ORIGINAL ARTICLE

Difficulty in detecting low levels of polymyxin resistance in clinical *Klebsiella pneumoniae* isolates: evaluation of Rapid Polymyxin NP test, Colispot Test and SuperPolymyxin medium

O. C. Conceição-Neto[1], B. S. da Costa[1], L. S. Pontes[1], I. C. O. Santos[1], M. C. Silveira[1], J. R. Cordeiro-Moura[2], N. F. Pereira[1], C. B. Tavares-Teixeira[1], R. C. Picão[2], C. M. Rocha-de-Souza[1] and A. P. D. Carvalho-Assef[1]

1) *Laboratório de Pesquisa em Infecção Hospitalar, Instituto Oswaldo Cruz-FIOCRUZ and* 2) *Laboratório de Investigação em Microbiologia Médica, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Abstract

Polymyxins are important therapeutic options for treating infections, mainly those caused by carbapenem-resistant *Klebsiella pneumoniae*. Specific chemical characteristics of polymyxins make it difficult to perform antimicrobial susceptibility testing, especially within the clinical laboratory. Here we aimed to evaluate the performance of three phenotypic methods: Rapid NP Polymyxin Test, ColiSpot test and the SuperPolymyxin medium. To accomplish this, 170 non-duplicate clinical *K. pneumoniae* isolates were analysed (123 colistin-resistant and 47 susceptible). The sensitivity and specificity obtained for Rapid Polymyxin NP Test, Colispot and SuperPolymyxin medium were, respectively, 90% and 94%, 74% and 100%, and 82% and 85%. Very major errors occurred more frequently in low-level colistin-resistant isolates (MICs 4 and 8 μg/mL). Rapid Polymyxin NP proved to be a method capable of identifying colistin-resistant strains in acceptable categorical agreement. However, major errors and very major errors of this method were considered unacceptable for colistin-resistance screening. Although the Colispot test is promising and easy to perform and interpret, the results did not reproduce well in the isolates tested. The colistin-containing selective medium (SuperPolymyxin) showed limitations, including quantification of mucoid colonies and poor stability. Nevertheless, Colispot and SuperPolymyxin medium methods did not present acceptable sensitivity, specificity and categorical agreement. It is essential to use analytical tools that faithfully reproduce bacterial resistance *in vitro*, especially in last-line drugs, such as polymyxins, when misinterpretation of a test can result in therapeutic ineffectiveness.

Keywords: Antibiotic resistance, Clinical isolates, *Klebsiella pneumoniae*, Phenotypic detection, Polymyxin resistance
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Corresponding author: A.P. D'Alincourt Carvalho-Assef, Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Avenida Brasil, 4365—Departamento de Bacteriologia, Rio de Janeiro, 21045900, Brazil.
E-mail: anapdca@ioc.fiocruz.br

Introduction

Polymyxins (colistin and polymyxin B) are important therapeutic options for treating infections, mainly those caused by carbapenem-resistant *Klebsiella pneumoniae* [1]. Resistance has been reported frequently, mainly mediated by chromosomal gene mutations [2–4]. In this context, the two-component systems PmrA/PmrB and PhoP/PhoQ and their negative regulatory gene *mgrB* are related to lipopolysaccharide modifications that decrease affinity for polymyxins, thereby reducing susceptibility [5]. More recently, mutations in the *crrB* gene, derived from a third two-component system, CrrAB, have been associated with changes in lipopolysaccharide, leading to resistance to polymyxins [6–8]. Resistance mediated by plasmid genes (*mcr*) encoding bacterial lipopolysaccharide-modifying enzymes has also been reported, starting with the characterization of the *mcr-1* gene in 2015 [9], which was followed by the description of numerous allelic variants (*mcr-2* to *mcr-9*) [10–17], including in carbapenem-resistant isolates and in high-risk clones [18,19].

