3rd EQAsia Matrix EQA on selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing Escherichia coli from cultures mimicking meat samples - 2023

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Photo: Colourbox

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World Organisation for Animal Health
The Peter Doherty Institute for Infection and Immunity, Australia
Pacific Pathology Training Centre, New Zealand
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Executive Summary

This report summarizes the results of the 3rd EQAsia Matrix EQA trial of the EQAsia project (Matrix EQA 2023), a Fleming Fund Regional Grant aiming to strengthen the provision of External Quality Assessment (EQA) services across the One Health sector among National Reference Laboratories / Centres of Excellence in South and Southeast Asia. EQAsia has been granted a 2nd phase (October 2023 to December 2025) to continue to deliver the established EQA for both the Human Health (HH sector) and Food and Animal Health (AH sector) laboratories in the region.

The trial was carried out in October-November 2023 and included isolation of *E. coli* presumptive of producing either ESBL, AmpC or carbapenemase enzymes from lyophilized cultures mimicking meat content, followed by antimicrobial susceptibility testing (AST) of the isolates.

A total of three HH and six AH laboratories participated and submitted results for the Matrix EQA. These laboratories are from seven countries situated in South and Southeast Asia (Bangladesh, Bhutan, Malaysia, Pakistan, Sri Lanka, Timor-Leste, and Vietnam).

The participants used the recommended methods for selective isolation of the presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli* isolates from the cultures mimicking meat content and applied biochemical tests for confirmation of the bacterial identification.

The four samples of presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli* were correctly identified as positive by at least 55.6% of the laboratories.

Eight of the participating laboratories submitted results for antimicrobial susceptibility testing and presented an average deviation of 13.4% (ranging from 2.7 to 29.2%) in terms of AST performance.

1. Introduction

The EQAsia project was launched in 2020 aiming to strengthen the provision of External Quality Assessment (EQA) services across the One Health sector among National Reference Laboratories / Centres of Excellence in South and Southeast Asia. EQAsia is supported by the Fleming Fund and strives to increase the quality of laboratory-based surveillance of WHO GLASS pathogens [1] and FAO priority pathogens [2]. EQAsia has been granted a 2nd phase to continue to deliver the established EQA for both the Human Health (HH) sector and the Food and Animal Health (AH) sector in the region from 2023 to 2025.

The EQAsia Consortium includes the National Food Institute, Technical University of Denmark (DTU Food) as the Lead Grantee, the International Vaccine Institute (IVI) in South Korea, and the Faculty of Veterinary Science, Chulalongkorn University (CUVET) in Thailand.

EQAsia provides a state-of-the-art EQA program free of charge for the South and Southeast Asian region through CUVET Thailand, an existing EQA provider in the region. The EQAsia program is designed to enable the laboratories to select and participate in relevant proficiency tests of both pathogen identification (ID) and antimicrobial susceptibility testing (AST), in line with the requirements of the WHO GLASS [1]. The EQA program is supported by an informatics module where laboratories can report their results and methods applied.

As suggested by FAO and in alignment with the scope of WHO Tricycle, the EQA trials taking place in 2021 ave included a Matrix-based specific EQA in each year, aiming at assessing the laboratories' ability to detect and isolate AmpC beta-lactamases (AmpC), extended-spectrum beta-lactamases (ESBL) and carbapenemase-producing *Escherichia coli* from food matrices, followed by identification and antimicrobial susceptibility testing. The purpose of the Matrix EQA is to monitor the capacity of the participating laboratories to perform isolation and AST of *E. coli* from food matrices and identify potential problems or focus areas for future training/education.

To prepare for the launch of the Matrix EQAs, several preliminary studies were conducted at CUVET Thailand, using meat samples spiked with *E. coli* isolates presumptive of producing either ESBL, AmpC or carbapenemase enzymes. However, due to the constraints in shipping such samples from Thailand to the participating countries, another approach was attempted. Shortly, a portion of pork-minced meat was spiked with an *E. coli* strain, the meat sample was pre-enriched and bacterial growth was allowed. The resulting bacterial culture (a mixture of the different bacteria present in the meat sample) was then lyophilized and a culture mimicking the meat content obtained (see section 2.2).

All *E. coli* isolates used for spiking the meat samples were assessed by DTU Food and the external partner (The Peter Doherty Institute for Infection and Immunity, Australia), and validated by CUVET Thailand. The assessment included both phenotypic minimum inhibitory concentration (MIC) determination by broth microdilution, and whole genome sequencing (WGS) to detect antimicrobial resistance (AMR) genes and chromosomal point mutations.

This report contains results from the 3rd EQAsia Matrix EQA trial of the EQAsia project (Matrix EQA 2023) carried out in October-November 2023. The trial included a total of four lyophilized cultures mimicking meat content spiked with an *E. coli* isolate presumptive of producing either ESBL, AmpC or carbapenemase enzymes. For each of the four isolated *E. coli* strains, results in relation to AST and Selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* were requested.

The evaluation of the participants' results is based on international guidelines, namely the Clinical and Laboratory Standards Institute

(CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Interpretative criteria referring to both disk diffusion and MIC determination are listed in the Matrix EQA protocol (**Appendix 1**) and allow for the obtained results to be interpreted into categories as resistant, intermediate, or susceptible depending on the method used. Results in agreement with the expected interpretation are scored '4' (correct), while results deviating from the expected interpretation are scored as either '0' (incorrect: very major), '1' (incorrect: major) or '3' (incorrect: minor), as explained in the Matrix EQA protocol (**Appendix 1**). This standardized interpretation of results is necessary to allow comparison of performance between laboratories. No thresholds were set in advance to evaluate the performance of the participating laboratories; thus, the results were evaluated case by case. Nevertheless, a laboratory performance of < 5% deviation from expected results would be considered ideal.

Evaluation of a result as "deviating from the expected interpretation" should be carefully analysed in a root cause analysis procedure performed by individual participants (self-evaluation) when the EQA results are disclosed. The methods applied have limitations in reproducibility, thus, on repeated testing, the same strain/antimicrobial combination can result in different MIC or inhibition zone diameter values differing by one-fold dilution or ± 3mm, respectively. If the expected MIC / zone diameter is close to the threshold for categorising the strain as susceptible or resistant, a one-fold dilution / ± 3mm difference may result in different interpretations. As this report evaluates the interpretations of MIC / zone diameter and not the values, some participants may find their results classified as incorrect (score of 0, 1 or 3) even though the actual MIC / zone diameter measured is only one-fold dilution / ± 3mm apart from the expected MIC / zone diameter. In these cases, the participants should be confident about the good quality of their AST performance.

In this report, results from laboratories affiliated with the HH or AH sectors are presented together. The laboratories are identified by codes and each code is known only by the corresponding laboratory and the organizers. The full list of laboratory codes is confidential and known only by the EQAsia Consortium.

This report is approved in its final version by a Technical Advisory Group composed by members of the EQAsia Consortium, and by the EQAsia Advisory Board members Ben Howden (The Peter Doherty Institute for Infection and Immunity, Australia), Monica Lahra (WHO Collaborating Centre for STI and AMR, NSW Health Pathology Microbiology, New South Wales, Australia) and Russel Cole (Pacific Pathology Training Centre, New Zealand).

