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Evaluation of the rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test for rapid colistin resistance detection in lactose non-fermenting Gram-negative bacteria

Hyunsul Jung[1], Johann D. D. Pitout[1,2], Barend C. Mitton[1,3], Kathy-Anne Strydom[1,4], Chanel Kingsburgh[4], Jennifer Coetzee[4], Marthie M. Ehlers[1,3] and Marleen Kock[1,3,*]

Abstract

Introduction. Colistin is one of the last-resort antibiotics for treating multidrug-resistant (MDR) or extensively drug-resistant (XDR) lactose non-fermenting Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Gap Statement. As the rate of colistin resistance is steadily rising, there is a need for rapid and accurate antimicrobial susceptibility testing methods for colistin. The Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test has recently been developed for rapid detection of colistin resistance in *P. aeruginosa* and *A. baumannii*.

Aim. The present study aimed to evaluate the performance of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test in comparison with the reference broth microdilution (BMD) method.

Methodology. The Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test was performed using a total of 135 *P. aeruginosa* (17 colistin-resistant and 118 colistin-susceptible) and 66 *A. baumannii* isolates (32 colistin-resistant and 34 colistin-susceptible), in comparison with the reference BMD method.

Results. The categorical agreement of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test with the reference BMD method was 97.5% with a major error rate of 0% (0/152) and a very major error (VME) rate of 10.2%. The VME rate was higher (23.5%) when calculated separately for *P. aeruginosa* isolates. The overall sensitivity and specificity were 89.8 and 100%, respectively.

Conclusion. The Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test performed better for *A. baumannii* than for *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa and *Acinetobacter baumannii* are lactose non-fermenting Gram-negative bacteria that cause serious healthcare-associated infections and mortality in critically ill and immunocompromised patients worldwide [1–3]. These bacteria are also part of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter cloacae*) that pose a global public health risk by showing high virulence and by 'escaping' the effects of several antibiotics *via* multiple antibiotic resistance mechanisms [4]. In addition, carbapenem-resistant *P. aeruginosa* and *A. baumannii* have been recognized as 'critical pathogens' by the World Health Organization (WHO) in 2017 and 'serious threats' and 'urgent

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Author affiliations: [1]Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa; [2]Division of Microbiology, Alberta Public Laboratories, Cummings School of Medicine, University of Calgary, Calgary, Canada; [3]Department of Medical Microbiology, Tshwane Academic Division, National Health Laboratory Service (NHLS), Pretoria, South Africa; [4]Ampath National Reference Laboratory, Centurion, South Africa.
***Correspondence:** Marleen Kock, marleen.kock@up.ac.za

Keywords: colistin; *Acinetobacter baumannii*; *Pseudomonas aeruginosa*; rapid resistance detection test.

Abbreviations: AST, antimicrobial susceptibility testing; BMD, broth microdilution; CA, categorical agreement; CA-MHB, cation-adjusted Mueller-Hinton broth; CDC, US Centers for Disease Control and Prevention; CLSI, Clinical and Laboratory Standards Institute; ESKAPE, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*; EUCAST, European Committee on Antimicrobial Susceptibility Testing; ISO, International Organization for Standardization; MDR, multi-drug resistant; ME, major error; MIC, minimum inhibitory concentration; VME, very major error; WHO, World Health Organization; XDR, extensively drug-resistant.
One supplementary table is available with the online version of this article.

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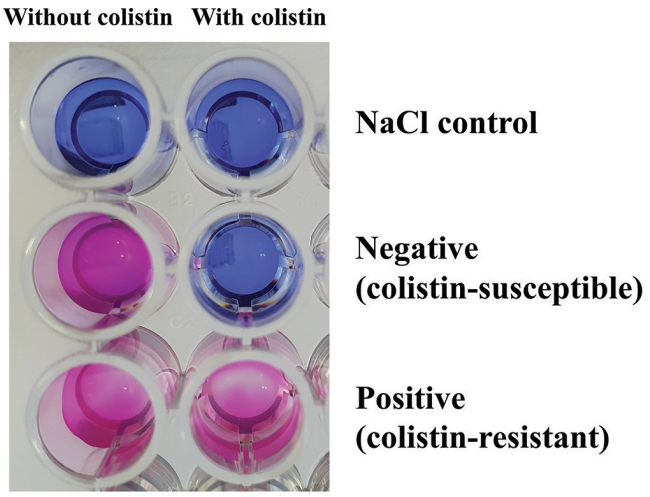

Fig. 1. Representative picture of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test results.

threats' by the US Centers for Disease Control and Prevention (CDC), respectively [5–7].

