The 2nd EQAsia Matrix EQA on selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing Escherichia coli from cultures mimicking meat samples – 2022

Photo: Colourbox

The 2nd EQAsia Matrix EQA on selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* from cultures mimicking meat samples – 2022

National Food Institute
Technical University of Denmark
Kemitorvet
Building 204
DK-2800 Kgs. Lyngby
Denmark

The 2nd EQAsia Matrix EQA on selective isolation of presumptive ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* from cultures mimicking meat samples – 2022

Patrícia Teixeira dos Santos[1], Freshwork A. Abegaz[2], Hiba Al Mir[1], Tomislav Kostyanev[1], Pattrarat Chanchaithong[3], Paulina Tamez-Hidalgo[1], Pitak Santanirand[4], Rangsiya Prathan[3], Taradon Luangtongkum[3], Tobin Guarnacci[2], Watcharaporn Kamjumphol[4], Nimesh Poudyal[2], Rungtip Chuanchuen[3], Wantana Paveenkittiporn[4], Rene S. Hendriksen[1]

[1]National Food Institute, Technical University of Denmark, Denmark
[2]International Vaccine Institute, South Korea
[3]Faculty of Veterinary Science, Chulalongkorn University, Thailand
[4]National Institute of Health, Department of Medical Sciences, Thailand

1st edition, March 2023

Index

Executive Summary

This report summarizes the results of the 2nd EQAsia Matrix EQA trial of the EQAsia project (Matrix EQA 2022), 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 (July 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 September-November 2022 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 two HH and three AH laboratories participated and submitted results for the Matrix EQA. These laboratories are from four countries situated in South and Southeast Asia (Bangladesh, Laos People Democratic Republic, Pakistan, and Sri Lanka).

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 expected to be positive for growth of presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli* were correctly identified as positive by at least 60% of the laboratories, whereas none of the participants correctly identified the negative sample.

Four of the participating laboratories submitted results for antimicrobial susceptibility testing and presented an average deviation of 5.8% (ranging from 0.5 to 12.9%) 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, the National Institute of Health (NIH), Department of Medical Sciences in Thailand 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 existing local providers (NIH Thailand and CUVET Thailand). The program, referred to as a "One-Shop EQA program", is designed to enable the laboratories to select and participate in relevant proficiency tests of both pathogen identification 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 2021-22 have 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 2nd EQAsia Matrix EQA trial of the EQAsia project (Matrix EQA 2022) carried out in September-November 2022. The trial included a total of five lyophilized cultures mimicking meat content, of which four were each 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 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 route 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 2022

A total of five laboratories participated in the second EQAsia Matrix EQA trial of the EQAsia project: two laboratories belonging to the HH Sector and three belonging to the AH Sector from Bangladesh, Laos People Democratic Republic, Pakistan, and Sri Lanka (**Figure 1**).

2.2 Samples preparation

Laboratories that registered for the Matrix EQA trial received five 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 contained some background flora.

Spiking of the meat samples

To prepare the five lyophilized cultures mimicking meat content, five 25 g pork minced meat portions from the same batch as in the pre-testing were used. Four of the portions were each spiked with an *E. coli* isolate, whereas the fifth portion was not spiked and, therefore, expected to be negative.

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.