2. Materials and Methods

2.1 Participants in EQAsia Matrix EQA 2023

A total of nine laboratories participated in the third EQAsia Matrix EQA trial of the EQAsia project: three laboratories belonging to the HH sector and six belonging to the AH sector from Bangladesh, Bhutan, Malaysia, Pakistan, Sri Lanka, Timor-Leste, and , Vietnam (**Figure 1**).

2.2 Samples preparation

Laboratories that registered for the Matrix EQA trial received four lyophilized cultures mimicking meat content for isolation of presumptive ESBL, AmpC- and carbapenemase-producing *E. coli*, including identification, and AST of the obtained isolates. The preparation of the cultures followed the official EURL-AR protocols [3]. The pre-testing and spiking of the meat samples are briefly described below:

Pre-testing

Firstly, pork minced meat samples were tested for the presence of ESBL-, AmpC-, and carbapenemase-producing *E. coli* to ensure that the meat does not naturally contain these type of bacteria. Meat portions of 25 g were mixed with 225 mL of buffered peptone water (BPW) and incubated at 37°C ± 1°C for 18-22 h (pre-enrichment step as referred to in the EURL-AR protocols). A loopful of the pre-enriched culture was plated onto a MacConkey agar plate containing 1 mg/L of cefotaxime and incubated overnight to assure that the batch used was negative for ESBL/AmpC/carbapenemase-producing *E. coli* and that it contained some background flora.

Spiking of the meat samples

To prepare the four lyophilized cultures mimicking meat content, four 25 g pork minced meat portions from the same batch as in the pre-testing were used, and all the portions were spiked with an *E. coli* isolate.

After spiking the meat with the *E. coli* isolates, all meat portions were mixed with BPW, incubated, and plated on selective agar as described in the pre-testing. The grown colonies, consisting in a mixture of the different bacteria present in the meat sample were then scrapped and lyophilized.

Figure 1: Countries participating in the 3rd EQAsia Matrix EQA 2023. Color indicates sector affiliation of the participating laboratory as Animal Health laboratory (yellow), Human Health laboratory (blue) or both Human and Animal Health laboratories (green).

2.3 Isolation and identification of ESBL-, AmpC- and carbapenemase-producing *E. coli*

The *E. coli* isolates used for this EQA were tested at DTU Food and by the external partner (The Peter Doherty Institute for Infection and Immunity, Australia), and additionally verified by CUVET Thailand. Expected MIC values can be found in **Appendix 2**. The reference strains *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were supplied during previous EQA rounds to all participants free of charge with instructions for storage and maintenance for quality assurance purposes and future EQA trials. The expected quality control ranges for the reference strain *E. coli* ATCC 25922 were retrieved from Clinical and Laboratory Standards Institute (CLSI) in document M100-33rd Ed. [4], tables 4A-1 and 5A-1, and for *E. coli* NCTC 13846 from EUCAST in document "Routine and extended internal quality control for MIC determination and disk diffusion" [5] (**Appendix 3**).

The protocols for selective isolation and identification of the ESBL-, AmpC- and carbapenemase-producing *E. coli* isolates contained in the lyophilized cultures were briefly described in the Matrix EQA protocol (**Appendix 1**) and are based on the official EURL-AR protocols [3]. For bacterial identification, the participants were asked to perform the methods routinely applied in their laboratories. Information about the methods used for selective isolation and species identification were requested when submitting results in the informatics module.

2.4 Antimicrobials

The antimicrobials recommended for AST in this trial are listed in the protocol (**Appendix 1**) and summarized in **Table 1**. These antimicrobials correspond to several antimicrobial class representatives important for surveillance, as well as antimicrobials required for detection and confirmation of ESBL-, AmpC- and carbapenemase-producing phenotypes.

The reference values used in this EQA for interpreting MIC and disk diffusion results are in accordance with current zone diameter and MIC breakpoint values developed by CLSI (M100, 33rd Ed.) [4]. When not available, EUCAST clinical breakpoints (Tables v. 13.0, 2023) [5] or epidemiological cut off values [6] were used instead. Cefotaxime/ clavulanic acid and ceftazidime/ clavulanic acid results were not scored, as these drug combinations are mostly important for confirmation of ESBL-, AmpC- and carbapenemase-producing phenotypes. Results for presumptive beta-lactam resistance mechanisms were interpreted according to the most recent EFSA (European Food Safety Authority) [7] recommendations also included in the Matrix EQA protocol (**Appendix 1**).
Participants were encouraged to test as many as possible of the antimicrobials listed, but always considering their relevance regarding the laboratory's routine work.

2.5 Distribution

CUVET Thailand dispatched the lyophilized cultures in October 2023 to all participating laboratories. The shipment (UN3373, biological substances category B) was done according to International Air Transport Association (IATA) regulations. Participating laboratories received information on how to open, revive and store these lyophilized cultures.

Table 1. Panel of antimicrobials and respective abbreviations for AST of *E. coli* included in the EQAsia Matrix EQA 2023. For the antimicrobials in grey, no interpretative criteria were available and/or scored in the informatics module.

Antimicrobials – *E. coli* AST
Amikacin (AMK)
Ampicillin (AMP)
Azithromycin (AZI)
Cefepime (FEP)
Cefotaxime (FOT)
Cefotaxime/clavulanic acid (F/C)
Cefoxitin (FOX)
Ceftazidime (TAZ)
Ceftazidime/clavulanic acid (T/C)
Chloramphenicol (CHL)
Ciprofloxacin (CIP)
Colistin (COL)
Doripenem (DOR)
Ertapenem (ETP)
Gentamicin (GEN)
Imipenem (IMI)
Levofloxacin (LEVO)
Meropenem (MERO)
Nalidixic Acid (NAL)
Piperacillin/tazobactam (P/T4)
Sulfamethoxazole (SMX)
Tetracycline (TET)
Tigecycline (TGC)
Tobramycin (TOB)
Trimethoprim (TMP)
Trimethoprim/sulfamethoxazole (SXT)

2.6 Procedure

Protocols and all relevant information were available at the EQAsia website [8], and accessible at any time throughout the EQA trial. The participants were recommended to store the lyophilized samples in a dark, dry, and cool place until performing selective isolation and AST.

Participating laboratories were advised to perform identification and AST of the test strains according to the methods routinely applied in their laboratory.

Procedures such as disk diffusion, gradient test, agar dilution and broth dilution were acceptable. For the interpretation of results, only the categorisation as resistant / intermediate / susceptible (R/I/S) was evaluated, whereas MIC

and inhibition zone diameter values were used as supplementary information.

All participants were invited to enter the obtained results into an informatics module designed for this trial. The informatics module could be accessed through a secured individual login and password. After release of the results, the participants were invited to login to retrieve an individual database-generated evaluation report.

3. Results

3.1 Methods used by the participants

Participants were asked to indicate the methods used for selective isolation of the *E. coli* strains, as well as the method used for bacterial identification, during results submission in the informatics module. **Figure 2** summarizes the methods reported by the participants.

