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Evaluation of the Rapid Polymyxin NP Test for Polymyxin B Resistance Detection Using *Enterobacter cloacae* and *Enterobacter aerogenes* Isolates

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ABSTRACT Polymyxin resistance is an increasing problem worldwide. Currently, determining susceptibility to polymyxins is problematic and lengthy. Polymyxins diffuse poorly into agar, potentially giving inaccurate disk diffusion and Etest results. A rapid screening test (2 h) for the detection of polymyxin resistance in *Enterobacteriaceae*, developed by P. Nordmann and L. Poirel (rapid polymyxin NP test) in 2016, detects glucose metabolization in the presence of polymyxin E (PE) and PB via pH-induced color change. The sensitivity and specificity were 99.3 and 95.4%, respectively, with results obtained in ≤2 h. Our goal was to evaluate this test using PB against larger numbers of *Enterobacter*. A total of 143 nonduplicate *Enterobacter* isolates (102 *E. cloacae* complex, 41 *E. aerogenes*) were tested, including 136 collected from Ochsner Health System patients from March to May 2016 and 7 previously determined PB-resistant *E. cloacae* isolates from JMI Laboratories. MICs were determined via broth microdilution. For the rapid polymyxin NP test, a color change from orange to yellow is positive; a weak/no color change is deemed negative after 4 h. Of 143 *Enterobacter* isolates, 25 were determined to be PB resistant by broth microdilution (MIC > 2 μg/ml), including all 7 JMI isolates. Of these 25, 7 were positive by the rapid polymyxin NP test (included 3/7 JMI isolates). All 118 isolates determined to be PB susceptible by broth microdilution were NP test negative. The sensitivity and specificity for the rapid polymyxin NP test were 25 and 100%, respectively, compared to broth microdilution. Although the rapid polymyxin NP test is a much faster method (2 to 4 h) for polymyxin resistance determination compared to broth microdilution (16 to 20 h), our study indicates that it may be subject to limitations when testing *Enterobacter*.

KEYWORDS microbial drug resistance, *Enterobacter*, polymyxin B, drug resistance

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The increase in antimicrobial resistance has created significant challenges for appropriate treatment. Rapid diagnostics for adequate antibiotic stewardship of antibiotics are needed (1).

With the 2016 discovery of the first plasmid-mediated colistin (polymyxin E) resistance gene, *mcr-1*, reported in the United States (2), it is now more important than ever to develop rapid methods to detect resistance to the polymyxins, the antibiotics of last resort for many multidrug-resistant Gram-negative bacteria. Susceptibility testing of polymyxins is challenging and time-consuming. These drugs diffuse poorly into agar, potentially giving inaccurate disk diffusion and Etest results (3–5). In addition, heteroresistant subpopulations found in *Enterobacter* species can cause "skipped wells" in broth microdilution, as well as growth inside the zone of inhibition with disk diffusion and Etest methods, leading to uninterpretable results (6). A rapid screening test (≤4 h) for detection of polymyxin resistance in *Enterobacteriaceae*, developed by P. Nordmann and L. Poirel (rapid polymixin NP test), detects glucose metabolization in the presence

