Impact of Variations in Test Method Parameters on *In Vitro* Activity of Surotomycin against *Clostridium difficile* and Surotomycin Quality Control Limits for Broth Microdilution and Agar Dilution Susceptibility Testing

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Test parameter variations were evaluated for their effects on surotomycin MICs. Calcium concentration was the only variable that influenced MICs; therefore, 50 µg/ml (standard for lipopeptide testing) is recommended. Quality control ranges for *Clostridium difficile* (0.12 to 1 µg/ml) and *Eggerthella lenta* (broth, 1 to 4 µg/ml; agar, 1 to 8 µg/ml) were approved by the Clinical and Laboratory Standards Institute based on these data.

In recent years, the incidence and severity of *Clostridium difficile*-associated diarrhea (CDAD) have increased (1–4). Despite the availability of antibiotic therapies, CDAD-related morbidity and mortality rates have remained high (5–8). In a recent study, the clinical response rates were 81.1% for vancomycin and 72.7% for metronidazole, with both drugs being associated with high rates of recurrence (~20 to 45%) (9–11). The more recently approved fidaxomicin demonstrated comparable clinical responses and reduced rates of recurrence (~13 to 15%), as compared with vancomycin (12, 13).

Surotomycin (CB-183,315) is an orally administered, minimally absorbed, selective, bactericidal cyclic lipopeptide being developed for treatment of patients with CDAD (14). It previously demonstrated selective potent bactericidal activity against various *C. difficile* strains and other Gram-positive bacteria *in vitro*, with minimal impact on the Gram-negative organisms of the intestinal microbiota *in vitro* and in CDAD patients (15–20). In a phase 2 trial, surotomycin was more sparing of gut microbes (primarily *Bacteroides* and *Prevotella* species) than vancomycin, with evidence for reduction in the risk of recurrence (20).

These studies were designed to evaluate the effects of changes in key test parameters, including pH, inoculum, calcium concentration ([Ca²⁺]), broth/agar lot, and incubation time, on the *in vitro* activity of surotomycin against three *C. difficile* strains (ATCC 700057 and the clinical isolates SID 7907612 and SID 2946830) and to generate MIC ranges for quality control (QC) organisms (*C. difficile* ATCC 700057, *Eggerthella lenta* ATCC 43055, and *Staphylococcus aureus* ATCC 29213). Broth microdilution and agar dilution were reported previously to be comparable and reproducible methods for testing surotomycin against *C. difficile* (21). Therefore, the test parameter and QC studies were performed using both methods, based on the data presented here.

Variations in key test parameters were tested in duplicate against *C. difficile* ATCC 700057 and two clinical isolates. *Brucella* agar, as recommended by the Clinical and Laboratory Standards Institute (CLSI) for MIC determinations, has various concentrations of divalent cations (22). The *in vitro* activity of surotomycin is dependent on the [Ca²⁺] of the test medium, which needs to be adjusted to a final value of 50 µg/ml, based

on previous observations with the similarly structured lipopeptide daptomycin (23). Inoculum size (5×10^3 to 5×10^5 CFU/ml), pH (pH 6 to 8), [Ca²⁺] (0 to 150 µg/ml), and incubation time (24 to 72 h) were varied relative to standard conditions, which were defined as incubation time of 48 h, [Ca²⁺] of 50 µg/ml, inoculum size of 5×10^4 CFU/ml, pH of 7.2 to 7.4, and serum level of 0% (Table 1). An ion-selective electrode method was used to assess the concentrations of both total and ionized Ca²⁺ in the media. Each isolate was tested using standard anaerobic broth microdilution and agar dilution methods, according to CLSI guidelines. The effects of test parameter variations on surotomycin MICs were assessed using tigecycline as a control for broth microdilution, vancomycin as a control for agar dilution (versus *C. difficile*), and clindamycin for both agar dilution (versus *C. difficile* and *E. lenta*) and broth microdilution (versus *E. lenta*) (24).

A CLSI M23-A3 tier 2 QC study (25) was performed in eight laboratories (see Acknowledgments). The methods described in CLSI document M11-A7 were used (22). Each laboratory tested 10 replicates of each QC strain on three lots of each medium, with the broth microdilution and agar dilution methods. Colony counts were performed at all test sites and were within the parameters of the CLSI guidelines for broth microdilution and agar dilution tests for anaerobic MICs (22, 24).