Six of the nine participating laboratories (#01, #27, #28, #34, #37 and #40) reported that selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing *E. coli* was performed exactly according to the protocol provided in the Matrix EQA protocol (**Appendix 1**), which is based in the official EURL-AR protocols [3], meaning that no changes in media, concentrations of antibiotics, etc. were referred (**Figure 2**, left column). Laboratory #22 reported that the protocol was used, but the incubation conditions in the selective plating were modified to 37°C for 20 h, instead of the suggested 44°C ± 0.5°C for 18-22 h (initial plating) or 37°C ± 1°C for 18-22 h (subculture of individual presumptive ESBL/AmpC-producing *E. coli* colonies). No information regarding the methods used was given by laboratory #44.

Regarding selective isolation of carbapenemase-producers (**Figure 2**, middle column), laboratories #01, #22, #27, #34, #37, #40, #42 and #44 reported that carbapenemase selective isolation was not performed, whereas laboratory #28 reported that carbapenemase selective isolation was performed.

Confirmation of *E. coli* species identification (**Figure 2**, right column) was performed by laboratories #01, #27, #28, #34, #40 and #44 using biochemical tests. Laboratories #22 and #37 used chromogenic media while laboratory #42 conducted MALDI-ToF.

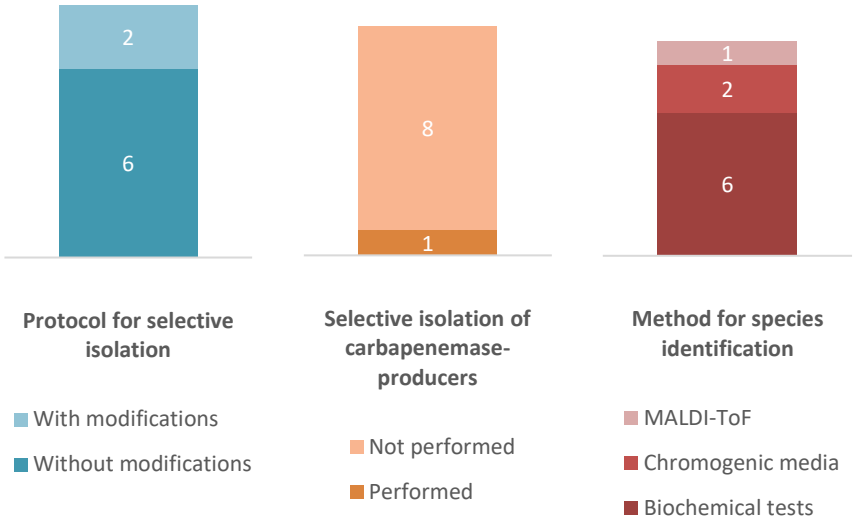


Figure 2. Methods used by the participants for selective isolation and confirmation of *E.coli* species identification.

3.2 ESBL-, AmpC- and carbapenemase-producing *E. coli* isolation and identification

Samples EQAsia 23.M1, EQAsia 23.M2, EQAsia 23.M3 and EQAsia 23.M4 were spiked with different *E. coli* isolates and, therefore, expected to be positive for growth of presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli*. Participants were asked to describe the growth observed in the selective plates (**Table 2**).

For samples EQAsia 23.M1, EQAsia 23.M3 and EQAsia 23.M4, 55.6% of the laboratories, described the presence of typical *E. coli* colonies as a pure culture. In contrast, for sample EQAsia 23.M2, this percentage increased to 66.7%. For samples EQAsia 23.M1 and EQAsia 23.M3, only one of the laboratories reported the absence of typical *E. coli* colonies respectively (**Table 2**).

Table 2. Expected and obtained results for ESBL/AmpC/carbapenemase-producing *E. coli* isolation. Number of obtained results (n) out of the total of reported results (N) is presented for each growth type and for each sample, as well as for the species identification. Obtained results in accordance with the expected result are shown in bold.

Growth observed on the selective plates	EQAsia 23.M1	EQAsia 23.M2	EQAsia 23.M3	EQAsia 23.M4
Mixed culture containing typical *E. coli* colonies	2/9 (22.2%)	2/9 (22.2%)	2/9 (22.2%)	3/9 (33.3%)
Pure culture of typical *E. coli* colonies	**5/9 (55.6%)**	**6/9 (66.7%)**	**5/9 (55.6%)**	**5/9 (55.6%)**
Pure culture without typical *E. coli* colonies	1/9 (11.1%)	--	1/9 (11.1%)	--
No growth	1/9 (11.1%)	1/9 (11.1%)	1/9 (11.1%)	1/9 (11.1%)

(n/N) number of responses (n) out of the total of reported results (N)

Results confirming the species identification were reported by laboratories #01, #22, #27, #34, #37, #40 and #42. Laboratory #44 reported that no growth was observed in the selective plates and, therefore, no results were reported for species identification (**Table 3**):

- EQAsia 23.M1: sample was confirmed as positive by five laboratories (#01, #22,

#27, #37 and #42) where laboratories (#01, #22, #27 and #42) have reported it as a pure culture of typical *E. coli* colonies. Laboratory #37, however, reported it as mixed culture containing typical *E. coli* colonies. Laboratory #40, which observed growth as 'Pure culture without typical *E. coli* colonies', reported the sample as negative. Laboratory #28 and #34 which observed growth as 'Pure culture of typical *E. coli* colonies' and 'Mixed culture containing typical *E. coli* colonies' ended up reporting the sample as negative as well.

- EQAsia 23.M2: sample was confirmed as positive by eight of the laboratories whereas laboratories (#01, #28, #22, #27, #40 and #42) have reported it as a pure culture of typical *E. coli* colonies. Laboratories #37 and #34 have reported it as mixed culture containing typical *E.coli* colonies.
- EQAsia 23.M3: sample was confirmed as positive by seven laboratories whereas five laboratories (#01, #27, #28, #40 and #42) have reported it as a pure culture of typical *E. coli* colonies. Two laboratories (#34 and #37) have reported it as mixed culture containing typical *E. coli* colonies. Laboratory #22, which observed growth as 'Pure culture without typical *E. coli* colonies', have reported the sample as negative.
- EQAsia 23.M4: sample was confirmed as positive by eight laboratories whereas five laboratories (#01, #22, #27, #28, #40 and #44) have reported it as a pure culture of typical *E. coli* colonies. Laboratories (#34, #37 and #42) have reported it as mixed culture containing typical *E. coli* colonies.

In summary (**Table 3**), four laboratories (#01, #27, #37 and #42) reported all samples as positive for presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli*. None of the participating laboratories correctly reported all four samples as positive pure culture of typical *E. coli*.

Table 3. Obtained results for ESBL/AmpC/carbapenemase-producing *E. coli* species identification reported by each laboratory for each sample. Presumptive ESBL/AmpC/carbapenemase isolates identified as *E. coli* are presented as 'Positive', and not *E. coli* or not tested samples are presented as 'Negative'. Obtained results in accordance with the expected result are shown in bold.

