EQAsia EQA7 trial

Protocol

Identification and antimicrobial susceptibility testing (AST) of *Salmonella spp., Enterococcus spp., Campylobacter spp.* and *Neisseria gonorrhoeae* test strains

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 trials are organized by the consortium of EQAsia and supported by the Fleming Fund.

The **EQAsia EQA7 trial** includes four EQA panels each composed of seven test strains – *Salmonella spp.*, *Enterococcus spp. (Enterococcus faecalis* and *Enterococcus faecium), Campylobacter spp. (Campylobacter coli* and *Campylobacter jejuni),* and *Neisseria gonorrhoeae*, respectively. Each of the four panels includes five strains of the targeted species and two non-target strains. Participating laboratories are asked to perform identification of all seven test strains from the panels they signed up for, as well as antimicrobial susceptibility testing (AST) only on the five target strains in each panel.

Additionally, AST of the relevant reference strains for quality control (QC) is also part of each EQA trial round. The QC reference strains supplied during previous EQA rounds are *Escherichia coli* ATCC 25922/CCM 3954 (for disk diffusion of Salmonella strains), *E. coli* NCTC 13846/CCM 8874 (for testing colistin), *Campylobacter jejuni* ATCC 33560/ CCM 6214, *Staphylococcus aureus* ATCC 25923/ CCM 3953 (for disk diffusion of the Enterococci), *Enterococcus faecalis* ATCC 29212/ CCM 4224 (for MIC).

The QC strains provided within EQA7 include *Neisseria gonorrhoeae* ATCC49226, WHO G, WHO L, WHO O and WHO P and will be sent along with the *N. gonorrhoea*e test strains to all the laboratories that requested to participate in this panel.

All of the reference strains are original CERTIFIED cultures provided free of charge and should be used for future internal quality control for antimicrobial susceptibility testing in your laboratory. Therefore, please take proper care of these strains.

2 OBJECTIVES

The main objective of this EQA is to support laboratories to assess and, if necessary, improve the identification and antimicrobial susceptibility testing of pathogens, specifically *Salmonella spp.*, *Enterococcus spp. (Enterococcus faecalis* and *Enterococcus faecium), Campylobacter spp. (Campylobacter coli* and *Campylobacter jejuni),* and *Neisseria gonorrhoeae*. Therefore, the laboratory work for this EQA should be performed using the methods routinely used in your own laboratory.

3 EQA7 OUTLINE

3.1 Shipping and receipt of strains

Your laboratory is one of the 37 human health and animal health laboratories from South and Southeast Asia participating in EQA7. In October 2023, you are expected to receive a parcel containing one or more of the following panels:

- ***Salmonella* panel** - seven test strains of which five are *Salmonella spp.* and two are non-target species. The *Escherichia coli* ATCC 25922/CCM 3954 and *E. coli* NCTC 13846/CCM 8874 (for colistin) reference strains have been provided in previous EQA rounds.

- ***Enterococcus* panel** - seven test strains of which five are *E. faecium* or *E. faecalis* and two are non-target species. The *Staphylococcus aureus* ATCC 25923/CCM 3953 (for disk diffusion) and *Enterococcus faecalis* ATCC 29212/ CCM 4224 (for MIC) reference strains have been provided in previous EQA rounds.

- ***Campylobacter* panel** - seven test strains of which five are *C. coli* or *C. jejuni* and two are non-target species. The *Campylobacter jejuni* ATCC 33560/ CCM 6214 reference strain has been provided in a previous EQA round.

- ***Neisseria gonorrhoeae* panel** - seven test strains of which five are *N. gonorrhoeae* and two are non-target species. The *Neisseria gonorrhoeae* ATCC49226, WHO G, WHO L, WHO O and WHO P reference strains are provided within this EQA round.

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

N.B.!!! The Enterococcus, Campylobacter and *N. gonorrhoeae* panel strains are shipped lyophilized. The Salmonella strains are shipped on media in transport tubes (swabs).

3.2 Reviving and storing the strains

The **lyophilized strains** must be stored in a dark, cool place. The strains must be sub-cultured and prepared for storage in your strain collection (e.g., in a -80°C freezer). The stored test strains 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 also serve as reference material available at a later stage, when needed.

- **Reviving Enterococcus and Campylobacter lyophilised cultures**

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:

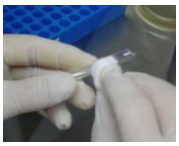
- An ampoule cutter or a file
- Sterile Luria Bertani (LB) broth
- LB agar plates (5 to 6 plates per one strain)
- Columbia broth for Campylobacter
- mCCDA agar plates (5 to 6 plates per one strain) for Campylobacter
- Autopipette with tips or Pasture pipettes
- Inoculating loop

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.

