



## LABORATORY PROTOCOL

### Isolation of *Vibrio parahaemolyticus* and *V. alginolyticus* from mussels

**DRAFT**

April 2026  
Version 1

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

DRAFT

## 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 the analysis of AMR data from aquaculture animals (3). The target organisms selected for the BLS include *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (3).

*V. parahaemolyticus* is regularly isolated from aquaculture products and is known to cause foodborne infections in humans and disease in aquatic animals (3). Besides its selection for the BLS by EFSA, *V. parahaemolyticus* has additionally been proposed as a surveillance target for AMR monitoring in aquaculture by the Food and Agriculture Organization (FAO) of the United Nations (4).

*V. alginolyticus* is also frequently isolated from aquaculture products, in some cases at much higher rates than *V. parahaemolyticus*, and is considered one of the *Vibrio* most common pathogenic species for humans (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 *V. parahaemolyticus* and *V. alginolyticus* from samples of mussels. The isolation procedure has been based on the methodology proposed by ISO 31872-1:2017 (3,4).

Specifically, the isolation procedure comprises four phases: two pre-enrichment steps, followed by two isolation steps with successive incubation on chromogenic vibrio agar (CVA) and indicative thiosulfate, citrate, bile and sucrose (TCBS) agar plates. Presumptive isolates then undergo species confirmation (3,4).

The present 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
- Identification of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* by PCR
- PCR detection of toxin genes in *V. parahaemolyticus* and *V. alginolyticus*

## Contents

|  |    |
|--|----|
| Isolation of <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> .....                    | 5  |
| Species identification (ID) of <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> .....  | 7  |
| PCR detection of toxin genes in <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> ..... | 8  |
| Antibiotic susceptibility testing (AST).....   | 8  |
| References.....  | 9  |
| Appendix 1: Composition and preparation of culture media and reagents.....                   | 10 |
| Appendix 2: Flowchart.....   | 11 |

DRAFT

| Procedure   | Theory/comments   |
|---|---|
| <p><b>1. Isolation of <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i></b></p>  | <p>The procedure is outlined in the flowchart of Appendix 2.</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 24 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 from healthy live mussels.</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>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 (5), 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 (6).</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 24 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, and the storage time should be minimised, to enhance the recovery of vibrios.</p>   |
| <p>1.3. <u>Primary enrichment in a liquid selective medium:</u></p> <p>Add 25 ± 0.5 g of mussel sample to 225 mL of the primary enrichment medium alkaline saline peptone water (ASPW), preheated to 37 ± 1 °C, in appropriate sterile tubes/beakers with lids. Alternatively, sterile Stomacher bags can also be used for incubation, if this is in accordance with the laboratory’s procedures.</p>   | <p><i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> are often present in small numbers and may represent only a small fraction of the total microbial community. Their successful detection requires four successive phases, including two pre-enrichments that increase the proportion of vibrios in the samples.</p> <p>The ASPW medium is commercially available. Alternatively, details on its composition and preparation can be found in Appendix 1 and ISO 31872-1:2017.</p>   |

