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Colistin and Polymyxin B Minimal Inhibitory Concentrations Determined by Etest Found Unreliable for Gram-Negative Bacilli

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Background: A reliable method of polymyxin B and E (colistin) susceptibility testing remains elusive. These drugs diffuse poorly into agar, creating potentially inaccurate Etest and disk diffusion results, and testing by these methods is not recommended. Broth microdilution is the reference testing method, although it can be sometimes difficult to interpret. Currently, when a colistin susceptibility test is ordered for a patient in the Ochsner Health System, our diagnostic microbiology laboratory performs the Etest. As an in-house quality assessment project, we compared colistin and polymyxin B minimal inhibitory concentrations (MICs) determined by Etest with MICs determined by broth microdilution to evaluate whether colistin MICs are accurately being reported by Etest.

Methods: A total of 143 nonduplicate clinical isolates from Ochsner patients during 2015-2016 were tested: *Acinetobacter baumannii* (n=60), *Pseudomonas aeruginosa* (n=44), and Enterobacteriaceae (n=39) (13 *Escherichia coli*, 15 *Klebsiella* spp, and 11 *Enterobacter* spp). Colistin and polymyxin B MICs were determined by Etest and broth microdilution.

Results: Using broth microdilution, 16/143 (11%) isolates were nonsusceptible to colistin, and 12/143 (8%) were nonsusceptible to polymyxin B. With Etest, 4/143 (3%) isolates were nonsusceptible to colistin, and 7/143 (5%) were nonsusceptible to polymyxin B. Essential agreement of colistin and polymyxin B MICs between broth microdilution and Etest was 84/143 (59%) and 87/143 (61%), respectively. Categorical agreement for colistin and polymyxin B was 127/143 (89%) and 126/143 (88%), respectively.

Conclusion: We found a high rate of discrepancy between colistin and polymyxin B Etest and broth microdilution MICs. Very major errors (colistin/polymyxin B-susceptible by Etest, colistin/polymyxin B-resistant by broth microdilution) were detected in 10% of isolates tested with colistin and 8% of polymyxin B-tested isolates. The data from this study confirm that broth microdilution should be performed for susceptibility testing of polymyxins.

Keywords: *Colistin, gram-negative bacteria, polymyxin B*

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INTRODUCTION

Resistance to polymyxins is being increasingly detected worldwide, and an accurate method of susceptibility determination remains elusive. Colistin (polymyxin E) and polymyxin B diffuse poorly into agar plates, potentially resulting in inaccurate Etest and disk diffusion results.[1] In addition, the adsorption of the drug to plastic and glass testing materials and prevalence of heteroresistant subpopulations in organisms like *Enterobacter* and *Acinetobacter* spp can cause skipped wells and trailing endpoints in broth microdilution, rendering this reference method sometimes difficult to interpret.[2] Currently, our hospital microbiology laboratory performs only the colistin Etest when a colistin minimal inhibitory concentration (MIC) is requested for a clinical bacterial isolate, and the results are reported as "research use only." Polymyxin B susceptibility testing is not performed. The goal of our in-house quality assessment study was to compare colistin and polymyxin B MICs from Etest with MICs from broth microdilution to determine if colistin MICs are accurately being reported by Etest.

METHODS

Microorganisms and Media

A total of 143 nonduplicate isolates (26% wound/abscess, 30% respiratory, 13% blood, 21% urine, 5% skin/tissue, 6% other) collected and identified from patients in the Ochsner Health System during 2015-2016 were tested, all of which had a "research use only" reported colistin Etest MIC from the hospital microbiology laboratory. Of these isolates, 60 were *Acinetobacter baumannii,* 44 were *Pseudomonas aeruginosa*, and 39 were from the Enterobacteriaceae family (13 *Escherichia coli*, 15 *Klebsiella* spp, and 11 *Enterobacter*

spp). Identification of isolates was performed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics Inc.). All isolates were stored frozen at −70°C in Remel Columbia broth (Thermo Fisher Scientific, Inc.) with 20% glycerol until further testing. Media included cation-adjusted Mueller Hinton II broth and agar plates (for MIC testing), as well as trypticase soy agar with 5% sheep blood plates (for subculturing isolates) (Becton-Dickinson Diagnostic Systems).

