



LABORATORY PROTOCOL

Isolation of *Aeromonas* from finfish

DRAFT

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Version 1**

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, including proposed harmonised approaches for the collection and analysis of AMR data from aquaculture animals (3). The target organisms selected for the BLSs include *Aeromonas* isolated from finfish (3).

Several features of *Aeromonas* support its selection as an AMR indicator. First, it is ubiquitous in various water environments, including freshwaters, marine waters, estuarine waters, wastewater, and drinking water (3, 4). In addition, it is an organism with great relevance for aquaculture production, as it has been frequently isolated from aquaculture animals, and is a known causative agent of infections in fish and, opportunistically, in humans as well. Detection of *Aeromonas* in finfish production is evenly distributed along the year, allowing the examination of possible seasonal effects (3). Finally, several *Aeromonas* species are naturally competent and commonly carry integrons and other mobile genetic elements (3,5).

Several different species of *Aeromonas* have been isolated from aquatic environments and have been associated with fish diseases, including *A. allosaccharophila*, *A. bestiarum*, *A. caviae*, *A. hydrophila*, *A. jandaei*, *A. salmonicida*, *A. schubertii*, *A. sobria* biovar *sobria* and *A. veronii* biovar *sobria* (3). Due to the large number of aquaculture-relevant *Aeromonas* species and the complexity of species identification in *Aeromonas*, in the aquaculture BLS *Aeromonas* will be examined at the genus level (3).

The present protocol forms part of a series of protocols that together with the “Technical specifications for a 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, including BLSs. Specifically, the protocol is intended for use by MSs for the isolation of *Aeromonas* spp. from samples of finfish gills. The protocol details the procedure step-by-step and includes explanations on the theory behind each step. It can be used in tandem with the following protocols:

- Overview of sampling, pre-enrichment and laboratory analysis of samples for aquaculture baseline surveys
- Identification of bacteria with MALDI-TOF (Matrix-Assisted Laser Desorption-Ionization Time Of Flight)
- Susceptibility testing by MIC of bacteria from aquaculture animals

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Procedure	Theory/comments
<p>1. Isolation of <i>Aeromonas</i></p>	
<p>1.1. Samples should be transported to the laboratory between 0 and 8 °C. Samples should arrive at the laboratory and be initially processed within 48 hours of sampling, where possible. The maximum acceptable time for initial processing is 72 hours from the time of sampling.</p> <p>Upon receiving the samples at the laboratory, inspect them visually and discard any samples with damaged packaging or incorrect labelling. Discard any samples that did not arrive to the laboratory within 72 hours of sampling as well as any samples which have not been kept at the appropriate temperature during transport or storage.</p>	<p>For instructions on sampling, refer to section 6.2 of the document “Technical specifications for a baseline survey on AMR in aquaculture animals” (3). Please note that the baseline study will focus on samples of gills from healthy aquaculture animals.</p> <p>It is necessary to keep the samples at a temperature approaching that of melting ice to avoid unreliable results. Samples should not be frozen.</p> <p>It is important that sample processing be initiated as soon as possible and no later than 48 h after sampling to optimise recovery of <i>Aeromonas</i>.</p> <p>During transport and storage prior to analysis, samples should be handled according to the ISO 7218 standard: “Microbiology of the food chain – General requirements and guidance for microbiological examinations”.</p> <p>For further details, refer to the Guidance document on official controls under Regulation (EU) 2017/625 (6), and the Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 that lays down specific hygiene rules for food of animal origin (7).</p>
<p>1.2. Store samples at the laboratory between 0 and 8 °C until microbiological analysis. Analyse samples within 72 h of sampling, preferably immediately after samples are received and within 48 hours of sampling. It must be ensured that the cold chain is maintained at all times between sample collection and analysis.</p>	<p>For storage prior to analysis, samples should be handled in accordance with the ISO 7218 standard: “Microbiology of the food chain – General requirements and guidance for microbiological examinations”.</p> <p>Samples should not be frozen.</p>
<p>1.3. Pre-enrichment: Add 25 ± 0.5 g of gill sample to 225 mL of buffered peptone water (BPW, Appendix 1) and homogenise.</p>	<p>Homogenization may be performed using a peristaltic blender or other comparable methods.</p>
<p>1.4. Incubate the pre-enrichment culture in appropriate sterile tubes/beakers with lids at 22 ± 1 °C for 48 h. Alternatively, sterile Stomacher bags can also be used for incubation, if this is in accordance with the laboratory’s procedures.</p>	<p>To minimize the risk of spillage, it is recommended to avoid shaking the tubes/containers.</p> <p>Please note that it has not yet been decided whether this step will be included in the final protocol</p>

