

## LABORATORY PROTOCOL

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

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Version 4

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HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
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

Resistance in Enterobacteriaceae producing extended spectrum beta-lactamases (ESBL), AmpC cephalosporinases or carbapenemases are of major public health significance.

In order to harmonise the antimicrobial resistance (AMR) monitoring systems in the European Union (EU), the European Commission (EC) adopted new legislation laying down detailed rules for the monitoring and reporting of AMR in zoonotic and commensal bacteria by Member States (MSs). The new legislation, “Commission Implementing Decision on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria” (2013/652/EU)<sup>1</sup> includes the obligation for the specific monitoring of ESBL and AmpC producing *E. coli* and the voluntary monitoring of carbapenemases producing *E. coli* in caecal samples and meat, according to the most recent version of the detailed protocol for standardisation of the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR). This protocol is intended for use at MS level to proceed to the isolation of *E. coli* producing either ESBL and AmpC and carbapenemase enzymes from meat samples as laid down in point 4.1 of Annex I of Commission Implementing Decision 2013/652/EU.

It has to be emphasized that the protocol to monitor ESBL and AmpC producing *E. coli* also have the potential to detect most variants of carbapenemases being produced in *E. coli*, as these normally also have reduced susceptibility to third generation cephalosporins (exceptions include Oxa-48-like producers, if they are not simultaneously co-producing an ESBL or AmpC enzyme). However, if a sample contains both an ESBL/AmpC producer as well as a carbapenemases producer, the method will only detect one of the two (which is most likely to be detected depends on the ratio between ESBL/AmpC and carbapenemase producers in a given sample).

The present protocol is a result of a larger validation of several methods and was validated for pig and cattle caecal samples by the EURL-AR at the Technical University of Denmark assisted by the Federal Institute for Risk Assessment (BfR) in Germany during autumn 2013/spring 2014. The protocol has been discussed in an expert meeting organized by the EC and held in Brussels with experts of ECDC, EFSA, EUCAST and some Member States (MS) and non-MS with both human and veterinary background. The protocol explains step by step the procedure and has some explanation of the theory behind each step.

Subsequently, the protocol in the present version 3 has also been validated by the EURL-AR on caecal samples from poultry origin during Autumn 2015.

## References

Commission Implementing Decision of 12 November 2013 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (2013/652/EU)<sup>1</sup>

Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food. EFSA Journal 2012;10(6):2742 [64 pp.]. doi:10.2903/j.efsa.2012.2742.

Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum  $\beta$ -lactamases and/or AmpC  $\beta$ -lactamases in food and food-producing animals. EFSA Journal 2011;9(8):2322 [95 pp.]. doi:10.2903/j.efsa.2011.2322.

Scientific Opinion on Carbapenem resistance in food animal ecosystems. EFSA Journal 2013; 11(12):3501 [70 pp.]. doi:10.2903/j.efsa.2013.3501.

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<sup>1</sup> OJ L 303, 14.11.2013, p. 26

<http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1417790423875&uri=CELEX:32013D0652>

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## Procedure

### 1. Isolation and identification of ESBL and AmpC producing *E. coli*

- 1.1. Samples arriving after the expiring date should be discarded. Also, samples which have not been stored appropriately (between + 2 °C and + 8 °C) under transportation or storage, should be discarded. Samples with damaged packaging should be discarded as well. Samples should arrive at the laboratory within 36 hours after sampling.
- 1.2. The stored samples shall be kept at a constant temperature between + 2 °C and + 5 °C until bacteriological examination at the laboratory. This should be initiated as soon as possible after receipt at laboratory, preferably within 24 hours. It is recommended as a rule that the analysis is started within 48 hours after collecting the sample.
- 1.3. A Quality Control (QC) procedure to validate the selective MacConkey agar plates should be performed according to EURL-AR recommendations<sup>2</sup> prior to initiating the enrichment procedure given below.
- 1.4. Pre-enrichment: 1 Gram +/- 0.1 gram of caecal sample is added to 9 mL of Buffered Peptone Water (BPW) in appropriate sterile tubes with lids or similar containers.
- 1.5. The tubes are incubated at 37 ± 1 °C for 18-22 h.

## Theory/comments

It is necessary to keep the samples refrigerated to avoid false results.

In general, samples shall be handled during transport and storage prior to analysis according to the ISO/DIS 7218 standard: Microbiology of food and animal feeding stuffs – General rules for microbiological examinations.

