The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent required to prevent growth of a microorganism under defined experimental conditions, giving a quantitative measure of bacterial susceptibility.
THE CLINICAL IMPORTANCE OF THE MINIMUM INHIBITORY CONCENTRATION

Disc testing can give unreliable or misleading results.

Clinical microbiology laboratories should be putting more emphasis on antimicrobial resistance testing (ART).

A method is chosen to either predict or test for small changes in the bacterial phenotype that may lead to clinical resistance.
The Clinical Importance of the Minimum Inhibitory Concentration

- Organisms are grouped into various categories:
  - Susceptible (S)
  - Intermediate (I)
  - Resistant (R)

- But, for serious infections, and those in sites where antibiotics penetrate poorly, and among immune-suppressed patients, categorical results have limited predictive value
Assessment of the MIC values of several agents will provide a selective choice as to which antibiotic possesses the widest margin between the MIC and the susceptible breakpoint. For the patient, this could be used both as a safety and efficacy margin.

This is particularly relevant for infections in sites into which antibiotics penetrate poorly. For example, in osteomyelitis or prosthetic joint infections, the MIC values are critical along with knowledge of penetration of different agents to select optimal therapy.
Clinical priorities for MIC testing include:

- Sterile site infections e.g. endocarditis
- Serious nosocomial infections
- Chronic infections e.g. cystic fibrosis
- ICU and other high risk patients
So, for:

- Resistance surveillances
- Drug evaluations
- Clinical trials

only quantitative MIC data can be used.
Methods for determination of MIC

1. Broth dilution method
2. Agar dilution method
3. E- test
Broth Dilution Method

- Macro broth dilution
- Micro broth dilution
Procedure

- **Antimicrobial Agents**
- Obtain antimicrobial standards or reference powders directly from the drug manufacturer
- **Acceptable powders**
  - Drug’s generic name
  - lot number
  - potency $[\mu g]$ or $[IU]$ per mg
  - expiration date
- **Store the powders at $\leq -20 ^\circ C$**
Weighing Antimicrobial Powders

\[ \text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (\(\mu g/mL\))}}{\text{Potency (\(\mu g/mg\))}} \]

• Preparing Solutions
<table>
<thead>
<tr>
<th>Drug</th>
<th>Solution</th>
<th>Drug</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>Water</td>
<td>Ampicillin</td>
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<td>Methanol</td>
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<td>Chloramphenicol</td>
<td>96% ethanol</td>
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<td>Cinoxacin</td>
<td>1/2 volume of water, then add 1 mol/L NaOH, dropwise to dissolve</td>
</tr>
<tr>
<td>Cinoxacin</td>
<td>1/2 volume of water, then add 1 mol/L NaOH, dropwise to dissolve</td>
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<td>Water</td>
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<tr>
<td>Clindamycin</td>
<td>Water</td>
<td>Colistin⁺⁺</td>
<td>Water</td>
</tr>
<tr>
<td>Colistin⁺⁺</td>
<td>Water</td>
<td>Dalbavancin</td>
<td>DMSO⁹⁺⁺</td>
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<tr>
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<td>DMSO⁹⁺⁺</td>
<td>Daptomycin</td>
<td>Water</td>
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<td>Water</td>
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<tr>
<td>Antimicrobial Agent</td>
<td>Solvent</td>
<td>Diluent</td>
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<tr>
<td>--------------------</td>
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<tr>
<td>Dinitromycin</td>
<td>Glacial acetic acid&lt;br&gt;0.85% physiological saline</td>
<td>Water&lt;br&gt;0.85% physiological saline</td>
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</tr>
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<td>Doxycycline</td>
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<td>Enoxacin</td>
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<td>Ertapenem</td>
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<td>Phosphate buffer, pH 7.2, 0.01 mol/L</td>
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<td>Erythromycin</td>
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<td>Faropenem</td>
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<td>Fleroxacin</td>
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<tr>
<td>Garenoxacin</td>
<td>Water&lt;br&gt;(with stirring)</td>
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<td></td>
</tr>
<tr>
<td>Gatiloxacin</td>
<td>Water&lt;br&gt;(with stirring)</td>
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<td>Imipenem</td>
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<td>Levofloxacin</td>
<td>1/2 volume of water, then 0.1 mol/L NaOH dropwise to dissolve</td>
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<td>Linezolid</td>
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<td>Loracarbef</td>
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<td>Mecillinam</td>
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<td>Methicillin</td>
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<tr>
<td>Metronidazole</td>
<td>DMSO&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Water</td>
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<tr>
<td>Moxifloxacin</td>
<td>Water</td>
<td>Water</td>
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<tr>
<td>Moxolactam (diammonium salt)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04 mol/L HCl (let sit for 1.5 to 2 h)</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
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<td>Moxifloxacin</td>
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<td>Nafcilin</td>
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<td>Nalidixic acid</td>
<td>1/2 volume of water, then add 1 mol/L NaOH, dropwise to dissolve</td>
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<td>Natamycin</td>
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<td></td>
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<tr>
<td>Nitrofurantoin&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Ofloxacin</td>
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<td>Pipemidin</td>
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<td>Polymyxin B</td>
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<td>Water</td>
<td></td>
</tr>
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<td>Quinupristin-dalfopristin</td>
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<td>Water</td>
<td></td>
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<tr>
<td>Rifampin</td>
<td>Methanol&lt;sup&gt;d&lt;/sup&gt; [maximum concentration = 640 µg/mL]</td>
<td>Water&lt;br&gt;(with stirring)</td>
<td></td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>Water</td>
<td>Water</td>
<td></td>
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<tr>
<td>Spectinomycin</td>
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</tr>
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<td>Streptomycin</td>
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<tr>
<td>Sulbactam</td>
<td>Water</td>
<td>Water</td>
<td></td>
</tr>
</tbody>
</table>
Inoculum Preparation for Dilution Tests

