Protocol for EQAsia Matrix EQAS

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

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. The EQA is organized by the EQAsia consortium and supported by the Fleming Fund.

The 2nd iteration of EQAsia EQAS includes a selective isolation of presumptive extended spectrum beta-lactamase (ESBL)-, AmpC- or carbapenemase-producing *Escherichia coli*, as well as antimicrobial susceptibility testing (AST) of obtained isolates from five cultures mimicking meat content. These 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°C ± 1°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, 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 strain' available on the EQAsia website.

2 OBJECTIVES

The main objective of this EQAS 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 minced meat 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 this EQAS 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 EQAS 2021

3.1 Shipping, receipt and storage of strains

In July/August 2021, participating laboratories located in South and Southeast Asia will receive a parcel from Dr. Rungtip Chuanchuen, Chulalongkorn University, Thailand 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 M.1 to M.5. Participants should expect that ESBL-, AmpC- and/or carbapenemase-enzymes producing strains will be included in some of the lyophilized cultures.

Upon arrival, the lyophilized cultures must be stored in a dark, 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 proceding 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 (Animal health laboratories)' on the EQAsia website.

2- After mixing gently the culture, subculture one loopful (10µL 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 1µL loop to ensure growth of single colonies. Incubate the plates at 44°C ± 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 ± 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°C ± 1°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.

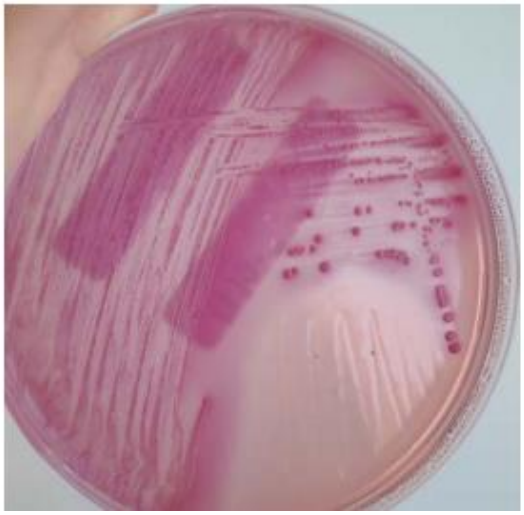

Figure 1: Typical appearance of *E. coli* on MacConkey agar supplemented with 1 mg/L cefotaxime.

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 mentioned in the test forms and in **Table 1**. However, in the impossibility of testing them all, the optional antimicrobials are marked with [a]. Please use the methods routinely used in your own laboratory.

The reference values used in this EQAS for interpreting MIC and disk diffusion results are in accordance with current epidemiological cut-off values developed by EUCAST. When not available, CLSI zone diameter and MIC breakpoint values are used instead.

Interpretation of MIC or disk diffusion results will lead to categorization of the result into one of two categories: **resistant** (R) or **susceptible** (S). In the evaluation report you receive upon the submission deadline, you can find that obtained interpretations in accordance with the expected interpretation will be evaluated as '1' (correct), whereas obtained interpretations not in accordance with the expected interpretation will be evaluated as '0' (incorrect).

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

Antimicrobials	Reference value MIC (µg/mL)	Reference value Disk diffusion (mm)
	Resistant	Resistant
Ampicillin, AMP	≥ 16	< 14
Azithromycin, AZI[a]	≥ 32*	≤ 12*
Cefepime, FEP[a]	≥ 0.50	< 28
Cefotaxime, FOT	≥ 0.50	< 21
Cefotaxime, FOT + clavulanic acid	NA	NA
Cefoxitin, FOX	≥ 16	< 17
Ceftazidime, TAZ	≥ 1	< 20
Ceftazidime, TAZ + clavulanic acid	NA	NA
Chloramphenicol, CHL	≥ 32*	≤ 12*
Ciprofloxacin, CIP	≥ 0.12	< 25
Colistin, COL	≥ 4	NA
Ertapenem, ETP[a]	≥ 0.06	< 24
Gentamicin, GEN	≥ 4	< 17
Imipenem, IMI	≥ 1	< 24
Meropenem, MERO	≥ 0.12	< 25
Nalidixic acid, NAL	≥ 16	< 19
Sulfamethoxazole, SMX	≥ 512*	≤ 12*
Tetracycline, TET	≥ 16*	≤ 11*
Tigecycline, TIG[a]	≥ 1	< 18
Trimethoprim, TMP	≥ 4	< 20

Reference values are based on *E. coli* epidemiological cut off values from www.eucast.org on June 2021.
*Reference values are based on Enterobacterales breakpoint values from CLSI M100, 30th Ed.
[a] Optional.

Beta-lactam and carbapenem resistance

The following tests are for the confirmation of ESBL-, AmpC-, and carbapenemase-producing *E. coli* isolates:

- 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, E-test or Disc 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 (E-test 3 dilution steps difference; MIC FOT : FOT/Cl or TAZ : TAZ/Cl ratio ≥ 8). A positive synergy testing for Disc 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 cefoxitin (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.

