

Antibiotic Technology Laboratory

Lab 2: Antibiotic Sensitivity Test

Diffusion Test: Antibiotic sensitivity test: is a laboratory method for determining the susceptibility of organisms to therapy with antibiotics, Antibiotic susceptibility testing is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. Aim is to measure susceptibility of an isolate to range of antibiotics, also, is vital for guiding effective antibiotic therapy and supporting antimicrobial stewardship. It identifies whether bacterial isolates are Susceptible (S), Intermediate (I), or Resistant (R) to specific antibiotics. Accurate AST is crucial for patient care and controlling resistance.

Purpose of AST:

- 1- Select the most effective antibiotic
- 2- Avoid antibiotic resistance
- 3- Guide clinicians for targeted therapy
- 4- Monitor emerging drug resistance

Antibiotic diffuses out of a disk placed on the surface of the agar. If bacteria are sensitive to the antibiotic, then a zone of growth inhibition forms around the disk after incubation. The zone size depends on several factors and two methods are available to control this process, comparative disk testing (where both a test and control organism are tested on the same plate), and standardized disk testing.

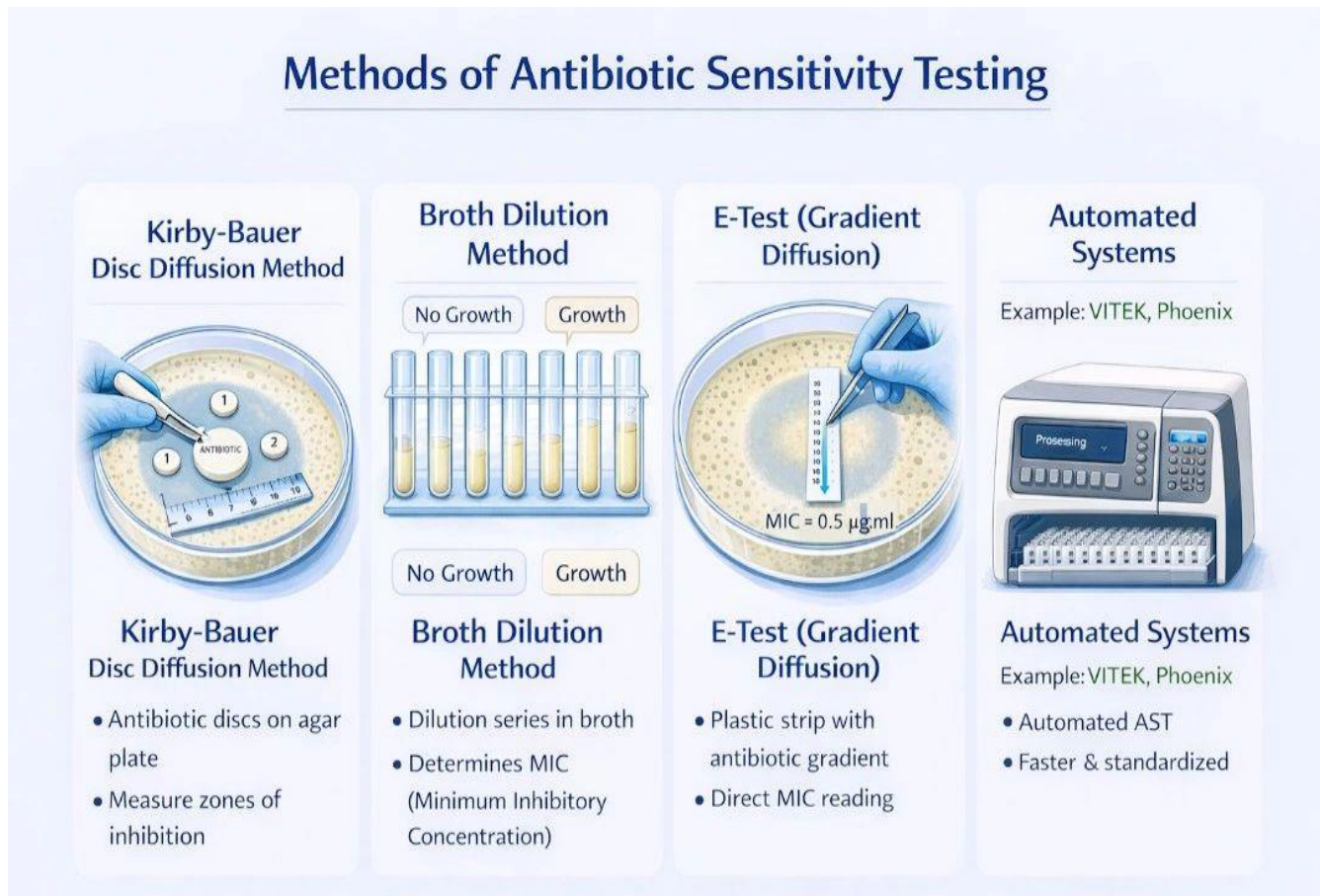
Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include:

