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IN VITRO COMPARISON OF DISK DIFFUSION METHOD AND AGAR DILUTION METHOD FOR SENSITIVITY OF POLYMYXIN B AGAINST MULTI DRUG RESISTANT ACINETOBACTER BAUMANNII

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ABSTRACT

Objective: To compare the in vitro disk diffusion method and agar dilution method for sensitivity of Polymyxin B against multi drug resistant Acinetobacter baumannii.
Study Design: Comparative cross sectional.
Place and Duration of Study: The study was carried out in the department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi from 1st Dec 2016 to 30th Dec 2017.
Methodology: Total 253 clinical specimens received from intensive care units with yielded growth of multidrug resistant Acinetobacter baumannii were evaluated for Polymyxin B susceptibility. Both Kirby bauer disk diffusion and agar dilution methods were compared with a reference method, broth microdilution.
Results: Among 253 multidrug resistant isolates, 180 (71%) were extensively drug resistant and 221 (87%) were carbapenem resistant. Comparison of the disk diffusion and the MIC method by Agar dilution showed 100% correlation. 251 isolates were sensitive and 2 were resistant to Polymyxin B with MICs and disk zone diameters within the range recommended by the CLSI (2014-17). MIC 50 and MIC 90 of Polymyxin B were found to be 0.5 and 1µg/ml with 99.2% susceptibility.
Conclusion: Both disk diffusion and agar dilution can be used together as initial screening methods in low income countries for susceptibility reporting of polymyxins. They are simple, reliable and economical. Also hetero resistance shown by some Acinetobacter baumanii strains can easily be picked up by agar dilution method.

Keywords: Agar dilution, Broth microdilution, Extensively drug resistant, Kirby bauer disk diffusion, Multi drug resistant, Minimum inhibitory concentration.

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INTRODUCTION

Acinetobacter baumannii (A. baumannii), a gram negative, cocco bacilli, is one of the known major opportunistic pathogen of nosocomial infections in the world. The factors that are responsible for causing multidrug resistance (MDR) include prolonged hospitalization, immune compromised health status, mechanical ventilator support, prolonged injudicious usage of broad spectrum antibiotics and catheterization[1]. It has an exceptional ability to adapt to unfavorable hospital environment due to emerging resistance to multiple antibiotics/ disinfectants and biofilm formation[2].

Due to increased prevalence of nosocomial infections by this MDR organisms and availability of limited therapeutic options, the old drug (polymyxin) is again being used as a last resort to treat such infections[2,3]. It is bactericidal in action that act primarily on gram negative cell wall, leading to rapid permeability changes in the cytoplasmic membrane and finally cell death occurs[4]. The most commonly used disk diffusion sensitivity method is not reliable because Polymyxin B diffuses poorly in agar based medium[5]. Consequently, clinical microbiology laboratory should be able to perform reliable susceptibility testing for polymyxins. Its increased usage in intensive care units (ICU) demands an up to date sensitivity data[5-7].

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Clinical and Laboratory Standard Institute (CLSI) recommended disc zones of polymyxin during 2007-2014 against Psuedomonas aeroginosa[8]. However, the current available guidelines of CLSI 2016-18 have recommended only MIC (Minimum Inhibitory Concentration) interpretation of polymyxins (Polymyxin B and Colistin). Therefore, no reliable agreement on breakpoints of disc zone diameters and MICs has been found between different societies of microbiology[9].

In view of paucity of reliable data regarding true resistance of this drug in our setup, in vitro antimicrobial activity of polymyxin B against multidrug resistant A.baumannii was conducted. In this study, we evaluated a correlation between disc diffusion and agar dilution method to determine reliable susceptibility criteria for polymyxin B.

METHODOLOGY

The study was carried out in Department of Microbiology, Armed Forces Institute of Pathology Rawalpindi. All clinical specimens received from intensive care units (ICU) of Combined Military Hospital (CMH) and Pak Emirates Military Hospital (PEMH), Rawalpindi were applied on 5% Sheep Blood Agar (SBA) and MacConkey agar. After overnight incubation, the samples yielding growth of Gram negative cocco bacilli resembling Acinetobacter baumannii were further identified as per the standard laboratory protocols. Confirmation of the organism was done by applying API 20 NE (Biomerieux, France) and VITEK 2 system.

