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TECHNICAL REPORT

Fourth external quality assessment on species identification and antimicrobial susceptibility testing of *Campylobacter*

2018

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Abbreviations

AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
DD	Disk diffusion inhibition zone
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological Cut-Off Value
EFSA	The European Food Safety Authority
EQA	External Quality Assessment
ESBL	Extended Spectrum Beta-Lactamase
EU/EEA	European Union/European Economic Area
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FWD	Food- and Waterborne Diseases and Zoonoses
FWD-Net	European Food- and Waterborne Diseases and Zoonoses Network
IATA	International Air Transport Association
MIC	Minimum inhibitory concentration
NA	Not applicable
ND	Not determined
NWT	Non-wild type
R	Resistant
S	Susceptible
SSI	Statens Serum Institut
TESSy	The European Surveillance System
WT	Wild type

Executive summary

Since 2008, the EU/ EEA countries have been able to report antimicrobial resistance (AMR) data to the European Surveillance System (TESSy) as part of the routine surveillance data for salmonellosis and campylobacteriosis. In 2014, ECDC published an EU protocol for harmonised monitoring of AMR in human *Salmonella* and *Campylobacter* isolates (updated in 2016). In addition, ECDC launched an external quality assessment (EQA) scheme for antimicrobial susceptibility testing (AST) for *Salmonella* and *Campylobacter* to support the implementation of the EU protocol in the EU Member States and EEA countries and to get an overview of the quality of the AMR data reported to ECDC.

This report presents the results of the fourth round of the EQA on antimicrobial susceptibility testing (AST) for national public health laboratories for *Campylobacter* (*Campylobacter* EQA4-AST) within the Food- and Waterborne Diseases and Zoonoses Network (FWD-Net). The objectives of this EQA4-AST were to determine the accuracy of quantitative AST results reported by participants; to identify common laboratory problems related to the guidance in the EU protocol, and to assess the overall comparability of routinely collected AST data from national public health reference laboratories across Europe.

The *Campylobacter* EQA4-AST covered species identification and AST in *Campylobacter* spp. Twenty-two national public health reference laboratories in the EU/EEA participated in the EQA that took place during the period March to December 2018. In addition, six EU candidate/potential candidate countries (EU enlargement countries) participated in the EQA. This report focuses only on the results and evaluation from the EU/EEA countries.

Strains for the EQA were selected according to their current relevance to public health in Europe and shipped to the participating laboratories. Participation in the EQA involved testing and reporting on three mandatory antimicrobials (ciprofloxacin, erythromycin and tetracycline), with an option to report on an additional antimicrobial, gentamicin. Test results from all participants were evaluated and individual feedback provided.

The test results for antimicrobial susceptibility were analysed using two different approaches. The laboratories reported their results as disk diffusion (DD) and minimum inhibitory concentration (MIC) values that were compared to the value established by the EQA provider, either by calculating mm difference for disk diffusion values or number of dilution differences for minimum inhibitory concentration values. Reported quantitative results were further interpreted as wild type or non-wild type (WT or NWT) based on the available Epidemiological Cut-Off Values (ECOFFs) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and compared to the expected results determined by the EQA provider. Reporting of the species identification (*C. jejuni* or *C. coli*) was mandatory as this is a requirement for the correct interpretation using ECOFFs.

Twenty laboratories reported the *Campylobacter* species and all identifications were correct, except for a single result for strain C18.0005 which one laboratory falsely reported as *C. coli*. Two laboratories did not submit results for species identification. All 22 participating national public health reference laboratories reported results for the mandatory antimicrobials ciprofloxacin, erythromycin, and tetracycline and thus fulfilled the requirement for participating in the EQA. Fourteen laboratories reported additional results for gentamicin.

Overall, there was a satisfactory correspondence between the expected results established by the EQA provider and the results reported by the participating laboratories. For the mandatory antimicrobials, the relative accuracy, (i.e. the percentage of DD and MIC results within the accepted range of the expected result) was 70% for DD and 76% for MIC results. When the results were interpreted using EUCAST ECOFFs, 89% of the DD results and 96% of MIC results were in accordance with the expected results. For disk diffusion, this is not such a good result as in the previous EQA round (EQA3-AST), 79% of DD results were within the expected range and all DD results were correct when interpreted with ECOFFs.

The performance of the individual laboratories varied considerably when comparing the reported results with the expected values. For the mandatory antimicrobials, the percentage of correct quantitative results ranged from 37% to 100% for DD results and from 33% to 100% for MIC results. This large range in results indicates that some laboratories may need further support to identify problems and correct them in their laboratory procedures. Two laboratories in particular were responsible for many of the incorrect quantitative results that also were incorrect when interpreted using ECOFFs.

No common laboratory problems were identified. However, a few laboratories did not entirely comply with the guidance in the harmonised EU AST protocol (e.g. using disk loads of antimicrobials that differed from those recommended or establishing MIC values using antimicrobial concentration ranges that did not comply with the recommendations set to cover both the ECOFFs and the clinical breakpoints).

The surveillance system implemented as part of TESSy relies on the capacity of the European Food- and Waterborne Diseases and Zoonoses Network (FWD-Net) laboratories to produce comparable AST results. Overall, the results from the *Campylobacter* EQA4-AST indicate that it is feasible to compare AST results from the national public health reference laboratories when applying ECOFFs. However, improvements are warranted at a few of the laboratories.

1. Introduction

1.1 Background

The European Centre for Disease Prevention and Control (ECDC) is a European Union (EU) agency with a mandate to operate European infectious disease networks and to identify, assess, and communicate current and emerging threats to human health from communicable diseases. As part of its mission, ECDC fosters the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents, which may threaten public health. One of the ways in which ECDC does this is by supporting the implementation of quality assurance schemes [1].

External quality assessment (EQA) is part of a quality management system. It evaluates the performance of laboratories by using material that is specifically prepared and supplied for this purpose.

ECDC supports a series of EQAs for EU/EEA countries within the disease networks. The aim of the EQAs is to identify areas requiring improvement in laboratory diagnostic capacity and further characterisation as relevant for the surveillance of the diseases listed in Commission Implementing Decision 2018/945/EU [2], and to ensure the reliability and comparability of results from laboratories in all EU/EEA countries. The main objectives of EQA schemes include:

- assessment of the general standard of performance ('state of the art');
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration);
- evaluation of individual laboratory performance;
- identification and justification of problem areas;
- provision of continuing education;
- identification of training activity requirements.

The Unit for Foodborne Infections at Statens Serum Institut (SSI) was awarded the framework service contract 'External quality assessment on antimicrobial susceptibility testing (AST) for national public health laboratories for *Salmonella* and *Campylobacter*' for the two lots covering Lot1 *Salmonella* and Lot2 *Campylobacter* for the period 2014–2018. The contract covers the organisation of an EQA exercise to test antimicrobial susceptibility and detect Extended Spectrum Beta-Lactamase (ESBL)-producers, acquired AmpC and carbapenemase-producers in *Salmonella* and identify species and test antimicrobial susceptibility in *Campylobacter*. This report presents the results of the fourth EQA exercise under the framework contract (*Campylobacter* EQA4-AST).

