PROTOCOL

For antimicrobial susceptibility testing of *Escherichia coli*, *Salmonella*, *Campylobacter* and *Staphylococcus*

1 INTRODUCTION

The organisation and implementation of an External Quality Assurance System (EQAS) on antimicrobial susceptibility testing (AST) of *Escherichia coli*, *Salmonella*, *Campylobacter* and *Staphylococcus* is among the tasks of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). The current EQAS 2022 will include AST of eight *E. coli*, *Salmonella*, *Campylobacter and Staphylococcus* strains and AST of reference strains *E. coli* ATCC 25922 (CCM 3954), *Campylobacter jejuni* ATCC 33560 (CCM 6214) and *Staphylococcus. aureus* ATCC 29213 (CCM 4223) together with AST of the internal EURL reference strain, *Acinetobacter baumannii* (2012-70-100-69).

The reference strains are included in the parcel only for new participants of the EQAS who did not receive them previously. The ATCC 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. The EURL-AR QC-strains are provided for the purpose of additional QC

of the broth microdilution plates. The reference strains will not be included in the years to come and we therefore encourage you to take proper care of these strains for example by handling and maintaining them as suggested in the manual 'Subculture and Maintenance of Quality Control Strains' available on the EURL-AR website (see https://www.eurl-ar.eu/eqas.aspx).

Various aspects of the proficiency test scheme may from time to time be subcontracted. When subcontracting occurs it is placed with a competent subcontractor and the National Food Institute is responsible to the scheme participants for the subcontractor's work.

2 OBJECTIVES

This EQAS aims to support laboratories to assess and, if necessary, to improve the quality of results obtained by AST of pathogens of food- and animal-origin, with special regard to *E. coli*, *Salmonella*, *Campylobacter* and *Staphylococcus*. Further objectives are to evaluate and improve the comparability of surveillance data on antimicrobial susceptibility of *E. coli*, *Salmonella*, *Campylobacter* and *Staphylococcus* reported to EFSA by different laboratories.

3 OUTLINE OF THE EC/SALM/CAMP/ENT EQAS 2022

3.1 Shipping, receipt and storage of strains

In September 2022, the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) will receive a parcel containing eight *E. coli*, *Salmonella*, *Campylobacter* and staphylococci strains, respectively from the National Food Institute (see Table 1). For participants who did not receive them previously, this parcel will also contain reference strains.

Table 1: Codes for the test strains included in the current EQAS

E. coli	***Salmonella***	***Campylobacter***	***Staphylococcus***
2022 EC-17.1	2022 S-17.1	2022 C-17.1	2022 ST-17.1
2022 EC-17.2	2022 S-17.2	2022 C-17.2	2022 ST-17.2
2022 EC-17.3	2022 S-17.3	2022 C-17.3	2022 ST-17.3
2022 EC-17.4	2022 S-17.4	2022 C-17.4	2022 ST-17.4
2022 EC-17.5	2022 S-17.5	2022 C-17.5	2022 ST-17.5
2022 EC-17.6	2022 S-17.6	2022 C-17.6	2022 ST-17.6
2022 EC-17.7	2022 S-17.7	2022 C-17.7	2022 ST-17.7
2022 EC-17.8	2022 S-17.8	2022 C-17.8	2022 ST-17.8

All strains belong to UN3373, Biological substance, category B. Extended spectrum beta-lactamase (ESBL)-producing strains as well as carbapenemase producing strains are included in the selected material. It is the recipients' responsibility to comply with national legislation, rules and regulation regarding the correct use and handling of the provided strains and to possess the proper equipment and protocols to handle these strains.

All test strains will be shipped as swabs of pure cultures in transport media and new laboratories to the network will receive lyophilised ATCC reference strains. Upon arrival to your laboratory, store the strains in a dark place at 5°C to 25°C until microbiological analysis. The *Campylobacter* test strains must be subcultured immediately upon arrival. A suggested procedure for reconstitution of the lyophilised reference strains is presented below.

3.2 QC reference strains

Include the ATCC reference strains as well as the internal EURL reference strain for the MIC testing and report results of these together with the isolates obtained from the EQAS samples. I.e. for the *E. coli* and *Salmonella* testing, include *E. coli* ATCC 25922 (CCM 3954) together with *Acinetobacter baumannii* (2012-70-100-69).

