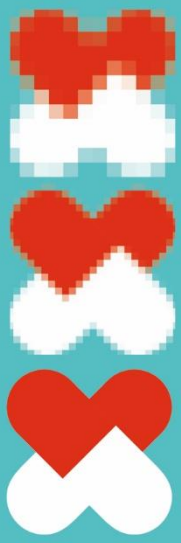
EU-JAMRAI
Joint Action
Antimicrobial Resistance and Healthcare-Associated Infections

Co-funded by the European Union

APPENDIX 3

EARS-Vet MANUAL
OF METHODS AND STANDARDS



BUILDING A ONE HEALTH WORLD

to reduce Antimicrobial Resistance (AMR)

WP8.2 | EARS-Vet Manual, version 1.0
Leader acronym | AEMPS, ANSES
Author(s) | Members of the EARS-Vet manual working group
Reviewer(s) | Members of the workpackage EARS-Vet
Dissemination level | Public
Delivery date | 26-06-2025

DETAILED DESCRIPTION OF BMD AND DD METHODS

TABLE OF CONTENT

1 QUALITY CONTROLS AND USE OF PANELS

1.1 Quality Control (QC)

The reference strains should be used for quality control (QC) purposes whenever a new lot of plates/panels is to be used and whenever required by the applicable standard operating procedures of each laboratory. The QC results need to be reviewed to verify that the minimum inhibitory concentration (MIC) values of the relevant strains are within acceptable ranges before reporting test results.

Lyophilized (freeze-dried) reference/QC strains should be handled and stored appropriately. They can be stored at ≤4 °C. Frozen QC strain cultures need to be immediately thawed and transferred onto appropriate growth media upon receipt. In this case, the culture of QC strain should be stored in aliquots at ≤-70 °C, in broth containing 15-50% glycerol.

The control strains, culture media and incubation specifications proposed to be used for antimicrobial susceptibility test (AST) against bacteria targeted within the EARS-Vet scope are shown in Table 1 which is based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) (ATCC bacteriology culture guide 2025, EUCAST v14.0 2024, EUCAST v7.0 2022).

Table 1: Description of control strains, culture media and incubation specifications employed for each bacterium included in the scope

Bacterial species	*Control strain*	Culture Media[a]	Incubation	AST method[b]
Mannheimia haemolytica	*Haemophilus influenzae ATCC 49766*	MH-F agar [d]	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h	BMD
Pasteurella multocida	*Haemophilus influenzae ATCC 49766*	MH-F agar [d]	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h	BMD
Escherichia coli	*Escherichia coli* ATCC 25922	MH agar	35 ± 1°C in air for 18 ± 2 h	BMD / DD
	Escherichia coli ATCC 35218[c]	MH agar	35 ± 1°C in air for 18 ± 2 h	DD
Klebsiella pneumoniae	*Escherichia coli* ATCC 25922	MH agar	35 ± 1°C in air for 18 ± 2 h	BMD / DD
	Escherichia coli ATCC 35218	MH agar	35 ± 1°C in air for 18 ± 2 h	DD
Pseudomonas aeruginosa	*Pseudomonas aeruginosa* ATCC 27853	MH agar	35 ± 1°C in air for 18 ± 2 h	DD
Staphylococcus aureus	*Staphylococcus aureus* ATCC 29213	MH agar	35 ± 1°C in air for 18 ± 2 h	BMD / DD
Staphylococcus hyicus	*Staphylococcus aureus* ATCC 29213	MH agar	35 ± 1°C in air for 18 ± 2 h	BMD / DD
Staphylococcus pseudintermedius	*Staphylococcus aureus* ATCC 29213	MH agar	35 ± 1°C in air for 18 ± 2 h	BMD / DD
Staphylococcus felis	*Staphylococcus aureus* ATCC 29213	MH agar	35 ± 1°C in air for 18 ± 2 h	BMD / DD
Streptococcus uberis	*Streptococcus pneumoniae* ATCC 49619	MH-F agar [d]	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h	BMD / DD
Streptococcus dysgalactiae	*Streptococcus pneumoniae* ATCC 49619	MH-F agar [d]	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h	BMD / DD
Streptococcus suis	*Streptococcus pneumoniae* ATCC 49619	MH-F agar [d]	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h	BMD / DD
Streptococcus equi subsp. *zooepidemicus*	*Streptococcus pneumoniae* ATCC 49619	MH-F agar [d]	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h	BMD / DD
Mannheimia haemolytica	*Mannheimia haemolytica* ATCC 33396	MH-F agar [d]	35 ± 2°C ambient air 18-24 hours	DD
Pasteurella multocida	*Mannheimia haemolytica* ATCC 33396	MH-F agar [d]	35 ± 2°C ambient air 18-24 hours	DD
Actinobacillus pleuropneumoniae	*Actinobacillus pleuropneumoniae* 27090	Chocolate M-H agar	35 ± 2°C in 5% CO2 20-24 hours	BMD / DD

[a]MH: Müller-Hinton, MH-F: MH-fastidious

[b]BMD: broth microdilution, DD: disk diffusion

[c](β-lactamase producer) for examination of β-lactamase inhibitor production.

