

PROTOCOL for

- serotyping and antimicrobial susceptibility testing of *Salmonella*
- serotyping and antimicrobial susceptibility testing of *Shigella*
- identification and antimicrobial susceptibility testing of *Campylobacter*
- identification of an unknown environmental bacterium

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1 INTRODUCTION

In 2000, the Global Foodborne Infections Network (formerly 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 be subcontracted from time to time. When subcontracting occurs, it is placed with a competent subcontractor and the National Food Institute is responsible for the subcontractor's work.

The WHO EQAS 2011 includes:

- serotyping and antimicrobial susceptibility testing of eight *Salmonella* strains,
- serotyping and antimicrobial susceptibility testing of four *Shigella* strains,
- antimicrobial susceptibility testing of the *Escherichia coli* ATCC 25922 (CCM 3954), reference strain for quality control,
- identification and antimicrobial susceptibility testing of two thermophilic *Campylobacter* isolates,
- antimicrobial susceptibility testing of *Campylobacter jejuni* ATCC 33560 (CCM 6214), reference strain for quality control,

- identification of one 'unknown' bacterial isolate.

All participants will receive the strains according to the information they reported in the sign-up form.

The above mentioned 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 WHO CC website (please see 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 antimicrobial susceptibility testing of enteric human pathogens, especially *Salmonella*. A further objective is to assess and improve the comparability of surveillance data on *Salmonella* serotypes and antimicrobial susceptibility reported by different laboratories. Therefore, the laboratory work for this EQAS should be done by using the methods routinely used in your laboratory.

3 OUTLINE OF THE EQAS 2011

3.1 Shipping, receipt and storage of strains

In August/September 2011, some 180 laboratories located worldwide will receive a parcel containing eight *Salmonella* strains, four *Shigella* strains, two *Campylobacter* strains and one 'unknown' bacterial isolate (according to information reported in the sign-up form). An *E. coli* ATCC 25922 reference strain and a *C. jejuni* ATCC 33560 reference strain will be included for participants who signed up to perform antimicrobial susceptibility testing (AST) and did not receive them previously. All provided strains are non-toxin-producing human pathogens Class II. ESBL-producing strains could be included in the selected material.

Please confirm receipt of the parcel through the confirmation form enclosed in the shipment

The *Salmonella* and *Shigella* strains and the 'unknown' bacterial isolate are shipped as agar stab cultures, whereas the reference strains and the *Campylobacter* strains are shipped lyophilized. On arrival, the agar stab cultures must be subcultured and prepared for storage in your strain collection (e.g. in a -80 °C freezer). This set of cultures should serve as reference if discrepancies are detected during tests (e.g. they can be used if errors such as mis-labelling or contamination occur). Lyophilized strains must be reconstituted, and you can find below a suggested procedure.

3.2 Serotyping of *Salmonella*

The eight *Salmonella* strains should be serotyped by using the method routinely used in the laboratory. If you do not have all the necessary antisera, please go as far as you can in the identification and report the serogroup since also serogroup results will be evaluated. Serogroups should be reported by using terms according to Kauffmann-White-Le Minor (Grimont and Weill, 2007. 9th ed. Antigenic formulae of the *Salmonella* serovars. WHO Collaborating Centre for Reference and Research on *Salmonella*).

Please fill-in information concerning the brand of antisera used for typing in the fields available in the database for entering results. In addition, we kindly ask you to report which antisera you think is required to complete the serotyping, if relevant.

3.3 Antimicrobial susceptibility testing of *Salmonella*, *Shigella* and *E. coli* ATCC 25922

The *Salmonella* and *Shigella* strains and the *E. coli* ATCC 25922 reference strain should be tested for susceptibility towards as many as possible of the antimicrobials mentioned in the test form. Please use the methods routinely used in your laboratory.

For reconstitution of the *E. coli* reference strain, please see the document ‘Instructions for opening and reviving lyophilized cultures’ on the WHO CC website (please find the link at www.antimicrobialresistance.dk).

Testing of gentamicin and streptomycin susceptibility may be valuable for monitoring purposes. Therefore, we kindly ask you to disregard, for the purpose of this proficiency testing, that the Clinical and Laboratory Standards Institute (CLSI) guidelines state that *Salmonella* and *Shigella* should not be reported as susceptible to aminoglycosides.

The breakpoints used in this EQAS for interpreting MIC results are in accordance with CLSI values, and are supplemented with values from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) and DTU Food (Table 1). Consequently, interpretation of MIC results will lead to categorization of strains into three categories: resistant (R), intermediate (I) and susceptible (S). In the evaluation report that you receive upon result submission, you can find that obtained interpretations in accordance with the expected interpretation will be defined as ‘correct’, whereas deviations from the expected interpretation will be defined as ‘minor’ (I ↔ S or I ↔ R), ‘major’ (S interpreted as R) or ‘very major’ (R interpreted as S).

Please report the breakpoints that you routinely use in your laboratory for interpretation of antimicrobial susceptibility test results in the fields available in the database (or in the test forms).

