

# Global Antimicrobial Resistance Surveillance System (GLASS) - The detection and reporting of colistin resistance

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# Acronyms and abbreviations

| | |
|---|---|
| AMR | antimicrobial resistance |
| CLSI | Clinical and Laboratory Standards Institute |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| GLASS | Global Antimicrobial Resistance Surveillance System |
| PCR | polymerase chain reaction |

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## Summary

The scope of this technical note is to review current methods for the detection of colistin resistance and to provide a framework for its investigation. The document highlights the critical distinction between phenotypic detection of colistin resistance and genotypic detection of specific colistin resistance mechanisms, such as *mcr* and chromosomal mutations. Colistin resistance in Enterobacteriaceae and *Acinetobacter baumannii* is included in the WHO Global Antimicrobial Resistance Surveillance System (GLASS). Currently, however, reliable tests for phenotypic detection of colistin resistance for clinical and surveillance purposes are not widely available.

The technical note describes existing phenotypic methods for detecting colistin resistance, genotypic methods for detecting specific colistin resistance mechanisms and surveillance strategies for monitoring colistin resistance.

This is a rapidly evolving field with new resistance genes being identified regularly and new methods for phenotypic resistance testing being described, hence this document will be updated, as needed, to reflect these developments.

## 1. Introduction

Colistin belongs to a group of antimicrobial agents known as polymyxins, which were originally isolated from the spore-forming soil organism *Paenibacillus polymyxa.* Molecules in this group are polymyxins A, B, C, D and E, of which only polymyxin E (colistin) and polymyxin B are used clinically in humans.

Colistin is a mixture of polymyxin E1 and E2, two bactericidal pentacationic lipopeptides. The mode of action of colistin is not fully elucidated but involves binding to lipopolysaccharides and phospholipids in the outer membrane of Gram-negative organisms, which results in membrane disruption and cell death. Colistin is active against a wide variety of Gram-negative bacteria and is not active against Gram-positive bacteria, which lack an outer membrane.

Colistin is used in both human and veterinary medicine. In humans, colistin is generally used to treat infections with multidrug-resistant, extensively drug-resistant and pan drug-resistant bacteria *(1)*. It is usually administered by injection or inhalation (the latter, for example, for patients with cystic fibrosis) as the sodium salt of colistin methanesulfonate, which is an inactive prodrug. It is considered less toxic than colistin sulfate, which is used orally (with very limited absorption) or topically *(2)*.

In veterinary medicine, colistin has been widely used in various food-producing animals (broiling and laying hens, pigs, calves, beef cattle, dairy cattle, meat- and milk-producing sheep and goats, rabbits and fish in aquaculture) for the prevention and treatment of infections caused by Enterobacteriaceae and other Gram-negative bacteria and for growth promotion. In veterinary medicine, colisitin is usually administered orally *(3)*. The wide use of colistin in food-producing animals is considered to have contributed extensively to the emergence of resistance to this drug *(3–5)*.

With the spread of highly resistant bacteria such as carbapenemase-producing Enterobacteriaceae and multi-drug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, colistin has been used increasingly as an agent of last resort. The emergence of colistin resistance in multidrug-resistant bacteria is therefore a significant clinical and public health concern *(6–8)*.

The spectrum of colistin (Table 1) includes activity in vitro against *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., *Enterobacter* spp., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Citrobacter* spp., *Yersinia pseudotuberculosis* and *Haemophilus*

*influenzae*. Furthermore, colistin has considerable activity against *Stenotrophomonas* spp., whereas it is not active against some Gram-negative aerobic bacilli, including *Burkholderia mallei*, *Burkholderia cepacia*, *Proteus* spp., *Providencia* spp., *Morganella morganii*, *Serratia* spp., *Edwardsiella* spp. and *Brucella* spp. *(9–12)*, Gram-negative and Gram-positive aerobic cocci, Gram-positive aerobic bacilli, anaerobes, fungi and parasites *(2, 13)*.

