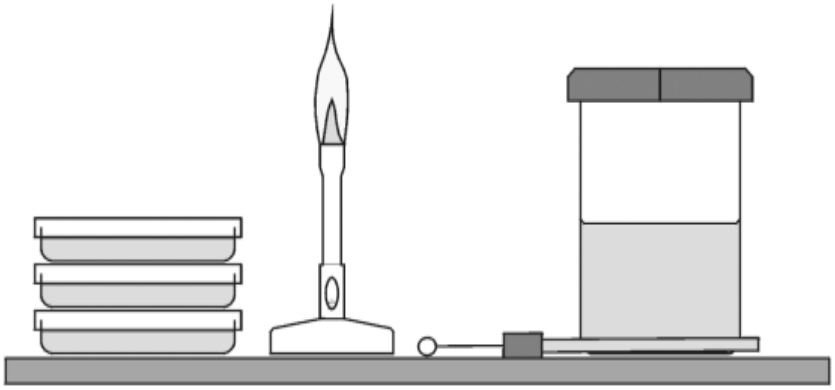
5. **Pouring Agar Plates:** Pour the cooled media into sterile Petri dishes inside a laminar airflow cabinet or other clean environments. Allow them to solidify at room temperature.. Seal and incubate the plate in an inverted position. (The base of the plate must be covered, agar must not touch the lid of the plate, and the surface must be smooth with no bubbles).
   Solid Media (Agar Slants): Fill the tubes halfway, then tilt them at an angle to create slants as the agar cools and solidifies.
   Broth Media: Pour the broth, filling the containers halfway to allow for gas exchange during bacterial growth.

6. **Post-Pouring**: Tighten caps once the media solidifies, and store them appropriately. Always use aseptic techniques to avoid contamination.

7. **Storage:** Once solidified, store the prepared plates upside down in the refrigerator (usually at 4°C) to prevent condensation on the agar surface.

Inoculation of agar plates, Slopes and Broth medium (Subculture)

Inoculation (Cultivation) of Pure Culture Media



Sterile (Aseptic) Technique: Always use aseptic technique to avoid contamination. This includes sterilizing tools like inoculating loops or needles by heating them in a flame until red hot.

Inoculation Steps:

1. **Obtain Isolate:** Using a sterile inoculating loop, needle, or swab, pick a colony, or dip (from a colony, broth, or clinical sample).
2. **Inoculate Media:**

- For **solid media (plates)**: Gently streak the loop across the surface in a zigzag pattern (streak plate method: https://www.youtube.com/shorts/_7uIB7jxV4w) to isolate colonies.
- For **liquid media**: Dip the inoculating loop or needle into the broth.

3. **Close the Container:** Immediately cover the plate or broth culture after inoculation to prevent contamination.
4. **Incubate:** Place the inoculated media in an incubator at the appropriate temperature (usually 37°C for human pathogens) for the recommended time (e.g., 24–48 hours).

Click on the following link to check aseptic technique & Culture media inoculation: https://www.youtube.com/watch?v=bRadiLXkqoU

Semi-Solid media Inoculation

The most commonly used test for motility in microbiology lab.

It depends on the ability of motile bacteria to move through semi-solid media.

Ordinary solid media contain 1.5-2.0% Agar

Semi solid media contain about 0.4% Agar

Steps:

- Using a sterile bacteriological needle (Straight wire), pick a colony of the test organism
- Stab quickly a tube of semi solid media. (avoid using bent needles).
- Incubate the semi solid media for 24 hours

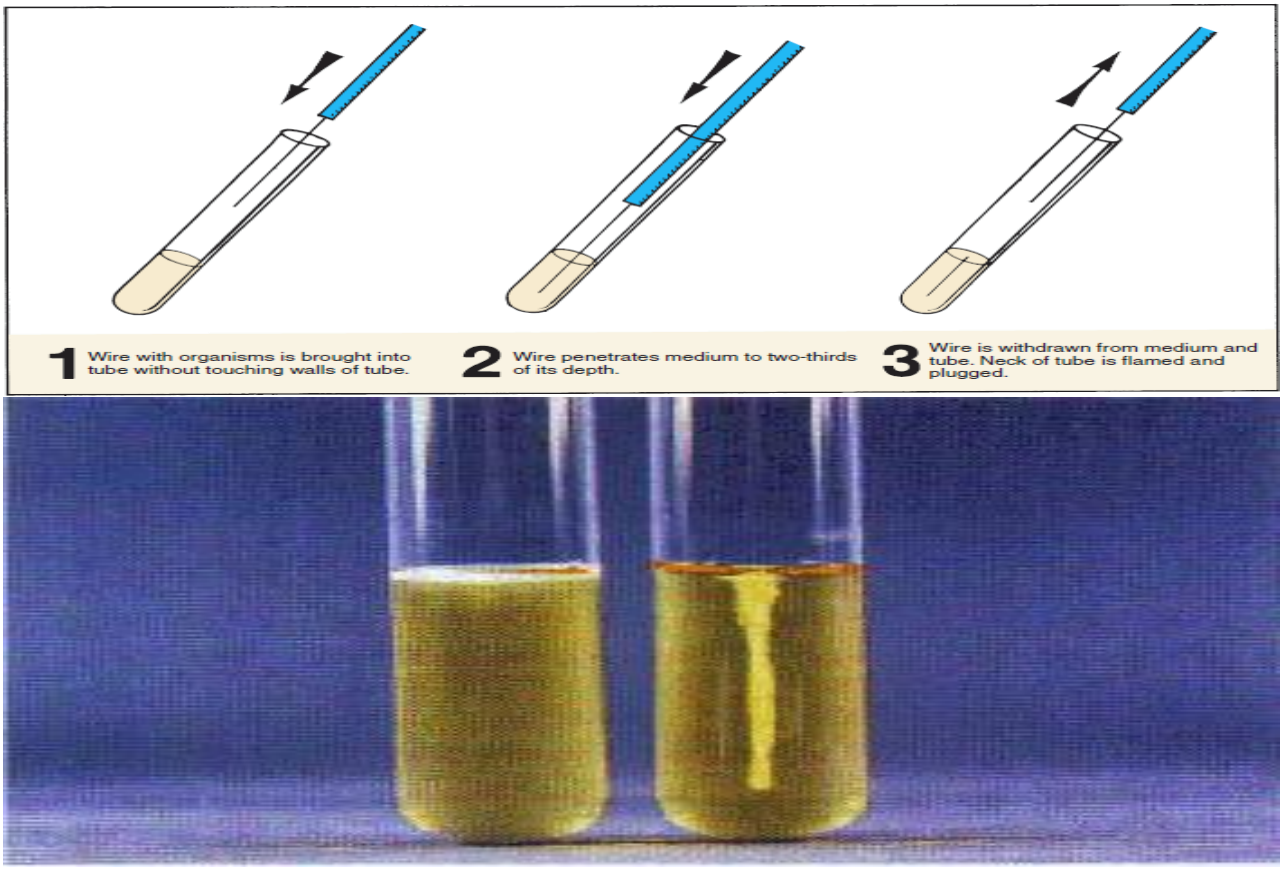


Figure (1) shows the stab technique demonstrating bacterial motility

Reading Results:

- If there will be growth going out away from the stab line, and test is positive (motile)
- If there was only growth along the stab line.

Practical 4: Pure Culture: Agar Isolation techniques

1. Streak plate

The loop is used for preparing a streak plate. This involves the progressive dilution of an inoculum of bacteria or

yeast over the surface of solidified agar medium in a Petri dish in such a way that colonies grow well separated from

each other.

The aim of the procedure is to obtain single isolated pure colonies[4].

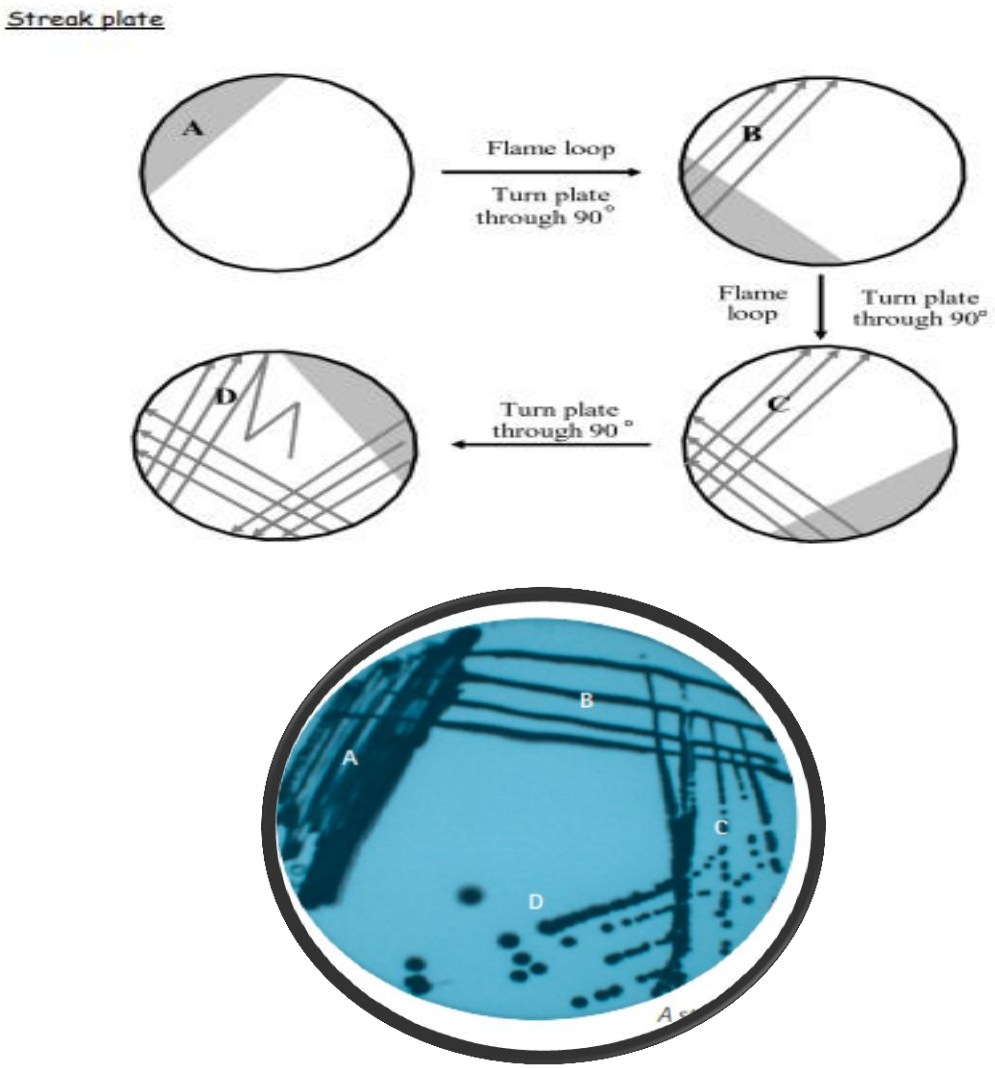


Figure (2): Streak plate technique

2. Pour plate

- A pour plate is achieved by adding a small volume of inoculum from a broth culture to a molten, cooled agar medium in a test tube or bottle.
- This mixture is thoroughly combined and then poured into a Petri dish to solidify. Pour plates allow microorganisms to grow both on the surface and within the medium.
- Most colonies form within the medium and tend to be small, while a few that develop on the surface appear similar in size and shape to those on a streak plate.
- By knowing the dilution and volume of the inoculum—typically 1 ml—the viable count of the sample can be calculated, revealing the number of bacteria or bacterial clusters per ml[4].

