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Evaluation of susceptibility testing methods for polymyxin

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SUMMARY

Background: The widespread resistance in Gram-negative bacteria has necessitated evaluation of the use of older antimicrobials such as polymyxins. In the present study we evaluated the different susceptibility testing methods for polymyxins B and E against Gram-negative bacteria using the new Clinical and Laboratory Standards Institute (CLSI) guidelines.

Methods: The susceptibility of 281 multidrug-resistant (MDR) Gram-negative bacteria (GNB) to polymyxin B was evaluated, comparing broth microdilution (BMD; reference method), agar dilution, E-test, and disk diffusion. Disk diffusion testing of polymyxin B was also performed against 723 MDR GNB.

Results: Twenty-four of 281 (8.5%) isolates were found to be resistant to polymyxin B by the reference BMD method. The rates of very major errors for agar dilution and E-test (for polymyxin B) were 0.7% and 1%, respectively, and those for disk diffusion (for polymyxin B and polymyxin E) were 1% and 0.7%, respectively. For the 257 isolates found sensitive by reference BMD, the rates of major errors by agar dilution and E-test (for polymyxin B) were 2.4% and 0%, respectively, and those for disk diffusion (polymyxin B and polymyxin E) were 0% and 0.7%, respectively. Twenty-six (3.6%) of the 723 Gram-negative isolates were resistant to polymyxin B by disk diffusion.

Conclusion: The E-test and agar dilution methods showed good concordance with BMD. The disk diffusion method can be useful for initial screening in diagnostic laboratories.

Introduction

The inexorable rise of antibiotic resistance and the paucity of new antimicrobials have led to a renewed interest in the use of the polymyxin group of antibiotics for the treatment of infections due to multidrug-resistant (MDR) bacteria.[1,2] Strains of *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* that exhibit resistance to almost all available antibiotics except polymyxins, have emerged as a common cause of hospital-acquired infections in critically ill patients.[3,4] Polymyxins are multicomponent polypeptide antibiotics that act primarily on the Gram-negative bacterial cell wall, leading to rapid permeability changes in the cytoplasmic membrane and ultimately to cell death.[5] Polymyxin E (colistin) and polymyxin B have occasionally been used to treat infections caused by Gram-negative bacteria (GNB) that are resistant to aminoglycosides, cephalosporins, anti-Pseudomonas penicillins, quinolones, monobactams and carbapenems.[6,7] Thus, they are being used as a last resort drug for the treatment of life-threatening infections.

The increased need for polymyxin treatment in critically ill patients in the intensive care unit (ICU) has also created a pressing need for up-to-date susceptibility data. The in vitro susceptibility testing of polymyxin group antimicrobials is hampered by several different factors. The accuracy of the disk diffusion assay is unsatisfactory because polymyxins diffuse poorly into agar, and consequently no reliable correlation of zone diameters and minimum inhibitory concentrations (MICs) has been found in some studies.[8] The interpretative criteria for quantitative in vitro testing also differ between nations.[8] Recently, the Clinical and Laboratory Standards Institute (CLSI) approved a standard document for the testing of polymyxins against *P. aeruginosa, Acinetobacter spp* and a few other non-fermenters using dilution

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methods.[9] It was only in 2007 that the interpretative criteria for disk susceptibility testing of polymyxins were published by the CLSI.[10] However, there is still no consensus regarding the breakpoints for defining resistance to polymyxins. Since relatively few surveys of antibiotic resistance have been performed on this group of antimicrobials, reliable data on true resistance levels are also lacking. Considering the increasing use and demand for polymyxins and the relative paucity of data regarding resistance, we evaluated different susceptibility testing methods for this class of antimicrobial in the present study.

Methods

The study was performed over a period of 1 year (October 2007 to September 2008) at the Microbiology Laboratory of the 190-bed level-1 Trauma Centre of the All India Institute of Medical Sciences (AIIMS), New Delhi, India. AIIMS is a 2500-bed, tertiary care, teaching and referral hospital.

