# PROTOCOL

for selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* from meat and caecal samples (Matrix EQAS)

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# 1 INTRODUCTION

The organisation and implementation of an External Quality Assurance System (EQAS) on selective isolation of presumptive extended spectrum beta-lactamase (ESBL)-, AmpC- or carbapenemase-producing *E. coli* is among the tasks of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR) and will include the selective isolation procedures and antimicrobial susceptibility testing (AST) of obtained isolates of eight samples of either meat or caecal content. In 2024, these eight samples will include four 25-g samples of chicken meat and four 1-g samples of chicken caecal content. These samples may contain *E. coli* presumptive of producing either ESBL-, AmpC- or carbapenemase-enzymes.

It is expected that the participating laboratories apply the same analysis procedures used in the monitoring, described by the regulation 2020/1729/EU, and perform the selective isolation following the by EU recommended methods, published on the EURL-AR website www.eurl-ar.eu.

# 2 OBJECTIVES

This EQAS aims to assess and, if necessary, to improve the quality of results obtained in the selective isolation of presumptive ESBL-, AmpC- or carbapenemase-producing isolates from meat and caecal samples. Further objectives are to evaluate and improve the comparability of surveillance data on ESBL-, AmpC- or carbapenemase -producing *E. coli* reported to EFSA by different laboratories.

# 3 OUTLINE OF THE MATRIX EQAS 2024

## 3.1 Shipping, receipt and storage of samples

In November 2024, the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) will receive a parcel containing eight samples from the National Food Institute. All strains used in the spiking of samples belong to UN3373, Biological substance, category B. Participants should expect that ESBL-, AmpC- and/or carbapenemase-enzymes producing strains will be included in (some of) the sample matrices.

The samples will be spiked matrices of either chicken meat or pooled chicken caecal content and will be distributed already weighed and ready to be tested, in tubes labelled from 10.1 to 10.8. Hereof 10.1 to 10.4 being samples of meat (each 25 g) and 10.5 to 10.8 being samples of caecal content (each 1 g).

The matrix samples will be shipped on 11 November in frozen/chilled state in separate tubes and contained in a cooling box with a temperature logging device and freezing elements.

Upon arrival, it is very important to open the parcel as soon as possible and proceed to the analysis (following the normal procedures for sample testing in the monitoring).

**It is required that participants:**

- **When opening the parcel, note the date and exact time at opening (this data is very important to follow the temperature data checks).**
- **Proceed to sample analysis immediately after opening the parcel.**
- **Register the date for start of analysis for each sample.**
- **Collect the temperature logging device from the parcel (small discoid device located in a bag inserted in a labelled tube);** open the tube and take out the bag with the device inside. Place this bag with the device in the labelled bubble envelope provided and return it to the EURL-AR as soon as possible. Please note that you will have to arrange for stamps/postage (the post systems differ from country to country, why this cannot be arranged and paid from the EURL-AR in advance).

### 3.2 QC reference strains

Include the *E. coli* ATCC 25922 and *Acinetobacter baumannii* 2012-70-100-69 reference strains in the MIC testing, and report results of these together with the isolates obtained from the EQAS samples. Note that, for the testing of the *E. coli* ATCC 25922 reference strain, the two compounds, sulfamethoxazole and sulfisoxazole, are regarded as comparable, i.e. the obtained MIC-value from the testing of sulfamethoxazole will be evaluated against the acceptance range listed in CLSI M100 for sulfisoxazole.