1.2 Surveillance of *Campylobacter* antimicrobial resistance

Antimicrobial resistance (AMR) is a serious threat to public health in Europe, leading to mounting healthcare costs, treatment failure and deaths. The issue calls for both concerted efforts at Member State level and close international cooperation in order to preserve future antimicrobial effectiveness and access to effective treatment for bacterial infections. Surveillance of AMR is a fundamental part of an effective response to this threat, and surveillance results constitute an essential source of information on the magnitude and trends of resistance.

Campylobacteriosis, followed by salmonellosis, is the leading cause of zoonotic foodborne diseases in the EU/EEA, with approximately 250 000 laboratory-confirmed cases reported in 2017 [3].

EU surveillance of AMR in foodborne human infections is carried out within the Food- and Waterborne Diseases and Zoonoses Network (FWD-Net), under the auspices of ECDC. Since 2008, the EU/EEA countries have been able to report AMR data to the European Surveillance System (TESSy) as part of the routine surveillance data for salmonellosis and campylobacteriosis. The European Food Safety Authority (EFSA) also collects AMR data on zoonoses and zoonotic agents in food-producing animals and food in accordance with Directive 2003/99/EC [4] and Implementing Decision 2013/652/EU [5]. Since 2012, both EFSA and ECDC have strived to harmonise the AMR monitoring in zoonoses and zoonotic agents within their respective areas but also between the areas in order to obtain data that can be compared across the sectors. This work was also requested by the European Commission in its Commission Action Plan on AMR and in 2014, ECDC published an EU protocol for harmonised monitoring of AMR in human *Salmonella* and *Campylobacter* isolates, which was further updated in 2016 (hereafter referred to as the harmonised EU AST protocol) [6]. The harmonised EU AST protocol is primarily designed for use by the national public health reference laboratories or other nationally recognised public health laboratories to guide the susceptibility testing needed for EU surveillance and reporting to ECDC. Since July 2018, in accordance with Commission Implementing Decision 2018/945/EU, the EU Member States have been required to test and report AST results for a representative subset of *Salmonella* and *Campylobacter* isolates according to the methods and criteria specified in the harmonised EU AST protocol [2].

The EU surveillance objectives for antimicrobial resistance in zoonotic bacteria, specifically *Salmonella* spp. and *Campylobacter* spp. [6] are:

- to monitor, in human clinical isolates, trends in the occurrence of resistance to antimicrobial agents relevant for treatment of human *Salmonella* and *Campylobacter* infections, including comparison with food/animal isolates;
- to monitor, in human clinical isolates, trends in the occurrence of resistance to other antimicrobial agents of public and animal health importance, including comparison with food/animal isolates;
- to monitor, in human clinical isolates, the prevalence of ESBL, plasmid-encoded Ambler class C β-lactamases (pAmpC) and carbapenemases;
- to use antimicrobial resistance patterns to characterise human clinical isolates (i.e. as an epidemiological marker), to support identification of outbreaks and related cases;
- to identify and monitor, in human clinical isolates, genetic determinants of resistance that are important for public health (e.g. to aid recognition of epidemic cross-border spread of multi-drug resistant *Salmonella* strains);
- to monitor, in human clinical isolates, trends in resistance to antimicrobial agents where these agents may be needed for future therapeutic use.

1.3 Objectives of the EQA4-AST on *Campylobacter*

The aim of the EQA4-AST was to support implementation of the harmonised EU AST protocol for monitoring antimicrobial resistance in human *Salmonella* and *Campylobacter* isolates and to assess the quality of the AST data obtained using minimum inhibitory concentration (MIC) and/or disk diffusion (DD) at national public health reference laboratories across Europe.

The *Campylobacter* EQA4-AST covered the laboratory procedure when producing AST data including species identification, as this is a prerequisite for interpreting quantitative data following the EUCAST ECOFFs. The objectives of the *Campylobacter* EQA4-AST scheme were:

- to determine the relative accuracy of quantitative AST results reported by participating laboratories;
- to identify common laboratory problems related to the testing of individual antimicrobials and the guidance in the harmonised EU AST protocol;
- to assess the overall comparability of routinely collected AST results from national public health reference laboratories across Europe based on the results of the EQA.

The term 'relative accuracy' used to describe the quantitative result means that the results from the participating laboratories are comparable with the expected result determined by the EQA provider.

2. Study design and methods

2.1 Organisation

The entire EQA process, from planning to final reporting, took place during the period March to December 2018 and included species determination and AST of eight *Campylobacter* spp. strains.

On 30 April 2018, SSI emailed invitations to the 27 laboratories in the FWD-Net that had been nominated as contact points for the EQA by the national focal points for food- and waterborne diseases and zoonoses in the FWD-Net. Twenty-two national public health reference laboratories in EU/EEA countries accepted the invitation to participate. In addition, six laboratories from EU candidate/potential candidate countries (EU enlargement countries) participated in the EQA. The list of participants is presented in Figure 1 and Annex 1.

The EQA test-strains were sent to the laboratories on 12 July 2018. The participants were asked to submit their results using a web-based electronic submission form. All laboratories were assigned an arbitrary laboratory number by the EQA provider and these numbers are used throughout this report to ensure the anonymity of the participating laboratories.

2.2 Selection of strain panel

Strains were selected for the EQA4-AST based on the following criteria:

- that they should represent strains commonly reported in the EU/EEA;
- that they should remain stable during the preliminary testing period at the organising laboratory.

The EQA-provider tested 16 *Campylobacter* spp. strains and selected eight (five *C. jejuni* and three *C. coli*), with different resistance profiles (Table 1).

In order to be able to determine the accuracy of the reported results, the EQA provider established expected results for MIC and DD values for the test strains. The expected values were established in accordance with the harmonised EU AST protocol [6]. The DD values were determined using disks from Oxoid and the MIC values were determined using the micro-broth-dilution-based MIC system from TREK diagnostic systems© (Thermo Scientific). The expected results were verified by EUCAST's Development Laboratory for Antimicrobial Susceptibility Testing of bacteria, c/o Clinical Microbiology, Central Hospital, Växjö, Sweden.

Table 1. Species and resistance profiles of the *Campylobacter* EQA4-AST test strains

Strain	Species	Resistance profile[1] (NWT)
EQA_AST.C18.0001	*Campylobacter jejuni*	Ciprofloxacin, tetracycline
EQA_AST.C18.0002	*Campylobacter jejuni*	Ciprofloxacin, erythromycin, tetracycline
EQA_AST.C18.0003	*Campylobacter jejuni*	Ciprofloxacin, tetracycline
EQA_AST.C18.0004	*Campylobacter jejuni*	Tetracycline
EQA_AST.C18.0005	*Campylobacter jejuni*	Ciprofloxacin, erythromycin, gentamicin, tetracycline
EQA_AST.C18.0006	*Campylobacter coli*	Ciprofloxacin, tetracycline
EQA_AST.C18.0007	*Campylobacter coli*	Wild type (i.e. no acquired resistance)
EQA_AST.C18.0008	*Campylobacter coli*	Tetracycline

[1] *Based on MIC values and according to EUCAST ECOFFs - see Annex 2.*

2.3 Preparation and shipment of the strains

Cultures of the test stains were grown on blood agar and transferred to Stuart's transport medium using charcoal swabs. The parcels with the strains were shipped on 12 July 2018 from SSI and labelled in accordance with the International Air Transport Association (IATA) regulations (UN 3373 Biological Substance, Category B).

