Service contract for the provision of EU networking and support for public health reference laboratory functions for antimicrobial resistance in *Salmonella* species and *Campylobacter* species in human samples

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TABLE OF CONTENTS

1. INTRODUCTION AND SCOPE

The purpose of the FWD AMR-RefLabCap project is to strengthen coordination, support and capacity building in national microbiology reference laboratory functions for testing and surveillance of antimicrobial resistance (AMR) in *Salmonella* and *Campylobacter* in human samples. The project aims at strengthening capacities for national surveillance and outbreak investigation of *Salmonella* and *Campylobacter* and improve the availability and quality of European-level molecular surveillance data. One of the main goals of the FWD AMR-RefLabCap project is to aid the National Reference Laboratories (NRL) in public health at implementing or improving their national surveillance networks. This includes ensuring that all or most local laboratories in each country are able to produce and provide reliable results that allow for proper characterization and surveillance of the local and national epidemiological situation of human *Salmonella* and *Campylobacter*.

This document provides guidance to the NRLs and their countries' local laboratories regarding the methods and processes that should be in place for routine internal quality control (IQC) of antimicrobial susceptibility testing (AST). This document also aims at describing the standardized and/or recommended methods for AST of *Salmonella* and *Campylobacter* in Europe, and the proposed methods for detecting relevant antimicrobial resistance (AMR) determinants. The document provides practical advice for routine IQC and includes examples of control strategies and examples of schemes for registering important testing details. Furthermore, it collects and presents, in a systematic way, the most recently available information from different regulatory agencies and other sources.

2. INTERNAL QUALITY CONTROL STRATEGIES

Drug- and multidrug-resistant bacterial pathogens might be involved in national or cross-border foodborne outbreaks, with capacity to cause severe disease and with supplementary investigation and healthcare costs. Thus, obtaining reliable, reproducible and internationally comparable quality assured AST results is of paramount importance, to allow for monitoring and surveillance of these pathogens at national and international levels. Furthermore, 'false susceptible' or 'false resistant' results can result in unintended consequences for patients receiving antimicrobial therapy and can lead to misleading evaluations of current epidemiological trends, as well as promote the spread of antimicrobial resistance genes.

Appropriate IQC procedures ensure that the analytical methods routinely used in reference, clinical, or other type of laboratories yield reliable and reproducible results, and perform within acceptable variation intervals. These procedures should be used in parallel with the quality control steps of each method, and not as a replacement.

The International Organization for Standardization (ISO) provides several standard documents that help laboratories ensure that their workflows are robust, and that results obtained through their routine methods are accurate. The ISO also develops standardized protocols that lead to robust and accurate results that are comparable between different institutions, within and between countries.

The ISO 15189:2022 standard, "Medical laboratories - Requirements for quality and competence" [1], sets out a number of requirements that guide medical laboratories in providing services aligned with the "best practices" in their field. Unlike other laboratory standards, ISO 15189 focuses on processes instead of procedures or protocols. In line with the standard, clinical laboratories should plan and implement actions to address risks and opportunities for improvement.
The standard describes different categories of requirements that should be respected, in order to facilitate cooperation between healthcare services, harmonization of procedures and comparability of results, with the ultimate goals of promoting welfare of patients.

These categories are general, structural and governance, resource, process, and management system requirements. The resource requirements, for example, are focused on staff competences (including training and posterior assessment of competence), working facilities, laboratory equipment (including calibration and adequate recording of uses), and reception and management of consumables and reagents. Process requirements include preparation, collection, reception, handling and testing of biological samples, as well as in-depth description of requirements for the examination of those samples (including their validation, documentation and reporting). Management system requirements include document control, preventive and corrective measures, risk management, performance of periodic internal audits and establishment of quality indicators.

An important section of the ISO 15189 is the description of IQC for monitoring examination result. Quality control recommendations include, but are not limited to: the use of quality control materials, which should be periodically examined; performing quality control of data before releasing results; participating in interlaboratory comparisons, which can take the form of external quality assurance (EQA) exercises of proficiency tests (PT), and implementing corrective measures when performance is not satisfactory.

The ISO/IEC 17025:2017 standard, "General requirements for the competence of testing and calibration laboratories" [2] contains similar recommendations to those presented in ISO 15189, although it has a broader focus because it also applies to laboratories operating in other areas besides clinical contexts. This standard aims at ensuring the consistency of operations within laboratories and "*enables laboratories to demonstrate that they operate competently and generate valid results, thereby promoting confidence in their work both nationally and internationally*". Thus, complying to this standard helps to facilitate cooperation between laboratories and other bodies, by generating wider acceptance of results between countries.

Laboratories that have applied the ISO 15189 or the ISO/IEC 17025 standard in their settings are usually also in compliance with the requirements described in the ISO 9001:2015, "Quality management systems - Requirements" [3].

