Laboratory Session 2

Bacteriological Examination

Preparing bacterial culture media is the basis for work in a microbiology laboratory. The core principle is to create an environment that mimics the natural habitat of the bacteria as closely as possible. The preparation process is divided into four main stages:

1. **Weighing:** Dry powders (extracts, peptones, salts) are weighed in specific amounts and dissolved in the required volume of distilled water (as specified on the label).
2. **Dissolving/Boiling:** The mixture is heated until completely dissolved (especially for agar-based media).
3. **Sterilization:** The most critical stage. Usually performed in an **autoclave** (121°C for 15-20 minutes) to destroy all foreign microorganisms and spores.
4. **Pouring:** The sterile medium is poured into Petri dishes or test tubes under sterile conditions.

Types of Culture Media

Based on consistency, media are classified as **solid, liquid, or semi-solid**. The primary difference is the concentration of **Agar-agar** (a solidifying agent derived from seaweed).

Characteristic	**Liquid Medium (Broth)**	**Semi-Solid Medium**	**Solid Medium (Agar)**
Agar Content	0%	0.2% to 0.5%	1.5% - 2%
Visual Appearance	Clear or turbid liquid	Jelly-like, soft mass	Firm jelly-like mass
Primary Purpose	Rapid accumulation of bacterial mass in large quantities.	Studying bacterial motility; cultivation of microaerophilic bacteria; storage of microbial cultures.	Isolation of pure cultures and the study of individual colonies.
Bacterial Growth Pattern	Bacteria predominantly grow throughout the volume (the liquid becomes turbid).	**Non-motile bacteria:** grow along the stab line; **Motile bacteria:** spread from the stab line into the depth of the medium.	Bacteria form isolated dots (**colonies**) on the surface.

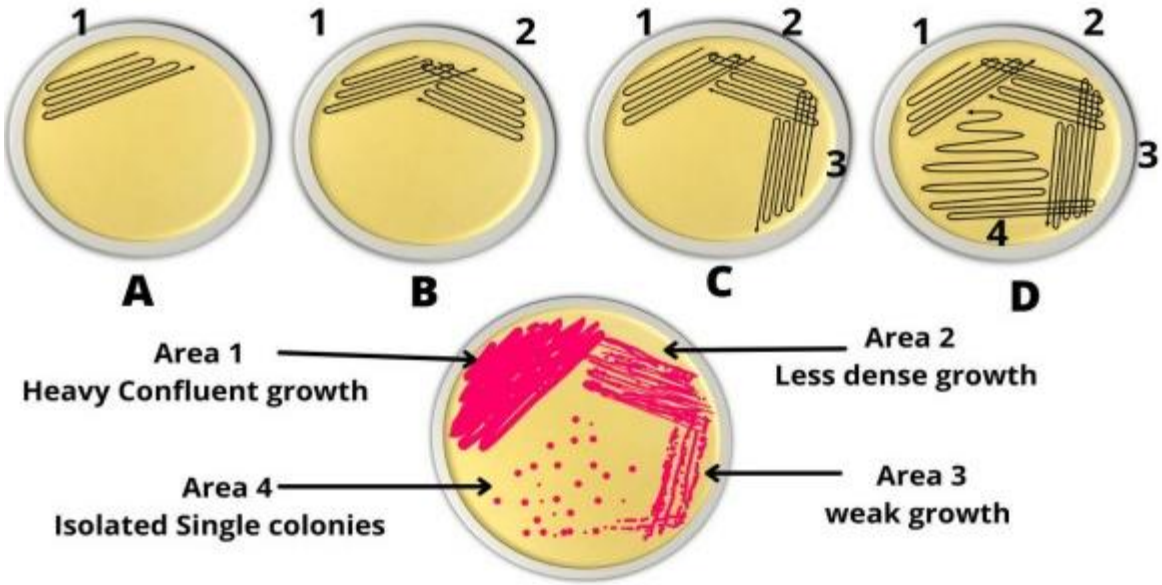
2. Inoculation Techniques

Timeline (By Day)