Concerning ciprofloxacin susceptibility test, please note that a low breakpoint has been used to determine the resistance category. This low breakpoint corresponds to the EUCAST

epidemiological cut-off value, which was established to take into consideration mechanisms of resistance like *qnr* genes or one point-mutation in the gyrase gene (Table 1; www.eucast.org). In this EQAS, microorganisms showing reduced susceptibility to ciprofloxacin are considered ciprofloxacin-resistant.

Table 1. Interpretive breakpoint for *Salmonella* and *Shigella* antimicrobial susceptibility testing

Antimicrobials	Reference value, MIC ($\mu\text{g/mL}$)			Reference value, Disk diffusion (mm)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Ampicillin, AMP	≤ 8	16	≥ 32	≥ 17	14-16	≤ 13
Cefotaxime, CTX	≤ 1	-	> 1	> 27	-	≤ 27
Ceftazidime, CAZ	≤ 1	-	> 1	> 22	-	≤ 22
Ceftriaxone, CRO	≤ 1	-	> 1	> 25	-	≤ 25
Chloramphenicol, CHL	≤ 8	16	≥ 32	≥ 18	13-17	≤ 12
Ciprofloxacin, CIP	$< 0.125^*$	-	$\geq 0.125^*$	$\geq 23\text{mm}$ ($1\mu\text{g}$) ^{***} or $\geq 30\text{mm}$ ($5\mu\text{g}$) ^{***}	-	$< 23\text{mm}$ ($1\mu\text{g}$) ^{***} or $< 30\text{mm}$ ($5\mu\text{g}$) ^{***}
Gentamicin, GEN	≤ 4	8	≥ 16	≥ 15	13-14	≤ 12
Nalidixic acid, NAL	≤ 16	-	≥ 32	≥ 19	14-18	≤ 13
Streptomycin, STR	$\leq 8^{**}$	16^{**}	$\geq 32^{**}$	≥ 15	12-14	≤ 11
Sulfonamides, SMX	≤ 256	-	≥ 512	≥ 17	13-16	≤ 12
Tetracycline, TET	≤ 4	8	≥ 16	≥ 15	12-14	≤ 11
Trimethoprim, TMP	≤ 8	-	≥ 16	≥ 16	11-15	≤ 10
Trimethoprim + sulfamethoxazole, TMP+SMX, SXT	$\leq 2/38$	-	$\geq 4/76$	≥ 16	11-15	≤ 10

Reference values used in this EQAS are according to CLSI, with the following exceptions:

* EUCAST (epidemiological cut-off values)

** DTU Food

*** In the absence of values provided by EUCAST, the article by Cavaco LM and Aarestrup FM (J. Clin. Microbiol. 2009. Sep;47(9):2751-8) provides the background for these interpretative criteria in the WHO GFN EQAS. In that article, *Shigella* was not included. However, the same interpretative criteria will be used in this context.

Important notes: *beta-lactam resistance*

The following tests for detection of Extended-Spectrum Beta-Lactamase (ESBL) production are optional:

All strains showing reduced susceptibility to cefotaxime (CTX), ceftazidime (CAZ) and/or ceftriaxone (CRO) could be tested for ESBL production by confirmatory test. 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.

Of note, MIC values and relative interpretation of cefotaxime (CTX), ceftazidime (CAZ) and/or ceftriaxone (CRO) used for detection of beta-lactamase-producing strains in this EQAS should be reported as found, which is in accordance with EUCAST expert rules.

3.4 Handling the *Campylobacter* strains

Freeze-dried cultures are supplied in vacuum-sealed ampoules. Care should be taken in opening the ampoule, and 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 of appropriate broth or sterile 0.9% NaCl solution by using a pipette. Mix carefully to avoid creating aerosols.
- f. Inoculate the suspension on a suitable agar plate with a 10 μ l loop or a cotton swab.
- g. Transfer the rest of the content of 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 10 μ l 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 subculture before they can be optimally used

- Unopened ampoules should be kept in a dark and cool place!

For reconstitution of *C. jejuni* ATCC 33560 reference strain: please see the document 'Instructions for opening and reviving lyophilised cultures' on the WHO CC website (please find the link at www.antimicrobialresistance.dk).

3.5 Identification of *Campylobacter*

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

3.6 Antimicrobial susceptibility testing of *Campylobacter* and *C. jejuni* ATCC 33560

The *Campylobacter* test strains and the *C. jejuni* reference strain should be tested for susceptibility to as many antimicrobials as possible among the ones mentioned in the test form. Please note that only MIC methods (i.e. broth or agar dilution methods) are recommendable for AST of *Campylobacter*. Neither the use of disk diffusion nor E-test is recommendable for AST of *Campylobacter*.

In this EQAS, the breakpoints used for interpretation of MIC results for *Campylobacter* are epidemiological cut-off values according to EUCAST (www.eucast.org; Table 2). Consequently, only two categories of characterization (resistant, R and sensitive, S) are allowed. In the evaluation report that you receive upon result submission, you can find that obtained interpretations that are in agreement with the expected interpretation will be categorized as 'correct', whereas deviations from the expected interpretation will be categorized as 'incorrect'.

