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Plasmid-mediated Colistin Resistance: What Do We Know?

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Abstract

Polymyxins (polymyxin E/colistin and polymyxin B) are considered the last-resort therapy against carbapenem-resistant *Enterobacterales*; however, the resistance of *Enterobacterales* to polymyxins is increasing worldwide. Until 2015, this resistance was related to chromosomal mutations, but in November 2015, it was described in China the transferable colistin resistance in animals and humans isolates of *E. coli* and *K. pneumoniae*, mediated by the *mcr-1* gene (mobile colistin resistance), located in a plasmid. Following the first description of the *mcr-1* gene, it has been reported in several regions of the world, in different bacterial species, from different sources and others *mcr* variants have been described. Moreover, the co-occurence of the *mcr* gene and other antimicrobial resistance genes was reported. This discovery changed the scenario of resistance to polymyxins, as this gene could be promptly disseminated among Gram negative bacilli becoming a major concern for public health. This review summarizes recent data about the plasmid-borne *mcr* colistin resistance gene.

Introduction

Antimicrobial resistance is considered as a major public health threat to human health mainly due to the widespread of carbapenem-resistant *Enterobacterales* (CRE) in the last decade. The colistin (polymyxin E) and polymyxin B belong to the class of polymyxins, which are considered the last-resort therapy against CRE; however, the resistance of *Enterobacterales* to polymyxins is increasing worldwide[1,2,3]. Overall, colistin resistance in *Enterobacterales* (excluding species with intrinsic resistance) is around 0.67–1.6%, with low rates in *Escherichia coli* (0.2–0.6%), moderate rates in *Klebsiella pneumoniae* (1.5–6.8%), and much higher rates in *Enterobacter* spp. (13.9–20.1%). Considering other resistance mechanisms associated with colistin resistance such as ESBLs the percentage of co-producers is: 2.3–5.5% for *Enterobacterales* (>11.5% are *K. pneumoniae*); for carbapenem-non-susceptible isolates the number is higher: 4.5–16.3% for *Enterobacterales* (32% are *K. pneumoniae*) and even higher is the number found for carbapenemase-producers, 6.2–12.0% for *Enterobacterales* (14–36.6% are *K. pneumoniae*)[4].

As mentioned above, polymyxins are active against most *Enterobacterales* and also have significant activity against nonfermentative Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. Some Gram-negative species are naturally resistant to polymyxins: *Proteus* spp., *Morganella morganii*, *Providencia* spp., *Serratia marcescens*, *Burkholderia mallei*, *Burkholderia cepacia* complex, *Chromobacterium* spp.,

Edwardsiella spp., *Brucella*, *Legionella*, *Campylobacter* and *Vibrio cholerae.*[5] Additionally, the polymyxins are not active against Gram-negative cocci, Gram-positive and anaerobic bacteria[5,6]. The natural resistance mechanism is hypothesized to be promoted by constitutive gene expression which adds cationic molecules to the LPS, leading to decreased affinity at the polymyxin site of action on the LPS, differently in Gram-positive bacteria lack of an LPS containing outer cell membrane promotes the resistance[7].

The colistin was obtained in 1947 from a soil bacteria *Paenibacillus polymyxa* subsp. *colistinus*[8] and has been cleared to use since 1959 by the Food and Drug Administration (FDA) for the treatment of infectious diseases caused by Gram-negative bacteria. However, in clinical use, colistin proved to be nephrotoxic and their use has declined from the early 1970s to the early 2000s, being replaced by more active and less-toxic antibiotics such as the aminoglycosides, quinolones and beta-lactams. However, due to increasing prevalence of multiresistant Gram-negative bacteria, the colistin were re-introduced into clinical practice as a valuable therapeutic option[5,9].

Unlike in human medicine, in veterinary medicine, colistin has been used extensively, and without interruption, for decades for the treatment and prevention of infectious diseases by *Enterobacterales*, as well as growth promoter in poultry and pigs[2,5,9]. However, due to the description of the colistin resistance mediated by a mobile genetic element and its widespread in animals in China in 2015, the Ministry of Agriculture of China decided to ban colistin as a feed additive for animals in 2016. Other countries also decided to ban colistin as growth animal promoter, due to the imminent impact on polymyxin resistance in human health[10].