Procedure	Theory/comments
<p>1.5. After mixing gently the incubated pre-enrichment culture in BPW (step 1.4), streak one loopful (10 µL loop) by applying a single streak onto a glutamate starch phenol red agar (GSP) plate. From this streak, make two additional streaks sequentially in a way that allows the development of well-isolated single colonies. Incubate the plates at 22°C ± 1°C for 48 h under aerobic conditions.</p>	<p>Glutamate starch phenol red agar is commercially available.</p>
<p>1.6. Based on colony morphology (presumptive <i>Aeromonas</i> colonies will appear yellow on glutamate starch phenol red agar), subculture individual colonies onto ChromAgar or nutritive agar (Tryptic Soy agar or Mueller-Hinton agar) plates. Up to three colonies should be individually subcultured. Incubate plates at 22 ± 1 °C for 24-48 h. Subsequently, select one of these subcultures for genus identification (see section 2). If the first subculture is not identified as <i>Aeromonas</i>, proceed with testing the second and, if needed, the third subculture.</p>	<p>In general, the number of subcultured colonies depends on the laboratory's success rate at recognizing and isolating <i>Aeromonas</i> from glutamate starch phenol red agar. <i>Aeromonas</i> colonies appear yellow on glutamate starch phenol red agar plates, as they produce acid when utilizing starch, thereby changing the phenol red pH indicator from red to yellow colour. <i>Pseudomonas</i> may also grow on the plates but will be distinguishable from <i>Aeromonas</i> because of the lack of yellow colour.</p> <p>It is recommended to subculture and store at least three colonies that exhibit a colony morphology typical for <i>Aeromonas</i>. Initially, perform genus identification on one subculture only. If this isolate is not confirmed as <i>Aeromonas</i>, proceed with testing the second and, if necessary, the third subculture. If none of the three subcultures is identified as <i>Aeromonas</i>, the sample may be considered negative.</p>
<p>1.7. Re-subculture one confirmed <i>Aeromonas</i> isolate to avoid contamination. To re-subculture, pick a colony from the subculture and streak it on a new nutritive (Tryptic Soy or Mueller-Hinton) agar plate. Incubate the plate at 22°C ± 1°C for 24-48h. The re-subcultured bacterial isolate should be stored under appropriate conditions.</p>	<p>To store a re-subcultured <i>Aeromonas</i> isolate, collect all bacterial growth from the incubated agar plate and suspend it in a suitable broth supplemented with a cryoprotectant such as glycerol. Store the suspension at -80 °C. Alternative methods of storage may be used, provided that they ensure both the viability and the preservation of the isolate's properties.</p>
<p>2. Genus identification (ID) of <i>Aeromonas</i></p>	
<p>2.1. The genus of presumptive <i>Aeromonas</i> isolates should be confirmed using an appropriate method, such as PCR, MALDI-TOF or sequencing.</p>	<p>It is necessary to confirm the genus of presumptive <i>Aeromonas</i> isolates using an appropriate identification method. Although there are several commercial systems for phenotypic identification of <i>Aeromonas</i>, their specificity is not adequate for confident identification (3).</p>