Note that, for the testing of the *E. coli* ATCC25922 reference strain, the two compounds, sulfamethoxazole and sulfisoxazole, are regarded as comparable, i.e. the obtained MIC-value from the testing of sulfamethoxazole will be evaluated against the acceptance range listed in CLSI M100 for sulfisoxazole.

For a suggested procedure for reconstitution of the lyophilised cultures, please refer to the document 'Instructions for opening and reviving lyophilised cultures' on the EURL-AR-website (see https://www.eurl-ar.eu/eqas.aspx).

3.3 Antimicrobial susceptibility testing

Participants should perform minimum inhibitory concentration (MIC) determination using the methods stated in the Commission Implementing Decision 2020/1729/EU (international reference method ISO standard 20776-1:2019). **Results should be produced according to the laboratory's routine procedures for antimicrobial susceptibility testing by MIC determination.** For interpretation of the results, please use the cut-off values listed in Tables 2, 3, 4, 5, 6 and 7 in this document. Except where specifically indicated, these values represent the current epidemiological cut-off values developed by EUCAST (www.eucast.org), and allow categorisation of bacterial

isolates into two categories: resistant and susceptible. A categorisation as intermediate is not accepted**.**

As the current regulation and recommendations focus on broth microdilution testing only, results obtained by other methods cannot be submitted for evaluation.

Beta-lactam and carbapenem resistance

Confirmatory tests for ESBL/AmpC/carbapenemase production are mandatory on all *E.coli* and *Salmonella* test strains resistant to cefotaxime (FOT), ceftazidime (TAZ) and/or meropenem (MERO) and should be performed by testing the second panel of antimicrobials (Table 3 and Table 5 of this document corresponding to Table 5 in Commission Implementing Decision 2020/1729/EU).

Confirmatory test for AmpC-, ESBL- and carbapenemase production requires use of both cefotaxime (FOT) and ceftazidime (TAZ) alone and in combination with a β-lactamase inhibitor (clavulanic acid). Synergy is defined as i) a ≥ 3 twofold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid vs. the MIC of the agent when tested alone (MIC FOT:FOT/Cl or TAZ:TAZ/Cl ratio ≥ 8) (CLSI M100 Table 3A, Tests for ESBLs). The presence of synergy indicates ESBL production.

Confirmatory test for carbapenemase production requires the testing of meropenem (MERO).

Detection of AmpC-type beta-lactamases can be performed by testing the bacterium for susceptibility to cefoxitin (FOX). Resistance to FOX could indicate the presence of an AmpC-type beta-lactamase.

The classification of the phenotypic beta-lactam resistance results should be based on the most recent EFSA recommendations (see appendix to this protocol). Importantly: Note that for *both E. coli* and *Salmonella*, two cut-off values apply for cefotaxime and ceftazidime: the EUCAST cut-off values, those that define R/S (see Tables 2, 3, 4 and 5), and the screening cut-off values (cefotaxime >1 and ceftazidime >1) which are those applied to categorise bacterial phenotypes as ESBL, AmpC, carbapenemase, etc., based on panel 2 results (see Appendix). Likewise this is the situation for the *E.coli* meropenem cut-off values/screening cut-off value.

3.3.2 E. coli

The interpretative criteria that should be applied for categorizing the *E. coli* test strain as resistant or susceptible are those listed in Tables 2 and 3.

Table 2: Panel 1 antimicrobials recommended for AST of *E. coli* spp. and interpretative criteria ((T)ECOFFs) according to latest updates from EUCAST (17.08.2022) supplemented with ECOFFs from the EFSA Technical Report 2021, Table B.1

Antimicrobial	MIC (µg/mL) (R>)
Amikacin (AMI)	8
Ampicillin (AMP)	8
Azithromycin (AZI)	16
Cefotaxime (FOT or CTX)	0.25
Ceftazidime (TAZ or CAZ)	0.5
Chloramphenicol (CHL)	16
Ciprofloxacin (CIP)	0.064
Colistin (COL)	2
Gentamicin (GEN)	2
Meropenem (MERO or MEM)	0.06
Nalidixic acid (NAL)	8
Sulfonamides (SMX)	64*
Tetracycline (TET)	8
Tigecycline (TGC)	0.5
Trimethoprim (TMP)	2

