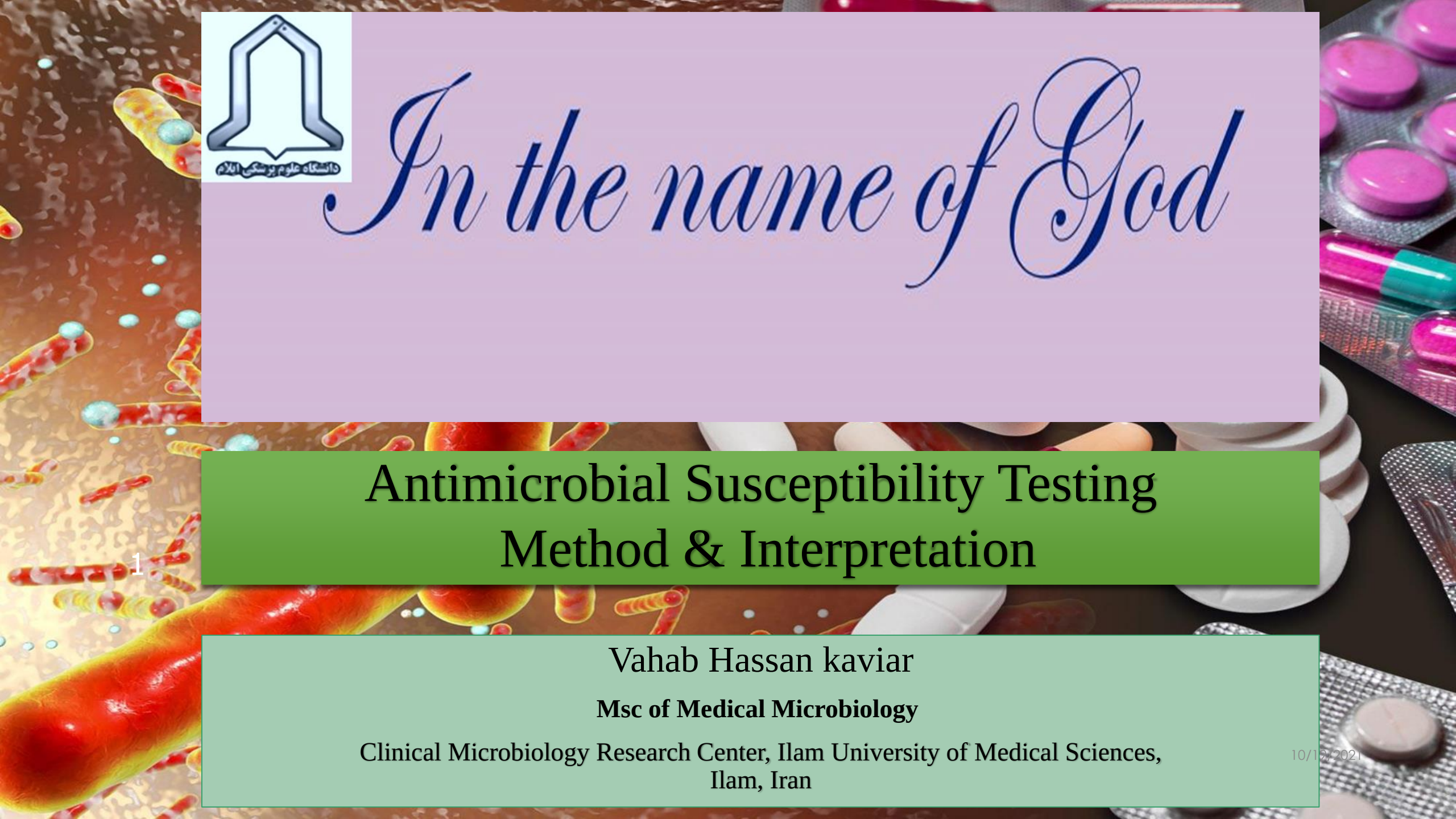



*In the name of God*

# Antimicrobial Susceptibility Testing Method & Interpretation



Vahab Hassan kaviar

**Msc of Medical Microbiology**

Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran

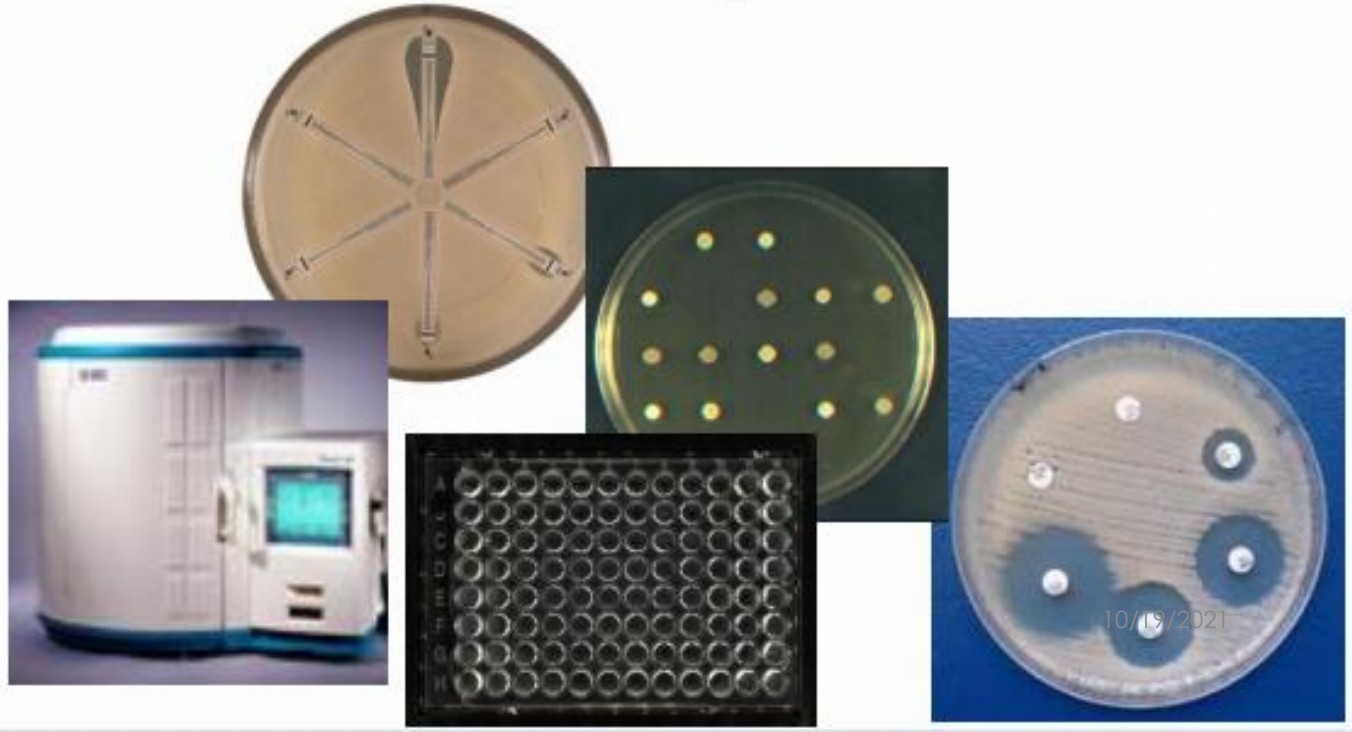
# How to determine susceptibility/resistance

Minimum Inhibitory Concentration

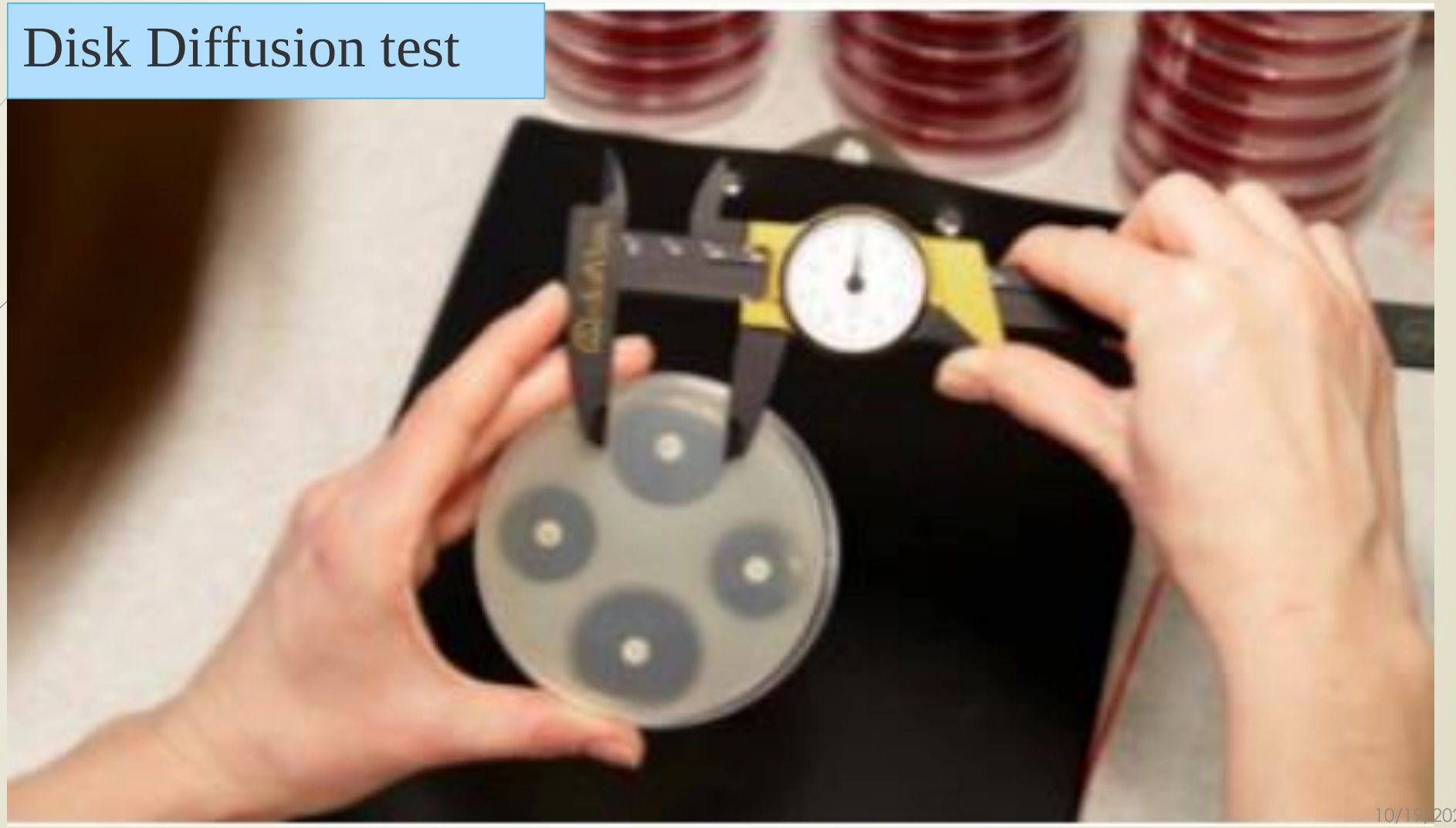
- Macro broth dilution
- Micro broth dilution
- Agar dilution
- Gradient Strips
- Automated systems

Category (S/I/R)

- Disc
- Breakpoint method



# Disk Diffusion test



# Susceptibility testing media

- Un-supplemented Mueller-Hinton (MH) agar is used for non-fastidious organisms.
- MH with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD (MH-F, Mueller-Hinton Fastidious) is used for fastidious organisms.
- Use β-Nicotinamide adenine dinucleotide (β-NAD) with a purity of ≥ 98%.