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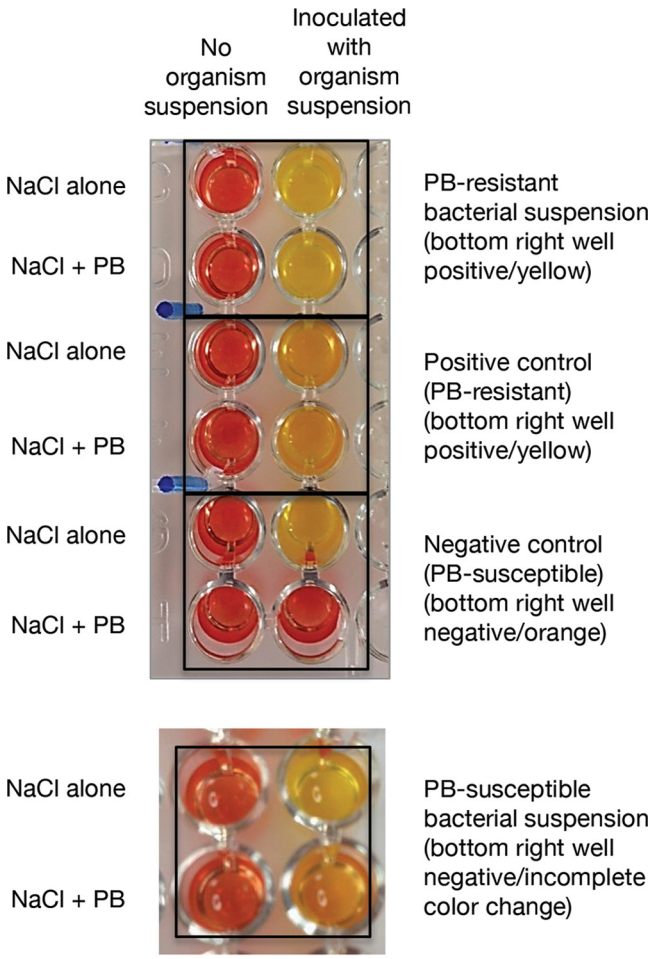

FIG 1 Results of the rapid polymyxin NP test. The first column consists of uninoculated wells (NaCl in place of isolate suspension) with or without polymyxin B (PB) in the indicator solution, and the second column consists of wells with the isolate suspension with or without polymyxin B in the indicator solution. Each square represents an individual test. The bottom square shows an incomplete color change in the bottom right corner (reported as a negative NP test or PB susceptible).

of a polymyxin via an orange-to-yellow color change of the pH indicator phenol red, signifying resistance to polymyxins (7). Their evaluation reported the test to be 99.3% sensitive and 95.4% specific for 200 *Enterobacteriaceae* isolates, including 22 *Enterobacter* (18 *Enterobacter cloacae*, 3 *E. aerogenes*, and 1 *E. asburiae*), compared to standard broth microdilution. Results were determined in <2 h. Nordmann and Poirel reported that their study was subject to several limitations, including the facts that (i) they did not assess the ability of the rapid polymyxin NP test to detect heteroresistant isolates with low polymyxin MICs (such isolates may be problematic to detect) and (ii) larger-scale studies by others were needed to fully evaluate the reliability of the new test's performance (7). The goal of the present study was to use this novel test with a larger collection of *Enterobacter* isolates, which are known to exhibit heteroresistance when performing susceptibility testing with polymyxins.

(Data from this study were presented at the 35th Annual Meeting of Southwestern Association of Clinical Microbiology, Fort Worth, TX, 7 to 10 September 2016.)

RESULTS

Rapid polymyxin NP tests were interpreted according to the original protocol: a color change from orange to yellow in the well containing the isolate suspension with polymyxin B indicated a positive result (polymyxin B resistance), and no or an incomplete color change indicated a negative result (polymyxin B susceptibility) (Fig. 1).

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TABLE 1 Polymyxin B broth microdilution and rapid polymyxin NP test results

PB broth microdilution (MIC)[a]	Rapid polymyxin NP test (no. of results)		
	Positive	Negative	Total
PB resistant, non-WT (>2 μg/ml)	7	18	25
PB susceptible, WT (≤2 μg/ml)	0	118	118
Total	7	136	143

[a]PB, polymyxin B; WT, wild type.

