D-1907

Quality Control Guidelines for Broth Microdilution and Disk Diffusion Susceptibility Testing for Polymyxin B and Colistin

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ICAAC 2004
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ABSTRACT

Background: The emergence of multi-drug resistant *P. aeruginosa* (PSA) and *Acinetobacter* spp. has restored the potential therapeutic indication for the parenteral use of polymyxin B and colistin. Consequently, there is a need for reliable susceptibility testing and quality control (QC) ranges, since no guidelines are provided by the NCCLS.
Methods: This study followed M23-A2, M7-A6 and M2-A8 NCCLS documents. The study used 8 laboratories, each testing 2 ATCC strains (*E. coli* 25922 and PSA 27853) in 4 cation-adjusted Mueller-Hinton (MH) broth lots, on 3 MH agar lots and applying 2 disk lots for each drug. For disk diffusion (DD), each site used 3 agar lots generating 2 zone diameters for each of the 10 replicates (480 values); while for broth microdilution (BMD) each laboratory generated 40 results (10 replicates X 4 MH broth lots), yielding 280 MIC values. Concurrent testing of gentamicin (DD only) and tetracycline (DD and BMD) were used as internal QC drugs with 720 results generated by DD and 320 by BMD methods.
Results: The results are summarized in the Table:

	MIC (µg/ml)		Zone diameters (mm)	
Drug/QC organism	Proposed range	% in range	Proposed range	% in range
Polymyxin B				
E. coli	0.25-2	100.0	13-19	99.8
PSA	0.25-2	99.4	14-18	97.9
Colistin				
E. coli	0.25-1	100.0	11-17	100.0
PSA	0.25-2	100.0	11-17	100.0

For the internal QC tests, 99.4% of DD and 100.0% of MIC results were within NCCLS published ranges.
Conclusions: Intra- and inter-laboratory variations of DD and BMD results were within acceptable limits for both QC evaluations. A slight variation on the results for the MH lots was noted for both DD and BMD. Also, the colistin disk lots differed in their modal zone diameter by only 1 mm.

INTRODUCTION

The polymyxin class antimicrobials (colistin and polymyxin B) are polycationic peptides that were originally synthesized from *Bacillus polymyxus*. The mechanism of action for the polymyxins is a surfactant-like effect that causes enhanced permeability of the bacterial cytoplasmic membrane leading to bacterial death. These agents were first described over five decades ago and were initially applied to the therapy of gram-negative bacilli before the discovery of other broad-spectrum agents such as the aminoglycosides, carboxypenicillins and cephalosporins. Toxicity issues and the emergence of alternative antimicrobial regimens resulted in the elimination of colistin and polymyxin B from NCCLS interpretive category and quality control (QC) tables in the early 1980's, although polymyxin B continued to be used in topical over-the-counter, triple-antibiotic ointment (neomycin-polymyxin B-bacitracin) preparations.

Recently the occurrence of multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. in several nations in epidemic proportions has necessitated the reconsideration of polymyxin therapies, and the subsequent need for accurate susceptibility testing by reference and standardized methods. Contemporary updates on polymyxin pharmacokinetics and pharmacodynamics have also been published. This report describes the multi-laboratory trial results to establish colistin and polymyxin B QC ranges for disk diffusion and MIC methods using a study design published in the NCCLS M23-A2 document.

MATERIALS AND METHODS

An eight laboratory QC study group was organized for the development of MIC and disk diffusion QC guidelines for the two polymyxins. The QC group consisted of laboratories at the Centers for Disease Control and Preventions (CDC; Atlanta, Georgia, USA); University of Alberta (Edmonton, Alberta, Canada); The Cleveland Clinic Foundation (Cleveland, Ohio, USA); University of Texas Medical Center (Houston, Texas, USA); University of Rochester Medical Center (Rochester, New York, USA); Denver Health Medical Center (Denver, Colorado, USA); University of Washington (Seattle, Washington, USA); and JMI Laboratories (North Liberty, Iowa, USA). Each laboratory followed a protocol based on the NCCLS M23-A2 document as well as procedural details found in the M2-A8 and M7-A6 test methods.

The MIC study utilized frozen-form, reference broth microdilution panels prepared by the CDC (Lot AC-6). The panels contained four lots of cation-adjusted Mueller-Hinton broth (Difco, Detroit, Michigan, USA [two lots #2198184 and #0325004]; Oxoid, Hampshire, United Kingdom [one lot; #258631]; BBL, Sparks, Maryland, USA [one lot, #2218968]) and colistin sulfate, polymyxin B, and tetracycline (Sigma Chemical Co., St. Louis, Missouri, USA). Colistin MICs were tested using reagent grade colistin sulfate (Sigma Chemical Co., St. Louis, Missouri, USA). Tetracycline was used as the MIC control agent. Each laboratory tested *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, generating 320 MIC QC results for each drug and organism. Colony counts were performed from the broth microdilution trays by subculturing in a quantitative manner onto drug-free solid media. The counts ranged from 1.6 x 10^5 to 8.0 x 10^5 CFU/ml and averaged 5.0 x 10^5 CFU/ml for all participating laboratories (target inoculum, 5.0 x 10^5 CFU/ml). All control MIC values were within those ranges published in M100-S14.

