DTU Food
National Food Institute

PROTOCOL

For antimicrobial susceptibility testing of *Escherichia coli*, enterococci and staphylococci

HISTORY OF CHANGES; Protocol, version 2
In Table 3, interpretative criteria corrected.

1 INTRODUCTION

The organisation and implementation of an External Quality Assurance System (EQAS) on antimicrobial susceptibility testing (AST) of *E. coli*, enterococci and staphylococci is among the tasks of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). The EC/Ent/Staph EQAS 2018 will include AST of eight *Escherichia coli*, eight enterococci and eight staphylococci

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strains and AST of reference strains *E. coli* ATCC 25922 (CCM 3954), *E. faecalis* ATCC 29212 (CCM 4224), and *S. aureus* ATCC 29213 (CCM 4223).

The reference strains are included in the parcel only for new participants of the EQAS who did not receive them previously. 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. The reference strains will not be included in the years to come. Therefore, please take proper care of these strains. Handle and maintain them as suggested in the manual 'Subculture and Maintenance of QC Strains' available on the EURL-AR website (see www.eurl-ar.eu).

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 for AST of pathogens of food- and animal-origin, with special regard to *E. coli*, enterococci and staphylococci. Further objectives are to evaluate and improve the comparability of surveillance data on antimicrobial susceptibility of *E. coli*, enterococci and staphylococci reported to EFSA by different laboratories.

3 OUTLINE OF THE EC/ENT/STAPH EQAS 2018

3.1 Shipping, receipt and storage of strains

In June 2018, the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) will receive a parcel containing eight *E. coli*, eight enterococci and eight staphylococci strains from the DTU National Food Institute. This parcel will also contain reference strains, but only for participants who did not receive them previously.

All strains belong to UN3373, Biological substance, category B. Extended-spectrum beta-lactamase (ESBL)-producing strains as well as carbapenemase-producing strains and methicillin-resistant *Staphylococcus aureus* (MRSA) will be 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. The reference strains are shipped lyophilised, while the test strains are stab cultures. On arrival, the stab cultures must be subcultured, and all cultures should be adequately stored until testing. A suggested procedure for reconstitution of the lyophilised reference strains is presented below.

3.2 Suggested procedure for reconstitution of the lyophilised reference strains

Please refer to the document 'Instructions for opening and reviving lyophilised cultures' reported on the EURL-AR-website (see www.eurl-ar.eu).

3.3 Antimicrobial susceptibility testing

Participants should perform minimum inhibitory concentration (MIC) determination using the methods stated in the Commission Implementing Decision 2013/652/EU (international reference method (ISO standard 20776-1:2006)). For staphylococci, MIC methods should be used as well, according to the EFSA recommendations and the antimicrobials to test are those stated under the EFSA technical specifications (see Table 3). For interpretation of the results, please use the cut-off values listed in Tables 1, 2, 3 and 4 in this document. These values (except where indicated) 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**.**

Participants will not be allowed to use disk diffusion as the current regulation and recommendations only focus on MIC determination.

3.3.1 *E. coli*

Table 1. Antimicrobials recommended for AST of *Escherichia coli* and interpretive criteria according to table 1 in Commission Implementing Decision 2013/652/EU

Antimicrobials for *E. coli*	MIC (µg/mL) **R is >**
Ampicillin, AMP	8
Azithromycin, AZI	16*
Cefotaxime, FOT	0.25
Ceftazidime, TAZ	0.5
Chloramphenicol, CHL	16
Ciprofloxacin, CIP	0.064
Colistin, COL	2
Gentamicin, GEN	2
Meropenem, MERO	0.125
Nalidixic acid, NAL	16
Sulfamethoxazole, SMX	64
Tetracycline, TET	8
Tigecycline, TGC	0.5*
Trimethoprim, TMP	2

* Tentative ECOFF

Beta-lactam resistance

Confirmatory tests for ESBL/AmpC/Carbapenemase production are mandatory on all strains resistant to cefotaxime (FOT), ceftazidime (TAZ) and/or meropenem (MERO) and should be performed by testing the second panel of antimicrobials (Table 2 in this document corresponding to Table 4 in Commission Implementing Decision 2013/652/EU).

