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Rapid Polymyxin/*Pseudomonas* NP test for rapid detection of polymyxin susceptibility/resistance in *Pseudomonas aeruginosa*

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Abstract
Polymyxins are becoming an added value for treating infections due to multidrug-resistant *Pseudomonas aeruginosa*. Therefore, the Rapid Polymyxin/*Pseudomonas* NP test was developed for the identification of polymyxin resistance in *P. aeruginosa* isolates. This test detects the bacterial growth in a medium containing a defined concentration of colistin. Formation of basic metabolites is visually detected by yellow to purple/violet color change of the bromocresol purple pH indicator. The test performance was evaluated by using 50 colistin-resistant and colistin-susceptible *P. aeruginosa* isolates, among which 10 were colistin resistant. The sensitivity and specificity of the test were found to be 100% (CI95 65.5–100%) and 95% (CI95 81.8–99.1%), respectively, by comparison with broth microdilution as the reference method. The Rapid Polymyxin/*Pseudomonas* NP test is easy to perform, specific and sensitive, allowing rapid visual observation of results without the requirement any special reading equipment, and results are obtained in 3 h.

Keywords Colistin · Rapid diagnostic test · *Pseudomonas aeruginosa* · Susceptibility testing

Introduction

Pseudomonas aeruginosa belongs to the ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species). It is one of the most important bacterial species in clinical settings in particular as a source of bacterial pneumonia in intensive care patients [1, 2]. Carbapenem-resistant *P. aeruginosa* isolates are increasingly reported in health care facilities that may lead to fatal outcomes due to limited therapeutic options [3–5]. The Center for Diseases Control of Atlanta in the USA and the World Health Organization classified the carbapenem-resistant *P. aeruginosa* among the most serious pathogens exhibiting multidrug resistance [6, 7].

Polymyxins are considered as a last resort treatment for treating multidrug-resistant *P. aeruginosa* infections [8]. Owing to the occurrence of colistin resistance in this species, reliable and rapid techniques for testing colistin susceptibility are needed to optimize antibiotic stewardship. The standard reference technique for determining polymyxin susceptibility in Gram-negative bacteria is the determination of the corresponding MIC by the broth microdilution method (BMD) [9], which is however time-consuming (24 h) and is impractical in routine microbiology. Other techniques for determining susceptibility to polymyxins such as disk diffusion and E-test are not recommended due to high rates of false-susceptibility results. Recently, we have developed the Rapid Polymyxin NP test that identifies colistin-susceptible/colistin-resistant isolates in 2 h for *Enterobacterales* [10]. This test is based on the visualization of glucose metabolization and consequently production of acidic components in the presence of a pH indicator. Here, we have developed a rapid test (the Rapid Polymyxin/*Pseudomonas* NP test) for identification of polymyxin resistance in *P. aeruginosa*. This test detects the bacterial growth in a medium supplemented with a defined concentration of colistin. Taking in account the production of alkaline

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products during *P. aeruginosa* metabolism, formation of basic metabolites was visually detected by yellow to purple/violet color change of the bromocresol purple taken as the pH indicator. The test is cost-effective, easy to perform, and interpreted through a rapid visual observation in 3 h. The objective of this study was to develop this novel test by comparison with the BMD method using a collection of colistin-susceptible and colistin-resistant *P. aeruginosa* clinical isolates.

Materials and methods

Bacterial strains This study was carried out using 50 well-characterized *P. aeruginosa* clinical isolates. Ten out of the 50 *P. aeruginosa* isolates were colistin resistant according to the results of the BMD testing (Table 1). The colistin-susceptible *P. aeruginosa* ATCC 27853 strain and the colistin-resistant *P. aeruginosa* R409 strain were used as negative and positive controls for the determination of MIC of colistin, respectively.

Antimicrobial susceptibility testing The BMD was performed, using homemade panels, for determination of MICs of colistin in cation-adjusted Mueller-Hinton broth (Bio-Rad, Marnes-La-Coquette, France, ref. 69444), and results were interpreted according to the EUCAST/CLSI joint guidelines [9]. Isolates were considered susceptible when MICs of colistin were ≤ 2 mg/L and resistant when MICs were > 2 mg/L [9]. Colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA) were tested over a range of dilutions (0.125–128 mg/L). These antibiotic powders can be stored at 4 °C before their use, whereas diluted polymyxin solutions may be kept at − 20 °C for up to 1 year.