Please see also chapter 5.7 of the Guidance document on official controls, under Regulation (EC) No 882/2004, concerning microbiological sampling and testing of foodstuffs<sup>3</sup> for details.

It is not recommended to have samples arriving close to weekends or holidays, as this protocol has not been validated for storage of samples in the laboratory for more than 24 hours.

The EURL-AR has provided negative and positive control strains and a special 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.

The tubes should not be filled completely to avoid development of gasses during incubation. It is recommended to use 50-ml tubes.

It is recommended to avoid shaking the tubes to minimize the risk of spillage.

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- 1.6. After gently mixing, inoculate one loop-full (10 µl loop) of the overnight culture by applying a single streak onto a MacConkey agar plate containing 1 mg/L cefotaxime (CTX). From this streak, further two streaks are applied using a 1 µl loop to ensure single colonies. The plates are then incubated for 18-22 hours at 44 ± 0.5 °C.
- 1.7. Based on colony morphology and appearance – presumptive ESBL/AmpC producing *E. coli* colonies will be coloured; purple/red on the MacConkey agar plates containing 1mg/L CTX (figure 1) – sub-culture up to three colonies by re-streaking onto MacConkey agar containing 1mg/L CTX. Incubate at 37°C, 18-22 h. Subsequently, select one of these colonies for species verification/identification (ID). In case the first isolate is not identified as *E. coli*, a second and eventually third isolate should be tested.
- 1.8. One confirmed *E. coli* presumptive ESBL/AmpC producing shall at this stage be stored under appropriate conditions for at least five years.

Prior to storing the strains, to avoid contamination and to confirm the growth of the isolated bacterial strain, the relevant colony grown on the selective medium should be re-isolated by sub-culturing it on a new plate of the relevant selective agar. This re-isolated bacterial strain is appropriate for storing in the strain collection.

- 1.9. Antimicrobial susceptibility testing (AST) utilizing the primary MIC panel containing essential first line antimicrobials, as described in Table 1 of Commission Implementing Decision 2013/652/EU. If resistant to cefotaxime and/or ceftazidime and/or meropenem the isolate should be further tested using the second MIC panel (Table 4 of Commission Implementing Decision 2013/652/EU). The AST of the isolates

## Theory/comments

OXA-48-like carbapenemase producers are not able to hydrolyse cefotaxime (or other cephalosporins). Thus, this method will not detect OXA-48-like producers. See specific method for isolation of OXA-48-like producers below.

Plates are incubated at 44 ± 0.5 °C to minimize the influence of natural background flora. This temperature is believed to be permissive for growth of most *E. coli* strains.

In general, the number of tested colonies is dependent on the laboratory's success rate of recognizing and isolating *E. coli* from MacConkey agar. It is recommended to isolate and store three colonies (with a colony morphology and appearance typical for *E. coli*) and initially perform species identification on only one isolate. In case this strain is not an *E. coli*, the second and eventually the third isolate can be tested. In case that none of the three isolates are 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 red/purple on MacConkey agar.

The isolates can potentially be stored by suspending them in a broth which contain a cryoprotectant such as glycerol and stored at -80 °C. Alternative methods of storage may be used provided that they ensure viability and absence of changes in strain properties.

It is mandatory to test presumptive ESBL/AmpC producing *E. coli* using the first MIC panel of antimicrobials listed in Table 1 of Commission Implementing Decision 2013/652/EU. If resistant to cefotaxime and/or ceftazidime and/or meropenem (in case the isolate is also a carbapenemases producer), it is mandatory to further test the isolate using the second MIC panel referred in Table 4 of the Decision. Resistance phenotypes including synergy between either cefotaxime

## Procedure

shall be performed either immediately after verification of the species ID or later from the storage stock for final reporting.

### 2. Species identification *E. coli*

2.1. It is always necessary to perform species ID on the presumptive ESBL/AmpC producing *E. coli* strains isolated as indicated above. The species ID of *E. coli* should be conducted by using an appropriate method.

### 3. Specific isolation of carbapenemase producing *E. coli*

3.1. To specifically isolate carbapenemase producing *E. coli* (including strains producing only OXA-48-like enzymes) from the caecal samples, a loop-full of 10 µl pre-enrichment (from the overnight BPW cultures as mentioned above in item 1.6 of the sample analysis protocol) can be inoculated onto suitable selective agar(s)<sup>4</sup>. The 10 µl of the overnight culture are plated for confluent growth on half of each plate section (1/4 of the plates; see figure 2). For each ¼ area, further streaking is performed to obtain single colonies using a 1 µl loop.