Laboratory ID Number	EQAsia 23.M1	EQAsia 23.M2	EQAsia 23.M3	EQAsia 23.M4
#01	**Positive**	**Positive**	**Positive**	**Positive**
#22	**Positive**	**Positive**	Negative	**Positive**
#27	**Positive**	**Positive**	**Positive**	**Positive**
#28	Negative	**Positive**	**Positive**	**Positive**
#34	Negative	**Positive**	**Positive**	**Positive**
#37	**Positive**	**Positive**	**Positive**	**Positive**
#40	Negative	**Positive**	**Positive**	**Positive**
#42	**Positive**	**Positive**	**Positive**	**Positive**
#44	Negative	Negative	Negative	Negative

3.3 Antimicrobial Susceptibility Testing

For all samples considered positive for ESBL- , AmpC- or carbapenemase-producing *E. coli*, one *E. coli* isolate per sample should be taken and further tested for susceptibility towards the antimicrobials suggested in the Matrix EQA protocol (**Appendix 1**) and outlined in **Table 1**.

Of the nine laboratories submitting results, only eight (#01, #22, #27, #34, #37, #40, #42 and #44) submitted results for AST. For sample EQAsia 23.M1 and its respective *E. coli* isolates, results were only available from six laboratories, since laboratories #34 and #40 considered the sample negative. For sample EQAsia 23.M3 and its respective *E. coli* isolates, laboratory #22 reported the sample as negative but submitted AST results. Laboratory #44 also submitted AST results despite reporting all samples as negative, observing no growth on selective plates.

The participants were invited to report inhibition zone diameters/MIC values and categorization as resistant ('R'), intermediate ('I') or susceptible ('S') for each strain/antimicrobial combination. Only the categorization was evaluated, whereas the inhibition zone diameters/MIC values were used as supplementary information.

The Matrix EQA set-up allowed laboratories to choose the antimicrobials to be tested among the panel of suggested antimicrobials (**Table 1**). All participating laboratories applied disk diffusion for testing the antimicrobials and, therefore, reported inhibition zone diameters (**Table 4**). Antimicrobials such as ampicillin, ciprofloxacin and gentamicin were tested by all eight laboratories. Tobramycin was not tested by any of the laboratories. Colistin was exclusively tested by laboratory #34 for samples EQAsia 23.M2, EQAsia 23.M3, and EQAsia 23.M4 using MIC-broth microdilution method. Other laboratories did not conduct this test as their chosen methodology is not suitable for testing this drug.

Table 4. Antimicrobial agents tested by the laboratories and by method applied. The number of participating laboratories that tested each antimicrobial is shown (n), as well as the percentage (%) of laboratories out of the total number of participating laboratories (N) for the trial (% of n/N).

Antimicrobial	Laboratories in total: n (% of n/N)	Laboratories in total: n (% of n/N)
	Disk Diffusion	MIC
AMK	6 (75.0)	--
AMP	8 (100.0)	--
AZI	4 (50.0)	--
FEP	5 (62.5)	--
FOT	7 (87.5)	--
FOX	6 (75.0)	--
TAZ	7 (87.5)	--
CHL	7 (87.5)	--
CIP	8 (100.0)	--
COL	--	1 (12.5)
DOR	2 (25.0)	--
ETP	5 (62.5)	--
GEN	8 (100.0)	--
IMI	6 (75.0)	--
LEVO	4 (50.0)	--
MERO	7 (87.5)	--
NAL	5 (62.5)	--
PT4	4 (50.0)	--
SMX	4 (50.0)	--
TET	7 (87.5)	--
TGC	3 (37.5)	--
TOB	0	--
TMP	4 (50.0)	--
SXT	7 (87.5)	--
Total	**8**	**--**

Disk Diffusion – inhibition zone diameter determination by disk diffusion

The AST performance of the laboratories can be analysed from a strain-, antimicrobial-, and laboratory-based perspective. From a strain-analysis point of view (**Figure 3**), the *E. coli* strain used to spike sample EQAsia 23.M4 presented the lowest deviation (7.3%), meaning that most of the susceptibility results obtained were in accordance with the expected **(Appendix 2)**. The other three samples revealed more than 10% deviation, where the highest deviation (26%) was observed for the *E. coli* strain isolated from EQAsia 23.M3.

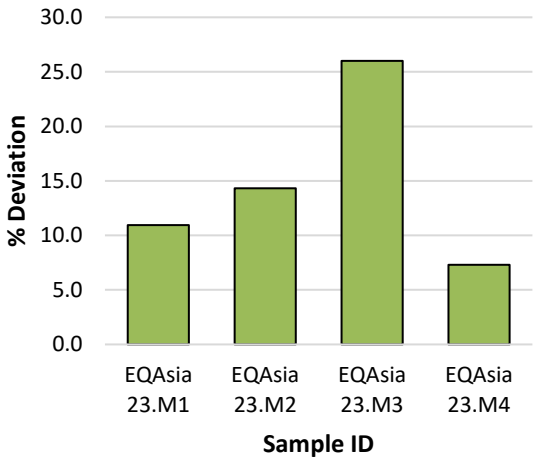


Figure 3. Percentage of deviation in the AST interpretation (R/I/S) per sample in the EQAsia Matrix EQA 2023.

Antimicrobials with highest deviations from the expected result were piperacillin/tazobactam (36.7%), as well as gentamicin (25.8%), whereas ampicillin revealed no deviation from the expected results and tetracycline had a deviation of less than 5% (**Figure 4**).

Piperacillin/tazobactam was only tested by laboratories #01, #37, #27 (four strains) and #40 (three strains), resulting in a total of only 15 tests performed towards this antimicrobial. Some of the strains expected to be susceptible to the drug were reported as intermediate, leading to light score penalties (score of 3 instead of 4) that contributed to the observed deviation.

The deviation observed in gentamicin testing was caused by an incorrect result, but that was a very major error, such as reporting a resistant strain as susceptible or a susceptible strain as resistant.