Minimal Inhibitory Concentration (MIC) Determination

Colistin and polymyxin B MICs were determined by Etest (bioMérieux, Inc.) and broth microdilution. *E coli* ATCC 25922 and *P aeruginosa* ATCC 27853 were included as control strains for both broth microdilution and Etest.[3]

Etest

Colistin Etest MICs were performed on fresh clinical isolates in the hospital's clinical diagnostic microbiology laboratory, and the results were used for comparison with MICs determined by broth microdilution. Polymyxin B Etest MICs were performed in our research laboratory by preparing a bacterial suspension equivalent to a 0.5 McFarland in Mueller Hinton broth and inoculating onto Mueller Hinton II agar plates. Polymyxin B Etest strips were aseptically placed on each inoculated plate. MICs were read after 16-20 hours of incubation at 35°C in ambient air. Neither colistin nor polymyxin B Etest strips are approved by the US Food and Drug Administration for in vitro diagnostic susceptibility testing and are to be used for research use only.

Broth Microdilution

Broth microdilution was performed to determine polymyxin B and colistin MICs in cation-adjusted Mueller Hinton II broth according to Clinical and Laboratory Standards Institute (CLSI) guidelines[4] using colistin sulfate and polymyxin B sulfate powders (Sigma-Aldrich, Inc.). The broth microdilution panels were examined and results were interpreted after incubation for 16-20 hours at 35°C.

Breakpoints and Definitions

CLSI interpretive guidelines used for each antimicrobial tested are (μg/mL) as follows: for *P aeruginosa*, ≤2 susceptible, 4 intermediate, ≥8 resistant (polymyxin B) and ≤2 susceptible, ≥4 resistant (colistin) and for *Acinetobacter* spp, ≤2 susceptible, ≥4 resistant (for polymyxin B and colistin).[3] Because the CLSI provides no breakpoints for Enterobacteriaceae when testing colistin or polymyxin B, we used the European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC breakpoints for colistin for interpretation: ≤2 susceptible, >2 resistant.[5]

The MICs from each method were considered in essential agreement if MICs were ±1 twofold dilution (eg, if the MIC from one method is 2, the compared MIC must be in the range of 1-4) and in categorical agreement if results were in the same interpretive category (susceptible, intermediate, or resistant). Acceptable performance rates for essential and categorical agreement are ≥90%.[6] Very major errors were defined as organisms determined susceptible by Etest but found to be resistant by broth microdilution, with an acceptable performance rate of ≤3%. Major errors were defined as a susceptible result by broth microdilution but a resistant result by Etest. A minor error was a resistant or susceptible result by broth microdilution but an intermediate result by Etest or vice versa.[6] Testing of *P aeruginosa* with polymyxin B is the only opportunity for minor errors to occur because *P aeruginosa*/polymyxin B is the only organism/drug combination with an intermediate MIC interpretation. Because our collection of isolates consisted primarily of polymyxin-susceptible isolates, to avoid overestimation of major and very major errors, we used the total number of isolates as the denominator for error rate determination.

RESULTS

Using broth microdilution, 16/143 (11%) isolates were nonsusceptible to colistin, and 12/143 (8%) were nonsusceptible to polymyxin B. Colistin and polymyxin B MICs by broth microdilution were in agreement (±1 twofold dilution) for 132/143 (92%) of isolates and in categorical agreement (same interpretive category) for 139/143 (97%) of isolates. The 4 isolates not in categorical agreement were all *A baumannii*, colistin-resistant but polymyxin B-susceptible by broth microdilution.

With Etest, 4/143 (3%) isolates were nonsusceptible to colistin and 7/143 (5%) were nonsusceptible to polymyxin B.

Overall, the rate of discordance between Etest and broth microdilution was high with both colistin and polymyxin B. Essential agreement between methods was unacceptable (<90%) for colistin (59%) and polymyxin B (61%). Categorical agreement performance rates were found to be acceptable (≥90%) between methods when testing *P aeruginosa* with colistin (95%) and *A baumannii* with polymyxin B (92%), but all other organism/drug combinations had unacceptable categorical agreements (Table 1).

