



PROTOCOL

For antimicrobial susceptibility testing of *Salmonella*, *Campylobacter* and optional genotypic characterisation of two test strains

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1 INTRODUCTION

One of the tasks as the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR) is to organise and conduct an External Quality Assurance System (EQAS) on antimicrobial susceptibility testing (AST) of *Salmonella* and *Campylobacter*. The *Salmonella* and *Campylobacter* EQAS 2011 will include susceptibility testing of eight *Salmonella* and eight *Campylobacter* strains together with susceptibility testing of the reference strains *E. coli* ATCC 25922 (CCM 3954) and *C. jejuni* ATCC 33560 (CCM 6214). Additionally, optional PCR-testing of a selected Gram-negative isolate and a selected Gram-positive isolate is offered.

For new participants of the EURL-AR network who have not already received the mentioned reference strains, these are included in the parcel. The reference strains will not be included in the years to come. The reference strains are original certified cultures and are free of charge. Please take proper care of the strains. Handle and maintain them as suggested in the manual 'Subculture and Maintenance of QC Strains'. Please use them for future internal quality control for susceptibility testing in your laboratory.



For this EQAS, members of the Food- and Waterborne Diseases and Zoonoses Programme (FWD) based at ECDC are also participating, however for these participants the EQAS has been slightly adjusted. Description of this can be found in this protocol, i.e. that QC reference strains are not offered, and that for antimicrobial susceptibility testing (AST) of *Campylobacter*, results obtained by in-house methods like disk diffusion or E-test are also accepted.

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

The main objective of this EQAS is to support laboratories to assess and if necessary improve the quality of susceptibility testing of pathogens originating from food and animal sources, especially *Salmonella* and *Campylobacter*. Furthermore, to assess and improve the comparability of surveillance and antimicrobial susceptibility data reported to EFSA by different laboratories on *Salmonella* and *Campylobacter* and to harmonise the breakpoints used within the EU.

3 OUTLINE OF THE EQAS 2011

3.1 Shipping, receipt and storage of strains

In October 2011, the EU appointed National Reference Laboratories will receive a parcel from the National Food Institute containing eight *Salmonella*, eight *Campylobacter* strains and two additional strain(s) for optional PCR (one *Salmonella* and one *Staphylococcus*). Reference strains will be included for participants who have not previously received these. There might be ESBL-producing strains among the selected material.

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 kept refrigerated 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 see the document 'Instructions for opening and reviving lyophilised cultures' on the EURL-AR-website (see www.eurl-ar.eu).

3.3 Susceptibility testing

The strains should be susceptibility tested towards as many as possible of the following antimicrobials by the method used in the laboratory when performing monitoring for EFSA. For



MIC the cut off values listed in Tables 1 and 2 should be used. The epidemiological cut-off values allow two categories of characterisation – resistant or sensitive.

Participants using disk diffusion are recommended to interpret the results according to their individual breakpoints, categorising them into the terms resistant and susceptible. A categorization as intermediary is not accepted; therefore **intermediary results should be interpreted as susceptible**. Interpretations in concordance with the expected value will be categorised as ‘correct’, whereas interpretations that deviate from the expected interpretation will be categorised as ‘incorrect’.

The cut off values used in the interpretation of the MIC results are developed by EUCAST (www.eucast.org).

With regard to MIC range and/or disc content we ask you to fill in these pieces of information in the database. Also, if you ***do not use*** the cut-off values listed in the protocol for interpretation of the susceptibility results, please fill in or update the breakpoints used, in the database.

3.3.1 *Salmonella*

Testing of gentamicin and streptomycin may be of value for monitoring. Please, do not take into account in this study, that the CLSI guidelines state that for aminoglycosides *Salmonella* should not be reported as susceptible.

Antimicrobials for <i>Salmonella</i>	MIC (µg/mL) R is >
Ampicillin (AMP)	8
Cefotaxime (CTX)	0.5
Ceftazidime (CAZ)**	2
Ceftiofur (XNL)**	2
Chloramphenicol (CHL)	16
Ciprofloxacin (CIP)	0.06
Gentamicin (GEN)	2
Nalidixic acid (NAL)	16
Streptomycin (STR)	16
Sulphonamides (SMX)*	256
Tetracycline (TET)	8
Trimethoprim (TMP)	2

Table 1: Interpretative guidelines for *Salmonella*

* CLSI

** Not part of the EFSA monitoring programme (used for confirmatory tests for ESBL production)

Also, when following EUCAST epidemiological cut-off values, *Salmonella* resistant to nalidixic acid should also be interpreted as resistant to ciprofloxacin. When using disc diffusion and CLSI



clinical breakpoints this connection between nalidixic acid and ciprofloxacin is not taken into account. Thus, the result in this situation with regard to ciprofloxacin will deviate from the expected result in this EQAS.