Rapid Polymyxin/*Pseudomonas* NP test

We first compared different parameters using a colistin-susceptible isolate (*P. aeruginosa* ATCC 27853) and a colistin-resistant isolate (*P. aeruginosa* R-409). These parameters included bacterial inoculum (0.5, 1.5, and 3.5 McFarland standards), colistin concentration (1.75, 2, 2.5, 3, 3.5, 4 mg/L), and time of incubation (1, 2, and 3 h). After comparison of the results with different parameters, all experiments were performed in triplicate by two different persons with the optimal conditions obtained, as described below.

Preparation of solutions For stock solutions of polymyxins, colistin sulfate tablets (Mast Diagnostics, Merseyside, UK) were dissolved into Mueller-Hinton (MH) solution (Bio-Rad, Marnes-La-Coquette, France, ref. 69444) in glass tubes to obtain a concentration of 0.256 mg/mL. These tablets can be stored at 4 °C before use, and the diluted polymyxin solutions can be kept at − 20 °C for 1 year.

The Rapid Polymyxin/*Pseudomonas* NP solution To prepare 250 mL of the Rapid Polymyxin/*Pseudomonas* NP solution, the culture medium and the pH indicator were mixed in a glass bottle as follows: 6.25 g of MHB-CA powder (cation-adjusted Mueller-Hinton broth, Bio-Rad, Marnes-La-Coquette, France, ref. 69444), 0.00625 g of bromocresol purple (Sigma-Aldrich), and 225 mL of distilled water. Several pH indicators were tested before retaining bromocresol purple that gave the optimal results (data not shown). The pH of the solution was adjusted to 5.25–5.4 by adding drops of 1 M HCl. Then, the final volume was adjusted to 250 mL, and the solution was autoclaved at 121 °C for 15 min. The final concentrations in the Rapid Polymyxin/*Pseudomonas* NP solution were consequently 2.5% of MHB-CA powder and 0.0026% of bromocresol purple indicator. This Rapid Polymyxin/*Pseudomonas* NP solution can be kept at 4 °C for 1 week or at − 20 °C for 1 year. This solution must be pre-warmed at 37 °C before use to prevent growth delay and therefore a delayed color change. Just before performing the experiment, colistin is added to the Rapid Polymyxin/*Pseudomonas* NP solution and mixed into sterile glass tubes to obtain the Rapid Polymyxin/*Pseudomonas* NP solution containing a colistin concentration of 4 mg/L. As an example, 15.6 μL of colistin stock solution at 0.256 mg/mL was added to 1 mL of Rapid Polymyxin/*Pseudomonas* NP solution for the testing of one clinical isolate and along with negative and positive controls.

Bacterial inoculum preparation For each isolate to be tested including the positive and negative controls, we prepared a standardized bacterial inoculum by using freshly obtained (overnight) bacterial colonies grown on UriSelect 4 agar plates (or Muller-Hinton agar plates). The bacterial colonies were resuspended into 5 mL of sterile NaCl (0.85%) to obtain a 3.0–3.5 McFarland standard optical density (~ 10^9 CFU/mL). This corresponds to about a 10-μL full loop of bacterial colonies diluted in 10 mL of NaCl 0.85%. We used as positive control (resistant isolate) *P. aeruginosa* R409 and as negative control (susceptible isolate) *P. aeruginosa* ATCC27853 (R1496). The bacterial suspensions should be used within 15 min of preparation and for no longer than 1 h after preparation, as recommended by the EUCAST guidelines for susceptibility testing [9].

Tray inoculation Inoculation and reading were performed similarly to what is recommended for the optimized Rapid Polymyxin NP test. We performed the testing in a 96-well polystyrene microplate (round base, with lid, sterile, Sarstedt Str. 1, D-51588 Nuembrecht, Germany, ref.