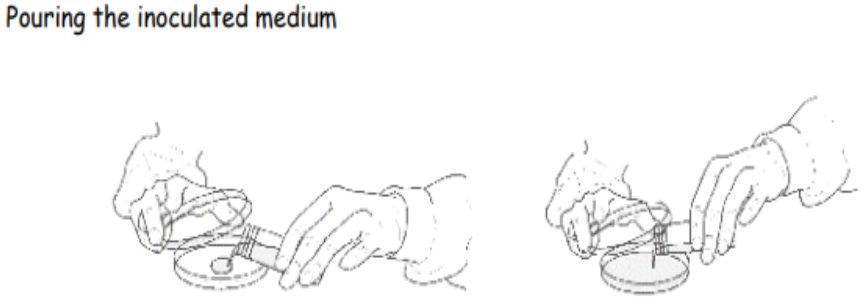


Figure (3): pour plate technique

3. Spread plate

Using a spreader: Sterile spreaders are used to distribute inoculum over the surface of already prepared agar plates. Spread plates, also known as lawn plates, should result in a culture spread evenly over the surface of the growth medium.

This means that they can be used to test the sensitivity of bacteria to many antimicrobial substances, for example mouthwashes, disinfectants and antibiotics.

The spread plate can be used for quantitative work (colony counts) if the inoculum is a measured volume, usually 0.1 ml, of each of a dilution series, delivered by pipette.

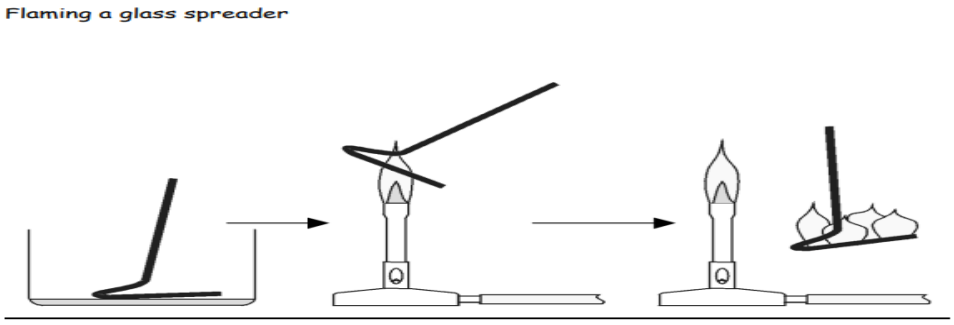


Figure (4): Spread plate technique

Incubation:

Incubator: typically simulate body temperatures, but many cultures can thrive at room temperature (22-25°C). Overnight incubation (18 hours) is effective, while optimal growth occurs at 37°C within 24-48 hours.

Water Bath: Water baths ensure precise temperature control for enzyme reactions and growth studies when incubators are inadequate. Use distilled or deionized water to prevent corrosion and remember to empty and dry them after use[4].

Colonial Characteristics (Morphology)

Colony morphology refers to the visible characteristics of a bacterial colony on an agar plate, including shape, size, color, surface appearance, and texture. Observing these traits is a key microbiological skill for identifying microorganisms. Well-isolated colonies are essential for accurate observation. Hemolysis, an important feature, occurs when bacteria lyse blood cells on Blood Agar.

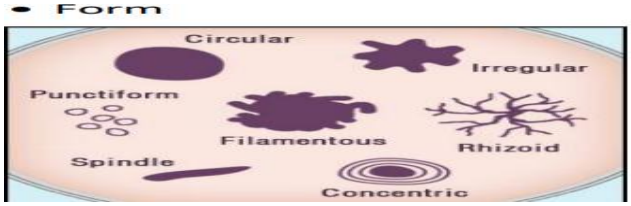
Bacterial colonies on solid media can be described by three key characteristics:

Texture: The surface appearance, which can be smooth, glistening, mucoid, slimy, dry, powdery, or flaky.

Transparency: Colonies may be transparent (see-through), translucent (partially clear), or opaque (solid).

Color or Pigmentation: Some bacteria produce pigments that color colonies yellow, pink, purple, or red, while others appear white or gray due to lack of pigmentation.

Size:• large (>1mm) • medium (approximately =1mm) • small (<0.5mm)[5].



On Broth :growth occurs throughout the container and can then present a dispersed, cloudy, or flaky appearance.5

Practical 5: Microscopy: Smear preparation & Wet preparation

How to use compound microscope[7]:

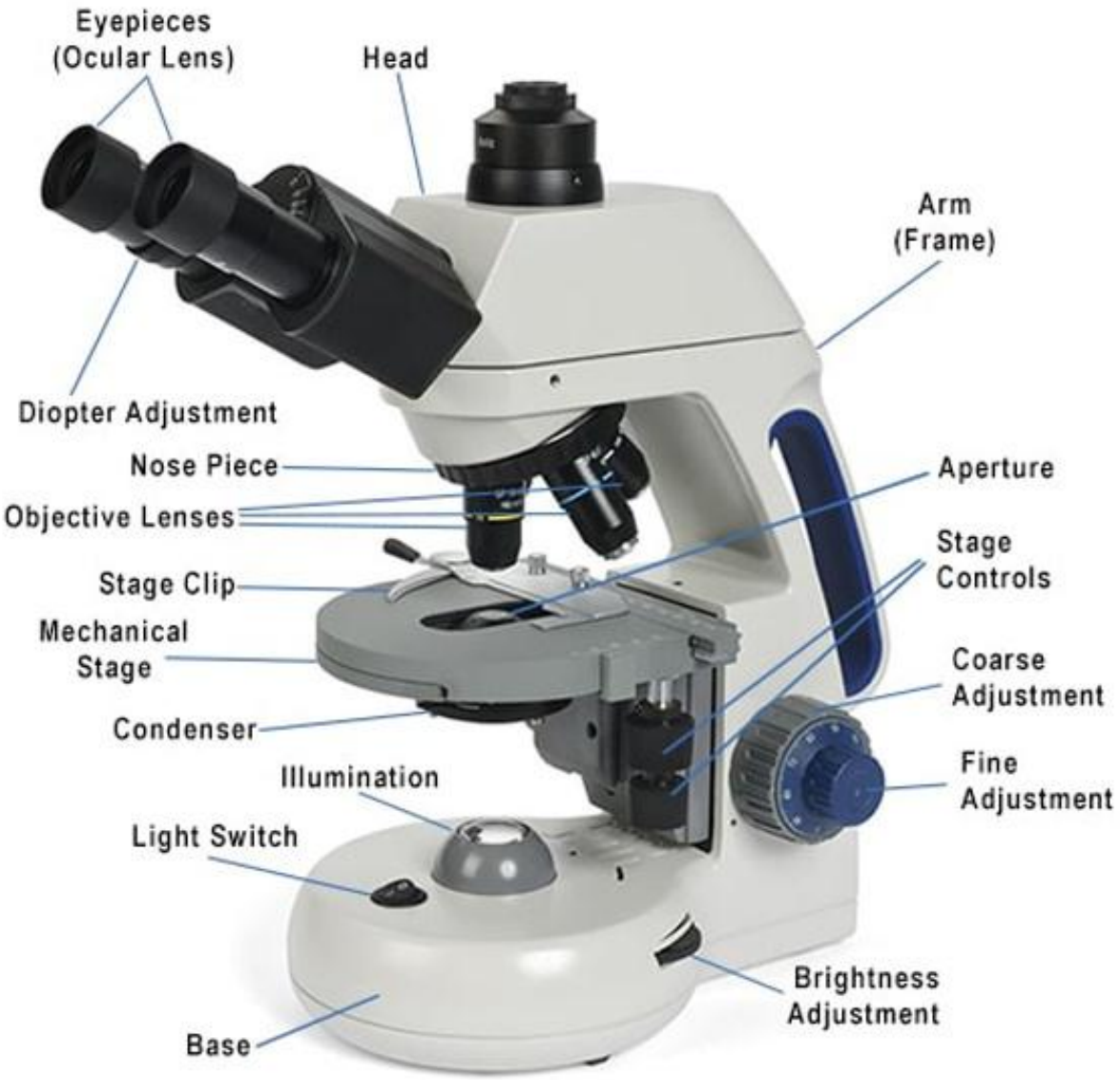


Figure (6) Bright field microscope parts

Please check the following link: https://www.youtube.com/watch?v=SUo2fHZaZCU

The Wet Mount (wet preparation):

Is a procedure primarily used for: – examining specimens and cultures for motile bacteria, – inspecting cerebrospinal fluid (CSF) for encapsulated yeast cells, – and observing specimens for fungi.

Requirements:

Personal protective equipment, Sterile microscope slides, Sterile pipettes, Glass coverslips

Steps:

1. Using a sterile dropper, gently mix and remove some of the specimen from the tube and place one drop (10µL) on a clean microscope slide.
2. Immediately put a coverslip over the sample for examination. A microscopic review of the slide should be performed as soon as possible to confirm the presence or absence of parasite (Direct from vaginal specimen).
3. Focus with low power (10X), low light, scan the entire slide, read at least 10 fields.
4. Identify objects with high power (40X). Record results based on your laboratory's criteria [6].

