DTU Food
National Food Institute

PROTOCOL

For antimicrobial susceptibility testing of *Salmonella*, *Campylobacter* and optional genotypic characterisation of AmpC-, ESBL- and carbapenemase-producing test strains

1 INTRODUCTION

The organisation and implementation of an External Quality Assurance System (EQAS) on antimicrobial susceptibility testing (AST) of *Salmonella* and *Campylobacter* is among the tasks of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). The *Salmonella*/*Campylobacter* EQAS 2018 will include AST of eight *Salmonella* and *Campylobacter* strains and AST of reference strains *E. coli* ATCC 25922 (CCM 3954) and *C. jejuni* ATCC 33560 (CCM 6214).

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

3 OUTLINE OF THE SALM/CAMP EQAS 2018

3.1 Shipping, receipt and storage of strains

In October 2018, the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) will receive a parcel containing eight *Salmonella* and *Campylobacter* strains from the 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 are included in the selected material and are part of the optional EQAS-item, consisting of characterization of genes conferring ESBL- or carbapenemase production. 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, the *Campylobacter* test strains are shipped as a charcoal swabs and the *Salmonella* test strains are stab cultures. On arrival, the stab cultures and the charcoal swabs 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 QC reference strains

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

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.

3.3 Antimicrobial susceptibility testing

The strains should be tested for susceptibility to the antimicrobials listed in Tables 1, 2 and 3, using the method implemented in your laboratory for performing monitoring for EFSA and applying the interpretative criteria listed below.

Participants should perform minimum inhibitory concentration (MIC) determination using the methods stated in the EC regulation EC 652/2013. For interpretation of the results, use the cut-off values listed in Tables 1, 2 and 3. Except where indicated, these represent the current epidemiological cut-off values developed by EUCAST (www.eucast.org), and allow categorisation of bacterial isolates into two categories; resistant or susceptible. A categorisation as intermediate is not accepted.

As the current regulation and recommendations focus on MIC testing only, results obtained by disk diffusion cannot be submitted.

3.3.1 *Salmonella*

The interpretative criteria that should be applied for categorizing the *Salmonella* test strain as resistant or susceptible are those listed in Tables 1 and 2.

Table 1: Antimicrobials recommended for AST of *Salmonella* spp. and interpretative criteria according to table 1 in EC regulation 652/2013

Antimicrobial	MIC (µg/mL) (R>)
Ampicillin (AMP)	8
Azithromycin (AZI)	16*
Cefotaxime (FOT)	0.5
Ceftazidime (TAZ)	2
Chloramphenicol (CHL)	16
Ciprofloxacin (CIP)	0.064
Colistin (COL)	2
Gentamicin (GEN)	2
Meropenem (MERO)	0.125
Nalidixic acid (NAL)	16
Sulfonamides (SMX)	256**
Tetracycline (TET)	8
Tigecycline (TGC)	1***
Trimethoprim (TMP)	2

* Tentative value
** CLSI M100 Table 2A
*** Data from EUCAST is available for *S.* Enteritidis, *S.* Typhimurium, *S.* Typhi and *S.* Paratyphi (for the purpose of this proficiency test, the ECOFF at 1 is applied)

Table 2: Antimicrobials recommended for additional AST of *Salmonella* spp. resistant to cefotaxime, ceftazidime or meropenem and interpretative criteria according to table 4 in EC regulation 652/2013

Antimicrobial	MIC (µg/mL) (R>)
Cefepime, FEP	Not available*
Cefotaxime, FOT	0.5
Cefotaxime + clavulanic acid (F/C)	Not applicable
Cefoxitin, FOX	8
Ceftazidime, TAZ	2
Ceftazidime+ clavulanic acid (T/C)	Not applicable
Ertapenem, ETP	0.06
Imipenem, IMI	1
Meropenem, MERO	0.125
Temocillin, TRM	32**

* Participants are requested to upload the MIC value obtained without selecting an interpretation
** Tentative value

Plasmid-mediated quinolone resistance

When performing antimicrobial susceptibility testing of the *Salmonella* test strains, the interpretative criteria listed in Table 1 for results obtained by MIC-determination should allow detection of plasmid-mediated quinolone resistant test strains.