Table 1 Rapid Polymyxin/*Pseudomonas* NP test for polymyxin-resistant and polymyxin-susceptible *P. aeruginosa*

Strain	Origin		Phenotype[a]	BMD MIC colistin (mg/L)	Rapid Polymyxin/*Pseudomonas* NP test	
					Result	Discrepancy with BMD MIC colistin result[b]
R1496	USA	Reference	S	0.125	Negative	–
R104	France	Clinical	S	0.125	Negative	–
R49	France	Clinical	S	0.125	Negative	–
R70	France	Clinical	S	0.125	Negative	–
R96	France	Clinical	S	0.125	Negative	–
R47	France	Clinical	S	0.25	Negative	–
R66	France	Clinical	S	< 0.125	Negative	–
R64	France	Clinical	S	0.25	Negative	–
R76	France	Clinical	S	0.5	Negative	–
R3407	France	Clinical	S	0.125	Negative	–
R3409	France	Clinical	S	0.125	Negative	–
R3408	France	Clinical	S	0.125	Negative	–
R3410	France	Clinical	S	0.125	Negative	–
R124	France	Clinical	S	0.125	Negative	–
R126	France	Clinical	S	0.125	Negative	–
R127	France	Clinical	S	0.125	Negative	–
R1202	Greece	Clinical	S	0.125	Negative	–
R196	France	Clinical	S	0.125	Negative	–
R606	France	Clinical	S	0.125	Negative	–
R15	France	Clinical	S	< 0.125	Negative	–
R128	France	Clinical	S	0.5	Positive	YES (ME)
R3411	France	Clinical	S	< 0.125	Negative	–
R3412	France	Clinical	S	< 0.125	Negative	–
R3413	France	Clinical	S	0.125	Negative	–
R3425	France	Clinical	S	0.125	Negative	–
N142	Switzerland	Clinical	S	0.5	Negative	–
N143	Switzerland	Clinical	S	0.125	Negative	–
R608	France	Clinical	S	0.25	Negative	–
R419	France	Clinical	S	0.5	Negative	–
N144	Switzerland	Clinical	S	0.125	Negative	–
N158	Switzerland	Clinical	S	0.125	Negative	–
N167	Switzerland	Clinical	S	0.125	Negative	–
N192	Switzerland	Clinical	S	0.125	Negative	–
N214	Switzerland	Clinical	S	0.125	Negative	–
R105	France	Clinical	S	0.125	Positive	YES (ME)
N254	Switzerland	Clinical	S	0.125	Negative	–
N301	Switzerland	Clinical	S	0.125	Negative	–
N69	Switzerland	Clinical	S	0.125	Negative	–
N170	Switzerland	Clinical	S	0.125	Negative	–
R275	France	Clinical	S	0.125	Negative	–
R3813	France	Clinical	R	> 128	Positive	–
R3422	France	Clinical	R	8	Positive	–
R1197	Turkey	Clinical	R	8	Positive	–
R3419	France	Clinical	R	32	Positive	–
R3418	France	Clinical	R	16	Positive	–
R3421	France	Clinical	R	16	Positive	–
R3427	France	Clinical	R	16	Positive	–
R409	France	Clinical	R	8	Positive	–
R3420	France	Clinical	R	64	Positive	–
N76	Switzerland	Clinical	R	4	Positive	–

S susceptible, *R* resistant, – no discrepancy, *ME* major error, *BMD* broth microdilution

82.1582.001). For each isolate, bacterial suspension was inoculated in parallel into 2 wells, with and without colistin, in separate wells. The following steps of the Rapid Polymyxin/*Pseudomonas* NP test were performed, as illustrated in Fig. 1:

Step 1: 150 µL of colistin-free Rapid Polymyxin/*Pseudomonas* NP solution was transferred to wells A1–A8.

Step 2: 150 µL of the Rapid Polymyxin/*Pseudomonas* NP solution containing colistin (4 mg/L to obtain a final

