UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

TRABALHO DE CONCLUSÃO DE CURSO

Polymyxins resistance: an overview of molecular mechanisms and genetic determinants

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Trabalho de Conclusão de curso apresentado por **Victória Martins Lima Cupertino** para obtenção do diploma de Farmacêutica.

Orientadora:
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APRESENTAÇÃO

Este trabalho foi elaborado no formato de artigo científico, seguindo as orientações para autores da revista *Brazilian Journal of Microbiology* (Anexo 1). As figuras e tabelas foram dispostas ao longo do texto para facilitar a leitura da banca examinadora.

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Polymyxins resistance: an overview of molecular mechanisms and genetic determinants

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ABSTRACT

Emergence of antimicrobial resistant bacteria is a majorworldwide public health issue. During the past decades, the misuse/overuse of antibiotics accelerated the development and dissemination of antimicrobial resistance, especially in multidrug-resistant Gram-negative bacilli (MDR-GN). Under this scenario, old drugs have been reconsidered. Since most available therapeutical options may fail in the treatment of some infections caused by MDR-GN, the polymyxins have arisen at the frontline of combating these highly resistant bacteria. As a consequence, resistance to polymyxins is growing in different geographical regions, compromising even more the treatment of infections caused by MDR-GN. These bacteria employ several strategies to protect themselves against polymyxins, such as mechanisms associated with changes in chromosomal genes, including lipopolysaccharide (LPS) modifications, overproduction of capsules, expression of efflux systems and enzymatic antibiotic inactivation. Worryingly, mechanisms related to plasmid-located genes have also been identified: the mobile colistin resistance gene (*mcr*), which threatens to increase the dissemination of resistance to polymyxins. The knowledge about mechanisms behind polymyxins resistance are useful to understand the epidemiology of this phenotype and to control the dissemination. For this purpose, this review aimed to discuss and update the main mechanisms involved in resistance to polymyxins.

Keywords: polymyxins, antimicrobial resistance, resistance mechanisms, gram-negative bacilli

SUMMARY

1. Introduction

The importance of antimicrobial resistance (AMR) is well-recognized worldwide. Indeed, according to the World Health Organization (WHO), AMR is considered one of the biggest challenges and threats to human health [1]. The available therapeutic arsenal may be no longer effective against some bacteria due to the development of multidrug resistance. This worrisome resistance reflects, at least partially, the negligent and inappropriate use of antimicrobials in human and animal care [2].

In this scenario, we must highlight gram-negative bacilli (GNB), which frequently present a multiresistant profile and cause infections associated with high morbidity and mortality [3,4]. At the present, carbapenem-resistant gram-negative bacteria (CRGNs) are one of the greatest worldwide concern, since carbapenems are considered one of the ultimate resort reserved for management infections caused by multiresistant pathogens [5]. And it was exactly considering CRGNs that polymyxins were massively reintroduced in therapeutic practice, aiming to combat infections caused by these bacteria [6].

β-lactams agents are historically combined with β-lactamase inhibitors, making them more effective against β-lactamase producers [7–9]. Recently, new combinations of β-lactams and β-lactamase inhibitors have been approved for clinical use in some countries around the world, such as ceftazidime/avibactam [10,11], meropenem/vaborbactam [12,13] and imipenem/cilastatin/relebactam [13,14], increasing the capacity of treating CRGN infections. However, they are not yet fully available in many countries. Moreover, it should be highlighted that ceftazidime/avibactam have activity against β-lactamase-producing GNB of classes A (ESBL and KPC, for example), C (AmpC) and some class D (OXA-48 for example), but presents no activity against class B metallo-β-lactamases (NDM, VIM, IMP, VEB, among others) [6,13]. In Brazil, only ceftazidime/avibactam has been available since 2018 and costs are definitely an issue, limiting its use [15,16]. Therefore, as these new combinations are not largely available in many regions, and also considering costs, polymyxins-centered therapeutical schemes are still widely used worldwide [17,18].

Polymyxins belong to a group of cationic antimicrobial peptides (CAPs), first isolated in 1947 from a gram-positive soil bacterium, *Paenibacillus polymyxa* (former *Bacillus polymyxa*), which is the natural source of colistin (polymyxin E) [19,20]. Other four polymyxins were also discovered from *P. polymyxa* metabolism, known as polymyxin A, B, C and D. Originally introduced in the 1950s, polymyxin B and colistin are the only two employed in veterinary and human medical therapy [4]. Structurally very similar, they differ only by a single amino acid change at the position 6 within the peptide ring, with a D-phenylalanine in polymyxin B and a D-leucine in colistin; however, both present same mechanisms of action [20,21]. By the mid-1970s, their parenteral use was gradually abandoned in most countries due to reports of severe toxicity, mainly nephrotoxicity and neurotoxicity [19,22,23]. Despite the adverse events, they remained in clinical practice as topical optic and ophthalmic solutions, and for the management of pseudomonal lung infections in patients with cystic fibrosis [24]. Besides, polymyxins persisted in veterinary medicine to treat infections, but also as prophylactic agent and as growth promoter

of farm animals. Nevertheless, the use of colistin as growth promoter was banned in November 2016 in Brazil. Over recent years, these "old antibiotics" reappeared as "last-resort" for emerging multidrug resistant gram-negative bacterial infections [25]. As a consequence of this increased use of polymyxins, the number of polymyxin resistance reports have also been rising, which represents a public health issue.

The objective of this review was to detailed discuss the mechanisms of resistance to polymyxins described so far, highlighting their impact on clinical/veterinary medicine and public health.

2. Mechanism of action

Polymyxins exhibit activity against most gram-negative bacteria. This relative selectivity is attributed to their amphipathic character, which allow interactions with both anionic and hydrophobic components of bacterial outer membrane (OM) [20,26]. The primary target of polymyxins is the lipopolysaccharide, which consists of three domains: O antigen chain, a core polysaccharide chain, and a lipid A that behaves as a hydrophobic anchor in the OM. LPS is negatively charged and this provides maintenance of the membrane integrity and stability (Figure 1) [27–29].

Initially, polymyxins, as other cationic peptides, bind to the bacterial surface through electrostatic and hydrophobic interactions with the anionic LPS molecules. The positively charged diaminobutyric acid (Dab) residues of polymyxins linkages electrostatically with negatively charged phosphate groups on lipid A moiety of LPS, leading to a competitive displacement of divalent cations (Ca^{2+} and Mg^{+2}), which are responsible to stabilize the OM. As a result, there is a rearrangement of the membrane potential (Figure 1) [22,30,31]. Once approached, polymyxins insert their hydrophobic domains into the bacterial OM by interaction with the fatty acyl chains of lipid A, resulting in a membrane disruption and polymyxins uptake. Finally, it has been suggested a merger of the inner leaflet of the OM with the outer leaflet of the cytoplasmic membrane, which leads to phospholipid exchange, osmotic imbalance and, thereby, cell death (Figure 1) [27,31,32].

