FWD-AMR-RefLabCap 1st Training Course

General introduction to antimicrobial susceptibility testing inc. micro-broth dilution, and disk diffusion

Tuesday, 17 May 2022
10:45 -11:30 CET at DTU Food

HaDEA Service Contract 20197409
Provision of EU networking and support for public health reference laboratory functions for antimicrobial resistance in *Salmonella* species and *Campylobacter* species in human samples

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Antimicrobial susceptibility testing - why is it important?

- Predict outcome of chemotherapy
- Monitor the development of resistance
- Detect novel and emerging resistance mechanisms
- Compare trends in a demographic and geographic context
- Develop and evaluate interventions and prevention strategies

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Antimicrobial susceptibility testing

Objectives

- Testing antimicrobial susceptibility of an isolate against selected antimicrobial drugs for determining resistance pattern
  - Research questions
    - Identification
    - Typing
    - Monitoring/ surveillance
  - Clinical treatment
    - Treatment choice
      - Specificities for antimicrobial drugs
      - Specificities for certain bacterial groups

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Antimicrobial susceptibility testing - limitations

- Susceptibility testing is still only a guideline for treatment
- Not all organs behave the same way *in vivo* and *in vitro*
- Patient response must ultimately confirm adequacy of treatment choice
- Clinical laboratory obligations include
  - Provide useful and appropriate clinical information
  - Provide clinician with most effective antimicrobials from which to choose
  - Control use of antimicrobials and prevent inappropriate usage
  - Minimize emergence of novel resistance by continued surveillance

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AST phenotypic testing - methodologies

- Selection of an AST method may be based on numerous factors
  - ease of performance, flexibility, adaptability to automated or semi-automated systems, cost, reproducibility, reliability, accuracy and preference

- Only few methods have been shown to be reproducible and repeatable:
  - Disk diffusion (Kirby Bauer method) **(limited application)**
  - Broth Micro-dilution (BMD) (Golden standard)
    - Broth dilution
  - Agar dilution

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AST harmonization - historical perspective

- Many public health and veterinary laboratories still use disk diffusion
- Laboratories are moving toward dilution methods for obtaining quantitative results
- Quality control/ quality assurance are critical and essential to warrant reliable data
- General lack of harmonization/ standardization between countries but improving

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Antimicrobial susceptibility testing - standardization

- Different Standards for methodology: EUCAST/ CLSI, ...
- All methods are extremely sensitive to variations in the performance!!
- Influencing factors and principle standardization of procedures for comparison of results
  - Standardized media (**Mueller Hinton Fastidious agar (MH-F)**)
    - Contents and acidity (pH) of medium (broth or agar)
    - Divalent cations (Ca2+, Mg2+)
    - Thymidine, thymine
  - Standardized inoculum size accordidng to MacFarland 0.5
  - **Incubation time and temperature**
  - **Incubation atmosphere / gaseous environment**
  - Reading procedures
  - Performance of tests under consistent QC standard conditions
  - Use of appropriated QC ATCC strains as validation of test parameters
    - ATCC 33560

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How do we measure resistance/ susceptibility?

- Molecular determination to detect the presence of genetic resistance determinants is already in the process to replace phenotypic testing by the introduction of whole genome sequencing for diagnostic and monitoring
  - Phenotypic and genotypic testing often complement – a high concordance
- Phenotypic testing - practical testing of strains in laboratory settings
- Standardized *in vitro* testing may provide qualitative or quantitative results
  - **Qualitative results**
    - Indicate how a drug may respond to a drug in vivo
    - Difficult to compare results unless zone inhibition (mm) diameters are measured
  - **Quantitative results**
    - Lowest antimicrobial concentration that will inhibit the growth or kill the test organism over a define range related to the organism's growth rate
      - Minimum inhibitory concentration (MIC) (mg/L)
    - Most basic measurement of antimicrobial activity against a target organism

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Disk diffusion – media prep

- Disk diffusion depends on the diffusion rate of the antimicrobial and the growth of the bacterium

- Performed on solid agar plates
  - **MH with 5% lysed horse blood and 20 mg/L β-NAD (MH-F, Mueller-Hinton Fastidious) is used for fastidious organisms.**
  - NOT appropriated for fastidious or slow-growing organisms

- Use β-NAD with a purity of ≥ 98%.
  - For MH-F, do not add blood or β-NAD until the medium has cooled to 42-45°C
  - Mix well after the supplements have been added to the cooled medium.

