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Guidance document on internal quality control schemes for clinical and reference laboratory antimicrobial susceptibility testing and molecular detection of antimicrobial resistance for carbapenem- and/or colistin-resistant *Pseudomonas aeruginosa* (C/CRPa) and *Acinetobacter baumannii* complex (C/CRAb)

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Health and Digital Executive Agency

Third EU Health Programme

Guidance document on internal quality control schemes for clinical and reference laboratory antimicrobial susceptibility testing and molecular detection of antimicrobial resistance C/CRPa and C/CRAb

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1. INTRODUCTION

The EURGen-RefLabCap project is complementary to the European Centre of Disease Prevention and Control (ECDC) European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net).

EURGen-RefLabCap aims to improve capacities of National Reference Laboratories (NRLs) in European countries for the identification and phenotypic and genotypic characterization of carbapenem-resistant *Enterobacterales* (CRE) and colistin-resistant CREs (CCRE), as well as carbapenem- and/or colistin-resistant *Pseudomonas aeruginosa* (C/CRPa) and carbapenem- and/or colistin-resistant *Acinetobacter baumannii* complex (C/CRAb). Furthermore, the project aims to strengthen capacities for national surveillance and outbreak investigation of CRE/CCRE, and to improve the availability and quality of European-level molecular surveillance data. One of the main goals of the EURGen-RefLabCap project is to support modernisation of diagnostic and molecular typing tests using whole-genome sequencing (WGS) analytical methods, to achieve these aims.

Carbapenems and colistin are important antimicrobial agents used for treatment of multidrug resistant bacterial infections. C/CRPa and C/CRAb can and have spread within and between healthcare facilities, regions and countries. Furthermore, at local levels, 'false susceptible' or 'false resistant' results can result in severe unintended consequences for the patient receiving antimicrobial therapy. 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.

2. SCOPE AND PURPOSE

This document provides guidance to NRLs and other laboratories regarding the methods and processes that should be in place for internal quality control (IQC) of antimicrobial susceptibility testing (AST). It also aims at:

- Providing practical advice for routine IQC and includes examples of control strategies and examples of schemes for registering important testing details;
- Collecting and presenting, in a systematic way, the most recently available information from different regulatory agencies and other sources;
- Describing the standardized and/or recommended methods for AST of C/CRPa and C/CRAb in Europe, and the proposed methods for detecting relevant antimicrobial resistance (AMR) determinants.

This guidance document should not be interpreted as a complete protocol to be followed for implementation of IQC in settings without already established quality control methods. Instead, it serves as a guide to update or expand currently used methods, and to easily share information with other laboratories in the NRL's networks.

Another document prepared in the context of EURGen-RefLabCap provide guidance for quality control of whole-genome sequencing-based investigation of antimicrobial resistance of C/CRPa and C/CRAb. This is Deliverable T4.4 "Agreed common WGS-based genome analysis methods and standard protocols for national surveillance and integrated outbreak investigations for use by participating NRL for the additional two pathogens for Workstream 2".

Also, guidance for quality control of phenotypic AST and molecular detection of AMR for CRE/CCRE was developed for EURGen-RefLabCap and shared as Deliverable T3.7 - Guidance document on internal quality control schemes for clinical and reference laboratory antimicrobial susceptibility testing and molecular detection of antimicrobial resistance.

3. TARGET AUDIENCE

This guidance document is directed at European clinical microbiology laboratories performing AST or molecular monitoring of AMR in healthcare-associated infections caused by C/CRAb and C/CRPa. These laboratories include NRLs or laboratories with similar functions, which are furthermore encouraged to share this guidance document, or parts of it, with local national laboratories performing the same type of analyses. The document was developed within the framework of Workstream 2 of the EURGen-RefLabCap project but is, in principle, valid for laboratories not participating in the project and also valid for other pathogens of importance for public health.

4. INTERNAL QUALITY CONTROL STRATEGIES

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.

In summary, and subsequently described, strategies for IQC include complying with standard documents from the International Organization for Standardization (ISO), securing accreditation of the method to document compliance to the chosen standard, and participating in relevant EQA exercises.

The 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 allow for validated and comparable results. The standards ISO 15189:2022 and ISO/IEC 17025:2017 are of particular relevance in clinical microbiology laboratories.

