Detection of *Enterobacterales* resistant to polymyxins using Rapid Polymyxins NP test

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Abstract

Two hundred isolates of *Enterobacterales* were tested by Rapid Polymyxins NP for the detection of polymyxin resistance and compared to the reference test broth microdilution (BMD). The sensitivity and specificity of the NP test were 98% and the results are faster than the BMD, decreasing from approximately 24 to 2 h.

Keywords Polymyxins · Susceptibility · *Enterobacterales* · Rapid Polymyxins NP test

Polymyxins (polymyxin B and colistin) are last resort antimicrobial agents for the treatment of serious infections caused by multidrug-resistant *Enterobacterales*, in particular carbapenem-resistant *Enterobacterales* (CRE) [1].

The main mechanisms related to acquired polymyxin resistance are associated to mutations in the regulatory systems that modify the lipopolysaccharide (LPS) of the bacterial outer membrane, resulting in a reduction in electrostatic interactions between the polymyxins and the bacterial membrane, triggering the resistance [2, 3]. Recently, Liu et al. (2016) described the first mobile polymyxin resistance mechanism, mediated by the gene *mcr-1* (mobile colistin resistance) located in a plasmid. The MCR-1 protein is a member of the phosphoethanolamine transferase family which promotes the addition of phosphoethanolamine to lipid A (lipopolysaccha-

ride component) leading to a reduction of the LPS affinity for polymyxins [4].

The description of the *mcr-1* gene caused a great concern regarding its dissemination, among polymyxin-susceptible *Enterobacterales*. Regardless of the resistance mechanism, the dissemination of polymyxin-resistant strains restricts the therapeutic options, leading to high mortality rates; therefore, the development of fast and reliable methods that allow the detection of polymyxin resistance is needed. The usual routine microbiology susceptibility tests (disk diffusion and gradient diffusion test) are not reliable to evaluate the susceptibility to polymyxins. The fact that the only method considered accurate for the detection of resistance to polymyxins (the broth microdilution—BMD) is laborious and expensive, is another important reason for the development of fast and simple methods to detect polymyxin resistance [5–7].

Recently, Nordmann et al. (2016) developed the Rapid Polymyxins NP test. This test consists of detecting bacterial growth on glucose, in the presence of a defined concentration of polymyxin (polymyxin B or colistin). Growth of the bacteria in the presence of polymyxins will lead to glucose metabolism which will acidify the medium altering the pH; hence, this change can be detected visually by a pH indicator color change. The final result of the Rapid Polymyxins NP test is obtained in up to 4 h, although the majority of the resistant isolates present positive results in around 2 h [7].

The aim of this study was to assess the performance of the Rapid Polymyxins NP test in the detection of polymyxin resistance among isolates of *Enterobacterales* from human and

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animal source when compared to the reference test, the broth microdilution.

The Rapid Polymyxins NP test was evaluated with 200 isolates of *Enterobacteriales* (100 resistant and 100 susceptible to the polymyxins by BMD) obtained from other surveillance studies between 2011 and 2017 in Southern Brazil. One hundred and eighty-three isolates were from human origin [8], 10 were from poultry slaughterhouse [9], 4 from food-producing animals [10], and 3 were transconjugants with the *mcr-1* gene [11, 12].

The resistant species to polymyxins (MICs 4 to > 64 µg/mL) included 74 *Klebsiella pneumoniae*, 4 *Enterobacter* spp., 4 *E. coli*, and 4 *Salmonella* spp. Additionally, isolates with intrinsic resistance to polymyxins (3 *Morganella morganii*, 1 *Proteus mirabilis*, 2 *Providencia rettgeri*, and 8 *Serratia marcescens*) were also subjected to the test. The polymyxin-susceptible isolates (MICs ≤ 0.125 to 2 µg/mL) included 49 *Klebsiella* spp., 28 *E. coli*, 20 *Enterobacter* spp., and 3 *Citrobacter freundii*.

The bacterial DNA was extracted by thermal lysis and detection of the carbapenemase genes (*bla_{IMP}*, *bla_{KPC}*, *bla_{GES}*, *bla_{NDM}*, *bla_{OXA-48-like}*, and *bla_{VIM}*) [13] and the *mcr-1* gene [4] was performed by a multiplex real-time PCR, analyzed by HRM software.

The Rapid Polymyxins NP test was performed according to Nordmann et al. [7]. The antibiotic solutions (polymyxin B and colistin at 0.2 mg/mL) were mixed with the Rapid Polymyxin NP solution (2.5% of MHB-CA powder, 0.005% of phenol red indicator and 1% of D (+)-glucose) into sterile glass tubes in a proportion of 1:40. Given that, the final

concentration containing colistin or polymyxin into tubes was 5 µg/mL [7].

The bacterial inoculum was standardized to obtain a 3.0–3.5 McFarland optical density ($\approx 10^9$ CFU/mL) in sterile solution of sodium chloride (NaCl) 0.85%. The test was performed in a 96-well polystyrene plate and for each isolate, 50 µL of the bacterial suspension was inoculated in 3 wells: first well only 150 µL of the Rapid Polymyxin NP solution (free of antibiotics), second well 150 µL of the Rapid Polymyxin NP solution with colistin, and third well 150 µL of the Rapid Polymyxin NP solution with polymyxin B. The final concentration of bacteria was $\approx 10^8$ CFU/mL in each well, and the final concentrations of colistin sulfate and polymyxin B were 3.75 µg/mL, respectively [7].

