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Why antibiotic susceptibility testing?

- Guide physicians in the selection of most appropriate agent
- Particularly important in the era of ATM resistance
- Can direct therapy towards the most narrow-spectrum & least expensive agents
The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism.
What is susceptibility to antibiotics?

- **Susceptible**: The microorganism is inhibited by a concentration of antimicrobial agent that can be attained in blood with the normally recommended dose of the ATM agent.

- **Resistant**: The microorganism is resistant to concentrations of the ATM agent that can be attained with normal doses.
Interpretation

The main concept is the “clinical categorisation”

- Strains are sorted according to level of MIC versus reference breakpoints
- c and C are the minor and major breakpoints

Sensitive       Intermediate       Resistant

\[ \text{MIC} < c < \text{MIC} < C < \text{MIC} \]
Definitions (cont’d)

- **Susceptible “S”**
  - Indicates an organism is inhibited by the recommended dose, at the infection site.

- **Intermediate “I”**
  - Represents an organism that may require a higher dose of antibiotic for a longer period of time to be inhibited.

- **Resistant “R”**
  - Indicates an organism is not inhibited by the recommended dose, at the infection site, of an antimicrobial agent.
Susceptibility testing methods

- **Solid media**
  - Disc diffusion
  - Agar dilution (MIC)
  - Gradient diffusion (E-test) - MIC

- **Liquid media (MIC)**
  -Macrobroth dilution
  - Microbroth dilution

- **Automated instrument methods**
Antibiotic Sensitivity Tests

- Diffusion
  - Kirby-Bauer Method
  - Stokes Method

- Dilution
  - Tube Dilution
  - Agar Dilution

- Diffusion & Dilution
  - E-Test

Qualitative Methods
Quantitative Methods
Is one method superior to the other?

- Misconception that MIC is superior!!!
- Selection based on:
  - Ease of performance
  - Cost
  - Selection of a system that can do both ID & susceptibility testing
Where is MIC indicated?

- Isolates from endocarditis/osteomyelitis patients
- Oxacillin & vancomycin for Staphylococcus
- Vancomycin for Enterococci
- Penicillin, cephalosporins & meropenem & vanco for S. pneumoniae
- Certain fastidious microorganisms
- Certain organisms with varying growth rates
Disk diffusion

- Antibiotic-impregnated discs placed on an agar plate
- Resulting zones of inhibition measured
- Vary among drugs depending on size of molecule & hydrophilicity
- Susceptibility is inferred (standard tables)
Selecting antimicrobial agents for testing & reporting

- Clinical Laboratory Standards Institute (CLSI document-M100)
  - Develop standards, methods, QC parameters, and interpretive criteria for sensitivity testing
  - If necessary, can alter the breakpoints of the SIR based on emerging resistance
  - Each laboratory should have a battery of ATMs ordinarily tested
  - Consider Hospital drug formulary

- Other reference guidelines
  - BSAC
  - EUCAST
Selection of antimicrobials

- Generally, labs choose 10-15 antibiotics to test susceptibility for GP organisms and another 10-15 for GN organisms.
- Too many choices can confuse physicians and be too expensive.
- Primary objective:
  - Use the least toxic, most cost-effective, and most clinically appropriate agents.
  - Refrain from more costly, broader-spectrum agents.
Table 2A. Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae

<table>
<thead>
<tr>
<th>Testing Conditions</th>
<th>Minimal QC Recommendations (See Table 3 for acceptable QC ranges.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium: Mueller-Hinton agar</td>
<td><em>Escherichia coli</em> ATCC® 25922</td>
</tr>
<tr>
<td>Inoculum: Growth method or direct colony suspension</td>
<td><em>Escherichia coli</em> ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations)</td>
</tr>
<tr>
<td>Incubation: 35 °C; ambient air; 16 to 18 hours</td>
<td></td>
</tr>
</tbody>
</table>

General Comments

(1) For *fecal* isolates of *Salmonella* and *Shigella* spp. only ampicillin, a quinolone, and trimethoprim-sulfamethoxazole should be tested and reported routinely. In addition, chloramphenicol and a third-generation cephalosporin should be tested and reported for extraintestinal isolates of *Salmonella* spp.