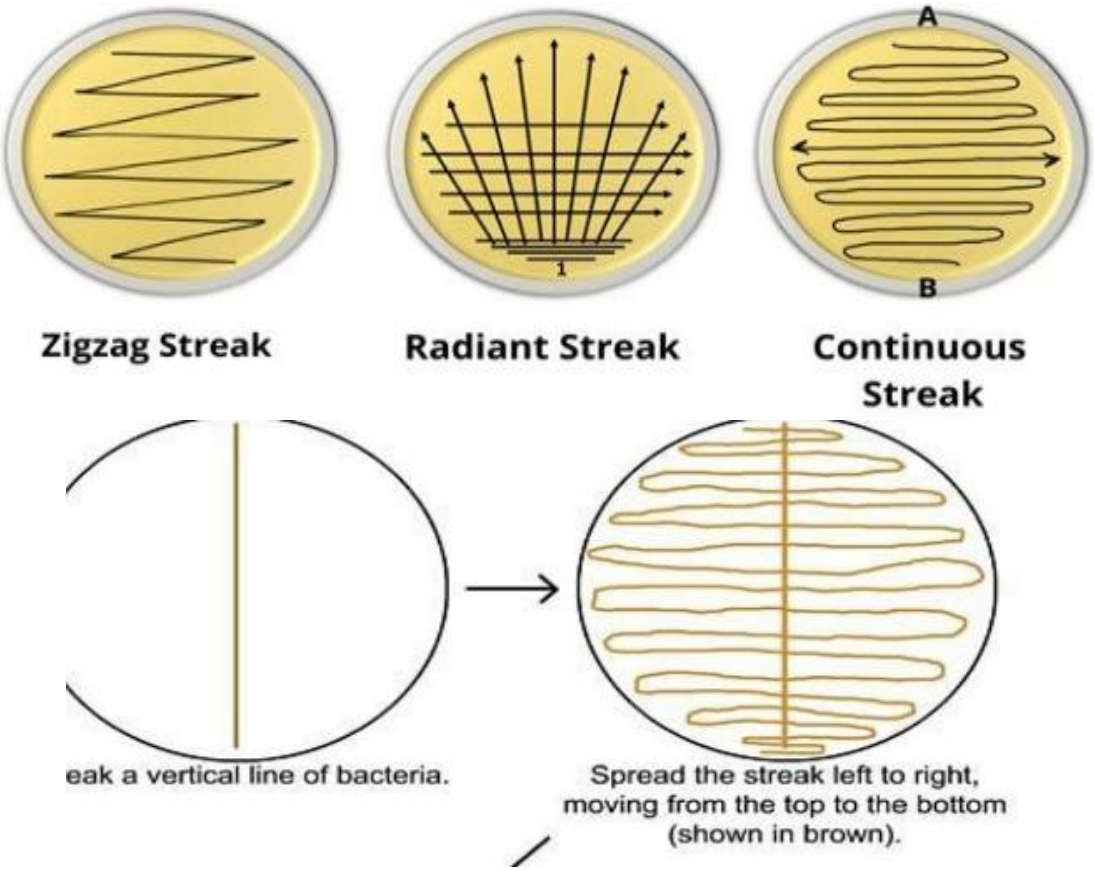
Day I: Inoculation (Streaking) The specimen is inoculated onto Petri dishes using a bacteriological loop. In microbiology, the primary goal of inoculation is to separate individual bacterial cells from a sample so they can grow into distinct, **isolated colonies**.

Methods: Sector streaking (Picture 1), zigzag, continuous, or vertical line streaking (Picture 2).

Picture 1



Picture 2



Day II: Identification
After **18–24 hours of incubation** in a thermostat at **37°C**, colonies from the solid media are used for:
Gram Staining: To observe morphology and cell wall properties.
Biochemical Testing: Conducted to identify the microbe (e.g., **API Systems** for identifying Enterobacteriaceae and other Gram-negative rods, as well as Oxidase and Catalase tests).
Day III: Antibiotic Susceptibility Testing. Kirby-Bauer Disk Diffusion Method.
The Kirby-Bauer Disk Diffusion Method is widely used to determine bacterial sensitivity to various antimicrobial compounds. This test is performed on Mueller-Hinton Agar.
Note: Mueller-Hinton agar is neither selective nor differential; it is a standardized medium that supports the growth of most organisms.

Procedural Tips for Success:

Sterilization: Always flame the loop until it is red-hot before and after every use.

Cooling: Ensure the loop is cool before touching the bacterial culture to avoid killing the sample.