The study was conducted in two parts. The first part of the study – evaluation of methodologies for determination of polymyxin B MICs – was carried out from October 2007 to March 2008. Representative MDR GNB isolated from the samples of patients admitted to the ICUs and wards of the AIIMS Hospital and its Trauma Centre (medical, pediatric, surgical, gastroenterology, oncology, nephrology, neurosurgery, cardiothoracic surgery and trauma care ICU) were included. Various techniques for the antimicrobial susceptibility testing of polymyxin B and polymyxin E (determination of MICs by broth microdilution (BMD), agar dilution, and E-test, along with disk diffusion testing) were evaluated in this part of the study.

The second part of the study – polymyxin B susceptibility testing – was done on consecutive MDR GNB isolated from various clinical samples of patients admitted to the ICUs of the Trauma Centre, AIIMS, from April to September 2008. Antimicrobial susceptibility testing for polymyxin B was performed on these isolates using the disk diffusion technique.

Evaluation of methodologies for the determination of polymyxin B MICs

In this part of the study, a total of 281 representative MDR GNB isolated from clinical samples as described above were included. The isolates represented various genera of *Enterobacteriaceae* and non-fermenters. MDR was defined as resistance to two or more of the most commonly used antimicrobial classes for the treatment of the indicated infection. All these GNB were identified by conventional microbiological methods[11] and by the Vitek 2 identification system (BioMerieux, France), using ID-GN cards. Only one isolate per patient was included. The clinical source of the isolates and patient ward were recorded. Antimicrobial susceptibility testing of these isolates was performed using the Vitek 2 system and the disk diffusion method, according to CLSI guidelines.[12] The following antibiotics were tested by the disk diffusion method: ceftazidime (30 μg), ceftazidime/clavulanic acid (30/10 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), cefoperazone/sulbactam (75/30 μg), piperacillin/tazobactam (100 μg/10 μg), ticarcillin/clavulanic acid (75/10 μg), amikacin (30 μg), gentamicin (10 μg), imipenem (10 μg), ertapenem (10 μg), meropenem (10 μg), and ciprofloxacin (5 μg) (BBL™, BD, USA). MICs were determined using the AST GN 13 cards by the Vitek 2 advanced expert system (BioMerieux, France). All the isolates were stored at −70 °C until further testing.

An evaluation of polymyxin B and polymyxin E susceptibility testing methods was done using these 281 isolates. For this, the following isolates were used as controls: *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603, *A. baumannii* ATCC 19606, *Stenotrophomonas maltophilia* ATCC 13636 and *Burkholderia cepacia* ATCC 25608.

MIC determination

The MIC of polymyxin B was determined for the 281 isolates by the following methods:

Broth microdilution (BMD). BMD with cation-adjusted Mueller–Hinton broth (BBL-Becton Dickinson) was performed in accordance with the CLSI recommendations and was used as the reference method.[9,10,13] Polymyxin B concentrations ranging from 0.0015 μg/ml to 1024 μg/ml were tested by the BMD method. The MIC was defined as the lowest concentration of polymyxin B at which no visible growth was obtained using CLSI recommended incubation conditions.[13] A polymyxin B MIC of ≤2 μg/ml was taken as the breakpoint for susceptibility.[14,15] Since to-date, the CLSI susceptibility breakpoints of polymyxin B have been available only for *P. aeruginosa, A. baumannii* and some other non-fermenters,[16] we used the same breakpoints for *Enterobacteriaceae* and other genera not covered in the CLSI document, as has been done in a few other studies.[14,15]

E-test. The MIC of polymyxin B was determined by the E-test method, performed according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The MIC was read where inhibition of growth intersected the E strip. When small colonies grew within the zone of inhibition or a haze of growth occurred around the MIC end-point, the highest MIC intersection was recorded. The MICs of the E-test were rounded up to the next highest two-fold dilution for comparison of results with the reference method.[17]

Agar dilution. The MIC of polymyxin B was also determined by agar dilution method,[13] in which polymyxin B powder (Sigma Aldrich, USA) in solution was added to molten Mueller–Hinton agar (BD, USA) to provide two-fold dilutions ranging from 0.25 μg/ml to 1024 μg/ml. Bacterial suspensions were applied on the agar plates and the results were read following incubation at 35 °C for 18–20 h.