Important notes: *beta-lactam resistance*:

Confirmatory tests for ESBL production is **mandatory** on all strains resistant to cefotaxime (CTX), ceftazidime (CAZ) and/or ceftiofur (XNL).

Confirmatory test for ESBL production requires use of both cefotaxime (CTX) and ceftazidime (CAZ) 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. its MIC when tested alone (E-test 3 dilution steps difference; MIC CTX : CTX/CL or CAZ : CAZ/CL ratio ≥ 8) or ii) a ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs. its zone when tested alone (CLSI M100 Table 2A; Enterobacteriaceae). The presence of synergy indicates ESBL production.

Confirmatory test for Metallo-beta-lactamase (MBL) production requires use of imipenem (IMI) and IMI/EDTA. Synergy is defined as a ≥ 3 twofold concentration decrease in the MIC for the combination IMI/EDTA vs. MIC for IMI alone (E-test 3 dilution steps difference, MIC IMI : IMI/EDTA ratio ≥ 8 ; CLSI M100, Table 2A; Enterobacteriaceae). The presence of synergy indicates MBL production.

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, that should be verified by PCR and sequencing.

The EURL-AR aims to harmonise with EUCAST expert rules. Accordingly, MIC values and relative interpretation of cefotaxime, ceftazidime and/or ceftiofur used for detection of beta-lactamase-producing strains in this EQAS should be reported as found.

3.3.2 *Campylobacter*

Please find information on the test forms showing which test strains are *C. jejuni* and *C. coli*, respectively.

For AST of *Campylobacter* only MIC methods are recommendable, i.e. broth or agar dilution methods. The EURL-AR does not recommend the use of either disk diffusion or E-test for AST of *Campylobacter*. Laboratories in the EURL-AR network should test the sub-cultured *Campylobacter* by the use of microbroth or agar dilution using incubation at 36-37°C for 48 hours or 42°C for 24 hours.



Antimicrobials for <i>Campylobacter</i>	MIC (µg/mL)	MIC (µg/mL)
	R is > <i>C. jejuni</i>	R is > <i>C. coli</i>
Chloramphenicol*	16	16
Ciprofloxacin	1	1
Erythromycin	4	16
Gentamicin	1	2
Nalidixic acid*	16	32
Streptomycin	2	4
Tetracycline	2	2

Table 2: Interpretative guidelines for *Campylobacter*

*Not part of the EFSA monitoring programme

For the laboratories of the FWD-network, results of AST of *Campylobacter* may be obtained by in-house methods like disk diffusion or E-test. In this case, in-house interpretative criteria must be applied.

3.4 Optional genotypic characterisation

An optional PCR-testing of a selected *S. aureus* (EURL GEN 3.1) as well as a *Salmonella* (EURL GEN 3.2) isolate is offered. If performing the genotypic characterisation of these test strains, the results requested are the genes harboured in the test strain. The genes listed in Tables 3 and 4 are those included in the test. The test strains may harbour resistance genes not present on these lists; these will not be evaluated by the database, but may be mentioned in the comments-field. When uploading the results in the database, the identified genes will be evaluated against the expected results. The results will be evaluated on the actual gene identified. The groups of TEM-, CTX-, SHV-, CMY-, OXA-genes as well as the *gyrA*-mutations and *parC*-mutations will additionally be evaluated on the group selected. For *gyrA* and *parC* the codon-no of the site of mutation will be evaluated in the same way as the genes.

The method used for the PCR-testing should be the one(s) used in your laboratory. The expected results listed in the database are those obtained by the EURL-AR.



Antimicrobial	Gene
Aminoglycosides	addD
	aphA3
	aacA-aphD
	aadE
Betalactamases	blaI
	blaR
	blaZ
	mecA
Chloramphenicol	cat
Glycopeptides	vanA
	vanB
	vanZ
Lincosamides	lnu(A)
Macrolides	erm(A)
	erm(B)
	erm(C)
	mef(A)
	mph(C)
	msr(A)
Quarternary ammonium compounds	qacA
Steroid antibacterial	farI
Streptogramin	vat(A)
	vat(B)
	vga(A)
	vgB(A)
Streptothricin	sat
Tetracycline	tet(K)
	tet(M)
	tet(O)
Trimethoprim	dfrA

Table 3: Genes included in the test of the *S. aureus*-strain (EURL GEN 3.1)