[d]5% mechanically defibrinated horse blood and 20 mg/L β-NAD

1.2 Panels used depending on the bacterial species tested

All bacteria defined in the EARS-Vet scope can be tested either using the broth microdilution (BMD) or disk diffusion (DD) methodology. Depending on the tested bacterial species, the BMD/DD panels described in Table 2 should be used.

Table 2: Antimicrobial panels to be used for each bacterial species

Bacterial species	Panel
Escherichia coli	Enterobacterales panel (BMD and DD)
Klebsiella pneumoniae	
Staphylococcus aureus	*Staphylococcus* panel (BMD and DD)
Staphylococcus hyicus	
Staphylococcus pseudintermedius	
Staphylococcus felis	
Streptococcus uberis	*Streptococcus/Enterococcus* panel (BMD and DD)
Streptococcus dysgalactiae	
Streptococcus suis	
Streptococcus equi subsp. *zooepidemicus*	
Mannheimia haemolytica	PMA[a] panel (BMD and DD)
Pasteurella multocida	
Actinobacillus pleuropneumoniae	

[a]PMA: Pasteurella / Mannheimia / Actinobacillus

2 DETAILED DESCRIPTION OF BROTH MICRODILUTION (BMD) METHOD

2.1 Use of quality control (QC)

QC for a specific broth microdilution (BMD) plate should take place whenever a new lot of plates/panels are to be used and at least once every week when the said plate is to be used. Additional QC can be organized when deemed necessary by the laboratory. The QC results need to be reviewed to verify that the MIC values of the relevant strains are within acceptable ranges before reporting test results.

The QC of the broth media should include pH measurement, and evaluation of whether it supports optimum growth of reference strains along with the examination of MIC values. The QC of the medium [either Müller-Hinton (MH), or MH-Fastidious (MH-F); section 2.3] should include pH measurement (7.2-7.4), evaluation of whether it supports optimum growth of reference strains and examining whether their MICs are within the control limits.

The inoculum of the test bacterial strain will be used as a positive control, into designated wells of each plate, to serve as positive (growth) control.

The purity of the culture should be checked in every case. Immediately after inoculating the positive (growth) control well, a quantity should be sub-cultured onto a non-selective agar plate (e.g., Columbia agar with 5% sheep blood) and incubated simultaneously. It is also suggested to verify the inoculum density (i.e., whether this is at 5 x 10^5 CFU/mL). For this purpose, immediately after inoculating the positive (growth) control well, 10 µL can be removed and diluted in 10 mL saline. After mixing, 100 µL is obtained to be plated onto a non-selective agar plate and simultaneously incubated. Following incubation, a number of approx. 20-80 CFU should be counted on the surface of the agar.

The QC strains should be stored preferably on beads or in ≥ 15% glycerol broth at –70 °C or below. Non-fastidious organisms can be stored at -20 °C for a short time. Please note that strains with plasmid-mediated resistance (such as *E. coli* ATCC 35218) have been shown to lose the plasmid if stored > -60 °C.

2.2 BMD (MIC) plates

Four MIC plates, covering the majority of bacteria-antibiotic combinations of interest to EARS-Vet, were designed within EU-JAMRAI2 and manufactured by ThermoScientific. Below are the four panels with the included antibiotics and each dilution range. In each panel, two wells are designated for positive and negative controls.

2.2.1 Enterobacterales panel

Antibiotics	Dilution range[a]	Number of wells
Ampicillin	0.06 - 1 / 4 - 32	9
Amoxiciilin + clavulanic acid	0.25 - 1 / 4 - 16	6
Cefalexin	4 - 32	4
Cefotaxime	0.125 - 4	5
Ceftazidime	0.5 - 8	5
Temocillin	4 - 32	4
Meropenem	0.06 - 0.5	4
Nalidixic acid	4 - 16	3
Enrofloxacin	0.06 - 2	6
Tetracycline	2 - 16	4
Doxycycline	0.12 – 0.5 / 2 - 16	7
Tigecycline	0.25 - 4	5
Colistin	1 - 4	3
Gentamicin	1 - 8	4
Neomycin	4 - 16	3
Kanamycin	2 – 32	5
Amikacin	4 – 16	3
SXT	0.25 – 2	4
Chloramphenicol	1 – 32	6
Florfenicol	4 – 32	4

[a]The "/" indicates a discontinuity in the dilutions

In case of ESBL/AmpC or carbapenemase suspicion, the phenotype should be confirmed either by performing a confirmatory MIC plate (Sensititre EU Surveillance ESBL EUVSEC2 Plate), or by whole-genome sequencing.

