DTU Food
National Food Institute

European Union Reference Laboratory Antimicrobial Resistance

LABORATORY PROTOCOL

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

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Version 7

Version 7 was reviewed and updated by: Rene S. Hendriksen and Valeria Bortolaia

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HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
7	Comment in relation to 1.2	The comment field was updated with the results of the 96h validation experiments performed at the EURL-AR in 2018	09 Dec 2019	Rene Hendriksen, Valeria Bortolaia
6	1.7, 3.1, 3.3, references, and Appendix 2	Improving the description of the used selective media	13 Feb 2018	Rene Hendriksen, Valeria Bortolaia
5	1.1 and 1.2	Sample collection shall be randomized equally over all five business days of the week.	03 Nov 2017	Rene Hendriksen, Valeria Bortolaia
5	Throughout the document	Editorial changes. Added acceptance interval for the incubation temperatures Specified the use of 1-µL or 10-µL loop		
4	1.8 and 3.4	Re-isolation step before storing described	10 Jan 2017	Lina Cavaco, Rene Hendriksen
3	Background Flow diagram	The protocol now also applies to caecal samples from poultry Correction of the concentration unit from mg/mL to mg/L	13 Oct 2015	Lina Cavaco, Rene Hendriksen
2	All through the document	Numbers adjusted for each item of the procedure. Max time of arrival to laboratory Editorial changes	08 Dec 2014	Authors
1	New document	-	21 Nov 2014	Authors

Background

Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs), AmpC cephalosporinases and carbapenemases are of major public health significance (1, 2). In order to harmonize the antimicrobial resistance (AMR) monitoring systems in the European Union (EU), the European Commission (EC) adopted legislation laying down detailed rules for the monitoring and reporting of AMR in zoonotic and commensal bacteria by Member States (MSs). The legislation, 'Commission Implementing Decision on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria' (2013/652/EU) (3), includes the obligatory monitoring of ESBL- and AmpC-producing *E. coli* and the voluntary monitoring of carbapenemase-producing *E. coli* in meat and caecal samples, according to the most recent version of the protocol of the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR). This protocol is intended for use at the MS level for isolation of *E. coli* producing ESBL, AmpC and/or carbapenemase enzymes from caecal samples as laid down in point 4.1 of Annex of Commission Implementing Decision 2013/652/EU.

It has to be emphasized that the protocol for monitoring ESBL- and AmpC-producing *E. coli* has the potential to detect also most variants of carbapenemases produced in *E. coli*, as these normally confer reduced susceptibility to third-generation cephalosporins. An exception is represented by OXA-48 and OXA-48-like producers, which will be undetected by using the ESBL/AmpC monitoring protocol unless they simultaneously co-produce an ESBL or an AmpC enzyme. Furthermore, if a sample contains both an ESBL/AmpC producer and a carbapenemase-producer, the method will only detect one of the two, likely depending on the ratio between ESBL/AmpC and carbapenemase-producers in a given sample.

The present protocol is the result of a large testing of several methods and validation was performed for cattle and pig caecal samples by the EURL-AR at the Technical University of Denmark assisted by the Federal Institute for Risk Assessment (BfR) in Germany. The protocol has been discussed in an expert meeting organized by the EC and held in Brussels, with experts from the ECDC, EFSA, EUCAST and some MSs and non-MSs with both human and veterinary background. The protocol explains the procedure step-by-step and provides explanation of the theory behind each step. Subsequently, the protocol has been validated on caecal samples of poultry by the EURL-AR.

