

## PROTOCOL for

- serotyping and susceptibility testing of *Salmonella*
- serotyping and susceptibility testing of *Shigella*
- identification and susceptibility testing of *Campylobacter*
- identification of an unknown enteric pathogen

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### 1 INTRODUCTION

In 2000, the Global Foodborne Infections Network (formely known as WHO Global Salm-Surv) launched an External Quality Assurance System (EQAS). The EQAS is organized by the National Food Institute, Technical University of Denmark (DTU Food), in collaboration with partners and Regional Sites in WHO GFN.

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.

The WHO EQAS 2009 includes

- serotyping and susceptibility testing of eight *Salmonella* strains,
- serotyping and susceptibility testing of four *Shigella* strains,
- susceptibility testing of the *E. coli* reference strain for quality control (ATCC 25922 (CCM 3954)),

- identification and susceptibility testing of two thermophilic *Campylobacter* isolates
- susceptibility testing of the *C. jejuni* reference strain for quality control (ATCC 33560 (CCM 6214)),
- and identification of one ‘unknown’ bacterial isolate.

All participants will receive the strains relevant to their laboratory according to the sign-up information.

For new participants of the EQAS 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 and should be used for future internal quality control for susceptibility testing in your laboratory. Please take proper care of the strains. Handle and maintain them as suggested in the manual ‘Subculture and Maintenance of QC Strains’ available on the WHO CC website (see [www.antimicrobialresistance.dk](http://www.antimicrobialresistance.dk)).

## 2 OBJECTIVES

The main objective of this EQAS is to support laboratories to assess and if necessary improve the quality of serotyping and susceptibility testing of enteric human pathogens, especially *Salmonella*. Furthermore, to assess and improve the comparability of surveillance data on *Salmonella* serotypes and antimicrobial susceptibility reported by different laboratories. The laboratory work for this EQAS should be done by the methods routinely used in your laboratory.

## 3 OUTLINE OF THE EQAS 2009

### 3.1 Shipping, receipt and storage of strains

In August/September 2009 around 190 laboratories from all parts of the world will receive a parcel containing eight *Salmonella* strains, four *Shigella*, two *Campylobacter* strains and one ‘unknown’ bacterial isolate (according to information when signing up). An *E. coli* reference strain and a *C. jejuni* reference strain will be included for participants who have signed up to perform antimicrobial susceptibility testing (AST) and who have not previously received these. All strains are non-toxin producing human pathogens Class II. There might be ESBL-producing strains among the selected material.

**Please confirm receiving the parcel by the confirmation form enclosed in the shipment**

The reference strains and the *Campylobacter* strains are shipped lyophilised, whereas the *Salmonella* and *Shigella* strains, as well as the ‘unknown’ isolate are stab cultures. On arrival, the stab cultures must be subcultured, and all cultures should be kept refrigerated until testing. A suggested procedure for reconstitution of lyophilized strains is presented below.

### 3.2 Serotyping of *Salmonella*

The eight *Salmonella* strains should be serotyped by the method routinely used in the laboratory. If you do not have all the antisera please go as far as you can, and please report the serogroup, since also serogrouping results will be evaluated. When reporting serogroups, please use terms according to Kaufman-White (Popoff and Le Minor, 2001. 8<sup>th</sup> ed. Popoff, M.U., Le Minor, L., 2001. Antigenic formulas of the *Salmonella* serovars. WHO Collaborating Centre for Reference and Research on *Salmonella*).

When uploading the data, please fill in the information on the brand of antisera used in the typing.

### 3.3 Susceptibility testing of *Salmonella*, *Shigella* and *E. coli* ATCC 25922

The *Salmonella* and *Shigella* strains as well as the *E. coli* reference strain should be susceptibility tested towards as many as possible of the antimicrobials mentioned in the test form. Please use the methods routinely used in the laboratory.

For reconstitution of the *E. coli* reference strain: Please see the document 'Instructions for opening and reviving lyophilised cultures' on the WHO CC website (see [www.antimicrobialresistance.dk](http://www.antimicrobialresistance.dk)).

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* and *Shigella* should not be reported as susceptible.

Antimicrobials	Reference value, MIC ( $\mu\text{g/mL}$ )		
	Sensitive	Intermediate	Resistant
Ampicillin, AMP*	$\leq 8$	16	$\geq 32$
Cefotaxime, CTX**	$\leq 0.5$	-	$> 0.5$
Ceftazidime, CAZ**	$\leq 2$	-	$> 2$
Ceftriaxone, CRO***	$\leq 0.25$	-	$> 0.25$
Chloramphenicol, CHL*	$\leq 8$	16	$\geq 32$
Ciprofloxacin, CIP**	$< 0.125$	-	$\geq 0.125$
Gentamicin, GEN*	$\leq 4$	8	$\geq 16$
Nalidixic acid, NAL*	$\leq 16$	-	$\geq 32$
Streptomycin, STR***	$\leq 8$	16	$\geq 32$
Sulfonamides, SMX*	$\leq 256$	-	$\geq 512$
Tetracycline, TET*	$\leq 4$	8	$\geq 16$
Trimethoprim, TMP*	$\leq 8$	-	$\geq 16$
Trimethoprim + sulfamethoxazole, TMP+SMX, SXT*	$\leq 2/38$	-	$\geq 4/76$