Antimicrobial	Group	Gene/Codon no.
Betalactams	TEM	List of gene numbers in the database
	CTX	List of gene numbers in the database
	SHV	List of gene numbers in the database
	CMY	List of gene numbers in the database
	OXA	List of gene numbers in the database
Chloramphenicol	-	cmlA
	-	catA1
Florphenicol	-	floR
Gentamicin	-	aac(3)-IV
	-	ant(2'')-I
	-	aac(3)-II
Neomycin	-	aph(3 ^{II})-III
	-	aph(3 ^{II})-II
	-	aph(3 ^{II})-I
Quinolones	gyrA	Codon 83
	gyrA	Codon 87
	parC	Codon 57
	parC	Codon 78
	parC	Codon 80
	parC	Codon 84
	-	qnrA
	-	qnrB
	-	qnrC
	-	qnrD
-	qnrS	
Streptomycin	-	strA
	-	strB
	-	aadA
Sulfonamides	-	sul1
	-	sul2
	-	sul3
Tetracycline	-	tetA
	-	tetB
	-	tetC
	-	tetD
	-	tetE
	-	tetF
	-	tetG

Table 4: Genes included in the test of the *Salmonella*-strain (EURL GEN 3.2)



4 REPORTING OF RESULTS AND EVALUATION

Test forms are available for recording your results before you enter them into the interactive web database. We kindly ask you to report in the database the tested MIC range and/or antimicrobial disk content. **If you did not use the cut-off values recommended in the protocol for interpretation of AST results, please report the breakpoints used.**

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 16th December 2011.** After the deadline, the database will be closed and you will be able 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 do not have access to the Internet, or if you experience difficulties in entering your results, try a few days later or, alternatively, return the completed test forms by e-mail, fax or mail to the National Food Institute, Denmark.

All results will be summarised in reports available to all participants. The data will be collected in an overall summary report in which anonymous laboratory results will be analyzed. This summary report will focus on comparing the results from the EURL-AR network, and public health laboratories (FWD-laboratories) to assess the level of harmonization need.

In addition, separate reports for the EURL-AR network (by DTU) and for public health laboratories (by ECDC) will be prepared.

The data in the report will be presented with laboratory codes. A laboratory code is only known to the individual laboratory, while the complete list of laboratories and their respective codes is confidential and only known to the EURL-AR (all participants), the ECDC (FWD-laboratories) and the EU Commission (NRL-ARs). All conclusions and all three reports will be publicly available.

If you have any 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 this passage before entering the web page. Before you go ahead, you need your test form by your side together with your breakpoint values.

You are able to browse back and forth by using the forward and back keys or click on the EURL logo.

You enter the EURL-AR EQAS web page (<http://thor.dfvf.dk/crl>) then write your username and password in low cases and press enter. Your username and password is the same as in the previous EQAS's arranged by the National Food Institute. If you have problems with the login please contact us.

Click on either “*Salmonella* test results” or “*Campylobacter* test results” depending on your results. The below description is aimed at *Salmonella* entry but is exactly the same as for *Campylobacter* entry.

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

In the next page you navigate to fields with the Tab-key and mouse.

Fill in what kind of method you have used for the susceptibility testing of *Salmonella* and the brand of discs, tablets, MIC trays etc.

Fill in the relevant information, either disk content or MIC range. If you use disk diffusion, please upload the breakpoints used.

You will find one more box to fill in on this page when testing *Campylobacter*: Fill in the actual incubation condition used 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 for each *Salmonella* and *Campylobacter* strain, you enter the obtained value and the interpretation as R or S.

For *Salmonella*, you also type in results for the ESBL tests.

If you have not used an antimicrobial, please leave the field empty.

Click on "save and go to next page"

When uploading data on the reference strains please enter the zonediameters in mm or MIC values in µg/ml. Remember to use the operator keys to show e.g. equal to, etc. If you do not use CLSI guidelines for AST on the reference strains, please add a comment on the method used.

Click on "save and go to next page"

This page is a menu, from where you can review the input pages, approve your input and finally see and print the evaluated results:



Browse through the pages and make corrections if necessary. Remember to save a page if you make any corrections. If you save a page without changes, you will see an error screen, and you just have to click on "back" to get back to the page and "go to next page" to continue.

Please fill in the evaluation form.

Approve your input. Be sure that you have filled in all the results before approval, as **YOU CAN ONLY APPROVE ONCE!** The approval blocks your data entry in the interactive database, but allows you to see the evaluated results.

If you have performed the optional genotypic characterisation:

Click on "Gene test" and follow the description in the database for upload of the optional PCR results. 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 evaluated results.