



PROTOCOL

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

1. INTRODUCTION-----	1
2. OBJECTIVES-----	1
3. OUTLINE OF THE EC/ENT/STAPH EQAS 2011-----	2
3.1 Shipping, receipt and storage of strains-----	2
3.2 Suggested procedure for reconstitution of the lyophilised reference strains-----	2
3.3 Antimicrobial susceptibility testing-----	2
4. REPORTING OF RESULTS AND EVALUATION-----	5
5. HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE-----	6

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 2011 will include AST of eight *E. coli*, eight enterococci and eight staphylococci strains and AST of reference strains *E. coli* ATCC 25922 (CCM 3954), *E. faecalis* ATCC 29212 (CCM 4224), *S. aureus* ATCC 25923 (CCM 3953) (for disk diffusion) and *S. aureus* ATCC 29213 (CCM 4223) (for MIC).

The above-mentioned reference strains are included in the parcel only for new participants in the EQAS who did not receive them previously. The reference strains are original certified cultures and are free of charge. Please take proper care of these strains, and handle and maintain them according to the instructions reported in the manual ‘Subculture and Maintenance of QC Strains’. Please use the reference strains for future internal quality control when performing AST in your laboratory.

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*, 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, and to harmonise the breakpoints for antimicrobial susceptibility used within the EU.

3. Outline of the EC/Ent/Staph EQAS 2011

3.1 Shipping, receipt and storage of strains

In June 2011, the EU-appointed National Reference Laboratories for Antimicrobial Resistance will receive a parcel containing eight *E. coli*, eight enterococci and eight staphylococci strains from the National Food Institute, Denmark. This parcel will also contain reference strains, but only for participants who did not receive them previously. All strains are non-toxin-producing human pathogens Class II, and extended-spectrum beta-lactamase (ESBL)-producing strains and methicillin-resistant *Staphylococcus aureus* (MRSA) strains could be included.

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 kept refrigerated until testing. Lyophilised reference strains should be revived by following the procedure reported in the link 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 (www.eurl-ar.eu).

3.3 Antimicrobial susceptibility testing

The strains should be tested for susceptibility to the antimicrobials listed in tables 1, 2 and 3 by using the method implemented in your laboratory for performing monitoring for EFSA.

Participants performing minimum inhibitory concentration (MIC) determination should use the values listed in tables 1, 2 and 3 for interpretation of results. These values represent the epidemiological cut-off values developed by EUCAST (www.eucast.org), and allow categorisation of bacterial strains into two categories: resistant and susceptible. A categorization as intermediate is not accepted, and **“intermediate strains” should be interpreted as susceptible**.

Participants using disk diffusion are recommended to interpret the results according to the breakpoints used routinely. Strains must be categorised into resistant and susceptible. Also in this case, a categorization as intermediate is not accepted, and **“intermediate strains” should be interpreted as susceptible**.



TABLE 1.
Antimicrobials recommended for AST of *Escherichia coli* and interpretative breakpoints

Antimicrobials for <i>E. coli</i> AST	MIC (µg/mL) R is >
Ampicillin, AMP	8
Cefotaxime, CTX	0.25
Cefoxitin, FOX	8
Ceftazidime, CAZ	0.5
Ceftiofur, XNL	1
Chloramphenicol, CHL	16
Ciprofloxacin, CIP	0.032
Florfenicol, FFN	16
Gentamicin, GEN	2
Nalidixic acid, NAL	16
Streptomycin, STR	8*
Sulfonamides, SMX	256**
Tetracycline, TET	8
Trimethoprim, TMP	2

*Based on studies performed by the EURL-AR network (manuscript accepted for publication in Microbial Drug Resistance)

**CLSI M100 Table 2A

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.

TABLE 2.
Antimicrobials recommended for AST of *Enterococcus* spp. and interpretative breakpoints

Antimicrobials for enterococci AST	MIC ($\mu\text{g/mL}$) R is >	MIC ($\mu\text{g/mL}$) R is >
	<i>E. faecium</i>	<i>E. faecalis</i>
Ampicillin, AMP	4	4
Chloramphenicol, CHL	32	32
Ciprofloxacin, CIP	4	4
Erythromycin, ERY	4	4
Gentamicin, GEN	32	32
Linezolid, LZD	4	4
Streptomycin, STR	128	512
Quinupristin-dalfopristin (Synercid), SYN	4*	Not applicable
Tetracycline, TET	4	4
Vancomycin, VAN	4	4

*DANMAP 2009 (www.danmap.org)

Important notes: *identity of the test strains*

Please refer to the test forms for the species (*E. faecalis* or *E. faecium*) of the test strains.



TABLE 3.

Antimicrobials recommended for AST of *Staphylococcus aureus* and interpretative breakpoints

Antimicrobials for <i>S. aureus</i> AST	MIC ($\mu\text{g/mL}$) R is >
Cefoxitin, FOX	4
Chloramphenicol, CHL	16
Ciprofloxacin, CIP	1
Erythromycin, ERY	1
Florfenicol, FFN	8
Gentamicin, GEN	2
Penicillin, PEN	0.125*
Streptomycin, STR	16
Sulfonamides, SMX	128
Tetracycline, TET	1
Trimethoprim, TMP	4

*CLSI M100 Table 2C

Important notes: MRSA

Some test strains may be methicillin-resistant. **Confirmation of *mecA* presence is mandatory** in this EQAS. For this purpose, you are welcome to use the method you prefer, and upload the result as ‘positive’ or ‘negative’. According to CLSI recommendations (M100, Table 2C), all MRSA should be regarded as resistant to all β -lactam antibiotics.

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 and/or antimicrobial disk content. Finally, if you **did not use the cut-off values recommended in the protocol for interpretation of AST results, please report the breakpoints used in the 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 9 September 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, please return the completed test forms by e-mail, fax or mail to the National Food Institute, Denmark.



All results will be summarised in a report available to all participants. 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 and the EU Commission. All conclusions will be public.

If you have any question, please do not hesitate to contact:

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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 2011 start web page (<http://thor.dfvf.dk/crl>), write your username and password in lower-cases and press enter. Your username and password are the same used in the previous EQAS's arranged by The National Food Institute, Denmark. Do not hesitate to contact us if you experience problems with the login.

You can browse back and forth by using the back and forward keys and by clicking on the EURL logo.

Click on either “*E. coli* test results”, “enterococci test results” or “staphylococci test results” based on the results you are going to upload. The description reported below is based on *Salmonella* test result entry, but it is the exact same procedure for entering *E. coli*, enterococci and staphylococci test results.

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

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 of *Salmonella* and the brand of discs, tablets, MIC trays, etc.

Fill in the fields related to either antimicrobial disk content or tested MIC range. If you used disk diffusion, please upload the breakpoints used for interpretation of results.



Click on "save and go to next page"

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 detection tests.

For *S. aureus* strains, remember to report also the results for presence/absence of *mecA*.

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 the zone diameters in mm and MIC values in µg/ml. Remember to use the operator keys to show symbols like "equal to", etc... If you do not use CLSI guidelines for AST of the reference strains, please add a comment on the method used.

Click on "save and go to next page"

This page is a menu that allows you to review the input pages and approve your input.

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