FWD-AMR-RefLabCap 1st Training Course

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

FWD AMR-RefLabCap

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

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

Funded by the European Union

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

Funded by the European Union

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

Funded by the European Union

DTU

STATENS SERUM INSTITUT

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

Funded by the European Union

DTU

STATENS SERUM INSTITUT

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)
  - Broth Micro-dilution (BMD) (Golden standard)
    - Broth dilution
  - Agar dilution

Funded by the European Union

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

Funded by the European Union

DTU

STATENS SERUM INSTITUT

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 (MH, Mueller Hinton Fastidious agar (MH-F) ..)
    - Contents and acidity (pH) of medium (broth or agar)
    - Divalent cations (Ca^{2+}, Mg^{2+})
    - 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

Funded by the European Union

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

Funded by the European Union

DTU

STATENS SERUM INSTITUT

Disk diffusion – media prep

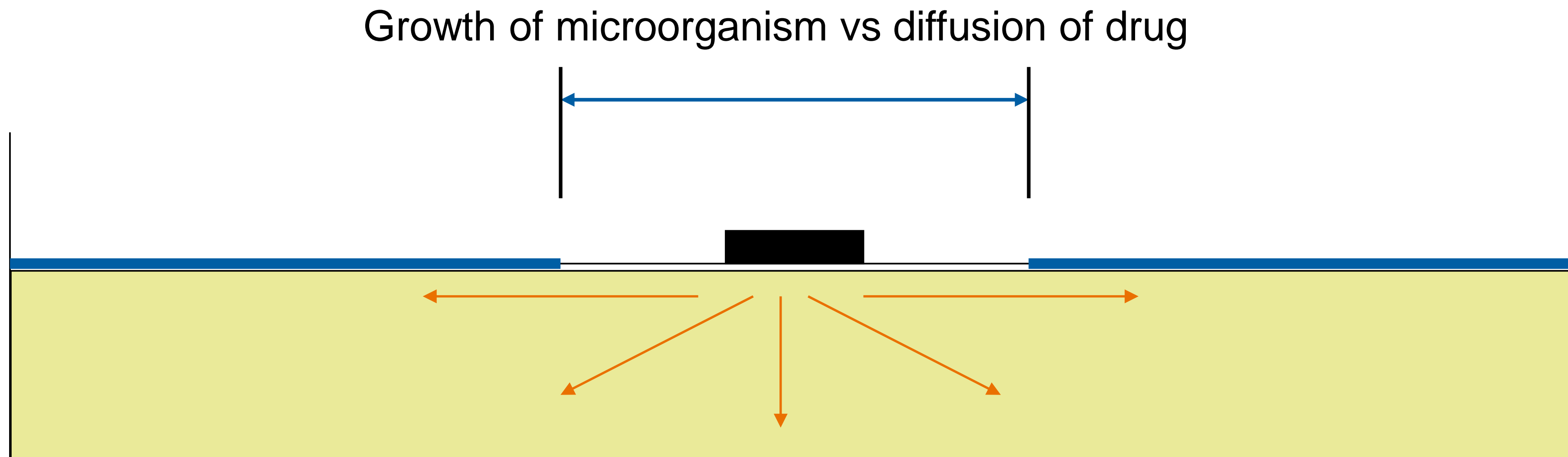
- Disk diffusion depends on the diffusion rate of the antimicrobial and the growth of the bacterium
- Performed on solid agar plates
  - Non-supplemented Mueller-Hinton (MH) agar is used for non-fastidious organisms
    - NOT appropriated for fastidious or slow-growing organisms
- MH with 5% lysed horse blood and 20 mg/L β-NAD (MH-F, Mueller-Hinton Fastidious) is used for fastidious 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.

Funded by the European Union

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.

