UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Trabalho de Conclusão de Curso de Farmácia

Polymyxin B Broth Disk Elution as a screening test to determine polymyxin B susceptibility in *Enterobacterales*

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Porto Alegre, Dezembro de 2019

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Trabalho de Conclusão de Curso apresentado como requisito parcial para obtenção do grau de farmacêutica pelo curso de Farmácia da Universidade Federal do Rio Grande do Sul.

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Orientadora

Porto Alegre, Dezembro de 2019

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo.”

(José de Alencar)

AGRADECIMENTOS

À Deus pela proteção;

À minha mãe, Nivia Canabarro da Silva, por ser meu porto seguro, meu exemplo de força e por ter vivido esse sonho comigo. Essa conquista é nossa;

Ao meu namorado, Jonatan Cezimbra Freitas, por todo amor, apoio e compreensão;

À minha orientadora, professora Juliana Caierão, pela disponibilidade, dedicação e por ter possibilitado a realização deste trabalho;

À todas do laboratório de bacteriologia pelo auxílio e companhia durante os experimentos;

Às minhas amigas e colegas de curso por terem tornado essa jornada mais leve.

APRESENTAÇÃO DE ARTIGO

Este artigo foi elaborado segundo as normas da *Diagnostic Microbiology and Infectious Disease* (DMID), apresentadas no apêndice.

STATEMENT OF DECLARATION OF INTEREST

The authors declare no conflicts of interest. There has been no financial support for this work that could have influenced its outcome. Any costs were covered with own funds.

Polymyxin B Broth Disk Elution as a screening test to determine polymyxin B susceptibility in *Enterobacterales*

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Abstract Word Count: 150

Body of Text Word Count: 2,298

Abbreviations:

BMD = Broth Microdilution, CBDE = Colistin Broth Disk Elution, CLSI = Clinical and Laboratory Standards Institute, EUCAST = European Committee on Antimicrobial Susceptibility Testing, MIC = Minimum Inhibitory Concentration, PBDE = Polymyxin Broth Disk Elution.

Abstract: Detection of polymyxins susceptibility by clinical laboratories is a nightmare mainly because of physicochemical properties of the drug. The aim of this study was to evaluate accuracy of Polymyxin B Broth Disk Elution (PBDE), as screening test, compared to Broth Microdilution (BMD). We evaluated 196 *Enterobacterales* resistant to carbapenems. BMD was performed as standardized by EUCAST and CLSI. PBDE was performed in 15-mL cation-adjusted Mueller-Hinton broth where one polymyxin B disk (300 IU) was eluted (2 µg/mL). Results were interpreted based on visual turbidity. Categorical Agreement (CA) was 99.5%, sensitivity and specificity were 98.9% and100%, respectively, after 16-20 hours of incubation. Sensitivity of PBDE with shorter incubation time were 63.6%, 69.7% and 78.8% after 6h, 7h and 8h, respectively. Those sensitivities were mainly compromised by isolates presenting borderline MICs. PBDE is a cheap and easy to perform methodology as reliable as BMD to evaluate the susceptibility to polymyxin B among *Enterobacterales*.

Keywords: Polymyxin B**,** Polymyxin Broth Disk Elution**,** antimicrobial susceptibility testing.

1. Introduction

Enterobacterales are the biggest and most heterogeneous group of clinically relevant gram-negative bacilli. They are responsible for 30 to 35% of sepsis cases and more than 70% of urinary tract infections, among other infections (Wilson *et al.,* 2004).

In the last few years, it has been observed the emergence of carbapenem-resistant gram-negative bacteria, which may be quite difficult to treat (Satlin, 2010) and search for new therapeutical options has been challenging (WHO, 2014). In this context, there has been a renaissance in the use of both polymyxin B and colistin (polymyxin E) over the last decade (Vasoo, 2017), years after they were abandoned as systemic antimicrobial agents because of their high nephrotoxicity and neurotoxicity (Falagas and Kasiakou, 2005; Koch-Weser *et al.*, 1970).

Polymyxins were first recognized to have broad-spectrum activity against gram-negative bacteria in the 1940s (Falagas and Kasiakou, 2005; Koch-Weser *et al.*, 1970). These molecules, which are large polycationic peptide, bind to the negatively charged lipopolysaccharide (LPS) of gram-negative bacteria, causing disruption of the outer membrane, eventually leading to cell death (Olaitan *et al.,* 2014).