As stated in the previously described standards, participation in EQA exercises strengthens the confidence in the methods used in each laboratory, and allows for comparison of the laboratory's performance with other national and international laboratories. Often, participation in EQA is also required for accreditation. In Europe, relevant EQA exercises are available. Examples are ECDC's EQA's on AST in *Salmonella* and *Campylobacter* (current programme terminated in 2022) and the EARS-Net EQA which, although not focused specifically on *Salmonella* and *Campylobacter*, targets the standard methods for AST of important healthcare-associated bacterial pathogens. Laboratories may also choose to participate in further EQA exercises provided by other institutions, although these might have associated financial costs. Examples of such EQAs are those organized by UK NEQAS (as the Antimicrobial Susceptibility EQA), ESfEQA, Labquality, Oneworld Accuracy, and others.

Another approach to guarantee that laboratorial procedures are performed adequately and that their respective results are of the highest quality is to secure accreditation of technical competence. Accreditation must be obtained from a specifically designated body which will, in principle, be a national institution. The designated accreditation bodies are, in turn, defined by the International Laboratory Accreditation Cooperation (ILAC). It is common that clinical microbiology laboratories procure accreditation related to their conformity to ISO 15189 and/or ISO/IEC 17025 standards.

In summary, strategies for IQC include:

- Complying with ISO 15189 or with ISO 17025;
- Securing accreditation of the method to document compliance to the chosen standard;
- Participating in relevant EQA exercises.

3. INTERNAL QUALITY CONTROL WHEN PERFORMING PHENOTYPIC ANTIMICROBIAL SUSCEPTIBILITY TESTING

3.1. Background

Phenotypic AST can be performed through a variety of methods, such as dilution or diffusion methods. However, it has been shown that reproducibility of results within and between methods is highly influenced by small variations in media compositions, inocula concentrations, approach towards reading of results, and other factors. AST methodologies are extremely sensitive to variations, and the slightest deviation from laboratory procedures can lead to error in the diagnostic test result [4–6]. Thus, it is important to follow standardized AST protocols that aim at minimizing these variations. This ensures that results are accurate and reproducible, and also allows for comparison of results obtained in different settings.

In Europe, guidance on phenotypic AST methods is developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The Committee recommends the use of broth microdilution or disk diffusion for AST, following specific and standardized protocols. Other methods (such as agar dilution or gradient strips) are not generally recommended due to the current lack of harmonisation in protocols and high variability of results.

3.2. Broth microdilution

EUCAST recommends to perform AST through broth microdilution (BMD), by following the protocol provided by ISO, document ISO 20776-1:2019 [7].

The ISO 20776-1:2019 standard is called "Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices - Part 1: Broth micro-dilution reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases". It contains detailed descriptions of all steps that are necessary to perform BMD of aerobic, non-fastidious bacteria. It is also the baseline document that should be employed when performing AST of *Campylobacter* isolates, with the change of using MH-F broth (MH broth supplemented with lysed horse blood and β-NAD). The special considerations provided by EUCAST are described in the following paragraphs.

The ISO document describes how to prepare stock and working solutions of antimicrobial agents, how to prepare the broth medium and microdilution trays for susceptibility testing. Furthermore, the standard includes two proposed methods for obtaining an adequate bacterial inoculum: the broth culture method and the direct colony suspension method. Either method ensures that a final concentration of 5 x 10^5 colony forming units per millilitre (CFU/ml) is achieved in the wells of the microdilution trays. The standard explains how to inoculate, incubate and read the minimum inhibitory concentrations (MIC) on the microdilution trays. Finally, it provides lists of situations that require special attention, including the adjustment of medium composition or incubation conditions for certain bacterial species and for certain antimicrobials. Relevant examples include the preparation of working solutions of tigecycline no more than 12 hours before testing, adjusting the zinc concentration of the broth medium for testing of carbapenems, and refraining from adding surfactants to the medium when testing colistin.

EUCAST recommends following the ISO standard 20776-1 exactly as described, except for the alterations listed in its website or described on the most recent EUCAST clinical breakpoints tables [8]. At the moment, the only general alteration suggested in the website

is to use supplemented broth medium for BMD of fastidious organisms (specifically MH-F broth [9] which is Mueller-Hinton broth supplemented with lysed horse blood and β-NAD), thus this medium should be employed when performing AST of *Campylobacter*. The clinical breakpoint tables contain some species-specific or antimicrobial-specific alterations. In *Campylobacter*, the tables indicate once again that the broth MH-F should be used, and furthermore that the microtiter plates should be incubated under the special conditions of 41± 1°C during 24 hours in microaerobic environment, and extension of incubation time up to 40-48 hours might be necessary if growth is insufficient. The tables also contain other important information about testing conditions not included in the ISO standard, about how to read the results and about interpretation of those results.

EUCAST provides a visual guide for determining MIC endpoints [10] and how to proceed in case of strange observations (such as skipped wells in the microdilution trays). Ensuring that growth is sufficient and uniform throughout wells, and confirming that there is no more than one skipped well in a row are important steps that allow for the results of the test to be accepted. Furthermore, occurrence of trailing (very reduced but persistent growth in the form of small "pinpoint buttons" in the bottom of the wells) is common for trimethoprim and tetracycline, and pinpoint growth should be disregarded for those antimicrobials.