The mechanism of action of polymyxins (colistin and polymyxin B) occurs by the electrostatic binding of the molecule to the lipopolysaccharide (LPS) and phospholipids in the outer membrane of Gram-negative bacteria. The polymyxins destabilize the LPS through the exchange of ions (Ca^{+2} and Mg^{+2}), increasing the permeability of the bacterial membrane, promoting the extravasation of the cytoplasmic content and consequent bacterial death. Despite the fact that the initial target for polymyxin is the LPS, the exact mode of action of polymyxins is still not totally clear. There are other possible mechanisms of action described for polymyxins: the endotoxin effect (during cell lysis the endotoxin molecule - the lipid A which is the main component of the LPS) is released and the polymyxins are able to bind and neutralize it and the inhibition of respiratory enzymes (type II NADH-quinone oxidoreductases [NDH-2]) in the bacterial membrane[5,11].

Until 2015, the reports of polymyxin resistance were all due to chromosomal mutations. These chromosomal mutations lead to modification of the LPS via cationic substitution that alter the lipid A, decreasing its affinity for the polymyxins, similarly to that observed in bacteria with intrinsic resistance to polymyxins. There are several mutation in genes and operons which are involved in the modification of the LPS: (i) mutations in genes (*pmrC, pmrE* and *pmrHFIJKLM*) responsible for synthesis of cationic groups and their addition to the LPS; (ii) mutations in regulatory genes that encode proteins involved in the PmrAB system in *K. pneumoniae*, *Enterobacter aerogenes* and *Salmonella enterica* (*pmrA* and *pmrB* genes) and the PhoPQ system in *K. pneumoniae* and *E. coli* (*phoP* and *phoQ* genes); (iii) and mutations in the regulators of these systems as the *mgrB* gene in *Klebsiella* spp. (regulates the PhoPQ system) and *crrAB* operon in *K. pneumoniae* (regulates the PmrAB system)[12].

In November 2015, Liu and colleagues described the transferable colistin resistance in animals and humans isolates of *E. coli* and *K. pneumoniae* recovered in China, mediated by the *mcr-1* gene (mobile colistin resistance). The *mcr-1* gene is located in a plasmid and its discovery changed the scenario of resistance to polymyxins, as this gene could be promptly disseminated among Gram-negative bacilli becoming a major concern for public health. MCR-1 protein is a member of the phosphoethanolamine transferase family, its acquisition promotes the addition of phosphoethanolamine (PEtN) to lipid A, similarly to the chromosomal mutations, resulting in reduction of polymyxin affinity[1].

Epidemiology of Plasmid-mediated Colistin Resistance

Following the first description of the *mcr-1* gene, it has been reported in several regions of the world, including countries of Asia, Africa, Europe and America[2,12,13]. Further studies indicated that bacteria carrying the *mcr-1* gene were identified in the 1980´s in China. The earliest report of the *mcr-1* gene among isolates from humans is from 2008 and it was described in a *Shigella sonnei* from Vietnam. These findings indicate that *mcr-1* gene has existed in *Enterobacterales* but remained not identified for a long time[9,14].

The *mcr-1* gene has been reported in several species of *Enterobacterales* but mostly in *E. coli.* The occurrence in *Salmonella, Klebsiella, Shigella, Vibrio* and *Enterobacter* was only sporadically reported, but indicates interspecies gene transfer[2,12].

The *mcr-1* gene has been mainly detected in *E. coli* isolates obtained from animals (livestock, wild animals and food of animal origin). Farm animals, in particular pigs and chickens, have been considered as reservoirs for *E. coli* isolates carrying the *mcr-1* gene. In humans, *Enterobacterales* carrying the *mcr-1* gene have

been obtained from clinical samples, as well as from asymptomatic patients, including international travellers. The possible dissemination of the *mcr-1* gene from bacteria obtained from animals to bacteria from humans is a serious concern, mainly whether this gene could be transferred into carbapenemase producing *Enterobacterales*[2,9,12].

MCR-1 producers may exhibit low level resistance to colistin or polymyxin B (minimum inhibitory concentration [MIC] of 4μg/mL) and a few cases of isolates carrying the *mcr-1* gene which are susceptible to colistin have already been described[15,16]. This may contribute to the silent dissemination of *mcr*-1 gene harbouring isolates. Other aspects may also play an important role in the dissemination of the *mcr*-1 gene: the technical problems related to the detection of resistance to polymyxins and the fact that most laboratories only evaluate the susceptibility profile to polymyxins among carbapenem non-susceptible isolates[15].