However, the widespread use of polymyxins, which are considered the last line drugs for treatment of infections caused by carbapenem-resistant gram-negative bacteria, lead to increasing prevalence of resistance to this antimicrobial. This resistance may be intrinsic or acquired, with the most common mechanism resulting from alterations in genes that lead to limited polymyxins binding to the outer membrane due to reduction of overall negative charge of LPS. Furthermore, after the first plasmid-mediated colistin resistance gene (*mcr-1*) was described in 2015, resistance to polymyxins became an even more worrying public health problem, given their potential to readily disseminate among clinical pathogens (AbuOun *et al.,* 2017; Borowiak *et al.,* 2017; Carattoli *et al.,* 2017; Pogue *et al.,* 2017; van Dorp *et al.,* 2018; Xavier *et al.,* 2016; Yang *et al.,* 2018; Yin *et al.,* 2017; Zhou *et al.,* 2018).

The physicochemical properties of the drug turn the detection of susceptibility to polymyxins a challenging task for laboratories**.** Techniques based on disc-diffusion are

not recommended by either Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) and gradient-based methods are not sufficiently accurate to evaluate susceptibility to polymyxins (Poirel *et al.,* 2017). Instead, both CLSI and EUCAST recommend Broth Microdilution (BMD) as reference method to determine the Minimal Inhibitory Concentration (MIC) and evaluate susceptibility to polymyxins.

BMD, however, is laborious and not performed in many clinical microbiology laboratories, which often rely on diffusion or automated systems for susceptibility testing (Vasoo, 2017)**.** Indeed, those laboratories probably miss a practical and reliable method to identify polymyxins susceptibility (Simner *et al.,* 2019).

In this scenario, Polymyxin B Broth Disk Elution (PBDE) has been recently proposed as an easier methodology to be performed in routine of microbiology laboratories. The principle of the test is the elution of antibiotic, at room temperature, from a commercial disc to Mueller-Hinton broth, where bacteria will be suspended, then. Polymyxin resistant isolates will be able to grow in this solution after 16 to 20 hours of incubation at 35°C (Simner *et al.,* 2019).

In this context, the aim of this study was to evaluate PBDE in a single antibiotic concentration, as a screening test to identify polymyxin B resistant *Enterobacterales* searching for a more practical method with results as reliable as BMD.

2. Material and Methods

2.1. Bacterial Isolates and susceptibility profile

We evaluated 196 non-duplicated isolates of *Enterobacterales* resistant to carbapenems recovered from inpatients of three hospitals in Porto Alegre city, South Brazil. One hundred and nine were obtained from Hospital 1, thirteen from Hospital 2 and the other seventy-four isolates were recovered from inpatients of Hospital 3.

Polymyxin B BMD was performed for all isolates as reference method and results obtained were interpreted based on CLSI and EUCAST guidelines, i.e., bacteria presenting MIC > 2 μg/mL were considered resistant.

Isolates included mainly *Klebsiella pneumoniae* (n= 173), but also *Klebsiella oxytoca* (n=6), *Escherichia coli* (n=5), *Enterobacter cloacae* complex (n=5), *Serratia marcescens* (n=2), *Providencia rettgeri* (n=2), *Citrobacter freundii* (n=2) and *Klebsiella ozanea* (n=1*). E. coli* ATCC 25922 and a *Morganella morganii* (identified by MALDI-TOF) from our personal collection, intrinsically resistant to polymyxins (polymyxin MIC > 64 μg/mL), were used as negative and positive controls, respectively.

2.2. Polymyxin B Broth Disk Elution (PBDE)

PBDE was performed according to Simner *et al*. (2019), with modifications. Briefly, it was used two 15-mL cation-adjusted Mueller-Hinton broth (Sigma-Aldrich) tubes per isolate. One tube was used as growth control and in the other it was added one 300 IU-polymyxin B disk (Oxoid), generating a final concentration of 2 μg/mL of polymyxin B in the 15 mL solution. This tube was maintained at room temperature for 30 minutes to allow polymyxin B to elute from the disk to the broth.

Bacterial suspensions were prepared by adding colonies from an overnight Tryptone Soy Agar (Oxoid) plate in sterile saline and adjusting the turbidity to match the 0.5 McFarland standard tube. A 75 μL aliquot of the suspension was added to each tube, reaching a final bacterial concentration of approximately 7.5 x 10^5 UFC/mL.

Results were read visually after 16 to 20 hours of incubation at 35°C and isolates were considered resistant when showing growth (visual turbidity) at the tube with 2 μg/mL of polymyxin B. Readers were blinded to MIC results.

A subset of 33 resistant bacteria were selected to perform readings in 6, 7 and 8 hours trying to reduce incubation time. Isolates included *Klebsiella pneumoniae* (n=32) *and Serratia marcescens* (n=1).