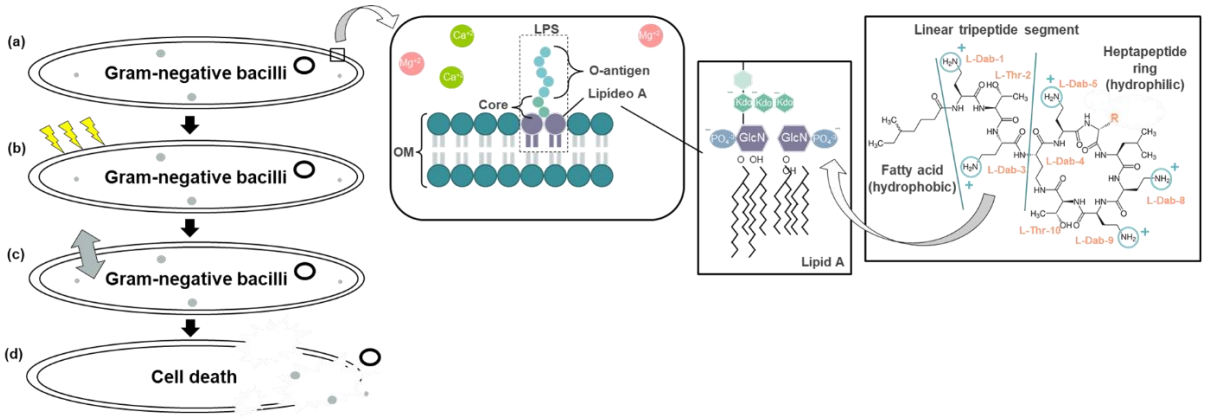

Fig 1. Mechanism of action of polymyxins. (a) Electrostatic and hydrophobic interaction between αγ-Dab^{+} of polymyxins and PO_4^{-3} of lipid A. (b) Competitive displacement of the divalent cations (Ca^{+2} e Mg^{+2}), generating the rearrangement of the membrane potential, consequently the LPS is destabilized. (c) Increased membrane permeability, subsequently polymyxins insert their hydrophobic domains in ME, leading to uptake of antibiotic molecule. (d) Membrane rupture and cell content leakage, causing bacterial death. LPS (lipopolysaccharide); OM (outer membrane); Dab (diaminobutyric acid); Kdo (3-deoxy-D-manno-oct-2-ulosonic acid).

3. Mechanism of resistance

Gram-negative bacteria present several strategies to self-protect against polymyxins [5,6,25]. Indeed, studies have elucidated many mechanisms of resistance to polymyxins, which may show some specie-specific characteristics but share the same global approach. Although they encompass mostly changes in outer membrane, through alterations of lipid A moiety of LPS [5,26,33,34], the mechanisms behind polymyxins resistance are not fully characterized to date. However, it is known that they are not restricted to a single pathway; on the contrary, they are vast and extremely complex [5,25]. These mechanisms may be associated with chromosomal or plasmid-located genes [35–37].

Some GNB are intrinsically resistant to polymyxins. The remaining species may develop, as a consequence of selective pressure, acquired resistance, which is a subject of major concern as it is unpredictable and transferable among bacteria [38,39]. Resistance mechanisms include LPS modifications or even loss of LPS; efflux pumps systems; capsule formation and enzymatic inactivation [5,39,40]. Below, these mechanisms will be described in detail.

3.1. INTRINSIC RESISTANCE

Among *Enterobacterales*, *Serratia marcescens* [41], *Proteus* spp. [42], *Providencia* spp., *Morganella morganii* [26,34], *Edwardsiella tarda* [43,44] and *Hafnia* spp. [45] are intrinsically resistant to polymyxins [25,39,46]. Moreover, other gram-negative bacilli such as *Burkholderia* spp. [47,48] and *Neisseria* spp. [49,50] also possess natural resistance to these cationic antimicrobials.

One of the main polymyxins resistance mechanism in GNB consists of alterations in the outer membrane, via addition of positively charged residues such as 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (PEtN) and/or galactosamine (GalN) to LPS moiety, which neutralizes, total or partially, the negative charge of LPS and reduces the interaction between cationic antimicrobial and bacterial OM [39,51]. Noteworthy, LPS-modifying genes are expressed under regulation of the two-component systems (TCS) PmrAB/PhoPQ in response to environmental conditions [5,52]. However, the aforementioned species intrinsically produce those residues as part of their LPS, reflecting their natural decreased susceptibility to polymyxins [39,53].

The *arn* operon (*arnBCADTEF*), recently named *pmr* operon (*pmrHFIJKLM*), is a seven-gene polycistronic unit closely linked to LPS modifications, which seems to be constitutively expressed in naturally resistant bacteria [39]. All *arn* genes, except for *arnF*, are responsible for the biosynthesis and addition of L-Ara4N to the 4'phosphate of lipid A. In *S. marcescens*, the intrinsic polymyxins resistance is due to the presence of *arnB* and *arnC* genes, which are under control of the two-component regulator PhoP [54,55]. Likewise, the response regulator *rppA* from RppA/RppB TCS, has been correlated to the natural resistance of *Proteus mirabilis* through induction of *pmrI*, *ugd* and *galU* expression [56,57]. Belonging to *pmr* operon, *pmrI* may encodes a UDP-glucuronic acid decarboxylase and contributes to alterations in LPS structure [58]. The *udg* and *galU* genes may respectively encode UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase, which are involved in maintenance of cell surface structure and also L-Ara4N production [59].

Furthermore, studies on *P. mirabilis* have shown the essential role of *eptC* gene in core LPS modification with PEtN [60]. Moreover, most *Burkholderia* species are inherently resistant to antimicrobial peptides through multifaceted mechanisms, but especially by constitutive production of L-Ara4N as part of their LPS molecule, where *arnT* (L -Ara4N transferase) and *lptG* (LPS transporter) genes play a critical role [48,53]. Complementarily, RpoE and BCAL2830/BCAL2831 TCS have been described as global regulatory systems associated with the increased resistance of *Burkholderia cenocepacia* to polymyxins [47]. *Burkholderia* innate polymyxins resistance repertoire also includes genes involved in the synthesis of isoprenoids and hopanoids, *ispH* (also called lytB) and *shc* (encoding for squalene-hopene cyclase), respectively [61].

3.2. ACQUIRED RESISTANCE

As mentioned previously, GNB, other than those intrinsically resistant, may develop acquired resistance to polymyxins through mutations in chromosomally located genes or acquisition of foreign resistance determinants via transferable plasmids [46,62]. Different bacteria, such as *Escherichia coli, Salmonella* spp., *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* have expressed this resistance trait, even in community or nosocomial settings [36]. The molecular and biochemical mechanisms developed to protect

themselves against polymyxins (Table 1) may present some peculiarity among different bacteria and will be discussed below.

3.2.1. Mechanisms associated with changes in chromosomal genes

3.2.1.1. Lipopolysaccharide modifications

It is well established that polymyxins resistance is mainly achieved by modifications of LPS structure, considering that this is the primary target of cationic peptide antimicrobials in bacterial cell. These modifications are a consequence of distinct processes, such as incorporation of positive elements into LPS structure, PmrCAB-mediated LPS modifications, action of a ferrous iron-binding protein (Omb), modifications in lipid A (deacylation, phosphorylation, dephosphorylation, glycylation and glucosylation), repression of phoPQ expression, addition of amide-linked acyl chains in lipid A, glucosamine modification or alternatively, loss of LPS (Table 1) [63,64]. In summary, these modifications cause reduction in net negative charge and/or in fluidity/permeability of LPS, decreasing self-promoted uptake of cationic peptides across the outer membrane [64].

TCS play an essential role in polymyxins resistance by regulating the expression of most genes involved in LPS modifications. Environmental stimuli and/or point mutations within TCS trigger their constitutive activation and subsequent overexpression of LPS-modifying genes. The upregulation of TCS leads to addition of cationic groups (L-Ara4N, PEtN and GalN) to lipid A of the LPS, reducing significantly antibiotic binding [5,36].

A wide range of operons and genes are directly involved in LPS modifications, including (i) genes responsible for biosynthesis and/or addition of cationic groups to LPS, such as the *pmrHFIJKLM* operon, the *pmrE* and *pmrC* genes (also called *eptA*); (ii) regulatory genes, which encodes proteins associated to PhoPQ and PmrAB two-component systems; and (iii) the regulators of these TCS, as the *mgrB* gene, which downregulates de PhoPQ system, and the CrrAB system, upregulating the PmrAB TCS [25,36,65].

Different TCS are distributed among bacterial species, contributing to their polymyxins resistance, such as PhoP/PhoQ (*K. pneumoniae*, *Salmonella* spp.), PmrA/PmrB (*A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella* spp. and *E. coli*), ParR/ParS (*P. aeruginosa*), ColR/ColS (*P. aeruginosa*), CprR/CprS (*Campylobacter jejuni*), CrrA/CrrB (*K. pneumoniae)* and VprA/VprB (*Vibrio cholerae*) [36,63]. Among these, PhoP/PhoQ and PmrA/PmrB stand out for their contribution to polymyxins resistance in most gram-negative bacteria. Both systems are composed of a transmembrane sensor kinase (PhoQ and PmrB) and a cognate cytoplasmic response regulator (PhoP and PmrA).

