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Review Article

Polymyxins: Antimicrobial susceptibility concerns and therapeutic options

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Abstract

The increasing prevalence of multidrug-resistant nosocomial pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* poses a great challenge to the treating physicians. The paucity of newer effective antimicrobials has led to renewed interest in the polymyxin group of drugs, as a last resort for treatment of gram-negative bacterial infections. There is a dearth of information on the pharmacological properties of colistin, leading to difficulties in selecting the right dose, dosing interval, and route of administration for treatment, especially in critically-ill patients. The increasing use of colistin over the last few years necessitates the need for accurate and reliable *in vitro* susceptibility testing methods. Development of heteroresistant strains as a result of colistin monotherapy is also a growing concern. There is a compelling need from the clinicians to provide options for probable and possible colistin combination therapy for multidrug-resistant bacterial infections in the ICU setting. Newer combination drug synergy determination tests are being developed and reported. There are no standardized recommendations from antimicrobial susceptibility testing reference agencies for the testing and interpretation of these drug combinations. Comparison and analysis of these reported methodologies may help to understand and assist the microbiologist to choose the best method that produces accurate results at the earliest. This will help clinicians to select the appropriate combination therapy. In this era of multidrug resistance it is important for the microbiology laboratory to be prepared, by default, to provide timely synergistic susceptibility results in addition to routine susceptibility, if warranted. Not as a favour or at request, but as a responsibility.

Key words: *Colistin, heteroresistance, in vitro susceptibility, monotherapy, synergy testing*

Introduction

The emergence of multidrug resistant (MDR) nosocomial pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae,* resistant to all currently available antibiotics, is coupled with the decline in the discovery and development of newer effective antibiotics over the last two decades. This has led to the depletion of most of the available therapeutic options for MDR bacterial infections. Currently there is renewed interest in the usage of polymyxins, as they are the only treatment option for these MDR and pan-drug resistant (PDR) gram-negative infections.[1] The use of colistin is also on the rise, due to the steady increase in bacterial resistance. Knowledge of the pharmacokinetics (PK) and pharmacodynamics (PD) of polymyxins is limited, resulting in inappropriate dosing, potential toxicity and development of resistance.[2] Laboratory testing plays an important role guiding therapy. This review, therefore, intends to throw light on the scope of colistin in the current scenario of emergence of MDR bacterial infections with focus on antimicrobial susceptibility testing methods, antimicrobial combination testing and emergence of resistance, with the aim to guide antimicrobial therapy.

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Origin

Polymyxins are polypeptide antibiotics comprised of five chemically different compounds (Polymyxins A-E). Polymyxin B was first isolated in Japan, in 1949, derived from *Bacillus polymyxa*. Polymyxin E also known as colistin is obtained from *Bacillus polymyxa subspecies colistinus*.[3] Polymyxins B and E have been used in clinical practice since 1959, while polymyxin A, C and D are not used because of toxicity. Polymyxin E (colistin) was initially used in intravenous and intramuscular formulations for the treatment of gram-negative bacterial infections. Later in the 1970s it fell out of favour for aminoglycosides, which were found to be less toxic.

Structure and mechanism of action

Polymyxins are surface-acting ampipathic agents. Each polymyxin molecule has a cationic polypeptide ring with a lipophilic fatty acid side chain.[4] The polypeptide ring binds

with the anionic phosphate moieties in the bacterial cell membrane, displacing Ca^{2+} and Mg^{2+}, which are needed for membrane integrity. This results in increased permeability of the cell membrane causing leakage of cellular contents, leading to cell death.[5] The disruption of membrane integrity also increases the susceptibility of the organism to hydrophilic antibiotics such as rifampicin, carbapenems, glycopeptides and tetracyclines, thus paving the way for both gram-negative and gram-positive antimicrobial synergistic combination therapy.[6]

In addition to bactericidal action, polymyxins have a potent *in vitro* anti-endotoxin activity.[3] The lipid portion of the molecule has a high affinity for the lipid-A present in the lipopolysaccharide layer of the gram-negative bacteria. Binding of the polymyxin rapidly inactivates the endotoxic action of lipid-A. However, its role in preventing septic shock *in vivo* is yet to be assessed.[3] A recent study has shown that co-incubation of bacterial culture supernatants with colistin significantly reduces LPS activity, with an associated decrease in cellular cytotoxicity. Colistin therapy is also found to significantly decrease both the production of inflammatory cytokines and LPS activity *in vivo*, even at a sub-therapeutic dose.[7]

Spectrum of activity

The polymyxins are active against a broad array of gram-negative aerobic bacilli with a few exceptions [Table 1]. Gram-positive organisms and most anaerobes are resistant.[1]

Pharmacological Properties of Colistin

Colistin is available in two forms, colistin sulphate and colistimethate sodium (CMS), also known as colistimethanesulphate, pentasodium colistimethanesulphate or colistin sulphonyl methate. CMS is the pro-drug that is hydrolyzed *in vivo,* to form the active colistin moiety. Both preparations are poorly absorbed orally. CMS is less potent, but less toxic than colistin sulphate. CMS can be used as intramuscular, intravenous and aerosolised formulations. Polymyxins exhibit concentration-dependent bactericidal activity. In adults, when CMS is given intramuscularly, at a dose of 2.5 mg / kg, a peak serum level of 5 to 7 µg / mL is achieved. When given intravenously, the peak level is 20 µg / ml at 10 minutes.[8] Accumulation of the drug can occur with repeated dosing. The PK of aerosolised preparations has not been extensively studied. Le Brun *et al.* studied the PK of aerosolised colistin in healthy volunteers and in cystic fibrosis patients and found the serum level of colistin to be higher following administration of colistin dry powder inhalation system, as compared to that of nebulised CMS solution.[9]