3. Results

We evaluated 196 isolates carbapenem-resistant *Enterobacterales*. Among them, ninety (45.9%) were resistant to polymyxin B (including 4 intrinsically resistant bacteria – 2 *S. marcescens* and 2 *P. rettgeri*), while the remaining 106 (54.1%) were susceptible to this drug when evaluated by BMD. Polymyxin B MICs varied from ≤ 0,125

to >64 μg/mL, with 34 isolates (17.3%) presenting borderline MICs (2 or 4 μg/mL), according to EUCAST breakpoints (Table 1).

PBDE demonstrated an excellent performance, as Categorical Agreement (CA) with BMD was 99.5%. Indeed, 89 out of 90 isolates resistant to polymyxin B (MIC > 2 μg/mL) did grow in tubes containing eluted antibiotic (sensitivity 98.9%). Only one *K. pneumoniae* with polymyxin B MIC of 4 μg/mL was negative by PBDE.

Among 106 susceptible *Enterobacterales* evaluated, all of them presented negative results by PBDE (specificity 100%), including those presenting MIC of 2 μg/mL. Polymyxin B susceptibility profile of isolates included in this study, defined by both the methods (considering ordinary incubation time for PBDE), is demonstrated in Figure 1.

Table 2 presents MICs and results of the subset of isolates evaluated after shorter incubation. We observed that 21 isolates (63.6%) had positive results after 6 hours of incubation, another 2 isolates after 7 hours, 3 other isolates after 8 hours. Seven isolates only had positive results after the usual 16 to 20 hours of incubation; six out of those 7 had borderline MICs (4 μg/mL). Therefore, sensitivity of PBDE with shorter incubation were 63.6%, 69.7% and 78.8% after 6h, 7h and 8h, respectively.

TABLE 1: Minimal Inhibitory Concentration distribution among *Enterobacterales*

Isolates	Total	MIC (μg/mL)											
		≤ 0,125	0,125	0,25	0,5	1	2	4	8	16	32	64	>64
K. pneumoniae	173	9	1	23	23	16	15	18*	10	18	27	9	4
K. oxytoca	6	3		1		1	1						
E. coli	5	1		2	2								
E. cloacae complex	5	1		2	1	1							
S. marcescens	2												2
P. rettgeri	2												2
C. freundii	2			1		1							
K. ozanea	1	1											

Columns highlighted in blue represent borderline MICs, according to EUCAST.
*Includes one *K. pneumoniae* presenting false negative result

Figure 1: Susceptibility profile to polymyxin B of the isolates according to BMD and PBDE methods.