### 3.3 Selective isolation of ESBL, AmpC or carbapenemase producing *E. coli* from the samples

The samples provided in each parcel are weighed beforehand and therefore no further weighing is required. Proceed immediately to the first enrichment step by adding the sample to the necessary volume of media (225 ml of Buffered Peptone water for the meat samples and 9 ml for the caecal samples) as referred in the official EURL-AR protocols. **Results should be produced according to the laboratory's routine procedures for antimicrobial susceptibility testing by MIC determination.** All the following procedures should follow the methods used in the monitoring for ESBL and AmpC *E. coli* according to the 2020/1729/EU Decision. If any changes are introduced to the official protocols, these changes should be described with details in the online database on the methods upload page. The participants are responsible for assuring the validity of the plates and therefore the protocol for "Validation of selective MacConkey agar plates supplemented with 1 mg/L cefotaxime for monitoring of ESBL and AmpC producing *E. coli* in meat and animals" should be run beforehand, as stated on the EURL-AR webpage (see https://www.eurl-ar.eu/protocols.aspx).

According to the 2020/1729/EU Decision, **the monitoring of carbapenemase-producing *E. coli* from the samples is now mandatory** and should be performed following the official protocols and plating on suitable agar plates. Similarly, the agar plates used for the carbapenemase isolation should be validated using the protocol for "Validation of selective and indicative agar plates for monitoring of carbapenemase-producing *E. coli*".

The officially recommended protocols are found on the EURL-AR webpage (http://eurl-ar.eu/233-protocols.htm):

- Follow the protocol for meat when testing samples 10.1 to 10.4.
- Follow the protocol for caecal content when testing samples 10.5 to 10.8.

As referred in these protocols, the isolates obtained from isolation procedure should be identified as *E. coli* using the procedures for *E. coli* species identification applied at the participant's laboratory for the specific monitoring of ESBL-, AmpC-, and carbapenemase producing *E. coli*.

Please store the isolates obtained in the isolation procedure and document the whole process as well as all the findings in each step.

As part of the results submission, you will be requested to describe the findings along the enrichment process and selective isolation including growth in the media, isolation of suspected colonies, species identification results and finally regarding the finding (or not) of presumptive *E. coli* isolates harbouring one of the selected resistances (this result will be evaluated in relation to the expected result as a qualitative result) (see details in the Test Form).

### 3.4 Antimicrobial susceptibility testing

If the sample is deemed positive for ESBL-, AmpC- or carbapenemase -producing *E. coli*, one *E. coli* isolate per sample should be taken further and tested for susceptibility to antimicrobials as stated in the EU regulation (antimicrobials listed in Appendix 1 to this document). Only one *E. coli* isolate is expected to be tested for AST and these results will be evaluated in the database by comparing to expected results.

AST results to be reported should be from:

- A presumptive carbapenemase positive isolate (from carbapenemase or OXA-48 selective plates, or a strain found resistant to meropenem in the MIC test), if a presumptive carbapenemase positive *E. coli* isolate was detected.
- An ESBL- or AmpC-presumptive isolate (if you do not have a carbapenemase positive isolate) if an ESBL- or AmpC-presumptive isolate was detected.

The testing should be performed using the same method as implemented in your laboratory for performing AST when monitoring for EFSA according to the Decision 2020/1729/EU (using the two-step approach, i.e. both testing panels) and applying the interpretative thresholds listed in Appendix 1.

Strains are categorised as "S" to a specific antimicrobial compound when the obtained MIC value for this compound is equal to, or less than, the respective ECOFF value, while strains are categorised as "R" when the obtained MIC value is greater than the ECOFF value.

**Beta-lactam resistance**

Confirmatory testing for ESBL and carbapenemase production is mandatory on all strains resistant to cefotaxime (FOT), ceftazidime (TAZ) and/or meropenem (MERO) and should be performed by testing the second panel of antimicrobials (beta-lactams; EUVSEC2).

Confirmatory test for ESBL production requires use of both cefotaxime (FOT) and ceftazidime (TAZ) alone and in combination with a β-lactamase inhibitor (clavulanic acid). Synergy is defined either as i) a ≥ 3 twofold concentrations decrease in a MIC for either antimicrobial agent tested in combination with clavulanic acid vs. the MIC of the agent when tested alone (MIC FOT: FOT/CL or TAZ: TAZ/CL ratio ≥ 8) (CLSI M100 Table 3A, Tests for ESBLs). The presence of synergy indicates ESBL production.