The half-life of CMS is around three hours. It is mainly excreted by glomerular filtration and 60% is excreted unchanged in the urine [Figure 1]. Reports show

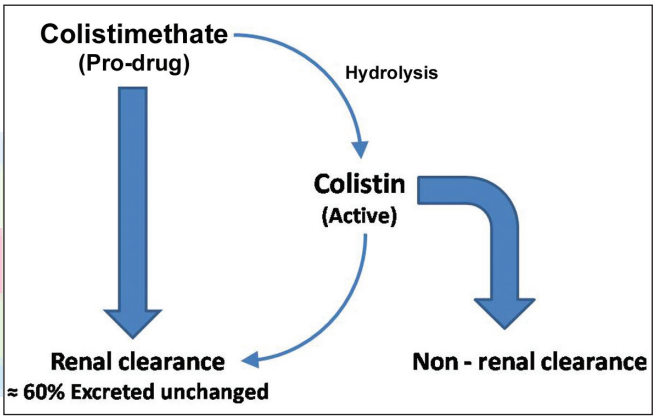

Figure 1: Schematic representation of excretion of Pro-drug and active colistin following administration of colistimethate Pharmacokinetic studies show that only 31% of the pro-drug was converted to active colistin even after 4 hours (slow conversion), resulting in the excretion of most of the pro-drug unchanged in urine

Table 1: Spectrum of action of polymyxins

Susceptible	Resistant	Variable for different species
Gram-negative bacilli:	All Gram-positive organisms	Gram-negative bacilli:
Pseudomonas aeruginosa	Gram-negative cocci:	*Stenotrophomonas maltophilia*
Acinetobacter spp	*Neisseria gonorrheae*	*Aeromonas* spp
Escherichia coli	*Neisseria meningitides*	*Vibrio* spp
Klebsiella spp	*Moraxella catarrhalis*	
Enterobacter spp	Gram-negative bacilli:	
Salmonella spp	*Proteus* spp	
Shigella spp	*Providencia* spp	
Haemophilus influenzae	*Morganella morgani*	
Bordetella pertussis	*Serratia* spp	
Anaerobic GNB:	*Edwardsiella tarda*	
Prevotella spp	*Burkholderia* spp	
Fusobacterium spp	*Brucella* spp	
	Other anaerobic GNB	

that colistin is poorly distributed to the other sites in the body such as CSF, biliary tract, pleural fluid and joint fluid.[10] However, intravenous colistin has been found to be efficacious in the treatment of MDR *A. baumannii* (MDRAB) meningitis.[2]

Dosing, Administration and Clinical Uses

As colistin was developed six decades ago, it was not subjected to the contemporary drug development procedures. Consequently, there is limited PK and PD data available. Based on this limited data, several dosing guidelines have been proposed, although the optimal dose and dosing intervals have not yet been clearly defined.[11]

Intravenous administration of colistin is widely used for PDR nosocomial infections with *Acinetobacter* spp, *Klebsiella* spp. and *Pseudomonas* spp. Current dosage recommendations are based on the manufacturer's instructions. The recommended systemic dose of CMS for a person weighing 60 kg is 6.67 – 13.3 mg / kg per day in —two to four doses in the US, and 4 – 6 mg / kg per day or 1 – 2 million IU / day in three doses, in the UK (12500 IU / 1 mg of CMS). In India, the only brand currently available is Xylistin® (Cipla, Bengaluru), which is labelled in international units (IU; 1 million IU and 2 million IU per vial); the dosage being similar to that followed in the UK. The upper limit of daily dosing recommendation varies from 400 mg / day in the US to 800 mg / day in the UK. As, colistin is measured in mg / L units in the US and in million international units (IU) in the UK and India, it creates confusion among treating physicians, resulting in suboptimal prescription.[12] Very recently, the results of the population analysis profile have been reported for the first time after testing 105 critically ill patients, including those with underlying renal dysfunction. When colistin is administered at a dose 50% higher than the recommended dose, it is observed that: the predicted maximum plasma concentration of the formed colistin after the first dose is substantially lower than that at the steady state after the fourth dose; the fourth dose is modestly above the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimum inhibitory concentration (MIC) breakpoint of 2 mg / L for *P. aeruginosa* and *A. baumannii*. This study finding strongly suggests that it is advantageous to administer CMS as a loading dose of 9 million IU (~720 mg) followed by a maintenance dose of 4.5 million IU (~360 mg), every 12 hours. This, however, remains to be tested clinically.[13] Recent studies are in favour of IV colistin in combination with other antibiotics, as against its use as monotherapy.

Inhaled or aerosolised colistin therapy is not FDA approved. However, many studies evaluating aerosolised colistin in the treatment of cystic fibrosis, ventilator-associated pneumonia (VAP) and other respiratory infections have proven it to be beneficial. However, the true efficacy of inhaled colistin cannot be ascertained as it is always coupled with intravenous colistin or other antibiotics. In addition, there is lack of scientific information on the appropriate inhaled dosage, duration, pharmaceutical formulation and bioavailability in the alveolar fluid.

