

Protocol for EQAsia Matrix EQA 2022

Selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* from cultures mimicking meat samples

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1 INTRODUCTION

The EQAsia project aims to strengthen the provision of External Quality Assessment (EQA) services across the **One Health** sector in South and Southeast Asia. Therefore, a comprehensive and high-quality EQA program for antimicrobial resistance (AMR) is offered to all the National Reference Laboratories/Centres of Excellence in the region during 2021-22. The EQA is organized by the EQAsia consortium and supported by the Fleming Fund.

Aligning with the scope of WHO Tricycle and as suggested by FAO, the EQAsia EQA5 2022 includes a **Matrix EQA** aiming to assess the laboratories' ability to detect AmpC beta-lactamases (AmpC), extended-spectrum beta-lactamases (ESBL) and carbapenemase producing *Escherichia coli* from food matrices.

The **Matrix EQA 2022** therefore entails the selective isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli*, as well as antimicrobial susceptibility testing (AST) of obtained isolates from five cultures mimicking meat content. These samples consist of five lyophilized bacterial cultures obtained from 25g samples of pork minced meat. Briefly, 25g of pork minced meat were spiked with an *E. coli* strain. The meat sample was then pre-enriched by the addition of 225 ml

of Buffered Peptone Water (BPW, **Appendix 1**) and incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-22 h (pre-enrichment step as referred in the official [EURL-AR protocols](#)). A loopfull of the pre-enriched culture was plated and incubated overnight. The grown cells (a mixture of the different bacteria present in the meat sample) were then scrapped and a lyophilized culture prepared. These lyophilized cultures may contain *E. coli* presumptive of producing either ESBL-, AmpC- or carbapenemase-enzymes.

The procedures described here, on how to perform the selective bacterial isolation, follow the EU recommended methods published on the [EURL-AR website](#).

Additionally, antimicrobial susceptibility testing of the reference strains *Escherichia coli* ATCC 25922/CCM 3954 and *E. coli* NCTC 13846/CCM 8874 (for colistin) for quality control (QC) in relation to antimicrobial susceptibility testing is included. These reference strains are original CERTIFIED cultures provided free of charge (or have been supplied in previous EQAs), and should be stored for future internal quality control for antimicrobial susceptibility testing in your laboratory. Therefore, please take proper care of these strains. Handle and maintain them as suggested in the manual '[Subculture and maintenance of quality control strains](#)' available on the [EQAsia website](#).

2 OBJECTIVES

The main objective of the Matrix EQA is to support laboratories to assess and if necessary, improve the quality of results obtained in the selective isolation of presumptive ESBL-, AmpC- or carbapenemase-producing isolates from mixed samples. A further objective is to assess and improve the comparability of surveillance data on ESBL-, AmpC- or carbapenemase-producing *E. coli* reported by different laboratories. Therefore, the laboratory work for the Matrix EQA should be performed using the methods routinely applied in your laboratory. Additional methodology for selective isolation is provided in section 3.2.

3 OUTLINE OF THE MATRIX EQA 2022

3.1 Shipping, receipt and storage of strains

In September 2022, participating laboratories located in South and Southeast Asia will receive a parcel containing five lyophilized cultures obtained from meat samples. The lyophilized cultures obtained from spiked matrix samples of pork meat content will be distributed in separate tubes labelled from EQAsia 22.M1 to M5. Participants should expect that ESBL-, AmpC- and/or carbapenemase-enzymes producing *E. coli* strains will be included in some of the lyophilized cultures.

Upon arrival, the lyophilized cultures must be stored in a dark, dry and cool place until microbiological analysis. This should be initiated as soon as possible after receipt in the laboratory.