One explanation for the reported *mcr-1* positive but colistin susceptible isolates was that the gene was truncated as described in a *Shigella sonnei* isolate[17]. Interestingly, the truncated *mcr-1* gene could be re-activated after conjugation experiments resulting in a colistin resistant phenotype[18]. Conversely, the report of an *E. coli* colistin susceptible and *mcr*-1 positive with an intact gene indicates that the gene truncation is not the only mechanism related to the susceptibility to polymyxins among *mcr*-1 harboring isolates[19].

According to an search assessment of the data at the GenBank (https://www.ncbi.nlm.nih.gov/genbank/ – 17th of June 2018), 13 *mcr-1* subgroups were already described in several countries, differing from *mcr*-1 by only one nucleotide: *mcr*-1.2 (*K. pneumoniae* from Italy)[20], *mcr*-1.3 (*E. coli* from China)[21], *mcr*-1.4 (*E. coli* from China), *mcr*-1.5 (*E. coli* from Argentina), *mcr*-1.6 (*Salmonella typhimurium* from China)[22] , *mcr-1.7* (*E. coli* from China), *mcr-1.8* (*E. coli* from Brunei)[23], *mcr-1.9* (*E. coli* from China)[24], *mcr-1.10* (*Moraxella* spp. from Great Britain)[25], *mcr-1.11* (KY853650.1), *mcr-1.12* (LC337668.1) and *mcr-1.13* (*E. coli* from Italy)[26].

To date, eight *mcr* variants have been described: *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, mcr-6*, *mcr-7* and *mcr-8* (Table 1). The *mcr-2* gene is 1,617 bp long, nine bases shorter than *mcr-1* (1,626 bp) and it has 76.7% nucleotide identity to *mcr-1*[27]. The *mcr-3* gene presented 45.0% and 47.0% nucleotide sequence identity to *mcr-1* and *mcr-2*, respectively. MCR-3 also showed 94.1% to 94.8% amino acid identity with proteins found in three *Aeromonas* species. Moreover, a truncated transposon element (TnAs2), which was characterized only in *Aeromonas salmonicida*, was located upstream of *mcr-3.* These findings suggest that *mcr-3* gene in *Enterobacterales* might have originated from *Aeromonas* species[28]. Unusually, the *mcr*-4 is inserted in a small and not self-conjugative plasmid. However, the addition of an auxiliary plasmid can promote conjugation[29].

Co-occurrence of *mcr* and Other Resistance Genes

There have been reports of co-production of the *mcr-1* gene and other resistance genes in several bacterial species. The presence of the *mcr-1* gene has been associated with ESBLs (CTX-M, SHV and TEM type) and AmpC cephalosporinase (CMY type). Moreover, there are descriptions of co-occurrence of the *mcr-1* gene and quinolone resistance genes (*qnrS* and *aac(6=)-Ib*-cr)[9,15,33,34].

Isolates carrying *mcr-1* and carbapenemase resistance genes have been described in *Enterobacterales*. Specifically, the co-production of *mcr-1* and carbapenem resistance genes is of great concern, since polymyxins represent the "last-line" therapeutic option for an infection caused by a carbapenemase-producing isolate. In the literature the *mcr-1* gene has been found mainly in isolates that produce the carbapenemase NDM in particular among *E. coli* isolates from animal and human origin[35,36,37]. However, it was also detected in *Cronobacter sakazakii*[38] from animal origin and *K. pneumoniae*[39] from human origin. Only one report about co-producer OXA-48 carbapenemase and *mcr-1*[40] and

Table 1. *mcr* variants available at the GenBank database (update in July 17, 2018- https://www.ncbi.nlm.nih.gov/genbank/)

Gene	Country	Origin	Species	Reference	Variants
mcr-1	China	Retail meat from chicken and pig, and inpatients[a]	*E. coli* and *K. pneumoniae*	1	*mcr-1.2 – mcr-1.13*
mcr-2	Belgium	Pig and bovine	*E. coli*	27	*mcr-2.2*
mcr-3	China	Pig	*E. coli*	28	*mcr-3.2-mcr-3.12*
mcr-4	Italy, Spain and Belgium	Pig	*Salmonella enterica* and *E. coli*	29	*mcr-4.2*
mcr-5	Germany	Faecal content of pigs and faecal samples from pigs	*S. enterica subsp. enterica serovar Paratyphi B*	30	*mcr-5.2*
mcr-6[b]	Great Britain	Faecal contents of healthy pigs	*Moraxella* sp.	25	-
mcr-7	China	Chickens	*K. pneumoniae*	31	-
mcr-8	China	Pig, chicken and inpatient	*K. pneumoniae*	32	

[a]The gene *mcr-1* was isolated from several other origin, after its first publication from Liu et al., 2016[1].

