Antimicrobial susceptibility testing (AST) using diffusion method

Purpose

To determine the antimicrobial susceptibility of bacteria by using disk and gradient test

Materials

1. Overnight culture of bacterial isolate on non-selective media (Tryptic soy agar (TSA), Sheep blood agar (SBA))
2. Sterile cotton swab
3. Sterile 0.85 % saline solution
4. Mueller-Hinton Agar (MHA)
5. **Mueller-Hinton Agar with 5% Sheep blood (MHSA) for Streptococci**
6. Antimicrobial agents (disks and MIC test strips)
7. Nephelometer
8. 0.5 McFarland standard
9. Sterile forceps
10. Vortex mixer
11. 35°C ±2°C ambient-air incubator
12. **35°C ±2°C 5% CO_2 incubator for Streptococci**

Procedure

1. Remove the MHA/MHSA plates from the refrigerator at least 15 mins before use to let them warm to room temperature. If agar surfaces contain excess moisture (e.g., large condensation droplets), place MHA/MHSA plates in an incubator (35°C ±2°C) or a laminar flow hood at room temperature with lids ajar until excess surface moisture is removed by evaporation (usually 10–30 mins).
2. Remove the sealed packages containing disk/MIC test strip cartridges from the refrigerator or freezer 1–2 hrs before use so they may equilibrate to room temperature before opening.
3. Make a direct MHB broth of isolated colonies selected from an 18 to 24-hrs agar plate.
4. Adjust the turbidity of culture with MHB broth to achieve a turbidity equivalent to a 0.5 McFarland standard. **Use the inoculum within 15 mins.**
5. Dip a sterile cotton swab into the 0.5 McFarland adjusted suspension.
6. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level.
7. Inoculate the dried surface of MHA/MHSA plate by streaking the swab over the entire sterile agar surface.
8. Repeat this procedure by streaking 2 more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum.
9. Swab the rim of the agar.
10. Leave the lid ajar for (ideally) 3–5 mins, but no more than 15 mins.
11. Dispense the antimicrobial disks/MIC test strips onto the surface of the inoculated agar plate.
12. Press each disk/MIC test strip down to ensure complete contact with the agar surface.

13. Invert the plates and place them in an incubator set to 35°C±2°C within 15 mins after the disks/MIC test strips are applied.
    Note: a) Do not incubate the plates in an atmosphere with increased CO_2
    b) **Streptococci on MHSA incubates in an atmosphere with 5% CO_2**
14. Incubate plate for optimal time for each organism.
15. Measure the zone diameter (mm.) of the antibiotic disk. For gradient test, the point of intersection of an ellipse (zone of inhibition) at the strip on the plate determines the MIC value.

Interpretation of results

Disk diffusion

- Each plate should be examined after incubating for 16 to 18 hrs. If the plate was satisfactorily streaked and the inoculum concentration correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, instead of a confluent lawn of growth, the inoculum concentration was too light and the test must be repeated.
- The diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk, must be measured to the nearest whole millimeter using sliding calipers or a calibrated ruler held on the back of the inverted Petri plate that is held a few inches above a black, nonreflecting background illuminated with reflected light. Exceptions include:
    - If MHSA was used (for streptococci), the zones must be measured from the upper surface of the agar illuminated with reflected light and with the cover removed.
    - For coagulase-negative *Staphylococcus* spp. with cefoxitin, 24 hrs of incubation are needed before reporting as susceptible. Other agents should be read and reported at 16 to 18 hrs. If cefoxitin is tested against *Staphylococcus* spp., the zone diameters need to be read with reflected, not transmitted light (plate held up to the light).
    - If testing vancomycin against *Enterococcus* spp., 24 hrs of incubation are needed before reporting as susceptible. Other agents should be read and reported at 16 to 18 hrs. For vancomycin against *Enterococcus* spp., the zone diameters need to be read with transmitted light.
    - If linezolid is tested against *Staphylococcus* spp., the zone diameters need to be read with transmitted light.
- The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth should be ignored. Other situations to consider include:
    - When discrete colonies grow within a clear zone of inhibition, the test should be repeated with a pure culture or subculture of a single colony from the primary culture plate. If discrete colonies continue to grow within the zone of inhibition, the colony-free inner zone should be measured.

- When blood-supplemented medium for testing streptococci is used, the zone of growth inhibition, not the zone of inhibition of hemolysis, should be measured.
- For trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, slight growth (20% or less of the lawn of growth) should be disregarded, and the more obvious margin measured to determine the zone diameter.

- The zone of inhibition sizes should be interpreted by referring to M100 using the current breakpoints, and the organisms should be reported as susceptible, susceptible-dose dependent, intermediate, or resistant to the agents that have been tested.

Gradient test

- After the required incubation period, and only when an even lawn of growth is distinctly visible, read the MIC value where the pointed end of the inhibition ellipse intersects the side of the strip. Do not read the plate if the culture appears mixed or if the lawn of growth is too light or too heavy; repeat the test.
- MIC endpoints are usually clear-cut although different growth/inhibition patterns may be seen.

References

CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 13th ed. CLSI supplement M02. Clinical and Laboratory Standards Institute; 2018.

CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. CLSI supplement M100. Clinical and Laboratory Standards Institute; 2022.

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