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ORIGINAL ARTICLE

Polymyxins: Re-considering the Disk Diffusion Susceptibility Testing Methodin MALDI–TOF-identified Gram Negative Bacilli

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ABSTRACT

Background: *The widespread multidrug-resistant Gram-negative bacilli (GNB)has necessitated the evaluation of the use of older antimicrobials such as polymyxins being the last resort drugs for the treatment of life-threatening nosocomial infections.* ***Objectives:*** *We conducted the present study to evaluate simple disk diffusion method (the routine method used in busy low resources hospitals)in comparison with MIC detection for polymyxin Band polymyxin E using the E-test according to the new Clinical and Laboratory Standards Institute (CLSI) guidelines and to find the level of polymyxins resistance of GNB in Kasr Alainy hospital.* ***Methodology****: A total of 50 Gram-negative bacterial isolates previously identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and classified as 12 MDR by being resistant to three or more antimicrobial classes and38 XDR by being non-susceptible to at least one agent in all but two or fewer antimicrobial categories. Antimicrobial susceptibility testing of polymyxin B and polymyxin E (colistin) v was done by the determination of MIC by E-test, along with disk diffusion testing v.* ***Results:*** *Comparison of the disk diffusion and the MIC method by E- test showed100% correlation between both methods for all isolates of Pseudomonas aeruginosa, Acinetobacter baumanii and Klebsiella spp. All isolates were sensitive to both polymyxin B and colistin with MICs and disk zone diameters within the range proposed by the CLSI 2014.MIC 50 and MIC 90 of both polymyxin B and colistin were found to be 1μg/ml.* ***Conclusion:*** *Polymyxins are a reliable option for the treatment of infections caused by MDR isolates of Gram-negative bacilli with proper dose adjustment. There is good concordance between the E-test and the disk diffusion. The disk diffusion method can be used for initial screening in busy hospital laboratories.*

INTRODUCTION

The extreme rise of antibiotic resistance and the need of new antimicrobials had led to a renewed interest in the use of the polymyxins group of antibiotics for the treatment of infections caused by multidrug-resistant (MDR) bacteria[1,2].

Isolates of *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* resistant to almost all available antibiotics except polymyxins, had emerged as an alarming cause of hospital acquired infections in critically hospitalized patients[3,4].

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Polymyxins are multicomponent polypeptide antibiotics which act primarily on the cell wall ofGram-negative bacteria, causing rapid permeability changes of the cytoplasmic membrane and finally causing cell death[5]. Polymyxin E (colistin) and polymyxin B had been recently used in the treatment of infections caused by Gram-negative bacteria (GNB) that are resistant to cephalosporins, anti-*Pseudomonas* penicillins, monobactams, quinolones, aminoglycosides and carbapenems[6,7] thus, constituting thelast resort drugs for the treatment of many life-threatening infections.

The increased demand for polymyxins in the treatment of those critically ill patients in the intensive care unit (ICU) has also created an urgency for up-to-date susceptibility data.

However the in vitro susceptibility testing of polymyxins is hindered by many different factors. The accuracy of the disk diffusion (DD) assay may be

unsatisfactory aspolymyxins diffuse poorly into the agar, and thus no reliable correlation between zone diameters and minimum inhibitory concentrations (MICs) had been found in previous studies[8]. Also the interpretative criteria for quantitative in vitro testing differs between communities[8].

The Clinical and Laboratory Standards Institute (CLSI) has provided a standard document for the testing of polymyxins against *P. aeruginosa, Acinetobacter* spp and a few other non-fermenters using dilutionmethods[9].

Only in 2007 the interpretative criteria for the disk susceptibility testing of polymyxins by the CLSI were published[10].

However, still there is no agreement regarding the break points for defining resistance to polymyxins between different medical societies. Also reliable data on true resistance levels on this group of antimicrobials are lacking.

Considering the increasing use and demand for polymyxins and the relative shortage of data regarding resistance, we conducted the present study to evaluate simple disk diffusion method the routine method used in busy low resources hospital against MIC detection for polymyxin E and polymyxin B using the E-test and to find the level of polymyxins resistance in GNB at our hospital.

METHODOLOGY

Clinical bacterial isolates:

A total of 50 Gram-negative bacterial isolates were included in this study. The isolates were identified as MDR by being resistant to three or more antimicrobial classes and extensively drug-resistant (XDR) by being non-susceptibile to at least one agent in all but two or fewer antimicrobial categories or bacterial isolates that remain susceptible to only one or two categories.

The specimens were collected from the ICU ward in Kasr Alainy Hospitals, Cairo University, in the period from May 2013 to September 2013.

