

Welcome to the NordicAST Basic AST webinar

Today's program:

- Welcome (Erika Matuschek)
- EUCAST Disk diffusion method (Erika Matuschek)
- Discussions/Mentimeter (Anna Hill)
 - 15 minutes' break
- Reading of zones (Jenny Åhman)
- Discussions/Mentimeter (Ingvild Haugan)
- Conclusions and evaluation (Nathalie Friberg)

For Mentimeter questions: Use your browser and go to menti.com or install the App "Mentimeter": Code: 8170 7583

Use the chat to ask questions.

The EUCAST disk diffusion method

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Standardisation of Antimicrobial Susceptibility Testing (AST)

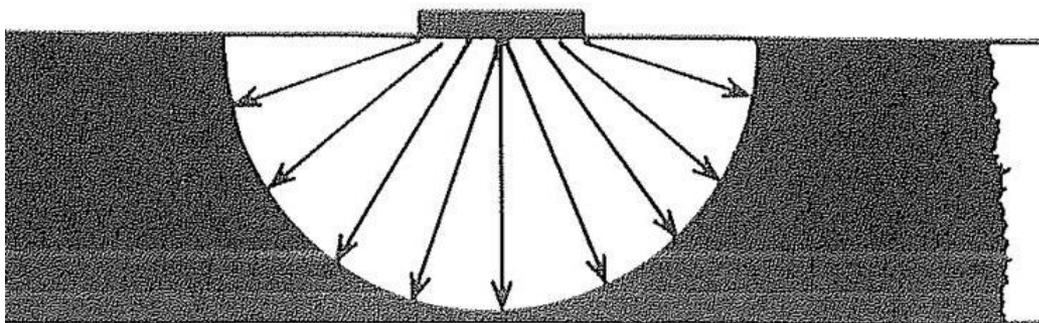
- Results change with changed parameters.
 - It is crucial to adhere to the standardised methodology to get reproducible and reliable results!
- Standardisation of:
 - Disk potency
 - Medium
 - Type of medium, supplements, pH, agar depth etc.
 - Inoculum
 - Incubation
 - Time, temperature and atmosphere
 - Reading of results

EUCAST AST methods

- MIC determination with broth microdilution (BMD) according to ISO standard 20776-1
 - Gold standard
- EUCAST disk diffusion method
 - Standardised method calibrated to gold standard BMD

General principle of disk diffusion

- Diffusion of the antibiotic from a disk to form a concentration gradient in the agar
- Must be calibrated to MIC values
 - Breakpoints needed for interpretation



Picture from Antibiotics in Laboratory Medicine (5th Ed) (2005), editor Victor Lorian.

Summary of the EUCAST disk diffusion method

Organism	Inoculum	Medium	Incubation
Non-fastidious organisms	McF 0.5	Un-supplemented MH agar	Air 35°C, 16-20 h
Fastidious organisms	McF 0.5	MH-F	5% CO ₂ 35°C, 16-20 h
Campylobacter	McF 0.5	MH-F	Microaerobic 41°C, 24 h
Anaerobic bacteria	McF 1.0	FAA-HB	Anaerobic 35-37°C, 16-20 h

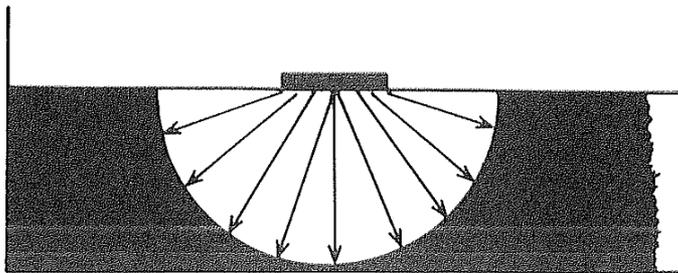
MH = Mueller-Hinton agar

MH-F = Mueller-Hinton agar + 5% defibrinated horse blood and 20 mg/L β -NAD

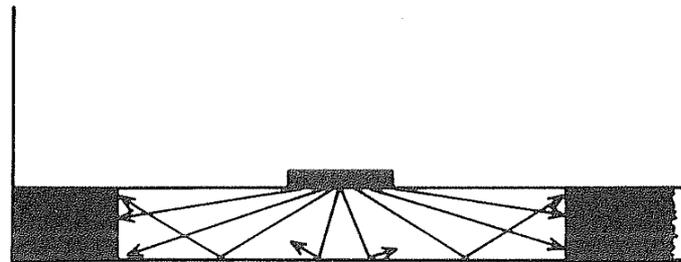
FAA-HB = Fastidious Anaerobe Agar + 5% defibrinated horse blood

