



LABORATORY PROTOCOL

Isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* 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, 3). 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 has been 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* producing extended-spectrum beta-lactamases (ESBLs), AmpC cephalosporinases, and/or carbapenemases (3).

In general, ESBL-, AmpC-, and carbapenemase-producing Enterobacteriales in food-producing animals present a major public health concern in the EU, as well as globally (2-4). As a result, ESBL-, AmpC- and carbapenemase-producing *E. coli* have been included in the EU mandatory monitoring of AMR in bovine animals, pigs and poultry, to complement the monitoring of commensal *E. coli*, and serve as an indicator of the level of circulating ESBL-, AmpC- and carbapenemase-encoding genes (1,2,5).

This 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. Specifically, the present protocol is intended for use by MSs for the isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* 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

Contents

Isolation of ESBL- and AmpC-producing <i>E. coli</i>	5
Specific isolation of carbapenemase-producing <i>E. coli</i>	7
Species identification (ID) of <i>E. coli</i>	9
Antibiotic susceptibility testing (AST).....	9
Figures	10
References.....	11
Appendix 1: Composition and preparation of culture media and reagents.....	12
Appendix 2: Flowchart.....	13

DRAFT

Procedure	Theory/comments
<p>1. Isolation of ESBL- and AmpC-producing <i>E. coli</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 on <i>E. coli</i> 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. Validate the selective MacConkey agar plates that will be used for selective isolation, using the Quality Control (QC) procedure recommended by the EURL-AMR.</p>	<p>A detailed protocol with instructions on how to perform the QC procedure can be found on the EURL-AMR website (https://www.food.dtu.dk/english/topics/antimicrobial-resistance/eurl-ar/protocols). The negative and positive control strains required for the QC are provided by the EURL-AMR.</p> <p>It is strongly recommended to complete the QC procedure for selective plate validation before starting the enrichment procedure (step 1.4). If, instead of in advance, the QC is performed in parallel with sample enrichment and plating, there is a risk that any results obtained may need to be rejected, if the plates fail validation.</p>

Procedure	Theory/comments
<p>1.4. 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>1.5. 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 tandem for the isolation of other indicator bacteria included in the BSL, such as <i>E. coli</i> commensal bacteria, <i>Klebsiella pneumoniae</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>
<p>1.6. After mixing gently the incubated pre-enrichment culture in BPW (step 1.5), subculture one loopful (10 µL inoculation loop) by applying a single streak onto a MacConkey agar plate containing 1 mg/L of cefotaxime (CTX). 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>The pre-enrichment culture in BPW (step 1.5) should be retained for the isolation of carbapenemase-producing <i>E. coli</i> (section 2.1).</p>	
<p>1.7. Based on colony morphology [presumptive ESBL-/AmpC-producing <i>E. coli</i> colonies will usually be red/purple on MacConkey agar (Figure 1)], subculture individual colonies onto new MacConkey agar plates containing 1 mg/L CTX to maintain selective pressure. Up to three colonies should be individually subcultured. Incubate all plates at 37 ± 1 °C for 18-22 h. Subsequently, select one of the subcultures for species identification (see section 3). 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>In general, the number of subcultured colonies depends on the laboratory's success rate at recognizing and isolating <i>E. coli</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.</p> <p>Note: 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>

Procedure	Theory/comments
<p>1.8. Re-subculture one confirmed <i>E. coli</i> isolate to avoid contamination. To re-subculture, pick a colony from the subculture and streak it on a new MacConkey plate containing 1 mg/L CTX to maintain selective pressure. 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>
<p>1.9. Perform antimicrobial susceptibility testing (AST) as described in section 4.</p>	<p>It is mandatory to test all presumptive ESBL/AmpC-producing <i>E. coli</i>, as described in the Commission Implementing Decision 2020/1729/EU (1).</p>
<p>2. Specific isolation of carbapenemase-producing <i>E. coli</i></p>	<p>The procedure is outlined in the flow diagram of Appendix 2.</p>
<p>2.1. To select for and isolate carbapenemase-producing <i>E. coli</i>, plate one loopful (10 µl loop) of the incubated pre-enrichment culture in BPW from step 1.5 on suitable selective agar(s), as described on the right column and presented in Figure 2.</p> <p>First, plate the 10 µL of the pre-enrichment culture as a confluent layer covering one-quarter of the selective agar plate. Then, perform streaking within an adjacent quarter of the plate using either the same loop or a sterile 1-µL loop to isolate single colonies. Using this streaking strategy, each plate can be used for plating two different samples (Figure 2).</p> <p>Incubate the selective agar plates according to the manufacturer's instructions.</p>	<p>The methodology presented enables the selective isolation of presumptive carbapenemase-producing <i>E. coli</i>, including strains that produce OXA-48 and OXA-48-like enzymes.</p> <p>While various types of selective agar can be used, commercially available chromogenic agars are generally preferred. When selecting agar, it is important to note that some commercial selective media exhibit low sensitivity and/or specificity for detection of OXA-244-producing strains. Given the variability in sensitivity and specificity among selective media, which may also depend on the type of carbapenemase produced, it may be necessary to complement the selective properties by using multiple media types in parallel. If more than one type of plate is used, spread 10 µL of the incubated pre-enrichment culture onto each type of plate.</p> <p>It is up to the user to decide whether to use half a plate or one plate per sample. When using one plate, 20 µL of the pre-enrichment culture in BPW should first be plated onto one half of the selective agar plate. From this area, streaks should be made across the second half using either the same loop or a sterile 1-µL loop to isolate single colonies.</p> <p>For successful isolation, it is important to use selective agar plates that have been validated for their specificity and sensitivity in detecting carbapenemase-producing <i>E. coli</i>. A protocol detailing the methodology for validating the selectivity of the agar plates is available on the EURL-AMR website (https://www.food.dtu.dk/english/topics/antimicrobial-resistance/eurl-ar/protocols). The positive and negative</p>