Procedure	Theory/comments
	<p>MALDI-TOF can be useful and is practical for identification at the genus level, but not at the species level, as the species database for the method is not yet comprehensive (3). For a detailed protocol on how to perform species ID by MALDI-TOF, please refer to the protocol “Identification of bacteria with MALDI-TOF (Matrix-Assisted Laser Desorption-Ionization Time Of Flight)”, which will be available on the EURL’s website.</p> <p>In terms of PCR identification of <i>Aeromonas</i>, for the BSL it is suggested to use the method developed by Khan et al. (8), which targets the conserved flanking regions of the gyrase B subunit (GyrB) gene (3).</p> <p>Alternatively, identification can be based on the sequencing of the housekeeping genes <i>gyrB</i> and <i>rpoD</i> (3).</p>
<p>3. Antibiotic susceptibility testing (AST)</p>	
<p>3.1. Perform antimicrobial susceptibility testing (AST) as described in the protocol “Susceptibility testing by MIC of bacteria from aquaculture animals”.</p>	<p>The AST of the isolates can be performed either immediately after species identification or later using the stored stock culture.</p> <p>The protocol “Susceptibility testing by MIC of bacteria from aquaculture animals” will be available on the EURL-AMR’s website.</p>

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
- (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) FAO, NParks and SFA, 2023. Monitoring and surveillance of antimicrobial resistance in bacterial pathogens from aquaculture – Regional Guidelines of the Monitoring and Surveillance of Antimicrobial Resistance, Use and Residues in Food and Agriculture. Volume 3. Bangkok.
- (5) Piotrowska M and Popowska M (2015) Insight into the mobilome of *Aeromonas* strains. *Front. Microbiol.* 6:494. doi: 10.3389/fmicb.2015.00494
- (6) Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation) <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02017R0625-20250105>
- (7) Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. <https://eur-lex.europa.eu/eli/reg/2004/853/oj/eng>
- (8) Khan, I. U., Loughborough, A., & Edge, T. A. (2009). DNA-based real-time detection and quantification of aeromonads from freshwater beaches on Lake Ontario. *Journal of Water and Health*, 7(2), 312–323. <https://doi.org/10.2166/wh.2009.041>

APPENDIX 1

Composition and preparation of culture media and reagents

The buffered peptone water (BPW), glutamate starch phenol red agar and reagents are available from several companies. The composition of the dehydrated media given below is an example and may vary slightly among the different manufacturers. Note that the media should be prepared according to the manufacturer's instructions, if they differ from the description given here.

Buffered peptone water (Example)

<i>Formula</i>	<i>g/L</i>
Enzymatic digest of casein	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	9.0
Potassium dihydrogen phosphate (KH_2PO_4)	1.5
pH 7.0 +/- 0.2 @ 25°C	

Dissolve the components in water by heating if necessary. Adjust the pH so that after sterilization it is 7.0 +/- 0.2 at 25 °C. Dispense the medium into flasks of suitable capacity to obtain the portions necessary for the test. Sterilize by autoclaving at 121 °C for 15 minutes.

APPENDIX 2

FLOWCHART

for isolation of *Aeromonas* from finfish samples

Non-selective pre-enrichment [steps 1.3-1.4]

25 g of sample in 225 mL of buffered peptone water
(incubate at 22 ± 1 °C for 48 h)



Isolation of presumptive *Aeromonas* [step 1.5]

Streak 10 µL of incubated pre-enrichment culture in BPW onto a **glutamate starch phenol red agar plate**.
(incubate at 22 ± 1 °C for 24-48 h)



Subculture of presumptive *Aeromonas* [step 1.6]

Subculture up to three individual presumptive colonies onto **ChromAgar or Tryptic Soy agar (TSA) or Mueller-Hinton agar plates**.
(incubate at 22 ± 1 °C for 24-48 h)



Identification and storage of isolates [steps 1.7 and 2.1]

Genus ID by appropriate method
Subculture to ensure purity
Storage: Suitable method for keeping isolates viable [see step 1.7 for details].



Antibiotic susceptibility testing [section 3]