\*CLSI \*\*EUCAST (epidemiological cut off values) \*\*\*DTU Food

In this EQAS the breakpoints used as a key to interpreting MIC results are a mixture of reference values from CLSI, EUCAST and DTU Food (see list above). This allows three categories of characterisation – resistant, intermediate or sensitive. Interpretations in concordance with the expected value will be categorised as ‘correct’, whereas deviations from the expected interpretation are categorized as ‘minor’ (I ↔ S or I ↔ R), ‘major’ (S interpreted as R) or ‘very major’ (R interpreted as S).

As to the breakpoints that you routinely use in your laboratories to determine the susceptibility category we ask you to fill in these breakpoints in the database (or in the test form).

For ciprofloxacin, please note that a low breakpoint has been used to determine resistance category. Considering the expected results of this EQAS, microorganisms are considered resistant to ciprofloxacin when showing reduced susceptibility to this antimicrobial.

#### ESBL production

It is optional to continue with the following tests regarding ESBL production:

All strains categorized reduced susceptible against cefotaxime (CTX), ceftazidime (CAZ) or ceftriaxone (CRO) could be confirmed by confirmatory tests for ESBL production.

The confirmatory tests require testing with a pure antimicrobial (CTX and CAZ) vs. a test with the same antimicrobial combined with a  $\beta$ -lactamase inhibitor (clavulanic acid). Synergy is defined as a 3 dilution steps difference between the two compounds in at least one of the two cases (MIC ratio  $\geq 8$ , E-test 3 dilution steps) or an increase in zone diameter  $\geq 5$  mm (CLSI M100 Table 2A; enterobacteriaceae). If the test shows signs of synergy it is an indication of the presence of ESBL.

Concerning cefotaxime (CTX), ceftazidime (CAZ) and/or ceftriaxone (CRO) used when detecting ESBL-producing strains in the EQAS: If a microorganism is resistant to one or two of these drugs, it should be regarded resistant to all three.

### **3.4 Handling the *Campylobacter* strains**

Freeze-dried cultures are supplied in vacuum-sealed ampoules. Care should be taken in opening the ampoule. All instructions given below should be followed closely to ensure the safety of the person who opens the ampoule and to prevent contamination of the culture.

- a. Check the number of the culture written on the label.
- b. Make a file cut on the ampoule just above the shoulder of the ampoule.
- c. Disinfect the ampoule with alcohol-dampened gauze or alcohol-dampened cotton wool.
- d. Crack the glass using sterile gauze or cotton to protect your fingers.
- e. Add to the dried suspension about 0.5 ml appropriate broth or a sterile 0.9% NaCl solution using a pipette. Mix carefully to avoid creating aerosols.

- f. Inoculate the suspension on a suitable agar plate with a 10µl loop or a cotton swab.
- g. Transfer the rest of the content in the ampoule to a test tube containing 5-6 ml of a suitable liquid media.
- h. Incubate the agar plate and liquid media at a temperature of 42°C at microaerobic conditions for 24-48 hours.
- i. Inoculate a second agar plate from the liquid media with a 10µl loop or a cotton swab if the initial plate had inadequate growth.
- j. Select a pure culture with vigorous growth from the agar plate for further work.

Please note that:

- Cultures may need at least one sub-culturing before they can be optimally used
- Unopened ampoules should be kept in a dark and cool place!

For reconstitution of the *C. jejuni* reference strain: Please see the document ‘Instructions for opening and reviving lyophilised cultures’ on the WHO CC website (see [www.antimicrobialresistance.dk](http://www.antimicrobialresistance.dk)).

### 3.5 Identification of *Campylobacter*

The two thermophilic *Campylobacter* isolates should be identified to species level.

### 3.6 Susceptibility testing of *Campylobacter* and *C. jejuni* ATCC 33560

The *Campylobacter* test strains as well as the *C. jejuni* reference strain should be susceptibility tested towards as many as possible of the antimicrobials mentioned in the test form. It should be noted that for AST of *Campylobacter* only MIC methods are recommendable, i.e. broth or agar dilution methods. Neither the use of disk diffusion nor E-test is recommendable for AST of *Campylobacter*.

In this EQAS the breakpoints used as a key to interpreting MIC results for *Campylobacter* are epidemiological cut off values. The reference values used are from EUCAST ([www.eucast.org](http://www.eucast.org); see list below). This allows only two categories of characterisation – resistant or sensitive. Interpretations in concordance with the expected value will be categorised as ‘correct’, whereas deviations from the expected interpretation are categorized as ‘incorrect’.

As to the breakpoints that you routinely use in your laboratories to determine the susceptibility category we ask you to fill in these breakpoints in the database (or in the test form).