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TABLE 1 Surotomyacin MICs with various test parameters, as demonstrated by anaerobic broth microdilution and agar dilution

Duplicate MICs (μg/ml)														
Assay method and <i>C. difficile</i> strain	Inoculum (CFU/ml)	24-h incubation	72-h incubation	0 μg/ml Ca ²⁺	25 μg/ml Ca ²⁺	Standard conditions ^a	75 μg/ml Ca ²⁺	150 μg/ml Ca ²⁺	Inoculum of 5 × 10 ⁵ CFU/ml	Inoculum of 5 × 10 ³ CFU/ml	pH 8	pH 6	5% human serum	10% human serum
Broth microdilution														
ATCC 700057	2 × 10 ⁴	0.25/0.5	1/1	4/4	2/2	1/1	0.5/0.5	0.25/0.25	2/2	1/1	1/1	— ^b	0.5/1	— ^c
SID 2907612	9 × 10 ³	0.25/0.25	0.5/0.5	2/2	2/2	0.5/0.5	0.5/0.5	0.25/0.25	1/1	0.5/0.5	0.5/0.5	— ^b	1/1	— ^c
SID 2946830	1.8 × 10 ⁴	0.5/0.5	0.5/0.5	2/2	2/2	0.5/0.5	0.5/0.5	0.25/0.25	1/1	0.5/0.5	0.5/0.5	— ^b	0.5/1	— ^c
Agar dilution														
ATCC 700057	1.2 × 10 ⁵	0.5/0.5	0.5/0.5	4/4	2/2	0.5/0.5	0.5/0.5	0.25/0.25	0.5/0.5	0.5/0.5	0.5/0.5	0.5/0.5	— ^d	— ^d
SID 2907612	4 × 10 ⁴	0.25/0.25	0.25/0.25	4/4	1/1	0.5/0.5	0.25/0.25	0.25/0.12	0.25/0.25	0.25/0.25	0.25/0.25	0.25/0.25	— ^d	— ^d
SID 2946830	1.8 × 10 ⁵	0.25/0.25	0.25/0.25	4/4	1/1	0.25/0.25	0.25/0.25	0.25/0.25	0.5/0.5	0.25/0.25	0.25/0.25	0.5/0.5	— ^d	— ^d

^a The standard test conditions included an incubation time of 48 h, $[\text{Ca}^{2+}]$ of 50 $\mu\text{g/ml}$, inoculum of 5×10^4 CFU/ml, pH of 7.2 to 7.4, and serum level of 0%.

^b No growth.

^c Difficult to read due to substantial background debris.

^d Agar dilution was not performed with 5% and 10% human serum.

All study strains were incubated under anaerobic conditions. Medium lot preparation information can be found in the supplemental material. For the QC study, tier 2 MIC ranges were selected based on CLSI M23-A3 guidelines (25) and confirmed by the alternate RangeFinder method described in the guideline (26) (see the supplemental material).

Based on surotomyacin MICs obtained for each of the three *C. difficile* isolates, the only parameter that affected surotomyacin activity was $[\text{Ca}^{2+}]$ (Table 1). The largest shift in surotomyacin MICs was an increase from 0.12 to 4 $\mu\text{g/ml}$ (32-fold MIC shift) for *C. difficile* ATCC 700057 as the $[\text{Ca}^{2+}]$ was decreased from 150 to 0 $\mu\text{g/ml}$. During broth microdilution, no organism growth occurred at pH 6, and it was not possible to determine MICs at 10% serum concentrations, due to substantial background turbidity. Parameter variations did not appear to notably affect the MICs of the control drugs (clindamycin, tigecycline, and vancomycin). At the time of testing, there were no resistant isolates available; therefore, the performance for detection of resistance was not addressed.

Surotomyacin MICs were all within 1 doubling dilution of each other for all three isolates tested in each of the three broth medium lots (Table 2). For all strains, minimal lot-to-lot variations were noted for surotomyacin MICs, as determined by agar dilution; for the two clinical isolates, however, surotomyacin MICs were 1 to 2 doubling dilutions lower in medium lot 1 when tested by agar dilution. The MICs of control drugs were not affected by different medium lots.

For the QC organism *C. difficile* ATCC 700057, all test results were within the range of 0.12 to 1 $\mu\text{g/ml}$ (Table 3). For *E. lenta* ATCC 43055, ranges of 1 to 4 $\mu\text{g/ml}$ (98.1% within range) and 2 to 8 $\mu\text{g/ml}$ (96.7% within range) were approved for microdilution and agar dilution, respectively (Table 4). A range of 0.25 to 1 $\mu\text{g/ml}$ (99.6% within range) was approved for broth microdilution for *S. aureus* ATCC 29213 incubated anaerobically (Table 5). For agar dilution, a range of 0.5 to 2 $\mu\text{g/ml}$ (100% within range) was approved for *S. aureus* ATCC 29213 incubated anaerobically, with the exclusion of laboratory 2 as a statistical outlier (Table 5). All surotomyacin QC ranges determined by methods proposed by the CLSI were identical to ranges determined by the RangeFinder method (Table 6) (26). CLSI-approved surotomyacin QC ranges plus the percentages of tests within range for testing under anaerobic conditions using supplemented *Brucella* broth or agar are also summarized in Table 6.