Preparation of β-NAD stock solution:

Dissolve β-NAD in sterile deionized water to a concentration of 20 mg/mL

Sterilize the solution through a 0.2 µm membrane filter.

The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

# Media for non-fastidious organisms

| Organisms | Medium |
| --- | --- |
| *Enterobacterales*<br>*Pseudomonas* spp.<br>*Stenotrophomonas maltophilia*<br>*Acinetobacter* spp.<br>*Staphylococcus* spp.<br>*Enterococcus* spp.<br>*Aeromonas* spp.<br>*Burkholderia pseudomallei* | Mueller-Hinton agar |

# Media for fastidious organisms

| Organisms | Medium |
| --- | --- |
| *Streptococcus pneumoniae*<br>Streptococcus groups A, B, C and G<br>Viridans group streptococci<br>*Haemophilus influenzae*<br>*Moraxella catarrhalis*<br>*Listeria monocytogenes*<br>*Pasteurella multocida*<br>*Campylobacter jejuni* and *coli*<br>*Corynebacterium* spp.<br>*Aerococcus sanguinicola* and *urinae*<br>*Kingella kingae* | Mueller-Hinton agar + 5% mechanically defibrinated horse blood + 20 mg/L β-NAD (MH-F) |
| Other fastidious organisms | Pending |

# Preparation of media

- Prepare media according to the manufacturer's instructions.
- For MH-F, do not add blood or β-NAD until the medium has cooled to 42-45°C and mix well after the supplements have been added to the cooled medium. For MH-F, aseptically add 50 mL mechanically defibrinated horse blood and 1 mL βNAD stock solution per litre medium. Mix well and dispense immediately.
- Pour plates on a level surface to give a uniform depth of 4.0 ± 0.5 mm. Adjust the volume if the agar depth is within the acceptable range but repeatedly above or below 4 mm.

# Quality control of Mueller-Hinton agar

- Particular problems:
- High or low concentrations of divalent cations (Ca2+, Mg2+) may be indicated by inhibition zones for aminoglycosides with *P.aeruginosa* ATCC 27853 below/above quality control limits,respectively.
- Excess thymine and thymidine may be indicated by inhibition zones ( >20mm) for trimethoprim-sulfamethoxazole and *E. faecalis* ATCC 29212 below quality control limits.
- Use a surface pH electrode to check that the pH is within the range 7.2-7.4.
- Check that the agar depth is 4 mm

# Drying and storage of agar plates

- Make sure that agar plates are at room temperature prior to inoculation.

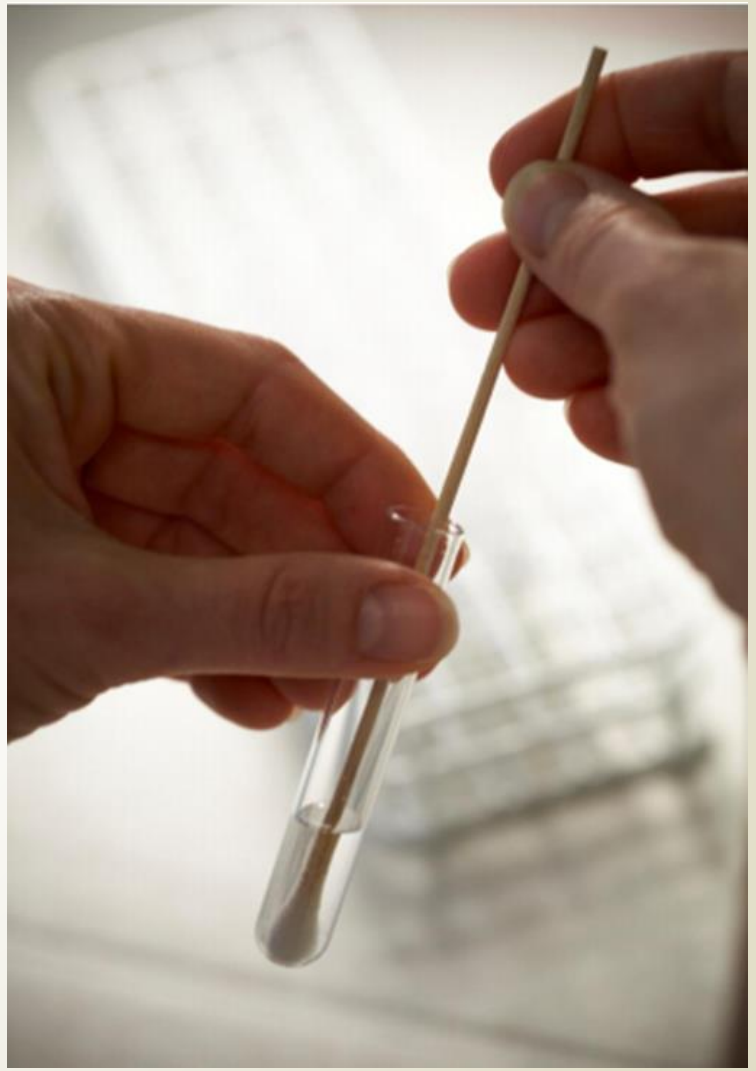
- The surface of the agar should be dry before use. Excess moisture may cause fuzzy zone edges and/or haze within zones.

No drops of water should be visible on the surface of the agar or inside the lid. This is often seen with plates stored in plastic bags or sealed containers.

- If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min.
- Do not over-dry plates.

# Inoculum

- The method requires an inoculum suspension with a turbidity equivalent to a 0.5 McFarland standard (Approximately corresponding to 1-2 x108 CFU/mL for E. coli).

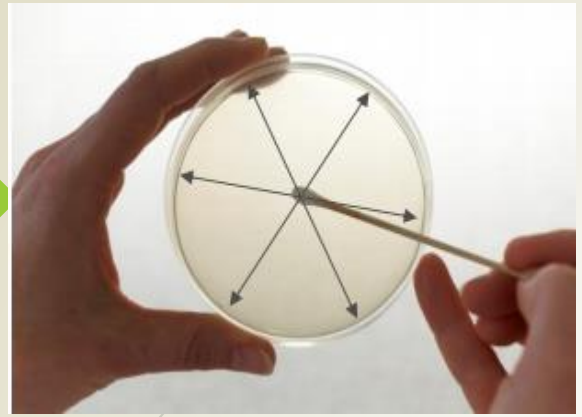


# Inoculum preparation

- Use a sterile loop or cotton swab ( or Dacron) to pick colonies from an overnight culture on non-selective media. If possible, use several morphologically similar colonies to avoid selecting an atypical variant.
- Suspend in saline and mix to an even turbidity.
- Adjust the density of the suspension to 0.5 McFarland by adding saline or more bacteria. Preferably use a photometric device to measure the turbidity.

**Exception**: *Streptococcus pneumoniae* is suspended to 0.5 McFarland from a blood agar plate, but to 1.0 McFarland from a chocolate agar plate.

| Table 2 | Preparation of 0.5 McFarland turbidity standard |
|---|---|
| 1 | Add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_20$) to 99.5 mL of 0.18 mol/L (0.36 N) $H_2S0_4$ (1% v/v) and mix thoroughly. |
| 2 | Check the density of the suspension in a spectrophotometer with a 1 cm light path and matched cuvettes. The absorbance at 625 nm should be in the range 0.08 to 0.13. |
| 3 | Distribute the suspension into tubes of the same size as those used for bacterial inoculum suspensions. Seal the tubes. |
| 4 | Store sealed standards in the dark at room temperature. |
| 5 | Mix the standard thoroughly on a vortex mixer immediately before use. |
| 6 | Renew standards or check their absorbance after storage for 6 months. |



# Inoculation of plates

- Optimally, use the inoculum suspension within 15 minutes of preparation.
- Make sure that agar plates are at room temperature prior to inoculation.
- Dip a sterile cotton swab into the suspension.
- For Gram-negative bacteria, remove excess fluid by pressing and turning the swab against the inside of the tube to avoid over-inoculation.
- For Gram-positive bacteria, do not press or turn the swab against the inside of the tube.
- For Gram-positive bacteria, take particular care to ensure that there are no gaps between streaks.
- When inoculating several agar plates with the same inoculum, dip the cotton swab into the suspension for each agar plate.

# Storage of antimicrobial disks

- Store stocks and working supplies of disks according to the manufacturers' instructions.
- Store disks in current use in sealed containers with a moisture-indicating desiccant and protected from light.
- To prevent condensation, allow disks to reach room temperature before opening containers. Rather keep disks at room temperature during the day than transfer repeatedly to and from cold storage.
- Do not use disks beyond the manufacturer's expiry date

# Application of antimicrobial disks

- Apply disks within 15 min of inoculation.
- Disks must be in close and even contact with the agar surface.
- The number of disks on a plate should be limited to avoid overlapping of zones and interference between agents. It is important that zone diameters can be reliably measured.
- Normally 6 and 12 disks are the maximum possible number on a 90 and 150 mm circular plate, respectively.
- To be able to detect inducible clindamycin resistance in staphylococci and streptococci, the erythromycin and clindamycin disks must be placed at a distance of 12-20 mm from edge to edge for staphylococci and 12-16 mm from edge to edge for streptococci

# Application of antimicrobial disks

- Loss of potency of antimicrobial agents in disks results in reduced inhibition zone diameters and is a common source of error. The following are essential:
- Store disks, including those in dispensers, in sealed containers with a moisture-indicating desiccant and protected from light (some agents, including metronidazole, chloramphenicol and the fluoroquinolones, are inactivated by prolonged exposure to light).
- Some agents are more labile than others (e.g. amoxicillin-clavulanic acid, cefaclor and carbapenems) and specific recommendations may be available from the manufacturers.
- .
- Perform frequent quality control of working supplies to control that the antimicrobial disks have not lost potency during storage.

# Incubation of plates

- Invert agar plates and make sure disks do not fall off the agar surface.
- Incubate plates within 15 min of disk application.
- Stacking plates in the incubator may affect results due to uneven heating. The efficiency of incubators varies, but for most incubators, a maximum of five plates per stack is appropriate.
- Incubation beyond the recommended time limits should not be performed as this may result in growth within inhibition zones and reporting isolates as false resistant.
- Incubate MH plates at 35±1°C in air.
- Incubate MH-F plates at 35±1°C in air with 4-6% CO2 (except for Campylobacter).

# The 15-15-15 minute rule

Follow these instructions for disk diffusion:

- Use the inoculum suspension optimally within **15 minutes** of preparation, and always within 60 minutes.
- Apply disks within **15 minutes** of inoculation.
- Incubate plates within **15 minutes** of disk application.