2.2.2 *Staphylococcus* spp. panel

Antibiotics	Dilution range	Number of wells
Benzylpenicillin	0.03 - 2	7
Cefoxitin	4 - 8	2
Oxacillin + 2% NaCl	0.25 - 2	4
Enrofloxacin	0.03 - 4	8
Clindamycin	0.125 - 4	6
Erythromycin	0.25 - 4	5
D test (clinda/ery)[a]	-	1

Tylosine	1 - 8	4
Tetracycline	0.125 - 2	7
Doxycycline	0.06 - 0.05	4
Gentamicin	0.125 - 4	6
Amikacin	2 - 32	5
Streptomycin	8 - 32	3
Kanamycin	4 - 16	3
SXT	0.25 - 4	5
Rifampicin	0.03 - 0.5	5
Linezolid	2 - 8	3
Fusidic acid	0.25 - 2	3
Vancomycin	1 - 8	4
Chloramphenicol	1 - 32	6
Tiamulin	1 - 4	3

[a]The D test allow the detection of the inducible MLS_B phenotype

2.2.3 Streptococcus/Enterococcus panel

Antibiotics	Dilution range	Number of wells
Benzylpenicillin[a]	0.03 - 4	8
Ampicillin[a]	0.06 - 16	9
Enrofloxacin	0.06 - 4	7
Clindamycin	0.125 - 4	6
Erythromycin	0.125 - 2	5
D test (clinda/ery)	-	1
Tetracycline	0.25 - 32	8
Gentamicin	2 - 128	7
Streptomycin	32 - 1024	6
Kanamycin	1 - 16	5
Amikacin	1 - 16	5
SXT	0.06 - 4	7
Vancomycin	0.5 - 8	5
Linezolid	1 - 8	4
Chloramphenicol	4 - 128	5

[a] Benzylpenicillin is used to test streptococci, ampicillin to test enterococci.

2.2.4 Pasteurella/Mannheimia/Actinobacillus (PMA) panel

Antibiotics	Dilution range	Number of wells
Benzylpenicillin	0.125 - 2	5
Ampicillin	0.25 - 2	4
Amoxiciilin + clavulanic acid	0.25 - 2	4
Ceftiofur	0.03 - 0.125 + 1 - 8	7
Nalidixic acid	4 - 32	4
Enrofloxacin	0.03 - 2	7
Tulathromycin	4 - 128	6
Tilmicosin	4 - 64	5
Gamithromycin	1 - 16	5
Tildipirosin	1 - 32	6
Tetracycline	0.25 - 8	6
Doxycyline	1 - 4	3
Gentamicin	2 - 64	6
Kanamycin	8 - 128	5
Spectinomycin	16 - 128	4
SXT	0.06 - 0.5	4
Tiamulin	8 - 128	4
Florfenicol	0.5 - 8	5
Chloramphenicol	0.5 - 4	4

2.3 Preparation and inoculation of isolates

Materials and equipment required

- Pure bacterial culture on agar plates.
- Appropriate BMD test plates (panels) (section 1.2).
- Equipment for media preparation (waterbath, autoclave, etc.).
- MH or MH-F broth.
- Sterile half-strength saline (0.45% NaCl) or deionized/distilled water.
- Agar plates with non-selective medium (e.g., Columbia agar with 5% sheep blood).
- Sterile, single-use inoculation loops and swabs.
- Turbidimeter / densitometer or similar photometric device for the standardization of the bacterial suspension, or alternatively, McFarland (McF) turbidity standards and comparison card.
- Pipettes and tips, including multichannel pipettes, or repetitive pipettes.
- Sterile reagent reservoirs for the use of multichannel pipette.
- Incubators capable of achieving and maintaining a temperature of 35 °C.

- Manual reading device (view box with mirror) or automated reader (e.g., microplate photometer capable of measuring absorbance values at 600 nm, digital imaging system, or automated instrument, such as Sensititre).

Preparation of media

For testing non-fastidious organisms (*E. coli, K. pneumoniae,* and staphylococci), the cation-adjusted MH broth should be used. MH broth can be prepared and autoclaved in-house according to the manufacturer's instructions or purchased commercially.

For testing fastidious Gram-negative bacteria and streptococci, MH-F broth should be used, i.e., MH broth supplemented with 5% lysed horse blood and 20 mg/L beta nicotinamide adenine dinucleotide (β-NAD).

The following steps need to be followed for the preparation of MH-F broth

Broth media can either be purchased directly or prepared in-house (EUCAST v7.0 2022, ISO standard method ISO 20776:1-2019).

An overnight culture on a non-selective agar plate should be available for the preparation of a homogeneous, standardized (0.5 McF) suspension, which is further diluted in broth (MH or MH-F, depending on the organism) to achieve the final inoculum for the whole test plate. The plate is inoculated, sealed and incubated. Specific details on the workflow of the technique, including inoculation and incubation conditions (temperature, atmosphere, and duration) are provided in the relevant ISO standard method (ISO 20776:1-2019).