The phoPQ system leads to the expression of genes that encode for magnesium transport, LPS-modifying enzymes, and enzymes that decrease cell stress, thus, allowing bacterial survival under conditions of low Mg^{2+}, low pH or sublethal concentrations of CAPs [66]. In response to the particular stress conditions aforementioned, the sensor kinase PhoQ autophosphorylates and, subsequently, transphosphorylates the response regulator, PhoP, which, in turn, binds the DNA and modulates the expression of specific genes [5,22,66]. Once activated, PhoP increases the

transcription of *pmrHFIJKLM* operon, responsible for the addition of L-Ara4N to LPS and can also activate directly or indirectly the PmrA response regulator by increasing the expression of PmrD connector protein, that leads to the addition of PEtN to LPS. In *P. aeruginosa* and *Klebsiella* spp., the response regulator, PhoP, acts directly on *pmrHFIJKLM* operon, in contrast with *Salmonella* spp. that has an indirect upregulation, via *pmrD* expression [36,51]. In *A. baumannii*, the PhoPQ component is absent, which drives the regulatory factor to another pathway [67].

Similar to PhoPQ system, environmental stimuli, such as high concentrations of Fe^{3+} and Al^{3+} and low pH turn on the tyrosine kinase activity of PmrB protein, which thus activates PmrA by phosphorylation. The pmrA then increases transcription of the *pmrCAB* operon, the *pmrHFIJKLM* operon and the *pmrE* gene (also known as *udg*). The phosphoethanolamine phosphotransferase, *pmrC*, is in charge of adding PEtN to lipid A. On the other hand, *pmrHFIJKLM* operon and *pmrE* gene are involved in the synthesis and fixation of L-Ara4N to LPS [51].

Polymyxins resistance may arise from chromosomal mutations in both systems, PhoPQ and PmrAB. Indeed, studies have found that point mutations in *pmrA* and *pmrB* genes are associated with acquired resistance in *K. pneumoniae* [68], *Salmonella enterica* [69,70], *P. aeruginosa* [71,72], *A. baumannii* [67,73] and *Enterobacter aerogenes*, by activating constitutively the PmrAB TCS [65]. Moreover, polymorphisms of *pmrAB* genes of colistin-resistant *E.coli* have been described, but the association of these mutations in resistance phenotype has not been fully understood [65,74,75].

Although the majority of *Enterobacterales* develops polymyxins resistance through general modifications in chromosomally-located genes responsible for expression and regulation of the outer membrane components, there are some mechanisms observed in an organism-specific manner [33]. For example, in *K. pneumoniae*, the addition of L-Ara4N to LPS is achieved by mutations and subsequently inactivation of *mgrB* (also known as *yobG*), which encodes a transmembrane protein that exerts negative feedback on PhoPQ regulatory system [25,36,76]. Upon activation of PhoP, *mgrB* is upregulated, and the translated MgrB protein represses the expression of the *phoQ* gene, leading to negative regulation of the kinase activity of PhoQ [77]. The inactivation of *mgrB* results in the overexpression of *phoPQ* operon, that in turns activates the *pmrHFIJKLM* operon, leading to the synthesis of L-Ara4N, and thus developing acquired resistance to polymyxins [65,78,79].

Studies have detected different alterations in *mgrB* coding sequence, including missense and/or nonsense point mutations, insertion sequences (IS), and small deletions [80]. IS observed in *mgrB* are genetically diverse, represented by several families, such as IS5-like, IS903B, IS1F-like and ISKpn14, and are inserted at multiple sites within *mgrB* [76,81,82]. Current reports have shown that, among *mgrB* of carbapenem-resistant *Klebsiella pneumoniae* (CRKp), IS5 family is the most frequent IS observed [80,83–85]. Furthermore, recent research describes a genetic element (ISEcp1) conferring resistance to carbapenem antibiotics, that leads to an insertional inactivation of *mgrB*, resulting in resistance to colistin, which proposes a model of development

of pandrug-resistant K. *pneumoniae* [86,87]. Able to mobilize adjacent genes and insert into new sites, the ISEcp1-like insertion sequences are located upstream several β-lactamase genes, promoting their overexpression. This mobile element has been generally associated with *bla*$_{ACC}$, *bla*$_{CMY}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-181}$ genes in *Enterobacterales* [86,88,89]. A study identified an ISEcp1-*bla*$_{OXA-181}$ transposon throughout the chromosome of a pandrug-resistant *K. pneumoniae* isolate, which has disrupted the *mgrB*, enhancing the colistin resistance [86,90]. Also, other studies have demonstrated a chromosomal insertion of the ISEcp1-*bla*$_{CTX-M-15}$ in tandem within the *mgrB*, resulting in its truncation and consequent polymyxins resistance in *K. pneumoniae* [87,91].

In a different way, CrrAB is a TCS that regulates positively the PmrAB system, and can also lead to polymyxins resistance [92,93]. The products of *crrAB* operon are a response regulator protein (CrrA) and a sensor kinase protein (CrrB) [36]. In *K. pneumoniae*, it has been reported amino acid substitutions in CrrB contributing to colistin resistance. These missense mutations of *crrB* increase the *crrC* transcription, which, through effects on PmrAB, upregulates the expression of *pmrHFIJKLM* operon, *pmrC* and *pmrE*, consequently results in the addition of L-Ara4N and PEtN to lipid A [65,94]. Moreover, it has been demonstrated that mutated CrrAB exhibits increased expression of a putative efflux pump, which could explain the higher colistin minimal inhibitory concentrations (MICs) displayed by isolates harboring CrrB missense mutants when compared to clinical isolates harboring mutations in *phoPQ*, *pmrAB* and *mgrB* [95].

Noteworthy, *A. baumannii*, unlike *Enterobacterales* and *P. aeruginosa*, lacks the genetic machinery required for L-Ara4N biosynthesis, since *pmrHFIJKLM* operon is absent [39]. Thereby, polymyxins resistance in *A. baumannii* is primarily achieved by mutations in *pmrA* and/or *pmrB* genes, through upregulation of *pmrCAB* operon, inducing the modification of LPS with PEtN. *A. baumannii* lipid A has been shown to be modified also with GalN in polymyxins-resistant strains [96,97]. Studies have identified a specific *pmrB*-regulated gene, *naxD*, which encodes a deacetylase required to convert N-acetylgalactosamine into galactosamine [96]. Additionally to LPS modifications, *A. baumannii* have demonstrated an unique polymyxins resistance mechanism, which implies the complete loss of lipid A or core of LPS, loosing colistin target and resulting in very high polymyxins MICs (128 mg/L) [98]. This phenotype is reached by the inactivation of lipid A biosynthesis genes (*lpxA*, *lpxC*, and *lpxD*) [99].

P. aeruginosa present five two-component systems mediating polymyxins resistance by LPS modifications described so far [79]. Similarly to *Enterobacterales*, alterations in PhoPQ and PmrAB systems have been associated with acquired polymyxins resistance via constitutive modifications on *P. aeruginosa* LPS by L-Ara4N addition [65]. Other three TCS (*parR/parS*, *colR/colS* and *cprR/cprS*) have been identified as a mediator of polymyxins resistance in *P. aeruginosa* [39]. The ParRS TCS has been related to polymyxins adaptive resistance: when mutated, causes the constitutive expression of *pmrHFIJKLM* operon, resulting in the addition of L-Ara4N to the LPS [39,100]. Besides, alterations in ColRS and CprRS TCS have shown major influence on *P. aeruginosa* polymyxins resistance, since involvement of ColRS and CprRS may

occur via interactions with the PhoPQ system, which enhances PhoQ regulatory system and therefore reaches a high level of colistin resistance [65].