Interpretation of MIC results should be done according to the most recent EUCAST clinical breakpoints tables [8]. One of the most relevant updates implemented in the latest versions of these tables (from 2019 onwards) is the change of the category "Intermediate" to the category "Susceptible, increased exposure", to better reflect that the antimicrobial agent(s) in question are still adequate options for antibiotherapy, albeit requiring adjustment of the administered dose.

It is important to be aware that EUCAST regularly updates the clinical breakpoints listed in the tables, and some recommendations for phenotypic AST. Laboratories should consult the website regularly to ensure that the most recent recommendations are followed, and to be up to date with potential warnings. These warnings mainly focus on problems detected in commercial products. An important example is the evaluation of commercial BMD products for AST of colistin [11,12]. Conclusions include the use of cation-adjusted Mueller- Hinton broth, not adding any additives (specifically surfactants), using non-treated polystyrene microdilution trays, and using colistin sulphate salts (and never the methanesulfonate derivative of colistin).

3.3. Disk diffusion

For *Salmonella* and *Campylobacter*, EUCAST also supports the use of disk diffusion (DD) for phenotypic AST. The exception is AST of colistin, which should be exclusively performed through BMD due to chemical properties of the antimicrobial that reduce the accuracy of results obtained with diffusion methods, including gradient strips which also use a solid agar medium.

There is no ISO standard focusing on DD but EUCAST has prepared a standardized protocol [13]. The protocol explains how to prepare solid Mueller-Hinton (MH) agar plates [9], and how to store and use these plates. Furthermore, it provides instructions for preparing the bacterial inoculum through the direct colony suspension method and for inoculating the surface of the agar. The handling, storage and application of the antimicrobial disks in the agar is also described. The incubation conditions are listed for each bacterial taxon, specifically incubation at 35 ± 1°C during 18 ± 2 hours for *Salmonella*, stacking no more than five agar plates. Finally, the document explains how to read results and determine the zone diameters for each antimicrobial, which should afterwards be interpreted according to the most recent clinical breakpoint tables. The EUCAST disk diffusion protocol contains one Appendix focusing exclusively on DD of *Campylobacter* isolates, which includes the use of MH-F agar and incubation of plates at 41± 1°C during 24 hours in microaerobic environment, and extension of incubation time up to 40-48 hours might be necessary if growth is insufficient.

The DD protocol lists several important quality control steps, including but not limited to confirming that the agar depth is 4 ± 0.5 millimetres and that the surface pH is within the range 7.2-7.4.

The main points of the DD protocol and visual guides for adequate growth are also available as a presentation [14], as well as a presentation [15] with pictures explaining how to determine the zone diameters.

As described for the BMD method, EUCAST frequently revises the protocols, presentations, clinical breakpoints and warning messages, thus users must ensure that their local method descriptions are kept up to date with the most recent information.

3.4. Quality control of phenotypic antimicrobial susceptibility testing

To ensure the accuracy and reproducibility of each iteration of AST in the laboratory, it is important that all workers follow the same standardized protocol. Besides following the instructions of the reference documents described thus far, it's necessary that the correct media, BMD panels and antimicrobial disks are used for each iteration of AST, and that material-specific differences are taken into account (for example differences in inoculation or transfer volume that are defined by manufacturers when using commercial BMD panels). Therefore, laboratories should create internal method overview documentation that contains specific and detailed information regarding the procedure to be applied when performing AST of *Salmonella* and *Campylobacter.* Appendix 1 presents an example of such a method overview document.

It is furthermore essential that the specific laboratory equipment, material and reagents that are used for each iteration of AST are well documented, both for traceability and also because this can assist in identifying problems related to a certain equipment or batch of reagents. Appendix 2 presents an example of internal laboratory documentation registering the batch of reagents, materials and equipment.

One of the most important considerations in the ISO standard and EUCAST guidelines is the use of control strains to perform quality control (QC) of each iteration of the method. The standard recommends that users choose from QC strain lists from EUCAST (available on the document "Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST" [16]) or from the Clinical Laboratory Standards Institute (CLSI) (available on the document CLSI M100 "Performance Standards for Antimicrobial Susceptibility Testing" [17]). These documents provide lists of characterized bacterial strains, with well-defined acceptable MIC ranges, belonging to the same, or similar species, of those being tested. Some specific antimicrobials will require the use of additional control strains, which is also described.