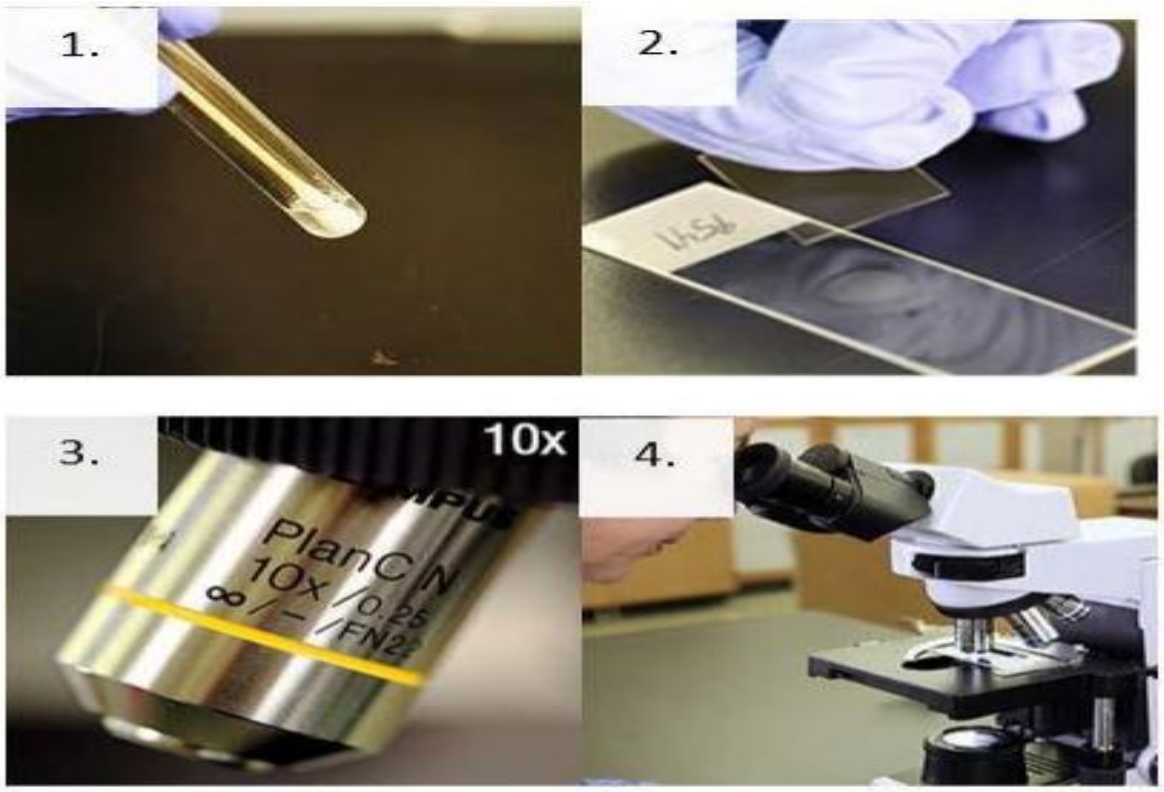


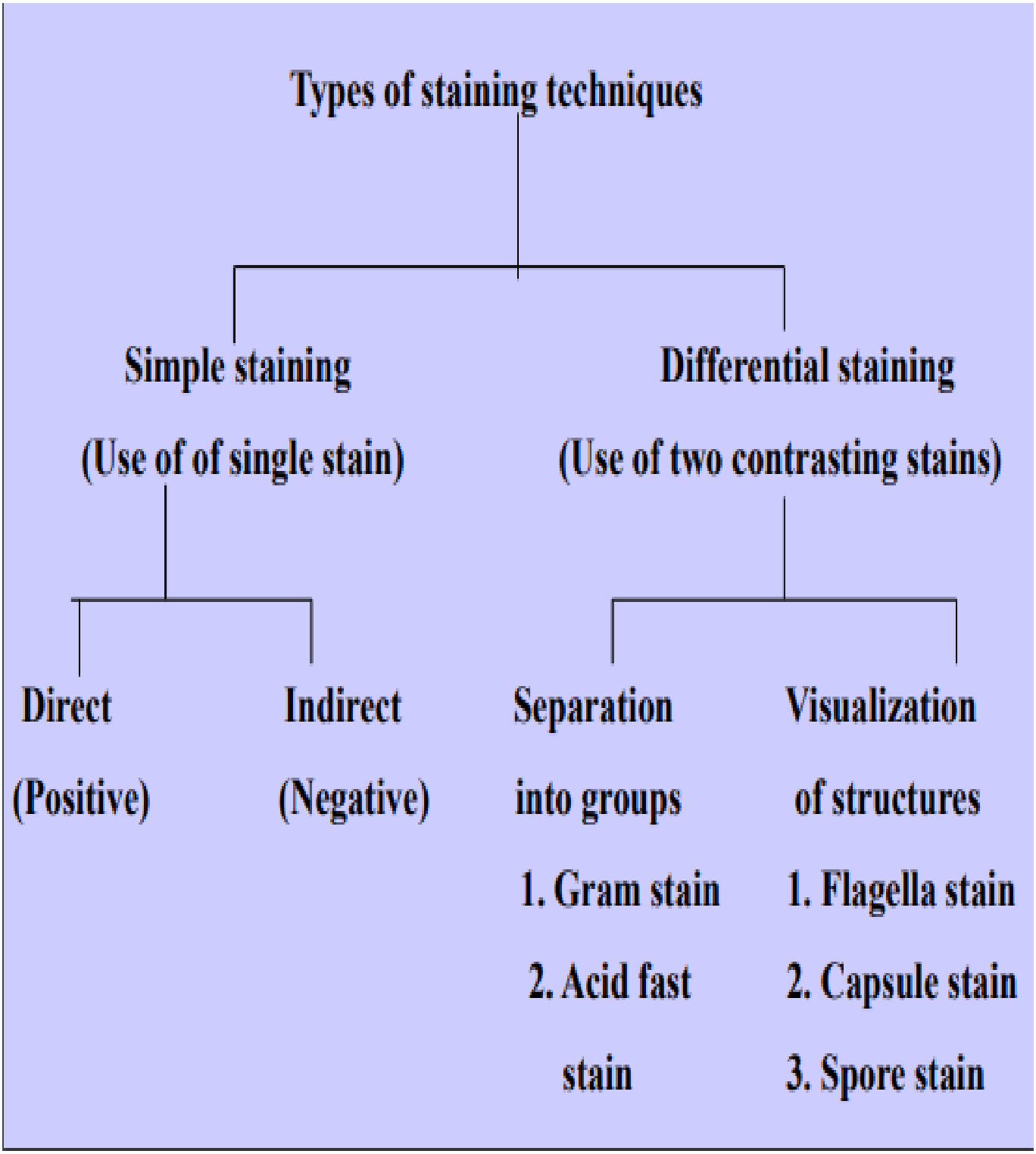
Figure (7) wet preparation steps

Smear Preparation

To prepare a microscope slide for examination, a smear of bacteria or yeast is made, fixed, stained, and dried without a coverslip. It's important to use aseptic techniques when sampling cultures. Using agar medium is preferable to liquid cultures for making smears. A thin, even smear allows for clear visualization of cell shape and arrangement, ensuring uniform application of stains.

For reliable smear results, proper preparation, labeling, and fixation are key:

1. **Labeling**: Use frosted-end slides and label with a pencil to prevent washing off during staining. Discard damaged or reused slides.
2. **Smear Preparation**: Use appropriate techniques based on the specimen type (purulent, non-purulent, culture, sputum, swabs, feces, or skin smears).
3. **Drying and Fixing**:
   - **Air-dry** the smear in a clean, dust-free environment.
   - **Heat fixation**: Pass the slide through a flame three times, avoiding overheating.
   - **Alcohol fixation**: Apply alcohol (methanol or ethanol) and let it evaporate for 2 minutes.
4. **Post-Fixation**: Proceed with staining after fixation[8].


Types of staining techniques
Simple staining
(Use of of single stain)
Differential staining
(Use of two contrasting stains)
Direct
(Positive)
Indirect
(Negative)
Separation
into groups
1. Gram stain
2. Acid fast
stain
Visualization
of structures
1. Flagella stain
2. Capsule stain
3. Spore stain

Simple Staining

A simple stain involves the application of one stain to show cell shape and arrangement and, sometimes, inclusions that do not stain, e.g. bacterial endospores;

Requires Basic dyes like Methylene blue, Crystal violet, or Safranin, dried fixed smear on a clean slide

Steps:

1. Apply the stain to the fixed, dried smear for 1 minute.
2. Rinse with clean water; if tap water is unclean, use filtered or boiled rainwater.
3. Clean the back of the slide and let it air dry on a draining rack.
4. Examine the smear microscopically first with a 40× objective to observe material distribution, then with an oil immersion objective to identify bacteria.

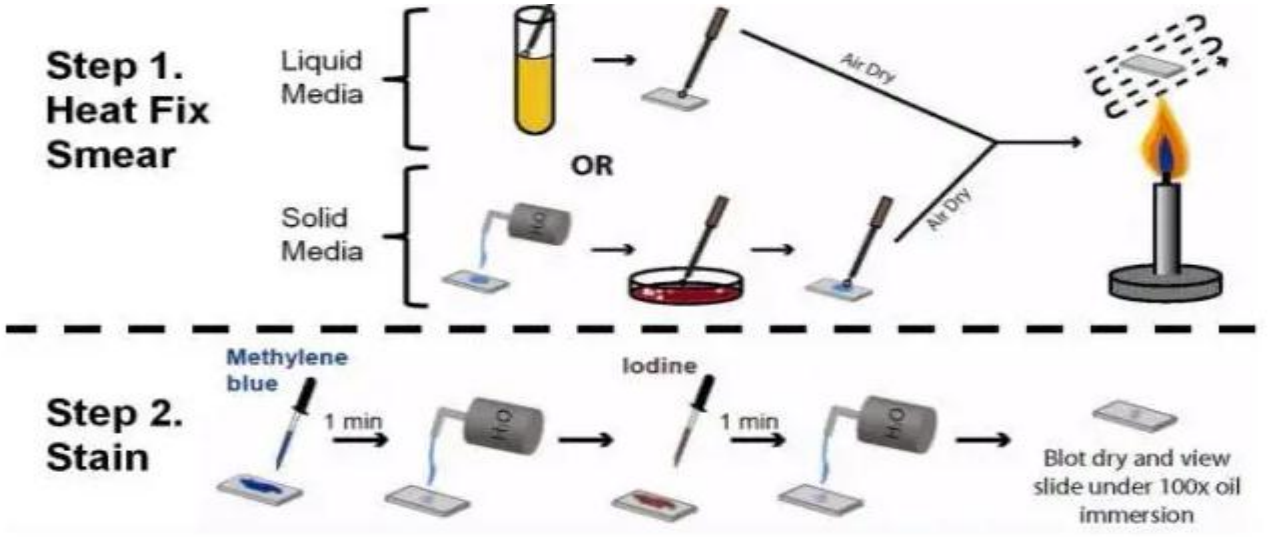


Figure (8- a): Steps for simple staining technique[12].

Results:

Bacterial cells: Blue

Nuclei of leukocytes: Blue[9].

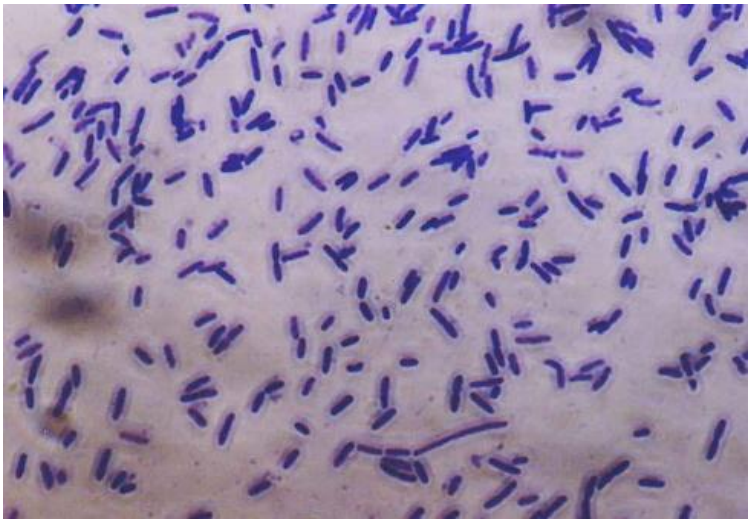


Figure (8- b): Crystal violet stained Bacilli[11].

Negative staining

In this staining method, the background is colored while the bacteria remain unstained. An acidic dye like nigrosin or Indian ink is used. Since both the dye and the bacteria have negative charges, they repel each other. This prevents the dye from coloring the bacteria. Instead, the dye creates a dark background, making the bacteria appear clear or transparent.

Steps:

1. Place a drop of stain (e.g., Nigrosin, India ink) on a slide.
2. Mix a bacterial sample into the stain.
3. Use another slide to spread the stain into a thin smear.
4. Air-dry the slide (without heat-fixing).
5. Observe under a microscope.

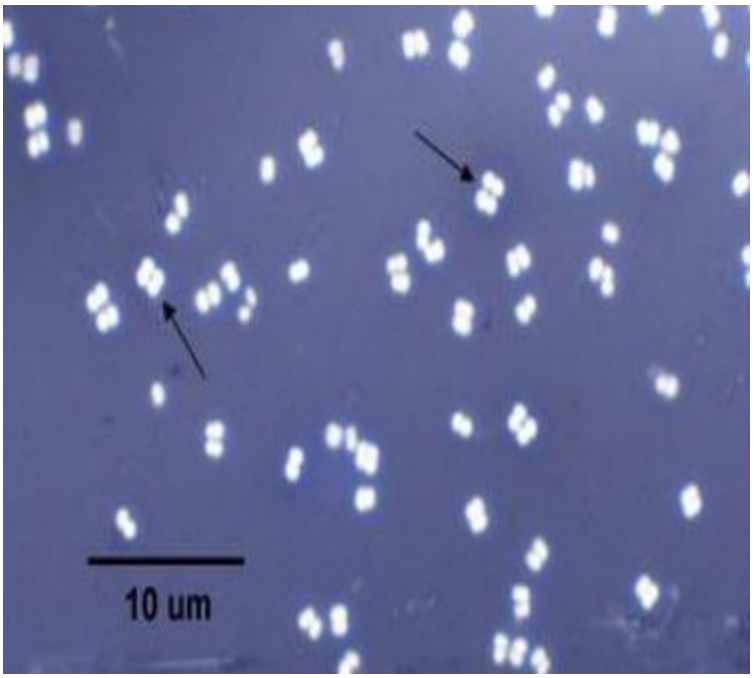


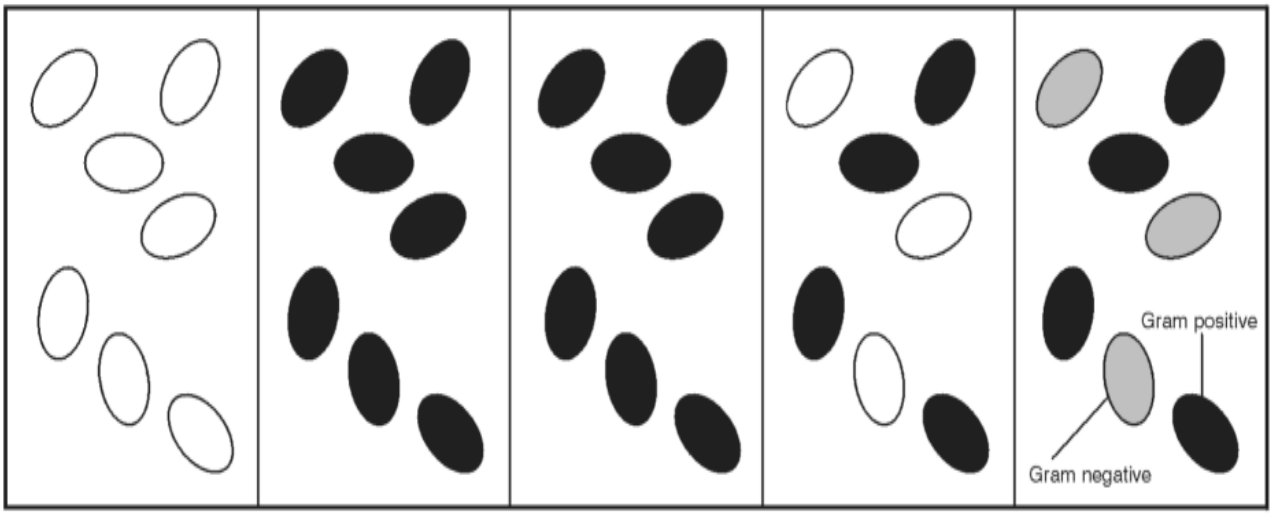
Figure (9): Stained Background – negative staining[11]