2.4 Testing and reporting

The EQA4-AST included AST of four first-priority antimicrobials listed in the harmonised EU AST protocol [6] and species identification. It was a prerequisite for the participating laboratories to test three of the first-priority antimicrobials (ciprofloxacin, erythromycin and tetracycline) and there was an additional option to report results for the fourth, gentamicin.

Instructions for AMR testing were given in the invitation letter, in an email following shipment of strains, and in the reporting forms. Participants were asked to follow the harmonised EU AST protocol and could submit results from

broth dilution and gradient strip methods (MIC results) or disk diffusion (DD). The participants were asked to report the test result as a value (mg/L or mm). The harmonised EU AST protocol mainly refers to the methods recommended by EUCAST, available on EUCAST's website [7] and it was expected that the participants would follow these instructions. No instructions were given with regard to species identification and it was anticipated that the laboratories would use their own standard method.

When the test strains were dispatched the laboratories received an email with a link to an electronic submission form, constructed using Enalyzer software (www.enalyzer.com), for the reporting of the results in a fixed format. The deadline for submitting the results was 28 July 2018. This deadline was extended to 3 August 2018 due to delays in the freight handling of packages to some of the laboratories. Data reporting included *Campylobacter* species, quantitative DD and/or MIC results, information about the methods used, growth media, brand of disks for DD and brand of strips or panels for MIC determination.

2.5 Data analysis

The participating laboratories provided test results (i.e. inhibition zones measured as diameter in mm for disk diffusion methods and MIC values for broth dilution and gradient strip methods.) It was mandatory to report the species identification (*C. jejuni* or *C. coli*) as this information is needed for the correct interpretation using EUCAST ECOFFs.

The test results were analysed using different approaches:

1. The laboratories reported their results and these values were compared with the expected results established by the EQA provider, either by calculating the difference in mm for DD values or the number of dilution differences for MIC values.
2. DD results (values in mm) generated with disk loads that deviated from the recommended disk loads were excluded from the analysis and classified as 'ND' (not determined).
3. MIC dilution differences between the reported and expected results were calculated taking into account several situations:
   - If the operator of the reported value was >, results were approximated to = the next dilution step.
   - If the operator of the reported value was <=, results were approximated to = the same dilution step.
   - If the operator of both the reported value and the expected value were > and the participant's range for a given antimicrobial was wider than the EQA provider's range, the dilution difference was designated as '0'.
   - If the expected result was outside of the range tested by the participant but within the EQA providers' range, it was not possible to calculate the dilution difference.

MIC values generated by the use of gradient strips for MIC determination were transformed on a base-2 log scale, rounded to the nearest two-fold dilution, and then retransformed to enable comparison with the results from dilution methods.

The quantitative results were categorised into three groups. The first group, designated correct, included DD results that were within ±4 mm difference of the expected result and MIC results that were within one dilution difference. The second group were results outside the accepted area (incorrect) and the third group included MIC results that were not within the relevant range for comparison with expected results (ND).

Reported qualitative results were interpreted based on the available EUCAST ECOFFs. This interpretation (wild type (WT) or non-wild type (NWT)) was compared with the expected result, as determined by the EQA provider. These qualitative results where categorised into three groups. The first group included results that complied with the expected interpretation (correct), the second group included the interpreted results that did not comply with the expected results (incorrect), and the third group included results where this comparison was impossible due to the lack of EUCAST ECOFFs for the antimicrobial (NA). The applied EUCAST ECOFFs can be found in Annex 1. In the event of incorrect or missing *Campylobacter* species identification, the reported DD and MIC data were analysed using the correct species result determined by the EQA provider.

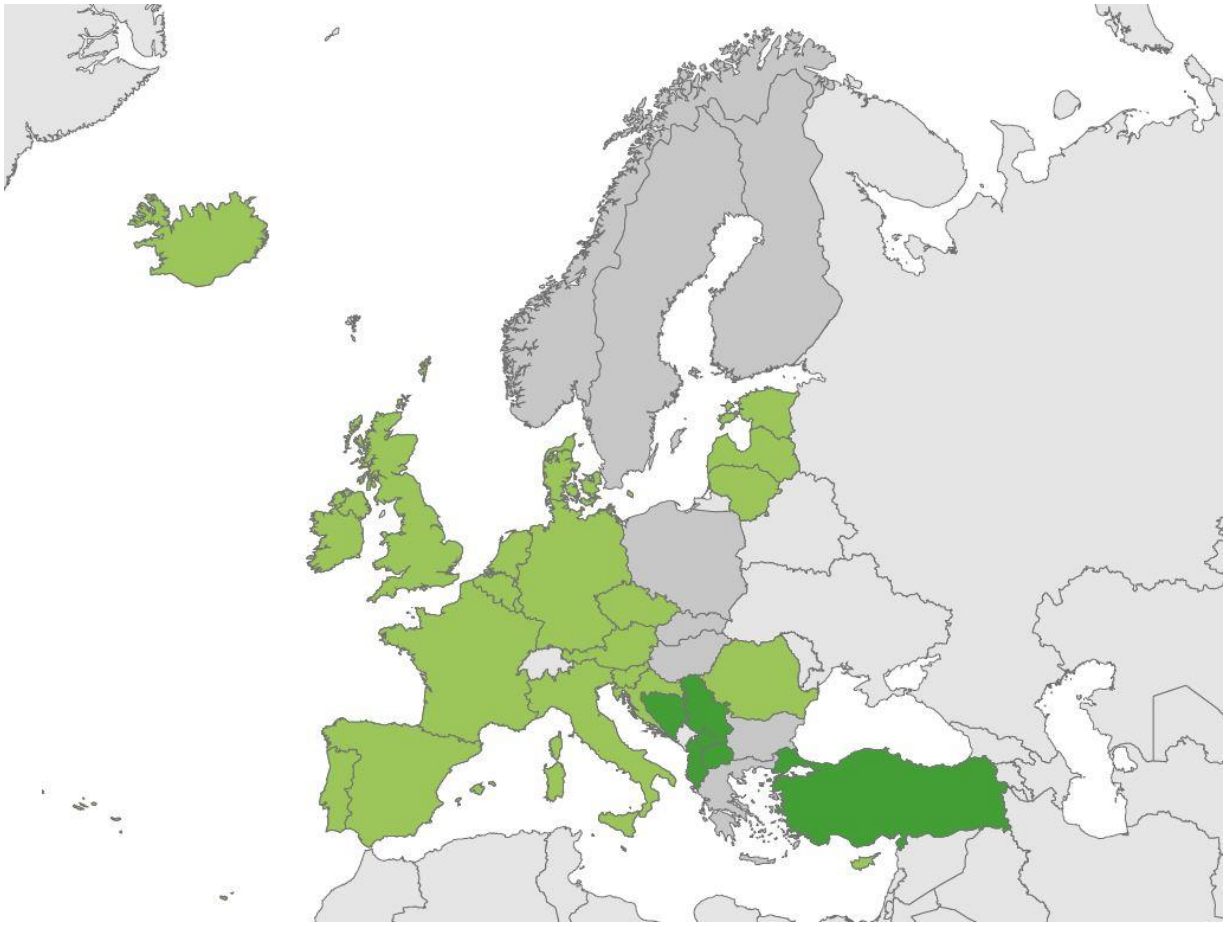
3. Results

3.1 Participation

Twenty-two laboratories from EU/EEA countries participated in the *Campylobacter* EQA4-AST (Figure 1), along with six EU candidate/potential candidate countries (EU enlargement countries). Test results from all participants were evaluated and individual feedback provided on 12 October 2018. All participants also received a file sent by e-mail on 19 October 2018, with the distributions of all reported MIC and DD (mm) values for all test strains/antimicrobials included in the EQA4-AST.

