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S-649266 MIC Quality Control Ranges in Iron-depleted cation-adjusted Mueller-Hinton Broth Using a Multi-laboratory Study Design

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Amended Abstract

Background: We conducted a study to establish broth microdilution quality control (QC) ranges for S-649266, a siderophore cephalosporin, in iron-depleted cation-adjusted Mueller-Hinton broth (MHB). Chelex treatment depletes iron from the medium and enhances the uptake of S-649266, which can enter the bacterial cell through iron transport mechanisms in Gram-negative (GN) bacteria. S-649266 exhibits potent efficacy against various GN bacteria including carbapenem-resistant strains.

Methods: An eight laboratory study design followed CLSI M23-A4 guidelines. Two QC strains were tested (*Escherichia coli* ATCC 25922 [EC 25922] and *Pseudomonas aeruginosa* ATCC 27853 [PSA 27853], using four media lots (three manufacturers) of MHB treated with a cation-binding resin. MHB was treated for 2 hours with Chelex, filtered, pH adjusted, and levels of calcium and magnesium were adjusted to CLSI specifications. Zinc was adjusted to 0.5 mg/mL; 10 µM. Ten replicate tests were performed for each QC organism generating 320 broth microdilution values/QC strain. Cefepime was used as a control agent.

Results: S-649266 MIC QC range of 0.06 – 0.5 µg/mL was approved for EC 25922, which included 99.7% of results and a mode at 0.25 µg/mL. A MIC "shoulder" at 0.12 µg/mL included 53.1% of the number of MIC results compared to the number of MIC values at the modal 0.25 µg/mL, thus causing a need for a four log_2 dilution range. PSA 27853 also needed a four log_2 dilution range of 0.06 – 0.5 µg/mL to include 95.0% of all reported results for S-649266. This distribution had a modal value at 0.25 µg/mL with a "shoulder" at 0.12 µg/mL which included 83.2% of the number of MICs compared to the modal value. One medium lot was identified as an outlier when tested against EC 25922 with S-649266 having a modal MIC value of two log_2 dilutions (0.06 µg/mL) lower than the other three lots (0.25 µg/mL). Medium variations occurred with the S-649266 compound only. All MIC values generated for cefepime were within the CLSI published QC range.

Conclusions: These approved MIC QC ranges for S-649266 when tested in iron-depleted cation-adjusted MHB (iron depleted by Chelex treatment) should accurately guide clinical or reference laboratories participating in the testing of clinical trial isolates, and facilitate the regulatory review process for this investigational antimicrobial agent.

Introduction

S-649266 is a novel siderophore cephalosporin discovered by Shionogi & Co., Ltd. (**Figure 1**) with potent *in vitro* antibacterial activity against Gram-negatives including multidrug-resistant strains. The siderophore moiety allows S-649266 to utilize the iron acquisition and transport systems of Gram-negative bacteria to gain entry into the cell during iron limiting conditions. As with other β-lactams, S-649266 binds to bacterial penicillin-binding proteins (PBPs), disrupting cell wall synthesis. Interestingly, S-649266 has also been shown to be stable to a variety of β-lactamases including carbapenemases.

This study established broth microdilution CLSI (Tier 2) quality control (QC) ranges for S-649266 in iron depleted Mueller Hinton broth (MHB). Chelex-pretreatment sequesters and removes iron from the medium allowing for enhanced uptake of S-649266 via bacterial iron acquisition and transport mechanisms. This broth microdilution QC study of S-649266 followed the CLSI M23-A4 (2016) guideline document and utilized eight qualified laboratories (minimum of seven), four lots of MHB obtained from 3 different manufacturers (minimum of 3 lots from 2 different manufacturers). Cefepime was used as the control agent. The results are presented as approved (January 2016) S-649266 MIC QC ranges in µg/mL for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 reference strains.

Methods

Participating Institutions: A total of eight laboratories participated in this CLSI M23 (Tier 2) QC study and provided broth microdilution susceptibility data for QC reference strains, as follow: JMI Laboratories, North Liberty, Iowa, USA (R.N. Jones, M.D.); Summa Health Systems, Akron, Ohio, USA (G. Kallstrom, Ph.D.); TREK Diagnostic Systems/ThermoFisher Scientific, Oakwood Village, Ohio, USA (C. Knapp, M.S.); University of Alberta, Edmonton, Canada (R. Rennie, Ph.D.); Wheaton Franciscan Laboratory, Wauwatosa, Wisconsin, USA (E. Munson, Ph.D.); Cleveland Clinic Foundation, Cleveland, Ohio, USA (G. Procop, M.D.); University of Texas Medical Branch, Galveston, Texas, USA (N. Williams-Bouyer, Ph.D) and Johns Hopkins Bayview Medical Center, Baltimore, Maryland, USA (S. Riedel, M.D., Ph.D.).

Chelex Treatment: Mueller-Hinton broth was treated with Chelex® (Bio-Rad [Lot #142-2842], Hercules, CA, USA) to remove existing cations. First, 100g of Chelex was added to one liter of MHB. It was stirred for two hours at room temperature and then filtered using a 0.2 micron filter to sterilize and remove the Chelex. Next, calcium (22.5 mg/L as Ca^{2+}), magnesium (11.25 mg/L as Mg^{2+}), and zinc sulfate ($ZnSO_4$, 10 µM) also removed by the Chelex treatment were added back into the broth. The pH was then adjusted to 7.2-7.4 using hydrochloric acid and filter-sterilized using a 0.2 micron filter. The concentrations of Ca, Fe, Mg and Zn ions were measured at baseline, after Chelex treatment and after cation supplementation by Test America (University Park, IL) to verify the success of the Chelex treatment (**Table 1**).