The ISO 15189:2022 standard, "Medical laboratories - Requirements for quality and competence", sets out a number of requirements that guide medical laboratories in providing services aligned with the "best practices" in their field. It 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. An important section of the ISO 15189 is the description of IQC for monitoring examination results. 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 or 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" contains similar recommendations to those presented in ISO 15189, but has not been adapted for laboratories operating in clinical contexts and has a stronger focus on ensuring the consistency of operations within laboratories. This standard "*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".

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.

Participation in EQA exercises strengthens the confidence in the methods used in each laboratory and corroborates the efficacy of the chosen IQC strategies, moreover allowing 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, and one example is the EARS-Net EQA which focuses on species identification and AST of important healthcare-associated bacterial pathogens. Participation of local laboratories in the EARS-Net EQA requires approval from the National Coordinator within each country. 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.

5. INTERNAL QUALITY CONTROL WHEN PERFORMING PHENOTYPIC ANTIMICROBIAL SUSCEPTIBILITY TESTING

5.1. 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 [1–3]. 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.

EUCAST recommends to perform AST through broth microdilution (BMD), by following the protocol provided by ISO, document ISO 20776-1:2019. The ISO standard 20776-1 should be followed exactly as described, except for the alterations listed in EUCAST's website or described on the most recent EUCAST clinical breakpoints tables.

EUCAST provides a visual guide for determining MIC endpoints 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 dilution range of an antimicrobial are important steps that allow for the results of the test to be accepted.

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 accuracy of results obtained with diffusion

methods. There is no ISO standard focusing on DD but EUCAST has prepared a standardized protocol.

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

Interpretation of BMD and disk diffusion results should be done according to the most recent EUCAST clinical breakpoints tables.

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.

5.2. Broth microdilution procedure and special situations

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. ISO 20776-1:2019 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 5×10^5 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 adjusting the zinc concentration of the broth medium for testing of carbapenems, depleting iron from the broth medium before testing cefiderocol, and refraining from adding surfactants to the medium when testing colistin.

At the moment, the only general alteration suggested in the EUCAST website relative to the ISO 20776-1:2019 is to use supplemented broth medium for BMD of fastidious organisms, thus not applicable in the context of this guidance document. The clinical breakpoint tables contain some species-specific or antimicrobial-specific alterations. For example, the tables indicate that susceptibility testing of penicillins for *A. baumannii* isolates yields unreliable results. Another relevant update implemented in the latest versions of the breakpoint tables (from 2019 onwards) is the change of the category "Intermediate" to the category "Susceptible, increased exposure". A final recent alteration is the change of the breakpoint for colistin in *P. aeruginosa*, from 2 mg/L (on v11.0 from January 2021) to 4 mg/L (on v12.0 from January 2022).

A crucial consideration when reading BMD results for *P. aeruginosa* and *A. baumannii* isolates is that turbidity, even without a visible pellet, should be considered as bacterial growth; thus, the MIC will correspond to the concentration of the first well with completely clear broth.

An important warning on EUCAST's website is the evaluation of commercial BMD products for AST of colistin [4]. 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).

5.3. Disk diffusion procedure and special situations

The EUCAST DD protocol explains how to prepare solid Mueller-Hinton agar plates, 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 manipulation, storage and application of the antimicrobial disks in the agar is also described. The incubation conditions are listed for each bacterial taxon, with incubation at 35 ± 1°C during 18 ± 2 hours for *P. aeruginosa* and *A. baumannii*, 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.

Examples of special situations are the possible presence of faint growth within the inhibition zone for trimethoprim, which should be disregarded. Similarly, it is possible that single colonies form within the inhibition zone for fosfomycin, which should also be ignored. It is also possible that variations in divalent cation concentration (specifically calcium cations, Ca^{2+}, and magnesium cations, Mg^{2+}) cause drifts in the inhibition zones observed for aminoglycosides.

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. It also lists the control strains to be used.

5.4. 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 *Pseudomonas* spp. and *Acinetobacter* spp. 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") or from the Clinical Laboratory Standards Institute (CLSI) (available on the document CLSI M100 "Performance Standards for Antimicrobial Susceptibility Testing"). 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. It is important to note that, within Europe, public health surveillance of AMR and surveillance in food and veterinary contexts requires the use of EUCAST guidelines [5,6].

