



## **LABORATORY PROTOCOL**

# **Susceptibility testing by broth microdilution (MIC) of bacteria from aquaculture animals**

**DRAFT**

**April 2026  
Version 0.5 -draft version**

HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
1	New document	First draft of the SOP	April 2026	

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## Background

To harmonise the antimicrobial resistance (AMR) monitoring systems in the European Union (EU), the European Commission (EC) has adopted legislation (1) laying down detailed rules for the monitoring and reporting of AMR in zoonotic and commensal bacteria by Member States (MSs). The legislation provides a framework for routine monitoring of AMR in bovine animals, pigs and poultry. Complementary to that, the European Food Safety Authority (EFSA) has additionally recommended to undertake baseline surveys (BLSs) to assess the epidemiological situation on specific AMR issues (2). One such highlighted topic is the assessment of the prevalence of AMR in bacteria from aquaculture animals. This is of importance, as, at present, there are only limited data on the occurrence of AMR in aquaculture production in Europe and the data that are available cannot easily be compared due to methodological differences.

To address this need, in accordance with Article 31 of Regulation (EC) No 178/2002, EFSA was requested to provide technical and scientific support for the development of a BLS on the prevalence of AMR in bacteria isolated from EU-produced aquaculture animals (finfish and mussels), including proposed harmonised approaches for the collection and analysis of AMR data from aquaculture animals (3). The BLS focuses on bacteria from the environment of healthy aquaculture animals (gut/gills/mucus), instead of clinical isolates of pathogens in aquaculture animals, as an indication of environmental risk factors associated with AMR introduction and spread. The target organisms selected for the BLS include *E. coli*, *K. pneumoniae*, Enterococci, *Vibrio* spp. and *Aeromonas* spp. isolated from aquaculture animals (3).

The present protocol forms part of a series of protocols that together with the “Technical specifications for an EU-wide baseline survey of antimicrobial resistance in bacteria from aquaculture animals” (3) aim to provide harmonised methods for AMR monitoring of bacteria in aquaculture productions. Specifically, the protocol is intended for use by MSs for the susceptibility testing of *Aeromonas* spp. from samples of fish and *Vibrio parahaemolyticus* and *V. alginolyticus* from mussels. The protocol details the MIC procedure, which is already well-known for the laboratories, and states the inoculum preparation, media and incubation conditions for both the *Vibrio* and *Aeromonas* spp. but also the additional bacteria which will be included in the aquaculture BSL; *E. coli*, *K. pneumoniae* and enterococci, to ensure that harmonised MIC results are achieved throughout the MS laboratories. The procedure is based on the reference method by ISO 20776-1:2019 (4).

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Procedure	Theory/comments
<p><b>1. MIC procedure</b></p>	
<p><b>1.1 Preparation of McFarland suspension</b></p> <p>Make a direct broth or saline suspension of 3-5 isolated colonies from an 18- to 24-hour agar plate culture. Mix well.</p> <p>Adjust to McFarland 0.5 suspension (using nephelometer): Calibrate the nephelometer before use and gently turn all suspensions upside-down before measuring. Adjust turbidity of inoculum to match that of standard.</p> <p>If a nephelometer is not available: Compare visually with the McFarland 0.5 standard using white paper with black lines as background.</p> <p>The McFarland 0.5 suspension is diluted as indicated in Table 1 for the different bacterial species (10-30 µl to 10-11 ml broth (CAMHB)). Mix well. The suspension should be used for inoculation within 15 minutes.</p>	<p>The bacteria should be grown on non-selective medium, such as blood agar.</p> <p>McFarland standard suspensions can be prepared in broth or saline according to ISO 20776 (4). Deionized water is not recommended, as some aquatic bacteria may experience osmotic stress in this medium.</p> <p>There can be some variation in the volume of CAMHB in tubes from Sensititre, but this variation is considered to be negligible.</p> <p>The adjusted inoculum should be diluted to give a final cell number of <math>5 \times 10^5</math> CFU/ml (with a target range of <math>2 \times 10^5</math> CFU/ml to <math>8 \times 10^5</math> CFU/ml). CFU counts should be conducted regularly, as confirmation (4).</p>
<p><b>1.2 Inoculation and incubation</b></p> <p>The MIC panels are inoculated with 50 µl or 100 µl (panel specific, depending on the manufacturers guidance) of the inoculum suspension using a multi-channel pipette or Sensititre autoinoculator.</p> <p>Panels are sealed with a sticker lid and incubated according to Table 1. Do not stack panels more than four high.</p>	<p>The antimicrobials to be included for MIC testing are specified in the EFSA Technical specifications (3), and the MIC panel content corresponds to the EU Surveillance panels. For <i>Aeromonas</i> and <i>Vibrio</i> spp. The panel EUAQUAN1 is tentatively suggested.</p> <p>To obtain the correct antibiotic concentration, it is important to follow the manufacturer's instructions. For the EUAQUAN1 panel, 100 µl should be inoculated per well.</p>
<p><b>1.3 Purity and quality control</b></p> <p>Purity control: Spread 10 µl of the inoculation-suspension on a nutrient agar plate. Incubate at the same temperature as the MIC panels overnight.</p> <p>Always run the quality control strains in parallel with the test strains.</p>	<p>An overview of which reference strain(s) to use for which test bacterium is listed in Table 1. The respective quality control (QC) target and range MICs for each antimicrobial are listed in the technical specifications (3). Further QC criteria and information can be found on the EUCAST website (5). Additional described and validated strains may be included as needed.</p>

