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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				
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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). The project aims at improving capacities of National Reference Laboratories (NRLs) in European countries for identification and for phenotypic and genotypic characterization of carbapenem-resistant *Enterobacterales* (CRE) and colistin-resistant CREs (CCRE), and other antimicrobial-resistant pathogens. Furthermore, the project aims at strengthening capacities for national surveillance and outbreak investigation of CRE/CCRE and 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 in order to achieve those respective aims.

This guidance document provides assistance to NRLs and other laboratories regarding the techniques that should be implemented, improved, or confirmed for Internal Quality Control (IQC) of their procedures, including requirements for competence and accreditation strategies. This document also aims at describing the standardized and/or recommended methods for antimicrobial susceptibility testing (AST) of CRE and CCRE in Europe, and the proposed methods for detecting relevant antimicrobial resistance (AMR) determinants. It collects and presents, in a systematic way, the most recently available information from different regulatory agencies and other sources, to ensure that all laboratories remain or become familiar with the guidance documents that should be employed in reference or clinical laboratories.

Guidance for quality control of whole-genome sequencing-based investigation of antimicrobial resistance is described in other documents prepared in the context of this Project, specifically in the Deliverable T3.4 "Agreed common WGS-based genome analysis methods and standard protocols for national CCRE surveillance and integrated outbreak investigations" and its Supporting Document.

2. INTERNAL QUALITY CONTROL STRATEGIES

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

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 allow for validated and comparable results.

The ISO 15189:2012 standard, "Medical laboratories - Requirements for quality and competence", sets out a number of management and technical requirements that guide medical laboratories in providing services aligned with the "best practice" in their field. Unlike other laboratory standards, ISO 15189 focuses on processes instead of procedures or protocols, with the aim of ensuring that "*the laboratory meets both the technical competence requirements and the management system requirements that are necessary for it to consistently deliver technically valid results*" [1].

In line with the ISO 15189 standard, clinical laboratories should aim at creating systems that are as failure resistant as possible, identify opportunities for improvement and involve and empower their staff by involving them in the solving of problems and implementation of solutions. The management requirements include regular management reviews and internal audits to assure that the laboratory's activities are effective, continually meet the users needs, and adhere to the quality management system (QMS). Management requirements aimed at preventing non-conformities include document control, preventive and corrective measures, risk management, performance of periodic internal audits and establishment of quality indicators.

The technical requirements are focused on staff competences, including training and posterior assessment of competence, working facilities, reception and management of consumables and reagents, and laboratory equipment including calibration and adequate recording of uses. Requirements for preparation, collection, reception, handling and testing of biological samples are also described. Furthermore, testing methods or examination procedures should be adequately validated and documented, and reporting of results should include at least the minimum parameters stated in the standard.

An important section of the ISO 15189 is the description of quality assurance of tests 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 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" 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".

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, 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.

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, instead, 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

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 [2–4]. 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.

3.1. Broth microdilution

EUCAST recommends to perform AST through broth microdilution (BMD), by following the protocol provided by ISO, document ISO 20776-1:2019 (or the less recent version ISO 20776-1:2006).

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.

The 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 5×10^5 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, depleting iron from the broth medium before testing cefiderocol, and refraining from adding surfactants to the medium when testing colistin.

One of the most important considerations in the ISO standard 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. According to EUCAST, when performing AST of *Enterobacterales*, 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.

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 has been made available by the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR).

Results from QC should be stored in the laboratory for long-term comparison. Punctual deviations in expected results for the QC strains might reveal an isolated 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, 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. 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.

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. At the moment, the only general alteration suggested in the website 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. In *Enterobacterales*, the tables indicate that BMD should not be used for fosfomycin nor mecillinam, but instead agar dilution. The tables also contain 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 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 is common for tigecycline and trimethoprim, 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. 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".

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 [5]. 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).

3.2. Disk diffusion

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 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, specifically incubation at 35 ± 1°C during 18 ± 2 hours for *Enterobacterales*, 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 applicable to *Enterobacterales* are the possible presence of faint growth within the inhibition zone for trimethoprim, ampicillin, ampicillin/sulbactam and amoxicillin/clavulanic acid, which should be disregarded. Additionally, it is possible that single colonies form within the inhibition zone for temocillin, mecillinam and fosfomycin, which should also be ignored.

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. Furthermore, the following control strains must be used: *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.

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.

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.

In summary, QC for phenotypic AST of CRE/CCRE includes:

- Following ISO 20776-1 for AST through BMD;
- Using the control strain *E. coli* ATCC 25922;
- 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 DD for AST of colistin.

3.3. Phenotypic detection of β-lactamase-producing *Enterobacterales*

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

The EUCAST guidelines also contain methods for detecting mechanisms of resistance towards β-lactam antimicrobials, 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.

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 appropriate cut-off values to consider isolates as putative 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. Although both antimicrobials can be used for screening, meropenem offers the best compromise between sensitivity and specificity for detecting putative carbapenemase-producing isolates. After identifying these isolates, the production of carbapenemase should be further confirmed by employing any of the methods described in the EUCAST guidelines, such as the combination disk testing method or colorimetric tests such as the CarbaNP test.

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. Isolates with MIC values >1 mg/L for cefotaxime or ceftriaxone, and simultaneously MIC >1 mg/L for ceftazidime should be considered as putative ESBL-producers and should undergo confirmatory testing for detection of ESBL-production. Isolates with MIC >1 mg/L for cefpodoxime should also be considered as putative ESBL-producers and undergo further

testing. The EUCAST guidelines also describe the inhibition zone diameters that should be used as thresholds in screening procedures, when using the DD method.

Confirmatory testing of ESBL production can be based on the fact that 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. The EUCAST guidelines describe the different possible methods for confirmation of ESBL-production. Two of those are the broth microdilution and the combination disk test, and examples of interpretative criteria for results obtained through broth microdilution are described in Table 1. The test should be performed with cefotaxime (as shown in Table 1) and also with ceftazidime and cefepime, and their results interpreted in the same way. 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 *Enterobacterales* according to results obtained through BMD

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

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 specific β-lactamase inhibitors (in particular clavulanic acid). However, the ratio between MIC values is much smaller than what is observed in ESBL-producing organisms, with MIC-values below 8 mg/L or DD zone diameters ≥ 19 mm. The isolates are also 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.

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 *Enterobacterales* 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	*Klebsiella pneumoniae* carbapenemase (KPC)

K. pneumoniae NCTC 13438	
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 extraordinary 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 for detection of *mcr*-genes and genes encoding β-lactamase-production.

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.

One of the most relevant mechanisms of resistance towards β-lactam antimicrobials is the expression of acquired genes encoding β-lactamase-production, including 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 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].

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.

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 [6].

So far, ten *mcr*-genes and their variants have been described, in *Enterobacterales* or other species [7–16]. 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.

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* [17]. Another multiplex PCR protocol exists for the *mcr-6*, *mcr-7*, *mcr-8* and *mcr-9* genes [18]. 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 S39I and PmrB V161G in *E. coli*, and PmrA G53CN, PmrB S85R and PhoP L26Q in *K. pneumoniae* [19–21]. At the moment, the only molecular method proposed for detection of these mutations is sequencing the bacterial DNA.

In summary, QC for molecular detection of AMR includes:

- Expanding the PCR protocols (or implementing PCR protocols) to include detection of *mcr*-genes;
- Expanding the PCR protocols (or implementing PCR protocols) to include detection of genes encoding β-lactamase 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.

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