

# MIC & Etest

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# MIC

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

# MIC

The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

# 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

# THE CLINICAL IMPORTANCE OF THE MINIMUM INHIBITORY CONCENTRATION

- 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 [  $\mu\text{g}$  ] or [ IU ] per
  - expiration date
- Store the powders at  $-20\text{ }^{\circ}\text{C}$

# Weighing Antimicrobial Powders

- All antimicrobial agents are assayed for standard units of activity
- The assay units may differ widely from the actual weight of the powder and often may differ between drug production lots.
- Thus, a laboratory must standardize its antimicrobial solutions based on assays of the lots of antimicrobial powders that are used to make stock solutions.

The value for potency supplied by the manufacturer should include consideration of :

- Measures of purity (usually by HPLC assay)
- Water content
- The salt/counter-ion fraction
- The potency may be expressed as a percentage, or in units of  $\mu\text{g}/\text{mg}$  (w/w).

# Weighing Antimicrobial Powders

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration } (\mu\text{g/mL})}{\text{Potency } (\mu\text{g/mg)}} \quad (1)$$

or

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Potency } (\mu\text{g/mg})}{\text{Concentration } (\mu\text{g/mL})} \quad (2)$$

## Preparing Solutions

Antimicrobial Agent	Solvent	Diluent
Amikacin	Water	
Amoxicillin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Azithromycin	95% ethanol or glacial acetic acid <sup>f</sup>	Broth media
Azlocillin	Water	
Aztreonam	Saturated solution sodium bicarbonate	Water
Carbenicillin	Water	
Cefaclor	Water	
Cefadroxil	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefamandole	Water	
Cefazolin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cefdinir	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefditoren	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefepime	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cefetamet	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefixime	Phosphate buffer, pH 7.0, 0.1 mol/L	Phosphate buffer, pH 7.0, 0.1 mol/L
Cefmetazole	Water	
Cefonicid	Water	
Cefoperazone	Water	
Cefotaxime	Water	
Cefotetan	DMSO <sup>e,g</sup>	Water
Cefoxitin	Water	
Cefpodoxime	0.10% (11.9 mmol/L) aqueous sodium bicarbonate	Water
Cefprozil	Water	
Ceftazidime	Sodium carbonate <sup>d</sup>	Water
Ceftibuten	1/10 vol DMSO <sup>g</sup>	Water
Ceftizoxime	Water	
Ceftobiprole	DMSO <sup>g</sup> plus glacial acetic acid. <sup>1</sup>	Water, vortex vigorously
Ceftriaxone	Water	
Cefuroxime	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cephalexin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephalothin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephapirin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephradine	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Chloramphenicol	95% ethanol	Water
Cinoxacin	1/2 volume of water, then add 1 mol/L NaOH, dropwise to dissolve	Water
Ciprofloxacin	Water	
Clarithromycin	Methanol <sup>e</sup> or glacial acetic acid <sup>f</sup>	Phosphate buffer, pH 6.5, 0.1 mol/L
Clavulanic acid	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Clinafloxacin	Water	
Clindamycin	Water	
Colistin <sup>a</sup>	Water	Water
Dalbavancin	DMSO <sup>g</sup>	DMSO <sup>g,h</sup>
Daptomycin	Water	Water

**Table 4. (Continued)**

<b>Antimicrobial Agent</b>	<b>Solvent</b>	<b>Diluent</b>
Dirithromycin	Glacial acetic acid <sup>f</sup>	Water
Doripenem	0.85% physiological saline	0.85% physiological saline
Doxycycline	Water	
Enoxacin	1/2 volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Ertapenem	Phosphate buffer, pH 7.2, 0.01 mol/L	Phosphate buffer, pH 7.2, 0.01 mol/L
Erythromycin	95% ethanol or glacial acetic acid <sup>f</sup>	Water
Faropenem	Water	Water
Fleroxacin	1/2 volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Garenoxacin	Water (with stirring)	
Gatifloxacin	Water (with stirring)	
Gemifloxacin	Water	
Gentamicin	Water	
Imipenem	Phosphate buffer, pH 7.2, 0.01 mol/L	Phosphate buffer, pH 7.2, 0.01 mol/L
Kanamycin	Water	
Levofloxacin	1/2 volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Linezolid	Water	
Lomefloxacin		
Loracarbef	Water	
Mecillinam	Water	
Meropenem	Water	
Methicillin	Water	
Metronidazole	DMSO <sup>e,g</sup>	Water
Mezlocillin	Water	
Minocycline	Water	
Moxalactam (diammonium salt) <sup>b</sup>	0.04 mol/L HCl (let sit for 1.5 to 2 h)	Phosphate buffer, pH 6.0, 0.1 mol/L
Moxifloxacin	Water	
Nafcillin	Water	
Nalidixic acid	1/2 volume of water, then add 1 mol/L NaOH, dropwise to dissolve	
Netilmicin	Water	
Nitrofurantoin <sup>c</sup>	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 8.0, 0.1 mol/L
Norfloxacin	1/2 volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Ofloxacin	1/2 volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Oxacillin	Water	
Penicillin	Water	
Piperacillin	Water	
Polymyxin B	Water	Water
Quinupristin-dalfopristin	Water	
Rifampin	Methanol <sup>e</sup> [maximum concentration = 640 µg/mL]	Water (with stirring)
Sparfloxacin	Water	
Spectinomycin	Water	
Streptomycin	Water	
Sulbactam	Water	



# Direct Colony Suspension Method

Direct colony suspension is the recommended method for testing the fastidious organisms

- *Haemophilus spp*
- *N. gonorrhoeae*
- *N. meningitidis*
- *Streptococci*
- *for testing* staphylococci for potential methicillin or oxacillin resistance.

# Growth Method

- The growth method can be used alternatively and is sometimes preferable when colony growth is difficult to suspend directly and a smooth suspension cannot be made.
- It can also be used for non fastidious organisms (except staphylococci) when fresh (24 hour) colonies, as required for the direct colony suspension method, are not available.
- Select at least three to five well-isolated colonies of the same morphologic type from an agar plate culture.