Disk diffusion

Disk diffusion testing was done using 300 U polymyxin B disk and 10 μg colistin disk (BBL, BD, USA), according to the CLSI guidelines.[10] The disk zone diameters were interpreted according to the CLSI guidelines for colistin (resistant ≤10 mm and susceptible ≥11 mm) and polymyxin B (resistant ≤11 mm and susceptible ≥12 mm).[10]

The bacterial inoculum was adjusted for disk diffusion, BMD, E-test, and agar dilution methods strictly according to CLSI guidelines[18] using a nephelometer (BioMerieux, France). All four methods were performed simultaneously for each isolate.

For comparison of performance, BMD was taken as the reference method. Agreement between BMD, agar dilution, and the E-test was defined as MICs that differed by ±1 log2 dilution or less. Categorical agreement was defined as test results within the same susceptibility. Errors were ranked as follows: very major error, false-susceptible result by the disk diffusion/agar dilution/E-test; major error, false-resistant result produced by the disk diffusion/agar dilution/E-test. Unacceptable levels were taken as ≥1.5% for very major errors and ≥3% for major errors, as recommended by CLSI document M23-A2.[19]

Repeat testing

A repeat testing of BMD, agar dilution, E-test, and disk diffusion of polymyxins B and E was done for all strains displaying resistance to polymyxin by the reference BMD method. Individual test

Table 1
Sources of the organisms included in the first part of the study

Organisms	Source						Total
	BAL	Blood	Pus	Urine	CSF	Tips	
Pseudomonas spp	63	21		5	1	1	91
Acinetobacter spp	64	18	2	6	1		91
Klebsiella spp	23	38	1	2		2	66
Enterobacter spp	5	2		2			9
Escherichia coli		1	1	7			9
Burkholderia spp	3	5					8
Stenotrophomonas spp	2	1					3
Proteus spp	2						2
Morganella spp					1		1
Providencia spp		1					1
Total	162	87	4	22	3	3	281

BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid.

procedures (agar dilution/E-test/disk diffusion) were repeated for reproducibility if they displayed very major or major errors.

Polymyxin B susceptibility of clinical isolates

In the second part of our study, consecutive MDR GNB obtained from clinical samples of patients admitted over a period of 6 months, as mentioned above, were included. Only one isolate per patient was included in the study. The clinical source of the isolates, ward of the patient, and antibiotic susceptibility profile of these isolates was recorded. The identification and antimicrobial susceptibility testing of the isolates was done as described in the first part of the study. All these isolates were subjected to polymyxin B disk diffusion susceptibility testing (using 300 U polymyxin B disks) as per the method described above. The isolates showing a polymyxin B-resistant phenotype were tested twice by the disk diffusion method. ATCC strains (as detailed above) were used as controls for disk diffusion testing.

Results

Evaluation of the methodologies for the determination of MIC

A total of 281 MDR GNB were included in the first part of the study. The distribution and sources of these isolates are shown in Table 1. All these isolates were extended-spectrum β-lactamase (ESBL)-positive and were also resistant to aminoglycosides and fluoroquinolones. Of the 281 isolates, 126 (45%) were resistant to all available antimicrobials.

Polymyxin B MICs

The MIC of polymyxin B was determined by three methods as described above. Of the 281 isolates, a total of 24 (8.5%) were resistant to polymyxin B by the reference BMD method. There was agreement (±1 log2 dilution difference or less) of MICs by all three methods in 20 of the 24 resistant strains. Details of the performance of the MIC methodologies for these 24 isolates are shown in Table 2.

Table 2
Comparison of E-test, agar dilution, and disk diffusion methods against the reference method in polymyxin B-resistant bacteria