Please report the breakpoints that you routinely use in your laboratory for interpretation of antimicrobial susceptibility test results in the fields available in the database (or in the test forms).

Please note that the interpretation of antimicrobial susceptibility test results for *Campylobacter* requires knowledge of the *Campylobacter* species. If you did not sign up for *Campylobacter* identification but you perform AST on *Campylobacter*, you are welcome to contact the EQAS Coordinator to obtain information regarding the identity of the *Campylobacter* test strains.



Table 2. Interpretive criteria for *Campylobacter* antimicrobial susceptibility testing

Antimicrobials for <i>Campylobacter</i>	<i>C. jejuni</i>	<i>C. coli</i>
	MIC ($\mu\text{g/mL}$) R is >	MIC ($\mu\text{g/mL}$) R is >
Chloramphenicol, CHL	16	16
Ciprofloxacin, CIP	1	1
Erythromycin, ERY	4	16
Gentamicin, GEN	1	2
Nalidixic acid, NAL	16	32
Streptomycin, STR	2	4
Tetracycline, TET	2	2

Reference values for interpretation of Campylobacter AST results according to EUCAST

The sub-cultured *Campylobacter* strains should be used for MIC testing after incubation at 36-37°C for 48 hours or at 42°C for 24 hours. Likely, two subcultures are needed prior to MIC testing to ensure optimal growth.

3.7 Identification of the unknown environmental bacterium

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

4 REPORTING OF RESULTS AND EVALUATION

Please write your results in the enclosed test forms, and enter your results into the interactive web database.

We recommend reading carefully the description reported in paragraph 5 before entering your results in the web database. For entering your results via the web, you will be guided through all steps on the screen and you will immediately be able to view and print a report evaluating your results. Results in agreement with the expected interpretation are categorized as 'correct', while results deviating from the expected interpretation are categorized as 'incorrect'.

Results must be submitted no later than 31 December 2011.

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 summarized in a report available to all participants. Individual results will be anonymous and will only be forwarded 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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Please note that it is also possible to communicate with the EQAS organizers in languages different from English. However, this is not a direct contact with the EQAS organizers since translation of the message is required. The following languages may be used: Chinese, French, Portuguese, Russian and Spanish.

5 HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE

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

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 (link available at <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 lower-case letters and click on 'Login'.
You can find your username and password in the letter accompanying your parcel.
Your username and password will remain unchanged in future trials.
- 2) Click on 'Materials and methods'
 - a. Fill-in the fields relative to brand of antisera (very important because we would like to compare results obtained with different brands of antisera)
 - b. Fill-in the fields relative to the method used for antimicrobial susceptibility testing
 - c. Enter the brand of materials, e.g. Oxoid
 - d. Fill-in the field asking whether your institute serves as a national reference laboratory
 - e. In the comment field, report which antisera you think is required to complete your serotyping, if relevant
 - f. Click on 'Save and go to next page' – REMEMBER TO SAVE EACH PAGE BEFORE LEAVING IT!
- 3) In the data entry page 'Routinely used breakpoints'



- a. Fill-in the fields relative to the breakpoints used routinely in your laboratory to determine the antimicrobial susceptibility category. Remember to use the operator keys in order to show: equal to (=), less than (<), less or equal to (\leq), greater than (>) or greater than or equal to (\geq).
- b. In the data entry pages ‘*Salmonella* strains 1-8’
- c. SELECT the serogroup (O-group) from the drop-down list, DO NOT WRITE – Wait a few seconds – the page will automatically reload, so that the drop-down list in the field “Serotype” only contains serotypes belonging to the chosen serogroup.
- d. SELECT the serotype from the drop-down list – DO NOT WRITE – wait a few seconds and you can enter the antigenic formula (e.g. 1,4,5,12:i:1,2)
- e. Enter the zone diameters in mm or MIC values in $\mu\text{g/ml}$. Remember to use the operator keys to show e.g. equal to (=), etc...
- f. Enter the interpretation as R (resistant), I (intermediate) or S (susceptible)
- g. If you performed confirmatory tests for ESBL production, please choose the appropriate result from the pick list.
- h. If relevant, fill-in the field related to comments (e.g. which antisera you miss for complete serotyping, etc...)
- i. Click on ‘Save and go to next page’

If you did not perform these tests, please leave the fields empty

4) In the data entry page ‘*E. coli* reference strain’:

- a. Enter the zone diameters in mm or MIC values in $\mu\text{g/ml}$. Remember to use the operator keys to show e.g. equal to (=), etc...
- b. Click on ‘Save and go to next page’

5) In the page ‘Identification of *Campylobacter* and unknown sample’:

- a. Choose the correct *Campylobacter* species from the pick list
- b. Fill-in the field concerning species and type of the unknown bacterial isolate, and report the method used for identification
- c. Click on ‘Save and go to next page’

If you did not perform these tests, please leave the fields empty

6) The next page is a menu that allows you to review the input pages and 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 into the interactive database, but allows you to see the evaluated results.
 - c. As soon as you have approved your input, an evaluation report will appear.
- 7) After browsing all pages in the report, you will find a new menu. You can choose ‘EQAS 2011 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!