According to EUCAST, when performing AST of *Salmonella* through BMD, laboratories must use the control strain *Escherichia coli* ATCC 25922. Furthermore, validation of AST results for colistin requires the use of the *mcr*-1-positive *E. coli* NCTC 13846 control strain, and validation of results for β-lactams in combination with β-lactamase inhibitors requires the use of *E. coli* ATCC 35218, or *Klebsiella pneumoniae* ATCC 700603, or *K. pneumoniae* ATCC BAA-2814. For AST of *Campylobacter* through BMD, the control strain *Staphylococcus aureus* ATCC 29213 should be employed. The reason why *Campylobacter jejuni* ATTC 33560 cannot be used for QC of BMD is that EUCAST has not established MIC QC ranges. However, CLSI (M45 methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria) has established QC ranges for *Campylobacter jejuni* ATCC 33560 and these ranges are applied in many European laboratories.

Moreover, the following control strains must be used when performing AST through DD for *Salmonella*: *E. coli* ATCC 25922, and additionally *E. coli* ATCC 35218, or *K. pneumoniae* ATCC 700603, or *K. pneumoniae* ATCC BAA-2814 for β-lactams in

combination with β-lactamase inhibitors. These are the same as previously listed for the BMD method. For *Campylobacter*, the control strain *Campylobacter jejuni* ATCC 33560 should be used, which is not the same strain proposed for the BMD method.

The reason that *C. jejuni ATTC 33560 cannot be used for BMD is that* EUCAST have not established MIC QC ranges. However, CLSI (M45 methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria have established QC ranges and these ranged are applied in many European laboratories

These QC strains should be employed in each iteration of AST that is performed in the laboratories, and should also be used to confirm every batch or lot of reagents that are used. In practical terms, this means that laboratories should:

i) Use the relevant QC strain(s) every day that AST is being performed;
ii) Additionally, use the relevant QC strain(s) every time a new batch or lot of materials is employed, even if this happens for the same group of isolates, at the same time, in the same location, with the same operator.

Furthermore, for even more strict QC, laboratories might choose to employ the relevant QC strain(s) multiple times in the same day if AST is performed at another time for different groups of isolates (for example, one set in the morning and one set in the afternoon). The QC strain(s) can also be used multiple times in the same day if AST is performed in another location or by different operators (for example a different room).

A suggested procedure for handling of reference strains [18] has been made available by the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR).

Another essential part of IQC procedures for microbiological work is to perform regular monitoring of culture media using reference strains. This is described in more details in the ISO standard 11133:2014 ("Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media") 19. The aim is to demonstrate the following three criteria:

- the acceptability of each batch of medium,
- that the medium is "fit for purpose", and
- that the medium can produce consistent results.

All new batches of culture medium (broth, agar or other), whether commercial or prepared in the laboratory, should be tested for reliability using reference strains. In addition, all new batches of discs should be tested on quality-approved MH agar plates before being accepted for routine use. The EUCAST website for warnings should be routinely checked for warnings related to quality issues with media, discs and other materials. Manufacturers' instructions should also be respected regarding storage and use of commercial products.

Appendix 3 presents an example of method control documentation that should be applied regularly (for example once a week) to ensure overall compliance with expected results, and that should also be applied to confirm that new batches of media, disks and BMD panels can be approved for use in the laboratory.

The laboratories can also choose to use a similar document as Appendix 3 for registering the AST results of the QC strains every time that these are employed (i.e., for each iteration of AST). However, this daily (or multiple times a day) confirmation can be also done in a simpler manner by registering the AST results in a document ("test form" for quality control) that contains the layout of the BMD panel and the accepted ranges for the respective QC strain(s). This will of course imply that the laboratory prepares a layout for each combination of BMD panel and QC strain. An example is provided in Appendix 4.

Results from QC should be documented and stored for long-term comparison. Punctual deviations in expected results for the QC strains might reveal a single mistake or

problems with a specific batch or lot of reagents. These are called random errors and their reason should be easily corrected. For this reason, it is also important to store information from the different batches or lots of materials and reagents that are used, such as the quality analysis certificates provided by the manufacturers. On the other hand, prevalent and persistent deviations from expected QC results are called systematic errors, and these are the ones that IQC measures attempt to avoid. They can be due, for example, to improperly calibrated instruments or standard solutions, such as autoinoculators dispensing incorrect volumes of broth into microtiter plates, poorly monitored incubators or refrigerator rooms with fluctuations in temperature, or incorrectly prepared McFarland standard solutions. They can also be due to changes implemented in critical steps of laboratory procedures (purposedly or by accident) such as improper incubation times or transferring wrong volumes of the demineralized water suspension into the broth medium. Systematic errors become apparent when analysing long-term QC results, because these will consistently drift in one specific direction. Both ISO 15189 and ISO 17025 describe that trends shall be recorded, for example this could be trends of QC results for each individual strain/antimicrobial.