This report focuses solely on the results and evaluation of data from the EU/EEA countries. The 22 participating countries susceptibility-tested the test strains for the mandatory antimicrobials, ciprofloxacin, erythromycin, and tetracycline and 13 countries also reported results for gentamicin. Due to delays during shipment, one laboratory was unable to recover the strains EQA_AST.C18.0001 and EQA_AST.C18.0005. After consultation with the EQA provider, it was decided that this laboratory should not provide results for these two test strains.

Figure 1. EU/EEA countries (light green) and EU candidate/potential candidate countries (dark green) participating in *Campylobacter* EQA4/AST, 2018



3.2 Applied methods

A total of 15 laboratories reported DD results. Most laboratories used the EUCAST recommended disk loads. Three laboratories used disk loads that did not comply with the recommendations in the harmonised EU AST protocol. One laboratory (L021) used a ciprofloxacin load of 10 µg instead of 5 µg, for erythromycin one laboratory (L024) used a disk load of 5 µg instead of 15 µg and for gentamicin one laboratory (L020) used a disk load of 30 µg instead of the recommended 10 µg.

Most of the DD results (86%) were produced on Mueller Hinton agar, supplemented with 5% horse blood and L ß-NAD at a concentration of 20 mg/L, and the remainder were produced on the same media without the NAD component.

Disks from Oxoid were used to produce 49% of the DD results and disks from Bio-Rad- and Becton Dickinson were used to generate 24% and 14% of the results, respectively. Disks from i2A diagnostics and Mastdiscs were used to make 8% and 6% of the results, respectively.

For AST DD testing of *Campylobacter*, EUCAST recommends an incubation temperature of 41±1°C for 24 hours in a micro-aerobic environment. Three laboratories did not follow this recommendation and used an incubation temperature of 35–37°C.

Eleven laboratories reported MIC results. Forty-one percent of the MIC results were obtained using gradient strips and all gradient strip results were obtained with Etest from bioMérieux. The remaining 59% of the MIC results were obtained using broth dilution methods; 31% were generated with the TREK sensititre microdilution system (also used by the EQA provider), 18% were made using in-house assays and 8% of the broth dilution results were reported as having been produced using the brand 'SVA'. Two laboratories used in-house assays that applied concentration ranges that did not comply with the recommendations in the harmonised EU AST protocol. This meant that it was impossible to calculate the dilution difference for some of the quantitative MIC results applying the principles described in section 2.5. Consequently, some of the results from these two laboratories were classified as ND.

3.3 *Campylobacter* species identification

Twenty laboratories reported *Campylobacter* species of the test strains. All species identifications were correct except for one result relating to strain *C. jejuni* C18.0005 which one laboratory, L032, reported as *C. coli*. Two laboratories, L12 and L19, did not report the species of any of the test strains.

3.4 Antimicrobial susceptibility testing of *Campylobacter*

The laboratories' DD and MIC results, along with the percentage of correct qualitative and quantitative results for the eight test strains, are presented in Table 2. Eleven of 22 laboratories tested all the mandatory antimicrobials only using DD, seven only used MIC determinations, and four laboratories reported both DD and MIC results. Six laboratories used broth dilution methods and five laboratories used gradient strip for MIC determinations.

Disk diffusion

Fifteen laboratories reported 432 DD results for the test strains. Twenty-four of these results were classified as ND due to deviating disk loads. Of the remaining 408 DD results, 292 (72%) were classified as correct and within ±4 mm of the expected value. For the mandatory antimicrobials, 253/360 (70%) of the DD results were classified as correct. The correct DD results from the individual laboratories ranged from 38% to 96%. After interpretation of the DD results using EUCAST ECOFFs, the overall proportion of correct qualitative DD results was 89% for the mandatory antimicrobials and 100% for gentamicin. Two laboratories had less than 90% correct qualitative results, L019 (54%) and L040 (83%), for the mandatory antimicrobials and L019 reported 11 of the total 25 incorrect qualitative results.

Dilution and gradient strip

Eleven laboratories reported 314 MIC results for the test strains. Twenty of these MIC results were classified as ND due to deviating test ranges. Of the 294 remaining MIC results, 229 (78%) were classified as correct and within ± one dilution rate of the expected value. For the mandatory antimicrobials, 189/237 (80%) were classified as correct. The correct MIC results for the individual laboratories ranged from 46% to 100%. After interpretation of the MIC results using EUCAST ECOFFs, the overall proportion of correct qualitative MIC results were 96% for the mandatory antimicrobials and 97% for gentamicin. All laboratories except one (L012) reported more than 90% correctly interpreted qualitative results. L012 reported 71% correct qualitative results and eight of the 12 MIC results that were incorrect when evaluated using the EUCAST ECOFFs (six of these were MIC results for tetracycline.) The 20 MIC results classified as ND were reported by two laboratories, L007 (12) and L012 (8). Seventeen of the ND MIC results were evaluated as correct when interpreted with ECOFFs and it was not possible to evaluate the remaining three MIC results as there was no ECOFF available for gentamicin/*C coli.*

Table 2. Laboratories participating in the *Campylobacter* EQA4-AST (represented by an arbitrary number) from the EU/EEA countries, participation with mandatory antimicrobials by method and percentage of correct results for the test strains (AST.C18.0001-0008)*