Organism[a] (*N* = 24)	Broth microdilution (μg/ml)	E-test (μg/ml)	Agar dilution (μg/ml)	Polymyxin B disk diffusion (mm)	Colistin disk diffusion (mm)
Burkholderia cepacia	>1024	>1024	>1024	0	0
Burkholderia cepacia	>1024	>1024	>1024	0	0
Burkholderia cepacia	>1024	>1024	>1024	0	0
Burkholderia cepacia	>1024	>1024	>1024	0	0
Burkholderia cepacia	>1024	>1024	>1024	0	0
Burkholderia cepacia	>1024	>1024	>1024	0	0
Burkholderia cepacia	>1024	>1024	>1024	0	0
Stenotrophomonas maltophilia	256	256	256	0	0
Stenotrophomonas maltophilia	8	8	16	0	0
Stenotrophomonas maltophilia[b]	8	64	128	0	0
Pseudomonas aeruginosa	>1024	>1024	>1024	0	0
Pseudomonas aeruginosa	>1024	>1024	>1024	0	0
Pseudomonas aeruginosa	>1024	>1024	>1024	0	0
Acinetobacter baumannii	>1024	>1024	>1024	0	0
Acinetobacter baumannii[c]	8	8	8	14	13
Acinetobacter baumannii[d]	8	2	2	16	0
Klebsiella pneumoniae	>1024	>1024	>1024	0	0
Klebsiella pneumoniae[e]	16	0.5	2	14	12
Klebsiella pneumoniae	8	8	8	0	0
Proteus mirabilis	1024	>1024	>1024	0	0
Proteus mirabilis	1024	>1024	>1024	0	0
Morganella morgannii	1024	>1024	>1024	0	0
Providencia spp	>1024	>1024	>1024	0	0
Enterobacter spp[f]	8	1	8	0	0

[a] Total number of bacteria tested by all methods: 281; polymyxin-resistant isolates: 24.
[b] Although the MICs differed by >3-fold between the reference and the other two methods, the isolate was found to be resistant by all the MIC methods and disk diffusion. Therefore, the results were not included in the 'error' category.
[c] Very major error for polymyxin B and colistin disk diffusion.
[d] Very major error for E-test, agar dilution and polymyxin B disk diffusion.
[e] Very major error for E-test, agar dilution and polymyxin B and E disk diffusion.
[f] Very major error for E-test.

Table 3
Agreement of polymyxin MICs among the three test methods

	Categorical agreement	Agreement (±1 log2 dilution variation)	>±1 log2 dilution variation[a]	Major error	Very major error
BMD and E-test	140 (50)	115 (41)	23 (8)	0	3 (1)
BMD and agar dilution	107 (38)	141 (50)	24 (8.5)	7 (2.4)	2 (0.7)

Results are *n* (%). MIC, minimum inhibitory concentration; BMD, broth microdilution.
[a] For isolates in this category, the MIC differed by ≥1 log2-fold. However, the interpretation was either sensitive or resistant by all three methods, although the MICs differed by ≥1 log2-fold between the methods.

Of the 257 isolates found sensitive by BMD, all were sensitive by the E-test. However, seven isolates displayed resistance by the agar dilution method (categorized as major error). The MIC_{90} of polymyxin B by broth microdilution was 1 μg/ml. The overall agreement level between the reference BMD and other two MIC methods is shown in Table 3. It was observed that the MICs by E-test were generally lower than by BMD, and agar dilution MICs were higher than by BMD. The rates of very major and major errors were within CLSI acceptable limits.

Disk diffusion testing

Of the 24 isolates resistant by the BMD, two were found to be sensitive by colistin and three were sensitive by polymyxin B disks (very major errors; Table 2). Of the three isolates sensitive by polymyxin B disks, two were also sensitive by E-test (Table 2). However, one strain of *A. baumannii* was resistant by all three MIC methods. This strain was also sensitive by colistin disk. Of the 257 strains sensitive by BMD, all were sensitive by polymyxin B disk. However, two displayed resistance with colistin (categorized as major error). Thus, the rates of very major errors for polymyxin B and E disks were 1% and 0.7%, respectively, whereas the rates of major errors were 0% and 0.7%, respectively. In 247 of the 257 strains, the zone diameter of polymyxin B was 1–5 mm larger than that of colistin. In nine strains, their zone diameters were equal and in one strain, the zone diameter of colistin was greater than that of polymyxin B.

The MICs and disk zone diameters for control strains were within the range proposed by the CLSI.

Polymyxin B susceptibility in clinical isolates

A total of 723 consecutive GNB obtained from various clinical specimens were included in this part of the study. Of these, *P. aeruginosa* was the most common (*n* = 409 (57%)). The distribution, clinical source and antibiotic susceptibility of these isolates are shown in Table 4. *Acinetobacter spp* had the highest prevalence of resistance to all antimicrobial classes. Of these 723 organisms, 697 (96.4%) were found to be sensitive to polymyxin B by the disk diffusion method. Of the 26 isolates found to be resistant to polymyxin, 14 were *P. aeruginosa*, seven were *B. cepacia* and five were *S. maltophilia* (Table 4).