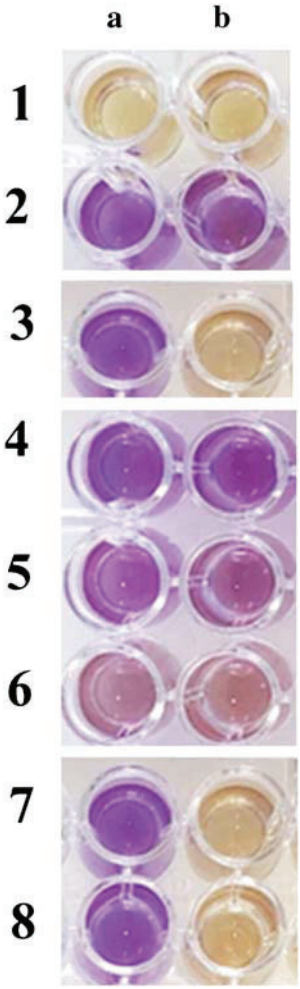

Fig. 1 Representative results of the Rapid Polymyxin/*Pseudomonas* NP test. Non-inoculated wells are shown as controls for possible and spontaneous color change (A1 and B1). Bacterial growth is evidenced by color change of the medium from yellow to purple or violet. The Rapid Polymyxin/*Pseudomonas* NP test was performed with a reference colistin-resistant *P. aeruginosa* isolate (A2 and B2) and with a reference colistin-susceptible *P. aeruginosa* isolate (A3 and B3) in a reaction without (first column, A) and with (second column, B) colistin at the defined concentration. The tested isolates (lines 4 to 6) that grew as well as in the absence and presence of colistin (wells A4–A6 and B4–B6), respectively) were colistin-resistant. The tested isolates (lines 7 to 8) that grew in the absence of colistin but not in its presence (wells A7–A8 and B7–B8), respectively) were colistin-susceptible isolates

concentration of 3 mg/L) was transferred to wells B1-B8.

Step 3: 50 μL of NaCl 0.85% was added to wells A1 and B1.

Step 4: 50 μL of the colistin-resistant isolate suspension used as positive control was added to wells A2 and B2.

Step 5: 50 μL of the colistin-susceptible isolate suspension used as negative control was added to wells A3 and B3.

Step 6: 50 μL of the tested isolate suspension was added to wells A4–A8 and B4–B8.

The bacterial suspension was mixed with the rapid solution by pipetting up and down. The final concentration of bacteria was ≈ 10^8 CFU/mL in each well, and the final concentration of colistin was 3.3 mg/L.

Tray incubation The inoculated tray was incubated for up to 3 h at 35 ± 2 °C in ambient air.

Tray reading Based on our previous experience of development of several rapid diagnostic tests [10, 11], We visually inspected the tray (checked for no spontaneous color change) after 10 min and then every hour for 3 h. The test was considered positive (i.e., purple or violet of the culture medium, indicating polymyxin resistance if the tested isolate grew in the presence of colistin), and negative (i.e., yellow or yellowish green of the culture medium, indicating no growth and consequently polymyxin susceptibility) if the tested isolate did not grow in the presence of colistin. We considered the test result interpretable if the following 4 conditions were met: (1) both wells (A1 and B1) with 0.85% NaCl and without bacterial suspension remained unchanged (absence of medium contamination); (2) the colistin-free wells A2–A8 with bacterial suspensions turned from yellow to purple or violet, confirming the growth of the isolate; (3) the well B2 with the colistin-resistant reference bacterial suspension (positive control) gave a positive result (purple/violet), confirming the viability of the isolate in the presence of colistin; and (4) the well B3 with the colistin-susceptible reference bacterial suspension (negative control) gave negative results (remaining yellow) confirming the absence of growth of this isolate. The tested isolates that grew in the absence and the presence of colistin (wells A4–A6 and B4–B6, respectively) were therefore reported to be colistin-resistant. The tested isolates that grew in the absence but did not grow in the presence of colistin (wells A7–A8 and B7–B8, respectively) were therefore reported as colistin-susceptible (Fig. 1).

The test result was positive when the well containing colistin (B2) and the isolate to be tested turned from yellow to purple or violet, both giving the exact same color as the well without colistin (A2), confirming the growth in the presence of colistin (colistin resistance) (Fig. 1). The test result was negative when the well containing colistin (B3) with the isolate to be tested remained yellow (unchanged color) (Fig. 1). Results were also interpreted by a technician in a blinded way.

Data analysis The results obtained with the Rapid Polymyxin/*Pseudomonas* NP test was compared to those obtained with the reference BMD method. Briefly, discrepancies were determined for each method to assess the performances of the test

to detect colistin resistance. Errors (very major errors [VME] and major errors [ME]) were calculated as described previously [12]. A VME was considered when isolates were categorized as susceptible using the Rapid Polymyxin/*Pseudomonas* NP test, being however resistant by the BMD method (false-susceptible result). A ME was considered when isolates were found to be resistant using the Rapid Polymyxin/*Pseudomonas* NP test, being however susceptible by using the BMD method (false-resistant result).