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Whether caused by plasmid or chromosomal genes, the detection of polymyxin resistance faces technical difficulties, because of its cationic nature and its propensity to adsorb to plastic surfaces [20]. Therefore, methods widely used in clinical laboratories such as disc diffusion assays, and Etests are not suitable [21–23]. Furthermore, MICs obtained with gradient strips are markedly lower than those observed with the reference method, which could result in the misinterpretation of resistant strains as susceptible, especially in low-level colistin-resistant bacteria [24]. Alternative phenotypic methods have been developed to enable the detection of polymyxin resistance in clinical microbiology laboratories. The Rapid NP Polymyxin Test, developed by Nordmann et al. in 2016, is based on verification of glucose metabolism associated with bacterial growth in the presence of polymyxin and phenol red as a pH indicator [25]. The Colispot Test is another phenotypic test published by Jouy et al. in 2017 that aims to determine susceptibility to polymyxins by analysing the diameter formed around a drop of a standardized antimicrobial solution on the agar surface [26]. Selective media containing polymyxin have also been developed for the detection of antimicrobial resistance [27–31].

Recently, broth microdilution (BMD), broth disc elution and agar dilution were recommended by the CLSI M100 30th edition guideline for determining colistin MICs. However, for polymyxin B, only BMD was approved [32]. When carrying out BMD, it is important to consider the use of polystyrene plates, as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group [33]. It is known that most clinical microbiology laboratories lack the human or financial resources to perform the broth microdilution test recommended.

In this context, the Laboratório de Pesquisa em Infecção Hospitalar (LAPIH), located at Oswaldo Cruz Institute (Rio de Janeiro, Brazil), which is part of the Brazilian National Programme for Monitoring the Prevalence of Bacterial Resistance, conducted by the National Health Surveillance Agency, routinely receives clinical bacterial isolates from Brazilian health-care services to investigate resistance mechanisms. In this study, we aimed to evaluate the performance of these three alternative phenotypic methods: Rapid NP Polymyxin Test, ColiSpot test and the SuperPolymyxin medium (Plast Labor, Rio de Janeiro, Brazil), especially for clinical isolates with MICs near the breakpoint.

Material and methods

Clinical strains

We evaluated 170 non-duplicate *K. pneumoniae* clinical isolates (blood, urine, tracheal aspirate, rectal swab and catheter tip) received by LAPIH from January to December 2016 from eight Brazilian states. The species identification was confirmed by conventional biochemical tests and the isolates were stored at −40°C in skimmed milk (DIFCO, Hants, UK) [34].

From these isolates, 123 were resistant to colistin (MIC 4–128 μg/mL) and 47 were susceptible (MIC ≤2 μg/mL). From all resistant isolates, only three carried the *mcr-1* gene (screened by conventional PCR) [9]. Furthermore, several isolates showed mutations in chromosomal genes, including disruption of *mgrB* by insertion sequences such as ISKpn13 and IS903B (data not shown), detected by sequencing and manual curation with Geneious v.1.6.8 (Biomatters Ltd, Auckland, New Zealand) and BLAST tool (https://www.ncbi.nlm.nih.gov). For each isolate, the BMD reference method and the three phenotypic tests evaluated were performed on the same day, starting from the same primary culture.

Broth microdilution

The reference method was performed using colistin sulphate powder (Sigma, St Louis, MO, USA). No additives were included (no polysorbate-80 or other surfactants). Colistin stock solutions were prepared and kept at −40°C and diluted in fresh cation-adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK) during assay only. The susceptibility to colistin was evaluated in duplicate, according to the ISO 20776-1 methodology [35]. Hence, two-fold serial dilutions of colistin were performed in a 96-well polystyrene plate, obtaining a concentration range of 0.25–256 μg/mL. The results were interpreted according to the EUCAST guideline (www.eucast.org) [36].