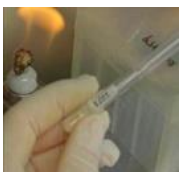
   Note: The ampoule can be cut in the middle or below the cotton wool plug.

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. Pipette 0.5 ml of sterile LB or Columbia broth into the dried cells. Mix gently and carefully to avoid creating aerosols.



6. Transfer one drop of each strain onto one LB agar plate for enterococci mCCDA agar plate for Campylobacter using autopipette or Pasteur pipette. Then, streak the isolate using inoculating loop to get single colonies on plate. The remaining suspension is stored in a screw cap test tube.

7. For enterococci, incubate the inoculated plates and the suspension tubes at 37^0C overnight and observe the bacterial growth. For Campylobacter, incubate the plates and the suspension tubes at 42^0C, 48 hours.

- **Reviving *N. gonorrhoeae* lyophilised cultures**

Needed material:

- Sterile nutrient broth (i.e. Tryptic Soy Broth)
- Sterile needles and syringes
- Chocolate agar plates
- Inoculating loop

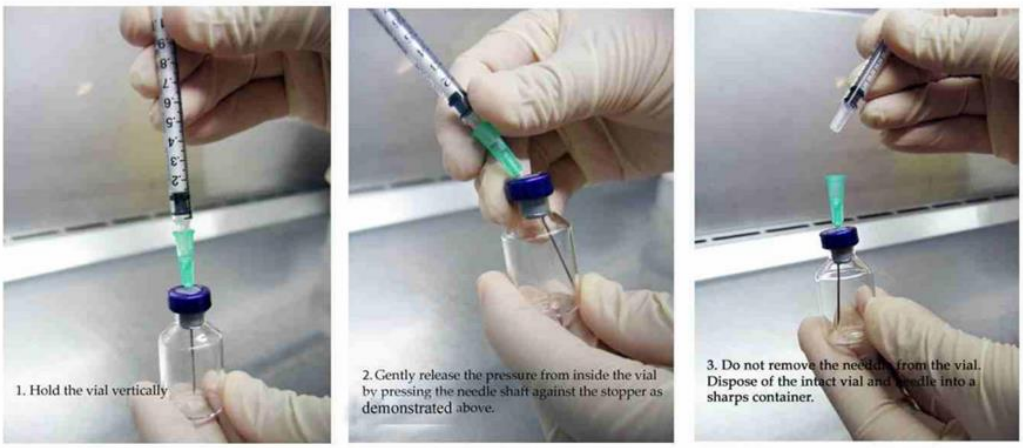
The lyophilized (freeze-dried) specimens with which you are provided must be rehydrated. When reconstituting them, exercise extreme caution not to create aerosols or spills which could cause infection. Please follow standard safety procedures and exercise all the usual precautions when dealing with this material. It is recommended that freeze dried specimens be stored out of direct light and refrigerated until the reconstitution process commences.

Do not mouth pipette and do not reconstitute the specimens until you are ready to plate them out.

1. Do **not** remove the whole cap - lift only the pre-cut section.
2. Sterilize the rubber stopper with a disinfectant swab as for inoculating a blood culture.
3. Add 1 ml of sterile Tryptic Soy Broth (or suitable substitute) to the vial with a needle and syringe.
4. Gently swirl the vial; allow 5 - 10 minutes for the dry material to rehydrate completely.
5. Gently release pressure inside the vial by pressing the needle shaft against the stopper.
6. Transfer an aliquot of the reconstituted specimen to the appropriate culture media using the syringe only.

   DO NOT REMOVE THE NEEDLE FROM THE VIAL. DISPOSE OF THE INTACT VIAL AND NEEDLE INTO A SHARPS CONTAINER

7. Hold the vial vertically.
8. Gently release the pressure from inside the vial by pressing the needle shaft against the stopper.
9. Draw the fluid up into the needle slowly.
10. Separate the needle tip from the syringe carefully.
11. Dispose of the intact vial and needle into a sharps container.
12. Plate one drop on a chocolate agar plate and spread.
13. Incubate for 16–18 hours at 36 ± 1°C in a 5 ± 1% CO_2-enriched humid atmosphere.