In summary, QC for phenotypic AST includes:

- Following ISO 20776-1 for AST through BMD;
- If performing AST through DD, using the standardized EUCAST protocol;
- Only use BMD for AST of colistin (do not use DD or gradient strip tests).
- Using the control strain *E. coli* ATCC 25922 for *Salmonella*;
- Using supplemented MH-F broth and the control strain *S. aureus* ATCC 29213 for *Campylobacter*, adjusting the incubation conditions;
- Respecting the specific recommendations or alterations proposed by EUCAST regarding AST of colistin: simultaneously use the control strain *E. coli* NCTC 13846, use cation-adjusted Mueller-Hinton broth, do not add any additives, use non-treated polystyrene microdilution trays, and use colistin sulphate salts. It should further be noted that certain serotypes of *Salmonella*, e.g. *S*. Enteritidis and *S*. Dublin typically exhibits MIC values of >2 and thereby exhibits a non-wild type phenotype.
- Respecting the specific recommendations or alterations proposed by EUCAST regarding AST of carbapenems: adjusting the zinc concentration of the broth medium;

3.5. Phenotypic detection of β-lactamase-producing bacteria

Several well-characterized phenotypic tests allow for the detection of specific mechanisms of AMR, and EUCAST has published guidelines for detection of these mechanisms [20].

These guidelines contain methods for detecting mechanisms of resistance towards β-lactam antimicrobials in *Enterobacteriaceae*, specifically the mechanisms of: production of carbapenemases, production of extended-spectrum β-lactamases (ESBL), and AmpC-mediated β-lactam resistance. A number of methods are described in the guidelines, including directly using results obtained through the standard AST methods of BMD and DD. The comparison of MIC values or zone diameters obtained for different β-lactam antimicrobials allows for the prediction of the specific resistance mechanism being expressed by the bacterial isolate. A brief summary of these methods is provided in Appendix 5.

The EUCAST guidelines propose several different positive control strains to be used when evaluating BMD or DD results for prediction of specific β-lactam resistance mechanisms (Table 2). To ensure reliable results, it is essential to also use negative control strains

(ideally *E. coli* ATCC 25922) and to follow the standardized protocols previously presented for performing BMD and DD.

Table 2. Positive control strains to use when evaluating broth microdilution results in *Enterobacteriaceae* with the purpose of predicting the mechanism of β-lactam resistance

Control strain	**Mechanism of resistance**
Enterobacter cloacae CCUG 59627	AmpC combined with decreased porin expression
K. pneumoniae CCUG 58547 or *K. pneumoniae* NCTC 13440	Metallo-β-lactamase (VIM)
K. pneumoniae NCTC 13443	Metallo-β-lactamase (NDM-1)
E. coli NCTC 13476	Metallo-β-lactamase (IMP)
K. pneumoniae CCUG 56233 or *K. pneumoniae* NCTC 13438	*Klebsiella pneumoniae* carbapenemase (KPC)
K. pneumoniae NCTC 13442	OXA-48 carbapenemase
K. pneumoniae ATCC 25955	Negative control for carbapenemase production
E. coli CCUG 58543	Acquired CMY-2 AmpC
E. coli CCUG 62975	Acquired CMY AmpC and CTX-M-1 group ESBL
K. pneumoniae CCUG 58545	Acquired DHA

The EUCAST guidelines also present other methods, with different protocols and requiring different materials than those that should be routinely used for standardized AST. These methods are approved for screening of β-lactamases, but might require the purchase of additional consumables or other laboratory materials.

4. INTERNAL QUALITY CONTROL WHEN PERFORMING MOLECULAR DETECTION OF ANTIMICROBIAL RESISTANCE DETERMINANTS

Molecular detection of AMR determinants is currently not standardized by EUCAST nor ISO. However, other reference institutions or research groups provide protocols for detection of relevant antimicrobial resistance genes, specifically polymerase chain reaction (PCR) protocols and whole-genome sequencing (WGS) approaches.

Quality control of the results obtained with any PCR protocol implies using all the control strains listed in the protocols, as well as a negative control (corresponding to the complete PCR mixture but excluding any control DNA). Primer sequences, control strains and cycle conditions should not be altered, since the protocols have been validated using the specific parameters stated in their text. Different protocols should not be combined into larger multiplex PCR tests, since specificity of primer sequences and interaction between primers have likely not been evaluated.

Reporting PCR results should never include the terms "susceptible" or "resistant", or similar expressions. Result should be reported as presence or absence of the genes included in the protocols.

Any gene fragments detected through PCR can be further analysed with, for example, Sanger sequencing, to detect variations within the nucleotide sequences. Alternatively, WGS followed by bioinformatics analysis using open-source and curated tools and databases can be used to identify genes or chromosomal point mutations in the bacterial genome.

Appendix 6 describes important molecular targets associated with AMR that can be investigated in *Salmonella* spp. and *Campylobacter* spp., as well as proposed PCR protocols for their detection.

In summary, QC for molecular detection of AMR through PCR includes:

- Expanding the PCR protocols (or implementing PCR protocols) to include detection

of relevant antimicrobial resistance genes;

- Using all positive control strains described in each PCR protocol;
- Always including a negative control;
- Not combining different PCR protocols into a larger multiplex;
- Create method overview documentation and record the batch of reagents, materials and equipment, as exemplified previously for phenotypic AST (Appendices 1 and 2).