As the number of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* and *A. baumannii* is increasing, polymyxins [polymyxin B and polymyxin E (colistin)] have been reintroduced into clinical practice although its use was abandoned in the past due to nephrotoxicity [8–10]. Colistin is a multicomponent cationic polypeptide antibiotic (composed of colistin A and B) that shows bactericidal activity against most Gram-negative bacteria [11]. However, colistin resistance by means of chromosomal mutations, target modification (lipopolysaccharide) and plasmid-mediated 'mobilized colistin resistance' (*mcr*) genes has emerged and has become common in *P. aeruginosa* and *A. baumannii* isolates [12–14]. The emergence of colistin resistance in *P. aeruginosa* and *A. baumannii* is worrisome, because colistin is often the only available effective drug for salvage therapy in critically ill patients with MDR or XDR *P. aeruginosa* or *A. baumannii* infections [15, 16]. Therefore, rapid detection of colistin resistance is crucial for effective control and management of infections caused by MDR or XDR *P. aeruginosa* and *A. baumannii*.

The current gold standard method for determining minimum inhibitory concentrations (MICs) of colistin for Gram-negative bacteria, as recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST), Clinical and Laboratory Standards Institute (CLSI) and the International Organization for Standardization (ISO) [ISO 20776-1:2019], is the broth microdilution (BMD) method [17, 18]. However, the BMD method is time-consuming (24 h), labour-intensive and not suitable for routine use in most clinical microbiology laboratories [19]. Other antimicrobial susceptibility testing (AST) methods commonly used in clinical microbiology laboratories are disc diffusion or gradient diffusion (Etest, MIC test strips) methods [19, 20]. These AST methods are not recommended for colistin, as colistin diffuses poorly into the agar due to its cationic nature and its large molecule size, which results in inaccurate AST results [20, 21]. In an attempt to fulfil the need for rapid, cost-effective and accurate colistin AST methods, several rapid tests [e.g. the rapid polymyxin NP test [22], the Rapid Polymyxin *Pseudomonas* test (ELITech Group, Puteaux, France), the Rapid Polymyxin *Acinetobacter* test (ELITech Group, Puteaux, France), Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test [23]] have been developed. The rapid resazurin-based screening assay developed by Lescat *et al.* [23] detects colistin resistance in lactose non-fermenting Gram-negative bacteria such as *P. aeruginosa* and *A. baumannii*. This test, named the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test, is based on the principle that metabolically active cells reduce resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide; blue colour by default) to resorufin, which is indicated by a colour change to pink [23]. The test allows for the rapid detection of colistin resistance within 4 h including the incubation time [23]. The present study aimed to evaluate the performance of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test for detecting colistin resistance in lactose non-fermenting Gram-negative bacteria by using a set of colistin-resistant or colistin-susceptible *P. aeruginosa* and *A. baumannii* isolates.

METHODS

Bacterial isolates

A total of 135 non-duplicate *P. aeruginosa* (133 clinical and two environmental) and 66 clinical *A. baumannii* isolates were included in this study. These bacterial isolates were collected from private and public diagnostic laboratories in Pretoria, South Africa. The species identification and AST were routinely performed using the VITEK 2 system (bioMérieux SA, Marcy l'Etoile, France) with the VITEK GN ID card (bioMérieux SA, Marcy l'Etoile, France). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as negative control strains (colistin-susceptible) for quality control of the colistin BMD plates, whereas *P. aeruginosa* ATCC 27853 and an in-house clinical *Proteus mirabilis* isolate (naturally resistant to colistin) was used as a negative and positive control strain for the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test respectively. The study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (Ethics reference no.: 671/2018).

Reference antimicrobial susceptibility testing

The BMD method was performed as a reference method for determination of the colistin MIC and the MIC was interpreted according to the joint CLSI/EUCAST recommendations for colistin MIC determination and the EUCAST Clinical Breakpoint Tables version 10.0 [17, 24]. The MIC breakpoints for *P. aeruginosa* and *A. baumannii* were ≤2 mg l^{-1} for colistin susceptibility and >2 mg l^{-1} for colistin resistance according to the EUCAST Clinical Breakpoint Tables version 10.0 [24]. The BMD method was performed as previously described [25]. Each batch of prepared BMD plates was quality controlled by using the reference strains

Table 1. Summary of the ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test results in comparison with the reference BMD method