Practical 7: Differential Staining Techniques: Gram's Staining reactions

Gram stain is a fundamental laboratory technique used to classify bacteria into two groups based on their cell wall composition: Gram-positive and Gram-negative.

Steps:

1. **Heat fixed Smear:** Place the slide with the smear facing up on a staining rack.
2. **Primary (Basic) Stain**: Apply crystal violet for 1 minute, then rinse with iodine solution.
3. **Mordant:** Apply iodine solution for 1 minute to fix the stain.
4. **Decolorizer:** Rinse with 95% ethanol until the wash is pale violet, then rinse with tap water.
5. **Secondary (Counter Stain):** Apply counterstain (e.g., safranin) for 30 seconds, then rinse with water.
6. Blot **dry** and examine under **oil immersion. X100.**
7. Dispose of the slide in a discard jar after use[4].



(a) before staining all bacteria are colourless

(b) after basic stain *(crystal violet)* all bacteria are stained violet

(c) after mordant *(Lugol's iodine)* stain is fixed more firmly into the cell

(d) after decolouriser (alcohol) some bacteria are colourless (Gram negative) while others are still violet (Gram positive)

(e) after counterstain (safrarin) colourless bacteria (Gram negative) have taken up stain and appear red: Gram positive bacteria remain violet

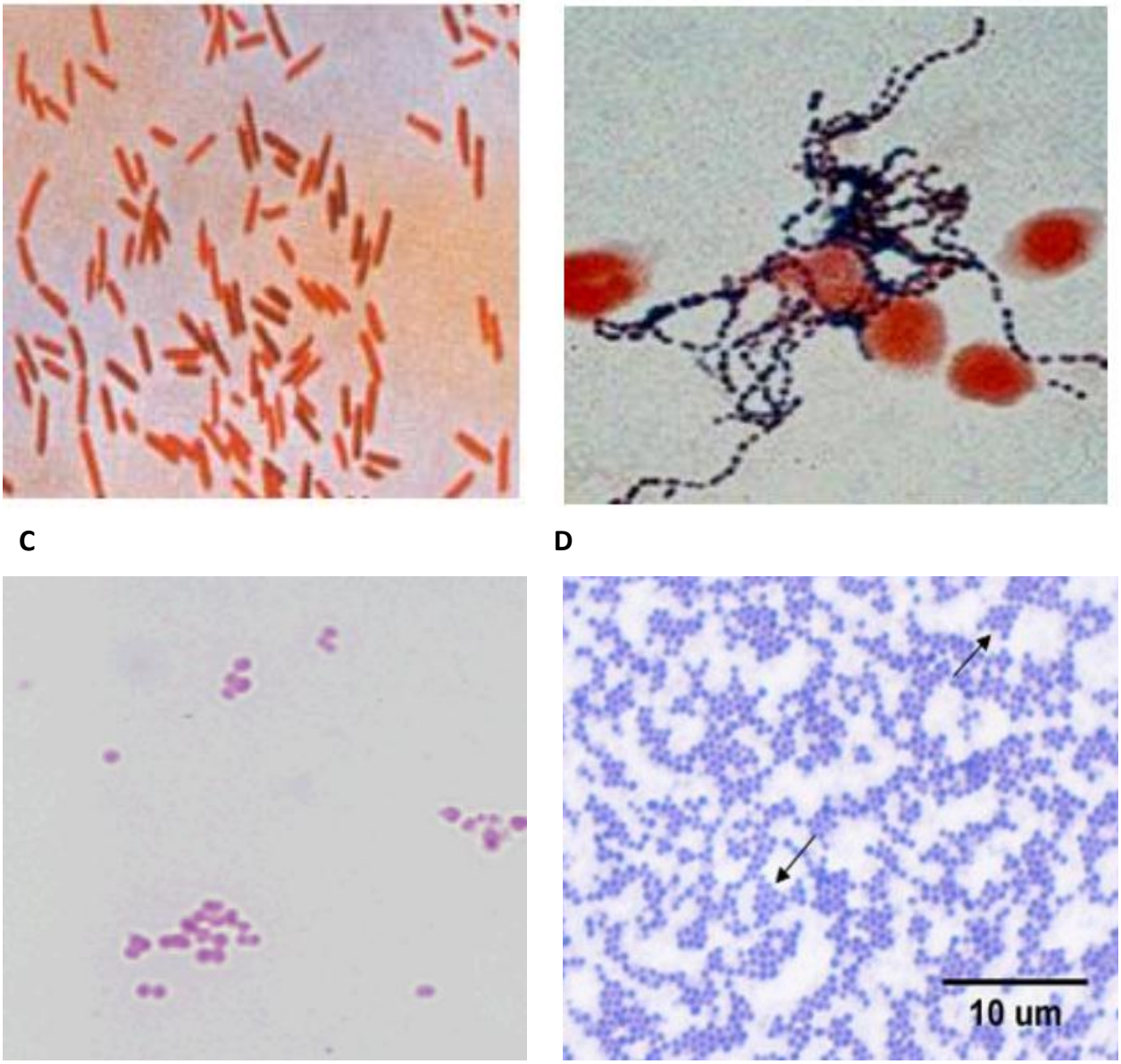
Figure (10): Steps of Gram stain

Results:

The report on Gram smears should contain the following details:

- **Gram Reaction**: Specify whether the bacteria are Gram-positive or Gram-negative.
- **Bacterial Morphology**: Describe the shape of the bacteria, categorizing them as cocci, diplococci, streptococci, rods, or coccobacilli
- **Arrangement:** determines how bacteria are arranged sometimes gives a hint about the suspected micro organism[8].

A **B**



C **D**

Figure (11): (A) Negative Bacilli, (B) Gram Positive Cocci in Chain (C) Gram negative diplo-cocci, (D) Gram Positive cocci in cluster

Practical 8: Differential Staining Techniques: Acid Fast stain

The **Ziehl-Neelsen technique** is used to stain Mycobacterium species, including M. tuberculosis, M. ulcerans, and M. leprae. Mycobacteria do not take up the Gram stain effectively; instead, they are stained with carbol fuchsin, which binds to the mycolic acid in their cell walls.

An acid decolorizing solution is then applied, removing the red dye from surrounding cells but leaving the mycobacteria stained red (termed acid-fast bacilli, or AFB). Finally, a counterstain of malachite green or methylene blue is added to provide contrast against the red AFB.

Types:

Hot ZN Technique:

- Involves heating the Phenolic-Carbol Fuchsin stain.
- Heating helps the dye penetrate the waxy cell wall of mycobacteria.

Cold ZN Technique:

- No heating is used.
- Higher concentrations of Basic Fuchsin and phenol are applied, often with a wetting agent.

Both *M. leprae* and *M. tuberculosis* stain less effectively with the cold method.

Smears stained using the cold technique fade more quickly.

***Auramine-Phenol Fluorochrome Staining Technique**

Requires: Auramine-phenol flourescent stain , 1% acid alcohol and Potassium permanganate (improves the contrast between acid-fast and non-acid-fast bacilli, reduces the background fluorescence) .

Steps:

Stain with auramine-phenol for 10 minutes, rinse.

Decolorize with 1% acid alcohol for 5 minutes, rinse.

Apply potassium permanganate for 10 seconds

Followed by several rinses with clean water.

Observe Examine the smear for acid-fast bacilli (AFB) using fluorescence microscopy with a 40× objective.

Result:

Acid-Fast Bacilli (AFB) appear **yellow-green** or **bright yellow** under a fluorescence microscope. Count as mentioned in ZN technique.

Non-Acid-Fast Bacilli appear **dark** or **black**, as they do not retain the dye[9].

Steps of Ziehl-Neelsen Staining Method

1. **Fix the Smear**: Alcohol-fixation is bactericidal, unlike heat-fixation for untreated sputum.
2. **Primary (Basic) Stain: Apply Carbol Fuchsin**: Cover the smear with the stain.
3. **Mordant: Heat the Stain**: Gently heat until vapors rise (around 60°C) and leave for 5 minutes. Be cautious as carbol fuchsin is flammable.
4. **Rinse**: Wash with clean water or filtered/boiled water if tap water isn't clean.
5. **Decolorization**: Apply 3% acid alcohol for 5 minutes or until pale pink.
6. **Rinse Again**: Wash thoroughly with clean water.
7. **Secondary (Counter) stain:** Apply Malachite Green/ Methylene Blue Stain for 1-2 minutes, longer for thin smears.
8. **Final Rinse**: Rinse with clean water.
9. **Dry**: Air-dry the slide without blotting.
10. **Examine Microscopically**: Use 100× oil immersion to systematically scan the smear, avoiding cross-contamination by cleaning the lens after examining positive slides9.

Result:

Acid-fast bacilli (**AFB**) appear as **Red**, straight or slightly curved rods, either solitary or in small clusters, and may look beaded.

The cells and background material appear **green**/ **Blue**.

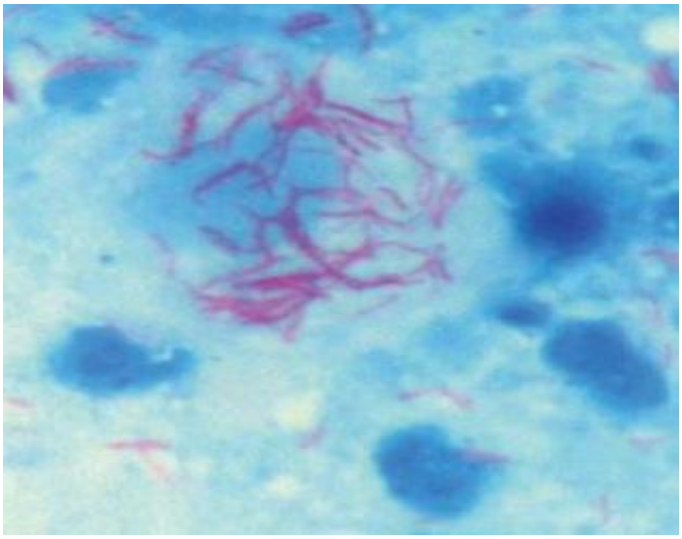


Figure (12): ZN Stain Carbol Fuchsin & Malachite green

*When red bacilli are observed, the smear is reported as "AFB positive" with an indication of the number of bacteria present:

- More than 10 AFB per field: report +++
- 1–10 AFB per field: report ++
- 10–100 AFB per 100 fields: report ++
- 1–9 AFB per 100 fields: report the exact number

Practical 9: Bacterial Structures Spore Stain & Capsule stain technique

Capsule stain (Burri's India ink method):

Bacterial capsules are primarily made of polysaccharides, though some genera produce polypeptide capsules.