# Incubation of plates

| Organism | Incubation conditions |
|---|---|
| *Enterobacterales* | 35±1ºC in air for 18±2 h |
| *Pseudomonas* spp. | 35±1ºC in air for 18±2 h |
| *Stenotrophomonas maltophilia* | 35±1ºC in air for 18±2 h |
| *Acinetobacter* spp. | 35±1ºC in air for 18±2 h |
| *Staphylococcus* spp. | 35±1ºC in air for 18±2 h |
| *Enterococcus* spp. | 35±1ºC in air for 18±2 h<br>(24 h for glycopeptides) |
| *Aeromonas* spp. | 35±1ºC in air for 18±2 h |
| *Burkholderia pseudomallei* | 35±1ºC in air for 18±2 h |

# Incubation of plates

| Organism | Incubation conditions |
|---|---|
| Streptococcus groups A, B, C and G | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| Viridans group streptococci | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| *Streptococcus pneumoniae* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| *Haemophilus influenzae* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| *Moraxella catarrhalis* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| *Listeria monocytogenes* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| *Pasteurella multocida* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h |
| *Campylobacter jejuni* and *coli* | 41±1°C in microaerobic environment for 24 h (40-48 h) |
| *Corynebacterium* spp. | 35±1°C in air with 4-6% $CO_2$ for 18±2 h (40-44 h) |
| *Aerococcus sanguinicola* and *urinae* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h (40-44 h) |
| *Kingella kingae* | 35±1°C in air with 4-6% $CO_2$ for 18±2 h (40-44 h) |
| Other fastidious organisms | Pending |

# Examination of plates after incubation

- A correct inoculum and satisfactorily streaked plates should result in a confluent lawn of growth.
- The growth should be evenly distributed over the agar surface to achieve uniformly circular (non-jagged) inhibition zones.
- If individual colonies can be seen, the inoculum is too light and the test must be repeated.

# Reading zones

- Zone edges should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye.

*E. coli*
Ciprofloxacin

*S. aureus*
Erythromycin

CoNS
Trimethoprim

*S. pneumoniae*
Rifampicin

# Reading zones

- Read **MH** plates from the back against a dark background illuminated with reflected light.

- Read **MH-F** plates from the front with the lid removed illuminated with reflected light.

# Reading zones

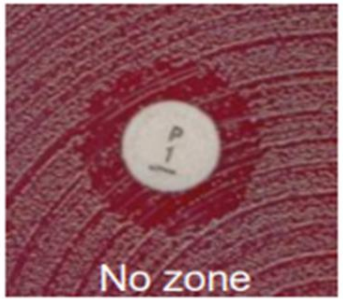
- Do not use transmitted light (plate held up to light) or a magnifying glass, unless otherwise stated.
- the zone edge should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye. Holding the plate at a 45-degree angle to the work bench may facilitate reading when zone edges are difficult to define.
- Measure zone diameters to the nearest millimetre with a ruler or a calliper. If an automated zone reader is used, it must be calibrated to manual reading.

# Colonies within zone

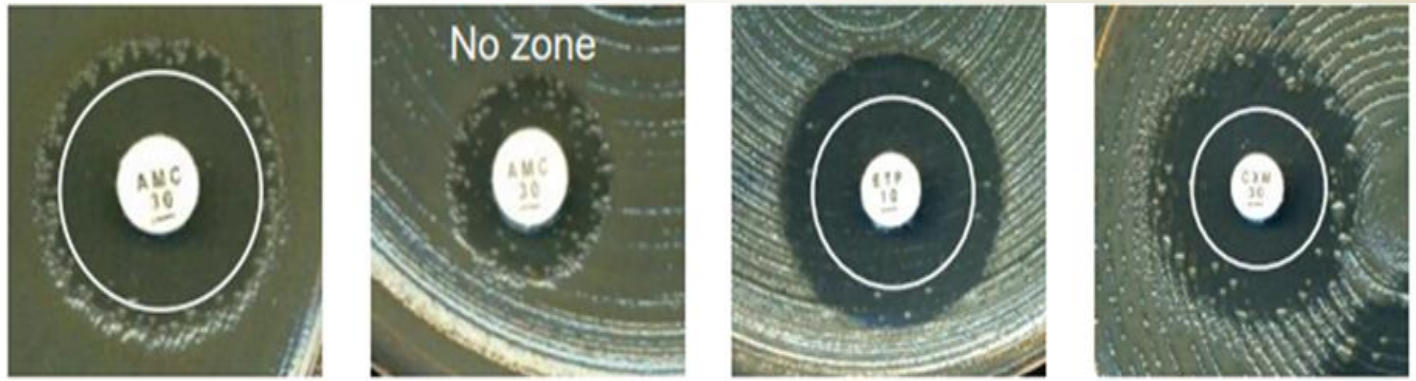
- In case of distinct colonies within zones, check for purity and repeat the test if necessary.
- If cultures are pure, colonies within zones should be taken into account when measuring the diameter.

*E. coli* with ESBL


*H. influenzae* with PBP mutations




Reading of zones with colonies within the zone.



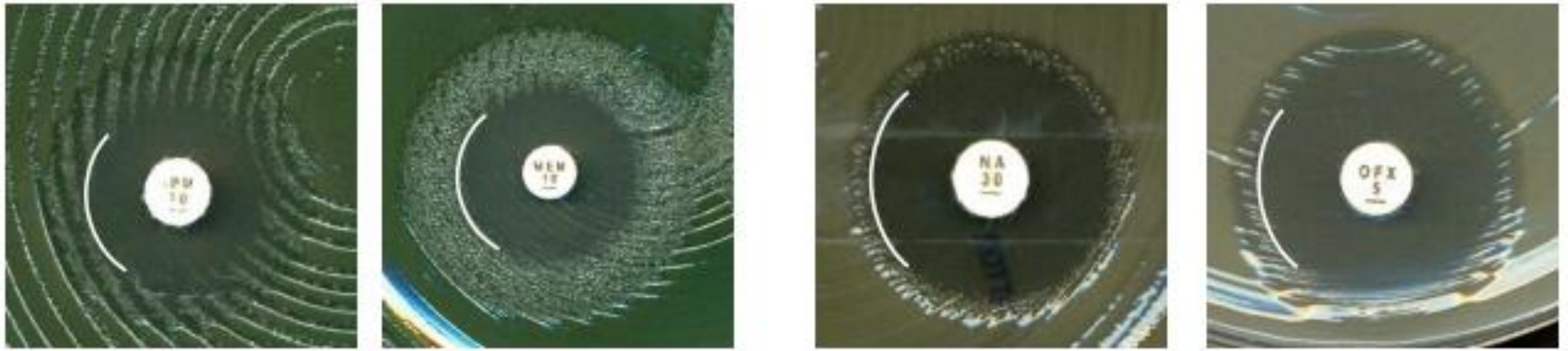

Reading of zones with colonies within the zone.

# Swarming

- For *Proteus* spp., ignore swarming and read inhibition of growth.

# Double zones

- In case of double zones, check for purity and repeat the test if necessary.
- If cultures are pure, read the inner zone.

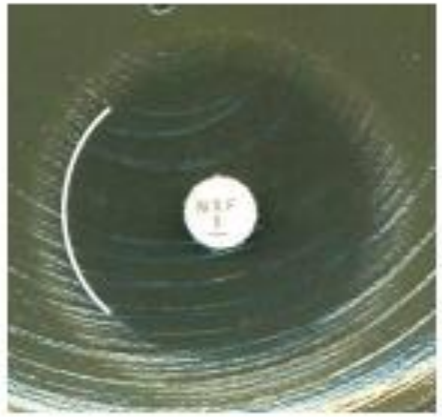





Reading of double zones.

# Fuzzy zone edges
## Enterobacterales

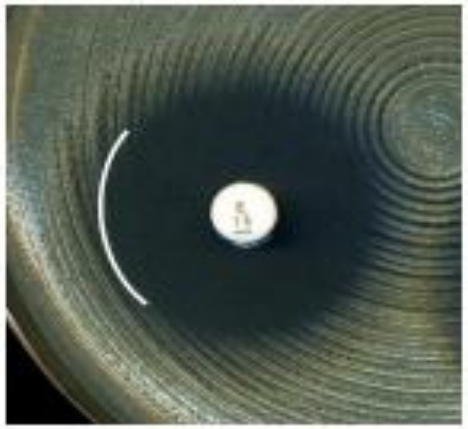
- Hold the plate against a dark background about 30 cm from the naked eye and estimate where the zone edge is. Do not hold the plate up to light (transmitted light) or use a magnifying glass.




Reading of zones with fuzzy zone edges for Enterobacterales.

# Fuzzy zone edges
## Staphylococci

- Hold the plate against a dark background about 30 cm from the naked eye and estimate where the zone edge is. Do not hold the plate up to light (transmitted light) or use a magnifying glass.





Reading of zones with fuzzy zone edges for staphylococci.

# Fuzzy zone edges
## *S. pneumoniae*

- Small colonies that are visible when the plate is hold about 30 cm from the naked eye should be taken into account when reading zones.
- The presence of small colonies close to the zone edge may be related to excess humidity in the MH-F media, and may be reduced by drying the plates prior to use.

Reading of zones with fuzzy zone edges for *S. pneumoniae*.

# β-haemolysis

- Tilt the plate back and forth to better differentiate between haemolysis and growth.
- β-haemolysis is usually free from growth.

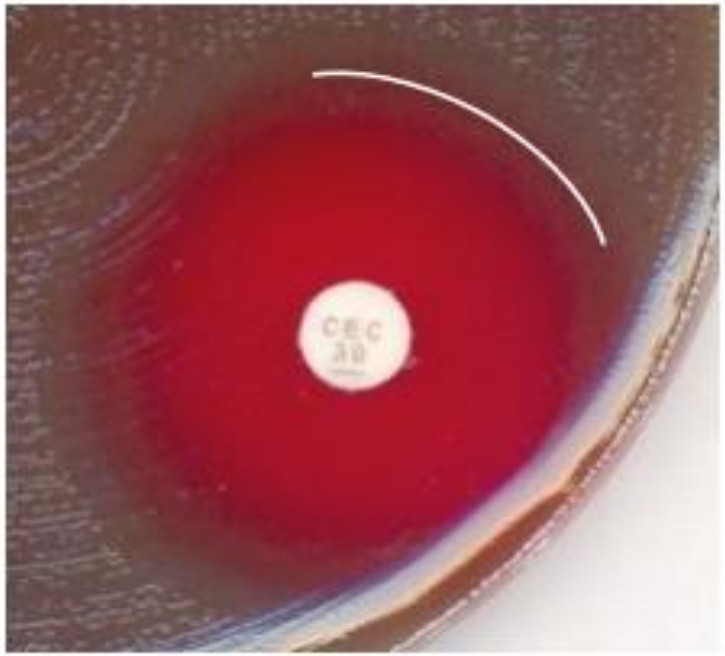
*S. pyogenes*

Streptococcus group C

# α-haemolysis

- Tilt the plate back and forth to better differentiate between haemolysis and growth.

There is usually growth in the whole area of α-haemolysis.



For some organisms, there is additional α-haemolysis without growth. Tilt the plate to differentiate between haemolysis and growth.

# Enterobacterales with ampicillin, ampicillin-sulbactam and amoxicillin-clavulanic acid

- Ignore growth that may appear as a thin inner zone on some batches of Mueller-Hinton agars. The inner zone is not seen with some batches of agar and when the outer zone is read there is no difference between batches.



# *Enterobacterales* and temocillin

- Ignore isolated colonies within the inhibition zone and read the outer zone edge.

**β-lactamase-resistant penicillin**


# *Enterobacterales* and mecillinam

- Ignore isolated colonies within the inhibition zone and read the outer zone edge.




**extended-spectrum penicillin**

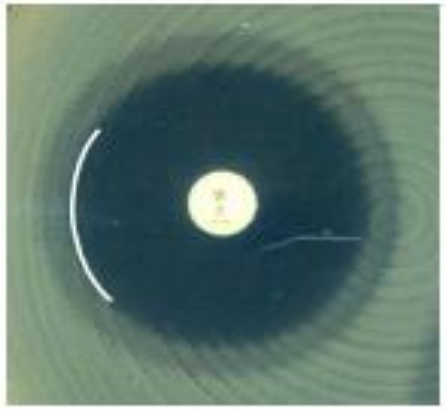
# *E. coli* and fosfomycin

- Ignore isolated colonies within the inhibition zone and read the outer zone edge.

