

## MCR: modern colistin resistance

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**Abstract** Recently, plasmid-mediated and, therefore, transferable bacterial polymyxin resistance was discovered in strains from both humans and animals. Such a trait may widely spread geographically, while simultaneously crossing microbial species barriers. This may ultimately render the “last resort” polymyxin antibiotics therapeutically useless. Colistin is currently used to treat infections caused by Gram-negative carbapenemase producers and colistin resistance may lead to practical pan-antibiotic resistance. We here analyzed the medical and diagnostic consequences of (emerging) colistin resistance and propose pathways toward adequate diagnostics for timely detection of both asymptomatic carriage and infection. Culture-based testing using chromogenic and selective media for screening clinical (and veterinary) specimens may constitute key tools for that purpose. Relevant molecular tests are also discussed.

### What is colistin?

Colistin, also known as polymyxin E, is produced by *Paenibacillus polymyxa* and belongs to the polymyxin class of antibiotics. It is a polycationic peptide with both

hydrophilic and lipophilic domains. The cationic regions interact with bacterial lipopolysaccharide (LPS) in the outer cell membrane, where it displaces magnesium and calcium counter ions. Colistin is not homogeneous at the molecular level and consists of a mix of the cyclic polypeptides A and B. Colistin essentially solubilizes the bacterial cell membrane, which is bactericidal in an aqueous environment. It is an older drug with significant nephrotoxicity.

Colistin is commonly used in agriculture in many countries for the control of infections in pigs and, in particular, cattle. Its usage in humans was, so far, rather limited, mainly because of its renal toxicity, but it has been recently re-introduced as a last-line option to treat extensively antibiotic-resistant bacteria such as carbapenem-resistant strains [i.e., NDM-1- and KPC-positive bacterial isolates or extensively drug-resistant (XDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*]. Colistin has been effective in treating infections caused by *P. aeruginosa*, *Escherichia coli*, and *Klebsiella* spp., for which the range of “normal” minimum inhibitory concentrations (MICs) is  $\leq 0.06$ –4  $\mu\text{g/ml}$ , 0.12–2  $\mu\text{g/ml}$ , and 0.25–2  $\mu\text{g/ml}$  for their wild-type populations, respectively. Colistin is also used for (a) prophylactic and selective decontamination of the digestive tract in intensive care unit (ICU) patients and (b) reducing respiratory tract infections [1]. “Classical” resistance to colistin is rare. However, the SENTRY surveillance network detected colistin resistance associated with the presence of the plasmid-mediated colistin resistance mechanism, involving the *mcr-1* gene, among *E. coli* and *K. pneumoniae* during 2014–2015 [2–4].

### Classical colistin resistance

Antibiotic susceptibility testing (AST) is normally performed using highly standardized microbiological technology and, in

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the case of colistin, the most common protocol has been jointly endorsed by both the Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the American and European groups overseeing standardization of AST laboratory protocols. Measurement of exact colistin MICs is associated with methodological issues, but the ISO-standard broth microdilution (BMD) method (20776-1) works well for Enterobacteriaceae, *P. aeruginosa*, and *Acinetobacter* spp. Specifically, additive-free cation-supplemented Mueller–Hinton broth needs to be employed. Trays must be made of plain polystyrene and not treated in any way before use, whereas sulfate salts of the antibiotic work best. Susceptibility testing by other methods, including agar dilution, disk diffusion, and gradient diffusion, cannot be recommended in the absence of regional historical colistin MIC data as a reference. Newer methods including the molecular ones are still in need of optimization.

Classical resistance to colistin can be explained by a variety of mechanisms. In *E. coli*, *Salmonella enterica* [3], *K. pneumoniae*, and *P. aeruginosa*, the net LPS charge can be reduced upon modification of lipid A through the chemical coupling of phosphoethanolamine or L-amino-arabinose through activation of the *pmrC* or *pmrF* operons. Also, the presence of a capsule has been shown to interfere with polymyxin susceptibility in *K. pneumoniae*. Resistance to colistin in *A. baumannii* may be associated with mutations in the PmrAB two-component system or with a progressive to full loss of LPS through mutations in the *lpxA*, *lpxC*, and *lpxD* genes [5]. A single mutation in the PmrB protein, which is a sensor kinase, can lead to the addition of phosphoethanolamine to lipid A, which causes high-level colistin resistance in *A. baumannii*. Studies in *P. aeruginosa* also suggested a potential role in the resistance to polymyxins for OprH, an outer membrane protein contributing to membrane stability [6]. Multidrug efflux systems can also be responsible for polymyxin resistance in *P. aeruginosa* [7]. Changes in the regulatory loci *pmrA* and *phoP* have also been shown to be responsible for polymyxin resistance in Enterobacteriaceae. With time, additional novel resistance mechanisms will be identified. Until very recently, colistin resistance was always thought to be chromosomally encoded allowing vertical transmission only. In addition, this type of resistance often appears to be quite unstable, self-limiting, and does not prevent the successful use of polymyxins.