Please confirm receipt of the parcel through the confirmation form enclosed in the shipment

All strains used in the spiking of samples are categorised as UN3373, Biological substance, category B. These strains can potentially be harmful to humans and pose a risk due to their possible pan-resistant profile, therefore becoming a challenge in the treatment of a potential human infection. It is the recipient laboratory's responsibility to comply with national legislation, rules and regulations regarding the correct use and handling of the provided test strains, and to possess the proper equipment and protocols to handle these strains. Nevertheless, it is recommended to handle the strains in a BSL2 containment facility using equipment and operational practices for work involving infectious or potentially infectious materials. The containment and operational requirements may vary with the species, subspecies, and/or strains, thus, please take the necessary precautions.

Please consult the [Pathogen Safety Data Sheets](#) (PSDSs) produced by the Public Health Agency of Canada. The PSDSs of each pathogen can be found in the bottom of the page. These PSDSs are technical documents that describe the hazardous properties of human pathogens, and provide recommendations for the work involving these agents in a laboratory setting.

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

Testing of meat samples requires a pre-enrichment step as referred in the official [EURL-AR protocols](#). As the provided samples are lyophilized cultures mimicking meat content, no pre-enrichment step is required (it has already been done by us). Instead, the lyophilized cultures should be revived before proceeding to the selective isolation. The steps are briefly described here (detailed information can be found on the official [EURL-AR protocols](#)):

- 1- Add 0.5 mL of sterile Luria Bertani broth into the lyophilized cells. Mix gently and carefully to avoid creating aerosols. Then, transfer all re-suspended cells into 5mL fresh Luria Bertani broth. For instructions on how to open and handle the **ampoules**, please see the document '[Instructions for opening and reviving lyophilised cultures of test strains](#)' on the [EQAsia website](#).
- 2- After mixing gently the culture, subculture one loopful (10 μ L loop) by applying a single streak onto a MacConkey agar plate containing 1 mg/L of cefotaxime (**Appendix 1**). From this streak, further two streaks are made using either the same loop or a 1 μ L loop to ensure growth of single colonies. Incubate the plates at 44°C \pm 0.5°C for 18-22 h.
- 3- 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 cefotaxime – see **Figure 1**), subculture individual colonies onto MacConkey agar containing 1 mg/L cefotaxime to maintain the selective pressure. Up to three colonies should be individually subcultured. Incubate at 37°C \pm 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.

- 4- 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 cefotaxime. This is performed by picking one single colony from the subculture and streaking it on a new plate of the relevant selective agar, which is then incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-22 h. This re-subcultured bacterial isolate should be stored under appropriate conditions in your strain collection (e.g. in a -80°C freezer). This set of cultures should serve as reference if discrepancies are detected during the testing (e.g. they can be used to detect errors such as mislabelling or contamination), and they can function as reference material available for reference at a later stage, when needed.

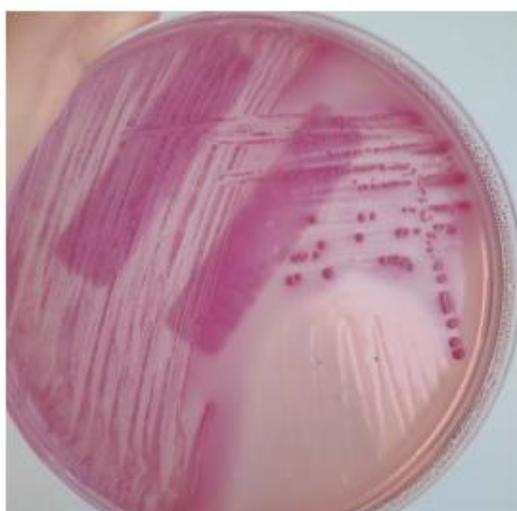


Figure 1: Typical appearance of *E. coli* on MacConkey agar supplemented with 1 mg/L cefotaxime.

The participants are responsible for assuring the validity of the plates by testing a positive (a known ESBL-/AmpC producing *E. coli*) and a negative (ESBL-/AmpC **non**-producing *E. coli*) control. A 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' is available on the [EURL-AR webpage](#).