- Adjust for thymidine, thymine
  - Excess thymine and thymidine may be indicated by inhibition zones for trimethoprim-sulfamethoxazole and E. faecalis ATCC 29212 below quality control limits.

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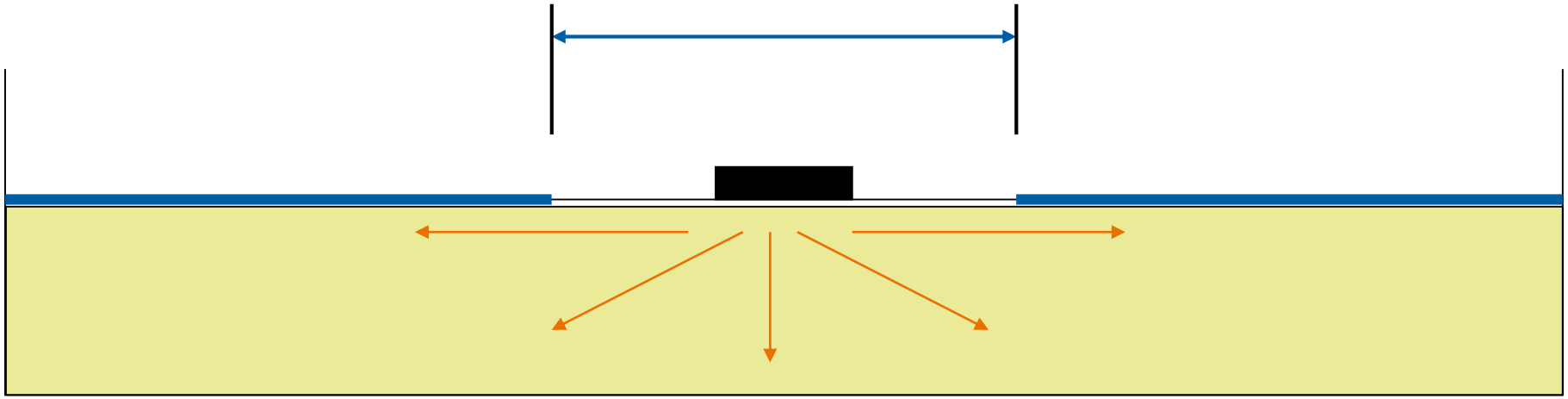
Disk diffusion – inoculum prep

- 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.