Plate Orientation: Always incubate Petri dishes **upside down** (lid-side down) to prevent condensation from dripping onto the agar surface and ruining the colonies.

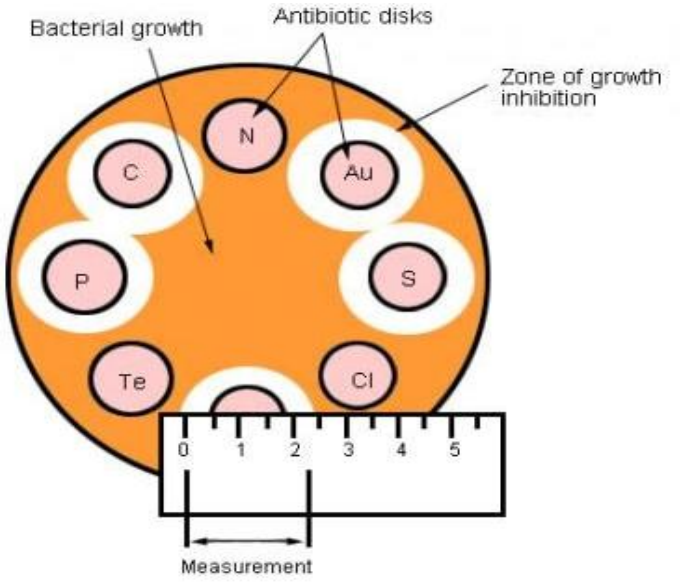
Step I – Preparation of Bacterial Suspension (Inoculum Preparation)

Add bacterial colonies to 2 ml of saline solution. Compare the resulting suspension to the **0.5 McFarland standard** (which (corresponds to one million bacteria). If the suspension is more turbid (cloudy) than the 0.5 McFarland standard, saline should be added to the inoculum tube incrementally. This is crucial because if an excessive amount of bacteria is introduced, the results will be unreliable.

Step II – Inoculation
Apply the bacterial suspension onto the **Mueller-Hinton agar** using a sterile cotton swab to ensure even coverage.
Step III – Disk Placement
1. Using forceps, carefully remove one antibiotic disk from the cartridge.
2. Partially lift the lid of the Petri dish. Place the disk on the agar and press it gently with the forceps to ensure **complete contact** with the surface. **Do not move the disk** once it has touched the agar surface, even if it is not in the ideal position. This is because some drugs begin to diffuse immediately upon contact with the agar.
3. Continue placing each disk onto the agar surface. Once all disks are in place, **invert the plates** and place them in a 35°C air incubator for 16–18 hours.

The results are read according to the standards provided by the **European Committee on Antimicrobial Susceptibility Testing (EUCAST)**.

Procedure: Using a ruler, measure the **diameter of the zone of inhibition** around each antibiotic disk and compare the results to the EUCAST standards.



1. ESBL (Extended-Spectrum Beta-Lactamase) Detection

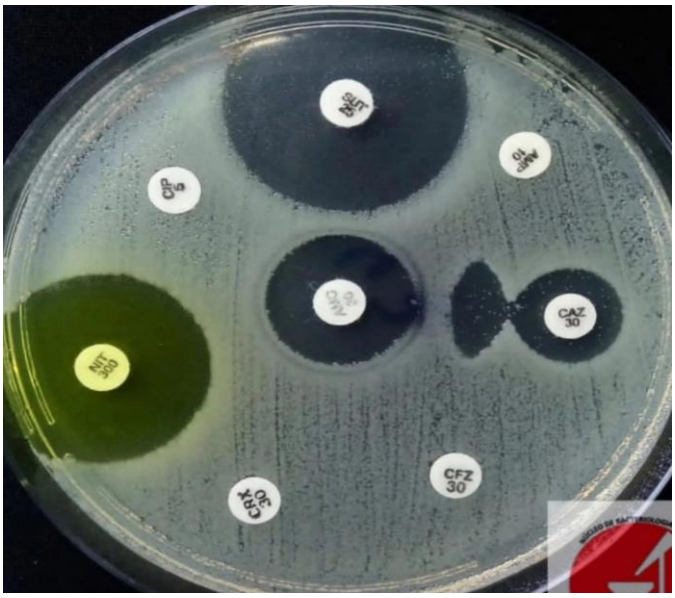
Extended-Spectrum Beta-Lactamase (ESBL) detection using the **Double Disk Synergy Test (DDST)** is a simple and reliable test used to determine whether a bacterium produces the ESBL enzyme. Third-generation cephalosporins are known as extended-spectrum beta-lactam antibiotics, while the enzymes that break down the beta-lactam ring of these third-generation antibiotics are called Extended-Spectrum Beta-Lactamases (ESBLs). The most common producers of ESBLs are *Klebsiella*, *Proteus*, and *E. coli*.