Table 2. Antimicrobials recommended for additional AST of *Escherichia coli* resistant to cefotaxime, ceftazidime and/or meropenem and interpretive criteria according to table 4 in Commission Implementing Decision 2013/652/EU

Antimicrobials for *E. coli*	MIC (µg/mL) **R is >**
Cefepime, FEP	0.125
Cefotaxime, FOT	0.25
Cefotaxime + clavulanic acid (F/C)	Not applicable
Cefoxitin, FOX	8
Ceftazidime, TAZ	0.5
Ceftazidime+ clavulanic acid (T/C)	Not applicable
Ertapenem, ETP	0.064
Imipenem, IMI	0.5
Meropenem, MERO	0.125
Temocillin, TRM	>32*

*Tentative ECOFF

Confirmatory test for ESBL 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 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 the Appendix to this protocol). It is important to notice that two

cut-off values apply for cefotaxime and ceftazidime: the EUCAST cut-off values (ECOFFs: FOT>0.25 and TAZ>0.5), which are those used to define R/S, and the screening cut-off values (FOT>1 and TAZ>1), which are those applied to categorise bacterial phenotypes as ESBL, AmpC, carbapenemase, etc. based on panel 2 results (see Appendix). The screening cut-off values are higher than the ECOFF values to increase sensitivity and specificity.

3.3.2 Enterococci

Table 3. Antimicrobials recommended for AST of *Enterococcus* spp. and interpretive criteria according to table 3 in Commission Implementing Decision 2013/652/EU

Antimicrobials for enterococci	MIC (µg/mL) **R is >** *E. faecium*	MIC (µg/mL) **R is >** *E. faecalis*
Ampicillin, AMP	4	4
Chloramphenicol, CHL	32	32
Ciprofloxacin, CIP	4	4
Daptomycin, DAP	4	4
Erythromycin, ERY	4	4
Gentamicin, GEN	32	32
Linezolid, LZD	4	4
Quinupristin-dalfopristin (Synercid), SYN	4*	Intrinsically resistant
Teicoplanin, TEI	2	2
Tetracycline, TET	4	4
Tigecycline, TGC	0.25**	0.25**
Vancomycin, VAN	4	4

*DANMAP 2009 (www.danmap.org); **Tentative ECOFF

Identification of *Enterococcus* spp.

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

3.3.3 Staphylococci

Table 4. Antimicrobials recommended for AST of *Staphylococcus aureus* and interpretive criteria according to EFSA technical specifications (EFSA Journal 2012;10(10):2897)

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	0.5
Penicillin, PEN	na
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

na, not available

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. Results should be uploaded as 'positive' or 'negative'.

4 REPORTING OF RESULTS AND EVALUATION

Please write your results in the test forms, and enter your results into the interactive web database. In addition, we kindly ask you to report in the database the tested MIC range for the staphylococci tests (for this organism only, as it is not included in the Commission Implementing Decision 2013/652/EU). Finally, if **you did not use the cut-off values recommended in the protocol for interpretation of *Staphylococcus* AST results**, please report the breakpoints used in the database.

4.1 General recommendations for data upload

We recommend reading carefully the description reported in paragraph 5 before entering your results in the web database. **Results must be submitted no later than September 14th, 2018.** After the deadline when all participants have uploaded results, you will be able to login to the database once again, and 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'.

If you experience difficulties in entering your results, please contact us directly.

Results will be summarised in a report which will be publicly available. Only MIC-results obtained by broth microdilution will be included in the report. All data 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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5 HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE

Please read carefully this paragraph before entering the web page.
Remember that you need by your side the completed test forms and the breakpoint values you used.