Broth microdilution (BMD) assay			Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test		
Colistin BMD MIC (mg l^{-1})	**MIC interpretation (S/R*)**	**No. of isolates**	**Positive**	**Negative**	**Discrepancies with BMD (type of error)**
P. aeruginosa					
0.125	S	6	0	6	No
0.25	S	6	0	6	No
0.5	S	49	0	49	No
1	S	24	0	24	No
2	S	33	0	33	No
4	R	3	2	1	Yes, 1 VME†
8	R	4	1	3	Yes, 3 VMEs†
16	R	0	0	0	No
32	R	5	5	0	No
64	R	1	1	0	No
>64	R	4	4	0	No
A. baumannii					
0.125	S	3	0	3	No
0.25	S	16	0	16	No
0.5	S	12	0	12	No
1	S	1	0	1	No
2	S	2	0	2	No
4	R	2	1	1	Yes, 1 VME†
8	R	5	5	0	No
16	R	2	2	0	No
32	R	2	2	0	No
64	R	5	5	0	No
>64	R	16	16	0	No

*S = susceptible; R = resistant
†VME = very major error

(*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) and a blank control. The purity of each bacterial inoculum was confirmed by inoculating an aliquot (5 µl) from the inoculated growth control well onto 5% blood agar plates (Diagnostic Media Products, Johannesburg, South Africa) and incubating aerobically at 37 °C for 18 h to 24 h.

The rapid resapolymyxin *Acinetobacter/Pseudomonas* NP test and result analysis

The Rapid ResaPolymyxin *Acinetobacter/Pseudomonas* NP test was performed as previously described in a study by Lescat *et al.* [23]. In brief, the colistin stock solution (made up with colistin sulphate powder, Abtek Biologicals, Liverpool, UK) was mixed with the cation-adjusted Mueller–Hinton broth (CA-MHB) (BBL Mueller Hinton II Broth, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to prepare a colistin-containing CA-MHB solution at a concentration of 4.16 mg l^{-1} (to reach a final concentration of 3.75 mg l^{-1} when mixed with bacterial inoculum). Colistin-free CA-MHB solution and colistin-containing CA-MHB solution were added to each well (180 µl) of first and second columns of a sterile 96-well microplate (clear, U-bottom, Greiner bio-one International GmbH, Kremsmünster, Austria), respectively. A volume of 20 µl 0.85% (w/v) sodium chloride (NaCl) solution (Merck KGaA, Darmstadt, Germany) was added to the wells A1 and A2. For control strains and bacterial isolates, the standardized bacterial inoculum (3.5 McFarland turbidity

Table 2. Result summary and performance of the ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test in comparison with the reference BMD method

	P. aeruginosa (*n*=135)	*A. baumannii* (*n*=66)	Total (*n*=201)
True positive*	13	31	44
True negative†	118	34	152
False negative‡	4	1	5
False positive§	0	0	0
Sensitivity	76.5%	96.9%	89.8%
Specificity	100%	100%	100%
Categorical agreement	97%	98.5%	97.5%
Very major errors	23.5%	3.1%	10.2%
Major errors	0%	0%	0%

*Resistant according to both the BMD method and the ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test.
†Susceptible according to both the BMD method and the ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test.
‡Resistant according to the BMD method and susceptible according to the ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test.
§Susceptible according to the BMD method and resistant according to the ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test.

standard;~1×10^9 c.f.u.·ml^{-1}) was prepared in a sterile saline using an overnight bacterial colonies with the DensiCHEK Plus instrument (bioMérieux SA, Marcy l'Etoile, France). A standardized bacterial inoculum (20 µl) of negative control strain *P. aeruginosa* ATCC 27853 was added to the wells B1 and B2, which was followed by addition of positive control strain (an in-house *P. mirabilis* isolate) inoculum (20 µl) in the wells C1 and C2. A volume of 20 µl of each bacterial inoculum was added to each well with colistin-containing CA-MHB solution or colistin-free CA-MHB. At each addition step, the solution was mixed by pipetting up and down. The inoculated microplate (with lid) was incubated at 35±2 °C for 3 h without being sealed. After incubation, 22 µl of the resazurin reagent (PrestoBlue Cell Viability Reagent, Thermo Fisher Scientific, Waltham, MA, USA) was added to each well [i.e. final concentration at 10% (v/v)] and was mixed by pipetting up and down. The colour change of the wells was visually inspected every 15 min until 1 h (i.e. 15 min, 30 min, 45 min and 1 h) and then at 2 h and 3 h after addition of the resazurin reagent (the microplate was kept at 35±2 °C during the time without being sealed). The test result was considered positive if the colour of the well with colistin-containing CA-MHB has changed from blue to purple or pink and negative if the colour remained blue (Fig. 1). The test results were considered valid and interpretable if: (i) the colour of the NaCl wells (A1 and A2) had remained blue; (ii) the colour of the well B2 (colistin-containing CA-MHB solution with negative control strain) had remained blue; (iii) the colour of the well C2 (colistin-containing CA-MHB solution with positive control strain) had turned from blue to purple or pink; and (iv) the colour of all the test wells with colistin-free CA-MHB medium had turned from blue to purple or pink.

The Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test results were compared to those obtained from the reference BMD method. For isolates with discrepant test results, the BMD assay and the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test was repeated in duplicate to confirm the results (for the BMD method, the median of the MIC results was retained as the final MIC). Categorical agreement (CA), major errors (MEs) and very major errors (VMEs) were calculated as previously described to evaluate the performance of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test to detect colistin resistance [26, 27]. Categorical agreement refers to the percentage of bacterial isolates that showed the same categorical results (susceptible or resistant) as those obtained from the reference BMD method over a total number of isolates tested [27]. It was considered as a ME when the reference BMD method obtained susceptible results and the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test obtained resistant results (false-resistant results). A VME was considered when the reference BMD method obtained resistant results, but susceptible results were obtained by the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test (false-susceptible results). A ≥90% level of CA and discrepancies (ME or VME) of ≤3% as described in the ISO standard 20776-2:2007 were considered as the acceptance criteria for colistin AST performance [27].

RESULTS AND DISCUSSION

As colistin is the last-resort antibiotic and often the only treatment option for treating MDR or XDR *P. aeruginosa* and *A. baumannii* infections, AST methods that rapidly and accurately detects colistin resistance are essential in formulating a treatment strategy and optimization of antimicrobial stewardship and infection control. The current 'gold standard' for colistin AST method – the BMD method – is however, labour-intensive and requires a long time (24 h) to obtain results, which challenges its use in routine clinical microbiology laboratory settings. There is thus an urgent need for rapid and cost-effective AST methods that accurately detects colistin resistance, especially in resource-limited settings where access to certain antibiotics is limited.

In the present study, the performance of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test was evaluated with 49 colistin-resistant (24.4%, 49/201; MICs ranging from 4 mg l^{-1} to >64 mg l^{-1}) and 152 colistin-susceptible isolates (75.6%; 152/201; MICs ranging from 0.125 to 2 mg l^{-1}) according to the reference BMD method (Table 1; full details of the isolates, MICs and test results are shown in Table S1, available in the online version of this article).

Table 3. The ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test cost calculation

Cost items	Cost per 1000 tests (USD*)	Cost per test (USD)	Notes
Cost of reagents and disposables			
Colistin sulphate powder	8.62	0.0086	214.41 USD per 1 g vial
Cation-adjusted Mueller-Hinton broth powder	1.23	0.0012	77.71 USD per 500 g
Resazurin solution	151.17	0.1512	341.04 USD per 100 ml
Saline (0.9 % NaCl)	3.38	0.0034	37.57 USD per 500 g
Microtitre plates (96-well)	39.68	0.0397	190.47 USD per 100 plates
Disposable glass tubes (5 ml)	104.37	0.1044	10.44 USD per 100 tubes
Sterile cotton swabs	21.30	0.0213	2.13 USD per 100 swabs
Pipette tips	153.21	0.1532	7.35 USD per 96 tips
Subtotal (reagents and disposables costs)	482.95	0.48	
Cost of labour			
Test preparation (60 min to prepare 100 tests)	200.00	0.20	20 USD per hour
Test inoculation (5 min hands-on-time per test)	1666.67	1.67	20 USD per hour
Subtotal (cost of labour)	1866.67	1.87	
Total cost	**2349.62**	**2.35**	

*USD: US Dollar.

The collection of colistin-resistant isolates consisted of 17 *P. aeruginosa* (34.7%; 17/49) and 32 *A. baumannii* isolates (65.3%; 32/49), whereas a collection of colistin-susceptible isolates consisted of 118 *P. aeruginosa* (77.6%; 118/150) and 34 *A. baumannii* isolates (22.4%; 34/150). Overall, the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test showed good performance in terms of categorical agreement (97.5%) and specificity (100%) with no ME (0%) when compared with the reference BMD method (Table 2). This is in agreement with the findings previously reported [23, 28, 29]. In terms of sensitivity, the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test performed better for *A. baumannii* (96.9%), while a considerably lower sensitivity rate (76.5%) was observed for *P. aeruginosa* (Table 2).