**used to treat lower UTI**

# Trimethoprim and trimethoprim-sulfamethoxazole

- Follow the instructions for reading and read the inner zone when double zones appear (see examples below).
- Ignore haze or faint growth up to the disk within a zone with otherwise clear zone edge.



*E. coli*

CoNS

*Moraxella*

*Haemophilus*

# *S. maltophilia* with trimethoprim-sulfamethoxazole

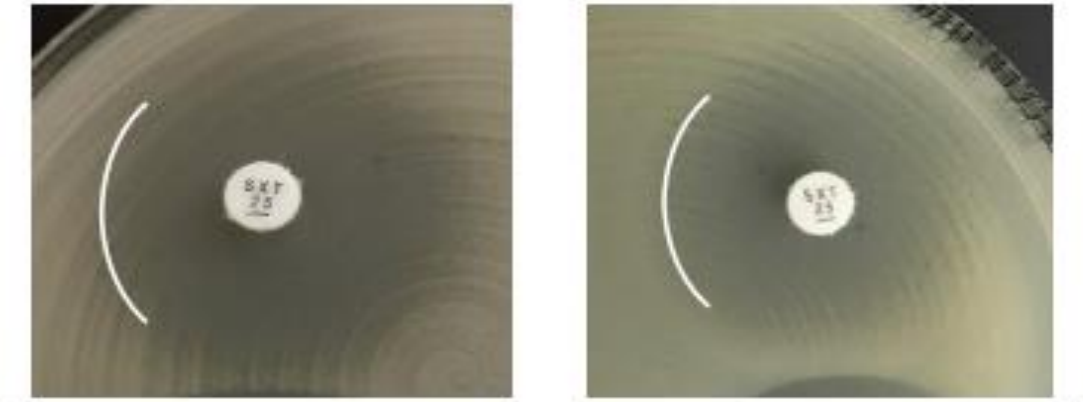
- Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
  - Read the outer zone edge and interpret according to the breakpoints.
- If there is growth up to the disk and no sign of inhibition zone, report resistant.

An outer zone can be seen


Growth up to the disk

# *B. pseudomallei* with trimethoprim-sulfamethoxazole

- Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
  - Read the outer zone edge and interpret according to the breakpoints.
- If there is growth up to the disk and no sign of inhibition zone, report resistant.

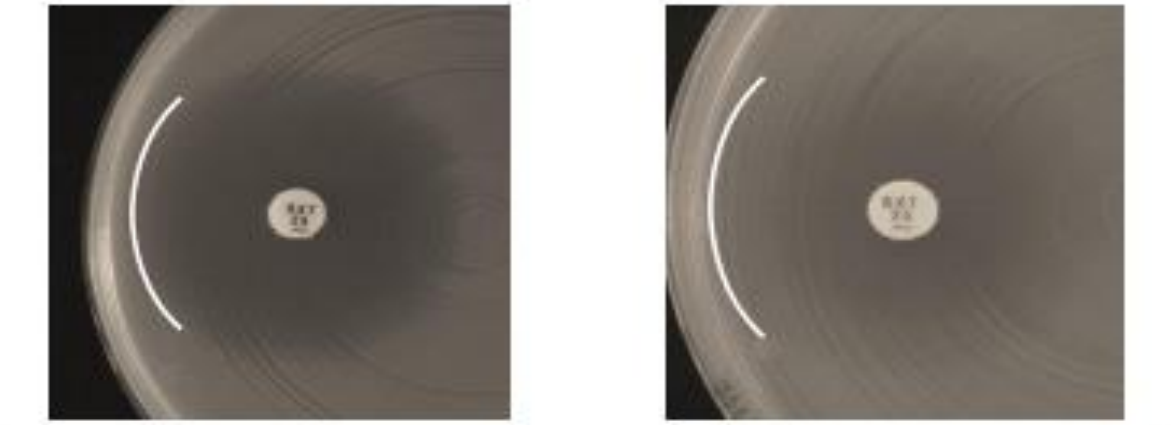



An outer zone can be seen


Growth up to the disk

# *A. xylosoxidans* with trimethoprim-sulfamethoxazole

- Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
  - Read the outer zone edge and interpret according to the breakpoints.
- If there is growth up to the disk and no sign of inhibition zone, report resistant.

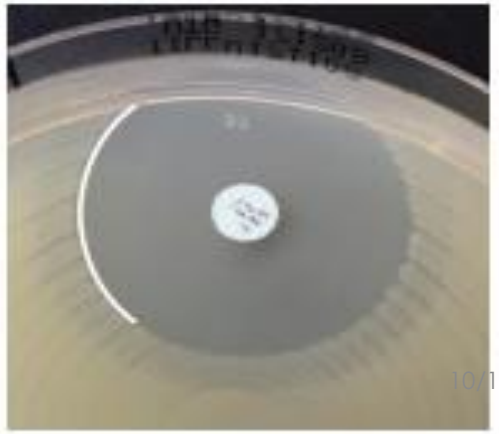


An outer zone can be seen

Growth up to the disk

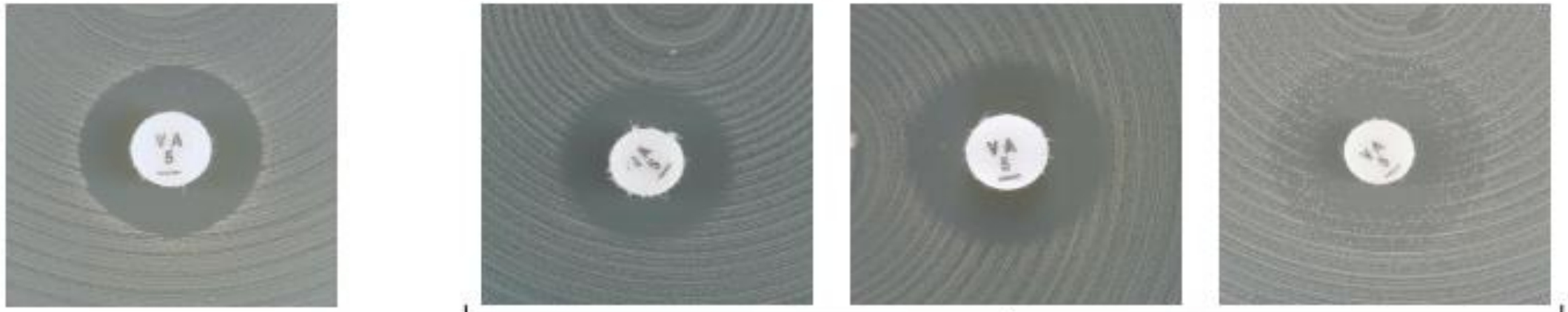
# *Aeromonas* spp. and trimethoprim-sulfamethoxazole

- Read the obvious zone edge and disregard haze or growth within the inhibition zone.
- If there is an obvious inner zone edge, read the inhibition zone as the inner zone.



# Enterococci and vancomycin

- Examine zone edge from the front of the plate with transmitted light (plate held up to light).
  - If the zone edge is sharp, report susceptible.
  - If the zone edge is fuzzy, colonies grow within the zone or if you are uncertain, suspect VRE and perform confirmatory testing, even if the zone diameter is ≥ 12 mm.
  - Isolates must not be reported susceptible before 24 h incubation.



non-VRE

VRE

# cefoxitin and *S. aureus*

- When using cefoxitin for the detection of methicillin resistance in Staphylococcus spp., measure the obvious zone, and examine zones carefully in good light to detect colonies within the zone of inhibition. These may be either a contaminating species or the expression of heterogeneous methicillin resistance.

# *S. aureus* and benzylpenicillin

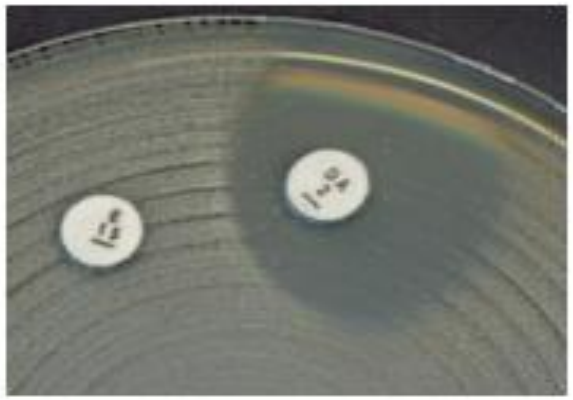
- Examine zone edge from the front of the plate with transmitted light (plate held up to light).
  - To detect penicillinase production, read the zone diameter AND examine the zone edge closely.
  - If the zone is ≥ 26 mm and the zone edge is sharp, the isolate is a pencillinase producer, report resistant.
  - If the zone is ≥ 26 mm and the zone edge is fuzzy, report susceptible.

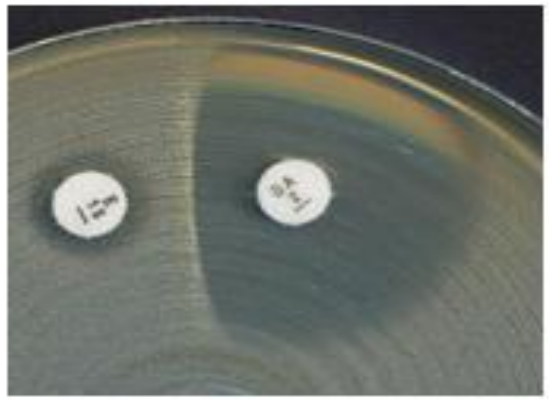
Zone ≥ 26 mm and
sharp zone edge= Resistant

Zone ≥ 26 mm and
fuzzy zone edge = Susceptible

# Detection of inducible clindamycin resistance in staphylococci

- Inducible clindamycin resistance can be detected by antagonism of clindamycin activity and a macrolide agent.
- Place the erythromycin and clindamycin disks **12-20 mm apart** (edge to edge) and look for antagonism (the D phenomenon).





Examples of D phenomenon for staphylococci.

# Detection of inducible clindamycin resistance in streptococci

- Inducible clindamycin resistance can be detected by antagonism of clindamycin activity and a macrolide agent.
- Place the erythromycin and clindamycin disks **12-16 mm apart** (edge to edge) and look for antagonism (the D phenomenon).

Examples of D phenomenon for streptococci.

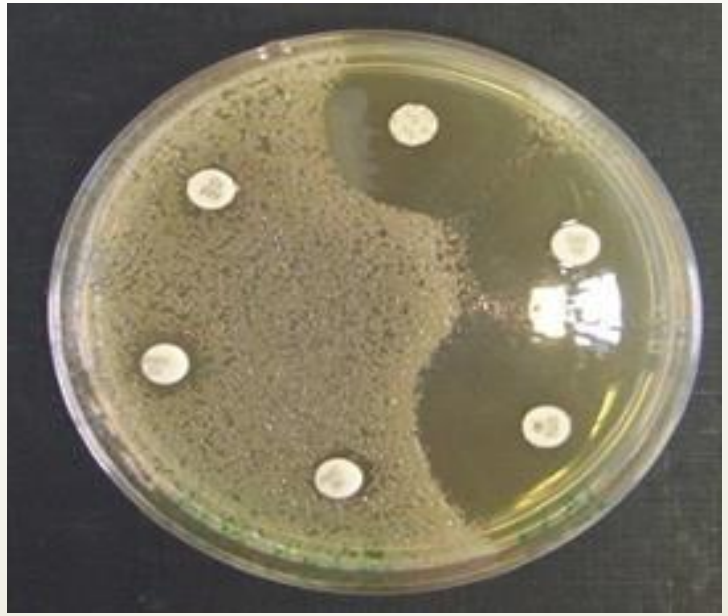
# *H. influenzae* and beta-lactam agents

- Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk.