Isolates that yielded a positive NP test result and a broth microdilution polymyxin B MIC > 2 μg/ml or a negative NP test and a polymyxin B MIC ≤ 2 μg/ml were considered in agreement (Table 1). Using these interpretations, 25/143 *Enterobacter* isolates were determined to be polymyxin B resistant by broth microdilution (24 *E. cloacae* complex, 1 *E. aerogenes*), which included the 7 previously determined polymyxin B-resistant JMI *E. cloacae* isolates. Seven of the 25 polymyxin B-resistant isolates were also NP test positive: 6 *E. cloacae* complex and 1 *E. aerogenes* (included 3/7 JMI isolates). Of these isolates, positive NP test results were observed at 1 h ($n = 1$), at 2 h ($n = 2$), at 3 h ($n = 2$), and at 4 h ($n = 2$). However, the remaining 18 polymyxin B-resistant *E. cloacae* complex isolates were NP test negative (false-negative results). Broth microdilution tests and NP tests (using fresh reagents) were repeated in parallel for these polymyxin B-resistant/NP test-negative isolates, and the results matched those of the original tests. All 118 *Enterobacter* isolates that were susceptible to polymyxin B by broth microdilution (MIC ≤ 2 μg/ml) produced a negative rapid polymyxin NP test result. There were no false-positive NP test results. Skipped wells in broth microdilution (Fig. 2) were seen with 22 *E. cloacae* complex isolates, all of which were NP test negative, and 11 of which were polymyxin B resistant; these tests were repeated and yielded the same MIC results. For our study, the test sensitivity and specificity were 25 and 100%, respectively, compared to broth microdilution. This is markedly different from Nordmann and Poirel's original findings of 100% sensitivity and specificity for the 22 *Enterobacter* isolates included in their study. We speculate that this difference may be attributable to the larger number of *Enterobacter*, including possible heteroresistant isolates, tested in our study.

DISCUSSION

The new rapid polymyxin NP test may be subject to important limitations when testing *Enterobacter* species. We agree with Nordmann and Poirel's speculation that heteroresistant isolates may be problematic to detect. The skipped wells we observed, a phenomenon often seen with *Enterobacter* in broth microdilution testing (6), as well as our false-negative (negative NP test result but resistant by broth microdilution), could possibly be attributed to isolate cluster-dependent heteroresistance described by Guérin et al. (8). Testing to determine cluster association and the genetic mechanism of polymyxin B heteroresistance for these isolates was not performed. In addition, a weak or incomplete color change was observed in 15 isolates, 4 of which were determined to be polymyxin B resistant by broth microdilution (Fig. 1). All isolates with a weak or incomplete color change were determined to be negative upon

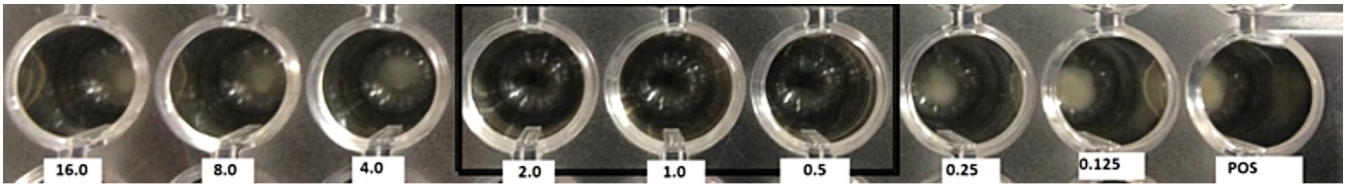

FIG 2 Broth microdilution wells in polymyxin B MIC (μg/ml) panel. The results represent the positive growth control (POS; rightmost well); growth in 0.125 and 0.25 wells; no growth in 0.5, 1.0, and 2.0 wells (which represents the skipped-well phenomenon); and growth in 4.0, 8.0, and 16.0 wells. The test was determined invalid and must be repeated when multiple skipped wells are present.

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repetition of the rapid NP test (no color change). Personal communication with P. Nordmann confirmed that any intermediate color change should be interpreted as a negative NP test result.

Furthermore, the polymyxin B-resistant *Enterobacter* isolates with a positive NP test result did not show the complete color change (positive result) until 3 to 4 h, compared to ≤2 h reported by Nordmann and Poirel. Warming of the NP indicator solution in a 37°C water bath during setup did not decrease the time in which results were seen. Overall, the rapid polymyxin NP test was easy to perform and yielded results much faster (3 to 4 h) than traditional broth microdilution (16 to 20 h). Our study indicates that this test may be subject to important limitations when testing *Enterobacter* species. Further testing with *Enterobacter* on a larger scale is needed.