Using WGS for detection of antimicrobial resistance genes or chromosomal point mutations also requires proper QC. Due to large number of DNA extraction kits, library preparation protocols and sequencing platforms that exist, it is difficult to propose general IQC strategies that are applicable to all methods. However, some recommendations that should always be followed are:

- Create method overview documentation and record the batch of reagents, materials and equipment, as exemplified previously for phenotypic AST (Appendices 1 and 2);
- Record the version and/or date of the bioinformatics tools and databases that are used for analysis of raw sequence data;
- Store the raw sequence data permanently;
- Apply well-defined QC thresholds for raw data and for assemblies (Appendix 7).

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6. APPENDICES

6.1. Appendix 1

Example of method overview documentation for internal quality control

Method overview for broth microdilution

Bacteria	Agar	Culture [1]	MIC panel	Solvent for McFarland suspension	Broth	Transfer from McFarland suspension	Inoculum to reconstitute wells	Inoculator programme [2]	Incubation
E. coli	TSA 5% blood	W	EUVSEC3	dem. water	CAMHB	10 µl	50 µl/ well	1	36-37°C 18-20 h
Salmonella	TSA 5% blood	W	EUVSEC3	dem. water	CAMHB	10 µl	50 µl/ well	1	36-37°C 18-20 h
Staphylococcus	TSA 5% blood	W	CAMPY	dem. water	CAMHB	10 µl	50 µl/ well	1	36-37°C 18-20 h
Campylobacter [3]	TSA 5% blood	F	CAMPY	CAMHB	CAMH-FB	100 µl	50 µl/ well	25	41°C 24 h [3]
ESBL suspect	TSA 5% blood	W	EUVSEC2	dem. water	CAMHB	10 µl	50 µl/ well	1	36-37°C 18-20 h
[Other relevant species]									

1) F: Fresh overnight culture must be used. W: The culture may be refrigerated up to 3 days before use.
2) Sensititre autoinoculator equipment number 1234 only.
3) Campylobacter is incubated microaerophilic (10% CO_2, 5% O_2, 85% N_2) in a CO_2-incubator or anaerobic container. NB: EUCAST recommends 41 ± 1°C for 24 hours to achieve better growth and more stable MIC-values, and suggests that isolates with poor growth may be re-incubated and re-read again after a total of 40-48 hours.

Document approved by:
Approval date:

6.2. Appendix 2

Example of batch of reagents, materials and equipment documentation for internal quality control

Batch and equipment documentation for internal quality control of broth microdilution

Batch and equipment control is carried out for every test iteration.

Table 1. Batch of reagents, materials and equipment

Date/initials						
Dem. water						
CAMHB						
CAMH-FB						
TSA 5% blood						
EUVSEC3						
EUVSEC2						
CAMPY						
Inoculator 1234						
Inoculator 5678						
McFarland std.						
Dosing heads						
Incubator AB12						
Incubator CD34						
CO_2-incubator						
[other]						
[other]						

Remarks:

6.3. Appendix 3

Example of method control documentation for internal quality control

Method control for broth microdilution

Method control is carried out every week of a test period.

Method control is performed for every new batch of panels or media.

Test forms for quality control must be attached.
Results sheets for all test isolates must be attached.

Table 1. Reference strains to be used for weekly method control and for control of new batches of panels or media

Reference strain		***E. coli* ATCC 25922**	***E. coli* NCTC 13846**	***C. jejuni* ATCC 33560**	***S. aureus* ATCC 29213**
Media		CAMHB	CAMHB	CAMH-FB	CAMHB
MIC panel (Sensititre™)	**EUVSEC3**	X	X		
	EUVSEC2	X			
	CAMPY			X	X

Table 2. Acceptance intervals (mg/L) for approval of method, panels or media

Antimicrobials	**Reference strain**			
	***E. coli* ATCC 25922**	***E. coli* NCTC 13846**	***C. jejuni* ATCC 33560**	***S. aureus* ATCC 29213**
Amikacin	0.5-4	--	--	1-4
Ampicillin	2-8	--	--	--
[…]	*[…]*	*[…]*	*[…]*	*[…]*
Ciprofloxacin	0.004-0.016	--	--	0.125-0.5
Clindamycin	--	--	--	0.06-0.25
Colistin	0.25-2	2-8	--	--
[…]	*[…]*	*[…]*	*[…]*	*[…]*

Purpose: [ ] weekly control [ ] panel batch control [ ] media batch control [ ] _____

Panel code: _____
Panel batch: _____
Panel expiration date: _____

Broth code: _____
Broth batch: _____
Broth expiration date: _____

Performed by: _____
Date: _____

Read by: _____
Date: _____

Remarks:

6.4. Appendix 4

Example of documentation for quality control of each AST iteration ("test form")

"Test form" for quality control for broth microdilution

Quality control is carried out at least once a day when testing is performed.