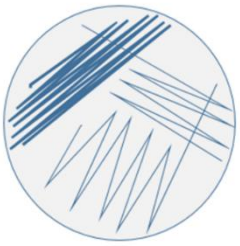


- **Reviving Salmonella isolates**

The **transport media swabs** must be stored in a dark place at 5°C to 25°C until microbiological analysis. We suggest that you subculture and process the strains within 48 hours from receipt of the parcel. Subculture the test strains onto non-selective media, e.g., a nutrient agar plate or blood agar plate, as illustrated below:

1. Inoculate it on one side of the agar plate using the swab to apply material gently and densely.

2. Turn the plate and use a sterile loop to streak once through the area first inoculated and allow further streaks to separate the culture aiming to obtain single colonies.



3. Turn the plate and use a sterile loop to streak once through the second area inoculated and allow further streaks to separate the culture aiming to obtain single colonies.

All provided strains are considered 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.

3.3 Identification of *Salmonella spp., Enterococci, Campylobacter spp.* and *Neisseria gonorrhoeae* test strains

Each of the four panels in this EQA round contains five target species. i.e. five *Neisseria gonorrhoeae* isolates in the *N. gonorrhoeae* panel. The remaining two isolates in each panel are non-target species – their identification is different from the five target species.

Please follow the routinely used methods in your own laboratory for **identification** of all panel strains.

3.4 Serotyping of *Salmonella spp*.

The five identified *Salmonella* strains should be serotyped by using the method routinely used in your own laboratory. In addition, serogroup results will be evaluated. Therefore, if you do not have all the necessary antisera for serotyping, please go as far as you can in the identification and report the serogroup. Serogroups should be reported using terms according to Kauffmann-White-Le Minor (Grimont and Weill, 2007. 9th ed. Antigenic formulae of the *Salmonella* serovars. WHO Collaborating Centre for Reference and Research on *Salmonella*).

3.5 Antimicrobial susceptibility testing of *Salmonella spp., Enterococci, Campylobacter spp.* and *Neisseria gonorrhoeae* test strains, and of the reference strains

The strains identified as *Salmonella spp., Enterococcus faecium, Enterococcus faecalis, Campylobacter coli, Campylobacter jejuni* and *Neisseria gonorrhoeae* (five isolates from each panel), as well as the appropriate reference strains, should be tested for susceptibility towards as many antimicrobials as possible indicated in the test form and in **Tables 1-4**. 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 range 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.). When not available, EUCAST clinical breakpoints (Tables v. 13.1, 2023) or epidemiological cut off values (https://mic.eucast.org/) were used instead. The breakpoint values for *Salmonella spp., Enterococci, Campylobacter spp.* and *Neisseria gonorrhoeae* can be found in **Tables 1-4**, respectively. **Please make sure to use the correct table for the interpretation**.

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

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

Antimicrobials	Reference value MIC (µg/mL)			Reference value Disk diffusion (mm)		
	S	I	R	S	I	R
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
Cefoxitin, FOX	≤ 8	16	≥ 32	≥ 18	15-17	≤ 14
Ceftazidime, TAZ	≤ 4	8	≥ 16	≥ 21	18-20	≤ 17
Chloramphenicol, CHL	≤ 8	16	≥ 32	≥ 18	13-17	≤ 12
Ciprofloxacin, CIP	≤ 0.06	0.12-0.5	≥ 1	≥ 31	21-30	≤ 20
Colistin, COL	-	≤ 2	≥ 4	NA	NA	NA
Ertapenem, ETP	≤ 0.5	1	≥ 2	≥ 22	19-21	≤ 18
Imipenem, IMI	≤ 1	2	≥ 4	≥ 23	20-22	≤ 19
Meropenem, MERO	≤ 1	2	≥ 4	≥ 23	20-22	≤ 19
Sulfamethoxazole, SMX	≤ 256	-	≥ 512	≥ 17	13-16	≤ 12
Tetracycline, TET	≤ 4	8	≥ 16	≥ 15	12-14	≤ 11
Trimethoprim, TMP	≤ 8	-	≥ 16	≥ 16	11-15	≤ 10

Reference values are based on Enterobacterales breakpoints from CLSI M100, 33rd Ed.

*****Aminoglycosides may appear active in vitro for *Salmonella spp.* but are not clinically effective and should not be reported as susceptible. They are not required to be reported for this EQA panel.