Growth of microorganism vs diffusion of drug



Result is an inhibition zone

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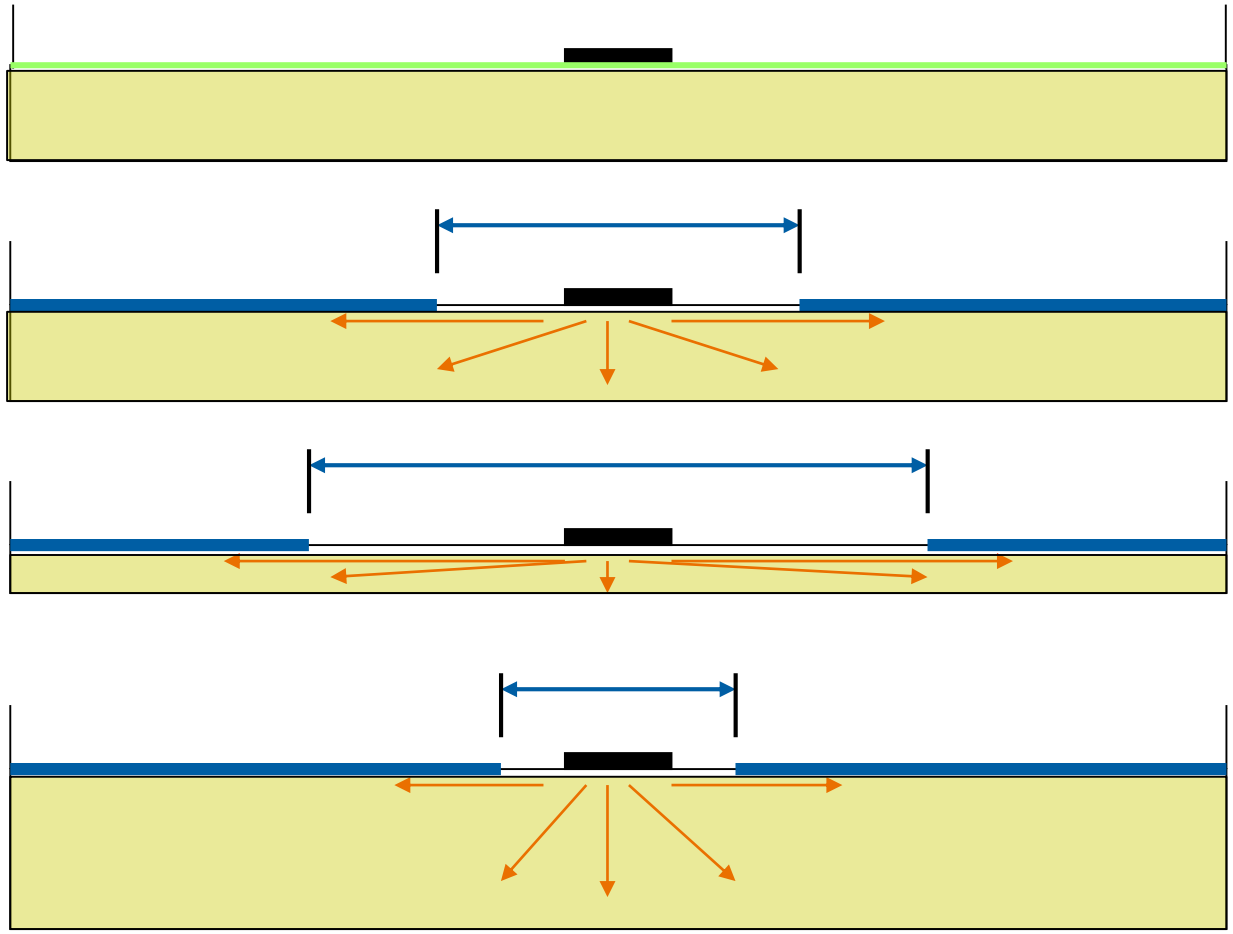
Effect of depth of agar



Media <4mm

Increase in zone of inhibition

Medium >4mm

Decrease in zone of inhibition

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Disk diffusion – inoculum prep

- Make sure that agar plates are at room temperature prior to inoculation.
- The surface of the agar should be dry before use.
- Standardize an inoculum suspension with a turbidity equivalent to a 0.5 McFarland standard (1-2 x10^8 CFU/mL *E. coli)* using adequate methods (calibrated densitometer or nephelometer)
- Select well-isolated colonies from overnight growth on non-selective medium
  - If possible, use several morphologically similar colonies to avoid selecting an atypical variant.
- Adjust the density of the suspension to 0.5 McFarland by adding saline or more bacteria

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Disk diffusion – inoculation

- 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.
  - Remove excess fluid by pressing and turning the swab against the inside of the tube to avoid over-inoculation.
- Spread the inoculum evenly over the entire surface by swabbing in three directions or by using a plate rotator.
- Apply disks within 15 min of inoculation.
  - Disks must be in close and even contact with the agar surface.
    - **Only Cip, Ery, Gen, and Tet accepted.**
  - Do not use more than 12 disks on 150mm or 5 disks on 90mm agar plate
  - Place evenly less than 24mm from center
- The number of disks on a plate should be limited to avoid overlapping of zones