### Emerging mobile colistin resistance markers

In late 2015, a plasmid-borne *mcr-1* gene encoding a phosphoethanolamine transferase giving rise to polymyxin resistance was first identified in bacterial strains of animal origin, but also in those cultured from humans and the environment [8]. The plasmid localization of that resistance trait

allows for horizontal transmission of the colistin resistance. Surveillance of *E. coli* strains from food animals in China showed that the plasmid was present in 15 % of strains found in meat samples and in 21 % of strains derived directly from animals. It was documented that the strains emerged locally between 2012 and 2014. MCR-1-producing *E. coli* and *K. pneumoniae* strains were detected in blood and urine specimens from hospitalized patients, showing that host species barriers had already been crossed by the plasmid. In addition, all strains were extended-spectrum  $\beta$ -lactamase (ESBL) producers [8] and similar strains were isolated from outpatients as well [9].

Transferable colistin resistance has been detected in 10 % of NDM-1-producing carbapenem-resistant Enterobacteriaceae (CRE) in the United Kingdom [10]. In Italy, the proportion of colistin resistance among KPC-producing *K. pneumoniae* was 57 % in 2013 [11]. Following these important findings, several international teams started looking for this new gene in existing collections of bacteria. In January 2016, *mcr-1*-positive strains were already found in about 19 different countries, including Malaysia [7], France [12, 13], Portugal [14], Laos [15], USA, Thailand, Algeria [16, 17], Germany [18], Switzerland [19, 20], Japan, Belgium, Vietnam [21, 22], the Netherlands [23], Spain [24], and Denmark [25]. The frequency of occurrence of *mcr-1*-positive bacterial isolates still seems relatively low in humans: in China, 1.4 % of the *E. coli* strains and 0.7 % of the *K. pneumoniae* isolates scored phenotypically positive. In Europe (Denmark), 0.2 % of the ESBL- and AmpC-positive *E. coli* strains are *mcr-1* PCR-positive. In animals, the detection of *mcr-1* was lower in Europe than in China, with 15 % of detection in the latter country compared to 1.5 % in the Netherlands and 2 % in Denmark [26].

In bacterial strains from humans, transferable colistin resistance was mainly identified in isolates from patients who were not treated with colistin, and it has been speculated that acquisition of such resistant strains might have resulted from the ingestion of contaminated meat, leading to asymptomatic gastrointestinal colonization [25]. Currently, *mcr-1* has been found in *E. coli*, *K. pneumoniae*, and *Salmonella*, and could be successfully maintained in *P. aeruginosa* [8]. Although many *mcr-1*-positive isolates were shown to co-produce an ESBL enzyme, it is noteworthy that many *mcr-1*-producing *E. coli* isolates are fully susceptible to  $\beta$ -lactams and are, therefore, far from multidrug-resistant [13, 23, 25]. Nevertheless, there have been reports of *mcr-1*-producing multidrug-resistant isolates, and, in particular, carbapenemase-producing ones. The first identified isolate was a VIM-1-producing *E. coli* recovered in Switzerland from a patient who did not have any history of foreign travel [19]. That isolate remained susceptible only to amikacin, fosfomycin, and tigecycline. Recently, an NDM-5- and *mcr-1*-producing *E. coli* has been recovered in the USA, but, still, several drugs were active against that organism

including amikacin, gentamicin, aztreonam, and nitrofurantoin [27]. In China, some cases of *K. pneumoniae* isolates co-producing *mcr-1* and NDM-5 have also been reported [9]. An association of *mcr-1*, *bla*<sub>NDM-5</sub>, and *fosA3* genes has also been reported in *E. coli* strains from chicken products, exhibiting high level of resistance to colistin [28]. The *mcr-1* gene has been found in KPC-producing and carbapenem-resistant *K. pneumonia* isolates in Italy and Greece as well.

Overall, the occurrence of such multidrug-resistant enterobacterial isolates underscores the importance of this major emerging problem and highlights that care needs to be taken to prevent further dissemination. However, recent studies showed that the occurrence of *mcr*-producing isolates remains relatively low in many countries. For instance, a very low prevalence of *mcr-1* and *mcr-1* producers was found in Switzerland among enterobacterial isolates recovered from urinary tract infections [29]. Similarly, no *mcr-1*-producing enterobacterial isolate was identified when screening a large collection of enterobacterial isolates recovered from bacteremia in the same country [30]. A significant increase in the detection of colistin resistance has been observed in isolates [22]: up to 50 % of *E. coli* isolated from swine with diarrhea or edema were *mcr-1*-positive. A high frequency of *mcr-1*-positive *E. coli* has been seen in livestock and food isolates, mainly in turkey (10.7 %) and broilers (5.6 %) in Germany [4]. Variants of the *mcr-1* gene have been identified, with *mcr-2* reported by Xavier et al. [31] in Belgium from porcine and bovine samples, and *mcr-1.2* by Di Pilato in Italy in *K. pneumoniae* [32].