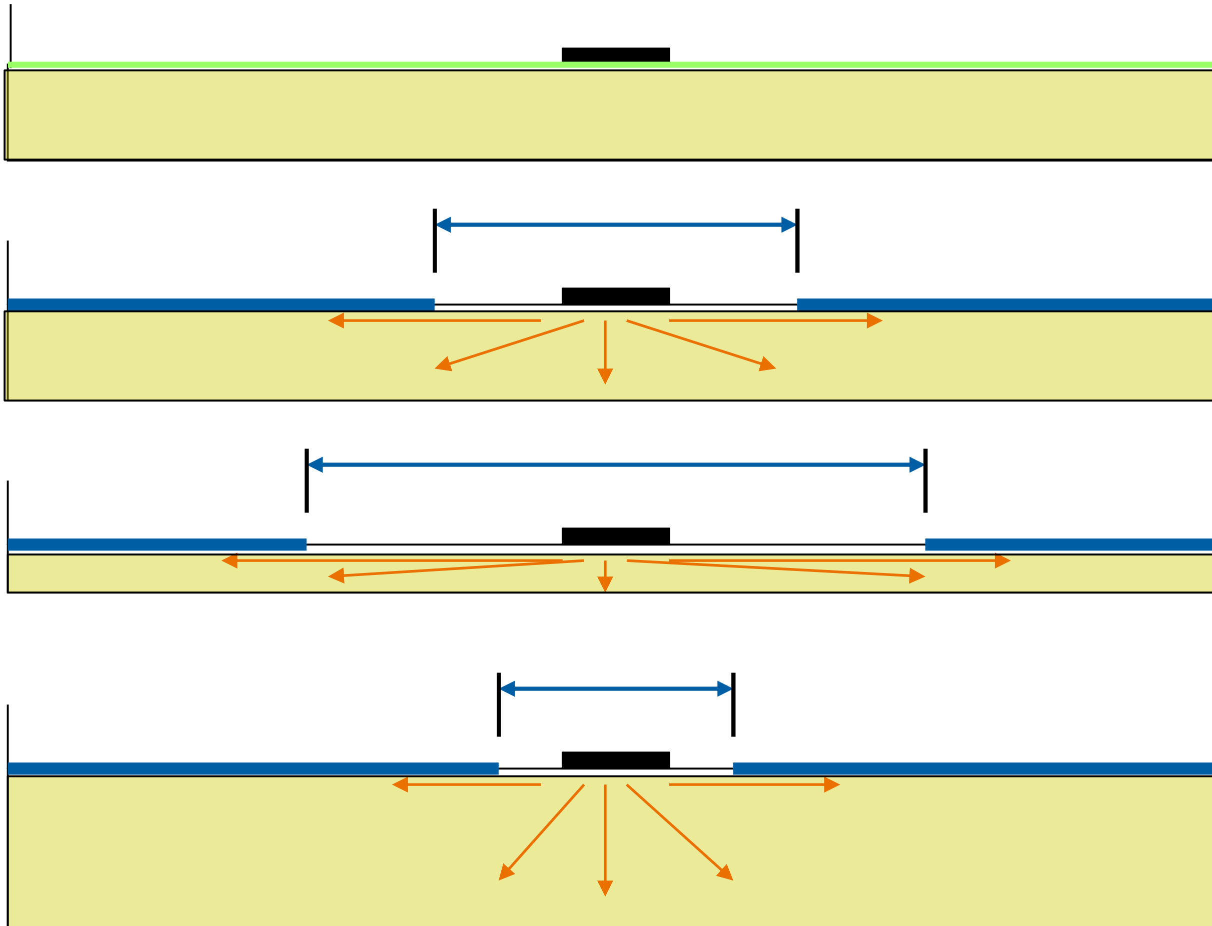
Growth of microorganism vs diffusion of drug

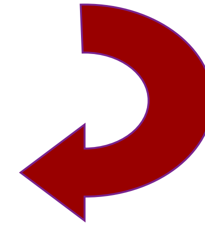


Result is an inhibition zone

Funded by the European Union

Effect of depth of agar





Media <4mm

Increase in zone of inhibition

Medium >4mm

Decrease in zone of inhibition

Funded by the European Union

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

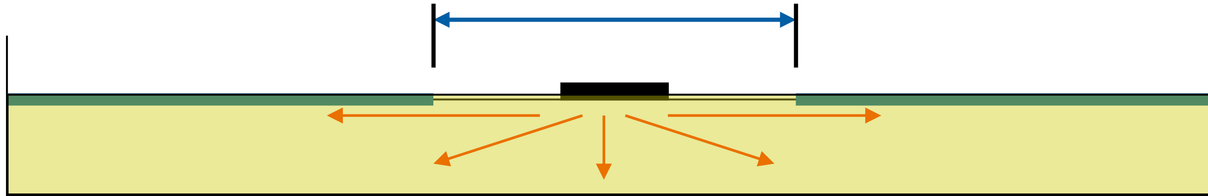
Funded by the European Union

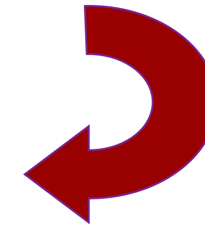
Disk diffusion – inoculation

- 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.
  - 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