Intrathecal (IT) or the intraventricular route of administration is possible, but not approved for the treatment of CNS infections. When used, the recommended dose is 3.2 – 10 mg per day IT, up to a maximum 20 mg per day.[2] Studies conducted recently suggest that intraventricular administration of CMS is effective in the treatment of ventriculitis caused by MDR *A. baumannii*.[2] The use of colistin sulphate is limited to oral and topical formulations.[3]

Adverse Reactions

Nephrotoxicity and neurotoxicity are the most common adverse effects of colistin. Toxicity is dose-dependent and reversible on discontinuation of treatment. Nephrotoxicity manifested as acute tubular necrosis is mostly of concern to the treating clinician. The exact molecular mechanism of toxicity is, however, not known. Recent studies have shown the incidence of nephrotoxicity to be 8 – 18%.[11] The lower incidence of nephrotoxicity with colistin use, at present, is attributed to the following factors: Close monitoring of renal function during treatment; modifying the dosage, dosing interval and duration of therapy; avoiding co-administration of nephrotoxic agents and greater supportive care to critically ill patients. It is also thought that older formulations of colistin probably contained a greater proportion of colistin sulphate. This combined with the confusion arising as a result of different nomenclatures such as CMS, colistin and colistin base, used in labelling products, led to inappropriate dosing, and hence, higher nephrotoxicity.

The incidence of neurotoxicity is low, around 7%, in patients receiving colistin therapy, with a higher risk in patients with cystic fibrosis. Some of the manifestations described are facial and peripheral paraesthesias, ataxia and neuromuscular blockade.[2] Inhaled therapy may cause bronchospasm.[3] Due to the potential renal and neurotoxicity of colistin, close monitoring is recommended during therapy.

Antimicrobial susceptibility testing — issues and challenges

The increasing use of polymyxins for treatment of MDR gram-negative bacterial infections compels the microbiologist to choose the most reliable standard *in vitro* susceptibility testing method. In the following section, the consensus and issues of various available antimicrobial

susceptibility testing (AST) methods and their interpretation are discussed.

Antimicrobial susceptibility testing for polymyxins can be performed using either diffusion or dilution techniques. The diffusion techniques include, disc diffusion, epsilometer test (E-test) and a recently described *disc prediffusion test*.[14] Methods employing the dilution technique are; broth microdilution (BMD) and agar dilution. Also, the MIC of colistin can be obtained using various automated instruments like Vitek 2C.

Disc diffusion testing

At present, the methodology and interpretations for the disc diffusion test are provided by the CLSI, USA. The CLSI recommends discs containing 10 µg colistin or 300 µg polymyxin B to be used on Mueller-Hinton agar with a 0.5 McFarland inoculum and incubated for 16 – 18 hours. Interpretive values are provided only for *P. aeruginosa* [Table 2]. However, the colistin disc diffusion interpretive criteria for Enterobacteriaceae and *A. Baumannii,* which are not given by CLSI, have been evaluated and proposed by Galani *et al*.[15]

Disc prediffusion test

This is a novel method that uses neosensitabs provided by Rosco, Denmark. This test comprises of placing the drug containing tablet on an uninoculated Mueller-Hinton agar plate and incubating it for two hours (first incubation period) at room temperature. The tablet is then removed and the plates are held for a further 18 hours at room temperature (second incubation period). The medium is then inoculated and incubated in the same way as for disc diffusion. The results are interpreted as per the manufacturer's recommendation.

E-test

The procedure is carried out as per the recommendation of the manufacturer (AB Biodisk, Sweden). On account of the concentration gradient of the antibiotic, an elliptical zone of inhibition is formed after incubation. The value at the point of intersection of the growth, with the strip, corresponds with the MIC of the organism.

Dilution tests and automated methods

The broth microdilution and agar dilution can be carried out using standard recommendations by the CLSI and Vitek 2C (bioMérieux, France), as per the manufacturer's instructions. Notably, different reference committees provide different interpretive breakpoints [Table 2].

Colistin susceptibility testing issues

Colistimethate sodium is not recommended for susceptibility testing because it is an inactive pro-drug, which undergoes variable hydrolysis in the medium, resulting in differential killing characteristics with varying

Table 2: Comparison of MIC breakpoints recommended by various National Antimicrobial Susceptibility Breakpoint Committees

MIC breakpoints for Colistin									Disc diffusion interpretive criteria for Colistin								
Enterobacteriaceae			*P. aeruginosa*			*A. baumannii*			*Committee*	*Enterobacteriaceae*			*P. aeruginosa*			*A. baumannii*		
S	I	R	S	I	R	S	I	R	Interpretation 2011	S	I	R	S	I	R	S	I	R
NR	NR	NR	≤ 2	4	≥ 8	≤ 2	-	> 2	CLSI	NR	NR	NR	≥ 11	-	≤ 10	NR	NR	NR
≤ 2	-	> 2	≤ 4	-	> 4	≤ 2	-	> 2	EUCAST	NR	NR	NR	NR	NR	NR	NR	NR	NR
≤ 2	-	>2	≤ 4	-	> 4	≤ 2	-	>2	BSAC	NR	NR	NR	NR	NR	NR	NR	NR	NR
									Galani *et al.* [a]	≥ 14	12 -13	≤ 11	NR	NR	NR	≥ 14	13	≤ 12

MIC breakpoints for Polymyxin B									Disc diffusion interpretive criteria for Polymyxin B								
S	I	R	S	I	R	S	I	R	Interpretation 2011	S	I	R	S	I	R	S	I	R
NR	NR	NR	≤ 2	4	≥ 8	≤ 2	-	> 2	CLSI	NR	NR	NR	≥ 12	-	≤ 11	NR	NR	NR
NR	NR	NR	NR	NR	NR	NR	NR	NR	EUCAST	NR	NR	NR	NR	NR	NR	NR	NR	NR
NR	NR	NR	NR	NR	NR	NR	NR	NR	BSAC	NR	NR	NR	NR	NR	NR	NR	NR	NR