3.2.1.2. Acylation of Lipid A

Complementary to L-Ara4N or PEtN additions, it has been reported other modifications related to chromosomally-encoded polymyxins resistance, which involve surface structural changes, such as acylation of lipid A. These alterations have been demonstrated to be capable of changing permeability properties of the OM [63]. The underacylation of lipid A results in an increased fluidity of LPS moiety and, consequently, an increased ability of CAPs to cross OM, leading to self-promoted uptake [64].

Considering the relevance of hydrophobic interactions with acyl chains of lipid A for polymyxins antibacterial activity, *K. pneumoniae* strains expressing LPS chemotypes with an underacylated lipid A present an increased polymyxins susceptibility [101]. The *lpxM* (former *msbB* or *waaN*) encodes the enzyme responsible for acylation of the immature structure of lipid A (penta-acylated lipid A) in hexa-acylated lipid A, which better interacts with polymyxins [39,101]. Therefore, a LpxM mutant of *K. pneumoniae* that mainly possess penta-acylated lipid A has been displayed 8- to 16- fold more sensitive to polymyxins than the wild type with hexa-acylated lipid A [102].

Furthermore, other mechanisms involving lipid A modifications have been evidenced in *S. enterica* strains, including deacylation of lipid A portion by *pagL*, which is activated via PhoP [39]. *pagL* encodes an outer membrane enzyme that normally appears latent given its inhibition by L-Ara4N or PEtN modifications, however, the pagL-dependent deacylation of lipid A may be detected in strains that are incapable to alter this LPS with L-Ara4N or PEtN [103,104]. As a result, these strains carry increased colistin resistance due to pagL-mediated deacylation of lipid A. These results indicate a possible compensatory mechanism of polymyxins resistance, where PagL is released from latency when there is loss of induced resistance by modifications of lipid A with aminoarabinose or phosphoethanolamine residues [103].

3.2.1.3. Capsule Polysaccharide

Another strategy of resistance has been characterized, where the polysaccharide capsule (CPS) mediates resistance to CAPs, including polymyxins, by limiting interaction with bacterial surface [36]. Mechanistically, anionic CPS acts as a protective shield against CAPs and blocks its antimicrobial activity by trapping them, thus reducing the amount of the compound reaching the membrane target [105]. This capsular mechanism has already been identified in some natural polymyxins-resistant strains, such as *Neisseria meningitidis*, which displayed higher levels of intrinsic resistance to polymyxin B when compared to unencapsulated strains [106].

In addition, studies reported that *K. pneumoniae* CPS may also play an important role in polymyxins resistance, even considering capsule function as a protective barrier, but also its overproduction in face of exposure to polymyxins. A study has evidenced mutants of *K. pneumoniae* lacking CPS exhibiting reduced resistance to CAPs than capsulated wild-type strains

[107]. Even though the CPS linked to the bacterium may not influence resistance to CAPs, a study hypothesized that free anionic CPS can trap CAPs, preventing them from reaching membrane targets and thus neutralizing their bactericidal activity. To confirm this, they tested three purified CPSs from *K. pneumoniae*, *Streptococcus pneumoniae* and *P. aeruginosa* strains and observed an increase of polymyxin B resistance of an unencapsulated *K. pneumoniae* mutant. It has also shown that CAPs released CPSs from the bacterial surface and this product acted in the same manner as purified CPSs [105].

Moreover, it was observed in *K. pneumoniae*, that the presence of polymyxin B and lactoferrin raised the amount of CPS attached to bacterial surface via induced transcription of capsule biosynthesis genes [107,108]. Furthermore, some critical regulators are involved in CPS biosynthesis, namely: *siaD*, OmpA and *cps* operon (*wca*) [63]. Indeed, the exposure of a wild-type *K. pneumoniae* has shown to upregulate the transcription of *cps* operon, where PhoPQ system proved to be a necessary mediator of polymyxin B-triggered induction of *cps* [109]. Additionally, the Rcs (regulator of capsule synthesis) phosphorelay system comprises three proteins; RcsC, RcsD (also known YojN), and RcsB (cytoplasmic response regulator), and mediates the expression of CPS in several *Enterobacterales*, thus also CAPs resistance [110]. In *S. enterica*, Rcs system has revealed a main function in regulating the expression of *ydeI* (also named *omdA*), which encodes a binding-fold protein, important for polymyxin B resistance [110].

Alternatively, in *Salmonella* spp., it has been established that the Rcs system can promote transcription of *udg* independently of PhoPQ and PmrAB systems, suggesting a role for *ugd* in capsule synthesis. Likewise, the expression of *udg* is induced in coordination with Cps genes via Rcs system and RcsA protein, which may be associated in colanic acid capsule synthesis [111]. On the other hand, in *N. meningitidis*, the presence of capsule relies on the expression of genes for the capsule synthesis, such as *siaA*, *siaB* and *siaC*, which are required for biosynthesis of the sialic acid capsule, and also *siaD*, that encodes a polysialyltransferase involved in capsule formation [106]. Moreover, the outer membrane protein, OmpA, of *K. pneumoniae* has implicated in the upregulation of previously systems committed to enhancing CAPs activity, specially polymyxins [112]. Finally, it has also been postulated that a multidrug efflux pump, named KpnEF, has a direct involvement in capsule synthesis, since a mutant KpnEF displayed a defect in capsular synthesis and also higher susceptibility to several antibiotics, including polymyxins, compared with the wild-type *K. pneumoniae* [113,114].

3.2.1.4. Efflux pump

It is well established that efflux pumps can confer resistance to multiple antibiotics, decreasing the intracellular concentration of these toxic agents [25,115]. Until now, five different families of efflux pump proteins have been identified that are associated with multidrug resistance: ATP-binding cassette (ABC) superfamily, major facilitator superfamily (MFS), resistance nodulation cell division (RND) family, multidrug and toxic compound extrusion (MATE) family, and the small multidrug resistance (SMR) family [116]. The upregulation of multidrug efflux pump activity was considered as an additional mechanism that also contributes

to polymyxins resistance [63]. Overall, the activation of these pumps leads to increased resistance to different antibiotics, including polymyxins. In gram-negative bacteria, several types of efflux pumps have been related to colistin and polymyxin B resistance, such as Sap (sensitive antimicrobial peptides) proteins, KpnEF, MtrCDE, RosAB, BrlR, MexXY/OprM and the AcrAB-TolC complex [25,63].

Indeed, efflux pumps play a crucial role in intrinsic resistance to CAPs of *Neisseria gonorrhoeae*, *N. meningitidis* and *Yersinia* species [51]. Gonococci present an energy-dependent efflux pump named Mtr (multiple transferable resistance) that contributes to resistance of diverse antimicrobial agents. The *mtr* efflux pump is encoded by a single transcriptional unit (*mtrCDE).* MtrCDE has proved to be responsible for enhancing resistance to cationic antibiotics, such as polymyxins [117]. Also, meningococcal resistance to polymyxin B has been associated with multiple mechanisms including the lipid A modification, but also the MtrCDE efflux pump [49].

Another mechanism of natural resistance involving efflux pumps has been reported in *Yersinia enterocolitica*, whereby RosA/RosB system mediates resistance to CAPs, since the *rosAB* locus encodes a temperature-regulated efflux pump that is coupled to potassium antiporter [118]. RosA shows similarity to drug resistance transport proteins, which was characterized as a proton motive force-driven efflux pump, while RosB is similar to some proteins involved in glutathione-regulated potassium efflux system [119]. Together, these elements are the key of the innate immune system and also contribute to resistance to antimicrobial peptides, especially polymyxin B [118]. Lastly, the multidrug efflux pump NorM has shown to be involved in polymyxins resistance among *Burkholderia vietnamiensis* [120].

