Plasmid-Mediated *mcr* Gene-Based Polymyxins

Subjects: Infectious Diseases

Contributor: Shakeel Shahzad , Mark D. P. Willcox , Binod Rayamajhee

The polymyxin antibiotics colistin and polymyxin B have been recently revitalized as bactericidal drugs due to the increase in bacterial resistance to many commonly used antibiotics. Polymyxins were originally derived from the bacterium *Paenibacillus polymyxa* as the products of fermentation in the form of amphipathic lipopeptide molecules. Polymyxins were discovered in the 1940s to be cyclic lipodecapeptide antibiotics and recognized for therapeutic use in the 1950s. Polymyxins contain conserved components that consist of a d-Phe^{6}-l-Leu^{7} segment, an N-terminal fatty acyl chain separated by cationic residues (l-α-γ-diaminobutyric acid (Dab)), and segments of the polar amino acid threonine (Thr). Polymyxins target the negatively charged outer membrane lipopolysaccharides (LPSs) of Gram-negative bacteria. Mobilized colistin resistance, *mcr*, genes are mainly associated with bacterial plasmids. These play an important role in the spread of colistin resistance because of their transferability among different strains in different environments. These *mcr* genes encode phosphoethanolamine-lipid A transferases that mediate the addition of PEA to the lipid A of an LPS at the 1′ and 4′ positions, causing a significant reduction in the overall negative charge on the bacterial outer membrane. This ultimately leads to the loss of binding affinity of an LPS to the cationic polymyxins and therefore resistance to their action.

polymyxin resistance | molecular evolution | resistance mechanisms | mcr

1. Global Dissemination of mcr among Different Bacteria in Different Environments

It is believed that sporadic outbreaks of *mcr* occurred in Chinese food-producing livestock in 1980 [1]. Since that time, *mcr-1*-carrying bacterial strains have been reported in several countries among five of the seven continents across the globe [1][2][3][4][5][6] including China [2], India [7], Pakistan [8], Vietnam [9], Laos [10], USA [11], Italy [12], and Japan [13].

The transmission of *mcr* genes carrying pathogens could occur from animals to humans via direct contact with food animals and pets [14][15][16]. Also, reservoirs for *mcr-1*-carrying bacteria have been identified in public beaches [17], hospital sewage, wastewater treatment plants [18][19], rivers [16], and water wells in rural areas [20], as well as from houseflies and blowflies [21]. Although data from some studies suggests that flies might be intermediate vectors for transmission of *mcr-1*-containing bacteria between companion animals and humans [22], the exact route for the spread of *mcr-1* and the bacteria carrying *mcr-1* needs more thorough investigation.

Several species of *Enterobacteriaceae* possess *mcr-1*, such as *E. coli* where the gene is carried on IncI2 and IncX4 plasmids [23], *Enterobacter aerogenes* on an IncX4 plasmid [24], *E. cloacae* on an IncFI plasmid [24], *Cronobacter sakazakii* on an IncB/O plasmid [25], *Citrobacter freundii* on an IncHI2 plasmid [26], *C. braakii* on an IncI2-type plasmid, *K. pneumoniae* on an IncX4 plasmid [27], *Salmonella enterica* on IncHI2-like plasmids [28], *Shigella sonnei* on IncHI2-like plasmids [29], and *Raoultella ornithinolytican* on an IncHI2 plasmid [30]. Also, *mcr-1* variants have been identified in strains co-harboring bla_{NDM-5} that confers carbapenem resistance to *E. coli* [8]. The *mcr-1.1* gene has been found in the chromosome of *E. coli* and plasmid p16BU137 of *K. pneumoniae* from environmental isolates in China [31]. Further details of recently discovered *mcr* variants and their respective transposons and plasmids are given in **Table 1**.

Table 1. The evolutionary divergence among *mcr* variants (*mcr-1* to *mcr-10*) (a score of 1 indicates no divergence between variants; a score of 0 indicates complete divergence).