Funded by the European Union

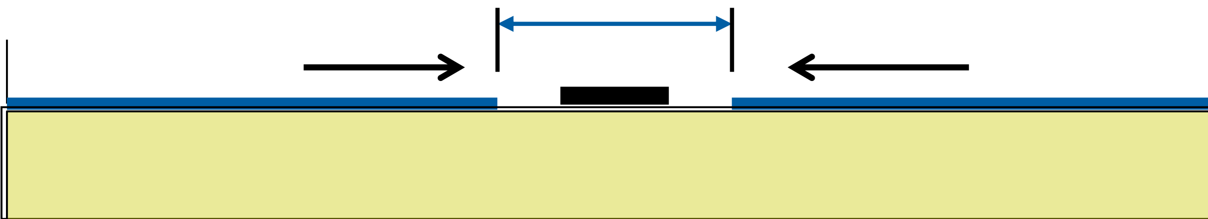
Inoculum effect





Inoculum too diluted

Increase in zone of inhibition



Inoculum too concentrated

Decrease in zone of inhibition

Funded by the European Union

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 plates for Enterobacterales at 35±1ºC in air for 18±2 h

Funded by the European Union

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.

Funded by the European Union

DTU

STATENS SERUM INSTITUT

Disk diffusion - reading

- Trimethoprim and sulfamethoxazole
  - Ignore faint growth up to the disk and measure at the more obvious zone edge.
- Ampicillin, Ampicillin-sulbactam, Amoxicillin-clavulanic acid
  - Ignore fine growth that may appear as an inner zone on some batches of MH agar.
- Temocillin
  - Ignore isolated colonies within the inhibition zone.
- Mecillinam
  - Ignore isolated colonies within the inhibition zone.

- 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

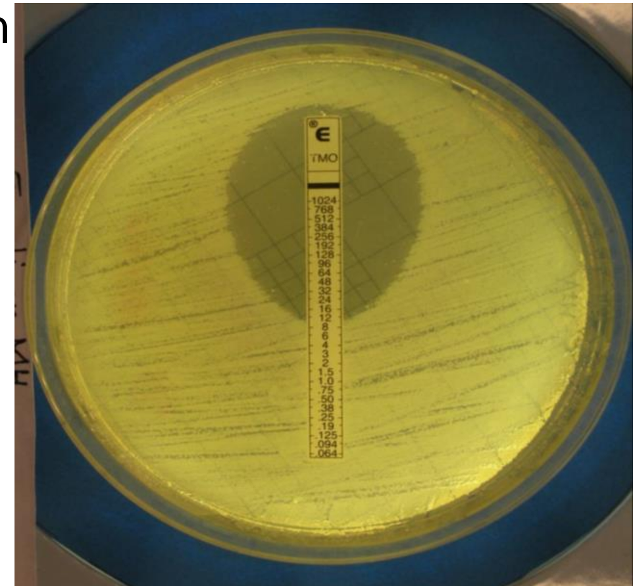
Funded by the European Union

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



Funded by the European Union

DTU

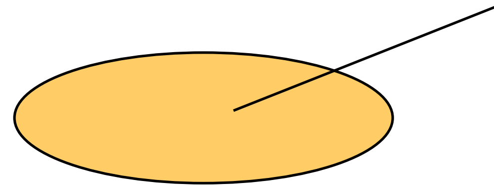
STATENS SERUM INSTITUT

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

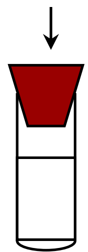
Funded by the European Union

Broth microdilution, cont.



