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Protocols for Antibiotic Susceptibility Testing and Laboratory Operating Procedure (LOP)

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A. Description

Antibiotic susceptibility of bacterial strains, collected in the context of the FED-AMR project, tested for 29 antibiotics (2 also in combination with a β-lactamase inhibitor, the clavulanate), are determined by different methods, being the Minimal Inhibitory Concentration (MIC) the reference method, by definition.

For the harmonisation of results obtained from different regions of Europe and to enhance their comparability: all methods are performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, and the interpretation of the respective results follows the epidemiological cut-off (ECOFF) values issued by EUCAST (www.eucast.org) in January 2021.

This protocol will allow answering to different questions from the FED-AMR project.

B. Antibiotics

Antibiotics to be tested are:

- **Gram negatives**:

Amoxycillin, Amoxycillin-clavunanic acid, Cefotaxime, Cefotaxime-clavunanic acid, Cefepime, Ertapenem, Meropenem, Imipenem, Ciprofloxacin and/or Nalidixic acid, Enrofloxacin, Streptomycin and/or Kanamycin, Colistin (must be by MIC, as Etest is not reliable), Chloramphenicol, Florfenicol, Tetracycline, Trimethoprim, Fosfomycin.

- ***S. aureus:***

Cefoxitin, Cefotaxime, Chloramphenicol, Ciprofloxacin, Erythromycin, Florfenicol (not available in Etest), Gentamycin, Linezolid, Oxacillin, Penicillin (Benzylpenicilin), Quinupristin, Sulfamethoxazole (in Etest only in combination with TMP), Tetracycline, Trimethoprim, Vancomycin.

- ***Enterococcus* spp*:***

Ampicillin, Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Florfenicol, Gentamycin, Linezolid, Quinupristin, Tetracycline, Teicoplanine, Tigecycline, Vancomycin.

C. Antibiotic susceptibility testing methods used, by country

1. Austria

MICs by Etest method:

Commercially available gradient E-test strips (bioMérieux) are used according to the manufacturer's instructions for each antibiotic tested, which leads to obtaining MIC values.

2. Estonia

MICs by broth microdilution method:

Commercially available 96-well broth microdilution plates (EUVENC, Sensititre, Trek Diagnostic Systems; Termo Scientifc, USA), purchased already with antibiotics, are used following the manufacturer's instructions.

3. Norway

MICs by broth microdilution method:

Commercially available 96-well broth microdilution plates (EUVENC, Sensititre, Trek Diagnostic Systems; Termo Scientifc, USA), purchased already with antibiotics, are used following the manufacturer's instructions.

Disk diffusion method:

This method is also used for some antibiotics.

EUCAST guidelines and interpretation are followed.

4. Great Britain

Disk diffusion method:

a) Materials

- Mueller-Hinton agar (Merck)
- 0.5 McFarland standard
- 15 mL culture tubes
- Sterile loops
- Sterile cotton swaps
- Sterile saline
- Antibiotics discs (Thermo Fisher)
- Control strains: *Escherichia coli* ATCC 25922 and ATCC 35218, *Klebsiella pneumoniae* ATCC 700603 *Staphylococcus aureus* ATCC 29213 & *Enterococcus faecalis* ATCC 29212

b) Procedure

1. Streak cultures to be tested overnight on LB agar.

2. Prepare MHB agar plates (check guidelines for selected organism and antibiotic) with an agar depth of 4.0± 0.5 mm (approximately 25 mL in a 90 mm circular plate, 31 mL in a 100 mm circular plate, 71 mL in a 150 mm circular plate or 40 mL in a 100 mm square plate).
3. Agar plates should be prepared freshly and stored in plastic bags until needed at 4 ºC.
4. Agar plates should be dry before use (the surface of the agar and inside the lid should not have any visible water drops), if necessary, dry plates at 20-25℃ overnight or at 35℃ for 15 min, aseptically.
5. Using sterile loop, pick few identical colonies and homogenise in sterile saline to obtain a bacterial suspension at a density of 0.5 McFarland.
6. Use the prepared bacterial suspension with 60 min, ideally within 15 min.
7. Make sure that agar plates are at room temperature prior to inoculation.
8. Dip a sterile cotton swap into the suspension (for Gram-negative bacteria only, remove excess fluid by pressing and turning the swab against the inside of the tube to avoid over-inoculation) and inoculate the plates by swabbing in three directions or use automatic plate rotator. Ensure there are no gaps between streaks.
9. Allow antibiotic discs to reach room temperature before opening and use.
10. Place a maximum of 6 or 12 discs for 90 or 150 mm circular plate, respectively.
11. Place antibiotic discs firmly on the plate within 15 min of inoculation and press carefully to remove any air bubbles.
12. Cover plate and incubate within 15 min of discs application at 35±1ºC for 18±2h, check guidelines for selected organism and antibiotic.
13. Check EUCAST breakpoints table for sensitive and resistance cutoffs and for guidelines on how to read the inhibition zone.