Detection of AmpC-type beta-lactamases can be performed by testing the bacterium for susceptibility to cefoxitin (FOX). Resistance to FOX could indicate the presence of an AmpC-type beta-lactamase.

Confirmatory test for carbapenemase production requires the testing of meropenem (MERO).

The classification of the phenotypic results should be based on the most recent EFSA recommendations (See the Appendix 2).

Importantly: Note that for *E. coli*, two cut-off values apply for cefotaxime and ceftazidime: the EUCAST cut-off values, those that define R/S (see Appendix 1 and 2), and the screening cut-off values (cefotaxime >1 and ceftazidime >1) which are those applied to categorise bacterial phenotypes as ESBL, AmpC, carbapenemase, etc., based on panel 2 results (see Appendix 1 and 2). Likewise, this is the situation for the *E. coli* meropenem cut-off values/screening cut-off value.

# 4 REPORTING OF RESULTS AND EVALUATION

Test forms are available for recording your results before you enter them into the web tool.

## 4.1 General recommendations for data upload

We recommend reading carefully the description reported in paragraph 5 before entering your results in the web database. **Results must be submitted no later than 13 January 2025.** After the deadline when all participants have uploaded results, you will be able to login to the database once again, and to view and print an automatically generated report evaluating your results. Results in agreement with the expected interpretation are categorised as 'correct', while results deviating from the expected interpretation are categorised as 'incorrect'.

If you experience difficulties in entering your results, please contact us directly.

All results will be summarized in a report which will be publicly available. The data in the report will be presented with laboratory codes. A laboratory code is known to the individual laboratory, whereas the complete list of laboratories and their codes is confidential and known only to the EURL-AR and the EU Commission. All conclusions will be public.

If you have questions, please do not hesitate to contact the EQAS Coordinator:

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## 5 HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE

The 'guideline for submission of results via webtool' is available for download directly from the EURL-AR website (https://www.eurl-ar.eu/eqas.aspx).

Access the webtool using this address: https://amr-eqas.dtu.dk. Please follow the guideline carefully and **remember to access the webtool via an 'incognito' website**.

When you submit your results, remember to have by your side the completed test forms.

Do not hesitate to contact us if you experience difficulties with the webtool.

Before finally submitting your input please ensure that you have filled in all the relevant fields as **you can only 'finally submit' once!** 'Final submit' blocks data entry.

⇨ About login to the webtool:

When first given access to login to the webtool, your **personal** loginID and password were sent to you by email. This is relevant for two email addresses connected to each NRL-AR (the EURL-AR defined a primary and a secondary contact).
Note that:

a) If the EURL-AR has only one contact person for an NRL, this person is registered both as primary and secondary contact. Should you like to add another person as the secondary contact, please contact jetk@food.dtu.dk.
b) If your laboratory has two or more contact points on the EURL-AR contact list, two have been defined as the primary and secondary contact. Should you like to make changes to the primary and secondary contact or should you like more than the two persons to be able to access the webtool, please contact jetk@food.dtu.dk.

All participants registered with an account in the submission webtool will receive a separate email presenting the relevant personal username and password. The email will be sent by the time when the webtool has gone through internal quality control and has been approved for user access. The EQAS Coordinator will let all participants know when to look out for it.

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## APPENDIX 1 Criteria for interpretation of *Escherichia coli* MIC, panel 1 and 2 results

The following tables present the concentration range to be tested for each antimicrobial compound as well as the Epidemiological Cut-off values for the AMR phenotype categorisation as Resistant or Susceptible, as presented in Appendix B of the EFSA (European Food Safety Authority), Amore G, Beloeil P-A, Garcia Fierro R, Guerra B, Rizzi V and Stoicescu A-V, 2024. Manual for reporting 2023 antimicrobial resistance data under Directive 2003/99/EC and Commission Implementing Decision (EU) 2020/1729. *EFSA supporting publication* 2024: 21(1):EN-8585. 41 pp. doi:10.2903/sp.efsa.2024.EN-8585.