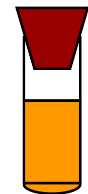
1) Prepare inoculum

Pick 3-5 colonies

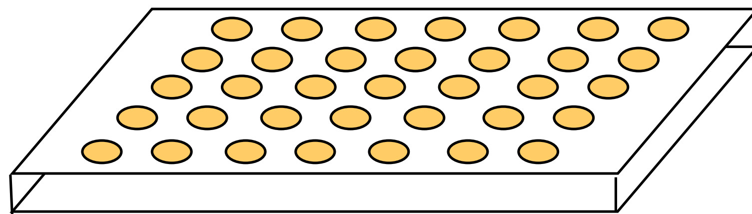


~0.5 McFarland adjusted with nephelometer

or other method



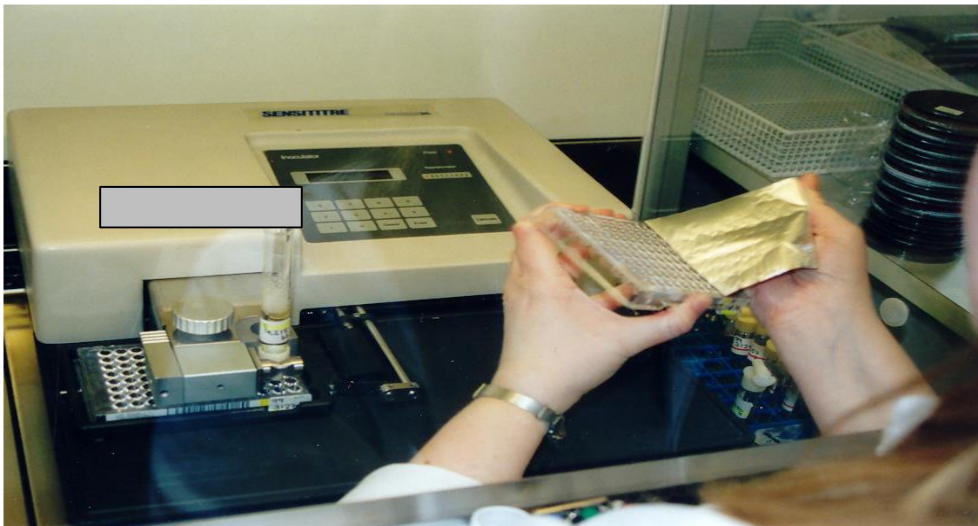
Dilution in MH broth and inoculation on MIC plates

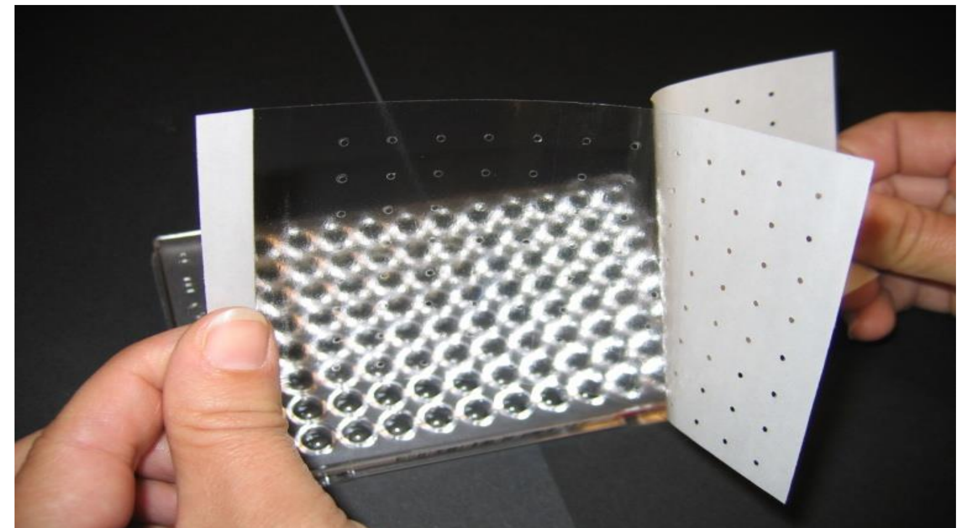


Funded by the European

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





Broth dilution procedures

- Incubate
  At standard temperature/time (depending on organism)
  I.e. at 35±2ºC for 16-20h (some exceptions)