[a] Galani *et al.* have evaluated and proposed the interpretive criteria Enterobacteriaceae and *A. baumanii* for Colistin Disc Diffusion, as there are no CLSI interpretive criteria available. The authors have suggested confirmation of all isolates with intermediate zone diameters with MIC testing.

time of incubation. Considering the drawbacks of CMS, all the national committees advocate the use of colistin sulphate for AST. Clinically for therapy, the pro-drug CMS is used. On the contrary, active colistin sulphate formulation is tested *in vitro*. It is unclear whether this translates correspondingly *in vivo*. The other laboratory concerns are the cation concentration in the medium and the inoculum effect. The bactericidal activity of polymyxins is directly proportional to the concentration of Mg^{2+} and inversely proportional to the Ca^{2+} concentration.[16,17] As shown in Table 2, the breakpoints of polymyxins are abrupt, so the inoculum and period of incubation must be precise. A slight increase or decrease in the inoculum (McFarland match) may overcall the resistance or susceptibility, respectively.

Studies evaluating various methods must ideally validate their test with broth microdilution (reference method). The concordance of the test with broth microdilution must be calculated with respect to the errors produced. The unacceptable levels are > 1.5% for very major errors, > 3% for major errors and 10% for minor errors, as recommended in the CLSI document, M23-A2. Prominent studies done using the different AST methods are compiled in Table 3.[18-20] Lo-Ten-Foe *et al.* have evaluated the performance of various tests for detecting colistin susceptibility and have concluded that, agar dilution, E-test and Vitek 2 have a high level of agreement with the BMD, although no statistical data are provided.[19] This has been contradicted by Tan *et al.* who have deemed Vitek 2 to be an unreliable method with unacceptable rates of very major errors (18%).[21] This controversy is yet to be resolved.

E-tests have the advantage of being easy to perform and less time consuming. It has been observed that polymyxin E-tests produce sharp end-points with Enterobacteriaceae. However, for Acinetobacter and other non-fermenting gram-negative bacilli (NFGNB), the clarity of the end-point is usually obscured by the presence of small colonies. The manufacturer addresses this issue by suggesting taking the readings for Enterobacteriaceae from the lower MICs and for NFGNB from higher MICs. Although it is a diffusion technique, the E-test is reported to perform remarkably well. Most studies have demonstrated the concordance of the E-test to be as high as 90 – 100% and have suggested it as a reliable and useful alternative to the dilution methods.[14,22] This is contradicted by the findings of Tan *et al.* where unacceptable rates of very major error, especially for *P. aeruginosa* and *S. maltophilia* have been detected.[21] Similarly, another study demonstrates the reduction in concordance of the E-test for higher MICs and has stressed the need to confirm with BMD for all isolates with MIC > 1 µg / ml.[23]

Almost all studies evaluating the efficacy of the disc diffusion test for polymyxins have consistently reported it to be unreliable for use. The reason attributed to the poor performance of the disc diffusion test is that, polymyxins are large molecules and diffuse inadequately into the medium to produce inconsistent zones of inhibition.[24] This can be overcome by the disc prediffusion test, where consistent zone diameters are obtained, because more time is provided for adequate diffusion of the drug into the medium. Disc prediffusion has a high concordance with BMD (96.8%) and can be used as an alternative to the E-test.[14] However, this method has been evaluated and reported only by the commercial manufacturer and is not approved by any of the AST committees. Also, increased chances of contamination and delay in obtaining the results due to the longer incubation time are the drawbacks of this method compared to the E-test. These disadvantages can be overcome by shortening the second incubation period, without compromising on the test performance.[14] The common pitfall of all the AST methods is that they miss out on the detection of heteroresistance.[25] Countries that do not have interpretive guidelines of their own, are unable to choose the best suited interpretive breakpoints among the various guidelines available. In this context, collection and compilation of data on laboratory and clinical outcome correlation, in that particular geographical area, may provide an insight on which guideline is to be adopted.

Heteroresistance and resistance to colistin

The rates of colistin resistance are relatively low, probably because of its infrequent use. Currently, colistin resistance in the form of increasing treatment failure is being reported all over the world. Rates of less than 28% colistin-resistant *Acinetobacter spp* are seen in Asia alone.[26]

Among the colistin-resistant bacteria, *A. baumannii* is the most common, followed by *K. pneumoniae* and *P. aeruginosa*.[3] Resistance to colistin can occur through mechanisms of mutation or adaptation , leading to bacterial cell membrane changes such as a decrease in the content of lipopolysaccharides, specific outer membrane proteins and Mg^{2+} and Ca^{2+} content.[27] Almost a complete cross-resistance exists between colistin and polymyxin B.[28] Increasing reports of colistin-susceptible isolates, harbouring resistant subpopulations are of great concern. This phenomenon known as heteroresistance has been described, for the first time, in the multidrug-resistant *Acinetobacter baumannii* (MDRAB) clinical isolates, by Li *et al.*[25] In their study, they found that a significant regrowth of a heteroresistant subpopulation occurred in the time-kill assay at 24 hours. Also, the population analysis profile revealed that heteroresistance to colistin occurred in almost all the clinical isolates tested.[25] A similar study demonstrated the degree of heteroresistance to be significantly higher in patients previously treated with colistin.[29] Of late, a decrease in the synergistic activity of colistin has been observed, in antimicrobial combinations tested against heteroresistant carbapenem-resistant *Acinetobacter baumanni* (CRAB) strains.[30]