Similarly, the disk diffusion tests were performed by the NCCLS M2-A8 method using three lots of Mueller-Hinton agar (BBL [two lots; #4014660 and #4021062] and Remel, Lenexa, Kansas, USA [one lot; #403187]). Two lots of disks were utilized versus each QC strain: colistin (10-µg; BBL lot #3119600 and Remel lot #281526) and polymyxin B (300-U; BBL lot #3209907 and Remel lot #290537). Single lots of gentamicin (10-µg) and tetracycline (30-µg) disks were applied as control agents. A total of 720 control zone diameters were generated, and 99.4% of reported results were within NCCLS QC ranges. All out-of-control results were repeated before analysis.

The study followed the NCCLS guidelines with the eight sites producing 320 MIC values for each polymyxin agent against the two QC strains. The total zone diameters generated for each polymyxin and QC organism was 480. The number of results produced was significantly greater than the minimal criteria specified for each method by the NCCLS. Analyses of data to determine MIC range limits were dictated by the NCCLS guideline, M23-A2. Selected ranges included 97.9 - 100.0% and 99.4 - 100.0% of participant results for the disk diffusion and broth microdilution tests, respectively. All proposed QC ranges were further optimized to encompass ≥ 95% of all reported results, as recommended by NCCLS M23-A2 guideline.

RESULTS

- Table 1 lists the distribution of results (zones of inhibition or MIC values) for both polymyxins tested against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. For the disk diffusion method, 5 or 7 mm zone ranges were calculated using the medians methods.
- For the disk diffusion test, 5 or 7 mm QC zone diameter ranges were calculated using the medians method.
  - Using *E. coli* ATCC 25922, the colistin QC range was proposed at 11 - 17 mm which incorporated 100.0% of participant results (Table 2); and for polymyxin B, the range proposed was 13 - 19 mm (99.8%).
  - Using *P. aeruginosa* ATCC 27853, the colistin range was proposed at 11 - 17 mm (includes 100.0% of reported zones); and the polymyxin B range was 14 - 18 mm (97.9%).
- The disk product package insert for *E. coli* ATCC 25922 suggests a 12 - 16 mm QC range for polymyxin B that would have only included 71.7% of zones reported in this study. Similarly for colistin, the suggested range for ATCC 25922 was 11 to 15 mm which would only encompass 84.0% of the study results. Prior published reports have also questioned the available QC ranges for the polymyxins [Gales et al., 2001].

Table 1. Distribution of quality control (QC) MIC and zone diameter results among participants in the polymyxin B and colistin (sulfate) study.

	Occurrences by QC strain:			
	E. coli ATCC 25922		*P. aeruginosa* ATCC 27853	
Method/result	Colistin	Polymyxin B	Colistin	Polymyxin B
Disk diffusion (mm)				
11	1[a]	0	4[a]	0
12	13[a]	0	46[a]	0
13	147[a]	0[a]	148[a]	0
14	154[a]	22[a]	160[a]	4[a]
15	88[a]	209[a]	95[a]	56[a]
16	66[a]	113[a]	25[a]	191[a]
17	11[a]	84[a]	2[a]	146[a]
18	0	38[a]	0	73[a]
19	0	13[a]	0	10
20	0	1	0	0
MIC (µg/ml)				
0.25	71[b]	43[b]	2[b]	0[b]
0.5	188[b]	135[b]	135[b]	100[b]
1	61[b]	135[b]	170[b]	146[b]
2	0	5[b]	13[b]	74[b]
4	0	2	0	0

a. Proposed ranges that included 97.9 - 100.0% of reported zone diameters from eight laboratories.
b. Proposed ranges of MIC values that included 99.4 - 100.0% of all reported results.

Table 2. Inter- and intra-laboratory comparisons of the colistin zone diameter results versus *E. coli* ATCC 25922 for an eight medical center protocol meeting the study design guidelines found in NCCLS M23-A2.

	Laboratory code (occurrences):								
Zone diameter (mm)	A	B	C	D	E	F	G	H	Total
11					1				1[a]
12				2	8			3	13[a]
13	1	20	23	21	24	14	18	26	147[a]
14	29	18	17	18	14	15	20	23	154[a]
15	10	11	11	11	7	19	11	8	88[a]
16	13	10	8	8	6	11	10		66[a]
17	7	1	1			1	1		11[a]
Total	60	60	60	60	60	60	60	60	480
Median	15	14	14	14	13	15	15	14	14
Range	5	5	5	5	6	5	5	4	7

a. 100.0% of qualified results in proposed QC range (11 - 17 mm).