1. MH broth needs to be prepared following the manufacturer's guidelines. However, the volume of deionized water per 1 L needs to be reduced by 10% (100 mL).
2. Following sterilization by autoclaving, the broth is cooled to approx. 45 °C.
3. Using an aseptic technique, 100 mL of 50% lysed horse blood (either purchased commercially or prepared in-house and stored at -20°C in aliquots) and 1 mL β-NAD (from a stock solution of 20 mg/mL, prepared in sterile water and filter-sterilized through a 0.2 µm syringe filter) are added (per 1 L) and thoroughly mixed.
4. The MH-F broth is distributed in sterile containers and stored under refrigeration (4-8 °C) for a period determined in the framework of the laboratory's QA schedule and no further than 3 months after preparation.

Preparation of bacterial suspension and inoculation

1. An overnight bacterial culture on a non-selective agar plate should be available for testing. A small number (typically 2-6, depending on their size) of morphologically similar colonies needs to be obtained from the agar plate using a single-use loop or a swab and transferred

into a sterile tube containing saline or water. Via the use of a vortex mixer, the preparation of a homogeneous suspension is ensured.

2. The density of the prepared suspension needs to be subsequently measured using a turbidimeter or a similar photometric device and adjusted to match the density of the 0.5 McF standard (corresponds to approx. 1-2 x 10^8 CFU/mL for *E. coli* ATCC 25922). Alternatively, in cases where no such device is available, the density of the prepared suspension can be estimated by visually comparing the tube to the 0.5 McF standard against a Wickerham card (i.e., a McF comparison card with black lines) and assessing visually both tubes in terms of turbidity and the lines' appearance through them.
3. Suspensions with higher density need to be diluted. On the other hand, more colonies can be added to suspensions with a density below 0.5 McF. This standardized suspension is then diluted in MH broth or MH-F broth to achieve a final inoculum of 5 x 10^5 CFU/mL. The total volume required for the dilution should enable the inoculation of the whole plate (96 wells) with a maximum volume of 200 µL per well.
4. Dispensing of the inoculum in the plate can be accomplished via the use of a multichannel pipette and a reagent reservoir, or a repetitive pipette.
5. After the inoculation, the purity of the culture as well as a verification of the inoculum density are performed, as already described.
6. The plate is subsequently sealed with a tight lid or adhesive seal to avoid evaporation. The microtiter plates should not be stacked more than 4 within the incubator, to enable even heating. It is also suggested to place an empty plate on top of each stack, to reduce condensation on the inside of the cover, which helps with proper reading.
7. Incubation takes place in air at 35 °C for 16-20 h. For vancomycin reading takes place after 24 h.

2.4 Reading, validation and interpretation

Manual reading is advised, whereas calibration is required prior to automated reading. Reading guidelines are provided by EUCAST (EUCAST v5.0 2024), which are especially important for particular cases (i.e., testing bacteriostatic drugs against staphylococci/streptococci, reading breakpoints of trimethoprim/sulfamethoxazole, as well as determining endpoints in wells with hemolysis). For the test to be valid, specific criteria also need to be verified.

- Manual reading is advised by employing a mirror view box.
- If an automated reader (e.g., photometric reader, camera-based instrument or another type of automatic instrument) is used, it should be first calibrated to manual reading.
- Growth appears as a deposit of bacterial cells at the bottom of the well (button or scattered sediment of cells) or as turbidity, depending on the antibiotic and the bacterium species.
- For the test to be valid, sufficient growth (turbidity or button) should be visible in the positive control well.
- The density of the inoculum and the purity of the culture should also be verified (section 1.1) for the results to be valid.

- Results are recorded as MIC (expressed in mg/L, or µg/mL), i.e., the lowest concentration of each antibiotic that inhibits completely the visible growth of the bacterium (i.e., its growth as detected by the naked eye). Specific care needs to be taken regarding results reading in the following cases:
  - Bacteriostatic drugs (fusidic acid, tetracycline) against staphylococci and streptococci: pinpoint growth (i.e., small buttons) should not be taken into consideration.
  - Trimethoprim/sulfamethoxazole: the MIC should be read at the lowest concentration that inhibits at least 80% of the growth, as compared to the positive control.
  - Haemolysis may be observed while testing fastidious organisms (MH-F broth). In this case, haemolysis with turbidity/pellet should be regarded as growth when determining endpoints.
- In case interpretation of MIC values into susceptibility categories (S-I-R) is required, this should be made according to the current breakpoint tables (EUCAST v15, 2025).