The clinical isolates were isolated from the following samples: 20(40%) from blood cultures, 18(36%) from respiratory samples including broncho-alveolar lavage (BAL), endotracheal tube (ETT) and sputum, 6 (12%) from wound swabs and 6(12%) isolates from urine samples. Only one isolate per patient was included. The clinical source of the isolates and patient ward were recorded.

The isolates were identified on the basis of conventional microbiological procedures; including colony morphology on blood agar and MacConkey's agar (Mast Diagnostics, Merseyside, UK), Gram's staining and biochemical reactions. In addition to the conventional phenotypic characterization, the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used asit provides a rapid, accurate and cost-effective method of microbial characterization and identification.

This technology generates characteristic mass spectral fingerprints, that are unique signatures for each microorganism and is thus ideal for an accurate microbial identification at the genus and species levels and has a potential to be used for strain typing and identification.[11]

All bacterial samples were smeared on the MALDI target as a thin film and overlaid with 1.5 µl of formic-acid (70%), air-dried and overlaid with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% TFA) which results in the crystallization of the sample within the matrix. The matrix is composed of small acid molecules that have a strong optical absorption in the range of the laser wavelength used.[12] Plates were analysed in positive linear mode with a MALDI-TOF MS Axima™ Confidence (Shimadzu, Japan). A minimum of 50 laser shots per sample were used to generate each ion spectrum analysed. Spectra were compared with the SARAMIS™ (spectral archive and microbial identification system) database application from Anagnostec GmbH (Potsdam, Germany).

Antimicrobial susceptibility was determined by the Kirby-Bauer disc diffusion method as per the CLSI 2014 recommendations[13].

The antibiotics tested by the disk diffusion method can be found in Table 1.

Antimicrobial susceptibility testing of polymyxin B and polymyxin E (colistin) was carried out by the determination of MICs by E-test, along with disk diffusion testing.

Table 1: The antimicrobial category and antimicrobial agent tested by the disk diffusion method

Antimicrobial category	Antimicrobial agent
Aminoglycosides	Gentamicin, Amikacin
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin-tazobactam
Carbapenems	Imipenem, Meropenem
Non-extended spectrumcephalosporins; 1st and 2nd generation cephalosporins	Cefazolin, Cefaclor
Extended-spectrumcephalosporins; 3rd and 4th generation cephalosporins	Cefotaxime or Ceftriaxone, Ceftazidime, Cefepime
Cephamycins	Cefoxitin
Fluoroquinolones	Ciprofloxacin, Levofloxcin, Ofloxacin
Folate pathway inhibitors	Trimethoprim-sulphamethoxazole
Glycylcyclines	Tigecycline
Penicillins	Ampicillin
Penicillins + β lactamase inhibitors	Amoxicillin+ clavulanic acid
Phenicols	Chloramphenicol
Polymyxins	Colistin, Polymyxin B

The following isolates were used as controls: *Escherichia coli* ATCC25922 and *P. aeruginosa* ATCC 27853.

E-test: The MIC of polymyxin B and colistin 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.

Disk diffusion:

Disk diffusion testing was done using 300 U polymyxin B disk and 10 mg colistin disk (BBL, BD, USA), according to the CLSI 2014guidelines[13].

The bacterial inoculum was adjusted for disk diffusion and E test strictly according to CLSIguidelines[14]. Both methods were performed simultaneously for each isolate.

There are no FDA breakpoints or FDA cleared commercial test for colistin and polymyxin B, thus the disk zone diameters were interpreted according to the CLSI guidelines for colistin and polymyxin B, since up to-date the CLSI[13] (table 2) susceptibility breakpoints of polymyxin B and polymyxin E have been available only for *P. aeruginosa, A. baumannii* and some other non-fermenters, we used the same breakpoints for *Enterobacteriaceae* and we supported our results by the EUCAST 2013 MIC breakpoint tables for Colistin[15] (table 3).

Table 2: The CLSI 2014[13] guidelines susceptibility breakpoints of polymyxin B and polymyxin E together with the available disk zone diameters

Organism	MIC (µg/ml)			Zone diameter (nearest whole mm)		
	S	I	R	S		R
Acinetobacter spp	**≤ 2**	_	**≥ 4**	NOT PROVIDED		
Pseudomonas spp	**≤ 2**	**4**	**≥ 8**	**≥ 11** **≥ 12**	**COLISTIN** **POLYMIXIN**	**≤10** **≤11**
Enterobacteriaceae	NOT PROVIDED					

Table 3: The EUCAST 2013[15]MIC breakpoint tables for Colistin

Bacterium n	**SUSCEPTIBLE**	**RESISTANT**
Pseudomonas aeruginosa	≤ 4 mg/L	≤ 4 mg/L
Acinetobacter baumanii	≤ 2 mg/L	> 2 mg/L
Klebsiella spp.	≤ 2 mg/L	> 2 mg/L

Data were statistically described in terms of percentages when appropriate. For comparison of performance, E-test was taken as the reference method. Categorical agreement was defined as tests results with the same susceptibility. Errors were ranked as follows: very major error; false-susceptible result by disk diffusion compared to the E-test, major error; false-resistant result produced by the disk diffusion compared to the E-test while minor errors as intermediate result by the DD and resistant or susceptible category by the E test. Unacceptable levels were taken as _1.5% for very major errors and _3% for major errors, as recommended by NCCLS document [16].

