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Review Article

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Comprehensive insights into colistin and colistin resistance: updated regulations and policies on colistin usage and alternative strategies for mitigation and prevention of colistin resistance

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Abstract

The increase in antimicrobial resistance (AMR) has a significant impact on healthcare, leading to longer hospital stays and higher economic costs. This is primarily caused by the over and inappropriate use of antibiotics. Microorganisms have developed various mechanisms to combat the effects of antibiotics, including modifying their genetic material to evade the drugs' harmful effects. Colistin disrupts bacterial cell membranes by interacting with lipopolysaccharides (LPS), causing cell death. Currently, colistin is regarded as a last-resort antibiotic for treating infections caused by Gram-negative bacteria (GNB). Overuse of colistin in animal feed and human medicine has led to the appearance of genetic mutations and the acquisition of mobile colistin resistance (*mcr*) genes associated with colistin resistance. The rapid emergence of the plasmid colistin-resistant gene further complicates treatment strategies, emphasizing the pressing need for improved management and development of novel antimicrobial agents. Thus, gaining an understanding of the mechanisms behind colistin resistance and surveying its global prevalence are crucial steps in managing the growing menace of antimicrobial resistance and safeguarding the effectiveness of existing antibiotics. This review aimed to provide an in-depth and up-to-date overview of the colistin antibiotic, detailing its updated modes of action and associated side effects. Additionally, it delves into various mechanisms of resistance to colistin, advanced susceptibility test methods used for detecting colistin resistance, and the challenges associated with these techniques. Furthermore, the review examines the risk factors contributing to the development of colistin resistance,

highlights updated regulations and policies governing colistin usage, and puts forth innovative strategies for addressing this resistance.

Keywords: Colisitn, Colistin resistance, *mcr* gene, Lipopolysaccharides, Gram-negative bacteria

1. Introduction

Emergence of antimicrobial resistance significantly impacts healthcare due to prolonged hospitalizations and heightened economic costs. This growing concern stems from the overuse and inappropriate use of antibiotics (Ahmed *et al*., 2024). Consequently, microorganisms employ diverse evolutionary mechanisms to effectively counteract the impact of antibiotics. In the presence of antibacterial agents, bacteria can modify their genetic materials with the objective of evading the deleterious effects of antibiotics (Ahmed *et al*., 2024). Colistin, an old non-ribosomal polypeptide antibiotic belonging to the polymyxin group; specifically polymyxin E, is a positively charged compound synthesized by the soil bacterium *Paenibacillus polymyxa* (Diani *et al*., 2024). When it interacts with the negatively charged LPS of the bacterial cell membrane, it disrupts the membrane, leading to the formation of pores in the bacterial membrane, causing the release of intracellular contents and ultimately resulting in cell death (Yang *et al*., 2024). Given its neurotoxicity and nephrotoxicity, colistin was discarded from clinical practice in the 1980s, in preference for novel antibiotics such as third-generation cephalosporins and large-spectrum β-lactams (Yaneja and Kaur, 2016). However, the escalating resistance stemming from carbapenem-resistant Gram-negative bacteria (GNB) has necessitated reintroduction of colistin as a last-line treatment option (Papazachariou *et al*., 2024). Subsequently, extensive use of colistin in both animal feed and human medicine has fostered the development of genetic mutations associated with its resistance. These mutations are manifested in chromosomal genes and acquisition of mobile plasmid genes. The most thoroughly studied of these genes are the plasmid-borne mobile colistin resistance (*mcr*) genes, which encode for phosphoethanolamine transferase enzymes responsible for adding phosphoethanolamine (PEtN) to lipid A of LPS. Notably, the transferability of these plasmids from one bacterium to another; known as horizontal transfer, increases their potential for resistance emergence compared to chromosomal genes (Mondal *et al*., 2024). Additionally, several chromosomal genes such as *PmrA*/*PmrB*, *PhoP*/*PhoQ*, and *mgrB* are also under investigation (Attalla *et al*., 2023). Conventional methods of antibiotic susceptibility testing such as agar diffusion, presents significant challenges for detection of colistin resistance (Satlin *et al*., 2020). Consequently, several novel rapid/specific methods and recommendations are under development to improve the detection of colistin resistance for accurate clinical diagnostics (Lescat *et al*., 2019). Furthermore, updated regulations and policies governing the use of colistin in both human and veterinary medicine have been instituted, restricting its utilization to critical situations where other alternatives have proven ineffective (EMA. 2019). Additionally, diverse strategies are being devised to counteract colistin resistance and curtail its swift emergence (Ebrahimi *et al*., 2021).

The objective of this review is to provide a comprehensive and contemporaneous assessment of the antibiotic colistin, elucidating its updated modes of action and associated adverse effects. Furthermore, it delves into various mechanisms of resistance to colistin, advanced methods for susceptibility testing to detect colistin resistance, and challenges inherent in these techniques. Moreover, the review scrutinizes the risk factors that contribute to the emergence of colistin

resistance, highlights contemporary regulations and policies governing colistin usage, and proposes innovative strategies for mitigating this resistance.

2. Proper probiotics and microbiota

Polymyxins are a group of fermentation products derived from a soil bacteria known as *Bacillus polymyxa*. Discovered in the 1940s, this group includes five distinct chemical compounds; mainly polymyxins A, B, C, D, and E (colistin) (Slingerland and Martin, 2024). Only polymyxin B and E are approved for clinical use due to their lower adverse effects. Colistin, authorized by the U.S. Food and Drug Administration in 1959, is available for treating infections caused by GNB resistant to most antibacterial classes (Ledger *et al*., 2022). Colistin is available in two forms: a prodrug that is colistin methanesulfonate sodium used for parenteral and aerosol therapy, and colistin sulfate for oral and topical uses (Yahav *et al*., 2012). Polymyxin B and colistin are two types of amphipathic polypeptide antibiotics. They are characterized by a highly basic nature with five free amino groups that are notably large; with colistin having a molecular weight of 1750 Da. Both molecules carry a positive charge due to the existence of five L-α, γ-diaminobutyric acid (Dab) residues (Su *et al*., 2019). While the specific composition of each polymyxin type may vary, their basic amphipathic structure remains largely consistent. All polymyxin members have a cyclic heptapeptide core linked to a linear tripeptide along with an N-terminal fatty acyl moiety. The latter may vary based on the length of the fatty acid chain, which ranges from seven to nine carbon atoms and can undergo methylation, hydroxylation, and/ or sulfonation. Polymyxin B and E can be differentiated by a single amino acid with a D-Phe/D-Leu switch at position 6 (Slingerland and Martin, 2024). Colistin exists in two forms, which have variations in the fatty acid group: 6-methyl-octanoic acid (polymyxin E1, also known as colistin A) and 6-methyl-heptanoic acid (polymyxin E2, also known as colistin B). Commercial colistin formulations usually consist of a mix of both colistin A and B (Dubashynskaya and Skorik, 2020).