- 1- Kirby–Bauer Disc Diffusion Method.
- 2- Broth Dilution Method: Minimum Inhibitory Concentration (MIC)

3- Epsilometer or E-Test (Gradient Diffusion)

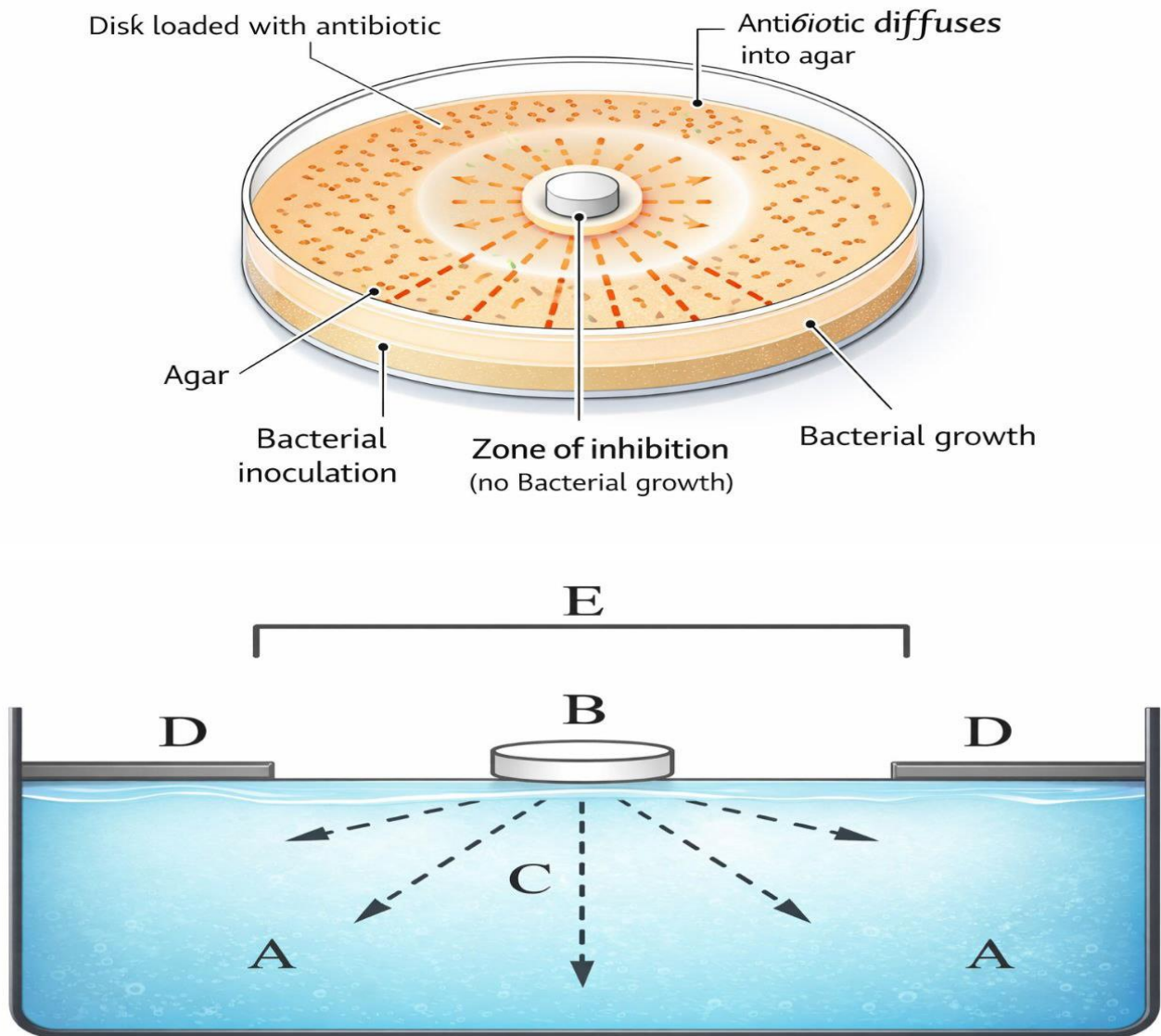
4- Automated Systems



The theory of zone formation:

Disk sensitivity tests are performed on agar plates. A small disk of filter paper, loaded with a defined quantity of antibiotic, is placed on the surface of an agar plate that has already been inoculated with a suspension of bacteria. The antibiotic diffuses out of the disk into the agar, along a concentration gradient, as the plates are incubated (for 16–18 h). If the bacterial strain is sensitive to the antibiotic, then a **zone of inhibition** (no growth) occurs around the disk

The Theory of Zone Formation



Disk sensitivity test. A – agar; B – antibiotic disc; C – antibiotic diffuses into agar along concentration gradient; D – bacterial growth on surface of agar after 18 hours of incubation; E – zone (diameter) of inhibition

The diameter of the zones of inhibition is measured in mm, and the organism Reported as sensitive or resistant based on defined cut-off points (for example <18 mm=resistant). One disadvantage of disk testing is that it is usually only possible to have a maximum of six different antibiotic disks on a standard agar plate

The diameter of the zone depends on a number of factors including

- 1- the quantity of antibiotic within the disk
- 2- the degree of susceptibility of the bacteria to the antibiotic
- 3- The physicochemical properties of the antibiotic
- 4- The depth (in mm) of the agar plate
- 5- The concentration of bacteria in the inoculum (semiconfluent growth is required)

The relationship between concentration of antibiotic and diameter of inhibition zone is logarithmic relationship and the shape of curve to be a straight line.



Factors that affecting in the results of the sensitivity test:

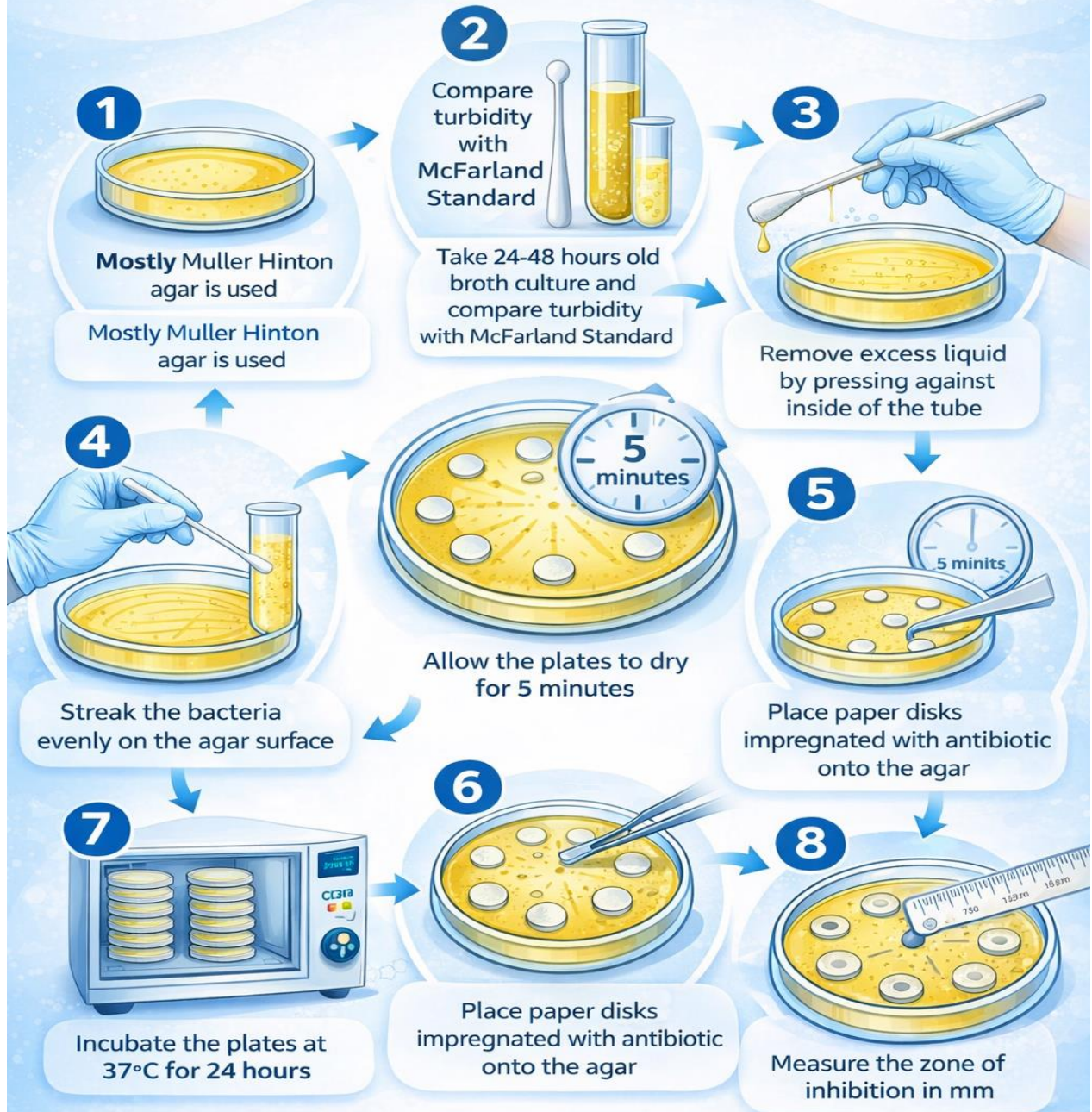
- 1- The pH of each batch of Mueller-Hinton agar should be checked when the medium is prepared. The agar medium should have a pH between 7.2 and 7.4 at room temperature, If the pH is too low, certain drugs will appear to lose potency (e.g., aminoglycosides, quinolones, and macrolides), while other agents may appear to have excessive activity (e.g., tetracyclines). If the pH is too high, the opposite effects can be expected.

- 2- Moisture: Just before use, excess surface moisture is present, the plates should be placed in an incubator (35°C) or a laminar flow hood at room temperature with lids ajar until excess surface moisture is lost by evaporation (usually 10 to 30 minutes). The surface should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the Petri dish covers when the plates are inoculated.
- 3- Inoculum density: Usually optimal results were obtained with an inoculum size that produces by compare McFarland standard tube.
- 4- Timing of disc application: If the plates, after being seeded with the test strain, are left at room temperature for periods longer than the standard time, multiplication of the inoculum may take place before the discs are applied. This causes a reduction in the zone diameter and may result in a susceptible strain being reported as resistant.
- 5- Temperature of incubation: Susceptibility tests are normally incubated at 35 °C for optimal growth. If the temperature is lowered, the time required for effective growth is extended and larger zones result. At higher temperatures, the entire culture appears to be susceptible.
- 6- Incubation Time: Most techniques adopt an incubation period of between 16 and 18 hours
- 7- Spacing of the antibiotic discs: If larger numbers of antibiotics have to be tested, two plates, or one 1 cm diameter plate, is to be preferred.