	DD									MIC									
	Mandatory						Optional			Mandatory						Optional			
Laboratory ID	Ciprofloxacin	Erythromycin	Tetracycline	Correct quantitative results	ND	Correct qualitative results	Gentamicin	Correct quantitative results	Correct qualitative results	Ciprofloxacin	Erythromycin	Tetracycline	Correct quantitative results	ND	Correct qualitative results	Gentamicin	Correct quantitative results	ND	Correct qualitative results
L003										B	B	B	92%		100%	B	100%		100%
L004				88%		96%		88%	100%	G	G	G	79%		96%	G	63%		80%
L006				96%		100%													
L007				88%		100%				B	B	B	46%	50%	100%				
L008										B	B	B	96%		100%	B	63%		100%
L011				67%		96%		38%	100%										
L012**										B	B	B	29%	4%	71%	B	0%	88%	80%
L015				58%		96%													
L016				96%		100%													
L017				38%		92%		38%	100%										
L019**				38%		54%													
L020				75%		92%		0%											
L021				50%	33%	94%		25%	100%	G	G		75%		94%				
L024				67%	33%	100%		88%	100%										
L028				67%		96%		88%	100%										
L030				88%		100%													
L032**										G	G	B	71%		96%	G	75%		100%
L034										B	B	B	92%		100%	B	100%		100%
L037										G	G	G	71%		100%	G	0%		100%
L039										G	G	G	83%		100%				
L040				75%		83%		63%	100%	B	B	B	100%		100%	B	100%		100%
L043				67%		96%		63%	100%										
Total				70%	4%	89%		54%	100%				76%	5%	96%		63%	11%	97%

: *Results for all test strains reported. B: Broth microdilution, G: Gradient strip.*

** Results classified as NA are excluded from the total number of results.*

*** The quantitative results were evaluated using species identification determined by the EQA provider.*

**** No gentamicin ECOFF has been established for C. coli and disk diffusion.*

3.4.1 Results by antimicrobial and by strain

Table 3 gives an overview of the DD and MIC results by antimicrobial and also presents the MIC results separately for gradient strip and broth dilution methods.

With regard to DD, the lowest scores for correct quantitative results were recorded for gentamicin and erythromycin (61% and 64% of the results respectively were correct.) The highest score was for ciprofloxacin with 80% of results correct. For MIC methods overall, between 70% and 86% of the quantitative results were correct for the four antimicrobials tested. When divided by type of MIC method, the lowest scores for gradient strips were observed for gentamicin and ciprofloxacin (46% and 55%, respectively), and with broth dilution for tetracycline (76%). The highest score for gradient strip results was for tetracycline (95%) and with broth dilution, similar results were achieved for ciprofloxacin, erythromycin and gentamicin (86–88%).

The proportion of correct EUCAST ECOFF interpreted qualitative results by antimicrobial ranged from 89% to 100%, where the lowest proportion of correct results, 89%, was observed for erythromycin DD results and for tetracycline broth dilution results.

The distribution of DD values (mm) and the distribution of MIC values (mg/L) for the participating laboratories are presented in Table 4 and 5, respectively.

Control strain

EUCAST has defined acceptance criteria relating to the size of the inhibition zone for the control strain, *C. jejuni* ATCC 33560, for ciprofloxacin, erythromycin and tetracycline [8]. For ciprofloxacin and tetracycline, two and three of the reported inhibition zones for the control strain, respectively, were too large (Table 4). For erythromycin, one DD result was too small and one was too large. For gentamicin, the reported values for the control strain were compared with the expected valued established by the EQA provider. Two DD values for gentamicin were outside the accepted range, with one value being too low and one being too high. One laboratory, L043, did not report any DD results for the control strain for any antimicrobial, L21 did not report DD results for ciprofloxacin, and L024 did not report DD results for erythromycin. The incorrect results for the control strains were submitted by six laboratories.

EUCAST has not defined acceptance criteria for MIC values for the control strain *C. jejuni* ATCC 33560 and therefore values established by the Clinical and Laboratory Standard Institute were used as guidance [9]. The accepted range included values based on incubation at both 37°C for 48 hours and 42°C for 24 hours. For erythromycin and gentamicin, all results for the control strain were within the accepted range (Table 5). For ciprofloxacin, one result was one dilution higher that the accepted range and for tetracycline, two values were one dilution lower than the accepted range.

Test strains

The reported DD values were generally in accordance with the expected values (Table 4). Strain C18.005 deviated markedly from the expected results for erythromycin as nine of the 13 results were incorrect and all nine incorrect results exhibited varying inhibition zones. In general, most of the incorrect results were due to the zones being too large, most noticeably for gentamicin. For three strain/antimicrobial combinations (C18.004/ciprofloxacin, C18.006/erythromycin, and C18.007/erythromycin), the accepted zone deviations included values on the 'wrong side' of the ECOFF and three such results were reported for erythromycin (i.e. quantitatively correct but qualitatively incorrect).

A high number of incorrect MIC values were observed for ciprofloxacin (Table 5). Most of the incorrect MIC values were generated using gradient strip methods (Table 3). The same phenomenon was observed for gentamicin where most of the incorrect values were also generated using gradient strip methods. The problem with erythromycin for strain C18.005 was also seen in the MIC results. For tetracycline, most of the incorrect DD values were due to the zones being too small. In addition, L012 performed rather poorly for MIC, with only 29% correct results using an in-house broth dilution method (Table 2).

Table 3. Performance per antimicrobial for DD and MIC for the three mandatory and one optional antimicrobial

Antimicrobial	Number of laboratories performing DD	Numbers of DD results within the accepted four mm difference of the total tested	Number of correct results when using EUCAST ECOFF
Disk diffusion			
Ciprofloxacin	14	90/112 (80%)	105/112 (94%)
Erythromycin	14	72/112 (64%)	100/112 (89%)
Tetracycline	15	91/120 (76%)	114/120 (95%)
Gentamicin*	8	39/64 (61%)	40/40 (100%)
Total DD		292/408 (72%)	359/384 (93%)
	Number of laboratories performing MIC (both gradient strips and broth-dilution)	**Numbers of MIC results within the accepted one dilution difference of the total tested**	**Number of correct results when using EUCAST ECOFF**
MIC total			
Ciprofloxacin	11	58/81 (72%)	85/86 (99%)
Erythromycin	11	72/84 (86%)	83/86 (97%)
Tetracycline	10	59/72 (82%)	72/78 (92%)
Gentamicin*	8	40/57 (70%)	38/40 (95%)
Total MIC		229/294 (78%)	278/290 (96%)
MIC gradient strips			
Ciprofloxacin	5	21/38 (55%)	38/38 (100%)
Erythromycin	5	32/38 (84%)	35/38 (92%)
Tetracycline	3	21/22 (95%)	22/22 (100%)
Gentamicin*	3	11/24 (46%)	14/15 (96%)
Total MIC gradient strips		85/122 (70%)	109/113 (96%)
MIC broth dilution			
Ciprofloxacin	6	37/43 (86%)	47/48 (98%)
Erythromycin	6	40/46 (87%)	48/48 (100%)
Tetracycline	7	38/50 (76%)	50/56 (89%)
Gentamicin*	5	29/33 (88%)	24/25 (98%)
Total MIC broth dilution		144/172 (84%)	169/177 (95%)

Results classified as NA and ND excluded.