- **Inoculum Preparation for Dilution Tests**

## Procedure

- **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  $5 \times 10^5$  CFU/mL
  - Dilute the 0.5 McFarland suspension 1:150
- **Mueller-Hinton Broth**

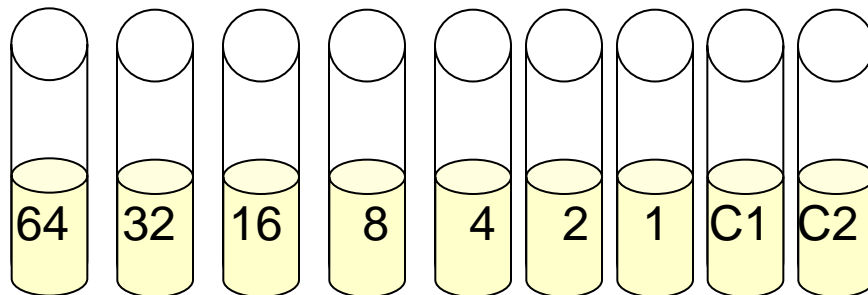
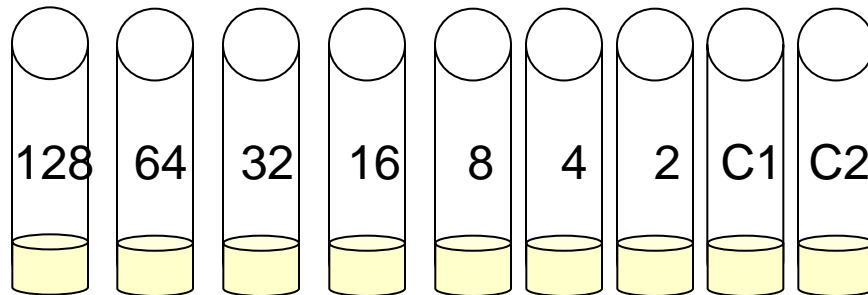
# Mueller-Hinton Broth

- Mueller-Hinton broth is recommended as the medium of choice for susceptibility testing of commonly isolated, rapidly growing aerobic, or facultative organisms.
- Mueller-Hinton broth demonstrates good batch-to-batch reproducibility for susceptibility testing
- Is low in sulfonamide, trimethoprim, and tetracycline inhibitors; and yields satisfactory growth of most pathogens.
- In addition, a large body of data and experience has been gathered about tests performed with this medium.

# Number of Concentrations Tested

- The concentrations to be tested for a particular antimicrobial agent should encompass the interpretive breakpoints shown in CLSI Tables
- But the actual number of concentrations tested is the decision of the laboratory

# Broth Dilution Method



Incubate 35 °C, o/n

## Day 1

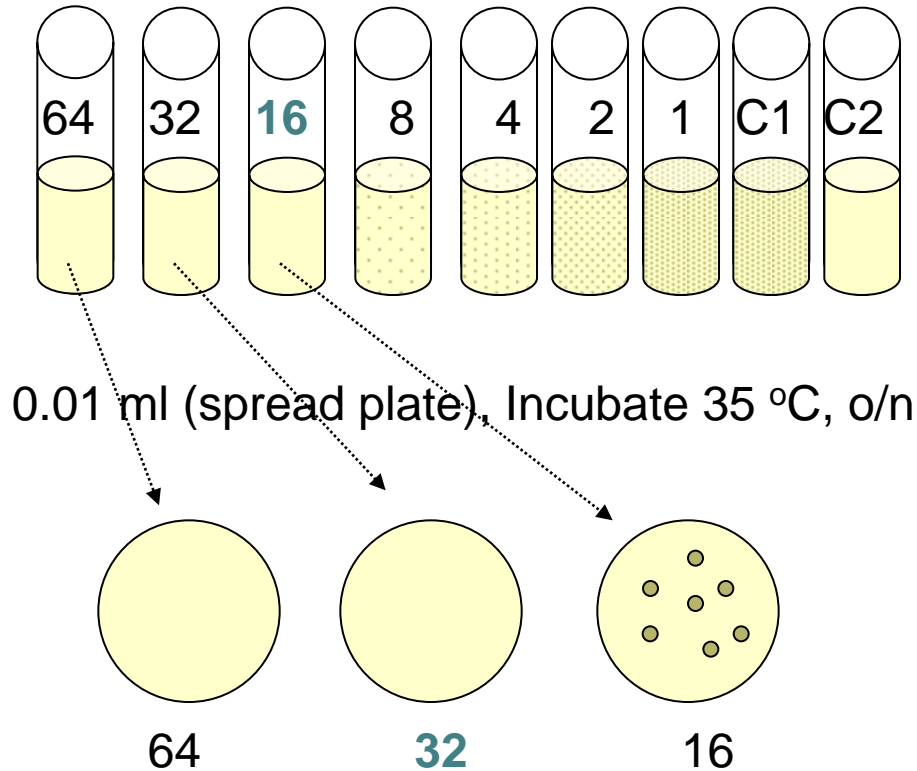
Add 1 ml of test bacteria ( $1 \times 10^6$  CFU/ml) to tubes containing 1 ml broth and concentration of antibiotic (mg/l)

## Controls:

C1 = No antibiotic, check viability on agar plates immediately

C2 = No test bacteria

# Broth Dilution Method



## Day 2

Record visual turbidity

Subculture non-turbid tubes to agar plates (use 0.01 ml standard loop)

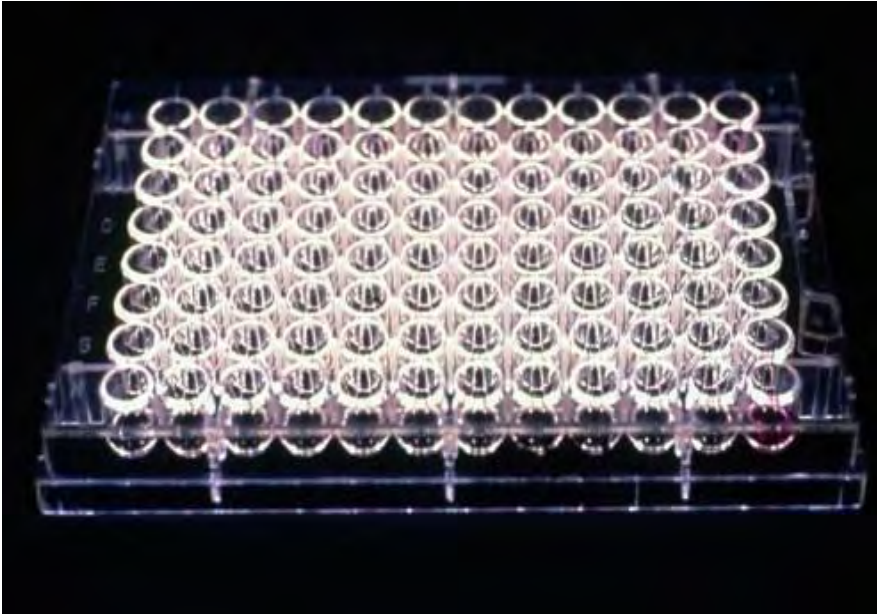
MIC = 16 mg/l

## Day 3

Determine CFU on plates:  
At 16 mg/l = 700 CFU/ml >  
0.1% of  $5 \times 10^5$  CFU/ml