* EFSA Technical Report (doi: 10.2903/sp.efsa.2021.EN-6652)

Table 3: Panel 2 antimicrobials recommended for AST of *E. coli* spp. resistant to cefotaxime, ceftazidime or meropenem in panel 1 antimicrobials and interpretative criteria ((T)ECOFFs) according to latest updates from EUCAST (17.08.2022)

Antimicrobial	MIC (µg/mL) (R>)
Cefepime (FEP)	0.25
Cefotaxime (FOT or CTX)	0.25
Cefotaxime + clavulanic acid (F/C or CTX/CLA)	0.25
Cefoxitin (FOX)	8
Ceftazidime (TAZ or CAZ)	0.5
Ceftazidime + clavulanic acid (T/C or CAZ/CLA)	0.5
Ertapenem (ETP)	0.03
Imipenem (IMI)	0.5
Meropenem (MERO or MEM)	0.06
Temocillin (TRM)	16

3.3.3 Salmonella

The interpretative criteria that should be applied for categorizing the *Salmonella* test strain as resistant or susceptible are those listed in Tables 4 and 5.

Table 4: Panel 1 antimicrobials recommended for AST of *Salmonella* spp. and interpretative criteria ((T)ECOFFs) according to latest updates from EUCAST (17.08.2022) supplemented with ECOFFs from the EFSA Technical Report 2021, Table B.1

Antimicrobial	MIC (µg/mL) (R>)
Amikacin (AMI)	4
Ampicillin (AMP)	4
Azithromycin (AZI)	16
Cefotaxime (FOT or CTX)	0.5
Ceftazidime (TAZ or CAZ)	2
Chloramphenicol (CHL)	16
Ciprofloxacin (CIP)	0.064
Colistin (COL)	2*
Gentamicin (GEN)	2
Meropenem (MERO or MEM)	0.125*
Nalidixic acid (NAL)	8
Sulfonamides (SMX)	256*
Tetracycline (TET)	8
Tigecycline (TGC)	0.5*
Trimethoprim (TMP)	2

* EFSA Technical Report (doi: 10.2903/sp.efsa.2021.EN-6652)

Table 5: Panel 2 antimicrobials recommended for AST of *Salmonella* spp. resistant to cefotaxime, ceftazidime or meropenem in panel 1 antimicrobials and interpretative criteria ((T)ECOFFs) according to latest updates from EUCAST (17.08.2022) supplemented with ECOFFs from the EFSA Technical Report 2021, Table B.1

Antimicrobial	MIC (µg/mL) (R>)
Cefepime (FEP)	0.25
Cefotaxime (FOT or CTX)	0.5
Cefotaxime + clavulanic acid (F/C or CTX/CLA)	0.5*
Cefoxitin (FOX)	8
Ceftazidime (TAZ or CAZ)	2
Ceftazidime + clavulanic acid (T/C or CAZ/CLA)	2*
Ertapenem (ETP)	0.06*
Imipenem (IMI)	1
Meropenem (MERO or MEM)	0.125*
Temocillin (TRM)	16*

* EFSA Technical Report (doi: 10.2903/sp.efsa.2021.EN-6652)

3.3.4 Campylobacter

The interpretative criteria to be applied for categorizing the *Campylobacter* test strain as resistant or susceptible are those listed in Table 6.

The obtained values of the *C. jejuni* QC reference strain will be evaluated according to the values listed in the CLSI document VET06, 1st ed., i.e. based on incubation at 36-37ºC for 48 hours or 42ºC for 24 hours.

Table 6: Antimicrobials recommended for AST of *Campylobacter jejuni* and *C. coli* and interpretative criteria ((T)ECOFFs) according to latest updates from EUCAST (17.08.2022) supplemented with ECOFFs from the EFSA Technical Report 2021, Table B.2

Antimicrobial	*C. jejuni*	*C. coli*
	MIC (µg/mL) (R>)	MIC (µg/mL) (R>)
Chloramphenicol (CHL)	16	16
Ciprofloxacin (CIP)	0.5	0.5
Ertapenem (ETP)	0.5*	0.5*
Erythromycin (ERY)	4	8
Gentamicin (GEN)	2	2
Tetracycline (TET)	1	2

* EFSA Technical Report (doi: 10.2903/sp.efsa.2021.EN-6652)

Identification of *Campylobacter* species

Species identification of the *Campylobacter* test strains must be performed by the NRLs using in-house methods or adopting the protocol available on the EURL-AR website under: http://eurl-ar.eu/233-protocols.htm.