Table 3: Comparison of different methods of AST for colistin / polymyxin B with BMD (2µg / ml as breakpoint) as gold standard

Author	Drugs tested	Organisms tested (n)	Remarks				Reference
			Agar dilution	E-test	Vitek-2	Disc diffusion	
Boyen *et al.*[a]	Colistin	*E. coli* (157)	-	No VME Concordance — 96.8% E-test is a reliable method	-	VME - 1.9% Concordance - 46.5% Unreliable method Disc prediffusion: VME - 1.3% Concordance - 96.8% Reliable method - Can replace E-test	[14]
Behera *et al.*	Polymyxin B	Mixed GNB (281)	Sensitivity — 92% Specificity — 97.6% VME — 0.7% Concordance — 97% MICs generally > BMD Can be used for batch testing of a large number of strains	Sensitivity — 87.5% Specificity — 100% VME — 1% Concordance — 99% MICs generally < BMD Can be used as an alternative to BMD, as it is easy to perform and interpret	-	Sensitivity - 92% Specificity - 100% VME - 1% Concordance - 99% Can be used for initial screening	[18]
Lo-Ten-Foe *et al.*	Colistin	Mixed GNB (102)	High level of agreement Heteroresistance in *E. cloacae* may be missed	High level of agreement Heteroresistance in *E. cloacae* may be missed	High level of agreement Low sensitivity in detecting heteroresistance Not suitable for *S. maltophilia*	High error rates Low reproducibility Iso-sensitest agar is better than Muller Hinton agar	[19]
Heijden *et al.*	Colistin and Polymyxin B	*P. aeruginosa* (78)	-	VME for PB — 1.2% Only minor errors for colistin Concordance for PB — 90% Concordance for PE — 100%	-	VME for PB - 1.2% No errors for colistin Unreliable method	[24]
Tan *et al.* [a]	Colistin	Mixed GNB (172)	-	VME — 4.7% Concordance — 87% Results must be confirmed by standard MIC methods High error rates for *P.aeruginosa* and *S. maltophilia*	VME — 18% Concordance — 82% Unreliable method	-	[21]

Table 3:Contd..

Author	Drugs tested	Organisms tested (n)	Remarks				Reference
			Agar dilution	E-test	Vitek-2	Disc diffusion	
Tan *et al.*[a]	Colistin	*A. baumannii* (44)	-	-	No VME Concordance - 100% Accuracy of Vitek2 could not be accurately determined	-	[20]
Arroyo *et al.*	Colistin	*A. baumannii* (115)	-	Sensitivity — 90.9% Specificity — 100% VME — 1.7% Concordance — 98.2% Poor concordance with higher MICs, confirm with BMD if MIC > 1 µg / ml	-	-	[23]
Bolmstrom *et al.*	Colistin and Polymyxin B	*A. baumannii* (327) *P. aeruginosa* (46)	-	Concordance for PB — 99% Concordance for PE — 99% E-test is equivalent to BMD	-	-	[22]

Laboratory detection of heteroresistant isolates has a low sensitivity with disc diffusion and commercial automated systems.[25] Colistin heteroresistance could be a concern akin to Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* (hVISA), where there is no consensus regarding treatment.[31] The frequency of occurrence of heteroresistance, its clinical impact and the antimicrobial treatment strategies have not yet been established. Heteroresistance can be effectively prevented if the appropriate dose, dosing interval and duration of therapy are followed and colistin is used as part of the combination therapy.

Drawbacks of monotherapy

Of late studies have shown that with the decreasing concentration of the drug, there is regrowth of the surviving bacteria. The MIC for these regrowing bacteria is significantly higher than that of the bacteria initially unexposed to polymyxins.[32] The optimal dose of polymyxins, to prevent development of these resistant mutants, is not known. Cai *et al.* estimated the Mutant Prevention Concentration (MPC) of colistin against MDRAB. The MPC at which 90% of the isolates are killed (MPC90), has been found to be ≥ 128 µg / ml, which is much higher than the plasma concentration of colistin (2.93 µg / ml), at the current recommended dosage.[33] There are various factors that prevent the attaining of a high serum concentration of polymyxins. A majority of the administered CMS is excreted through the urine, before conversion to its active form. Kwa *et al.* have noted that polymyxins bind to the α1-acid glycoprotein, an acute-phase reactant present in high levels in critically ill patients. This binding results in the neutralization of polymyxin activity.[2] The facts mentioned above suggest that polymyxins can be best used as a combination, which can act synergistically to clear the infection and possibly slow down the emergence of resistance. There is a compelling need for the microbiology laboratory to get familiarized and acquainted with the knowledge and techniques of performing synergy testing. To determine the synergistic combination, various *in vitro* and *in vivo* tests have been described.

Combination drug synergy testing

In vivo testing methods

There are very limited *in vivo* studies to prove the superiority of combination therapy over polymyxin monotherapy. Aoki *et al.* have demonstrated a mouse pneumonia model using BALB / c mice and have shown that the combination of polymyxins with rifampicin is more effective than polymyxins used alone.[7]

In vitro testing methods

There are various *in vitro* synergy testing methods described such as time-kill assay, checkerboard assay and different modifications of the E-test.