3.2. The selective agar plates are incubated according to the manufacturer's instructions.

3.3. At least one presumptive carbapenemase and one presumptive OXA-48 producing *E. coli* is re-streaked onto MacConkey agar (without antibiotic supplements) and incubated at 37°C, 18-22 h. After incubation, these are subjected to species verification/identification (ID) and either processed immediately or stored under appropriate conditions (as described below in item 3.4) for later processing in relation to species identification and phenotypic antimicrobial susceptibility testing as

## Theory/comments

or ceftazidime and clavulanic acid can be assessed using the guidelines given in Chapter 4.1, Part A of Annex of Commission Implementing Decision 2013/652/EU.

Different laboratories may have different methods (biochemical, mass spectroscopy, chromogenic agar or genetic based methods) for performing species identification of *E. coli*. Chromogenic agar can be useful to distinguish presumptive *E. coli* from other bacteria that may form similar colour colonies on MacConkey agar.

It is important to choose selective agar plates, which have been validated in regard to specificity and sensitivity towards carbapenemase producing *E. coli* using the control strains described below. Preferably, a chromogenic agar aimed for isolation of carbapenemases producing *E. coli* including strains, which are only producing OXA-48-like enzymes, shall be used. If two different plates are required to accommodate this, 10 µl are spread on each type of plate. It is feasible to plate on ½-plates but a single plate for each isolate is also feasible. Here, 20 µl should be plated on ½ the plate and streaks made on the second half of the plate.

Positive and negative control strains and a protocol for validation of the method can be requested from the EURL-AR and should be included in parallel with the sample testing.

Note: In general, most media containing a carbapenemases selective agent have a short shelf-life, which should be followed strictly. In addition, it is important to ensure storage of the plates is done according to the manufacturer's recommendation.

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<sup>4</sup> For more information about the suitable selective agars, please contact the EURL-AR (rshe@food.dtu.dk)

## Procedure

described above in item 1.9 of the sample analysis protocol.

- 3.4. One presumptive carbapenemase producing *E. coli* and/or one presumptive OXA-48-like producing *E. coli* (if these are detected on different plates than the other carbapenemase producing *E. coli*) with confirmed species ID shall at this stage be stored under appropriate conditions for at least five years as described above in item 1.8 of the sample analysis protocol.

Prior to storing the strains, to avoid contamination and to confirm the growth of the isolated bacterial strain, re-isolate the relevant colony grown on the selective medium by sub-culturing it on a new plate with the selective medium. This re-isolated bacterial strain is appropriate for storing in the strain collection.

## Theory/comments

The presumptive carbapenemase producers (based on the MIC results) often require further validation in relation to presence of a carbapenemase, either phenotypically or genotypically and according to the recommendations in the EFSA scientific opinion on carbapenems resistance in food animal ecosystems (referenced above). Thus, it is strongly recommended to validate presumptive carbapenemase producing *E. coli* prior to reporting or to dispatch these to the EURL-AR for confirmative testing to avoid reporting of false positive results. Guidelines on how to send isolates will be provided by the EURL-AR.