Medium: Agar depth

- The agar depth affects the diffusion of the antibiotic and thereby the zone sizes.
- The agar depth should be 4.0 ± 0.5 mm.
 - Agar depth < 4 mm: Larger zone diameters
 - Agar depth > 4 mm: Smaller zone diameters



Correct agar depth



Agar depth too low

Medium: Storage and handling

- Store plates according to the manufacturer's instructions.
 - In-house prepared plates should be stored at 4-8°C.
- Make sure that agar plates are at room temperature prior to inoculation.
- The surface of the agar should be dry before use.
 - Excess humidity may cause fuzzy zone edges and/or haze within zones.

Medium: Excess humidity

- Problems related to excess humidity in agar plates are most often seen on MH-F and FAA-HB plates.
- 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.

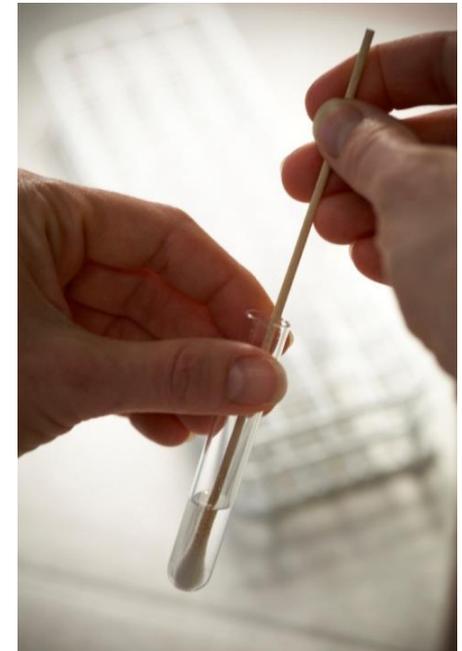


Excess humidity and fuzzy zone edges Example with MH-F agar plates



Inoculum preparation

- Use an overnight culture on non-selective media.
- Pick several morphologically similar colonies to avoid selecting an atypical variant.
- Suspend the colonies in saline and mix to an even turbidity.