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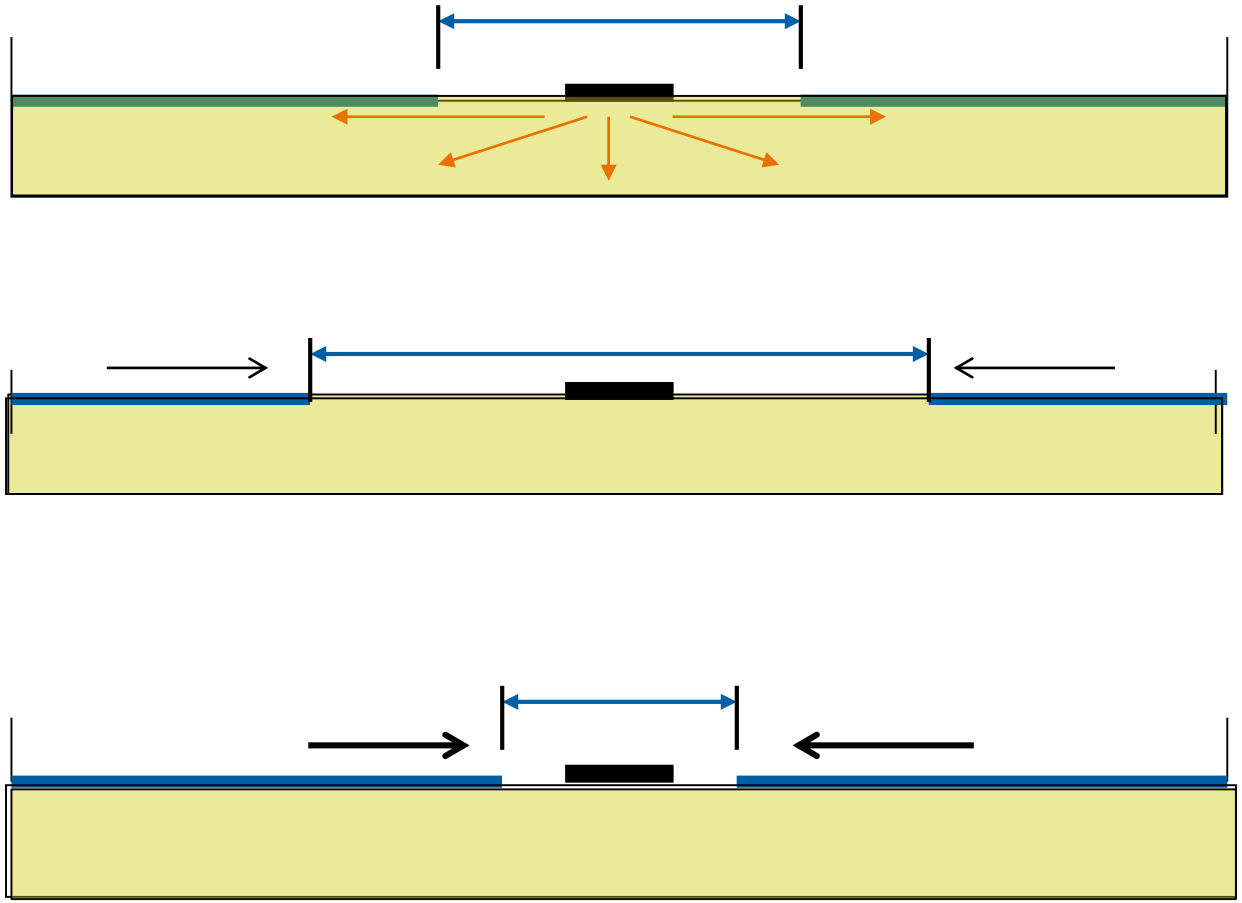
Inoculum effect



Inoculum too diluted

Increase in zone of inhibition

Inoculum too concentrated

Decrease in zone of inhibition

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Disk diffusion - incubation

- 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.
  – a maximum of five plates per stack is appropriate.
- **Incubate MH-F plates for Campylobacter at 41±1ºC in microaerobic environment for 24 h (40-48 h)**

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Disk diffusion - reading

- 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.

- Read MH plates from the back against a dark background illuminated with reflected light.
  - Do not use transmitted light (plate held up to light) or a magnifying glass, unless otherwise stated.
  - 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.
  - In case of double zones, or 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.

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Disk diffusion - reading

- Check that zone diameters for quality control strains are within acceptable ranges before interpreting tests.
  - Interpret zone diameters into susceptibility categories (S, I and R) according to the current EUCAST Breakpoint

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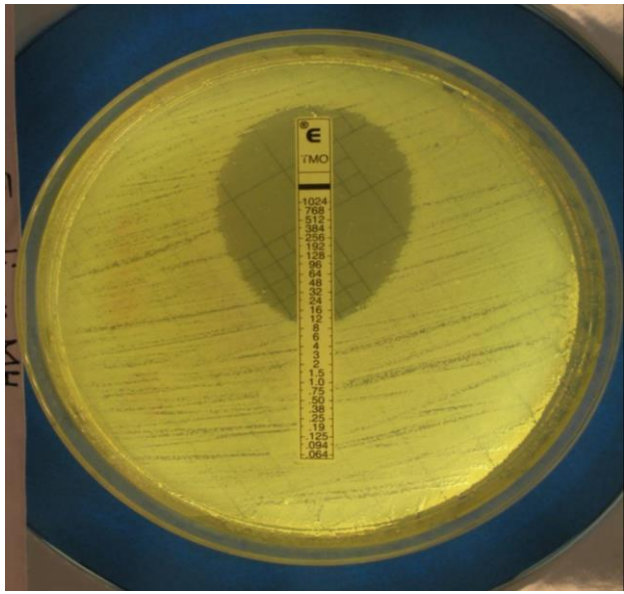
Antimicrobial gradient testing