	***mcr* Gene Number**									
mcr gene number and source	1	2	3	4	5	6	7	8	9	10
mcr-1 Escherichia coli KU886144.1		0.18	0.67	0.57	0.54	0.22	0.47	0.68	0.71	0.71
mcr-2 Pseudomonas aeruginosa MW811418.1	0.18		0.68	0.58	0.56	0.12	0.49	0.69	0.7	0.72
mcr-3 Escherichia coli MW811424.1	0.67	0.68		0.62	0.75	0.68	0.7	0.76	0.38	0.38
mcr-4 Escherichia coli MW811433.1	0.57	0.58	0.62		0.56	0.58	0.49	0.65	0.65	0.61
mcr-5.1 Salmonella enterica NG055658.1	0.54	0.56	0.75	0.56		0.55	0.43	0.64	0.72	0.73
mcr-6.1 Moraxella sp. NG055781.1	0.22	0.12	0.68	0.58	0.55		0.51	0.72	0.72	0.74
mcr-7 Pseudomonas aeruginosa MW811434.1	0.47	0.49	0.7	0.49	0.43	0.51		0.65	0.71	0.68
mcr-8 Klebsiella pneumoniae MT815555.1	0.68	0.69	0.76	0.65	0.64	0.72	0.65		0.69	0.72
mcr-9 Uncultured bacterium MW478857.1	0.71	0.7	0.38	0.65	0.72	0.72	0.71	0.69		0.22
mcr-10.1 Enterobacter cloacae MN044989.1	0.71	0.72	0.38	0.61	0.73	0.74	0.68	0.72	0.22	
Average evolutionary divergence	**0.53**	**0.52**	**0.62**	**0.59**	**0.61**	**0.54**	**0.57**	**0.69**	**0.61**	**0.61**

In Australia, colistin resistance was reported among poultry isolates of *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Myroides odoratus*, *Hafnia paralvei*, and *Pseudochrobactrum* spp. from a chicken processing unit in the state of

	***mcr* Gene Number**									
Standard Deviation	0.20	0.23	0.14	0.05	0.11	0.23	0.11	0.04	0.18	0.19

[32] [33] [34] [35]

s from isolates in and *mcr-3* were found among MDR isolates of *Salmonella enterica* 4 from human and animal sources in NSW [34][35]. An evolutionary analysis of multiple drug-resistant *Salmonella enterica* serovar 4 indicated that the spread of the *mcr-3* variant in lineages 1 and 3 was associated with overseas travel to Southeast Asia [36]. Lineage 1 included *mcr-3.1*- and $bla_{CTX\text{-}M\text{-}55}$-positive isolates of *Salmonella enterica* sequence type 34 from Europe and Asia that were resistant to colistin and third-generation cephalosporins [36][37]. Whilst *mcr*-3.2 in lineage 3 was associated with IncHI2 pST3 and IncAC plasmids, wherein the colistin resistance genes were part of *dgkA* (diacylglycerol kinase) [36][38], which is a small transposable unit associated with IS elements circularized and integrated into *Enterobacterales* genomes [39].

2. Evolution of mcr Gene Variants from mcr-1 to mcr-10

In the current study, the phylogeny among *mcr* variants was determined using Molecular Evolutionary Genetics Analysis (MEGA 11) and is shown in **Table 1**. This shows the pair-end number of substitutions between *mcr-1* and *mcr-10*, with the number of base differences per site indicated. An estimate of evolutionary divergence between the sequences of *mcr-1* and *mcr-10.1* was performed using MEGA 11. Overall, the average divergence among *mcr* ranged from 52 ± 20% for *mcr-2* compared to all others to 69 ± 4% for *mcr-8*.

Moreover, phytogenic analysis of *mcr-3* also demonstrated that most occurred and evolved among *Aeromonas* species. This suggested the origin of *mcr-3* was *Aeromonas* species with gradual evolution and transmission of *mcr-3* variants to *E. coli* and *K. pneumoniae*, while other *mcr* gradually evolved among *E. coli* and *K. pneumoniae.* Interestingly, after the emergence of *mcr-4*, the identification of *mcr*-4.3 in *A. baumannii* represented a gradual evolution of *A. baumannii* against colistin with a distinct type of *mcr* gene in the form of a novel plasmid carrying *mcr-4.3* [40].

The analysis of evolutionary probabilities in *mcr* variants used a previously described method [41] using modified evolutionary probabilities (EPs) [42]. A user-specified tree topology was analyzed using the maximum likelihood method and the general time reversible model [43]. The evolutionary time depths used in the EP calculation can be obtained using the real-time [44] method. This analysis involved using the 10 nucleotide sequences of *mcr*. Codon positions included the first + second + third plus the noncoding positions. All positions containing gaps and missing data were eliminated (complete deletion option). The results, which represent the number of base differences per site for each *mcr* variant, are depicted in (**Figure 1**).