As previously mentioned, mutations in *K. pneumoniae kpnEF*, efflux pump from SMR family, has reflected in impairment of capsular synthesis and in 2-fold reduction of colistin MIC, which supports its influence in polymyxins resistance [113]. Also in *K. pneumoniae*, missense mutations of *crrB* increasesthe expression of H239_3064, which encodes a putative RND-type efflux pump that leads to decreased susceptibility to colistin, tetracycline and tigecycline [95]. Moreover, it has been reported the AcrAB multidrug efflux system in *K. pneumoniae*, which is encoded by the *acrRAB* operon, where *acrR* encodes the AcrAB repressor, while *acrA* and *acrB* encode a periplasmic protein [121]. AcrB binds a particular outer membrane, TolC, that belongs to a family of envelope proteins in GNB, and revealed an essential function exporting a wide range of compounds, specially antibacterial agents [121].

Indeed, an *acrB K. pneumoniae* mutant exhibited significantly higher susceptibility to polymyxin B than the wild-type strain, which indicates that polymyxin B is a potential substrate for AcrAB-TolC (RND-type efflux pump) [121]. Most recently, a study presented the first report of clonal KPC-2-producing isolates with different susceptibility profiles that was attributed to previously unknown regulatory system of the AcrAB-TolC [122]. Based on that, distinct behavior from isolates with differential expression of a genetically identical pump system emphasizes the versatility of resistance mechanisms in GNB [122]. Additionally, the reduction in polymyxins susceptibility was associated with the RamA-dependent regulation of AcrAB efflux pump system of *K. pneumoniae* [123]. RamA is a transcriptional activator and its overexpression

results in increased *acrAB* expression and also lipid A alterations, increasing resistance to polymyxins among *K. pneumoniae* [124].

P. aeruginosa possess several multidrug efflux pumps, kwon as Mex pumps, considered an efficient mechanism in increasing resistance level [123]. The constitutively expressed MexAB/OprM system was the first RND efflux pump characterized in *P. aeruginosa* and has been associated with MDR isolates [123,125]. It has also been reported involvement of MexAB/OprM efflux pump in phenotypic tolerance development to colistin in *P. aeruginosa* biofilms [126]. Furthermore, it was demonstrated the role of BrlR, a MerR family member of multidrug efflux pump activators, where its inactivation is correlated with increased colistin resistance of *P. aeruginosa* biofilms, whilst its overexpression resulted in elevated susceptibility of colistin and also in reduced expression of *phoP*, *phoQ*, and *arnC* [127].

In addition, previous studies described another tripartite pump, named MexXY/OprM, which implies an active efflux mechanism against various antibiotics, particularly providing low to moderate levels of polymyxins resistance in *P. aeruginosa* [25,128]. Importantly, the upregulation of MexXY efflux system has been commonly linked to mutations in *mexZ*, a local repressor gene of the *mexXY* operon that increases polymyxins resistance when inactivated [128]. However, it was characterized a mexZ-independent pathway regulation in *P. aeruginosa*, where the activated ParRS system leads to upregulation of MexXY/OprM efflux pump and also downregulation of OprD (carbapenem-selective porin), which generates a multidrug resistance phenotype [129]. Likewise, the small RNA-binding protein RsmA has been involved polymyxins resistance among *P. aeruginosa* through its posttranscriptional regulation of the virulence-associated type III secretion system (TTSS) and the expression of some Mex multidrug efflux pumps [63,130].

The upregulation of RDN efflux transporters proteins (AdeABC and HlyD) also seems to be implicated in polymyxins resistance in *A. baumannii*, and along with overexpression of protein complexes involved in membrane homeostasis, causes damage in integrity and barrier function of the OM in polymyxins-treated strains [63,131]. The AdeRS TCS, sensor AdeS and regulator AdeR proteins, regulates the expression of *adeABC* genes. Mutations in *adeSR* were found in *A. baumannii* clinical isolates showing overexpressed AdeAB efflux pump, and therefore displaying reduced polymyxins susceptibility when compared with wild-type strains [116,132]. Furthermore, it was identified in *A. baumannii* the presence of four pairs of *emr*-like genes (*emrB* and *emrA*), which encodes transporter proteins named EmrB and EmrA [133]. The absence of *emrB* gene resulted in impaired ability to pump out and thus increases colistin susceptibility, while increased expression of *emrB*-like genes induces colistin resistance in *A. baumannii* [67,79]. These findings have explained the association between the EmrAB efflux system and polymyxins resistance in *A. baumannii*.

3.2.1.5. Enzymatic Inactivation

Besides the mechanisms of polymyxins resistance discussed above, an additional strategy is the enzymatic inactivation of the drug by hydrolysis [25,51]. Studies from four decades ago

have evidenced a colistin-inactivating enzyme that inactivates colistin via degradation of the cationic peptide molecule in *P. polymyxa* [134]. The gram-positive bacterium *P. polymyxa* produces both colistin and the putative serine alkaline protease, designated colistinase, that is responsible to degrade colistin by specific cleavage of colistin peptide molecule, between the Dab (2,4-diaminobutyric acid) of the side chain and Dab adjacent in the cyclic peptide portion [134]. Although gram-positive bacteria lack the LPS, it has demonstrated that polymyxins can also kill their producer, since its induction of toxic free radical production (oxidative stress) and intracellular enzymes damage [135,136].

A recent report has identified polymyxin-inactivating enzyme from *Bacillus licheniformis*, an alkaline protease Apr responsible for polymyxins inactivation by hydrolysis [137]. The *B. licheniformis* Apr displayed the ability of cleaving peptide bonds, one between the tripeptide side chain and the cyclic heptapeptide ring, and the other between L-Thr and L-Dab in the cyclic heptapeptide ring [134,137]. It is important to note that the Apr enzyme is strongly conserved among gram-positive bacteria, specially *Paenibacillus* and *Bacillus* species. Interestingly, two peptidases S8 from Gram-negative bacteria (*Sphingobacterium* spp. and *Pseudomonas* spp.) shared relatively high level (64%) of sequence identity with the *B. licheniformis* Apr [137]. Results demonstrated that the Apr is necessary for bacterial survival under polymyxins exposure, during the stationary phase, where an extensive amount of antimicrobial peptides are synthesized [137–139]. In this circumstance, the alkaline proteases play an essential role in the protection of bacterial cell against biosynthesized polymyxins

Table 1. Mechanisms of resistance to polymyxins related to alterations in chromosome-located genes

Resistencia mechanism	Modifications	Function	Genes/determinants involved	Bacteria	References
Lipopolysaccharide modifications	L-Ara4N and/or PEtN addition on lipid A	Two-component system	*phoP/phoQ*	*K. pneumoniae*, *Salmonella* spp., *S. enterica*, *E. coli* and P. *aeruginosa.*	36; 51; 63; 66; 72
			pmrA/pmrB	*A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella* spp., *S. Typhimurium* and *E. coli*	71; 72; 73; 74
			crrA/crrB	*K. pneumoniae*	65; 94
			parR/parS colR/colS	*P. aeruginosa*	39; 65; 100
			vprA/vprB	*Vibrio cholerae*	36; 63
			cprR/cprS	*Campylobacter jejuni* and *P. aeruginosa.*	36; 63; 65
			rppA/rppB	*P. mirabilis*	56;
		Activator of PmrAB	*pmrD*	*P. aeruginosa*, *K. pneumoniae* and *S. Typhimurium*	36; 51; 69; 70
		UDP-glucose dehydrogenase	*pmrE (ugd or pagA)*	*P. mirabilis* and *S. Typhimurium*	25; 36; 51; 65
			arnBCADTEF (*pmrHFIJKLM*)	*K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *S. Typhimurium, S. enterica* and *E. coli*	25; 36; 39; 51; 65
		L-Ara4N transferase	*arnT* (*pmrK*)	*Burkholderia* species, *E. coli* and *S. Typhimurium*	48; 53
		Transmembrane transport	*lptG*	*Burkholderia* species	53
	PEtN addition on lipid A	PEtN transferase	*pmrC* (*eptA* and *lptA*)	*S. Typhimurium*, *A. baumannii* and *E. coli*	51; 63; 65
			eptB	*E. coli* and *S. Typhimurium*	25; 64
			eptC	*P. mirabilis*	60
	Overexpression of *phoPQ*	Negative feedback regulator	*mgrB*	*K. pneumoniae*	76; 78; 79; 80; 81; 82; 83; 84; 85

