Susceptibility Testing of Isolates Displaying Elevated Colistin MIC Values Using Polysorbate-80 (P-80)

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M CASTANHEIRA, JM STREIT, RR DIETRICH, RN JONES
JMI Laboratories, North Liberty, Iowa

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JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370, fax 319.665.3371
mariana-castanheira@jmilabs.com

AMENDED ABSTRACT

Background: Susceptibility (S) testing of polymyxins is challenging and the differentiation of resistant and S strains, difficult to read endpoints due to skipping and lack of consistent guidelines contribute to the problem testing these compounds increasingly used in the clinical setting. We tested a collection of strains displaying elevated colistin (Co) MICs using variations of the reference CLSI broth microdilution method, including ± P-80.

Methods: During 2012, 15,487 isolates from the following genus/species were submitted to the SENTRY Program: *A. baumannii* (ACB; n=1,167), *Citrobacter* spp. (CBSP; 808), *E. coli* (EC; 5,705), *K. oxytoca* (KOX; 648), *K. pneumoniae* (KPN; 3,266) and *P. aeruginosa* (PSA; 3,893). Isolates were initially S tested by CLSI reference method using dry-from (DF) panels and isolates showing elevated Co MIC values (>2 µg/ml for Enterobacteriaceae [ENT] and >4 µg/ml for ACB and PSA) were re-tested in frozen-form (FF) panels against Co ± P-80 and polymyxin B (PB) ± P-80. All isolates displaying elevated Co MICs had identification confirmed by MALDI-TOF MS.

Results: Co elevated MIC values were observed among 4.4% for ACB (21 isolates displayed lower [<4 µg/ml] upon re-testing), 1.6% for CBSP, 0.7% for EC, 0.9% for KOX, 4.4% for KPN and 0.4% for PSA in initial tests. Compared to Co FF, 122 (45.2%) of the isolates had the same or 1 dilution greater MICs for Co+P-80 and 43 (16.0%), 35 (13.0%), 45 (16.8%), 22 (8.2%) and 2 (0.7%) isolates had MICs with P-80 lower by two-, four-, eight-, 16- and 32-fold when compared to Co FF, respectively. All isolates displaying >eight-fold decrease in the presence of P-80 were ACB and ENT. Co DF displayed 59.3% results concordant to Co FF and another 87 (32.5%) results within the ± two-fold difference, considered acceptable. 165 results for PB FF + P-80 were identical or ± two-fold of PB alone. 29, 40, 25 and 6 isolates had four-, eight-, 16-, and 32-fold lower PB + P-80 MICs compared to PB, respectively. Among 60 KPN from the USA, 40 were carbapenem-resistant and 21 were KPC-positive.

Conclusions: We demonstrated that in this collection of isolates displaying elevated Co MIC values, some could not be confirmed upon re-testing, and those were mainly ACB and ENT. Overall, P-80 decreased MIC results, including numerous isolates that have high MIC values for both polymyxins using different testing methods that do not include P-80.

INTRODUCTION

The polymyxins are polypeptides that have activity against a wide variety of Gram-negative bacilli, including Enterobacteriaceae and non-fermentative species. The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter* spp. and especially carbapenem-resistant Enterobacteriaceae (CRE), including KPC-producing isolates has required the expanded systemic use of these antimicrobial agents. As polymyxins (colistin and polymyxin B) usage increases, the development of polymyxin resistance becomes a clinical concern and standardization of an accurate susceptibility testing method for these compounds is urgently needed.

Polysorbate-80 (P-80; also known as Tween-80) is a surfactant widely employed as a dispersing agent in the preparation of broth microdilution panels used in susceptibility testing; however, its' use is controversial and various studies have been attempting to demonstrate if surfactant use would be beneficial or not for testing the polymyxins. We evaluate the effect of the addition of P-80 (0.02%) to Mueller-Hinton broth (MHB) when testing colistin and polymyxin B by the broth microdilution method using frozen-form panels and comparing to dry-form validated broth microdilution panels for a collection of Gram-negative bacilli initially displaying elevated MIC values for colistin by broth microdilution using dry-form panels. Susceptibility testing was repeated using reference frozen-form broth microdilution panels for colistin and polymyxin B with and without P-80 and results were compared for 268 isolates of Enterobacteriaceae (n=202), *A. baumannii* (n=51) and *P. aeruginosa* (n=15).