3 DETAILED DESCRIPTION OF DISK DIFFUSION (DD) METHOD

3.1 Use of quality control (QC)

Maintaining QC strains

- The QC strains should be stored preferably on beads or in ≥ 15% glycerol broth at -70 °C or below. Non-fastidious organisms can be stored at -20 °C for a short time. Please note that strains with plasmid-mediated resistance (such as *E. coli* ATCC 35218) have been shown to lose the plasmid if stored > -60 °C.
- QC strains presented in Table 1 (or alternative known QC strains) should be set up every week that the relevant antibiotic panel is used, with every test and whenever a new batch/lot of disks or media is used. In addition, QC strains with known resistance may be used for confirmatory purposes and should be set up with each new batch of disks or media, or monthly.
- Every time QC strains are set up, the inhibition zone diameter (mm) should be within the ranges stated by EUCAST QC tables. Please note, if different QC strains are included, ranges should be available.
- QC strain ranges should be checked to be within acceptable ranges before interpreting results of test strains.
- Examine the results of the last 20 consecutive QC tests for trends (inhibition zones falling consistently above or below the target). Investigate if: two consecutive results of the last 20 tests are outside the acceptable ranges, multiple results are out of range on the same day or resistance in a resistant QC strain is not detected.

Preparation and storage of reagents

Agar

1. Follow the manufacturer's instructions if media are to be prepared in-house, with supplementation as indicated in Table 1 if needed.
2. Drying, storage and shelf life of plates prepared in-house should be determined by the quality assurance programme of the laboratory.
3. Plates should be stored at 4-8 °C, unless otherwise indicated by the manufacturer.
4. Commercially prepared plates should be stored as per manufacturer's instructions and used within the expiry date indicated.
5. Plates should have a uniform depth of 4.0 ± 0.5 mm and the surface of the agar should be dry before use.
6. Please note that variation in divalent cations in the agar, like magnesium and calcium, affects the results for aminoglycosides and tetracycline tests with *P. aeruginosa*, and can therefore produce an out-of-range result of the QC strain.

7. Each new batch, shipment or lot of MH agar or supplemented MH agar should be tested with appropriate QC strains before use for testing isolates and zone diameter measurements should fall within the acceptable zones.

Antimicrobial disks

1. Unopened cartridges should be stored at 4-8 °C.
2. In use cartridges should be stored at 4-8 °C in a disk dispenser (if applicable) with an active dessiccant.
3. Unopened disc cartridges should be allowed to come to room temperature before removal from packaging and placement into dispenser for use.
4. In use disk cartridges must be discarded within the time limit specified by the manufacturer
5. Disk dispensers should be put at room temperature 10-15 minutes before use.
6. Disc dispensers should be returned to 4-8 °C immediately after use to avoid exposure of the disks to moisture. The potency of the disks is greatly reduced in the presence of moisture.
7. Dessiccant should be replaced regularly, as indicated by loss of the colour.
8. Each new batch, shipment or lot of disks should be tested with appropriate QC strains before use for testing isolates and zone diameter measurements should fall within the acceptable zones.

3.2 DD panels

The panels for disk diffusion are given below and are largely consistent with the MIC plates described in section 2.2. For *Pseudomonas aeruginosa*, due to expected resistance to many antimicrobial agents, testing should include primarily the aminoglycosides (amikacin, gentamicin) and fluoroquinolones (ciprofloxacin, enrofloxacin, marbofloxacin) and colistin.

3.2.1 Enterobacterales panel

Antimicrobial category	Antimicrobial agent	Disk	ECOFF (>)
1st Generation Cephalosporin	Cefalexin	30 µg	*E. coli:* 15 *K. pneumoniae:* 17
3rd Generation Cephalosporin	Cefotaxime	5 µg	*E. coli:* 24 *K. pneumoniae:* 21
Aminoglycoside	Amikacin	30 µg	*E. coli:* 19 *K. pneumoniae: 16*
Aminoglycoside	Gentamicin	10 µg	*E. coli:* 18 *K. pneumoniae:* 14
Aminoglycoside	Neomycin	10 µg	*E. coli:* (13) *K. pneumoniae:* NI
Aminoglycoside	Kanamycin	30 µg	*E. coli:* NI *K. pneumoniae:* NI

Aminopenicillins	Ampicillin	10 µg	*E. coli:* 14 *K. pneumoniae:* intrinsic R
Aminopenicillin + β-lactamase inhibitor	Amoxicillin-clavulanic acid	20/10 µg	*E. coli:* 18 *K. pneumoniae:* 17
Amphenicol	Chloramphenicol	30 µg	*E. coli: 20* *K. pneumoniae:* NI
Carbapenem	Meropenem	10 µg	*E. coli:* 29 *K. pneumoniae:* 25
Carboxypenicillin	Temocillin	30 µg	*E. coli: 16* *K. pneumoniae:* NI
Fluoroquinolone	Enrofloxacin (USE CIP)	5 µg	*E. coli:* CIP 29 *K. pneumoniae:* CIP 22
Glycylcycline	Tigecycline	15 µg	*E. coli:* 20 *K. pneumoniae:* NI
Sulfamethoxazole-Trimethoprim	Sulfamethoxazole-Trimethoprim	23.75 / 1.25µg	*E. coli: 24* *K. pneumoniae:* 18
Quinolone	Nalidixic acid	30 µg	*E. coli:* 19 *K. pneumoniae*: (16)
Tetracycline	Tetracycline	30 µg	*E. coli:* 21 *K. pneumoniae:* NI
Tetracycline	Doxycycline	30 µg	*E. coli:* NI *K. pneumoniae:* NI