When isolates were tested with colistin by Etest, very major errors (ie, false susceptibility) were found in 10% (14/143) of isolates (or 88% false-susceptible results among the 16 resistant isolates by broth microdilution). For polymyxin B MICs determined by Etest, the overall very major error rate was 8% (11/143) (or 92% false-susceptible results among the 12 resistant isolates by broth microdilution). No very major errors were detected in the 44 *P aeruginosa* isolates (Tables 2 and 3).

DISCUSSION

Emerging bacterial resistance to most commonly used antibiotics has necessitated the increased use of polymyxins for treatment of multidrug-resistant gram-negative bacteria. With this resurgence of polymyxin usage, it is important to establish reliable, standardized, and reproducible susceptibility testing methods for these drugs. While broth microdilution and agar dilution have been established by CLSI as reference methods for susceptibility testing of the polymyxins,[4] the current standards and breakpoints are under review by CLSI and EUCAST with the Transatlantic Taskforce on Antimicrobial Resistance.[7] Broth microdilution has been shown to occasionally produce unreliable and unreadable MICs because of resistant bacterial subpopulations,[2] and agar dilution is time-consuming and infrequently used.[7] The Etest method of susceptibility determination is widely recognized as unreliable when used with polymyxins, as the large cationic peptides of polymyxins diffuse poorly in agar.[2] A number of studies have found that

Table 1. Colistin (COL) and Polymyxin B (PB) Isolates (n=143) Found Nonsusceptible (NS) by Etest and Broth Microdilution (BMD) and Essential and Categorical Agreement Between Minimal Inhibitory Concentrations by Organism for Each Method

Organism	# COL-NS by Etest (% R)	# COL-NS by BMD (% R)	Essential Agreement (%)	Categorical Agreement (%)	# PB-NS by Etest (% I or R)	# PB-NS by BMD (% R)	Essential Agreement	Categorical Agreement
Enterobacteriaceae (n=39)	1/39 (3) R	7/39 (18) R	22/39 (56)	33/39 (85)	1/39 (3) R	7/39 (18) R	17/39 (44)	33/39 (85)
Pseudomonas aeruginosa (n=44)	2/44 (5) R	0/44 (0)	21/44 (48)	42/44 (95)	6/44 (14) I/R	0/44 (0)	26/44 (59)	38/44 (86)
Acinetobacter baumannii (n=60)	1/60 (2) R	9/60 (15) R	41/60 (68)	52/60 (87)	0/60 (0)	5/60 (8) R	44/60 (73)	55/60 (92)

I, intermediate; R, resistant.

comparison of broth microdilution and Etest methods for polymyxins produces unacceptably high rates of very major errors, with some as high as 39.3%,[8] and that concordance between Etest and the reference method of choice is often below the acceptable standard of ≥90%.[8,9] Tan and Ng found that most major errors occurred with *P aeruginosa* and that varying the incubation time by as little as 4 hours can have an impact on Etest MICs with these organisms.[9] We speculate that the higher readings we observed with Etest compared to broth microdilution with *P aeruginosa* could be attributed to the reading of Etest plates during variable times throughout the 16-20 hour window recommended by the manufacturer, as well as the spreading colony morphology that could have been inadvertently