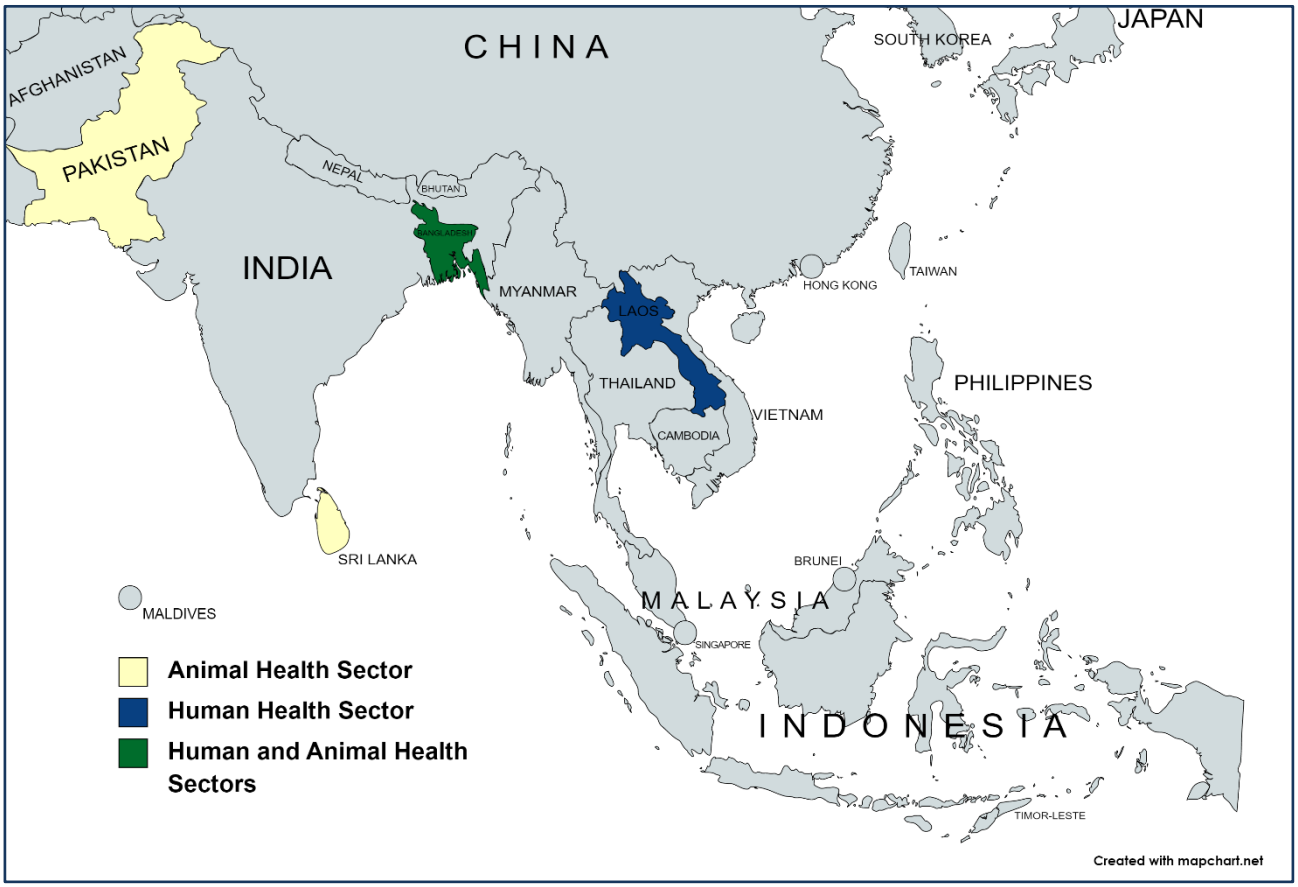

Figure 1: Countries participating in the 2nd EQAsia Matrix EQA 2022. Color indicates sector affiliation of the participating laboratory as Animal Health laboratory (yellow), Human Health laboratory (blue) or both Human and Animal Health laboratories (green).

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 on **Appendix 2**. The reference strains *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were provided 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 M100-32nd 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.

2.3 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, 32nd Ed.) [4]. When not available, EUCAST clinical breakpoints (Tables v. 12.0, 2022) [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**).

Table 1. Panel of antimicrobials and respective abbreviations for AST of *E. coli* included in the EQAsia Matrix EQA 2022. 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)

Participants were encouraged to test as many as possible of the antimicrobials listed, but always considering their relevance regarding the laboratory's routine work.

2.4 Distribution

NIH and CUVET Thailand dispatched the lyophilized cultures in September 2022 to the HH and AH laboratories, respectively. The shipment (UN3373, biological substances category B) was sent according to International Air Transport Association (IATA) regulations. Participating laboratories received information on how to open, revive and store these lyophilized cultures.

2.5 Procedure

Protocols and all relevant information were available at the EQAsia website [8], to allow access to all the necessary information at any time. 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 as disk diffusion, gradient test, agar dilution and broth dilution were all valid. 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.