5. Portugal

MICs by broth microdilution method, made in house (accredited method):

This procedure uses 96-well broth microdilution plates, prepared at INSA with antibiotics. It follows the EUCAST guidelines, as well as the 'MIC testing' according to EN/ISO 17025.

D. Quality Control procedures

Assure the quality of the testing results:

1. Using Control strains

Results from control strains with selected antibiotics are compared against EUCAST table to confirm and quality check your methodology and results.

As for any MIC determination or disk diffusion method, run control strains to detect any problem with the testing procedure, the panels or plate and disks, the media or any deviation in results.

Do always a purity control.

2. Controlling the conditions

1. Check strain: purity, how long it has been on plate (culture should be fresh), morphology consistent with expected, re confirm ID with some phenotypic tests.
2. Check the growth control. Read susceptibility only if growth control has growth and if weak and results seem low, do not read
3. Check the panel for skips in the test ranges (for MICs).
4. Check growth pattern (observe if any signs of contamination)
5. Do some testing from the wells were growth would be unexpected (for MICs), or check colonies from the inhibition zone (for disk diffusion)
6. Be careful when pipetting and transferring the suspension of 0.5 MacFarland (use long tips to avoid contamination of the pipette)
7. Perform all procedures of MIC panel or disk diffusion plate inoculation in a Laminar Flow Hood and be careful not to contaminate the panel, or the plate and disks.
8. Assure sterility of saline and broth.
9. Re-test to confirm results
10. Contamination can be avoided by:
    - Using pure cultures (if in doubt re-isolate)
    - Streak out well on a non selective agar so you can see if culture on plate is pure (single colonies can be observed and picked)

3. Evaluating the results

Look at phenotype, is it expected? For example, the MRSA obtained from the screenings with selective media are expected to display resistance to beta-lactam antibiotics.

Are resistances observed rare?

Try to check for contamination.

Check reading.

Discard contaminations

Resistance pattern

Look for possible genetic determinants that might explain the observed resistances.

Annotate the endpoints: consider MIC the smallest concentration causing inhibition of growth; for disk diffusion read the mm of diameters as EUCAT recommendations.

Interpret the results using the recommended EUCAST cut-off values (are based on the distribution of susceptibilities in the wildtype and non-wildtype populations, and are used mostly for monitoring purposes to increase the sensitivity of the detection of resistance determinants).

Isolates of the non-wild type are considered microbiologically resistant, for brevity referred to as resistant.

E. References

- DTU protocols for susceptibility testing
- Österberg J, Wingstrand A, Nygaard Jensen A, Kerouanton A, Cibin V, Barco L, et al. (2016) Antibiotic Resistance in Escherichia coli from Pigs in Organic and Conventional Farming in Four European Countries. PLoS ONE 11(6): e0157049. doi:10.1371/journal.pone.0157049
- Cite the used/latest EUCAST table version in

https://www.eucast.org/clinical_breakpoints/

- Check antibiotic susceptibility testing guidelines and videos in https://www.eucast.org/ast_of_bacteria/
- ETEST® https://www.biomerieux-usa.com/etesterv
- Sensititre Antimicrobial Susceptibility Testing System https://www.thermofisher.com/pt/en/home/clinical/clinical-microbiology/antimicrobial-susceptibility-testing/sensititre-antimicrobial-susceptibility-testing-system.html