

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 enteric pathogen

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HISTORY OF CHANGES; protocol version 2

Interpretative criteria for meropenem adjusted in Table 1 (changes from protocol version 1 indicated with bold and italics)

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 from time to time be subcontracted. 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 2016 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 (NCIMB 12210) reference strain for quality control (QC),
- identification and antimicrobial susceptibility testing of two thermophilic *Campylobacter* isolates,
- antimicrobial susceptibility testing of *Campylobacter jejuni* ATCC 33560 (NCTC 11351) reference strain for QC,
- 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 QC reference strains are included in the parcel only for new participants of the EQAS who did not receive them previously. The QC 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 QC 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 Collaborating Centre website (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 2016

3.1 Shipping, receipt and storage of strains

In September 2016 around 200 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 belong to UN3373, Biological substance category B. AmpC-, Extended-Spectrum Beta-Lactamase (ESBL)-, and carbapenemase-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 for QC and the *Campylobacter* strains are shipped lyophilised (LYFO DISK®). See section 3.1.1 below for additional info on handling and reconstitution of the lyophilised cultures.

On arrival, the bacterial cultures must be stored in a dark place at 2°C to 8°C until handling in the laboratory.

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 the testing (e.g. they can be used to detect errors such as mis-labelling or contamination).

3.1.1 Instructions related to handling of LYFO DISK®

The microorganisms supplied as LYFO DISK® are packaged in re-sealable vials that contain a lyophilized pellet and a desiccant to prevent adverse accumulations of moisture.

The following instructions can be downloaded from the manufacturer’s website (<http://microbiologics.com/Support-Center/KWIK-STIK-trade>):

1. Remove the unopened LYFO DISK® vial from 2°C to 8°C storage and allow the unopened vial to equilibrate to room temperature.
2. Aseptically remove the pellet with sterile forceps from the vial. Do not remove desiccant.
3. Place the pellet in 0.5 mL of sterile fluid (water, saline, TSB, or BHIB).
4. Crush the pellet with a sterile swab until the suspension is homogenous. Immediately heavily saturate the same swab with the hydrated material and transfer to agar medium.
5. Inoculate the primary culture plate(s) by gently rolling the swab over one-third of the plate.
6. Using a sterile loop, streak to facilitate colony isolation.
7. Using proper biohazard disposal, discard the remaining hydrated material.
8. Immediately incubate the inoculated media at temperature and conditions appropriate to the microorganism.

Materials required but not provided:

- Microorganisms require sterile tubes and 0.5 ml of sterile liquid such as, Tryptic Soy Broth, Brain Heart Infusion Broth, saline, or deionized water to hydrate the lyophilized preparation.
- Sterile swabs or inoculating loops are needed to transfer the hydrated preparation to an agar plate.
- Non-selective, nutrient or enriched agar media and specific incubation times and conditions to optimize growth and recovery.

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 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 are required to complete the serotyping, if relevant.

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

The *Salmonella* and *Shigella* strains as well as the *E. coli* ATCC 25922 QC 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* QC reference strain (NCIMB 12210) which is supplied in the form of a LYFO DISK®, see instructions in section 3.1.1 above.

Testing of gentamicin susceptibility may be valuable for monitoring purposes. Therefore we kindly ask you to disregard, for the purpose of this proficiency trial, 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 (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 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).