MBC = 32 mg/l

# Prepare inoculum suspension

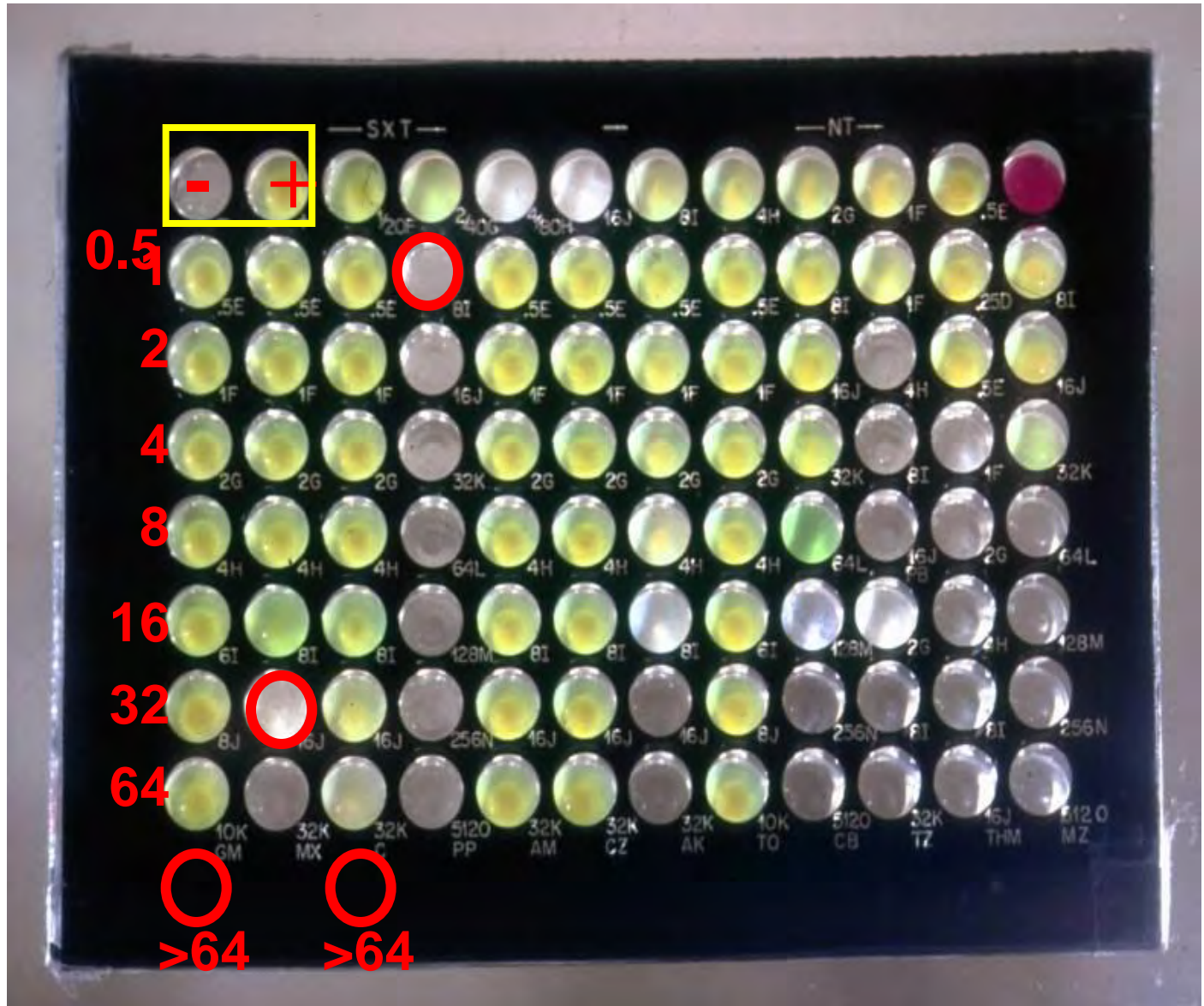


Microdilution MIC tray



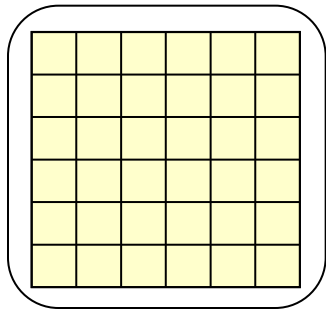


# MICs

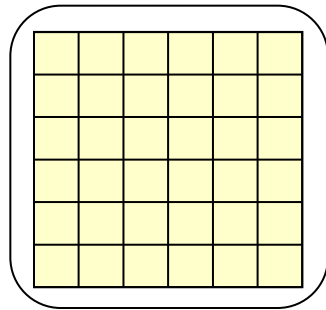


# Agar Dilution Method

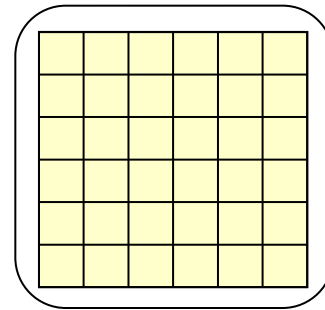
- Procedure
  - Making dilutions of antimicrobial agent in melted media and pouring plates
    - One concentration of antibiotic/ plate
    - Possible for several different strains/plate



64 ug/ml



32 ug/ml



16 ug/ml

# Agar Dilution Method

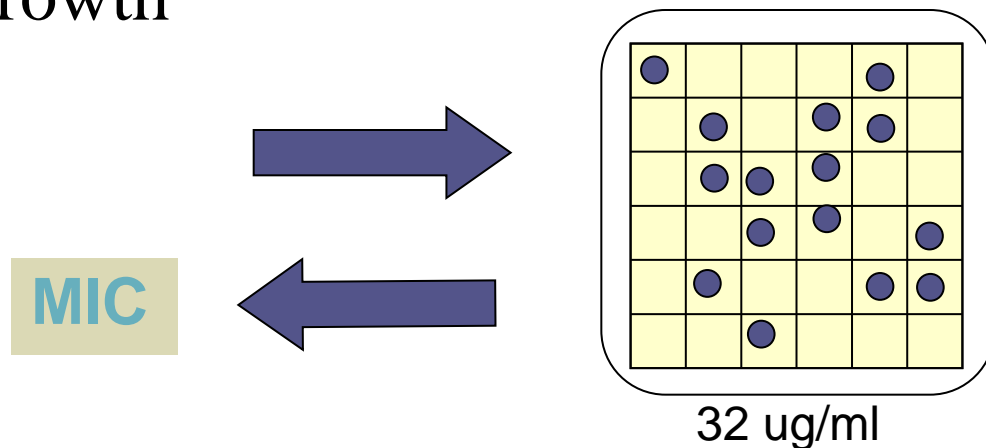
- › Procedure

- › Inoculation of bacterial inoculum (McFarland No. 0.5)

- › Delivers 0.001 ml of bacterial inoculum

- › Incubation

- › Spot of growth



# Interpretation

- The MIC, however, does not represent an *absolute value*.
- *The true MIC is somewhere between the lowest test concentration that inhibits the organism's growth (that is, the MIC reading) and the next lower test concentration.*
- If, for example, twofold dilutions were used and the MIC is 16  $\mu\text{g}/\text{mL}$ , the “**true**” MIC would be between 16 and 8  $\mu\text{g}/\text{mL}$ .