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Procedure (4, 5, 6, 7)		**Theory/comments**
1. Isolation and identification of ESBL-, AmpC-, and carbapenemase-producing *E. coli*		
1.1. Sample collection shall be randomized equally over all five business days of the week. It is recommended that samples should arrive at the laboratory within 36 hours after sampling. Samples which have not been stored appropriately (5°C ± 3°C) under transportation or storage shall be discarded.		It is necessary to keep the samples refrigerated to avoid unreliable results. During transport and storage prior to analysis, samples shall be handled according to the ISO/DIS 7218 standard: Microbiology of food and animal feeding stuffs – General rules for microbiological examinations. For further details, see chapter 5.7 of the Guidance document on official controls, under Regulation (EC) No 882/2004, concerning microbiological sampling and testing of foodstuffs (8).
1.2. The samples shall be stored at the laboratory at a temperature between 5°C ± 3°C until microbiological analysis. This should be initiated as soon as possible after receipt in the laboratory, preferably within 24 hours. It is recommended that analysis is started, as a rule, within 48 hours after collecting the sample. Exceptions to this rule are samples collected on Thursdays and Fridays, these samples may be analyzed on the following Monday at the latest, i.e. within 96 hours after collecting the sample. It must be ensured that the cold chain is kept at all times between sample collection and analysis.		For further details, see chapter 5.7 of the Guidance document on official controls, under Regulation (EC) No 882/2004, concerning microbiological sampling and testing of foodstuffs (8). This protocol was initially validated for storage of samples in the laboratory for up to 24 hours. In 2018, the EURL-AR validated the 96-hour time interval between collection and analysis of samples to be applied to samples collected on Thursdays and Fridays.
1.3. A Quality Control (QC) procedure to validate the selective MacConkey agar plates should be performed according to EURL-AR recommendations (see www.eurl-ar.eu/protocols.aspx) prior to initiating the enrichment procedure provided below.		The EURL-AR has provided all NRL-AR with negative and positive control strains and a specific protocol on how to perform the QC procedure. It is strongly recommended to have completed the QC procedure to validate the plates prior to performing the enrichment procedure. Otherwise, if the QC procedure is carried out in parallel with the sample enrichment and plating, there is a risk of having to reject the results of the enrichment, if the plates fail the validation.

Procedure (4, 5, 6, 7)		**Theory/comments**
1.4. Pre-enrichment: 1 g ± 0.1 g of caecal sample is added to 9 mL of buffered peptone water (BPW) in appropriate sterile tubes/beakers with lids.		To avoid spillage, the tubes/containers should not be filled completely. It is recommended to use 50 mL tubes.
1.5. Incubate the tubes (pre-enrichment culture) at 37°C ± 1°C for 18-22 h.		To minimize the risk of spillage, it is recommended to avoid shaking the tubes.
1.6. After mixing gently the incubated pre-enrichment culture in BPW [item 1.5]. Subculture by streaking one loopful (10 µL loop) over the surface of the MacConkey agar containing 1 mg/L of cefotaxime (CTX) (Appendix 1). As indicated in Figure 1, to thin out the bacterial culture, from the first streak, make further two streaks using either the same loop or a 1 µL loop to ensure growth of single colonies. Incubate the plates at 44°C ± 0.5°C for 18-22 h.		OXA-48 and OXA-48-like carbapenemase producers do not consistently hydrolyze cefotaxime (and other third-generation cephalosporins). Thus, this method will likely overlook most OXA-48 and OXA-48-like producers. Please consult the specific method for isolation of OXA-48 and OXA-48-like producers below. Plates are incubated at 44°C ± 0.5°C to minimize the influence of natural background flora. This temperature appears to be permissive for the growth of most *E. coli* strains.
1.7. Based on colony morphology [presumptive ESBL-/AmpC-producing *E. coli* colonies will usually be red/purple on the MacConkey agar plates containing 1 mg/L CTX (Figure 1)] – sub-culture individual colonies onto MacConkey agar containing 1 mg/L CTX to maintain the selective pressure. Up to three colonies should be individually subcultured. Incubate at 37°C ± 1°C for 18-22 h. Subsequently, select one of these subcultures for species identification (ID). In case the first subculture is not identified as *E. coli*, the second and eventually the third subculture shall be tested.		In general, the number of sub-cultured colonies is dependent on the laboratory's success rate of recognizing and isolating *E. coli* from MacConkey agar. It is recommended to subculture and store at least three colonies with a colony morphology typical for *E. coli*. Initially, perform species identification on one subculture only. In case this isolate is not an *E. coli*, the second and eventually the third subculture can be tested. In case none of the three subcultures is identified as *E. coli*, the sample can be regarded as negative for ESBL-/AmpC-producing *E. coli*. Non-lactose fermenting *E. coli* can occur but will not be detected by this method as they will appear with a neutral color (not red/purple) on MacConkey agar.
1.8. One confirmed *E. coli* isolate presumptively producing ESBL-/AmpC shall be re-subcultured to avoid contamination and to confirm the growth in presence of 1 mg/L CTX. This is performed by picking a colony from the subculture and streaking it on a		The isolate could be stored by suspending a loopful of colony material in a broth containing a cryoprotectant such as glycerol and kept at -80˚C. Alternative methods of storage may be used provided that they ensure viability and absence of changes in isolate properties.