Note that the interpretation requires knowledge about the species. If you do not identify *Campylobacter* but perform AST on *Campylobacter*, you may contact the EQAS Coordinator to obtain information regarding the identity of the *Campylobacter* test strains.

Antimicrobials for <i>Campylobacter</i>	MIC ( $\mu\text{g/mL}$ )	MIC ( $\mu\text{g/mL}$ )
	<b>R is &gt;</b> <i>C. jejuni</i>	<b>R is &gt;</b> <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

The sub-cultured *Campylobacter* should be used for the MIC-testing after incubation at 36°C for 48 hours or 42°C for 24 hours; possibly two subcultures are needed to ensure good growth before testing.

### 3.7 Identification and of the unknown enteric pathogen

The 'unknown' isolate should be identified to species level and further typed if relevant.

## 4 REPORTING OF RESULTS AND EVALUATION

Fill in your results in the enclosed test form and enter your results into the interactive web database. Please read the detailed description below before entering your results. When you enter the results via the web, you will be guided through all steps on the screen and you will immediately be able to view and print an evaluation report of your results. **Please submit results by latest December 31<sup>st</sup>, 2009.** If you do not have access to the Internet or if you experience difficulties entering the data, please return results by fax or mail to the National Food Institute.

All results will be summarized in a report which will be made available to all participants. Individual results will be anonymous and will only be passed on to the official GFN Regional Centre in your region.

We are looking forward to receiving your results.

**If you have any questions or concerns, please do not hesitate to contact the EQAS Coordinator:**

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It is possible to communicate with the EQAS organisers in other languages than English. However, this is not a direct contact with the EQAS organisers since translation of the message is required. The following languages may be used: Russian, Chinese, French, Spanish or Portuguese.

## 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.

In general you navigate in the database with the Tab-key and mouse, and at any time a click on the WHO logo takes you back to the main menu.

- 1) Enter the WHO CC website (from <http://www.antimicrobialresistance.dk>), then
  - a. Click on 'EQAS'
  - b. Click on the link for the interactive database
  - c. Write your username and password in low letters and click on 'Login'.  
In the letter following your parcel you can find your username and password.  
Your username and password will be the same in future trials.
- 2) Click on 'Materials and methods'
  - a. Fill in the brand of antisera (very important as we would like to compare results with the brand of antisera)
  - b. Fill in the method used for susceptibility testing
  - c. Enter the brand of accessories, e.g. Oxoid
  - d. Fill in whether your institute serves as a national reference laboratory
  - e. Click on 'Save and go to next page' – REMEMBER TO SAVE EACH PAGE LIKE THIS!
- 3) In the data entry page 'Routinely used breakpoints'
  - a. Fill in the breakpoints that you routinely use in your laboratory to determine the susceptibility category. Remember to use the operator keys in order to show – equal to, less than, less or equal to, greater than or greater than or equal to.
- 4) In the data entry pages '*Salmonella* strains 1-8', you
  - a. SELECT the serogroup (O-group) from the pop-up list, DO NOT WRITE – Wait a few seconds – the page will automatically reload, so that the pop-up in the field "Serotype" only contains serotypes belonging to the chosen serogroup.

- b. SELECT the serotype from the pop-up list – DO NOT WRITE – wait a few seconds and you can enter the antigenic formula (e.g. 1,4,5,12:i:1,2)
- c. Enter the zonediameters in mm or MIC values in µg/ml. Remember to use the operator keys to show e.g. equal to, etc.
- d. Enter the interpretation as R, I or S
- e. If you have performed confirmatory tests for ESBL producing strains, please choose the test result from the pick list.
- f. Fill in comments if relevant e.g. which antisera you miss for complete serotyping
- g. Click on ‘Save and go to next page’

**If you have not performed these tests please leave the fields empty**

- 5) In the data entry page ‘*E. coli* reference strain’:
  - a. Enter the zonediameters in mm or MIC values in µg/ml. Remember to use the operator keys to show e.g. equal to, etc.
  - b. Click on ‘Save and go to next page’

- 6) In the page ‘Identification of *Campylobacter* and unknown sample’:
  - a. Choose the correct *Campylobacter* species from the pick list
  - b. Fill in the species and type of the unknown bacterial isolate, and fill in the method used
  - c. Click on ‘Save and go to next page’

**If you have not performed these tests please leave the fields empty**

- 7) The next page is a menu, from where you can review the input pages or approve your input *and finally see and print the evaluated results*
  - a. Browse through the input pages and make corrections if necessary. Remember to click on ‘save and go to next page’ if you make any corrections.
  - b. 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.
  - c. As soon as you have approved your input, an evaluation report will show.
- 8) When you have seen all pages in the report, you will find a new menu. You can choose ‘EQAS 2009 start page’, ‘Review evaluated results’ (a printer friendly version of the evaluation report is also available) or ‘Go to Global Salm-Surv homepage’.

**End of entering your data – thank you very much!**