# Fastidious Organisms



# *Haemophilus influenzae and H. parainfluenzae*

- MIC testing *using Haemophilus Test Medium (HTM)*
- Only for broth dilution as described below
- The agar dilution method using HTM has not been studied

# *HTM*

- Mueller-Hinton broth
- $\beta$ -nicotinamide adenine dinucleotide (NAD)
- Bovine hematin
- Yeast extract
- Thymidine phosphorylase

# *Neisseria gonorrhoeae*

MIC testing of *N. gonorrhoeae* has been developed only for

- *agar dilution*
- *using GC agar base*
- *growth supplement*

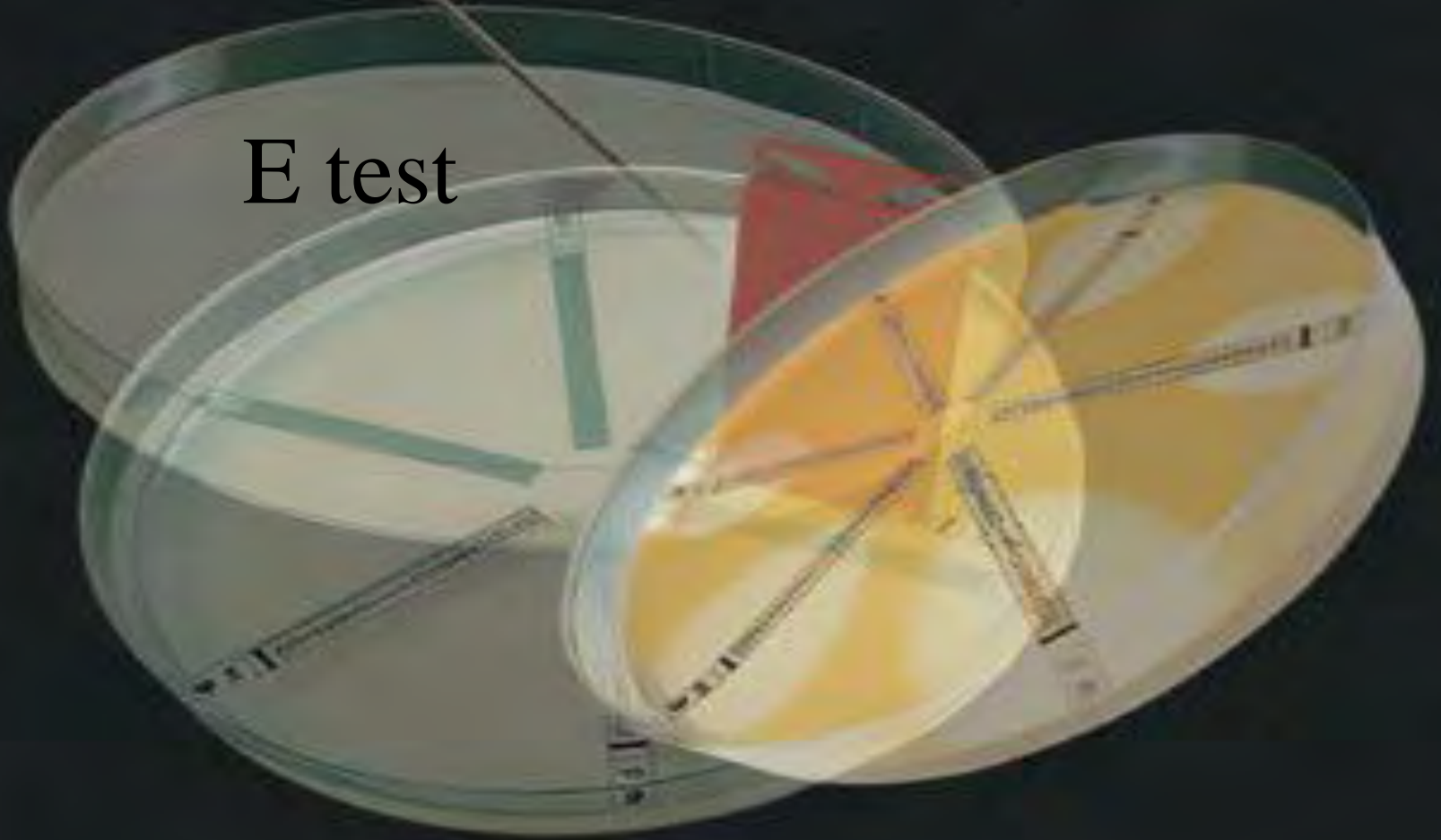
Broth microdilution and agar dilution susceptibility testing of *N. meningitidis* have been validated



## *Streptococcus pneumoniae and Other Streptococcus spp*

- MIC testing of *Streptococcus spp.*
- *Using CAMHB with 2.5 to 5% lysed horse blood has been developed*
- Only for broth dilution

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

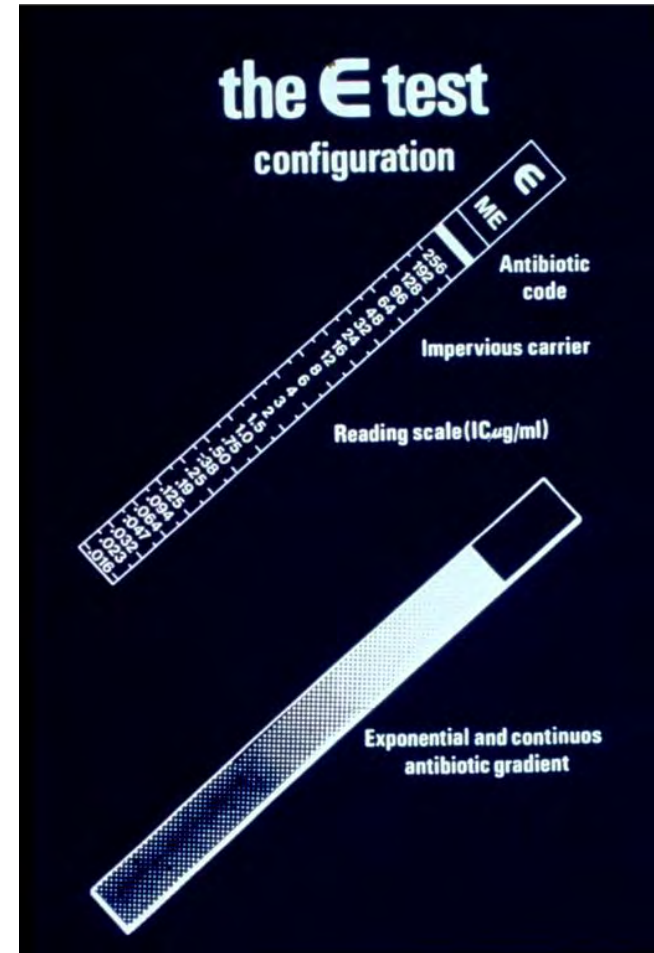
# Robust to fastidious

- Easily adaptable to different test conditions
  - robust aerobes to anaerobes
  - *Pneumococci*
  - *Meningococci*
  - *H. pylori*
  - *Bartonella*
  - *Bordetella*
  - *Franscisella*
  - *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  $\mu\text{g}/\text{ml}$  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  $4.0 \pm 0.5$  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.



**Remove sample**

**Swab plate**

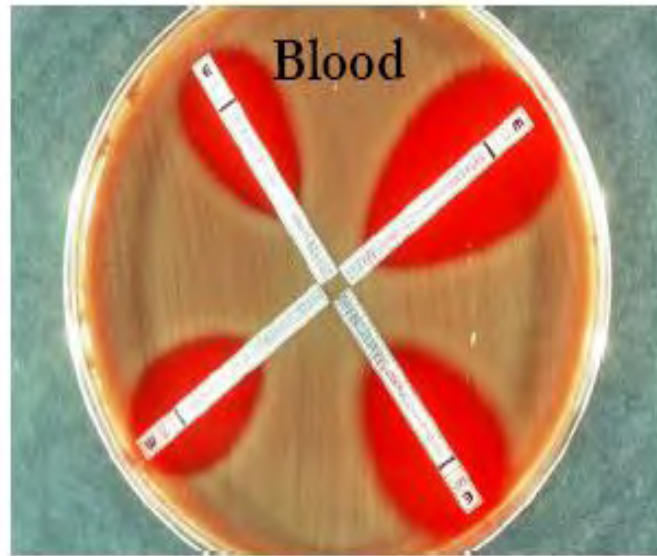


# Template for application of MIC antibiogram

15 cm

8 cm

# INTERPRETATION OF RESULTS



Intersection in between markings, read at the upper value. MIC  $0.19 \mu\text{g} / \text{m}^3$ .



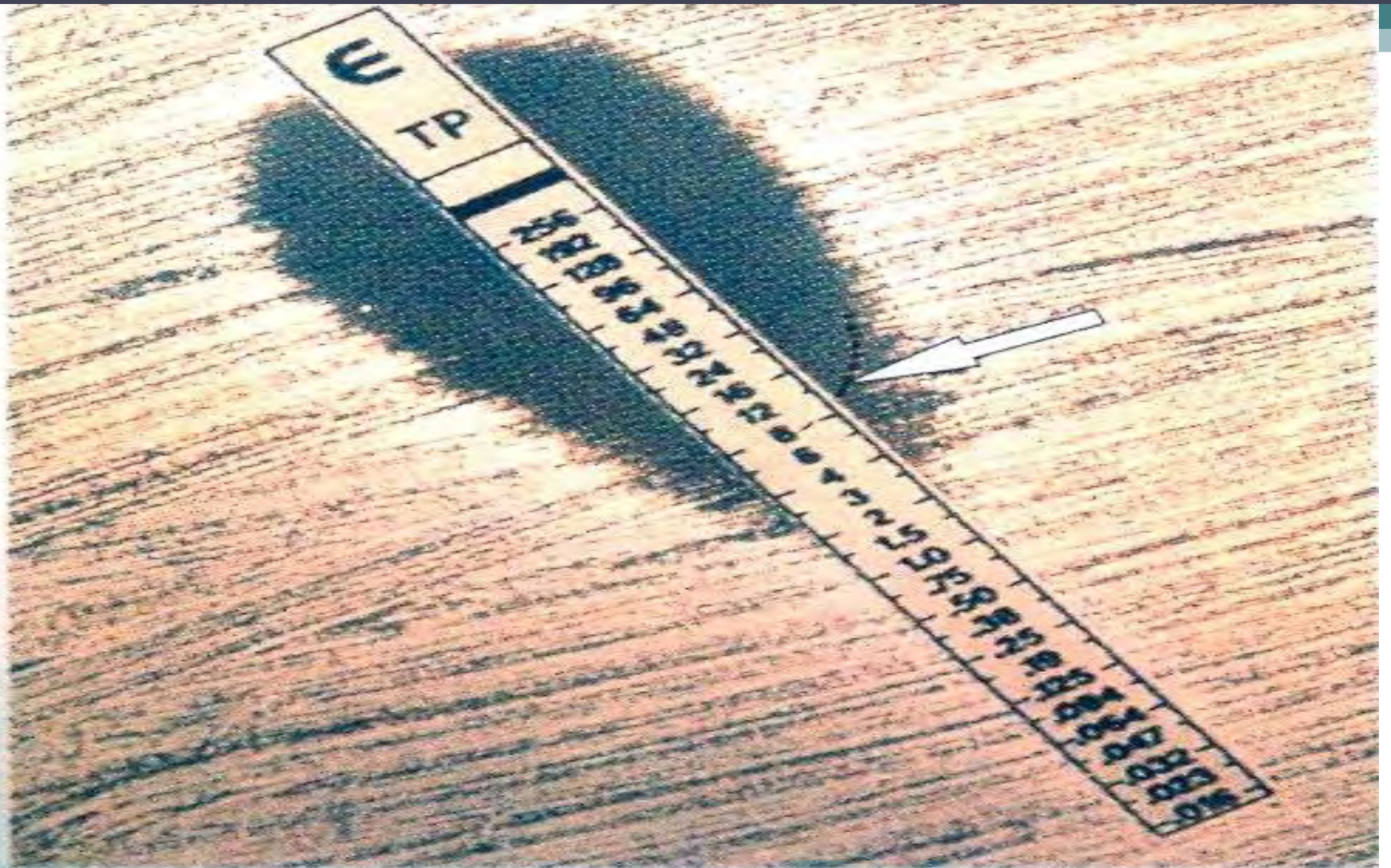
Bactericidal agents like aminoglycosides give sharp ellipses. MIC  $0.064 \mu\text{g} / \text{m l}$