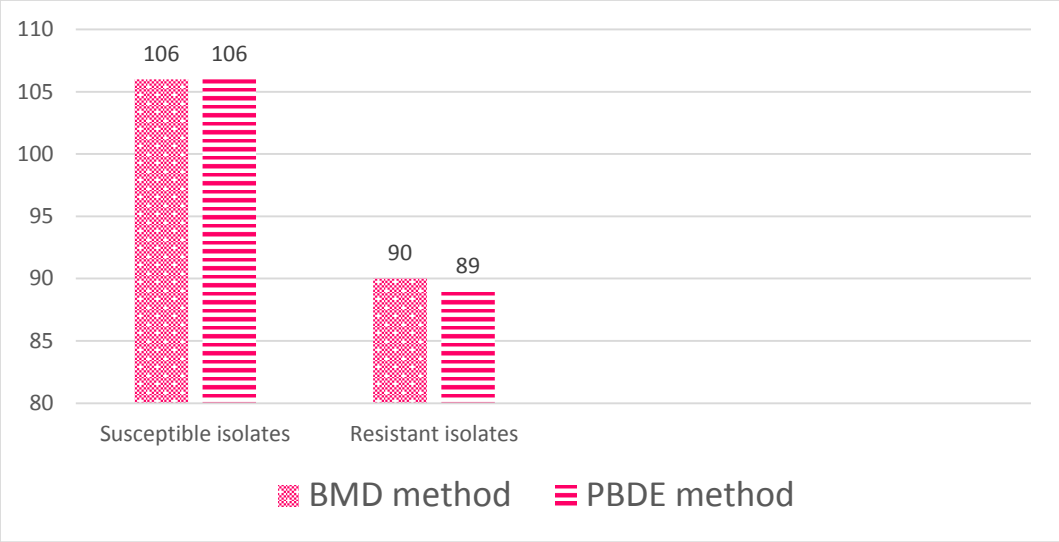

TABLE 2: Results of PBDE applying reduced incubation time

Isolate	MIC	Incubation time			
		6h	7h	8h	Standard*
S. marcescens	>64	POS	POS	POS	POS
K.pneumoniae	>64	POS	POS	POS	POS
K.pneumoniae	64	POS	POS	POS	POS
K.pneumoniae	64	POS	POS	POS	POS
K.pneumoniae	64	POS	POS	POS	POS
K.pneumoniae	64	**NEG**	POS	POS	POS
K.pneumoniae	32	POS	POS	POS	POS
K.pneumoniae	32	POS	POS	POS	POS
K.pneumoniae	32	POS	POS	POS	POS
K.pneumoniae	32	POS	POS	POS	POS
K.pneumoniae	32	POS	POS	POS	POS
K.pneumoniae	32	POS	POS	POS	POS
K.pneumoniae	32	**NEG**	**NEG**	POS	POS
K.pneumoniae	32	**NEG**	**NEG**	POS	POS
K.pneumoniae	32	**NEG**	**NEG**	**NEG**	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	POS	POS	POS	POS
K.pneumoniae	16	**NEG**	POS	POS	POS
K.pneumoniae	8	POS	POS	POS	POS
K.pneumoniae	8	**NEG**	**NEG**	POS	POS
K.pneumoniae	4	POS	POS	POS	POS
K.pneumoniae	4	POS	POS	POS	POS
K.pneumoniae	4	**NEG**	**NEG**	**NEG**	POS
K.pneumoniae	4	**NEG**	**NEG**	**NEG**	POS
K.pneumoniae	4	**NEG**	**NEG**	**NEG**	POS
K.pneumoniae	4	**NEG**	**NEG**	**NEG**	POS
K.pneumoniae	4	**NEG**	**NEG**	**NEG**	POS
K.pneumoniae	4	**NEG**	**NEG**	**NEG**	POS

* 16 to 20 hours of incubation

4. Discussion

Dissemination of carbapenem-resistant gram-negative bacteria may compromise treatment, raising mortality rates, hospital stay duration and hospital costs (Federico and Furtado, 2018). In such situation, the use of polymyxins has spread dramatically over the last years, followed by increasing resistance to this antibiotic (ECDC, 2017; ESPAUR, 2017; Giamarellou, 2016; Monaco *et al*., 2014; Otter *et al.,* 2017).

The methodology recommended by CLSI and EUCAST to detect resistance to polymyxins (BMD) is laborious and may be difficult to insert on routine of microbiology laboratories, which sometimes end up performing easier methodologies demanding ordinary materials, such as antibiotic disks or antibiotic gradient strips, despite the recognized inaccuracy (Dafopoulou *et al.,* 2015; Hindler and Humphries, 2013).

Broth Disk Elution methods have been described for antibiotics such as carbenicillin, chloramphenicol, clindamycin, penicillin, tetracycline (Jorgensen *et al*., 1980) and beta-lactams (Redding *et al.,* 1986; Shungu *et al.,* 1985) since the 1980s, and now emerges as a practical and cheap methodology for polymyxins, costing around US$ 0.42 per isolate.

To the best of our knowledge this is the fourth study performing elution to determine susceptibility to polymyxins. Simner *et al*. (2019) used Colistin Broth Disk Elution (CBDE) to evaluate 172 isolates (77.9% susceptible and 22.1% resistant), which is quite similar to the number we tested (196), although our population presented a higher proportion of resistant isolates (54.1% susceptible and 45.9% resistant). Furthermore, Simner *et al*. (2019) performed the method in concentrations of 1, 2 and 4 µg/mL of colistin. On the other hand, we used elution as a screening methodology with a unique concentration of polymyxin B, i.e. 2 µg/mL.

Authors mentioned above found a categorical agreement (CA) between the elution method and BMD of 98%, very similar to ours (99.5%). According to their results, three *mcr-1*-producing *Escherichia coli* had MICs of 4 µg/mL by BMD and 2 µg/mL by CBDE. These false-negative results in isolates with borderline MICs were the reason why the sensitivity of CBDE in this study was not higher than 98.3%.

Dalmolin *et al.* (2019b) also performed elution test to evaluate colistin susceptibility for 85 isolates (40% susceptible and 60% resistant) with a unique concentration of colistin, i.e. 2 μg/mL. They observed sensitivity of 88.2% (6 false-negative results for isolates with MIC of 4 μg/mL) and specificity of 91.2% (3 false-positive results: two isolates with MIC of 2 μg/mL and one isolate with MIC ≤ 0.5 μg/mL), with a general CA of 91.2% for *Enterobacterales.*

Overall, isolates presenting borderline MICs are the ones that compromise sensitivity and specificity of laboratorial techniques, once ± 1 dilution are universally accepted when MIC values are considered and this variations for borderline isolates implies in categorical changes (from susceptible to resistant or vice-versa). Indeed, the only isolate with false-negative result by PBDE in our study was a borderline *K. pneumoniae* with polymyxin B MIC of 4 μg/mL, giving a sensitivity of 98.9%, which is similar to CBDE performed by Simner *et al.* (2019) (98.3%).