- Gradient testing is a quantitative diffusion method that generates MIC-values

- Plastic strips are impregnated with an continuous gradient of the antimicrobial
  - Essentially 15 reference MIC dilutions
  - MIC values in-between two-fold dilutions

- In principle, the application is very similar if not identical to disk diffusion
  - Determine the MIC based on where the ellipse intersects the scale.
    - If this is in-between two values, round up to the higher value.
    - If the intersect differs on either side of the strip read the MIC as the higher value

- There are a number of commercially available surrogate MIC determination methods, such as commercial broth microdilution methods, gradient tests, semi-automated devices, etc.
  - It is the responsibility of the manufacturer to guarantee the accuracy of their systems but it is the responsibility of the user to quality control the results obtained with these systems.
    - Consult the EUCAST Warnings page where some of these products listed.



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Micro-Broth dilution (MBD)

- Performed on 96 wells trays
- Commercially available
  - Antimicrobials dehydrated and dissolved in broth at fixed concentrations (two-fold dilution range)
- Golden AST standard - Quantitative method
- High degree of standardization
- Preparation of plates is quite cumbersome if produced manually
  - Not recommended
- Inoculum preparation is in principle, is very similar if not identical as for disk diffusion

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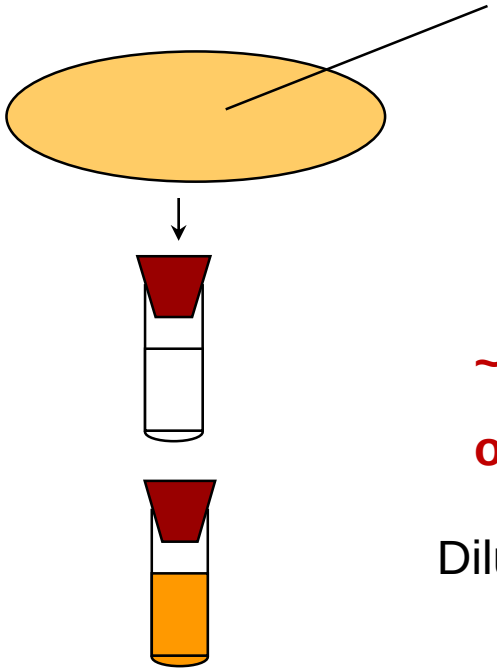
Broth microdilution, cont.