NOTE: Information in boldface type is considered tentative for one year.

<table>
<thead>
<tr>
<th>Test/Report Group</th>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter, Nearest Whole mm</th>
<th>Equivalent MIC Breakpoints (µg/mL)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>I</td>
<td>S</td>
</tr>
<tr>
<td>PENICILLINS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Ampicillin</td>
<td>10 µg</td>
<td>≤ 13</td>
<td>14-16</td>
<td>≥ 17</td>
</tr>
<tr>
<td>B</td>
<td>Mezlocillin or</td>
<td>75 µg</td>
<td>≤ 17</td>
<td>18-20</td>
<td>≥ 21</td>
</tr>
<tr>
<td>B</td>
<td>piperacillin</td>
<td>100 µg</td>
<td>≤ 17</td>
<td>18-20</td>
<td>≥ 21</td>
</tr>
<tr>
<td>B</td>
<td>Ticarcillin</td>
<td>75 µg</td>
<td>≤ 14</td>
<td>15-19</td>
<td>≥ 20</td>
</tr>
<tr>
<td>U</td>
<td>Carbenicillin</td>
<td>100 µg</td>
<td>≤ 19</td>
<td>20-22</td>
<td>≥ 23</td>
</tr>
<tr>
<td>U</td>
<td>Mecillinam</td>
<td>10 µg</td>
<td>≤ 11</td>
<td>12-14</td>
<td>≥ 15</td>
</tr>
</tbody>
</table>

β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS

| B                 | Amoxicillin-clavulanic acid or ampicillin-sulbactam | 20/10 µg | ≤ 13 | 14-17 | ≥ 18 | ≥ 32/16 | ≤ 8/4 |
| B                 | Piperacillin-tazobactam | 10/10 µg | ≤ 11 | 12-14 | ≥ 15 | ≥ 32/16 | ≤ 8/4 |
| B                 | Ticarcillin-clavulanic acid | 100/10 µg | ≤ 17 | 18-20 | ≥ 21 | ≥ 128/4 | ≤ 16/4 |
| B                 |                        | 75/10 µg | ≤ 14 | 15-19 | ≥ 20 | ≥ 128/2 | ≤ 16/2 |
Standardization of Inoculum

- **Inoculum Preparation**
  - Use 4-5 colonies of well-isolated, 18-24 hour old organism
  - Transfer organism to a broth
  - Either tryptic soy/sterile saline

Growth method

Direct colony suspension method

Inoculum Standardization using 0.5 McFarland std
Inoculate MH agar by swabbing in three different directions + the rim “Lawn of growth” within 15 minutes after adjusting the turbidity.

Place filter paper disks impregnated with antimicrobial agents on the agar within 15 mnts after plating.

Should not be closer than 24 mm from center to center.

No longer than 15 mnts after applying the disks.
Reading of results

- Invert and incubate for 16-18 hours at 35°C in non-CO₂
- 24hrs for staph for oxacillin & enterococci for vancomycin
- During incubation, drug diffuses into agar
- Depending on the organism and drug, areas of no growth form a **zone of inhibition**
- Zones are measured – zone margin is the area where no obvious growth is visible
Special areas need to be addressed

- In case of Staph (oxacillin) and enterococci (vancomycin), any discernible growth within the zone of inhibition is considered as resistant.
- Discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified, and retested.
- With *Proteus* spp., the thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored.
- For trimethoprim/sulfamethoxazole, 80% inhibition is considered.
- When using blood-supplemented medium for testing streptococci, the zone of growth inhibition should be measured, not the zone of inhibition of hemolysis.
### Advantages

- Technically simple to perform
- Reproducible
- Relatively inexpensive
- Doesn’t require any special equipment
- Provides susceptibility category results easily understood by the clinicians
- Flexible regarding the selection of agents

### Disadvantages

- Not standardised for many bacteria
  - Needs special media
  - Variable growth rate
- Inadequate in certain situations
  - Detection of VISA
  - Oxacillin hetero-resistant staphylococci
  - Van-B type VRE
- Provides qualitative result
  - MIC may be desirable for *S. pneumoniae*
Dilution methods