The activity of these enzymes is inhibited by **clavulanic acid** (β-lactamase inhibitor).

The Principle:

If the bacterium produces ESBL, the presence of clavulanic acid will cause an expansion of the cephalosporin disk's zone of inhibition toward the **Amoxicillin-Clavulanic Acid (AMC)** disk. In other words, the clavulanic acid suppresses the ESBL enzyme as it diffuses toward the 3rd generation

cephalosporin, restoring the antibiotic's activity and creating a classic zone of synergy (often called the "keyhole effect").



On the Petri dish, an Amoxicillin + Clavulanic acid (AMC) impregnated disk is placed in the center, while third-generation cephalosporins (Cefotaxime, Cefixime, Ceftazidime, Ceftriaxone) and a monobactam (Aztreonam) are placed around it.
The results are read after 18–24 hours of incubation at 35–37°C.

Comparison of Results

Observation	**Interpretation**
If the zone of inhibition around the cephalosporin disk is wider towards the **AMC** disk (expanding like a "wing").	**ESBL Positive** — The bacterium produces extended-spectrum beta-lactamase.
If the cephalosporin zone is symmetrical, distinct, and does not touch the other zones, with no expansion towards the **AMC** disk.	**ESBL Negative** — The bacterium does not produce ESBL.

Figure 1

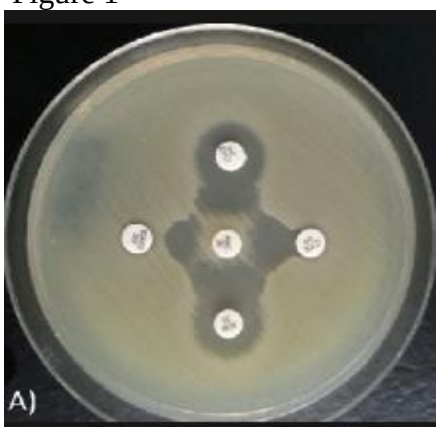


Figure 2

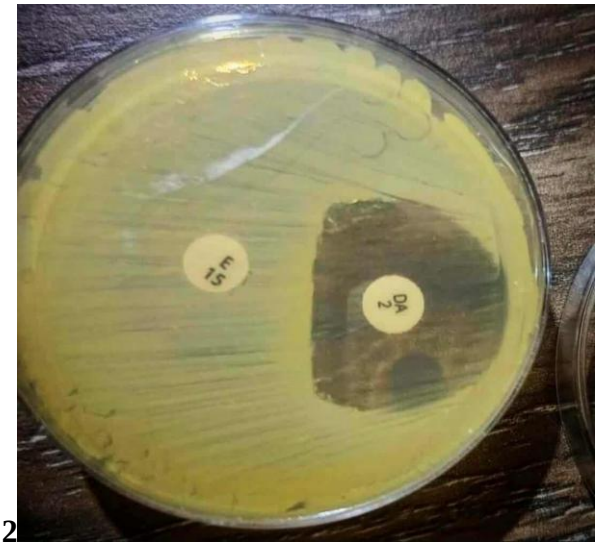
In **Figure 1**, the inhibition zones of all surrounding disks (left, right, top, and bottom) are expanded toward the **AMC** (Amoxicillin-Clavulanic acid) disk. This means a synergy effect, known as the in **"keyhole effect,"** is visible with all of them. This indicates a positive result: **ESBL (+)**.

In **Figure 2**, the zones are symmetrical, and there is no expansion toward the AMC disk. This means that the zone of inhibition around the cephalosporin disk spreads rapidly and evenly in all directions, showing no expansion toward the Amoxicillin-Clavulanic acid disk—as a result, the test is **ESBL negative**.