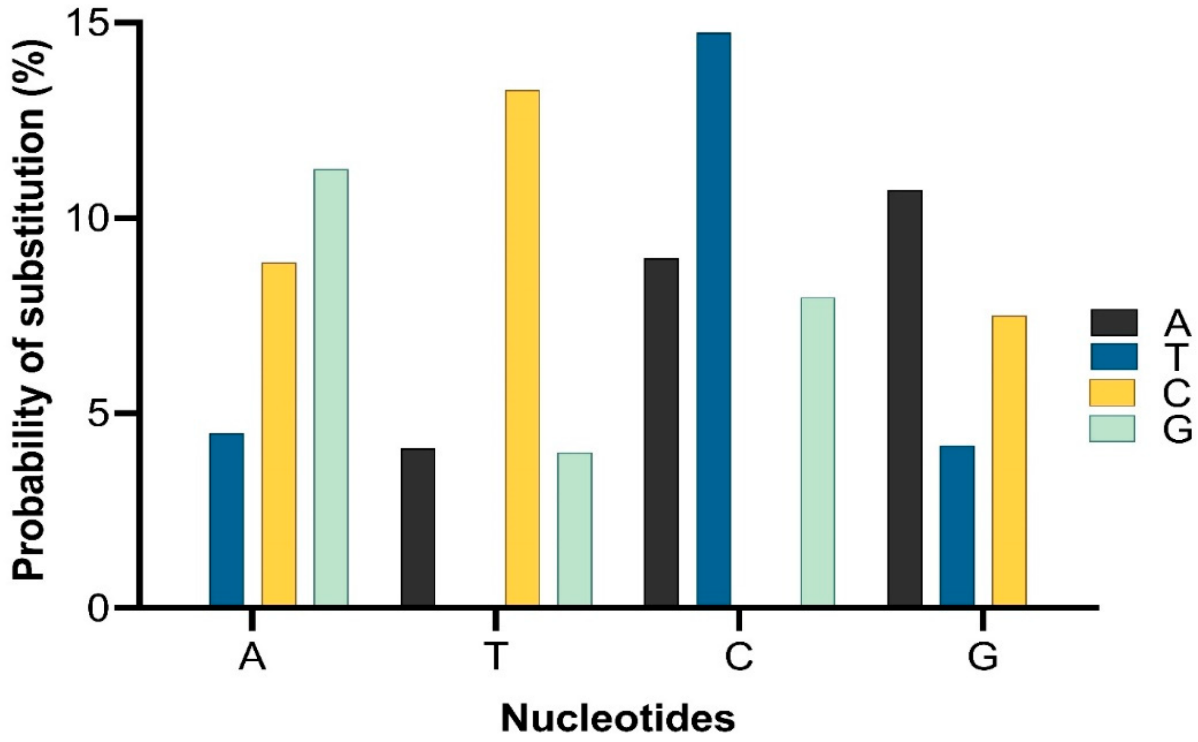

Figure 1. The probability of substitution of one base for another base. Substitution patterns and rates were estimated using the general time reversible model [45]. The maximum log-likelihood for this computation was 2655.269. This analysis involved all 10 nucleotide sequences of *mcr*. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated (complete deletion option).

The probability of substitution of nucleotides to *mcr*-1 is demonstrated in **Figure 1**, which shows that the most likely substitution of adenine was with guanine (12%), of thymine was with cytosine (15%), of cytosine was with thymine (15%), and of guanine was with adenine (11%). The positions of substitution of nucleotides (A, T, G, and C from position 1 to 262 of different sites) for *mcr*-1 (*E. coli* strain ZZ1409 KU886144) are shown in **Figure 2**, respectively. In terms of positioning, cytosine (C) is predominately present at positions 1 to 257, followed by adenine (A) from positions 1 to 253, guanine (G) from positions 1 to 261, and thymine (T) from positions 5 to 261. In terms of probability and position of substitution, guanine was mostly likely to be present at position 27 with a probability of 0.95, and least likely to be present at position 28 with a probability of substitution of 0.007; thymine was most likely to be present at position 30 with a probability of 0.95 and least likely to be present at position 28 with a probability of 0.007; adenine was most likely to be present at position 220 with a probability of 0.94 and least likely to be present at position 27 with a probability of 0.007; cytosine was most likely to be present at position 160 with a probability of 0.93 and least likely to be present at position 262 with a probability of 0.014.