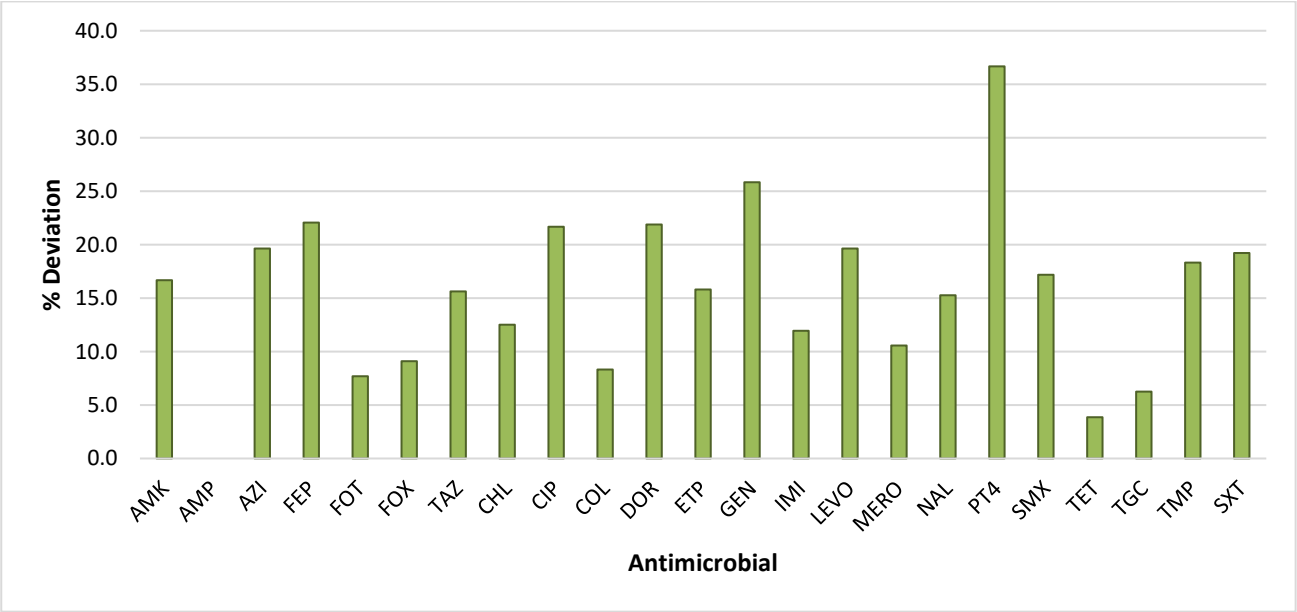


Figure 4. Percentage of deviation in the AST interpretation (R/I/S) among the *E. coli* strains used to spike the matrix samples in EQAsia Matrix EQA 2023. Results are categorized according to antimicrobial agent. Bars represent the average distribution of the deviation.

A deviation below 5% of laboratory performance in terms of interpretation of the results (R/I/S) was observed for laboratories #34, #40 (results from three strains assessed) and #22 (results from all four strains) (**Figure 5**). In average, the deviation was 13.4% (ranging from 2.7 to 29.2%).

Laboratory #01' deviations were mostly caused by major errors, in particular for the strain used to spike samples EQAsia 23.M2 and EQAsia 23.M3.

Laboratory #27' deviations were mainly attributed to major errors for antimicrobials such as piperacillin/tazobactam, gentamicin and

ciprofloxacin.

Laboratory #37 presented several deviations from the expected results, notably regarding the strain used to spike sample EQAsia 23.M3 as mentioned above.

Laboratory #42' deviations were due to major errors in testing gentamicin and ciprofloxacin, while laboratory #44 showed several deviations caused by minor errors. In this case, some strains expected to be susceptible to the drug were reported as intermediate.

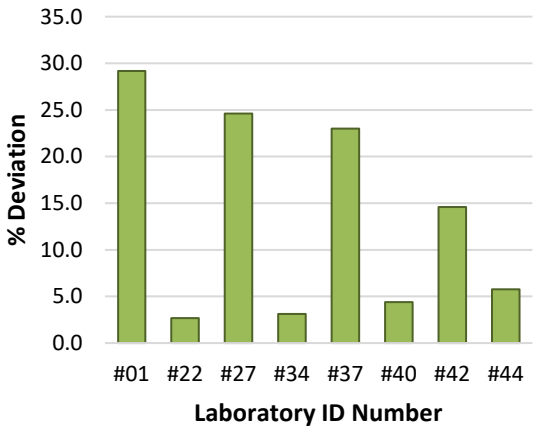


Figure 5. Percentage of deviation in the AST interpretation (R/I/S) among the *E. coli* strains used to spike the matrix samples in EQAsia Matrix EQA 2023. Results are categorized by laboratory ID number.

3.4 ESBL, AmpC and Carbapenemase phenotypic testing

Four lyophilized samples mimicking meat content were included in this Matrix EQA. The sample EQAsia 23.M1 contained an *E. coli* isolate expressing ESBL+AmpC producer phenotype (**Table 5**). Four of the participating laboratories correctly classified the phenotype (#01, #22, #27 and #42), Whereas laboratory #37 classified the strain as ESBL-producer as they observed synergy and reported the strain susceptible to cefoxitin.

The *E. coli* isolate from sample EQAsia 23.M2 had an AmpC-producer phenotype (**Table 5**). Four of the laboratories reported the correct phenotype (#34, #37, #40 and #42), whereas laboratories #27 and #28 classified the strain as an ESBL producer even though neither cefotaxime nor ceftazidime in combination with clavulanic acid were tested and, therefore, synergy could not be observed. Laboratory #01 classified the strain as a carbapenemase-producer as they reported the strain resistant to meropenem. Laboratory #22 classified the strain as ESBL + AmpC phenotype as they observed synergy between cefotaxime alone and in combination with clavulanic acid (a 5 mm increase of diameter for FOT/Cl compared to FOT alone) and reported the strain resistant to cefoxitin.

Sample EQAsia 23.M3 was spiked with an *E. coli* isolate with a carbapenemase-producer phenotype (**Table 5**). Five of the laboratories reported the correct phenotype (#22, #27, #34 #40, and #44), whereas laboratory #01 reported it as an AmpC-producer (synergy observed and sample reported as resistant to cefoxitin and susceptible to meropenem). Laboratory #42 reported the sample as an ESBL-producer and indicated that the selective isolation procedures were modified (using cefpodoxime 10 mg/L) while laboratory #37 classified the sample as "other phenotype".

Lastly, the *E. coli* isolate from sample EQAsia 23.M4 had an ESBL-producer phenotype (**Table 5**), which was correctly identified by the five laboratories submitting results (#01, #22, #27, #34 and #40). Laboratory #42 reported the sample as an ESBL+AmpC-producer and laboratory #37 classified it as "other phenotype" despite that they observed synergy.

In summary, laboratories #01, #22, #27, #28, #37 and #42 presented one incorrect classification of the different ESBL / AmpC / carbapenemase phenotypes among the four *E. coli* strains and laboratories #34, #40 and #44 correctly identified the phenotypes for all the strains assessed.

Table 5. Expected and obtained classification of ESBL-, AmpC- and carbapenemase-producing *E. coli* strains used to spike the matrix samples. Number of obtained results (n) out of the total of reported results (N) is presented for each phenotype and for each strain. Obtained results in accordance with the expected result are shown in bold.

Sample ID		EQAsia 23.M1	EQAsia 23.M2	EQAsia 23.M3	EQAsia 23.M4
Expected results		ESBL+AmpC	AmpC	Carbapenemase	ESBL
Obtained results (n/N)	ESBL	1/5 (20%)	2/8 (25%)	1/8 (12.5%)	**5/7 (71.4%)**
	AmpC	--	**4/8 (50%)**	1/8 (12.5%)	--
	ESBL + AmpC	**4/5 (80%)**	1/8 (12.5%)	--	1/7 (14.3%)
	Carbapenemase	--	1/8 (12.5%)	**5/8 (62.5%)**	--
	Other phenotypes	--	--	1/8 (12.5%)	1/7 (14.3%)
	Susceptible*	--	--	--	--

*no AmpC, ESBL and Carbapenemase;
(n/N) number of responses (n) out of the total of reported results (N)

3.5 Quality control strains

The quality control strains *E. coli* ATCC 25922 and *E. coli* NCTC 13846 (for colistin) were sent free of charge to all participating laboratories in previous trials to be used as reference strains.