MATERIALS AND METHODS

Bacterial isolates, media, and antimicrobial susceptibility testing. A total of 143 clinical *Enterobacter* isolates from individual patients (102 *E. cloacae* complex, 41 *E. aerogenes*) from urine (54%), wound/abscess (22%), respiratory system (7%), skin/tissue (5%), blood (3%), and other sources (9%) were tested. Isolates included 136 collected from Ochsner Health System patients from March to May 2016 and 7 genetically unique (as determined by Rep-PCR; Diversilab, bioMérieux, Inc., Durham, NC) polymyxin B-resistant *E. cloacae* isolates obtained from JMI Laboratories (North Liberty, IA). Identification of *Enterobacter* isolates was performed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker Daltonics, Inc., Billerica, MA). Isolates were subcultured to BBL Trypticase soy agar with 5% sheep blood (Becton-Dickinson Diagnostic Systems, Sparks, MD) and tested after overnight incubation at 35°C.

Broth microdilution was performed for determination of polymyxin B MICs in cation-adjusted Mueller-Hinton II broth (Becton-Dickinson Diagnostic Systems) according to Clinical and Laboratory Standards Institute guidelines (9). A direct colony suspension in Mueller-Hinton broth using 18 to 24 h growth was adjusted to the turbidity of a 0.5 McFarland standard (1×10^8 to 2×10^8 CFU/ml). The suspension was diluted with sterile saline, and the 96-well polystyrene panel MIC panel was inoculated to give a final concentration of approximately 5×10^5 CFU/ml in each well. Panels were incubated 16 to 20 h at 35°C in ambient air. Polymyxin B sulfate powder (Sigma-Aldrich Inc., St. Louis, MO) was used, and the concentration range tested was 0.125 to 128 μg/ml. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as control strains on each day of testing (10). In addition, two *E. coli* isolates (0346 and 0349), possessing the *mcr-1* gene for colistin resistance, were obtained from the FDA-CDC Antimicrobial Resistance Isolate Bank and included as controls. In the absence of polymyxin B interpretations for the *Enterobacteriaceae*, MICs were interpreted according to the colistin epidemiological cutoff value for *Enterobacteriaceae* of ≤2 μg/ml (10). Broth microdilution and the rapid NP test were performed on the same day using the same suspension of organism.

Rapid polymyxin NP test. According to the rapid polymyxin NP test protocol, a rapid polymyxin NP solution containing 6.25 g of cation-adjusted Mueller-Hinton II powder, 0.0125 g of phenol red (Sigma-Aldrich), and 225 ml of distilled water was prepared, and the solution was adjusted to a pH of 6.7 with 1 mol/liter HCl and then autoclaved at 121°C for 15 min. After cooling, 25 ml of filter-sterilized 10% anhydrous D(+)-glucose (Sigma-Aldrich) was added. The solution was made fresh each week, 250 ml each time, and it was prewarmed to 37°C before each use to prevent delays in bacterial growth and color change. Because of the volume of isolates being tested, the solution being used was kept warm in a water bath during setup of the tests. Stock solution of polymyxin B was added to a portion of the rapid polymyxin NP solution before each batch of isolates was tested, for a final polymyxin B concentration of 3.75 μg/ml in each applicable well. Each isolate tested was set up alongside uninoculated wells, using saline in place of a bacterial isolate suspension, with or without polymyxin B as built-in negative controls. In addition, a well containing the isolate suspension without polymyxin B was used as a built-in positive growth control. Bacterial suspensions were prepared in 10 ml of sterile 0.85% saline to obtain turbidity corresponding to a 3.0 to 3.5 McFarland standard. The final concentration of each isolate suspension per well was ~10^8 CFU/ml. The same procedure was used for the positive (*Proteus vulgaris* ATCC 6380) and negative (*E. coli* ATCC 25922) controls, which were included each day of testing. In addition, two *E. coli mcr-1* isolates (0346 and 0349) were included as controls. The tests were incubated uncovered in ambient air at 35°C and observed at 1, 2, 3, and 4 h for color changes.

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