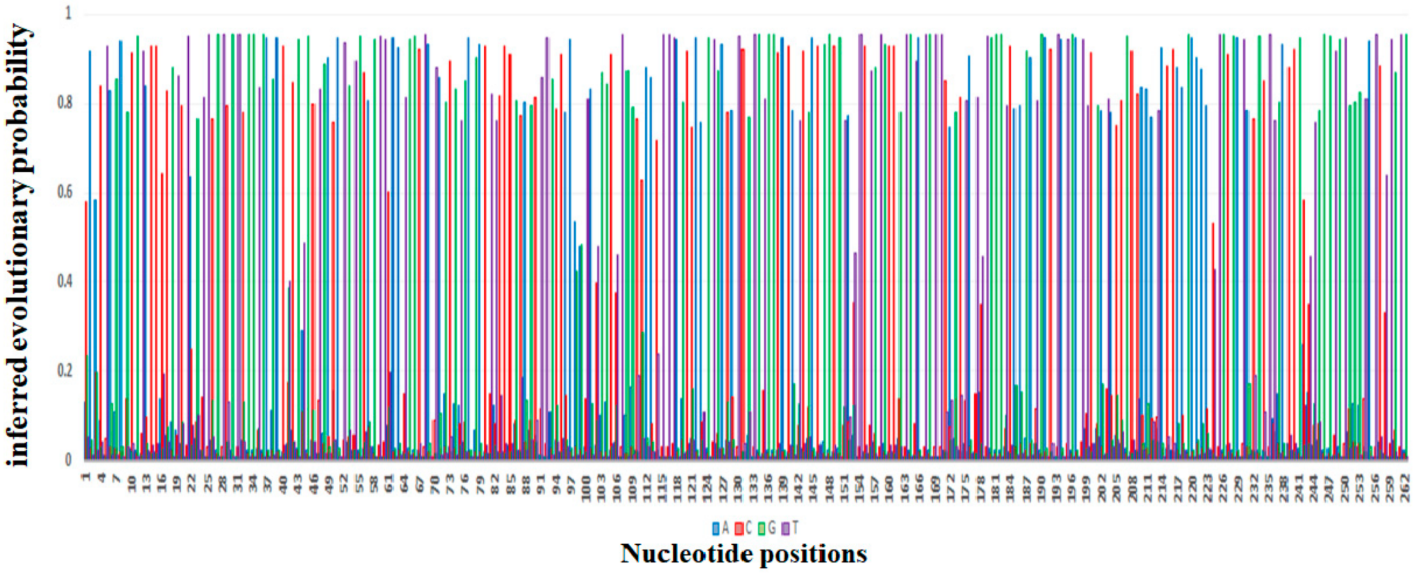

Figure 2. Depiction of the evolutionary probabilities of nucleotide substitution with respect to positions 1 to 262 for *mcr-1* in *Escherichia coli* strain ZZ1409 KU886144.

The Processes and Molecular Vehicles Responsible for the Transmission of *mcr* Variants

Studies have comprehensively analyzed the genetic environments of *mcr*-carrying genomes using bioinformatics tools such as Geneious R8 [46] and ISfinder software [47] to demonstrate the insertion of *mcr* variants. The structures of recently reported insertion sequences and the names of their associated transposons are given in **Table 2**.