Turbidity Standard for Inoculum Preparation (0.5 McFarland standard)
- Direct Colony Suspension Method
- Growth Method
- Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in broth so that, after inoculation, each tube contains approximately 5 x 10^5 CFU/mL
- Dilute the 0.5 McFarland suspension 1:150

Mueller-Hinton Broth
Broth Dilution Method

Day 1
Add 1 ml of test bacteria (1*10^6 CFU/ml) to tubes containing 1 ml broth and concentration of antibiotic (mg/l)

Controls:
C1 = No antibiotic, check viability on agar plates immediately
C2 = No test bacteria

Incubate 35 ºC, o/n
**Broth Dilution Method**

**Day 2**
- Record visual turbidity
- Subculture non-turbid tubes to agar plates (use 0.01 ml standard loop)
- MIC = 16 mg/l

**Day 3**
- Determine CFU on plates:
  - At 16 mg/l = 700 CFU/ml > 0.1% of 5*10^5 CFU/ml
- MBC = 32 mg/l
Prepare inoculum suspension

Microdilution MIC tray
Agar Dilution Method

Procedure

- Making dilutions of antimicrobial agent in melted media and pouring plates
  - One concentration of antibiotic/plate
  - Possible for several different strains/plate

64 ug/ml 32 ug/ml 16 ug/ml
Agar Dilution Method

- **Procedure**
  - Inoculation of bacterial inoculum (McFarland No. 0.5)
    - Delivers 0.001 ml of bacterial inoculum
  - Incubation
  - Spot of growth

![MIC](32 ug/ml)
E test
E test

Etest is a quantitative technique for determining the Minimum Inhibitory Concentration (MIC) of antimicrobial agents against microorganisms and for detection of resistance mechanisms.
ART method Resistance Testing

Needs to be flexible and should fulfill some of the following characteristics:

- Allowing the use of heavier inoculum to detect low level resistance

- Adaptable to conditions optimal for resistance extended incubation (glycopeptide resistance)
Applicable to fastidious, uncommon and slow growing organisms

Provide quantitative MIC values over a wide concentration range (>10 dilutions)

Decreases in susceptibility to be reliably detected
E test

- Concentration range across 15 dilutions
- Precise, continuous and stable gradient
- Visual recognition of resistant phenotypes on agar
E test

This test may have

- It has a continuous concentration gradient and is able to show subtle changes in susceptibility
- The wide concentration gradients cover the MIC ranges of a wide variety of pathogens
- Allow both low level and high level resistance to be detected.
- The Etest is reportedly easy to use in most laboratory settings and requires no complicated procedures
Robust to fastidious