Procedure:

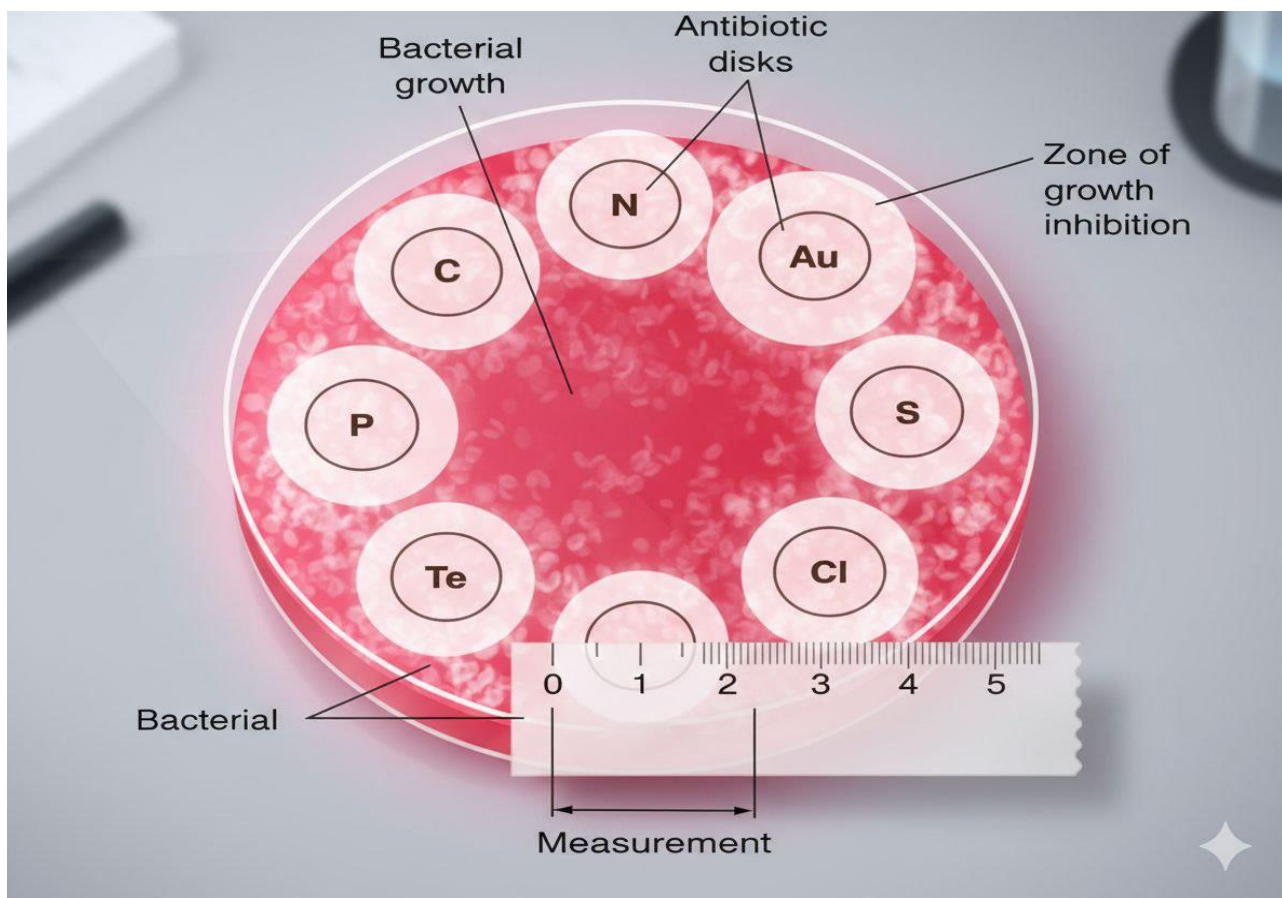
1. Mostly Muller Hinton agar is used in this antibiotic susceptibility test.
2. Take 24-48 hours old broth (Liquid) culture of bacteria to be tested and then do the suspension of inoculum bacteria and compared with McFarland standard.
Turbidity standard for inoculum preparation: to standardize the inoculum density for a susceptibility test, a BaSO₄ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used.
3. Place a sterile cotton swab in the bacterial suspension and remove the excess fluid by pressing and rotating the cotton against the inside of the tube above the fluid level.
4. The swab is streaked in three directions over the surface of the Mueller-Hinton agar to obtain uniform growth. A final sweep was made around the rim of the agar.
5. Allow the plates to dry for five minutes.
6. Using sterile forceps or a suitable disc dispenser, place paper disks impregnated with a fixed concentration of an antibiotic, on the surface agar plates at equal distance.
7. Incubate the plates at 37°C for 24 hours.
8. Following overnight incubation, measure the diameter of the zone of inhibition in millimeter (mm) around each disk.