Discussion

The emerging multi-drug resistance in nosocomial GNB has necessitated the use of parenteral polymyxins for the treatment of life-threatening infections. Therefore, there is an increased need for reliable susceptibility testing methods to predict clinical response. Susceptibility testing for polymyxin B and E is plagued with problems, such as the lack of consensus regarding break-points for resistance between the CLSI, the British Society for Antimicrobial Chemotherapy (BSAC), the French Société Française

Table 4
Sources and sensitivity of the isolates in the second part of the study

Organisms (*n*)	Samples							Antimicrobial resistance, *n*[a] (%)							
	Tracheal aspirate	Blood	Pus	Urine	CSF/sterile fluid	Drain fluid	Tips	ESBL+	IMI	MER	PIP/TZB	TIC/CLA	AMK	CIP	POLY
Pseudomonas spp (181/409 = 44%)	181 (44)	82 (20)	77 (19)	39 (10)	10 (2)	11 (3)	9 (2)	372 (91)	249 (61)	261 (64)	181 (45)	319 (78)	270 (66)	278 (68)	14 (3.4)
Acinetobacter spp (52/184 = 28%)	92 (50)	56 (30)	22 (12)	2 (1)	1 (0.5)	7 (4)	4 (2)	160 (87)	127 (69)	52 (28)	118 (64)	147 (80)	140 (76)	152 (83)	0
Klebsiella spp (21/71 = 30%)	21 (30)	30 (42)	4 (6)	15 (21)	0	1 (1)	0	71 (100)	0	30 (42)	5 (7)	56 (79)	35 (49)	61 (86)	0
Escherichia coli (32)	18 (56)	6 (19)	4 (13)	4 (13)	0	0	0	30 (94)	2 (6)	7 (22)	2 (6)	4 (13)	4 (13)	7 (22)	0
Enterobacter spp (14)	2 (14)	5 (36)	6 (43)	0	0	0	1 (7)	12 (86)	0	0	0	10 (71)	7 (50)	7 (50)	0
Citrobacter spp (1)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Stenotrophomonas spp (5)		5 (100)						7 (100)	3 (43)	3 (43)	4 (57)	5 (71)	3 (43)	4 (57)	5 7
Burkholderia spp (7)	2 (29)	5 (71)													
Total: 723	316 (44)	189 (26)	114 (16)	60 (8)	11 (1.5)	19 (3)	14 (2)	652 (90)	381 (53)	353 (49)	310 (43)	541 (75)	459 (63)	509 (70)	26 (3.6)

Results are *n* (%). ESBL, extended-spectrum β-lactamase; IMI, imipenem; MER, meropenem; PIP/TZB, piperacillin/tazobactam; TIC/CLA, ticarcillin/clavulanic acid; AMK, amikacin; CIP, ciprofloxacin; POLY, polymyxin.
[a] The numbers indicate the number of isolates resistant to the particular antimicrobial.

de Microbiologie (SFM), and the German Deutsches Institut für Normung (DIN); the reported poor diffusion of polymyxins in the agar; and the lack of correlation between different dilution methods, as well as lacunae in studies done on this group of antimicrobials, most of which have been done using colistin.[2,15,17] Our institute has a very high prevalence of ESBL-producing GNB[20] and metallo-β-lactamase-producing *P. aeruginosa*.[21] At the Trauma Centre of AIIMS, patients admitted to the ICUs frequently suffer from ventilator-associated pneumonia due to MDR *P. aeruginosa* and *Acinetobacter spp*, necessitating the use of intravenous/aerosolized polymyxins.