The classification of the phenotypic results should be based on the adaptation of the most recent EFSA recommendations (**Figure 2** below) – 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

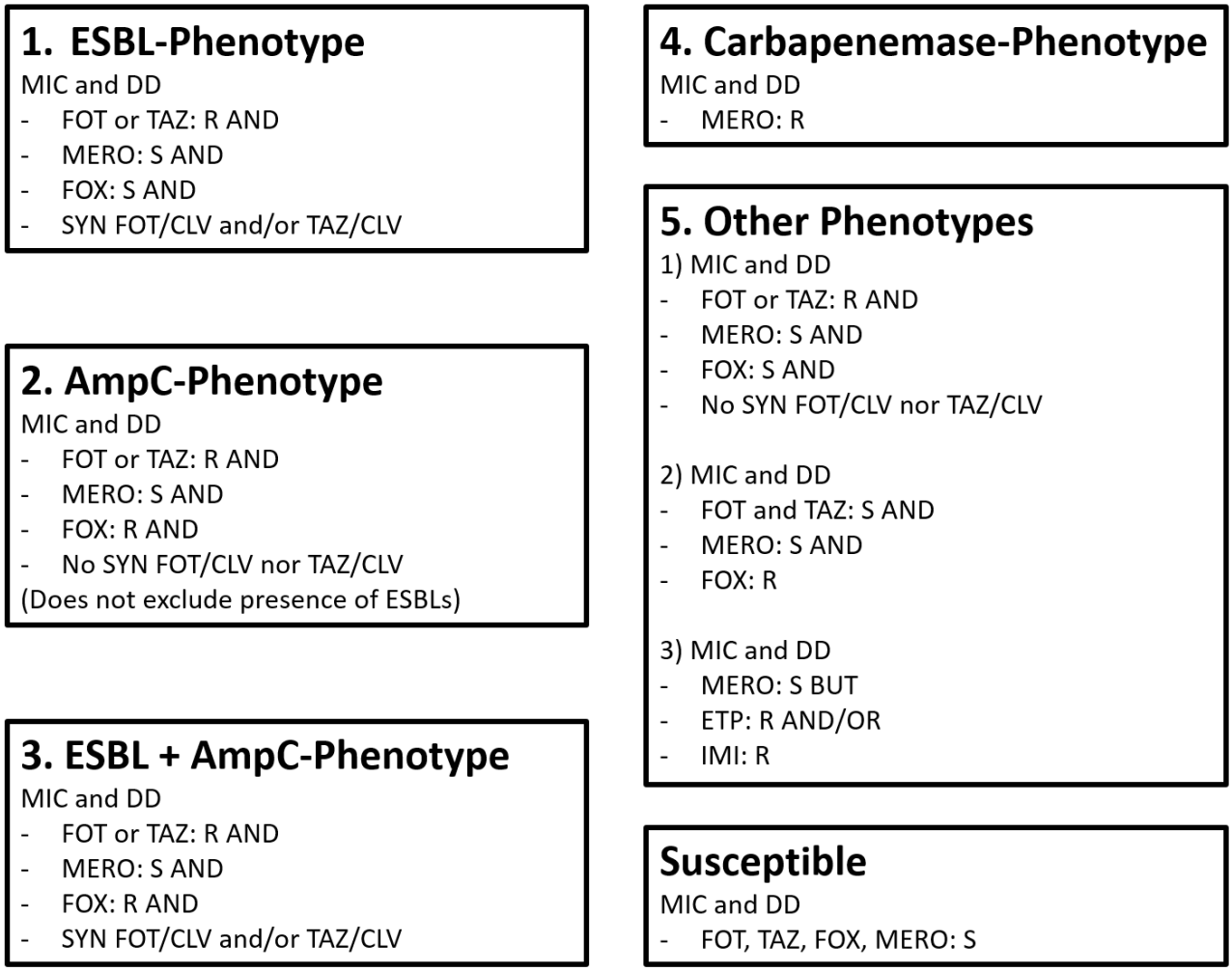

Figure 2: Adapted from EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2020. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018.

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 EQAS.

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 EQAS.

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 web database. The web database 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 where you will be able to access the evaluation of your results. Results in agreement with the expected interpretation are categorised as '1' (correct), while results deviating from the expected interpretation are categorised as '0' (incorrect).

Results must be submitted no later than September 15th 2021.

If you have trouble in entering your results, please contact the EQAsia Project Manager directly, explaining the issues that you encountered:

Rikke Braae
National Food Institute, Technical University of Denmark
Kemitorvet, Building 204, DK-2800 Lyngby – DENMARK
E-mail: rikb@food.dtu.dk

Direct communication with the EQAsia Project Manager 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 webtool using this address. See below how to login to the webtool.

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 webtool.

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 webtool:

When first given access to login to the webtool, 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 rikb@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 ($Na_2HPO_4 \cdot 12H_2O$)	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.