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Short Communication

Performance of polymyxin B Etest in a setting of high prevalence of KPC-producing *Klebsiella pneumoniae*

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ABSTRACT

Objectives: Polymyxin resistance has been increasing in many regions, and appropriate determination of polymyxin susceptibility is now a major challenge worldwide. Many clinical laboratories rely on gradient diffusion methods to assess polymyxin susceptibility, although broth microdilution (BMD) is the only method currently recommended by the CLSI and EUCAST. The aim of this study was to assess the performance of the polymyxin B (PMB) Etest in a setting with a high prevalence of KPC-producing *Klebsiella pneumoniae* (KPC-KP).

Methods: A commercial Etest susceptibility testing method was evaluated and compared with the reference BMD method, considering isolates with a minimum inhibitory concentration (MIC) ≤2 mg/L for PMB as susceptible to this drug. A total of 310 clinical KPC-KP isolates were evaluated.

Results: Susceptibility was significantly higher by Etest compared with BMD (82.6% vs. 75.8%). The MIC_{50}, MIC_{90} and modal MICs for PMB were 0.25, 32 and 0.25 mg/L (27.1%) by BMD and 0.5, 16 and 0.5 mg/L (49.7%) by Etest, respectively. Although categorical agreement was 90.0%, there was poor essential agreement (50.6%). A high rate (34.7%) of very major errors (VMEs) and a relatively low rate (2.1%) of major errors were found.

Conclusion: The considerable number of resistant isolates in this study allowed an accurate estimation of VME rates and, consequently, a more comprehensive assessment of susceptibility testing for polymyxins. Etest did not meet fully the acceptance criteria for US FDA requirements. These data do not support the use of this commercial method for determining PMB MICs in carbapenem-resistant Enterobacterales populations.

1. Introduction

Klebsiella pneumoniae is among the most important causes of nosocomial infections, and resistance to carbapenems mediated by carbapenemase production, especially *K. pneumoniae* carbapenemase (KPC), is highly prevalent in several countries [1]. Mainstream therapy for KPC-producing *K. pneumoniae* (KPC-KP) has been the polymyxins, either polymyxin B (PMB) or colistin, in combination with a second antimicrobial agent [2]. Although new antimicrobial drugs showing promising activity against KPC-KP isolates have recently been launched, the polymyxins will remain the cornerstone of therapy where these new drugs are not available as well as for non-KPC-producing carbapenem-resistant Enterobacterales [3].

Resistance to polymyxins has been increasing in many regions and has been associated with higher mortality in patients infected by KP-KPC isolates [4]. For this reason, appropriate determination of susceptibility to this class of drugs is of paramount importance.

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There is no standard for polymyxin disk diffusion testing, which has not shown acceptable performance, possibly due to poor diffusion of polymyxins in agar. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) Polymyxin Breakpoint Working Group recommends that methods other than broth microdilution (BMD) should not be used for susceptibility testing of polymyxins [5]. Although this recommendation as well as other published studies have been mostly based on colistin, it has been assumed that same results would be found for PMB since colistin and PMB are highly similar molecules [6,7].

Use of the reference BMD method for susceptibility testing may not be practical in many diagnostic microbiology laboratories, thus Etest remains an attractive option in these facilities. The aim of this work was to assess the performance of PMB Etest in a setting with a high prevalence of KPC-KP.

2. Materials and methods

2.1. Bacterial isolates

From April 2017 to April 2018, *K. pneumoniae* isolated from patients admitted to Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) that were intermediate or resistant to meropenem or ertapenem by disk diffusion according to the CLSI were evaluated for the presence of carbapenemase-encoding genes. The presence of carbapenemase genes ($bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$, $bla_{\text{VIM-type}}$, $bla_{\text{GES-type}}$, $bla_{\text{OXA-48-like}}$ and $bla_{\text{IMP-type}}$) was evaluated by multiplex high-resolution melting real-time PCR [8]. Only one isolate per patient (the first positive for the presence of $bla_{\text{KPC-2}}$) was included in this analysis. Bacterial identification was performed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (bioMérieux, Marcy-l'Étoile, France). All isolates included in this study were recovered from the same medical centre and were stored in 16% glycerol at −80 °C.

