



Antimicrobial Susceptibility Testing for Polymyxins: Challenges, Issues, and Recommendations

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ABSTRACT Polymyxins, including polymyxin B and polymyxin E (colistin), are now increasingly being used worldwide to treat patients with multidrug-resistant (MDR) Gram-negative bacterial infections. This necessitates that laboratories employ an accurate and reliable method for the routine performance of polymyxin susceptibility testing. A number of reasons have accounted for the difficulties with susceptibility testing for the polymyxins, including their multicomponent composition, poor diffusion in the agar medium, adsorption to microtiter plates, the lack of a reliable susceptibility test, the lack of a specific breakpoint from professional organizations, the synergistic effect of polysorbate 80, and the development of heteroresistance. This minireview discusses such problems that impact the results of currently available susceptibility testing methods. We also provide emerging concepts on mechanisms of polymyxin resistance, including chromosomally and plasmid-mediated *mcr*-related resistance. Broad-range investigations on such critical issues in relation to polymyxins can be beneficial for the implementation of effective treatment against MDR Gram-negative bacterial infections.

KEYWORDS antimicrobial susceptibility, colistin, Gram-negative bacteria, polymyxin resistance

The increase in antimicrobial resistance has currently become a serious threat to public health worldwide, having a significant impact on infection control practices. If this trend continues, simple infections will no longer be treatable (1, 2). This is particularly true for carbapenems, which used to be the first-line antimicrobials but which have been increasingly compromised and which no longer constitute salvage therapy against the Gram-negative bacilli isolated in hospitals (3–6).

POLYMYXINS

Polymyxins B and E (also known as colistin) are cyclic polypeptide antibiotics that are synthesized by nonribosomal enzymes by members of the *Paenibacillus* genus (7, 8). Polymyxin B was discovered in 1947 from *Paenibacillus polymyxa*, whereas colistin was produced by the growth of *P. polymyxa* subsp. *colistinus* in 1949 (9) (Fig. 1). These antibiotics were widely used to treat serious infections caused by Gram-negative bacilli until the mid-1980s, when they were banned in clinical practices because of their nephrotoxicity and neurotoxicity adverse events and also the availability of less toxic drugs, mainly antipseudomonal aminoglycosides (10–12). The rise of multiresistant Gram-negative bacilli and also the paucity of new effective antibiotics led to the reemergence of polymyxins as a last-resort treatment option by the mid-1990s (13, 14). Polymyxins are now a key part of the antibiotic armamentarium and serve as the last therapeutic option to confront life-threatening infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens, including *Pseudomonas aerugi-*

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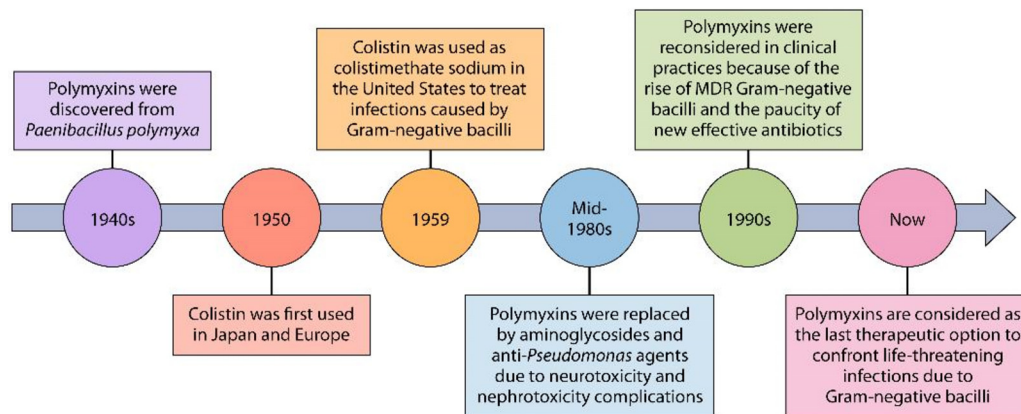


FIG 1 A polymyxin timeline from 1940s to today. Polymyxin B was discovered in 1947, whereas colistin was produced in 1949. Colistin has been available since 1959 for the treatment of infectious diseases caused by Gram-negative bacteria. Polymyxins were gradually abandoned in most parts of the world in about 1980. In recent years, polymyxins have been reconsidered in clinical practices for use against MDR strains of bacteria.

nosa, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* (15–18). Such antibiotic-resistant microorganisms are described by world authorities as “nightmare bacteria” that pose a “catastrophic threat” to people worldwide (19). They are also so important that the mortality rate in individuals infected with these bacteria can be as high as over 50% (17, 19).

Chemical Structure. Polymyxins are polycationic peptides consisting of a cyclic heptapeptide, a linear tripeptide segment, and a fatty acid tail linked to the N terminus of the tripeptide (Fig. 2) (7, 8). They consist of both hydrophilic and hydrophobic regions that are essential for their antimicrobial activity. Two commercially available polymyxins, polymyxin B and polymyxin E (colistin), are very similar structurally. They differ by only a single amino acid in the heptapeptide ring, with a phenylalanine in polymyxin B and a leucine in colistin (7, 8). In addition, at least four major components of polymyxin B, B1, B2, B3, and B4, and two major types of colistin, A and B (also known as polymyxins E1 and E2, respectively), have been described, differing only in the structure of their N-terminal fatty acyl moiety (7, 8, 20).

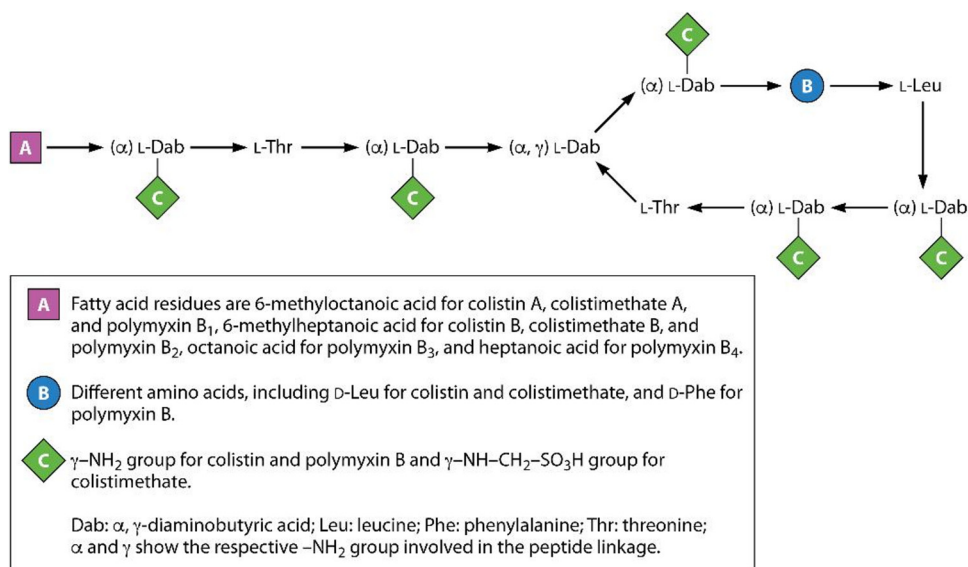


FIG 2 Structures of colistin, polymyxin B, and colistimethate.

Polymyxin B is administered parenterally, orally, and topically as sulfate salts. Two different forms of colistin are available for clinical practices: colistin sulfate and colistin methanesulfonate (CMS; or colistimethate). Colistin sulfate is used topically and orally, while CMS is administered by the parenteral and intravenous routes and by nebulization. CMS is less toxic than colistin sulfate when it is used parenterally (21). Indeed, CMS is formed by the reaction of colistin with formaldehyde and sodium bisulfite (22), leading to the addition of a sulfomethyl group to the primary amines of colistin. It is an inactive prodrug form of colistin that undergoes hydrolysis for conversion to the active compound, colistin, as well as a complex mixture of partially sulfomethylated derivatives in both *in vitro* aqueous media and *in vivo* biological fluids, taking several hours to achieve effective colistin concentrations (22–24).

Chemical Stability. Importantly, the long-term storage of polymyxins can serve as a source of loss of potency and can provide untoward physicochemical modifications (25). Neither the Clinical and Laboratory Standards Institute (CLSI) nor the European Committee on Antimicrobial Susceptibility Testing (EUCAST) addressed the stability of or storage time for polymyxins in aqueous solutions. Polymyxin B was used as a component of a permeabilization reagent reported to be stable in aqueous solution for at least 2 months at 4°C (2 to 8°C) and more than 3 weeks at room temperature (26). Polymyxin B was found to be stable for at least 1 day when stored at 4 or 25°C in infusion bags containing 0.9% sodium chloride injection (pH 5.6). Stability did not differ significantly between the two storage temperatures (27). In addition, Li et al. (24) demonstrated in a study that solutions of colistin sulfate were very stable in water at 4°C. After 60 days, the percentages of colistin A (polymyxin E1) and B (polymyxin E2) remaining were 97.4% and 105.3%, respectively. In addition, there were no decreases in the concentrations of colistins A and B stored in water at 37°C for up to 120 h. However, they, especially colistin A, were less stable in isotonic phosphate buffer (pH 7.4) and human plasma at 37°C.

Mechanism of Action. Due to the nature of the positive charge of polymyxins, they bind to the negatively charged phosphate groups of membrane lipids of Gram-negative bacilli. This displaces divalent Ca^{2+} and Mg^{2+} cations and destabilizes the lipopolysaccharide (LPS), consequently changing the permeability of the bacterial cell membranes, leading to leakage of the cytoplasmic content, and eventually causing cell death (8, 13, 28). It has been also demonstrated that polymyxins inhibit vital respiratory enzymes, such as type II NADH-quinone oxidoreductases in the bacterial cell membrane (29).

Spectrum of Activity. Polymyxins have a narrow antibacterial spectrum, mainly most clinically significant Gram-negative bacilli, including members of the *Enterobacteriaceae* (*Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., and *Shigella* spp.) and nonfermentative bacteria (*Acinetobacter* spp., *P. aeruginosa*, and most strains of *Stenotrophomonas maltophilia*) (12, 30–33). As described above, polymyxins have now become the last-line choice against infections caused by multi-resistant Gram-negative bacilli, especially *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* (34–36). They are not active against Gram-negative cocci (*Neisseria* spp.), Gram-positive organisms, and most anaerobic bacteria (12, 30).

Mechanisms of Resistance. Fortunately, the resistance of Gram-negative bacilli to the polymyxins is yet uncommon. However, there are a number of reports of infections due to polymyxin-resistant bacteria that were normally susceptible to these drugs (5, 37–39). Modification of the LPS molecules is the primary mechanism responsible for resistance to polymyxins in Gram-negative bacteria (Fig. 3) (40–43). This resistance mechanism is as a result of chromosomal mutations and occurs frequently by the synthesis, transmembrane transport, and attachment of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtN) to a phosphate group in lipid A. Subsequently, the increased net charge of modified lipid A reduces the affinity of positively charged polymyxins, leading to resistance (44, 45). Attachment of L-Ara4N and/or PEtN units to the lipid A moiety of LPS has been identified in polymyxin-resistant Gram-

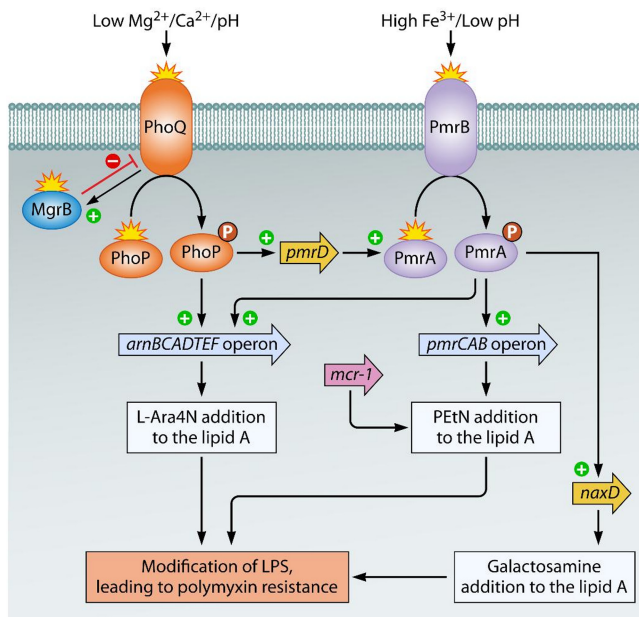


FIG 3 Mechanisms of LPS modification involved in polymyxin resistance in Gram-negative bacilli. In *Salmonella* spp., *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, the sensing of various stress conditions, such as the presence of cationic compounds (polymyxins), low Mg^{2+} and Ca^{2+} concentrations, acidic pH, and high Fe^{3+} concentrations, by the histidine kinases PhoQ and PmrB activates the two-component systems (TCSs) PhoP-PhoQ and PmrA-PmrB, respectively. Subsequent activation of the *arnBCADTEF* and *pmrCAB* operons leads to the synthesis and addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) to lipid A, respectively. In addition, PmrAB is activated by PhoP-PhoQ via the product of the *pmrD* gene, which in turn activates *pmrA* for activation of the *arnBCADTEF* operon. Inactivation of MgrB (a negative regulator of the PhoP-PhoQ system) by amino acid substitutions leads to overexpression of the *phoP-phoQ* operon as well, causing activation of the *pmrHFUJLKM* operon, thus leading to the production of L-Ara4N. On the other hand, polymyxin resistance in *Acinetobacter baumannii* is due to alterations in PmrB or the response regulator PmrA, which activates the PmrAB TCS. The subsequent upregulation of the *pmrCAB* operon and the *naxD* gene promotes the addition of PEtN and galactosamine to lipid A, respectively. In addition, a recently identified *mcr-1* gene, encoding phosphoethanolamine transferase, has been shown to be the main mechanism of polymyxin resistance.

negative bacilli, including *Salmonella enterica* serotype Typhimurium, *Escherichia coli*, *K. pneumoniae*, and *P. aeruginosa* (46–50). However, *A. baumannii* possesses no genetic apparatus necessary for the biosynthesis and transport of L-Ara4N; therefore, in addition to modification of lipid A with PEtN, glycosylation of the phosphate group of lipid A with D-galactosamine units is also associated with resistance to polymyxins (51, 52).

Two-component systems (TCSs), mainly PhoP-PhoQ and PmrA-PmrB, regulate the expression of most of the genes involved in LPS modification in Gram-negative bacilli. In *Salmonella* spp., *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, activation of these TCSs under specific stress conditions generally leads to LPS modification. The inner membrane sensor histidine kinase PhoQ and/or PmrB is activated under conditions of bacterial growth in the presence of low concentrations of Mg^{2+} and Ca^{2+} and high concentrations of Fe^{3+} or in the presence of cationic compounds, including polymyxins. When sensor kinases (PhoQ, PmrB) are activated, they phosphorylate their cognate cytoplasmic response regulator (PhoP, PmrA), which in turn modulates the expression of the target genes *arnBCADTEF* (also called *pmrHFUJLKM*) and *pmrCAB*, responsible for LPS modifications by L-Ara4N and PEtN, respectively. Furthermore, constitutive activation of the systems of enteric bacteria and *P. aeruginosa* can be caused by missense mutations, leading to the upregulation of the corresponding operons, resulting in LPS modification (53). The histidine kinase *pmrB* seems to be a more common site for mutations than the response regulator gene *pmrA*. Polymyxin resistance in *A. baumannii* is primarily due to missense or small insertion/deletion mutations in *pmrA* and/or *pmrB* genes (51, 53). Similar to the findings for enteric bacteria, *pmrB* appears to be the

most commonly mutated gene in *A. baumannii*. The complete loss of lipid A or the LPS core by mutations in the first three lipid A biosynthesis genes, *lpxA*, *lpxB*, and *lpxC*, and in the *lpsB* gene, encoding aglycosyltransferase (involved in the biosynthesis of the LPS core), has also been implicated in colistin resistance in *A. baumannii* (54).

A distinct resistance phenomenon, namely, heteroresistance, has appeared as a new challenge faced by antimicrobial therapy. This type of resistance to polymyxins has been described in several Gram-negative pathogens, including *A. baumannii* (55, 56), *K. pneumoniae* (57), *Salmonella* spp. (58), *Enterobacter* spp. (59), as well as *P. aeruginosa* (60). Heteroresistance is defined as resistance to certain antibiotics expressed by a subset of isolates of a microbe that are generally categorized as susceptible based on *in vitro* susceptibility tests (61). Heterogeneous colistin-resistant *A. baumannii* is known as a subpopulation of colistin-susceptible isolates with MICs in the range of 0.25 to 2 $\mu\text{g/ml}$ and with the ability to grow in the presence of colistin at 3 to 10 $\mu\text{g/ml}$ (62). These resistant subpopulations can be developed into highly or completely resistant isolates following colistin administration (63). In such cases, antibiotic therapy leads to elimination of the sensitive members of the bacterial subpopulation and, it is speculated, the selection of more resistant cells, which would, consequently, impede the therapeutic efficiency of antibiotics. Although the underlying molecular mechanism is unknown, data suggest that heteroresistance may occur as a result of mutations within the *phoPQ* or *mgrB* regulatory system (57, 64), as well as the complete loss of LPS production due to a mutation in or the insertional inactivation of lipid A biosynthesis genes *lpxA*, *lpxC*, and *lpxD* (54, 65). It is important to note that the heterogeneity of resistance to antibiotics is different from bacterial persistence, which was first described as the capacity of bacteria to tolerate exposure to lethal concentrations of penicillin (66). Persisters are mutant cells that neither die nor grow in the presence of an antibiotic, suggesting that they are in a state of dormancy (67), and they grow only after removal of the antibiotic. Additionally, the progeny of persisters does not display increased resistance to the antibiotic but shows the same pattern of susceptibility to the antibiotic as the original bacterial population (68).

Liu et al. discovered for the first time, in November 2015, transmissible polymyxin resistance in an *E. coli* isolate recovered from food animals and raw meat in China (69). The plasmid-borne gene *mcr-1*, which mediates the addition of PEtN moiety to lipid A (69), has since been described in different species of the family *Enterobacteriaceae*, such as *K. pneumoniae*, *Shigella sonnei*, *Salmonella* spp., and *Enterobacter* spp., from patients, animals, and environmental samples in different regions of the world (70–72). In a short time, 13 *mcr-1* subgroups (*mcr-1.1* to *mcr-1.13*) were identified in various species of *Enterobacteriaceae* in different countries (73). To date, eight *mcr* variants (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7*, and *mcr-8*) showing nucleotide sequence identity to each other have been described in *Enterobacteriaceae* and other genera (73–77). Notably, a colistin-nonsusceptible *P. aeruginosa* strain carrying a chromosomal copy of the *mcr-5* gene which was embedded within a Tn3-family transposon has recently been reported (78). This new mechanism confers a degree of resistance lower than that conferred by the chromosomally mediated mechanism of polymyxin resistance (79). However, this is a major global issue, where such transferable mechanisms are responsible for the rapid and ongoing dissemination of polymyxin resistance among clinical pathogens.

CHALLENGES IN POLYMYXIN SUSCEPTIBILITY TESTING

Due to the growing menace of multiresistant pathogens (80) and, consequently, the increased need for polymyxin treatment in critically ill patients with bacteremia and ventilator-associated pneumonia (9), a significant effort is required to maintain the antibacterial properties of these antibiotics. Optimization and standardization of *in vitro* polymyxin susceptibility testing and definition of the correct breakpoints are critical issues for both patient care and epidemiological surveillance purposes. In other words, because of their increased clinical use, the development of an accurate and reliable method for determining the susceptibility of isolates to polymyxins is now an urgent

need. There are several issues associated with *in vitro* susceptibility testing of polymyxins, such as interpretive categories (criteria) and breakpoints, quality control (QC) strains, the reliable reference susceptibility testing method, the multicomponent composition of polymyxins, their poor diffusion into agar, their cationic nature, the effect of polysorbate 80 (P-80), and the development of heteroresistance, as well as the impact of the medium, subcultures, and storage (81). This minireview attempts to describe the different challenges, technical issues, and recommendations associated with polymyxin susceptibility testing, with the aim of guiding antimicrobial therapy.

Interpretive Criteria/Categories and Quality Control Strains. There is no consensus between the two professional organizations establishing breakpoints for polymyxins, the Clinical and Laboratory Standards Institute (CLSI) (82) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (83) (Table 1), although attempts have recently been made to coordinate these. Neither CLSI nor EUCAST has recommended the disk diffusion (DD) method for polymyxin susceptibility testing, so there are currently no established zone diameter breakpoints for colistin or polymyxin B (82–84). Furthermore, MIC breakpoints for colistin and polymyxin B are available in CLSI guidelines for *Pseudomonas* spp. and *Acinetobacter* spp. but not for the *Enterobacteriaceae*, and also, no interpretative breakpoint for polymyxin B has been established by EUCAST guidelines (82, 83). CLSI has established a MIC epidemiological cutoff value (ECV) for colistin applicable to five members of the *Enterobacteriaceae*, including *E. coli*, *K. pneumoniae*, *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*), *Enterobacter cloacae*, and *Raoultella ornithinolytica* (82). The ECV is generally the MIC or zone diameter value that divides bacterial populations into those with and those without phenotypically detectable resistance (non-wild-type [NWT] and wild-type [WT], respectively). An ECV for WT strains describes the isolates with no detectable resistance (due to acquired and/or mutational mechanisms) or reduced susceptibility toward the antimicrobial agent tested, while the ECV for NWT strains defines isolates with detectable resistance and reduced susceptibility to the antimicrobial agent being evaluated. It is noteworthy that ECVs are based on *in vitro* data only, using MIC or zone diameter distributions, and must not be used as clinical breakpoints of susceptible, intermediate, or resistant (82). Here, if the colistin MIC for the above-mentioned members of the *Enterobacteriaceae* is at or below the ECV ($\leq 2 \mu\text{g/ml}$), it can be assumed that the isolate is a WT strain. If the colistin MIC is $\geq 4 \mu\text{g/ml}$, the isolate should be retested, and if the result is confirmed, the isolate can be considered NWT.

Both CLSI and EUCAST recommend use of the quality control (QC) organisms *E. coli* ATCC 25922 (MIC range, 0.25 to 2 $\mu\text{g/ml}$) and *P. aeruginosa* ATCC 27853 (MIC range, 0.5 to 4 $\mu\text{g/ml}$) for polymyxin susceptibility testing to standardize the procedures (82, 85). Although there are many conflicting results between broth microdilution (BMD) and the gradient diffusion test, e.g., Etest, at MICs of $\geq 2 \mu\text{g/ml}$, the suggested MICs of the QC strains are lower. In addition, EUCAST has currently advised laboratories to include colistin-resistant *E. coli* NCTC 13846 (*mcr-1* positive) as a resistant QC strain. The recommended MIC target value of colistin for this strain is 4 $\mu\text{g/ml}$ and should only occasionally be 2 $\mu\text{g/ml}$ or 8 $\mu\text{g/ml}$ (85). So, it is important that laboratories be aware of the possibility of the presence of *mcr* in isolates with colistin MICs of $\geq 2 \mu\text{g/ml}$, indicating that further confirmation testing of isolates may be warranted.

Susceptibility Testing Methods for Polymyxins. Despite the increasing use of polymyxins in clinical practices, the appropriate method for determining polymyxin susceptibility in routine clinical laboratories remains unclear (86). So far, several studies evaluating the performance of methods for polymyxin susceptibility testing have obtained inconsistent results; hence, there is a great challenge for the scientific community to develop a rapid and reliable method to determine the susceptibility of isolates to polymyxins (87).

The disk diffusion (DD) test, or Kirby-Bauer procedure, is a simple and inexpensive method for screening a large number of isolates to determine the antimicrobial susceptibility in many clinical laboratories (88). However, the poor and slow diffusion of

TABLE 1 Polymyxin B and colistin interpretative breakpoints according to CLSI and EUCAST guidelines in 2018

Microorganism	EUCAST ^a												
	CLSI ^{a,b}						EUCAST ^a						
	Disk content		Zone diameter interpretative criteria (mm)		MIC interpretative criteria (μg/ml)		Disk content		Zone diameter interpretative criteria (mm)		MIC interpretative criteria (μg/ml)		
Polymyxin B	Colistin	S	I	R	S	I	R	S	I	R	S	I	R
<i>Enterobacteriaceae</i>	Polymyxin B	—	—	—	—	—	—	—	—	—	—	—	—
	Colistin	—	—	—	—	—	—	—	—	—	≤2	—	>2
<i>Pseudomonas</i> spp.	Polymyxin B	—	—	—	≤2	4	≥8	—	—	—	—	—	—
	Colistin	—	—	—	≤2	—	≥4	—	—	—	≤2	—	>2
<i>Acinetobacter</i> spp.	Polymyxin B	—	—	—	≤2	—	≥4	—	—	—	—	—	—
	Colistin	—	—	—	≤2	—	≥4	—	—	—	≤2	—	>2

^aS, susceptible; I, intermediate; R, resistant; —, not determined.

^bCLSI has recently established the MIC epidemiological cutoff value (ECV) for colistin applicable to five members of the *Enterobacteriaceae*, including *E. coli*, *K. pneumoniae*, *K. aerogenes* (formerly *Enterobacter aerogenes*), *E. cloacae*, and *Raoultella ornithinolytica*. ECVs are generally the MIC or zone diameter value that divides bacterial populations into those with and those without phenotypically detectable resistance (non-wild-type [NWT] and wild-type [WT], respectively). It is noteworthy that ECVs must not be used as clinical breakpoints for susceptible, intermediate, or resistant. If the colistin MIC for the above-mentioned members of the *Enterobacteriaceae* is at or below the ECV (≤2 μg/ml), it can be assumed that the isolate is a WT strain. If the colistin MIC is ≥4 μg/ml, the isolate should be retested, and if the result is confirmed, the isolate can be considered NWT.

polymyxins through agar is associated with small zones of growth inhibition and significant assay variation, negating use of this method for susceptibility testing (89, 90). In fact, the predictive accuracy of the DD test is unacceptable, and consequently, no reliable correlation of zone diameters and MICs has been found in many previous studies (89–92). The difficulty in differentiating the inhibition zone diameters has been illustrated by the study of Jerke et al. (93), where a resistant *P. aeruginosa* isolate with a zone diameter of 10 mm was compared to a susceptible isolate with a zone diameter of 11 mm; both isolates were categorized as susceptible with BMD MICs of $\leq 0.25 \mu\text{g/ml}$. Additionally, studies have reported very major errors (VMEs), indicating false susceptibility, obtained from the DD test at rates ranging from 0.7% (92) to 3.5% (91) for colistin and from 1.2% (94) to 5% (95) for polymyxin B and with a low level of reproducibility (90) in comparison with the results of the BMD reference method. These findings explain why no zone diameter breakpoints are currently provided by CLSI and EUCAST for polymyxins (82, 83) and, thus, why the joint CLSI-EUCAST Polymyxin Breakpoints Working Group does not recommend use of the DD method for susceptibility testing of polymyxins (84).

The agar dilution (AD) susceptibility method relies on various concentrations of polymyxins in Mueller-Hinton agar (MHA) plates (2-fold dilution series). A bacterial inoculum corresponding to a 0.5 McFarland standard concentration is added as a spot to a plate with 10^4 CFU per spot. After incubation of the plates for 18 to 24 h at $35 \pm 2^\circ\text{C}$, the bacterial growth in the inoculated spots is examined (88). This technique allows replicate spots of one bacterial strain or spots of different strains to be tested so that the MIC of the antibiotic against multiple strains can be tested on the same plate. However, AD is very laborious and is rarely used by many clinical laboratories.

Although AD may theoretically avoid the adsorption of polymyxins to the plates, no studies have measured the antibiotic concentration in agar dilution plates. One study (96) evaluating colistin stability in agar found that there were no differences in the MICs of two ATCC control strains obtained from 1-week-old colistin agar plates and from freshly used plates. In particular, the MICs obtained from all plates were $0.25 \mu\text{g/ml}$ and $2 \mu\text{g/ml}$ for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, respectively. The results for colistin-resistant *K. pneumoniae* isolates on the plates that been stored for 1 week were also uniform and showed MICs of $128 \mu\text{g/ml}$. These findings suggest that AD is a reliable and reproducible method to determine the colistin MIC, even when colistin-containing agar plates are stored for 1 week at 4°C . Additionally, the main findings of the study by Moskowitz et al. (97) indicated that AD is the most sensitive method to detect polymyxin resistance in clinical *P. aeruginosa* isolates. Other susceptibility testing methods, including BMD, DD, and Etest strips, exhibited high rates of significant false susceptibility for both *P. aeruginosa* and *S. maltophilia* clinical isolates compared with those achieved with AD.

Previous studies have also found a good accordance between the results of AD and BMD (90, 92, 98). In the study of 61 carbapenem-nonsusceptible clinical isolates, including 41 *K. pneumoniae* and 20 *baumannii* isolates (99), AD showed colistin susceptibility rates similar to those of BMD (4.9% versus 3.3%), with an acceptable categorical agreement (CA) (91.8%) being observed for both pathogens. In addition, relatively limited errors, including 3.3% VMEs and 4.9% major errors (MEs), were found by AD. Similarly, Behera et al. (92) found a concordance of 97% between AD and BMD for testing of the susceptibility to polymyxin B in 257 MDR Gram-negative bacteria, with 0.7% VMEs and 2.4% minor errors (mEs) for AD. However, trends toward higher MICs resulting from AD than from BMD have been described previously (99, 100). This can be due to the different concentrations of cations present in MHA, which is not suggested for use by CLSI guidance. Altogether, the joint CLSI-EUCAST Polymyxin Breakpoints Working Group recently did not recommend the use of AD for susceptibility testing of colistin (84).

BMD is widely employed as the reference test method for MIC susceptibility testing of all antimicrobials (88). As the CLSI-recommended method, BMD is performed with cation-adjusted Mueller-Hinton broth (CA-MHB), a range of doubling dilutions of

antibiotic, and a final inoculum size of 5×10^5 CFU/ml in round or conical wells of a sterile plastic microdilution plate or tray. The MIC value is determined to be the lowest concentration of antibiotic that stops visible bacterial growth after 18 to 24 h of incubation at $35 \pm 2^\circ\text{C}$ (88). In particular, each well should contain 0.1 ml of broth, and the inoculum volume should be 0.01 ml. To prepare the inoculum, a 0.5 McFarland suspension should be diluted 1:20 to yield 5×10^6 CFU/ml. When 0.01 ml of this suspension is inoculated into the broth, the final test concentration of bacteria is approximately 5×10^5 CFU/ml (88).

Recently, the joint CLSI-EUCAST Subcommittee on Polymyxin Susceptibility Testing and Breakpoints recommended the ISO-20776 standard BMD method (with sulfate salts of colistin in plain polystyrene trays without additives, like polysorbate 80) as the ideal method to evaluate susceptibility to polymyxins (84). It has been claimed that BMD is time-consuming and requires expert staff for manual preparation of the antibiotic solutions for a routine clinical laboratory. Additionally, some technical issues for testing have been reported using this assay (101, 102). Polymyxins readily adhere to the plastic trays, leading to decreased antibiotic concentrations actually being present in the MHB dispensed in the wells (98, 103). The addition of a surfactant, such as polysorbate 80 (P-80), limits antibiotic adhesion to the BMD panels (104). Nonetheless, CLSI or EUCAST guidelines do not currently recommend the use of P-80 for colistin or polymyxin B susceptibility testing by BMD (84). As will be discussed further, it has been demonstrated that P-80 displays a synergistic effect with polymyxins (105) and has antibacterial activity of its own (106).

Another technical issue with polymyxin susceptibility testing using BMD is the presence of a "skipped well," which makes it difficult to determine the true MIC. The phenomenon of a skipped well, which refers to the well without growth, despite the occurrence of growth in wells with higher concentrations, has been reported for *Enterobacter* species (107), *P. aeruginosa* (108), and *A. baumannii* (109). As presented in a study of *Enterobacter* species by Landman et al. (107), isolates with a multiple-skipped-well phenotype are associated with uninterpretable MIC results using the BMD assay. According to CLSI guidance (88), a single skipped well is acceptable and the well with the highest MIC value should be read. Isolates exhibiting more than one skipped well should be considered to have an uninterpretable polymyxin MIC; therefore, interpretation of susceptibility test results should be done with great care until the clinical relevance of this particular phenotype is defined. The phenomenon of a skipped well may represent heteroresistance and, in turn, might result from mutations affecting the response regulator PhoP (57, 107). This is supported by an experiment conducted by Jayol et al., where a colistin-resistant population of *K. pneumoniae* isolates that had a MIC of $128 \mu\text{g/ml}$ and that harbored the amino acid change Asp191Tyr in PhoP was found to coexist with a colistin-susceptible subpopulation (MIC = $0.12 \mu\text{g/ml}$) (57). Furthermore, an increase in the expression of the *phoQ*, *arnB*, and *pmrAB* genes was demonstrated in the skipped-well isolates of *P. aeruginosa*, resulting in LPS modifications and a reduction of polymyxin-binding sites (108).

Nevertheless, BMD is currently the gold standard method for determination of the MICs of antimicrobial agents, including polymyxins, due to its reproducibility, reliability, and possibility of automation. A number of semiautomated devices for colistin BMD testing have been suggested for routine clinical practices (99, 110), although some problems have been reported with colistin susceptibility testing on these systems (111, 112). A study evaluating five commercially BMD products (Sensititre, Micronaut-S, Micronaut MIC-Strip, SensiTest, and UMIC) in comparison with the BMD reference method reported the rate of essential agreement (EA) to range from 82% to 99%, the rate of CA to range from 89% to 95%, the major error (ME) rate to range from 3% to 7%, and the VME range to be up to 3% (110). Another study by Chew et al. indicated that commercial and automated BMD systems may obtain results relatively comparable to those of the BMD reference method (79). Conversely, Vourli et al. (112) assessed the performance of two automated systems (Phoenix 100 and Vitek 2) for colistin susceptibility testing among carbapenem-resistant *A. baumannii* isolates. High rates of false

susceptibility or VMEs were observed for both the Phoenix 100 (41.4%) and Vitek 2 (37.9%) systems. VMEs were much more frequent for isolates that showed MICs of 2 $\mu\text{g}/\text{ml}$ than for isolates that showed MICs of $\leq 1 \mu\text{g}/\text{ml}$, indicating that MIC results by automated systems within the susceptible range should be validated by a BMD assay. Until now, the U.S. Food and Drug Administration (FDA) has not approved the commercial automated assays for use for routine polymyxin susceptibility testing at clinical laboratories. This is because there are no FDA-recognized breakpoints for Gram-negative bacilli, so these methods are recommended only for research use.

Broth macrodilution (BMAD) is a method for the determination of MICs that is identical to BMD in terms of the growth medium (CA-MHB), the bacterial suspension used as the inoculum, the preparation of serial 2-fold dilutions of antimicrobial agent, the incubation conditions, and the reading of the plate; the methods differ by the volume of growth medium used and the use of sterile 13- by 100-mm test tubes instead of plastic trays (88). To prepare an adjusted inoculum, the 0.5 McFarland standard suspension is diluted 1:150, resulting in a tube containing approximately 5×10^6 CFU/ml. Addition of 1 ml of the standardized inoculum to each tube containing 1 ml of antimicrobial agent in the dilution series brings the final inoculum to 5×10^5 CFU/ml.

As the reference method, BMAD has been used to evaluate the accuracy of a commercially available BMD tray for detecting colistin and polymyxin B resistance in carbapenem-resistant *K. pneumoniae* isolates (113). Hindler and Humphries (98) evaluated the colistin BMAD method against the BMD method with *P. aeruginosa* ($n = 60$), *K. pneumoniae* ($n = 20$), and *A. baumannii* ($n = 27$) clinical isolates. They found no false-susceptible results and the highest essential agreement (EA) of 83% compared with the results of the other methods tested.

The gradient diffusion test, such as the Etest (bioMérieux) or M.I.C.Evaluator (Oxoid), is a variation on MIC determination. This test uses an inert and a nonporous plastic strip impregnated with the increasing concentrations of antibiotic (ranging from 0.016 to 256 $\mu\text{g}/\text{ml}$ and 0.064 to 1,024 $\mu\text{g}/\text{ml}$ for colistin and polymyxin B, respectively) to determine the MICs of different organisms. This method is performed by applying the strip to the surface of an MHA plate inoculated with a 0.5 McFarland standard. After incubation for 18 to 24 h at $35 \pm 2^\circ\text{C}$, a symmetrical inhibition ellipse centered along the strip is formed, and the MIC value is defined from where the ellipse edge intersects the test strip (114, 115).

A variety of previous studies demonstrated a good correlation between the Etest method and the reference BMD technique (90–92, 116). Recently, a study with 202 carbapenem-resistant *A. baumannii* isolates found a high CA of 99.5% between the colistin Etest and BMD with P-80, whereas the CA between Etest and AD was 90.6% (117). In contrast, different studies have reported high error rates of the Etest method for the determination of polymyxin susceptibility (97–99, 107). Chew et al. (79) compared Etest for polymyxin susceptibility testing to BMD with 66 carbapenem-resistant isolates of the *Enterobacteriaceae*. EAs of 75% and 48.7% between these two methods were reported for colistin and polymyxin B, respectively. VME rates of 12% for colistin and 26.1% for polymyxin B were also found. In addition, the Etest method may misestimate the MIC values of polymyxins for test bacteria. Hindler and Humphries (98) revealed that Etest MICs compared to those obtained by BMD with P-80 were significantly lower among the colistin-resistant isolates (MICs $\geq 4 \mu\text{g}/\text{ml}$), whereas MICs for susceptible isolates were raised (MICs $< 4 \mu\text{g}/\text{ml}$). Similar to what has been known for disk diffusion, this problem could be due to the poor diffusion of polymyxins into agar. Therefore, even though the gradient diffusion test is simple to perform, it is rather expensive and fails to reliably detect polymyxin-resistant strains.

More recently, a simplified approach, namely, colistin broth disk elution (CBDE), for colistin susceptibility determination has been described by Simner et al. (118). This method uses four, 10-ml CA-MHB tubes per isolate to which 0, 1, 2, and 4 colistin 10- μg disks are added, generating a final concentration of 0 (growth control), 1, 2, and 4 $\mu\text{g}/\text{ml}$ in the tubes, respectively. After 30 min of incubation at room temperature, each tube is inoculated with a 50- μl aliquot of a 0.5 McFarland standard suspension for

a final inoculum of 7.5×10^5 CFU/ml. MIC values are determined following 16 to 20 h of incubation at $35 \pm 2^\circ\text{C}$. Data from the study of Simner and coworkers (118) revealed that CBDE yielded a CA and an EA of 98% and 99%, respectively, compared to the results of colistin BMD for the 172 Gram-negative bacilli tested. In addition, a CA and an EA of 100% each was achieved by CBDE compared to the results of BMAD. The overall VME rate was 8% because 3 of the *E. coli mcr-1* isolates showed a MIC of $2 \mu\text{g/ml}$ by CBDE and a MIC of $4 \mu\text{g/ml}$ by BMD (118). These findings recommend that colistin MICs of $2 \mu\text{g/ml}$ by CBDE be confirmed by the reference BMD method and that isolates with MICs of $\geq 2 \mu\text{g/ml}$ be assessed for *mcr* genes.

Multicomponent Composition of Polymyxins. Earlier works have suggested considerable variability in the chemical compositions of colistin and polymyxin B from the different manufacturers (119, 120). The heterogeneity in polymyxin formulations could affect their pharmacodynamic activity and, therefore, impact preclinical studies, including time-kill experiments and MIC testing, as well as clinical treatment. In a study by Diep et al. (121), there was a significant difference in the *in vitro* activity between several polymyxin B products, including those from Sigma-Aldrich, AK Scientific, USP, and MP Biomedicals. For instance, concentrations of 1 and $4 \mu\text{g/ml}$ of all products showed the same early killing activity against *A. baumannii* ATCC 19606. However, at $16 \mu\text{g/ml}$, the Sigma-Aldrich polymyxin B exhibited more rapid bacterial killing at 2 h and 4 h than did the other products. Furthermore, the MP Biomedicals and USP polymyxin B products at 1 and $4 \mu\text{g/ml}$ resulted in the more rapid killing of *K. pneumoniae* ATCC BAA1705 by 2 h than the AK Scientific and Sigma-Aldrich products. At a $16\text{-}\mu\text{g/ml}$ concentration of polymyxin B, the MP Biomedicals product demonstrated more rapid killing at 2 h than the AK Scientific product.

Another important issue is the use of the inactive prodrug colistimethate sulfate (CMS) instead of colistin sulfate for MIC measurement. Due to doubts over whether CMS has antimicrobial activity itself, *in vitro* studies determining MICs have been performed by using colistin sulfate and CMS (23, 120). In a study investigating the *in vitro* pharmacodynamic properties of colistin and colistin methanesulfonate against clinical isolates of *P. aeruginosa*, Li et al. (120) found that the MICs of colistin for the susceptible strains ranged from 1 to $4 \mu\text{g/ml}$, while the MICs of colistin methanesulfonate were significantly higher and ranged from 4 to $16 \mu\text{g/ml}$. In addition, Bergen et al. (23) demonstrated that the concentration of colistin generated in 240 min after spiking of the samples with CMS at $32 \mu\text{g/ml}$ had the same killing effects as the colistin generated after spiking of the samples with CMS at $6 \mu\text{g/ml}$ by 15 min. An *in vitro* study (24) showed the hydrolysis of CMS to colistin in water, isotonic phosphate buffer, and human plasma within 24 to 48 h at 37°C . No formation of colistin was also observed when a $1,024\text{-}\mu\text{g/ml}$ concentration of CMS was stored for 30 min in MHB at 37°C . From these evidences, it is inappropriate to use CMS for MIC measurement, as antibacterial activity is due to the formation of colistin and not to the CMS in its own right.

Cationic Properties of Polymyxins. It is well-known that the cationic nature of polymyxins causes them to adhere to a variety of laboratory plastic devices, including microtiter plates, leading to different interlaboratory results for the MIC by the BMD method (101, 102). The rate of drug binding depends on various factors, such as the nature of the plastic products and the coating used on the plates (81, 93, 101, 122). For example, tissue culture-coated microplates resulted in an overall 5.3-fold increase in the MIC values of colistin for all tested isolates (*P. aeruginosa*, *Enterobacteriaceae*, and *Acinetobacter* spp.), maybe due to the reduced free drug concentration within the microwells (101). Plastics can also be manufactured as microtubes with the ability to minimize any interactions between the analytes and the tubes, and these are often marketed as low-protein/DNA-binding microtubes. In recent experiments with low-protein-binding polypropylene microtubes (122), the initial concentrations of colistin were higher than those in other materials (a standard polypropylene tube, polystyrene tube, glass tube, and standard polystyrene microplate), at between 63% and 99%. These type of microtubes showed the least adsorption, followed by standard polypro-

pylene and glass. Similar results have also been obtained from other studies, demonstrating that BMD with glass-coated plates gives more reliable MIC results for polymyxins than BMD with polystyrene plates (96, 123). Furthermore, Karvanen et al. (103) previously showed that colistin adsorption was proportionally higher at a lower concentration of 0.125 $\mu\text{g/ml}$ after 24 h at 35°C, with the estimated colistin concentration in MHB being 8%, 13%, and 25% in polystyrene, polypropylene, and glass-coated wells, respectively. However, at an initial concentration of 4 $\mu\text{g/ml}$, 75%, 62%, and 62% of the colistin remained following 24 h in polystyrene, polypropylene, and glass, respectively. These findings indicate that polymyxins are extensively lost under normal experimental conditions in a concentration- and material-dependent manner; thus, it is important to carefully monitor drug concentrations during the experiment course in order to gain more accurate results for the antibacterial effect of antibiotic.

Effect of Polysorbate 80. Polysorbate 80 (P-80 or Tween 80) is a nonionic surfactant and emulsifier often used in foods and cosmetics. Structurally, this surfactant contains both a hydrophilic polyether group and a lipophilic oleic acid chain (93). It is also used in bacterial broth cultures to prevent foam formation and as an excipient in many medications and vaccines against influenza to stabilize aqueous formulations (124). The antimicrobial properties of microemulsions containing surfactant molecules, in particular, P-80, have been reported in some studies published in the literature, where such microemulsions caused a complete loss of viability of *Staphylococcus aureus* and *E. coli* (125) and effectively decreased the viability of an established biofilm population of *P. aeruginosa* (124). Furthermore, the synergistic effect of P-80 with various chemical agents, such as benzalkonium chloride, chlorhexidine diacetate, and polymyxin B, which leads to reduced resistance to these agents, has been previously described in *P. aeruginosa* (105, 126). P-80 also has a mild antimicrobial activity, especially when it is combined with other antimicrobial agents; increases cell permeability; and lyses spheroplasts (106). It is feasible that the primary damage to the outer membrane caused by polymyxins allows the surfactant to enter the cell and promote cell lysis. Therefore, polymyxin-resistant bacteria would not be affected by P-80, as their cell membrane remains intact, giving higher MICs (127). This can be an explanation for the findings that P-80 mainly affects isolates showing MICs of ≤ 1 or ≤ 2 $\mu\text{g/ml}$ (98, 104).

A CLSI guideline has recommended the use of P-80 as a dispersing agent at a final concentration of 0.002% for the testing of some lipoglycopeptides, including dalbavancin, oritavancin, and telavancin, to prevent the binding of drug to plastics (82, 88). Studies have also demonstrated that the addition of P-80 either to the bacterial inoculum or directly to MHB minimizes polymyxin adhesion to microplates, improving MIC susceptibility testing results for both polymyxin B and colistin (98, 128, 129). In a study with 247 organisms, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Acinetobacter* isolates, Sader et al. (104) found a significant downward shift (4- to 8-fold) in the MICs of polymyxins measured by BMD with the addition of a 0.002% final concentration of P-80. The decreases were also the most appreciable for susceptible organisms having MICs of ≤ 2 $\mu\text{g/ml}$ compared with those for nonsusceptible strains with MICs of > 2 $\mu\text{g/ml}$, indicating that the reduction in the MIC in the presence of surfactant does not change remarkably the susceptibility category when the current CLSI breakpoints are applied to polymyxins. Regardless, the CLSI subcommittee decided in January 2014 to adopt polymyxin (colistin) BMD testing without the addition of P-80 (87). The outcomes of this decision will hinge mostly on the breakpoints adopted for the polymyxins. If the present susceptibility breakpoints of ≤ 2 or ≥ 4 $\mu\text{g/ml}$ are retained, the MIC interpretation is unlikely to be compromised. However, the ability of BMD to detect susceptible isolates without using P-80 may be weakened, if a susceptible breakpoint of ≤ 1 $\mu\text{g/ml}$ is chosen. Because the MIC results obtained by BMAD in glass tubes, which have the lowest colistin adsorption, are comparable to those obtained by BMD with P-80, determination of polymyxin MICs in glass-coated plates could address this concern (98).

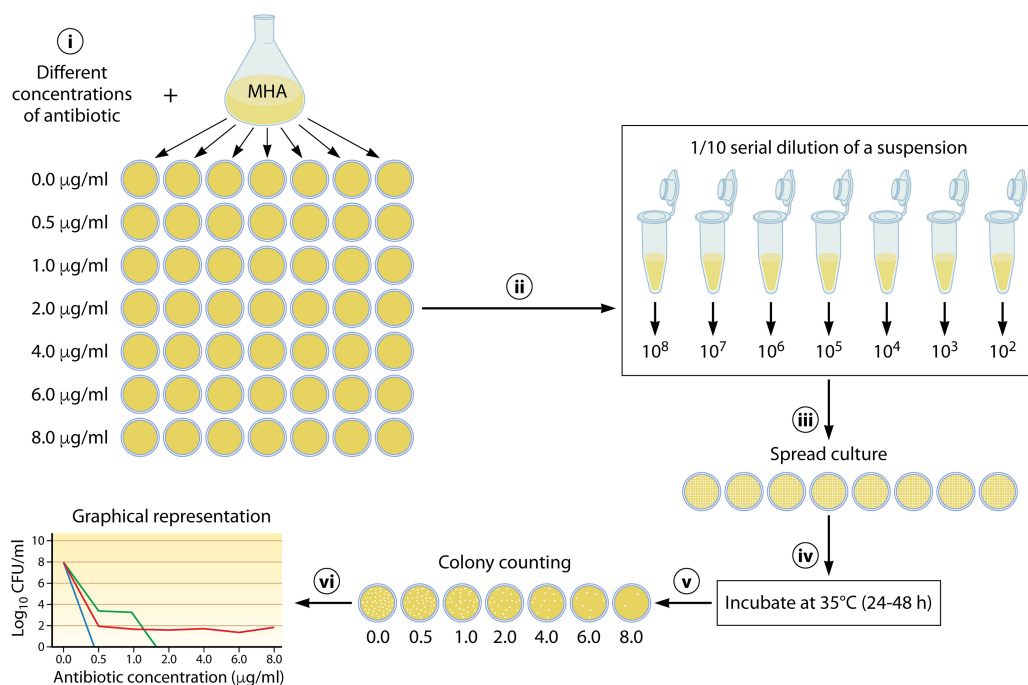


FIG 4 Population analysis profile (PAP) protocol to detect the heteroresistance phenomenon in bacteria. PAP is usually performed by inoculation of a bacterial population onto MHA plates containing increasing concentrations of antibiotic, quantifying the bacterial growth at each antibiotic concentration, and, finally, graphical analysis of the results.

Altogether, since the potential antimicrobial activity of Tweens alone was not exactly explored, the use of P-80 to evaluate the susceptibility of Gram-positive pathogens (vancomycin- and methicillin-resistant staphylococci, vancomycin-resistant enterococci, and penicillin-resistant streptococci) to some lipoglycopeptides, including dalbavancin, oritavancin, and telavancin, is recommended to be continued (82, 130, 131).

The Heteroresistance Phenomenon. The phenomenon of heteroresistance could pose a new challenge to antimicrobial therapy, especially from the point of view that it is not detected by the traditional susceptibility testing methods and may also be misinterpreted on susceptibility categorization (61). While population analysis profiling (PAP) is considered the gold standard method broadly used to characterize heteroresistant bacteria (60, 62, 107, 132–134), other susceptibility assays, such as DD, BMD, the gradient diffusion test, and commercial automated systems, have also been employed (59, 90, 109, 135, 136). PAP analysis is usually performed by inoculation of a bacterial population onto MHA plates containing gradient concentrations of antibiotic and quantifying the bacterial growth at each of these concentrations. To facilitate counting of the number of CFU in each plate after 48 h of incubation at 35°C, serial saline dilutions of an overnight culture are spread on MHA plates (Fig. 4) (62, 133). The detection of heteroresistance by the PAP method for clinical laboratories is difficult and time-consuming. Furthermore, there is no standard guideline to perform PAP; in particular, there is no guideline for the selection of antibiotic concentration increments, such as 1- to 2-µg/ml steps (55, 60, 62, 137) and even steps as low as 0.1 µg/ml (138), or the starting concentration of the bacterial inoculum (0.5 McFarland and even higher) (55, 58, 133, 139, 140), leading to categorization errors (137). DD and gradient diffusion strip tests have been used for determining heteroresistance, as recommended for traditional *in vitro* susceptibility testing (57–59, 133, 135). A criterion for heteroresistance is the growth of colonies within the inhibition zone of disks and/or gradient diffusion strips (135). In a study by Lo-Ten-Foe et al. (90), Etest and DD were found to be methods advantageous for the detection of colistin-heteroresistant *E. cloacae* and *A. baumannii* isolates. Jayol et al. (57) showed that Etest strips can sufficiently identify

heteroresistance to colistin in *K. pneumoniae* isolates with PhoP-PhoQ alterations. On the other hand, Gravey et al. (139) evaluated the performance of various techniques for detecting colistin-heteroresistant *E. cloacae* clinical isolates. By comparison with PAP, DD and Etest showed low sensitivities of 22.9% and 54.2%, respectively, although the specificity for both methods was 100% (139). The low sensitivity of DD and Etest to detect heteroresistant strains might be due to the low bacterial load used in these methods, in that the load is improper for detecting the resistant subpopulations present at a low frequency. In addition, automated systems did not exhibit appropriate performance with bacteria with resistant subpopulations. Supporting this claim is the finding of a study by Lo-Ten-Foe et al. (90), where the Vitek 2 susceptibility test failed to detect colistin-heteroresistant *E. cloacae* isolates. The authors considered it necessary to use an alternative testing method with the capacity to detect resistant subpopulations for isolates in which occasional heteroresistance has been described.

The Impact of Medium. The cation composition of Mueller-Hinton (MH) medium has been shown to vary depending on the commercial brand (141). According to CLSI, a calcium concentration ranging from 20 to 25 mg/liter and a magnesium concentration ranging from 10 to 12.5 mg/liter in MH medium ensures reliable antimicrobial susceptibility results (88). A study by Girardello et al. (142) demonstrated that the calcium and magnesium concentrations measured for four MHB brands (Oxoid, Difco, Merck, HiMedia) were far below the recommendations of the CLSI guidance. None of the MHA commercial brands tested showed the correct concentrations of calcium and magnesium recommended by the CLSI. Although an EA of 100% was found for the Difco medium used, the EA rates between BMD and Etest for determination of the polymyxin B MIC was reported to be 80% for the Merck and Oxoid MHAs and 90% for the HiMedia MHA. In addition, the Merck MHA produced zones of inhibition larger than those produced by the other MHA brands tested. A poor EA of 46%, 64%, and 68% between the Etest and BMD with P-80 was reported by Hindler and Humphries (98) for the BBL, Hardy, and Remel MHA brands, respectively. However, CA was 78% for the BBL MHA, 78% for the Hardy MHA, and 84% for the Remel MHA. Such discrepancies in antimicrobial susceptibility testing results may be due to the distinct cation concentrations in the MHA or MHB brands tested (141, 143, 144). This is why the CLSI recommends the use of cation-adjusted Mueller-Hinton (CA-MH) medium or supplementation of the culture medium with cations for antimicrobial susceptibility testing purposes (88). Moreover, a study by Matzneller et al. (145) revealed that colistin MIC values against *P. aeruginosa* and *A. baumannii* increased significantly in a cation-dependent manner. In contrast, colistin activity against *E. coli* showed a linear increase with ascending cation concentrations. These findings indicate that the antibacterial activity of colistin is cation dependent and might be misestimated. A medium, namely, Iso-Sensitest agar (ISA), was introduced by the British Society for Antimicrobial Chemotherapy (BSAC) to overcome problems associated with the traditional media used for antimicrobial susceptibility testing procedures (146, 147). This medium allows the growth of the vast majority of microorganisms without additional supplementation. For fastidious organisms, it can also be supplemented with 5% whole horse blood and 20 mg/liter beta-NAD (148). Furthermore, ISA medium has been shown to be better than other media for the detection of heteroresistance. Lo-Ten-Foe et al. (90) compared the AD and Etest methods for colistin on MHA and ISA media. Their study demonstrated that the Etest on MHA failed to detect resistant subpopulations of heteroresistant *E. cloacae* isolates, while these resistant subpopulations were detected on ISA by both methods, reflecting the higher sensitivity of ISA for the detection of resistant subpopulations. For these reasons, there is still no consensus on the use of cation-adjusted or non-cation-adjusted medium, and thus, a consensus is needed.

Detection of *mcr-1*-carrying isolates by phenotypic methods using CA-MH medium with BMD remains a challenge for routine microbiology laboratories, where many isolates exhibit a MIC of 2 μ g/ml to colistin and are thus categorized as sensitive. More recently, Gwozdinski et al. developed a medium with a novel formulation called

calcium-enhanced Mueller-Hinton (CE-MH) medium containing 200 mg/liter Ca^{2+} ions with the capability of the simple and reliable determination of the colistin MIC in *mcr-1*-producing *Enterobacteriaceae* (149). In addition, this medium allowed the identification of colistin-resistant isolates that were incorrectly classified as susceptible by the reference BMD method in CA-MH. Further genomic analysis of these isolates revealed specific alterations in the *pmrCAB* operon, confirming the efficacy of CE-MH for colistin resistance testing compared to that of the reference medium, CA-MH (149).

The Impact of Bacterial Storage. The loss of colistin resistance following ≤ 12 months of storage in glycerol-supplemented brucella broth at -70°C has been reported by Hindler and Humphries (98). They found that 5 out of 14 *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* isolates that were initially resistant to colistin became susceptible after frozen storage. In study by Barin et al. (55), the polymyxin B MICs of all 4 *A. baumannii* isolates tested growing at $64\ \mu\text{g/ml}$ was reduced to 0.125 to $0.25\ \mu\text{g/ml}$ after 60 days of storage at -80°C . These findings indicate that polymyxin resistance might be lost following long-term storage.

The Impact of Bacterial Subcultures. The loss of resistance has been shown after the serial passaging of *in vitro*-selected colistin-resistant mutants without selective pressure (55, 62). Li et al. (62) demonstrated the loss of adaptive resistance to colistin in about 98% and 97% of colistin-resistant *A. baumannii* subpopulations, including ATCC 19606 and a clinical isolate, respectively, following 16 successive passages in colistin-free CA-MHB medium. Adaptive resistance to polymyxins, which is characterized by the induction of resistance in the presence of drug and reversal to the susceptible phenotype in its absence, has been identified in other Gram-negative bacilli (150, 151). Reversal to colistin susceptibility in *P. aeruginosa* has been observed in one study (150) by serial passage of strains with a phenotype of adaptive resistance to colistin in drug-free medium. Structural analysis revealed that the lipid A modification with L-Ara4N and palmitate observed in the evolved colistin-resistant strains did not appear in the revertant strains. Sequence analysis also demonstrated the lack of some genetic alterations within several TCSs of the colistin-susceptible revertants; all of those alterations were observed in colistin-resistant mutants. Reversions to the wild-type amino acid in the colistin-susceptible *P. aeruginosa* revertants under drug-free conditions might explain the restoration of colistin susceptibility (150). This acquisition and loss of polymyxin resistance might be one of the reasons for the variable results obtained from laboratory susceptibility testing, leading to the clinical ineffectiveness of the antibiotic.

CONCLUSIONS

The growing threat of infections caused by multiresistant Gram-negative bacilli, particularly in vulnerable patients, has currently forced many physicians to turn to polymyxins. This emphasizes the need for the effective evaluation of critical issues associated with testing for susceptibility to these compounds. Because of the unreliable results obtained from both DD and gradient diffusion susceptibility tests, BMD remains the only option for the *in vitro* assessment of polymyxin susceptibility. The adherence of polymyxins to the plastics used for BMD trays is an issue leading to significant variability in MIC results. Although addition of the surfactant P-80 alleviates antibiotic adsorption, the joint CLSI-EUCAST Polymyxin Breakpoints Working Group does not recommend that because of the synergistic activity of P-80 with polymyxins. Moreover, whether cation-adjusted or non-cation-adjusted medium should be used and whether BMD should be performed with or without P-80 have not yet been fully defined. The issue of brand-to-brand and batch-to-batch heterogeneity in polymyxin formulations is also a source of difference in MIC results. Resistance to polymyxins is increasingly being reported among MDR clinical isolates worldwide. A variety of LPS modifications, mainly mediated by *phoP-phoQ* and *pmrA-pmrB* TCSs, are strategies to protect bacteria from polymyxins. However, there is still a knowledge gap in a full understanding of polymyxin resistance, particularly in areas of heteroresistance and the conditions required for the activity of *mcr* genes. Further research is required to clarify (i) the optimal

method for polymyxin susceptibility testing in routine diagnostic settings, (ii) the impact of heteroresistance on susceptibility testing results and, (iii) the correlation between polymyxin MICs and clinical outcomes to guide decision-making for the treatment of MDR Gram-negative bacterial infections.

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