3.3.6 Staphylococci

The interpretative criteria that should be applied for categorizing the Staphylococci test strain as resistant or susceptible are those listed in Table 7.

Table 7. Antimicrobials recommended for AST of *Staphylococcus aureus* and interpretive criteria ((T)ECOFFs) according to latest updates from EUCAST

Antimicrobials for *S. aureus*	MIC (µg/mL) **R is >**
Cefoxitin, FOX	4
Chloramphenicol, CHL	16
Ciprofloxacin, CIP	1
Clindamycin, CLN	0.25
Erythromycin, ERY	1
Fusidic acid, FUS	0.5
Gentamicin, GEN	2
Kanamycin, KAN	8
Linezolid, LZD	4
Mupirocin, MUP	1
Penicillin, PEN (benzylpenicillin)	0.125
Quin.-Dalf. (Synercid), SYN	1
Rifampicin, RIF	0.032
Streptomycin, STR	16
Sulfamethoxazole, SMX	128
Tetracycline, TET	1
Tiamulin (TIA)	2
Trimethoprim, TMP	2
Vancomycin, VAN	2

Identification of MRSA

Confirmation of *mec*A and/or *mecC* presence is mandatory in this EQAS and should be performed by the NRLs using in-house methods or adopting the protocol available on the EURL-AR website at www.eurl-ar.eu/233-protocols.htm. Test strains for which mecA and/or mecC have been confirmed should be reported as 'mecA/mecC positive' whereas test strains for which neither mecA nor mecC have been confirmed should be reported as 'mecA/mecC negative'.

5 REPORTING OF RESULTS AND EVALUATION

Test forms are available for recording your results before you enter them into the web tool.

We recommend reading carefully the web tool manual before submitting your results.

Results must be submitted no later than December 9th 2022.

After the deadline, when all participants have uploaded results, you will be able to login to the webtool once again to view and print an automatically generated report evaluating your results. Results in agreement with the expected interpretation are categorised as 'correct', while results deviating from the expected interpretation are categorised as 'incorrect'.

All results will be summarized in a publically available report. The data in the report will be presented with laboratory codes. A laboratory code is known to the individual laboratory, whereas the complete list of laboratories and their codes is confidential and known only to the EURL-AR and the EU Commission. All conclusions will be public.

If you have questions, please do not hesitate to contact the EQAS Coordinator:

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6 HOW TO SUBMIT RESULTS VIA THE WEBTOOL

The 'guideline for submission of results via webtool' is available for download directly from the EURL-AR website (https://www.eurl-ar.eu/eqas.aspx).

Access the webtool using this address: https://amr-eqas.dtu.dk. Please follow the guideline carefully and **remember to access the webtool via an 'incognito' website**.

When you submit your results, remember to have by your side the completed test forms.

Do not hesitate to contact us if you experience difficulties with the webtool.

Before finally submitting your input for *E. coli*, *Salmonella*, *Campylobacter* and staphylococci, respectively, please ensure that you have filled in all the relevant fields as **you can only 'finally submit' once for each organism!** 'Final submit' blocks data entry.

⇨ About login to the webtool:

When first given access to login to the webtool, your **personal** loginID and password were sent to you by email. This is relevant for two email addresses connected to each NRL-AR (the EURL-AR defined a primary and a secondary contact).
Note that:

a) If the EURL-AR has only one contact person for an NRL, this person is registered both as primary and secondary contact. Should you like to add another person as the secondary contact, please contact suska@food.dtu.dk
b) If your laboratory has two or more contact points on the EURL-AR contact list, two have been defined as the primary and secondary contact. Should you like to make changes to the primary and secondary contact or should you like more than the two persons to be able to access the webtool, please contact suska@food.dtu.dk.

All participants registered with an account in the submission webtool will receive a separate email presenting further information related to the personal username and password. The email will be sent by the time when the webtool has gone through internal quality control and has been approved for user access. The EQAS Coordinator will let all participants know when to look out for it.