3. Colistin activity spectrum

Colistin exhibits a narrow antibacterial spectrum, predominantly targeting the common species of *Enterobacteriaceae* such as *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Salmonella* spp., and *Shigella* spp. It is also active against the none-fermentative GNB such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter* species (Gogry *et al*., 2021). However, it is proved to be ineffective against GNB, acid-fast bacteria, Gram-negative cocci, and anaerobes. Some specific Gram-negative aerobic bacilli, including *Proteus* spp., *Brucella* spp., *Hafnia* spp., *Edwardsiella tarda*, and *Morganella morganii*, intrinsically resist colistin (Gogry *et al*., 2021).

4. Modes of action of colistin

Efficacy of colistin as an antibacterial agent depends on the presence of LPS components in the GNB cell membrane. More specifically, colistin's mode of action primarily involves interaction with the hydrophobic lipid A component of LPS (Fig. 1). However there exist various pathways by which colistin affects bacteria:

(i) Classical membrane lysis pathway: Colistin's antibacterial activity occurs through a two-step mechanism with a detergent-like effect. The first step involves initial binding of the cationic ring of colistin to the negatively charged cell envelope; displacing calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions, which are bound to the anionic phosphate groups of the lipid A portion of LPS through an electrostatic repulsion. Following binding to lipid A, colistin molecules are inserted into the outer leaflet of the outer membrane owing to its amphipathic nature. This initial insertion causes destabilization of membrane integrity, resulting in its increased permeabilization "self-promoted uptake" but not cell death. Only when colistin gets through the periplasmic space and binds to LPS located in outer leaflet of the inner membrane, it leads to bacterial cell death (Fig. 1) (Diani *et al*., 2024).

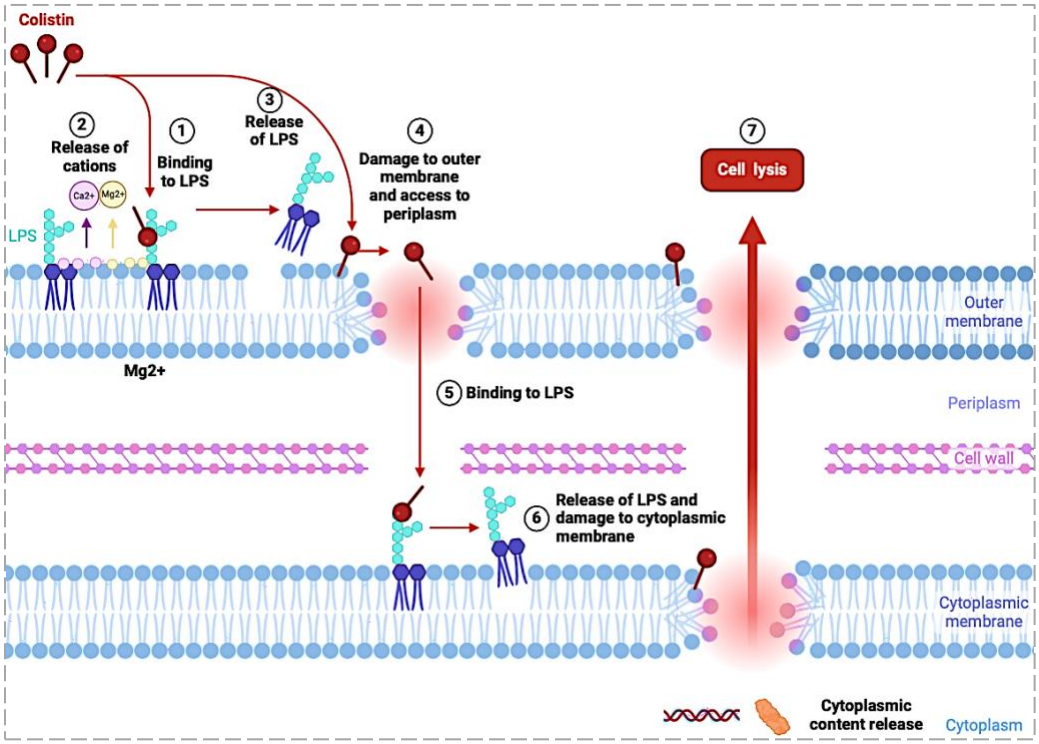

Fig. 1. Schematic representation of the classical membrane lysis pathway of colistin against GNB, adopted by Diani *et al.*, (2024)

(ii) Vesicle–vesicle contact pathway: Interaction of colistin with anionic phospholipid vesicles of the inner and outer bacterial membranes results in fusion of both membranes, leading to loss of specificity in phospholipids composition. Consequently, the bacterial cell experiences loss of osmotic balance, which leads to cell lysis and eventual death (Fig. 1) (Moubareck, 2020).

(iii) Respiratory enzyme inhibition pathway: Recently, it has been discovered that colistin may affect bacterial cells by inhibiting the activity of a respiratory enzyme called NADH-quinon oxydoréductase (NDH-2) located in the inner bacterial membrane, thus enhancing its bactericidal impact (Panta and Doerrler, 2021).

(iv) Hydroxyl radical death pathway: Colistin is postulated to generate reactive oxygen species (ROS) specifically superoxide (O^{2-}), peroxide (H_2O_2), and hydroxyl radicals (OH^*) within GNB. Upon permeating the bacterial cell, colistin instigates the production of O^{2-}, which subsequently undergoes conversion to H_2O_2 facilitated by the superoxide dismutase enzyme. The presence of H_2O_2 prompts the conversion of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), initiating the Fenton reaction and engendering the production of OH^*. The latter is capable of instigating oxidative damages to cell membrane lipids, protein degradation, and DNA fragmentation, ultimately leading to cell death (Mondal *et al*., 2024).

(v) Anti-endotoxin activity of colistin: Unlike most of the other antibiotics, colistin displays anti-endotoxin activity; as its binding to lipid A prevents the liberation of LPS (GNB endotoxin), preventing the occurrence of endotoxin chocks during bacterial lysis (Mondal *et al*., 2024).

5. Adverse effects of colistin

Colistin treatment was largely abandoned in clinical practices, principally owing to its significant kidney toxicity. Nephrotoxic adverse effects of colistin are

largely attributed to reabsorption of colistin by proximal tubule cells through an endocytotic process. Intracellular colistin accumulation precipitates mitochondrial and endoplasmic reticulum stresses, culminating in adverse cellular effects, cellular lysis, and acute tubular necrosis (Gai *et al*., 2019). The nephrotoxic potential of colistin is directly proportional to its treatment duration, which is more pronounced in older patients. Acute kidney injury (AKI) resulting from colistin treatment is typically manifested approximately 5 to 7 d post-exposure (Campbell *et al*., 2023). The predominant etiology of AKI attributed to colistin appears to be acute tubular necrosis. Risk factors for AKI encompass elevated daily colistin doses (>5 mg/ kg), older age, severe underlying illness, comorbidities such as chronic kidney and/ or liver disease, and concurrent usage of nephrotoxic agents (Campbell *et al*., 2023).

Unlike general antibiotics nephrotoxicity, the neurotoxicity of colistin is much less documented. Discontinuation of colistin treatment typically leads to restoring normal neuronal cell functions following association with nephrotoxic events. However, neurotoxicity is commonly associated with heightened drug exposure, including prolonged treatment duration and/ or increased dosage (Lírio *et al*., 2018). Neuronal cells, characterized by high lipid contents, are notably vulnerable to colistin's potential influence, resulting in neurological manifestations, including ataxia, confusion, paresthesia, seizures, visual disturbances, peripheral and orofacial paresthesias, vertigo, and mental confusion. Of particular concern is the potential development of neuromuscular blockade, which is presented as a syndrome akin to myasthenia or paralysis of respiratory muscles, culminating in apnea (Spapen *et al*., 2011). A previous study conducted by Lírio *et al*., (2018) examining the mechanism of colistin-induced neurotoxicity revealed axonal degeneration and demyelization in mice following intravenous administration of 15 mg/ kg/ d colistin for 7 d.

The concern over nephrotoxicity and neurotoxicity led to dismissal of colistin from clinical use in 1980s (Binsker *et al*., 2022). Paradoxically, recent clinical studies have revealed that patients treated with colistin have a reduced risk of kidney damage compared to what was previously thought. This contrast may be attributed to lack of standardized criteria used for defining renal functions impairment, increased utilization of higher purity, improved drug preparations such as using colistimethate instead of colistin sulphate, and efficient dosage adjustments based on renal functions and enhanced intensive care unit monitoring (Gai *et al*., 2019). Additionally, the varied dosing regimens employed globally; often stemming from historical empirical approaches without accurate pharmacokinetic and pharmacodynamic (PK/PD) data, further complicate the matter. Recent clinical investigations demonstrated that most of the currently recommended colistin regimens are sub-optimal and much higher doses should be administered to maximize antibiotic activity and reduce development of resistance (Gai *et al*., 2019). Similar to nephrotoxicity, recent retrospective studies on the neurotoxicity of colistin have not conclusively established a definitive link between colistin and neurotoxic events in those patients subjected to colistin treatment. Furthermore, diagnosis of neurotoxicity predominantly relies on clinical observations, thus posing challenges in discerning of potential colistin-induced neurotoxicity from the more frequently observed "critical illness polymyoneuropathy" in the intensive care unit patients (Honore *et al*., 2013).

6. Mechanisms of resistance to colistin

Rise of colistin antibiotic as a last-defense line has sparked extensive researches into the mechanisms behind bacterial resistance to such treatment. Key findings have suggested that bacteria can develop resistance to colistin by (i) interfering with the electrostatic interaction between colistin and lipid A and/ or (ii) obstructing the insertion of its hydrophobic domains into the inner and outer bacterial membranes. However, it is noteworthy that these two forms of resistance can be manifested in various ways, including:

6.1. Intrinsic resistance to colistin

Bacillus polymyxa subspecies *colistinus*, the species that produces polymyxins, is naturally resistant to colistin due to the production of an enzyme that hydrolyzes colistin, colistinase. No similar enzymatic resistance mechanism has been described in other bacterial species (Aghapour *et al*., 2019). An additional type of natural resistance to colistin occurs in many GNB spp. such as *Proteus mirabilis* (*P. mirabilis*), *S. marcesens*, and *M. morganii*, among others, through LPS modification involving cation substitution. This alteration is enabled by *arnBCADTEF* operon and *eptB* gene expression. As a result, cationic entities such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) are incorporated into the LPS colistin's target. Additionally, involvement of *EptC* gene has been identified in modification of *P. mirabilis* LPS mediated by addition of PETN to LPS.

These modifications increase the overall positive charge of LPS, thereby diminishing binding affinity of colistin and establishing intrinsic resistance in these bacteria (Aghapour *et al*., 2019).

6.2. Acquired resistance to colistin: chromosomal-mediated resistance

Emergence of resistance mechanisms to colistin is thought to be associated with chromosomal mutations that are non-transferrable through a horizontal gene transfer. Multiple genes and operons are involved in LPS modification, resulting in colistin resistance. Synthesis of *PEtN* and *L-Ara4N* is mainly under the regulation of the two-component systems *PmrAB*, *PhoPQ*, and *CrrAB* among others (Fig. 2) (Aghapour *et al*., 2019).

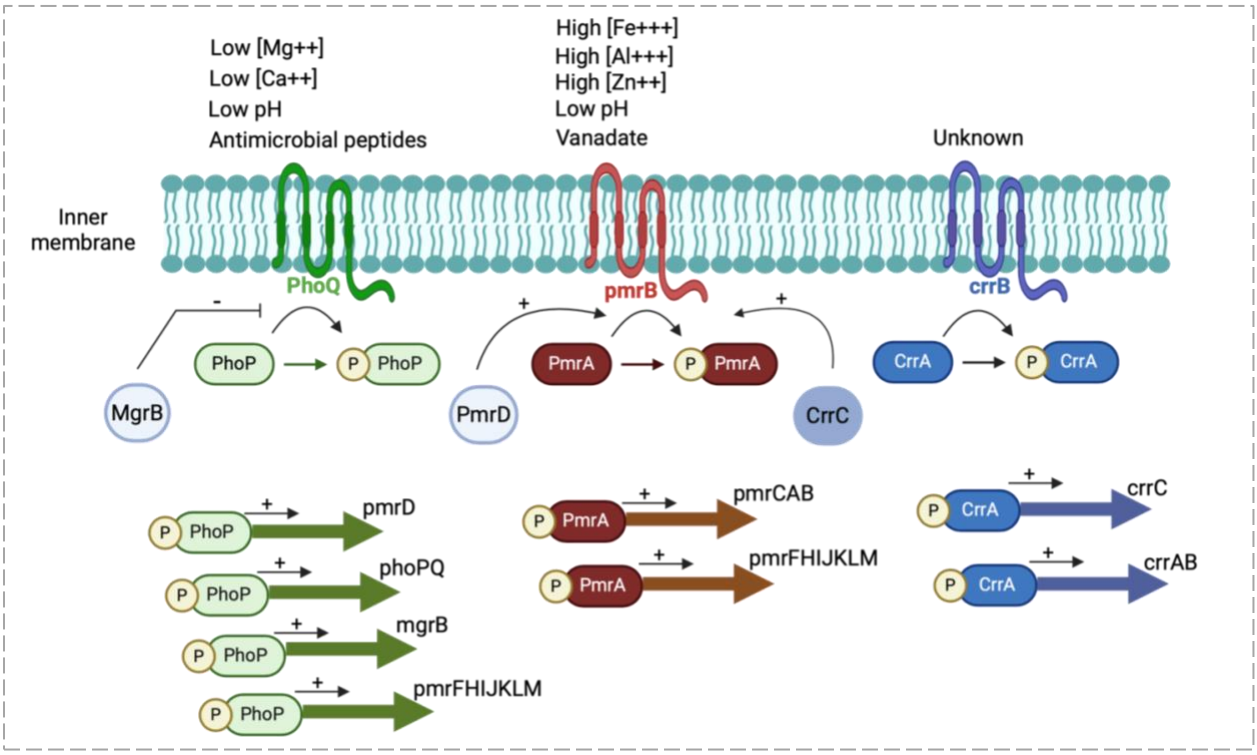

Fig. 2. Chromosomal mutations mediated colistin resistance in two-component systems, *CrrA/B*, *PhoP/PhoQ*, and *PmrA/PmrB*, resulting in modification of LPS. The inactivation or deletion of *mgrB* gene results in overexpression of *PhoP/PhoQ*, ultimately leading to LPS modification, adopted by Aghapour *et al*., (2019)

(i) *PmrABC* operon: consists of three key components; mainly *PmrA*, a regulatory protein; *PmrB*, a sensor kinase located in the cytoplasmic membrane; and *PmrC*, a putative membrane protein. Under specific conditions such as those within macrophage phagosomes, elevated concentrations of iron (Fe^{3+}); aluminum (Al^{3+}), and Zinc (Zn^{2+}), acidic pH, and certain specific chromosomal mutations in the genes encoding for *PmrAB TCS*, trigger the activation of *PmrA*. Subsequently, *PmrA* initiates the regulation of *pmrABC*, *pmrHFIJKLM* operons, and *pmrE* gene. Ultimately, this cascade results in modification of LPS through the addition of *pEtN* and *L-Ara4N* to lipid A (Fig. 2) (Wang *et al*., 2024).

(ii) PhoPQ two-component system (TCS): consists of several genes encoding for *PhoP* as a regulatory protein and *PhoQ* as a sensor kinase (Fig. 2). When exposed to different stimuli such as low Mg^{2+} or Ca^{2+}, acidic pH, and/ or antimicrobial peptides, the *PhoPQ* TCS becomes activated. This activation leads to modifications in lipid A, as *PhoQ* phosphorylates *PhoP*, subsequently activating the transcription of the *pmrFHIJKLM* operon. Furthermore, *PhoP* indirectly activates *pmrA* through *PmrD* connector protein and triggers the transcription of *pmrHFIJKLM* operon, leading to *pEtN* synthesis and its addition to lipid A (Fig. 2). *mgrB* gene encodes for a diminutive transmembrane protein, which functions by negatively regulating *PhoPQ TCS* by inhibiting phosphorylation of *PhoP* by *PhoQ* through repression of *PhoQ* gene expression. Mutations or inactivation of *mgrB* gene led to upregulation of *phoPQ* operon, subsequently activating *pmrHFIJKLM* operon. Finally, this activation results in synthesis of *L-Ara4N*, leading to modification of lipid A and conferring colistin resistance (Fig. 2) (Wang *et al*., 2024).

(iii) CrrAB TCS encodes CrrA: a regulatory protein and *CrrB*; a sensor kinase protein (Fig. 2). A mutation in *CrrB* gene causes mutation in *CrrB* protein, which in turn regulates a *CrrAB*-adjacent gene that encodes for a glycosyltransferase-like protein, leading to lipid A modification and subsequent colistin resistance. Interestingly, it has been shown that specific mutations in *CrrB* gene may lead to activation of *pmrHFIJKLM* operon and *pmrC* and *pmrE* genes via overexpression of *pmrAB* operon. As a result, this causes modification of lipid A through addition of *L-Ara4N* and *pEtN* moieties, leading colistin resistance (Gogry *et al*., 2021).

6.3. Acquired resistance to colistin: plasmid-mediated resistance

Historically, it was commonly held that chromosomal mutations were the sole factors contributing to the development of colistin resistance, as there were no known plasmid-borne mechanisms capable of horizontally transferring resistance genes. However, in 2015, a novel gene named mobile colistin resistance (*mcr-1*) was discovered in China, which is carried on plasmids and encodes for colistin resistance. Importantly, the plasmid harboring this gene displayed remarkable stability, surviving even in the absence of selection pressure by colistin (Liu *et al*., 2016). The *mcr-1* gene is typically mobilized by a transposon known as *Tn6330.2*, which comprises the *mcr-1* gene flanked by two *ISApl1* insertion sequences. This transposon exhibits a preference for targeting AT-rich sequences. *mcr-1* gene can be carried by diverse array of plasmids, including *IncX4*, *IncI2*, *IncHI2*, *IncF*, *IncY*, and *IncP*. Specifically, initial identification of *mcr-1* gene has occurred within *IncI2* plasmid known as *pHNSHP45* (Liu *et al*., 2021). This particular finding has unveiled a novel mode of resistance transfer for colistin, highlighting the potential significant involvement of plasmids in the dissemination of such resistance. Since then, *mcr-1* gene has been detected in various GNB species, including *K. pneumoniae*, *E. coli*, *Acinetobacter* sp., *P. aeruginosa*, and *Enterobacter* spp. (Ferjani *et al*., 2022). At present, as many as ten *mcr* genes, designated as *mcr-1* to *mcr-10*, have been identified, with each presenting different variants. *mcr-1* remains by far the most commonly reported plasmid gene

worldwide (Gong *et al*., 2024). This *mcr-1* gene encodes for a *PEtN* transferase enzyme, sharing structural properties with *LptA* and *EptC PEtN* transferases found in *Neisseria meningitidis* and *Campylobacter jejuni*, respectively. These enzymes catalyze modification of lipid A within LPS by incorporating *PEtN*, thereby conferring resistance to colistin (Mmatli *et al*., 2022).

6.4. Other types of resistance to colistin

(i) Lack of LPS biosynthesis: Research findings have indicated that in *A. baumannii*, resistance to colistin can arise from complete absence of LPS biosynthesis. This absence results from spontaneous mutations causing nucleotide substitutions, deletions, and insertions in *lpxA*, *C*, *D*, and *lpsB* genes, which encode for enzymes involved in lipid A biosynthesis. Consequently, loss of LPS has rendered colistin ineffective in targeting its intended site (*i.e*. LPS), leading to colistin resistance. Despite being an effective colistin resistance mechanism, lack of LPS carries significant fitness costs, explaining rare occurrence of these mutants in clinical settings (Diani *et al*., 2024).

(ii) Role of capsules: A capsule serves a crucial function in protecting bacteria against antibacterial agents by acting as a physical barrier that impedes direct contact between drugs and bacterial targets. The negative charge of capsule facilitates electrostatic interaction with the positive charge of antimicrobial peptides such as colistin, thereby preventing colistin from reaching its target (*i.e*., LPS) (El-Sayed Ahmed *et al*., 2020). Interestingly, *K. pneumoniae*, which possesses multiple layers of a capsule, exhibits greater resistance to colistin compared to single-layer isolates. Various genes responsible for regulating capsule formation, including conjugative pilus expression (*Cpx*) and regulator of capsule synthesis (*Rcs*) have been implicated in conferring colistin resistance. For instance, *Cpx* gene activates the efflux pump *KpnEF*, while *Rcs* gene regulates the *PhoP/PhoQ TCS* (Gogry *et al*., 2021). In some cases, *K. pneumoniae* and *P. aeruginosa* adopt a clever strategy to escape the action of colistin. Due to the anionic nature of bacterial capsules, bacteria release the capsule structures, causing capture of the cationic antibacterial peptides, a phenomenon known as trapping. Consequently, there is a drastic reduction in quantities of antibacterial peptides that reach the bacterial surface, thus preventing their interaction with the bacterial surface LPS. This ultimately causes a decreased susceptibility to colistin (Llobet *et al*., 2008).

(iii) Overexpression of efflux pumps: Efflux pumps are transmembrane proteins found in both eukaryotic and prokaryotic cells. They play a crucial role in cellular detoxification by expelling harmful substances such as antibiotics, heavy metals, and drugs outside of the cell. These pumps are powered by energy (*i.e*., energy-dependent) in the form of ATP or an electrochemical gradient. In the context of colistin resistance, several pumps including *AcrAB-TolC*, *KpnEF*, *MexXY/OprM*, *RosAB*, and *RND*-type efflux systems are implicated. For instance, *AcrAB-TolC* pump is assumed to confer colistin resistance in *K. pneumoniae* isolate from a neonatal unit. Ongoing researches are focused on understanding the involvement of other efflux systems, including *MexXY/OprM*, *KpnEF*, *RosAB*, and *RND-type* in conferring colistin resistance (Ding *et al*., 2023).

(iv) Role of porins: Porins are integral membrane proteins that create channels in the GNB outer membrane. As their name suggests, these proteins are responsible for diffusion of small hydrophilic molecules across the cell membrane. Within *S. enterica*, specific periplasmic proteins such as *YdeI* and *YgiW* could interact with *OmpD* and *OmpF* porins, causing an increase in bacterial resistance to antibacterial peptides, including colistin (Pilonieta *et al*., 2009).

(v) Role of biofilm-formation: Biofilm-forming bacteria are the subject of extensive researches due to their significant multidrug-resistant (MDR) profiles against various antibacterial treatments. A previous study revealed that colistin can promote resistance by enhancing biofilm formation in *E. coli* through

increased expression of *phoQ* (Park *et al*., 2021). Another recent study demonstrated that mutations in *mgrB* gene may result in impairment of *phoP/Q TCS*, consequently contributing to colistin-induced resistance through collective expression of several genes involved in biofilm formation and quorum-sensing (García-Romero *et al*., 2024).

7. Detection of susceptibility to colistin and encountered challenges

In accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratories Standards Institute (CLSI) guidelines, the disc diffusion method is deemed unsuitable for evaluating colistin susceptibility. This limitation has risen from the poor diffusion of colistin in agar, causing inadequate correlation between diameter of the inhibition zone and minimum inhibitory concentrations (MICs). The large molecular weight and cationic nature of colistin results in a disproportionately small inhibition zone, which may not accurately reflect the true impact of colistin, thus yielding false results (Satlin *et al*., 2020). Accordingly, both EUCAST. (2020); CLSI. (2020) have embraced the Broth Micro-dilution assay (BMD) outlined in ISO 20776-1 as the sole acceptable method of assessing colistin susceptibility. Additionally, determination of MIC requires a pure culture, and it is imperative to note that samples directly obtained from the environment or clinical settings are unsuitable for this purpose (CLSI. 2020). Furthermore, it is recommended to conduct this assessment assay using non-polystyrene plates and substitute the commonly utilized colistimethate version of colistin. Moreover, caution is advised regarding the use of polysorbate 80 (Mondal *et al*., 2024).

8. Novel rapid/specific methods used to detect resistance/susceptibility to colistin

Challenges related to the diffusion of colistin in agar, the extended time required to achieve colistin susceptibility results using broth micro-dilution, which usually takes 18 to 24 h, and the inadequate detection rate of *mcr-1* positive isolates at a threshold of ≤ 2 µg/ml have led the researchers to consider the development of rapid testing methods. The principal aim is to optimize time efficiency and enhance efficacy of the extant testing methodologies.

• Rapid Polymyxin NP test in *Enterobacteriaceae*:

This test focuses on ascertaining the presence of bacterial resistance to colistin in a growth medium by observing the microorganism's capability to metabolize glucose in the presence of a pH indicator (phenol red). In the event of resistance, bacteria have displayed growth in the presence of colistin at a concentration of 3.75 ug/ ml, leading to glucose metabolization and subsequent release of acidic metabolites, resulting in a color change in the medium from orange to yellow due to the indicator's pH sensitivity (Fig. 3). Conversely, sensitive bacteria exhibited inhibited growth in the presence of colistin, thereby retaining the orange coloration of the medium. This assay has expressed a remarkable correlation with MIC values attained through broth micro-dilution (BMD), boasting a sensitivity and specificity of 99.3 % and 95.4 %; respectively, in detecting resistant bacterial strains. In summary, this test presents a user-friendly, cost-effective, and rapid diagnostic solution (< 2h). Moreover, it enables direct detection of colistin polymyxin-resistant isolates from infected samples or selective media, prior to conducting any antimicrobial susceptibility testing. However, this assay exclusively provides qualitative determinations of resistance without affording insights into the underlying mechanism of action. Consequently, its application is fitting for diagnostic laboratories, albeit unsuitable for research settings primarily concerned with elucidating the mechanism of action (Nordmann *et al*., 2016b).

• Rapid Resa Polymyxin *Acinetobacter*/ *Pseudomonas* NP test:

The test is designed to identify *Acinetobacter* and *Pseudomonas* strains that remain viable after incubation in a medium containing a defined concentration of colistin.

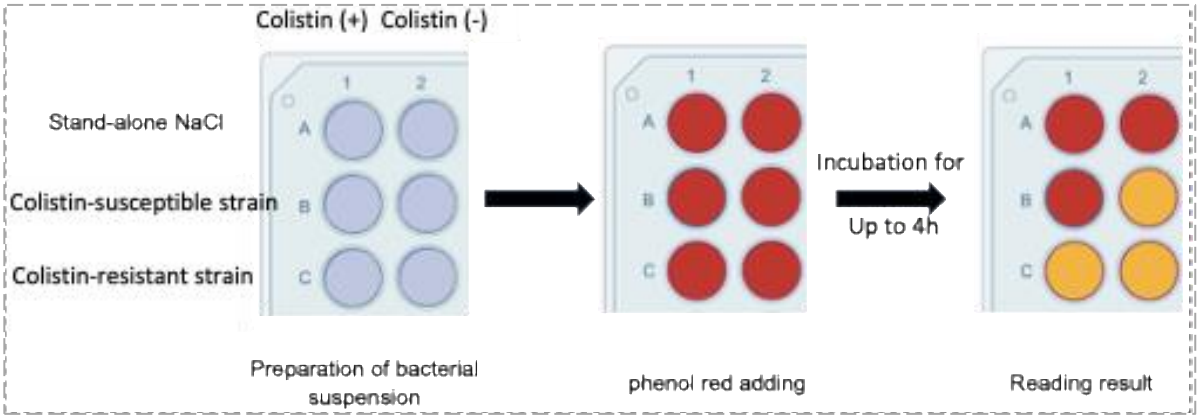

Fig. 3. Schematic representation of the principles of Rapid Polymyxin NP test used for rapid detection of colistin resistance, adopted by Nordmann *et al*., (2016b). Where; (+) indicates presence of colistin while (-) indicates absence of colistin in the bacterial suspension. Red color observed in A1, A2, and B1 wells indicated no bacterial growth while yellow color in C1, B2, and C2 wells indicated bacterial growth

The assessment is based on visual detection of resazurin, transitioning from a blue (no bacterial growth) to purple or pink color (bacterial growth) (Fig. 4) (Lescat *et al*., 2019). Comparative analysis with the standard BMD revealed a sensitivity of 100 % and a specificity of 95 %. Noteworthy, the attributes of this test include its rapid completion within less than 3-4 h, cost-effectiveness, and suitability for implementation across various clinical laboratories. This test serves as a comprehensive component of the Rapid Polymyxin NP assay (Lescat *et al*., 2019).

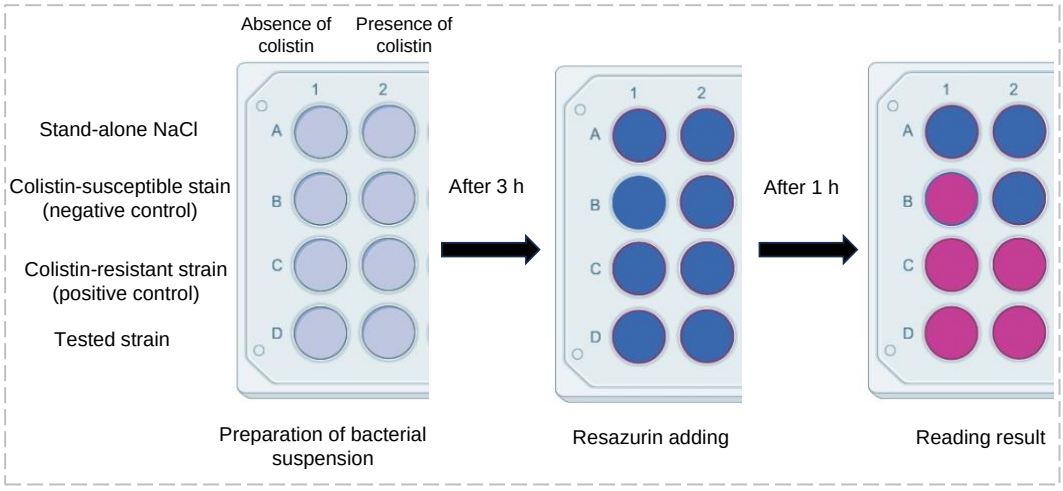

Fig. 4. Schematic representation of the principle of Rapid Resa Polymyxin *Acinetobacter*/ *Pseudomonas* NP test used for detection of colistin resistance, adopted by Lescat *et al*., (2019). Where; blue color in A1, A2, A2, and B2 wells indicated no bacterial growth, purple/ pink color in B1, C1, D1, C2, and D2 wells indicated bacterial growth

• **Super Polymyxin agar for screening of colistin-resistant GNB**: This agar medium is employed for isolation of colistin-resistant GNB from clinical specimens such as rectal swabs and stool samples. It contains colistin (1 to 5 mg/ l), antifungals such as amphotericin B (5 µg/ ml), and antibiotics like daptomycin (10 µg/ ml). Interestingly, the medium has exhibited 100 % sensitivity and specificity in identifying colistin-resistant strains; irrespective of the resistance mechanism (*i.e*., plasmid or chromosome), and facilitates direct detection of such strains from stool samples (Nordmann *et al*., 2016b). However, it is essential to acknowledge the potential impact of employing multiple antimicrobials, including daptomycin, colistin, and amphotericin B, which may introduce result distortion in cases of synergistic interactions among these agents. This is evidenced by the documented synergy that existed between colistin and vancomycin in combating *A. baumannii* (Nordmann *et al*., 2016b).

• **Phenotypic detection of plasmid-mediated colistin resistance in *Enterobacteriaceae* using EDTA based test:** The test is based on the concept of using ethylenediaminetetraacetic acid (EDTA) supplemented in the growth medium (1 mM EDTA), which serves to hinder the activity of lipid A phosphoethanolamine transferase enzyme (arising from the expression of *mcr-1* gene). This has occurred by chelating zinc ions, vital for activity of the latter enzyme, subsequent to a 24-h incubation period at 35 ˚C. Combination of EDTA and colistin yields an expanded zone of inhibition, denoting resistance to colistin that is mediated by *mcr* gene (*i.e*., plasmid gene). Conversely, in cases where the bacterial isolate exhibits susceptibility to colistin or resistance attributable to chromosomal mutations, co-application of EDTA and colistin does not engender a notable augmentation in the zone of inhibition compared to colistin alone (Gonzales Escalante *et al*., 2020).

In summary, this test is highly effective in differentiating between plasmid and chromosomal-related resistance. It has demonstrated 100 % sensitivity and specificity in detecting *mcr*-producing *Enterobacteriaceae*, while enabling simultaneous screening of a substantial number of samples (21 samples) on a single plate (Gonzales Escalante *et al*., 2020).

• **Rapid matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS):** Studies have shown that the bacterial surface lipids can serve as specific biomarkers for detecting bacterial resistance. In response to this, a method utilizing matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) has been developed to discern changes in lipid A of GNB associated with colistin resistance; particularly *mcr-1* gene resistance (Fig. 5). The presence of an additional peak at a mass-to-charge ratio (m/z) of 1920.5; exclusively found in *mcr-1*-producing isolates, confirms the incorporation of PEtN in lipid A of LPS by PEtN transferase enzyme, thus indicating a bacterium's *mcr-1* positive status (Mmatli *et al*., 2022).

9. Risk factors for colistin resistance development

Development of colistin resistance can be influenced by a multitude of risk factors, encompassing various aspects of antibiotic usage, microbial genetics, and clinical practices; mainly:

• Antibiotic usage in agriculture and animals: Notably, extensive administration of polymyxins, particularly colistin form in veterinary medicine has imposed significant selection pressure, prompting resistance evolution (EMA. 2013). Oral administration of colistin in either a powder or solution form demonstrates poor absorption within the digestive tract, resulting in its excretion in elevated levels through animal feces. Subsequent presence of colistin or its derivatives in the environment enhances selective pressure on the bacterial species. This concern is particularly significant when utilizing animal manure as a fertilizer

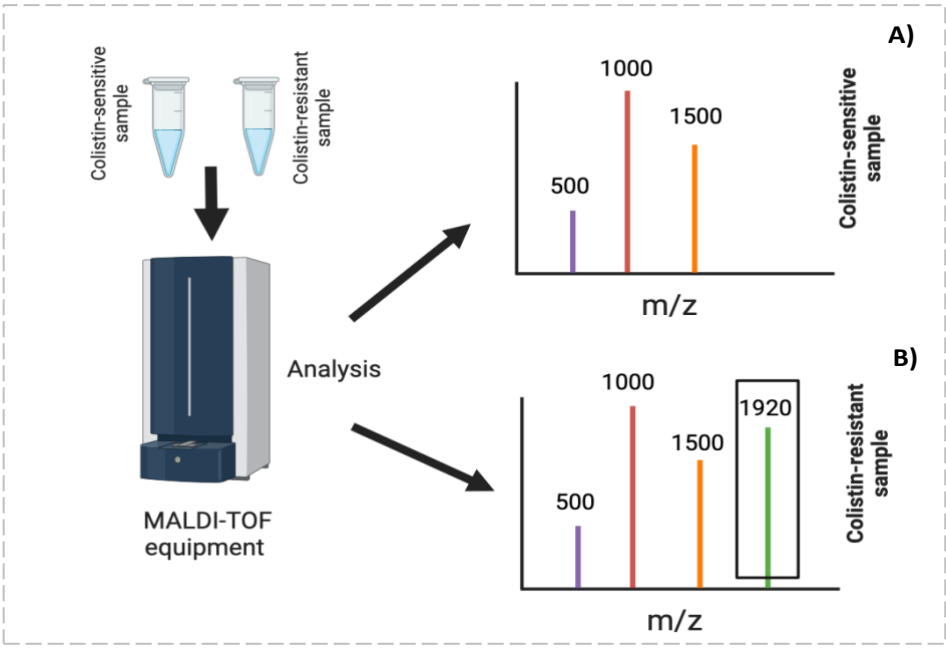

Fig. 5. Schematic representation of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) used for detection of *mcr-1*-colistin resistant isolates. Where; colistin-resistant isolate (*mcr-1* positive) (A), while colistin-susceptible isolate (*mcr-1* negative) (B). The presence of a peak at m/z = 1920.5 indicated that the isolates is *mcr-1* positive, adopted by Mmatli *et al*., (2022)

in agricultural practices, as it can lead to agricultural contamination and pollution of rivers and lakes in certain instances (Shen *et al*., 2019). In 2010, polymyxins were ranked among the top five best-selling antibiotics in the European veterinary medicine (EMA. 2013), with colistin sulfate predominantly employed in poultry and pork production; accounting for over 96 % of global usage. Extensive use of colistin; particularly in sub-therapeutic doses, as a growth factor for enhancing animal production, poses a substantial risk for resistance development, which is evident in certain regions such as Asia; notably China (Zhang *et al*., 2017).

• **Microbial genetics and healthcare personnel practices**: Emergence of colistin resistance genes such as *mcr-1* to *mcr-10*, located on mobile genetic elements like plasmids, emphasizes the potential of rapid dissemination of resistance through mechanisms of horizontal gene transfer such as conjugation. For instance, the plasmids *IncX4* and *IncI2* are commonly cited as "epidemic" owing to their capacity to globally disseminate *mcr-1* gene among GNB (Mondal *et al*., 2024). Additionally, co-occurrence of colistin resistance *mcr* genes with other antibiotic resistance genes; particularly carbapenemases such as *K. pneumoniae* carbapenemases (KPC) on the same conjugative plasmid, results in co-selection of isolates carrying *mcr*-plasmid. This co-selection process promotes extensive spread of *mcr*-plasmid within bacterial populations, presenting a marked challenge to the efficacy of available treatment options for bacterial infections (Long *et al*., 2019). Hospital environments such as intensive care units, serve as hotspots for transmission of colistin-resistant bacteria, often facilitated by nursing and medical personnel practices, which elucidate the heightened prevalence of colistin resistance in hospital settings as opposed to community environments, even in absence of plasmid-mediated resistance mechanisms (Papadimitriou-Olivgeris *et al*., 2014).

• **Dosing regimen**: Extended exposure to colistin exceeding 14 d in clinical settings consistently leads to the development of resistance. Research studies have demonstrated that prolonged treatment durations significantly elevate the likelihood of resistance emergence. For instance, 88.2 % of patients who developed resistance to colistin had been administered the drug for an average of 14 d before displaying resistance. Moreover, despite bacterial decontamination, there remains a risk of bacterial regrowth; even in cases where initial susceptibility to colistin has been observed (Karageorgopoulos *et al*., 2008). Several dosing studies have indicated that the bactericidal effect remains consistent regardless of whether colistin has been administered at 8, 12, or 24 h intervals. These findings suggest that the optimal dosing regimen is the administration of colistin at 8 h intervals, which has shown promising results in mitigating resistance development; particularly in *P. aeruginosa* infections (Ah *et al*., 2014). Prior colonization with carbapenemase-producing *K. pneumoniae* has also been identified as a risk factor for acquiring colistin resistance (Karageorgopoulos *et al*., 2008). Use of colistin for selective decontamination of the digestive tract (SDD) poses a considerable risk for resistance development. Prolonged exposure of intestinal bacteria to colistin during SDD increases the likelihood of selecting resistant strains, as colistin does not effectively traverse the gastrointestinal barrier (European Parliament and Council. 2016). As a result, resistance to colistin is frequently correlated with suboptimal empirical antibiotic therapy and absence of combination of antibiotic regimens (Giacobbe *et al*., 2020). In the ongoing efforts to combat the escalating threat of colistin resistance, it is imperative to comprehensively understand and address the multifaceted risk factors involved. Effective management of these intricate risk elements is essential for safeguarding the efficacy of this crucial antibiotic in the context of clinical practice.

10. Updated regulations/ policies on colistin usage in animals

In the wake of identification of the *mcr-1* gene, there has been a marked upsurge in enforcement of novel regulations and policies designed to safeguard the effectiveness of colistin. Compared to humans, the incidence of isolates harboring *mcr-1* gene originating from animals underscores the role of animal production as a primary reservoir of *mcr* gene-harboring bacteria (Rhouma and Letellier, 2016). In response to this concern, various regulatory adjustments have been instituted in veterinary medicine to oversee utilization of colistin.

In 2016, the World Health Organization (WHO) classified colistin as a critically-important antibacterial agent intended to be accessible worldwide; with a requirement for a customized use based on patients and environmental circumstances. Its usage has to be reserved for cases where alternative options have been exhausted (WHO. 2017). In May 2016, the European Medicines Agency (EMA) issued updated guidelines pertaining the use of colistin products in animals across the European Union (EU) (EMA. 2016). Subsequently, in July 2016, the Ministry of Agriculture of China implemented a ban on utilization of colistin in animal feed as a growth promoter for farm animals (Walsh, 2016). Contrastingly, in most of the other African countries, colistin is readily available as an over-the-counter drug within the animal production sector; often lacking proper veterinary oversight. Similarly, in 2015, Morocco saw colistin emerge as the most commonly employed antibiotic for treating animals in the broiler sector, in some cases, in excessive doses (Rahmatallah *et al*., 2018).

In December 2019, EMA revised its 2014 advice, categorizing colistin from a first-choice antibiotic to a second-choice antibiotic (category B, Restrict), restricting its administration solely to clinical infections in absence of alternatives in a lower category (C, Caution or D, Prudence) (EMA. 2019). As of January 2022, updated EU regulations regarding veterinary medicines and medicated feed (Regulations 2019/6 and 2019/4) have implemented a prohibition on the routine application of antibiotics in farming practices. The commission has further recommended

withdrawing all marketing authorizations for colistin-based veterinary medicinal products when combined with other antimicrobial substances for oral administration, citing the absence of discernible benefits compared to monotherapy (More, 2020). In the Chinese market, the manufacture of colistin sulfate premix, which is primarily employed in the treatment of specific infections in poultry and livestock, has contracted as a result of prohibition on using colistin as a growth promoter in 2017. Furthermore, the latest report on veterinary antimicrobial usage in Europe indicated a decline of over 77 % in the quantity of colistin sold for food-producing animals from 2011 to 2020 (EMA. 2021).

11. Strategies to combat colistin resistance

In response to the need to decrease reliance on colistin and other currently active antibiotics, it is critically important to pursue the development of innovative strategies and alternatives. These alternatives encompass a wide range of approaches, including but not limited to the following:

• **Developing new colistin derivatives**: Investigations of new derivatives of colistin aim at enhancing its effectiveness. For instance, compounds such as polymyxin (NAB739 and NAB815); derivatives of polymyxin B, exhibit promising attributes such as heightened potency, improved cellular penetration, and identification of novel targets. Notably, NAB739 derivative carries only three of the five amino groups of colistin, strategically positioned (Vaara *et al*., 2018). Furthermore, this derivative has shown synergistic activity with other antibiotics such as meropenem and retapamulin against GNB polymyxin-resistant strains.

• **Combined therapy**: Researches into the effects of colistin in combination with other active molecules present a promising avenue of inquiry. Studies have demonstrated synergistic interactions between colistin and clarithromycin against *mcr-1*-positive *K. pneumoniae* (MacNair *et al*., 2018), and with vancomycin against extensively drug-resistant strains of *A. baumannii*. Despite limited efficacy of vancomycin against GNB, this synergy is attributed to colistin's role in facilitating the entry of vancomycin into the bacterial cells (Gordon *et al*., 2010). Furthermore, combining colistin with antimicrobial peptides such as *MSI-78*; an analog of the magainin antimicrobial peptide, or *OTD-244*; a modified human β-defensin-2, has shown significant promise. This notably reduced the MIC of colistin for a substantial proportion of isolates, including resistant strains, while exhibiting no adverse effects on red blood cells, thus suggesting potential clinical utility (Witherell *et al*., 2020). Exploring the synergy between colistin and natural extracts such as pterostilbene found in plants has shown a synergistic impact against *mcr-1*-positive *K. pneumonia* (Zhou *et al*., 2018). Moreover, co-administration of colistin and curcumin has displayed notable effectiveness in enhancing bacterial membrane permeability, facilitating curcumin uptake, and inhibiting colistin expulsion via suppression of efflux pumps (Kaur *et al*., 2018).

• **Looking into existing molecules used to treat non-bacterial diseases**: Exploring the potential of repurposing of already existing drug molecules used to treat non-bacterial diseases presents an intriguing avenue for combating antibiotic resistance. For instance, Ticagrelor, an antiplatelet medication employed in cardiovascular disease management, has exhibited potent antimicrobial activity against antibiotic-resistant Gram-positive bacteria (GPB) (Lancellotti *et al*., 2019). Additionally, incorporation of organic acids and zinc oxide into animal feed represents a promising strategy to reducing the necessity for antibiotic usage. However, careful consideration of the associated benefits and risks is imperative, considering the potential emergence of cross-resistance between zinc and antibiotics, as exemplified by methicillin-resistant *Staphylococcus aureus* in specific instances (Madec, 2017).

• **Vaccination**: Vaccination stands out as a promising strategy for addressing colistin resistance and other forms of antibiotic resistance. Using immunization against bacterial infections, such as pneumococcal

vaccination, the incidence of infectious diseases can be minimized, consequently reducing the reliance on antibiotic therapy (Buchy *et al*., 2020).

• **Exploring the mechanisms behind colistin resistance**: Ongoing researches into the mechanisms that underlie colistin resistance are imperative, as elucidating these mechanisms holds the potential to guide the development of targeted therapeutic strategies. For instance, exploring inhibitors of efflux pumps, such as 3-chlorophenylhydrazone cyanide type (CCCP), exemplifies a promising avenue to combat antibiotic-resistant GNB (Ding *et al*., 2023). Furthermore, discovering plasmid-mediated colistin resistance (*mcr*-like gene) encoding phosphoethanolamine transferase enzymes has opened up possibilities for using the ion chelators such as EDTA to inhibit the function of these enzymes (Gonzales Escalante *et al*., 2020).

• **Bacteriophage therapy**: In contemporary strategies, emphasis is placed on utilization of the bacteriophages, which are highly specialized viruses targeting bacterial cells, causing their lysis. Notable examples include the lytic phage *A. baumannii*, YMC 13/03/R2096 ABA BP (phage Bφ-R2096); known for its specific lysis of carbapenem-resistant *A. baumannii* (CRAB), and phages like IsfAB78 that is capable of reducing biofilms formation of MDR *A. baumannii* by 87 %, including strains resistant to colistin. Alternative strategies are based on using antisense peptide nucleic acids (ANPs) such as *Anti-lpxB pPNA*, which has demonstrated efficacy against *A. baumannii*, either as a monotherapy or in combination with colistin (Ebrahimi *et al*., 2021). These innovative strategies hold promise for addressing colistin resistance and warrant further investigation for optimal clinical application.

• **Nano-drug delivery vesicles**: Ongoing research is underway to develop liposomes and delivery vesicles capable of co-delivering colistin and ciprofloxacin into the pulmonary epithelial cells. Preliminary findings have suggested improved drug retention on the pulmonary epithelial surfaces (Chai *et al*., 2019), indicating the potential utility of colistin, either alone or in combination, for treating lung-associated infections using liposomal formulations. Furthermore, colistin has been encapsulated within vesicles known as ABC-micelles, which displayed improved storage stability and enhanced biocompatibility in murine models (Yang *et al*., 2021).

• **Antibiotic adjuvants**: Given the known high-dose toxicity of colistin, researchers are exploring antibiotic adjuvants as potential therapeutic adjuncts. These compounds act by enhancing antibiotic efficacy through inhibition of resistance mechanisms and augmentation of intracellular antibiotic accumulation rather than by directly eradicating the bacteria. Among these adjuvants, those based on 2-aminoimidazole that have demonstrated the ability to potentiate colistin activity against colistin-sensitive *A. baumannii* strains. *In vitro* studies have shown a remarkable 1000-fold reduction in colistin's MIC when used in conjunction with this adjuvant. Furthermore, *in vivo* experiments utilizing *Galleria mellonella* models have validated these findings, indicating a potential avenue for utilizing colistin without associated cytotoxic effects (Minrovic *et al*., 2018). These multifaceted approaches hold promise in mitigating colistin resistance and warrant further investigation to optimize their clinical applicability.

Conclusion

Colistin is often regarded as a final resort in combating specific MDR GNB. Nonetheless, its widespread application and other factors have led to a notable surge in resistance. The emergence of novel forms of resistance, inclusive of both plasmid and chromosomal, further complicates the identification and management of resistance owing to the distinct structural characteristics of colistin molecules. Addressing this pronounced resistance quandary necessitates the pursuit of efficacious solutions, encompassing the exploration of novel active compounds, and the preservation of existing antibiotics as part of our medical legacy. This strategic

approach is imperative in effectively addressing the advent of unforeseen new resistances.

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Conflict of interests

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Ethical approval

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Author's Contributions

Zine El Abidine Bzazou EL Ouazzani: Conceptualization and Methodology, Formal analysis, Investigation, Resources, Writing-Original Draft. Houda Benaicha: Conceptualization and Methodology, Writing-Review & Editing. Laila Reklaoui: Formal analysis, Investigation, Said Barrijal: Conceptualization and Methodology, Resources, Writing-Review & Editing, Supervision.

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