Rapid Polymyxin NP test

Fresh colonies grown on Mueller–Hinton agar (Oxoid) were used to perform the Rapid Polymyxin NP Test as described by Nordmann et al. [25]. A bacterial suspension with an optical density of 3.0–3.5 McFarland (≈10^9 CFU/mL) was prepared for each isolate and, after mixing with the Rapid NP colistin medium, resulted in a bacterial concentration of ~10^8 in each well of the plate. They were considered resistant when the colour of the developing solution changed from orange/red to yellow after incubation at 35°C ± 2°C for 20 minutes to 4 hours, which indicated bacterial metabolism in the presence of a 3.75 μg/mL colistin concentration (Fig. 1).

Colispot test

ColiSpot was performed as described by Jouy et al. [26] and consisted of applying a single drop (10 μL) of 8 μg/mL colistin solution onto the surface of a previously inoculated Mueller–Hinton agar with a 0.5 McFarland bacterial suspension (1.5 × 10^8 CFU/mL). Resistance was revealed when the inhibition zone did not exceed 5 mm (Fig. 2).

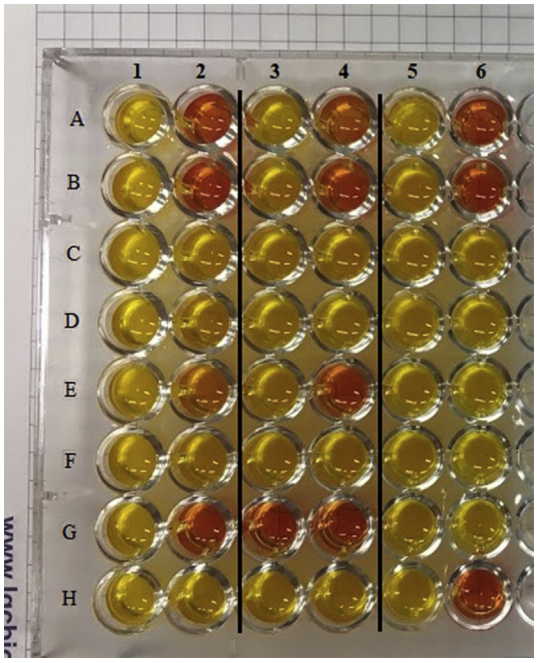

FIG. 1. Representative results of the Rapid Polymyxin NP test. Columns 1, 3 and 5 contain colistin-free solution and columns 2, 4 and 6 contain 5 μg/mL colistin-solution wells. A1-A2 wells were inoculated with the susceptible control strain *Escherichia coli* ATCC25922. Wells C1–C2 and D1-D2 were inoculated with the resistant strains *Klebsiella pneumoniae* CCBH22220 and *Proteus mirabilis* CCBH6854, respectively. G3-G4 wells were inoculated with NaCl solution only (no growth control). Wells D3-D4, F3–F4 and E5-E6 are examples of isolates considered resistant by the test. Wells A5-A6, B5–B6 and H5–H6 are examples of isolates considered susceptible by the Rapid Polymyxin NP test.

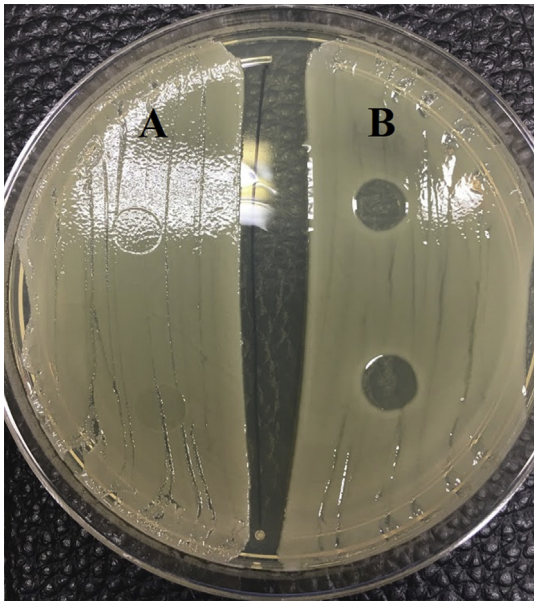

FIG. 2. Colispot Test results for two clinical isolates of *Klebsiella pneumoniae* evaluated in this study. In 'A', a resistant isolate (absence of zone of inhibition). In 'B', a susceptible isolate (7-mm inhibition zone). Two drops of colistin solution (8 μg/mL) were added separately to each isolate for comparison.