	Inactivation of lipid A biosynthesis	Biosynthesis enzymes	*lpxA*, *lpxC* and *lpxD*	*A. baumannii*	99
	Acylation of lipid A	Acyloxyacyl hydrolase	*pagL*	*S. enterica*	39; 103; 104
		Myristoyltransferase	*lpxM* (*msbB* or *waaN*)	*K. pneumoniae*	39; 101; 102
Capsule polysaccharide	Overproduction of CPS	Polysialyltransferase	*siaD*	*K. pneumoniae*	63; 106
		Porin activity	OmpA	*K. pneumoniae*	63; 112
		Colanic acid polysaccharide capsule biosynthetic operon	*cps* (*wca*)	*K. pneumoniae*	63; 109
		Regulator of capsule synthesis	*rcs*	*S. enterica*	110
Efflux pump	Multidrug efflux pump	Efflux transmembrane transporter activity	*mtrCDE*	*N. gonorrhoeae* and *N. meningitidis*	49; 51; 117
			kpnEF	*K. pneumoniae*	113
			acrAB		121; 122
			rosAB	*Y. enterocolitica*	118; 119
			norM	*B. vietnamiensis*	120
			mexAB	*P. aeruginosa*	123; 125; 126
			mexXY	*P. aeruginosa*	25; 128
			adeABC	*A. baumannii*	63; 131; 132
		Transmembrane transporter	*hlyD*	*A. baumannii*	63; 131
			emrB and *emrA*	*A. baumannii*	133
		Ribosomal RNA small subunit methyltransferase A	*rsmA*	*P. aeruginosa*	63; 130
	Overexpression of *acrAB*	Transcriptional activator of AcrAB	RamA	*K. pneumoniae*	123; 124;
		Repressor of AcrAB	*acrR*	*K. pneumoniae*	121
	Overexpression of *phoPQ*	Mer-like efflux pump activator	*brlR*	*P. aeruginosa*	127
	Overexpression of *mexXY*	Repressor of MexXY	*mexZ*	*P. aeruginosa*	128; 129
	Upregulation of *mexXY*	Two-component system	*parR/parS*	*P. aeruginosa*	129
Enzymatic inactivation	Hydrolysis of polymyxins	Alkaline serine protease	*apr*	*B.licheniformis*, *B. polymyxa*	134; 137

L-Ara4N: 4-amino-4-deoxy-L-arabinose; PEtN: phosphoethanolamine; CPS: capsule polysaccharide.

3.2.2. Mechanisms associated with plasmid-located genes

3.2.2.1. Plasmid-mediated resistance

Although most genes related to polymyxins resistance are chromosomally located, recently, it was discovered a mobilized colistin resistance gene, plasmid-located and horizontally transferred [25,140]. In November 2015, during a routine surveillance in China, the first *mcr* (mobile colistin resistance) gene, termed *mcr-1*, was detected in *E. coli* recovered from food animals and humans, presenting colistin resistance [141]. Given its transmission by a variety of conjugative plasmid, *mcr-1* may be easily disseminated among various GNB from animal and human sources, which represents a serious threat to the clinical and veterinary utility of polymyxins [140].

The *mcr-1* encodes a phosphoethanolamine transferase that catalyzes the addition of PEtN moiety to the phosphate groups in lipid A, decreasing negative charges of LPS and, thereby, reducing polymyxins binding [25]. A previous study demonstrated that the expression of *mcr-1* in *E. coli* led, in general, to 4- to 8-fold increases in colistin MICs [65]. It is worth mentioning that some isolates carry *mcr*, but do not present clinical resistance to polymyxins, as MICs remain below the breakpoint (4 μg/mL). Although these isolates have clear epidemiological importance, as they work as *mcr* reservoirs, the clinical significance of these bacterial are not clear understood so far.

From the first isolation in China, the *mcr-1* has been found in various genera of *Enterobacterales* (*E. coli*, *K. pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Cronobacter sakazakii*, *S. enterica*, *Raoultella ornithinolytica*, *Citrobacter freundii*, *Citrobacter braakii*, *Shigella sonnei*, *Kluyvera ascorbata*, and *Moraxella* spp.), recovered from environment, foods, animals and humans, and disseminated worldwide (Table 2) [140]. So far, different variants of *mcr* genes have been identified: *mcr-1* to *mcr-9* among *Enterobacterales*;*mcr*-1 and *mcr-4* in *Acinetobacter* spp., and only *mcr-1* in *Pseudomonas* spp. (Table 2) [140,142–153]. All *mcr* homologues encode PEtN transferases and MCR-2, -3, -4, -5, -6, -7 and -8 share 81%, 34%, 33%, 31%, 82%, 29%, and 31% amino acid sequence identity, respectively, with MCR-1 [25]. More recently, a novel *mcr*, *mcr-10*, was described in IncFIA plasmid of an *Enterobacter roggenkampii* clinical strain [154].

As mentioned before, *mcr* genes are carried by several transferable plasmids belonging to different incompatibility groups, indicating an increased capacity of dissemination among bacteria [25,140]. Molecular investigations have characterized *mcr* on three predominant types of plasmids: IncI2, IncHI2 and IncX4 [79]. Other plasmids were also observed carrying *mcr*, including IncHI1, IncF, IncFI, IncFIB, IncFII, IncP, IncP-1, IncK2, phage-like IncY [140], and most recently a small ColE-type plasmid has been identified carrying *mcr-4* and *mcr-5* (Table 2) [155–157]. Of note, the IncHI2-type has often been linked with diverse antimicrobials resistance determinants [140]. This variety of plasmid types that may carry *mcr* genes highlights the great facility of dissemination of these genetic determinant, which is a nightmare, epidemiologically speaking.

Noteworthy, some *mcr-1*-carrying plasmids harbor other antimicrobial resistance genes encoding resistance to relevant antibiotics in clinical practice, such as β-lactams, aminoglycosides, quinolones, fosfomycin, sulfonamides, and tetracyclines [79]. Therefore, the location of *mcr-1* on MDR-plasmids is a serious issue [25]. Indeed, studies have detected in highly drug-resistant *Enterobacterales*, plasmid co-carring *mcr-1* and cabapenamase genes (*bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *bla*$_{OXA-48}$, *bla*$_{KPC-2}$, and *bla*$_{VIM-1}$) [158–160] and/or other β-lactamase genes (*bla*$_{CTX-M-1}$) [161].

Considering the genetic context, *mcr-1* frequently appears accompanied by *ISApl1* insertion sequence that is flanked upstream the gene, which suggests that this gene was mobilized by an *ISApl1* composite transposon [79]. Originally reported as a plasmid-mediated colistin resistance gene, *mcr* has also integrate into the bacterial chromosome and/or non-conjugative plasmids through vertical transmission to their progenies, and thus stabilizing the genome of clonal lineages [140]. Studies have detected a chromosomally-located *mcr-1* in *E. coli* and revealed a ISApl1-*mcr-1* chromosomal region, indicating that *ISApl1* may be involved in *mcr-1* acquisition [162,163].

Epidemiological data evidenced that *mcr* genes have been reported from six continents in 47 different countries: Asia (Bahrain, China, Cambodia, Hong Kong, Japan, Laos, Malaysia, Oman, Pakistan, Russia, Saudi Arabia, Singapore, South Korea, Taiwan, Thailand, United Arab Emirates and Vietnam), Africa (Algeria, Egypt, Tunisia, Morocco and South Africa), Europe (Austria, Belgium, Denmark, Estonia, France, Germany, Hungary, Italy, Lithuania, Norway, Poland, Portugal, Russia, Spain, Switzerland, Sweden, The Netherlands and UK), Oceania (Australia and New Caledonia), North America (Canada and USA) and South America (Argentina, Brazil, Colombia and Ecuador) (Table 2) [140]. The global average prevalence of *mcr* genes was 4,7% (0.1–9.3%), with the largest number of *mcr*-positive strains found in China. To date, *mcr-1* represents approximately 95% of the *mcr* genes described in literature [79]. Overall, the most frequent carrier of *mcr* genes was the pathogenic *E. coli* (54%) isolated from animals (52%) and harboring an IncI2 plasmid (34%).