Full genome sequencing and analysis for identification of replication origin (*oriC*) in *mcr-1*-harboring plasmids from colistin-resistant isolates have identified a novel hybrid IncI2/IncFIB plasmid pGD17-2 [48]. Moreover, the co-occurrence of pGD17-2 with plasmids pGD65-3, IncI2, and pGD65-5, IncX4 has been reported in a single drug-resistant isolate (GD65), and this co-occurrence might promote the dissemination of *mcr-1* under environmental selection pressure [48]. *mcr*-1 and other clinically significant resistant genes such as extended-spectrum β-lactamase (ESBL) *blaCTX-8* and *blaCTX-M-1* are related to globally identified sequence types ST10, ST46, and ST1638 in pathogenic strains of *E. coli* responsible for infections in humans and animals [49][50][51]. *E. coli* ST10 stains carrying *mcr-1* have been isolated from water at a public beach in the USA where the same ST10 strain had been isolated from an infected migratory Magellanic penguin with pododermatitis [49], suggesting that the ST10 strains carrying *mcr-1* can disseminate in the marine environment. *E. coli mcr-1*-positive environmental isolates have been isolated from German swine farms [52] and in diseased food animals in China [53], Italy, and France [54]. A plastidome analysis of *mcr-1* of *Enterobacterales* human isolates suggested that the spread of *mcr-1* among commensals such as *K. pneumoniae*, *E. coli*, and other clinical isolates could be facilitated by various promiscuous diverse plasmids [55].

Insertion sequences (ISs) or integrons can also facilitate the spread of *mcr*. An analysis of *mcr-1* from various sources using whole genome sequencing supported a single *mcr-1* mobilization event in IS*Apl1*-*mcr-1*-*orf*-IS*Apl1*

transposon [56]. This transposon has been immobilized on different plasmids such as IncI2, IncHI2, and IncX4 [57]. Plasmids pGD65-3, IncI2, and pGD65-5, IncX4 contain two insertion sequences, IS*Ecp1* and IS*Apl1*, that facilitate the mobilization of *mcr-1* [48]. The insertion sequence IS*Apl1*, which originated in *Actinobacillus pleuropneumoniae*, is located upstream of *mcr-1* in the IncI2-type *mcr-1*-harboring plasmid Phnshp45 [58][59][60]. However, the IS*Apl1* element is not always found associated with *mcr-1* on most IncX4 plasmids [59][60][61]. A reason for this may be that the translocation of an *mcr-1-pap2* element by integration of an IS*Apl1* cassette (a member of the IS*30* family) [38][59] into plasmids such as pMCR1-IncI2, and pMCR1-IncX4 may induce the formation of circular intermediates by recognizing inverted repeat sequences, which ultimately results in loss of IS*Apl1* after integration of *mcr-1* [38][62][63].

The *mcr-2* gene is not associated with IS*Apl1*, but there are two IS*1595*-like insertion sequences predicted to surround *mcr-2* in the IncX4 plasmid pKP37-BE [64]. The short IS*1595*-like element carries a transposase gene flanked by two inverted repeats surrounding *mcr*-2. This transposase-encoding gene is similar (75% identity) to a fragment found in *Moraxella bovoculi* strain 58069, which suggests the origin of *mcr*-2 was from *M. bovoculi* [62]. The occurrence of duplicate target sites adjacent to a spacer sequence suggests that the spacer sequence is the most probable hot site in IncX4 plasmids for integration and transposition of *mcr-2* variants [65]. Transfer of *mcr-2* can occur through IS1595-containing transposons [62][63][65][66].

Table 2. Recently reported insertion sequences and transposon elements associated with *mcr* genes transmission.

mcr Variants	Insertion Sequences Structure	Transposon	Plasmids	Organism	Host (Isolated from)	Year of Discovery	References
mcr-1	(IS*Apl1-mcr-1-pap2*-IS*Apl1* and Tn*7511*)	Novel transposon Tn*7511*	IncI1 plasmid, pMCR-E2899	*E. coli* DH5α	Turkey meat	2022	[67]
mcr-1	Combination of IS*Apl1* and IS*91* (IS*Apl1-mcr-1*-IS*91*)	Chromosomal Tn*6330* transposon	IncI2 plasmid	*E. coli*	Community and hospital settings	2022	[58]
mcr-1	IS*26-mcr-1-PAP2*, and IS*APl1-mcr-1-PAP2* and IS*Ecp1-blaCTX−M−55-mcr-1-PAP2*	---	IncI2, IncX4, and IncHI2 plasmids	*E. coli* and *Salmonella* spp.	Food products, food supply chain, and clinical samples	2021	[68][69]
mcr-1.1	IS*26-parA-mcr-1.1-pap2*	---	IncX4-type plasmid	*E. coli*	Dog feces	2020	[56]
mcr-1	*I* IS*Apl1-mcr-1-orf* IS*Apl1*	IS*Apl1* transposon	IncHI2 and IncX4	*Enterobacteriaceae*	Livestock	2018	[70]