***Table 1. Activity of colistin against selected pathogenic bacteria***

| Organism | Natural state (resistant or susceptible to polymyxins) |
|---|---|
| *Escherichia coli* | Susceptible |
| *Klebsiella* spp. | Susceptible |
| *Enterobacter* spp. | Susceptible |
| *Pseudomonas aeruginosa* | Susceptible |
| *Acinetobacter* spp. | Susceptible |
| *Salmonella* spp. | Susceptible |
| *Shigella* spp. | Susceptible |
| *Pasteurella* spp. | Susceptible |
| *Haemophilus* spp. | Susceptible |
| *Proteus* spp. | Intrinsically resistant |
| *Providencia* spp. | Intrinsically resistant |
| *Morganella morganii* | Intrinsically resistant |
| *Serratia* spp. | Intrinsically resistant |
| *Brucella* spp. | Intrinsically resistant |
| *Neisseria* spp. | Intrinsically resistant |
| *Chromobacterium* spp. | Intrinsically resistant |
| *Burkholderia* spp. | Intrinsically resistant |
| Gram-positive bacteria | All Gram-positive bacteria are intrinsically resistant to polymyxins because of the absence of an outer membrane. |

# 2. Acquired resistance to colistin

## *2.1 Mutational colistin resistance*

Acquired resistance to polymyxins in naturally susceptible species is usually the result of modifications of the bacterial cell surface, including alterations to the lipopolysaccharide structure. Other reported resistance mechanisms include shedding of capsular polysaccharides, which trap or bind polymyxins, as found in some isolates of *Klebsiella pneumoniae (14)*. In *A. baumannii*, two primary mechanisms of chromosomally mediated colistin resistance have been described. The first is due to loss of lipopolysaccharide production and the second to modification of the system that allows bacteria to respond to environmental conditions, affecting lipid modification and reducing bacterial membrane permeability *(14, 15)*. Resistance arising from chromosomal mutations is not usually transferred horizontally among bacteria *(16)*.

## *2.2 Transferrable colistin resistance*

A plasmid-mediated gene that confers resistance to colistin, *mcr-1*, was first reported in late 2015 in *E. coli* isolates from food animals and their meat collected in China during 2011–2014 and in *E. coli* and *K. pneumoniae* isolates collected from human patients in China in 2014 *(17)***.** Although colistin had been used extensively in food-producing animals globally for many years, it was not available for human clinical use in China until 2017, suggesting

that the selection pressure for the spread of *mcr-1* was driven by veterinary use of colistin *(18)*.

Shortly after this first description, it was observed that the *mcr-1* gene had spread globally in *K. pneumoniae, E. coli*, *Enterobacter cloacae* and *Salmonella* spp. isolates of animal, environmental and human origin *(17, 19–25).* The global spread of *mcr-1* was probably also facilitated by human travel, as suggested by the finding of the gene in enteric bacteria from travellers returning to Europe after visiting countries with a high prevalence of *mcr-1* in South America, Asia and Africa *(18, 26)*.

After identification of *mcr*-1, wide scientific attention led to recognition of multiple *mcr-1* variants *(27–35)* and seven additional (at the time of writing) *mcr* gene families *(36).* The latter include *mcr-2* in *E. coli* and *Salmonella* spp. from pigs, cattle and chickens in Belgium *(37, 38), mcr-3* in human and animal *E. coli, Proteus* spp. and *Aeromonas* spp. in countries in Asia, Europe and South America *(39), mcr-4* in *E. coli* and *Salmonella enterica* serovar Typhimurium from pigs and humans in Belgium, Italy and Spain *(40, 41*), *mcr-5* in poultry and poultry meat isolates of *S. enterica* serovar Paratyphi B d-tartrate fermenting and in porcine *E. coli* in Germany *(42, 43),* mcr-6 in *Moraxella* sp. isolates from pigs in the United Kingdom *(35)*, *mcr-7* in *K. pneumoniae* from chickens in China *(44, 45)* and *mcr-8* in *K. pneumoniae* from humans and pigs in China *(46)*. The co-occurrence of more than one of these genes has been reported in *E. coli* and *Salmonella* spp. isolates of human and animal origin in Europe and China, such as the simultaneous presence of *mcr-1* and *mcr-3* and of *mcr-1* and *mcr-4 (47–51).*