These capsule polymers are usually uncharged and difficult to stain, necessitating the use of a negative staining technique.

This method involves staining the background while leaving the capsule uncolored for visibility. Burri's India ink method uses India ink to darken the background and crystal violet (or safranin) to stain the bacterial cells.

Capsule Stain Procedure:

1. Place a drop of India ink on a clean microscope slide near the frosted edge.
2. Using a flamed loop and sterile technique, mix a sample of *K. pneumoniae* (or the desired organism) into the India ink drop.
3. Angle another clean slide against the first slide to spread the drop into a thin film.
4. Allow the film to air dry.
5. Saturate the slide with crystal violet for 1 minute.
6. Rinse the slide gently with water.
7. Allow the slide to air dry completely.
8. Observe the slide under a microscope.

Result:

The background will be dark.

The bacterial cells will be stained purple.

The capsule (if present) will appear clear against the dark background[8].

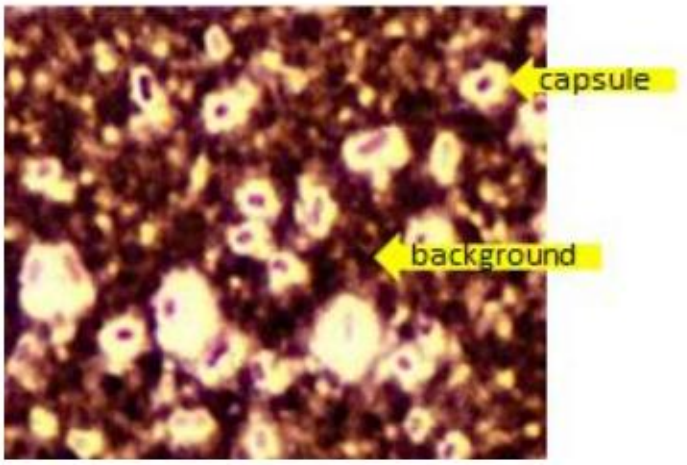


Figure (13): Burris India ink capsular stain

Spore Stain (Schaeffer-Fulton Method)

Bacterial endospores are highly resilient structures designed for survival in harsh conditions. They consist of several protective layers, including a thick outer coat made of keratin-like proteins, an inner coat of peptidoglycan, and a cortex that aids in dehydration and dormancy. The core contains dehydrated bacterial DNA, ribosomes, and proteins, which contribute to metabolic inactivity, enhancing resistance to heat and desiccation. Together, these features enable endospores to endure extreme environments.

Steps:

1. **Prepare Smears:** Create air-dried, heat-fixed smears of *Bacillus* and/or *Clostridium* species, containing both spores and vegetative cells.
2. **Set Up Staining Apparatus:** Cover the work area with paper, then assemble a steaming apparatus with a Bunsen burner and a beaker of boiling water.
3. **Heat and Stain:** Balance the slide over the steam, cover the smear with blotting paper, and saturate it with malachite green for 3 minutes, adding stain as needed.
4. **Rinse:** Carefully remove the slide, peel off the blotting paper, and wash with water.
5. **Safranin Staining:** Flood the smear with safranin for 1 minute, rinse, and blot dry.
6. **Microscopic Examination:** Use low (10×), high (40×), and oil immersion (100×) lenses

Result:

Observe **Green**-stained spores and **red-orange** vegetative cells.

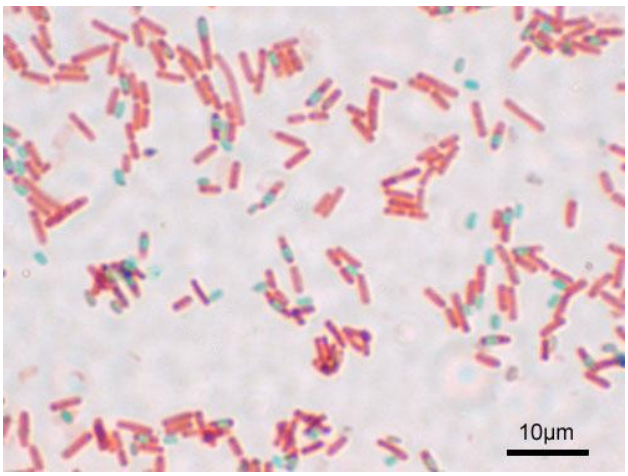


Figure (14), Schaeffer-fulton Spore Stain

Note the position of the spores and draw representations in the Results section[10].

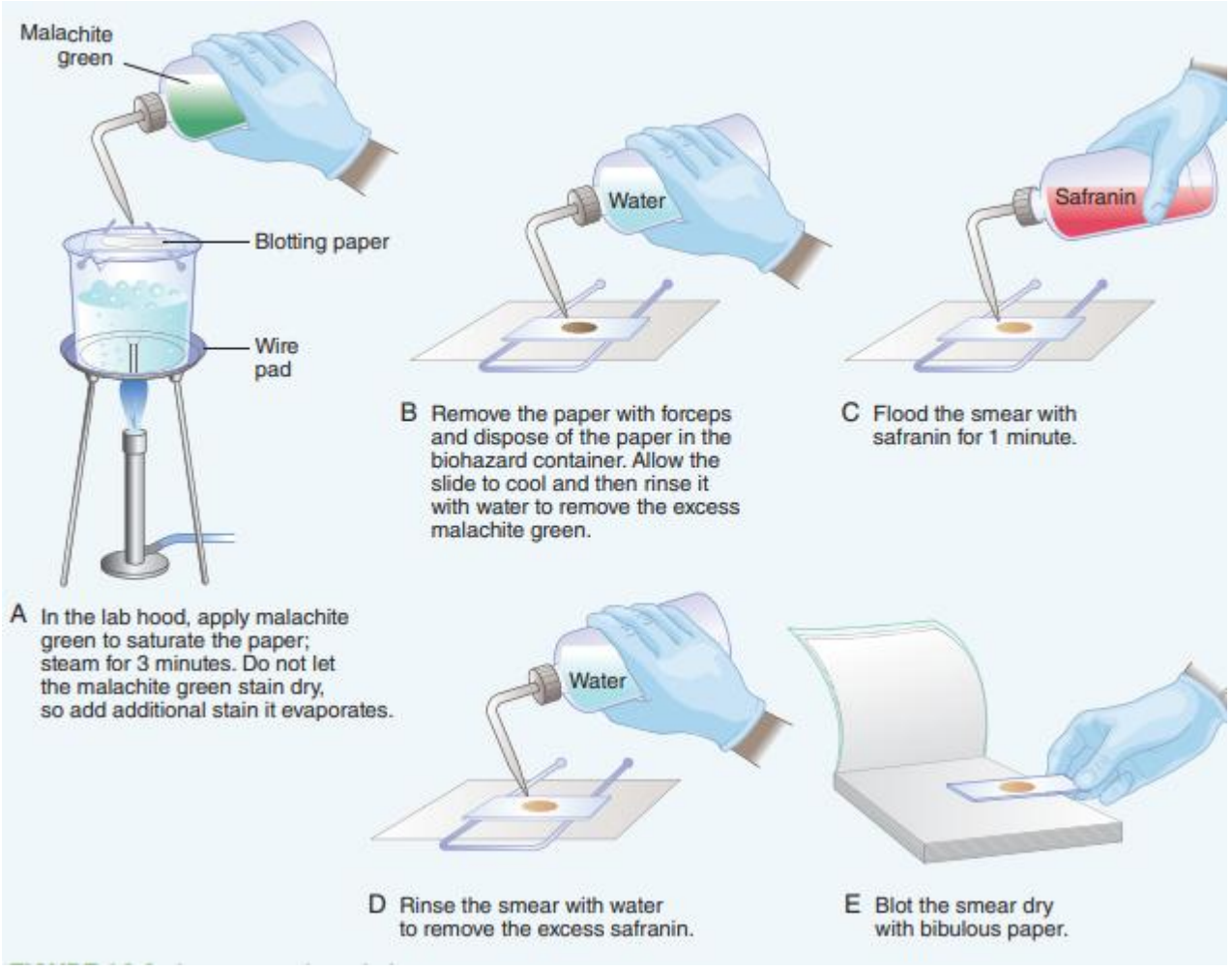


Figure (15): Result of Spore staining technique

Practical 10: Metabolism, Physiology, and Growth Characteristics of Gram-Positive Bacteria – Biochemical tests

1. Catalase test

Detects the ability of bacteria to produce an enzyme called catalase which is found in cells that live where there is air. If the Organism produces catalase enzyme it will break down the substrate of the catalase enzyme which converts hydrogen peroxide to water and oxygen. Used to differentiate Gram Positive Bacteria as a primary test.

Staphylococci is +ve & Streptococci & enterococci species are -ve

Steps:

1. Place a drop of hydrogen peroxide (3% H_2O_2) in a clean slide.
2. Mix a small amount of a bacterial culture with a drop of hydrogen peroxide on a slide.

Result:

Active bubbling . . . . . Positive catalase test

No bubbles . . . . . …Negative catalase test([8,9])

Figure (16): Negative Catalase (No bubbles) vs. Positive Catalase test (Bubbles)

2. Coagulase test

The coagulase test is used to identify *Staphylococcus* species, mainly to distinguish *Staphylococcus aureus* from coagulase-negative staphylococci (e.g., *Staphylococcus epidermidis*)Coagulase is an enzyme that clots plasma in a way similar to how thrombin converts fibrinogen to fibrin.

Types of Coagulase:

1. Cell-bound coagulase- clumping factor (detected by the **slide test**)
2. Free coagulase (detected by the **tube test**)

Slide Test for Bound Coagulase

1. Place distilled water on each end of a slide.
2. Emulsify a colony of the test organism in the drops to form thick suspensions. *Use nutrient or blood agar colonies, not mannitol salt agar.*
3. Add plasma to one suspension and mix. Observe for clumping within 10 seconds.
4. Use the second suspension (without plasma) as a control to differentiate granular appearance from true coagulase activity.

Results:

- Clumping in 10 seconds = **S. aureus** .
- No clumping = **no bound coagulase**.

Go to tube method to detect free coagulase.

Tube Test for Free Coagulase

1. Label three tubes: **T** (Test),

Pos (Positive control, S. aureus),

Neg (Negative control, sterile broth).

1. Add 0.2 ml plasma to each tube.
2. Add 0.8 ml test broth to **T**, 0.8 ml

S. aureus broth to **Pos**, and 0.8 ml sterile broth to **Neg**.

1. Mix gently, incubate at 35–37°C, and check

for clotting after 1 hour, 3 hours.

Results:

- **Clotting** = **S. aureus** (positive).
- **No clotting** = Negative test[8].

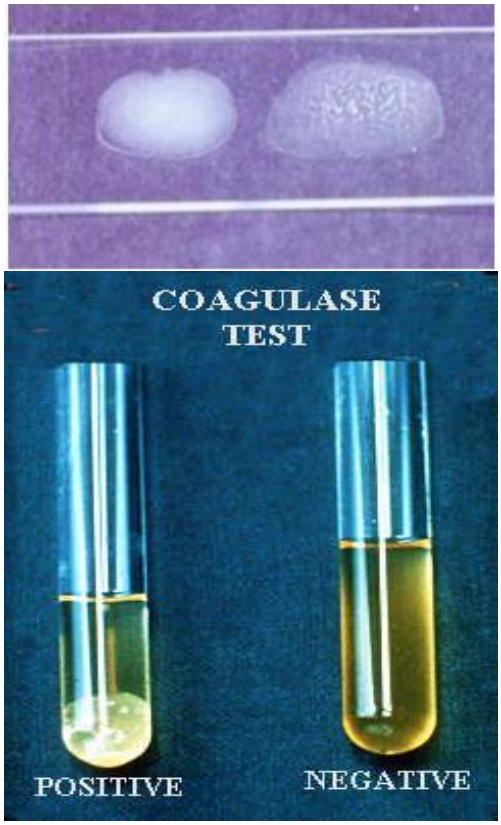


Figure (17): A .Slide method, B .Coagulase tube Method

3. DNAse test:

The identification of Staphylococcus aureus can be confirmed by the deoxyribose nuclease (DNase) test, as this organism is DNase-positive and the coagulase-negative staphylococci are DNase-negative.