Moreover, recently, Humphries *et al.* (2019) described the evaluation of CBDE performed by the CLSI Antimicrobial Susceptibility Testing Subcommittee, in order to validate it as an alternative reference susceptibility test for colistin. The method was performed as described by Simner *et al.* (2019) with minor modifications and found a CA of 98.6% for *Enterobacterales,* an overall sensitivity of 96.8% and specificity of 99.1%. Results obtained in this study led to the endorsement of the CBDE as colistin susceptibility testing method by CLSI and will be published in the 30th edition of CLSI document M100 in January 2020. Interestingly, the authors reinforced that their study did not evaluate elution with polymyxin B, which was exactly what was performed in our study.

We mainly analyzed *K.pneumoniae* (88.3%), decreasing representativeness of *Enterobacterales* in this study. However, among this species we have a varied population considering MIC values, including 17.3% (n = 34) of isolates presenting borderline MICs. Besides, one may consider that use of PBDE, as a screening test proposed by us, when compared to BMD is impaired by the qualitative characteristic of its results, i.e., "susceptible" or "resistant", without MIC values. Meantime, due to polymyxin-induced nephrotoxicity, the therapeutic window of polymyxins is very narrow (Nation *et al.,*

2019), making it difficult to perform significant adjustments on antibiotic dosage according to MIC values. In this case, identification of resistant isolates, even without MIC values, might not be harmful to patient's treatment. Nevertheless, PBDE can be described as a simple and accurate screening method to determinate susceptibility to polymyxin B. Moreover, this methodology may be easily adapted and, as performed elsewhere (Simner *et al.,* 2019; Humphries *et al.,* 2019; Dalmolin *et al.,* 2019b), more than one concentration may be used in order to determine MIC values.

Because BMD is time-consuming, other simplest methodologies such as Rapid Polymyxin NP test have been tested in multiple studies for determining susceptibility to polymyxins and usually presents very good sensitivity (98 to 100%) and a little lower specificity (82-100%)(Dalmolin *et al.,* 2019a; Jayol *et al.,* 2018; Malli *et al.,* 2019; Poirel *et al.,* 2018; Yainoy *et al.,* 2018). The major advantage is the reduced incubation time (up to 4h). However, this is a method that can only be performed for *Enterobacterales* because it detects glucose metabolism related to bacterial growth. Although our population included only *Enterobacterales,* Simner *et al*. (2019), Humphries *et al.* (2019) and Dalmolin *et al.* (2019b) performed Broth Disk Elution for *Acinetobacter* spp. and *Pseudomonas aeruginosa.* Furthermore, Broth Disk Elution tests for polymyxins showed specificity between 91.2% and 100%, while Rapid Polymyxin NP test can show a worrying specificity as low as 82%, depending of the bacterial population evaluated.

Based on our results, PBDE seems to be a very accurate method. However, prolonged incubation time may be a disadvantage. Therefore, we evaluated shorter incubation for a subgroup of 33 resistant isolates and, as expected, isolates presenting borderline MICs were the main responsible for the false-negative results, representing almost all (n=6; 85.7%) isolates with negative results when reduced reading time was taken into consideration. Therefore, MIC values seems to interfere directly in sensitivity of test in this case. Indeed, taking into account exclusively isolates with MICs of 8 μg/mL or more, sensitivity of PBDE with shorter incubation would have a significant increase to 96%: only one of those isolates was not positive in incubations up to 8h. The lower number of isolates evaluated (n=33) may also have compromised sensitivity of the method with shorter incubation time.

5. Conclusion

We concluded that Polymyxin B Broth Disk Elution is a cheap and easy to perform methodology. It proved to be an accurate method as reliable as BMD to evaluate the susceptibility to polymyxin B among *Enterobacterales*. Besides, our results demonstrate that shorter incubation compromises sensitivity, although it seems to be in a MIC-dependent way. Moreover, the reduced number of isolates evaluated after shorter incubation may also have compromised sensitivity.

Acknowledgements

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

Declarations of interest: none

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APPENDIX - DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE STANDARDS FOR SUBMISSION OF ARTICLES

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