[b] *mcr-2.2* (1617 bp) has been renamed as *mcr-6*.

VIM carbapenemase and *mcr-1*[41] have been related in the literature, both from human sources. There are a few KPC and *mcr-1* co-producers reported, in particular those genes were identified in *E. coli*[42,43,44,45,46] and less frequently in *K. pneumoniae* isolates[20,47,48].

Genetic Context of the *mcr* Gene

The first plasmid carrying the *mcr-1* gene belonged to the plasmid incompatibility type IncI2[1]. Others studies indicated that *mcr-1* gene is not restricted to the IncI2 plasmid group and various plasmids may carry the *mcr-1* gene including those belonging to the IncX4, InHI2, IncF, IncHI1, IncY and IncP[33,49]. These incompatibility groups of plasmids have been implicated in the global spread of others resistance genes among *Enterobacterales* from human and animal sources[50,51]. As the *mcr-1* gene is not associated with a specific incompatibility group of plasmids, it can be considered that this molecular flexibility promotes global dissemination of the gene[52].

Noteworthy, there are intriguing descriptions of coexistence of two *mcr* bearing plasmids in a single bacterial isolate. Nevertheless, the MIC for colistin of the isolates presenting more than one *mcr* gene in different plasmids was not increased[52,53].

Often the *mcr* gene is flanked by an insertion sequence designated IS*Apl*1 and the *nikB* gene, in this order. It is suggested that the IS*Apl*1 is a key component in the mobilization of the *mcr* gene. IS*Apl*1 belongs to the IS30 family and was first identified in *Actinobacillus pleuropneumoniae*, a Gram-negative bacterium of the *Pasteurellaceae* family, causing pig necrotic pleuropneumonia[42,49,54].

It is also suggested that the *mcr-1* gene had been initially mobilized by two copies of IS*Apl*1 from an unknown progenitor. Over the course of evolution, this complex transposon has lost one or both copies of IS*Apl*1, since it is common for IS30 family member to lose a copy of the IS element by transposition or illegitimate recombination events, in order to stabilize the *mcr-1* bearing plasmid. Sometimes the *mcr-1* gene can be mobilized by only a single copy of IS*Apl*1 upstream the gene in combination to an incomplete sequence of the IS*Apl*1 downstream. IS*Apl*1 is presents in multiple copies in the genome and these insertion sequence sites are notable for their high AT content[49,52,55,56].

As already mentioned, the *mcr-1* gene was originally described mostly among *E. coli* isolates from animal sources and this indicates that this gene is of animal origin. The genetic context of the *mcr-1* gene reinforces the theory that the gene was originally from isolates obtained from animals. The first evidence is that the *mcr-1* gene is very often associated to the insertion sequence IS*Apl*1 and this insertion sequence is usually identified in bacteria of animal origin. Another indicative of the animal origin and the genetic context of the *mcr-1* is the presence of other antibiotic resistance genes specific to veterinary medicine, such as the floR gene which confers resistance to florfenicol[57].

Moreover, the *Moraxella* genus was identified as potential reservoirs of *mcr*-like genes that might be mobilized from their original host to become an acquired resistance mechanism in clinically significant species. The *mcr-1* and *mcr-2* genes present a significant identity with intrinsic chromosomal genes of the *Moraxella* species. The exact species of the progenitors of *mcr-1* and *mcr*-2 genes remains to be determined, but the most closely-related variant compared to MCR-1 was identified in *Moraxella porci*, while the most closely-related variant of MCR-2 was identified in *Moraxella osloensis*[57].

Detection of MCR-Producers

Broth microdilution (BMD) is considered the reference test for determining the susceptibility profile of polymyxins regardless the fact that BMD do not establish the underlying mechanism of resistance[58]. There are a few phenotypic tests which are supposedly able to detect MCR producers, including: the Colistin MAC Test (CMT)[59]; Combined Disc Test (CDT); Colistin MIC Reduction (CMR); Modified Rapid Polymxin NP Test (modified-RPNP) and alteration of Zeta potential tests[60]. Nevertheless, the molecular tests are considered the reference tests for the detection of the *mcr* gene; the molecular techniques using sequencing can also specify the gene variants.