Considering the source of recovery, a study revealed that the prevalence of *mcr-1* was higher in environment (22%; 2.8–47.8%), followed by animals (11%; 0.3–22.4%), food (5.4%; 0.6–11.6%), and humans (2.5%; 0.1–5.1%) [164]. Also, this epidemiological study demonstrated that the estimated prevalence of *mcr-1* among *E. coli* was higher in food-animals than in humans and food-products, supporting the theory of foodborne transmission and the importance of One Health in managing this resistance trait [164].

The potential transmission of plasmids carrying *mcr* from isolates of animal origin to humans via food chain was proved in experiments *in vitro* [141]. Indeed, livestock has been considered the major reservoir of *mcr* as a result of selective pressure caused by the long-term use of polymyxins in veterinary medicine for prophylaxis, metaphylaxis, therapeutic purposes and also as a growth promoter [140]. Furthermore, there are particular evidences to support the hypothesis that animals are the original source of *mcr-1* genes, as follow: (i) the large number of reports of *mcr-1* isolated from animals; (ii) the extensive use of polymyxins in veterinary

medicine, which boost the selection of *mcr-1* producing strains; (iii) the genetic finding of *mcr-1* associated with a *ISApl1* originanting from *Pasteurella multocida*, which is a common animal pathogen, particularly in pigs; (iv) the identification of the florfenicol resistance gene (*floR*), an antibiotic used exclusively in veterinary practice; (v) the co-expression of a β-lactamase gene (bla_{CMY-2}) and *mcr-1*, which is known to be widely disseminated in animal isolates. Based on this data, it has been speculated the major role of *mcr*-carrying animal isolates in the mobilization and emergence of *mcr* genes in humans being [140,165,166].

Regardless the genetic mobility of *mcr-1*, polymyxins resistance among isolates recovered from nosocomial settings remains mostly associated with alterations in chromosomal-located genes. Global surveillance studies have demonstrated that the frequency of clinical isolates of *Enterobacterales* carrying *mcr-1*- is less than 2% [167–172]. Nevertheless, the selective pressure exerted by the use of polymyxins in the nosocomial environment can change this scenario, increasing the frequency of isolation of *mcr* in hospital environment, which highlights the importance of systematic surveillance programs.

Table 2. Mechanisms of resistance to polymyxins related to plasmid-located genes

***mcr* gene type**	**Bacterial species**	**Plasmid Type**	**Country**	**Source**	**References**
mcr-1	*A. baumannii*, *Cronobacter sakazakii*, *Citrobacter freundii*, *Citrobacter braakii*, *E. coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *K. pneumoniae*, *Kluyvera ascorbate*, *Moraxella spp.*, *Providencia alcalifaciens*, *P. aeruginosa*, *Raoultella ornithinolytica*, *S. enterica*, *Shigella sonnei and Shigella flexneri*	IncX4, IncX3, IncI, IncI2, IncI1, Inc2, IncHI2/HI2A, IncHI1, IncHI2A, IncN, IncF, IncP, IncP-1, IncQ, IncX, IncY, IncPO111, ColRNAI, IncFIB, IncQ1, IncFI, IncFII	Argentina, Algeria, Brazil, Brunei, Bangladesh, Belgium, Cambodia, China, Canada, Dominican Republic, Denmark, Egypt, Estonia, France, Germany, India, Italy, Japan, Lebanon, Lithuania, Laos, Malaysia, Norway, Pakistan, Portugal, Switzerland, Spain, South Africa, Tunisia, Thailand, The Netherlands, Taiwan, UK, USA and Vietnam	Livestock (pig, chicken, poultry, cattle), human, sewage and meat (pork)	25; 79; 140; 141; 142; 143; 157; 162
mcr-2	*E. coli*, *K. pneumoniae* and *Moraxella pluranimalium*	IncX4	Belgium and Spain	Livestock (pig)	25; 79; 140; 142; 143; 157
mcr-3	*Aeromonas veronii*, *Aeromonas allosaccharophila*, *Aeromonas media*, *Aeromonas jandaei*, *Aeromonas hydrophila*, *Aeromonas caviae*, *E. coli* and *Shigella sonnei*	IncHI2, IncP	Brazil, China, Denmark, France, Germany, Japan, Spain and Thailand	Livestock (pig, cattle, turkey, duck), human, animal (fish) and meat (chicken)	25; 79; 140; 143; 144; 145; 146; 147; 148
mcr-4	*A. baumannii*, *E. coli*, *Enterobacteria cloacae*, *S. enterica*, and *Shewanella frigidimarina*	ColE	Italy and Spain	Livestock (pig) and human	25; 79; 140; 149; 156
mcr-5	*A. hydrophila*, *Cupriavidus gilardii*, *E. coli*, *M. pluranimalium*, *P. aeruginosa* and *S enterica*,	IncX1, ColE	China, Colombia, Germany, Japan, Spain and UK	Livestock (pig, chicken)	25; 79; 140; 149; 155
mcr-6	*M. pleuranimalium*	-	UK	Livestock (pig)	25; 79; 140; 153
mcr-7	*K. pneumoniae*	IncI2	China	Livestock (chicken)	25; 79; 140; 150
mcr-8	*K. pneumoniae*, *Raoultella* spp. and *Stenotrophomonas* spp	IncFII	China	Livestock (pig) and human	25; 79; 140; 151
mcr-9	*C. sakazakii*, *E. coli*, *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Leclercia* spp., *Phytobacter ursingii*, *Raoultella* spp. and *S. Typhimurium*	IncHI2/HI2A, IncFII	USA	Human	25; 79; 140; 152; 157
mcr-10	*Enterobacter roggenkampii*	IncFIA	China	Human	79; 154

4. Conclusions

Resistance to polymyxins is complex, involving several different pathways and may have peculiarities depending on the species. Although most mechanisms are related to alterations in chromosomally-located genes, the discovery of plasmid-located ones increased the epidemiological importance of this resistance trait. The rational use of antibiotics, in human and veterinary practices, is an adequate measure in attempt to control the occurrence and dissemination of this resistance. Moreover, the surveillance of isolates carrying *mcr-1* is also important in order to break the transmission chain of this resistance, from animals/food to humans in light of One Health approach. Indeed, One Health-based strategies are key points in order to minimize polymyxins resistance. As long as new combinations of β-lactams and β-lactamases inhibitors are not widely available worldwide, polymyxins will continue to be a paramount therapeutic option of life-threatening infections by MDR GNB. Hence, it is extremely important to maintaining its effectiveness through the rational use of antibiotics.

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6. Annexes

6.1. ANNEX 1. PUBLICATION STANDARDS FOR THE SELECTED SCIENTIFIC JOURNAL.

Brazilian Journal of MicrobiologySubmission Guidelines

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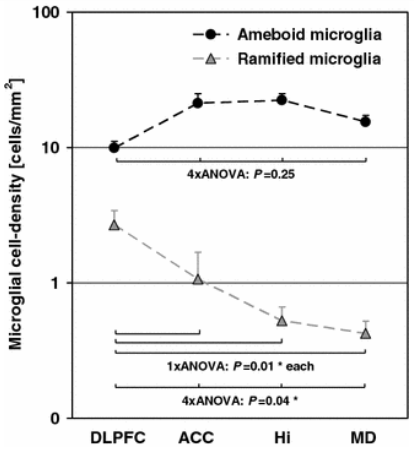

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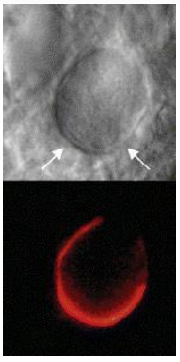

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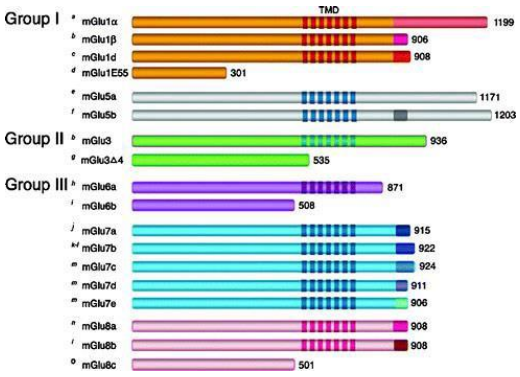

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Exact requirements may vary depending on the journal; please refer to the journal's Instructions for Authors.