Antibiotic Susceptibility Test



How to Measure Zone Diameters Correctly:

1. Use a calibrated ruler (in millimeters).
2. Measure the entire diameter, including both sides of the clear zone around the disk.
3. Measure from the back of the plate with reflected light for best visibility.
4. If the zone is irregular, measure the shortest diameter.
5. Record in mm, never convert or approximate.

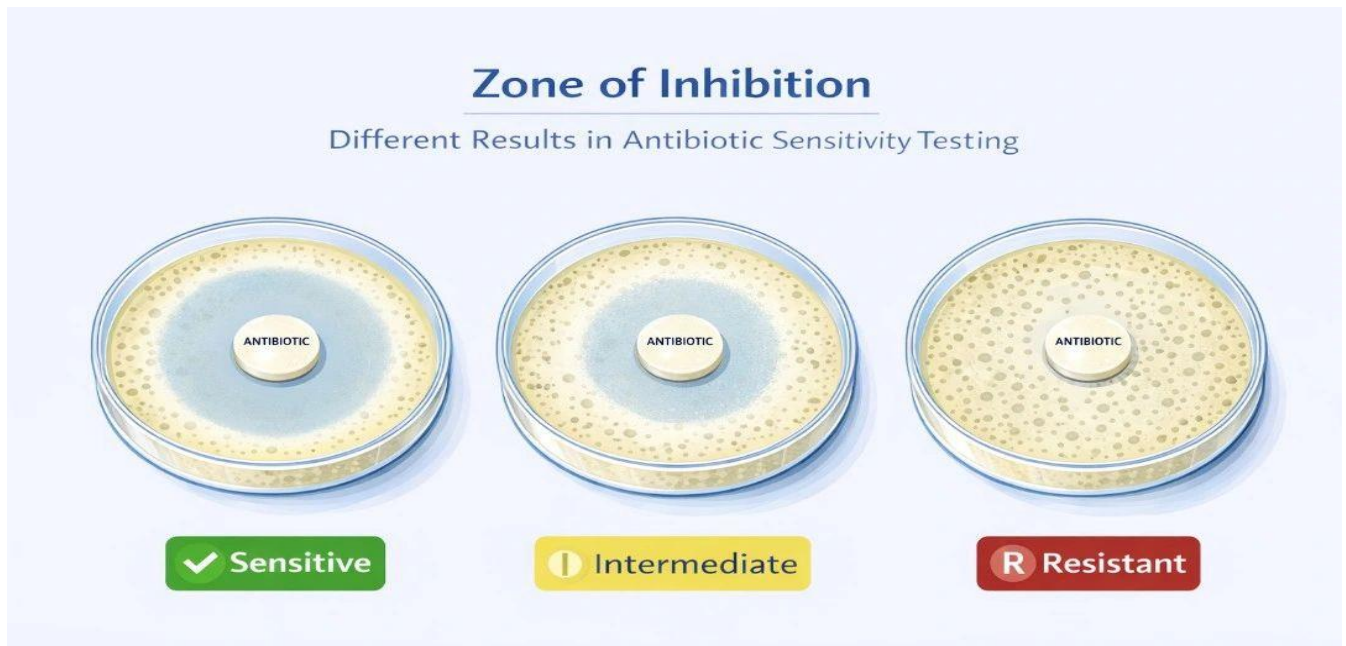


What the Zone Tells You

- Large zone → Bacteria are susceptible
- Small or no zone → Bacteria are resistant

Compare your measurements with CLSI/EUCAST breakpoints to report:

S (Susceptible), I (Intermediate), R (Resistant)



What are McFarland Standards?

They are turbidity reference standards used to visually match the cloudiness of a bacterial suspension to a known cell concentration—ensuring consistent, reproducible inoculum density across labs worldwide.

Quality Control (QC) in Antibiotic Susceptibility Testing (AST)

QC is a cornerstone and essential to ensure accuracy, reproducibility, and reliability of AST results and refers to a set of standardized procedures used to verify that:

1. Culture media are suitable for bacterial growth
2. Antibiotic discs are potent and active
3. Inoculum density and incubation conditions are correct.
4. Incubation at $35 \pm 2^\circ\text{C}$ for organism-specific duration
5. ATCC reference strains

The testing system performs according to international standards

QC is not a **routine formality**, but a **critical measure** against misleading results.