Four of the five participating laboratories (#01, #22, #33 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). The fifth laboratory (#05) reported that the protocol was used, but the incubation conditions in the selective plating were modified to 35°C for 24 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).

Regarding selective isolation of carbapenemase-producers (**Figure 2**, middle column), laboratories #01, #22 and #33 reported that carbapenemase selective isolation was not performed, whereas laboratory #05 reported that carbapenemase selective isolation was performed, but no information regarding the plates used was given; laboratory #40 did not provide a response.

Confirmation of *E. coli* species identification (**Figure 2**, right column) was performed by all five laboratories using biochemical tests.

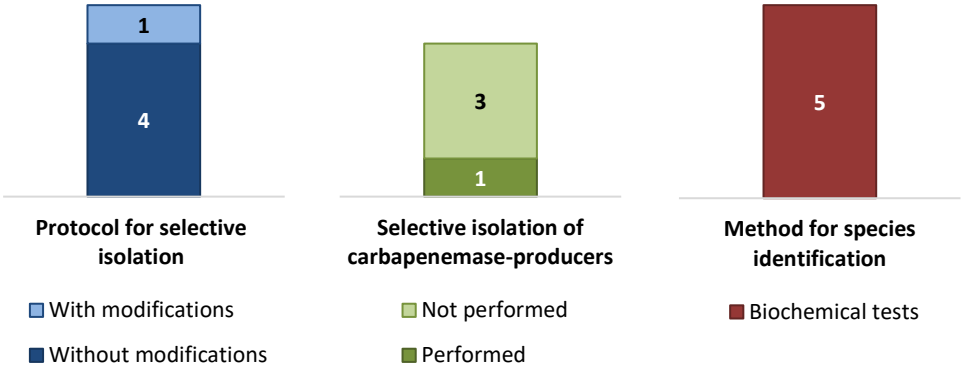

Figure 2. Methods used by the participants for selective isolation and confirmation of *E. coli* species identification.

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

Samples EQAsia 22.M1, EQAsia 22.M2, EQAsia 22.M4 and EQAsia 22.M5 were spiked with different *E. coli* isolates and, therefore, expected to be positive for growth of presumptive ESBL-, AmpC- and carbapenemase-producing *E. coli*, whereas sample EQAsia 22.M3 was kept blank and thus expected to be negative. Participants were asked to describe the growth observed in the selective plates (**Table 2**).

For samples EQAsia 22.M1, EQAsia 22.M2, EQAsia 22.M4 and EQAsia 22.M5, 100%, 80%, 60% and 60% of the laboratories, respectively, described the presence of typical *E. coli* colonies as a pure culture. For sample EQAsia 22.M3, only one of the laboratories reported the absence of typical *E. coli* colonies (**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 22.M1	EQAsia 22.M2	EQAsia 22.M3	EQAsia 22.M4	EQAsia 22.M5
Mixed culture containing typical *E. coli* colonies	--	--	1/5 (20%)	--	--
Mixed culture without typical *E. coli* colonies	--	--	--	1/5 (20%)	1/5 (20%)
Pure culture of typical *E. coli* colonies	**5/5 (100%)**	**4/5 (80%)**	3/5 (60%)	**3/5 (60%)**	**3/5 (60%)**
Pure culture without typical *E. coli* colonies	--	--	**1/5 (20%)**	1/5 (20%)	1/5 (20%)
No growth	--	1/5 (20%)	--	--	--

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

Results confirming the species identification were reported by all five laboratories (**Table 3**):

- EQAsia 22.M1: sample was confirmed as positive by all laboratories, which have reported it as a pure culture of typical *E. coli* colonies;
- EQAsia 22.M2: sample was confirmed as positive by four of the laboratories, whereas laboratory #05 reported that no growth was observed in the selective

plates and, therefore, no results were reported for species identification;

- EQAsia 22.M3: sample was not confirmed as negative by any of the laboratories. Instead, four of the laboratories reported it as either a pure or mixed culture of typical *E. coli* colonies. Laboratory #05, which observed growth as 'Pure culture without typical *E. coli* colonies', ended up reporting the sample as positive as well.
- EQAsia 22.M4: sample was confirmed as positive by three of the laboratories (#01, #33 and #40) reporting a pure culture of typical *E. coli* colonies. Laboratories #05 and #22, which observed growth as 'Pure/Mixed culture without typical *E. coli* colonies', respectively, considered the sample negative;
- EQAsia 22.M5: sample was confirmed as positive by two of the laboratories reporting pure culture of typical *E. coli* colonies (#33 and #40), whereas the third laboratory (#05) did not submit results for species identification. Laboratory #01 reported a pure culture without typical *E. coli* colonies, but considered the sample as positive when performing species identification. Laboratory #22 observed mixed growth without typical *E. coli* colonies and reported the sample as negative.