Checklists are available for a number of study designs, including:

- Randomised trials (CONSORT) and Study protocols (SPIRIT)
- Observational studies (STROBE)
- Systematic reviews and meta-analyses (PRISMA) and protocols (Prisma-P)
- Diagnostic/prognostic studies (STARD) and (TRIPOD)
- Case reports (CARE)
- Clinical practice guidelines (AGREE) and (RIGHT)
- Qualitative research (SRQR) and (COREQ)
- Animal pre-clinical studies (ARRIVE)
- Quality improvement studies (SQUIRE)
- Economic evaluations (CHEERS)

Summary of requirements

The above should be summarized in a statement and included on a title page that is separate from the manuscript with a section entitled "Declarations" when submitting a paper. Having all statements in one place allows for a consistent and unified review of the information by the Editor-in-Chief and/or peer reviewers and may speed up the handling of the paper. Declarations include Funding, Conflicts of interest/competing interests, Ethics approval, Consent, Data and/or Code availability and Authors' contribution statements. Please use the following template title page for providing the statements.

Once and if the paper is accepted for publication, the production department will put the respective statements in a distinctly identified section clearly visible for readers.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

• Provide “Ethics approval” as a heading (see template)

Examples of ethics approval obtained:

• All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Medical University of A (No....).

• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No....).

• Approval was obtained from the ethics committee of University C. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

• The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of C (Ethics approval number: ...).

Examples of a retrospective study:

• Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

• This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.

• This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

Examples no ethical approval required/exemption granted:

• This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

• The data reproduced from Article X utilized human tissue that was procured via our Biobank AB, which provides de-identified samples. This study was reviewed and deemed exempt by our XYZ Institutional

Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Informed consent

All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. This is especially true concerning images of vulnerable people (e.g. minors, patients, refugees, etc) or the use of images in sensitive contexts. In many instances authors will need to secure written consent before including images.

Identifying details (names, dates of birth, identity numbers, biometrical characteristics (such as facial features, fingerprint, writing style, voice pattern, DNA or other distinguishing characteristic) and other information) of the participants that were studied should not be published in written descriptions, photographs, and genetic profiles unless the information is essential for scholarly purposes and the participant (or parent or guardian if the participant is incapable) gave written informed consent for publication. Complete anonymity is difficult to achieve in some cases. Detailed descriptions of individual participants, whether of their whole bodies or of body sections, may lead to disclosure of their identity. Under certain circumstances consent is not required as long as information is anonymized and the submission does not include images that may identify the person.

Informed consent for publication should be obtained if there is any doubt. For example, masking the eye region in photographs of participants is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic profiles, authors should provide assurance that alterations do not distort scientific meaning.

Exceptions where it is not necessary to obtain consent:

• Images such as x rays, laparoscopic images, ultrasound images, brain scans, pathology slides unless there is a concern about identifying information in which case, authors should ensure that consent is obtained.

• Reuse of images: If images are being reused from prior publications, the Publisher will assume that the prior publication obtained the relevant information regarding consent. Authors should provide the appropriate attribution for republished images.

Consent and already available data and/or biologic material

Regardless of whether material is collected from living or dead patients, they (family or guardian if the deceased has not made a pre-mortem decision) must have given prior written consent. The aspect of confidentiality as well as any wishes from the deceased should be respected.

Data protection, confidentiality and privacy

When biological material is donated for or data is generated as part of a research project authors should ensure, as part of the informed consent procedure, that the participants are made what kind of (personal) data will be processed, how it will be used and for what purpose. In case of data acquired via a biobank/biorepository, it is possible they apply a broad consent which allows research participants to consent to a broad range of uses of their data and samples which is regarded by research ethics committees as specific enough to be considered "informed". However, authors should always check the specific biobank/biorepository policies or any other type of data provider policies (in case of non-bio research) to be sure that this is the case.

Consent to Participate

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript. In the case of articles describing human transplantation studies, authors must include a statement declaring that no organs/tissues were obtained from prisoners and must also name the institution(s)/clinic(s)/department(s) via which organs/tissues were obtained. For manuscripts reporting studies involving vulnerable groups where there is the potential for coercion or where consent may not have been fully informed, extra care will be taken by the editor and may be referred to the Springer Nature Research Integrity Group.

Consent to Publish

Individuals may consent to participate in a study, but object to having their data published in a journal article. Authors should make sure to also seek consent from individuals to publish their data prior to submitting their paper to a journal. This is in particular applicable to case studies. A consent to publish form can be found here. (Download docx, 36 kB)

Summary of requirements

The above should be summarized in a statement and included on a title page that is separate from the manuscript with a section entitled "Declarations" when submitting a paper. Having all statements in one place allows for a consistent and unified review of the information by the Editor-in-Chief and/or peer reviewers and may speed up the handling of the paper. Declarations include Funding, Conflicts of interest/competing interests, Ethics approval, Consent, Data and/or Code availability and Authors' contribution statements. Please use the template Title Page for providing the statements.

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Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Provide “Consent to participate” as a heading

Sample statements consent to participate:

- Informed consent was obtained from all individual participants included in the study.
- Informed consent was obtained from legal guardians.
- Written informed consent was obtained from the parents.
- Verbal informed consent was obtained prior tothe interview.
- The patient has consented to the submission of the case report for submission to the journal.

Provide “Consent to publish” as a heading

- The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.
- The participant has consented to the submission of the case report to the journal.
- Patients signed informed consent regarding publishing their data and photographs.
- Sample statements if identifying information about participants is available in the article:
- Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.
- Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.
- If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.
- Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

- Images will be removed from publication if authors have not obtained informed consent or the paper may be removed and replaced with a notice explaining the reason for removal.

Research involving animals

Experimental research on vertebrates or any regulated invertebrates must comply with institutional, national, or international guidelines, and where available should have been approved by an appropriate ethics committee. The Basel Declaration outlines fundamental principles to adhere to when conducting research in animals and the International Council for Laboratory Animal Science (ICLAS) has also published ethical guidelines.

A statement detailing compliance with relevant guidelines (e.g. Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU in Europe) and/or ethical approval (including the name of the ethics committee and the reference number where appropriate) must be included in the manuscript.

For experimental studies involving client-owned animals, authors must also document informed consent from the client or owner and adherence to a high standard (best practice) of veterinary care.

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Utilization of plants, algae, fungi

This journal values stewardship, transparency, and adhering to governance with regards to collecting and utilizing specimens and conducting experiments and/or field studies. Therefor the journal sets out the following guidelines:

Field studies involving genetically engineered plants must be conducted in accordance with national or local legislation and, if applicable, the manuscript needs to include a statement specifying the appropriate permissions and/or licences.

Authors utilizing genetic plant resources received via local suppliers/collectors, such as species collected from protected areas or endangered species with medical importance, must conduct their experiments following the Nagoya Protocol (as part of the Convention on Biological Diversity).

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- The International Union for Conservation of Nature (IUCN) Policy Statement on Research Involving Species at Risk of Extinction and consult the IUCN red list index of threatened species
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* Based on/adapted from:

ICMJE, Defining the Role of Authors and Contributors,

Transparency in authors' contributions and responsibilities to promote integrity in scientific publication, McNutt at all, PNAS February 27, 2018

Disclosures and declarations

All authors are requested to include information regarding sources of funding, financial or non-financial interests, study-specific approval by the appropriate ethics committee for research involving humans and/or animals, informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals (as appropriate).

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All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

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