	<p>control strains required for the validation are provided by the EURL-AMR.</p> <p>Note: In general, most media containing carbapenemase-selective agents have a short shelf-life, which should be strictly observed. Additionally, it is important to store the plates according to the manufacturer's recommendations to ensure optimal performance.</p>
<p>2.2. Subculture at least one colony of presumptive carbapenemase-producing <i>E. coli</i> onto either (i) a commercially available selective chromogenic agar for isolation of carbapenemase-producing <i>E. coli</i>, including isolates producing only OXA-48 and/or OXA-48-like enzymes, or (ii) a MacConkey agar plate without antibiotic supplementation, or (iii) a MacConkey agar plate supplemented with meropenem (0.125 mg/L).</p> <p>If more than one colony is selected, each colony should be subcultured separately. If additional selective plates are used to target presumptive OXA-48/OXA-48-like-producing <i>E. coli</i>, at least one colony from each plate should also be subcultured. Again, if multiple colonies are selected from a certain plate, each colony should be subcultured separately.</p>	<p>Ideally, a commercial chromogenic agar selective for carbapenemase-producing <i>E. coli</i>, including isolates producing only OXA-48 and/or OXA-48-like enzymes, should be used for subculturing. However, if there is a need to reduce costs, MacConkey agar plates without antimicrobial supplementation represent a valid alternative. The addition of cefotaxime is not recommended, as this cephalosporin is not an optimal substrate for many carbapenemases. It is, instead, possible to supplement with a carbapenem.</p>
<p>2.3. Incubate at 37 ± 1 °C for 18-22 h.</p>	<p>After incubation, the obtained bacterial colonies can either be analysed immediately or stored under appropriate conditions for later analysis (see step 2.5).</p>
<p>2.4. Perform species identification (section 3) to confirm the species of subcultured isolates.</p>	
<p>2.5. To avoid contamination and confirm carbapenem resistance, re-subculture isolates confirmed to be <i>E. coli</i> onto appropriate selective agar plates.</p> <p>Store the re-subcultured bacterial isolate(s) 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>2.6. Perform antimicrobial susceptibility testing (AST) as described in section 4.</p>	<p>Isolates that are resistant to carbapenems, based on MIC results, should undergo further phenotypic or genotypic</p>

		testing to confirm the presence of a carbapenemase, in accordance with EFSA recommendations (1,5).
3. Species identification (ID) of <i>E. coli</i>		
3.1. Species identification should be performed for all presumptive ESBL-, AmpC- and carbapenemase-producing <i>E. coli</i> isolates, as indicated above. The species ID should be conducted using an appropriate method.		<p>Different laboratories may have different methods (biochemical tests, mass spectrometry, chromogenic agar, genotypic methods, etc.) for performing species ID. Chromogenic agar can be useful for distinguishing presumptive <i>E. coli</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 made available on the EURL’s website.</p>
4. Antibiotic susceptibility testing (AST)		
4.1. 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>All presumptive ESBL/AmpC/carbapenemase-producing <i>E. coli</i> must be tested using the first panel of antimicrobials listed in Table 2 of the Commission Implementing Decision 2020/1729/EU (1). If an isolate is found to be resistant to cefotaxime, ceftazidime and/or meropenem, it must be tested further using the second panel of beta-lactam antimicrobials listed in Table 5 of the Commission Implementing Decision 2020/1729/EU.</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>
For a detailed protocol on how to perform the AST, please refer to the protocol “Susceptibility testing by MIC of bacteria from aquaculture animals”.		The protocol “Susceptibility testing by MIC of bacteria from aquaculture animals” will be available on the EURL-AMR’s website.