Time-kill assay

In this technique, the bacterium is allowed to grow in a liquid medium incorporated with the test antimicrobial and is checked for viability at different time intervals. Synergy between the drug combinations can be detected by performing the test in two sets, one with a single drug and the other with the additional drug intended for use in the combination therapy. Specific volume is sub-cultured from both sets onto an agar medium at definite time intervals and counted for the number of colonies. Synergy (secondary end point) is defined as a ≥ 2 $\log_{10}$ decrease in the colony count between the drug combination and its most active antibiotic alone. A ≥ 2 $\log_{10}$ increase is interpreted as antagonism and < 2 $\log_{10}$ decrease or increase is considered indifferent. The bactericidal activity (primary end point) is defined as ≥ 3 $\log_{10}$ decrease in the colony count in the drug combination compared to the most active antibiotic alone.[30]

Checkerboard assay

In this method, the MICs of the test drug-A, test drug-B and that of the combination (A + B) are determined using the broth microdilution technique in a 96-well microplate. The interaction between drugs A and B is interpreted using the total fractional inhibitory concentration (ΣFIC). This is calculated by the formula, ΣFIC = FIC of drug-A + FIC of drug-B; where the FIC of drug-A = MIC of drug A / MIC of (A + B) combination and FIC of drug-B = MIC of drug B / MIC of (A + B). The ΣFIC values ≤ 0.5 are interpreted as synergy, 0.5 – 4 indicates indifference, while values > 4 indicate antagonism.[34]

E-test methods

To date, three different methods have been described for performing synergy detection in drug combinations using E-test strips. In all the three methods, initially the MIC of drug-A and drug-B are identified separately. Thereafter, in the first method (fixed ratio method), the E-test strip containing drug-A is placed on the agar streaked with the test organism. The area corresponding to its MIC is marked on the agar surface using a sterile needle. This is incubated for one hour to allow the diffusion of the drug from the strip into the medium. The strip is then removed and the strip containing drug-B placed exactly over the place of the previous strip in such a way that the MIC of the drug-B exactly coincides with the mark on the agar surface; that is, MICs of both drug-A and drug-B lie at the same point [Figure 2]. The plate is incubated for 18 – 20 hours, after which the reading is taken.[35] The second method comprises of placing both the E-test strips together at a 90° angle in such a way that the strips intersect at their respective MICs for the organism.[36] In the third method, the most active drug is incorporated into the medium in the specified concentration and the E-test strip containing the second drug is placed on the medium.[35] All the three modifications of the E-test results can be interpreted in the same line as that of the checkerboard assay.

Performance of Different Synergy Testing Methods

Among the *in vitro* methods, the time-kill assay performs the best and is considered as the reference method. The time-kill assay detects synergy at a much higher frequency when compared to the other tests described. Furthermore, it also measures the bactericidal activity of the drug combination. Lim *et al.* point out that the true efficacy of the drug is reflected by bactericidal activity (primary end point), which is a more meaningful and reliable pharmacological indicator than synergy (secondary end point).[37] The checkerboard assay produces fairly reliable results, but it may require confirmation with the time-kill assay.[38] This is based on the observation that there are discordant findings between the assays. For example, Sheng *et al.*, have reported 42% synergy for an imipenem

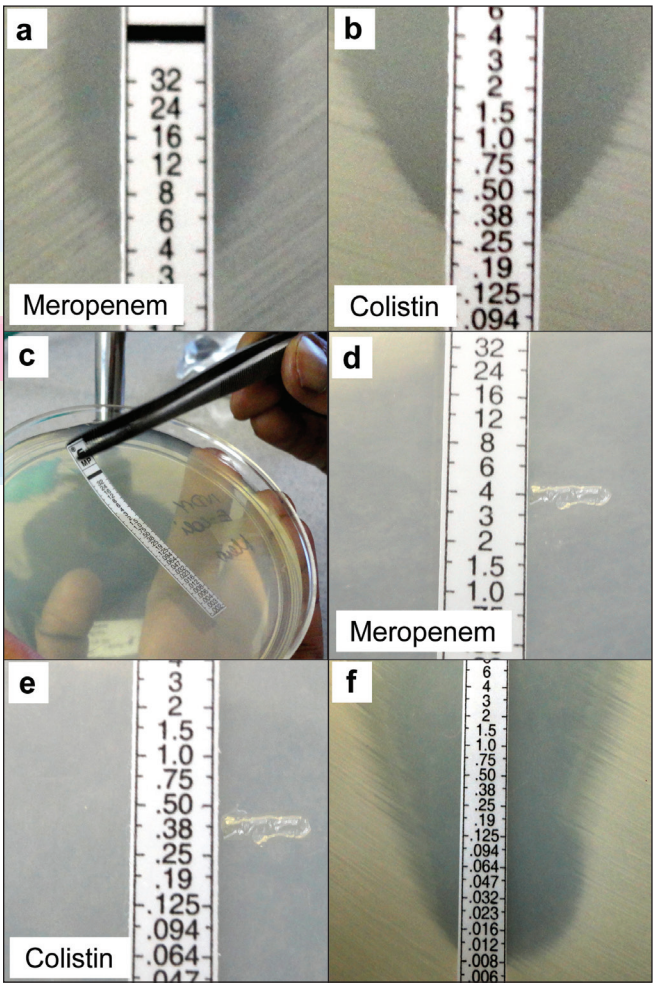

Figure 2: *In vitro* synergy testing between meropenem and colistin for carbapenem resistant *Klebsiella pneumoniae* using the fixed ratio method (E-test method 1) (a), (b) Determine MICs of mero and colistin individually (4µg / ml and 0.38µg / ml) (c) Place mero E-test strip (d) Mark on the agar surface coinciding with the MIC value of mero, incubate for one hour (e) Place colistin E-test strip after removing mero strip. MIC value of colistin coinciding with the mark (f) Read MIC after 18 hours, >2 fold reduction (0.012µg / ml) compared to colistin alone suggests synergy

and colistin combination by checkerboard titration, but 75% synergy in the time-kill assay of CRAB isolates.[39] A similar discrepancy in results between these two methods has also been reported in two earlier studies.[35,40] The advantage of the checkerboard assay is better detection of antagonism.[36] Similar to the time-kill assay, the checkerboard assay also faces the disadvantage of being too labour intensive for routine diagnostic work.[35]