# Zones overlap

- Too many disks per plate

**In these cases, the zone radius is considered and multiplied by 2**



# Reading zones – exceptions

| Organism | Antimicrobial agent | Reading inhibition zones |
|---|---|---|
| *Enterobacterales* | Ampicillin<br>Ampicillin-sulbactam<br>Amoxicillin-clavulanic acid | Ignore fine growth that may appear as an inner zone on some batches of MH agar. |
| *Enterobacterales* | Mecillinam | Ignore isolated colonies within the inhibition zone. |
| *E. coli* | Fosfomycin | Ignore isolated colonies within the inhibition zone and read the outer zone edge. |
| *Proteus* spp. | Any | Ignore swarming. |
| *S. maltophilia* and *B. pseudomallei* | Trimethoprim-sulfamethoxazole | Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial. |
| *S. aureus* | Benzylpenicillin | Examine zone edge from the front of the plate with transmitted light (plate held up to light). |

# Reading zones – exceptions

| Organism | Antimicrobial agent | Reading inhibition zones |
|---|---|---|
| Staphylococci | Cefoxitin | Examine zones carefully to detect colonies within the inhibition zone. |
| *Enterococcus* spp. | Vancomycin | Examine zone edge from the front of the plate with transmitted light (plate held up to light). |
| *Streptococcus* spp. | Any | Read inhibition of growth and not the inhibition of haemolysis. |
| *H. influenzae* | Beta-lactam agents | Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk. |
| *Aeromonas* spp. | Trimethoprim-sulfamethoxazole | Read the obvious zone edge and disregard haze or growth within the inhibition zone |
| Any | Trimethoprim<br>Trimethoprim-sulfamethoxazole | Ignore faint growth up to the disk and measure at the more obvious zone edge. |

# Quality control

- When sub-culturing a control strain, use several colonies to avoid selecting a mutant.

- Aminoglycosides may disclose unacceptable variation in divalent cations in the medium,
- tigecycline may disclose variation in magnesium,
- trimethoprimsulfamethoxazole will reveal problems with the thymine and thymidine,
- erythromycin can disclose an unacceptable pH.
- An agar depth above or below acceptable limits will result in smaller or larger zone diameters.

# EUCAST routine quality control strains

| Organism | Culture collection numbers | Characteristics |
|---|---|---|
| *E. coli* | ATCC 25922; NCTC 12241; CIP 76.24 DSM 1103; CCUG 17620; CECT 434 | Susceptible, wild-type |
| *E. coli* | ATCC 35218; NCTC 11954; CIP 102181 DSM 5923; CCUG 30600; CECT 943 | TEM-1 β-lactamase producer |
| *K. pneumoniae* | ATCC 700603; NCTC 13368 CCUG 45421; CECT 7787 | ESBL producer (SHV-18) |
| *K. pneumoniae* | ATCC BAA-2814 | KPC-3, SHV-11 and TEM-1 |
| *P. aeruginosa* | ATCC 27853; NCTC 12903; CIP 76.110 DSM 1117; CCUG 17619; CECT 108 | Susceptible, wild-type |
| *S. aureus* | ATCC 29213; NCTC 12973; CIP 103429 DSM 2569; CCUG 15915; CECT 794 | Weak β-lactamase producer |
| *E. faecalis* | ATCC 29212; NCTC 12697; CIP 103214 DSM 2570; CCUG 9997; CECT 795 | Susceptible, wild-type |

# EUCAST routine quality control strains

| Organism | Culture collection numbers | Characteristics |
|---|---|---|
| *S. pneumoniae* | ATCC 49619; NCTC 12977<br>CIP 104340; DSM 11967<br>CCUG 33638 | Reduced susceptibility to benzylpenicillin |
| *H. influenzae* | ATCC 49766; NCTC 12975<br>CIP 103570; DSM 11970<br>CCUG 29539 | Susceptible, wild-type |
| *Campylobacter jejuni* | ATCC 33560; NCTC 11351<br>CIP 70.2T; DSM 4688<br>CCUG 11284 | Susceptible, wild-type |

# EUCAST strains for detection of specific resistance mechanism

| Organism | Culture collection numbers | Characteristics |
|---|---|---|
| *K. pneumoniae* | ATCC 700603; NCTC 13368 CCUG 45421; CECT 7787 | ESBL producer (SHV-18) |
| *S. aureus* | NCTC 12493; CCUG 67181 | *mecA* positive, methicillin resistant (MRSA) |
| *E. faecalis* | ATCC 51299; NCTC 13379 CIP 104676; DSM 12956 CCUG 34289 | High-level aminoglycoside resistant (HLAR) and vancomycin resistant (*vanB* positive) |
| *H. influenzae* | ATCC 49247; NCTC 12699 CIP 104604; DSM 9999 CCUG 26214 | Reduced susceptibility to β-lactam agents due to PBP mutations |

# Culture Collections

- ATCC American Type Culture Collection, USA http://www.atcc.org.
- NCTC National Collection of Type Cultures, Public Health England, UK https://www.phe-culturecollections.org.uk/collections/nctc.
- CIP Collection de l`Institut Pasteur, France https://www.pasteur.fr/en/public-health/biobanks-and-collections/collectioninstitut-pasteur-cip.
- DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) https://www.dsmz.de.
- CCUG Culture Collection University of Gothenburg, Sweden http://www.ccug.se.
- CECT Colección Española de Cultivos Tipo, Spain http://www.cect.org

# Potential sources of error

| | |
|---|---|
| **Medium** | Storage of plates |
| | Not prepared according to instructions |
| | Batch to batch variation or change of supplier of agar |
| | Supplements (batch to batch variations, incorrect amount or expired) |
| | pH |
| | Agar depth/Agar volume |
| | Expiry date |
| **Test conditions** | "15-15-15 minute"-rule not adhered to (suspension used within 15 min, disks applied within15 min, incubation within 15 min) |
| | Incubation (temperature, atmosphere and time) |
| | Incorrect inoculation (too light, too heavy or uneven) |
| | Reading conditions (background, light) |
| | Reading zone edges |

# Potential sources of error

| | |
|---|---|
| **Disks** | Incorrect disk (wrong agent or wrong disk strength) |
| | Disk potency (incorrect storage, labile agent, expiry date) |
| | Disks not at room temperature when containers opened |
| | Too many disks on plate (interference between agents) |
| **Control organisms** | Incorrect QC strain |
| | Mutation |
| | Contamination |
| | Age of culture |

# Troubleshooting

- Aminoglycosides

➢ Zone too small

I. pH of media too low

II. Ca++ and/or Mg++ content too high

➢ Zone too large

I. pH of media too high

II. Ca++ and/or Mg++ content too low

Acceptable pH range =7.2–7.4

Avoid CO2 incubation

# Troubleshooting

- Amoxicillin-clavulanate

➢ Zone too small

✓ Clavulanate is labile, Disk has lost potency

❑ Check storage conditions and package integrity

# Troubleshooting

- Penicillins, Tetracyclines

➢ Zone too large
✓ pH of media too low
❑ Acceptable pH range=7.2–7.4
❑ Zone too small
✓ pH of media too high

- Clindamycin, Quinolones , Macrolides

In contrast to penicillins

# Troubleshooting

- If zones too large

✓ Inoculum too light

✓ Error in inoculum preparation

✓ Media depth too thin

✓ MHA nutritionally unacceptable

➢ Repeat

1. McFarland 0.5 turbidity standard
2. Check expiration date and proper storage
3. Use agar with depth approximately 4 mm

# Minimum Inhibitory Concentration (MIC)

# Media for MIC determination

- **MH broth:** un-supplemented cation-adjusted Mueller-Hinton broth, is used for testing of non-fastidious organisms.

- **MH-F broth:** cation-adjusted MH broth supplemented with 5% lysed horse blood and 20 mg/L β-NAD, is used for testing Streptococcus spp. (including S. pneumoniae), Haemophilus influenzae, Moraxella catarrhalis, Listeria monocytogenes, Campylobacter jejuni and coli, Pasteurella multocida, Corynebacterium spp., Aerococcus sanguinicola and urinae, Kingella kingae and several other fastidious organisms.

# Preparation MH-F broth

- Prepare and autoclave cation-adjusted MHB according to the manufacturer's instructions, but with 100 mL less deionized water per litre to allow for the addition of lysed horse blood.
- Cool medium to 42-45°C. Aseptically add 100 mL 50% lysed horse blood and 1 mL β-NAD stock solution per litre medium and mix well. Dispense MH-F broth in sterile containers with screw caps.
- Store MH-F broth at 4-8°C. A shelf life of 3 months can be expected.
- Check that the pH is within the range 7.2-7.4.

# Preparation of Stock Solution

$$\text{Weight of powder (mg)} = \frac{\text{Volume of solution (mL)} \times \text{Concentration (mg/L)}}{\text{Potency of powder (mg/g)}}$$

$$M_1V_1 = M_2V_2.$$

$$10{,}000\ \mu g/mL \times V_1\ mL = 256\ \mu g/mL \times 5\ mL.$$

# Preparation of Stock Solution

- Concentrations of stock solutions should be 1000 mg/L or greater.
- Sterilization of solutions is not usually necessary. If required, sterilization should be by membrane filtration, and samples before and after sterilization should be compared by assay to ensure that adsorption has not occurred.
- Unless otherwise instructed by the manufacturer, stock solutions may be stored in aliquots at −20 °C or below. Most agents will keep at −60 °C for at least 6 months. Use stock solutions promptly on defrosting and discard unused solutions.

# Solvent of Antibiotics

| Antimicrobial agent | Solvent | Diluent |
|---|---|---|
| Amoxycillin | Phosphate buffer 0.1 M, pH 6.0 | Phosphate buffer 0.1 M, pH 6.0 |
| Ampicillin | Phosphate buffer 0.1 M, pH 8.0 | Phosphate buffer 0.1 M, pH 6.0 |
| Azithromycin | Ethanol 95% | Water |
| Aztreonam | Saturated sodium bicarbonate solution | Water |
| Cefepime | Phosphate buffer 0.1 M, pH 6.0 | Phosphate buffer 0.1 M, pH 6.0 |
| Cefpodoxime | 0.1% sodium bicarbonate solution | Water |
| Ceftazidime | Saturated sodium bicarbonate solution | Water |
| Cephalothin | Phosphate buffer 0.1 M, pH 6.0 | Water |
| Chloramphenicol | Ethanol 95% | Water |
| Clarithromycin | Methanol | 0.1 M phosphate buffer, pH 6.5 |
| Clavulanic acid | Phosphate buffer 0.1 M, pH 6.0 | Phosphate buffer 0.1 M, pH 6.0 |
| Erythromycin | Ethanol 95% | Water |
| Fusidic acid | Ethanol 95% | Water |
| Imipenem | Phosphate buffer 0.01 M, pH 7.2 | Phosphate buffer 0.01 M, pH 7.2 |
| Levofloxacin | Half volume water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water | Water |
| Meropenem | Phosphate buffer 0.01 M, pH 7.2 | Phosphate buffer 0.01 M, pH 7.2 |
| Naladixic acid | Half volume water, a minimum volume 1 M NaOH to dissolve then make up to total volume with water | Water |
| Nitrofurantoin | Minimum volume dimethylformamide to dissolve, then make up to total volume with phosphate buffer 0.1 M, pH 8.0 | Phosphate buffer 0.1 M, pH 8.0 |
| Norfloxacin | Half volume of water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water | Water |
| Ofloxacin | Half volume water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water | Water |
| Rifampicin | Methanol | Water |
| Sulbactam | Phosphate buffer 0.1 M, pH 6.0 | Phosphate buffer 0.1 M, pH 6.0 |
| Sulfonamides | Half volume water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water | Water |
| Ticarcillin | Phosphate buffer 0.1 M, pH 6.0 | Phosphate buffer 0.1 M, pH 6.0 |
| Trimethoprim | Half volume water, a minimum volume 0.1 M lactic acid or 0.1 M HCl to dissolve, then make up to a total volume with water. | Water |

# Preparation of inoculum

- Standardization of inoculum is vital for accurate and reproducible susceptibility testing.
- The inoculum may be prepared by diluting a broth culture or by emulsifying overnight colonies from an agar medium in broth or saline.
- For either method four or five colonies of a pure culture on agar are chosen to avoid selecting an atypical variant.