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

Antimicrobials	Reference value, MIC (µg/mL)			Reference value, Disk diffusion (mm)		
	Sensitive	Intermediate	Resistant	Resistant	Intermediate	Sensitive
Ampicillin, AMP	≤8	16	≥32	≤13	14-16	≥17
Cefotaxime, CTX*	≤1	-	>1	≤27	-	>27
Cefoxitin, FOX	≤8	16	≥32	≤14	15-17	≥18
Ceftazidime, CAZ*	≤1	-	>1	≤22	-	>22
Ceftriaxone, CRO*	≤1	-	>1	≤25	-	>25
Chloramphenicol, CHL	≤8	16	≥32	≤12	13-17	≥18
Ciprofloxacin, CIP	≤0.06**	0.12-0.5**	≥1**	≤20mm (5µg)** or <23mm (1µg)***	21-30mm (5µg)** or - (1µg)***	≥31mm (5µg)** or ≥23mm (1µg)***
Gentamicin, GEN	≤4	8	≥16	≤12	13-14	≥15
Meropenem, MER*	≤0.12	-	>0.12	<27	-	≥27
Nalidixic acid, NAL	≤16	-	≥32	≤13	14-18	≥19
Sulfonamides, SMX	≤256	-	≥512	≤12	13-16	≥17
Tetracycline, TET	≤4	8	≥16	≤11	12-14	≥15
Trimethoprim, TMP	≤8	-	≥16	≤10	11-15	≥16
Trimethoprim + sulfamethoxazole, TMP+SMX, SXT	≤2/38	-	≥4/76	≤10	11-15	≥16

Reference values used in this EQAS are according to CLSI (M100-S25), with the following exceptions:

* For the cephalosporins and meropenem, the application of the interpretative criteria is intended to indicate if the microorganism is a presumptive ESBL- or carbapenemase-producer. Reference values for the cephalosporins are according to CLSI M100-S25 Table 3A. These interpretative criteria are also applied for *Salmonella* and *Shigella* test strains for interpretation of AST results in this EQAS. **Reference values for meropenem are based on epidemiological cut off values from www.eucast.org.**

** These breakpoints should also be applied for *Shigella* test strains for interpretation of AST results in this EQAS

*** The publication 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. These interpretative criteria are also applied for *Shigella* test strains for interpretation of AST results in this EQAS.

Concerning ciprofloxacin susceptibility tests, please note that for results obtained in this proficiency test, the breakpoints for *Salmonella* are applied for *Shigella* also. These breakpoints for ciprofloxacin take into consideration mechanisms of resistance due to plasmid-mediated quinolone resistance genes (e.g. *qnr*-genes) and one-point-mutation in the gyrase gene.

Important notes: beta-lactam resistance

The following tests for detection of ESBL-, AmpC-, and carbapenemase-producing phenotypes are optional in relation to the current WHO GFN EQAS.

If choosing to participate in this component of the EQAS, all strains displaying reduced susceptibility to cefotaxime (CTX), ceftazidime (CAZ), and/or ceftriaxone (CRO) should be tested for ESBL-, AmpC, or carbapenemase-production by confirmatory tests. Reduced susceptibility to any of the above-mentioned antimicrobials indicates that the bacterial strain is an ESBL-, AmpC, or carbapenemase-producing phenotype.

Confirmatory test for ESBL production requires the 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) by microbroth dilution methods or E-test; 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) by disk diffusion; 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.

Detection of AmpC-type beta-lactamases can be performed by testing the bacterial culture for susceptibility to ceftiofloxacin (FOX). Resistance to FOX indicates the presence of an AmpC-type beta-lactamase.

Confirmatory test for carbapenemase production requires the testing of meropenem (MER). Reduced susceptibility to MER indicates that the bacterial strain is a carbapenemase-producer.

The classification of the phenotypic results should be based on the most recent EFSA (European Food Safety Agency) recommendations (EURL-AR Workshop 2016, http://www.crl-ar.eu/data/images/ws_april-2016/f11_efsa_criteria.pdf). The following summary of these recommendations indicate how the phenotypes should be categorized:

ESBL-phenotype:

- CTX or CAZ > 1 mg/L **AND**
- MER ≤ 0.12 mg/L **AND**
- FOX ≤ 8 mg/L **AND**
- Synergy for CTX : CTX/Cl and/or CAZ : CAZ/Cl

ESBL+AmpC-phenotype:

- CTX or CAZ > 1 mg/L **AND**
- MER ≤ 0.12 mg/L **AND**
- FOX > 8 mg/L **AND**
- Synergy for CTX : CTX/Cl and/or CAZ : CAZ/Cl

AmpC-phenotype:

- CTX or CAZ > 1 mg/L **AND**
- MER ≤ 0.12 mg/L **AND**
- FOX > 8 mg/L **AND**
- No synergy for CTX : CTX/Cl nor CAZ : CAZ/Cl
(note, presence of ESBLs is not excluded)

Carbapenemase-phenotype:

- MER > 0.12 mg/L
(note, presence of ESBLs or AmpCs is not excluded)

Other-phenotype:

- Not covered by any of the above categories **AND**
- CTX, CAZ, FOX, or MER > interpretative criteria as susceptible in Table 1 (i.e. exhibits reduced susceptibility)

No ESBL-, AmpC-, or carbapenemase:

- CTX, CAZ, FOX, and MER ≤ interpretative criteria as susceptible in Table 1 (i.e. exhibits susceptibility)

The genotype obtained by PCR and/or sequencing may be necessary to correctly categorize a bacterial test strain as either of the categories, ESBL-, AmpC, and/or carbapenemase-producer, but is not requested as part of this WHO GFN EQAS.

3.4 Handling the *Campylobacter* strains

The *Campylobacter* test strains as well as the *C. jejuni* reference strain (NCTC 11351) are supplied in the form of LYFO DISK®. To revive the strains, see instructions in section 3.1.1 above

3.5 Identification of *Campylobacter*

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

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

The *Campylobacter* test strains and the *C. jejuni* reference strain ATCC33560 should be tested for susceptibility to as many antimicrobials as possible among the ones mentioned in the test form. It should be noted 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 (European Committee on Antimicrobial Susceptibility Testing; www.eucast.org; Table 2). Consequently, only two categories of characterisation (resistant, R or susceptible, S) are allowed. In the evaluation report that you receive upon result submission, you can find that obtained interpretations in agreement with the expected interpretation, will be categorised 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 form).

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 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>	MIC (µg/mL)	MIC (µg/mL)
	R is > <i>C. jejuni</i>	R is > <i>C. coli</i>
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

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 enteric pathogen

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

4 REPORTING OF RESULTS AND EVALUATION

We recommend that you write your results in the enclosed test forms and that you read carefully the description 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 categorised as 'correct', while results deviating from the expected interpretation are categorised as 'incorrect'.

Results must be submitted no later than 31 December 2016.

Results must be submitted directly via the Internet based database. Should you not be able to access the Internet, you may return the completed test forms scanned by e-mail to the National Food Institute, Denmark.

All results will be summarized in a report which will be publicly available. 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 WHO GFN EQAS Coordinator:

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6 HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE

Please carefully 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 can browse back and forth in the pages of the database. Always remember to save your input before leaving a page.

- 1) Enter the WHO Collaborating Centre website (from <http://www.antimicrobialresistance.dk>), then
 - a. Click on 'EQAS'
 - b. Click on the link for the interactive database (<http://eqas.food.dtu.dk/who>)
 - c. Write your username and password in lower-case letters and click on 'Login'.
You can find your username and password in the letter following your strains.
Your username and password will remain unchanged in future trials. Do not hesitate to contact us if you experience problems with the login.
- 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' – ALWAYS 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 (≤), greater than (>) or greater than or equal to (≥).
- 4) In the data entry pages '*Salmonella* strains 1-8',
 - a. 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.
 - b. 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)

- c. Enter the zone diameters 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 (resistant), I (intermediate) or S (susceptible)
- e. If you performed confirmatory tests for ESBL production, select the appropriate result.
- f. If relevant, fill in the field related to comments (e.g. which antisera you miss for complete serotyping)
- g. Click on 'Save and go to next page'

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

- 5) In the data entry page '*E. coli* reference strain':
 - a. Enter the zone diameters 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 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

- 7) 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.
- 8) After browsing all pages in the report, you will find a new menu. You can choose 'EQAS 2016 start page', 'Review evaluated results' (a printer friendly version of the evaluation report is also available) or 'Go to WHO GFN homepage'.

End of entering your data – thank you very much!