The E-test is easy to perform and the results can be provided earlier. However it should be noted that the MIC has to be read from the higher to the lower concentration as recommended by the manufacturer (AB Biodisk, Sweden). Among the three described E-test methods, the third method using incorporated drug in the medium has a good correlation with the time-kill assay.[41] This is because it overcomes the problem of poor diffusion of polymyxins. The results generated by the first two E-test methods do not correlate well with the time-kill assay.[35,42]

Combination of Drugs as a Therapeutic Option: Laboratory perspective

There are very few prospective clinical studies evaluating the efficacy of the combination of polymyxins with other antibiotics. The majority of the peer-reviewed publications are on *in vitro* synergy testing and more recently literature is accumulating on the retrospective clinical outcome studies. This review intends to highlight only the results of the *in vitro* combination studies. [Table 4][37,43-47] Among the various drugs tested for synergy with polymyxins, the efficacy of rifampin is notable. The colistin–rifampin combination showed a 100% synergy for MDRAB in most studies.[48,49] However, the same combination showed highly variable synergy (14 – 100%) for *P. aeruginosa*.[50] The Polymyxin B–rifampin combination for *P. aeruginosa* also showed a wide variation, but the addition of a third drug imipenem to the combination, improved the synergy to 100%.[51] The Polymyxin B–rifampin combination was highly synergistic for *K. pneumoniae*.[38] Recent studies have shown a promising synergy for the colistin–meropenem combination (100%) in MDRAB / CRAB.[49] Sheng *et al.* have demonstrated varied synergy of the colistin–imipenem combination (75–100%) among different species of *Acinetobacter* tested.[39] The addition of a third drug, sulbactam, to the meropenem–colistin combination has further improved the antibacterial activity against CRAB.[52] When choosing a carbapenem for the synergistic combination with colistin, meropenem seems to be a better choice than imipenem for the following reasons; meropenem is a smaller molecule and can enter into the target organism in sufficient amounts even with a little increase in membrane permeability facilitated by colistin; it is safer in higher doses (increased up to 6 gm / day); less neurotoxic and unlike imipenem, porin loss is not a major problem for meropenem resistance.

Interestingly, glycopeptides, which individually have no action on gram negatives, have shown a high degree of synergy with polymyxins. Gordon *et al*., demonstrated 100% synergy of the colistin–vancomycin combination against MDRAB.[32] As both drugs are nephrotoxic, their usage *in vivo* is unlikely. This issue was overcome by Wareham *et al.*, who evaluated a less nephrotoxic agent, teicoplanin, in a similarly designed experiment and showed equal results.[41] However, this is yet to be tested clinically. Daptomycin is another promising, less nephrotoxic alternative for vancomycin. Other effective combinations with good synergy worth mentioning are colistin–minocycline for MDRAB, polymyxin B–doxycycline / tigecycline for MDR, *K. pneumoniae* and colistin–ceftazidime for MDR *P. aeruginosa*. All these combinations show 100% synergy with their respective organisms. Elemam *et al.* demonstrated the absence of synergy of cephalosporins and gentamicin with polymyxins.[38] Studies have shown poor synergy of fosfomycin and tigecycline when combined with polymyxins for non-fermenters.[53,54] Antagonism was rarely noted in combinations with tigecycline, azithromycin and imipenem.[11,53] However, consistent reproducibility of all these findings should be established to translate it into a prescription. There are many retrospective clinical outcome studies favouring combination therapy in adults, but very less data exists on therapeutic options for neonatal infections. The only choice reported as of now is colistin and meropenem, with or without rifampicin.

It should be noted that, at present, there are no standardized recommendations for drug combination testing and interpretation from any of the existing national antimicrobial susceptibility testing committees. In this circumstance, it is imperative that the *in vitro* studies evaluated on synergy must standardise and report clinically relevant concentrations and ratios of the drug combination. Merely proving *in vitro* reduction in MIC with a drug combination may be of no use if the same concentration cannot be attained *in vivo*. Also while performing a time kill assay, the bactericidal effect needs to be considered along with synergy.[37] Hypothetically, the organism must be susceptible to polymyxin so that it disrupts the cell membrane and facilitates its companion drug to act. Most *in vitro* studies have tested polymyxin-susceptible isolates and observed synergy. However, Elemam *et al.,* have demonstrated synergy using polymyxin-resistant isolates.[38] More studies are needed to substantiate this observation.

Conclusions

Antimicrobial treatment of multidrug-resistant pathogens presents an increasing challenge to patient care. Clinicians are increasingly compelled to use an unusual combination of drugs, in the hope that it may be efficacious. The usefulness of polymyxins has been clearly documented along with evidence of less nephrotoxicity than earlier believed.

