



LABORATORY PROTOCOL

Isolation of *Escherichia coli* and *Klebsiella pneumoniae* from mussels

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 the analysis of AMR data from aquaculture animals (3). Target organisms selected for the BLS include *Escherichia coli*, as well as and *Klebsiella pneumoniae* (on a voluntary basis) (3).

E. coli was selected as a target organism on the basis of its indicator and monitoring properties. Specifically, *E. coli* can serve as an indicator of faecal contamination and anthropogenic environmental pollution in aquaculture products and its monitoring in live bivalve molluscs is already required by EU rules, as an estimate of the risk of food-borne exposure to faecal pathogens (3-5). *K. pneumoniae* was selected as a second indicator, complementary to *E. coli*, to be included on a voluntary basis, as it is recovered more frequently than *E. coli* from the aqueous milieu and its inclusion may be particularly relevant when the recovery rate of *E. coli* is low (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 *E. coli* and *K. pneumoniae* from samples of mussels.

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
- Isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from mussels

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Procedure	Theory/comments
<p>1. Isolation of <i>E. coli</i> and <i>K. pneumoniae</i></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 (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 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.</p>
<p>1.3. Pre-enrichment: Add 25 ± 0.5 g of mussel 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 37 ± 1 °C for 18-22 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>Note: The pre-enrichment culture can be used in parallel for the isolation of other indicator bacteria included in the BSL, such as ESBL/AmpC/carbapenemase-producing <i>E. coli</i> and enterococci. For an overview on how to consolidate this step, please refer to the protocol “Overview of sampling, pre-enrichment and laboratory analysis of samples for aquaculture baseline surveys”.</p>

Procedure	Theory/comments
<p>1.5. After mixing gently the incubated pre-enrichment culture in BPW (step 1.4), subculture one loopful (10 µL inoculation loop) by applying a single streak onto a MacConkey agar plate. From this streak, make two additional streaks sequentially, using either the same loop or a new loop, to ensure growth of single colonies. Incubate at 37 ± 1 °C for 18-22 h.</p> <p>As a voluntary measure, the MacConkey plate may be supplemented with an additional Simmons citrate agar plate with inositol to target recovery of <i>Klebsiella</i> (8).</p>	
<p>1.6. Subculture presumptive colonies</p> <p>For <i>E. coli</i></p> <p>Based on colony morphology [presumptive <i>E. coli</i> colonies will usually be red/purple on MacConkey agar (Figure 1)], subculture individual colonies onto new MacConkey agar plates. Up to three colonies should be individually subcultured. Incubate all plates at 37 ± 1 °C for 18-22 h. Subsequently, select one of these subcultures for species identification (see section 2). If the first subculture is not identified as <i>E. coli</i>, proceed with testing the second and, if needed, the third subculture.</p> <p>For <i>K. pneumoniae</i></p> <p>In case the MacConkey agar plate or the Simmons citrate agar plate with inositol yields colonies resembling <i>Klebsiella</i> spp., these colonies may also, on a voluntary basis, be subcultured as described above for <i>E. coli</i>.</p>	<p>In general, the number of subcultured colonies depends on the laboratory's success rate at recognizing and isolating <i>E. coli</i> and/or <i>Klebsiella</i> from MacConkey agar.</p> <p>For <i>E. coli</i>, it is recommended to subculture and store at least three colonies that exhibit a colony morphology typical for <i>E. coli</i>. Initially, perform species identification on one subculture only. If this isolate is not confirmed as <i>E. coli</i>, proceed with testing the second and, if necessary, the third subculture. If none of the three subcultures is identified as <i>E. coli</i>, the sample may be considered negative. It is important to note that non-lactose-fermenting <i>E. coli</i> may occur but will not be detected by this method, as they will appear with a neutral colour (not red/purple) on MacConkey agar.</p> <p>For <i>K. pneumoniae</i>, the same procedure should be followed, focusing on isolates with a colony morphology typical for <i>Klebsiella</i>. <i>Klebsiella</i> appear as red or pink mucoid colonies on MacConkey agar plates and as yellow, dome-shaped, often mucoid, colonies on Simmons citrate agar plates with inositol (8).</p>
<p>1.7. Re-subculture one confirmed isolate (<i>E. coli</i> or <i>K. pneumoniae</i>, as applicable) to avoid contamination. To re-subculture, pick a colony from the subculture and streak it on a new MacConkey plate. Incubate the plate at 37 ± 1 °C for 18-22 h. The re-subcultured bacterial isolate should be stored under appropriate conditions.</p>	<p>The isolate 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>