Procedure (4, 5, 6, 7)		**Theory/comments**
new plate of the relevant selective agar, which is then incubated at 37°C ± 1°C for 18-22 h. This re-subcultured bacterial isolate should be stored under appropriate conditions for at least five years.		
1.9. Perform antimicrobial susceptibility testing (AST) utilizing the first panel of antimicrobials, as described in Table 1 of Commission Implementing Decision 2013/652/EU. If resistant to cefotaxime, ceftazidime and/or meropenem, the isolate must be further tested using the panel of beta-lactam antimicrobials (Table 4 of Commission Implementing Decision 2013/652/EU). The AST of the isolates can be performed either immediately after verification of the species ID or later from the storage stock for final reporting.		It is mandatory to test presumptive ESBL-/AmpC-producing *E. coli* using the first panel of antimicrobials listed in Table 1 of Commission Implementing Decision 2013/652/EU. If the isolate is resistant to cefotaxime, ceftazidime and/or meropenem, it is mandatory to further test it using the second panel of antimicrobials reported in Table 4 of the Decision 2013/652/EU. If performing the voluntary monitoring of carbapenemase-producing *E. coli* and the isolate is resistant to meropenem in the first panel of antimicrobials, the second panel of antimicrobials should be tested. Resistance phenotypes including synergy (i.e. a ≥ 3 twofold concentration decrease in the MIC for cefotaxime and/or ceftazidime tested in combination with clavulanic acid vs the MIC of cefotaxime and/or ceftazidime tested alone) can be assessed using the guidelines given in Chapter 4.1, Part A of Annex of Commission Implementing Decision 2013/652/EU.
2. Species identification *E. coli*		
2.1. It is always necessary to perform species ID on the presumptive ESBL-/AmpC-producing *E. coli* isolates as indicated above. The species ID of *E. coli* should be conducted by using an appropriate method.		Different laboratories may have different methods (biochemical tests, mass spectrometry, chromogenic agar, genetic-based methods, etc.) for performing species identification of *E. coli*. Chromogenic agar can be useful to distinguish presumptive *E. coli* from other bacterial species that may have similar colony appearance on MacConkey agar.

Procedure (4, 5, 6, 7)		**Theory/comments**
3. Specific isolation of carbapenemase-producing *E. coli*		
3.1. To specifically isolate carbapenemase-producing *E. coli* (including strains producing OXA-48 and OXA-48-like enzymes) from the caecal samples, inoculate one loopful (10 µL loop) of the incubated pre-enrichment culture in BPW [item 1.5] onto suitable selective agar(s) as shown in Figure 2 (see also flow diagram in Appendix 2). In details, the 10 µL of the pre-enrichment culture are plated for confluent growth on ¼ of a plate and further streaking is performed using either the same loop or a 1 µL loop within an additional ¼ of the plate to obtain single colonies. In this way, each plate can be used for cultures from two samples (Figure 2).		It is important to choose selective agar plates that have been validated with regard to specificity and sensitivity of detection of carbapenemase-producing *E. coli* using the control strains described below. Preferably, a commercially available chromogenic agar for isolation of carbapenemase-producing *E. coli* (including isolates producing only OXA-48 and/or OXA-48-like enzymes) shall be used. If two different plates are required to accomplish this, 10 µL are spread on each type of plate. It is up to the user to decide if using ½ plate or 1 plate per sample. If using 1 plate per sample, 20 µL of pre-enrichment culture in BPW should be plated on half of the plate and streaks made on the second half of the plate using either the same loop or a 1-µL loop. A protocol for validation of the method is available from the EURL-AR website (https://www.eurl-ar.eu/protocols.aspx). Positive and negative control strains have been forwarded to all National Reference Laboratories for Antimicrobial Resistance (NRL-AR) and should be included in parallel with the sample testing. Note: In general, most media containing a carbapenemase-selective agent have a short shelf-life, which should be strictly followed. In addition, it is important to ensure that storage of the plates is done according to the manufacturer's recommendation.
3.2. The selective agar plates are incubated according to the manufacturer's instructions.		
3.3. Subculture one colony of presumptive carbapenemase-producing *E. coli* onto a MacConkey agar plate without antibiotic supplements and incubate at 37°C ± 1°C for 18-22 h. If selecting more than one		Ideally, a commercially available chromogenic agar for isolation of carbapenemase-producing *E. coli* (including isolates producing only OXA-48 and/or OXA-48-like enzymes) could be used but, to contain costs, use of MacConkey agar plates without