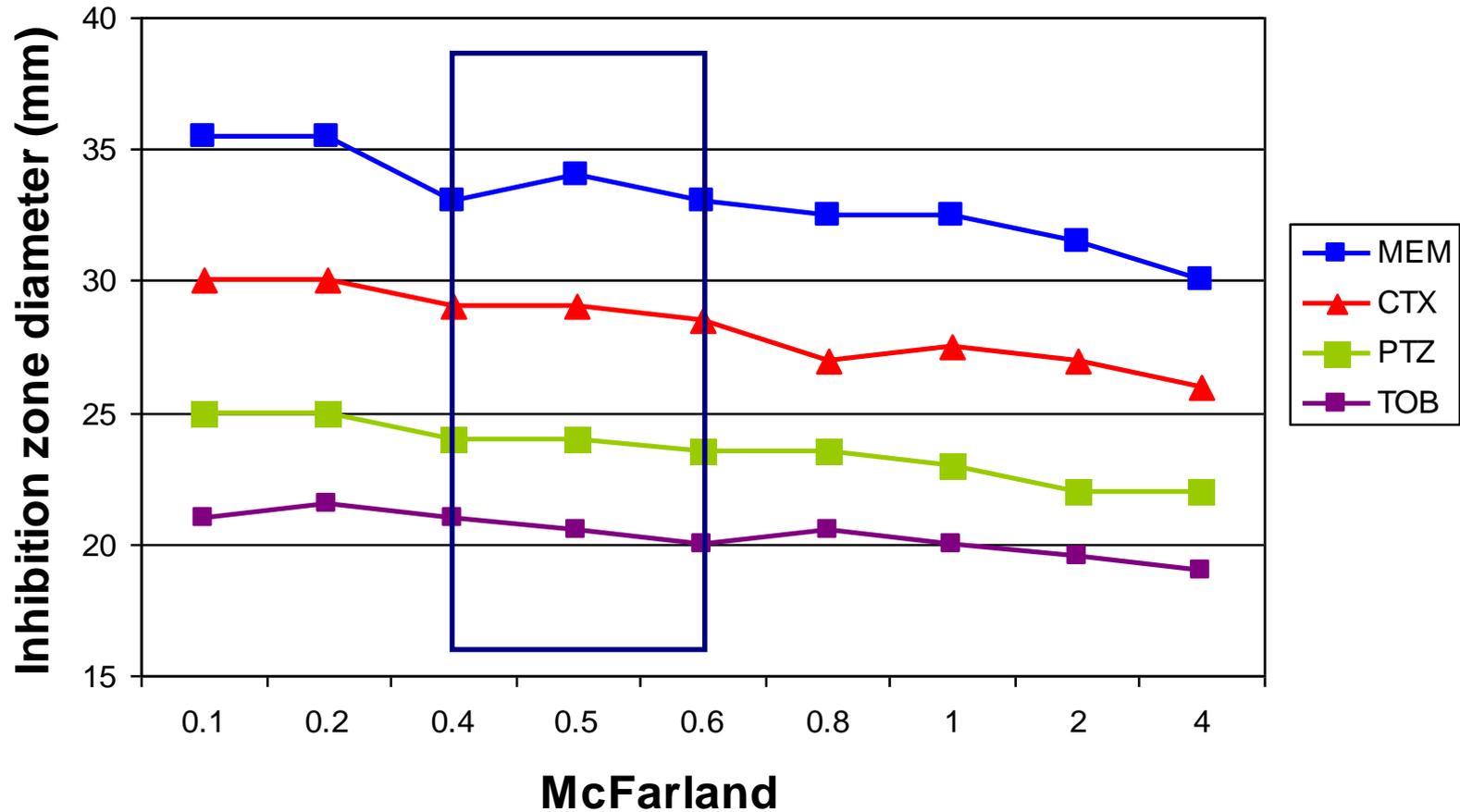


Inoculum preparation

- It is recommended that the turbidity is measured with a photometric device.
- The inoculum should be equivalent to a 0.5 McFarland standard (McF 1.0 for anaerobes)
 - If the density is too low, add more bacteria.
 - If the density is too high, add more saline.
- If *Streptococcus pneumoniae* is suspended from a chocolate agar plate and not a blood agar plate, the inoculum must be equivalent to a McFarland 1.0 turbidity standard.

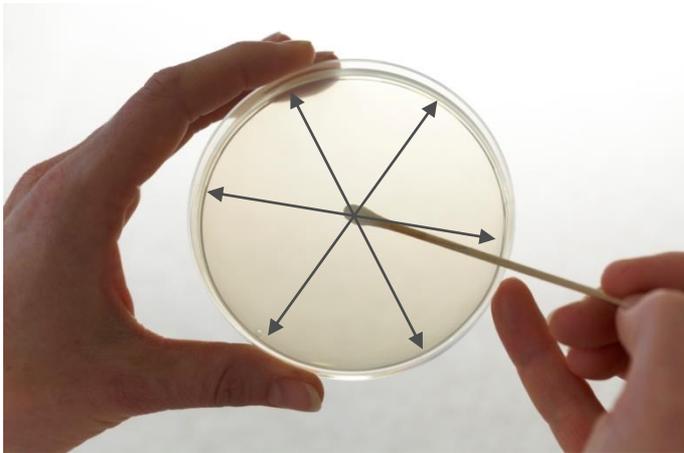
Effect of inoculum size on zone sizes

E. coli ATCC 25922



Inoculation of plates

- Dip a sterile cotton swab into the suspension.
- Spread the inoculum evenly over the entire surface by swabbing in three directions or by using a plate rotator.
- When inoculating several agar plates with the same inoculum, dip the cotton swab into the suspension for each agar plate.



Inoculation of plates

- 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 the swab against the inside of the tube and take particular care to ensure that there are no gaps between streaks.

The more carefully the plates are streaked, the easier it is to read the zone diameters!

Application of disks

- Apply disks within 15 min of inoculation.
- Disks must be in close and firm contact with the agar surface and must not be moved once they have been applied.
- The number of disks per plate should be limited:
 - To avoid overlapping of zones and interference between agents.
 - To allow sensitive organisms to grow.