# Broth culture method

- The colonies are touched with a loop and the growth transferred to broth such as tryptic soy broth or brain heart infusion.
- The broth used must not be antagonistic to the agent tested. The broth is incubated at 35–37 °C until the growth reaches a turbidity equal to or greater than that of a 0.5 McFarland standard.
- 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.

# Direct colony suspension method

- Cultures must be less than 30 h old. The colonies are touched with a loop and the growth transferred to sterile broth or saline. The suspension is adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard.

- 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 all organisms the precise concentration of cells in the final inoculum will depend on the state of the culture.

# Colony suspension method

- Plates or tubes must be inoculated within 30 min of standardizing the inoculum to maintain viable cell density.
- The inoculum prepared above is diluted in broth to give a final organism density of $5 \times 10^5$ cfu/mL (range $3–7 \times 10^5$ cfu/mL).
- The dilution required depends on the method being used for testing, e.g. transfer of 0.1 mL of organism suspension to a tube containing 9.9 mL of broth will give an inoculum density of $1 \times 10^6$ cfu/mL which, when mixed with an equal volume of antimicrobial solution in tubes or wells will result in a final inoculum of $5 \times 10^5$ cfu/mL.
- Periodically viable counts should be performed on inoculum suspensions to ensure that inocula contain approximately $5 \times 10^5$ cfu/mL. This may be done by removing 10 µL from the growth control well or tube immediately after inoculation and diluting it in 10 mL of broth or saline

100 µL of this dilution is spread over the surface of a suitable agar plate which is then incubated overnight. Fifty colonies would be expected from an original inoculum of $5 \times 10^5$ cfu/mL.

# Incubation of tubes and microdilution plates

- Incubate tubes or plates at 35–37 °C in air for 16–20 h for most antimicrobial agent/organism combinations.
- Incubation time should be extended to 24 h when testing *Haemophilus* spp. and streptococci and before interpreting results from suspected oxacillin resistant *staphylococci* or glycopeptide resistant *enterococci*.
- Incubation at 30–35 °C will increase the likelihood of detection of oxacillin resistance and should be used if testing oxacillin susceptibility.
- In order to avoid uneven heating do not stack microdilution plates more than four high.

# Reading results

- Broth microdilution is the reference method for antimicrobial susceptibility testing of rapidly growing aerobic bacteria, except for mecillinam and fosfomycin, where agar dilution is the reference method
- Results must only be read when there is sufficient growth of the test organism (i.e. obvious button or definite turbidity in the positive growth control), no growth in the uninoculated or negative growth control (where present) and when a purity plate shows that the test organism was pure.
- The amount of growth in each tube or well is compared with that in the positive growth control and the MIC recorded as the lowest concentration of the agent that completely inhibits growth.
- for sulfonamides, the lowest concentration that inhibits 80% of growth.
- When testing glycopeptide susceptibility of enterococci, any faint turbidity should be classified as growth.
- When testing fastidious organisms an appropriate quality control organism must also be tested

# Oxacillin on staphylococci

- Resistance to oxacillin can be difficult to detect. The following conditions will aid detection of resistant strains:

1. Incorporation of NaCl at a final concentration of 2% w/v in the broth.

2. Use of the direct suspension method for preparing bacterial suspensions rather than the growth method.

3. Incubation of tests for a full 24 h.

4. Incubation temperature of not more than 35 °C.

# Tests on β-lactamase-producing organisms

- When testing organisms that produce extracellular β-lactamases against penicillins and cephalosporins, the MIC may be significantly affected by the density of inoculum. The standard inoculum may result in MICs only slightly higher than obtained with susceptible strains.

- β-Lactamase production can be more reliably detected in *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Moraxella catarrhalis* by definitive tests such as nitrocefin-based techniques.
- Among Gram-negative organisms the effect of inoculum depends on the amount of enzyme produced, and the activity of the enzyme against the particular penicillin or cephalosporin under test. Effective standardization of the inoculum is necessary to avoid large variations in MIC results.

# Reading broth microdilution

- Results are only valid when the following criteria are met:
- Sufficient growth, i.e. obvious button or definite turbidity, in the positive growth control.
- Pure culture − Check for purity by sub-culturing from the growth-control well immediately after inoculation onto a non-selective agar plate for simultaneous incubation.
- Correct inoculum 5 x 105 CFU/mL − Viable colony counts can be performed by removing 10 µL from the growth-control well or tube immediately after inoculation and diluting in 10 mL of saline. Mix and spread 100 µL onto a non-selective agar plate. After incubation, the number of colonies should be approximately 20-80.

# Growth appearance

- Growth appears as turbidity or as a deposit of cells at the bottom of the well. The appearance of growth differs depending on the microorganism and the antimicrobial agent tested.
- For round-bottom wells, growth will most often appear as a button/pellet centered in the middle. For flat-bottom wells, growth may be scattered.
- Growth in antibiotic-containing wells may differ from growth seen in the positive growth control, even for pure cultures.

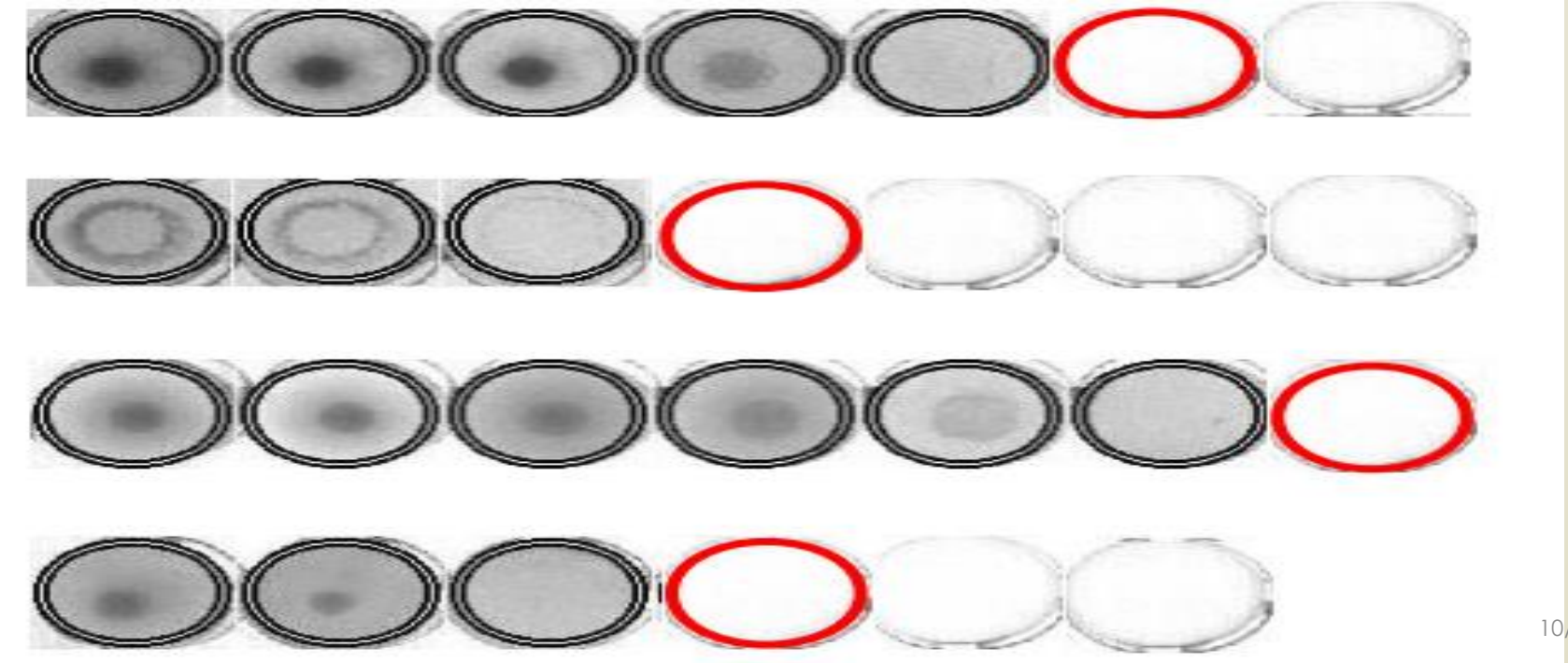
# Reading MIC endpoints

- Read the MIC as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye.

- Unless otherwise stated, endpoints should be read at complete inhibition of growth

# Turbidity without pellet

- Haze or turbidity without a pellet is often seen for *Pseudomonas* spp. and *Acinetobacter* spp. This should be regarded as growth and the endpoint read at the first well with complete inhibition (clear broth).