MATERIALS AND METHODS

Bacterial isolates. Clinical isolates submitted to the SENTRY Antimicrobial Surveillance Program during 2012 of the following bacterial species/genus: *A. baumannii* (n=1,167), *Citrobacter* spp. (808), *E. coli* (5,705), *K. oxytoca* (648), *K. pneumoniae* (3,266) and *P. aeruginosa* (3,893) and displaying elevated colistin MIC values were further evaluated. Isolates were initially susceptibility tested using validated dry-form broth microdilution panels produced by ThermoFisher Scientific (formerly TREK Diagnostics, Cleveland, Ohio, USA). Enterobacteriaceae isolates displaying MIC values >2 µg/ml and *A. baumannii* or *P. aeruginosa* displaying MIC values at >4 µg/ml for) were re-tested. Bacterial identification of these selected organisms was confirmed using the MALDI-TOF Biotyper (Bruker Daltonics, Billerica, Massachusetts, USA) according to the manufacturer instructions. Only one isolate per patient per episode that was deemed by the participant investigator to be the cause of infection was included in the surveillance study.

Susceptibility testing. Isolates were susceptibility tested using the reference broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) against colistin and polymyxin B (acquired through United States Pharmacopeia [USP]) with and without P-80 (Sigma-Aldrich, St. Louis, Missouri, USA). P-80 was used at panel preparation by adding 0.02% to Mueller-Hinton broth used to dilute antimicrobial solutions. Dry-form testing was repeated using the same bacterial cell inocullum used for inoculation of reference methods and other modifications. Results were compared with reference broth microdilution method without P-80 and essential agreement (EA) was considered to be within ± one $\log_2$ dilution step. Quality control (QC) was performed using *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents.

RESULTS

- A total of 202 Enterobacteriaceae displayed elevated colistin MIC values (>2 µg/ml) on the initial testing and among these isolates were: 143 *K. pneumoniae*, 40 *E. coli*, 13 *Citrobacter* spp. and 6 *K. oxytoca*. Colistin MIC values were reproducibly elevated upon re-testing using reference broth microdilution method for 140 (69.3% of the initial isolates) isolates of which, 112 (78.3%) were *K. pneumoniae* (**Table 1**).
- Among 51 *A. baumannii* and 15 *P. aeruginosa* initially displaying elevated colistin MIC results (>4 µg/ml), 30 (58.8%) *A. baumannii* and only four (26.6% of the initial numbers) *P. aeruginosa* had elevated colistin MIC values upon re-testing by reference frozen-form broth microdilution method (**Table 1**).
- A total of 27.7 to 30.7% of the Enterobacteriaceae isolates displayed MIC values at ≤2 µg/ml for colistin or polymyxin B when re-tested using frozen-form and/or dry-form methods. Interestingly, 19.6 to 23.1% of the *K. pneumoniae* strains displayed lower results. Colistin results of ≤2 µg/ml were observed for 41.2 to 56.9% *A. baumannii* and 60.0 to 73.3% *P. aeruginosa* upon re-testing depending on the confirmation method used (**Table 1**).
- The $MIC_{50/90}$ values using P-80 were at least two-fold lower when compared to the reference method for *E. coli* (2/4 and 4/8 µg/ml colistin with and without P-80, respectively), *A. baumannii* (0.5/>8 vs. 8/>8 µg/ml for colistin and 1/8 vs. 4/>8 µg/ml for polymyxin B) and *P. aeruginosa* (2/>8 vs. 4/>8 µg/ml for colistin and 1/>8 vs. 4/>8 µg/ml for polymyxin B). Overall, for *K. pneumoniae* all $MIC_{50/90}$ were ≥8 µg/ml and polymyxin B ± P-80 displayed identical $MIC_{50/90}$ results.
- The comparison of the various methods employed is displayed in **Table 2**. An acceptable method variation of ± one $\log_2$ dilution was noted for 85.7 to 90.1% of the isolates for frozen- versus dry-form results for colistin; and EA was lower for *P. aeruginosa* and greater for *A. baumannii*. *K. pneumoniae* isolates displayed 90.0% EA among these methods.
- Colistin and polymyxin B displayed excellent EA, ranging from 92.1% for *A. baumannii* to 100.0% for *P. aeruginosa*. Enterobacteriaceae isolates displayed an overall EA of 96.6% for these two compounds.
- As expected, the use of P-80 in the testing of these two polymyxins lowered the MIC values for all organisms groups tested. For colistin, only 27.4% of the *A. baumannii* displayed MIC values within ± one $\log_2$ dilution (**Table 2**); and 19.6% of the isolates tested had MIC results >4 µg/ml (**Table 1**). Percentage of EA ranged from 60.1 to 65.8% for other organisms (**Table 2**).
- Percentage of EA for polymyxin B ± P-80 ranged from 33.3% for *P. aeruginosa* to 76.9% for *K. pneumoniae* (**Table 2**).