Procedure	Theory/comments
<p><b>2. Reading and interpretation of MIC</b></p>	
<p>2.1 Reading of MIC results</p> <p>Reading of MIC can be performed using a mirror cabinet (SensiTouch) with manual recording of the readings, or by using the VIZION equipment or equivalent.</p> <p>2.2 Interpretation of MIC results</p> <p>Interpretation of MIC results (S/R) should follow the EFSA Technical specifications (3), and further be used for WT/Non-WT evaluation.</p>	<p>Other types of mirrors than the SensiTouch may be used for reading, but are not optimal, as the design of the mirror and the lighting may cause differences in the MIC values. Mirrors used should not magnify the MIC panel.</p> <p>The EUCAST reading guide (6) should be consulted, in case of doubt of details of the MIC reading.</p> <p>The interpretation of MIC results is based on EUCAST, relevant literature and the CLSI document VET04. Thresholds will be reviewed prior to the implementation of the BLS; and further guidance will be provided by the EURL-AR and EFSA.</p>
<p><b>3. Materials</b></p> <p><b>3.1 Equipment</b></p> <p>McFarland standard 0.5 Nephelometer MIC panels with dehydrated antibiotics in two-fold dilutions (Sensititre -Trek Diagnostic System) Disposable loops (1 µl and 10 µl) Multichannel pipette MIC reader with mirror Disposable reservoir for reagents Graduated pipettes (20 µl - 1000 µl)</p> <p><b>3.2 Media</b></p> <p>Sterile saline or broth, 4 ml volumes in tubes for nephelometer 10-11 ml cation adjusted Mueller-Hinton II broth in sensititre tubes Nutrient agar plates for purity control</p> <p><b>3.3 Bacterial strains for QC</b> See Table 1 for further details</p>	

#### 4 Tables

**Table 1: Overview of inoculum and incubation conditions and QC strains -TENTATIVE INCUBATION CONDITIONS**

Bacteria	Solvent for McFarland inoculum	Volume to transfer from McFarland inoculum to broth <sup>1</sup>	Broth	Incubation conditions	QC strain
<i>Escherichia coli</i>	Saline or CAMBH	30 µl	CAMHB	35±1°C, 18±2 h	<i>E. coli</i> ATCC 25922
<i>Klebsiella pneumoniae</i>	Saline or CAMBH	30 µl	CAMHB	35±1°C, 18±2 h	<i>E. coli</i> ATCC 25922
ESBL/carbapenem resistant Enterobacterales	Saline or CAMBH	30 µl	CAMHB	35±1°C, 18±2 h	<i>P. aeruginosa</i> ATCC 27853
<i>Vibrio spp.</i>	Saline or CAMBH	30 µl	CAMHB	28±2°C, 46 ±2 h	<i>E. coli</i> ATCC 25922
<i>Aeromonas spp.</i>	Saline or CAMBH	30 µl	CAMHB	28±2°C, 46 ±2 h	<i>Aeromonas salmonicida</i> ATCC 33658, <i>E. coli</i> ATCC 25922
<i>Enterococcus spp.</i>	Saline or CAMBH	30 µl	CAMHB	35±1°C, 18±2 h and 24 h <sup>2</sup>	<i>E. faecalis</i> ATCC 29212

- 1) Suggestion from the EURL to use 10-50 µl, but the volume should be verified by each laboratory, dependent on their McFarland preparation
- 2) Reincubation of *Enterococcus* strains for 24 h for glycopeptides, excluding the ATCC type strain.

**Table 2 List of MIC panels and inoculum volume (TENTATIVE PANELS)**

MIC-panel	Inoculum volume*	Suggested use	Notes
EUVSEC3	50 µl/well	<i>E. coli</i> , <i>K. pneumoniae</i>	
EUVSEC2	50 µl/well	<i>E. coli</i> , <i>K. pneumoniae</i> (ESBL/carbapenem resistant)	
EUVENC	50 µl/well	<i>Enterococcus spp.</i>	
EUAQUAN1	100 µl/well	<i>Aeromonas</i> and <i>Vibrio spp.</i>	Tentative panel

\*According to Sensititre

## 5 References

- (1) Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria and repealing Implementing Decision 2013/652/EU. [https://eur-lex.europa.eu/eli/dec\\_impl/2020/1729/oj/eng](https://eur-lex.europa.eu/eli/dec_impl/2020/1729/oj/eng)
- (2) Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food, EFSA Journal Volume 17, Issue 6, e05709, Jun 2019. DOI: <https://doi.org/10.2903/j.efsa.2019.5709>
- (3) Technical specifications for a EU-wide baseline survey of antimicrobial resistance in bacteria from aquaculture animals. EFSA Journal Volume 22, Issue 7, e8928, Jul 2024. DOI: <https://doi.org/10.2903/j.efsa.2024.8928>
- (4) ISO 20776-1:2019 Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases.
- (5) EUCAST - Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. [https://www.eucast.org/fileadmin/eucast/pdf/disk\\_test\\_documents/archive/v\\_15.0 EUCAST QC tables\\_routine\\_and\\_extended\\_QC.pdf version 15.0](https://www.eucast.org/fileadmin/eucast/pdf/disk_test_documents/archive/v_15.0_EUCAST_QC_tables_routine_and_extended_QC.pdf_version_15.0) valid from January 2025
- (6) EUCAST reading guide for broth microdilution v. 5.0, January 2024 [https://www.eucast.org/fileadmin/eucast/pdf/MIC/Reading\\_guide\\_BMD\\_v\\_5.0\\_2024.pdf](https://www.eucast.org/fileadmin/eucast/pdf/MIC/Reading_guide_BMD_v_5.0_2024.pdf)