## Figures



Figure 1: presumptive *E. coli* on MacConkey agar supplemented 1mg/L CTX

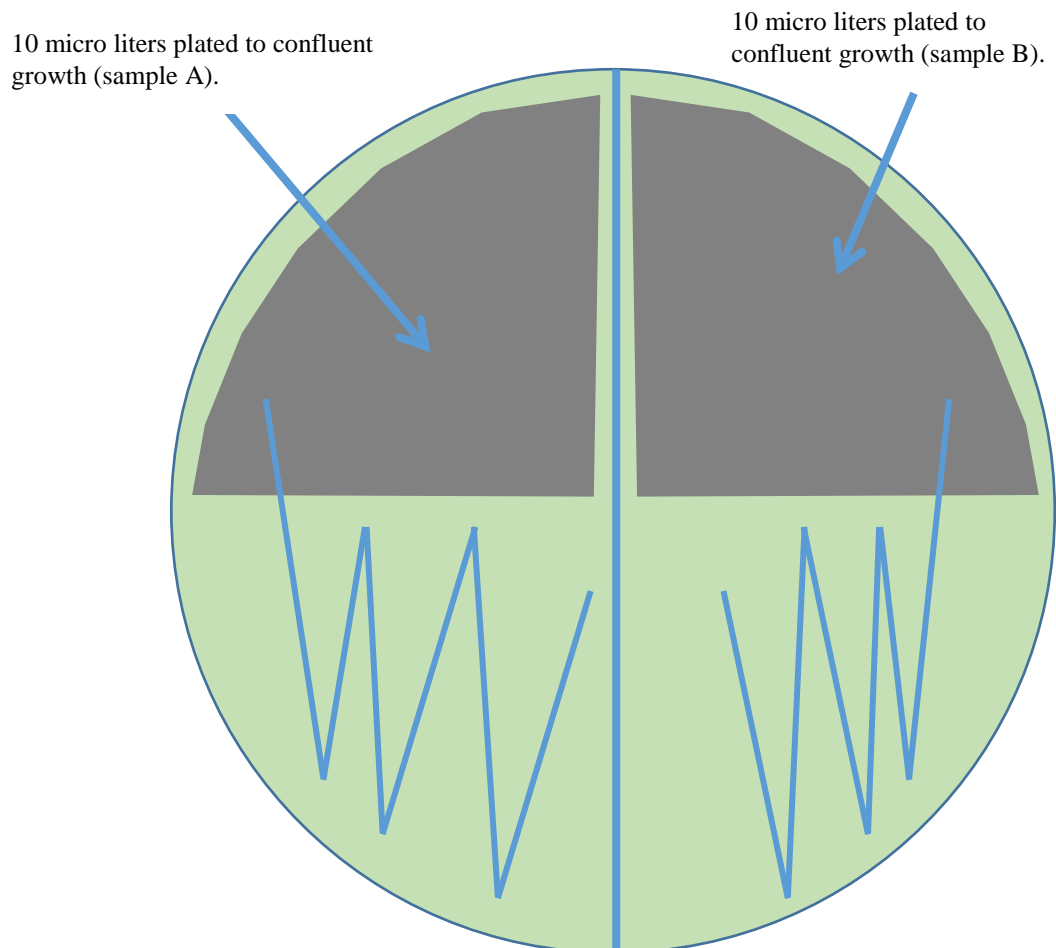


Figure 2: Plating on selective plates to detect presumptive carbapenemase producing *E. coli*.



## APPENDIX 1

### Composition and preparation of culture media and reagents

The Buffered peptone Water and MacConkey Agar media and reagents are available from several companies. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturer's description if it differs from the description given here.

#### Buffered peptone water (Example)

<i>Formula</i>	<i>g/litre</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 ( $\text{KH}_2\text{PO}_4$ )	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 for 15 min in the autoclave set at 121 °C.

#### MacConkey agar (Example)

<i>Formula</i>	<i>g/litre</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 liter of distilled water (Optional: Add 6.5 g agar to increase the solidity of the agar plates). Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

#### Selective Supplements

<i>Formula</i>	<i>mg/mL</i>
1. cefotaxime sodium salt stock solution prepared in bi-distilled water	1

It is important to take the activity of the drug into account 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 given an activity of 50%, 2 mg/mL should be prepared as the active concentration will then be 1 mg/mL.

## APPENDIX 2

### FLOW DIAGRAM

#### for detection ESBL/AmpC/carbapenemases and OXA-48 and carbapenemases from caecal samples

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

1 gram of caecal sample in 9 ml of buffered peptone water (37°C, 18-22 h)



##### Selective isolation

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

10 µL overnight culture streaked on MacConkey agar plate with 1 mg/L cefotaxime (44°C, 18-22 h)

→ of presumptive OXA-48 and carbapenemase producing *E. coli* [item 3.1 + 3.2]

10 µL overnight culture streaked on each selected suitable selective agar plate<sup>5</sup>  
(incubation according to manufacturer's instructions)



##### Sub-cultivation

Presumptive *E. coli* colonies from [item 1.6] onto MacConkey agar plate with 1 mg/L cefotaxime. Incubate at 37°C, 18-22 h [item 1.7]

Presumptive *E. coli* colonies from [item 3.2] onto MacConkey agar (without antibiotic supplements). Incubate at 37°C, 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

Storage: Suitable method for keeping isolates for at least five years<sup>6</sup>.



##### Susceptibility testing [item 1.9]

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

<sup>5</sup> For more information about the suitable selective agars, please contact the EURL-AR (rshe@food.dtu.dk)

<sup>6</sup> See item 1.8 on for details.