Table 1. MIC distribution for colistin and polymyxin B by different testing methods for isolates initially displaying elevated colistin results when tested in dry-form panels.

Organism/group	No. of isolates at MIC (µg/ml; cumulative %)									MIC (µg/ml)	
Antimicrobial agent/method	≤0.06	0.12	0.25	0.5	1	2	4	8	>8	50%	90%
All Enterobacteriaceae (202)											
Colistin frozen-form	--	--	6 (3.0)	24 (14.9)	16 (22.8)	16 (30.7)	41 (51.0)	22 (61.9)	77 (100.0)	4	>8
Colistin dry-form	--	--	31 (15.3)	12 (21.3)	3 (22.8)	14 (29.7)	27 (43.1)	26 (55.9)	89 (100.0)	8	>8
Colistin frozen-form +P-80	32 (15.8)	9 (20.3)	9 (24.8)	4 (26.7)	14 (33.7)	25 (46.0)	28 (59.9)	14 (66.8)	67 (100.0)	4	>8
Polymyxin B frozen-form	--	--	--	16 (7.9)	24 (19.8)	15 (27.2)	47 (50.5)	29 (64.9)	71 (100.0)	4	>8
Polymyxin B frozen-form +P-80	18 (8.9)	19 (18.3)	14 (25.2)	5 (27.7)	9 (32.2)	22 (43.1)	29 (57.4)	25 (69.8)	61 (100.0)	4	>8
***K. pneumoniae* (143)**											
Colistin frozen-form	--	--	3 (2.1)	9 (8.4)	10 (15.4)	9 (21.7)	19 (35.0)	19 (48.3)	74 (100.0)	>8	>8
Colistin dry-form	--	--	15 (10.5)	4 (13.3)	2 (14.7)	12 (23.1)	10 (30.1)	18 (42.7)	82 (100.0)	>8	>8
Colistin frozen-form +P-80	15 (10.5)	5 (14.0)	5 (17.5)	2 (18.9)	9 (25.2)	11 (32.9)	19 (46.2)	13 (55.2)	64 (100.0)	8	>8
Polymyxin B frozen-form	--	--	--	9 (6.3)	11 (14.0)	8 (19.6)	26 (37.8)	21 (52.4)	68 (100.0)	8	>8
Polymyxin B frozen-form +P-80	6 (4.2)	11 (11.9)	7 (16.8)	3 (18.9)	8 (24.5)	8 (30.1)	19 (43.4)	23 (59.4)	58 (100.0)	8	>8
***E. coli* (40)**											
Colistin frozen-form	--	--	2 (5.0)	7 (22.5)	2 (27.5)	5 (40.0)	21 (92.5)	3 (100.0)	--	4	4
Colistin dry-form	--	--	8 (20.0)	2 (25.0)	1 (27.5)	1 (30.0)	17 (72.5)	7 (90.0)	4 (100.0)	4	8
Colistin frozen-form +P-80	11 (27.5)	0 (27.5)	2 (32.5)	1 (35.0)	3 (42.5)	14 (77.5)	8 (97.5)	1 (100.0)	--	2	4
Polymyxin B frozen-form	--	--	--	4 (10.0)	6 (25.0)	4 (35.0)	18 (80.0)	8 (100.0)	--	4	8
Polymyxin B frozen-form +P-80	7 (17.5)	3 (25.0)	5 (37.5)	0 (37.5)	1 (40.0)	13 (72.5)	9 (95.0)	2 (100.0)	--	2	4
***A. baumannii* (51)**											
Colistin frozen-form	--	--	--	6 (11.8)	8 (27.5)	5 (37.3)	2 (41.2)	12 (64.7)	18 (100.0)	8	>8
Colistin dry-form	--	--	--	4 (7.8)	7 (21.6)	7 (35.3)	5 (45.1)	5 (54.9)	23 (100.0)	8	>8
Colistin frozen-form +P80	5 (9.8)	7 (23.5)	7 (37.3)	10 (56.9)	4 (64.7)	3 (70.6)	4 (78.4)	1 (80.4)	10 (100.0)	0.5	>8
Polymyxin B frozen-form	--	--	--	--	10 (19.6)	6 (31.4)	13 (56.9)	13 (82.4)	9 (100.0)	4	>8
Polymyxin B frozen-form +P-80	2 (3.9)	11 (25.5)	5 (35.3)	7 (49.0)	7 (62.7)	6 (74.5)	3 (80.4)	6 (92.2)	4 (100.0)	1	8
***P. aeruginosa* (15)**											
Colistin frozen-form	--	--	--	--	4 (26.7)	2 (40.0)	5 (73.3)	0 (73.3)	4 (100.0)	4	>8
Colistin dry-form	--	--	--	--	7 (46.7)	0 (46.7)	3 (66.7)	0 (66.7)	5 (100.0)	4	>8
Colistin frozen-form +P-80	--	--	4 (26.7)	2 (40.0)	1 (46.7)	2 (60.0)	1 (66.7)	2 (80.0)	3 (100.0)	2	>8
Polymyxin B frozen-form	--	--	--	--	3 (20.0)	3 (40.0)	3 (60.0)	2 (73.3)	4 (100.0)	4	>8
Polymyxin B frozen-form +P-80	--	--	4 (26.7)	3 (46.7)	1 (53.3)	2 (66.7)	2 (80.0)	1 (86.7)	2 (100.0)	1	>8