Control strain: *Escherichia coli* ATCC 25922
Panel: EUVSEC3
Broth medium: CAMHB
Volume per well: 50 µl
Accepted ranges: Green (EUCAST QC tables v13.0, valid from 01/01/2023)

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMP 32	AZI 64	AMI 128	GEN 16	TGC 8	TAZ 8	FOT 4	COL 16	NAL 64	TET 32	TMP 16	SMX 512
B	AMP 16	AZI 32	AMI 64	GEN 8	TGC 4	TAZ 4	FOT 2	COL 8	NAL 32	TET 16	TMP 8	SMX 256
C	AMP 8	AZI 16	AMI 32	GEN 4	TGC 2	TAZ 2	FOT 1	COL 4	NAL 16	TET 8	TMP 4	SMX 128
D	AMP 4	AZI 8	AMI 16	GEN 2	TGC 1	TAZ 1	FOT 0.5	COL 2	NAL 8	TET 4	TMP 2	SMX 64
E	AMP 2	AZI 4	AMI 8	GEN 1	TGC 0.5	TAZ 0.5	FOT 0.25	COL 1	NAL 4	TET 2	TMP 1	SMX 32
F	AMP 1	AZI 2	AMI 4	GEN 0.5	TGC 0.25	TAZ 0.25	CHL 8	CHL 16	CHL 32	CHL 64	TMP 0.5	SMX 16
G	MERO 0.03	MERO 0.06	MERO 0.12	MERO 0.25	MERO 0.5	MERO 1	MERO 2	MERO 4	MERO 8	MERO 16	TMP 0.25	SMX 8
H	CIP 0.015	CIP 0.03	CIP 0.06	CIP 0.12	CIP 0.25	CIP 0.5	CIP 1	CIP 2	CIP 4	CIP 8	POS CON	POS CON

Code	**Antimicrobial agent (15)**	**Test range (mg/L)**
AMI	AMIKACIN	4-128
AMP	AMPICILLIN	1-32
AZI	AZITHROMYCIN	2-64
FOT	CEFOTAXIME	0.25-4
TAZ	CEFTAZIDIME	0.25-8
CHL	CHLORAMPHENICOL	8-64
CIP	CIPROFLOXACIN	0.015-8
COL	COLISTIN	1-16
GEN	GENTAMICIN	0.5-16
MERO	MEROPENEM	0.03-16
NAL	NALIDIXIC ACID	4-64
SMX	SULFAMETHOXAZOLE	8-512
TET	TETRACYCLINE	2-32
TGC	TIGECYCLINE	0.25-8
TMP	TRIMETHOPRIM	0.25-16
POS	POSITIVE CONTROL	2x

Performed by: _____
Date: _____

Read by: _____
Date: _____

Remarks:

6.5. Appendix 5

Description of phenotypic detection of β- lactamase-producing bacteria

The EUCAST guidelines for detection of β-lactamase-producing bacteria [20] contain instructions for predicting production of carbapenemases and ESBL, and AmpC-mediated β-lactam resistance. This can be done by directly analysing results obtained through the standard AST methods of BMD and DD.

Carbapenemase-producing organisms will present MIC values above the epidemiological cut-off values (ECOFF) for carbapenem antimicrobials. Meropenem and ertapenem are recommended as screening agents, and the cut-off values to consider isolates as carbapenemase-producers are MIC > 0.125 mg/L for either of the antimicrobials. Similarly, DD results will be lower than the ECOFFs, and carbapenemase-producers will present zone diameters < 28 mm for meropenem or < 25 mm for ertapenem.

ESBL-producing organisms will be resistant to a cephalosporin by itself, such as cefotaxime, but will be susceptible towards the same antimicrobial when in combination with a β-lactamase inhibitor, or will present a much lower MIC value. The ratio between the MIC values of the cephalosporin, and the cephalosporin in combination with the inhibitor will be equal to or higher than 8. Table 1 exemplifies situations where ESBL production is predicted, and situations where it is not. Other cephalosporins and cephalosporins in combination with clavulanic acid can be used, specifically ceftazidime or cefepime. If using the DD method for AST, ESBL-producers will present zone diameters at least 5 mm larger for the disk containing the cephalosporin with inhibitor, than the diameter observed for the cephalosporin by itself.

Table 1. Example of prediction of ESBL production in *Enterobacteriaceae* according to results obtained through BMD

Isolate	Cefotaxime MIC (mg/L)	Cefotaxime/clavulanic acid[1] MIC (mg/L)	Ratio between MIC values	ESBL production
Isolate A	32	0.5/4	64	Yes
Isolate B	32	8/4	4	No
Isolate C	32	32/4	1	No

[1] The concentration of clavulanic acid is fixed at 4 mg/L.