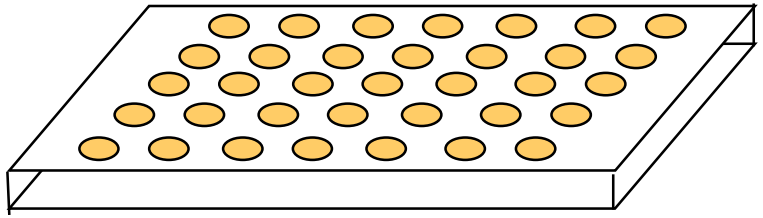
1) Prepare inoculum

Pick 3-5 colonies

~0.5 McFarland adjusted with nephelometer

or other method – dilute by 100ul to the saline

Dilution in MH broth and inoculation on MIC plates



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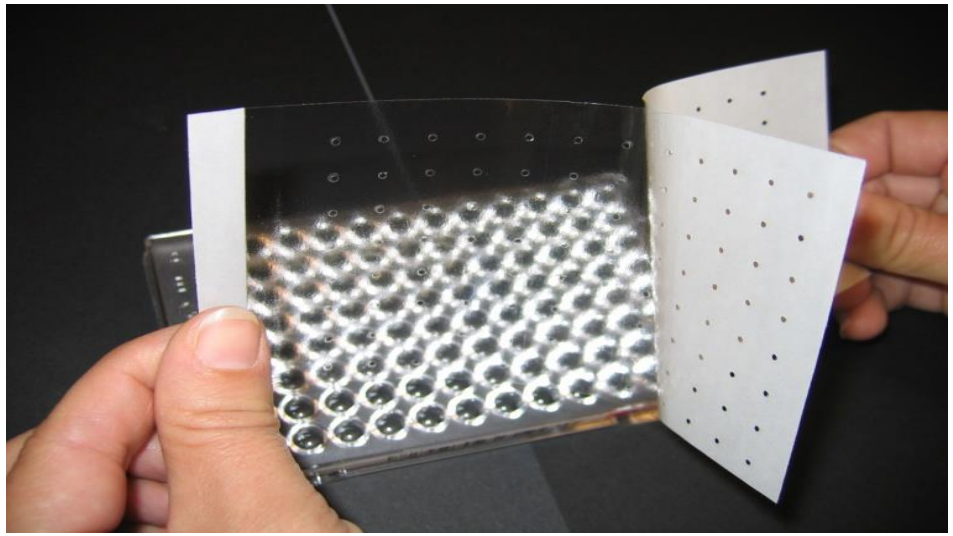
Broth dilution procedures

– Inoculate plate
  - Use the autoinoculator or a multichannel pipette and dispense a fixed volume into each well (depends on plate design/concentrations)
  - Prepare purity control
  - Seal the plates (**punctured**)



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Broth dilution procedures

- Incubate
  41±1ºC in microaerobic environment for 24 h (40-48 h)
- Check purity control

- Read plates after incubation to determine end-points (first; observe control wells and check plate for skips)

- Disregard slight growth for sulphonamides and trimethoprim as these are bacteriostatic, otherwise read using manual or automatic devices

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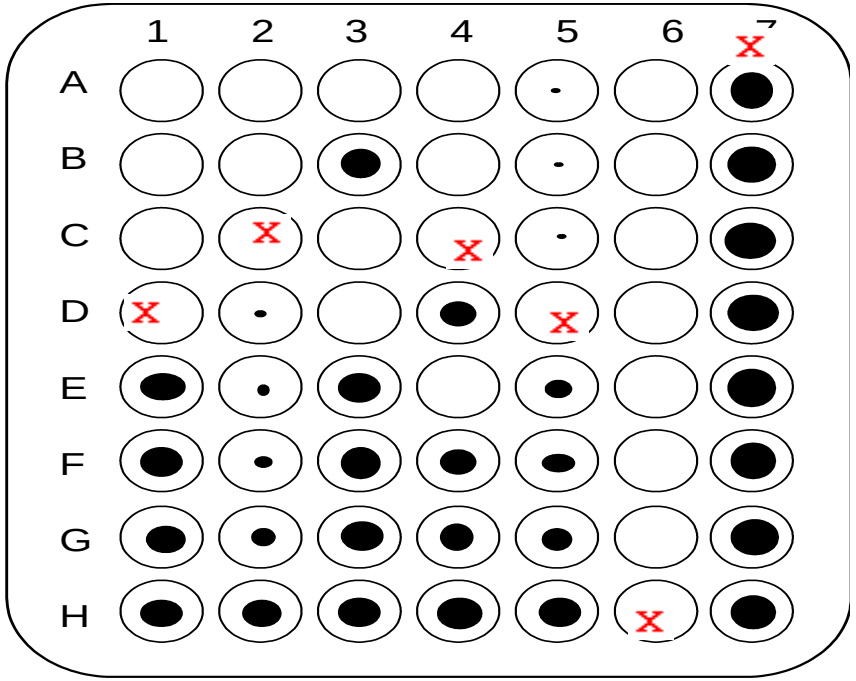
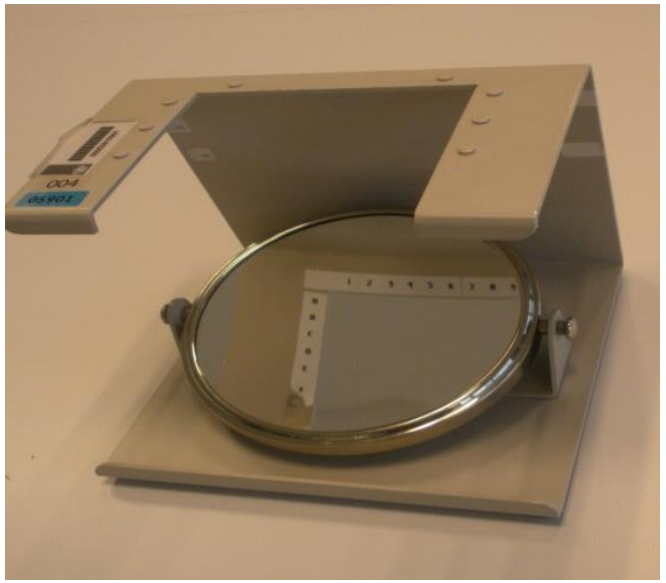
Broth microdilution