CONCLUSIONS

- EA for reference broth microdilution method using frozen-form panels was very acceptable when compared to dry-form panels (upon repeat testing) for colistin. Additionally, colistin and polymyxin B also displayed very good correlation for all organism groups analyzed.
- Addition of P-80 to the media lowered colistin and polymyxin B MIC results. However, the effect varied by species, strain and between the two polymyxins tested; this requires further study.
- The lack of reproducibility of colistin MIC results from initial to repeat testing may be related to the adaptative nature of polymyxin resistance mechanisms, mainly in non-fermentative organisms such as *A. baumannii* and *P. aeruginosa*. Results for *K. pneumoniae* isolates seemed more stable over time.

Table 2. Variation in colistin and polymyxin B results for dry-form panels and frozen-form broth microdilution using P-80 when compared with reference CLSI method. Values within the acceptable variation of ±1 $\log_2$ dilution are highlighted in blue.

Methods compared/Organism/ Organism group (no. tested)	% of isolates by $\log_2$ dilution variation in MIC results							
	-4	-3	-2	-1	0	+1	+2	+3
Colistin frozen-form vs. Colistin dry-form								
Enterobacteriaceae (202)	0.5	1.0	5.0	18.8	50.5	18.8	4.5	1.0
K. pneumoniae (143)	0.7	1.4	4.2	15.4	55.9	16.8	4.9	0.7
E. coli (40)			2.5	20.0	40.0	30.0	5.0	2.5
A. baumannii (51)			3.9	7.8	58.8	23.5	3.9	2.0
P. aeruginosa (15)				14.3	71.4	0.0	14.3	
Colistin frozen-form vs. Colistin frozen-form + P-80								
Enterobacteriaceae (202)	5.4	16.3	11.9	16.8	47.0	2.0	0.5	
K. pneumoniae (143)	4.9	14.0	9.8	11.2	56.6	2.8	0.7	
E. coli (40)	7.5	20.0	10.0	40.0	22.5			
A. baumannii (51)	25.5	25.5	21.6	7.8	19.6			
P. aeruginosa (15)		6.7	33.3	26.7	26.7	6.7		
Colistin frozen-form vs. Polymyxin B frozen-form								
Enterobacteriaceae (202)			1.5	10.4	63.4	22.8	1.5	0.5
K. pneumoniae (143)			2.1	12.7	70.4	14.1		0.7
E. coli (40)				7.5	50.0	40.0	2.5	
A. baumannii (51)			2.0	35.3	39.2	17.6	3.9	2.0
P. aeruginosa (15)					80.0	20.0		
Colistin frozen-form vs. Polymyxin B frozen-form + P-80								
Enterobacteriaceae (202)	0.5	3.0	14.9	11.4	22.3	42.6	5.0	0.5
K. pneumoniae (143)	2.8	11.9	9.1	18.9	51.0	5.6	0.7	
E. coli (40)	7.5	17.5	10.0	37.5	25.0	2.5		
A. baumannii (51)	19.6	27.5	27.5	17.6	7.8			
P. aeruginosa (15)	6.7	6.7	46.7	13.3	20.0	6.7		
Polymyxin frozen-form vs. Polymyxin B frozen-form + P-80								
Enterobacteriaceae (202)	8.4	14.4	7.9	20.8	46.0	2.0	0.5	
K. pneumoniae (143)	4.9	10.5	7.0	16.1	58.0	2.8	0.7	
E. coli (40)	17.5	12.5	10.0	45.0	15.0			
A. baumannii (51)	25.5	23.5	15.7	21.6	13.7			
P. aeruginosa (15)	6.7	6.7	53.3	13.3	20.0			

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