Easily adaptable to different test conditions

- robust aerobes to anaerobes
- *Pneumococci*
- *Meningococci*
- *H. pylori*
- *Bartonella*
- *Bordetella*
- *Francisella*
- *Brucella*
- *Actinomycetes* such as nocardia and rhodococcus
- Fungi including yeasts, moulds
- *Mycobacteria*
E test

- Etest consists of a thin, inert and non-porous plastic strip
- 5 mm wide and 60 mm long
- One side of the strip carries the MIC reading
- Scale in $\mu$g/ml and a two-letter code on the handle to designate
- The gradient covers a continuous 15 two-fold dilutions of a conventional MIC method
STORAGE

All packages, must be stored in a freezer at -20°C until the expiry date.

Etest strips left over from an opened package must be stored at -20°C in an airtight storage container or tube.

Strips in storage containers can be used until the expiry date if correctly stored and handled.

Store only one antibiotic type per storage tube.

Prevent moisture from penetrating into or forming within the package or storage tubes. Etest strips must be kept dry.
Procedure

Medium
- Ensure that the agar plate has a depth of 4.0 ± 0.5 mm and pH 7.2 - 7.4
- The medium and required supplements will depend on the bacterial species being tested

Inoculum preparation

Inoculation
- The surface is completely dry before applying the Etest strips.
Swab plate

Remove sample
Template for application of MIC antibiogram

15 cm

8 cm
INTERPRETATION OF RESULTS
Intersection in between markings, read at the upper value. MIC 0.19 μg/ml.
Bactericidal agents like aminoglycosides give sharp ellipses. MIC 0.064 μg/ml
Microcolonies at endpoints of bactericidal agents. MIC 4 μg/ml.
Coagulase negative staphylococci can show trailing at the endpoint due to glycopeptide resistant subpopulations. MIC 12 μg/ml.
Read where the resistant subpopulation is completely inhibited. MIC > 32 μg/ml.

Isolated resistant colonies due to low-level mutation. MIC > 256 μg/ml.
β-lactamase inhibitors at constant levels can extend the ellipse below the MIC due to intrinsic activity. Extrapolate the upper curvature towards the strip to get the MIC. MIC 0.75 μg/ml.

"Dip" effect due to inducible macrolide resistance. Extrapolate the ellipse towards the strip to get the MIC, i.e. 0.38 μg/ml. This strain also had colonies at the upper range of the strip. MIC > 256 μg/ml.
Ignore swarming by Proteus. MIC 0.064 μg/ml.
Tilt the plate to see pin-point colonies and hazes, especially with *enterococci, pneumococci, fusobacteria, Acinetobacter* and *Stenotrophomonas* spp. MIC 1 μg/ml.
A resistant subpopulation in a *pneumococci* MIC $>32$ $\mu$g/ml.
Ignore haemolysis of the blood and read at inhibition of growth. MIC 0.032 μg/ml.
Different intersections at the strip, read the higher value (MIC 0.5 μg/ml). If >1 dilution difference, repeat the test.
Ignore a thin line of growth at the edge of the strip. 
MIC 0.25 μg/ml
Special Applications

- ESBLs
- Anaerobes
- Helicobacter and Campylobacter
- Haemophilus
- Mycobacteria and Actinomycetes
Susceptibility testing of anaerobes can be problematic.

Disc diffusion is unreliable and not recommended.

Almost 15 to 40% of anaerobes do not grow well in broth microdilution systems.

Broth microdilution may be suboptimal.
Susceptibility testing limitations

- The low inoculum used may underestimate resistance.
- Metronidazole testing is problematic.
- Reference agar dilution is cumbersome and expensive to set up for a few strains.
Susceptibility testing of Anaerobes

The continuous and stable antibiotic gradient in Etest has been documented to be suitable for testing anaerobes.

The use of MIC data has been shown to give therapy change in up to 56% of clinical cases.

The lack of routine susceptibility testing to guide physicians, leads to the use of expensive broad spectrum antibiotics, in order to cover all potentially significant organisms.
Why Etest for anaerobes?