In summary (**Table 3**), three laboratories (#01, #33 and #40) reported all samples as positive for presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli*, and one laboratory (#05) only reported species identification results for three of the samples. None of the participating laboratories correctly reported all five samples: EQAsia 22.M1, EQAsia 22.M2, EQAsia 22.M4 and EQAsia 22.M5 samples as positive and EQAsia 22.M3 sample as negative.

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'. Missing data is presented as --. Obtained results in accordance with the expected result are shown in bold.

Laboratory ID Number	EQAsia 22.M1	EQAsia 22.M2	EQAsia 22.M3	EQAsia 22.M4	EQAsia 22.M5
	Positive	Positive	Negative	Positive	Positive
#01	**Positive**	**Positive**	Positive	**Positive**	**Positive**
#05	**Positive**	--	Positive	Negative	--
#22	**Positive**	**Positive**	Positive	Negative	Negative
#33	**Positive**	**Positive**	Positive	**Positive**	**Positive**
#40	**Positive**	**Positive**	Positive	**Positive**	**Positive**

3.3 Antimicrobial Susceptibility Testing

For the 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 five laboratories submitting results, only four (#01, #22, #33 and #40) submitted results for AST. For samples EQAsia 22.M4 and EQAsia 22.M5 and respective *E. coli* isolates, only results from three laboratories were available, since laboratory #22 considered the samples as negative.

The participants were invited to report Inhibition Zone Diameters/MIC values and categorisation as resistant ('R'), intermediate ('I') or susceptible ('S') for each strain/antimicrobial combination. Only the categorisation 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**). Nearly half of the antimicrobials were tested by all four laboratories. Antimicrobials such as doripenem, ertapenem, tobramycin and trimethoprim/sulfamethoxazole were tested by only one of the participating laboratories. Colistin was not tested as the methodology chosen by the laboratories cannot be used to test 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)
	Disk Diffusion
AMK	3 (75.0)
AMP	4 (100.0)
AZI	4 (100.0)
FEP	4 (100.0)
FOT	4 (100.0)
FOX	4 (100.0)
TAZ	4 (100.0)
CHL	4 (100.0)
CIP	3 (75.0)
COL	0
DOR	1 (25.0)
ETP	1 (25.0)
GEN	4 (100.0)
IMI	4 (100.0)
LEVO	3 (75.0)
MERO	4 (100.0)
NAL	4 (100.0)
PT4	2 (50.0)
SMX	2 (50.0)
TET	3 (75.0)
TGC	2 (50.0)
TOB	1 (25.0)
TMP	2 (50.0)
SXT	1 (25.0)
Total	**4**

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* isolates used to spike samples EQAsia 22.M1, EQAsia 22.M2 and EQAsia 22.M5 presented deviations below 5%, meaning that most of the susceptibility results obtained were in accordance with the expected (**Appendix 2**). In contrast, the strain used to spike sample EQAsia 22.M4 presented a deviation of 16.5%; the deviation was mostly caused by the results reported by laboratory #40, which reported antimicrobials such as cefepime, imipenem, meropenem and trimethoprim as resistant when the expected outcome was susceptible, and, on the contrary, reported chloramphenicol and gentamicin as susceptible instead of resistant (**Appendix 2**).