Antimicrobial susceptibility test results for the quality control strain *E. coli* ATCC 25922 were submitted by all eight participating laboratories, which applied the disk diffusion method and reported inhibition zone diameters. As colistin should not be tested by disk diffusion, no results were submitted for this drug and, therefore, the quality control strain *E. coli* NCTC 13846 was not tested.

The highest proportion of test results outside of the expected range were observed for cefotaxime (3 out of 7) and trimethoprim/sulfamethoxazole (3 out of 7), whereas no deviations were observed for ampicillin, ceftazidime, ciprofloxacin, doripenem, levofloxacin, meropenem, nalidixic acid, piperacillin/tazobactam, sulfamethoxazole, tetracycline, and trimethoprim (**Table 6**).

These incorrect results led to the laboratories' deviation summarized in **Figure 6**. Laboratories #22, #34 and #37 presented no deviation. Inversely, laboratory #27 deviation was as high as 43.8%.

Table 6. AST of the reference strain *E. coli* ATCC 25922. Proportion of test results outside of expected range is presented by methodology used.

Antimicrobial	Proportion outside of range
	Disk Diffusion
AMK	1/6
AMP	0/8
FEP	1/5
FOT	3/7
FOX	1/6
TAZ	0/7
CHL	1/7
CIP	0/8
DOR	0/2
ETP	1/4
GEN	1/8
IMI	1/6
LEVO	0/4
MERO	0/7
NAL	0/5
PT4	0/4
SMX	0/2
TET	0/7
TGC	1/3
TMP	0/3
SXT	3/7

Disk Diffusion – inhibition zone diameter determination by disk diffusion

Laboratory #27 obtained 7 out of 16 results outside of range. Specifically, results for cefotaxime and cefoxitin were just slightly (1

mm) below the acceptance interval, whereas results for amikacin, chloramphenicol, imipenem, tigecycline and trimethoprim/sulfamethoxazole were above (1, 5, 3, 2 and 6 mm, respectively) the accepted range. A similar finding is observed for laboratory #40, whereas 4 out of 16 test results were outside of range. Result for Trimethoprim/sulfamethoxazole was 1 mm above the acceptance interval, whereas results for cefepime, ertapenem and gentamicin were below (6, 2 and 1 mm respectively) the accepted range.

Laboratories #01 and #44 reported inhibition zone diameters for cefotaxime below (1 and 4 mm, respectively) the acceptance interval, and laboratory #42 reported results for trimethoprim/sulfamethoxazole slightly below (1 mm) the expected range.

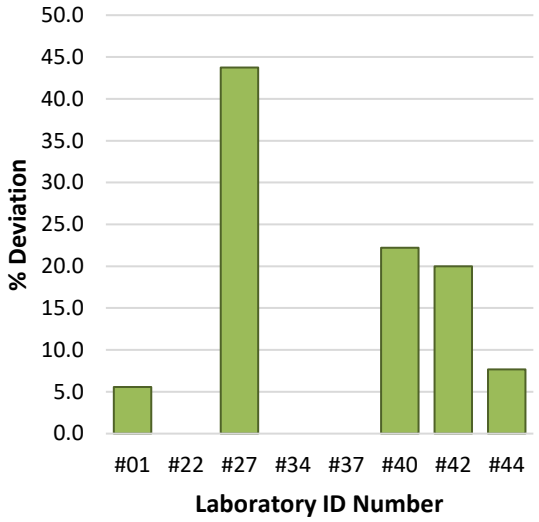


Figure 6. Percentage of deviation in the AST of the quality control strain *E. coli* ATCC 25922 by the laboratories.

4. Discussion

A total of nine laboratories from both the HH and AH sectors participated in the 3rd EQAsia Matrix EQA on selective isolation of presumptive ESBL-AmpC- and carbapenemase-producing *E. coli* from cultures mimicking meat samples. In general, the participants used the recommended methods for selective isolation, which are based on the official EURL-AR protocols [3]. Only one of the participating laboratories performed carbapenemase selective isolation using specific selective plates. Bacterial identification was achieved using biochemical tests, chromogenic media and MALDI-ToF.

All samples EQAsia 23.M1, EQAsia 23.M2, EQAsia 23.M3 and EQAsia 23.M4 were expected to be positive for growth of presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli*. Regarding the positive samples, at least 55.6% of the laboratories correctly identified the samples as positive for the presence of *E. coli* colonies. Sample EQAsia 23.M1 seemed to cause more problems as several laboratories (5 out of 9) reported the sample as negative instead of positive. In fact, none of the laboratories identified correctly all the samples as positive, which demonstrates the need for more education and training in the selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing bacteria from complex matrices.

Of the nine laboratories submitting results, only eight submitted results for antimicrobial susceptibility testing. The AST performance was assessed from different perspectives to better identify deviations from the expected results. Hence, the strain-based analysis revealed that the *E. coli* strain isolated from sample EQAsia 23.M3 presented the highest deviation from the expected results in comparison to the other three isolates. One possible explanation could be the high level of resistance of this strain towards cefotaxime (MIC > 64), which assured the growth of this strain on the selective MacConkey agar plate containing 1 mg/L cefotaxime. The other isolates, which are less resistant to cefotaxime **(Appendix 2)** may be overgrown by contaminating bacteria and more challenging to isolate. Nevertheless, it is not completely clear if the submitted results for the remaining *E. coli* strains are due to the isolation and testing of other bacteria than the target one used to spike the samples with. Therefore, the possibility of performance issues cannot be discarded.

The several incorrect results observed for sample EQAsia 23.M3 were the main contributor for the highest deviation in terms of laboratories' AST performance, which presented an average deviation of 13.4% (ranging from 2.7 to 29.2%).

A few incorrect results were reported by the laboratories for the classification of the *E. coli* phenotypes into ESBL, AmpC or carbapenemase-producers. These seem to have been caused by the incorrect results obtained for relevant antimicrobials, rather than by incorrect classification. For instance, one laboratory incorrectly classified one of the strains as carbapenemase-producer because the strain was found to be resistant to meropenem. This observation demonstrates the importance of accurate testing, as it may lead to inappropriate antibiotic treatment.

Lastly, regarding AST of the quality control strains, the majority of the results outside the quality control range were just slightly (1-2mm) below/above the acceptance interval. It demonstrates possible technical problems in performing AST but may also suggest problems in maintenance of the quality control strains.

5. Conclusion

This report presented the results of the third EQAsia Matrix EQA trial 2023. This EQA assessed the performance in 1) isolation and identification of presumptive ESBL-, AmpC-, and carbapenemase-producing *E. coli* from cultures mimicking meat content, 2) AST determination and interpretation and 3) detection and classification of β-lactam resistance phenotypes mediated by ESBL, AmpC and carbapenemase enzymes.