Accuracy was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 17 for Microsoft Windows.

RESULTS

The present study included 50 Gram-negative bacterial isolates that were presented to the Microbiology Laboratory in the period from May 2013 to September 2013. Among the 50 bacterial isolates, *Acinetobacter baumanii* was the most common pathogen isolated from the tested samples, constituting 21/50 (42%) of the total isolates, followed by *Pseudomonas aeruginosa* 19/50 (38%), *and lastly Klebsiella* spp.10/50 (20%). The sources of the organisms included in the study are represented in table 4.

Table 4: Sources of the organisms included in the study

Clinical sample	n (%)	*Pseudomonas aeruginosa*	*Acinetobacter baumanii*	*Klebsiella* spp.
Blood	20 (40%)	7 (35%)	10(50%)	3 (15%)
Endotracheal tube	10(20%)	3 (30%)	3 (30%)	4(40%)
Sputum	6 (12%)	3(50%)	2 (33.3%)	1 (16.66%)
Broncho-alveolar lavage	2 (4%)	0	1(50%)	1(50%)
Urine	6(12%)	4 (66.66%)	2 (33.3%)	0
Wound	6(12%)	2(33.3%)	3(50%)	1 (16.66%)
Total	50(100%)	19/50 (38%),	21/50 (42%)	10/50 (20%).

n = number

The 50 Gram-negative bacterial isolates constituted 38 (76%) isolates identified as XDR by being non-susceptible to at least one agent in all but two or fewer antimicrobial categories, while 12 (24%) were identified as MDR by being non-susceptible to at least one agent in three or more antimicrobial categories. Among the 38 XDR isolates *Acinetobacter baumanii* was the most common, constituting 18/38 (47.36%) followed by *Pseudomonas aeruginosa* 12/38 (31.5%) and lastly *Klebsiella* spp.8/38 (21.05%).Among the 12 MDR isolates *Pseudomonas aeruginosa* was the most common, constituting 7/12 (58.33%) followed by *Acinetobacter baumanii* 3/12 (25%), and lastly *Klebsiella* spp.2/12 (16.66%).

The MICs and disk zone diameters of both polymyxin B and colistin for all strains were within the range proposed by the CLSI 2014[13].

MIC 50 and MIC 90 of both polymyxin B and colistin were found to be 1µg/ml and can be found in table 5.

Table 5: MIC 50 and MIC 90 of both polymyxin B and colistin

Antibiotic	Colistin				PolymyxinB			
	MIC50 µg/ml	MIC90 µg/ml	Range µg/ml	% Susceptibility	MIC50 µg/ml	MIC90 µg/ml	Range µg/ml	% Susceptibility
Pseudomonas aeruginosa	1	1	<0.25– 2	100%	1	1	<0.5–8	100%
Acinetobacter baumanii	1	1	<0.25–2	100%	1	1	<0.5–4	100%
*Klebsiella*spp.	1	1	<0.25–2	100%	1	1	<0.5–2	100%

Comparison of the DD and the MIC method by E-testis shown in table 6.These results indicate that correlation was 100% between both methods for all isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumanii and Klebsiella* spp.

Accuracy between both methods is represented in table 7.

Table 6: Concordant results between the DD and the MIC method by E-test presented according to the error category testing

	DD versus MIC by E – test in %		
Bacterium n	**Very major error**	**major error**	**minor error**
Pseudomonas aeruginosa 19	0%	0%	0%
Acinetobacter baumanii 21	0%	0%	0%
Klebsiella spp. 10	0%	0%	0%

Table 7: Accuracy rates between DD and the MIC method by E-test as the gold standard

Measure	Colistin	Polymyxin B
Agreement with gold standard	100%	100%
Sensitivity	100%	100%
Positive predictive value	100%	100%
Accuracy	100%	100%
Percent of sensitive cases	100%	100%
Percent of resistant cases	0%	0%

DISCUSSION

The ongoing emergence of multi-drug resistant GNB causing nosocomial infection poses a treatment challenge that renewed interest in the use of parenteral polymyxins for treating these

life threatening infections. Thus it is important to evaluate different susceptibility testing methods to this class of antibiotic to predict clinical response. Susceptibility testing for polymyxin B and E is plagued with problems, such as the lack of agreement regarding break points resistance between the CLSI, German Deutsches Institut fur Normung (DIN), the British Society for Antimicrobial Chemotherapy (BSAC) and the Société Francaise de Microbiologie (SFM) together with the reported poor diffusion of polymyxins in the agar; and the lack of correlation between different dilution methods, [17, 18,19]

We conducted the present study to evaluate the performance of disc diffusion method for both polymyxin B and E which is the routine method used in our hospital against MIC detection by the E-test, and also to find the level of polymyxin B and E resistance of GNB in our hospital.