- Agar or broth based
- Tested at twofold serial dilutions
- MIC: lowest concentration that inhibits visible growth of an organism
- Break point susceptibility testing
- Single drug concentration screens
Advantages

- Std medium may be supplemented or even replaced with another medium to allow fastidious bacteria
- Any drug available in powdered form may be used
- Applicable to automated systems
- Quantitative results (MIC) or category results (SIR) or both can be used
Agar dilution method

- Mueller-Hinton agar
- Atb added to tubes at 48-50°C temp after autoclaving
- 100 mm plates, 3 to 4 mm depth
  - 1 ml concentration of the drug + 24 ml MHA
- May store at 4 to 8°C for 5 days
- Imipenem, cefaclor, clavulanic acid combinations can’t be stored
Agar dilution method…..

- **Inoculum:** 1:10 dilution of 0.5 Mcfarland standard which delivers $10^7$ CFU/ml
  - Direct colony suspension method
  - Growth method
    - When smooth suspension cannot be made
    - Non- fastidious organisms
- **Using pipette/calibrated loop** deliver 1-2µl of suspension on the surface of agar giving final inoculum of $10^4$CFU/spot
- **Inoculate a control plate**
- **Incubate at 35°C for 16 to 20h in non CO$_2$ atmosphere**
Agar dilution method....
Interpretation

- Examine the drug free control for viability & purity
- Examine the plates on a dark background, look for lowest concentration that inhibits visible growth
- A single colony or faint haze is ignored
- 80% inhibition of growth is taken as the end point for trimethoprim & sulfonamides
- Trailing end point may occur with
  - chloramphenicol, tetracyclines, linezolid, quinupristine-dalfopristin)
Advantages

- Well standardized, reliable
- Simultaneous testing of large no of isolates for few drugs
- Microbial contamination or heterogeneity is more readily detected than broth method
- Reference method in most of Europe

Disadvantages

- Preparation of plates is time consuming & labor intensive
- Strict adherence to protocol is required
- The MIC value is not the sole predictor for clinical outcome
Broth dilution methods

Macro
- Broth volume is $\geq 1$ ml (usually 2 ml)
- Contained in 13 by 100 mm tubes

Micro
- 0.1 ml volumes
- In wells of microtitre plates
Broth macrodilution method

- Becs of 1:2 dilution, all final drug concentrations must be prepared at twice the desired testing conc
- CAMHB
  - For H. influenzae – HTM
  - For TMP-SMX – Thymidine free medium
  - For Oxacillin resistance – MH broth with 2% NaCl
  - Storage at 4-8°C for 5 days
Broth macrodilution method..

- **Inoculum**
  - $5 \times 10^5$ CFU/ml
  - 0.5 McFarland std is diluted 1:100
    - $(10^6)$ CFU/ml
  - When inoculated in 1ml of CAMHB, final inoculum of $5 \times 10^5$ CFU/ml

- **Incubate at 35°C for 16-20hrs non Co₂ atmosphere**
Broth macrodilution method...

TUBE-BASED MIC PHOTO (Post-Incubation)

- Highly diluted quaternary ammonium
- Same antimicrobial agent in both rows

Range of product dilutions are analyzed

Point at which growth is inhibited = Minimum Inhibitory Concentration (MIC)

Cloudy Tubes Indicate Growth

S. aureus

E. coli
Advantages

- Well standardised & reliable
- Suitable for testing one drug against one isolate

Disadvantages

- Only one ATM & one organism can be tested each time
- Time consuming
Broth microdilution method...

- 96 well plates
  - May be prepared in-house
  - Commercially prepared
    - Frozen or dried/lyophilised
    - Consistent performance, but high cost
    - May suffer from degradation of antibiotics during shipping & storage

- 0.05ml or 0.1ml atm suspension dispensed into each well.

- CAMHB
Broth microdilution method...