The plate was incubated for up to 4 h at 35 °C ± 2 °C and the first reading was made at 15 min and then every hour. The test was considered positive (polymyxin resistance) when the isolate grew in presence of colistin or polymyxin B, changing the color of the Rapid Polymyxin NP solution containing antibiotic from orange to yellow, i.e., the same color of the well containing only NP solution. However, the test was considered negative (polymyxin susceptible) when the isolate did not grow in the presence of antibiotics and the color of the solution remained orange [7].

Enterobacterial isolates with minimal inhibitory concentration (MIC) for colistin and/or polymyxin B ≤ 2 µg/mL were considered susceptible and those with MIC > 2 µg/mL were considered resistant according to the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST) guidelines [14].

Table 1 Rapid Polymyxins NP test results among *Enterobacteriales* isolates

	Susceptibility to polymyxins	Isolates	NP Polymyxin test	Sensitivity and specificity of NP Polymyxin test
<i>K. pneumoniae</i> (121 isolates—60.5%)	Resistant ^{a,b}	74 (37%)	72 positive result and 2 negative results	97.3% and 100%
	Susceptible	47 (23.5%)	47 negative results	
<i>E. coli</i> (32 isolates—16%)	Resistant ^b	4 (2%)	4 positive results	100% and 92.86%
	Susceptible	28 (14%)	26 negative results and 2 positive results	
<i>Enterobacter</i> spp. (24 isolates—12%)	Resistant ^a	4 (2%)	4 positive results	100%
	Susceptible	20 (10%)	20 negative results	
<i>S. marcescens</i> (8 isolates—4%)	Resistant ^c	8 (4%)	8 positive results	Sensitivity 100%
<i>Salmonella</i> spp. (4 isolates—2%)	Resistant ^b	4 (2%)	4 positive results	Sensitivity 100%
<i>C. freundii</i> (3 isolates—1.5%)	Susceptible	3 (1.5%)	3 negative results (100%)	Specificity 100%
<i>M. morganii</i> (3 isolates—1.5%)	Resistant ^c	3 (1.5%)	3 positive results (100%)	Sensitivity 100%
<i>K. oxytoca</i> (2 isolates—1%)	Susceptible	2 (1%)	2 negative results (100%)	Specificity 100%
<i>P. rettgeri</i> (2 isolates—1%)	Resistant ^c	2 (1%)	2 positive results (100%)	Sensitivity 100%
<i>P. mirabilis</i> (1 isolates—0.5%)	Resistant ^c	1 (0.5%)	1 positive results (100%)	Sensitivity 100%

^a Chromosomal resistance mechanism not evaluated; ^b Plasmidial resistance; ^c Intrinsic resistance

From 100 resistant isolates by BMD, a total of 98 presented positive result in the Rapid Polymyxins NP test, and only two isolates, both *K. pneumoniae* of human origin, KPC producers, with MIC of 4 µg/mL and 32 µg/mL by BMD, presented negative results in the Rapid Polymyxins NP test. Among the 100 susceptible polymyxin isolates by BMD, two *E. coli* isolates from poultry slaughterhouse presented positive results in the Rapid Polymyxins NP test; these isolates harbored the *mcr-1* gene, and presented MIC of 1 µg/mL and 2 µg/mL by BMD (Table 1).

In general, the time needed to the test to become positive was 2 h ($n = 87$), although 10 isolates turned positive in 1 h and only 1 isolate needed 3 h to become positive. The sensitivity and specificity of the Rapid Polymyxins NP test were 98%.

The Rapid Polymyxins NP test was easy to perform and considerably fast like other studies [5, 7]. Our findings confirm that the test produces results much faster than the BMD, which can contribute to the rapid identification of polymyxin-resistant isolates. In addition, the test showed excellent sensitivity and specificity, similarly to the results found by Nordmann et al. [7], which reported 99.3% and 95.4% of sensitivity and specificity, respectively. Bakthavatchalam et al. [15] also evaluated the Rapid Polymyxins NP test and found even higher levels (100%) of sensitivity and specificity.

Poirel [16] found 100% sensitivity for the Rapid Polymyxins NP when it was performed with polymyxin resistance isolates mediated by plasmid carrying the *mcr-1* or *mcr-2* genes recovered from the environment, animals, and human origin. On the other hand, we evaluated 19 isolates carrying *mcr-1* gene from animal and human origin (11 resistant to polymyxins and 8 susceptible) and the results were in agreement with the standard method (BMD); however, 2 isolates harboring *mcr-1* gene, but susceptible to polymyxins, presented false-positive results.

The Rapid Polymyxins NP reported low sensitivity (25%) for polymyxin resistance detection in *Enterobacter* spp., probably due to heteroresistant phenomenon [17]. Unlikely, we did not have problems in detecting the susceptibility of *Enterobacter* spp. in our study.

The Rapid Polymyxins NP test showed excellent performance for the detection of resistant isolates and susceptible enterobacteria for either polymyxin B or colistin, when compared to the standard method. In fact, the Rapid Polymyxins NP test had no difference between the two antimicrobials tested. Additionally, the time needed to obtain the result reduce from approximately 24 h (BMD test) to around 2 h (Rapid Polymyxins NP test), besides, being less laborious. The rapid identification of polymyxin-resistant isolates may contribute for

more precise therapy choices, as well as the rapid implementation of contact precaution measures, preventing the development of outbreaks with multidrug-resistant isolates.

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