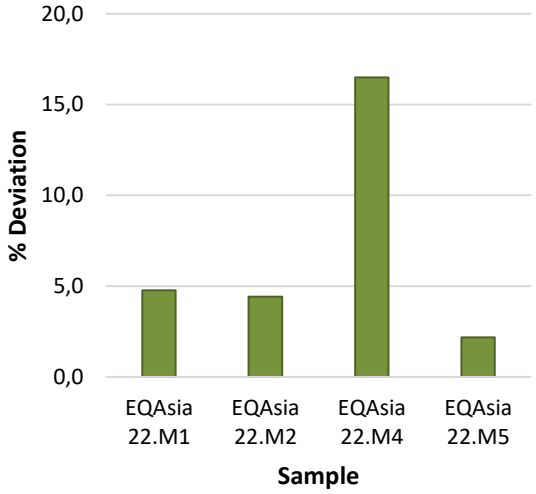

Figure 3. Percentage of deviation in the AST interpretation (R/I/S) per sample in the EQAsia Matrix EQA 2022.

Antimicrobials with highest deviations from the expected result were piperacillin/tazobactam (29.2%), azithromycin (23.2%), trimethoprim/sulfamethoxazole (18.8%), as well as sulfamethoxazole (16.7%), whereas ampicillin, cefotaxime, doripenem, ertapenem, levofloxacin, nalidixic acid, tetracycline, tigecycline and tobramycin revealed no deviation from the expected results (**Figure 4**).

Piperacillin/tazobactam was only tested by laboratories #01 (four strains) and #22 (two strains), resulting in a total of only six tests performed towards this antimicrobial. Some of the strains expected to be susceptible to the drug where reported as intermediate, leading to light

score penalties (score of 3 instead of 4) that contributed to the observed deviation.

Regarding azithromycin deviation, some of the incorrect reported results were close to the threshold for categorising the strain as susceptible or resistant (intermediate option is not available), which resulted in a heavy score penalty (score of 0 or 1 out of the possible full score 4).

Trimethoprim/sulfamethoxazole was tested by laboratory #01 only, which reported an incorrect result for sample EQAsia 22.M2 (resistant instead of susceptible).

Lastly, sulfamethoxazole deviation was only caused by an incorrect result, but that was a very major error (resistant strain reported as susceptible).

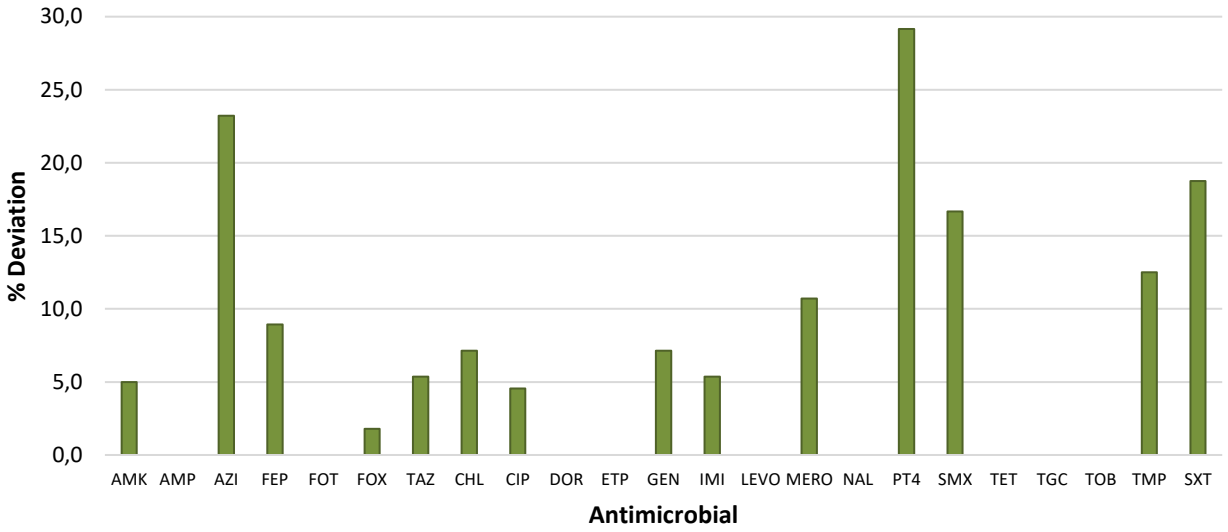

Figure 4. Percentage of deviation in the AST interpretation (R/I/S) among the *E.coli* strains used to spike the matrix samples in EQAsia Matrix EQA 2022. Results are categorized according to antimicrobial agent. Bars represent the average distribution of the deviation.