Bacteria isolated from food-producing animals to date appear to carry the *mcr* genes more frequently than bacteria isolated from humans, which is probably a consequence of the selective pressure exerted by the wide use of colistin in veterinary practice *(14)*. Importantly, *mcr*-mediated colistin resistance can be transferred among bacterial strains, species and genera. To limit further dissemination of such genes, accurate identification of colistin-resistant, *mcr*-encoding isolates is of critical importance.

# 3. Laboratory detection of colistin resistance

## *3.1 Phenotypic methods*

Testing for antimicrobial susceptibility by phenotypic methods is currently the cornerstone of AMR surveillance and is necessary to determine the occurrence of colistin resistance within GLASS. Phenotypic testing for antimicrobial susceptibility to colistin is, however, complex. The technical difficulties in phenotypic testing for colistin susceptibility include poor diffusion of polymyxins through agar, which compromises the performance of both disc diffusion and gradient diffusion methods, and the tendency of polymyxins to bind to the surface of plastics (e.g. the plastic of microtitre trays used for broth microdilution and of pipette tips) *(52)*.

International standard-setting organizations such as the Clinical and Laboratory Standards Institute (CLSI)[1] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[2] formed a joint polymyxin breakpoints working group in March 2016, which recommended only broth microdilution for testing of colistin susceptibility. Thus, the susceptibility of Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp. to colistin should be tested according to the International Organization for Standardization standard broth microdilution method (ISO 20776-1), in which cation-adjusted Mueller-Hinton broth is used. No additives may be included in any part of testing (in particular no polysorbate-80 or other

[1] https://clsi.org/

[2] http://www.eucast.org/

surfactants), because, for example, polysorbate 80 can act synergistically with polymyxins and artificially lower the minimum inhibitory concentration *(53).* Trays should be made of plain polystyrene, and sulfate salts of polymyxins must be used (the methane sulfonate derivative of colistin cannot be used as it is an inactive pro-drug that breaks down slowly in solution). A review of the performance of different broth microdilution-based systems used for colistin susceptibility testing is available *(54).*

Quality control is essential in phenotypic detection of colistin resistance. To ensure that the method used provides reliable results, inclusion of a strain with low-level colistin resistance (such as *mcr-1*-positive *E. coli* NCTC 13846) is recommended by EUCAST *(55)*.

Phenotypic results can be interpreted and reported as either the epidemiological cut-off (according to EUCAST or CLSI), which define wild-type and non-wild-type populations, or as clinical breakpoints, which define clinical susceptibility and resistance (Table 2) (56, 57).

***Table 2. Epidemiological cut-off values and clinical breakpoints***

| Bacteria | Wild type or sensitive | Non-wild type or resistant | Comment |
|---|---|---|---|
| Enterobacteriaceae | ≤ 2 μg/mL | ≥ 4 μg/mL | For CLSI, this is an epidemiological cut-off to distinguish between wild and non-wild types. |
| *Acinetobacter* spp. | ≤ 2 μg/mL | ≥ 4 μg/mL | Clinical breakpoints |
| *Pseudomonas aeruginosa* | ≤ 2 μg/mL | ≥ 4 μg/mL | Clinical breakpoints |

Clinical trials, including ones in which new antibiotics are compared, are providing new data on the clinical effectiveness of colistin for the treatment of infections caused by Gram-negative bacteria. According to the clinical outcomes, colistin appears to be a poor mono-therapeutic option, especially when other drugs are available *(58)*. In particular, the dosing of colistin is complex, as its pharmacokinetics differs widely among patients *(59)*. Therefore, further review of clinical outcomes is necessary to determine whether currently established breakpoints and epidemiological cut-off values accurately inform clinical decisions. See CLSI[1] and EUCAST[2] documents for the latest information.