***mcr* Variants**	**Insertion Sequences Structure**	**Transposon**	**Plasmids**	**Organism**	**Host (Isolated from)**	**Year of Discovery**	**References**
			plasmids				
mcr-1	IS*Apl1-mcr-1-pap2*-IS*Apl1*	Tn*6330*	IncI2 and IncX4 plasmids	Novel *Moraxella* spp.	Pig	2018	[46]
mcr-1	*mcr-1-orf*, IS*Apl1-mcr-1-orf* and Tn*6330*	Novel transposon Tn*6330*	IncX4 and IncI2 plasmids	*E. coli*	Pig farms in China	2017	[69]
mcr-2	(IS*Ec69-mcr-2-ORF-*IS*Ec69*	Tn*7052*	IncX4 conjugative plasmid	*Moraxella osloensis*	---	2021	[71]
mcr-2	IS*Ec69-mcr-2*-IS*Ec69*	---	IncX4 plasmid	*M. bovoculi*	Pigs, pork and chicken meat, and humans	2017	[72]
mcr-3.1	Tn*As2-mcr-3.1-dgkA-*IS*Kpn40*	Novel transposon Tn*6330*	pCP61-IncFIB plasmid	*E. coli*	Pigs	2021	[73]
mcr-3.5	IS*4321R*-Tn*As2-mcr-3.5-dgkA-*IS*15*	Novel transposon Tn*6330*	IncFIItype plasmid pCP55-IncFII	*E. coli*	Pigs	2021	[73]
mcr-3.7	Tn*As2-mcr-3.7-dgkA-*IS*26*	---	IncP1 plasmid	*E. coli*	Dogs	2020	[56]
mcr-8	IS*903B-ampC-hp-hphp-Giy-T-dgkA-baeS-copR*-IS*3-mcr-8-Gly-T-*IS*5*	_ Δ IS*66* transposases	IncFIA plasmid	*K. pneumoniae*	Patients from intensive care	2022	[74]
mcr-8	IS*903B-ymoA-inhA-mcr-8-copR-baeS-dgkA-ampC*	Composite transposon	pABC264-OXA-181 plasmid	*K. pneumoniae*	Patient with bacteremia	2022	[75]

***mcr* Variants**	**Insertion Sequences Structure**	**Transposon**	**Plasmids**	**Organism**	**Host (Isolated from)**	**Year of Discovery**	**References**
mcr-8.2	IS*Ecl1-mcr-8.2-orf-*IS*Kpn26*	---	IncFII/FIA	*K. pneumoniae*	Patient's Intestinal sample	2022	[76]
mcr-9.1	IS*903B-mcr-9.1-wbuC-*IS*26*	Tn*6360*	IncHI2/2A plasmid	*E. cloacae* complex	Clinical isolates	2022	[77]
mcr-10	IS*Kpn26* is present at upstream of *xerC-mcr-10* and an IS*26*	Transposon Tn*1722*	IncFIA plasmid	*Enterobacter roggenkampii*	Clinical isolate	2020	[78][88]
mcr-10.1	*hsdSMR-*IS*Ec36-mcr-10.1-xerC*	---	IncFII$_K$ plasmids	*K. pneumoniae*	Clinical isolates	2022	[77]

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Based on cost, sensitivity and specificity, turnaround time, and the skills required to perform the test, the use of culture media or the Rapid Polymyxin Nordmann–Poirel (RPNP) test are recommended for low-resourced laboratories, while Multiplex PCR or Taqman/SYBR Green real-time PCR assays along with RPNP or novel culture media are applicable for well-resourced laboratories [93][94].

To study the evolution in *mcr*-positive bacterial strains, different sequencing techniques can be used including Sanger sequencing and the identification of single nucleotide polymorphisms [95] for mutational analysis or identification of new *mcr*- variant(s) [96]. For detailed studies of intrinsic determinants of resistance, whole genome sequencing (WGS) [97], nanopore sequencing, and transposon-directed insertion site sequencing [72] can give insights into the interactions of genetic elements associated with polymyxins resistance. To study coevolution among pairs of *mcr* or multiple *mcr* elements within a single bacterial cell, *mcr*-coevolution assays could be used [72].

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