A deviation below or equal to 5% of laboratory performance in terms of interpretation of the results (R/I/S) was observed for laboratories #22 (results from two strains assessed) and #33 (results from all four strains) (**Figure 5**). In average, the deviation was 5.8% (ranging from 0.5 to 12.9%).

Laboratory #40 presented several deviations from the expected results, in particular for the strain used to spike sample EQAsia 22.M4 as mentioned above.

Laboratory #01' deviations were mostly caused by minor errors and a few major errors for antimicrobials such as azithromycin, meropenem, piperacillin/tazobactam and trimethoprim/sulfamethoxazole.

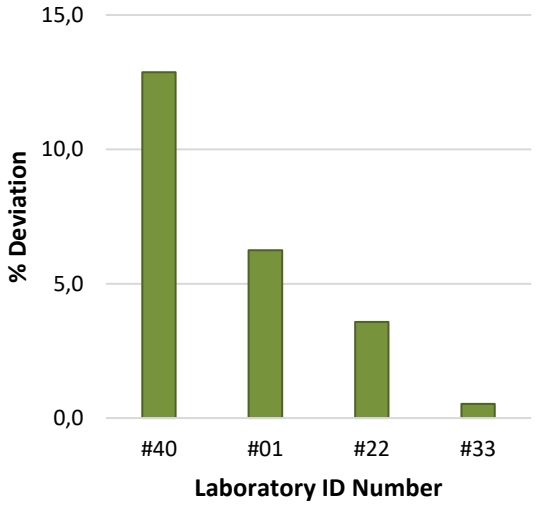

Figure 5. Percentage of deviation in the AST interpretation (R/I/S) among the *E.coli* strains used to spike the matrix samples in EQAsia Matrix EQA 2022. Results are categorized by laboratory ID number.

3.4 ESBL, AmpC and carbapenemase phenotypic testing

Four lyophilized samples mimicking meat content were included in this Matrix EQA. The sample EQAsia 22.M1 contained an *E. coli* isolate expressing ESBL phenotype (**Table 5**). All four participating laboratories correctly classified the phenotype.

The *E. coli* isolate from sample EQAsia 22.M2 expressed an ESBL + AmpC phenotype (**Table 5**). Three of the laboratories reported the correct phenotype (#01, #22 and #40), whereas laboratory #33 classified the strain as AmpC phenotype, possibly because neither cefotaxime nor ceftazidime in combination with clavulanic acid were tested and, therefore, synergy could not be observed.

Sample EQAsia 22.M4 was spiked with an *E. coli* isolate expressing AmpC phenotype (**Table 5**). Laboratory #33 reported the correct phenotype, while the remaining two laboratories (#01 and #40) reported carbapenemase phenotype. Both laboratories found the strain to be resistant to meropenem (Inhibition Zone Diameters of 7 and 0mm, respectively), which led to the submitted classification.

Lastly, the *E. coli* isolate from sample EQAsia 22.M5 expressed a carbapenemase phenotype (**Table 5**), which was correctly identified by the three laboratories submitting results (#01, #33 and #40).

In summary, laboratories #01, #33 and #40 all presented one incorrect classification of the different ESBL / AmpC / carbapenemase phenotypes among the four *E. coli* strains, and laboratory #22 correctly identified the phenotype for the two 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 22.M1	EQAsia 22.M2	EQAsia 22.M4	EQAsia 22.M5
Expected results		ESBL	ESBL + AmpC	AmpC	Carbapenemase
Obtained results (n/N)	ESBL	**4/4 (100.0%)**	--	--	--
	AmpC	--	1/4 (25.0%)	**1/3 (33.3%)**	--
	ESBL + AmpC	--	**3/4 (75.0%)**	--	--
	Carbapenemase	--	--	2/3 (66.7%)	**3/3 (100.0%)**
	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 this trial or 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 four participating laboratories, which applied the disk diffusion method and reported Inhibition Zone Diameters. As colistin cannot 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.

Test results outside of the expected range were only observed for ampicillin (1 out of 4), levofloxacin (1 out of 3) and sulfamethoxazole (1 out of 2) (**Table 6**).