Disk Diffusion Antimicrobial Susceptibility Testing (DD)

It was done by Kirby-Bauer method on Mueller Hinton agar (MHA). Discs (Oxoid, UK) with following concentrations were used. Amikacin 30 µg, Ceftazidime 30 µg, Ciprofloxacin 5 µg, Gentamicin 10 µg, Cefipime 30 µg, Imipenem 10 µg, Meropenem 10 µg, Moxifloxacin 5 µg, Ampicillin-sulbactam 105 µg, Pipercillin-Tazobactam 110 µg, Minocycline 30µg , Doxycycline 30µg and Polymyxin B 300 µg. The disk zone diameters were interpreted according to the CLSI guidelines 2014 for polymyxin B (resistant ≤11 mm and susceptible ≥12 mm). P.aeruginosa ATCC 27853 and A. baumannii ATCC BAA 747 (American Type Culture Collection, Rockville, MD) were used as controls.

Agar Dilution Method (AD)

A standard concentration of Polymyxin B sulfate salt powder (Sigma-Aldrich, Germany) was dissolved in sterile distilled water to make stock solution (2.56mg/ml) and stored at -70°. Prior to each susceptibility testing, an aliquot of the drug was thawed and diluted to the desired concentration. The drug was added in molten Mueller-Hinton agar (Oxoid, UK) to make twofold concentrations ranging from 0.25 to 128 µg/ml, which was subsequently poured into standard petri dishes of 90 mm. The pH of the medium was maintained at 7.2 to 7.4 and the agar was then allowed to solidify. Bacterial suspension equivalent to 0.5 McFarland standard was made and inoculated onto each agar plate using a denley multi point inoculator to yield a final inoculum of 104 colony-forming units (CFU) per spot. The inoculum was used within 15 min of preparation. Results were obtained after incubation at 35-37°C for 16-20 hrs and were interpreted according to the criteria laid down in CLSI guidelines[9].

Broth Micro Dilution (BMD)

A primary reference method, BMD was carried out in 96-well microtitre plates using Cation-adjusted Mueller Hinton broth (CA-MHB BBL-Becton Dickinson) in accordance with the CLSI recommendations (M07-A9). Polymyxin B Sulfate salt (Sigma-Aldrich, Germany) concentrations ranging from 0.25 µg/ml to 128 µg/ml were tested. The initial inoculum of bacterial suspension made from overnight culture in a broth equivalent to 0.5 MacFarland turbidity, was further diluted to achieve the final inoculum containing approx. 5×10^5 CFU/ml. Then within 15 min of this diluted bacterial inoculums preparation was added in each well of specified row. No drug was added in growth control well and no inoculum was added in sterility control.

The plates were incubated at 35 ± 2°C for 18 hrs aerobically[10]. Streak a 10 µl loopful of suspension from the growth control well onto SBA plate and incubate overnight to check the purity of the test isolate and correct inoculum density. The presence of approximately 50 colonies would indicate an inoculum density of 5×10^5 CFU/mL. The MIC was defined as the lowest concentration of Polymyxin B at which there is no visible growth. A Polymyxin B MIC of ≤2 and ≥4 µg/ml was taken as the breakpoint for susceptibility (CLSI 2016-17).

Quality Control testing: The CLSI-recommended quality-control (QC) strains, P.aeruginosa ATCC 27853 was tested by all methods. The MICs were within the acceptable QC range of 0.5 to 2µg/ml by all test methods[8,9].

The data obtained was entered in SPSS software (version 24) for statistical evaluation. Descriptive statistics were calculated for both qualitative and quantitative variables. Mean zone diameter and MIC of Polymyxin B were calculated. For quantitative variables like age, mean and SD were calculated. A *p*-value ≤0.05 was considered as significant.