Table 2. Breakpoints for interpretation of MICs and zone diameters for *E. faecium / E. faecalis*

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

Antimicrobials		Reference value MIC (µg/mL)			Reference value Disk diffusion (mm)		
		S	I	R	S	I	R
Ampicillin, AMP		≤ 8	-	≥ 16	≥ 17	-	≤ 16
Chloramphenicol, CHL		≤ 8	16	≥ 32	≥ 18	13-17	≤ 12
Ciprofloxacin, CIP		≤ 1	2	≥ 4	≥ 21	16-20	≤ 15
Daptomycin, DAP	*E. faecium*	-	-	≥ 8	NA	NA	NA
	E. faecalis	≤ 2	4	≥ 8	NA	NA	NA
Erythromycin, ERY		≤ 0.5	1-4	≥ 8	≥ 23	14-22	≤ 13
Gentamicin, GEN*		≤ 128	-	≥ 256	≥ 8	-	≤ 7
Linezolid, LZD		≤ 2	4	≥ 8	≥ 23	21-22	≤ 20
Quinupristin/dalfopristin, SYN		≤ 1	2	≥ 4	≥ 19	16-18	≤ 15
Teicoplanin, TEI		≤ 8	16	≥ 32	≥ 14	11-13	≤ 10
Tetracycline, TET		≤ 4	8	≥ 16	≥ 19	15-18	≤ 14
Tigecycline, TGC*	*E. faecium*	≤ 0.25	-	≥ 0.5	≥ 22	-	≤ 21
	E. faecalis	≤ 0.25	-	≥ 0.5	≥ 20	-	≤ 19
Vancomycin, VAN		≤ 4	8-16	≥ 32	≥ 17	15-16	≤ 14

Reference values are based on *Enterococcus spp.* breakpoints from CLSI M100, 33rd Ed.
*Reference values are based on *Enterococcus* spp. 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."

Table 3. Breakpoints for interpretation of MICs and zone diameters for *C. jejuni / C. coli*

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

Antimicrobials	Reference value MIC (µg/mL)			Reference value Disk diffusion (mm)		
	S	**I**	**R**	**S**	**I**	**R**
Chloramphenicol, CHL*	≤ 16	-	≥ 32	NA	NA	NA
Ciprofloxacin, CIP	≤ 1	2	≥ 4	≥ 24	21-23	≤ 20
Ertapenem, ETP**	≤ 0.5	-	≥ 1	NA	NA	NA
Erythromycin, ERY	≤ 8	16	≥ 32	≥ 16	13-15	≤ 12
Gentamicin, GEN*	≤ 2	-	≥ 4	≥ 21	-	≤ 20
Tetracycline, TET	≤ 4	8	≥ 16	≥ 26	23-25	≤ 22

Reference values are based on *Campylobacter jejuni/coli* breakpoints from CLSI M45, 3rd Ed.
*Reference values are based on *C. jejuni* and *C. coli* epidemiological cut off values from https://mic.eucast.org/ in August 2023.
**Reference values are based on EFSA (European Food Safety Authority) recommendation.

Table 4. Breakpoints for interpretation of MICs and zone diameters for *N. gonorrhoeae*

Antimicrobials	Reference value MIC (µg/mL)			Reference value Disk diffusion (mm)		
	S	I	R	S	I	R
Azithromycin, AZI	≤ 1	-	-	≥ 30	-	-
Cefixime, CFM	≤ 0.25	-	-	≥ 30	-	-
Ceftriaxone, CRO	≤ 0.25	-	-	≥ 35	-	-
Ciprofloxacin, CIP	≤ 0.06	0.12-0.5	≥ 1	≥ 41	28-40	≤ 27
Penicillin, PEN	≤ 0.06	0.12-1	≥ 2	≥ 47	27-46	≤ 26
Tetracycline, TET	≤ 0.25	0.5-1	≥ 2	≥ 38	31-37	≤ 30

Reference values are based on *N. gonorrhoeae* breakpoints from CLSI M100, 33rd Ed.

N.B. For the interpretation of the AST results for *N. gonorrhoeae* quality control strains provided with this EQA panel (ATCC49226, WHO G, WHO L, WHO O and WHO P) please refer to Table 4B and 5C (Disk diffusion and MIC QC ranges for ATC49226) in CLSI M100, 33rd Ed, as well as Table 1 in the publication by Unemo M et al.. The novel 2016 WHO *Neisseria gonorrhoeae* reference strains for global quality assurance of laboratory investigations: phenotypic, genetic and reference genome characterization. *J Antimicrob Chemother*. 2016 Nov;71(11):3096-3108. doi: 10.1093/jac/dkw288. PMID: 27432602; PMCID: PMC5079299.

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.

N.B. For all susceptibility testing results for which there are no breakpoints identified, please enter the susceptibility category that you interpret, i.e. if a *N. gonorrhoeae* isolate has an MIC > 1 µg/mL or zone inhibition diameter < 30mm for azithromycin, interpret either as resistant (R) or decreased susceptibility (DS).

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!