The goal of the EQAsia Matrix EQAs is to ensure that all participating Human and Animal Health laboratories are able to provide quality data to be used for the comparability of surveillance data on ESBL-, AmpC- or carbapenemase-producing *E. coli* reported by different laboratories.

This Matrix EQA trial allowed the EQAsia Consortium to have once again an overview of the laboratories' capacity for a complete participation in such a proficiency test. Firstly, only nine laboratories participated in the trial, even though 13 laboratories initially signed up for it. The reason for not participating was mainly the lack of essential resources, such as selective media/plates. Secondly, not all the participating laboratories submitted results for all the components (only eight laboratories reported AST results). Thirdly, it seems that the laboratories can classify the resistance phenotypes, however incorrect results obtained for certain antimicrobials will lead to incorrect classification. Lastly, some laboratories may lack resources required for this type of proficiency test, such as cefotaxime/clavulanic acid or ceftazidime/clavulanic acid combination required for confirmatory testing.

On a final note, even though this trial was initially meant for laboratories of the Animal Health sector, since ESBL-, AmpC- and carbapenemase-producing *E. coli* continue to spread in food-producing animals, we were pleased to see the interest from the Human Health laboratories in participating, aligned with the concept of the WHO, FAO, WOHA tripartite Tricycle project. In fact, the increasing number of this type of strains is concerning and it is of high importance to support all type of laboratories with technical guidance and capacity building. In future EQAs, the EQAsia Consortium will aim at providing samples from other complex matrices that could be more relevant for the Human Health Sector.

6. References

[1] Annex 8: Pathogen-antimicrobial combinations under GLASS-AMR surveillance. Global antimicrobial resistance and use surveillance system (GLASS) report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.

[2] FAO. 2019. Monitoring and surveillance of antimicrobial resistance in bacteria from healthy food animals intended for consumption. Regional Antimicrobial Resistance Monitoring and Surveillance Guidelines – Volume 1. Bangkok.

[3] EURL-AR protocols: https://www.eurl-ar.eu/protocols.aspx

[4] CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*. 33rd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2023.

[5] The European Committee on Antimicrobial Susceptibility Testing. *Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST*. Version 13.0, 2023.

[6] EUCAST Website: https://www.eucast.org/

[7] 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.

[8] EQAsia Website: https://antimicrobialresistance.dk/eqasia.aspx

7. Appendices

Appendix 1: Matrix EQA 2023 Protocol

EQAsia Matrix EQA 2023

Protocol

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 since 2021. 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 EQA7 2023 includes a **Matrix EQA** aiming at assessing 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 2023** therefore entails the selective isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli*, as well as antimicrobial susceptibility testing (AST) of obtained isolates from four cultures mimicking meat content. These samples consist of four lyophilized bacterial cultures obtained from 25g samples of minced pork meat. Briefly, 25g of minced pork 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 contain *E. coli* that may be producing 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 in previous EQAsia 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 2023

3.1 Shipping and receipt of strains

In October 2023, participating laboratories located in South and Southeast Asia will receive a parcel containing four lyophilized cultures obtained from meat samples. The lyophilized cultures obtained from spiked matrix samples of pork meat content will be distributed in separate vials labelled as EQAsia 23.M1 to M4. Participants should expect that ESBL-, AmpC- and/or carbapenemase-enzymes producing *E. coli* strains will be included in some of the lyophilized cultures.

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 Reviving and storage of strains

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.

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 prior to shipping the samples). Instead, the lyophilized cultures should be revived before proceding to the selective isolation.

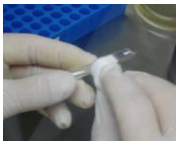
Aseptic technique must be applied throughout. All testing should be performed in a BSL2 level laboratory or in a biosafety cabinet class II.

- Needed material:
    - An ampoule cutter or a file
    - Sterile Luria Bertani (LB) broth
    - Agar plates (5 to 6 plates per one strain)
    - Autopipette with tips or Pasteur pipettes
    - Inoculating loop

1. Carefully take the ampoule out of the wrap.

   Note: To maintain the vacuum condition, do not break the tip of the ampoule. Otherwise, the air will enter the ampoule and the cotton wool plug will be pushed down and in contact with dried bacterial culture. If it happens, please simply remove the cotton plug with forceps.

   Note: The ampoule can be cut in the middle or below the cotton wool plug.

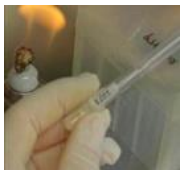


2. Wipe the ampoule neck with 70% alcohol-dampened cotton wool.

3. Make a deep score on the around the circumference of the ampoule near the middle of the plug using ampoule cutter or a file. The ampoule should be cut in the middle or below the cotton wool plug.

4. Wrap thick cotton wool around the ampoule and break at the marked area.

5. Remove the pointed end of the ampoule and cotton into a biohazard container. Add 0.5 mL of sterile LB broth into the lyophilized cells. Mix gently and carefully to avoid creating aerosols.



6. Then, transfer all re-suspended cells into 5mL fresh LB broth.

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

7. 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.
8. 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.
9. 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.

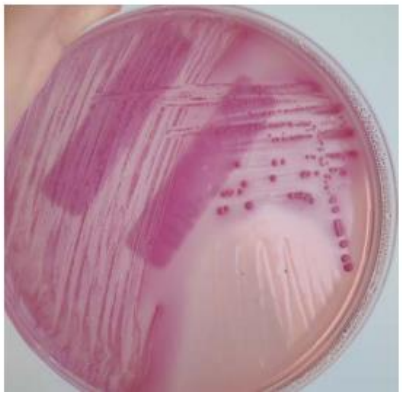


Figure 1: Typical appearance of *E. coli* on MacConkey agar supplemented with 1 mg/L cefotaxime.

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.

3.4 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, 33rd Ed.). When not available, EUCAST clinical breakpoints (Tables v. 13.1, 2023) or epidemiological cut off values (https://mic.eucast.org/) are used instead.

Table 1. Breakpoints for interpretation of MICs and zone diameters for *E. coli*

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

Antimicrobials	Reference values MIC (µg/mL)			Reference values Disk diffusion (mm)		
	S	I	R	S	I	R
Amikacin, AMK	≤ 4	8	≥ 16	≥ 20	17-19	≤ 16
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	≤ 2	4	≥ 8	≥ 18	15-17	≤ 14
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	≤ 2	4	≥ 8	≥ 17	13-16	≤ 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, 33rd 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 13.1, 2023. 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 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.