Acceptable performance was evaluated according to criteria established by the International Organization for Standardization: ≥90% for essential or category agreement and ≤3% for VMEs or MEs. Essential agreement (EA) was defined as the percentage of MICs within ± 1 log2 dilution of the MIC determined by BMD. Categorical agreement (CA) was defined as the percentage of isolates classified in the same susceptibility category by BMD and the method under evaluation. Very major errors (VMEs) defined as false-susceptible result, and major errors (MEs) denoted a false-resistant result. Minor errors (ME) are reported as intermediate results when either resistant or susceptible[12]. Undesirable levels as recommended in the CLSI document, M23-A2 are >1.5% for very major errors, >3% for major errors and 10% for minor errors[13].

RESULTS

A total 253 MDR (resistant to three or more drugs) isolates from various clinical specimens were dealt in our study. Most of the isolates were from respiratory specimens and majority of patients were on ventilatory support. There was a male predominance and mean age was 47 ± 20.7 years. Out of total 253 isolates, 180 (71%) were extensively drug resistant (XDR, resistant to at least one drug in all classes except one or two drugs) and 221 (87%) carbapenem resistant strains (CRAB i.e resistant to either one or any two carbapenem drugs). These isolates were further tested for Polymyxin B susceptibility by two methods (DD & AD) and their results were compared with each other in relation to reference method (BMD) as shown in table-I.

Table-I: Demographic data and clinical sources of MDR A.baumannii isolates (n=253).

Demographic Data		Clinical Samples (n=253)	MIC Range (≤0.5-4 µg/ml)
Age (0 to 86 years)	Gender	*Respiratory = 156 (62%) Blood = 35 (14%) Pus = 22 (8.7%) CVP tip = 13 (5.1%) Urine = 11 (4.3%) Tissue = 8 (3.1%) CSF = 8 (3.1%)	MIC 50 = 0.5 MIC 90 = 1 Susceptibility% = 99.2%
0-45 = 104 (41%) 46-86 = 149 (59%) Mean = (47 ± 20.7)	Males = 160 (63%) Female = 93 (37%)		

*Respiratory Samples: Non directed Bronchoalveolar Lavage, Bronchoalveolar Lavage, Sputum, Endobronchial washings, Endotracheal tubes.

In our study, the MICs and disk zone diameters of Polymyxin B were within the range as recommended by the CLSI 2014. The diameter of the inhibition zone is proportional to the bacterial susceptibility to polymyxins and inversely correlates with the MIC of the bacterial strain. MIC 50 and MIC 90 were found to be 0.5µg/ml and 1ug/ml respectively (table-I).

Over all (100%) categorical agreement (CA) was found in AD and DD method with BMD. 100% accuracy with no categorical error has been found in our study. Considering different ranges of BMD, some inconsistency can be appreciated between AD (73% to 100%) and DD (70% to 100%). Our results were within desirable limits that showed 100% essential agreement (EA) with BMD (table-II & III).

Table-II: Polymyxins MICs by varioussusceptibility testing methods and their categorical agreement with reference method (BMD) (n=253).

A.b types	Test met hod	No. of isolates with colistin MIC (ug/ml) (S≤2 µg/ml & R ≥4 µg/ml)				No. of isolates with zone diameter (S≥12 mm & R ≤11mm)				No. of isolates with results		% of category errors			% Categorical agreement with BMD				
		≤0.5	1	2	≥4	≤ 11	13-14	15-16	17-18	Sensitive	Resistant	V.major	major	minor	≤0.5 ≤18	1 ≤ 16	2 ≤ 14	≥ 4 ≤ 11	Overall %(253)
1XDR = 71% (180) 2CRAb = 87% (221)	AD	48	102	101	2	-	-	-	-	251	2	0	0	0	73%	93%	89%	100%	100%
	BMD	66	95	90	2	-	-	-	-	251	2	-	-	-	-	-	-	-	-
	DD	-	-	-	-	2	67	136	48	251	2	0	0	0	73%	70%	74%	100%	100%

1XDR= Extensively drug resistant, 2CRAb = Carbapenem resistant Acinetobacter baumannii

Table-III: Accuracy rates of Polymyxin B by DD and AD method in relation with gold standard, BMD method.