We have observed almost perfect agreement between identifications obtained by MALDI-TOF MS and those provided by conventional, biochemical methods. The present study shows that species identification by MALDI-TOF MS provides reliable and rapid identification of microorganisms grown on solid media and in liquid media. A noticeable acceleration of the identification process was obtained for blood cultures. This was in agreement with many recent studies which concluded that MALDI-TOF MS has proven to be a reliable alternative for traditional identification methods, and is on its way to become a new standard for the identification of microorganisms in high throughput laboratories[20,21,22.]

In our study high resistance was observed to aminoglycosides, first and second cephalosporins, third and fourth generation cephalosporins, penicillins and even to monobactams and ampicillin/sulbactum**.** Thirty-eight (76%) isolates were identified as XDR, while twelve (24%)were identified as MDR. In our study MIC 90 of colistin for, *P. aeruginosa, A. baumannii and K. pneumoniae* was found to be 1µg/ml similar to the studies from Singapore and EUCAST studies [23,24,25,26] MIC 50 of colistin was also found to be 1µg/ml which was similar to other studies[23,24]. 100% of the isolates were found susceptible to colistin with MIC break points S ≤2 and R>2[27]. Maximum isolates were found to be sensitive at 1µg/ml with sensitive MIC ranges 0.25-2µg/ml.

MIC 50 and MIC 90 of polymyxin B were found to be 1µg/ml similar to the sentry report USA[26,27]. All isolates were found sensitive at 1ug/ml with susceptible MIC range <0.5–8 for *P aeruginosa,* <0.5–4 for *A baumannii and* <0.5–2 *for K pneumonia*. We noticed 0% resistance to polymyxin B which is different from the result of the sentry report USA that reported 2.5% resistance [26.]

This may be explained by the limited and recent usage of polymyxins in our hospital. Polymyxins resistance among *P aeruginosa* isolates has been described in patients with cystic fibrosis which is not a common disease in Egypt, however, data on acquired resistance to polymyxins among isolates causing nosocomial infection are scanty [28,29].

Comparison of the DD and the MIC method by E – test (table 5) showed the concordant results between the DD and the MIC method by E – test as presented according to the error category testing. These results indicate that correlation was 100% between both methods for all isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumanii and Klebsiella* spp. for both colistin and polymyxin.

E-tests have the advantage of being easy to perform and less time consuming compared to the reference broth microdilution (BMD). 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 with reference (BMD)to be as high as 90 - 100% and have suggested it as a reliable and useful alternative to the dilution methods[30,31]. This is contradicted by the findings of Tan *et al.* where unacceptable rates of very major error, especially for *P. aeruginosa* have been detected[18]. 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 [32].

The results of the present study were in accordance of the results of previous study by Behera*et al*[33]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 where they 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, it is impractical and cumbersome for most busy clinical microbiology laboratories. Therefore, the E-test could be used as a valuable alternative, since it is very easy to perform and interpret. Also they found good concordance of disk diffusion results by polymyxin B and colistin with the reference BMD and the E-test stating that disk diffusion can be used for screening a larger number of GNB.[33]

However, this was contradictory to previous data which suggested that the accuracy of disc diffusion assays was unsatisfactory because polymyxins diffuse poorly, which may be due to its large molecules and thus producing inconsistent zones of inhibition [19]and reported that the efficacy of the disc diffusion test for polymyxins to be unreliable for use and consequently results of a diffusion test should be confirmed with a dilution method[23].

CONCLUSION

Polymyxinsare a reliable option for the treatment of infections caused by MDR isolates of Gram-negative bacilli with proper dose adjustment. Clinical use must be based on validated *in vitro* susceptibility results. The disk diffusion method can be an appealing option to be used for initial screening in busy hospital laboratories. However, the burden of the selective pressure caused by extensive polymyxins use may contribute to the emergence of polymyxins resistance among gram-negative bacilli, thus increasing morbidity and mortality rates in critically ill patients. For this reason, the empiricuse of polymyxins should be limited to institutions with recognized infections caused by MDR gram-negative bacilli.

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