AmpC-overproducing organisms are phenotypically resistant to cefoxitin (MIC > 8 mg/L or zone diameter < 19 mm). Furthermore, these isolates will be resistant to some cephalosporins (specifically ceftazidime and/or cefotaxime), as well as the cephalosporins in combination with β-lactamase inhibitors (but with a much smaller ratio between MIC values than what is observed in ESBL-producing organisms). They are generally susceptible to cefepime. Phenotypic results do not allow for distinction between organisms that are overexpressing intrinsic AmpC due to chromosomal point mutations in promoter regions or other regulatory mechanisms, from those that have acquired additional plasmid-mediated *ampC* genes.

This direct interpretation of BMD or DD results is also described in the "EU protocol for harmonised monitoring of antimicrobial resistance in human *Salmonella* and *Campylobacter* isolates", where it is presented as a successive flowchart of confirmatory and synergy tests [21]. Laboratories might use the procedure described in the EUCAST guidelines or in the EU protocol for harmonised monitoring, according to their preference and/or already implemented approach. The biological principle is the same, only the visual representation of the procedure changes between these documents.

6.6. Appendix 6

Description of molecular mechanisms of resistance and relevant PCR protocols for their detection

In *Salmonella* spp. isolates it is important to adequately characterize if isolates present mechanisms of resistance towards β-lactam antimicrobials, particularly the expression of acquired genes encoding β-lactamase-production. These include the specific categories of ESBL [22,23], carbapenemases [24,25], and AmpC β-lactamases [26,27]. Acquired genes encoding β-lactamase-production belonging to these and other groups are described in several open-access databases, and one of the most comprehensive ones is the Beta-Lactamase DataBase (BLDB) [28].

PCR protocols generally focus on β-lactamase genes which have been well characterized as leading to resistant phenotypes. One example is the multiplex PCR protocol (Protocol 7) described in the EuSCAPE laboratory manual, which includes the carbapenemases *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$ and *bla*$_{NDM}$ [29]. Other protocols are available, as the set of six multiplex and one simplex PCR assays for detection of β-lactamase genes of different classes including *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{VEB}$, *bla*$_{GES}$, *bla*$_{PER}$ and plasmid-mediated AmpC β-lactamase genes [30].

Colistin resistance is also an important and increasing concern in *Salmonella* spp. The currently known genetic determinants of colistin resistance are the plasmid-mediated *mcr*-genes, and chromosomal point mutations in the genes affecting PmrAB or PhoPQ two-component systems, or other systems leading to lipopolysaccharide modification [31].

So far, ten *mcr*-genes and their variants have been described, in *Enterobacteriaceae* or other species [32–41]. It is predicted that almost all *mcr*-genes confer phenotypic resistance towards colistin; the exception is *mcr-9* and its variants. Isolates harbouring *mcr-9* will exhibit a susceptible phenotype. The EURL-AR provides a list of the currently know *mcr*-genes and their variants, which is regularly updated.

The EURL-AR has published a multiplex PCR protocol for detection of colistin-resistance genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* [42]. Another multiplex PCR protocol exists for the *mcr-6*, *mcr-7*, *mcr-8* and *mcr-9* genes [43]. The EURL-AR is currently adapting the protocol from Borowiak *et al*., 2020, to include the *mcr-10* gene.

Some chromosomal point mutations have been described as being correlated with phenotypic resistance towards colistin, such as PmrA R81H and PmrB V161M [44–46]. At the moment, the only molecular method proposed for detection of these mutations is sequencing the bacterial DNA.

For prediction of AMR in *Campylobacter* spp. isolates, some multiplex PCR protocols are published to detect acquired ARGs. For example, Eryıldız *et al*., 2021, developed a multiplex PCR that detects the *tet(O)* and *erm(B)* genes, mediating resistance towards tetracycline and erythromycin, respectively [47].

For *Campylobacter* spp. isolates, chromosomal point mutations in the 23S rRNA gene and in *gyrA*, responsible for resistance towards macrolides and fluoroquinolones, respectively [48], are best detected through sequencing of the bacterial DNA. Some PCR methods such as mismatch amplification mutation assay PCR have also been proposed to detect these mutations [49,50].

6.7. Appendix 7

Suggested quality control thresholds for whole-genome sequencing data

The complete protocol for WGS analysis of *Salmonella* and *Campylobacter* is available at the FWD AMR-RefLabCap website.

The protocol proposes the following QC thresholds for raw sequence data:

- Average read length: Should correspond to that expected from the sequencing platform and kit (e.g. Illumina NextSeq read length is approximately 150 base-pairs (bp));
- Number of reads: Should be as high as possible and at least enough to obtain the desired depth of coverage;
- Depth of coverage: Should as a minimum be 30X, and preferably even higher;
- Species identification: If more than 5% of the reads match another species the sequence could be contaminated.

The protocol proposes the following QC thresholds for assembled data:

- Size of assembled genome: Should be within the range for the targeted organism (4.4-5.8 million bp for *Salmonella* and 1.5-1.9 million bp for *Campylobacter*);
- N50: Should be higher than 30,000 bp;
- Total number of contigs: Should be less than 500 (*Campylobacter* will typically be assembled into less than 100 contigs and *Salmonella* to less than 300 contigs).


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