Table 2. Error Distribution by Organism, Type of Error, and Drug Tested

No.	Organism	Colistin Etest MIC	Colistin BMD MIC	Error Type	Polymyxin B Etest MIC	Polymyxin B BMD MIC	Error Type
3	*Enterobacter cloacae*	0.19 (S)	>128 (R)	VME[a]	1.5 (S)	>128 (R)	VME
11	*Klebsiella pneumoniae*	0.25 (S)	16 (R)	VME	1 (S)	8 (R)	VME
13	*Enterobacter cloacae*	0.25 (S)	>128 (R)	VME	0.5 (S)	>128 (R)	VME
16	*Enterobacter aerogenes*	0.125 (S)	8 (R)	VME	0.5 (S)	8 (R)	VME
35	*Escherichia coli*	0.25 (S)	>128 (R)	VME	0.5 (S)	>128 (R)	VME
37	*Klebsiella pneumoniae*	0.5 (S)	>128 (R)	VME	0.75 (S)	>128 (R)	VME
A16	*Acinetobacter baumannii*	0.25 (S)	4 (R)	VME			
A18	*Acinetobacter baumannii*	0.19 (S)	16 (R)	VME			
A22	*Acinetobacter baumannii*	0.25 (S)	4 (R)	VME	0.5 (S)	6 (R)	VME
A23	*Acinetobacter baumannii*	0.19 (S)	4 (R)	VME	0.5 (S)	4 (R)	VME
A29	*Acinetobacter baumannii*	0.25 (S)	>128 (R)	VME	0.75 (S)	32 (R)	VME
A30	*Acinetobacter baumannii*				1 (S)	4 (R)	VME
A35	*Acinetobacter baumannii*	0.25 (S)	4 (R)	VME			
A45	*Acinetobacter baumannii*	0.75 (S)	4 (R)	VME			
A64	*Acinetobacter baumannii*	1.5 (S)	32 (R)	VME	1 (S)	16 (R)	VME
P4	*Pseudomonas aeruginosa*				4 (I)	1 (S)	mE[b]
P8	*Pseudomonas aeruginosa*	4 (R)	1 (S)	ME[c]	3 (I)	0.5 (S)	mE
P32	*Pseudomonas aeruginosa*				6 (R)	0.5 (S)	ME
P33	*Pseudomonas aeruginosa*				6 (R)	0.5 (S)	ME
P39	*Pseudomonas aeruginosa*	3 (R)	0.5 (S)	ME	3 (I)	1 (S)	mE
P45	*Pseudomonas aeruginosa*				8 (R)	1 (S)	ME

BMD, broth microdilution; I, intermediate; ME, major error; mE, minor error; MIC, minimal inhibitory concentration; R, resistant; S, susceptible; VME, very major error.

Note: MIC values are in μg/mL.

[a]Very major error (VME) is defined as a false-susceptible result.

[b]Minor error (mE) is defined as a resistant or susceptible result by one method but an intermediate result by another method and can only be determined in polymyxin B testing with *Pseudomonas aeruginosa*.

[c]Major error (ME) is defined as a false-resistant result.

Table 3. Error Rates for Etest Compared to Broth Microdilution for Each Drug Tested

Organism Tested	No. Tested	Minor Error (% of total)	Major Error (% of total)	Very Major Error (% of total)
Colistin				
Enterobacteriaceae	39	0	0	6 (15)
Pseudomonas aeruginosa	44	0	2 (5)	0
Acinetobacter baumannii	60	0	0	8 (13)
Total	143	0	2 (1)	14 (10)
Polymyxin B				
Enterobacteriaceae	39	0	0	6 (15)
Pseudomonas aeruginosa	44	3 (7)	3 (7)	0
Acinetobacter baumannii	60	0	0	5 (8)
Total	143	3 (2)	3 (2)	11 (8)

Note: Minor error is defined as a resistant or susceptible result by one method but an intermediate result by another method and can only be determined in polymyxin B testing with *Pseudomonas aeruginosa*. Major error is defined as a false-resistant result. Very major error is defined as a false-susceptible result.

interpreted as a slightly higher MIC. Despite the problems associated with broth microdilution and heteroresistance, we found that the broth microdilution method more reliably identified resistant populations in our isolate collection and therefore should be used in lieu of Etest.

CONCLUSION

Our study highlights a high rate of discrepancy between polymyxin susceptibility test methods. Etest very major error rates for colistin were 10% overall (88% false susceptible results among the 16 resistant isolates by broth microdilution) and 8% overall for polymyxin B (92% false susceptible results among the 12 resistant isolates by broth microdilution). These data strongly support other published findings that any colistin or polymyxin B MIC should not be determined by Etest (because of major problems with false susceptibilities) and results should not be reported for clinical use. If polymyxin susceptibility is requested, testing by broth microdilution should be performed.

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