SuperPolymyxin medium

The SuperPolymyxin medium (PlastLabor, Rio de Janeiro, Brazil) was manufactured as described by Nordmann, Jayol and Poirel in 2016 [27]. Based on the eosin methylene blue agar with a final concentration of 3.5 μg/mL colistin, the medium also contained 10 μg/mL daptomycin for inhibition of *Streptococcus* and *Staphylococcus* species, and 5 μg/mL amphotericin B, an anti-fungal agent. An inoculum with an optical density of 0.5 McFarland standard (1.5×10^8 CFU/mL) was used. Then, 1 μL of each bacterial suspension (~1.5×10^5 CFU/mL) was plated on the medium using calibrated bacteriological handles. The number of viable colonies was counted after 24 hours of incubation in an aerobic atmosphere at 35°C ± 2°C. To verify isolate viability, eosin methylene blue agar was inoculated concomitantly. Preliminary data from our laboratory show that the SuperPolymyxin medium tested in the present study allowed the growth of sensitive reference strains, and these data corroborate with others described in the literature [28]. Hence, we proposed a cut-off point of five colonies, to increase specificity, above which the isolate was considered resistant to colistin.

Control strains, replicates and analysis of results

Escherichia coli ATCC25922 was included as a susceptible strain. *Klebsiella pneumoniae* CCBH22220 (colistin MIC 8 μg/mL) carrying the *mcr*-1 gene (obtained from the CCBH collection, http://ccbh.fiocruz.br/) and *Proteus mirabilis* CCBH6854, an intrinsically polymyxin-resistant species, were used as resistant controls.

The BMD reference method was performed in duplicate, with concordant values for most isolates. In case of disagreement, a third determination was performed, and the MIC obtained twice was accepted. The phenotypic methods were evaluated twice when they diverged from the reference method, and in case of disagreement with the first run, a third analysis was performed. In case of inconsistent results, the final interpretation of the test was based on the result as suggested by the majority of the three test runs.

For the analysis of the results, the values of sensitivity and specificity of each phenotypic method were calculated. The Category Agreement (CA), which is considered the percentage of isolates that produce the same categorical result (susceptible, intermediate or resistant) when compared with the reference method, was obtained. Major errors (ME, resistant results by the new method and susceptible results by the reference standard

method) and very major errors (VME, susceptible result by the new method and a resistant result by the reference standard method) were also calculated [36,37]. The analysis of the results followed the recommendations of CLSI guideline M23 [38] and the criteria required by the ISO 20776-1 standard [35].

Results

Klebsiella pneumoniae isolates

The 123 clinical isolates of colistin-resistant *K. pneumoniae* had MICs ranging from 4 to >128 μg/mL (MIC_{50} 32 μg/mL; MIC_{90} >128 μg/mL). The 47 randomly selected susceptible isolates (MIC ≤2 μg/mL) showed MICs that ranged from 0.25 to 2 μg/mL (MIC_{50} 0.5 μg/mL; MIC_{90} 2 μg/mL). Of all the isolates tested, 45 showed MICs close to the breakpoint (MICs 1–8 μg/mL). Most of all resistant isolates were recovered from patients' blood, urine or tracheal aspirate, where the presence of *K. pneumoniae* may require the use of antimicrobial therapy (see Supplementary material, Table S1). In this context, we emphasize that 121 (98%) of the resistant isolates showed co-resistance to at least one carbapenem (data not shown), which makes the use of polymyxin a probable alternative.