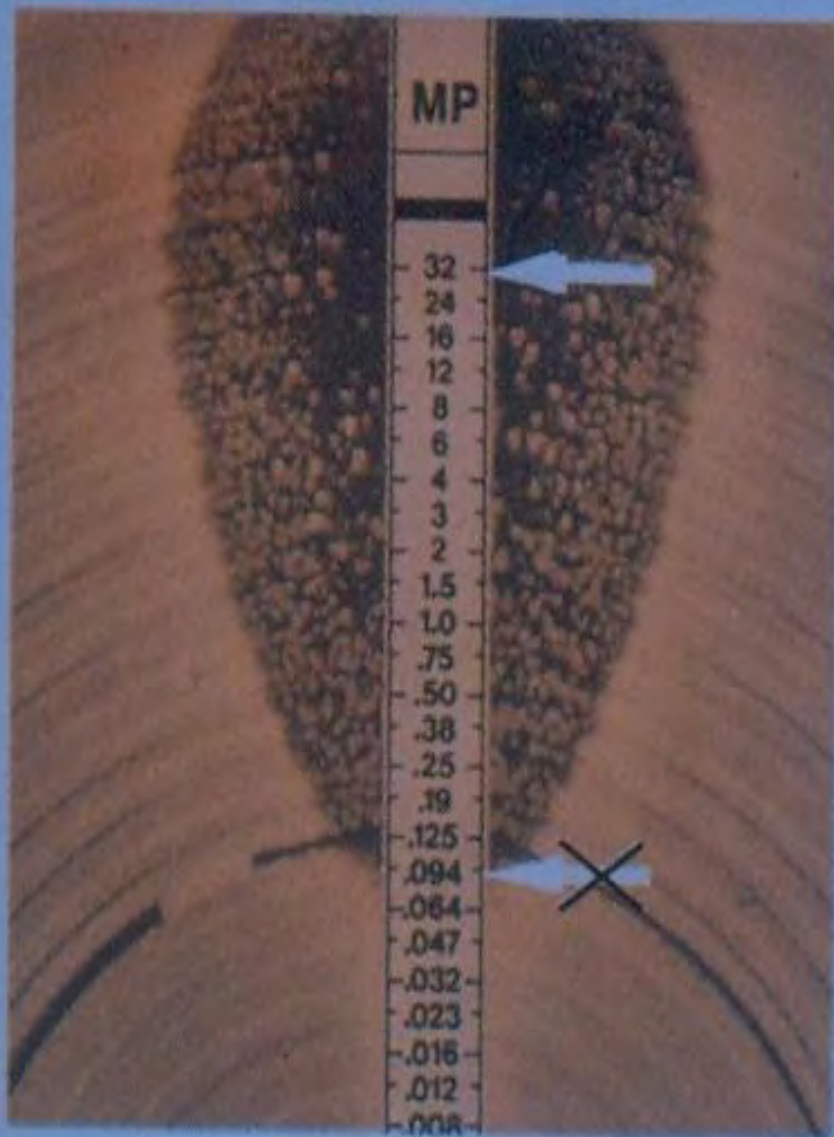


Microcolonies at endpoints of bactericidal agents.  
MIC 4  $\mu$  / ml.

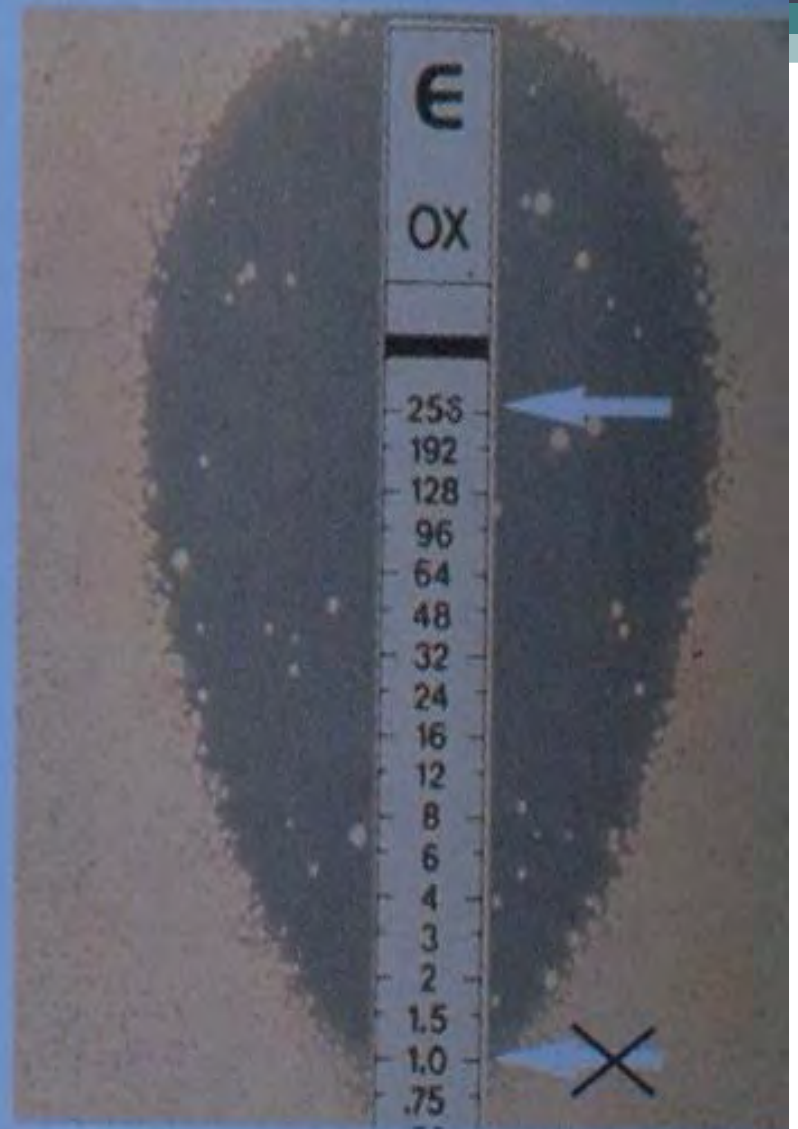




Coagulase negative staphylococci can show trailing at the endpoint due to glycopeptide resistant subpopulations. MIC 12  $\mu\text{g/ml}$ .