Beta-lactam- and carbapenem resistance

Confirmatory tests for ESBL production are mandatory on all strains resistant to cefotaxime (FOT), ceftazidime (TAZ) and/or meropenem 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).

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 either 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 results should be based on the most recent EFSA recommendations (available in The European Union summary report on antimicrobial resistance in

zoonotic and indicator bacteria from humans, animals and food in 2015, EFSA Journal 2017;15(2):4694,212 pp. (page 43), and in 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>0.5 and TAZ>2), 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).

3.3.2 *Campylobacter*

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 3: Antimicrobials recommended for AST of *Campylobacter jejuni* and *C. coli* and interpretative criteria according to table 1 in EC regulation 652/2013

Antimicrobial	*C. jejuni*	*C. coli*
	MIC (µg/mL) (R>)	MIC (µg/mL) (R>)
Ciprofloxacin (CIP)	0.5	0.5
Erythromycin (ERY)	4	8
Gentamicin (GEN)	2	2
Nalidixic acid (NAL)	16	16
Streptomycin (STR)	4	4
Tetracycline (TET)	1	2

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.

3.4 Optional genotypic characterisation

For the optional genotypic characterisation of the AmpC-, ESBL- or carbepenemase producing *Salmonella* test strains, the requested results are the genes encoding AmpC-, ESBL- or carbepenemase –production. AmpC-, ESBL- or carbapenemase types included in the test are the following: ACC, ACT, CARB, CMY, CTX-M, DHA, FOX, GES, IMP, KPC, MOX, NDM, OXA, PER, SCO, SHV, TEM, VEB, and VIM. The database lists the relevant variants of each type.

When uploading the results in the database, the identified genes will be evaluated against the expected results. The results will be evaluated on the detected type (ACC-, ACT-, CARB-, etc.) as well as the variant identified.

The method used for the genotypic characterisation should be your laboratory's routine method. The expected results listed in the database are those obtained by the EURL-AR.

4 REPORTING OF RESULTS AND EVALUATION

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

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 December 7th 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.

All results will be summarized in a report which will be publicly available. 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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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.

Enter the EURL-AR EQAS start web page (http://eurl-ar.food.dtu.dk), write your username and password (lower-case) and press enter. Your username and password are indicated in the letter following 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.

Click on either "*Salmonella* test results" or "*Campylobacter* test results" for input of test results.

Click on "Start of Data Entry - Methods"

In the next page, you 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.

When submitting *Campylobacter* results, fill in the incubation conditions applied for susceptibility testing of *Campylobacter* – 36°C/48h or 42°C/24h.

Click on "save and go to next page"

In the data entry pages, you enter the species (for *Campylobacter* only), the obtained MIC-value and the interpretation (R, resistant or S, susceptible) for each *Salmonella* and *Campylobacter* strain.

For *Salmonella*, remember to also report the results for the ESBL detection tests.

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

Click on "save and go to next page"

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 them and make corrections if necessary. Remember to save a page if you make corrections. If you press home 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 as YOU CAN ONLY APPROVE ONCE! The approval blocks your data entry in the interactive database.

If you have performed the optional genotypic characterisation:

Click on "Gene test" and follow the description in the database for upload of the results of the optional genotypic characterization. Approve your input. Be sure that you have filled in all the results before approval. The approval blocks your data entry in the interactive database, but allows you to see the submitted results.

APPENDIX

Criteria for interpretation of *Salmonella*, 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), 2017. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015. EFSA Journal 2017;15(2):4694, 212 pp. doi:10.2903/j.efsa.2017.4694 (page 43).