- Check purity control

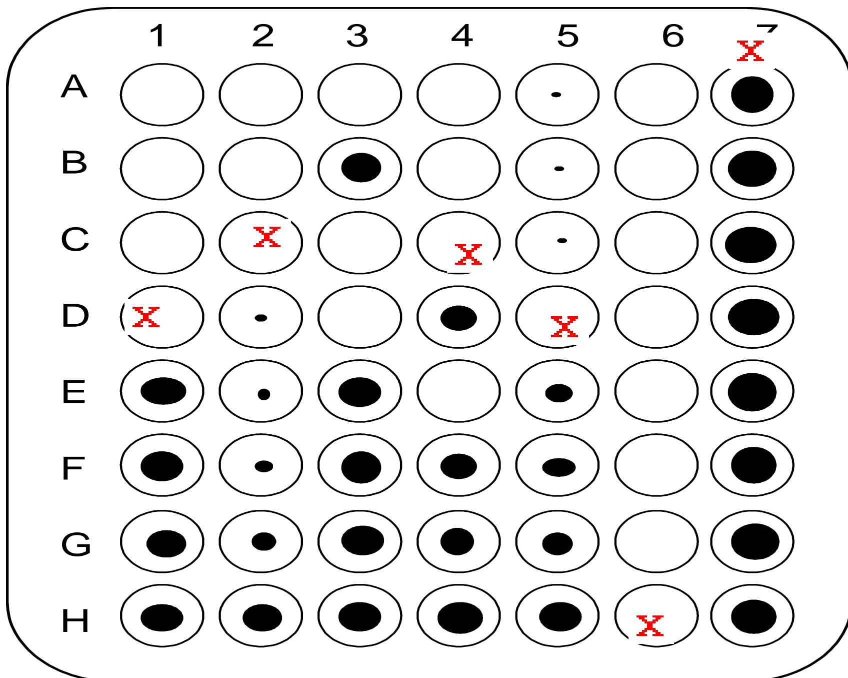
- Read plates after incubation to determine end-points (first; observe control wells and check plate for skips)

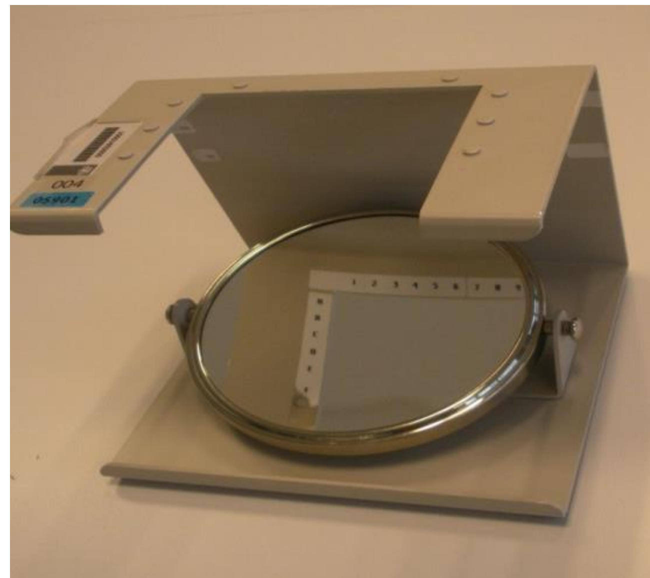
- Disregard slight growth for sulphonamides and trimethoprim as these are bacteriostatic, otherwise read using manual or automatic devices

Funded by the European Union

Broth microdilution

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





Funded by the European Union

Broth microdilution, cont.

Drug	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	MIC
A													2
B													Repeat (?)
C													Repeat
D													8
E													≤0.03
F													>64
G													Repeat
H													0.06

Controls

Funded by the European Union

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

Funded by the European Union

Reasoning behind the new Gram neg panel

- Complement the harmonized panel of substances with amikacin
  - one of the **most commonly used aminoglycosides** in hospitals for the treatment of infections by Gram-negative bacteria in a number of MSs
  - **large differences** in use across the EU, with very high use in some MSs with high levels of resistance in Gram-negatives opposed to it is not used at all
  - **cross-resistance with other aminoglycosides** - marker with gentamicin
  - **improve the detection of the 16S rRNA methyltransferases** associated with carbapenemases, AmpC or ESBLs and FQ resistance in Gram-negative Enterobacteriaceae

- Allow for inclusion of amikacin by
  - slightly reduce some of the dilution ranges in the upper end of the scales:
    - in particular those for ampicillin, nalidixic acid, tetracycline, gentamicin, trimethoprim, sulfamethoxazole and chloramphenicol