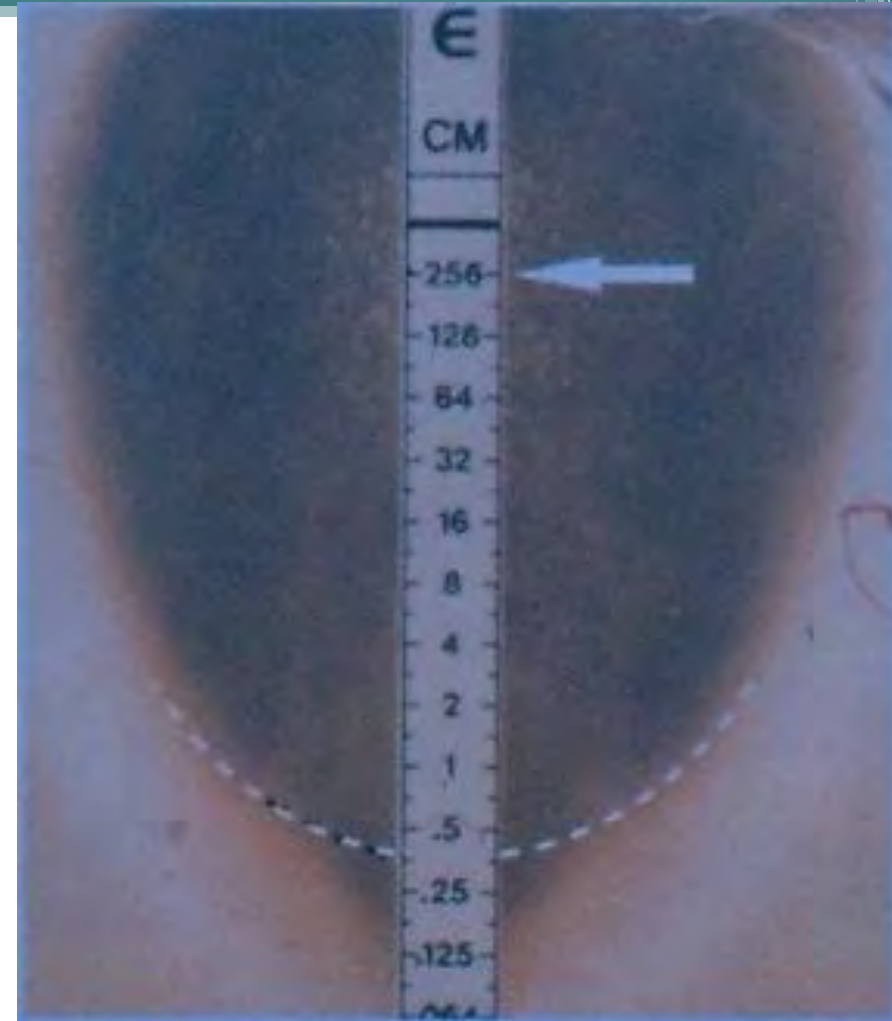
Read where the resistant subpopulation is completely inhibited. MIC > 32 µg/ml.



Isolated resistant colonies due to low-level mutation. MIC > 256 µg/ml.



$\beta$ -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  $\mu\text{g/ml}$ .



"Dip" effect due to inducible macrolide resistance. Extrapolate the ellipse towards the strip to get the MIC, i.e. 0.38  $\mu\text{g/ml}$ . This strain also had colonies at the upper range of the strip. MIC > 256  $\mu\text{g/ml}$ .

Ignore swarming by Proteus. MIC  
 $0.064 \mu\text{g} / \text{m l} .$



Tilt the plate to see pin-point colonies and hazes, especially with *enterococci*, *pneumococci*, *fusobacteria*, *Acinetobacter* and *Stenotrophomonas* spp. MIC 1  $\mu$ g / ml .



A resistant subpopulation in a *pneumococci*  
MIC >32  $\mu$  / ml

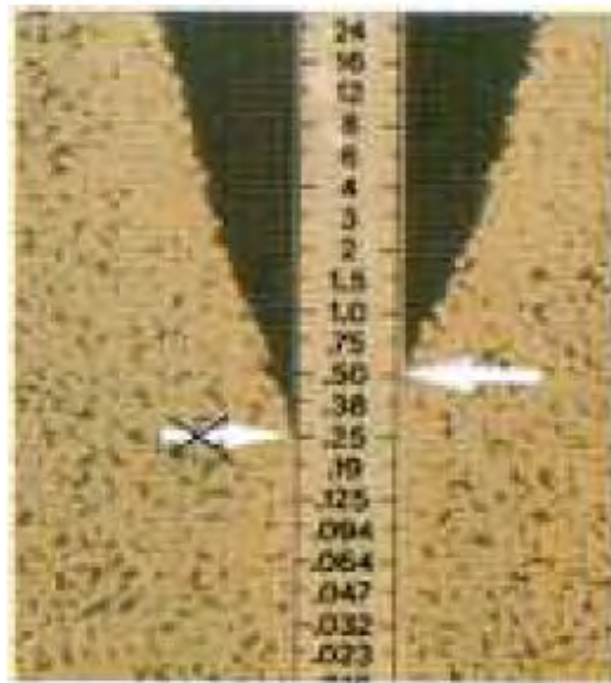


Ignore haemolysis of the blood and read at inhibition of growth. MIC 0.032  $\mu\text{g} / \text{m l}$  .





Different intersections at the strip, read the higher value (MIC 0.5  $\mu$ g / ml dilution difference, repeat the test



Ignore a thin line of growth at the edge of the strip.  
MIC 0.25  $\mu$ g / ml



# Special Applications

- ESBLs
- *Anaerobes*
- *Helicobacter* and *Campylobacter*
- *Haemophilus*
- *Mycobacteria* and *Actinomyces*
- .....

# Susceptibility testing of Anaerobes

- 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?

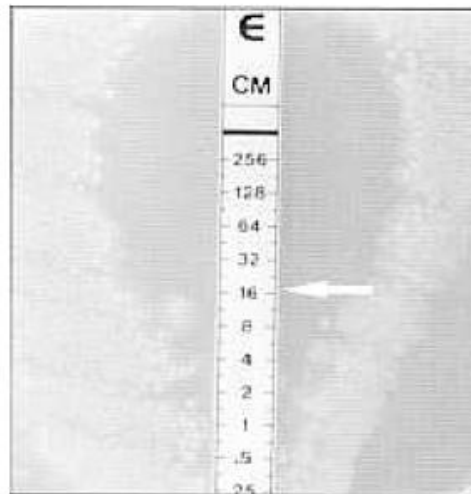
- Agar based growth supporting good growth.
- Stable antibiotic gradient minimally influenced by varying growth rates.
- Extensively validated for anaerobes(120 studies).
- Can provide 24h MIC for critical situations

# E test procedure

- Brucella agar with 5% blood, 5 g/ml hemin and 1g/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