The test relies on the fact that unhydrolyzed native deoxyribonucleic acid (DNA) is insoluble and precipitates in strong acid.

Agar containing DNA is 'stabbed' with staphylococci and incubated overnight at 37°C. If bacteria in the developing colony secrete the enzyme, DNA will be degraded into soluble nucleotides. After incubation, the plate is flooded with hydrochloric acid. A clear area around a colony, indicating hydrolysis of the DNA, identifies the organism as *Staphylococcus aureus.*

The test requires: DNA-ase agar plate (can test up to 6 organisms), &1 mol/l HCL acid.

Method:

1. Divide the DNA-ase plate into strips and label.
2. Inoculate the test and control organisms onto the plate.
3. Incubate at 35–37°C overnight.
4. Cover the plate with hydrochloric acid, then remove excess acid.
5. Observe for clearing around colonies within 5 minutes.

Results:

- **Clearing around growth** = DNA-ase positive strain.
- **No clearing around growth** = DNA-ase negative strain[9].

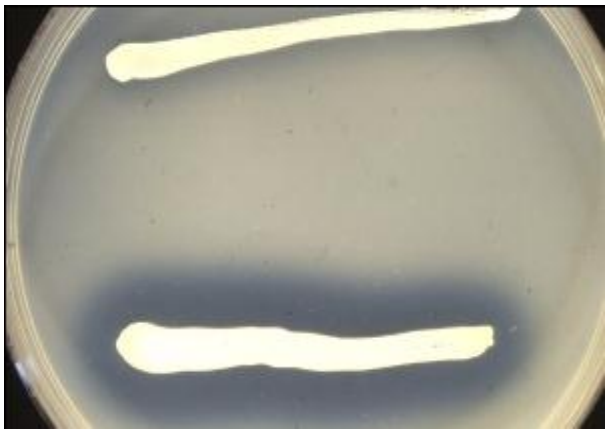


Figure (18): DNAse test

4. Hemolysis

Most species of streptococci are classified according to hemolytic patterns then genus and species: complete (β) hemolysis, incomplete (α) hemolysis, and no hemolysis (γ);

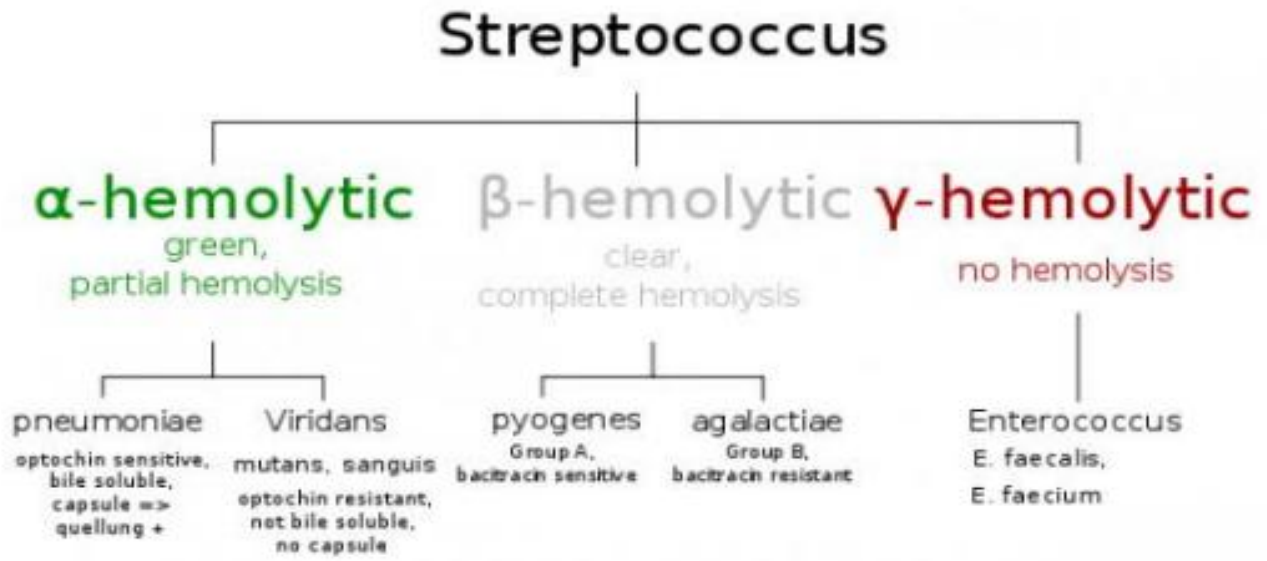


Figure (19): Hemolytic patterns

a. **α-Hemolysis**: Green pigment formed by the partial hemolysis on blood & Chocolate blood agar. (incomplete hemolysis and partial hemolysis. Alpha hemolysis is caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobin to green met-hemoglobin).
b. **β- Hemolysis:** Is a complete lysis of red blood cells in the media around and under the colonies; the area appears lightened (yellow) and transparent.
c. **γ-Hemolysis:** If an organism does not induce hemolysis, he agar under and around the colony is unchanged, and the organism is called non-hemolytic or said to display gamma hemolysis[8].

Figure (20) : α, β, & γ-Hemolysis

5. Litmus Milk Test

This test is a rapid inexpensive technique to assist in the identification of enterococci. It is based on the ability of most strains of Enterococcus species to reduce litmus milk by enzyme action as shown by decolorization of the litmus. Reduction of the litmus is indicated by a color change from mauve to white or pale yellow .

Requires: Litmus milk medium.

Steps: :

1. Inoculate 0.5 ml of sterile litmus milk with a heavy inoculum of the test organism (scrape three times across a heavily grown area).
2. Incubate at 35–37°C for up to 4 hours, checking every 30 minutes for a color change from mauve to white or pale yellow (compare to positive control).

Results:

White or pale yellow-pink = Suggestive of Enterococcus.

No change or pink = Likely not Enterococcus.

6. Bile esculin hydrolysis

The esculin hydrolysis test helps Differentiates enterococci and group D streptococci from other non-group D streptococci. Esculin hydrolysis in the presence of bile produces esculetin, which reacts with ferric ions to form a black precipitate.

Requires: Bile esculin agar (with bile salts, esculin, and ferric citrate).

Steps:

Inoculate the organism on bile esculin agar.

Incubate at 35–37°C for 24–48 hours.

Observe for color change.

Results:

Blackening of the medium …… +ve, esculin hydrolysis.

No blackening ……..-ve ,no esculin hydrolysis[9].

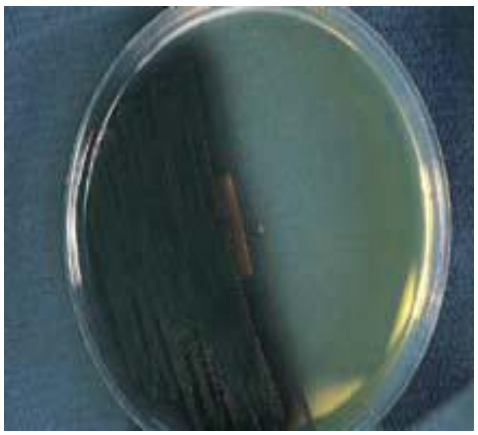


Figure (21): Bile esculin hydrolysis test

Question: What is Optichin, Bacitracin, & novobiocin sensitivity ?

What is PYR test?

Practical 11: Metabolism, Physiology, and Growth Characteristics of Gram- Negative Bacteria – Biochemical tests

Identification relies on biochemical tests detecting sugar metabolism (fermentation), Enzymes activity (urease), and metabolic byproducts production (indole). or MALDI-TOF technology,

Commercial Systems: API strips or enterotube test strip are used, where bacterial cultures are inoculated, incubated, and read to generate a code for organism identification.

1. Oxidase Test

Primary Biochemical test to Differentiates between oxidase-positive and oxidase-negative Gram-negative bacteria. Oxidase-positive bacteria contain cytochrome c, which oxidizes tetramethyl phenylenediamine (colorless) into a blue/purple compound.

Requires: Oxidase reagent (tetramethyl phenylenediamine),Filter paper or oxidase test strip.

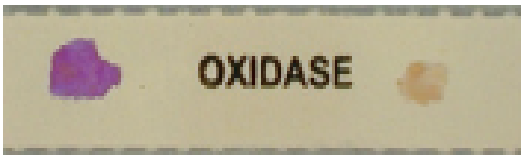
Steps:

1. Place a drop of oxidase reagent on filter paper or use an oxidase test strip.
2. Using a sterile applicator or loop, pick a colony of the test organism.
3. Smear the colony onto the reagent-soaked paper or test strip.
4. Observe for color change within 10-30 seconds.

Results:

Positive test: Blue or purple color within 10-30 seconds

Negative test: No color change (no cytochrome c present)[1]



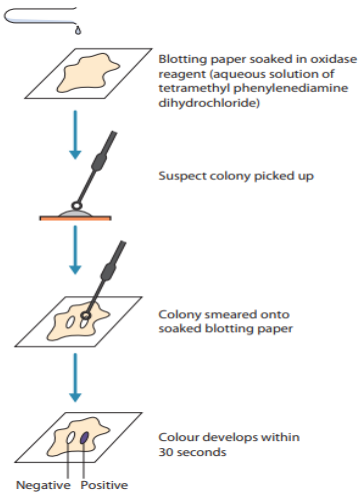


Figure (22): Oxidase test steps , Oxidase test +ve on the left side in the purple color, on the right side no change of color indicates –ve oxidase test

2. Indole test:

Indole production testing is essential for identifying enterobacteria, as most strains of *E. coli*, *P. vulgaris*, *P. rettgeri*, *M. morganii*, and *Providencia* species can degrade tryptophan to release indole ring.

The test organism is cultured in a medium containing tryptophan. Indole production is detected using Kovac's reagent (4-(p)-dimethylaminobenzaldehyde). This reagent reacts with indole to produce a red-colored compound.

Steps:

1. Inoculate 3 ml of sterile tryptone water with the test organism in a bijou bottle.
2. Incubate at 35–37°C for up to 48 hours.
3. Add 0.5 ml of Kovac's reagent, shake gently, and observe for a red color in the surface layer within 10 minutes.

Results:

Positive Indole Test: Red surface layer.

Negative Indole Test: No red surface layer[9].

Figure (23-b) A. +ve Indloe red ring,

B -ve Indole test

3. Urease test

The test organism is cultured in a medium containing urea and the pH indicator phenol red. If the organism produces urease, the enzyme hydrolyzes urea to ammonia and carbon dioxide, resulting in a color change to pink-red due to increased alkalinity. *Proteus* strains are strong urease producers, while *Y. enterocolitica* exhibits weak urease activity

Steps:

1. Inoculate 3 ml of sterile Christensen's modified urea broth/ slant heavily with the test organism.
2. Incubate at 35–37°C for 3–12 hours (preferably in a water bath).

Results:

Positive Test: Pink color.

Negative Test: No pink color[11].

Figure (23-b):+ve urease test organism and –ve urease test organism, non- inoculated Slant respectively from left to right

4. Citrate utilization test

The test is used to assess an organism's (Gram negative bacteria) ability to utilize citrate as its sole carbon source. The enzyme citrase hydrolyzes citrate into oxaloacetic acid and acetic acid, carbon dioxide (CO2) is produced, it reacts with the medium to form an alkaline compound, raising the pH, bromthymol blue changes color from green to blue, indicating a positive result.