3.3 Antimicrobial susceptibility testing

If the sample is considered positive for ESBL- , AmpC- or carbapenemase-producing *E. coli*, one *E. coli* isolate per sample should be taken further and tested for susceptibility towards as many as possible of the antimicrobials listed in **Table 1**, but always considering their relevance regarding the laboratory's routine work. Note that some of the antimicrobials (**highlighted**) could be omitted by the Human Health laboratories. Please use the methods routinely used in your own laboratory.

The reference values used in this Matrix EQA for interpreting MIC and disk diffusion results are in accordance with current zone diameter and MIC breakpoint values developed by CLSI (M100, 32nd Ed.). When not available, EUCAST clinical breakpoints (Tables v. 12.0, 2022) or epidemiological cut off values (<https://mic.eucast.org/>) are used instead.

Interpretation of MIC or disk diffusion results will lead to categorization of the result into one of the categories: **resistant (R)**, **intermediate (I)** or **susceptible (S)**. In the evaluation report you receive upon the submission deadline, the obtained interpretations in comparison with the expected interpretation will be evaluated/scored as follows:

SCORES		Obtained Interpretation		
		Susceptible	Intermediate	Resistant
Expected Interpretation	Susceptible	4	3	1
	Intermediate	3	4	3
	Resistant	0	3	4

0	Incorrect: very major
1	Incorrect: major
3	Incorrect: minor
4	Correct

Table 1. Interpretive criteria for *E. coli* antimicrobial susceptibility testing

The highlighted antimicrobials could be omitted by the Human Health laboratories.

Antimicrobials	Reference values			Reference values		
	MIC (µg/mL)			Disk diffusion (mm)		
	S	I	R	S	I	R
Amikacin, AMK	≤ 16	32	≥ 64	≥ 17	15-16	≤ 14
Ampicillin, AMP	≤ 8	16	≥ 32	≥ 17	14-16	≤ 13
Azithromycin, AZI	≤ 16	-	≥ 32	≥ 13	-	≤ 12
Cefepime, FEP	≤ 2	4-8	≥ 16	≥ 25	19-24	≤ 18
Cefotaxime, FOT	≤ 1	2	≥ 4	≥ 26	23-25	≤ 22
Cefotaxime + clavulanic acid, F/C	NA	NA	NA	NA	NA	NA
Cefoxitin, FOX	≤ 8	16	≥ 32	≥ 18	15-17	≤ 14
Ceftazidime, TAZ	≤ 4	8	≥ 16	≥ 21	18-20	≤ 17
Ceftazidime + clavulanic acid, T/C	NA	NA	NA	NA	NA	NA

Chloramphenicol, CHL	≤ 8	16	≥ 32	≥ 18	13-17	≤ 12
Ciprofloxacin, CIP	≤ 0.25	0.5	≥ 1	≥ 26	22-25	≤ 21
Colistin, COL	-	≤ 2	≥ 4	NA	NA	NA
Doripenem, DOR	≤ 1	2	≥ 4	≥ 23	20-22	≤ 19
Ertapenem, ETP	≤ 0.5	1	≥ 2	≥ 22	19-21	≤ 18
Gentamicin, GEN	≤ 4	8	≥ 16	≥ 15	13-14	≤ 12
Imipenem, IMI	≤ 1	2	≥ 4	≥ 23	20-22	≤ 19
Levofloxacin, LEVO	≤ 0.5	1	≥ 2	≥ 21	17-20	≤ 16
Meropenem, MERO	≤ 1	2	≥ 4	≥ 23	20-22	≤ 19
Nalidixic acid, NAL	≤ 16	-	≥ 32	≥ 19	14-18	≤ 13
Piperacillin/tazobactam, PT4	≤ 8/4	16/4	≥ 32/4	≥ 25	21-24	≤ 20
Sulfamethoxazole, SMX	≤ 256	-	≥ 512	≥ 17	13-16	≤ 12
Tetracycline, TET	≤ 4	8	≥ 16	≥ 15	12-14	≤ 11
Tigecycline, TGC*	≤ 0.5	-	≥ 1	≥ 18	-	≤ 17
Tobramycin, TOB	≤ 4	8	≥ 16	≥ 15	13-14	≤ 12
Trimethoprim, TMP	≤ 8	-	≥ 16	≥ 16	11-15	≤ 10
Trimethoprim/sulfamethoxazole, SXT	≤ 2/38	-	≥ 4/76	≥ 16	11-15	≤ 10

Reference values are based on Enterobacterales breakpoints from CLSI M100, 32nd Ed.