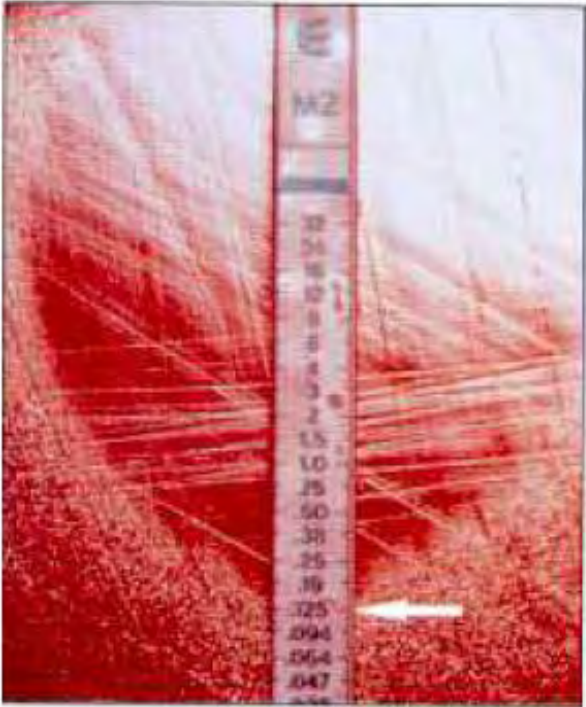
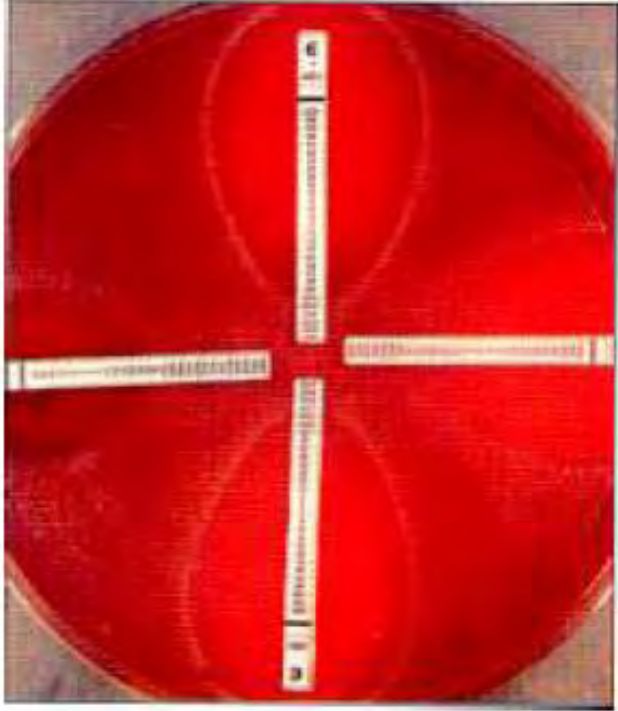


Read all colonies in the clindamycin ellipse  
for anaerobes. MIC 16  $\mu$ g / ml



# 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



Thank you

- 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  $1 \mu\text{g} / \text{m l}$   $\geq 2 \text{mm}$  zones
- Therapy failure despite in vitro susceptibility

# Limitations may occur with disc diffusion

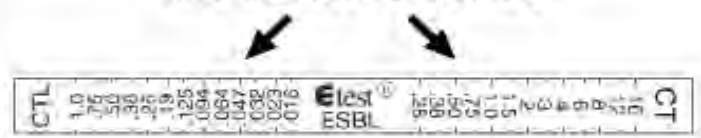
- If positioning of the ESBL disc is not proper ,approximation test is sub-optimal
- Low level ESBL will not be detected by using disc diffusion and automation
- The clavulanic acid level in double disc testing is suboptimal at the lower MIC region



# PRINCIPLE

- 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  $\mu\text{g} / \text{ml}$ ) gradient and CTL the cefotaxime (0.016-1  $\mu\text{g} / \text{ml}$ ) plus 4  $\mu\text{g} / \text{ml}$  clavulanic acid.
- TZ codes for the ceftazidime (0.5-32  $\mu\text{g} / \text{ml}$ ) gradient and TZL the ceftazidime (0.064-4  $\mu\text{g} / \text{ml}$ ) plus 4  $\mu\text{g} / \text{ml}$  clavulanic acid.

### MIC READING SCALE



Cefotaxime

Cefotaxime +  
clavulanic acid



Ceftazidime

Ceftazidime +  
clavulanic acid



Cefotaxime or  
Ceftazidime  
gradient + 4 µg/ml  
clavulanic acid

Cefotaxime or  
Ceftazidime  
gradient

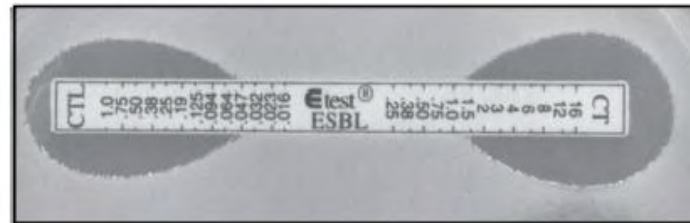
The presence of ESBL is confirmed by the appearance of :

- Phantom zone or deformation of the CT or TZ ellipse
- When either CT or TZ MIC is reduced by  $3 \log_2$  dilutions in the presence of clavulanic acid.

# Interpretation

ESBL	MIC $\mu\text{g/ml}$ Ratio	Reporting
Positive	$\text{CT} \geq 0.5$ and $\text{CT}/\text{CTL} \geq 8$ O R $\text{TZ} \geq 1$ and $\text{TZ}/\text{TZL} \geq 8$ O R “Phantom” zone or deformation of the CT or TZ ellipse.	ESBL producer and resistant to all penicillins, cephalosporins and aztreonam (NCCLS M100-S series).
Negative	$\text{CT} < 0.5$ or $\text{CT}/\text{CTL} < 8$ AND $\text{TZ} < 1$ or $\text{TZ}/\text{TZL} < 8$	ESBL non-producer and report actual MIC of all relevant drugs as determined by an MIC method.
Non determinable (ND)	$\text{CT} > 16$ and $\text{CTL} > 1$ AND $\text{TZ} > 32$ and $\text{TZL} > 4$ O R When one strip is ESBL negative and the other ND.	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.

# E test for ESBL

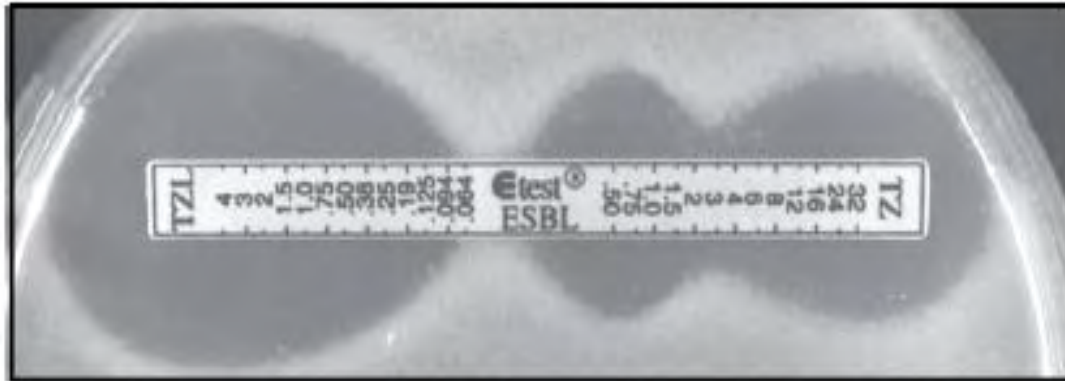


O c c a s i o n a l l y , a “ r o u n d e d ” z o n e  
CTL or TZL gradients  
while no ellipse may be seen around the CT or TZ ends



*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.*