2.2. Minimum inhibitory concentration (MIC) determination

MICs of PMB were determined by Etest and BMD. The PMB Etest was performed on fresh clinical isolates using Mueller–Hinton agar (NewProv, Pinhais, PR, Brazil) according to the manufacturer's protocol. Etest MICs were rounded up to the nearest two-fold dilution as read by BMD (i.e. MIC = 1.5 mg/L was considered 2 mg/L). Isolates were stored at −80 °C and were subsequently tested by BMD.

The BMD method was performed in duplicate using polystyrene plates with cation-adjusted Mueller–Hinton broth (Becton Dickinson, Franklin Lakes, NJ, USA). The PMB solution was obtained from Sigma-Aldrich (St Louis, MO, USA). PMB was tested at two-fold dilutions over the concentration range 64–0.125 mg/L. Supplementation with polysorbate-80 was not performed [5]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Isolates with an MIC of ≤2 mg/L for PMB were considered susceptible [5].

2.3. Definitions and analysis

Rates of essential agreement (EA), categorical agreement (CA), very major error (VME) and major error (ME) were determined. EA was defined as an MIC result within a two-fold dilution of the gold-standard (BMD) result. CA occurred when the interpretation of the MIC results was the same (i.e. susceptible or resistant). VME was defined as false susceptibility and ME as false resistance by Etest.

3. Results

A total of 310 KPC-KP isolates were included in the study, among which 75 (24.2%) and 54 (17.4%) were resistant to PMB by BMD and Etest, respectively. The MICs of PMB ranged from 0.125–64 mg/L by BMD and from 0.125–512 mg/L by Etest (Fig. 1). The MIC_{50} and MIC_{90} values for PMB were 0.25 mg/L and 32 mg/L for BMD and 0.5 mg/L and 16 mg/L for Etest, respectively. The modal MICs were 0.25 mg/L (84 isolates; 27.1%) and 0.5 mg/L (154 isolates; 49.7%). CA was 90.0% (279/310). EA was observed in 157 (50.6%) of tests. Of 75 resistant isolates by BMD, 26 were categorised as susceptible by Etest, resulting in a VME rate of 34.7% Of 235 susceptible isolates by BMD, 5 were categorised as resistant by Etest, resulting in a ME rate of 2.1% (Fig. 1).

4. Discussion

Clinical laboratories often rely on gradient diffusion methods or automated systems for susceptibility testing for polymyxins as they are less laborious [8]. These methods have been shown to be unreliable for colistin (polymyxin E) compared with the gold-standard method of BMD [9]. However, very few studies have assessed gradient tests for PMB [10–13].

In the present study, the antimicrobial activity of PMB was evaluated against selected contemporary bacterial pathogens consisting exclusively of KPC-KP (n = 310). Although CA was 90.0%, the results presented unacceptably high and low rates of VME and EA, respectively. Using the US Food and Drug Administration (FDA) requirements for commercial antimicrobial susceptibility testing systems (EA ≥ 90%, CA ≥ 90%, VME ≤ 1.5%, ME ≤ 3.0%) [13], Etest did not meet the acceptance criteria for EA and VME, presenting EA and VME values well below and above the FDA requirements, respectively.

Noteworthy, eight VMEs occurred in isolates with an MIC of 4 mg/L by BMD (Fig. 1). However, this did not impact the finding of an unacceptably high VME rate since only two of these presented an MIC of 2 mg/L by Etest, which would be in EA with the

MIC - BMD (mg/L) \ MIC - Etest® (mg/L)	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
64	0	0	0	0	0	0	0	2	3	2	3	0	1
32	0	**2**	**2**	**1**	0	0	1	4	9	1	0	1	0
16	0	0	**4**	0	**1**	0	0	12	3	2	0	0	0
8	0	**2**	**3**	**2**	**1**	1	1	1	0	0	0	0	0
4	0	0	**3**	**3**	**2**	0	0	2	0	0	0	0	0
2	0	1	7	2	2	2	0	0	0	0	0	0	0
1	0	1	9	2	1	0	0	0	0	0	0	0	0
0.5	0	2	26	8	3	0	0	2	0	0	0	0	0
0.25	1	6	48	18	11	0	0	0	0	0	0	0	0
0.125	0	8	52	14	8	0	0	0	1	0	0	0	0