According to EUCAST, when performing AST of either *P. aeruginosa* or *A. baumannii* isolates through BMD, laboratories must use the control strain *P. aeruginosa* ATCC 27853. Furthermore, validation of AST results for colistin requires the use of the *mcr*-1-positive *Escherichia 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. Finally, when performing AST through BMD of trimethoprim-sulfamethoxazole for *A. baumannii* isolates, the control strain *E. coli* ATCC 25922 should be used.

For DD, the following control strains must be used for AST of *P. aeruginosa* and *A. baumannii* isolates: *P. aeruginosa* ATCC 27853 for routine testing, and also *E. coli* ATCC 35218, or *Klebsiella pneumoniae* ATCC 700603, or *K. pneumoniae* ATCC BAA-2814 for validation of results for β-lactams in combination with β-lactamase inhibitors. When performing DD of trimethoprim-sulfamethoxazole for *A. baumannii* isolates, the control strain *E. coli* ATCC 25922 should be used. All these control strains are the same as previously listed for the BMD method. However, the additional control strain *E. coli* ATCC 25922 should be employed for DD testing of piperacillin and ticarcillin in *P. aeruginosa*.

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.

Additionally, 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 has been made available by the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR). The EURL-AR competences are the organisation, implementation and evaluation of monitoring schemes for AMR in bacterial isolates recovered from food and food-producing animals, specifically those from the *Escherichia*, *Salmonella*, *Campylobacter*, *Enterococcus* and *Staphylococcus* genera. However, the procedure for handling reference strains is also applicable within public health and clinical settings.

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 medium 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 in the laboratory 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 cause 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, or to implemented changes in critical steps of laboratory procedures. Systematic errors become apparent when analysing long-term QC results, because these will consistently drift in one specific direction. According to ISO 15189 and ISO 17025 these trends could be observed, for example, on the QC results for individual strains or for individual antimicrobials, thus those standards advocate that results recorded for each individual strain/antimicrobial combination would be relevant trends to follow.

In summary, QC for phenotypic AST of C/CRPa and C/CRAb includes:

- Following ISO 20776-1 for AST through BMD;
- Using the control strain *P. aeruginosa* ATCC 27853;
- Use other relevant control strains for specific antimicrobials and for the DD protocol;
- 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;
- Respecting the specific recommendations or alterations proposed by EUCAST regarding AST of carbapenems: adjusting the zinc concentration of the broth medium;
- If performing AST through DD, using the standardized EUCAST protocol;
- Avoiding the use of DD for AST of colistin;
- Creating method overview documentation and record the batch of reagents, materials and equipment (Appendices 1 and 2);
- Registering and storing all results from QC (Appendices 3 and 4).

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

Molecular detection of AMR determinants in C/CRPa and C/CRAb is currently not standardized by EUCAST nor ISO. However, reference institutions and research groups provide protocols for detection of relevant antimicrobial resistance genes, specifically polymerase chain reaction (PCR) protocols for detection of genes encoding carbapenemases.

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. Results should be reported as presence or absence of the genes included in the protocols.

P. aeruginosa and *A. baumannii* isolates can be resistant towards carbapenems due to the expression of acquired genes that encode carbapenemases, although other important mechanisms of resistance also exist. In these species, *bla*$_{KPC}$ and *bla*$_{OXA}$ enzymes have been reported, as well as Ambler class B carbapenemases (such as *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{NDM}$) [7–10]. Carbapenemases (and other enzymes or genes) are described in several open-access databases, and one of the most comprehensive ones is the Beta-Lactamase DataBase (BLDB) [11].

PCR protocols generally focus on carbapenemase-encoding genes which have been well characterized as leading to resistance phenotypes. One example is the combination of three multiplex PCR protocols, developed by Poirel*, et al.*, which includes the carbapenemases *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{SPM}$, *bla*$_{AIM}$, *bla*$_{DIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{KPC}$, *bla*$_{BIC}$, and *bla*$_{OXA-48}$ [12]. These protocols do not target other *bla*$_{OXA}$ genes that are prevalent in *A. baumannii*, but another multiplex PCR, developed by Woodford, *et al.*, focuses on these genes (specifically *bla*$_{OXA-23-like}$, *bla*$_{OXA-24-like}$ and *bla*$_{OXA-58-like}$) [13]. Other examples are the real-time PCR protocols for detection of different combinations of relevant carbapenemases, such as the ones developed by Monteiro, *et al.* (*bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$ and *bla*$_{NDM-1}$) [14], Smiljanic, *et al.* (*bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{OXA-48-like}$ and *bla*$_{OXA-23-like}$) [15], and Mentasti, *et al.* (*bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48-like}$) [16] .