Funded by the European Union

Salmonella / E. coli panel

EUVSEC3 - 2020/1729

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMP 32	AZI 64	AMI 128	GEN 16	TGC 8	TAZ 8	FOT 4	COL 16	NAL 64	TET 32	TMP 16	SMX 512
B	AMP 16	AZI 32	AMI 64	GEN 8	TGC 4	TAZ 4	FOT 2	COL 8	NAL 32	TET 16	TMP 8	SMX 256
C	AMP 8	AZI 16	AMI 32	GEN 4	TGC 2	TAZ 2	FOT 1	COL 4	NAL 16	TET 8	TMP 4	SMX 128
D	AMP 4	AZI 8	AMI 16	GEN 2	TGC 1	TAZ 1	FOT 0.5	COL 2	NAL 8	TET 4	TMP 2	SMX 64
E	AMP 2	AZI 4	AMI 8	GEN 1	TGC 0.5	TAZ 0.5	FOT 0.25	COL 1	NAL 4	TET 2	TMP 1	SMX 32
F	AMP 1	AZI 2	AMI 4	GEN 0.5	TGC 0.25	TAZ 0.25	CHL 8	CHL 16	CHL 32	CHL 64	TMP 0.5	SMX 16
G	MERO 0.03	MERO 0.06	MERO 0.12	MERO 0.25	MERO 0.5	MERO 1	MERO 2	MERO 4	MERO 8	MERO 16	TMP 0.25	SMX 8
H	CIP 0.015	CIP 0.03	CIP 0.06	CIP 0.12	CIP 0.25	CIP 0.5	CIP 1	CIP 2	CIP 4	CIP 8	POS CON	POS CON

EUVSEC2 - 2013/652

	1	2	3	4	5	6	7	8	9	10	11	12
A	SMX 1024	TMP 32	CIP 8	TET 64	MERO 16	AZI 64	NAL 128	CHL 128	TGC 8	COL 16	AMP 64	GEN 32
B	SMX 512	TMP 16	CIP 4	TET 32	MERO 8	AZI 32	NAL 64	CHL 64	TGC 4	COL 8	AMP 32	GEN 16
C	SMX 256	TMP 8	CIP 2	TET 16	MERO 4	AZI 16	NAL 32	CHL 32	TGC 2	COL 4	AMP 16	GEN 8
D	SMX 128	TMP 4	CIP 1	TET 8	MERO 2	AZI 8	NAL 16	CHL 16	TGC 1	COL 2	AMP 8	GEN 4
E	SMX 64	TMP 2	CIP 0.5	TET 4	MERO 1	AZI 4	NAL 8	CHL 8	TGC 0.5	COL 1	AMP 4	GEN 2
F	SMX 32	TMP 1	CIP 0.25	TET 2	MERO 0.5	AZI 2	NAL 4	FOT 1	TGC 0.25	TAZ 2	AMP 2	GEN 1
G	SMX 16	TMP 0.5	CIP 0.12	CIP 0.03	MERO 0.25	MERO 0.06	FOT 4	FOT 0.5	TAZ 8	TAZ 1	AMP 1	GEN 0.5
H	SMX 8	TMP 0.25	CIP 0.06	CIP 0.015	MERO 0.12	MERO 0.03	FOT 2	FOT 0.25	TAZ 4	TAZ 0.5	POS CON	POS CON

ECOFF Salmonella EU surveillance 2021

ECOFF E. coli EU surveillance 2021

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

Funded by the European Union

Techniques - Pros and Cons

MIC determination

- Golden standard for AST
- Data more reproducible
- Better separation of R/S
- More information
- Expensive
- Only pure cultures
- Contaminations more difficult to detect
- Possible to use automated systems

Diffusion techniques

- Cheaper
- Primary material
- See contaminations
- Quick screening (4 hours)
- Qualitative information
- Less reproducible data
- Standardization more difficult

Funded by the European Union

Thank you for your attention

Prof. Rene S. Hendriksen, PhD

Head of Research Group Global Capacity Building

WHO Collaborating Centre for Antimicrobial Resistance in Food borne Pathogens and Genomics

European Union Reference Laboratory for Antimicrobial Resistance

FAO Reference Laboratory for Antimicrobial Resistance

National Food Institute, Technical University of Denmark

rshe@food.dtu.dk

Funded by the European Union