Fig. 1. Comparison of minimum inhibitory concentrations (MICs) by the reference standard method broth microdilution (BMD) and Etest for KPC-producing *Klebsiella pneumoniae* clinical isolates (n = 310). Numbers represent the occurrences observed at each point. Grey boxes indicate essential agreement (EA) measurements ± 1 $\log_2$ MIC limits between testing methods. Dashed lines indicate the resistant MIC breakpoints (≥4 mg/L) according to CLSI-EUCAST criteria [5]. Very major errors (VMEs) are indicated in bold.

corresponding BMD result. In contrast, five isolates presenting an MIC of 32 mg/L by BMD demonstrated MIC $\leq$ 1.0 mg/L by Etest.

Four other studies have compared Etest with BMD for PMB in carbapenem-resistant Enterobacterales [10–13]. Only Lat et al. found that the PMB Etest overestimates resistance in comparison with BMD; indeed, their study evaluating 48 KPC-KP also reported a low EA (19%) but only 2% VME and, in contrast to the current study, the ME rate was 23% [10]. On the other hand, the other three studies are consistent with the findings of the current study [11–13]. Chew et al. tested 76 carbapenem-resistant Enterobacterales, including only 2 *K. pneumoniae* isolates, and reported an EA of 48.7%, a VME rate of 26.1% and ME rate of 1.9% [11], which were all similar to those observed in the current study (50.6%, 34.7% and 2.1%, respectively). Another study tested 39 Enterobacterales isolates, including 15 *K. pneumoniae*, and reported an EA of 61%, a VME rate of 8% and a CA of only 88%; no ME was reported [12]. Finally, Kulengowski et al. assessed 70 isolates of carbapenem-resistant Enterobacterales, including 34 *K. pneumoniae*, and reported overall very poor results, with only 80% and 10% of CA and EA, respectively, and an astonishing 88% of VME; no ME was reported [13]. Interesting, the vast majority of PMB MICs by Etest in the sample of that study were 0.5 mg/L [13], which is similar with the modal MIC of 0.5 mg/L by Etest in the current study. It is important to state that these previous reports had a limited sample size with a relatively small number of resistant isolates and this might have affected a more accurate estimation of VME rates. Moreover, only the report of Chew et al. [11] mentioned that polysorbate-80 (P-80) was not used as supplementation to the BMD methodology, but the other studies did not mention it. BMD without P-80 supplementation is currently recommended as the reference method [5] as P-80 in itself has some antibacterial activity and may act synergistically with polymyxins to spuriously lower MICs, especially for organisms for which MICs are near the breakpoints and/or epidemiologic cut-off values of 1–2 mg/L [14,15].

In the present study, a high prevalence of PMB resistance with a wide range of polymyxins MICs was found. The considerable number of resistant isolates allows an accurate estimation of VME rates and, consequently, a more comprehensive assessment of susceptibility testing for polymyxins.

Some limitations of this study must be acknowledged. All isolates were recovered from the same medical centre, thus clonal relatedness cannot be ruled out. In addition, it should be mentioned that Etest and BMD were performed at two different times: Etest was tested initially and BMD was subsequently performed on isolates that had been stored in 16% glycerol at −80 °C. Therefore, occasionally thaw–frozen cycles and passage bias might have affected some BMD MIC results. Finally, as in previous studies, only one brand of PMB was evaluated. Although we would not expect a distinct result, it may be worthwhile to compare different brands in additional studies.

In conclusion, in a high number of KPC-KP isolates with a broad MIC distribution, this study found an unacceptably high rate of VME of Etest for PMB. The high level of false susceptibility presented by Etest for PMB has great clinical impact since it may lead to inadequate treatment and clinical failure. The current data do not support the continued use of Etest strips by clinical laboratories for determining PMB MICs in carbapenem-resistant Enterobacterales.

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Competing interests

APZ has received honoraria for speaking engagements and consultancy from Merck, Cipla and Pfizer and a research grant from Pfizer. All other authors declare no competing interests.

Ethical approval

This study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) [16-0444].

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