** EUCAST has not determined a gentamicin ECOFF value for C. coli.*

Table 4. Distribution of *Campylobacter* DD values (mm) among the participating laboratories

Antibiotic	Strain	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
Ciprofloxacin	ATCC 33560																														2	1	2	2		1	2	1	1		1		
	C18.0001	11						1																																	1		1
	C18.0002	9	1	2		1																																					1
	C18.0003	10	1	1					1																																		1
	C18.0004	1																					1	2	1		1	2		2	2		1			1							
	C18.0005	13																																									1
	C18.0006	14																																									
	C18.0007	1																				**1**				1	1	3	1			3				2				1		
	C18.0008																										1		1	2	4			1		3				1			1
Erythromycin	ATCC 33560																				1		1	1		1	2		2	3	1					1							
	C18.0001																									2	1	3	1		2	2		2									1
	C18.0002	12	1																																								1
	C18.0003																				1					1	1	4	1			2	1			1							2
	C18.0004																	1			1	1	1	1	1	3		1	1					2	1								
	C18.0005	4									1	1			1	1						1		1		1		1			1												1
	C18.0006	1														1		1		1	1	3	1	1	2			1															
	C18.0007																	1		2	1		1	2	2	4		1															
	C18.0008																		1				1			3	1	3			1	1				2		1					
Tetracycline	ATCC 33560																											2	1	4	1		2	1		1					1	1	
	C18.0001					2					1	1		1	1	1	1	4					1																				2
	C18.0002	3					2	1				3	1	2	1		1																										1
	C18.0003	8	1			1	3					1																															1
	C18.0004	13	1			1																																					
	C18.0005	12	1							1																																	1
	C18.0006	12		2				1																																			
	C18.0007																											1		1		1	1		1		4	1	1	1		2		1
	C18.0008	1				1			1	1			2		1	2	1		2		2					1																	
Gentamicin	ATCC 33560															1					1			2		1			1		1												
	C18.0001																	1							1	2		1			1		1			1							
	C18.0002																				1					3	1			1					1	1							
	C18.0003																		1	1		1		1		1			1			2										
	C18.0004																		1					2		1			1	1		1		1								
	C18.0005	3		1		1		1					1		1																												
	C18.0006																		1		2				2	1			1					1								
	C18.0007																		1					1				2	3						1							
	C18.0008																			1				2		1	2		1						1							

: Expected value : Accepted range

The red line indicates ECOFF according to EUCAST for the respective antimicrobial, wild type/susceptible to the right of the red line.
The expected values for the control strain ATCC 33560 were from EUCAST, except for gentamicin, where the expected values were determined by the EQA provider.

Table 5. Distribution of MIC values (mg/L) among the participating laboratories

Antimicrobial	Strain	ND	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
		MIC results for the *Campylobacter* strains tested															
Ciprofloxacin	ATCC 33560		2	1	3	3	1										
	C18.0001	1								1	3	2		4			
	C18.0002	1							1	1	5	1		3			
	C18.0003	1								2	4	1	1	3			
	C18.0004			1	2	6	1		1								
	C18.0005	1						1			1		3	5			
	C18.0006	1									1	3	1	6			
	C18.0007				3	6	2										
	C18.0008			3	3	4	1										
Erythromycin	ATCC 33560					1	2	4	3								
	C18.0001				1	2	1	6									
	C18.0002	1									1		1		1	3	5
	C18.0003					1	3	7									
	C18.0004						3	4	4								
	C18.0005	1					1	1	1		1	1	1		1	3	
	C18.0006						1	2	4	4							
	C18.0007							6	3	2							
	C18.0008				1		1	6	3								
Tetracycline	ATCC 33560				2		2	1	4								
	C18.0001						1			1	1	3	3				
	C18.0002	1						1				2	4	2	1		
	C18.0003	1						1				1		2	5		1
	C18.0004	1									1	1		1	3		4
	C18.0005	1						1				1			4		3
	C18.0006	2										2			4	1	3
	C18.0007				1		7	1		1							
	C18.0008							1		1	2	2	3			1	
Gentamicin	ATCC 33560	1				3	3	2									
	C18.0001	1			3	2	2	1									
	C18.0002	1			2	2	3	1									
	C18.0003	1				2	5	1									
	C18.0004	1			2	3	2	1									
	C18.0005					1	1					5		1			
	C18.0006	1				1	5	2									
	C18.0007	1				2	4	2									
	C18.0008	1				1	3	4									

: Expected value : Accepted range

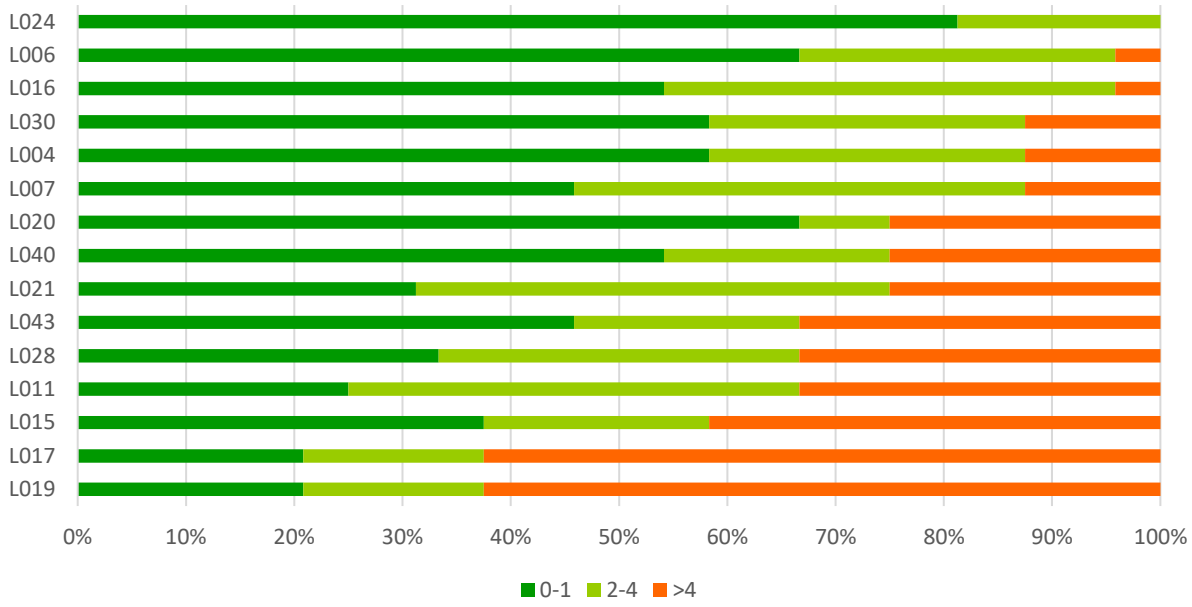
The red line indicates ECOFF according to EUCAST for the respective antimicrobial, wild type/susceptible to the left of the red line.
The expected values for the control strains ATCC 33560, are from the Clinical and Laboratory Standard Institute CLSI [9] and include values based on incubation at both 37°C for 48 hours and 42°C for 24 hours.

3.4.2 Individual laboratory results

Disk diffusion

The proportion of correct DD results for the mandatory antimicrobials reported by individual laboratories varied from 38% to 100% (Figure 2). One laboratory (L024) reported 100% correct DD results and two laboratories (L006 and L016) had more than 90% correct results. The lowest proportion of correct results, 37%, was reported by two laboratories (L017 and L019).