2. Induced Clindamycin Resistance Test - D-Test

Bacteria may possess either **constitutive resistance**, where the bacterium is resistant to the antibiotic from the start, or **induced resistance**. In the latter case, the bacterium initially appears sensitive to the antibiotic, but after a certain stimulus (often the action of another antibiotic), it "activates" genes that lead to Clindamycin resistance.

The zone around the **Clindamycin (DA-2)** disk should normally be circular; however, in this case (see image below), it takes on a **"D" shape**. This phenomenon is called a **positive D-test result (D-test positive)**.

2 

The reason is the presence of the **erm (Erythromycin Ribosome Methylase)** gene, which determines resistance. **Erythromycin (E-15 disk)** stimulates the *erm* gene, which subsequently alters the structure of the ribosome so that the antibiotic can no longer bind to it.
In the body, Clindamycin may initially appear effective, but the bacterium eventually figures out how to "protect itself." After a certain period (several days or weeks), the bacterium recognizes the threat and activates the dormant *erm* gene. This gene modifies the ribosome so that Clindamycin can no longer bind, leading to treatment failure.
This is precisely why the **D-test** is performed - to determine whether the bacterium possesses this hidden (inducible) resistance. If the D-test is positive, Clindamycin should **not** be prescribed, as it will only work temporarily before the treatment fails.
Interpretation of D-test Results:

- If the zones around both disks are uniform: The D-test is negative, the *erm* gene is absent, and Clindamycin can be prescribed.
- If the inhibition zone around Clindamycin is "blunted" toward Erythromycin, taking a "D" shape: This means the bacterium possesses the *erm* gene, indicating inducible resistance. In this case, Clindamycin must not be prescribed.

Similar **inducible or ribosomal methylation mehanisms** are not limited to macrolides; other examples include:

Antibiotic Group	**Genes**	**Mechanism**	**Induction**
Macrolides / Lincosamides / Streptogramins (MLSB)	*erm*	23S rRNA Methylation	Induced by Erythromycin
Tetracyclines	*tet(M), tet(O)*	Ribosomal Protection	Induced by Tetracycline
Aminoglycosides	*aac, aph*	Enzymatic Inactivation	Sometimes Inducible

In the laboratory, the **D-test**, **tet-test**, and other inducibility tests are used to detect these complex resistance mechanisms.

3. Methicillin-Resistant Staphylococcus Aureus (MRSA) Screening

Screening for MRSA in the nasal cavity is one of the most common procedures for preventing hospital-acquired (nosocomial) infections and surgical site infections in orthopedic surgery.

Nasal MRSA Screening (SOP)

Objective and Scope: To detect MRSA colonization in a patient's nasal cavity before or during hospitalization. This screening is essential for epidemiological control and the prevention of post-operative wound infections.

Required Materials and Equipment:

Sterile swab (wood-cotton).
Sterile saline solution (if the swab is dry).
Culture media: Mannitol Salt Agar (MSA).

Procedural Stages:

A) Sample Collection

Moisten the sterile swab in sterile saline.
Collection: Insert the moistened swab into one nostril (approximately 1–2 cm deep).
Rotate the swab 5 times against the inner wall of the nostril using a gentle circular motion.
Repeat the procedure in the second nostril using the **same** swab.

B) Laboratory Analysis:

Inoculation: Transfer the sample onto Mannitol Salt Agar.
Incubation: Place the plate in an incubator at 35–37°C for 18–24 hours.
Identification:
Mannitol Salt Agar changes color from red/pink to yellow in the presence of *Staphylococcus aureus*, as the bacteria ferment mannitol and produce acid.

For full identification, traditional methods are used: **Identification of *S. aureus*** (Catalase test, Coagulase test).

C) Determination of Resistance To confirm MRSA, an antibiogram is performed:

Disk Diffusion Method: (Considered the best predictor for the presence of the ***mecA*** gene). Introduce the bacterial suspension onto Mueller-Hinton agar and place a Cefoxitin disk. After 18-20 hours, if the zone of inhibition is **≥ 22 mm**, the result is negative for MRSA.

Result	**Interpretation**
No growth (or zone ≥ 22 mm)	MRSA colonization is not confirmed.
Positive (MRSA) (zone < 22 mm)	The patient is a carrier of MRSA. Isolation and a decolonization protocol are required.