No lot-to-lot variations in *Brucella* base medium were observed with any of the broth or agar media, and the tests were highly reproducible among the eight laboratories involved. The QC ranges for the control agents with *C. difficile* ATCC 700057 and *E. lenta* ATCC 43055 listed in Table 6 were presented to the CLSI Subcommittee on Antimicrobial Susceptibility Testing in January 2012 and were approved, based on the data presented here. These ranges were published in document M100-S24 in January 2014 (24). *S. aureus* ATCC 29213 anaerobic ranges were approved as supplemental QC ranges and are not listed in document M100-S24.

In summary, the *in vitro* activity of surotomyacin against *C. difficile* was not affected by altered pH, inoculum size, incubation time, or medium lot variations. The only parameter that appeared to change the *in vitro* activity of surotomyacin notably with the three *C. difficile* strains studied was $[\text{Ca}^{2+}]$. This result suggests that accurate $[\text{Ca}^{2+}]$ determination is crucial for

TABLE 2 Medium lot comparisons of surotomycin MICs for anaerobic broth microdilution and agar dilution

<i>C. difficile</i> strain	Inoculum (CFU/ml)	Duplicate MICs ($\mu\text{g/ml}$)					
		Broth microdilution			Agar dilution		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
ATCC 700057	2.5×10^4	0.5/0.5	1/1	0.5/0.5	0.5/0.5	0.5/0.5	0.5/0.5
SID 2907612	1.0×10^4	1/1	1/1	0.5/0.5	0.12/0.25	0.5/0.5	0.5/0.5
SID 2946830	2.2×10^4	0.5/0.5	1/0.5	0.5/0.5	0.12/0.25	0.5/0.5	0.5/0.5

TABLE 3 Number of strains with surotomycin-specified QC ranges for anaerobic broth microdilution and agar dilution for *C. difficile* ATCC 700057^a

Assay method	Lot 1	Lot 2	Lot 3	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Laboratory 7	Laboratory 8	All
Anaerobic broth microdilution												
No. with MIC of:												
0.12 $\mu\text{g/ml}$	2	7	3	0	0	0	0	12	0	0	0	12
0.25 $\mu\text{g/ml}$	30	32	37	0	0	30	16	18	8	0	27	99
0.5 $\mu\text{g/ml}$	48	38	36	30	30	0	14	0	22	23	3	122
1 $\mu\text{g/ml}$	0	3	4	0	0	0	0	0	0	7	0	7
Total no.	80	80	80	30	30	30	30	30	30	30	30	240
MIC geometric mean ($\mu\text{g/ml}$)	0.372	0.343	0.356	0.500	0.500	0.250	0.345	0.186	0.416	0.588	0.268	0.357
Agar dilution												
No. with MIC of:												
0.12 $\mu\text{g/ml}$	10	12	0	0	0	0	0	0	22	0	0	22
0.25 $\mu\text{g/ml}$	64	70	75	52	20	59	10	30	38	0	0	209
0.5 $\mu\text{g/ml}$	65	59	69	8	37	1	50	0	0	60	37	193
1 $\mu\text{g/ml}$	11	9	6	0	3	0	0	0	0	0	23	26
Total no.	150	150	150	60	60	60	60	60	60	60	60	450
MIC geometric mean ($\mu\text{g/ml}$)	0.341	0.336	0.351	0.274	0.411	0.253	0.445	0.170	0.191	0.500	0.652	0.328

^a Shading indicates quality control (QC) ranges.TABLE 4 Number of strains with surotomycin-specified QC ranges for anaerobic broth microdilution and agar dilution for *E. lenta* ATCC 43055^a