| Procedure   | Theory/comments  |
|---|--|
|   | <p>The ASPW primary enrichment medium should be preheated to <math>37 \pm 1^\circ\text{C}</math> prior to inoculation with the test sample.</p> <p>To avoid spillage, the tubes/containers should not be filled completely.</p>  |
| <p>1.4. Homogenise the suspension of the primary enrichment culture and incubate at <math>37 \pm 1^\circ\text{C}</math> for <math>6 \pm 1</math> h.</p>   | <p>Homogenization may be performed using a peristaltic blender or other comparable methods.</p> <p>To minimize the risk of spillage, it is recommended to avoid shaking the tubes/containers.</p>  |
| <p>1.5. <u>Secondary enrichment in liquid selective medium:</u></p> <p>Transfer 1 ml (taken from the surface) of the incubated primary enrichment culture to 10 mL alkaline saline peptone water (ASPW, Appendix 1), preheated to <math>37 \pm 1^\circ\text{C}</math>, in appropriate sterile tubes.</p>  | <p>For transfer to the secondary enrichment medium, take 1 mL from the surface of the incubated primary enrichment culture from step 1.4. The primary enrichment culture should <b>not</b> be agitated before taking the aliquot.</p>  |
| <p>1.6. Incubate at <math>37 \pm 1^\circ\text{C}</math> for <math>18 \pm 1</math> h.</p>  |  |
| <p>1.7. <u>First isolation on solid selective media:</u></p> <p>Spread one loopful (1 <math>\mu\text{L}</math> inoculation loop) of the incubated secondary pre-enrichment culture from step 1.6 on two solid selective media plates (use a fresh sampling loop for each of the plates): 1) an indicative thiosulfate, citrate, bile and sucrose (TCBS) agar plate and 2) a chromogenic vibrio agar (CVA) plate. Spread/streak the culture in a way that allows the development of well-isolated single colonies. Incubate the plates at <math>37 \pm 1^\circ\text{C}</math> for <math>24 \pm 3</math> h.</p> | <p>TCBS agar plates are commercially available. Alternatively, details on the composition and preparation of TCBS plates can be found in Appendix 1 and ISO 31872-1:2017.</p> <p>The type of chromogenic vibrio agar (CVA) plate used for isolation can be selected by the laboratory. There are several commercially available CVA plates, such as the CHROMagar™ Vibrio.</p>   |
| <p>1.8. <u>Second isolation (selection) on solid selective media:</u></p> <p>After incubation, examine the two selective plates for the presence of presumptive <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> colonies (see instructions in the right column). Based on colony morphology, subculture individual presumptive colonies of <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> onto new TCBS and CVA agar plates. Use one plate of each type for each subcultured colony. At least five colonies should be</p>  | <p>On TCBS agar typical colonies of <i>V. parahaemolyticus</i> are smooth, green (negative sucrose) and of 2 to 3 mm in diameter. Instead, typical <i>V. alginolyticus</i> colonies appear as smooth, yellow (sucrose positive) and opaque.</p> <p>On CHROMagar™ Vibrio plates, presumptive <i>V. parahaemolyticus</i> colonies appear as mauve in colour, whereas presumptive <i>V. alginolyticus</i> colonies are typically colourless.</p> <p>Since the agar plates are not fully selective for the target species, it is important to carefully assess</p> |

| Procedure  | Theory/comments  |
|--|--|
| <p>individually subcultured. If both presumptive <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> are observed, subculture at least five colonies of each species separately. Incubate plates at <math>37 \pm 1</math> °C for <math>24 \pm 3</math> h. Subsequently, select one of these subcultures for species identification (see section 2). If the first subculture is not identified as <i>V. parahaemolyticus</i> or <i>V. alginolyticus</i>, respectively, proceed with testing the second and, if needed, the third subculture etc.</p> | <p>and identify any presumptive colonies based on their morphology. At least five presumptive colonies of each of the two targeted species should be subcultured and assessed. If none of the subcultures is found to be <i>V. parahaemolyticus</i> or <i>V. alginolyticus</i> the sample can be considered negative.</p> <p>It should be noted that the appearance of <i>Vibrio</i> species can sometimes vary from one batch of culture medium to another. Thus, the success in recognising the colonies of interest will largely depend on the laboratory's experience. Increasing the number of subcultured and screened colonies can help increase the rate of detection.</p>   |
| <p>1.9. Re-subculture one confirmed isolate of each species (<i>V. parahaemolyticus</i> and/or <i>V. alginolyticus</i>, as applicable) to avoid contamination. To re-subculture, pick a colony from the subculture and streak it on a new TCBS or CVA agar plate. Incubate the plate at <math>37 \pm 1</math> °C for <math>24 \pm 3</math> h. The re-subcultured bacterial isolate(s) should be stored under appropriate conditions.</p>   | <p>Isolates can be stored by suspending a loopful of colony material in a broth containing a cryoprotectant such as glycerol and storing 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><b>2. Species identification (ID) of <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i></b></p>   |  |
| <p>2.1. <u>MALDI-TOF MS screening</u></p> <p>Screen at least five presumptive <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> isolates, subcultured to purity in step 1.8, for species identification using MALDI-TOF mass spectrometry (MS).</p>   | <p>It is necessary to perform species ID on the presumptive <i>Vibrio</i> isolates, as indicated above. As it may be necessary to test several presumptive isolates, it is suggested to perform species ID in two steps: i) as a first step (step 2.1), to screen all presumptive isolates via MALDI-TOF MS, and ii) subsequently, in the second step, to confirm the ID of all isolates identified as <i>V. parahaemolyticus</i> or <i>V. alginolyticus</i> via PCR (step 2.2).</p> <p>If MALDI-TOF MS is not available, it is possible to move directly to identification by PCR.</p> <p>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)". The protocol will be made available on the EURL's website.</p> <p>Note that it is advisable to use freshly grown bacteria (16-26 h at 37 °C) for the MALDI-TOF MS</p> |

