Service contract for the 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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Determination of antimicrobial resistance by disk diffusion

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FWD AMR· RefLabCap

STATENS SERUM INSTITUT

DTU

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1. Introduction

This protocol has been prepared for the purpose of presenting and describing the laboratory activities covered by the FWD-AMR-RefLabCap Training Course hosted at DTU Food, Denmark in May 2022.

Disk diffusion is one of several phenotypic assays which can be used to determine the antimicrobial resistance profile of a bacterial organism. Disk diffusion tests estimate in vitro antimicrobial susceptibility.

An agar plate is inoculated with a standardized inoculum of the bacteria and a paper disk impregnated with a standardized, known amount of an antimicrobial is placed on the inoculated plate. The antimicrobial diffuses into the agar when in contact with the agar surface. The plate is incubated under standardized conditions, e.g. following EUCAST guidelines. During incubation, the antimicrobial agent diffuses into the agar and inhibits growth of the inoculated bacteria, thereby creating an inhibition zone.
After incubation the inhibition zone is measured reflecting the antimicrobial susceptibility status of the bacteria. The size of the zone indicates the level of resistance and may also be indicative for the resistance mechanism. Zone diameters can be interpreted by EUCAST guidelines.

Highly standardized methods are essential for all types of antimicrobial susceptibility testing. The test results are highly sensitive to variations in inoculum density, media formulation, agar thickness an moisure, potency of the disk, correct storage of the disks, incubation time and how you read and interpretate the inhibition zones.

For a EUCAST guide on reading zones see
https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2022_manuals/Reading_guide_v_9.0_EUCAST_Disk_Test_2022.pdf

References
https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2022_manuals/Manual_v_10.0_EUCAST_Disk_Test_2022.pdf

Publications Office

1. Equipment and reagents

- Nephelometer
- McFarland standard 0.5
- Vortex mixer
- Sterile forceps or disk dispenser
- Loops
- Sterile cotton swabs
- 0,85% sterile saline
- Ruler or calipers
- MH or MH-F agar plates (where MH is for *Salmonella* (Mueller Hinton agar) and MH-F is for *Campylobacter* (Mueller Hinton agar for fastidious organisms, i.e. Mueller Hinton supplemented with 5% lysed horse blood and 20 mg/L β-NAD
- Disks

3. Procedure day 1

- Allow your disks to reach room temperature
- Prepare your disks in an empty petri dish or in a disk dispenser
- Make sure your MH or MH-F agar plates are not too wet from condensation (if they are, they can be dried 15 minutes in an incubator. This is especially important for MH-F plates)
- Standardize the inoculum: From a pure overnight culture, pick material from at least 3-4 colonies. Suspend in 5 ml saline in a tube of the same type as the one for the McFarland 0.5 standard. Mix. Adjust to McFarland 0.5 using a nephelometer. Calibrate the nephelometer before use using the McFarland 0.5 standard and gently invert your test suspension a couple of times by turning the tube upside-down before measuring. If necessary, adjust turbidity of inoculum to match the standard by adding either more colony material or more saline to the inoculum.
- Place a cotton swab in your 0.5 McFarland suspension
- Remove excess fluid from the cotton swab by pressing the swab against the inside of the tube above the inoculum level (this applies to Gram negatives, only)
- Cover the agar plate with streaks in three different directions to ensure an even growth or use a plate rotator
- Apply your disks using sterile forceps or use a disk dispenser
- Turn your plates upside down and incubate them at 35° +/- 1° for 18 +/- 2 h for *Salmonella* and 41° in a microaerophile environment for 24 h for *Campylobacter*.

Observe the 15-15-15 minutes rule, meaning that from the time you make your suspension you should inoculate your agar plate within 15 minutes. From the time you have inoculated your agar plate you should apply the disks no more than 15 minutes after. From the time you applied your disks to the agar plate, your plate should be in the incubator within 15 minutes.

4. Procedure day 2

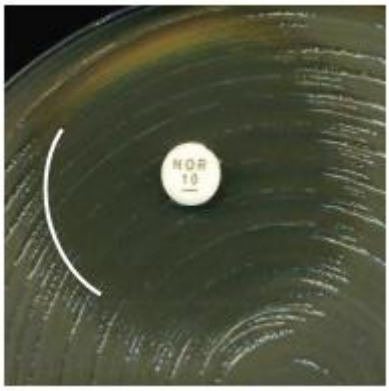
- Take your plates out of the incubator
- Examinate your plates. A confluent lawn of growth is the result of a correctly inoculated and satisfactorily streaked plate. If individual colonies can be seen, the inoculum is too light for the result to be read and the test must be repeated
- MH plates (*Salmonella*) are read from the back of the plate with reflected light and a dark background. MH-F plates (*Campylobacter*) are read from the front with the lid removed and reflected light. Holding the plates at 45 degree angle can facilitate reading when zone edges are difficult to determine. The inhibitons zones should be read at the point of complete inhibiton as judged by the naked eye unless otherwise stated in the guidelines
- Thet zone diameters can be interpreted into susceptibility category according to current breakpoint tables at https://www.eucast.org/clinical_breakpoints/

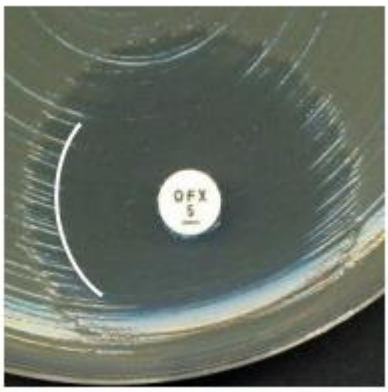
5. Reading examples

In case of distinct colonies within zones, check for purity and repeat if necessary. If the culture is pure, colonies within zones should be taken into account when measuring the zones.

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

You may observe fuzzy zone edges for *Enterobacterales.* If pure, read the inner zone.





6. Quality control

To ensure that you obtain reliable results when performing antimicrobial susceptibility testing, quality control (QC) is crucial.

At the current training course, the quality control of the test results encompasses testing of ATCC 25922 Escherichia coli.

Traceability is key when performing quality control. Ensure that you perform check of new batches of media etc. and ensure that your document and track your QC results allowing you to trace back if you need to troubleshoot, for example if you observe that your QC strain is one step out of range you can trace back to check if this might have started when you started to use a new batch of something.

If you do not perform a particular type of test routinely, ensure that you consider which QC-measures are relevant, for example including a QC strain in parallel to your test strains. If you obtain results from the QC strain that are within the acceptance range, you have an indication that your test strain results are reliable.

Annually, in January, EUCAST update their breakpoint tables and QC tables. This might include updated ranges or the addition of breakpoints and ranges for new antimicrobial agents. Keep updated on the newest version when interpretating your results by looking into the most recent QC table. The current QC table is V.12 and can be found here
EUCAST: Quality Control

Storage of disks should always follow the manufacturer's instructions. After opening, disks should be stored in sealed containers with a moisture-indicating dessicant and protected from light. Perform frequent quality control to make sure that the antimicrobial disks have not lost potency during storage.

7. Media preparation

For media preparation, commercial media can be used if they follow EUCAST recommendations.

For EUCAST media preparation guide see EUCAST: Media preparation