- Reading the micro-broth dilution plates
  - Manual, semi- or full automatic reading





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Background for update

- Suggestions of **inclusion of additional substances** were based on:
  - discussed in the **EFSA expert group** incl. the EURL
  - through a **specific questionnaire** survey to consider less significance substances for the first panel
  - all were **carefully assessed and addressed**

- Antimicrobials listed in **previous recommendations** should remain in future testing requirements
  - emphasis on **last-resort antimicrobials** in the treatment of certain infections with highly resistant Gram-negative bacteria in humans, such as the **carbapenems** and **colistin**

EFSA (European Food Safety Authority), Aerts M, Battisti A, Hendriksen R,Kempf I, Teale C, Tenhagen B-A, Veldman K, Wasyl D, Guerra B, Liebana E, Thomas-Lopez D and Belœil P-A, 2019. Scientific report on the technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. EFSA Journal 2019;17(6):5709, 122 pp. https://doi.org/10.2903/j.efsa.2019.5709

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Reasoning behind the panel for the Campy. panel

- Recent findings related to **emerging mechanisms of resistance in Campylobacter spp. need to be addressed**:
  - the presence of the **erm(B) gene** in Campylobacter spp. has been reported
    - detection is critical as often present on mobile genetic elements
    - usually responsible for a very high level of macrolide resistance (> 128 mg/L)
  - current harmonised panel does **not allow detection of the precise ciprofloxacin** MIC of isolates not inhibited by 16 mg/L
  - **chloramphenicol** or florfenicol absent but included to allow detection of CmeABC pump
  - **carbapenem-non-susceptible** Campylobacter strains have already been reported and this should be addressed by incl. a carbapenem

- Allow for inclusion of ertapenem and chloramphenicol by
  - **omit streptomycin** which is optional for testing
  - resistance to ciprofloxacin parallels resistance to **nalidixic acid, thus omitted**

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Campylobacter panel

EUVSEC3 - 2020/1729

	1	2	3	4	5	6
A	CHL 64	ERY 512	GEN 16	CIP 32	TET 64	ETP 4
B	CHL 32	ERY 256	GEN 8	CIP 16	TET 32	ETP 2
C	CHL 16	ERY 128	GEN 4	CIP 8	TET 16	ETP 1
D	CHL 8	ERY 64	GEN 2	CIP 4	TET 8	ETP 0.5
E	CHL 4	ERY 32	GEN 1	CIP 2	TET 4	ETP 0.25
F	CHL 2	ERY 16	GEN 0.5	CIP 1	TET 2	ETP 0.12
G	ERY 2	ERY 8	GEN 0.25	CIP 0.5	TET 1	POS CON
H	ERY 1	ERY 4	CIP 0.12	CIP 0.25	TET 0.5	POS CON

EUVSEC2 - 2013/652

	1	2	3	4	5	6
A	ERY 128	CIP 16	TET 64	GEN 16	NAL 64	STR 16
B	ERY 64	CIP 8	TET 32	GEN 8	NAL 32	STR 8
C	ERY 32	CIP 4	TET 16	GEN 4	NAL 16	STR 4
D	ERY 16	CIP 2	TET 8	GEN 2	NAL 8	STR 2
E	ERY 8	CIP 1	TET 4	GEN 1	NAL 4	STR 1
F	ERY 4	CIP 0.5	TET 2	GEN 0.5	NAL 2	STR 0.5
G	ERY 2	CIP 0.25	TET 1	GEN 0.25	NAL 1	STR 0.25
H	ERY 1	CIP 0.12	TET 0.5	GEN 0.12	POS CON	POS CON

Red boxes indicate those that have been removed in the new plate

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Thank you for your attention

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