- For the MIC QC ranges, three or four $\log_2$ dilution steps were proposed for each polymyxin with modal MIC values of 0.5 or 1 µg/ml for each agent.
  - Proposed MIC ranges for *E. coli* ATCC 25922 were 0.25 to 2 µg/ml (polymyxin B) and 0.25 to 1 µg/ml (colistin) (Table 2).
  - The proposed MIC range for *P. aeruginosa* ATCC 27853 was 0.25 to 2 µg/ml for both polymyxins (Table 2).
- These MIC results contrast with earlier published "expected colistin MIC values" (not a range) of 0.5 - 1 and 2 - 4 µg/ml when tested against *E. coli* and *P. aeruginosa* QC strains, respectively (NCCLS PSM-7, 1980). Other early NCCLS documents (1981 and 1982) had also suggested polymyxin B disk diffusion QC ranges of 7 - 13 mm when testing *Staphylococcus aureus* ATCC 25923; this organism was not tested during this protocol.
- The multi-center study results were also tabulated and compared by intra- and inter-laboratory analysis to determine potentially unacceptable technical variations occurring at any study site. Different reagent lots were compared to determine variations among manufacturer's products. No significant variations among laboratories (Tables 2 and 3) or reagent lots were observed (Table 4).

Table 3. Inter- and intra-laboratory comparisons of the polymyxin B zone diameter results versus *E. coli* ATCC 25922 for an eight medical center protocol meeting the study design guidelines found in NCCLS M23-A2 (2001).

	Laboratory code (occurrences):								
Zone diameter (mm)	A	B	C	D	E	F	G	H	Total
13									0[a]
14		2	2	3	10		1	4	22[a]
15		27	36	31	27	19	30	39	209[a]
16	36	12	2	8	8	21	9	17	113[a]
17	4	11	14	18	13	11	13		84[a]
18	6	8	6		2	9	7		38[a]
19	13								13[a]
20	1								1
Total	60	60	60	60	60	60	60	60	480
Median	16	16	15	15	15	16	15	15	16
Range	5	5	5	4	5	4	5	3	7

a. 99.8% of qualified results in proposed QC range (13 - 19 mm).

Table 4. Comparison of the polymyxin B and colistin (in parenthesis) zone diameter distributions for two lots of disks when testing *E. coli* ATCC 25922.

	Disk lot:		
Zone diameter (mm)	A	B	Total
11		(4)	(4)[b]
12	(1)	(45)	(46)[b]
13	(26)	(122)	0[a] (148)[b]
14	10 (107)	12 (53)	22[a] (160)[b]
15	103 (79)	106 (16)	209[a] (95)[b]
16	55 (25)	58	113[a] (25)[b]
17	44 (2)	40	84[a] (2)[b]
18	21	17	38[a]
19	6	7	13[a]
20	1		1
Total	240 (240)	240 (480)	480 (480)
Median	16 (14)	16 (13)	16 (14)
Range	7 (6)	6 (5)	7 (7)

a. Proposed range includes 99.8% of reported and qualified zone diameters and no significant variation (> 2 mm) between disk lots.
b. Proposed range that included all reported colistin zone diameters. A one mm difference in the colistin modal zones was observed.

CONCLUSIONS

- These summarized results from a multi-center study provide the initial structured QC ranges for colistin and polymyxin B to be considered for inclusion in NCCLS tables.
- All proposed ranges incorporated ≥ 97.9% of study generated zone diameters and MIC values without significant occurrence of inter-laboratory variations or medium quality issues.
- These QC ranges will allow clinical microbiology laboratories to test these polymyxin agents for possible therapeutic guidance, particularly against multidrug-resistant Gram-negative strains.
- The NCCLS Subcommittee on Antimicrobial Susceptibility Testing has recently approved these QC ranges for publication in 2005 associated with susceptible interpretive criteria of ≤ 2 µg/ml for the MIC method, the preferred test because of the poor agar diffusion characteristics of polymyxins that limits the predictive accuracy of the disk test for some species of bacteria.

ACKNOWLEDGMENTS

Members of the Quality Control Working Group included the following: Centers for Disease Control and Prevention (J. Swenson), Atlanta, Georgia; University of Alberta (R. Rennie), Edmonton, Canada, The Cleveland Clinic Foundation (G. Hall), Cleveland, Ohio; University of Texas Medical Center (A. Wagner), Houston, Texas; University of Rochester Medical Center (D. Hardy), Rochester, New York; Denver Health Medical Center (M. Wilson), Denver, Colorado; University of Washington (A. Limaye), Seattle, Washington; and JMI Laboratories (T. Fritsche), North Liberty, Iowa.

The co-authors also express their gratitude to the participating technologists at each study site and the following persons that significantly contributed to manuscript preparation: J. Ross and K. Meyer. The study was supported by an educational/research grant from bioMerieux Vitek (Hazelwood, Missouri, USA).

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