- Store at -20°C for 6 weeks, -60-70°C for months
- Inoculum: final $5 \times 10^5$ CFU/ml
  - When 0.001-0.005 ml is used: 0.5 McFarland std is diluted 1:10 ($10^7$ CFU/ml)
  - When 0.05 ml of inoculum is used: 1:100 dilution of 0.5 McFarland ($10^6$ CFU/ml)-final inoculum $5 \times 10^5$ CFU/ml

- Incubate at 35°C for 16-20h
Broth microdilution method...
Advantages

- Standardised reference method
- Simultaneous testing of several agents against individual isolates
- Simultaneously performed with many isolates
- Less reagent required
- Inoculation & reading procedures relatively convenient.

Disadvantages

- More difficult & expensive than disk diffusion for routine usage
- Trailing growth-difficulty in end-point determination for certain drugs
Breakpoint susceptibility tests

- Atm agents are tested only at the specific concentrations (break point)
- Greater no: and variety of atm may be incorporated in the panel

- Resistance screens
  - Testing of a single drug concentration
  - Staphylococcal resistance to oxacillin
  - Vancomycin resistance to Enterococcus
E- test/ Gradient Diffusion Method

- Epsilometer test
- An exponential gradient testing methodology
- A predefined stable antimicrobial gradient is present on thin inert carrier strip (polymer)
- Commercially prepared by microdispensing robotic machines that can deliver nano liters volumes of antibiotic concentrations along the strip
E- test/ Gradient Diffusion Method

- Following incubation, the E strip releases drug and a symmetrical inhibition ellipse is produced.
- MIC is the intersection of the inhibitory zone edge and the calibrated carrier strip.
- Easy to use.
- Useful for infrequently tested drugs.
- Useful for fastidious & anaerobic bacteria.
- Storage at -20°C.
- Short shelf life.
- Expensive.
Complete inhibition of macrocolonies at MIC > 32 μg/ml.

Different intersections on either side of the strip. Read the higher value; if the difference is > 1 dilution, repeat the test. MIC 0.5 μg/ml.
## Susceptibility test methods: special cases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Medium</th>
<th>Incubation atm</th>
<th>Incubation length</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Pneumoniae &amp; Streptococcus spp.</td>
<td>Disk diffusion</td>
<td>MHA+5%sheep blood</td>
<td>5-7% CO₂</td>
<td>20-24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broth microdilution</td>
<td>CAMHB-LHB</td>
<td>Ambient air</td>
<td>20-24</td>
</tr>
<tr>
<td>Haemophilus spp.</td>
<td>Disk diffusion</td>
<td>HTM agar</td>
<td>5-7% CO₂</td>
<td>16-18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broth microdilution</td>
<td>HTM broth</td>
<td>Ambient air</td>
<td>20-24</td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>Disk diffusion</td>
<td>GC agar base + supplemet</td>
<td>5-7% CO₂</td>
<td>20-24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agar dilution</td>
<td>GC agar base + supplemet</td>
<td>5-7% CO₂</td>
<td>20-24</td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>Disk diffusion</td>
<td>MHA+5%sheep blood</td>
<td>5-7% CO₂</td>
<td>20-24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broth microdilution</td>
<td>CAMHB-LHB</td>
<td>Ambient air</td>
<td>20-24</td>
</tr>
</tbody>
</table>
S. pneumoniae

- MIC method for penicillin, cefotaxime/ceftriaxone, meropenem & vancomycin (may be by disk diffusion also)
- One representative from Macrolide, fluoroquinolone, tetracycline – MHA with 5% sheep blood
- Meningitic & nonmeningitic break points separately for penicillin, cefotaxime & ceftriaxone
- Oxacillin screening procedure should be used only for non-lifethreatening infections
  - \( \leq 19 \text{mm MIC} \) for penicillin & cefotaxime/ceftriaxone should be done
Quality Control in Susceptibility Testing

- CLSI has recommended QC strains (ATCC)
  - Depending on method, media & organism to be tested

- Perform testing for 30 consecutive days
  - Yes
    - Continue daily testing until this criteria is satisfied
  - No
    - Reduce testing to once per week
      - Are all zone diameters within control limits?
        - Yes
          - Continue with weekly testing
        - No
          - Resume daily testing for 5 consecutive days
            - Are all consecutive zone diameters within control limits?
              - Yes
                - Resume weekly testing
              - No
                - Resume daily testing until another 30 consecutive days of satisfactory performance is documented