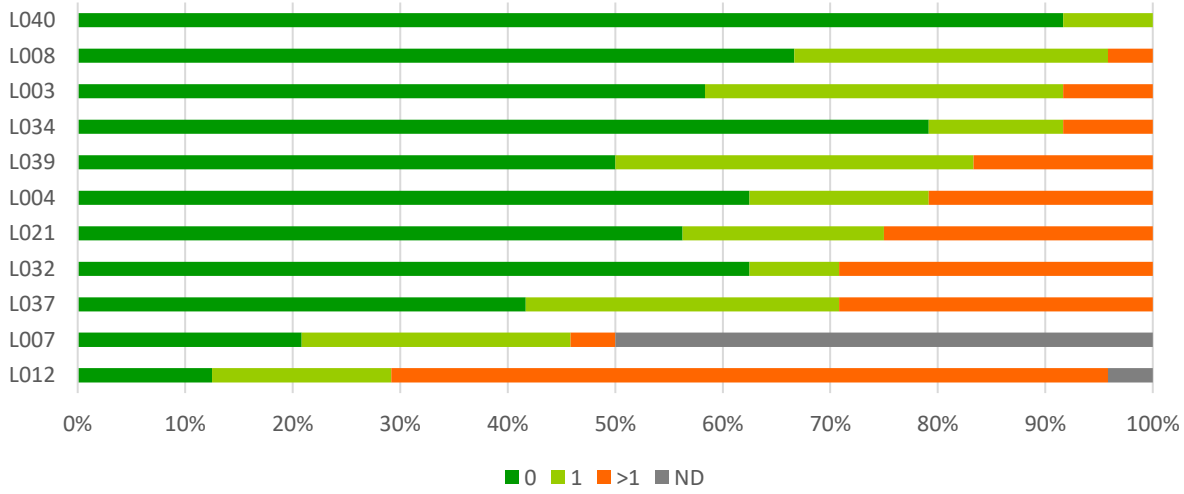
Figure 2. Distribution of DD (mm) differences compared to the expected result for the mandatory antimicrobials for *Campylobacter* by laboratory



Dilution and gradient strip

The proportion of correct MIC results for the mandatory antimicrobials reported by individual laboratories ranged from 29% to 100% (Figure 3). One laboratory (L040) reported 100% correct MIC results and three laboratories (L008, L003 and L034) reported more than 90% correct MIC results. Two laboratories (L007 and L012) reported MIC results that were classified as ND due to deviations in the testing ranges, one of them (L007) for half of the test results. However, it should be noted that all results from the latter laboratory were categorised correctly using ECOFFs (Table 2).

Figure 3. Distribution of MIC dilution differences compared to the expected results for the mandatory antimicrobials for *Campylobacter* by laboratory



4. Discussion

Since 2008, the EU Member States and EEA countries have been able to report AMR data to TESSy as part of the routine surveillance data for campylobacteriosis. In 2014, ECDC published the harmonised EU AST protocol (updated in 2016) with guidance on laboratory procedures and the interpretation of data [6]. The purpose of the EQA4-AST on *Campylobacter* was to evaluate the quality of the AST data generated in the FWD laboratory network when following the harmonised EU AST protocol. The data submitted were used to determine the relative accuracy of AST data and to assess its overall comparability. An additional aim was to collect information on the methods used by each laboratory to produce data on antimicrobial susceptibility.

Twenty-seven national public health reference laboratories in EU/EEA countries were invited to participate in the EQA and 22 countries accepted the invitation, meaning that the number of participating laboratories was similar to that for the previous *Campylobacter* EQAs. All laboratories submitted results for the mandatory antimicrobials and fulfilled the requirement for participating in the EQA.

Overall the logistics of the EQA went well. All laboratories, except one, were able to recover the test strains and the submission of results on the Enalyzer platform was efficient.

Some laboratories did not fully adhere to the recommendations given in the harmonised EU AST protocol but in general, the laboratories used disks with the recommended concentration of antimicrobials, and the media, incubation conditions and test ranges prescribed by EUCAST.

When interpreting the AST values for *C. jejuni* and *C. coli* different criteria apply and correct species identification is essential for the correct interpretation of AST data in relation to *Campylobacter*. For the test strains in this EQA the reported species was in line with the expected results, although two laboratories did not report any species for the test strains.

Overall, there was a satisfactory agreement between the quantitative results reported by the participants and the expected results established by the EQA provider. With a few exceptions, the test strains exhibited DD zones and MIC values that were distinct from the ECOFF values and, consequently, the interpreted qualitative results were generally better than the quantitative results.

The results submitted for erythromycin for strain C18.005 deviated from the expected results, both for the DD and MIC values. The strain was consistently resistant to erythromycin when tested by the EQA provider. High-level macrolide resistance in *Campylobacter* is commonly caused by a base substitution in the 23S rRNA gene, specifically A2075G, and less frequently A2074C/G [10]. DNA sequencing of C18.005 revealed that the strains carried the A2075G substitution in two of three copies of the 23S rRNA gene. It has been reported that ribosomal substitutions may not always occur in all three copies of the gene, and this can result in a lower level of resistance [10]. This could be one explanation for the deviation in the result for erythromycin obtained in relation to strain C18.005. Generally the laboratories demonstrated proficiency in erythromycin AST testing for the other test strains.

Most of the quantitative DD results for the wild type strains that differed from the expected results had inhibition zones that were too large. Some laboratories reported a number of DD results with very large inhibition zones, and a single laboratory, L19, reported 12 DD results that were 60 mm.

As with the EQA3-AST on *Campylobacter* [11], the number of correct MIC results for ciprofloxacin were rather low, as only 72% of the results were in accordance with the expected value. All incorrect MIC results generated by broth dilution were reported by the same laboratory that submitted results for six strains using an in-house method. All the other 17 incorrect results except one were gradient strip results, reported with MIC values of >32 where the expected broth dilution results were 8 or 16. The reason for this difference is unknown, but the difference in the MIC results did not affect the qualitative results.

The harmonised EU AST protocol recommends (micro-) broth dilution as the preferred testing method for monitoring purposes. However, validated methods of gradient strip diffusion or disk diffusion according to EUCAST protocols are also accepted. In the present EQA, 72% and 70% of the diffusion-based methods (DD and gradient disk results respectively) were correct when compared with the expected values, whereas 84% of the reported MIC results were correct. This is similar to the findings in the previous *Campylobacter* EQAs which support the recommendation that broth dilution should be the preferred testing method.

The performance of the individual laboratories varied substantially. For DD, the percentage of correct results for the mandatory antimicrobials varied from 37% to 100% by laboratory and for MIC results, the variation was from 33% to 100% correct results. This large range in results indicates that some laboratories may need further support to identify problems and, if necessary, adjust their laboratory procedures. Two laboratories in particular, one submitting MIC results and one submitting DD results, were responsible for many of the incorrect quantitative results that also were incorrect when interpreted using ECOFFS.

When interpreting the results with ECOFFs, the laboratories reported a total of 89% and 96% correct DD and MIC results, respectively for the mandatory antimicrobials. For the optional antimicrobial, gentamicin, they reported 100% and 97% respectively. These results are slightly lower than the results obtained in the EQA3-AST performed in 2017. They are also slightly lower than the results in the most recent EU reference laboratory network proficiency test for antimicrobial resistance [12], where the qualitative MIC results for the four antimicrobials tested in this EQA were all above 98.7%. Overall, the results from the *Campylobacter* EQA4-AST indicate that it is feasible to compare AST results from the national public health reference laboratories when applying ECOFFs. Nevertheless, improvements are warranted at a few of the FWD-Net laboratories.