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 β-lactamase (and other) genes or chromosomal point mutations in the bacterial genome [17,18].

There are other important mechanisms mediating resistance towards carbapenems, including over-expression of efflux pumps, decreased porin expression or alterations in configuration, and over-expression of intrinsic β-lactamases [10,19–24]. These resistance mechanisms are often multi-factorial and difficult to detect through conventional PCR and, at the moment, the only molecular method proposed for their detection is sequencing the bacterial DNA. Due to this variety of genetic mechanisms leading to carbapenem resistance, especially relevant in *P. aeruginosa*, it is important that laboratories are able to detect carbapenemases and that they apply IQC strategies when performing those PCR protocols.

In *P. aeruginosa* and *A. baumannii* colistin resistance is most often mediated by chromosomal point mutations in the genes affecting PmrAB, PhoPQ, or other two-component systems leading to lipopolysaccharide modification [25]. To a lesser extent, colistin resistance has also been attributed to mutations in the lipid A biosynthesis genes *lpxA*, *lpxC* and *lpxD* [26–29]. The presence of plasmid-mediated *mcr*-genes is less frequent in these species than what is observed for *Enterobacterales.*

Some of the chromosomal point mutations that have been described in two-component systems as being correlated with phenotypic resistance towards colistin in *P. aeruginosa* isolates are PmrB M48L, V15I, V281I and A248T, PhoQ A143V and R214H, and ParS G361R [30–32]. In *A. baumannii* isolates, the mutations PmrB T192I, A227V, P233S, and others have

been reported as being associated with colistin resistance [30,33,34]. At the moment, the only molecular method proposed for detection of these mutations is sequencing the bacterial DNA.

So far, ten *mcr*-genes and their variants have been described, in different bacterial species [35–44]. For screening purposes and future references, the EURL-AR provides a list of the currently know *mcr*-genes and their variants, which is regularly updated. EURL-AR has also published a multiplex PCR protocol for detection of colistin-resistance genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*, although only validated for *Enterobacterales* [45]. Another multiplex PCR protocol exists for the *mcr-6*, *mcr-7*, *mcr-8* and *mcr-9* genes [46].

In summary, QC for molecular detection of AMR includes:

- Expanding the PCR protocols (or implementing PCR protocols) to include detection of genes encoding carbapenemase production;
- Using all positive control strains described in each PCR protocol;
- Always including a negative control;
- Not combining different PCR protocols into a larger multiplex;
- Creating method overview documentation and record the batch of reagents, materials and equipment, as exemplified for phenotypic AST.

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8. APPENDICES

8.1. 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	Bouillon	Transfer from McFarland	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
Pseudomonas	TSA 5% blood	W	EUVSEC3	dem. water	CAMHB	10 µl	50 µl/ well	1	36-37°C 18-20 h
Acinetobacter	TSA 5% blood	W	EUVSEC3	dem. water	CAMHB	10 µl	50 µl/ well	1	36-37°C 18-20 h
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.

Document approved by:
Approval date:

8.2. 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						
TSA 5% blood						
EUVSEC3						
EUVSEC2						
Inoculator 1234						
Inoculator 5678						
McFarland std.						
Dosing heads						
Incubator AB12						
Incubator CD34						
[other]						
[other]						

Remarks:

8.3. 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**	***P. aeruginosa*** **ATCC 27853**
Media		CAMHB	CAMHB	CAMHB
MIC panel (Sensititre™)	**EUVSEC3**	X	X	X
	EUVSEC2	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**	***P. aeruginosa*** **ATCC 27853**
Amikacin	0.5-4	--	1-4
Ampicillin	2-8	--	--
[…]	*[…]*	*[…]*	*[…]*
Ciprofloxacin	0.004-0.016	--	0.125-1
Clindamycin	--	--	0.5-4
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:

8.4. 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:

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