Susceptibility test methods: fastidious bacteria

Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline
<table>
<thead>
<tr>
<th>Table No.</th>
<th>Organism/Organism Group</th>
<th>Broth Microdilution MIC Test Medium</th>
<th>Broth Microdilution MIC Incubation Conditions</th>
<th>Disk Diffusion Test Medium/Incubation Conditions</th>
<th>QC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Abiotrophia spp., Granulicatella spp.</td>
<td>CAMHB-LHB (2.5-5% v/v) + 0.001% pyridoxal HCl</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>2</td>
<td>Aeromonas hydrophila complex, Plesiomonas shigelloides</td>
<td>CAMHB</td>
<td>35 °C; ambient air; 18-20 h</td>
<td>MHA (unsupplemented)/35 °C; ambient air 16-18 h</td>
<td>E. coli ATCC® 25922</td>
</tr>
<tr>
<td>3</td>
<td>Bacillus spp. (not B. anthracis)</td>
<td>CAMHB</td>
<td>35 °C; ambient air; 16-20 h</td>
<td>NA</td>
<td>S. aureus ATCC® 29213</td>
</tr>
<tr>
<td>4</td>
<td>Campylobacter jejuni/coli</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>36 °C/48 h or 42 °C/24 h; 10% CO₂, 5% O₂, 85% N₂ (microaerobic)</td>
<td>MHA with 5% sheep blood/36-37 °C; 48 h or 42 °C 24 h; 10% CO₂, 5% O₂, 85% N₂</td>
<td>C. jejuni ATCC® 33560 for microdilution</td>
</tr>
<tr>
<td>5</td>
<td>Corynebacterium spp.</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 24-48 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 40619</td>
</tr>
<tr>
<td>6</td>
<td>Erysipelothrix rhusiopathiae</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>7</td>
<td>HACEK group</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; 5% CO₂; 24-48 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>8</td>
<td>Lactobacillus spp.</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>9</td>
<td>Leuconostoc spp.</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>10</td>
<td>Listeria monocytogenes</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>11</td>
<td>Moraxella catarrhalis</td>
<td>CAMHB</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. aureus ATCC® 29213</td>
</tr>
<tr>
<td>12</td>
<td>Pasteurella spp.</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 18-24 h</td>
<td>MHA with 5% sheep blood/35 °C; ambient air; 18-24 h</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>13</td>
<td>Pediococcus spp.</td>
<td>CAMHB-LHB (2.5-5% v/v)</td>
<td>35 °C; ambient air; 20-24 h</td>
<td>NA</td>
<td>S. pneumoniae ATCC® 49619</td>
</tr>
<tr>
<td>14</td>
<td>Vibrio spp. (not V. cholerae)</td>
<td>CAMHB</td>
<td>35 °C; ambient air; 16-20 h</td>
<td>MHA (unsupplemented)/35 °C; ambient air 16-18 h</td>
<td>E. coli ATCC® 25922</td>
</tr>
</tbody>
</table>

Footnotes:

1. CAMHB-LHB (2.5-5% v/v) + 0.001% pyridoxal HCl
2. CAMHB
3. CAMHB
Common sources of error

1. Test system & components
   - Disk diffusion suitable only for rapidly growing organisms with consistent growth rates

2. Test procedure
   - pH & cation content of medium
   - Proper amount of NaCl for oxacillin resistant Staphylococci for dilution testing
   - Depth of media, Storage of media & disks
   - Inoculum, incubation condns
   - Staph for oxa & vanco, entero for vanco 24 hrs in incubation

3. Peculiar to certain organisms & drug combinations
   - Testing of some bacteria against certain agents

4. Transcription errors
Laboratory strategies for susceptibility testing

- Communication
- Prompt and thorough review of results
- Prompt resolution of unusual results
- Augment susceptibility reports with messages that help clarify and explain potential therapeutic problems not necessarily evident by data alone
Thank You