Figures



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

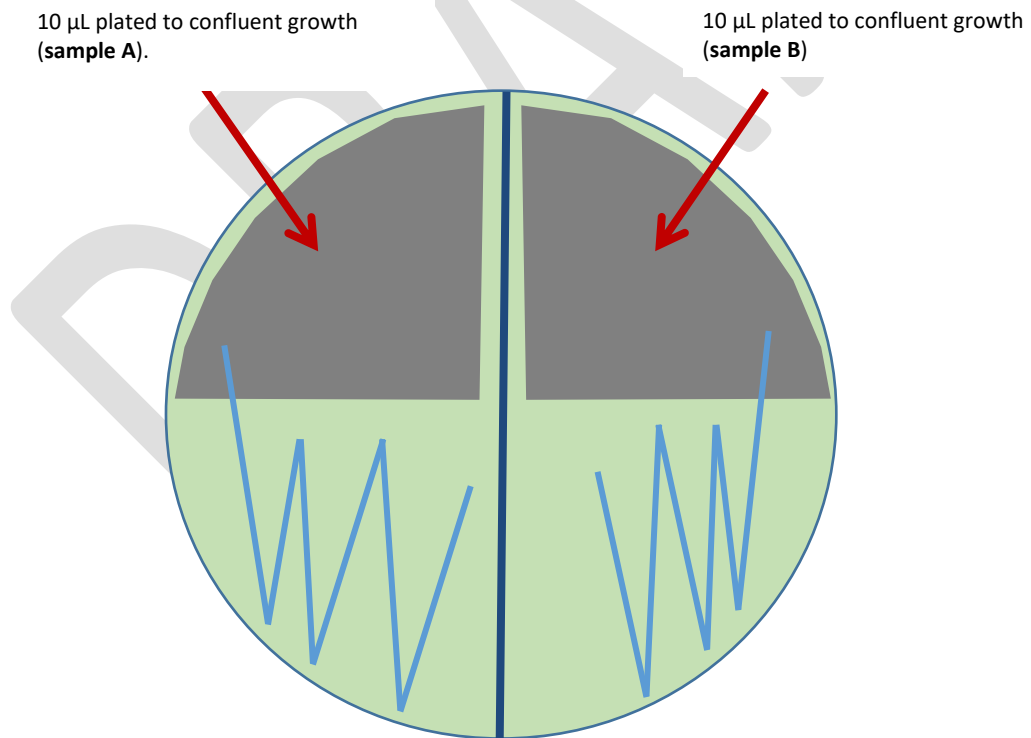


Figure 2: Plating on selective plates to detect presumptive carbapenemase-producing *E. coli*.

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>

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* producing ESBL/AmpC/carbapenemases (including OXA-48 and OXA-48-like enzymes) from mussels

Non-selective pre-enrichment [steps 1.4-1.5]

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



Selective isolation

→ of presumptive ESBL-/AmpC-/carbapenemase-producing *E. coli* [step 1.6]

Streak 10 µL of the incubated pre-enrichment culture in BPW onto a **MacConkey agar plate supplemented with 1 mg/L cefotaxime**
(incubate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-22 h)

→ of presumptive carbapenemase (including OXA-48 / OXA-48-like)-producing *E. coli* [steps 2.1]

Streak 10 µL of the incubated pre-enrichment culture in BPW onto **suitable selective agar plate(s)**
(commercially available chromogenic agar for isolation of carbapenemase-producing *E. coli*, including isolates producing only OXA-48 and/or OXA-48-like enzymes)
(incubate according to manufacturer's instructions)



Subculture

→ of presumptive ESBL-/AmpC-/carbapenemase-producing *E. coli* [step 1.7]

Subculture individual presumptive colonies onto **MacConkey agar plate(s) supplemented with 1 mg/L cefotaxime** to maintain selective pressure
(incubate at 37 ± 1 °C for 18-22 h)

→ of presumptive carbapenemase (incl. OXA-48 / OXA-48-like)-producing *E. coli* [steps 2.2-2.3]

Subculture individual presumptive colonies onto either i) **commercially available selective chromogenic agar** for isolation of carbapenemase-producing *E. coli*, including isolates producing only OXA-48 and/or OXA-48-like enzymes, or ii) **MacConkey agar without antibiotic supplementation**, or iii) **MacConkey agar with meropenem supplementation (0.125 mg/L)**
(incubate at 37 ± 1 °C for 18-22 h)



Identification and storage of isolates [section 3; steps 1.8 + 2.5]

Species ID by appropriate method

Subculture to ensure purity

Storage: Suitable method for keeping isolates viable

Antibiotic susceptibility testing [section 4]

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)