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Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes

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(continued)

SUMMARY Polymyxins are well-established antibiotics that have recently regained significant interest as a consequence of the increasing incidence of infections due to multidrug-resistant Gram-negative bacteria. Colistin and polymyxin B are being seriously reconsidered as last-resort antibiotics in many areas where multidrug resistance is observed in clinical medicine. In parallel, the heavy use of polymyxins in veterinary medicine is currently being reconsidered due to increased reports of polymyxin-resistant bacteria. Susceptibility testing is challenging with polymyxins, and currently available techniques are presented here. Genotypic and phenotypic methods that provide relevant information for diagnostic laboratories are presented. This review also presents recent works in relation to recently identified mechanisms of polymyxin resistance, including chromosomally encoded resistance traits as well as the recently identified plasmid-encoded polymyxin resistance determinant MCR-1. Epidemiological features summarizing the current knowledge in that field are presented.

KEYWORDS Gram-negative bacteria, MCR-1, lipopolysaccharide, polymyxins, toxicity

INTRODUCTION

Colistin (also known as polymyxin E) is a polypeptide antibiotic that was originally isolated in 1947 from the soil bacterium *Paenibacillus polymyxa* subsp. *colistinus* (1). Colistin and polymyxin B belong to the class of polymyxins, which is one of the primary classes of antibiotics with activity against most Gram-negative bacteria.

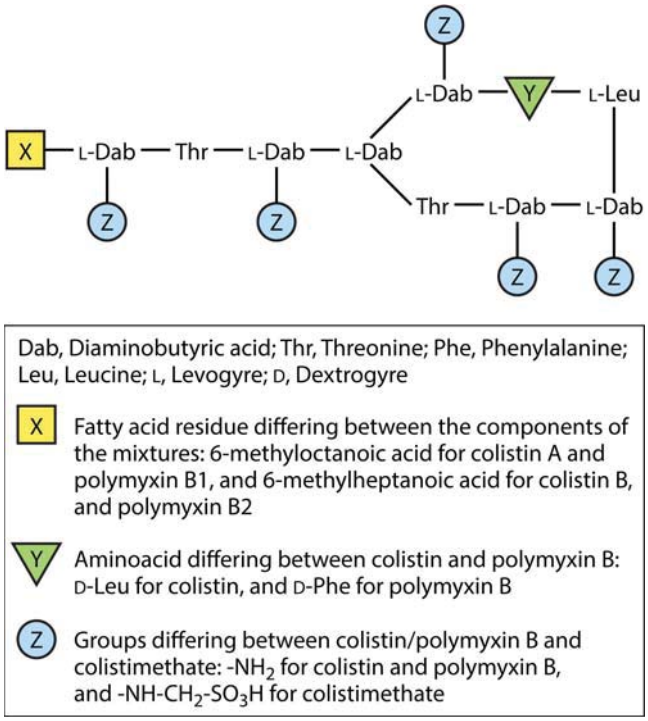

FIG 1 Structures of colistin A and B, colistimethate A and B, and polymyxin B1 and B2.

Structure

The chemical structure of polymyxins is similar to that of cationic antimicrobial peptides (CAMPs) (defensins and gramicidins), which represent the first line of defense against bacterial colonization in eukaryotic cells (2). Polymyxins are cationic polypeptides that consist of a cyclic heptapeptide possessing a tripeptide side chain acylated at the N terminus by a fatty acid tail (3, 4) (Fig. 1). The inherent toxicity of colistin may be explained by the hydrophobic properties of the N-terminal fatty acyl segment, which also accounts significantly for its antimicrobial activity, and also by positions 6 and 7, which are very important (5, 6).

Colistin and polymyxin B differ by only a single amino acid in the peptide ring, with a phenylalanine in polymyxin B and a leucine in colistin (Fig. 1) (7). Polymyxin B is administered directly as an active antibiotic, whereas colistin is administered as an inactive prodrug, colistin methanesulfonate (also known as colistimethate [CMS]) (Fig. 1) (7).

The terms "colistin" and "colistimethate" are not interchangeable, since they correspond to different forms of colistin available for clinical use (4). Indeed, colistimethate sodium is a polyanionic inactive prodrug that is less toxic than colistin sulfate (Fig. 1) (4, 8). Colistimethate is formed by the reaction of colistin with formaldehyde and sodium bisulfite (9). This prodrug is transformed in aqueous media, and also *in vivo* in biological fluids, and is converted into colistin and several inactive methanesulfonated compounds (10, 11).

Mechanism of Action

The target of polymyxins is the outer membrane of Gram-negative bacteria. Because of an electrostatic interaction occurring between the α,γ-diaminobutyric acid (Dab) residue of the positively charged polymyxin on one side and the phosphate groups of the negatively charged lipid A membrane on the other side, divalent cations (Ca^{2+} and Mg^{2+}) are displaced from the negatively charged phosphate groups of membrane lipids (12). The lipopolysaccharide (LPS) is therefore destabilized, consequently increasing the permeability of the bacterial membrane, leading to leakage of the cytoplasmic

content and ultimately causing cell death (4, 13). Note that even though the LPS is the initial target, the exact mode of action of polymyxins still remains unclear.

Another antibacterial mechanism is the endotoxin effect. The endotoxin of Gram-negative pathogens corresponds to the lipid A portion of the LPS; polymyxins have the ability to bind to and neutralize this LPS molecule released during cell lysis (14).

Finally, another mode of action of the polymyxins is the inhibition of vital respiratory enzymes (inhibition of type II NADH-quinone oxidoreductases [NDH-2]) in the bacterial inner membrane (15).

Spectrum of Activity

Polymyxins have a narrow antibacterial spectrum, mainly against common Gram-negative bacteria. They are active against most members of the *Enterobacteriaceae* family, including *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp. Polymyxins also have significant activity against common nonfermentative Gram-negative bacteria, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* (13).

Conversely, some species are naturally resistant to polymyxins, including *Proteus* spp., *Morganella morganii*, *Providencia* spp., *Serratia marcescens*, *Pseudomonas mallei*, *Burkholderia cepacia*, *Chromobacterium* spp., *Edwardsiella* spp., *Brucella*, *Legionella*, *Campylobacter*, and *Vibrio cholerae*. Polymyxins are not active against Gram-negative cocci (*Neisseria* spp.), Gram-positive bacteria, and anaerobic bacteria (13).

Pharmacodynamics

The antibacterial effect of colistin is concentration dependent (4, 16–18). The pharmacokinetic-pharmacodynamic (PK-PD) index that best predicts the antibacterial activity against *A. baumannii* and *P. aeruginosa* is the ratio of the area under the concentration-time curve for free drug from 0 to 24 h to the MIC ($f\text{AUC}_{0-24}$/MIC), with this index being superior to the maximum concentration of drug in serum (C_{max})/MIC relationship, suggesting that time-averaged exposure to colistin is more important than the achievement of high peak concentrations (19–21). An average steady-state plasma colistin concentration of 2 μg/ml has been suggested as a reasonable target value for isolates with MICs of $\leq$1 μg/ml, maximizing the antimicrobial activity while minimizing the risk of nephrotoxicity (22). An inadequate AUC/MIC ratio likely leads to treatment failure. The colistin antibacterial effect is extremely rapid, occurring as early as 5 min after exposure (17, 18, 23, 24).

A postantibiotic effect was observed against *Klebsiella pneumoniae*, *P. aeruginosa*, and *A. baumannii* (25). However, it is important to highlight that polymyxins have minimal postantibiotic effects at clinically relevant concentrations. Despite the major initial killing rate observed against colistin-susceptible strains exposed to colistin alone, regrowth has been reported for *A. baumannii* (17) and *K. pneumoniae* (18) in static time-kill studies. Colistin heteroresistance, a phenomenon corresponding to the emergence of a colistin-resistant subpopulation (that can grow in the presence of $\geq$4 μg/ml of colistin) within a susceptible population (i.e., with a MIC of $\leq$2 μg/ml), has been observed for *A. baumannii* (26, 27), *K. pneumoniae* (18, 28), and *P. aeruginosa* (23).

USE OF COLISTIN IN HUMAN AND VETERINARY MEDICINE

Use in Human Medicine

After its discovery in 1947, colistin was used in Japan and Europe during the 1950s (29). Then, after its approval by the U.S. FDA in 1959, colistimethate (CMS), the inactive prodrug of colistin, replaced colistin for parenteral administration (29). Colistin and CMS have been used widely for decades for treatment of infections caused by Gram-negative bacteria. However, in the 1970s, because of their toxicity, especially nephrotoxicity (30), their use was reconsidered. They were then replaced by novel, more active and less toxic antibiotics, such as aminoglycosides, quinolones, and β-lactams. For 20 years, the use of colistin was restricted to ophthalmic and topical uses. Systemic or nebulized colistin was used only for cystic fibrosis patients.

However, the increasing prevalence of multidrug-resistant (MDR) Gram-negative bacteria (31), particularly *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, has forced physicians to reintroduce systemic polymyxin as a valuable therapeutic option (4, 13, 32).

Considering the paucity of novel antibiotics, colistin is currently often the only effective antibiotic agent against MDR organisms, particularly carbapenemase-producing bacteria.

Commercial formulations. There are more than 30 polymyxin molecules, among which there are five main chemical compounds (polymyxins A to E), each containing multiple components. Although colistin (polymyxin E) and polymyxin B are both used in clinical practice (33), colistin is the most widely used polymyxin (23). The two most common commercially available parenteral formulations of the colistin prodrug, CMS, are Colomycin (Forest Laboratories UK Limited, Dartford, United Kingdom), primarily employed in Europe, and Coly-Mycin M Parenteral (Monarch Pharmaceuticals, Inc., Bristol, TN), primilarily employed in the United States (34). Unfortunately, the vials of both formulations contain different dry powder quantities, and the two products are differently labeled, with Colomycin being labeled in international units (IU) of CMS and Coly-Mycin M Parenteral being labeled in milligrams of CMS or colistin base activity (CBA) (34). The conversion is as follows: 1 million units (MU) CMS = 80 mg CMS = 30 mg CBA (35). To add to the confusion, some other brands corresponding to generic products are now available (36). The multiplicity of terms used to express contents of vials and dose regimens unfortunately creates confusion and does not allow any meaningful comparison of data collected from studies performed in different parts of the world.

Routes of administration. Colistin sulfate can be administered orally as tablets and syrup for selective digestive tract decontamination (no absorption) and topically for the treatment of bacterial skin infections (13). CMS, the less toxic prodrug, has different administration routes, i.e., parenteral (including intravenous) and intramuscular, but intrathecal or intraventricular administration is also possible (13). The intramuscular injection is rarely used in clinical practice because it may be very painful locally, and also because its absorption is variable (33). Both colistin sulfate and CMS can be delivered through inhalation by aerosol therapy, but there is a higher frequency of bronchoconstriction with colistin sulfate (33). Delivery of CMS by inhalation and by the intrathecal and intraventricular routes allows much higher concentrations in lung fluid and cerebrospinal fluid, respectively, than those seen with systemic administration. Moreover, those routes of administration lead to negligible plasma exposure and are less toxic (in particularly less nephrotoxic) (22).

In aqueous solutions, colistimethate sodium is transformed into colistin; therefore, it should be administered shortly after reconstitution to avoid the toxicity associated with colistin (37).

Pharmacokinetics. Because of their discovery and their introduction into clinical use more than 50 years ago, polymyxins were never subjected to the drug development approval process currently required by international drug regulatory authorities. Consequently, the PK and PD data on the rational use of polymyxins (maximizing antibacterial activity and minimizing toxicity and development of resistance) were not available until recently. The fact that, until recently, plasma concentrations of CMS and formed colistin could not be differentiated because of a lack of suitable techniques was another obstacle limiting progress in this area. The recent development of chromatographic methods allowing quantitative assessment of each compound separately significantly contributed to the renewed interest in prescribing colistin and colistimethate (38, 39). It was clearly demonstrated that the observed antimicrobial activity results from the action of colistin itself, which is generated *in vivo* when CMS is given. For accurate PK information, a prerequisite is to quantify separately the inactive prodrug (CMS) and the active entity (colistin) (34).

After its parenteral administration, a large proportion of CMS is eliminated mainly through the kidneys by glomerular filtration and tubular secretion (Fig. 2A) (11).

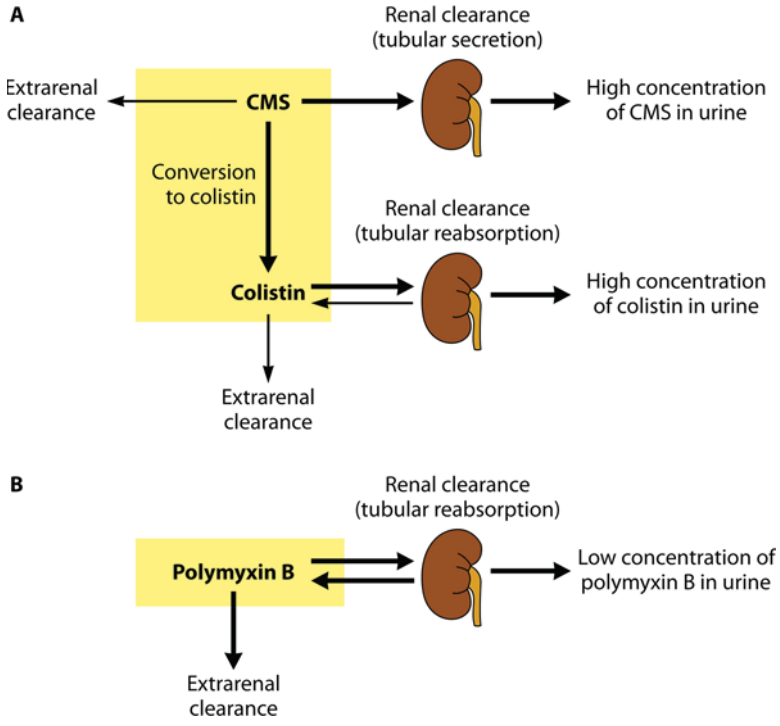

FIG 2 Overview of the pharmacokinetic pathways for colistimethate (CMS) and colistin (A) and for polymyxin B (B). The thicknesses of the arrows indicate the relative magnitudes of the respective clearance pathways when kidney function is normal. CMS includes fully and all partially methanesulfonated derivatives of colistin. After administration of CMS, extensive renal excretion of the prodrug occurs, with some of the excreted CMS being converted to colistin within the urinary tract. (The figure is based in part on data from reference 7.)

Because in a healthy individual the clearance of CMS by the kidneys is much higher than its conversion clearance to colistin, no more than 20 to 25% of a CMS dose is hydrolyzed *in vivo* into an active colistin entity (7). Consequently, the colistin concentrations resulting from the original CMS administration are low. In contrast to CMS, colistin is eliminated predominantly by a nonrenal way because of its extensive renal tubular reabsorption (Fig. 2A) (11, 40). Although colistin is poorly excreted in urine, the urinary concentration of colistin may be relatively high after administration of CMS due to the conversion of CMS (highly excreted by the kidneys) into colistin within the urinary tract (7).

In contrast to colistin, polymyxin B is administered directly in its active antibacterial form. As for colistin formed from CMS, polymyxin B is subject to very extensive renal tubular reabsorption and is thus eliminated mainly by a nonrenal clearance mechanism(s) (Fig. 2B) (7).

Dosing regimen. Due to renewed interest in its use in the context of infections caused by multidrug-resistant bacteria, and considering the increasing rates of resistance to colistin currently observed, CMS has to be administered carefully. In particular, the regimens allowing maximal antibacterial activity and minimal development of resistance have to be defined accurately, since the regimens need to minimize adverse effects (23). A study analyzing product data characteristics of intravenous CMS revealed a lack of uniformity between manufacturers, with quite broad variations in term of indications, dose regimens (3 to 12 MU/day), and PK (36). Moreover, dosing regimens given by manufacturers are often discordant with the dosing regimens recommended by the recent literature (21, 34, 41).

(i) Patients with normal renal function. The currently used dosage regimens of CMS generate suboptimal exposure to colistin in many critically ill patients, in particular in renally competent patients. Two studies reported low plasma colistin C_{max} values

following administration of 174 mg to 250 mg (2 to 3 MU) of CMS every 8 or 12 h, with steady-state levels of 1.15 to 5.14 μg/ml or 0.68 to 4.65 μg/ml, respectively (42). Moreover, a significant delay in obtaining steady-state plasma concentrations of formed colistin was reported for CMS treatment started without administration of a loading dose (43). In the latter study, concentrations of colistin in the plasma were reported to be below the MIC breakpoint (2 μg/ml), which is a main drawback considering that a delayed initiation of appropriate antibiotic therapy has been shown to be associated with increased mortality rates, in particular for critically ill patients (44). In addition, low colistin concentrations may induce the amplification of colistin-resistant subpopulations (18, 45). Interestingly, on consideration of the current dose range product recommendations for CMS, it was confirmed that its administration at the upper limit to patients with normal renal function resulted in low and potentially suboptimal plasma colistin concentrations, especially when the MIC for the infecting bacterial strain was in the upper range (2 μg/ml) or if the infection was associated with a high bacterial inoculum (21). That study also revealed that steady-state plasma colistin concentrations are highly variable, with up to a 10-fold range achieved across patients at a given creatinine clearance (21).

In contrast, there is a relatively low interpatient variability (3.3-fold) across a wide range of creatinine clearance values following administration of polymyxin B (46). Considering that polymyxin B is not given as a prodrug, it is easier to rapidly achieve a desired plasma concentration of polymyxin B (46).

There is no consensus about dosing regimens, even though recently published dosing suggestions seem to be widely accepted (19). Compared to those suggested by the manufacturers, the regimens in recent studies support the administration of a loading dose and of higher doses of CMS in order to achieve adequate colistin concentrations leading to a better therapeutic effect (21, 41, 47). The dosing regimen currently recommended by the recent literature (for patients with good renal function) is a loading dose of 4.5 MU of CMS followed by maintenance doses of 4.5 MU twice daily (48–50). A colistin-containing combination therapy has to be considered if the infecting pathogen shows an MIC of colistin above 1 μg/ml, if there is a high inoculum, or in dealing with deep-seated infections (e.g., in lungs). One therefore has to consider adding antibiotics to colistin regimens, especially for patients with relatively normal renal function (21, 22).

Data about the pharmacokinetics, effectiveness, and safety of polymyxins were recently reviewed by the European Medicines Agency (EMA). There have been recommended changes in terms of product information in order to ensure the safer use of polymyxins (51). According to the EMA, polymyxins should be reserved for the treatment of serious infections due to aerobic Gram-negative pathogens with limited treatment options (51). Also, they should be given with another suitable antibiotic when possible. The recommended dose for CMS in adults is 9 MU daily in 2 or 3 divided doses as a slow intravenous infusion. For dealing with critically ill patients, a loading dose of 9 MU should be given. For patients with renal impairment, doses should obviously be reduced, with consideration of the creatinine clearance.

Because the efficacy and toxicity of colistin are dose dependent, it is crucial that optimal dose regimens be used to maximize the antimicrobial activity and to minimize adverse effects and the development of resistance. This is especially important for critically ill patients, as they are most at risk for high morbidity and mortality (52).

(ii) Patients with renal insufficiency. A study showed that colistin levels were elevated in patients with renal insufficiency, presumably due to decreased elimination of the antibiotic generating a higher rate of conversion of CMS to colistin (43). Development of nephrotoxicity is consequently higher in patients with renal insufficiency than in patients with normal renal function (53).

Dalfino et al. (54) suggested a new dose adjustment for high-dose colistin therapy for patients with renal insufficiency. For patients with creatinine clearance of 20 to 50 ml/min, they recommend a loading dose of 9 MU and maintenance doses of 4.5 MU

every 24 h. For patients with creatinine clearance of <20 ml/min, they recommend a loading dose of 9 MU and maintenance doses of 4.5 MU every 48 h (21, 55).

Toxicity. Rates of toxicity following intravenous administration of CMS are considered lower today than those observed in previous studies, and it has to be mentioned that the criteria for defining toxicity have also been updated (56). The lower toxicity may be related to the fact that there are fewer chemical impurities in CMS but also to the fact that monitoring in intensive care units (ICUs) is better nowadays and the coadministration of other nephrotoxic drugs is significantly avoided (33).

Colistin has a narrow therapeutic window, and major adverse effects related to its parenteral use are neurotoxicity and nephrotoxicity. Neurotoxicity is dose dependent and reversible (55) and may cause peripheral and facial paresthesia, weakness, dizziness/vertigo, visual disturbances, confusion, ataxia, and neuromuscular blockade, even leading to respiratory failure or apnea (56). The most common neurotoxicological effect is paresthesia (occurring in 27% of patients), and there is no report of neuromuscular blockade or apnea in the recent literature (56). Nephrotoxicity is the most common and concerning adverse effect, especially with the newly recommended high-dose regimen. Similarly to neurotoxicity, nephrotoxicity is dose dependent. The risk of colistin-associated nephrotoxicity increases with plasma colistin concentrations above 2.5 to 3 μg/ml, as revealed by recent PK-PD analyses (57). Other risk factors for nephrotoxicity include coadministration of other drugs that are also nephrotoxic (anti-inflamatory drugs, vancomycin, or aminoglycoside antibiotics) and patient-related factors (advanced age, male sex, hypoalbuminemia, hyperbilirubinemia, preexisting chronic kidney disease, and severity of illness) (33, 56). Nephrotoxicity is reported to be a rapid-onset effect, with most cases occurring within the first week of treatment, and is mostly reversible (33, 55). Rates of nephrotoxicity in recent studies ranged from 6% to 55% (33). The large range of nephrotoxicity rates may be explained partially by different definitions of renal failure, the dosing regimens used, the concomitant administration of nephrotoxic drugs, and the use of colistin monitoring to adapt dosing regimens. The RIFLE (risk–injury–failure–loss–end-stage renal disease) classification is used to determine colistin-associated nephrotoxicity (58).

Two recent comparative studies involving large numbers of patients showed that the nephrotoxicity rates were lower for polymyxin B than for CMS/colistin (59, 60).

Use in Veterinary Medicine

As opposed to the case in human medicine, in veterinary medicine colistin has been used extensively for decades for the treatment and prevention of infectious diseases. The majority of polymyxin consumption corresponds to orally administered forms, with different formulations (premix, powder, or oral solutions). The main usage is related to enterobacterial infections, and in particular to gastrointestinal infections caused by *E. coli* in poultry and pigs within intensive husbandry systems (61). Apart from this common usage for treating infections caused by *Enterobacteriaceae*, another usage corresponds to growth promotion, which is a common practice worldwide. Furthermore, the fact that only a thin line exists between oral metaphylactic therapy, preventive starter rations, and growth promotion adds to the problem. In 2011, polymyxins were the fifth most sold class of antimicrobials (7%) for treating food-producing animals in Europe (61).

Despite this extensive use in veterinary medicine, the resistance rate to colistin in *E. coli* strains recovered from healthy animals remains <1% in many European countries (62). However, resistance to colistin has increasingly been reported (10%) among porcine-pathogenic *E. coli* strains in Belgium (63), and the emergence of resistance has been described for cattle (64). Moreover, some recent data revealed the possibility of horizontal transmission from farm animals to humans in Asia (65). Given the increasing need to retain the efficacy of colistin to treat MDR infections in humans, the potential for spreading colistin-resistant isolates from animals to humans, and the recent identification of colistin-resistant *Enterobacteriaceae* organisms harboring a plasmid-borne colistin resistance determinant in animals and food products (see below), the use of colistin in veterinary medicine is being reevaluated. As a very recent example, the

formal Ministry of Agriculture of China decided to ban colistin as a feed additive for animals (66). Also, the European Medicines Agency provided a position paper in June 2016, in which updated advice on the use of colistin products in animals within the European Union is provided (67).

METHODS FOR SUSCEPTIBILITY TESTING

Despite such a long term of clinical use (decades), the optimal method for polymyxin susceptibility testing still remains undefined. However, the recent emergence of MDR Gram-negative bacteria and the subsequent increased use of colistin prompted the scientific community to develop rapid and reliable methods to determine the susceptibility of isolates to polymyxins, as this is now an urgent need in clinical laboratories. Polymyxin susceptibility testing is now a major challenge, as human infections with colistin-resistant Gram-negative bacteria are associated with higher patient mortality (68). The difficulties in testing susceptibility to polymyxins are diverse, including poor diffusion of polymyxins into agar, the inherent cationic properties of polymyxins, the occurrence of heteroresistance to polymyxins in many species, and the lack of a reliable reference method that may allow reliable comparisons of commercial tests (69, 70).

Dilution Methods

The aim of dilution methods is to determine the MIC, corresponding to the lowest concentration of polymyxin that inhibits visible bacterial growth after an incubation of 16 to 24 h at 35 $\pm$ 2°C.

Broth dilution methods. Broth dilution is a technique in which a bacterial suspension at a predetermined concentration is tested against various concentrations of antimicrobial agent in a liquid medium with a predetermined formulation. Two types of broth dilution methods are available: (i) the broth macrodilution method, performed with a minimum volume of 2 ml in standard test tubes; and (ii) the broth microdilution (BMD) method, performed with a volume of 0.05 to 0.1 ml in microtitration trays.

(i) Broth microdilution method. BMD is the reference susceptibility test method. It is currently the only method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (71, 72) for polymyxin antimicrobial susceptibility testing.

According to CLSI recommendations, BMD is performed with cation-adjusted Mueller-Hinton broth (CA-MHB), a range of 2-fold dilutions of polymyxins (ranging from 0.12 to 512 μg/ml), and a final bacterial inoculum of 5×10^5 CFU/ml in each well (73). BMD is considered to be the optimal method and is currently recommended for susceptibility testing in the recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf).

However, BMD is quite laborious, and manual preparation (if the technique used is not an automated one) of antibiotic solutions may lead to significant errors. It is therefore not adaptable for most clinical microbiology laboratories. Furthermore, nonreproducible and noninterpretable MIC results have been reported due to the presence of skip wells (i.e., wells that exhibit no growth, whereas growth is observed in wells with higher antibiotic concentrations) for *Enterobacter* species (69), *P. aeruginosa* (72), and *A. baumannii* (73). This phenomenon might be caused by heteroresistant subpopulations for *Enterobacter* spp. (69). In parallel, "skip well" isolates of *P. aeruginosa* have been found to have increased expression of the *pmrAB*, *phoQ*, and *arn* genes related to changes in the LPS structure, reducing the potential binding sites of polymyxins (74).

Nevertheless, BMD currently remains the reference method for determination of MICs because of its reproducibility, reliability, and possibility of automation.

(ii) Broth macrodilution method (or tube dilution method). The growth medium (CA-MHB), the inoculum bacterial suspension, the preparation of 2-fold dilutions of polymyxins, the incubation conditions, and the reading of the plate are identical to those for the broth microdilution method. The only difference is the volume of growth

medium and the use of test tubes instead of trays. When evaluated against BMD results, the results of the broth macrodilution method showed the highest agreement (83%) compared to other available methods, and no false susceptibility was observed (70).

Agar dilution method. Agar dilution is another reference method that relies on various concentrations of polymyxin molecules in Mueller-Hinton agar (usually 2-fold serial dilutions), followed by the seeding of a defined bacterial inoculum onto the agar plate. In accordance with the CLSI recommendations, the polymyxin powder is dissolved in sterile water and added to molten MH agar to provide 2-fold dilutions (usually ranging from 0.12 μg/ml to 512 μg/ml) (70, 71). A bacterial inoculum corresponding to a 0.5 McFarland standard (approximately 10^8 CFU/ml) is prepared, and then 10-fold dilutions are performed. One microliter of this dilution is spotted manually or with an automated system, and each spot consequently inoculates 10^4 CFU of bacteria.

Agar dilution may theoretically avoid the adsorption of colistin to the plates, but no study has measured the colistin concentration in agar dilution plates to confirm this hypothesis. Numerous studies have demonstrated a strong correlation between agar dilution and BMD (70, 75, 76), with the exception of results obtained with *P. aeruginosa* and *S. maltophilia* isolates from cystic fibrosis patients (77, 78). One advantage of the agar dilution method is the ability to test multiple strains on the same plate and the possibility to semiautomate the method. However, the agar dilution method also presents some disadvantages, as it is very laborious if not automated and the plates (not available from commercial sources) must be used within a week of preparation.

Many studies have employed the agar dilution method as a standard; however, BMD remains the primary reference method for polymyxin MIC testing. In a recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group, it is stated that agar dilution is not recommended for susceptibility testing (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf).

Routine Susceptibility Testing Methods

Nonautomatic systems. (i) DD test (Kirby-Bauer procedure). The disk diffusion (DD) test refers to the diffusion of a given concentration of polymyxin from disks into MH agar that has been seeded with a defined bacterial inoculum. According to the CLSI and EUCAST guidelines, the disk diffusion test is performed by applying a bacterial inoculum corresponding to a 0.5 McFarland standard (approximately 10^8 CFU/ml) suspended in 0.85% NaCl onto the entire surface of an MH agar plate by use of a sterile cotton swab. Paper disks impregnated with polymyxin are placed on the inoculated agar surface. Following the CLSI guidelines, the contents of colistin and polymyxin B on the paper disks are 10 μg and 300 U, respectively (72), while following the EUCAST recommendations, the colistin content is 50 μg (71). The growth inhibition zone diameter around the disk is measured after incubation for 16 to 24 h at 35 $\pm$ 2°C. The diameter of the inhibition zone is proportional to the bacterial susceptibility to polymyxins and inversely correlates with the MIC of the bacterial strain.

The DD test is easy and cheap and does not require specific equipment. These advantages explain why this method is commonly used as a primary test method to screen large numbers of isolates. However, the poor and slow diffusion of polymyxins through agar gives small zones of inhibition and limits the predictive accuracy of the DD test. In fact, many studies showed that the DD test is a nonreliable method for measuring susceptibility to colistin for Gram-negative rods, giving an unacceptable and very high rate of false susceptibility (up to 35%) compared to that with dilution methods (76, 78–80). A higher concentration of colistin in the disk (50 μg as recommended by EUCAST versus 10 μg as recommended by CLSI) does not improve the reliability of the test (80). Susceptible results should therefore be confirmed by dilution tests. On the other hand, no false resistance results are found with this method (80).

This method is not reliable and should be abandoned. For human medicine, EUCAST recommends that precise MIC determination be mandatory before clinical use and no longer provides disk breakpoints (71). For veterinary medicine, EUCAST recommends

precise determination of the MIC each time that the diameter of the inhibition zone is between 15 and 18 mm for a given strain.

(ii) Etest strips. Etests are thin plastic test strips impregnated with increasing antibiotic concentrations. MICs are read with the concentration scale marked on the upper surface. According to the manufacturer's recommendations, this method is performed by applying a bacterial inoculum of approximately 10^8 CFU/ml (turbidimetry of a 0.5 McFarland standard) suspended in 0.85% NaCl onto the entire surface of an MH agar plate by use of a sterile cotton swab. Etest strips containing a colistin concentration gradient (ranging from 0.016 to 256 μg/ml) are placed on the inoculated agar surface, and the MIC is determined after incubation for 16 to 24 h at 35 $\pm$ 2°C. The MIC value is defined by the intersection of the lower part of the ellipse-shaped growth inhibition area with the test strip. When the intersection occurs around the MIC endpoint, the highest MIC intersection is recorded (75). When small colonies grow within the zone of inhibition, the strain must be considered heteroresistant to colistin, and the highest MIC intersection is recorded (75, 81).

Several studies, notably including few resistant isolates, found an excellent correlation between the Etest and reference techniques (75, 76, 80, 82). However, studies including larger numbers of resistant isolates reported high rates of false susceptibility (up to 32%) for Gram-negative rods compared to those with dilution methods (69, 70, 78, 83). The Etest method may fail to detect resistance to colistin even when isolates exhibit high MICs by dilution methods (70, 83). In addition, there are discrepancies between MICs measured by Etest and MICs measured by dilution methods (70, 82). It has been reported that the Etest strip method underestimates the level of resistance of polymyxin-resistant strains (MIC $\geq$ 4 μg/ml) and overestimates the MIC values for susceptible strains (MIC $<$ 4 μg/ml) (70).

This method is easy to perform but is relatively expensive and does not reliably detect colistin-resistant isolates. As for the disk diffusion test, susceptibility results obtained by Etest require a 24-h delay.

(iii) UMIC system. The UMIC system (Biocentric) consists of broth microdilution unitary panels in which the wells contain prediluted lyophilized colistin at concentrations ranging from 0.06 to 64 μg/ml. The inoculation is performed manually, and the required incubation time ranges from 18 to 24 h. The performance of this system has not yet been evaluated.

Automatic systems. Use of instruments may allow susceptibility testing to be performed in a shorter period than that required for manual methods, as the sensitive optical detection systems of current instruments measure subtle changes in bacterial growth. To date, four automated instruments capable of measuring susceptibility to polymyxins are available. Two of them generate overall rapid (4 to 16 h) susceptibility test results (Vitek 2 and Phoenix), while the others (MicroScan and Sensititre) are overnight systems. These systems are associated with computer software to interpret susceptibility results.

(i) Vitek 2 system. The Vitek 2 system (bioMérieux) uses plastic reagent cards that contain microliter quantities of antibiotics and test media in wells (84). It tests colistin concentrations ranging from 0.5 to 16 μg/ml and monitors turbidimetry to determine bacterial growth during a period of 4 to 10 h. Compared to dilution methods, the Vitek 2 system displays a low sensitivity for detecting colistin-resistant Gram-negative isolates (83) and is not reliable for detecting heteroresistant subpopulations (76).

(ii) Phoenix automated microbiology system. The BD Phoenix automated microbiology system (BD Diagnostics) has a large incubator reader. Panels test colistin concentrations ranging from 0.5 to 4 μg/ml, and the inoculation is manual or automatic (84). MIC results are generated in 6 to 16 h. No study has evaluated the performance of Phoenix for detection of colistin resistance among Gram-negative bacteria. The only published study evaluating polymyxin susceptibility by using the Phoenix system unfortunately did not include colistin-resistant strains (85). Nevertheless, we recently evaluated the accuracy of this system by testing 100 enterobacterial isolates (60 colistin-resistant and 40 colistin-susceptible isolates) and found a high rate (15%) of

false-susceptible results. We observed a low sensitivity for detecting colistin heteroresistance in *K. pneumoniae* and *Enterobacter cloacae* isolates (our unpublished data) but a good sensitivity for detecting plasmid-mediated colistin resistance.

(iii) MicroScan system. The MicroScan system (Beckman Coulter Diagnostics) uses microdilution trays with colistin concentrations of 2 and 4 μg/ml. The trays are inoculated manually and incubated in the instrument for 16 to 20 h (84). Compared to dilution methods, the categorical agreement of the MicroScan system is 87% for *Acinetobacter* isolates (86), and the sensitivity is 88% for detection of polymyxin B resistance in *K. pneumoniae* isolates (87).

(iv) Sensititre system. The Sensititre system (Thermo Fisher Scientific) is an automated incubation and reading system (84). The tests are standard broth microdilution panels containing prediluted ranges of lyophilized colistin within the wells (0.12 to 128 μg/ml). Inoculation may be performed by using a Sensititre autoinoculator. Growth is measured after an incubation of 18 to 24 h. A single study has evaluated the Sensititre method, and a 96% categorical agreement with BMD was found, with no false susceptibility results reported (70).

Impact of Materials on MIC Determination

Impact of medium. Polymyxin resistance is regulated by the two-component systems PhoP/PhoQ and PmrA/PmrB (88), which respond to cation (calcium, iron, and magnesium) concentrations and pH variations. These systems are involved in the LPS modifications leading to polymyxin resistance.

There is a high variability of cation concentrations in MH medium depending on the commercial brand, and calcium and magnesium concentrations measured for each brand tested are far below the recommendations of the CLSI (89). This is why the CLSI recommends cation-adjusted MH or supplementation of the culture medium with cations (72, 73).

Iso-Sensitest agar is a well-defined medium with stabilized mineral content that was developed to overcome problems associated with traditional media used for antimicrobial susceptibility tests. Comparison of the agar dilution and Etest methods on MH and Iso-Sentitest agar (76) revealed a lack of detection of the resistant subpopulation of heteroresistant *E. cloacae* isolates for the Etest performed on MH agar, while Iso-Sensitest agar was more sensitive for detecting the resistant subpopulation with both methods (76).

However, a cation-dependent inhibition of antimicrobial activity has been reported for polymyxin antibiotics (90, 91). In fact, it is suspected that the colistin antimicrobial activity might be overestimated if tested using conventional cation-adjusted MH as recommended by the CLSI. Note that the calcium concentration recommended by the CLSI for determining colistin susceptibility *in vitro* is 2-fold higher than the concentration found in human interstitial space fluid *in vivo* (92). A recent study revealed that the MIC of colistin might be misestimated if tested with conventional cation-adjusted growth media (overestimation for *P. aeruginosa* and *A. baumannii* and underestimation for *E. coli*) (92). The use of cation-adjusted or non-cation-adjusted medium therefore remains questionable, and a consensus is still needed.

Impact of powder composition. MIC testing is performed using commercially available polymyxin B and colistin sulfate powders. The variability in the relative proportions of the mixture components between powder batches and manufacturers is a potential source of variability of the results (93, 94). In parallel, MICs obtained using BMD with purified forms of the major compounds of polymyxin B were within a $\log_2$ dilution of the MICs obtained using the U.S. Pharmacopoeia polymyxin B sulfate powder mixture (95). These data suggest that the powder composition may not have an impact on polymyxin susceptibility testing. Note that CMS, as a prodrug, cannot be used for susceptibility testing, as it yields erroneously high MIC values (96).

Impact of the composition and treatment of plates. Due to their cationic properties, polymyxins adhere to the negative charges of the microtiter trays commonly used for BMD. Karvanen et al. (97) measured the colistin concentrations following incubation

in polypropylene, polystyrene, and glass tubes. The adsorption was proportionally higher at lower concentrations of the drug. Consequently, the results of colistin BMD measurements significantly differ if tests are conducted in microtiter plates with different coated wells (98). The amount of colistin adsorbed to the plate surface therefore depends on various factors, such as the coating applied to the plate, and is not consistent from well to well (K. Sei, presented at the January 2012 Meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing, Tempe, AZ, 22 to 24 January 2012). Since the nature and treatment of plastics are not addressed in the CLSI recommendations, significant variability is observed between laboratories performing the reference BMD method.

Presence or absence of P-80. Polysorbate 80 (P-80 or Tween 80) is a surfactant used for the preparation of BMD panels used for susceptibility testing (99). This surfactant has been recommended by the CLSI to prevent or at least mitigate binding of lipoglycopeptides to plastics (72, 73). The presence of 0.002% P-80 mitigates colistin adsorption to polystyrene microplates (Sei, presented at the January 2012 Meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing). When P-80 is added to a final concentration of 0.002% in the well, the polymyxin MICs are 4- to 8-fold lower than those obtained without P-80 among isolates with low MICs by BMD testing (70, 99).

It is noteworthy that the effect of P-80 on bacterial viability has not been well evaluated. Also, another concern is that P-80 may act synergistically with polymyxins, consequently giving artificially lower MICs (100). Also, in *P. aeruginosa*, P-80 increases cell permeability and lyses spheroblasts (101). On the other hand, polymyxins destabilize the outer membrane, allowing P-80 to access the inner membrane and induce cell lysis. Therefore, isolates resistant to polymyxins would not be affected significantly by P-80. Therefore, only isolates with polymyxin MICs of ≤ 1 μg/ml might be affected (94).

In January 2014, the CLSI subcommittee decided to pursue a recommendation of polymyxin BMD testing without P-80. However, if a susceptibility breakpoint of ≤ 1 μg/ml is chosen, the ability to detect susceptible isolates without using P-80 may be compromised (94). Since the use of P-80 is still questionable, a solution might be to determine MICs in glass plates, as colistin fixation on glass is less extensive (94). However, glass plates are fragile and expensive.

Impacts of Subcultures and Storage on MICs

Impact of subcultures. A study by Li et al. (26) revealed a loss of colistin resistance when resistant isolates were subcultured without selective pressure. For instance, about 98% of a colistin-resistant *A. baumannii* population lost the resistance phenotype after a single passage in a colistin-free medium.

Impact of storage. A loss of colistin resistance was also observed after 6 to 8 months of storage at -70°C (70). Among 25 isolates that initially tested resistant by a dilution method, five (20%) tested susceptible by the same method after freezing. The availability of easy, rapid, and inexpensive techniques allowing screening of colistin resistance on fresh cultures in routine laboratories is consequently a real clinical need.

Interpretive Criteria

There is a lack of consensus between the two organizations setting up breakpoints for polymyxins, namely, the CLSI in the United States (72) and EUCAST in Europe (71). The zone diameter and MIC interpretive criteria given by those two organizations for colistin and polymyxin B are shown in Table 1. However, recent data related to PK suggest that the current breakpoints might be too high (21).

Quality Controls

Quality control organisms are required during susceptibility testing in order to ensure accuracy and standardization of the procedures. Quality control can be assessed using the *E. coli* ATCC 25922 (NCTC 12241; CIP 76.24) and *P. aeruginosa* ATCC 27853

TABLE 1 Colistin and polymyxin B breakpoints according to CLSI and EUCAST in 2014

Criteria and bacterial group	Colistin					
	Disk content (μg)	Zone diam interpretative criteria (mm)[a]		MIC interpretative criteria (μg/ml)[a]		
		Sensitive (S)	Resistant (R)	S	Intermediate (I)	R
CLSI criteria						
Enterobacteriaceae		—	—	—	—	—
Acinetobacter spp.		—	—	≤2	—	≥4
Pseudomonas spp.	10	≥11	≤10	≤2	4	≥8
EUCAST criteria						
Enterobacteriaceae	50	≥18[b,c]	<15[b,c]	≤2	—	>2
Acinetobacter spp.		—[c]	—[c]	≤2	—	>2
Pseudomonas spp.		—[c]	—[c]	≤4	—	>4

[a]—, not determined or absent.
[b]Zone diameter interpretative criteria for *Enterobacteriaceae* given by EUCAST are only for veterinary medicine; M from 15 to 18 mm.
[c]No zone diameter interpretative criteria for human medicine; the MIC must be determined before use.

(NCTC 12903; CIP 76110) reference strains. The disk diffusion and MIC quality control ranges for these strains determined by the CLSI are shown in Table 2 (72).

Correlation between MICs of Colistin and Polymyxin B

Despite the high similarity of the molecular structures of colistin and polymyxin B, a recent study including 15,377 Gram-negative bacteria revealed differences between the MICs of colistin and polymyxin B (102). MIC values determined by the Sensititre system were 2-fold higher for polymyxin B than for colistin for 55% and 53% of *Klebsiella* species isolates (n = 4,177) and *E. coli* isolates (n = 6,311), respectively. However, a categorical agreement of >99% was obtained for enterobacterial strains when breakpoints of ≤2/≥4 for both colistin and polymyxin B were applied. That study showed a high level of agreement between MICs of colistin and polymyxin B for *P. aeruginosa* and *Acinetobacter* spp.

Qualitative Detection Techniques

Rapid detection of heterogeneous populations among colistin-resistant Gram-negative bacteria by use of capillary electrophoresis. Sautrey et al. (103) proposed a capillary electrophoresis method for rapid detection of heterogeneous populations of colistin-resistant strains. However, further development is required for such applications to be used in clinical laboratories on a daily basis.

Rapid detection of colistin-resistant *A. baumannii* isolates by use of the Micromax assay. The Micromax assay is based on the detection of released nucleotides, indicating cell wall damage, in the presence of colistin (104). After incubation with 0.5 μg/ml of colistin, strains are considered resistant to colistin if ≤11% of bacteria present cell wall damage. Bacteria are incubated for 90 min in Mueller-Hinton broth to achieve exponential growth and then incubated for 60 min with colistin at concentrations of 0 and 0.5 μg/ml, respectively. Bacteria embedded in agarose are incubated with a lysis solution removing only weakened cell walls. The released fragmented DNA may be stained with the fluorochrome SYBR gold (Molecular Probes, Eugene, OR) and visualized by fluorescence microscopy (45 min to 60 min of technical processing and scoring under the microscope). This method is faster than the routine automatic microdilution

TABLE 2 Zone diameter and MIC quality control ranges for polymyxins according to CLSI guidelines

Strain	Zone diam (mm) range		MIC (μg/ml) range	
	Colistin	Polymyxin B	Colistin	Polymyxin B
E. coli ATCC 25922	11–17	13–19	0.25–2	0.25–2
P. aeruginosa ATCC 27853	11–17	14–18	0.5–4	0.5–2

procedure (3 h 30 min versus 6 to 8 h) and is accurate for detecting colistin resistance in *A. baumannii* (100% sensitivity and 96% specificity). Another advantage is that it can be automated. However, the manual task and the cost of the materials (fluorochrome and epifluorescence microscope) are disadvantages for its routine use.

Rapid detection of colistin-resistant *Enterobacteriaceae* isolates by use of the Rapid Polymyxin NP test. We developed the Rapid Polymyxin NP test, which is based on the detection of bacterial growth in the presence of a defined polymyxin concentration (105). Detection is based on detection of glucose metabolism upon bacterial growth. Glucose metabolism induces the formation of acid, leading to a color change of the red phenol used as a pH indicator. The test is performed with a final concentration of bacteria of ca. 10^8 CFU/ml in each well (or tube), and the final concentration of polymyxin is 3.75 μg/ml. Visual inspection of the tray is made after 10 min and then every hour for 2 h. The test is considered positive (indicating polymyxin resistance) if the isolate grows in the presence of colistin (color change from orange to yellow), while it is considered negative (indicating polymyxin susceptibility) if the isolate does not grow in the presence of polymyxin (no color change). This test is rapid (less than 2 h) and easy to perform.

By testing a total of 200 enterobacterial isolates exhibiting either resistance (intrinsic or acquired, or various) or susceptibility to polymyxins, the specificity and sensitivity of this test were evaluated at 99.3% and 95.4%, respectively, compared to BMD as the reference method (105). Note that the Rapid Polymyxin NP test identified the isolates exhibiting a heteroresistance phenotype as well as those producing the plasmid-mediated MCR-1 determinant (see below).

For the Rapid Polymyxin NP test, the adequate culture media for culturing the bacteria prior to the test were Mueller-Hinton agar, Luria-Bertani agar, Columbia agar plus 5% sheep blood, chocolate agar, UriSelect 4 agar, and eosin methylene blue agar.

The Rapid Polymyxin NP test may also detect colistin-resistant *Enterobacteriaceae* directly from blood cultures (106). Results are obtained within 4 h.

Selective medium. So far, no selective medium allowing screening for any type of polymyxin-resistant Gram-negative isolates (with intrinsic, chromosomally encoded, or plasmid-mediated polymyxin resistance) has been available. Neither commercial nor in-house screening culture media had been designed that might permit screening of patients possibly colonized by polymyxin-resistant isolates. Therefore, we developed SuperPolymyxin, a selective culture medium for detection of any type of polymyxin-resistant Gram-negative organism (107). The SuperPolymyxin medium prevents swarming of *Proteus* spp. (intrinsically resistant to polymyxins) and also the growth of Gram-positive bacteria and fungi, by addition of daptomycin and amphotericin B, respectively. Its base corresponds to the eosin methylene blue medium (Levine's medium) (108) selective for Gram-negative bacteria, which differentiates lactose fermenters (black colonies) from nonfermenters (colorless or light lavender colonies). In addition, differentiation of lactose fermenters is also possible to some extent. The SuperPolymyxin medium contains a colistin concentration (3.5 μg/ml) that allows clear categorization between polymyxin-resistant and -susceptible isolates. The sensitivity and specificity of this medium have been found to be 100% (107).

Genotypic Methods

Although the mechanisms underlying resistance to polymyxins have not all been elucidated, acquisition of colistin resistance in Gram-negative bacteria has been attributed to lipopolysaccharide (LPS) modifications via diverse routes, including (i) the addition of cationic groups to the LPS reducing the overall negative charge of the LPS and consequently preventing the fixation of polymyxins; (ii) loss of the LPS and, consequently, loss of the polymyxin target; (iii) the overproduction of capsule polysaccharide (CPS) hiding polymyxin binding sites; and (iv) the release of CPS trapping polymyxins. Specific modifications of outer membrane porins and overexpression of efflux pump systems have also been described (88).

Several molecular mechanisms have been associated with colistin resistance in Gram-negative bacteria, such as alterations in the PmrA/PmrB, PhoP/PhoQ, ParR/ParS, ColR/ColS, and CprR/CprS two-component systems and alterations in the *mgrB* gene, which encodes a negative regulator of PhoPQ. Mutations leading to the addition of cationic groups on lipid A result in a less anionic lipid A and, consequently, to less fixation of polymyxins (88).

Similarly, alterations in the *lpxA*, *lpxC*, and *lpxD* genes of *A. baumannii* result in inactivation of lipid A biosynthesis, leading to a complete loss of LPS and, consequently, to a loss of the polymyxin target (109).

The mechanisms of polymyxin resistance can be identified by sequencing those specific genes. However, molecular techniques cannot be envisioned in the near future considering that (i) many chromosomally encoded mechanisms of resistance remain to be identified, (ii) it is difficult to extrapolate whether some substitutions identified in proteins known to be involved in LPS biosynthesis lead to resistance, and (iii) the levels of expression of the corresponding genes may vary and consequently influence the level of resistance to polymyxins.

There is an exception that corresponds to the recent identification of the plasmid-borne *mcr-1*/*mcr-2* genes, whose products confer resistance to polymyxins (see below). According to the current knowledge on the topic, identification of these genes may be considered a signature of resistance or reduced susceptibility to polymyxins. This is why identifying the gene makes sense in this case, since qualitative genetic results may be translated directly into a nonsusceptibility phenotype. Screening of both *mcr-1* and *mcr-2* can be performed by using a standard PCR protocol using the primers MCR-1/2-Fw (5′-TAT CGC TAT GTG CTA AAG CC-3′) and MCR-1/2-Rv (5′-TCT TGG TAT TTG GCG GTA TC-3′), giving rise to a 715-bp amplicon. Also, a SYBR green-based real-time PCR assay that provides a simple, specific, sensitive, and rapid molecular tool for detection of *mcr-1*-positive isolates was recently published (110). That technique was validated on human and animal isolates and may be applied to extensive surveillance studies.

Porin mutations and overexpression of efflux pump systems may also be involved in colistin resistance (88), and it is very likely that phenotypic resistance to polymyxins in clinical isolates often results from combined resistance mechanisms (e.g., defects in outer membrane proteins combined with structural modification of the LPS). Phenotypic methods, such as the Rapid Polymyxin NP test, are consequently very relevant for determining the subsequent therapeutic decision, since they actually concretely determine the susceptibility or lack thereof of isolates, in contrast to genotypic methods, which detect only potential resistance and require sequencing of multiple genes.

RESISTANCE MECHANISMS IN *ENTEROBACTERIACEAE*

Intrinsic Resistance Mechanisms in *Proteus mirabilis* and *Serratia marcescens*

In *P. mirabilis* and *S. marcescens*, naturally occurring resistance to polymyxins is linked to the constitutive expression of the *arnBCADTEF* operon and/or the *eptB* gene, causing addition of phosphoethanolamine (pEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) cationic groups to the LPS. This modification increases the charge of the LPS, which is the initial target of the polymyxins, and therefore decreases polymyxin binding, giving rise to intrinsic resistance of these species (111–113).

Mechanisms Responsible for Acquired Resistance in *Enterobacteriaceae*

Acquired resistance to polymyxins has been identified in several genera of the *Enterobacteriaceae*, such as *Klebsiella*, *Escherichia*, *Enterobacter*, and *Salmonella*. Colistin resistance mechanisms remain unknown for some bacterial species, but several molecular mechanisms have been identified. The most common is modification of the LPS via cationic substitution, similar to that observed in bacteria with intrinsic resistance to polymyxins. A single transferable mechanism of resistance has been identified so far (see below), with most of the resistance mechanisms being encoded chromosomally.

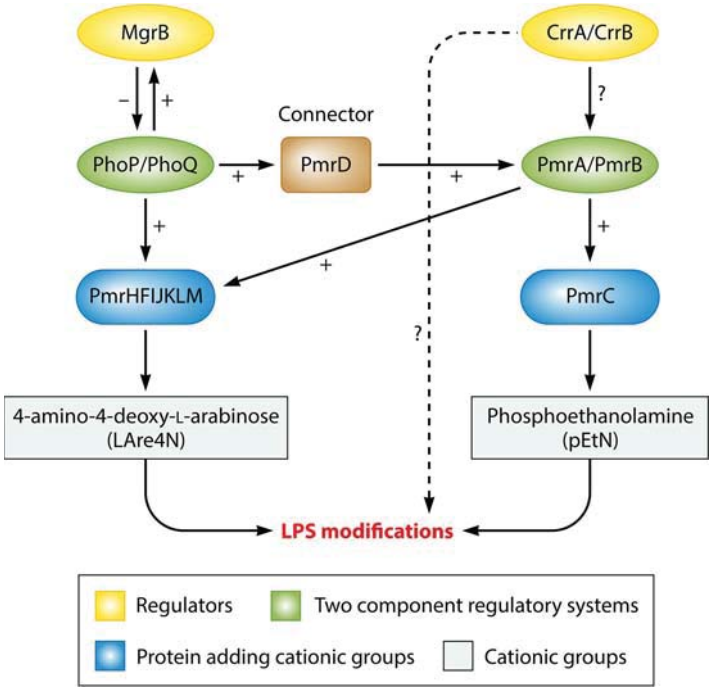

FIG 3 Regulation pathways of LPS modifications in *Klebsiella pneumoniae*.

Similar to what is observed in strains that are naturally resistant to colistin, addition of cationic groups (L-Ara4N and pEtN) to the LPS is responsible for acquisition of colistin resistance in *Enterobacteriaceae*. A large panel of genes and operons are involved in qualitative modification of the LPS (Fig. 3), including genes and operons coding for enzymes that are directly involved in LPS modifications (genes responsible for synthesis of cationic groups and/or their addition to the LPS), i.e., the *pmrC* gene, the *pmrE* gene, and the *pmrHFIJKLM* operon; regulatory genes, such as those encoding proteins involved in the PmrAB and PhoPQ two-component systems; and the regulators of these two-component systems, i.e., the *mgrB* gene, which negatively regulates the PhoPQ system, and the newly described *crrAB* two-component regulatory system, which regulates the PmrAB system.

Genes encoding LPS-modifying enzymes. (i) The *pmrC* gene. The *pmrCAB* operon codes for three proteins, namely, the phosphoethanolamine (pEtN) phosphotransferase PmrC, the response regulator PmrA (also called BasR), and the sensor kinase protein PmrB (also called BasS) (114). The phosphoethanolamine phosphotransferase PmrC adds a pEtN group to the LPS (Fig. 3) (114).

(ii) The *pmrHFIJKLM* operon and the *pmrE* gene. The *pmrHFIJKLM* operon (also called the *arnBCADTEF* or *pbgPE* operon) codes for a total of seven proteins (115). The *pmrE* gene and the *pmrHFIJKLM* operon are responsible for the synthesis of the L-aminoarabinose group (L-Ara4N) and its fixation to lipid A (Fig. 3) (115).

(iii) The *pmrA* and *pmrB* genes, which encode the PmrAB two-component system. Environmental stimuli, such as macrophage phagosomes, ferric (Fe^{3+}) iron, aluminum (Al^{3+}), and low pH (e.g., pH 5.5), mediate activation of PmrB through its periplasmic domain (114). The PmrAB and PhoPQ two-component systems are normally activated when bacteria are phagocytized into macrophages, allowing bacterial survival (114).

PmrB is a protein with tyrosine kinase activity that activates PmrA by phosphorylation. PmrA in turn activates the transcription of the *pmrCAB* operon, the *pmrHFIJKLM* operon, and the *pmrE* gene involved in LPS modification (pEtN and L-Ara4N addition) (Fig. 3) (114).

Specific mutations within the *pmrA* and *pmrB* genes have been described as being responsible for acquired colistin resistance in *K. pneumoniae* (105, 116–120), *Enterobac-*

ter aerogenes (121), and *Salmonella enterica* (122, 123) (Table 3). These mutations are responsible for constitutive activation of the PmrAB two-component system, leading to upregulation of the *pmrCAB* operon, the *pmrHFIJKLM* operon, and the *pmrE* gene, and thus to the synthesis of pEtN and L-Ara4N and their transfer to lipid A (Fig. 3).

Some polymorphism in the *pmrAB* genes of colistin-resistant *E. coli* has been reported (124, 125), but the involvement of these mutations in the colistin resistance phenotype has not formally been demonstrated, since no complementation or site-directed mutagenesis has been performed.

(iv) The *phoP* and *phoQ* genes, which encode the PhoPQ two-component system. The *phoPQ* operon codes for two proteins, namely, the regulator protein PhoP and the sensor protein kinase PhoQ. Environmental stimuli, such as macrophage phagosomes, low magnesium (Mg^{2+}), and low pH (e.g., pH 5.5), mediate activation of PhoQ through its periplasmic domain (114). The PhoPQ two-component system allows the expression of genes that code for magnesium transport, enzymes that modify the LPS to allow resistance to cationic antimicrobial peptides, and enzymes that decrease the cell stress caused by acidic pH or some virulence factors (126, 127). The PhoPQ two-component system therefore allows bacterial survival under conditions of low magnesium or acidic pH or in the presence of cationic antimicrobial peptides.

PhoQ is a protein with tyrosine kinase activity that activates PhoP by phosphorylation. PhoP in turn activates the transcription of the *pmrHFIJKLM* operon, involved in the addition of L-Ara4N to the LPS (Fig. 3) (126, 127). PhoP can also activate the PmrA protein, either directly or indirectly via the PmrD connector protein, causing the addition of pEtN to the LPS.

Several mutations in the *phoP* and *phoQ* genes are responsible for acquired resistance to polymyxins in *K. pneumoniae* (81, 105, 117, 118, 120, 128) (Table 3). One mutation potentially involved in colistin resistance in *E. coli* has also been described (65). These mutations are responsible for constitutive activation of the PhoPQ two-component system, leading to upregulation of the *pmrHFIJKLM* operon and thus to the synthesis of L-Ara4N and its transfer to lipid A (Fig. 3).

Regulators of the PmrAB and PhoPQ Two-Component Systems

The *mgrB* gene. MgrB (also called YobG) is a small transmembrane protein of 47 amino acids (129). Upon activation of PhoP, the *mgrB* gene is upregulated. The MgrB protein in turn represses the expression of the PhoQ-encoding gene, leading to negative regulation of the PhoPQ two-component system (Fig. 3) (129). Inactivation of the *mgrB* gene (the negative regulator of the PhoPQ two-component system) leads to overexpression of the *phoPQ* operon, thus causing *pmrHFIJKLM* operon activation, leading to the production of L-Ara4N responsible for the acquisition of colistin resistance.

Several missense mutations resulting in amino acid substitutions and nonsense mutations and therefore leading to a truncated MgrB protein may be responsible for acquired resistance to colistin in *K. pneumoniae* (Table 3). Other alterations, such as insertions or deletions of small nucleotide sequences in the *mgrB* gene, or even some complete deletions of the *mgrB* locus, have been reported (120, 130, 131). Insertional inactivation caused by diverse insertion sequences (IS), belonging to several families and inserted at different locations within the *mgrB* gene, is often responsible for acquired resistance to colistin in *K. pneumoniae* (105, 117, 120, 130–132) and *Klebsiella oxytoca* (133, 134). Recently, the transposition of genes encoding extended-spectrum β-lactamases (ESBLs) or carbapenemases, leading to disruption of the chromosomal *mgrB* gene, was reported as a source of resistance to colistin (135, 136). Notably, selective pressure with β-lactams leading to the acquisition of β-lactamase genes may therefore be responsible for coselection of colistin resistance. Despite the high homology observed among *mgrB* gene sequences of *Enterobacteriaceae* organisms (129), disruption of this gene has so far not been found to be responsible for acquired resistance to colistin in genera other than *Klebsiella*.

The *crrAB* operon. The *crrAB* (colistin resistance regulation) operon codes for two proteins, namely, the regulatory protein CrrA and the sensor protein kinase CrrB. The

TABLE 3 Chromosomal mutations and amino acid deletions responsible for acquired colistin resistance in *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella enterica*, *P. aeruginosa*, and *A. baumannii* isolates

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues)[a,b]	Amino acid change[d]	Reference(s)
Enterobacteriaceae				
K. pneumoniae	PmrA (223)	REC (1–112)	S42N	120
			G53C	105, 120
			G53S	105
		Trans_reg_C (145–216)		
	PmrB (365)	TM (13–35)	ΔR14	118
			L17Q	105
		HAMP (90–142)	L82R	116
			S85R	120
			T140P	120
		HisKA (143–203)	T157P	117–119
			S208N	118
			ΔY209	118
		HATPase_c (250–358)	R256G	117
	PhoP (223)	REC (1–112)	V3F	117
			L26Q	120
			S86L	117
		Trans_reg_C (145–220)	D191Y	81
	PhoQ (488)	PhoQ sensor (10–189)	R16C	105
			L26P	117
			L96P	120
			D150G	117
			S174N	118
		HAMP (195–263)	V258F	117
		HisKA (267–330)		
			L348Q	120
		HATPase_c (375–482)	G385S	120
			D434N	128
	MgrB (47)		K3*	105
			L9*	120
			I13*	120
			A14S	120
			W20R	105
			L24H	130
			V26*	120
			M27K	105
			C28F	120
			C28Y	117, 120, 128, 130
			C28*	105, 120
			Q30*	105, 120
			D31N	120
			Q33*	105
			F35I	120
			G37S	130
			C39Y	105
			N42Y/K43I	105
			I45T	105
			W47R	105
			W47*	105
			*48Y	117
	CrrB (353)		Q10L	128, 137
		TM (12–34)	Y31H	137
		HAMP (81–135)	L94 M	128
		HisKA (136–200)	W140R	137
			N141I	137
			P151S	137
			S195N	137
E. aerogenes	PmrA		G53S	121
E. coli	PmrA (222)	REC (1–112)	R81S[c]	125
		Trans_reg_C (145–216)		
	PmrB (363)		Δ7–12[c]	124
		TM1 (15–37)		
		TM2 (69–91)		

(Continued on next page)

TABLE 3 (Continued)

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues)[a,b]	Amino acid change[d]	Reference(s)
		HAMP (92–144)		
		HisKA (145–205)	T156K[c]	124
			A159V[c]	124
			V161G[c]	125
		HATPase_c (252–360)		
		PhoQ_sensor (10–189)		
		HAMP (195–263)		
		HisKA (267–330)		
	PhoP (223)	REC (1–112)		
		Trans_reg_C (145–220)		
	PhoQ (486)	PhoQ_sensor (10–189)		
		HAMP (195–263)		
		HisKA (267–330)		
		HATPase_c (374–480)	E375K[c]	65
S. enterica	PmrA (222)	REC (1–112)	G15R[c]	123
			G53E[c]	123
			G53R[c]	123
			R81C[c]	123
			R81H[c]	123
		Trans_reg_C (145–216)		
	PmrB (356)	TM (13–35)	Δ11–14[c]	122
			L14F[c]	123
			L14S[c]	123
			M15L[c]	122
			L22P[c]	123
			S29R[c]	123
		HAMP (89–141)	T92A[c]	123
			P94Q[c]	123
			E121A[c]	123
			S124P[c]	123
			N130Y[c]	123
		HisKA (142–202)	T147P[c]	123
			R155P[c]	123
			T156M[c]	123
			T156P[c]	123
			V161G[c]	123
			V161L[c]	123
			V161M[c]	123
			E166K[c]	123
			M186I[c]	123
		HATPase_c (249–356)	G206R[c]	123
			G206W[c]	123
			S305R[c]	123
Nonfermentative bacilli				
P. aeruginosa	PmrA (221)	REC (1–112)		
		Trans_reg_C (145–216)	L157Q	166
	PmrB (477)		L14P	167
		TM1 (15–37)		
		PD (38–160)	ΔD45	74, 167
			A54V	167
		TM2 (161–183)	L167P	166
		HAMP (186–238)	G188D	167
			F237L	118
		HisKA (239–304)	L243Q	167
			A247T	168
			A248V	167
			S257N	167
			M292I	167
			M292T	169
		HATPase_c (348–459)		
	PhoQ (448)		R6C	170
		TM1 (7–29)		

(Continued on next page)

TABLE 3 (Continued)

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues)[a,b]	Amino acid change[d]	Reference(s)
			ΔV57–Q332	170
		PD (30–166)	N104I	118
			K123Q	166
			K123E	118
			Q133E	118
			A143V	166
			V152*	168
		TM2 (167–189)	V184G	118
			A207R	118
			R214H	118
			H223R	168
		HisKA (238–300)	V260G	163, 247
		HATPase_c (343–448)	ΔL364–G365	170
			I421*	170
			Fr at I421	170
			D433*	170
			R444C	170
	ParR (235)	REC (7–117)	L18I	118
			N24S	118
			S24N	118
			M59I	171
		Trans_reg_C (152–228)	E156K	171
	ParS (428)	TM1 (5–27)	L14Q	171
		PD (28–131)	V101 M	171
		TM2 (132–154)	L137P	171
		HAMP (155–207)		
		HisKA (208–273)	Q232E	118
		HATPase_c (318–428)	G361R	118
			H398R	247
	ColS		A106V	172
	CprS		R241C	172
A. baumannii	PmrA (224)	REC (2–112)	E8D	177, 180
			M12I	174
			P102H	173
			S119T	174
		Trans_reg_C (150–221)		
	PmrB (444)	TM1 (10–29)	T13N	173
			S14L	175
			S17R	177
			Fr at F26	176
		PD (30–141)	ΔA32–E35	174
			D64V	174
			A80V	174
			L87F	175
			Y116H	177
			I121F	178
		TM2 (142–164)	M145K	175
			ΔL160	174
			P170L	174, 179
			P170Q	174
			A183T	178
			A184V	178
			P190S	178
			T192I	178
			L208F	174
		HisKA (218–280)	A226V	174
			A227V	173, 175, 176
			Q228P	178
			R231L	174
			T232I	177
			P233S	118, 173–176, 179
			P233T	173
			T235I	174
			N256I	174

(Continued on next page)

TABLE 3 (Continued)

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues)[a,b]	Amino acid change[d]	Reference(s)
			A262P	173
			R263C	174
			R263L	177
			R263P	174
			Q277H	174
			G315D	174
		HATPase_c (326–437)	N353Y	175
			P377L	174
			F387Y	175
			S403F	175
	LpxA (262)		Fr at I25	109
			G68D	109
			Q72K	109
			Fr at H121	109
			Fr at D130	109
			H159D	109
			Q234*	109
	LpxC (276)		P30L	109
			Fr at D45	109
			Fr at T285	109
	LpxD (356)		Fr at K317	109

[a]Domains predicted in SMART by using protein sequences of *Escherichia coli* K-12 substrain MG1655, *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578, *Salmonella enterica* serovar Typhimurium LT2, *P. aeruginosa* PAO1, and *A. baumannii* AB0057. REC, CheY-homologous receiver domain; Trans_reg_C, transcriptional regulatory protein, C-terminal domain; TM, transmembrane domain; TM1, first transmembrane domain; TM2, second transmembrane domain; PD, periplasmic domain; HAMP, histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases; HisKA, histidine kinase A (phosphoacceptor) domain; HATPase_c, histidine kinase-like ATPases; PhoQ sensor, phosphorelay signal transduction system.
[b]A periplasmic domain (PD) was not predicted in SMART but was assumed to be between TM1 and TM2.
[c]The involvement of the mutation in the colistin resistance profile was determined by *in silico* analysis.
[d]Δ, deletion; Fr, frameshift; *, stop codon.

physiological role of the *crrAB* operon is still unknown. However, inactivation of the *crrB* gene leads to overexpression of the *pmrAB* operon, thus causing activation of the *pmrHFIJKLM* operon and of the *pmrC* and *pmrE* genes, consequently leading to the production of L-Ara4N and pEtN, both of which are responsible for the acquisition of colistin resistance (Fig. 3) (128). CrrB inactivation may also modify lipid A through activation of a glycosyltransferase-like protein (128).

Six amino acid substitutions in the CrrB protein have been identified as being responsible for acquired resistance to polymyxins in *K. pneumoniae* (Table 3) (128, 137).

The Intrinsic Regulator RamA

The intrinsic regulator RamA of *K. pneumoniae* is known to play a significant role in the overall response to antimicrobials. It regulates genes that are linked to permeability barriers and therefore may be involved in reduced susceptibility to antibiotics. It was recently shown that increased levels of this regulator caused LPS alterations and consequently reduced susceptibility to polymyxins (138).

Plasmid-Mediated Resistance to Polymyxins

The plasmid-mediated *mcr-1* gene, responsible for horizontal transfer of colistin resistance, was described first for *E. coli* and *K. pneumoniae* isolates recovered in China between 2011 and 2014 (139). The encoded MCR-1 protein is a member of the phosphoethanolamine transferase enzyme family, as its acquisition results in the addition of phosphoethanolamine to lipid A, and consequently in a more cationic LPS, similarly to the chromosomal mutations mentioned above.

Overall, production of MCR-1 in *E. coli* leads to 4- to 8-fold increases of the MICs of polymyxins. Therefore, without additional resistance mechanisms, production of MCR-1 is enough to confer resistance to colistin in *E. coli* and other enterobacterial species, such as

K. pneumoniae (our unpublished data). Note that despite the fact that polymyxins actually share the same mechanism of action as that of the cationic antimicrobial peptides (CAMPs) cathelicidin LL-37, α-defensin 5 (HD5), and β-defensin 3 (HDB3), which are normal components of the immune system, coresistance to CAMPs and polymyxins has not beeen observed (J. Dobias, L. Poirel, and P. Nordmann, submitted for publication).

Apart from resistance to polymyxin antibiotics, production of MCR-1 was shown to confer resistance to lysozyme (140). The structure of MCR-1 was recently solved at a 1.32-Å resolution, revealing that its active site is similar to that of related phosphoethanolamine transferases (141). Threonine 285 was identified as the putative nucleophile for catalysis, as it was phosphorylated in the catalytic domain of MCR-1 (cMCR-1). Four zinc ions were identified in the active site of cMCR-1, which is thus a metalloenzyme. The binding sites for the lipid A and phosphatidylethanolamine substrates were not apparent in the cMCR-1 structure, likely indicating that they were present in the membrane domain.

Following these initial findings, the *mcr-1* gene was reported worldwide and beyond China, on all continents (Fig. 4; Table 4). The earliest *mcr-1*-positive strain was collected from chickens in China 3 decades ago (142), when colistin first started to be used in food-producing animals. The *mcr-1* gene has been found in various genera of the *Enterobacteriaceae* (*Escherichia*, *Klebsiella*, *Enterobacter*, *Cronobacter*, *Salmonella*, *Shigella*, and *Kluyvera*) isolated from the environment, vegetable and meat foods, animals, and human beings (Fig. 4; Table 4). Note that the occurrence of MCR-1-producing *E. coli* in the environment in Switzerland but also in Asian imported vegetables in the same country highlights the likely wide occurrence of that resistance trait in many different environments (143).

The hypothesis that animals, particularly pigs and cattle, might be a main source of MCR-1 producers is very strong. Indeed, several features are in accordance with such a hypothesis, including the high selective pressure in veterinary practice and the wide occurrence of that resistance trait in isolates recovered from animals (144).

The genetics of acquisition of the *mcr-1* gene has been investigated extensively. This gene was found in plasmids possessing various backbones (IncI2, IncHI2, IncP, IncX4, IncFI, and IncFIB) and of various sizes (58 to 251 kb). Upstream of the *mcr-1* gene, the IS*Apl1* insertion sequence element is inconstantly identified (Table 4) (139). Thanh et al. (145) described an *mcr-1* gene disrupted by a 22-bp duplication in a *Shigella sonnei* isolate. This isolate was colistin susceptible, but under selective pressure with colistin, one copy of the 22-bp tandem repeat could be deleted, restoring the open reading frame of *mcr-1* and leading to colistin resistance. This deactivated version of the colistin resistance gene *mcr-1* suggests a fitness cost for the active *mcr-1* gene. Some but not all plasmids bearing the *mcr-1* gene carry other antimicrobial resistance genes encoding resistance to clinically relevant antibiotics for human medicine, such as β-lactams, aminoglycosides, quinolones, fosfomycin, sulfonamides, and tetracyclines. The location of the *mcr-1* gene on multidrug resistance plasmids is worrying because the use of antimicrobials other than polymyxins can participate in the coselection of isolates carrying *mcr-1* and in their spread. More worryingly, the plasmid-mediated *mcr-1* gene has been identified in highly drug-resistant *Enterobacteriaceae* isolates harboring plasmids encoding carbapenemase genes ($bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$, $bla_{\text{NDM-9}}$, $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{VIM-1}}$) (146–152). Note that the *mcr-1* gene was recently identified on the chromosome of an *E. coli* strain in Switzerland, suggesting that this resistance gene might be integrated and therefore stabilized in the genome in some isolates (153).

Further investigations are required to better understand the process of acquisition of the *mcr-1* gene; however, we recently showed that it was located within a 2,600-bp genetic structure, defined as the "*mcr-1* cassette," that might have been mobilized by transposition (154). The cassette was found to carry its own promoter sequences driving the expression of *mcr-1*. In addition, it was shown that several isolates may possess the *mcr-1* gene located in a composite transposon structure made of two copies of IS*Apl1* (155). However, that structure has not been identified systematically, and therefore further investigations are still required to better understand the process

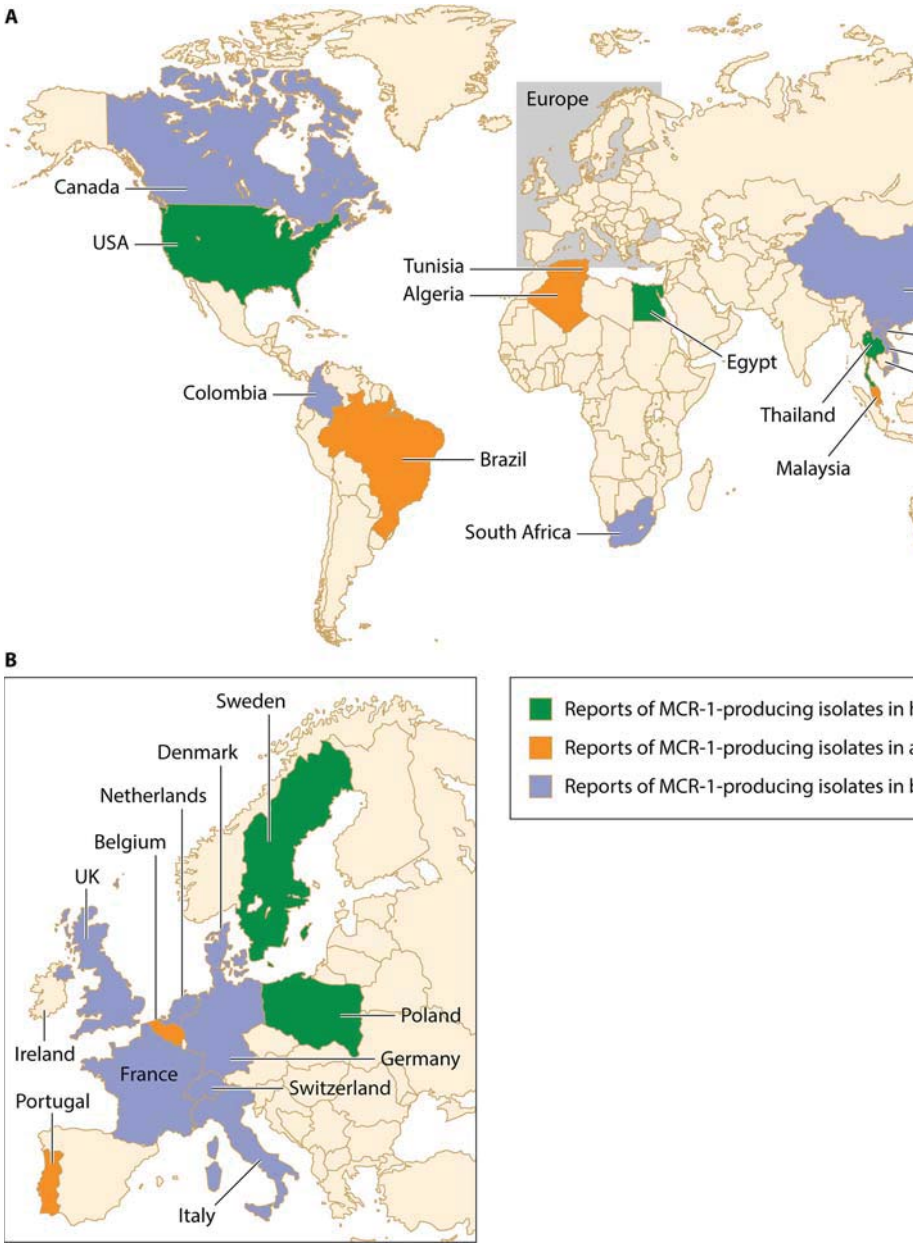

FIG 4 Reports of MCR-1-producing isolates in humans, animals, and both humans and animals.

of acquisition of that gene from an unknown progenitor in plasmids replicating in *Enterobacteriaceae*.

MCR-1-producing enterobacterial isolates have often been identified as colonizers in either humans or animals. Nevertheless, there are some reported cases of infections, including two patients with bacteremia in Switzerland (156).

A functional variant of *mcr-1* (Q3L) encoding MCR-1.2 was detected in KPC-3-producing *K. pneumoniae* in Italy (157), likely sharing the same activity as MCR-1.

In addition, the plasmid-mediated colistin resistance gene *mcr-2* was identified in *E. coli* strains recovered from piglets in Belgium (158). It shared 77% nucleotide identity with *mcr-1* and was carried on an IncX4 plasmid.

Other Mechanisms Contributing to Polymyxin Resistance in *Enterobacteriaceae*

Hyperproduction of CPS. A study showed that the capsule polysaccharide (CPS) acts as a protective barrier against polymyxins in *K. pneumoniae* (159). The upregulation

TABLE 4 Worldwide reports of *Enterobacteriaceae* isolates harboring a plasmid-mediated *mcr-1* gene

Species	Country of isolation	Country of origin or traveled region	Sample origin	Period (yr)	Plasmid features[a]				Reference(s)
					Inc type	Size (kb)	Mobile element	Other antimicrobial resistance(s)	
E. coli	China	China	Chickens	1980–2014	—	—	—	—	142
	China	China	Human (fecal carriage)	Before 2011	—	—	—	—	218, 219
	China	China	Chickens, pigs, and humans (infections)	2011–2014	IncI2	64	IS*Apl1*	No	139
	China	China		2011–2014	IncHI2	251	IS*Apl1*	Resistance to cefotaxime ($bla_{CTX\text{-}M\text{-}14}$), aminoglycosides, florfenicol, olaquindox, cotrimoxazole, fosfomycin (*fosA3*), and ciprofloxacin (*oqxAB*)	220
	China	China	Chicken meat	2014	IncI2	65	—		146
	China	China	Human (blood)	2014–2015	—	—	—	—	147
	China	China	Human (fecal carriage)	2015	—	—	—	—	221
	China	USA	Human (fecal carriage)	2016	IncFI	33	—	Resistance to β-lactams ($bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$)	148
	Laos	Laos	Human (fecal carriage) and pigs	2012	—	—	—	—	222
	Thailand	Thailand	Human (fecal carriage)	2012	—	—	—	—	223
	Vietnam	Vietnam	Pigs (fecal carriage)	2014–2015	—	—	IS*Apl1*	Resistance to cefotaxime ($bla_{CTX\text{-}M\text{-}55}$), trimethoprim (*dfrA12*), tetracycline (*tetA*), aminoglycosides [*aadA3*, *aph*(3′)-*Ia*], phenicol (*cmlA1*), quinolones (*qnrS1*, *oqxA*), lincosamides [*inu*(*F*)], and sulfonamides (*sul2*, *sul3*)	224
	Cambodia	Cambodia	Human (feces)	2012	—	—	IS*Apl1*	—	224
	Malaysia	Malaysia	Water, chickens, pigs	2013	—	—	IS*Apl1* or not	—	225
	Japan	Japan	Cattle (mastitis)	2008–2013	IncI2	60–61	—	—	226
	France	France	Veal calves (feces)	2005–2014	IncHI2	—	—	Resistance to cefotaxime ($bla_{CTX\text{-}M\text{-}1}$), sulfonamides, and tetracyclines	227
	France	France	Broiler, turkeys, pigs	2007–2014	—	—	—	—	228
	Italy	Italy	Human (urine, surgical wound)	2013–2015	—	—	—	—	229
	Italy	Italy	Human (rectal swabs)	2015	NT	35	—	—	230
	United Kingdom	United Kingdom or Egypt	Human (blood, stools)	2012–2015	IncHI2, IncI2	—	IS*Apl1* or not	—	231
	Switzerland	—	River water	2012	—	—	—	—	143
	Switzerland	Thailand and Vietnam	Vegetables	2014	—	—	—	—	143

TABLE 4 (Continued)

Species	Country of isolation	Country of origin or traveled region	Sample origin	Period (yr)	Plasmid features[a]				Reference(s)
					Inc type	Size (kb)	Mobile element	Other antimicrobial resistance(s)	
	Netherlands	Travel in Tunisia, South America, China, Southeast Asia	Human (fecal carriage)	2012–2013	—	—	—	—	234
	Netherlands	Germany	Veal valves, broilers, and turkey	2010–2015	IncHI2 or IncX4	—	IS*Apl1*	—	235
	Germany		Pigs and human (wound infection)	2010–2015	IncHI2 or IncX4	—	IS*Apl1* or not	—	150
	Denmark	Europe	Chicken meat	2012–2014	IncI2 or IncX4	—	—	—	236
	Denmark	Denmark	Human (bloodstream infection)	2015	IncI2	—	—	—	236
	Canada	Unknown	Beef meat	2010	IncHI2A	—	—	—	151
	Canada	Lived in Egypt for 5 years	Human (gastrostomy site and rectum)	2011	IncI2	—	—	—	151
	USA	USA	Human (urine)	2016	IncF	225	IS*Apl1*	Resistance to β-lactams ($bla_{CTX\text{-}M\text{-}55}$)	237
	Algeria	Algeria	Chickens	2015	—	—	—	—	222
	Tunisia	Tunisia/France	Chickens	2015	IncHI2	—	—	Resistance to β-lactams ($bla_{CTX\text{-}M\text{-}1}$)	238
	Egypt	Egypt	Human (sputum)	2015	—	>90	—	—	239
	South Africa	South Africa	Human	2014–2015	IncI2	65, 70	IS*Apl1* or not	—	154
					IncHI2	150			154
					IncX4	30			154
	South Africa	South Africa	Broiler chicken	2008–2014	IncI2	62	IS*Apl1*	—	240
	Brazil	Brazil	Chicken, swine	2003–2015	—	—	—	—	241
K. pneumoniae	China	China	Human (surgical wound, peritoneal fluid)	2015	—	—	—	—	147
	China	China	Food animals and human (infections)	2011–2014	IncI2	64	IS*Apl1*	No	139
	Denmark	Denmark	Human (unknown)	2014	—	—	—	—	224
Enterobacter cloacae	China	China	Human (urine)	2014	IncFI	70	—	—	242
Enterobacter aerogenes	China	China	Human (vaginal secretion)	2014	IncFI	65	—	Resistance to β-lactams ($bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$)	242
Cronobacter sakazakii	China	China	Chicken (diarrhea)	2015	IncI2	65	IS*Apl1*	—	152
Salmonella enterica serotype Typhimurium	Portugal	Portugal	Food animals	2011	IncHI2	—	IS*Apl1*	—	218, 225, 243
S. enterica	Japan	Japan	Swine (septicemia)	2013	IncI2	58	—	—	226

of capsular biosynthesis genes indeed reduces the interactions of polymyxins with the bacterial surface, leading to polymyxin resistance.

K. pneumoniae is able to release anionic capsular polysaccharides from its surface (160). This release leads to the trapping of cationic antimicrobial peptides, such as polymyxins, thus decreasing the amount of antibiotic reaching the bacterial surface. The CPS is connected to the bacterial surface through an ionic interaction with the LPS, and this interaction is stabilized by divalent cations (161). As a consequence, the release of CPS in the presence of polymyxins is likely due to perturbation of the cation-dependent bridges between the molecules of LPS.

Role of porins. It has been shown that a periplasmic protein (YdeI) regulated by the PhoPQ and PmrAB two-component systems can interact with the OmpD porin to increase bacterial resistance to polymyxins in *Salmonella enterica* (162).

Role of efflux pumps. The role of efflux in colistin resistance is not well understood, but several studies suggested the involvement of efflux pumps in colistin resistance. Mutations in *kpnEF* and *acrAB*, encoding components of efflux pumps, may actually lead to a 2-fold decrease of the MIC of colistin and increase bacterial survival in the presence of a low concentration of polymyxins (163, 164). Addition to the test medium of low doses of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) decreased the MICs for resistant strains (128- to 512-fold reductions) and partially or completely inhibited the regrowth of resistant subpopulations (165). However, this observation should be considered with caution owing to the nonspecific effect of CCCP on efflux systems, with a likely wider impact on bacterial metabolism.

Mechanisms of Polymyxin Resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

***Pseudomonas aeruginosa*.** The colistin resistance in *P. aeruginosa* is mediated by five two-component systems that regulate LPS modifications. As for the *Enterobacteriaceae*, alterations in the PmrAB (74, 118, 166–169) and PhoPQ (118, 166, 168, 170) two-component systems have been shown to be responsible for acquired resistance to colistin. Mutations in these two-component systems cause constitutive alterations and consequently activate transcription of the *pmrHFIJKLM* operon and the subsequent addition of L-Ara4N to the LPS, finally leading to colistin resistance. Notably, unlike what is observed in *K. pneumoniae*, the colistin resistance mediated by PhoPQ modifications does not depend on the PmrAB system.

Three other two-component systems have been proved to contribute to colistin resistance in *P. aeruginosa*, namely, ParRS, ColRS, and CprRS. The ParRS (polymyxin adaptive resistance) two-component system is involved in adaptative resistance to polymyxins (118, 166, 171). Mutations in this system cause constitutive expression of the *pmrHFIJKLM* operon and thus lead to the addition of L-Ara4N to the LPS, leading to colistin resistance. Additionally, mutations in the ColRS and CprRS two-component regulatory systems may also contribute to polymyxin resistance, since the association of mutations in the *phoQ* gene and mutations in the *colS* or *cprS* gene confers a high level of colistin resistance (172). The action of the ColRS and CprRS systems may occur through the activation of the *phoQ* gene and/or through other genes that have not yet been identified.

***Acinetobacter baumannii*.** The main mechanism of colistin resistance in *A. baumannii* corresponds to the addition of cationic groups to the LPS (qualitative modification of the LPS); nevertheless, acquired resistance to colistin may also be the consequence of a complete loss of LPS production (quantitative modification of the LPS).

The addition of cationic groups in *A. baumannii* is mediated by mutations in PmrAB (118, 173–180). Mutations in the *pmrA* and *pmrB* genes have been shown to cause colistin resistance through upregulation of the *pmrCAB* operon, leading to pEtN synthesis but not to L-Ara4N synthesis (unlike in *Enterobacteriaceae*).

The second mechanism of colistin resistance in *A. baumannii* corresponds to the complete loss of LPS caused by alterations of the lipid A biosynthesis genes, namely, the *lpxA*, *lpxC*, and *lpxD* genes. Mutations identified in those genes were either

substitutions, truncations, frameshifts (109), or insertional inactivation by the insertion sequence IS*Aba11* (181).

EPIDEMIOLOGY OF RESISTANCE TO POLYMYXINS

General Epidemiology of Resistance to Polymyxins

Although polymyxins currently retain significant *in vitro* activity against most Gram-negative organisms, resistance to these antibiotics is increasingly being reported among clinical isolates (29, 182).

The SENTRY antimicrobial surveillance program carried out a worldwide survey in 2009 and reported low rates of resistance to polymyxins among Gram-negative pathogens (*Acinetobacter* spp., *P. aeruginosa*, *E. coli*, and *Klebsiella* spp.) (<0.1% to 1.5%) (183). However, a rising trend was observed in a 2006-2009 study period focusing on *K. pneumoniae* isolates (resistance rates of 1.2% in 2006 and 1.8% in 2009), probably because of the extensive and/or inadequate usage of polymyxins worldwide for treating infections with MDR Gram-negative bacteria.

Colistin resistance in *K. pneumoniae* represents a growing public health concern, since this bacterial species is one of the main pathogens of nosocomial infection and has gathered a wide range of resistance mechanisms to broad-spectrum antibiotics over the years. Table 5 shows the populations studied (mainly carbapenem-resistant *K. pneumoniae* [CR-KP] clinical isolates), along with the methods that have been used to determine the rate of colistin resistance, since some methods are now known to underestimate the level of colistin resistance and therefore may significantly bias the proposed rates. The occurrence of colistin resistance in *K. pneumoniae* has been reported in surveillance studies and clinical case reports worldwide (Table 5) (183). Many studies report an increase of the resistance rate among multidrug-resistant *K. pneumoniae* isolates, particularly among CR-KP isolates, with high colistin resistance rates reported (Table 5). More worryingly, multiple outbreaks with carbapenem- and colistin-resistant isolates have been reported in North America and Europe (Table 6; Fig. 5).

North America. Multicenter surveys showed low rates of resistance among *K. pneumoniae* isolates in Canada (2.9%) (184) and the United States (4%) (185) (Table 5). However, the colistin resistance rate was higher (6.7 to 18%) among carbapenemase-producing isolates (185–187). In addition, outbreaks with colistin-resistant, KPC-producing *K. pneumoniae*, mostly attributed to the international epidemic clone type ST258, have been reported in the United States (188, 189) and Mexico (190) (Table 6; Fig. 5).

South America. The results from the SENTRY antimicrobial surveillance program showed a moderate resistance rate in Latin America in 2009 (3%) (183); however, the emergence of colistin-resistant *K. pneumoniae* isolates has been reported in Argentina (191), Colombia (119), and Brazil (87).

Europe. Multiple outbreaks of both carbapenem- and colistin-resistant *K. pneumoniae* isolates have been reported in Europe (Table 6; Fig. 5). Outbreaks with KPC-producing *K. pneumoniae* isolates attributed to the international epidemic clone type ST258 have been reported in the Netherlands (192), Hungary (193), Greece (194, 195), and Italy (196, 197).

In addition to the two outbreaks attributed to the ST258 clone (196, 197), a large nosocomial outbreak of colistin-resistant and KPC-producing ST512 *K. pneumoniae* isolates was reported in Italy (198) (Table 6; Fig. 5). More worryingly, colistin resistance was recently reported at a high level (>20%) among carbapenemase-producing isolates in ICUs of two Italian hospitals (199, 200), with an even higher rate (36.1%) in hospitals in Rome (68) (Table 6). Moreover, a national study reported a countrywide level of colistin resistance among KPC-producing *K. pneumoniae* isolates, with 43% of isolates being resistant to colistin (201) (Table 5).

In Greece, several outbreaks caused by KPC-producing, colistin-resistant *K. pneumoniae* isolates have been reported (194, 195) (Table 6; Fig. 5). Studies performed in two Greek hospitals reported a huge increase in colistin resistance within a few years (<3.5% incidence before 2010 and >20% incidence after 2010) (202, 203).

TABLE 5 Studies reporting prevalences of colistin resistance among *K. pneumoniae* clinical isolates[d]

Study type and period	Country	Setting	Test method	Total no. of isolates	Carbapenemase producers (no. [%])	Polymyxin-resistant isolates (no. [%])	Reference(s)
Studies reporting colistin resistance rates among overall clinical isolates							
2006–2009	Worldwide	Worldwide surveillance	BMD	9,774	NA	(1.5)	183
2007–2008	Canada	National	BMD	515	NA	15 (2.9)	184
2013–2014	United States	Multicenter	BMD	1,205	NA	(4)	185
2006–2007	South Korea	Multicenter (9 hospitals)	BMD	221[a]	15 (13.3)	24 (10.9)[b]	206
2004–2005	Singapore	Single center	Agar dilution	16	NA	1 (6)	207
Studies reporting colistin resistance rates among CR-KP isolates							
2008–2011	Canada	Multicenter	Etest	30	30 (100)	2 (6.7)	184, 186
2013–2014	United States	Multicenter	BMD	69	69 (100)	(18)[c]	185
2003–2004	United States	Multicenter	BMD or agar dilution	96	96 (100)	(9)	187
2010–2013	Greece	Single center (only ICU)	Vitek2, Etest	92	92 (100)	20 (21.7)	202
2010	Greece	Single center	Vitek2, Etest	120	120 (100)	25 (20.8)	203
2014	Italy	Single center (only ICU)	Vitek2, Etest	214	214 (100)	47 (21.9)	199
2013	Italy	Single center (only ICU)	BMD	25	24 (100)	6 (24.0)	200
2013–2014	Italy	National	BMD	178	178 (100)	76 (43)	201
2010–2011	Italy	Multicenter (9 hospitals)	Vitek2, BMD	97	97 (100)	35 (36.1)	68
2010–2012	Spain	Hospital	Agar dilution	79	79 (100)	18 (22.8)	204
2014	France	National	BMD	561	561 (100)	35 (6.2)	205
2012–2013	Turkey	Single center	Vitek2, Etest	37	36 (98)	(2.7)	245
2006–2007	Israel	Single center	?	88	88 (100)	(4.5)	246
2009–2010	China	Single center	Agar dilution	68	68 (100)	3 (4.4)	208
2012	Taiwan	National	Sensititre	247	55 (22.3)	(12.1)	209

[a]Isolated from blood samples.
[b]Number of colistin-resistant isolates according to EUCAST breakpoints.
[c]Eighteen percent colistin resistance among 4 *E. coli* and 69 *K. pneumoniae* isolates harboring carbapenemases.
[d]NA, not applicable.

TABLE 6 Studies reporting outbreaks of colistin-resistant and carbapenemase-producing isolates[e]

Study period	Country	Setting	Test method	Colistin MIC (μg/ml)	Resistance mechanism	Total no. of cases	Beta-lactamase(s)	Sequence type	Reference
2010	United States	One single hospital (ICU and medical ward)	Agar dilution	>128	ND	5	KPC-2	ST258	188
2009	United States	Two hospitals and a long-term acute care setting in Detroit, MI	Etest	8–64	ND	4	KPC	ND	189
2012–2013	Mexico	One single hospital in Mexico City	BMD	4	ND	15	KPC-2	ST258	190
2013	Netherlands	One hospital and a nursing home	Vitek2, Etest	ND	ND	6	KPC-2, SHV-12	ST258	192
2008–2009	Hungary	Three hospitals in Miskolc	Etest	16–32	ND	8	KPC-2, SHV-12, TEM-1, SHV-11	ST258	193
2008	Greece	ICUs of two distinct hospitals	Etest	12–128	ND	6	KPC-2, SHV-12	ST258	194
2004–2005	Greece	One single hospital in Athens (ICU)	Etest	12–>1,024	ND	13 (multiclonal)	ND	ND	195
2010	Italy	Two hospitals in Catania, Sicily	BMD	8–64	ND	8	KPC-3, SHV-11, TEM-1, OXA-9	ST258	196
2011	Italy	One single hospital in Palermo, Sicily	Etest			28 (multiclonal)[a]			197
				3–128	ND	24	KPC-3, SHV-11, TEM-1, OXA-9	ST258	
				3–32	ND	3	KPC-3, SHV-12, TEM-1	ST273	
				4–12	ND	2	KPC-3, SHV-28, TEM-1, CTX-M-15	ST15	
2010–2013	Italy	One single hospital (22 different wards)	Vitek2, Sensititre	4–>16	*mgrB* Δnt109/119	Multiclonal[b]			198
					Unknown[c]	50	KPC-3	ST512	
					Unknown[c]	5	KPC-3	ST512	
						2	KPC-2	ST101	
2010–2012	Spain	One hospital	Agar dilution	ND	ND	14	VIM-1	ST22	204
2014	France	Hospital in Picardie	BMD	4–64	Unknown[d]	15	OXA-48, CTX-M-15	ST11	205

[a]One patient with two colistin-resistant clones (belonging to ST-258 and ST-273).
[b]There was a total of 93 bloodstream infections, but only isolates recovered in 2013 were investigated further.
[c]No mutation in the *mgrB*, *pmrA*, or *pmrB* gene was responsible for colistin resistance.
[d]No mutation in the *mgrB* gene was responsible for colistin resistance.
[e]ND, not determined.

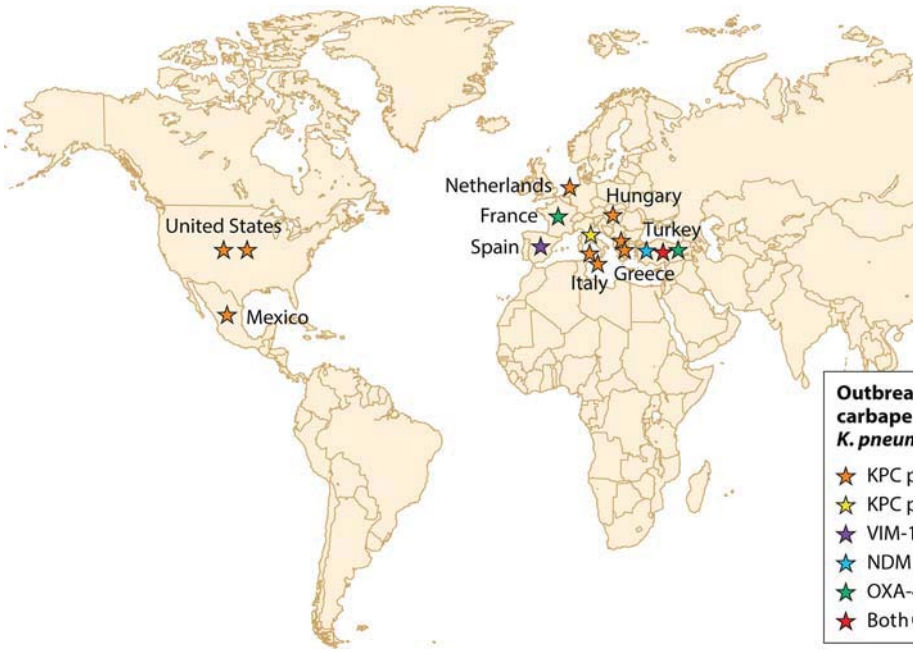

FIG 5 Outbreaks caused by colistin-resistant, carbapenemase-producing *K. pneumoniae* isolates. Each star

During a 2-year period (2010 to 2012) in Spain, a study showed an increase of the prevalence of colistin resistance among carbapenemase-producing *K. pneumoniae* isolates, from 13.5 to 31.7%, and an outbreak of colistin-resistant, VIM-1-producing *K. pneumoniae* was reported (204).

In France, a national survey revealed a low rate of colistin resistance (6.2%) among carbapenemase-producing *K. pneumoniae* isolates; neverthess, an outbreak of OXA-48 carbapenemase-producing and colistin-resistant *K. pneumoniae* isolates was reported (205).

Middle East. In Turkey and Israel, low rates of resistance to colistin among CR-KP isolates have been reported (2.7% and 4.5%, respectively). However, multiclonal outbreaks with OXA-48-, NDM-, and both OXA-48- and NDM-producing *K. pneumoniae* are currently ongoing in Turkey (our unpublished data).

Africa. A very low colistin resistance rate was reported among *K. pneumoniae* isolates in Tunisia (1.2%), but this rate was probably underestimated because susceptibility to colistin was primarily screened using a DD method generating high false susceptibility rates (205). The emergence of colistin resistance was reported for *K. pneumoniae* isolates recovered in South Africa (81, 119) and Nigeria (120).

Asia. Moderate rates of colistin resistance (about 6 to 11%) have been reported for *K. pneumoniae* isolates from South Korea (206) and Singapore (207), and similar resistance rates (4.4 to 12.1%) were found among CR-KP isolates in China (208) and Taiwan (209). Colistin-resistant *K. pneumoniae* isolates have also been reported in Laos and Thailand (120).

Risk Factors

The use of colistin was found to be an independent risk factor for the occurrence of resistance in Gram-negative bacteria (210, 211). Important increases of colistin resistance rates among ESBL-producing *K. pneumoniae* isolates (from 0 to 71% and from 11.1 to 75%) were reported after the introduction of selective digestive tract decontamination in two intensive care units (212, 213). Moreover, this decontamination failed to prevent colonization by ESBL-producing *Enterobacteriaceae*, and such a strategy should be abandoned. Note that the inappropriate use of colistin (such as suboptimal dosing or prolonged monotherapy) has been shown to be a source of colistin resistance selection (214, 215). The occurrence of colistin resistance in *P. aeruginosa* was most

effectively prevented by 8-h dosing intervals compared to 12- or 24-h dosing intervals (45).

Specific Epidemiology of the Plasmid-Mediated *mcr-1* Resistance Gene

Notably, plasmid-borne resistance to polymyxins has been reported for few different enterobacterial species so far, mainly among *E. coli* isolates and rarely for *Salmonella enterica*, *Enterobacter* spp., and *K. pneumoniae*. There are also some scattered reports of MCR-1-producing isolates in other species, such as *Cronobacter sakazakii* (152) and *Kluyvera ascorbata* (216). According to the current literature on the subject, the distribution of MCR-1 appears to be worldwide, covering all continents (217). It remains to be determined if the identification of the *mcr-1* gene worldwide corresponds to subsequent spread from an original source (China?) or to simultaneous gene mobilization events in different parts of the world. Ongoing epidemiological surveys should provide some important clues.

It is actually speculated that the original source of the gene, or at least of its mobilization and emergence, might be the animal world. This speculation is based on the fact that MCR-1-producing *E. coli* isolates have been identified in several animals and animal food products, including chickens and chicken meat, pigs and piglets, cattle, calves, and turkeys, but also in humans (Fig. 4). The corresponding samples were collected from many Asian countries (Cambodia, China, Japan, Laos, Malaysia, Taiwan, Singapore, and Vietnam) but also from Europe (Belgium, Denmark, France, Germany, Portugal, Italy, the Netherlands, Spain, Sweden, Switzerland, and the UK), the Americas (Argentina, Brazil, and Canada), and Africa (Algeria, Egypt, South Africa, and Tunisia) (Table 4). Note that a study conducted in Switzerland identified MCR-1-producing *E. coli* isolates in vegetables imported from Asia (143), and positive isolates were also identified in environmental water samples in Switzerland and Malaysia (Table 4).

The speculation of an animal origin of the *mcr-1* gene is also based on genetic features, since this gene is often associated with the insertion sequence IS*Apl1*, identified in *Pasteurella multocida*, which is a common animal pathogen, and also with the bla_{CMY-2} and *florR* genes, which are often identified in animal enterobacterial isolates (144). Finally, another feature suggesting an animal source of the problem is the heavy usage of polymyxins in veterinary medicine, with usage on many different animal species.

Dating the emergence of MCR-1-positive strains remains quite difficult; however, a Chinese study retrospectively identified positive isolates recovered from chickens during the 1980s (142), and they were discovered as early as 2005 in veal calves in France (144). It therefore seems that the emergence of MCR-positive isolates, at least in animals, is not a recent event. Very likely, there has been some silent dissemination of that resistance mechanism throughout the last few decades, and the current situation shows an ongoing further dissemination rather than an emerging phenomenon.

CONCLUSIONS

Polymyxins are gaining increasing interest because of the current epidemiological situation, with MDR Gram-negative bacteria spreading worldwide and with a paucity of novel marketed antibiotics. In some areas where infections caused by carbapenem-resistant *Enterobacteriaceae* are now common (such as Greece or Italy), the use of polymyxins (alone or often in combination with other antibiotics) is becoming crucial and may even be considered first-line therapy. The reevaluation of some critical issues in relation to polymyxins (accurate susceptibility testing, defining correct breakpoints, and better appreciating the toxicity issues) now opens new perspectives for its use. Studies that may permit a better evaluation of the PK-PD data, the toxicity level, and appropriate drug combinations are therefore crucial.

The recent identification of plasmid-mediated mechanisms of resistance to polymyxins also modifies the perspective. Indeed, epidemiological studies have to be initiated in order to better evaluate the extent of dissemination of this resistance in human and veterinary medicine and the impact of its occurrence. The perspective of

nosocomial dissemination of MDR Gram-negative organisms possessing resistance determinants to all main antibiotics is frightening, in particular for *K. pneumoniae*, which is one of the main nosocomial pathogens. Whether veterinary medicine is affecting the epidemiological situation by providing selective pressure with polymyxins has to be precisely determined. Whether discontinuing some specific usages of these drugs (prophylaxis or metaphylaxis in animals and decontamination of MDR bacteria in humans) should be considered is therefore an open debate.

Ultimately, reinforcing the detection of polymyxin-resistant isolates must be encouraged. Prospective epidemiological surveys are needed, since the current knowledge on this issue is very scarce. Actually, the recent development of a rapid diagnostic test for detection of polymyxin resistance, along with the development of a screening agar medium, will contribute to facilitating those surveys.

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