Why is Quality Control Important?

Small error → Incorrect zone → Wrong interpretation → Incorrect antibiotic choice for the patient.

Explain Causes of Absent Growth in this plate:

Causes of Absent Growth or Abnormally

Zones in QC Tests, Failure of bacterial

growth may occur due to:

Weak or incorrect inoculum not matching 0.5 McFarland.

Non-viable bacteria due to old cultures, improper transport, heat exposure, disinfectants, or use of MHA for fastidious organisms.

Expired or improperly prepared culture media.

Incorrect pH or poor storage of the medium.

Improper incubation temperature, time, or atmospheric conditions.

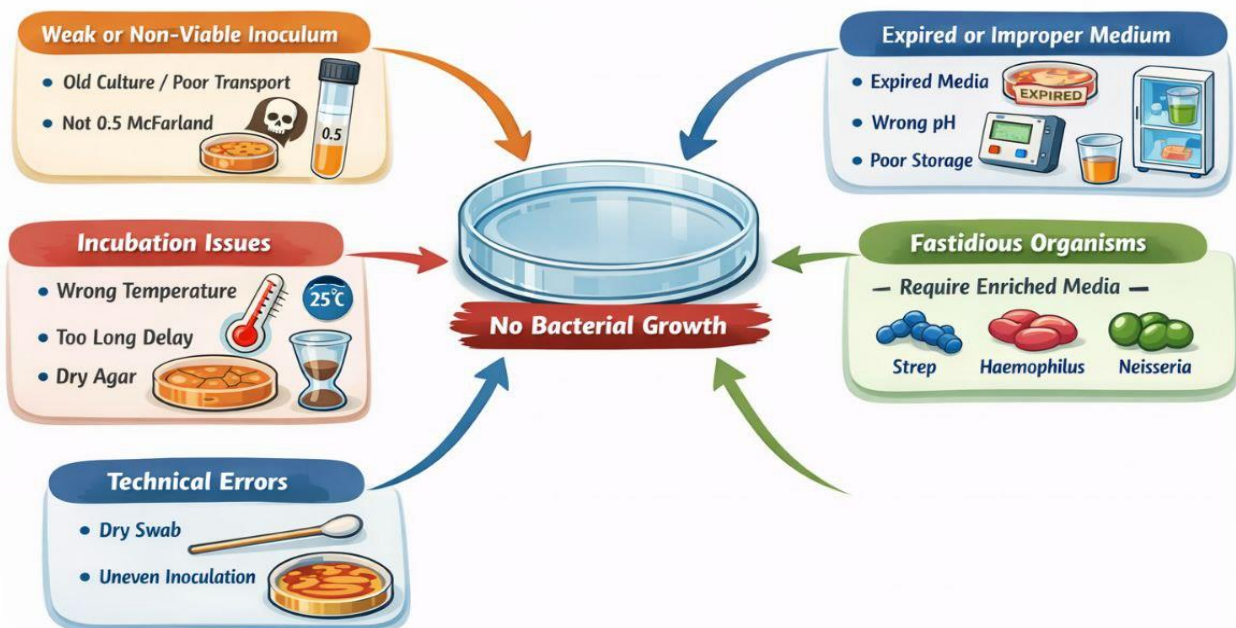
Delay before incubation causing agar surface drying.

Use of Mueller–Hinton agar for fastidious organisms.



Why No Growth?

Possible Causes of Absence of Bacterial Growth



Note: Media Selection and Its Impact on AST

Not all bacteria grow on the same media.

- Using fungal media (e.g., Sabouraud dextrose agar) will not support bacterial growth.
- Using MacConkey or CLED agar for AST may allow growth, but acidic conditions from lactose fermentation can reduce the activity of certain antibiotics.
- Using fastidious organisms and can not growth on MHA but growth on specific media like chocolate agar media.

Therefore, media selection must strictly follow CLSI/EUCAST guidelines.