Performance evaluation of phenotypic tests

The Rapid Polymyxin NP Test showed 90% sensitivity and 94% specificity. Colispot Test showed 74% and 100% and SuperPolymyxin medium showed 82% and 85% sensitivity and specificity, respectively. Table 1 shows the results of the three phenotypic tests for the 170 isolates of *K. pneumoniae*. As required by CLSI and the ISO 20776-1 standard [35,38], Categorical Agreement (CA) has been determined. Our study showed that for susceptible isolates, Rapid-NP and Colispot had CA >90%. The method using the polymyxin previously incorporated into the culture medium (SuperPolymyxin) presented low CA (70%) for the susceptible isolates with MICs close to the breakpoint (MIC 1 or 2 μg/mL). In the 123 resistant isolates, the accuracy of the tests increased with increasing MIC, >80% of CA is observed in high-level polymyxin-resistant isolates. It is important to highlight that VME occurred more frequently in low-level polymyxin-resistant isolates (MICs 4 and 8 μg/mL), meaning a false-susceptible result (Table 1). Especially for *K. pneumoniae* isolates, which characteristically have mucoid colonies in solid media [34], the quantification became difficult and lacked precision. The Rapid Polymyxin NP test had a CA >90% in isolates with MIC ≥16 μg/mL, which was considered acceptable (>90%) [35,38]. Despite this, the ME and VME calculated were 6.4% and 9.7%, respectively, which is not acceptable for colistin-resistance screening, required by the ISO 20776-1 standard (<3%). For resistant isolates, Colispot and SuperPolymyxin medium only showed CA >90% at MICs ≥128 μg/mL and 32 μg/mL, respectively. For low resistant MICs (4 and 8 μg/mL) (data underlined in Table 1), CA was insufficient for acceptance (<90%) for all three methods. All phenotypic methods evaluated were able to detect resistance in the three *mcr-1*-positive isolates, as shown in Table 1.

Discussion

In 2017, the World Health Organization ranked carbapenemase-producing *Enterobacterales* as a priority 1 for

TABLE 1. Performance evaluation of three phenotypic tests to determine resistance to polymyxins

	MIC [a] (μg/mL)	No. of isolates (n = 170)	Rapid Polymyxin NP Test			Colispot Test			SuperPolymyxin Medium		
			S	R	CA	S	R	CA	S	R	CA
Resistant isolates [b]	>128	20	0	20	100%	0	20	100%	3	17	85%
	128	8	0	8	100%	0	8	100%	0	8	100%
	64	33	0	33	100%	4	29	87.9%	3	30	90.9%
	32	25	0	25	100%	4	21	84%	3	23	92%
	16	13	1	12	92.3%	6	7	53.8%	3	10	76.9%
	8	11 [c]	3	8 [c]	72.7%	6	5 [c]	45.4%	4	7 [c]	63.6%
	4	13 [d]	8	5 [d]	38.5%	12	1 [d]	7.7%	6	7 [d]	53.8%
Susceptible isolates [b]	2	11	10	1	90,9%	11	0	100%	8	3	72.7%
	1	10	10	0	100%	10	0	100%	7	3	70%
	0.5	22	20	2	90.9%	22	0	100%	21	1	95.4%
	0.25	4	4	0	100%	4	0	100%	4	0	100%
All isolates		170	CA = 91.2% No. of ME = 3 (6.4%) No. of VME = 12 (9.7%)			CA = 81.2% No. of ME = 0 (0%) No. of VME = 32 (26%)			CA = 82.9% No. of ME = 7 (14.9%) No. of VME = (17.9%)		

Abbreviations: S, susceptible; R, resistant; CA, categorical agreement; ME, major errors; VME, very major errors. The underlined data represent low-level resistance isolates (MICs 4 or 8 μg/mL), where the lowest categorical agreement was observed.
[a]MICs were determined using the broth microdilution test according to European Committee on Antimicrobial Susceptibility Testing (EUCAST guidelines.
[b]Antimicrobial susceptibility tests for colistin were interpreted according to EUCAST guidelines.
[c]Including two *mcr-1*-positive isolates.
[d]Including one *mcr-1*-positive isolate.

research and development of new antibiotics—emphasizing the scarcity of therapeutic options [39]. In these scenarios, polymyxins are widely prescribed as a therapeutic option and then the clinical microbiology laboratory plays an important role in antimicrobial therapy. In Brazilian hospitals, carbapenem and polymyxin co-resistance has been described [18].