5. Conclusions

A total of 22 national public health reference laboratories from the FWD network participated in the EQA. All laboratories, except two, performed *Campylobacter* species identification and all reported results for the mandatory antimicrobials ciprofloxacin, erythromycin, and tetracycline, thus fulfilling the requirement for participating in the EQA. In addition, fourteen laboratories reported results for gentamicin.

There were no issues identified linked to the content of the harmonised EU AST protocol. The problems arose among the few laboratories that did not entirely comply with the protocol (e.g. using disk loads of antimicrobials that differed from those recommended or establishing MIC values using test ranges for antimicrobials that did not comply with the recommendations.)

Overall, there was a satisfactory correspondence between the expected results established by the EQA provider and the results reported by the participating laboratories. For the mandatory antimicrobials the relative accuracy, (i.e. the percentage of DD and MIC results that were within the accepted range from the expected result) was 70% for disk diffusion and 76% for MIC results. When the results were interpreted with EUCAST ECOFFs, 89% of the DD results and 96% of MIC results were in accordance with the expected results.

When compared with the expected values, the performance of individual laboratories varied substantially. For the mandatory antimicrobials, the percentage of correct quantitative results ranged from 37% to 100% for DD results and from 33% to 100% for MIC results. This large range in results indicates that some laboratories may need further support to identify problems and, if necessary, to adjust their laboratory procedures. Two laboratories in particular were responsible for many of the incorrect quantitative results that also were incorrect when interpreted using ECOFFs.

Overall, the results from the *Campylobacter* EQA4-AST indicate that it is feasible to compare AST results from the national public health reference laboratories when applying ECOFFs. Nevertheless, improvements are warranted at a few FWD-Net laboratories.

6. Recommendations

6.1 Laboratories

The laboratories should comply with the recommendations set out in the harmonised EU AST protocol which stipulates that the EUCAST guidelines should be followed. These guidelines include specifications for control strains, media, incubation temperature, disk loads for DD testing, concentration range for MIC determination, etc. The results from this and other EQAs show that the results generated using MIC broth dilution methods are generally better than the diffusion-based methods (disk diffusion and gradient strip based methods). Therefore, if possible, laboratories should consider implementing broth dilution methods.

6.2 FWD-Net

In order to ensure the comparability of AST data reported to TESSy it is important to apply standardised testing and interpretation of data in the Member States. ECDC and the EQA provider will continue to provide consultancy facilities for the FWD-Net laboratories, however specific support may also be required by individual laboratories.

6.3 The EQA provider

In order to assist with troubleshooting, the current reporting scheme will be further developed for a more detailed and uniform collection of results, method, manufacturer, growth medium and incubation temperature used by the participating laboratories.

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Annex 1. List of participants

Country	EU status	Name of laboratory	Name of institution
Albania	Enlargement	Laboratory of Enterobacteriology	Institute of Public Health
Austria	EU/EEA	NRL *Campylobacter*	Austrian Agency for Health and Food Safety
Belgium	EU/EEA	LHUB-ULB site Porte de Hal - Microbiologie	CHU Saint-Pierre
Croatia	EU/EEA	Department for Clinical Microbiology	University Hospital for Infectious Diseases
Cyprus	EU/EEA	Reference Laboratory for *Salmonella* and other Enteric Pathogens	Nicosia General Hospital
Czech Republic	EU/EEA	National reference laboratory for antibiotics	National Institute of Public Health
Denmark	EU/EEA	Diagnostic and Typing of Gastrointestinal Bacteria	Statens Serum Institut
England	EU/EEA	Gastrointestinal Bacteria Reference Unit	National Infection Service
Estonia	EU/EEA	Laboratory of Communicable Diseases	Health Board
France	EU/EEA	French National Reference Center for *Campylobacter*	CHU Pellegrin
Germany	EU/EEA	FG11	Robert Koch Institute
Iceland	EU/EEA	Dept. of Clinical Microbiology	Landspítali University Hospital
Ireland	EU/EEA	National *Salmonella, Shigella* and *Listeria* Reference Lab (NSSLRL)	NSSLRL
Italy	EU/EEA	Antimicrobial resistance and special pathogens	Istituto Superiore di Sanità
Kosovo	Enlargement	Microbiology	National Institute of Public Health of Kosovo
Latvia	EU/EEA	National Microbiology Reference Laboratory of Latvia	Riga East University Hospital
Lithuania	EU/EEA	National Public Health Surveillance Laboratory	National Public Health Surveillance Laboratory
Luxembourg	EU/EEA	Bacteriologie-Mycologie-Antibiorésistance-Hygiéne hospitalière	Laboratoire National de Santé
Macedonia	Enlargement	Laboratory of bacteriology and AMR	Institute of Public Health of Macedonia
Malta	EU/EEA	Bacteriology Laboratory	Mater Dei Hospital
Portugal	EU/EEA	NRL for Gastrointestinal Infections	National Institute of Health Dr Ricardo Jorge
Republic of Serbia	Enlargement	Referee laboratory for *Campylobacter* and *Helicobacter*	Center for Microbiology
Republic of Srpska, Bosnia and Herzegovina	Enlargement	Department of Microbiology	Public Health Institute, Republic of Srpska, Bosnia and Herzegovina
Romania	EU/EEA	Bacterial Enteric Infections Laboratory	National Institute of Medico-Military Research and Development Cantacuzino
Slovenija	EU/EEA	Oddelek za medicinsko mikrobiologijo Nova Gorica	NLZOH
Spain	EU/EEA	Unidad de Enterobacterias	Centro Nacional de Microbiología
The Netherlands	EU/EEA	NRL on AMR in animals	Wageningen Bioveterinary Research (WBVR)
Turkey	Enlargement	National Reference Laboratory for Enteric Pathogens	General Directorate of Public Health

Annex 2. EUCAST ECOFFs used for *Campylobacter* EQA4-AST

	MIC determination (µg/mL)				Disk Diffusion (mm)			
Antimicrobial agent	**EUCAST ECOFF**		**EUCAST Clinical breakpoint**		**EUCAST ECOFF**		**EUCAST Clinical breakpoint**	
Mandatory	**WT ≤**	**NWT >**	**S ≤**	**R >**	**WT ≥**	**NWT <**	**S ≥**	**R <**
Ciprofloxacin	0.5	0.5	0.5	0.5	26	26	26	26
Erythromycin *(C. jejuni*)	4	4	4	4	22	22	20	20
Erythromycin (*C. coli)*	8	8	8	8	24	24	24	24
Tetracycline *(C. jejuni*)	1	1	2	2	30	30	30	30
Tetracycline (*C. coli)*	2	2	2	2	30	30	30	30
Optional								
Gentamicin (*C. jejuni*)	2	2			20	20		
Gentamicin (*C. coli*)	2	2						

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