Procedure	Theory/comments
<p>2. Species identification (ID) of <i>E. coli</i> and <i>K. pneumoniae</i></p>	
<p>2.1. It is always necessary to perform species ID on the presumptive <i>E. coli</i> and <i>K. pneumoniae</i> isolates, as indicated above. The species ID should be conducted using an appropriate method.</p>	<p>Different laboratories may have different methods (biochemical tests, mass spectrometry, chromogenic agar, genotypic methods, etc.) for performing species identification. Chromogenic agar can be useful for distinguishing presumptive <i>E. coli</i> and presumptive <i>K. pneumoniae</i> from other bacterial species that may have similar colony appearance on MacConkey agar.</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)”, which will be available on the EURL’s website.</p>
<p>3. Antibiotic susceptibility testing (AST)</p>	
<p>3.1. Perform antimicrobial susceptibility testing (AST) using the first panel of antimicrobials, as described in Table 2 of the Commission Implementing Decision 2020/1729/EU. If resistant to cefotaxime, ceftazidime, and/or meropenem, the isolate must be further tested using the panel of beta-lactam antimicrobials (Table 5, Decision 2020/1729/EU). The AST of the isolates can be performed either immediately after species identification or later using the stored stock culture.</p> <p>For a detailed protocol on how to perform the AST, please refer to the protocol “Susceptibility testing by MIC of bacteria from aquaculture animals”.</p>	<p>If the isolate is found to be resistant to cefotaxime, ceftazidime and/or meropenem in the first panel of antimicrobials (Table 2 of the Commission Implementing Decision 2020/1729/EU), the isolate must be tested further using the second panel of beta-lactam antimicrobials listed in Table 5 (1).</p> <p>Resistance phenotypes, including synergy (i.e. a ≥ 3 twofold-concentration decrease in the MIC for cefotaxime and/or ceftazidime when tested in combination with clavulanic acid compared to the MIC of cefotaxime and/or ceftazidime when tested alone), can be assessed using the EFSA guidelines (5).</p> <p>The protocol “Susceptibility testing by MIC of bacteria from aquaculture animals” will be available on the EURL-AMR’s website.</p>

Figures



Figure 1: Typical appearance of *E. coli* on MacConkey agar plates.

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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) WHO integrated global surveillance on ESBL-producing *E. coli* using a “One Health” approach: implementation and opportunities. Geneva: World Health Organization; 2021.
- (5) EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2023–2024. EFSA Journal, 23(3), e9237, Feb 2026.
- (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) Van Kregten E, Westerdal NA, Willers JM. New, simple medium for selective recovery of *Klebsiella pneumoniae* and *Klebsiella oxytoca* from human feces. J Clin Microbiol. 1984 Nov;20(5):936-41.
doi: 10.1128/jcm.20.5.936-941.1984. PMID: 6392324; PMCID: PMC271478.

APPENDIX 1

Composition and preparation of culture media and reagents

The buffered peptone water (BPW), MacConkey 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.

MacConkey agar (Example)

<i>Formula</i>	<i>g/L</i>
Pancreatic Digest of Gelatin	17.0
Peptones (meat and casein)	3.0
Lactose	10.0
Bile salts No. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	13.5
pH 7.1 +/- 0.2 @ 25 °C	

Suspend 50 g in 1 L of distilled water (Optional: Add 6.5 g agar to increase the hardness of the agar plates). Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 minutes.

APPENDIX 2

FLOWCHART

for isolation of *E. coli* and *K. pneumoniae* from mussels

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

25 g of sample in 225 mL of buffered peptone water
(homogenise and incubate at 37 ± 1 °C for 18-22 h)



Isolation of presumptive *E. coli* and *K. pneumoniae* [step 1.5]

Streak 10 µL of incubated pre-enrichment culture in BPW onto a **MacConkey agar plate**.

Optional: Additionally streak 10 µL of incubated pre-enrichment culture onto a **Simmons citrate agar plate with inositol**.

(incubate at 37 ± 1 °C for 18-22 h)



Subculture of presumptive *E. coli* or *K. pneumoniae* [step 1.6]

Subculture up to three individual presumptive colonies onto **MacConkey agar plate**.

(incubate at 37 ± 1 °C for 18-22 h)



Identification and storage of isolates [section 2; step 1.7]

Species ID by use of appropriate method

Subculture to ensure purity

Storage: Suitable method for keeping isolates viable for at least five years [see step 1.7 for details].



Antibiotic susceptibility testing [section 3]

Testing on the first panel (Table 2 of Commission Implementing Decision 2020/1729/EU) and, if resistant to cefotaxime, ceftazidime and/or meropenem, further testing on the second panel (Table 5 of Commission Implementing Decision 2020/1729/EU)