| Procedure  | Theory/comments  |
|--|--|
|  | analysis in order to obtain results with better scores.  |
| <p>2.2. <u>PCR confirmatory testing</u></p> <p>Perform polymerase chain reaction (PCR) to confirm the species of isolates identified as <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i> in the MALDI-TOF MS screening (step 2.1).</p> <p>For a detailed protocol on how to perform the PCR confirmatory testing, refer to the protocol “Identification of <i>Vibrio parahaemolyticus</i> and <i>Vibrio alginolyticus</i> by PCR”.</p> | <p>The protocol “Identification of <i>Vibrio parahaemolyticus</i> and <i>Vibrio alginolyticus</i> by PCR” will be made available on the website of the EURL-AMR.</p>   |
| <p>2.3. All confirmed isolates should be re-subcultured to purity and stored, as described in step 1.9.</p>  |  |
| <p>2.4. Confirmed isolates should be subjected to toxin gene typing (section 3) and to antibiotic susceptibility testing (section 4).</p>  |  |
| <p><b>3. PCR detection of toxin genes in <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i></b></p>  |  |
| <p>3.1. Perform PCR detection using the protocol “PCR detection of toxin genes in <i>V. parahaemolyticus</i> and <i>V. alginolyticus</i>”.</p>   | <p>The protocol will be made available on the website of the EURL-AMR.</p>   |
| <p><b>4. Antibiotic susceptibility testing (AST)</b></p>   |  |
| <p>4.1. 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](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 & SFA, 2023. Monitoring and surveillance of antimicrobial resistance in bacterial pathogens from aquaculture – Regional Guidelines for the Monitoring and Surveillance of Antimicrobial Resistance, Use and Residues in Food and Agriculture. Volume 3. Bangkok. <https://doi.org/10.4060/cc3512en>
- (5) 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>
- (6) 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>

## APPENDIX 1

### Composition and preparation of culture media and reagents

The alkaline saline peptone water (ASPW), the thiosulfate citrate bile and sucrose (TCBS) agar, the chromogenic vibrio agar (CVA) and all relevant reagents are available from several companies. The composition of the dehydrated media given below is an example based on the ISO 2187201:2017 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.

#### Alkaline saline peptone water (ASPW)

| <i>Composition</i> |         |
|--------------------|---------|
| Peptone            | 20.0 g  |
| Sodium chloride    | 20.0 g  |
| Water              | 1000 mL |

Dissolve the components in water, by heating if necessary. If necessary, adjust the pH so that after sterilization it is 8.6 +/- 0.2 at 25 °C. Dispense the medium in quantities required for the examination. Sterilize by autoclaving at 121 °C for 15 minutes.

#### Thiosulfate citrate bile and sucrose agar (TCBS)

| <i>Composition</i> |                 |
|--------------------|-----------------|
| Peptone            | 10.0 g          |
| Yeast extract      | 5.0 g           |
| Sodium citrate     | 10.0 g          |
| Sodium thiosulfate | 10.0 g          |
| Iron (III) citrate | 1.0 g           |
| Sodium chloride    | 10.0 g          |
| Dried bovine bile  | 8.0 g           |
| Sucrose            | 20.0 g          |
| Bromothymol blue   | 0.04 g          |
| Thymol blue        | 0.04 g          |
| Agar-agar          | 8.0 to 18.0 g * |
| Water              | 1000 mL         |

\*Depending on the gel strength of the agar-agar.

Dissolve the components or the complete dehydrated medium in the water, by bringing it to a boil. If necessary, adjust the pH so that it is 8.6 +/- 0.2 at 25 °C. Do not autoclave.

Dispense 15 to 20 mL of the medium, cooled down to approximately 50 °C, into sterile Petri dishes and leave to solidify.

## APPENDIX 2

### FLOWCHART

for isolation of *Vibrio parahaemolyticus* and *V. alginolyticus* from mussels