	DD	AD
Agreement with gold standard	100%	100%
Sensitivity	98.5 to 100%	99% to 100%
Positive predictive value	100%	100%
Percent of sensitive cases	97 to 99.2%	99.2%
Accuracy	98.56 to 100%	98.6 to 100%
Percent of resistant cases	0.79%	0.8%
Mean	15.4mm (zone diameter)	1.3ug/ml

DISCUSSION

In recent years the prevalence of MDR A. baumannii infection (especially carbapenem resistant) as a leading cause of mortality and morbidity has increased mainly in Asia[11]. This developed clinicians' interest more towards the polymyxins usage either alone or in combination. In some setups, it is being used empirically in critical patients of ICUs, on the basis of contemporary epidemiological state[12]. Due to increased usage of polymyxins in clinical settings in recent years, there is a need to know and test its various susceptibility methods and defining accurate break points of both zone diameters and MICs[13].

In this study, we evaluated the AD method which provides a quantitative result in the form of MIC. The DD method is an indirect measure of susceptibility and provides a qualitative interpretative result in form of zones of inhibition[13]. It is an easy and cost effective but unreliable method due to its false susceptibility rate up to 35% cases[14]. The AD is laborious method and its plates must be used in a week but multiple isolates can be tested at a time in same plate. Also, it can

detect mixed cultures or heterogeneous populations that can be missed by BMD method[14].

BMD is the primary gold standard method recommended by both European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI because of its reproducibility, reliability, and possibility of automation[15]. However, it may lead to significant error, if carried out manually and quite laborious as well[16]. Still AD method has been used as reference method in many parts of the world[17]. The present study showed agreement among both methods, AD and DD. The results of AD were in acceptable range in relation to reference method, BMD as shown by other studies[18,19]. MIC 50 and MIC 90 by AD method were in comparable ranges with the susceptibility rate of 99.2% like other studies[20, 21].

However there was high error rate with DD method in various studies which is in contradiction to our findings[19]. In our study, we also observed variability in CA (70 to 100%), when we consider different concentrations of susceptibility in terms of disk zones and MICs by AD. Majority of isolates (136) were inhibited at 15-16 mm zones showed 75% CA with MIC of 1ug/ml by AD and 70% with BMD. Only 48 isolates were inhibited at 17-18 mm range with 100% CA (0.5ug/ml) AD and 73% with BMD. Higher MICs (2ug/ml) equivalent to 13-14mm zones were noted in 101 cases by AD (CA 89%) and 67 isolated on DD (CA 74%) respectively. But there was 100% CA in all methods in resistant cases as documented by other studies as well[19-22]. Mean MIC and zone of inhibition were 1.3ug/ml and 15.4mm respectively. The findings of studies by Behera *et al* and El Sherif *et al*, regarding DD method were in accordance with our results[22,23]. The limitation of Egyptian and present study was inadequate sample size with few resistant cases reported[23]. Therefore it is difficult to comment about the efficacy of DD test for polymyxins as CLSI has not recommended it's zones of inhibition after 2014[23,24]. Moreover, our study had 71% of XDR and 87% CRAb from 62% of respiratory source in 63% male adults patients aged ≥46 years (59%),which are comparable to results of Sallam *et al*[23].

Despite a good correlation demonstrated by all the tested methods, the accuracy of DD method remained questionable due to small zone sizes of inhibition and disparity with MIC[24,25]. Therefore it is recommended to carry out more studies across the board focusing on all the factors that can improve accuracy of DD method for this drug[25]. This can help us in correlating the disk zones with the provided MICs breakpoints by CLSI and locally establishing reliable interpretation of inhibitory zones for our lab to follow with ease and self-confidence.

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CONCLUSION

Disc diffusion has a very good agreement with agar dilution and broth micro dilution. However laboratories must be aware of the fact that polymyxins may show some disparity in results with different methods due to its poor diffusion in agar. Reporting of polymyxins by disk diffusion method should be done carefully specially in critically ill patients with no clinical response, even with polymyxins. However in a resource poor country like ours, disk diffusion method can be used as screening method. Further studies on a large scale with polymyxin resistant isolates as well are needed for better understanding of both methods. This enabled us to achieve more precise, correct and reproducible results.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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