Requires: Simmon's citrate agar in slant test tube containing citrate as the sole carbon source, & Bromthymol blue as the pH indicator.

Steps:

1. Inoculate the defined citrate medium with the test organism.
2. Incubate the tube at the appropriate temperature (usually 35–37°C) for 24-48 hours.

Results:

Positive Result: Blue color indicates the organism can utilize citrate.

Negative Result: Green color indicates the organism cannot utilize citrate[9].

Figure (24): left side non- inoculated Simmon's citrate slant, right side +ve Citrate test[11].

5. Kligler Iron agar

KIA is a differential medium used to identify enteric bacteria based on carbohydrate fermentation and hydrogen sulfide (H_2S) & gas production. It contains lactose, glucose, peptone, phenol red (pH indicator), and iron salts.

Steps:

1. Prepare and sterilize KIA medium in test tubes.
2. Inoculate with the bacterial isolate using a sterile loop.
3. Incubate at 35-37°C for 18-24 hours.
4. Observe for color changes and gas production.

Results:
Gas Production: Indicated by bubbles or lifting of the agar.
H_2S Production: Black precipitate in the butt (e.g., *Proteus*, *Salmonella*).
Lactose and Glucose fermentation are interpreted according to the below table (2)

Table (2):Kligler Iron agar Results

Slant	Butt	Gas	H_2S	Interpretation	Suspected Organism
Yellow(A)	Yellow(A)	+	-	Fermentation of G/L	*E. coli*
Red (K)	Yellow(A)	-	+	Fermentation of G	*Salmonella, Proteus*
Red (K)	Red (K)	-	-	No Fermentation	*Pseudomonas*
Red (K)	Yellow(A)	-	-	Fermentation of G only	Shigella

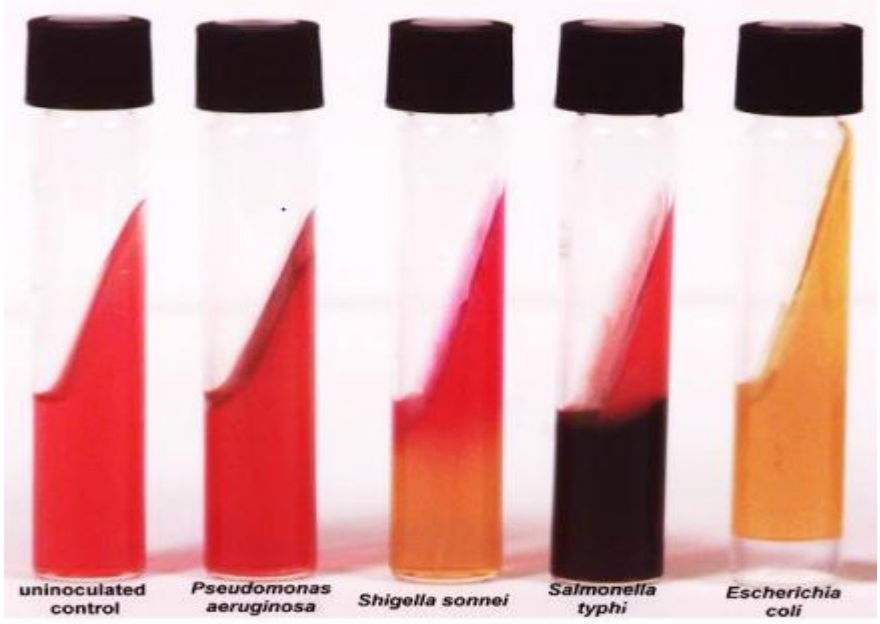


Figure (25): shows different results for Glucose, lactose fermentation, H2S, and Gas production

Methyl red (MR), Voges Proskauer test (VP)

The MR and VP test : tests for the ability of the microorganism to ferment glucose with the mixed acid products (Acidic) pathway or the alkaline byproduct acetyle methyl carbinol (acetoin) production (alkaline) pathway.

Requires: glucose phosphate (MR-VP) broth

Steps:

1. Inoculate 5 ml of glucose phosphate (MR-VP) broth with the test organism
2. Incubate at 37˚C for 48 hours.
3. First test tube: to the remaining 2.5 ml add 2-3 drops of Methyl red anf observe color change immediately, The culture suspension turns red instantly and remains stable if the test is positive or turns yellow if it is negative.
4. Second test tube: 2.5 ml of this suspension is transferred to another tube, 0.6 ml of alcoholic solution of α-naphthol (Barrit's solution A) is added and shaken. To this, 0.2 ml of 40% KOH (Barrit's solution B) is added, the tube is shaken and let to stand at room temperature for 15 minutes. Appearance of red color indicates positive test. If negative, the tube must be held for another 45 minutes[3].

Result:

- First test tube observe immediately: +ve MR test ---Red , -ve MR test ---Yellow
- Second test tube after 15 mins of 2 reagents addition: +ve VP test --- Red,

-ve VP test ---Yellow

Note: microorganism can only be positive to 1 test and negative to the other , both can never be positive.

Figure (26): Glucose Phosphate broth with MR added to the left tube, indication MR +ve test

Commercial Biochemical tests

API E20 – Analytical Profile Index

The API E20 strip contains 20 biochemical tests that assess various metabolic capabilities of the bacteria, such as fermentation of sugars, production of gas, and enzymatic activity.

Procedure steps:

1. Inoculation: A pure culture of the organism is prepared and inoculated into the API E20 strip wells.
2. Incubation: The strip is incubated for a specified time, typically 24-48 hours, at 30-37°C.
3. Reading Results: After incubation, each well is examined for color changes or other reactions, which indicate specific biochemical activities.

Results Interpretation

Each test produces a specific result (positive or negative), which is recorded as a profile.

The resulting profile is then compared to a database or identification software to determine the bacterial species.

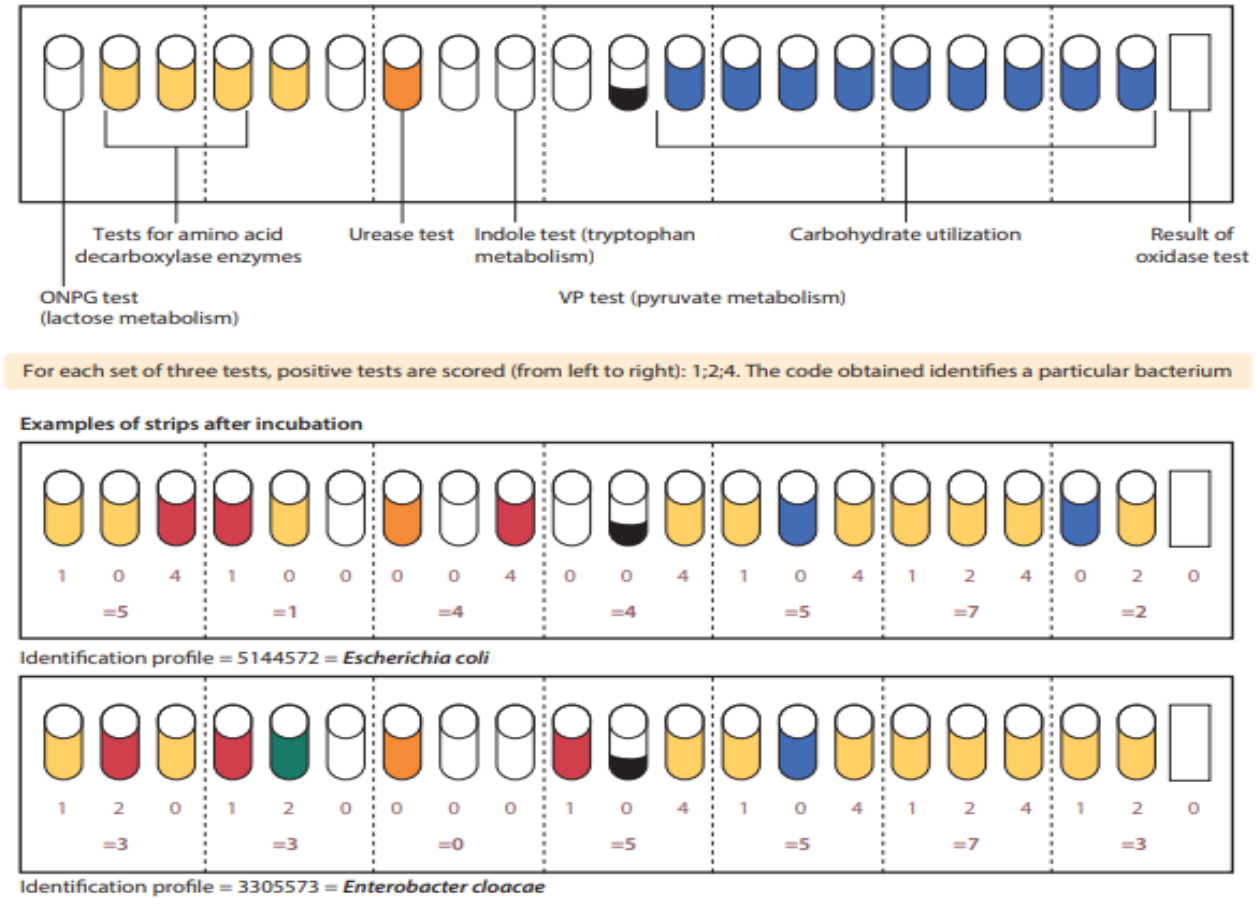


Figure (27): an example of a commercially available identification system for bacteria such as coliforms. [11]

Practical 12: Other diagnostic tests for microbial Identification – Serotyping and serology

Bacterial Serotyping

Serotyping (or serological typing) is a method to differentiate microorganisms of the same species based on variations in the antigenic determinants present on their cell surfaces.

Requires Antisera Types:

- **Polyvalent Antisera:** Contains antibodies for multiple serotypes (e.g., *E. coli* serotypes 026, 055, 086, etc.).
- **Monovalent Antisera:** Contains antibodies for a specific serotype (e.g., *E. coli* serotype 0111).

Principle:

Antigen-antibody complexes form when a bacterial culture is mixed with specific antiserum, leading to visible agglutination. This allows for the identification of O and H antigens through slide agglutination.

Start with polyvalent antisera, followed by specific monovalent antisera if needed.

Slide Agglutination Procedure

1. Place a small drop of antiserum on a glass slide.
2. Mix it with the bacterial culture (e.g., *Salmonella*).
3. Tilt the slide for 5-10 seconds.

Results:

- **Positive Reaction:** Visible agglutination.
- **Negative Reaction:** Homogeneous milky turbidity.

Figure (28): Slide agglutination serotyping[11].

Serology

Tube Agglutination procedure:

A stained, standardized, smooth suspensions of killed bacteria (brucella) prepared by manufacturer, are agglutinated when mixed with samples containing specific antibodies to Brucella.

1. Prepare serial dilution of sample/ reagent , Diluent

Tube number	1	2	3	4	5	6	7	8
Diluent ml	1.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Patient serum	0.1	1 ml serial dilutions						0
Final dilution	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Control

2. Mix and incubate at 37 C for 24 hour.
3. Examine for agglutination.

Interpretation of results:

1. Agglutinations are found in a high proportion of normal individuals and titers of less than 1 in 80 are of doubtful significance. A rising or falling titer is more singificant than a single high titer.
2. It is difficult to differentiate between species infection by serological tests, but from the point of view of treatment, this distinction is not necessary.
3. False positive reactions may occur with sera from patients infected with Pasteurella tularensis or vaccinated with Vibrio cholerae.