NI = no interpretive criteria

Confirmatory:

- 2nd Generation cephalosporin: Cefoxitin (ECOFF, *E. coli*: NI / *K. pneumoniae* >18)
- 3rd Generation cephalosporins in combination with β-lactamase inhibitor: cefotaxime/ ceftazidime alone and in combination with clavulanic acid (ECOFF, Cefotaxime-clavulanic acid NI. Ceftazidime-clavulanic acid NI)
- 4th Generation cephalosporin: cefepime (ECOFF, *E. coli* >28 / *K. pneumoniae* > (26))
- Carboxypenicillin: temocillin (ECOFF, *E. coli*: NI / *K. pneumoniae* NI /)
- Carbapenems: meropenem/imipenem/ertapenem (ECOFF, Meropenem *E. coli* > 27 / K. pneumonia >25. Imipenem *E. co*li > 24 / *K. pneumoniae* >22. Ertapenem *E. coli* > 23 / K. pneumoniae >21 /)

3.2.2 *Staphylococcus* spp. panel

Where disk concentration is in brackets or is blank, this may be because although there may be disks commercially available containing the antimicrobial there may not be an approved method for testing in this bacterial species in animals.

Antimicrobial category	Antimicrobial agent	Disk	ECOFF (>)

2nd Generation Cephalosporin	Cefoxitin	30 µg	*S. aureus:* 20 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
	Oxacillin (+2% NaCl) for *S. pseudointermedius*?	1 µg	
Aminoglycoside	Amikacin	30 µg	*S. aureus:* 17 *S. hyicus:* NI *S felis:* NI *S pseudintermedius:* NI
Aminoglycoside	Gentamicin	10 µg	*S. aureus:* 18 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Aminoglycoside	Kanamycin	30 µg	*S. aureus :* NI *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Aminoglycoside	Streptomycin	(25 µg)	*S. aureus:* NI *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Amphenicol	Chloramphenicol	30 µg	*S. aureus:* 17 *S. pseudointermedius:* NI *S. felis:* NI *S. pseudointermedius:* NI
Fluoroquinolone	Enrofloxacin (USE CIP)	5 µg	*S. aureus*: CIP 17 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Fusidanes	Fusidic acid	10 µg	*S. aureus:* 23 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Glycopeptide	Vancomycin	(30 µg)	*S. aureus:* NI *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Lincosamide	Clindamycin	2 µg	*S. aureus:* 21 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Macrolide	Erythromycin	15 µg	*S. aureus:* 22 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Macrolide	Tylosin	-	
Oxazolidinone	Linezolid	10 µg	*S. aureus:* 18 *S. hyicus:* NI

			S. felis: NI *S. pseudointermedius:* NI
Penicillin	Penicillin	1 unit	*S. aureus:* NI *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Pleuromutilin	Tiamulin	-	*S. aureus :* NI *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Rifamycin	Rifampicin	5 µg	*S. aureus:* 24 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Sulfamethoxazole-Trimethoprim	Sulfamethoxazole-Trimethoprim	23.75 / 1.25 µg	*S. aureus:* 23 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Tetracycline	Doxycycline	-	*S. aureus:* NI *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI
Tetracycline	Tetracycline	30 µg	*S. aureus:* 20 *S. hyicus:* NI *S. felis:* NI *S. pseudointermedius:* NI

[a]Only *S. aureus* has ECOFFs available

NI = no interpretive criteria

3.2.3 *Streptococcus/Enterococcus* panel

Where disk concentration is in brackets or is blank, this may be because although there may be disks commercially available containing the antimicrobial there may not be an approved method for testing in this bacterial species in animals.

Antimicrobial category	Antimicrobial agent	Disk	ECOFF (>)
Aminoglycoside	Amikacin	-	*S. dysgalactiae*: NI *S. uberis*: NI *S. suis*: NI *S. equi subsp. zooepidemicus*: NI
Aminoglycoside	Gentamicin HC	120 µg	*S. dysgalactiae*: NI *S. uberis*: NI *S. suis*: NI

