Disk Diffusion Susceptibility Testing

I. Principle and Clinical Significance:

In vitro susceptibility testing for rapidly growing non-fastidious aerobic bacteria may be determined by the agar disk diffusion method described by Bauer er al. in 1966. This simple but strictly controlled technique yields a qualitative report that is based on the relationship of MIC breakpoints to therapeutically achievable levels of drug in blood in systemic infections or levels of drug concentrated in urine for urine-only antimicrobial agents. Testing with a selected battery of antimicrobial agents will assist the physician in choosing an appropriate drug for therapy.
A standard inoculum of the organism is swabbed onto the surface of a Mueller-Hinton agar plate. Filter paper disks impregnated with antimicrobial agents are placed on the agar. After overnight incubation, the diameter of the zone of inhibition around each disk is measured. The size of the zone is inversely proportional to the MIC of the organisms. By referring to NCCLS recommendations for interpretation, a qualitative report of susceptible, intermediate, or resistant can be obtained.

II. Specimen:

A. Four or five isolated colonies of similar colony morphology.
B. Isolates from frozen, lyophilized, or other stock conditions require two subcultures prior to testing.

III. Materials:

A. Media and reagents (storage conditions)
   1. Mueller-Hinton agar plates (2 to 8º C)
   2. Antimicrobial disks (-20 to 8º C)
   3. TSB broth (2 to 8º C)
   4. Prompt® inoculators (RT)
B. Supplies
   1. Sterile cotton-tipped swabs
   2. McFarland 0.5 turbidity standard
C. Equipment
   1. Ruler
   2. 34 to 35º C ambient air incubator
      CO2 incubation is necessary for some organisms.
   3. Multi-disk disk-dispensing apparatus

IV. Quality Control:

A. QC strains
   1. Organisms
      a. *Escherichia coli* ATCC 25922
      b. *Staphylococcus aureus* ATCC 25923
      c. *Pseudomonas aeruginosa* ATCC 27853
      d. *E. coli* ATCC 35218
      e. *Enterococcus faecalis* ATCC 29212
      f. *Haemophilus influenzae* ATCC 49247
      g. *Streptococcus pneumoniae* ATCC 49619
   2. Maintenance of QC strains
      a. Maintain permanent stock cultures at –70º C in sheep blood for up to 3 years.
      b. A new stock of each organism is pulled from the -70°C monthly.
      c. Maintain working stock cultures on SBA.

B. Frequency of QC testing
   1. Perform QC weekly.

2. Perform QC testing prior to or concurrent with testing of patient isolates each time a new lot or new shipment of materials is put into use.
3. Each new antibiotic added to a panel must be tested for 30 consecutive days with no more than 3 results out of range, before testing can be done weekly.

V. Procedure:

A. Bring agar plates and disks to room temperature before use.
   1. Using a loop or swab, pick 4 or 5 similar colonies from the primary plate.
   2. Inoculate into a TSB tube and adjust the turbidity with sterile 0.85% NaCl or broth to equal that of a 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL).
   3. As an alternative to the above procedure, use a commercial Prompt volumetric wand-type device for inoculum preparation. Pick 4 or 5 similar colonies on the wand tip and then reinsert back into the Prompt. Mix well.
   4. Dip a sterile cotton swab into the inoculum (TSB or Prompt) and rotate it against the wall of the tube to remove excess inoculum.
   5. Swab the entire surface of the MH plate three times, rotating the plate approximately 60º between streaking to ensure even distribution.
   6. Allow inoculated plates to stand for at least 3 min but no longer than 15 min before applying disks.
   7. Apply the disks using the disk dispenser.
   8. Apply gentle pressure if necessary with sterile forceps to each disk to ensure complete contact with the agar.
   9. Once a disk is on the media, do not relocate it because antimicrobial diffusion begins instantly.
   10. Invert the plates and incubate for 16 to 18 hr or up to 24 hr at 34 to 35º C in an ambient-air incubator. *Streptococcus pneumoniae* and *Haemophilus influenzae* require increased CO_2 for growth.

B. Reading plates
   1. Read plates only if the lawn of growth is confluent or nearly confluent.
   2. Illuminate plate with reflected light directed from above at a 45º angle.
   3. Measure the diameter of inhibition zone to the nearest whole millimeter by holding the measuring device against the back of the plates.
   4. For staphylococci and penicillinase-resistant penicillins, use transmitted light and examine closely. Consider any growth as evidence of resistance.
   5. For staphylococci exhibiting penicillin-susceptibility based on disk diffusion, transfer a loopful of organism growing closest to the oxacillin disk to a cefinase disk to test for beta-lactamase production. Observe the cefinase disk for up to 1 hour or until it become positive, whichever occurs first. Beta-lactamase positive isolates must be reported as penicillin-resistant.
   6. Opaque media requires light directed from above at a 45º angle.

C. Reporting
   1. If QC is acceptable, report categoric result as susceptible, intermediate, or resistant according to laboratory policy and NCCLS guidelines.
   2. Report oxacillin resistant staphylococci as resistant to all beta-lactam drugs (including beta-lactam-B-lactamase inhibitor combinations, all cephalosporins, all penicillins, and imipenem) regardless of in vitro susceptibility testing.
   3. Refer to Susceptibility Pattern reporting table.

VI. Reference:

CLSI. January 2006, Performance Standards for Antimicrobial Disk Susceptibility Tests, 9th ed. Approved standard M2-A9. NCCLS, Wayne, PA.

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