However, the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test did not meet the acceptance criteria for colistin AST performance (≤3% ME or VME rate) in accordance with the ISO standard 20776-2:2007 [27], due to high VME rates observed among *P. aeruginosa* isolates (23.5%) and *A. baumannii* (3.1%) (Table 2). This observation is contrary to a few or no VMEs observed elsewhere [23, 28, 29], but is not unexpected as the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test uses a final colistin concentration of 3.75 mg l^{-1} and all discrepant *P. aeruginosa* and *A. baumannii* isolates had MICs close to the colistin MIC breakpoint (4 or 8 mg l^{-1}) (Table 1). The discrepancy (VMEs or MEs) of the AST results in isolates with the MICs close to the breakpoint ('difficult isolates') is commonly observed in lactose non-fermenters like *P. aeruginosa* and *A. baumannii*, as well as in *Enterobacterales* [30–33]. It could be hypothesized that the VMEs observed in this study could be due to heteroresistant subpopulations or a low proportion of resistant subpopulations as demonstrated in a study by Rodriguez *et al.* [31]. Isolates with low colistin resistance MICs (4 to 8 mg l^{-1}) may contain a low frequency of resistant subpopulations that may require approximately 6 h to 24 h (or longer) to show regrowth after exposure to colistin [31, 34]. Thus, it is possible that the incubation time of 3 h for the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test was not inadequate to allow detectable growth of resistant subpopulations of these 'difficult' isolates, whereas 18 h to 20 h of incubation time for the reference BMD method allow resistant subpopulations to grow at the colistin MIC of 4 to 8 mg l^{-1}. Further studies are needed to confirm whether colistin heteroresistance in *P. aeruginosa* and *A. baumannii* plays a role in these discrepant test results (i.e. MEs or VMEs).

In this study, most *A. baumannii* isolates showed the obvious colour change at between 15 min to 30 min after dye addition, while some isolates took 1 h to show the colour change. In contrast, an extra hour (i.e. 2 h) or longer time (up to 3 h for some isolates) was required for *P. aeruginosa* to obtain a distinctive colour change. These observations are different from the time (1 h for all strains) reported by the original authors who invented the test [23], but agrees with the observations (15 min for *A. baumannii* and 2 h for *P. aeruginosa*) reported by Jia *et al.* [29]. According to Jia *et al.* [29], difference in the incubation time between *A. baumannii* and *P. aeruginosa* isolates for obtaining the distinctive colour change could be due to the slower growth rate of *P. aeruginosa* than other Gram-negative bacteria, resulting in slower reduction from resazurin to

resorufin. Thus, while 1 h incubation time could allow most *A. baumannii* isolates to obtain accurate results for the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test, up to 3 h incubation time might be required when testing *P. aeruginosa*. Alternatively, another newly developed rapid test for *P. aeruginosa* with a faster turnaround time, named the Rapid Polymyxin/*Pseudomonas* NP test [35], could be evaluated in future for its performance and rapidity.

The strength of this study is that a large set of *P. aeruginosa* isolates was used to evaluate the performance of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test. The authors acknowledge a few limitations. First, the number of colistin-resistant *P. aeruginosa* and *A. baumannii* isolates were low compared to the number of colistin-susceptible isolates. This is due to a low prevalence of colistin resistance in *P. aeruginosa* and *A. baumannii* isolates in South Africa [36–38]. Second, the colistin resistance mechanisms of *P. aeruginosa* and *A. baumannii* were not investigated in this study. Thus, future studies that investigate colistin resistance mechanism with a larger sample size of colistin-resistant isolates will ensure more definitive conclusions regarding the performance of the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test.

In conclusion, the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test is a rapid, cost-effective (at a cost of US$0.48 per test without the cost of labour as shown in Table 3) and easy-to-perform test that shows reliable performance in terms of categorical agreement and specificity but did not meet the acceptance criteria set by the ISO 20776-2:2007 due to VMEs. Since the Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP test showed good performance for detecting *A. baumannii* with MICs at different ends of susceptibility spectrum (i.e. 2 mg l^{-1} or lower for susceptible isolates; 16 mg l^{-1} or higher for resistant isolates), individual laboratories could consider using this test to screen for colistin resistance in *A. baumannii* isolates after a proper validation.

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Author contributions
H.J. conceptualization, formal analysis, investigation, writing – original draft, review and editing; J.D.D.P. writing – review and editing; B.C.M. investigation, writing – review and editing; K.S. resources, writing – review and editing; C.K. resources, writing – review and editing; J.C. resources, writing – review and editing; M.M.E. writing – review and editing; M.M.K. conceptualization, writing – review and editing, funding acquisition.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (Ethics reference no.: 671/2018).

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