- Agar based growth supporting good growth.
- Stable antibiotic gradient minimally influenced by varying growth rates.
- Extensively validated for anaerobes (120 studies).
- Can provide 24h MIC for critical situations.
E test procedure

- Brucella agar with 5% blood, 5 g/ml hemin and 1 g/ml vitamin K, supports growth.

- A broth suspension of viable colonies with turbidity equivalent to 1 Mcfarland.

- Anaerobic incubation for 24 to 48 hours, or longer for slow growers.
Read all colonies in the clindamycin ellipse for anaerobes. MIC 16 μg/ml
Susceptibility testing of *Helicobacter pylori* and *Campylobacter*

- **Medium**
  - Brucella, Columbia or Mueller Hinton

- An inoculum suspension equivalent to 3 Macfarland

- Incubate the plates for 3 to 5 days.
Etest ESBL

Enzymes produced by Gram negative aerobic bacteria mainly in *K. pneumoniae* and *E. coli*

- Enzymes generally inhibited by beta-lactamase inhibitor e.g. clavulanic acid
- Often cross resistance to quinolones, aminoglycosides and trimethoprim/sulfamethoxazole
To be selected by heavy use of expanded spectrum cephalosporins (ESC) e.g. ceftazidime

- In nosocomial pathogens from ICUs, oncology, burn and neonatal wards
- In infections associated with indwelling devices
- Increasing prevalence and as outbreaks worldwide
Testing is indicated for:

- Isolates from ICUs and other high risk patients
- Isolates with reduced susceptibility to ESCs i.e. MICs 1 μg/ml or zones 22 mm
- Therapy failure despite in vitro susceptibility
Limitations may occur with disc diffusion

- If positioning of the ESBL disc is not proper, approximation test is sub-optimal.
- Low level ESBL will not be detected by using disc diffusion and automation.
- The clavulanic acid level in double disc testing is suboptimal at the lower MIC region.
The Etest ESBL CT/CTL and TZ/TZL strips consist of a thin, inert and non-porous plastic carrier (5 x 60 mm).

CT codes for the cefotaxime (0.25-16 μg/ml) gradient and CTL the cefotaxime (0.016-1 μg/ml) plus 4 μg/ml clavulanic acid.

TZ codes for the ceftazidime (0.5-32 μg/ml) gradient TZL the ceftazidime (0.064-4 μg/ml) plus 4 μg/ml clavulanic acid.
The presence of ESBL is confirmed by the appearance of:

- Phantom zone or deformation of the CT or TZ ellipse
- When either CT or TZ MIC is reduced by ≥3 log2 dilutions in the presence of clavulanic acid.
# Interpretation

<table>
<thead>
<tr>
<th>ESBL</th>
<th>MIC µg/ml Ratio</th>
<th>Reporting</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive</strong></td>
<td>CT $\geq 0.5$ and CT/CTL $\geq 8$ OR TZ $\geq 1$ and TZ/TZL $\geq 8$ OR “Phantom” zone or deformation of the CT or TZ ellipse.</td>
<td>ESBL producer and resistant to all penicillins, cephalosporins and aztreonam (NCCLS M100-S series).</td>
</tr>
<tr>
<td><strong>Negative</strong></td>
<td>CT $&lt;0.5$ or CT/CTL $&lt;8$ AND TZ $&lt;1$ or TZ/TZL $&lt;8$</td>
<td>ESBL non-producer and report actual MIC of all relevant drugs as determined by an MIC method.</td>
</tr>
<tr>
<td><strong>Non determinable (ND)</strong></td>
<td>CT $&gt;16$ and CTL $&gt;1$ AND TZ $&gt;32$ and TZL $&gt;4$ OR When one strip is ESBL negative and the other ND.</td>
<td>ESBL non determinable and report actual MIC of all relevant drugs as determined by an MIC method. If ESBL is suspected, confirm results with an NCCLS method and/or genotyping.</td>
</tr>
</tbody>
</table>
E test for ESBL
Occasionally, a “rounded” zone (phantom zone) may be seen below the CTL or TZL gradients while no ellipse may be seen around the CT or TZ ends.

Figure 5. A “rounded” phantom inhibition zone below CT indicative of ESBL.
The CT or TZ inhibition ellipse may also be deformed at the tapering end.

Figure 6. Deformation of the TZ inhibition ellipse indicative of ESBL.
Thank you