Number of disks on a plate

- The maximum possible number of disks on a 90-mm circular plate is 6, but for many organisms, 4-5 disks per plate is appropriate.
- For bacteria that are very sensitive, the appropriate number of disks might be as low as 2 disks on a 90-mm plate.
 - Examples: *Corynebacterium* spp., *Aerococcus sanguinicola* and *urinae* and *Cutibacterium acnes*

Storage and handling of disks

- Store disks according to the manufacturers' instructions.
 - Some agents are more labile than others and may have specific recommendations (e.g. amoxicillin-clavulanic acid and carbapenems).
- Store disks in current use in sealed containers with a moisture-indicating desiccant and protected from light.
- Do not use disks beyond the manufacturer's expiry date.
- Allow disks to reach room temperature before opening cartridges or containers used for disk storage. This is to prevent condensation, leading to rapid degradation of some agents.
 - It is better to keep disks at room temperature during the day than transfer repeatedly to and from cold storage.

Incubation of plates

- Invert plates and make sure disks do not fall off the agar surface.
- Keep stacks of plates as small as possible to avoid uneven heating.
 - For most incubators, a maximum of five plates per stack is appropriate.
- Incubation time is 16-20 hours unless otherwise stated in the EUCAST disk diffusion manual.
 - Prolonged incubation is allowed for *Corynebacterium* spp., *Aerococcus*, *Kingella kingae* and *Campylobacter* if growth is non-sufficient after standard incubation.

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.

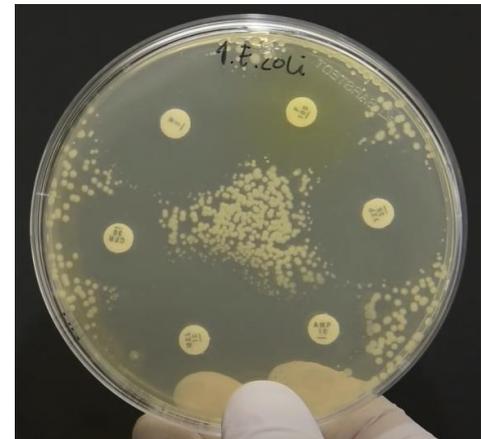
Why the 15-15-15 minute rule?

- Leaving inoculum suspensions at room temperature before inoculation:
 - Increased inoculum for fast-growing bacteria
 - Decreased inoculum for fastidious organisms
- Leaving inoculated plates at room temperature before the disks are applied:
 - The organism may begin to grow, resulting in erroneously small zones.
- Leaving the plates at room temperature after disks have been applied:
 - Pre-diffusion may result in erroneously large zones.

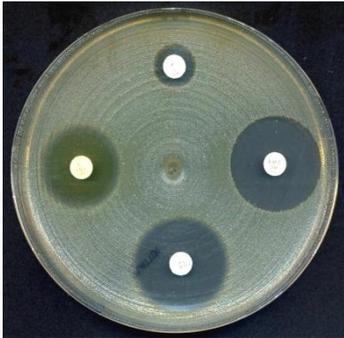
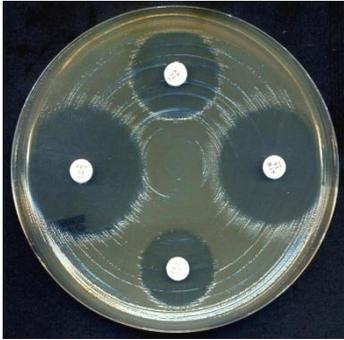
Examination of plates after incubation

- 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.

Example of a plate with too light growth



The growth should be confluent and evenly spread over the plate



Plates should look like this..

..and NOT like this!

Reading of zones and interpretation of results

- Measure zone diameters to the nearest millimetre with a ruler or a or a calliper.



- Measured zone diameters are interpreted into S, I and R according to EUCAST breakpoints
 - Susceptible, standard dosing regimen (S)
 - Susceptible, increased exposure (I)
 - Resistant (R)

Factors affecting the zone size

- Medium (agar depth, pH, supplements)
- Size of inoculum (McF 0.5)
- Age of inoculum (overnight culture)
- Streaking of plates (technique, speed, removing excess fluid)
- Antimicrobial disks (correct potency, storage and handling)
- Incubation environment (air/CO₂/microaerobic/anaerobic)
- Incubation temperature and time
- ...and the reported zone diameter also by reading!