Testing for antimicrobial susceptibility by broth microdilution requires greater laboratory capacity than standard disc diffusion and gradient diffusion methods and other phenotypic methods. Nevertheless, other methods are either insufficiently accurate or have not yet undergone rigorous multicentre evaluation with large collections of bacteria, including different species (i.e. most Enterobacteriaceae and Gram-negative non-fermenters), that express all known colistin resistance mechanisms. Thus, these methods cannot yet be recommended as best practice.

The methods that appear promising but are still to undergo the rigorous evaluation described above are: (i) agar-based screening media for bacteria *suspected* to be colistin-resistant *(60, 61*), which should always be followed by further investigation of the colonies growing on such media by broth microdilution and/or molecular methods; (ii) the Rapid Polymyxin NP test (*62*); (iii) Colispot *(54*); and (iv) disc prediffusion (*63*). The performance of these methods depends on the bacterial species and the colistin resistance mechanisms.

The recognized need for simpler, more reliable in vitro testing of colistin susceptibility and for methods to selectively identify colistin-resistant, *mcr*-positive isolates is driving technical development, and improved phenotypic testing products are expected to become available.

## *3.2 Genotypic methods*

During its early implementation stage *(64)*, GLASS only collects data on antimicrobial susceptibility of target pathogens acquired by phenotypic testing; it does not yet collect data on the distribution of specific resistance mechanisms. Nevertheless, genotypic surveillance can be used as a basis for selection of isolates for subsequent phenotypic testing for antimicrobial susceptibility and the results submitted to GLASS, or it can be used as a supplementary investigation of resistance mechanisms for isolates with unusual phenotypic resistance(s). Such studies can help in understanding the epidemiology of AMR and the relative contributions of the human clinical, animal health and agricultural sectors to the problem of AMR. They may also improve reporting to the Emerging Antimicrobial Resistance module in GLASS (the GLASS-EAR system).

Genotypic results should be reported as the presence or absence of the specified colistin resistance mechanism or gene. At present, there is insufficient understanding of acquired colistin resistance mechanisms to design a molecular test that would be sufficiently sensitive or specific to be recommended as best practice. In particular, genotypic methods would probably not detect all the diverse chromosomal mutations known to be responsible for most phenotypic colistin resistance in clinical settings *(14)* but would be limited to the detection of acquired colistin resistance genes. A few published and/or commercially available colistin molecular tests are available for detecting transferrable colistin resistance, including a multiplex polymerase chain reaction (PCR) for screening for *mcr-1* to *mcr-5*.

It should be noted that a negative result in a PCR molecular test cannot be used to predict susceptibility to colistin, because the test cannot exclude the presence of chromosomal mechanisms of resistance or even of novel *mcr* genes that are not included in the test. As evidence of this limitation, high rates of colistin resistance are reported among strains of *K. pneumoniae* producing carbapenemase in Brazil and Italy but which lack *mcr* genes *(65)*. In these settings, a negative PCR result for *mcr* genes would have poor predictive value for a colistin-susceptible phenotype. Whole-genome sequencing would allow screening for *mcr* genes and known chromosomal mutations that confer colistin resistance *(40)*. Bioinformatics analysis could be conducted with, among others, the Center for Genomic Epidemiology Web tools *(*66), and ResFinder 3.1.0 (database updated on 10 September 2018 to include all known *mcr*-genes and chromosomal point mutations) (*67*). Although the sensitivity and negative predictive value would be affected by inclusion of strains with novel mechanisms of resistance, this is the most comprehensive method for detecting all currently known putative colistin resistance mechanisms. It will also enable retrospective analysis of sequencing data as new resistance mechanisms are described.