Assay method	Lot 1	Lot 2	Lot 3	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Laboratory 7	Laboratory 8	All
Anaerobic broth microdilution												
No. with MIC of:												
1 $\mu\text{g/ml}$	1	0	1	0	0	0	0	— ^b	1	0	1	2
2 $\mu\text{g/ml}$	57	54	50	30	11	30	30	— ^b	29	20	11	161
4 $\mu\text{g/ml}$	11	14	18	0	19	0	0	— ^b	0	8	16	43
8 $\mu\text{g/ml}$	1	2	1	0	0	0	0	— ^b	0	2	2	4
Total no.	70	70	70	30	30	30	30	— ^b	30	30	30	210
MIC geometric mean ($\mu\text{g/ml}$)	2.252	2.390	2.414	2.000	3.102	2.000	2.000	— ^b	1.954	2.639	3.102	2.351
Agar dilution												
No. with MIC of:												
2 $\mu\text{g/ml}$	17	7	10	0	0	0	27	— ^b	0	0	7	34
4 $\mu\text{g/ml}$	114	117	89	40	50	60	33	— ^b	60	24	53	320
8 $\mu\text{g/ml}$	9	7	36	20	10	0	0	— ^b	0	22	0	52
16 $\mu\text{g/ml}$	0	9	5	0	0	0	0	— ^b	0	14	0	14
Total no.	140	140	140	60	60	60	60	— ^b	60	60	60	420
MIC geometric mean ($\mu\text{g/ml}$)	3.845	4.373	4.780	5.040	4.490	4.000	2.928	— ^b	4.000	7.127	3.689	4.315

^a Shading indicates quality control (QC) ranges.^b Unable to obtain sufficient growth.

TABLE 5 Number of strains with surotomycin-specified QC ranges for anaerobic broth microdilution and agar dilution for *S. aureus* ATCC 29213^a

Assay method	Lot 1	Lot 2	Lot 3	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Laboratory 7	Laboratory 8	All
Anaerobic broth microdilution												
No. with MIC of:												
0.06 µg/ml	0	0	1	1	0	0	0	0	0	0	0	1
0.12 µg/ml	0	0	0	0	0	0	0	0	0	0	0	0
0.25 µg/ml	14	12	16	0	0	30	0	2	0	2	8	42
0.5 µg/ml	65	66	60	27	30	0	28	27	29	28	22	191
1 µg/ml	1	2	3	2	0	0	2	1	1	0	0	6
Total no.	80	80	80	30	30	30	30	30	30	30	30	240
MIC geometric mean (µg/ml)	0.447	0.459	0.431	0.485	0.500	0.250	0.524	0.489	0.501	0.477	0.416	0.445
Agar dilution												
No. with MIC of:												
0.25 µg/ml	14	11	13	0	38 ^b	0	0	0	0	0	0	38
0.5 µg/ml	48	54	51	50	22	0	2	0	60	3	16	153
1 µg/ml	87	93	93	10	0	60	48	60	0	51	44	273
2 µg/ml	11	2	3	0	0	0	10	0	0	6	0	16
Total no.	160	160	160	60	60	60	60	60	60	60	60	480
MIC geometric mean (µg/ml)	0.755	0.726	0.726	0.561	0.322	1.000	1.097	1.000	0.500	1.035	0.831	0.735

^a Shading indicates quality control (QC) ranges.^b Laboratory 2 was identified as an outlier for both the mean and the mode by the RangeFinder method.**TABLE 6** MIC ranges determined using confirmatory RangeFinder method and CLSI-approved surotomycin MIC QC ranges

QC organism and method ^a	MIC range (µg/ml)	No. of dilutions	% of tests in range
RangeFinder			
<i>C. difficile</i> ATCC 700057 ^b			
Broth microdilution	0.12–1	4	100
Agar dilution	0.12–1	4	100
<i>E. lenta</i> ATCC 43055 ^b			
Broth microdilution	1–4	3	98.1
Agar dilution	2–8	3	96.7
<i>S. aureus</i> ATCC 29213 ^b			
Broth microdilution	0.25–1	3	100
Agar dilution	0.25–2 ^c	4	100
CLSI approved			
<i>C. difficile</i> ATCC 700057 ^{b,d}			
Broth microdilution	0.12–1	4	100
Agar dilution	0.12–1	4	100
<i>E. lenta</i> ATCC 43055 ^{b,d}			
Broth microdilution	1–4	3	98.1
Agar dilution	2–8	3	96.7
<i>S. aureus</i> ATCC 29213 ^{b,d}			
Broth microdilution	0.25–1	3	99.6
Agar dilution	0.5–2 ^c	3	100

^a QC, quality control.^b Incubated under anaerobic conditions.^c Laboratory 2 was identified as an outlier for both the mean and the mode.^d Supplemental QC, not to be published in CLSI tables.^e Excluding laboratory 2 as a statistical outlier; however, including laboratory 2 and accepting a 56% shoulder as a bimodal distribution resulted in a range of 0.25 µg/ml to 2 µg/ml.

reliable surotomycin susceptibility testing and that a Ca²⁺ concentration of 50 µg/ml is necessary.

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