Considering the importance of early detection of polymyxin resistance for a positive patient outcome and the difficulty of performing the microdilution method in routine microbiology laboratories, phenotypic tests such as those evaluated in this study have been developed and evaluated worldwide [26,27,40–43]. All three methods evaluated proved to be easy to prepare and perform. The performance of the Rapid Polymyxin NP Test was comparable to that observed by other authors [40–43]. However, here we emphasize the difficulty of detecting resistance in isolates with MICs close to the breakpoint (4 and 8 μg/mL) for all evaluated methods. These findings have been described even for other methods, such as Colistin Broth Disk Elution [30,40,41]. Detection of polymyxin resistance in low MICs is important as the spread of resistance by plasmid mechanisms, such as the *mcr* gene, has been associated with bacteria that exhibit low resistance levels (i.e. 4–16 μg/mL) [44–46].

Rapid Polymyxin NP proved to be a faster and less laborious technique than conventional BMD. Limitations for the use of this technique in some clinical laboratories are the preparation of the NP solution, which requires pH adjustment, and the standard polymyxin solution, which requires the use of analytical balances, which may be unavailable. For the Colispot Test, developed in 2016 [26], as far as we know, there are no published papers that aimed to evaluate its performance. Although the method is promising and easy to perform and interpret, the results did not reproduce well in the isolates tested, especially in strains with MICs close to the breakpoint. Regarding the SuperPolymyxin medium, other authors have observed satisfactory results, including the use of chromogenic media [29] and the Colistin Agar Test, which proposes to use Mueller–Hinton agar with colistin added as a screening [30]. However, in the present study, the categorical agreement observed was not satisfactory for SuperPolymyxin medium. Limitations included quantification of mucoid colonies and poor medium stability, evidenced by the growth of the susceptible control strain.

Although a recent study showed an adequate distribution of colistin in the agar, the authors reported that the culture medium could only be stored for 7 days [24]. This may have been one of the reasons for the low performance observed in the culture medium evaluated in our study. The storage conditions recommended by the manufacturer were strictly followed in the present study, but some tests were carried out after 7 days of storage.

In a screening for colistin-resistant bacteria in stool samples with SuperPolymyxin medium, 1851 phenotypically different colonies were recovered. After excluding intrinsically resistant species (n = 421), resistance to colistin was confirmed by BMD in 218 (15.3%) of the remaining 1430 isolates [28]. On the other hand, some studies have found a high sensitivity [31], and this variation may be related to the manufacturer or to the characteristics of the evaluated isolates.

Due to the wide variation in performance observed in these methods, the development of new tools, including phenotypic methods and genotyping, has been discussed [47], including selective culture media [25,27,29,48] and specific screening for detection of *mcr-1* [49]. Two phenotypic methods, colistin broth disc elution and colistin agar test, not evaluated in the present study, were described recently and included in the CLSI Guideline 2020 [32]. Although promising, they are still considered provisional, because they were established with limited discs and/or media manufacturers, endorsing the need for continuous evaluation of tests for the detection of polymyxin-resistant strains.

Conclusion

As noted by other authors, Rapid NP proved to be a method capable of identifying colistin-resistant strains in acceptable categorical agreement, but in clinical *K. pneumoniae* isolates evaluated in this work, ME and VME were considered unacceptable for colistin-resistance screening. Although very easy to perform, Colispot and SuperPolymyxin medium methods did not show acceptable sensitivity, specificity or categorical agreement. All methods have failed to detect resistance at low levels, which requires a more careful analysis in these cases, so avoiding the use of an ineffective therapeutic agent to treat an infection.

Conflicts of interest

The authors have stated that there are no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2020.100722.

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