We conducted the present study to evaluate the methods of MIC detection for polymyxin B and to find the level of polymyxin B resistance in GNB at our hospital. Our initial aim was to assess the performance of agar dilution and E-test as well as of the simple disk diffusion methods against the reference BMD. All the isolates found to be sensitive by the reference BMD were also detected to be sensitive by the E-test (specificity 100%). However, the agar dilution method (major error rate of 2.4%) had a specificity of 97.6%. The sensitivity of the E-test (21/24 detected as resistant) and agar dilution (22/24 detected as resistant) were 87.5% and 92%, respectively. A concordance of 99% was found between the E-test and BMD (three very major errors) and 97% was found between agar dilution and BMD (seven major and two very major errors). The resistant isolates included *B. cepacia, Proteus mirabilis* and *Providencia spp*, which are known to be intrinsically resistant to polymyxins.[2] Similarly, the sensitivities of polymyxin B and polymyxin E disk diffusion testing were 92% and 87.5%, respectively, and the specificities were 100% and 99%, respectively. A concordance of 99% and 98.5% was found between polymyxin B and polymyxin E disk diffusion, respectively, with BMD.

Of the limited number of studies on polymyxin resistance, the majority have been done on *Pseudomonas* and *Acinetobacter spp*. The rate of resistance in these genera is reported to vary from 0% to 5% for Pseudomonas and 0% to 19% for Acinetobacter. However, different interpretative guidelines have been used in these studies making it difficult to compare the results.[15,17,22] Only one study has reported resistance in *Stenotrophomonas spp, Morganella spp*, and *Klebsiella spp*.[15] Due to the emergence of pan-resistant Klebsiella and other genera, polymyxins are being used as life-saving antimicrobials in serious infections caused by these organisms. In our study, isolates of Acinetobacter, Pseudomonas, Stenotrophomonas, Morganella, Klebsiella, Enterobacter and Burkholderia were found to be resistant to polymyxin by quantitative MIC and disk diffusion techniques. A limitation of our study was the relatively small number of organisms representing some genera. Therefore, more studies are needed to assess the true magnitude of polymyxin resistance in various Gram-negative organisms.

The E-test has been evaluated for polymyxin susceptibility testing in a few studies. A concordance of 98.2% between BMD and the E-test for colistin, with 1.7% very major error 22 was found in *Acinetobacter spp*. in one study.[22] In another study comparing the E-test and agar dilution for colistin, a concordance of 87% was found with 5% very major errors and 9% major errors.[15] A concordance of 90% was found between the E-test and BMD for polymyxin B in 109 isolates of *P. aeruginosa*.[17]

To our knowledge, this is the first study comparing BMD, agar dilution, and the E-test for polymyxin B along with disk diffusion for polymyxins B and E for a range of MDR Gram-negative genera using the new CLSI guidelines. We found a very good concordance between the reference BMD and the E-test. Although determination of MIC by BMD is considered to be the optimum method, it is impractical and cumbersome for most busy clinical microbiology laboratories. Therefore, the E-test could be used as a viable alternative, since it is very easy to perform and interpret.

We also found a good concordance between agar dilution and BMD in our study. In a study on *P. aeruginosa* from cystic fibrosis patients, agar dilution was taken as the gold standard and it was found that BMD was most reliable at a susceptibility breakpoint of $\leq$4 mg/l, with a specificity of 91% and sensitivity of 89%.[8] However, in agreement with Hogardt et al.,[8] a slightly higher polymyxin B MIC (1–2 log2 dilution difference) was seen with agar dilution as compared to the BMD in our study. Nevertheless, the method is suitable for batch testing of a large number of strains. We also found good concordance of disk diffusion results by polymyxin B and colistin disks. Since disk diffusion was found to have an acceptable rate of errors, we used this simple method for screening a larger number of GNB in the second half of the study. We found a resistance rate of 3.6% by the disk diffusion method. However, of the resistant strains, 27% were *B. cepacia*, which are intrinsically resistant to polymyxins. A significant finding was the detection of polymyxin resistance (3%) in strains of Pseudomonas. A limitation of our study was that we did not determine the polymyxin MICs for these isolates of *P. aeruginosa*. However, all the isolates were tested twice and there was no zone around polymyxin B disks. Therefore, we presume these to be actually resistant to polymyxin B, based on our results in the initial part of this study.

To conclude, in view of the paucity of available antimicrobials and the potential toxicity of polymyxin group antimicrobials, their clinical use must be based on validated in vitro susceptibility results. The disk diffusion method can be used for initial screening in busy hospital laboratories.

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