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	0/3
AMP	1/4
FEP	0/4
FOT	0/4
FOX	0/4
TAZ	0/4
CHL	0/4
CIP	0/3
DOR	0/1
ETP	0/1
GEN	0/4
IMI	0/4
LEVO	1/3
MERO	0/4
NAL	0/4
PT4	0/2
SMX	1/2
TET	0/3
TGC	0/2
TOB	0/1
TMP	0/2
SXT	0/1

Disk Diffusion – Inhibition Zone Diameter determination by Disk Diffusion

These incorrect results resulted in the laboratories' deviation summarized in **Figure 6**. Laboratories #01 and #33 presented no deviation, whereas laboratory #22 reported Inhibition Zone Diameters for ampicillin and levofloxacin slightly below (2 and 1 mm, respectively) the acceptance interval, and laboratory #40 reported results for sulfamethoxazole above (6 mm) the expected range.

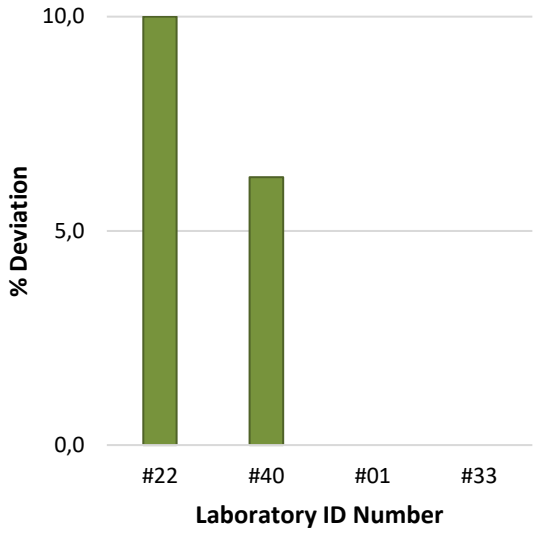

Figure 6. Percentage of deviation in the AST of the quality control strain *E. coli* ATCC 25922 by the laboratories.

4. Discussion

A total of five laboratories from both the HH and AH Sectors participated in the 2nd 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 in the official EURL-AR protocols [3]. None of the participating laboratories performed carbapenemase selective isolation using specific selective plates. Bacterial identification was achieved by the use of biochemical tests.

Samples EQAsia 22.M1, EQAsia 22.M2, EQAsia 22.M4 and EQAsia 22.M5 were expected to be positive for growth of presumptive ESBL-, AmpC- or carbapenemase-producing *E. coli*, whereas sample EQAsia 22.M3 was expected to be negative. Regarding the positive samples, at least 60% of the laboratories correctly identified the samples as positive for the presence of *E. coli* colonies. Sample EQAsia 22.M3 seemed to cause more problems as several laboratories (80%) reported the sample as positive instead of negative. In fact, none of the laboratories correctly identified all the samples as positive/negative, 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 five laboratories submitting results, only four 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 22.M4 presented the highest deviation from the expected results in comparison to the other three isolates. One possible explanation could be the higher level of resistance of the strains from samples EQAsia 22.M1, EQAsia 22.M2 and EQAsia 22.M5 towards cefotaxime (MIC of 32, 64 and > 64, respectively) in comparison to the strain from sample EQAsia 22.M4 (MIC = 8, **Appendix 2**), which assured their growth on the selective MacConkey agar plate containing 1 mg/L cefotaxime. This observation could suggest that the isolate used to spike sample EQAsia 22.M4 may have been overgrown by contaminating bacteria and more challenging to isolate, and that the incorrect submitted results are due to the isolation and testing of other bacteria other than the one used to spike the sample. Nevertheless, the possibility of performance issues cannot be discarded.

The several incorrect results observed for sample EQAsia 22.M4 were the main contributor for the highest deviation observed in terms of laboratories' AST performance, which presented an average deviation of 5.8% (ranging from 0.5 to 12.9%).

A few incorrect results were reported by the laboratories for the classification of the *E. coli* phenotypes into ESBL, AmpC or carbapenemase production. These seem to have been caused by the incorrect results obtained for relevant antimicrobials, rather than by incorrect classification. For instance, two laboratories 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 innapropriate antibiotic treatment.