Enter the EURL-AR EQAS 2018 start web page (http://eurl-ar.food.dtu.dk/01), write your username and password in lower-cases and press enter. Your username and password are indicated in the letter accompanying your strains. Do not hesitate to contact us if you experience problems with the login.

You can browse back and forth by using the Home or back keys, but please remember to save your inputs before changing pages.

5.1 AST of *E. coli*, enterococci and staphylococci

Click on either "*E. coli*", "enterococci" or "staphylococci" for input of test results based on the results you are going to upload.

Click on "Start of Data Entry - Methods and Breakpoints".

In the next page, you can navigate among fields with the Tab-key and the mouse.

Complete the fields related to the method used for antimicrobial susceptibility testing and the brand of MIC trays, etc.

Click on "save" and then go back using the tab "home" and enter another test page to upload results.

In the data entry pages, enter the obtained values and the interpretation (R, resistant or S, susceptible) for each *E. coli*, *Enterococcus* and *Staphylococcus* strain.

For *E. coli* strains, remember to report also the results for the ESBL/AmpC/Carbapenemase detection tests.
For *S. aureus* strains, remember to report also the results for presence/absence of methicillin resistance.

If you did not test for susceptibility to a given antimicrobial, please leave the field empty.

Click on "save" and then go back using the tab "home" and enter another test page to upload results.

When uploading data on the reference strains, please enter MIC values in µg/ml. Remember to use the operator keys to show symbols like "equal to", etc.

Click on "save".

Review the input pages by browsing through the pages and make corrections if necessary. Remember to save a page if you make corrections. If you press home to leave a page without saving changes, you will see an error screen. In this case, click on "save" to save your results, browse back to the page and then continue.

Please complete the evaluation form.

Before approving your input, please be sure that you have filled in all the relevant fields because YOU CAN ONLY APPROVE ONCE! The approval blocks your data entry in the interactive database.

APPENDIX

Criteria for interpretation of *Escherichia coli*, panel 2 results

1. ESBL-Phenotype
- FOT or TAZ > 1 mg/L AND
- MERO ≤ 0.12 mg/L AND
- FOX ≤ 8 mg/L AND
- SYN FOT/CLV and/or TAZ/CLV

2. AmpC-Phenotype
- FOT or TAZ > 1 mg/L AND
- MERO ≤ 0.12 mg/L AND
- FOX > 8 mg/L AND
- No SYN FOT/CLV nor TAZ/CLV
- (Not excluded presence of ESBLs)

3. ESBL + AmpC-Phenotype
- FOT or TAZ > 1 mg/L AND
- MERO ≤ 0.12 mg/L AND
- FOX >8 mg/L AND
- SYN FOT/CLV and/or TAZ/CLV

4. Carbapenemase-Phenotype
- MERO > 0.12 mg/L
- Needs confirmation
- (Not excluded presence of ESBLs or AmpC)

Susceptible

FOT-TAZ-FOX-MEM ≤ ECOFF

5. Other phenotypes

1) If FOT or TAZ > 1 mg/ml AND
- MEM ≤ 0.12 mg/L AND
- FOX ≤ 8 mg/L AND
- NO SYN FOT/CLV nor TAZ/CLV
- Not excluded CPs (consult EURL)

2) If FOT and/or TAZ ≤ 1 mg/L AND > ECOFF AND
- MERO ≤ 0.12 mg/L
- FOX ≤ 8 mg/L

3) If FOT and TAZ ≤ 1 mg/L
- MERO ≤ 0.12 mg/L
- FOX > 8 mg/L

*cAmpCs could be included here

4) If MERO ≤ 0.12 mg/L BUT
- ETP > ECOFF AND/OR
- IMI > ECOFF
- Not excluded CPs, needs confirmation (consult EURL)

5) Any other combinations not described in previous boxes (consult EURL)

Please refer to: EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. EFSA Journal 2018;16(2):5182, 270 pp. doi:10.2903/j.efsa.2018.5182 (page 46).