	Procedure (4, 5, 6, 7)		**Theory/comments**
	colony, separate subcultures should be performed. If presumptive OXA-48-/OXA-48-like-producing *E. coli* were isolated on a separate selective plate compared to other carbapenemase-producing *E. coli* (item 3.1), one colony from this plate should also be subcultured onto a MacConkey agar plate without antibiotic supplements and incubate at 37°C ± 1°C for 18-22 h. Also in this case, if selecting more than one colony, separate subcultures should be performed. After incubation, the obtained bacterial colonies are either processed immediately or stored under appropriate conditions (as described below in item 3.4) for later analysis. Independent of the time of analysis, the colonies grown on the MacConkey agar plate should be subjected to species identification and antimicrobial susceptibility testing as described above in item 2.1 (species ID) and 1.9 (AST), respectively.		antimicrobial supplement represents a valid alternative. Addition of cefotaxime is not recommended as this cephalosporin is not the optimal substrate for various carbapenemases.
3.4	One confirmed *E. coli* isolate presumptively producing a carbapenemase shall, at this stage, be stored under appropriate conditions after a re-subculture as explained below. Isolates should be stored for at least five years as described above in item 1.8. Prior to storing the *E. coli* isolate, to avoid contamination and to confirm carbapenem resistance, re-subculture the relevant colony on the relevant selective agar plate. This re-subcultured bacterial isolate is appropriate for storing in the strain collection.		The isolates resistant to carbapenems based on the MIC results should be further phenotypically or genotypically tested to verify the presence of a carbapenemase, according to the recommendations in the EFSA scientific opinion on carbapenem resistance in food animal ecosystems (2).

Figures

Figure 1. Typical appearance of *E. coli* on MacConkey agar supplemented with 1 mg/L cefotaxime.