Lastly, AST of the quality control strains presented only a few results outside the quality control range, which the majority were just slightly (1-2mm) below the acceptance interval. This can be considered as a quite successful outcome.

5. Conclusion

This report presented the results of the second EQAsia Matrix EQA trial 2022. 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 five laboratories participated in the trial, even though eight laboratories initially signed-up. The reason for not participating was mainly the lack of essential resources, such as selective media/plates. Secondly, not all of the participating laboratories submitted results for all the components (only four 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 insterest 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*. 32nd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.

[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 12.0, 2022. http://www.eucast.org.

[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 2022 Protocol

Protocol for EQAsia Matrix EQA 2022

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 2021-22. 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°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 (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 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' 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.

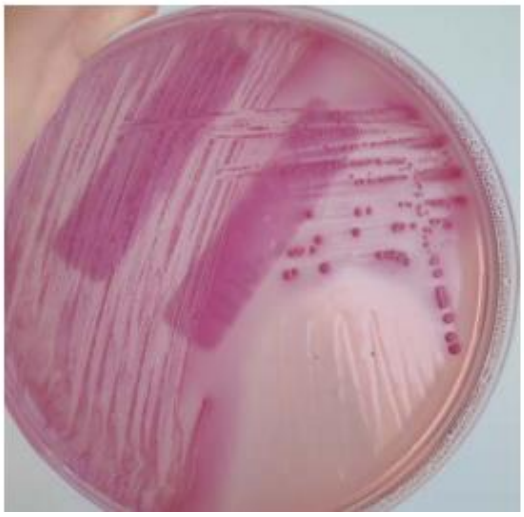

Figure 1: Typical appearance of *E. coli* on MacConkey agar supplemented with 1 mg/L cefotaxime.

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 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 MIC (µg/mL)			Reference values 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 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 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.

--- --- ---

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 22.M1	≤ 4	S	> 32	R	64	R	4	I	32	R	≤ 0.06/4	2	S	1	S	≤ 0.12/4
EQAsia 22.M2	≤ 4	S	> 32	R	16	S	16	R	64	R	2/4	32	R	16	R	8/4
EQAsia 22.M4	≤ 4	S	> 32	R	16	S	0.25	S	8	R	8/4	64	R	16	R	4/4
EQAsia 22.M5	> 128	R	> 32	R	> 64	R	> 32	R	> 64	R	> 64/4	> 64	R	> 128	R	> 128/4

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 22.M1	> 64	R	0.12	S	≤ 0.25	I	≤ 0.12	S	≤ 0.015	S	≤ 0.5	S	≤ 0.12	S	≤ 0.5	S	≤ 0.03	S
EQAsia 22.M2	> 64	R	> 8	R	≤ 0.25	I	≤ 0.12	S	0.06	S	≤ 0.5	S	≤ 0.12	S	> 8	R	≤ 0.03	S
EQAsia 22.M4	> 64	R	2	R	≤ 0.25	I	≤ 0.12	S	0.03	S	> 16	R	≤ 0.12	S	2	R	≤ 0.03	S
EQAsia 22.M5	≤ 8	S	> 8	R	≤ 0.25	I	> 2	R	> 4	R	> 16	R	> 16	R	> 8	R	> 16	R

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 22.M1	≤ 4	S	≤ 8/4	S	> 512	R	> 32	R	≤ 0.25	S	≤ 1	S	> 16	R	> 4/76	R
EQAsia 22.M2	> 64	R	≤ 8/4	S	16	S	> 32	R	≤ 0.25	S	≤ 1	S	> 16	R	≤ 0.5/9.5	S
EQAsia 22.M4	> 64	R	≤ 8/4	S	> 512	R	> 32	R	≤ 0.25	S	> 8	R	0.5	S	≤ 0.5/9.5	S
EQAsia 22.M5	> 64	R	> 64/4	R	> 512	R	> 32	R	≤ 0.25	S	> 8	R	> 16	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 32nd 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."

National Food Institute
Technical University of Denmark
Building 202
Kemitorvet
2800 Lyngby
Denmark

Tel: +45 35 88 70 00

www.food.dtu.dk

ISBN: 978-87-7586-015-9