Assays based on colistin MIC reduction in the presence of dipicolinic acid (DA) or ethylene diamine tetra-acetic acid (EDTA) have been evaluated to detect *mcr*-producing *Enterobacterales*[59,60]. These tests are based on the fact that the catalytic domains of the MCR enzymes resemble that of zinc metalloproteins[61]. DA or EDTA are able to chelate zinc (necessary for the enzymatic activity of the PEtN transferase) and consequently can inhibit the enzymatic activity of MCR-1 and finally reduce colistin resistance in MCR-producing strains.

The CMT method is a broth microdilution method, in which colistin MIC (0.125 to 8.0 μg/mL) is tested in absence and in presence of fixed concentration of the DA (900 μg/mL). If the MIC increases ≥8-fold, in the presence of DA, the result is interpreted as *mcr*-positive while an MIC reduction of ≤2-fold is interpreted as *mcr*-negative. The test does not present satisfactory results with *K. pneumoniae*, probably due to a reduced permeability to DA and/or the presence of additional unknown mechanisms. In addition, the authors tested the effect of DA in increasing colistin susceptibility of *mcr*-1 positive isolates, in a disc-diffusion format, however no significant difference was detected as the inhibition zones between *mcr*-1-positive and *mcr*-negative isolates were very similar[59].

Similarly, Esposito *et al.* (2017)[60] evaluated four EDTA-based assays to detect MCR-1-positive isolates. The CDT test compare the inhibition zones of colistin (10μg) and colistin plus EDTA (100mM). An incremental difference of ≥ 3mm between the colistin impregnated disc and the colistin-EDTA-impregnated disc was interpreted as MCR-1-positive. The CDT presented sensitivity and specificity of 96.7% and 89.6%, respectively[60].

The CMR test was designed according to the BMD method with or without the addition of 80μg/ml EDTA, into the wells containing 0.06–32μg/ml of colistin. A ≥ 4-fold colistin MIC reduction in EDTA-containing wells was interpreted as MCR-1-positive. The sensitivity and specificity of this method was 96.7% and 83.3%, respectively[60].

The original RPNP test consists of detecting bacterial growth on a glucose based medium, in the presence of a defined concentration of colistin. The bacterial growth leads to acid formation in the medium which alters the pH and this can be detected visually by a pH indicator color change[62]. If the well containing colistin, change its colour from orange to yellow, it is considered positive (colistin resistance). The modified RPNP is based on the incorporation of two extra wells, one containing 80μg/mL EDTA (without colistin) and the other one containing 80μg/ml EDTA plus 5μg/ml colistin. In this case, the MCR-1 positive result is observed when the colistin containing solution supplemented with EDTA remained orange (i.e., absence of glucose metabolization due to EDTA inhibition). The sensitivity and specificity of this test are higher (96.7% and 100.0%, respectively) compared to CDT and CMR tests[60].

Another method for phenotypic detection of *mcr* producers is the alteration of Zeta potential in the absence and presence of 80 μg/ml EDTA, which increase the anionic charges on the surface membrane of MCR-1-positive isolates. However, according to the authors, this method can only be used in well-resourced microbiology laboratories[60].

Polymerase Chain Reaction (PCR) and Whole Genome Sequence (WGS) are considered the reference tests to identify the *mcr* gene from cultured bacteria as well as in clinical, fecal, environmental and food samples[58,63]. While PCR can only detect known *mcr* genes, due to specific primers and probes, the WGS can identify all known or unknown colistin resistance mechanisms within 2 days[58]. Further molecular tests are the commercial microarray that can simultaneously detect both β-lactamases and *mcr-1/-2* genes, even though those tests are inaccessible to under-resourced laboratories[64].

Conclusion

Considering the importance of polymyxins in human and veterinary medicine, there is an urgent need to limit the propagation of *mcr-1* harboring plasmid. Given the constant exchange of resistance genes in all microbiomes (animals, environment and human population), surveillance programs to monitor the diversity of reservoirs, plasmids and to evaluate the real dimension of resistance mediated by the *mcr* gene are necessary.

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