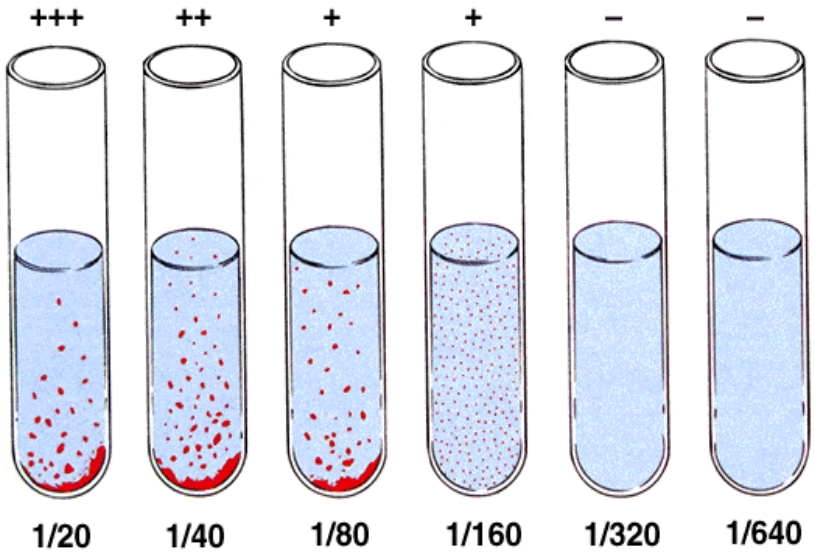


Figure (29) Standard tube agglutination test

Advanced Identification techniques

1. MALDI-TOF mass spectroscopy:

Identifies bacteria and yeasts by analyzing their protein profiles. A single colony sample mixed with a matrix (e.g., sinapinnic acid) is bombarded with a UV laser, ionizing the matrix and the organism's proteins. These ionized proteins are accelerated through a vacuum, and their time of flight (proportional to mass) is measured by a detector. The resulting protein profile is compared with a database to identify the organism.

Requirements:

Sample of the organism (colony from a culture plate), Matrix substance (e.g., sinapinnic acid), MALDI-TOF instrument, UV laser, Protein profile database

Steps:

1. Mix a sample of the organism with a matrix substance.
2. Bombard the mixture with a UV laser to ionize the matrix and proteins.
3. Accelerate the ionized proteins through a vacuum and measure their time of flight.
4. Compare the protein profile with a reference database for identification.

Results:

Qualitative and quantitative protein profile: Dominated by ribosomal proteins.

Comparison with database: Identifies the organism, providing species-level identification within minutes[11].

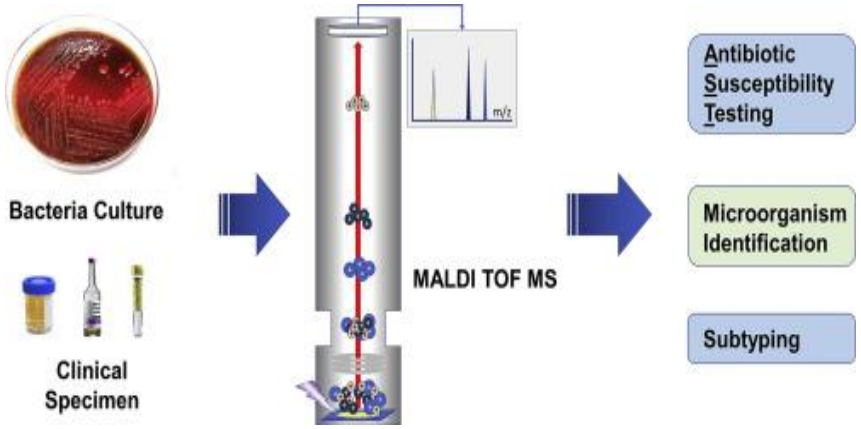


Figure (30) MALDI-TOF instrument

Practical 13:Antimicrobial susceptibility tests (AST)

Antibiotic susceptibility testing is crucial for determining the effectiveness of antibiotics against bacteria. While tube MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) tests are accurate, they are cumbersome for routine high-volume testing. Instead, laboratories commonly use disc susceptibility testing, automated systems, or E. TEST methods.

Methods:

1. Disc Diffusion Test:

Cellulose discs impregnated with antibiotics are placed on agar plates inoculated with the test organism.

As bacteria grow, the antibiotic diffuses into the agar, creating a zone of inhibition proportional to the organism's susceptibility. Results are reported as 'resistant' or 'sensitive,' with 'intermediate' sometimes used.

Zone Sizes: Determined by breakpoints based on MIC values that differentiate susceptible and resistant populations[9, 11].

A. Kirby-Bauer Disc Diffusion (NCCLS Modified):

- Standardized method using specific disc content, inoculum size (Mcfarland Standard), and Mueller-Hinton agar.
- Results are interpreted after incubation at 35°C for 16–18 hours.
- Stokes Disc Diffusion: Compares the zone sizes of test organisms directly with control organisms on the same plate.

Steps:

a. Preparation of Inoculum:

Select a pure bacterial colony and suspend it in sterile saline or broth.

Adjust the turbidity of the suspension to match the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL).

b. Inoculation of Agar Plate:

Use Mueller-Hinton agar (a standardized medium).

Dip a sterile swab into the prepared bacterial suspension.

Evenly streak the entire surface of the agar plate in three directions to ensure a uniform bacterial lawn[9].

c. Placement of Antibiotic Discs:

Allow the inoculum to absorb into the agar for about 5 minutes.

Place antibiotic-impregnated paper discs onto the surface of the inoculated agar using sterile forceps or a disc dispenser.

Ensure that the discs are spaced adequately to avoid overlapping zones of inhibition.

d. Incubation:

Incubate the plates at 35°C for 16–18 hours in an ambient air incubator (not CO_2 unless specifically required for certain organisms).

e. Measurement of Zones of Inhibition:

After incubation, measure the diameter of the clear zone of inhibition around each disc in millimeters using a ruler or caliper.

Compare the zone sizes to the Clinical and Laboratory Standards Institute (CLSI) or National Committee for Clinical Laboratory Standards (NCCLS) guidelines to interpret susceptibility (susceptible, intermediate, or resistant).

f. Result Interpretation:

Correlate the inhibition zone diameters with MIC values using CLSI/NCCLS tables.

Based on the results, categorize the bacterial strain as **susceptible**, **intermediate**, or **resistant** to each antibiotic tested.

g. Report Findings:

Provide the final susceptibility report to guide appropriate antimicrobial therapy.

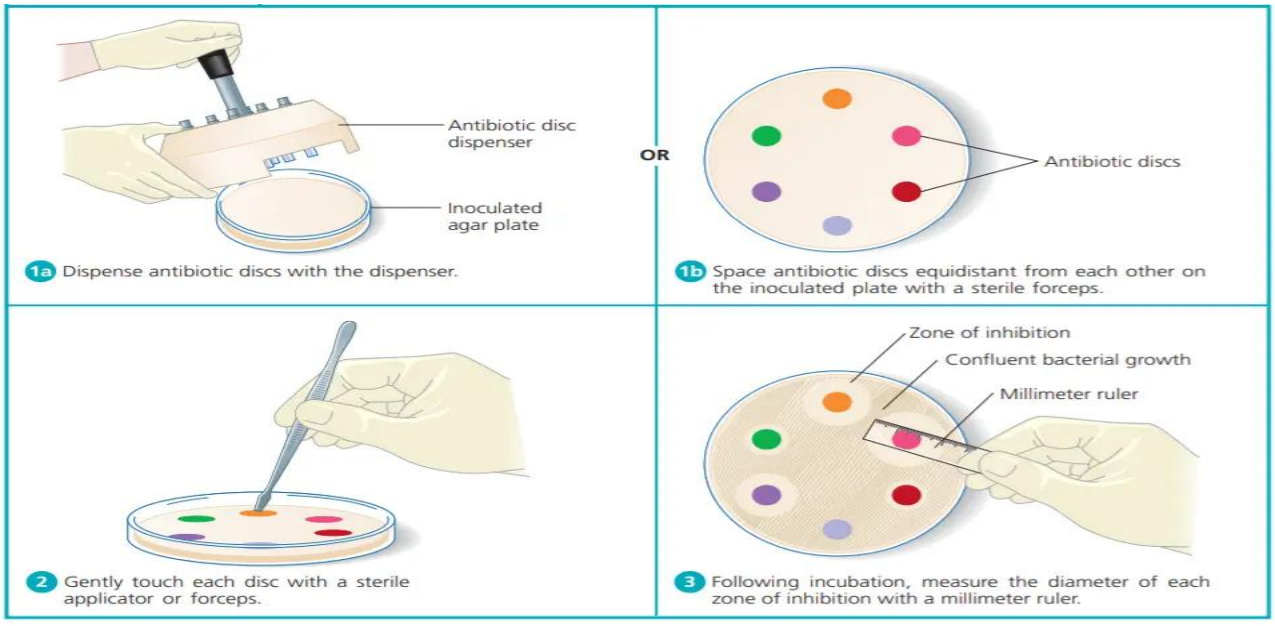


Figure (31): Kirby-Bauer Disc Diffusion method:

2. Automated Testing (e.g.,Vitek):

Procedure: A cassette with varying antibiotic concentrations is inoculated with a diluted organism suspension. The machine monitors growth electronically by measuring turbidity.

Interpretation: The growth rate inversely correlates with antibiotic susceptibility, and an MIC value can also be obtained.

3. E TEST:

Procedure: A strip with an exponential gradient of an antibiotic is placed on an inoculated agar plate. The antibiotic diffuses as the organism grows, creating a zone of inhibition.

Interpretation: The MIC is determined from the scale on the strip. While more expensive and labor-intensive, it provides precise MIC values for specific organisms[9,11].

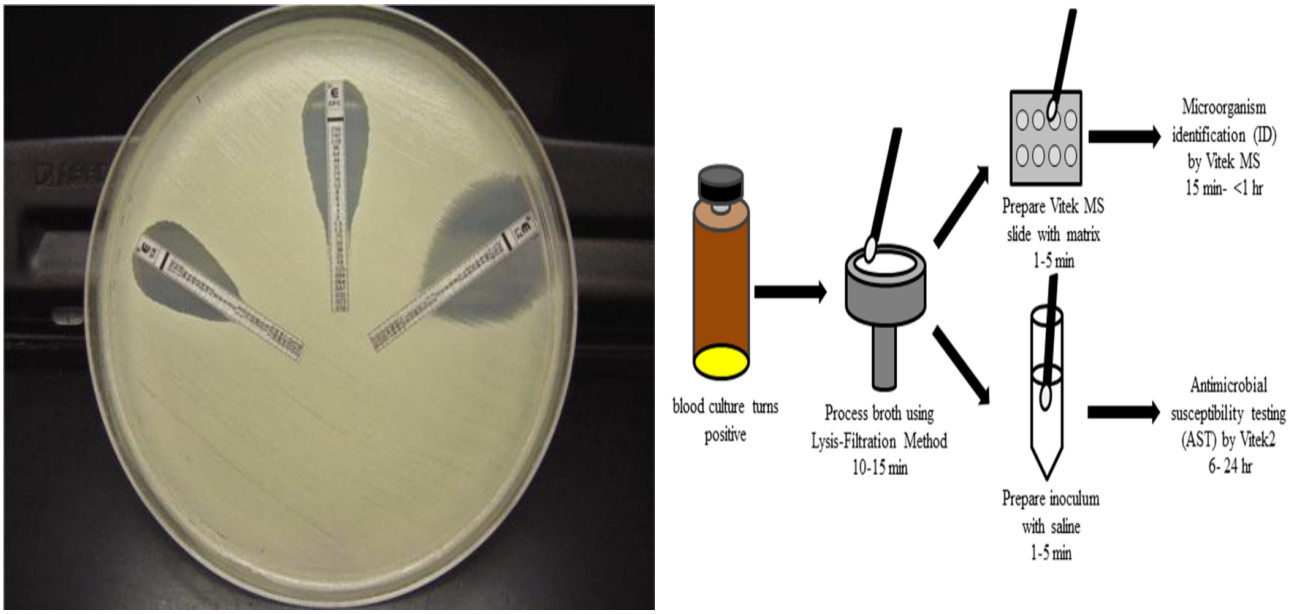


Figure (32): E test on the left, steps of Vitek on the right

Check the following link for the complete procedure:

https://www.youtube.com/watch?v=Il9vcYZlVs8 &
https://www.youtube.com/watch?v=NO3C5TBo1KY

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