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

	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

2. AmpC-Phenotype

	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	NO SYNERGY	NO SYNERGY

3. ESBL + AmpC-Phenotype

	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

4. Carbapenemase-Phenotype

	MIC (mg/L)	Zone Diameter (mm)
MERO	> 0.12	< 25

5. Other Phenotypes

	MIC (mg/L)	Zone Diameter (mm)
1)		
FOT or TAZ	> 1	< 21 (FOT); < 22 (TAZ)
MERO	≤ 0.12	≥ 25
FOX	≤ 8	≥ 19
FOT/CLV and/or TAZ/CLV	NO SYNERGY	NO SYNERGY
2)		
FOT or TAZ	≤ 1	≥ 21 (FOT); ≥ 22 (TAZ)
MERO	≤ 0.12	≥ 25
FOX	> 8	< 19

Susceptible

	MIC (mg/L)	Zone Diameter (mm)
FOT or TAZ	≤ 1	≥ 21 (FOT); ≥ 22 (TAZ)
MERO	≤ 0.12	≥ 25
FOX	≤ 8	≥ 19

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 SUBMISSION OF RESULTS VIA THE INFORMATICS MODULE

We recommend that you write down your results in the enclosed test forms as it will help you when transferring results onto the online platform.

The detailed 'Guideline for reporting results in the EQAsia Informatics Module' is available for download directly from the EQAsia website. Please follow the guideline carefully.

Login to the Informatics Module:

Access the Informatics Module (incognito window) via the following link https://eqasia-pt.dtu.dk/

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 hiami@food.dtu.dk

When you submit your results, remember to have by your side the completed test forms (template available for download from the EQAsia website). If the same reference strain is used for different pathogens, please enter the results (even if the same) for all the pathogens.

Results must be submitted no later than November 24th, 2023.

If you have troubles entering your results or if you experience technical problems with the informatics module, please contact the DTU team directly at eqasia@food.dtu.dk, explaining the issues that you encountered.

Before submitting your final 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 further data entry.

After submission, the Informatics Module will allow you to view and print a report with your submitted results.

5 EVALUATION OF RESULTS

The scores for the submitted results will be released after the submission deadline has passed. 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).

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

Once the results have been evaluated, you will be able to access your certificate via the EQAsia Informatics Module. You will be notified by email when the certificate is available. The certificate will contain score for identification and for susceptibility testing for each of the panels for which you submitted results. Performance rate for each panel will also be shown on the certificate.

The EQAsia project team would like to thank you once again for your participation in this EQA round!

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.

Appendix 2: Reference values (MIC) – *Escherichia coli*

Reference values (MIC values and interpretation) – *Escherichia coli*

	Amikacin AMK		Ampicillin AMP		Azithromycin AZI		Cefepime FEP		Cefotaxime FOT		FOT+Cl F/C	Cefoxitin FOX		Ceftazidime TAZ		TAZ+Cl T/C
EQAsia 23.M1	≤ 4	S	> 32	R	>64	R	16	R	>64	R	0.25/4	8	S	8	I	0.12/4
EQAsia 23.M2	≤ 4	S	> 32	R	8	S	0.25	S	8	R	8/4	64	R	16	R	8/4
EQAsia 23.M3	> 128	R	> 32	R	>64	R	> 32	R	>64	R	> 64/4	> 64	R	> 128	R	> 128/4
EQAsia 23.M4	≤ 4	S	> 32	R	>64	R	4	I	64	R	≤ 0.06/4	4	S	1	S	≤0.12/4

R, Resistant; I, Intermediate; S, Susceptible

	Chloramphenicol CHL		Ciprofloxacin CIP		Colistin COL		Doripenem DOR		Ertapenem ETP		Gentamicin GEN		Imipenem IMI		Levofloxacin LEVO		Meropenem MERO	
EQAsia 23.M1	> 64	R	> 8	R	>4	R	≤ 0.12	S	0.12	S	>16	R	≤ 0.12	S	>8	R	≤ 0.03	S
EQAsia 23.M2	8	S	≤ 0.015	S	>4	R	≤ 0.12	S	0.03	S	0.5	S	≤ 0.12	S	< 1	S	≤ 0.03	S
EQAsia 23.M3	16	I	> 8	R	≤ 0.25	I	> 4	R	>8	R	> 16	R	> 16	R	> 8	R	> 16	R
EQAsia 23.M4	> 64	R	0.12	S	≤ 0.25	I	≤ 0.12	S	0.03	S	≤ 1	S	≤ 0.12	S	< 1	S	≤ 0.03	S

R, Resistant; I, Intermediate; S, Susceptible

	Nalidixic acid NAL		Piperacillin/ tazobactam P/T4		Sulfamethoxazole SMX		Tetracycline TET		Tigecycline TGC		Tobramycin TOB		Trimethoprim TMP		Trimethoprim/ sulfamethoxazole SXT	
EQAsia 23.M1	>64	R	≤ 8/4	S	> 512	R	> 32	R	≤ 0.25	S	8	I	> 32	R	> 4/76	R
EQAsia 23.M2	2	S	≤ 8/4	S	≤ 8	S	> 32	R	≤ 0.25	S	≤ 1	S	≤ 0.25	S	≤ 0.5/9.5	S
EQAsia 23.M3	> 64	R	> 64/4	R	> 512	R	> 32	R	≤ 0.25	S	> 8	R	> 32	R	> 4/76	R
EQAsia 23.M4	≤ 4	S	≤ 8/4	S	> 512	R	> 32	R	≤ 0.25	S	≤ 1	S	> 32	R	> 4/76	R

R, Resistant; I, Intermediate; S, Susceptible

Appendix 3: Quality control ranges for reference strains

E. coli ATCC 25922		
Antimicrobial	**MIC (mg/L)**	**Inhibition Zone Diameter (mm)**
Amikacin, AMK	0.5-4	19-26
Ampicillin, AMP	2-8	15-22
Azithromycin, AZI	--	--
Cefepime, FEP	0.016-0.12	31-37
Cefotaxime, FOT	0.03-0.12	29-35
Cefotaxime and clavulanic acid, F/C	--	--
Cefoxitin, FOX	2-8	23-29
Ceftazidime, TAZ	0.06-0.5	25-32
Ceftazidime and clavulanic acid, T/C	--	--
Chloramphenicol, CHL	2-8	21-27
Ciprofloxacin, CIP	0.004-0.016	29-38
Doripenem, DOR	0.016-0.06	27-35
Ertapenem, ETP	0.004-0.016	29-36
Gentamicin, GEN	0.25-1	19-26
Imipenem, IMI	0.06-0.5	26-32
Levofloxacin, LEVO	0.008-0.06	29-37
Meropenem, MERO	0.008-0.06	28-35
Nalidixic acid, NAL	1-4	22-28
Piperacillin and tazobactam, P/T4	1-4	24-30
Sulfamethoxazole, SMX	8-32	15-23
Tetracycline, TET	0.5-2	18-25
Tigecycline, TGC	0.03-0.25	20-27
Tobramycin, TOB	0.25-1	18-26
Trimethoprim, TMP	0.5-2	21-28
Trimethoprim and sulfamethoxazole, SXT	≤ 0.5	23-29

MIC ranges and disk diffusion ranges are according to CLSI M100 33rd edition, Tables 4A-1 and 5A-1

E. coli NCTC 13846		
Antimicrobial	**MIC (mg/L)**	**Inhibition Zone Diameter (mm)**
Colistin, COL	2-8	--

MIC range in accordance to “The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. Version 12.0, 2022. http://www.eucast.org.”

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