### Clinical consequences and medical needs

Continued spread of the plasmid-borne *mcr-1* gene will compromise the clinical utility of last-resort polymyxins, which may lead to more frequent treatment failure and substantial additional morbidity and mortality. If establishing itself in successful pandemic clones such as *E. coli* ST131 or *K. pneumoniae* ST11/ST258, acquisition of *mcr-1* will likely lead to the global presence of pan-drug-resistant pathogens. Consequently, the European Medicines Agency (EMA), in response to a European Commission request, is currently updating the clinical guidelines on the use of colistin. The EMA is considering to upgrade colistin as an antibiotic of category 2, dedicated to critical medicine and to be used only if no alternatives actually exist. The World Health Organization (WHO) already considers colistin of crucial importance in human medicine ([http://who.int/iris/bitstream/10665/77376/1/9789241504485\\_eng.pdf](http://who.int/iris/bitstream/10665/77376/1/9789241504485_eng.pdf)). The European Centre for Disease Prevention and Control (ECDC) recently released a medical risk assessment on plasmid-mediated colistin resistance in Enterobacteriaceae (ECDC rapid risk assessment. Plasmid-mediated colistin

resistance in Enterobacteriaceae, June 13th, 2016; <http://www.ecdc.europa.eu>). The document describes a short series of specific actions to help reduce colonization and infection risks:

- Develop improved laboratory methods for colistin AST and molecular *mcr-1* detection. Microbroth dilution is the recommended reference method, while several diagnostic PCR tests have already been described.
- Implement integrated surveillance in hospitals, the community, food, animals, and environment (the One Health approach).
- Screening of multidrug-resistant enterobacterial isolates from patients who recently visited healthcare facilities in foreign countries.
- Perform targeted surveillance to detect reservoirs and include individuals who underwent selective decontamination of the gastrointestinal tract using polymyxins [1].
- Carefully manage patients infected with colistin-resistant strains and reinforce infection prevention and control measures [33].
- Assure that colistin treatment is always accompanied by adequate AST of clinical isolates.
- Apply prudent antimicrobial therapy [17, 34] and screening for carriage at hospital admission [35, 36].

### Recommendations

Various pragmatic aspects must be considered to help improve the current diagnostic and clinical needs:

**The use of selective culture media as an initial screening tool** Culture media are well accepted in clinical microbiology and quite well available and affordable on a worldwide scale. The development of novel selective (and possibly chromogenic) culture media will facilitate screening for colonization or infection of high-risk patients: those previously hospitalized, those hospitalized in endemic zones, those with a known history of infection or colonization with colistin-resistant bacteria, or those who were recently exposed to antibiotics. One needs to keep in mind that future work might establish if hospitalization is, indeed, the most significant risk factor since, even to date, several community cases have already been reported. This will eventually help to implement appropriate infection control measures [37, 38]. Such screening should include patients who were previously subjected to colistin-based decolonization [1].

Besides the potential use in human medicine, selective culture media are also relevant to veterinary surveillance considering that screening may soon become a regulatory requirement (e.g., screening of fecal samples from livestock), as part of the ongoing monitoring programs for antibiotic resistance

in bacteria from animals (<http://www.healthcanal.com/infections>). To date, the exact prevalence of the *mcr-1* gene remains unknown. Benefits of culture-based polymyxin resistance screening and rapid detection of polymyxin resistance in Enterobacteriaceae have been pointed out [39, 40]. To render the search for colistin resistance more clinically useful, simultaneous detection of carbapenem resistance should be considered. A suitable culture medium facilitating such a combined diagnosis could be of significant relevance in countries facing coinciding and possibly extensive spread of carbapenem resistance.

The recently developed Super Polymyxin selective medium provides a useful tool for the screening of polymyxin-resistant Gram-negative isolates [39]. Using a large collection of polymyxin-resistant isolates, it has been shown to possess close to 100 % sensitivity and specificity. Noteworthy, it may detect both intrinsic and acquired polymyxin resistance, either using replicates of isolated colonies or even direct stool or rectal swabs samples, depending on local surveillance needs.