			S. equi subsp. zooepidemicus: NI
Aminoglycoside	Kanamycin	-	*S. dysgalactiae*: NI *S. uberis*: NI *S. suis*: NI *S. equi subsp. zooepidemicus*: NI
Aminoglycoside	Streptomycin HC	300 µg	*S. dysgalactiae*: NI *S. uberis*: NI *S. suis*: NI *S. equi subsp. zooepidemicus*: NI
Amphenicol	Chloramphenicol	30 µg	S. dysgalactiae: NI *S. uberis*: NI *S. suis:* NI *S. equi subsp. zooepidemicus:* NI
Fluoroquinolone	Enrofloxacin	5 µg	*S. dysgalactiae:* NI *S. uberis:* NI *S. suis*: NI *S. equi subsp. zooepidemicus:* NI
Glycopeptide	Vancomycin	5 µg	*S. dysgalactiae:* NI *S. uberis:* NI *S. suis:* NI *S. equi subsp. zooepidemicus:* NI
Lincosamide	Clindamycin	2 µg	*S. dysgalactiae:* 17 *S. uberis:* NI *S. suis:* NI *S. equi subsp. zooepidemicus:* NI
Macrolide	Erythromycin	15 µg	*S. dysgalactiae*: 20 *S. uberis*: NI *S. suis*: NI *S. equi subsp. zooepidemicus*: NI
Oxazolidinone	Linezolid	10 µg	*S. dysgalactiae:* NI *S. uberis:* NI *S. suis:* NI *S. equi subsp. zooepidemicus:* NI
Penicillin	Ampicillin (For *Enterococcus faecalis*)	2 µg	*E. faecalis:* 15
Penicillin	Penicillin	1 unit	*S. dysgalactiae:* 20 *S. uberis*: NI *S. suis:* NI

			S. equi subsp. zooepidemicus: NI
Sulfamethoxazole-Trimethoprim	Sulfamethoxazole-Trimethoprim	23.75 / 1.25 µg	*S. dysgalactiae:* NI *S. uberis:* NI *S. suis:* NI *S. equi subsp. zooepidemicus:* NI
Tetracycline	Tetracycline	30 µg	*S. dysgalactiae*: 20 *S. uberis*: NI *S. suis:* NI *S. equi* subsp. *zooepidemicus*: NI

[a]Only *S. dysgalactiae* has ECOFFs available

NI = no interpretive criteria

3.2.4 Pasteurella/Mannheimia/Actinobacillus (PMA) panel

Where disk concentration is in brackets or is blank, this may be because although there may be disks commercially available containing the antimicrobial there may not be an approved method for testing in this bacterial species in animals.

Antimicrobial category	Antimicrobial agent	Disk	ECOFF (>)
3rd Generation Cephalosporin	Ceftiofur	30 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Aminoglycoside	Gentamicin	-	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Aminoglycoside	Kanamycin	-	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Aminoglycoside	Spectinomycin	100 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Aminopenicillin + β-lactamase inhibitor	Amoxicillin-clavulanic acid	3 µg	*M. haemolytica*: NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Amphenicol	Chloramphenicol	-	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Amphenicol	Florfenicol	30 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Fluoroquinolone	Enrofloxacin	5 µg	*M. haemolytica:* NI *P. multocida:* NI

			A. pleuropneumoniae: NI
Macrolide	Gamithromycin	15 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Macrolide	Tilmicosin	15 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Macrolide	Tildipirosin	60 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Macrolide	Tulathromycin	30 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Penicillin	Ampicillin	2 µg	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Penicillin	Penicillin	1 unit	*M. haemolytica:* NI *P. multocida:* 15 *A. pleuropneumoniae:* NI
Quinolone	Naladixic acid	30 µg	*M. haemolytica:* NI *P. multocida:* 19 *A. pleuropneumoniae:* NI
Sulfamethoxazole-Trimethoprim	Sulfamethoxazole-Trimethoprim	23.75 / 1.25 µg	*M. haemolytica:* NI *P. multocida:* 19 *A. pleuropneumoniae:* NI
Tetracycline	Tetracycline	30 µg	*M. haemolytica:* NI *P. multocida:* 22 *A. pleuropneumoniae:* NI
Tetracycline	Doxycycline	-	*M. haemolytica:* NI *P. multocida:* NI *A. pleuropneumoniae:* NI
Pleuromutilin	Tiamulin	-	*M. haemolytica:* NI *P. multocida: NI* *A. pleuropneumoniae:* NI

[a]Only *P. multocida* has ECOFFs available

NI = no interpretive criteria

3.3 Preparation and inoculation of isolates

Reagents and equipment

- Pure overnight bacterial culture on solid media.
- Sterile swabs or single-use loops.
- Sterile saline or demineralised water.
- Densitometer or 0.5 McF turbidity standard.
- Sterile bacterial inoculum tubes.

- Spectrophotometer.
- Vortexer.
- MHA (supplements required are indicated in Table 1).
- Antimicrobial disks (section 2.3).
- Disk dispenser / forceps.
- Incubator 35 °C (and/or 5% ±2% CO_2) according to the bacteria being tested (see Table 1).
- Plate reader/ callipers.
- Bacterial control cultures - see section 1.1 of this manual (other control strains may also be used, please refer to EUCAST guidelines).

Control strains, culture media and incubation specifications

QC strains should be maintained and stored as described in section 3.1 and should be set up every week that the relevant antibiotic panel is used, with every test and whenever a new batch/lot of disks or media is used. In addition, QC strains with known resistance may be used for confirmatory purposes and should be set up with each new batch of disks or media, or monthly.