Table 4: Results of various *in-vitro* studies carried out for synergy in combination of drugs with polymyxins at physiologically attainable concentrations

Author	Organism	n	Polymyxin tested	Method used	Drug combined	Synergy (%)	Indeterminate (%)	Reference
Lim *et al.*	Polymyxin B susceptible MDRAB	31	Polymyxin B	Time-kill assay	Rifampicin	41.9[a]	58.1	[37]
					Tigecycline	29[a]	71	
Liang *et al*. 2011	Colistin and minocycline susceptible CRAB	14	Colistin	Time-kill assay	Meropenem	100	-	[49]
					Minocycline	100		
					Rifampicin	100		
Sheng *et al*. 2011	Carbapenem resistant *Acinetobacter* spp.	17	Colistin	Time-kill assay	Imipenem	75 – 100 [b]	Up to 25	[39]
				Checkerboard	Imipenem	42 – 100 [b]	Up to 58	
Wareham *et al*. 2011	Colistin susceptible MDRAB	6	Colistin	Time-kill assay	Teichoplanin	100	-	[41]
				Checkerboard	Teichoplanin	100		
				E-test 3	Teichoplanin	100		
Souli *et al.* 2011	KPC producing *K. pneumoniae*	17	Colistin	Time-kill assay	Fosfomycin	11.8	98.2	[54]
Gordon *et al*. 2010	Colistin susceptible MDRAB	39	Colistin	E-test 3	Vancomycin	100	-	
Elemam *et al*. 2010	Polymyxin B resistant, KPC producing *K. pneumoniae*	12	Polymyxin B	Checkerboard	Rifampin	100	-	[38]
					Doxycycline	100	-	
					Tigecycline [c]	100	-	
					Cefazolin	-	100	
					Ceftriaxone	-	100	
					Cefepime	-	100	
					Gentamicin	-	100	
					Imipenem	-	100	
Pankey *et al*. 2009	CRAB	8	Polymyxin B	Time-kill assay	Meropenem	100	-	[46]
				E-test 1	Meropenem	63	37	
Principe *et al.*	Colistin susceptible CRAB	22	Colistin	Checkerboard	Tigecycline	8.3	86.4[d]	[53]
Dizbay *et al.*	XDR *A. baumannii,* susceptible to colistin	25	Colistin with Polymyxin B	E-test 1	-	72	28	[43]
Lopez *et al.*	Carbapenem resistant, colistin susceptible *P. aeruginosa*	12	Colistin	Checkerboard	Doxycycline	66.6	33.4	[45]
					Rifampicin	16.6	83.4	
					Azithromycin	25	75	
Tan *et al.*	Colistin susceptible MDRAB	13	Colistin	Time-kill assay	Minocycline	92	8	[20]
				E-test 2	Minocycline	0	100	

n = Number of isolates tested [a]Bactericidal activity was used as the pharmacological measurement of efficacy, as the synergistic definition may no longer be applicable for carbapenem-resistant *A. baumannii,* [b]Lower % for *A. baumannii* and high % for *A. genospecies* 3 and 13TU, [c]Less pronounced synergy, [d]One out of 22 isolates showed antagonism for colistin–tigecycline combination, [e]Triple drug combination

Table 4: Contd..

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Author	Organism	n	Polymyxin tested	Method used	Drug combined	Synergy (%)	Indeterminate (%)	Reference
Timurkaynak *et al.*	Multidrug resistant, colistin susceptible *P. aeruginosa*	5	Colistin	Checkerboard	Rifampin	40	60	[47]
					Meropenem	-	100	
					Azithromycin	-	100	
Landman *et al.*	Carbapenem resistant, polymyxin B resistant *P. aeruginosa*	10	Polymyxin B	Time-kill assay	Rifampin	90	10	[51]
					Imipenem	80	20	
					Azithromycin	40	60	
Gunderson *et al.*	Carbapenem resistant, colistin susceptible *P. aeruginosa*	2	Colistin	Time-kill assay	Rifampin + Imipenem [e]	100	-	[44]
					Ceftazidime	100	-	
					Ciprofloxacin	-	100	
Balaji *et al.*	Carbapenem resistant	31	Colistin	E-test 1	Meropenem	32	68	-
Unpublished data	*K. pneumoniae*							

n = Number of isolates tested [a]Bactericidal activity was used as the pharmacological measurement of efficacy, as the synergistic definition may no longer be applicable for carbapenem-resistant *A. baumannii*, [b]Lower % for *A. baumannii* and high % for *A. genospecies* 3 and 13TU, [c]Less pronounced synergy, [d]One out of 22 isolates showed antagonism for colistin–tigecycline combination, [e]Triple drug combination

However, the susceptibility of polymyxins is predicted to be short-lived, due to inappropriate use, resulting in the development of heteroresistant pathogens, thus raising the question — "Are we nearing the end of the colistin era?" The clinicians are dependent on the microbiology laboratory for guidance in choosing a therapy. Although various AST methods are available, there is no uniformity in the interpretation provided by the different committees across the world. More so, it becomes difficult to choose the recommendations to suit a given geographical region. Different synergy testing methods have been described and many *in vitro* studies have demonstrated highly synergistic drug combinations. It is therefore the primary responsibility of the microbiologist to assist the clinician in choosing the appropriate combination therapy. It is mandatory for the microbiologist to be knowledgeable and skilled in testing synergistic combinations and its validation. The failure to collate and report the cumulative antibiogram periodically is partly the reason for not containing the occurrence and for the spread of antimicrobial resistance. Appropriate testing and timely reporting can help choose appropriate drug combinations and preserve the therapeutic value of colistin, the last line of antimicrobials until the arrival of newer effective antimicrobials.

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