**Criteria for interpretation of *E. coli* from** *EFSA supporting publication* 2024: 21(1):EN-8585):

Table B.1: Panel of antimicrobial substances to be included in AMR monitoring, interpretative thresholds for interpreting resistance and concentration ranges to be tested in *Salmonella* spp. and indicator commensal *E. coli*

| Antimicrobial | *Salmonella* EU surveillance 2023 | | | *E. coli* EU surveillance 2023 | | | Concentration range, mg/L (no of wells) |
|---|---|---|---|---|---|---|---|
| | ECOFF | EUCAST | EFSA | ECOFF | EUCAST | EFSA | |
| Amikacin[a] | 4 | x | | 8 | x | | 4–128(6) |
| Ampicillin | 8 | x | | 8 | x | | 1–32 (6) |
| Azithromycin | 16 | x | | 16 | | x | 2–64 (6) |
| Cefepime[b] | 0.125 | | x | 0.125 | | x | 0.064–32 (10) |
| Cefotaxime | 0.5 | x | | 0.25 | x | | 0.25–4 (5)[c] 0.25–64 (9)[d] |
| Cefotaxime + clavulanic acid | 0.5 | | x | 0.25 | x | | 0.064–64 (11) |
| Cefoxitin | 8 | x | | 8 | x | | 0.5–64 (8) |
| Ceftazidime | 2 | x | | 0.5 | x | | 0.25–8 (6)[c] 0.25–128 (10)[d] |
| Ceftazidime + clavulanic acid | 2 | | x | 0.5 | x | | 0.125–128 (11) |
| Chloramphenicol | 16 | x | | 16 | x | | 8–64 (4) |
| Ciprofloxacin | 0.064 | x | | 0.064 | x | | 0.015–8 (10) |
| Colistin | 2 | | x | 2 | x | | 1–16 (5) |
| Ertapenem[e] | 0.064 | | x | 0.064 | | x | 0.015–2 (8) |
| Gentamicin | 2 | x | | 2 | x | | 0.5–16 (6) |
| Imipenem | 1 | x | | 0.5 | x | | 0.125–16 (8) |
| Meropenem | 0.125 | | x | 0.125 | x | | 0.03–16 (10) |
| Nalidixic acid | 8 | x | | 8 | x | | 4–64 (5) |
| Sulfamethoxazole | 256 | | x | 64 | | x | 8–512 (7) |
| Temocillin | 16 | | x | 16 | x | | 0.5–128 (9) |
| Tetracycline | 8 | x | | 8 | x | | 2–32 (5) |
| Tigecycline | 0.5 | | x | 0.5 | x | | 0.25–8 (6) |
| Trimethoprim | 2 | x | | 2 | x | | 0.25–16 (7) |

(a): EUCAST epidemiological cut-off (ECOFF) value for *Salmonella* is tentative.
(b): EUCAST epidemiological cut-off (ECOFF) value for *E. coli* is 0.25.
(c): Range to be used when the substance is tested in panel 1.
(d): Range to be used when the substance is tested in panel 2.
(e): EUCAST epidemiological cut-off (ECOFF) value for *E. coli* is tentative at 0.03.

## APPENDIX 2 Criteria for categorisation of beta-lactam resistance phenotypes

Please use the scheme below to phenotypically identify presumptive ESBL-, AmpC-, and/or CP-producers. Five main categorizations of phenotypes are made: 1. Extended-Spectrum Beta-Lactamase-producing (ESBL phenotype), 2. AmpC Beta-Lactamase-producing (AmpC phenotype), 3. ESBL+AmpC phenotype, 4. Carbapenemase-producing (CP phenotype) and 5. Other.

The figure is from *EFSA and ECDC, 2024. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2021/2022. EFSA Journal. 2024;22:e8583. https://doi.org/10.2903/j.efsa.2024.8583, Appendix F, Figure F1.*