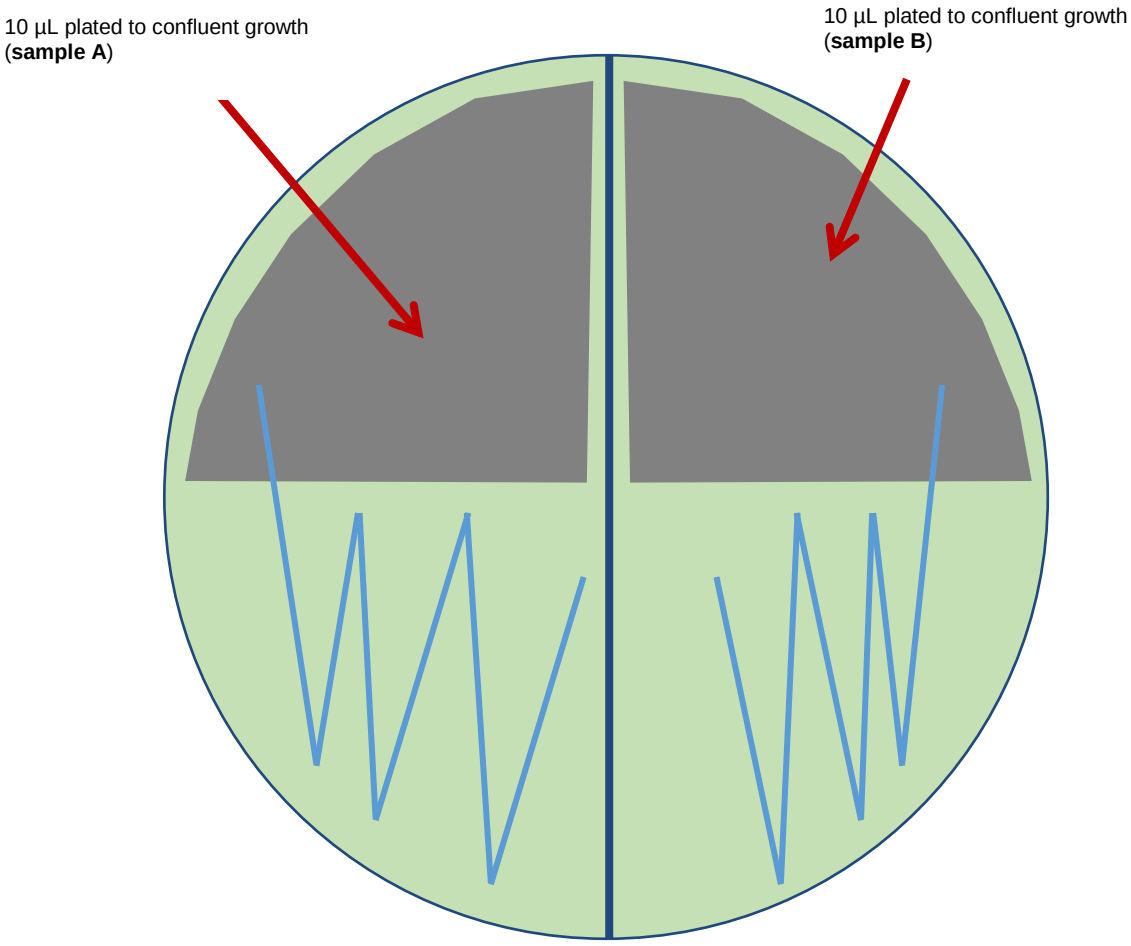

Figure 2. Plating on selective plates to detect presumptive carbapenemase-producing *E. coli*.

References

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(8) Guidance document on official controls, under Regulation (EC) No 882/2004, concerning microbiological sampling and testing of foodstuffs (available for download from https://ec.europa.eu/food/sites/food/files/safety/docs/oc_leg_guidance_sampling_testing_en.pdf)

APPENDIX 1

Composition and preparation of culture media and reagents

The buffered peptone water (BPW), MacConkey agar media 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. Of note, the media should be prepared according to the manufacturer's instructions, if they differ from the description given here.

Buffered peptone water (Example)

Formula	g/L
Enzymatic digest of casein	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	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)

Formula	g/L
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.

Selective Supplements

Formula	mg/mL
Cefotaxime sodium salt stock solution prepared in bi-distilled water	1

It is important to take into account the potency of the drug to ensure that 1 mg/mL active compound is used. Aliquots of aqueous cefotaxime stock solution (concentration 1 mg/mL) can be stored at -20°C.
Example: If the manufacturer has stated a potency of 50%, 2 mg of antibiotic powder should be added to 1 mL of sterile dH2O (or the solvent routinely used) to reach a final concentration of 1 mg/mL of active compound.

APPENDIX 2

FLOW DIAGRAM
for detection ESBL/AmpC/carbapenemases
(including OXA-48 and OXA-48-like enzymes) in caecal samples

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

1 g of caecal sample in 9 mL of buffered peptone water (37°C ± 1°C, 18-22 h)

↓

Selective isolation

➔ of presumptive ESBL-/AmpC-/carbapenemase-producing *E. coli* [item 1.6]
Streak 10 µL of the incubated pre-enrichment culture in BPW onto **MacConkey agar plate supplemented with 1 mg/L of cefotaxime** (incubate at 44°C ± 0.5°C for 18-22 h)

➔ of presumptive carbapenemase (including OXA-48- and OXA-48-like)-producing *E. coli* [item 3.1 + 3.2]
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). (incubation according to manufacturer's instructions)

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Sub-cultivation

Presumptive *E. coli* colonies from [item 1.6] onto **MacConkey agar plate supplemented with 1 mg/L cefotaxime to maintain the selective pressure** (incubate at 37°C ± 1°C for 18-22 h) [item 1.7]

Presumptive *E. coli* colonies from [item 3.2] onto commercially available chromogenic agar for isolation of carbapenemase-producing *E. coli* (including isolates producing only OXA-48 and/or OXA-48-like enzymes)" or MacConkey agar without antibiotic supplements (**as cefotaxime would not be the optimal substrate for all carbapenemases potentially present)** (incubate at 37°C ± 1°C for 18-22 h) [item 3.3]

↓

Identification and storage of isolates [item 1.8; 2.1 + 3.4]

Species ID by use of appropriate method
Subculture to ensure purity
Storage: Suitable method for keeping isolates viable for at least five years [see item 1.8 for details]

↓

Antimicrobial susceptibility testing [item 1.9]

Testing on the first panel (Table 1 of Commission Implementing Decision 2013/652/EU) and, if resistant to cefotaxime, ceftazidime and/or meropenem, further testing on the second panel (Table 4 of Commission Implementing Decision 2013/652/EU).