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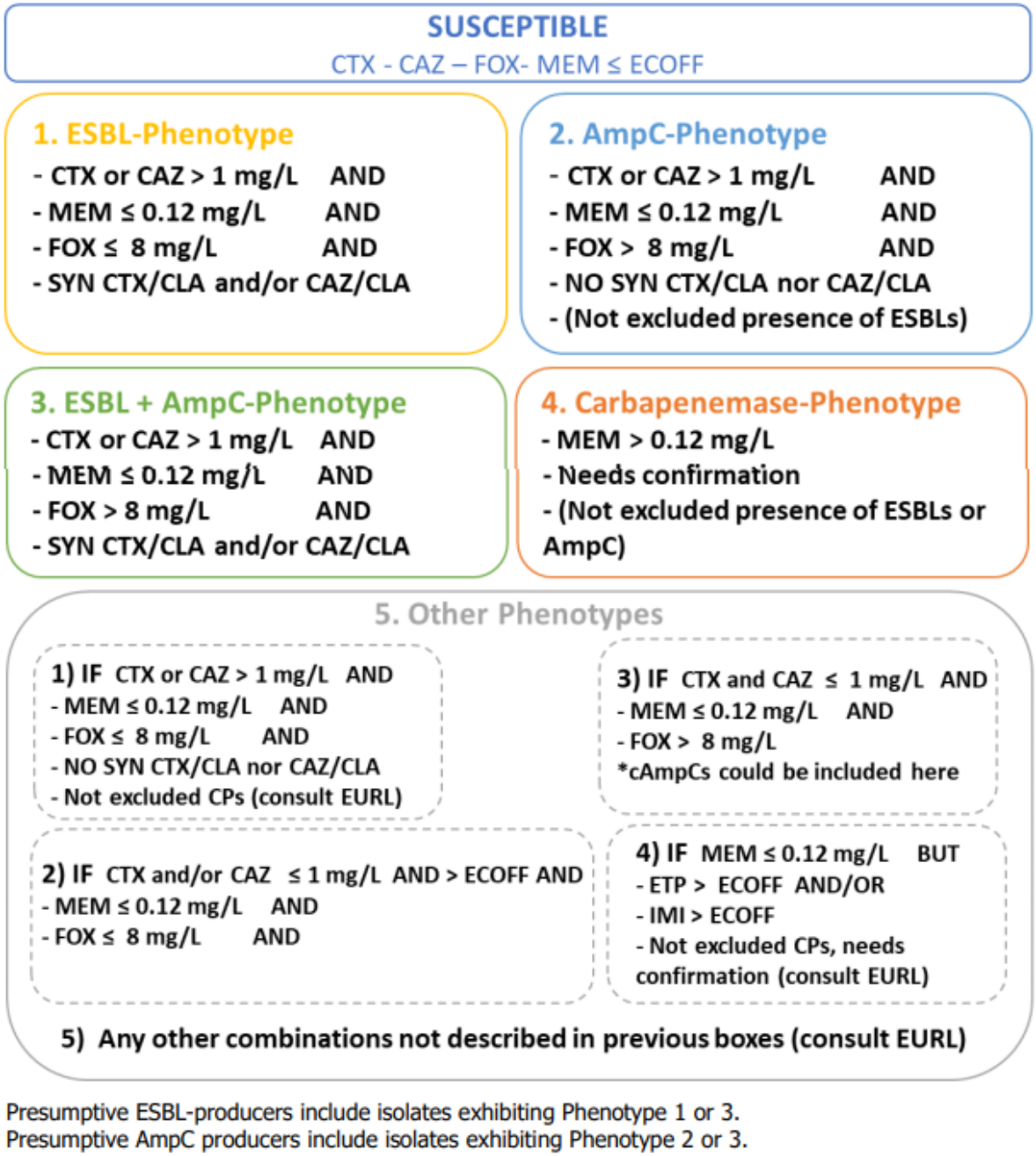
APPENDIX

Criteria for interpretation of *E. coli* and *Salmonella*, panel 2 results



Presumptive ESBL-producers include isolates exhibiting Phenotype 1 or 3.
Presumptive AmpC producers include isolates exhibiting Phenotype 2 or 3.

Please refer to: EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2022. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2019–2020. EFSA Journal 2022;20(3):7209, 197 pp. https://doi.org/10.2903/j.efsa.2022.7209, Figure F.1.