Culture media, either MH or supplemented MH depending on the requirements of the organism, should be prepared and stored as described in section 3.1. Media should be brought to room temperature before use.

Inoculum preparation and inoculation

- A 0.5 McF standard should be prepared as described in the EUCAST manuals.
- Preparation of the inoculum
    1. Make a suspension of the culture to be tested by mixing sufficient colonies (usually 3-5) of an overnight culture on a non-selective agar in a tube of sterile water or saline to match the density of the 0.5 McF standard (corresponds to approx. 1-2 x 10^8 CFU/mL for *E. coli* ATCC 25922). Colonies may be picked using a sterile loop or sterile cotton swab. Vortex to ensure an even suspension. Adjust the density of the colony suspension by adding saline or further colonies as necessary. The density of the suspension can be checked in a photometric device that has been calibrated with the 0.5 McF standard. Alternatively, the density of the suspension can be compared visually with the standard by holding the test suspension and the McF standard against a white page with black lines/type and assessing the turbidity of both by examining the appearance of the lines/type through both tubes. The test suspension should be used within 15 minutes of preparation.
    2. Alternatively, organisms that do not form a smooth suspension can be prepared by inoculating 4-5 colonies into a suitable broth such as tryptone soya broth and incubating at 35+2 °C for 2-6 hours until a turbidity of approximately equivalent to or greater than 0.5 McF standard is reached. Adjust to the correct turbidity of 0.5 by adding saline if required and check the density as described above.

3. Inoculation of MH agar (or MHA supplemented as described in Table 1 for fastidious organisms).

- Dip a sterile cotton tip swab into the suspension of the test organism and rotate and press against the side of the tube to remove excess liquid. Swab the entire surface of the plate, taking care not to leave any space between streaks; repeat this procedure twice more, turning the plate approximately 60° each time. Swab the rim of the plate. This ensures an even lawn of growth on the plate following incubation.

Application of disks and incubation

Disks should be brought to room temperature before use. Disks may be applied using a disk dispenser or manually using a forceps. Ensure disks are spaced evenly on the plate, sufficiently far from the edge of the plate and from each other to allow full circular zones of inhibition to develop. It is best to place disks with likely smaller zones of inhibition beside those with predictably larger zone sizes. No more than 6 disks should be placed on 90 mm plates or 12 disks on 150 mm plates.

Plates are incubated in air at 35+1 °C for 18+1 hours. *Pasteurella multocida, Actinobacillus pleuropneumoniae* and streptococci require supplementation with 4 –6 % CO_2.

The '15-15-15 minute' rule should be adhered to: the inoculum should be used within 15 minutes, disks should be applied within 15 minutes, and plate incubation should begin within 15 minutes of disk application.

3.4 Reading plates and result interpretation

(Raw data – MICs or Zone Diameters; Breakpoints, interpretation)

Plates may be read manually or, if an automated plate reader is used, it should be calibrated to manual reading.

1. Check that the lawn of growth is confluent and evenly distributed across the surface of the plate; zones of inhibition should be uniformly circular.
2. Ensure that the inhibition zones for the quality control strains are within acceptable ranges.
3. In general, read the zones of inhibition using a caliper or ruler held to the back of the plate, using reflected light and with the plate held above a dark surface. Supplemented plates should be read from the front with the lid removed. The zone diameter should be read to the nearest millimeter, with the edge of the zone defined as the point where there is complete inhibition of growth as seen by the naked eye in good light. The plate is held approximately 30 cm from the eye, at a 45-degree angle to the bench.
4. Specific cases
   - Care should be taken when reading disk diffusion results for streptococci that the zone of haemolysis is not read instead of the zone of inhibition.

- For trimethoprim and trimethoprim-sulfamethoxazole, faint growth up to the disk can be ignored.
- If colonies are observed within zones, check for purity and repeat the test if necessary. If the growth is pure, colonies within the zone should be taken into account when measuring the zone size.
- Enterobacterales tested with ampicillin, ampicillin-sulbactam and amoxicillin-clavulanic acid may show a thin film of growth inside the obvious zone of inhibition on some batches of MH media; this can be ignored. The colonies observed within the inhibition zone for temocillin can also be ignored.
- When testing staphylococci against cefoxitin for detection of methicillin resistance, measure the obvious zone of inhibition; colonies detected within the zone may represent contaminants or expression of heteroresistance.

EU-JAMRAI

Joint Action
Antimicrobial Resistance and
Healthcare-Associated Infections

EU-JAMRAI Partners involved in the elaboration of this document:

1. Right-click your mousse
2. Choose change picture
3. Select your Logo

If not used delete grey squares

1. Right-click your mousse
2. Choose change picture
3. Select your Logo

If not used delete grey squares

1. Right-click your mousse
2. Choose change picture
3. Select your Logo

If not used delete grey squares