Reliable disk diffusion results

- Follow the described disk diffusion methodology!
- Use disks and media of good quality!
 - Use EUCAST quality control strains to evaluate the materials used
- Perform frequent quality control!
 - Daily or at least 4 times per week to control the materials, equipment and procedures



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[Bacteria](#)

[Mycobacteria \(AMST\)](#)

[Fungi \(AFST\)](#)

[VetCast](#)

[Industry](#)

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Bacteria [Overview](#) →

Clinical Breakpoints and Interpretation →

[Clinical Breakpoint Tables](#) →

[Definition of S, I and R](#) →

[When there are no breakpoints?](#) →

[Breakpoints in Brackets](#) →

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[MIC Determination](#) →

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Guidance Documents →

Rapid AST in blood culture →

[Rapid AST directly from blood culture bottles](#) →

[Methods and Quality Control](#) →

[Breakpoints for Rapid AST](#) →

[Resistance Detection in RAST](#) →

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Important Additional Information →

[Expert Rules](#) →

[Expected Phenotypes](#) →

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Development of Clinical Breakpoints and ECOFFs →

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MIC and Zone Distributions, ECOFFs →

FAQ and EDL Services →

[FAQ](#) →

[EDL MIC Testing Services](#) →

Methodology and Instructions

Here you find information on the EUCAST disk diffusion methodology and on MIC testing: Testing Instructions, Quality Control (QC) and Warnings. In addition, Warnings are issued by EUCAST for commercially available products, which are not performing to the expected standard.

Disk Diffusion and Quality Control

Discover More →

MIC Determination

Discover More →

Disk Diffusion Implementation

Discover More →

Media Preparation

How to prepare in-house broth and agar

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MIC:Zone Diameter correlations

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EUCAST panels of phenotypically defined strains

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Warnings

Concerning antimicrobial susceptibility testing product

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www.youtube.com



EUCAST disk diffusion antimicrobial susceptibility testing method

Preparation of inoculum

0:02 / 2:33

EUCAST videos (English)

Gunnar Kahlmeter • 1/5 videoer

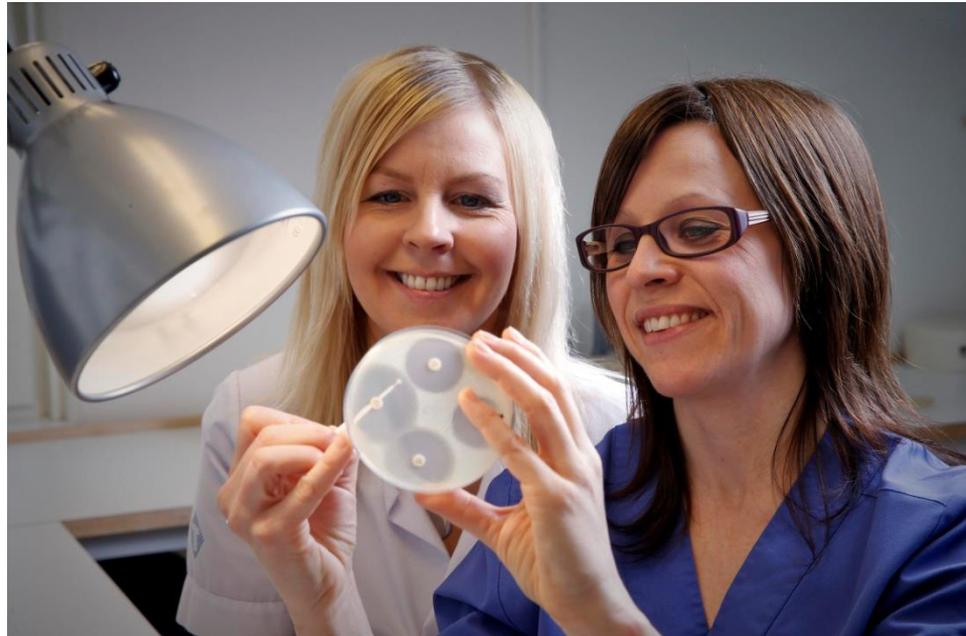


-  **1 Preparation of inoculum (english)**
Gunnar Kahlmeter
-  **2 Inoculation english**
Gunnar Kahlmeter
-  **3 Application of disks and incubation english**
Gunnar Kahlmeter
-  **4 Reading of zones english**
Gunnar Kahlmeter
-  **5 Guidance on breakpoint table english**
Gunnar Kahlmeter

Questions and/or problems?

Contact us!

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EUCAST

EUROPEAN COMMITTEE
ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases