Polymyxin Susceptibility Testing and Breakpoint Setting

9

John Turnidge, Katherine Sei, and Johan Mouton

Abstract

Susceptibility testing of polymyxins has been subject to intensive review and revision in recent years. A joint working group was established by the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing to establish a reference method. Issues examined included the effects of divalent cations, binding to laboratory materials, and addition of polysorbate 80. The working group recommended the use of broth microdilution without the addition of polysorbate 80 as the reference method. Published studies have shown that other testing methods, including agar dilution, disk diffusion and gradient diffusion, have unacceptably high levels of very major errors compared to the reference method, and are not recommended for routine laboratory use. Most data were for the testing of colistin; less information was available for polymyxin B. The joint working group was also asked to consider the setting of clinical breakpoints for relevant pathogens. This task involved examination of the available pharmacokinetic-pharmacodynamic, pharmacokinetic-toxicodynamic and population clinical pharmacokinetic data. All current pharmacokinetic-pharmacodynamic targets are based on MICs generated using the reference broth dilution procedure.

Keywords

Colistin · Polymyxin B · Susceptibility testing methods · Need for stringent control of conditions · Clinical breakpoints

Given that the polymyxin class has been in clinical use for more than 50 years, it would normally be assumed that susceptibility testing and the associated breakpoints would have been adequately resolved many years ago. However, because this class fell into disuse for many years, scant attention was paid to either susceptibility testing or clinical breakpoints. The resurgence in use driven by the emergence and spread of multiresistant Gram-negative bacteria has resulted in a critical re-appraisal of susceptibility testing, the standardization thereof and clinical breakpoints. Thus, the last decade has seen a substantial upgrade in the types of data and methods to be applied in setting clinical breakpoints.

J. Turnidge (✉)
Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia
e-mail: jturnidge@gmail.com

K. Sei
Microscan, West Sacramento, CA, USA

J. Mouton (deceased)
Department of Medical Microbiology and Infectious Diseases, Erasmus Medical Centre, Rotterdam, The Netherlands

J. Li et al. (eds.), *Polymyxin Antibiotics: From Laboratory Bench to Bedside*, Advances in Experimental Medicine and Biology 1145, https://doi.org/10.1007/978-3-030-16373-0_9

Importantly, a clear distinction is now being made between breakpoints that distinguish the natural population without resistance mechanisms ('wild type') and those that distinguish between a high probability of cure and a low probability of cure. The former is now defined as an epidemiological cut-off value (ECOFF), is independent of any therapeutic intervention, and involves the application of *in vitro* phenotypic data only. The latter are called "breakpoints" or "clinical breakpoints" (the words are used synonymously), and are set only when there are sufficient in vitro, animal model and human pharmacokinetic-pharmacodynamic (PK-PD) and clinical outcome data [1]. Clinical breakpoints are used in the clinical laboratory to indicate whether treatment with an antibiotic is feasible or not, provided that the dosing regimen given to the patient is adequate.

A Joint Working Group was established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) to determine the most appropriate reference method for susceptibility testing and the setting of clinical breakpoints for the polymyxins.

9.1 In Vitro Susceptibility Testing

A standard reference method for susceptibility testing of antimicrobial agents is only of recent origin and was published in 2006. The accepted reference method for MIC testing of polymyxins is broth microdilution (BMD), as described in ISO 20776-1 [2], and is by and large the same as those described by CLSI [3] and EUCAST [4] with subtle differences. The vast majority of published MIC distributions have used this method, although a variety of other methods including agar dilution and Etest® have been used as well (see below for comments). Below, a number of issues are described that have a direct effect on the MIC value of polymyxins, indicating that standardization of testing is extremely important, as the conclusions that can be drawn from the test results would otherwise be invalid and highly misleading.

9.1.1 Formulations of Test Compounds

It is important to realize that for testing of colistin (polymyxin E) the colistin sulfate salt is used as the test reagent. The parenteral methanesulfonate formulation has almost no activity on its own; it is a prodrug [5]. The use of this prodrug will therefore give misleadingly high MIC values. Since slow conversion of colistin methanesulfonate to free colistin in aqueous solution does occur, MICs will be dependent on the circumstances that result in more or less conversion [6]. There are no such issues for polymyxin B, because the injectable product and the test reagent are the same, namely the sulfate salt.

9.1.2 Effects of Components of Polymyxins

Both polymyxin B and colistin contain mixtures of components. Colistin is predominantly a mixture of colistin A (E1) and colistin B (E2), which differ only in the length of the fatty acyl tail (by one carbon) [7]. Polymyxin B is also predominantly a mixture of polymyxin B1 and polymyxin B2, with two other components accounting for around 12% [8].

9.1.2.1 Colistin

Very recent work at MicroScan Microbiology systems, Beckman Coulter Inc. in California has shown that the USP standard is predominantly colistin A, while that of the Sigma-Aldrich chemical supply company, the most widely used reagent for in vitro studies, is predominantly colistin B [Sei, personal communication]. This suggests that there are substantial differences between manufacturers in the balance between the two major components of colistin and these in turn may result in different MIC values, although recent data suggest that these differences are likely to be negligible [9].

9.1.2.2 Polymyxin B

Tam et al. [10] have shown that MICs obtained against a variety of Gram-negative bacteria using Sigma-Aldrich brand polymyxin B components do not differ significantly from those observed with the USP standard. These investigators also reported only modest differences in the MICs of polymyxin B1 and B2. This was confirmed by the more recent work of Roberts et al. [9].

9.1.3 Influence of Cations

Calcium ions were shown many years ago to reduce the in vitro activity of colistin and polymyxin B against *Pseudomonas aeruginosa* [11–13]. Magnesium and some other divalent cations (strontium, barium) share this property, at least for polymyxin B [11, 13]. Added Ca^{++} and Mg^{++} also reduced the activity of polymyxin B against several other species of *Pseudomonas* and *Stenotrophomonas maltophilia* [14]. Colistin activity has also been shown to be affected when adding Ca^{++} and Mg^{++} ions to Mueller-Hinton broth. The effect may result from the interaction of these divalent cations with the outer membrane of these target species [11, 13]. It is stated without proof that this effect is not observed with *Escherichia coli*, *Klebsiella* spp., or *Proteus* spp. [12]. Using different experimental conditions, however, Chen and Feingold [13] suggested that there was an observable effect against *E. coli*. There is no published work examining the effect of other cations likely or possibly present in Mueller-Hinton broth, such as iron, zinc or manganese which have been documented to vary between manufacturers [15] and been shown to affect the MIC for some other drugs such as tigecycline [16].

The documented concentration of calcium and magnesium ions known to abolish the bactericidal effect of polymyxin B is 2 mM [13]. This translates to concentrations of 80 and 24 mg/L, respectively. ISO 16782 stipulated final cation concentrations of 20–25 mg/L of calcium and 10–12.5 mg/L of magnesium in cation-adjusted Mueller-Hinton broth used for reference and routine susceptibility testing [17]. Based on these concentrations, partial inhibition of the antibacterial activity of polymyxins against *P. aeruginosa* can be expected [13]. ISO 16782 did not stipulate final concentrations of either of these cations in Mueller-Hinton agar [17].

As the concentrations of calcium and magnesium are controlled in cation-adjusted Mueller-Hinton broth, this should not present a problem for reference or routine broth susceptibility testing. If there is some antagonism of polymyxin action, then at least it will occur consistently. However, it could potentially affect susceptibility testing using agar dilution where calcium and magnesium concentrations are not controlled and where calcium and magnesium concentrations are known to vary between brands [15]. This may also pose a problem for disk diffusion testing.

9.1.4 Binding to Plastic and Other Materials

Recent evidence has emerged that many polycationic molecules, including colistin and polymyxin B, adhere to plastics and other surfaces. Because BMD susceptibility testing is conventionally conducted in 96-well plastic microtiter trays, concern has arisen that this adherence may have a variable and deleterious effect on the accuracy of MIC measurements. Microtiter trays can be made from polystyrene, polypropylene or polycarbonate, and can have their surface charge enhanced for tissue culture work by the method discussed below. Polystyrene is by far the most commonly used for MIC and commercial panel testing.

Binding to plastic and glass has been examined to some extent by investigators at Beckman Coulter Inc. (previously Siemens Healthcare Diagnostics), manufacturers of Microscan® panels [Sei, unpublished observations]. The most influential effect on binding of colistin to typical polystyrene trays is surface charge. This was examined by measuring MICs of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 29753 in plates exposed to no external charge, and two levels of "corona" (plasma, the fourth state of matter) which ionises the plate surface (Table 9.1). Exposure to "full corona" could shift MIC values upward by at least fourfold.

In another experiment by these investigators supported by the US Centers for Disease Control and Prevention, MICs of 12 strains of Gram-negative bacteria were compared in five testing formats. Geometric mean MICs for the different formats are shown in Table 9.2.

As part of this experiment, they also undertook assays of the well/tube contents at different colistin concentrations and demonstrated that binding was concentration-dependent and saturable (Fig. 9.1).

Table 9.1 Distributions of MIC[a] replicates according to surface charge (corona strength)

Test strain	Corona strength	Number at MIC (mg/L)			
		≤0.25	0.5	1	2
E. coli ATCC 25922	None	21			
	Half	5	6	6	4
	Full		4	12	5
P. aeruginosa ATCC 29753	None	18	2		
	Half	1	11	8	
	Full			5	15

[a]Replicates were done 6 or 7 times in 3 separate brands of Mueller-Hinton broth

Table 9.2 Effect of different surfaces on MICs of 12 bacterial strains

	Microscan BMD panels	Trek BMD panels	Trek BMD Panels + Polysorbate 80	Macrobroth glass tubes	Macrobroth polypropylene tubes
Geo mean	0.11	0.94	0.22	0.40	0.42
Range	0.06–2	0.5–4	0.06–8	0.125–4	0.125–4

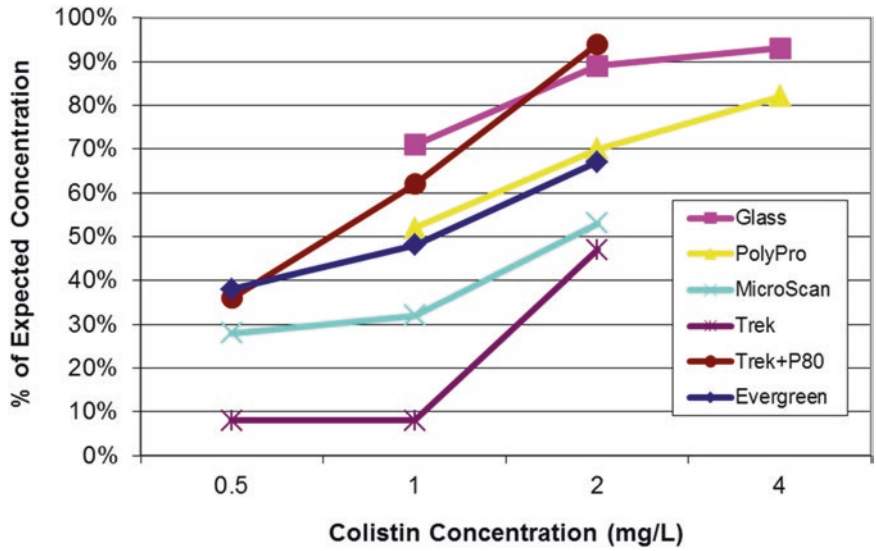

Fig. 9.1 Binding of colistin to the surface of trays and tubes made from different materials[a] and by various manufacturers[a], over a range of concentrations
[a]PolyPro = polypropylene microtiter tray; MicroScan = polystyrene in Beckman Coulter Inc. Microscan® brand tray; polystyrene in ThermoFisher's Trek brand tray; P80 = addition of polysorbate 80; Evergreen = brand of polystyrene tray

Another study from the United Kingdom examined the impact of using "tissue-culture treated" polystyrene microtiter trays (Corning brand) on colistin MICs. These trays are treated with corona discharge, resulting in a strong negative surface charge, to ensure maximum cell adhesion in cell cultures [18]. Corona treatment had a major effect on colistin binding, ranging from a 5.5-fold increase for *P. aeruginosa* to an 8.1-fold increase for Enterobacteriaceae.

Karvanen et al. examined the concentration- and time-dependent effects of binding to plastics (including polystyrene microtiter trays) and glass [19]. An exponential reduction in unbound colistin was observed, worst at the lowest concentration tested (0.125 mg/L) in glass, polypropylene and polystyrene tubes. Concentration-dependent binding was observed at 0 h and after 24 h of incubation at 37 °C; most of the loss occurred within 4–8 h (data not shown). How much this impacts on susceptibility testing is not known. Karvanen and co-workers also documented the loss of colistin during the preparation of stock solutions [19]. A drop of up to 57% was noted at the lower end of the stock solutions when prepared using the ISO-prescribed dilution method. Losses were even higher when stocks were prepared by straight serial dilution.

There is currently no information on binding to other materials such as Silastic®, rubber or other materials used in the preparation of drug stock solutions and dilutions.

9.1.5 Effect of Polysorbate 80 (P80) on 'Binding' and MICs of Polymyxins

It is known that some other antimicrobial classes have high binding to plastic and other materials, especially the lipoglycopeptides such as dalbavancin [20], and oritavancin [21]. Binding can be reduced or even eliminated by the addition of a non-ionic surfactant such as polysorbate 80 (P80, often known by one of its brand names, Tween® 80).

A number of experiments have been conducted at MicroScan Microbiology Systems, Beckman Coulter Inc. to determine the efficacy of P80 in reducing binding of colistin, the most recent of which examined this in the plates used to develop quality control ranges. P80 was included in the broth at 0.002% but not in drug dilutions. The most important features of this study were the demonstration that binding to plastic is concentration-dependent and saturable (Fig. 9.2), and influenced by brands/lots of Mueller-Hinton broth. They showed that P80 at 0.002% reduces binding but does not eliminate it.

Hindler and Humphries [22] have published a comparison of BMD MICs conducted using Evergreen brand microtiter trays with and without P80 at a concentration of 0.002%. They clearly demonstrated that the addition of P80 lowered the MICs of the 50 strains they tested, an expected effect if P80 increased free active drug in the well. They also demonstrated that the effect was dependent on the MIC in the absence of P80, with smaller differences between the MICs measured with and without P80 for less susceptible strains.

Sader et al. [23] have recently also shown the same phenomenon of reduced MICs in the presence of P80 at 0.002% for both colistin (Fig. 9.3a) and polymyxin B (Fig. 9.3b) against 124 strains of Enterobacteriaceae, 60 strains of *Acinetobacter* spp. and 63 strains of *P. aeruginosa*. Again, a concentration-dependent effect was shown.

Unpublished data have kindly been provided to the Joint EUCAST-CLSI Working Group on Polymyxins by IHMA (http://www.ihmainc.com/), the US-based company heavily involved in international surveillance programs such as the "SMART" study. They have conducted some in-house work comparing MICs generated with and without the presence of 0.002% P-80. Because of the truncation of MIC values at the lower end, most pertinent to Enterobacteriaceae, it is only possible to make general observations about their data (Table 9.3).

A recent experiment conducted at MicroScan Microbiology Systems, Beckman Coulter Inc., has thrown a cloud over all previous P80 findings, showing in fact that if anything the addition of P80 made colistin MICs higher. The only notable difference from previous experiments con-

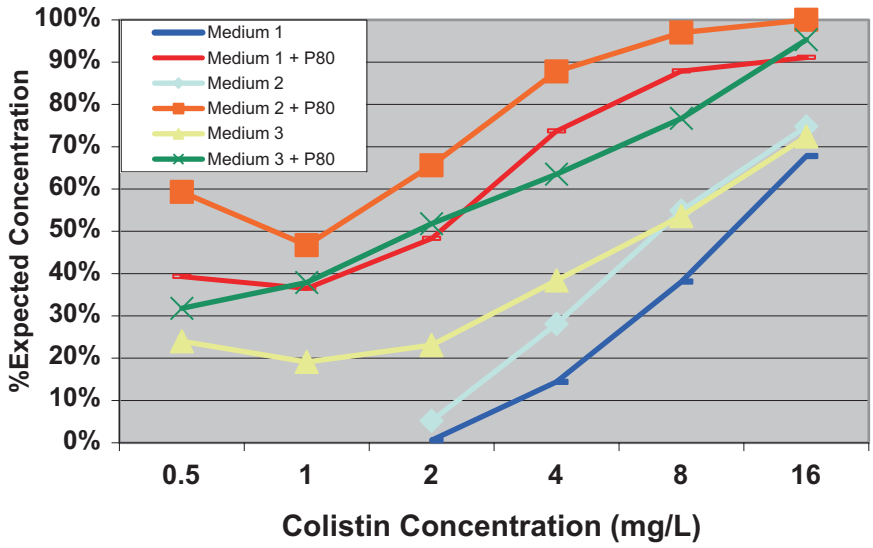

Fig. 9.2 Effect of polysorbate 80 on binding of colistin to polystyrene trays using three lots of Mueller-Hinton media

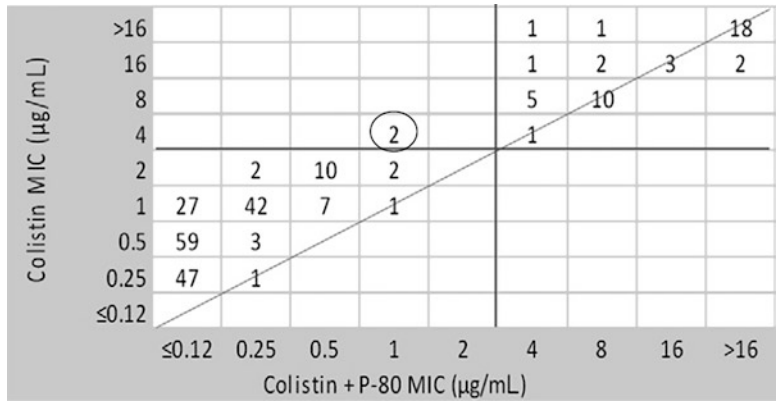

Fig. 9.3a Colistin MICs in the presence and absence of polysorbate 80 [23]

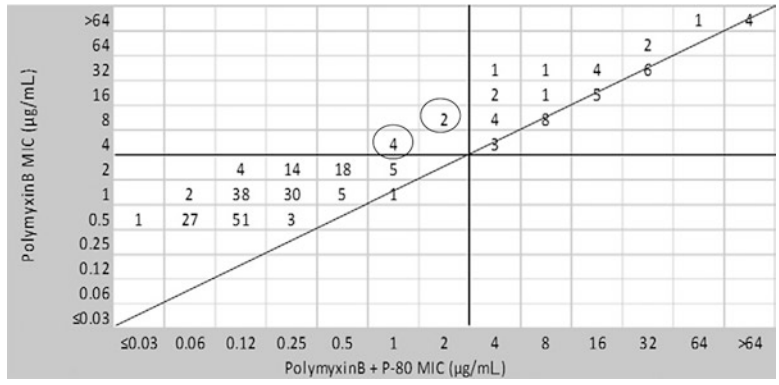

Fig. 9.3b Polymyxin B MICs in the presence and absence of polysorbate 80 [23]

Table 9.3 Distribution of MICs with and without polysorbate 80

Species	P-80	MIC (mg/L)						
		≤0.125	0.25	0.5	1	2	4	>4
Selected Enterobacteriaceae[a]	Without	8	65	324	61	5	4	20
	With	454	2	3	4		1	18
P. aeruginosa	Without		1	5	52	98	16	
	With	54	38	39	41			
A. baumannii†	Without			44	34	8	5	4
	With	75	11	4	3		1	1

[a]*E. coli*, *K. pneumoniae*, *E. cloacae*, *E. aerogenes*, *C. freundii*, *C. koseri*. All other Enterobacteriaceae species had MICs >4 mg/L

Table 9.4 Effects of corona treatment and polysorbate 80 on colistin MICs determined against a range of gram-negative bacteria

Group	Geometric Mean MIC (mg/L)			
	Corona-treated without P80	Corona-treated with P80	Untreated without P80	Untreated with P80
Enterobacteriaceae (n = 29)	1.54	2.10	0.12	0.40
A. baumannii (n = 10)	2.46	3.48	0.23	0.47
P. aeruginosa (n = 11)	1.66	2.00	0.16	0.18
Other non-fermenters (n = 5)	5.28	5.28	1.74	1.74

ducted there was that the drug dilutions were dispensed into their own brand microtiter trays using stainless steel equipment and Teflon™-coated tubing, rather than Silastic® tubing. Subsequent testing suggested significant loss of colistin when run through Silastic® tubing. The effect of corona treatment was again confirmed (Table 9.4).

These findings are difficult to explain. Communications with other investigators who have generated data described above has shown that the use of Silastic® tubing for dispensing drug solutions would not explain colistin 'binding'. For instance, Hindler and Humphries [22] only added P80 to the inoculum added to the wells in the trays and not to the drug solutions.

9.1.5.1 Optimum Polysorbate 80 Concentration

There are no data on the effect of different concentrations of P80 on binding of polymyxins. However, it has been shown for dalbavancin that the optimum range of P80 concentrations, as measured by the greatest effect on lowering MICs for *S. aureus*, was 0.002–0.02% [20]. For such antimicrobials a concentration of 0.002% is the most widely used in susceptibility testing.

9.1.5.2 Micelle Formation with Polysorbate 80

P80 is known to form micelles above the critical concentration of 0.0014% [24]. Thus the typical 0.002% concentration (=20 mg/L) used in susceptibility testing is above the critical micelle concentration. How this affects drug activity is not known. Its potential importance is that micelles may sequester drug and reduce the concentration of free drug in the test system.

9.1.5.3 Quality Control Range Studies and the Influence of Polysorbate 80

At the January 2013 meeting of the CLSI Antimicrobial Susceptibility Testing Subcommittee, the results of a formal 8-laboratory

CLSI study designed to establish QC ranges for colistin and polymyxin B in BMD testing were presented [25]. The study included MIC testing in the absence and presence of P80. The important components of the study were (i) source of drug, Sigma-Aldrich; (ii) source of trays, Sarstedt brand polystyrene; (iii) source of polysorbate 80, Spectrum Chemical; (iv) addition of P80 to media, but not to drug dilutions performed before dispensing into trays; (v) dissolution and dilution of stock drugs in glass tubes, followed by filter sterilization, and dispensing into trays through Silastic® and/or rubber tubes. The inclusion of P80 resulted in ~5.5-fold reduction in MICs for *E. coli* and ~fourfold for *P. aeruginosa* (Tables 9.5 and 9.6).

The study was the first clear demonstration that Mueller-Hinton medium brand/lot could also have an impact on MICs. In 4 of 8 instances, one of the three medium lots gave significantly lower MICs than those observed with the other two medium lots (as tested by Analysis of Variance). Importantly, this was only observed with *P. aeruginosa* ATCC 27853.

Disappointingly, the addition of P80 did not reduce the overall assay variance observed in the QC study for either colistin or polymyxin B, so in this respect did not offer an advantage over performing MIC testing in the absence of P80 (Table 9.6). The QC study was performed with a single set of plastic trays of unknown surface charge.

Table 9.5 Effect of polysorbate 80 (P80) on MIC distributions from the CLSI quality control study of colistin and polymyxin B [25]

Agent	Quality control strain	Additive	MIC (mg/L)							
			0.03	0.06	0.13	0.25	0.5	1	2	4
Colistin	*E coli* ATCC 25922	P80	14	134	82	13	2			
		None			2	62	152	23	7	
	P. aeruginosa ATCC 27853	P80		12	53	155	23			
		None			1	9	93	129	11	
Polymyxin B	*E coli* ATCC 25922	P80	2	109	103	22	7			
		None				61	102	71	8	1
	P. aeruginosa ATCC 27853	P80		12	52	139	36	7		
		None				4	44	159	33	6

Table 9.6 Assay variance (SD) for QC strains from the CLSI QC study

Agent	Strain	Polysorbate 80	Geometric mean MIC (log_2)	Geometric SD (log_2)
Colistin	*E. coli* 25922	Present	−4.089	0.716
		Absent	−1.618	0.693
		Fold difference	5.54	
	P. aeruginosa 27853	Present	−2.722	0.680
		Absent	−0.924	0.660
		Fold difference	3.47	
Polymyxin B	*E. coli* 25922	Present	−3.817	0.768
		Absent	−1.381	0.837
		Fold difference	5.41	
	P. aeruginosa 27853	Present	−2.606	0.811
		Absent	−0.528	0.690
		Fold difference	4.22	

9.1.6 Adherence to Plastic or Synergy?

P80 is thought to have antibacterial properties at certain high concentrations, although concentrations of 0.05% have been shown to have no effect on the short-term viability of *P. aeruginosa* [26]. Results from the same study suggested that P80 is synergistic with polymyxin B at concentrations of P80 as low as 0.001%. However, these studies were conducted at a time when the binding to plastic and other surfaces was not appreciated, and the effect was ignored.

Recently, investigators at Microscan Microbiology Systems, Beckman Coulter Inc. undertook a novel experiment by conducting broth dilution testing in Teflon™-coated trays (mini-muffin pans for kitchen use). After confirming that there was minimal adherence of colistin to the Teflon™ surface of these trays, the investigators showed a fourfold drop in MIC in the ATCC control strains of *E. coli* and *P. aeruginosa* with the addition of 0.002% P80. This experiment was repeated and expanded in Australia using similar kitchen-use Teflon™-coated mini-muffin pans [Bell, Li and Nation, unpublished observations]. While this brand of tray did bind colistin to a small extent, about 20–30% in the presence or absence of bacteria, this amount of binding did not account for the 2- to 128-fold reduction in MICs observed with addition of 0.002% P80 to 5 of 6 strains of bacteria, the *P. aeruginosa* control strain and 2 each of clinical isolates of *K. pneumoniae* and *A. baumannii* complex.

These MIC studies confirmed that the dominant effect on MIC measurements of colistin is due to a synergistic activity of this surfactant on that antimicrobial agent. MicroScan Microbiology Systems, Beckman Coulter Inc., have shown the same effect with another non-ionic surfactant, Pluronic® P-104 [Sei, unpublished observations]. We hypothesise that the synergy of non-ionic surfactants with polymyxins is due to a direct action of the surfactants on the bacterial outer membrane.

9.1.7 Summary of Issues Relating to Broth Microdilution Testing

Colistin and polymyxin B bind to the plastics and probably other materials used in reference BMD susceptibility tests and commercial systems. The binding is dependent on surface charge, type of plastic, brand of plate, and the tubing and pipette materials used for plate preparation. The effect is concentration dependent and saturable from little binding at concentrations of 4 mg/L and higher, and up to 90% binding after 24 h at the lowest test concentrations (0.03–0.06 mg/L).

Of these factors, the most important by far appears to be surface charge, particularly the plastic in microtiter trays. The addition of P80 surfactant appears to reduce the binding of colistin and polymyxin B in most instances, but does not completely eliminate it, nor does it appear to offer a great advantage or disadvantage from the assay point of view, as it does not reduce the assay variance.

More important is the evidence that P80 synergises with colistin and polymyxin B. The effect is more potent than that of reducing binding, and called into question the value of adding P80 to MIC test systems. As a consequence, both EUCAST and CLSI have agreed that reference MIC testing of polymyxins should not include the addition of P80 or other non-ionic surfactants.

9.1.8 Disc Diffusion Testing

Soon after the introduction of polymyxins into clinical practice disc diffusion susceptibility testing was introduced because of the great popularity with the method at the time. Although still used in many laboratories, the major change over time has been that MIC microdilution testing has become the standard of susceptibility testing. The immediate consequence is that any susceptibility testing method should be referenced to the standard, including automated methods, gradient tests and disk diffusion. With respect to disk sus-

ceptibility testing of polymyxins, results have been disappointing, probably because the size and charge of the molecules results in poor diffusion through agar. Very soon after introduction of the BMD MIC method, it was shown by Matsen et al. that there was very poor correlation between disk zone size and BMD MIC [27].

In more recent years, in an extensive study involving 200 bloodstream isolates, Gales and colleagues [28] documented clearly that there is a serious problem with very major errors associated with disk diffusion testing of both colistin and polymyxin B. They concluded that clinical laboratories should exclusively use MIC methods to assist the therapeutic application of colistin or polymyxin B. These observations were later again confirmed in comparative studies by Tan and Ng [29], Lo-Ten-Foe et al. [30], Moskowitz et al. [31] and Maalej et al. [32] who all observed significant rates of false susceptibility with disc diffusion. In addition, in a recent study with 10, 25 or 50 μg colistin disks, resistant and susceptible isolates could not be reliably separated [Kahlmeter and Matuschek, personal communication]. The current view is that disk susceptibility testing is unreliable and should not be used for susceptibility testing in the clinical laboratory.

9.1.9 Gradient Diffusion Methods

Two commercial strips for gradient diffusion testing are available, those of bioMérieux (Etest®) and Liofilchem (MTS®). The Etest in particular has been compared to other testing methods by several authors and has shown conflicting results [30, 32–34], but when compared to reference BMD MIC testing, always showed significant proportions of very major errors [30, 33]. Both brands were recently compared to the standard method and both showed significant major errors in a recent comparison by the EUCAST Development Laboratory [35]. The EUCAST has placed a warning on their website in 2016 against using the gradient methods (http://www.eucast.org/ast_of_bacteria/warnings/) until such time as the manufacturers are able to address the problems (at the time of writing, one brand was still commercially available).

9.1.10 MIC Distributions and ECOFFs

Current methods to determine the susceptibility of micro-organisms are not very reproducible when compared with other clinical tests. This is due to both the inherent biological variation of micro-organisms and assay variation. MICs are normally determined using a twofold dilution series of the antimicrobial agent and the MIC distribution of the wild-type strains tested is log-normally distributed. Moreover, repeated measurement of the same strain will provide MICs that show at least a 50–100% coefficient of variation. MICs of strain collections therefore always show a log-normal distribution in the wild type, and the variation within that distribution is due to both intra- and inter-laboratory variation. MIC distributions are specific to each combination of species and antimicrobial agent. MIC distributions for a very broad range of species/antimicrobial combinations can be found at the website of EUCAST (http://mic.eucast.org/Eucast2/).

Methods have been sought to describe MIC distributions statistically, and in particular to determine whether strains are wild type or non-wildtype. This has led to the introduction of the concept of epidemiological cutoff values (ECOFFs) which are MIC values that mark the high end of the wild-type distribution.

9.1.10.1 Colistin MIC Distributions

BMD MIC distribution data and ECOFFs for colistin are on the EUCAST website: http://mic.eucast.org/Eucast2/, and were updated (February, 2016) using more stringent rules of data acceptance that are under development by EUCAST.

9.1.10.2 Polymyxin B MIC Distributions

There are few published data on MIC distributions of polymyxin B, and none currently listed in the EUCAST website. Only three publications currently provide on-scale MIC distribution data

for polymyxin B by species [33, 36, 37]. The data from two of these studies, as well as data obtained from the SENTRY surveillance program [Sader, personal communication, 2015], where BMD was used, are shown in Table 9.7. Sader et al. [38] have shown that polymyxin BMD MICs tend to be higher for polymyxin B when compared directly with colistin for the wild-types of three species, *E. coli*, *K. pneumoniae* and *P. aeruginosa*.

9.2 Breakpoint Setting

Since 2000, the methods for selecting interpretive criteria (breakpoints) for susceptibility tests have undergone profound change. Prior to that time, much weight was applied to MIC distribution data, although pharmacokinetic and some pharmacodynamic data were taken into account in some European committees but not elsewhere [39]. Clinical data were used where available, although clinical trial design improved considerably after 2000. Since that time, the science of antimicrobial pharmacokinetic-pharmacodynamics (PK-PD) has come to provide a suite of tools to integrate susceptibility data with pharmacokinetics, based on knowledge of PK-PD indices (fT>MIC, fAUC$_{24}$/MIC and fC$_{max}$/MIC) and their respective target values associated with efficacy. PK-PD is now an integral part of the breakpoint setting standards applied by EUCAST and CLSI committees.

The process of setting clinical breakpoints involves several steps and procedures, both preclinical and clinical, as described Mouton, et al. [39]. Ideally, each step is known and taken into account when setting the clinical breakpoint, and for new drugs this information is generally available, or becomes available during the development of the drug. However, for polymyxins a substantial amount of this information is not available. At the time of registration of the polymyxins, the PK-PD of antimicrobial agents as a science did not exist and there was no reference method for susceptibility testing.

Polymyxins, like other 'old antibiotics', therefore needed redevelopment using modern standards in order to determine breakpoints. Although much information has become available in recent years, there are still many gaps that need to be filled. Below, we discuss the most important issues: the pharmacodynamic target, pharmacokinetics in patients and the modelling to determine the probability of target attainment (PTA). These processes ultimately lead to the setting of clinical breakpoints.

9.2.1 The Pharmacodynamic Target of Polymyxins

The pharmacodynamic target (PT) of an antimicrobial involves two types of studies. In the first, time-kill experiments are conducted to determine whether the drug shows primarily time-dependent

Table 9.7 Distributions of polymyxin B (sulfate) MIC

Ref	**Species**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥ 16**
a	*P. aeruginosa*				20	26	26	4		1	
Ref	**Species**		**≤ 0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
b	*E. coli*		29	14	8	2	6	2			
	K. pneumoniae		39	9	2	1	0	4	6		1
	P. aeruginosa		2	29	18	4	1	2	2	1	4
Ref	**Species**			**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥ 16**
c	*E. coli*			4	466	4463	1340	15	20	5	
	Klebsiella spp.			1	43	2368	1561	35	30	40	100
	P. aeruginosa			3	12	95	1850	1854	4	0	3

a. van der Heijden et al. [33]
b. Vaara et al. [37]
c. Sader, personal communication, 2015

or concentration-dependent killing. Maximum kill at relatively low concentrations proceeding over time is usually associated with time-dependent effects, and efficacy thus primarily correlated with the time the concentration of the drug remains above the MIC, usually expressed as *%f*T>MIC, where "*f*' refers to the unbound fraction of drug. In contrast, increased killing as a result of increasing concentrations is usually associated with area under the time-concentration curve (AUC), most often taken over 24 h, divided by the MIC of the target organism ($fAUC_{24}$/MIC). Killing curves for polymyxins show concentration-dependent killing [40], which generally predicts that bacterial killing in vivo is associated with AUC.

As suggested above, protein binding of an antimicrobial agent must be accounted for in determination of PK-PD indices. The initial experiments with protein binding indicated that it might be concentration-dependent [41]. This subsequently proved to be an artefact of the assay systems used, due to the adherence of colistin and polymyxin B to laboratory plastics and surfaces. When this process was controlled for, it was shown that protein binding was not concentration-dependent, and values were found for the percent binding in human volunteers and in infected patients, both approximately 50% [42].

Studies to determine the PK-PD indices that predict killing have been undertaken so far for colistin against *P. aeruginosa* and *Acinetobacter baumannii* in murine thigh and lung infection models [42] and against *Klebsiella pneumoniae* in an in vitro PK-PD model [43]. There is a report of a PK-PD study with polymyxin B against *P. aeruginosa* in an in vitro model [44] and a report of a study in murine thigh and lung infection models [45]. All of the above-mentioned studies indicated that the *f*AUC/MIC ratio is the PK-PD index that is most predictive of efficacy. For polymyxin B, notwithstanding the qualitative similarity in the nature of the PK-PD relationship between the two studies, there was a substantial quantitative difference. For example, over the same range of *f*AUC/MIC (exposure) values, up to six-log_{10} bacterial killing was achievable in the in vitro model [43] but less than two-log_{10} bacterial killing was possible in the murine thigh infection model and no killing was observed in the lung infection model [45]. In addition, in the latter study, there was a relatively wide range in the *f*AUC/MIC target values for stasis and one-log_{10} kill in the thigh model. Clearly, more PK-PD data are required for *K. pneumoniae* and for polymyxin B.

The most recent of the colistin studies [42], conducted in the neutropenic mouse thigh and lung models of *P. aeruginosa* and *A. baumannii* infection, established target values for $fAUC_{24}$/MIC for stasis and one- and two-log_{10} killing at the site of infection. For thigh infection, a mean target $fAUC_{24}$/MIC ratio of ~9 for one-log_{10} kill and ~12 for two-log_{10} kill was observed. For lung infection, target $fAUC_{24}$/MIC ratios were much higher, and were considered to be unachievable in clinical practice based upon the finding that there is a substantially increased risk of nephrotoxicity in critically ill patients when the average steady-state plasma colistin concentration exceeds ~2.5 mg/L [46, 47].

9.2.2 Human Pharmacokinetics

The next step in breakpoint setting, after PK-PD targets have been established, is to choose appropriate human estimates of the pharmacokinetic parameter of interest, in this case, $fAUC_{24}$. Most commonly, this is done using population PK studies, either from human volunteer studies, or preferably PK studies in patients with infections. A number of such studies have now been published [48–52]. An important feature of colistin is that the parenteral preparation is the methanesulfonate, an inactive prodrug which is cleared by the kidney and slowly broken down in plasma and tissue to the active colistin molecule. As a consequence, colistin exposure is very strongly influenced by the degree of renal function [52, 49, 51].

The most useful data for assisting in breakpoint setting that are available is from a large international multicenter trial of colistin treat-

ment in infected intensive care patients [53, 52]. Due to the nature of the types of patients treated with colistin, the study found patients with an extremely broad range of creatinine clearances, which meant that estimates of target attainment had to be calculated across groups comprising different degrees of renal function. In order to do this, both the FDA [54] and the EMA [55] dosing recommendations for patients with different levels of renal function must be considered because they differ somewhat in their recommendations, but reflect the dosing regimens most widely used internationally. The analyses have assumed that there is 50% protein binding in humans, and that the appropriate target value for $fAUC_{24}$/MIC is 12, based on the mouse thigh infection model study for at least two $\log_{10}$ of killing [42]. Note that the parameter chosen by Nation et al. [53, 52], namely average steady-state plasma colistin concentration of total drug, relates to $fAUC_{24}$ as follows. At 50% protein binding a $fAUC_{24}$ of 12 is equivalent to a total drug AUC_{24} of 24, which in turn is equal to an average steady-state plasma colistin concentration of total drug of 1 mg/L (i.e. 24 mg*h/L divided by 24 h). In essence, this means that the target average steady-state plasma colistin concentration is equal to the MIC of the infecting organism.

It is clear from these analyses that at a colistin MIC of 0.5 mg/L adequate target attainment (>90%) is likely to be achieved with both the FDA and the EMA dosing recommendations [53]. At a colistin MIC of 1 mg/L the attainment percentages are more variable: using FDA dosing recommendations there is low target attainment (<30%) at the lowest and highest levels of renal function, while with the EMA recommendations, low target attainment is seen only with patients having creatinine clearance >80 mL/min (<40% target attainment). For a colistin MIC of 2 mg/L, only the EMA dosing recommendations were able to achieve satisfactory target attainment for the three lowest renal function categories (i.e. patients with creatinine clearance <80 mL/min). As 2 mg/L is the epidemiological cut-off value for *A. baumannii*, the Joint CLSI-EUCAST Working Group on Polymyxins recommended a colistin breakpoint for this species of 2 mg/L, accompanied by the recommendation of using maximum recommended dose. It also led EUCAST to lower the *P. aeruginosa* breakpoint from the previous value of ≤4 mg/L, even though a small proportion of the wild-type population has an MIC of 4 mg/L. In addition, because of the less than optimum attainment for some degrees of renal function, the Working Group also recommended that there be no "Intermediate" category for the interpretive susceptibility test criteria.

In making its breakpoint recommendations to CLSI and EUCAST, the Joint Working Group was aware that these recommendations were based on murine thigh and lung infection models only, and that validation of the PK-PD targets will be required from prospective clinical studies. Furthermore, the data from the murine models would suggest that target attainment is suboptimal in pulmonary infections caused by these two species, and thus the recommended breakpoints may not apply in this setting.

9.2.3 Future Goals

There is further work to be done on breakpoint setting. For example:

- Data on clinical response rates by colistin MIC for *P. aeruginosa* and *A. baumannii* are lacking.
- More information is awaited on the PK-PD target *f*AUC/MIC ratios for Enterobacteriaceae. In the meantime, both EUCAST and CLSI will work with an epidemiological cut-off value of 2 mg/L for this group of micro-organisms.
- There are insufficient data in all the areas for polymyxin B: MIC distributions, PK-PD target data, and human PK data.

These are all eagerly awaited.

References

1. Turnidge J, Paterson DL (2007) Setting and revising antibacterial susceptibility breakpoints. Clin Microbiol Rev 20(3):391–408. https://doi.org/10.1128/cmr.00047-06
2. ISO 20776-1 (2006) Clinical laboratory testing and in vitro diagnostic test systems – Susceptibility testing of infectious agents and evaulation of peformance of antimicrobial susceptibility test devices. Part 1: Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. Internation Organization for Standardization, Geneva
3. Clinical and Laboratory Standards Institute (CLSI) (2015) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard, 10th edn. Clinical and Laboratory Standards Institute, Wayne
4. EUCAST DISCUSSION DOCUMENT E. Dis 5.1 Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution (2003). Clin Microbiol Infect 9:1–7
5. Bergen PJ, Li J, Rayner CR, Nation RL (2006) Colistin methanesulfonate is an inactive prodrug of colistin against Pseudomonas aeruginosa. Antimicrob Agents Chemother 50(6):1953–1958. https://doi.org/10.1128/AAC.00035-06
6. Li J, Milne RW, Nation RL, Turnidge JD, Coulthard K (2003) Stability of colistin and colistin methanesulfonate in aqueous media and plasma as determined by high-performance liquid chromatography. Antimicrob Agents Chemother 47(4):1364–1370
7. Orwa JA, Govaerts C, Busson R, Roets E, Van Schepdael A, Hoogmartens J (2001) Isolation and structural characterization of colistin components. J Antibiot (Tokyo) 54(7):595–599
8. He J, Ledesma KR, Lam WY, Figueroa DA, Lim TP, Chow DS, Tam VH (2010) Variability of polymyxin B major components in commercial formulations. Int J Antimicrob Agents 35(3):308–310. https://doi.org/10.1016/j.ijantimicag.2009.11.005
9. Roberts KD, Azad MA, Wang J, Horne AS, Thompson PE, Nation RL, Velkov T, Li J (2015) Antimicrobial activity and toxicity of the major lipopeptide components of polymyxin B and colistin: last-line antibiotics against multidrug-resistant gram-negative bacteria. ACS Infect Dis 1(11):568–575. https://doi.org/10.1021/acsinfecdis.5b00085
10. Tam VH, Cao H, Ledesma KR, Hu M (2011) In vitro potency of various polymyxin B components. Antimicrob Agents Chemother 55(9):4490–4491. https://doi.org/10.1128/AAC.00119-11
11. Newton BA (1954) Site of action of polymyxin on Pseudomonas aeruginosa: antagonism by cations. J Gen Microbiol 10(3):491–499
12. Davis SD, Iannetta A, Wedgwood RJ (1971) Activity of colistin against Pseudomonas aeruginosa: inhibition by calcium. J Infect Dis 124(6):610–612
13. Chen CC, Feingold DS (1972) Locus of divalent cation inhibition of the bactericidal action of polymyxin B. Antimicrob Agents Chemother 2(5):331–335
14. D'Amato RF, Thornsberry C, Baker CN, Kirven LA (1975) Effect of calcium and magnesium ions on the susceptibility of Pseudomonas species to tetracycline, gentamicin polymyxin B, and carbenicillin. Antimicrob Agents Chemother 7(5):596–600
15. Girardello R, Bispo PJ, Yamanaka TM, Gales AC (2012) Cation concentration variability of four distinct Mueller-Hinton agar brands influences polymyxin B susceptibility results. J Clin Microbiol 50(7):2414–2418. https://doi.org/10.1128/JCM.06686-11
16. Veenemans J, Mouton JW, Kluytmans JA, Donnely R, Verhulst C, van Keulen PH (2012) Effect of manganese in test media on in vitro susceptibility of Enterobacteriaceae and Acinetobacter baumannii to tigecycline. J Clin Microbiol 50(9):3077–3079. https://doi.org/10.1128/jcm.01485-12
17. ISO/TS 16782 (2016) Clinical laboratory testing -- Criteria for acceptable lots of dehydrated Mueller-Hinton agar and broth for antimicrobial susceptibility testing. International Organization for Standardization, Geneva
18. Albur M, Noel A, Bowker K, Macgowan A (2014) Colistin susceptibility testing: time for a review. J Antimicrob Chemother 69(5):1432–1434. https://doi.org/10.1093/jac/dkt503
19. Karvanen M, Malmberg C, Lagerback P, Friberg LE, Cars O (2017) Colistin is extensively lost during standard in vitro experimental conditions. Antimicrob Agents Chemother 61(11). https://doi.org/10.1128/aac.00857-17
20. Rennie RP, Koeth L, Jones RN, Fritsche TR, Knapp CC, Killian SB, Goldstein BP (2007) Factors influencing broth microdilution antimicrobial susceptibility test results for dalbavancin, a new glycopeptide agent. J Clin Microbiol 45(10):3151–3154. https://doi.org/10.1128/jcm.02411-06
21. Arhin FF, Sarmiento I, Belley A, McKay GA, Draghi DC, Grover P, Sahm DF, Parr TR Jr, Moeck G (2008) Effect of polysorbate 80 on oritavancin binding to plastic surfaces: implications for susceptibility testing. Antimicrob Agents Chemother 52(5):1597–1603. https://doi.org/10.1128/aac.01513-07
22. Hindler JA, Humphries RM (2013) Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. J Clin Microbiol 51(6):1678–1684. https://doi.org/10.1128/JCM.03385-12
23. Sader HS, Rhomberg PR, Flamm RK, Jones RN (2012) Use of a surfactant (polysorbate 80) to improve MIC susceptibility testing results for polymyxin B and colistin. Diagn Microbiol Infect Dis 74(4):412–414. https://doi.org/10.1016/j.diagmicrobio.2012.08.025
24. Wan LS, Lee PF (1974) CMC of polysorbates. J Pharm Sci 63(1):136–137
25. Brown S, Traczewski M (2013) Quality control limits for microdilution susceptibility tests of colistin against Escherichia coli ATCC 25922 and Pseudomonas

aeruginosa ATCC 278563. Clinical and Laboratory Standards Institute
26. Brown MR, Winsley BE (1971) Synergism between polymyxin and polysorbate 80 against Pseudomonas aeruginosa. J Gen Microbiol 68(3):367–373
27. Matsen JM, Koepcke MJ, Quie PG (1969) Evaluation of the Bauer-Kirby-Sherris-Turck single-disc diffusion method of antibiotic susceptibility testing. Antimicrob Agents Chemother (Bethesda) 9:445–453
28. Gales AC, Reis AO, Jones RN (2001) Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. J Clin Microbiol 39(1):183–190. https://doi.org/10.1128/JCM.39.1.183-190.2001
29. Tan TY, Ng LS (2006) Comparison of three standardized disc susceptibility testing methods for colistin. J Antimicrob Chemother 58(4):864–867. https://doi.org/10.1093/jac/dkl330
30. Lo-Ten-Foe JR, de Smet AM, Diederen BM, Kluytmans JA, van Keulen PH (2007) Comparative evaluation of the VITEK 2, disk diffusion, etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant Enterobacter cloacae and Acinetobacter baumannii strains. Antimicrob Agents Chemother 51(10):3726–3730. https://doi.org/10.1128/AAC.01406-06
31. Moskowitz SM, Garber E, Chen Y, Clock SA, Tabibi S, Miller AK, Doctor M, Saiman L (2010) Colistin susceptibility testing: evaluation of reliability for cystic fibrosis isolates of Pseudomonas aeruginosa and Stenotrophomonas maltophilia. J Antimicrob Chemother 65(7):1416–1423. https://doi.org/10.1093/jac/dkq131
32. Maalej SM, Meziou MR, Rhimi FM, Hammami A (2011) Comparison of disc diffusion, Etest and agar dilution for susceptibility testing of colistin against Enterobacteriaceae. Lett Appl Microbiol 53(5):546–551. https://doi.org/10.1111/j.1472-765X.2011.03145.x
33. van der Heijden IM, Levin AS, De Pedri EH, Fung L, Rossi F, Duboc G, Barone AA, Costa SF (2007) Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant P. Aeruginosa to polymyxins. Ann Clin Microbiol Antimicrob 6:8. https://doi.org/10.1186/1476-0711-6-8
34. Tan TY, Ng SY (2007) Comparison of Etest, Vitek and agar dilution for susceptibility testing of colistin. Clin Microbiol Infect 13(5):541–544. https://doi.org/10.1111/j.1469-0691.2007.01708.x
35. Matuschek E, Ahman J, Webster C, Kahlmeter G (2018) Antimicrobial susceptibility testing of colistin – evaluation of seven commercial MIC products against standard broth microdilution for Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Clin Microbiol Infect 24(8):865–870. https://doi.org/10.1016/j.cmi.2017.11.020
36. Hogardt M, Schmoldt S, Gotzfried M, Adler K, Heesemann J (2004) Pitfalls of polymyxin antimicrobial susceptibility testing of Pseudomonas aeruginosa isolated from cystic fibrosis patients. J Antimicrob Chemother 54(6):1057–1061. https://doi.org/10.1093/jac/dkh470
37. Vaara M, Sader HS, Rhomberg PR, Jones RN, Vaara T (2013) Antimicrobial activity of the novel polymyxin derivative NAB739 tested against Gram-negative pathogens. J Antimicrob Chemother 68(3):636–639. https://doi.org/10.1093/jac/dks438
38. Sader HS, Rhomberg PR, Farrell DJ, Jones RN (2015) Differences in potency and categorical agreement between colistin and polymyxin B when testing 15,377 clinical strains collected worldwide. Diagn Microbiol Infect Dis 83(4):379–381. https://doi.org/10.1016/j.diagmicrobio.2015.08.013
39. Mouton J, Ambrose P, Kahlmeter G, Wikler M, Craig W (2007) Applying pharmacodynamics for susceptibility breakpoint selection and susceptibility testing. In: Nightingale CAP, Drusano GL, Murakawa T (eds) Antimicrobial pharmacodynamics in theory and clinical practice, 2nd edn. Informa Healthcare/CRC Press, New York, pp 21–44
40. Li J, Turnidge J, Milne R, Nation RL, Coulthard K (2001) In vitro pharmacodynamic properties of colistin and colistin methanesulfonate against Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Antimicrob Agents Chemother 45(3):781–785. https://doi.org/10.1128/AAC.45.3.781-785.2001
41. Dudhani RV, Turnidge JD, Coulthard K, Milne RW, Rayner CR, Li J, Nation RL (2010) Elucidation of the pharmacokinetic/pharmacodynamic determinant of colistin activity against Pseudomonas aeruginosa in murine thigh and lung infection models. Antimicrob Agents Chemother 54(3):1117–1124. https://doi.org/10.1128/AAC.01114-09
42. Cheah SE, Wang J, Nguyen VT, Turnidge JD, Li J, Nation RL (2015) New pharmacokinetic/pharmacodynamic studies of systemically administered colistin against Pseudomonas aeruginosa and Acinetobacter baumannii in mouse thigh and lung infection models: smaller response in lung infection. J Antimicrob Chemother 70(12):3291–3297. https://doi.org/10.1093/jac/dkv267
43. Tsala M, Vourli S, Georgiou PC, Pournaras S, Tsakris A, Daikos GL, Mouton JW, Meletiadis J (2018) Exploring colistin pharmacodynamics against Klebsiella pneumoniae: a need to revise current susceptibility breakpoints. J Antimicrob Chemother 73(4):953–961. https://doi.org/10.1093/jac/dkx522
44. Tam VH, Schilling AN, Vo G, Kabbara S, Kwa AL, Wiederhold NP, Lewis RE (2005) Pharmacodynamics of polymyxin B against Pseudomonas aeruginosa. Antimicrob Agents Chemother 49(9):3624–3630. https://doi.org/10.1128/AAC.49.9.3624-3630.2005
45. Landersdorfer CB, Wang J, Wirth V, Chen K, Kaye KS, Tsuji BT, Li J, Nation RL (2018) Pharmacokinetics/pharmacodynamics of systemically administered polymyxin B against Klebsiella pneumoniae in

mouse thigh and lung infection models. J Antimicrob Chemother 73(2):462–468. https://doi.org/10.1093/jac/dkx409

46. Sorli L, Luque S, Grau S, Berenguer N, Segura C, Montero MM, Alvarez-Lerma F, Knobel H, Benito N, Horcajada JP (2013) Trough colistin plasma level is an independent risk factor for nephrotoxicity: a prospective observational cohort study. BMC Infect Dis 13:380. https://doi.org/10.1186/1471-2334-13-380
47. Forrest A, Garonzik SM, Thamlikitkul V, Giamarellos-Bourboulis EJ, Paterson DL, Li J, Silveira FP, Nation RL (2017) Pharmacokinetic/Toxicodynamic analysis of colistin-associated acute kidney injury in critically ill patients. Antimicrob Agents Chemother 61(11). https://doi.org/10.1128/aac.01367-17
48. Plachouras D, Karvanen M, Friberg LE, Papadomichelakis E, Antoniadou A, Tsangaris I, Karaiskos I, Poulakou G, Kontopidou F, Armaganidis A, Cars O, Giamarellou H (2009) Population pharmacokinetic analysis of colistin methanesulfonate and colistin after intravenous administration in critically ill patients with infections caused by gram-negative bacteria. Antimicrob Agents Chemother 53(8):3430–3436. https://doi.org/10.1128/AAC.01361-08
49. Garonzik SM, Li J, Thamlikitkul V, Paterson DL, Shoham S, Jacob J, Silveira FP, Forrest A, Nation RL (2011) Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. Antimicrob Agents Chemother 55(7):3284–3294. https://doi.org/10.1128/AAC.01733-10
50. Karaiskos I, Friberg LE, Pontikis K, Ioannidis K, Tsagkari V, Galani L, Kostakou E, Baziaka F, Paskalis C, Koutsoukou A, Giamarellou H (2015) Colistin population pharmacokinetics after application of a loading dose of 9 MU colistin methanesulfonate in critically Ill patients. Antimicrob Agents Chemother 59(12):7240–7248. https://doi.org/10.1128/aac.00554-15
51. Jacobs M, Gregoire N, Megarbane B, Gobin P, Balayn D, Marchand S, Mimoz O, Couet W (2016) Population pharmacokinetics of colistin methanesulphonate (CMS) and colistin in critically ill patients with acute renal failure requiring intermittent haemodialysis. Antimicrob Agents Chemother 60(3):1788–1793. https://doi.org/10.1128/aac.01868-15
52. Nation RL, Garonzik SM, Thamlikitkul V, Giamarellos-Bourboulis EJ, Forrest A, Paterson DL, Li J, Silveira FP (2017) Dosing guidance for intravenous colistin in critically ill patients. Clin Infect Dis 64(5):565–571. https://doi.org/10.1093/cid/ciw839
53. Nation RL, Garonzik SM, Li J, Thamlikitkul V, Giamarellos-Bourboulis EJ, Paterson DL, Turnidge JD, Forrest A, Silveira FP (2016) Updated US and European dose recommendations for intravenous colistin: how do they perform? Clin Infect Dis 62(5):552–558. https://doi.org/10.1093/cid/civ964
54. Food-and-Drug-Administration (2013) Approved drug products. Label and approval history for ColyMycin M, NDA 050108. http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/050108s030lbl.pdf. Last accessed 16 June 2019
55. European-Medicines-Agency (2014) Assessment report on polymyxin-based products. Referral under Article 31 of Directive 2001/83/EC. http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Polymyxin_31/WC500179664.pdf. Last accessed 16 June 2019