*Reference values are based on Enterobacterales clinical breakpoints from “The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0, 2022. <http://www.eucast.org>.”

Beta-lactam and carbapenem resistance

The following tests for detection of ESBL-, AmpC-, and carbapenemase-producing phenotypes for *E. coli* are recommended:

- Reduced susceptibility to cefotaxime (FOT) and/or ceftazidime (TAZ): it indicates that the bacterial strain is an ESBL-, AmpC, or carbapenemase-producing phenotype. These strains should be tested for ESBL-, AmpC, or carbapenemase-production by confirmatory tests.
- Confirmatory test for ESBL production: it requires the use of both cefotaxime (FOT) and ceftazidime (TAZ) alone, as well as in combination with a β -lactamase inhibitor (clavulanic acid). Synergy can be determined by broth microdilution methods, Gradient Test or Disk Diffusion:
 - It is defined as a ≥ 3 twofold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid vs. its MIC when tested alone (Gradient Test 3 dilution steps difference; MIC FOT : FOT/Cl or TAZ : TAZ/Cl ratio ≥ 8).
 - A positive synergy testing for Disk Diffusion is defined as ≥ 5 mm increase of diameter of FOT or TAZ in combination with clavulanic acid (FOT/Cl or TAZ/Cl) compared to testing them alone. The presence of synergy indicates ESBL production.
- Detection of AmpC-type beta-lactamases: it can be performed by testing the bacterial culture for susceptibility to ceftiofuran (FOX). Resistance to FOX indicates the presence of an AmpC-type beta-lactamase.
- Confirmatory test for carbapenemase production: it requires the testing of meropenem (MERO). Resistance to MERO indicates that the bacterial strain is a carbapenemase-producer.

It should be noted that some resistance mechanisms do not always confer clinical resistance.

Therefore, the classification of the phenotypic results (**Figure 2** below) should be based on the “EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance”, Version 2.0, July 2017, and the most recent EFSA recommendations – The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018. EFSA Journal 2020;18 (3) <https://doi.org/10.2903/j.efsa.2020.6007>

1. ESBL-Phenotype			4. Carbapenemase-Phenotype		
	MIC (mg/L)	Zone Diameter (mm)		MIC (mg/L)	Zone Diameter (mm)
FOT or TAZ	> 1	< 21 (FOT); < 22 (TAZ)	MERO	> 0.12	< 25
MERO	≤ 0.12	≥ 25	5. Other Phenotypes		
FOX	≤ 8	≥ 19		MIC (mg/L)	Zone Diameter (mm)
FOT/CLV and/or TAZ/CLV	SYNERGY	SYNERGY	1)		
2. AmpC-Phenotype			FOT or TAZ	> 1	< 21 (FOT); < 22 (TAZ)
	MIC (mg/L)	Zone Diameter (mm)	MERO	≤ 0.12	≥ 25
FOT or TAZ	> 1	< 21 (FOT); < 22 (TAZ)	FOX	≤ 8	≥ 19
MERO	≤ 0.12	≥ 25	FOT/CLV and/or TAZ/CLV	NO SYNERGY	NO SYNERGY
FOX	> 8	< 19	2)		
FOT/CLV and/or TAZ/CLV	NO SYNERGY	NO SYNERGY	FOT or TAZ	≤ 1	≥ 21 (FOT); ≥ 22 (TAZ)
3. ESBL + AmpC-Phenotype			MERO	≤ 0.12	≥ 25
	MIC (mg/L)	Zone Diameter (mm)	FOX	> 8	< 19
FOT or TAZ	> 1	< 21 (FOT); < 22 (TAZ)	Susceptible		
MERO	≤ 0.12	≥ 25		MIC (mg/L)	Zone Diameter (mm)
FOX	> 8	< 19	FOT or TAZ	≤ 1	≥ 21 (FOT); ≥ 22 (TAZ)
FOT/CLV and/or TAZ/CLV	SYNERGY	SYNERGY	MERO	≤ 0.12	≥ 25
4. Carbapenemase-Phenotype			FOX	≤ 8	≥ 19
	MIC (mg/L)	Zone Diameter (mm)			
FOT or TAZ	> 1	< 21 (FOT); < 22 (TAZ)			
MERO	≤ 0.12	≥ 25			
FOX	> 8	< 19			
FOT/CLV and/or TAZ/CLV	SYNERGY	SYNERGY			