**Disk diffusion assays, antibiotic gradient testing, and automated AST** Disk diffusion methods are not appropriate for the sensitive and specific detection of colistin resistance and, hence, there needs to be a specific search for alternative, fast, reproducible, and, to date, preferably culture-based AST methods. The same problem holds true for gradient testing (e.g., the Etest, which is available in research-use-only format for colistin), which seems to underestimate resistance levels, following evaluation studies conducted by the EUCAST. Currently, only BMD methods are recommended as reliable tests for colistin resistance. However, this method is quite cumbersome and labor-intensive to perform. A reliable colistin MIC test is of utmost importance for patient management, since colistin is a drug with significant adverse effects, which requires precise therapeutic drug monitoring (TDM) to avoid toxic effects while maintaining sufficient antibacterial activity. In addition, precise assessment of serum dosage is important as well, and dosing needs to be compatible with the MIC(s) of the infecting strain(s). Innovative propositions for rapid culture-based testing are still urgently needed. In order to increase the throughput, precision, and speed of colistin resistance testing, it would be good if (additional) assays for the commercially available automated AST systems could be developed or improved. The recently developed Rapid Polymyxin NP test is a tool that is available for accurate distinction between susceptible or resistant strains [39]. It may be implemented worldwide and is affordable. Results are obtained in less than 4 h and both specificity and susceptibility are high. This colorimetric test may be used for testing colonies and positive blood cultures, therefore providing an interesting tool for the rapid implementation of adequate therapies in the context of bacteremia and sepsis [40]. The interest in rapid confirmation of polymyxin resistance using phenotypic tests

has been highlighted by Jayol et al. [41], especially if it can be done directly from blood culture broth in a few hours and enable early adaptation of antimicrobial therapy in case of multidrug resistance.

**Molecular diagnostics** Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be used for specific protein molecule detection. Currently, however, there is no such test that would directly detect and/or identify the MCR-1 protein, but such tests might be developed, especially if more advanced MS methods could be used. Assays to detect the *mcr-1* enzymatic activities underlying the resistance or direct detection of the modified LPS molecules might also become interesting options with the progression of MS technology. This may, in the end, still remain difficult to implement diagnostically, since resistance may depend upon rather subtle up- or down-regulation of LPS-modifying enzymes.

Molecular biological detection of the acquired *mcr*-like genes at the nucleic acid level is relatively easy and can be done with simple and straightforward PCR tests, either developed in-house or being part of FDA-approved approaches. Rapid real-time PCR techniques have recently been developed for that purpose and validated using not only pure bacterial cultures, but also stool samples [42, 43]. Such tests could be easily added to existing multiplex panels, usually in addition to genes encoding other key resistance mechanisms. Nevertheless, it is noteworthy that several polymyxin resistance mechanisms are chromosomally encoded and depend upon the substitution of amino acids in diverse regulatory proteins involved in LPS biosynthesis. Therefore, a single PCR approach alone cannot be an adequate tool for an overall diagnosis due to the high diversity of sometimes even co-existing resistance mechanisms. Not only is subsequent sequencing required, but the issue is that correlating amino acid substitutions to corresponding susceptibility or resistance changes (and, hence, distinct MIC values) remains almost impossible.

## Conclusion

Colistin resistance poses a universal problem and rapid detection of the pheno- or genotype is of core importance. Phenotyping would initially be preferred given the diverse, potentially co-existing and complex resistance mechanisms, and since phenotyping takes into account features that are hard to detect by alternative, usually proteomic, genetic, or genomic methodologies [44]. This short review focused on phenotyping, since the many alternative test formats that do exist at the proof of principle level do not (yet) meet quality and validation requirements as imposed by the FDA and other regulatory agencies. Hence, the development of innovative,

accurate, and rapid tests, although clinically very important, remains quite challenging in the context of polymyxin resistance, owing to the diversity and complexity of the mechanisms involved. Molecular tests cannot be reliably implemented as long as a careful assessment of the correlation between phenotype and genotype has been accurately and precisely performed. This underscores the necessity to reinforce the current fundamental investigations aiming to exactly decipher the fundamental polymyxin resistance mechanisms and their genetic and proteomic bases.

In fact, the renewed interest in polymyxins observed to date reinforces the need to better evaluate the local and international frequency of occurrence of polymyxin-resistant strains, regardless of the underlying resistance mechanisms, whether plasmid or chromosomally encoded. The recent identification in France of a major outbreak caused by OXA-48-producing and colistin-resistant *K. pneumoniae* strongly underscores the relevance of such monitoring [45] and urges toward a real One Health approach investigating not only human infections and colonization, but including research of food, the environment, and animal reservoirs. Finally, the recent observation that colistin resistance may emerge as a consequence of chlorhexidine use [46] again demonstrates the complexity of antibiotic resistance and the need for concerted actions towards the optimal use of antibiotics and antiseptics.

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