Whatever the molecular method used, it is critically important to ensure that the PCR either detects all currently known *mcr* genes or that the databases used to impute resistance mechanisms from whole-genome sequencing data are up to date.

As our understanding of colistin resistance mechanisms improves, so will the concordance between phenotypic and genotypic test results. As for many other classes of antimicrobial agent, molecular testing may eventually offer an alternative to phenotypic testing for the surveillance of colistin resistance. Nevertheless, if the results are intended to guide clinical management, inference of phenotype based solely on a genotypic result may be valid only when the genotypic result is positive (i.e. mechanisms or genes detected), with a cautionary note that the resistance phenotype is likely but not guaranteed. If the results of the genotypic test are negative, no inference should be drawn about phenotype.

## 4. Surveillance strategies

In view of the many technical challenges to colistin susceptibility testing, clear strategies are required for clinical diagnosis, sampling and testing for surveillance. In local health care settings, colistin resistance should be tested if the recommended technology is available, and samples should be sent to laboratories with the necessary technical capacity if the technology is not available. The cost of shipping isolates is an important aspect to be considered in confirmatory testing. Testing should be performed on:

- carbapenem-resistant Enterobacteriaceae (except for intrinsically colistin-resistant genera),
- other Gram-negative bacteria resistant to carbapenems (except intrinsically colistin-resistant genera) and,
- when treatment with colistin must be considered (except infections caused by intrinsically colistin-resistant genera).

Health care facilities with a high prevalence or outbreaks of infections caused by carbapenem-resistant and other multidrug-resistant Enterobacteriaceae and/or non-fermenting bacteria such as *Acinetobacter baumannii* spp. and *P. aeruginosa*, could consider conducting periodic surveys to assess the occurrence of colistin resistance to inform local antimicrobial use policies.

At national level, at least one AMR reference laboratory should be established to monitor colistin resistance reliably, with appropriate controls and standardized methods. Such an AMR reference laboratory could perform confirmatory testing upon request from local laboratories. Samples for confirmatory testing should be selected carefully, with priority given to isolates suspected to have acquired transferrable (*mcr*-mediated) colistin resistance. A reasonable strategy for detecting *mcr*-mediated colistin resistance would be to screen for phenotypic resistance (for example, using selective agar plates), CHROMID® Colistin R agar, Superpolymyxin or CHROMagar COL-APSE) *(*60, 61, 68, 69*)*, followed by PCR to detect one or more of the *mcr* genes. Such molecular testing could be performed at a reference laboratory or at a WHO collaborating centre.

Ideally, colistin susceptibility testing should be included in national plans for AMR surveillance. Countries with strong national AMR surveillance programmes and laboratory capacity could extend them to include monitoring for colistin resistance in bacteria isolated from food-producing animals and also from food and environmental samples, at least periodically.

Currently, the detection of colistin resistance is too technically complicated to recommend as a phenotypic screening method for resource-limited settings. It is therefore essential that laboratories store isolates suspected of being colistin-resistant and collaborate with AMR reference laboratories and/or WHO collaborating centres to confirm colistin resistance and determine the possible presence of *mcr* genes.

The GLASS protocol *(64)* for sample-based surveillance is recommended for colistin susceptibility testing. In sample-based surveillance case-finding is focused on priority specimens sent routinely to laboratories for clinical purposes. In addition, epidemiological information should be clearly linked to microbiological information for each sample and the standard AMR indicators recorded for positive cultures. The sample-based approach allows acquisition of information on the proportion of susceptible, intermediate and resistant isolates and the total number of patients sampled, making it possible to deduce the rates or frequencies of specific types of resistance in the tested population. Information on the incidence of this type of resistance among tested patients could provide an indication of the extent of colistin resistance in the population. The sample population must be carefully defined and described in any report in order not to overestimate resistance rates if only highly resistant strains are tested.

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