Figure 2: Adapted from EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2020 – The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018 – and in accordance with the EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance, Version 2.0, July 2017.

The genotype obtained by PCR and/or sequencing may be necessary to correctly categorize a bacterial test strain as either of the categories, ESBL-, AmpC, and/or carbapenemase-producer, but is NOT requested as part of this Matrix EQA.

Even though this 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. Therefore, to specifically isolate carbapenemase-producing *E. coli* (including strains producing OXA-48 and OXA-48-like enzymes) from the cultures mimicking meat samples, it may be required to choose selective agar plates that have been validated with regard to specificity and sensitivity of detection of carbapenemase-producing *E. coli*. For example, commercially available chromogenic agar for isolation of carbapenemase-producing *E. coli* (including isolates producing only OXA-48 and/or OXA-48-like enzymes) can be used. A protocol for 'Validation of selective and indicative agar plates for monitoring of carbapenemase-producing *E. coli*' is available on the [EURL-AR webpage](#). We encourage you to perform the validation, but it is optional and NOT requested as part of this EQA.

4 REPORTING OF RESULTS AND EVALUATION

We recommend that you write your results in the enclosed test forms and that you read carefully the description in paragraph 5 before entering your results in the informatics module. If the same reference strain is used for different pathogens, please enter the results (even if the same) for all the pathogens. The informatics module will allow you to view and print a report with your reported results. The scores for the results will be released after the result submission deadline; then, you will be able to access the evaluation of your results. Results in agreement with the expected interpretation are categorised as ‘4’ (correct), while results deviating from the expected interpretation are categorised as ‘3’ (incorrect, minor), ‘1’ (incorrect, major) or ‘0’ (incorrect, very major).

Results must be submitted no later than November 4th 2022.

If you have trouble in entering your results, please contact the EQA Coordinator directly, explaining the issues that you encountered:

Patrícia T. dos Santos
National Food Institute, Technical University of Denmark
Kemitorvet, Building 204, DK-2800 Lyngby – DENMARK
E-mail: pado@food.dtu.dk

Direct communication with the EQA Coordinator must be in English.

5 HOW TO SUBMIT RESULTS VIA THE WEBTOOL

The ‘Guideline for reporting results in the EQAsia Informatics Module’ is available for download directly from the [EQAsia website](#). Please follow the guideline carefully.

Access the Informatics Module (**incognito window**) using <https://eqasia-pt.dtu.dk>. See below how to login to the Informatics Module.

When you submit your results, remember to have by your side the completed test forms (template available for download from the [EQAsia website](#)).

Do not hesitate to contact us if you have trouble with the Informatics Module.

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

Login to the informatics module:

When first given access to login to the Informatics Module, your **personal loginID and password** is sent to you by email.

Note that the primary contact person for a participating institution is registered both as primary and secondary contact. Should you like to add another person as the secondary contact, please contact pado@food.dtu.dk.

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APPENDIX 1

Composition and preparation of culture media and reagents (available on [EURL-AR protocols](#))

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 ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	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)

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.