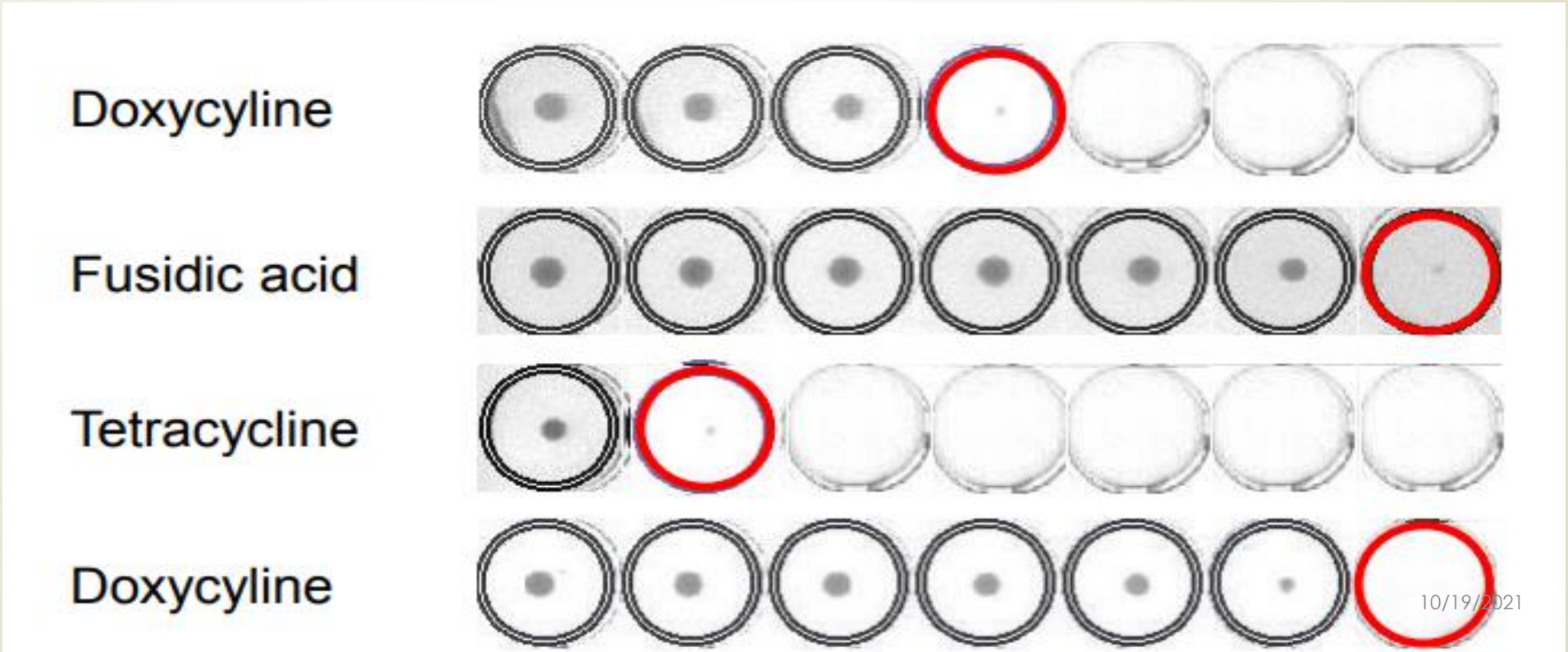


# Hemolysis

- For fastidious organisms tested in MH-F broth, haemolysis of the blood can be seen. This is often accompanied by turbidity or a deposit of growth (pellet).
- Haemolysis with turbidity or pellet should be regarded as growth when determining endpoints.

# Gram-positive cocci with bacteriostatic antimicrobial agents

- Disregard pinpoint growth (tiny buttons) when trailing growth occurs.

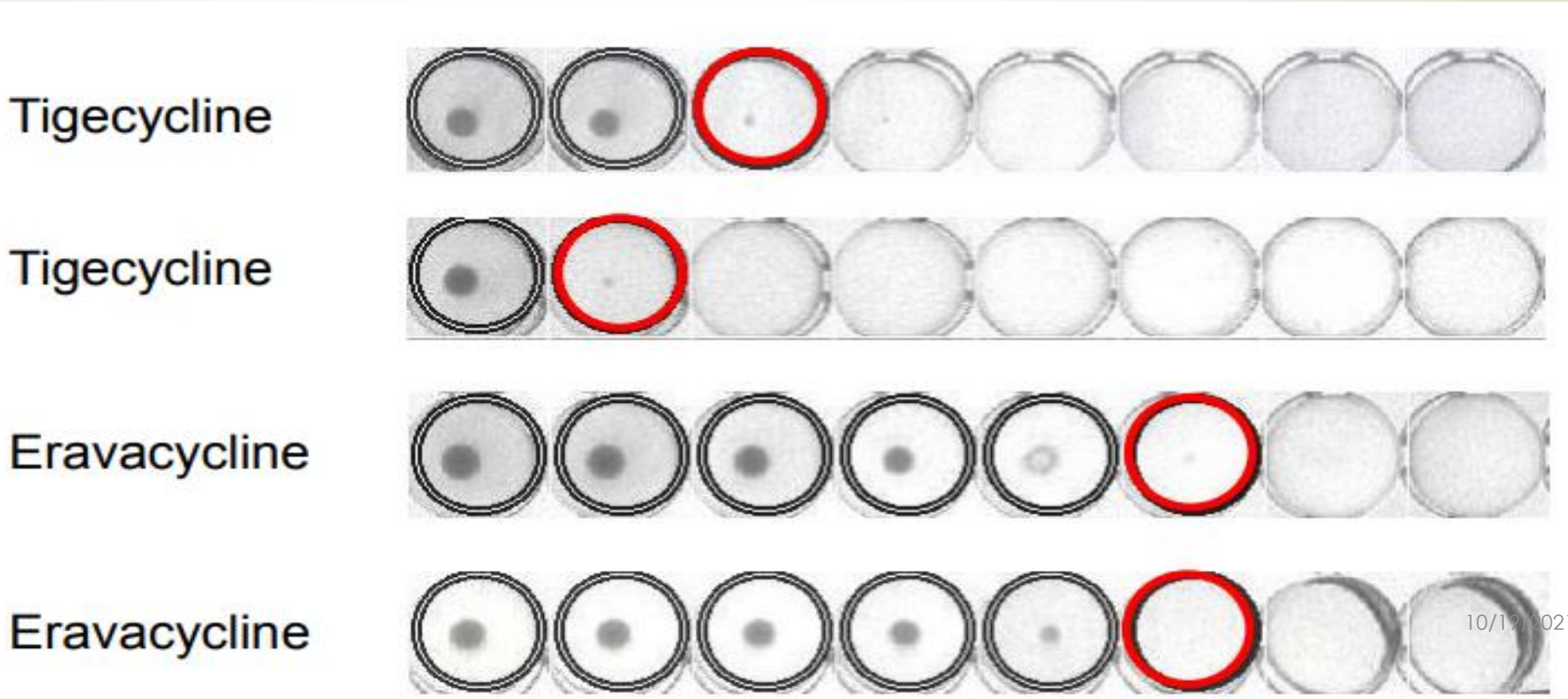


# Gram-positive cocci with bacteriostatic antimicrobial agents

- Disregard pinpoint growth (tiny buttons) when trailing growth occurs.

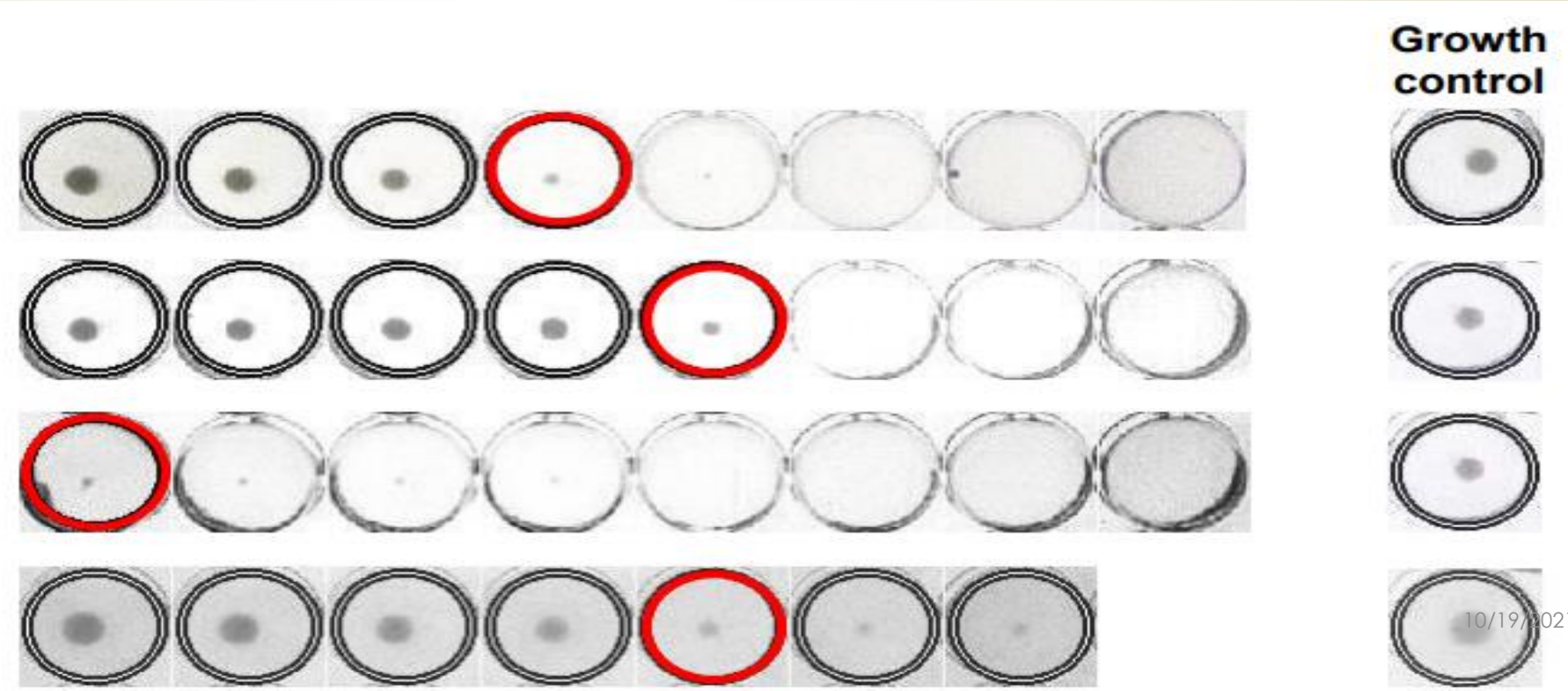
# Gram-negative organisms with tigecycline and eravacycline

- Disregard pinpoint growth (tiny buttons) when trailing growth occurs.



# Trimethoprim and trimethoprim-sulfamethoxazole

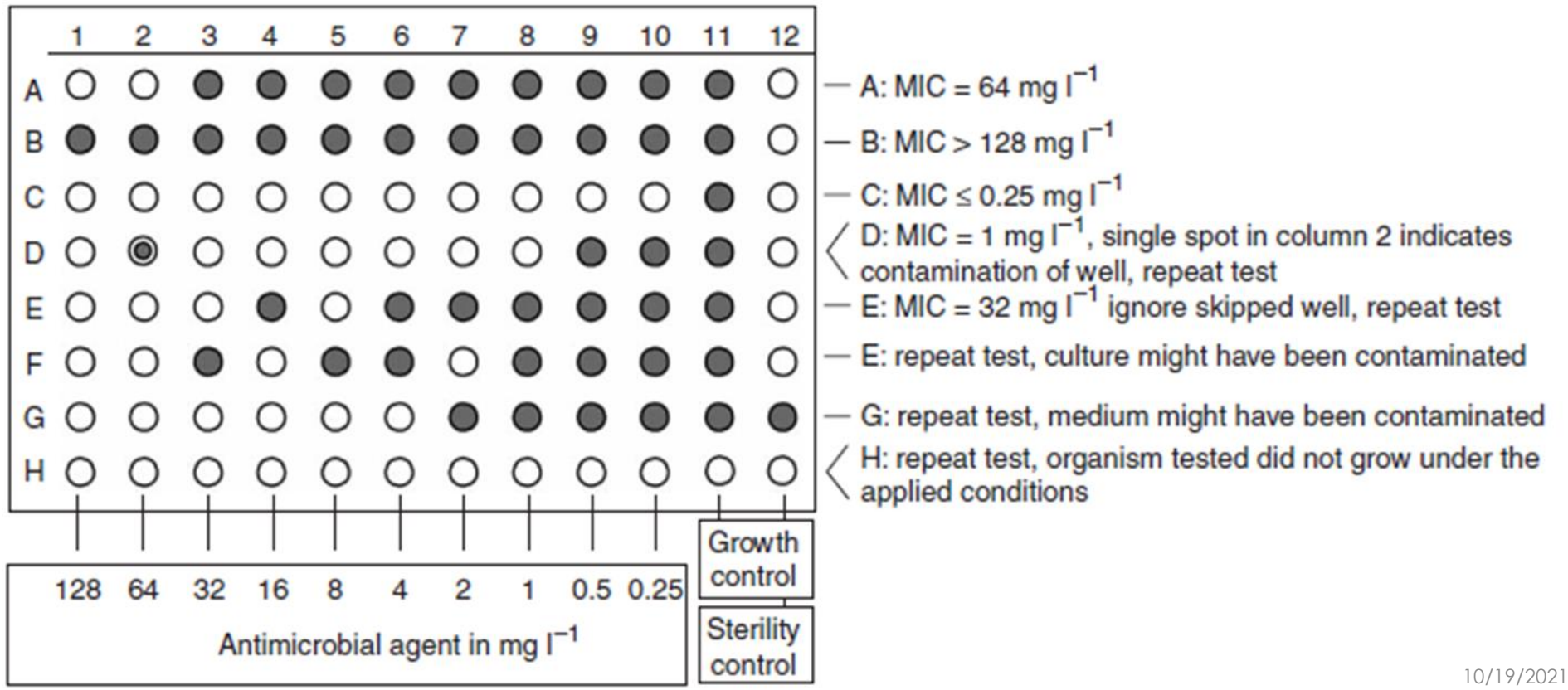
- Read the MIC at the lowest concentration that inhibits ≥80 % of growth as compared to the growth control.