Results

Among the fifty *P. aeruginosa* isolates included to assess the performance of the Rapid Polymyxin/*Pseudomonas* NP test (Table 1), 40 were colistin susceptible (MICs of colistin ranging from less than < 0.125 to 0.5 mg/L) and ten isolates were resistant (MICs of colistin ranging from 4 to 128 mg/L), according to the results of the BMD method. The 40 colistin-susceptible *P. aeruginosa* isolates gave negative results according to the Rapid Polymyxin/*Pseudomonas* NP test, except for two isolates (MICs of 0.125–0.5 mg/L) which gave positive (false-positive) results (Table 1). The colistin-resistant *P. aeruginosa* isolates ($n = 10$) (MICs of colistin ranging from 4 to 128 mg/L) were all correctly identified as colistin resistant by using the Rapid Polymyxin/*Pseudomonas* NP test (Table 1). Overall, two ME (false resistance) but no VME (false susceptibility) were observed. Therefore, a strong concordance was found between the results of the susceptibility test and of the Rapid Polymyxin/*Pseudomonas* NP test results, both for susceptible and resistant isolates (Table 1). Sensitivity (100%, CI95 65.5–100%) and specificity (95%, CI95 81.8–99.1%) of the test were therefore high compared with the BMD method. By reading the color change of the wells every hour, we determined that final results shall be best read at 3 h after incubation at 35 ± 2 °C under an ambient atmosphere.

Discussion

This study showed that the Rapid Polymyxin/*Pseudomonas* NP test is a reliable technique for detecting polymyxin resistance in *P. aeruginosa*. Development of this test is based on detection of the bacterial growth in a medium containing a defined concentration of colistin. Detection of alkaline constituents in the growth medium (and not acidic components as for the Rapid Polymyxin NP for *Enterobacterales*) is made by adding a pH indicator, bromocresol purple, which color change occurs when the culture medium becomes more alkaline upon bacterial growth. This test is rapid, inexpensive, and easy to implement. It offers the possibility of detecting polymyxin resistance from bacterial cultures in 3 h, which is at least 16 h earlier than with the reference BMD method, and faster than a test we previously developed (so-called Rapid ResaPolymyxin *Acinetobacter/Pseudomonas* NP test [11], requiring at least 4 h). In addition, the Rapid Polymyxin/*Pseudomonas* NP test requires a single methodological step while the Rapid ResaPolymyxin *Acinetobacter/Pseudomonas* NP test requires an additional one including growth period of time of 3 h and then addition of resazurin in the medium in another 1 h revelation period of time [11]. Even though a few discrepancies were observed (two ME), the sensitivity and specificity of the Rapid Polymyxin/*Pseudomonas* NP test were high (100%, CI95 65.5–100% and 95%, CI95 81.8–99.1%, respectively), making it a potential useful technique for clinical laboratories. This test complements the Rapid Polymyxin NP test, which works with *Enterobacterales* but is not appropriate for non-fermenters such as *P. aeruginosa*. Altogether, such newly developed approach fits with what is expected from a rapid diagnostic test (besides accuracy with excellent sensitivity and specificity), namely optimal fastness and easiness.

Owing to the relatively small sample size of our collection which may be considered as a limitation, which is indicated by the wide the confidence interval (CI95) especially for sensitivity, further studies with a broader set of resistant isolates will be needed to further validate the accuracy of that test. In addition, validation of this test may be performed also with other *Pseudomonas* species, besides *P. aeruginosa*. The Rapid Polymyxin/*Pseudomonas* NP test may be useful for a first-step screening of polymyxin resistance, considering the diversity (and sometimes still unknown) mechanisms of resistance to polymyxins in *P. aeruginosa*. The turn-around time of such test is short, which may lead to improve the rapid implementation of adequate hygiene measures to control the spread of such multidrug-resistant bacteria. Ultimately, implementation of such test may significantly contribute to optimize the rationale use of polymyxins by differentiating rapidly infections due to polymyxin-susceptible or polymyxin-resistant *P. aeruginosa* isolates.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not needed.

Informed consent All authors agreed on the submitted version.

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