# Skipped wells

- Occasionally a skip may be seen, i.e. a well showing no growth bordered by wells showing growth. There are several possible explanations including incorrect inoculation, contaminations, heterogenous resistance etc.
- When a single skipped well occurs, retest the isolate or read the highest MIC value to avoid reporting isolates as false susceptible.
- Do not report results for antimicrobial agents for which there is more than one skipped well.

# Troubleshooting



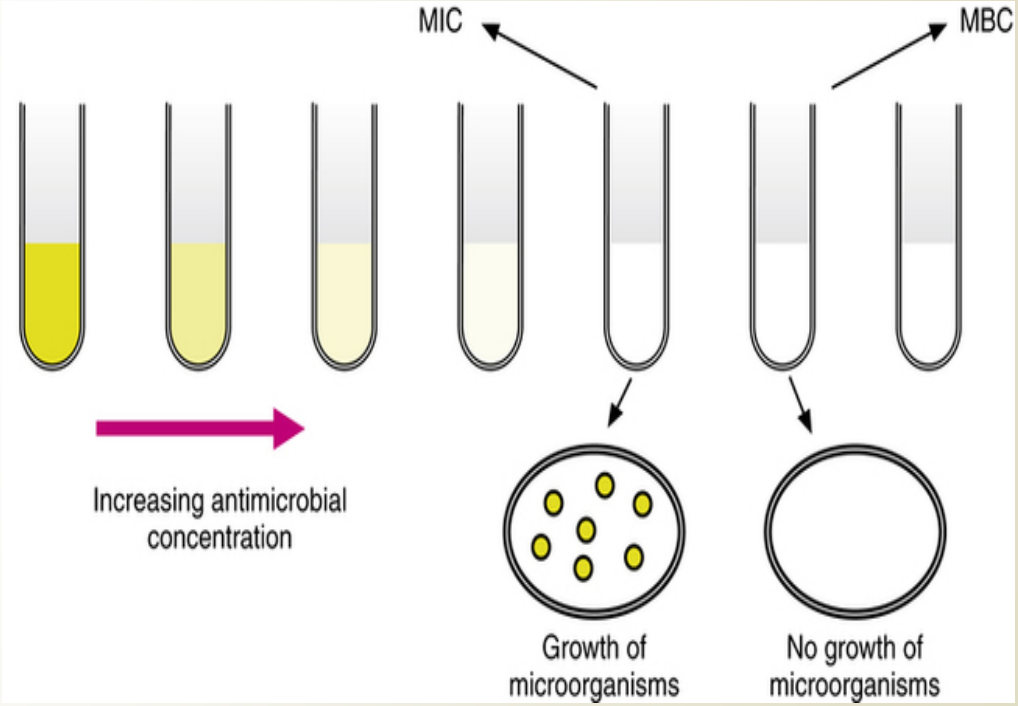
# Interpretation of results

- Make sure that MIC values for relevant Quality Control strains are within acceptable ranges before reporting results for clinical isolates. See quality control criteria in EUCAST QC Tables (www.eucast.org).

- Interpret MIC values into susceptibility categories (S, I and R) according to the current EUCAST Breakpoint Tables.

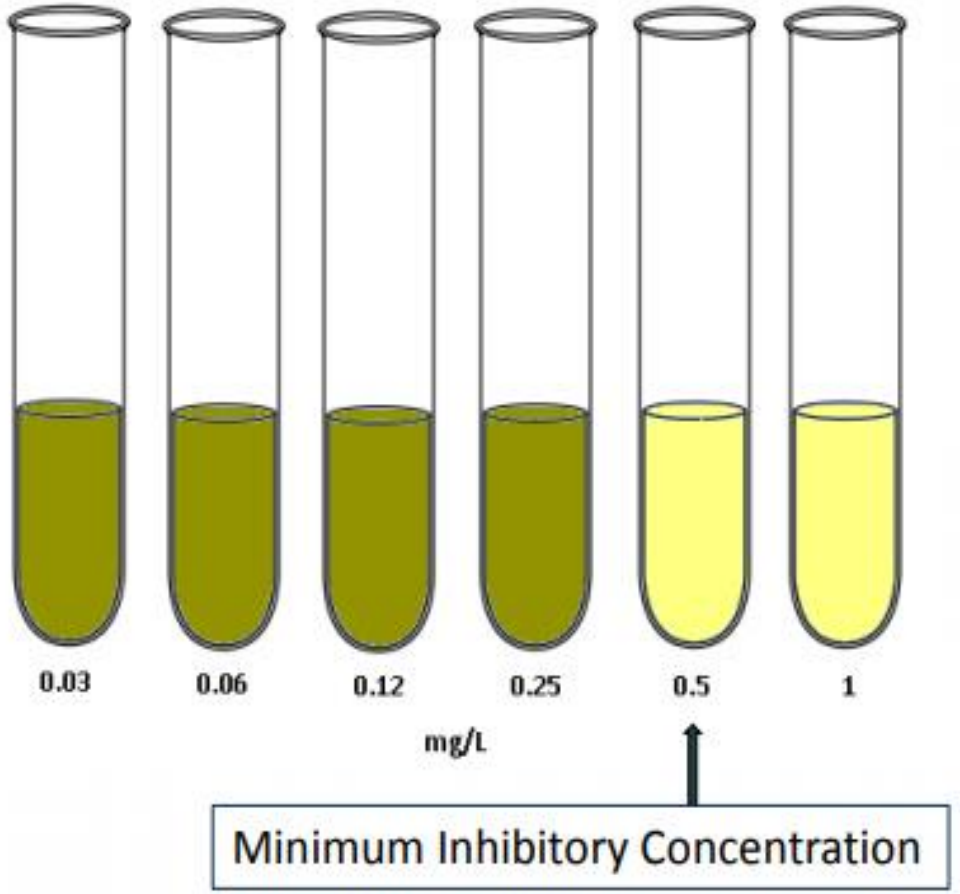
# Minimum Bactericidal Concentration

- The lowest concentration of an antibiotic killing the majority (99.9%) of a bacterial inoculum.

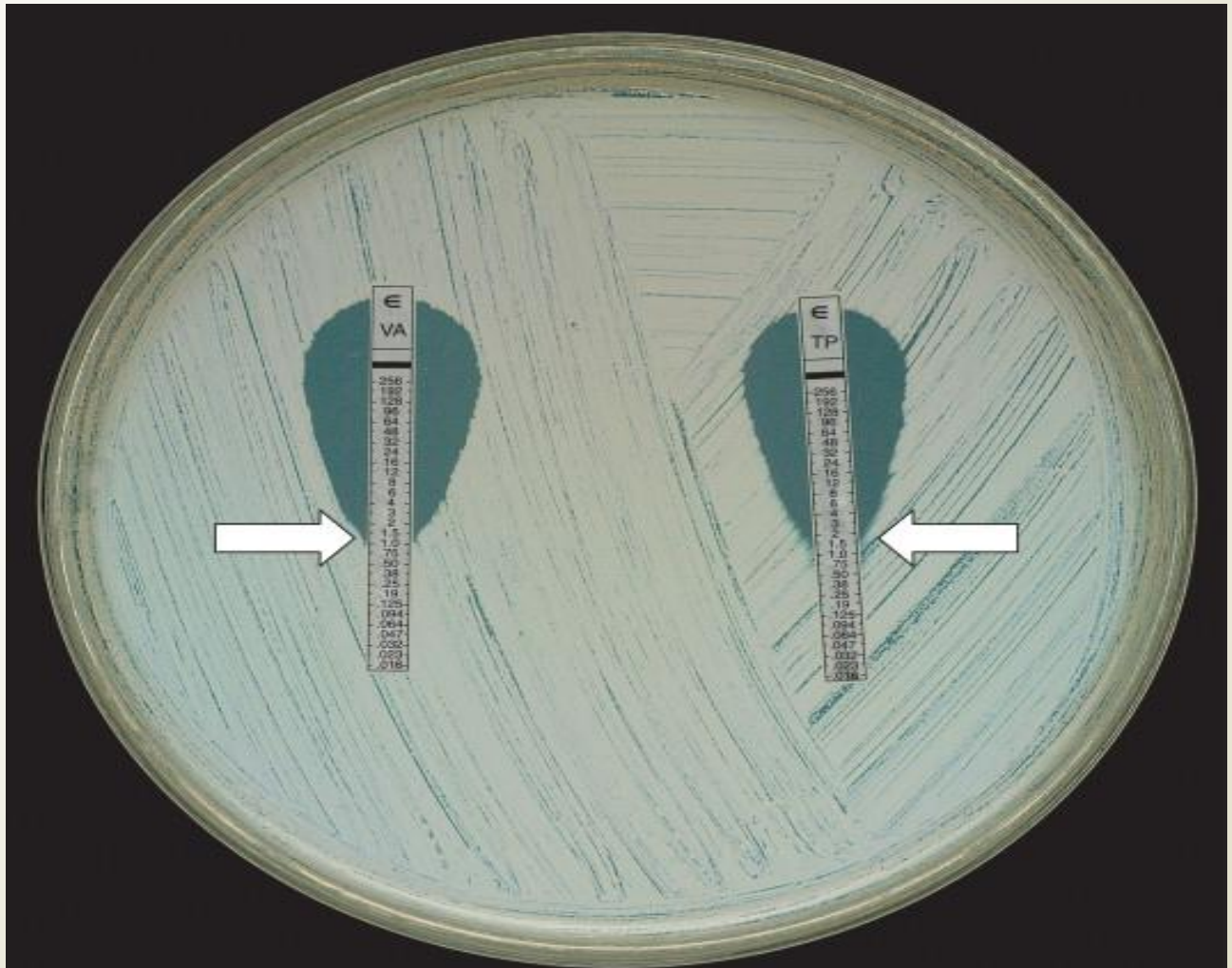


# Macrobroth dilution (tube dilution)

- Broth media (1-2mL)
- Antimicrobial dilution: log2
  - Control tube
- Inoculum; direct or growth
  - touching 2 to 5 morphologically similar colonies
  - 0.5 McFarland ($10^8$); dilute to achieve $10^5$
  - Control organisms
- Incubation
- Reading



# Etest



# THANK YOU FOR YOUR KIND ATTENTION!