Susceptibility Testing: Broth microdilution panels were prepared at a certified GMP facility (Trek Diagnostic Systems/ThermoFisher Scientific) using four MHB media lots produced by Difco Laboratories (Detroit, Michigan, USA), Becton Dickinson (BD; Sparks, Maryland, USA [cation-adjusted]), and Oxoid (Hampshire, United Kingdom). S-649266 (Lot #12M01) was provided by Shionogi & Co., Ltd (Osaka, Japan) and cefepime (Lot A00714060616) was obtained from Toku-E (Bellingham, WA). Broth microdilution susceptibility testing was performed as described in CLSI document M07-A10; 2015. Inoculated panels were incubated for 16-20 hours at 35°C in ambient atmosphere prior to reading susceptibility results. S-649266 and cefepime were tested against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains over a minimum of three days to meet M23-A4 guidelines, generating one MIC value per each media lot for 10 replicates (1 x 4 x 10 = 40 determinations). Appropriate inoculum concentrations were verified by performing colony counts from broth in the microdilution panels which were subcultured in a quantitative manner onto drug-free agar plates.

Results

- Chelex treatment effectively reduced iron concentrations to below the limit of detection (<0.10 µg/mL) in all four lots of MHB tested (**Table 1**). Iron concentrations remained below the limit of detection after cation supplementation with Ca, Mg and Zn.
- Applying CLSI M23-A4 analysis criteria to S-649266, >95% of MIC results from the eight participating laboratories using iron depleted cation-adjusted MHB were within the proposed/recently approved (January 2016) QC ranges for both *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (**Tables 2 - 4** and **Figures 2 - 3**).
- Based on CLSI M23-A4 analysis criteria, the approved QC range for S-649266 of 0.06 – 0.5 µg/mL against *E. coli* ATCC 25922 included 99.7% of all MIC results (**Tables 2** and **4** and **Figure 2**).
- A QC range of 0.06 – 0.5 µg/mL was approved for S-649266 against *P. aeruginosa* ATCC 27853 using iron depleted cation-adjusted MHB (**Tables 3** and **4**). A four log_2 dilution range was necessary in order to include ≥95.0% of S-649266 MIC values (**Table 3**). The bimodal distribution of S-649266 MIC values at 0.12 and 0.25 µg/mL including the 83.2% shoulder at 0.12 µg/mL also called for the four dilution QC range (**Table 3** and **Figure 3**).
- Using the CLSI Range Finder statistical program, Media lot C was determined to be an outlier for the S-649266 modal MIC value against *E. coli* ATCC 25922 (0.06 µg/mL) which was two log_2 dilutions lower than that obtained with medium lots A, B, and D (0.25 µg/mL).
- Cefepime control compound MIC results were within CLSI published QC ranges for both *E. coli* ATCC 25922 (480/480; 100.0%) and *P. aeruginosa* ATCC 27853 (480/480; 100.0%), providing validated internal controls for this study (**Figures 4 - 5**).
- Colony counts were performed on each day of testing and averaged 3.2 x 10^5 CFU/mL for *E. coli* ATCC 25922 and 3.6 x 10^5 CFU/mL for *P. aeruginosa* ATCC 27853.

Table 1. Cation analysis of Mueller-Hinton broth media lots used to produce S-649266 broth microdilution panels for the CLSI M23 (Tier 2) eight laboratory QC study.

Mueller-Hinton broth[b]	Cation Concentrations (µg/mL)[a]			
	Calcium (Ca)	Iron (Fe)	Magnesium (Mg)	Zinc (Zn)
Difco Lot A - Baseline	3.1	0.4	3.6	0.44
Difco Lot B - Baseline	4	0.41	3.7	0.41
BD Lot C - Baseline	22	0.18	9.8	1.1
Oxoid Lot D - Baseline	3.5	0.39	4.5	0.94
Difco Lot A - After Chelex Treatment	<0.059	<0.10	<0.034	<0.0093
Difco Lot B - After Chelex Treatment	<0.059	<0.10	<0.034	<0.0093
BD Lot C - After Chelex Treatment	0.08	<0.10	<0.034	<0.0093
Oxoid Lot D - After Chelex Treatment	<0.059	<0.10	<0.034	0.0099
Difco Lot A - After cation supplementation	21	<0.10	9.3	0.67
Difco Lot B - After cation supplementation	22	<0.10	9.6	0.67
BD Lot C - After cation supplementation	20	<0.10	8.8	0.65
Oxoid Lot D - After cation supplementation	22	<0.10	9.8	0.69

a. Detection limits were 0.059 µg/mL for calcium, 0.1 µg/mL for iron, 0.034 µg/mL for magnesium and 0.0093 µg/mL for zinc.
b. Lot A = Difco (#4045148), Lot B = Difco (#3120127), Lot C = BD (#4044343) and Lot D = Oxoid (#1433705).

Table 2. Media lot and inter- and intra-laboratory comparisons of S-649266 MIC results versus *E. coli* ATCC 25922 using iron depleted cation-adjusted Mueller Hinton broth in an eight medical center protocol meeting the study design guidelines found in CLSI M23-A4 (2016).

MIC (µg/mL)	Occurrences by lot[a]:				Laboratory code (occurrences):								Total
	A	B	C	D	A	B	C	D	E	F	G	H	
0.015													
0.03			1								1		1
0.06			40		9	3	8	4	3	3	9	1	40[b]
0.12	17	26	38	12	20	8	5	18	9	6	18	9	93[b]
0.25	61	50	1	63	11	29	27	18	22	26	12	30	175[b]
0.5	2	4		5					6	5			11[a]
1													
Total	80	80	80	80	40	40	40	40	40	40	40	40	320
Median	0.25	0.25	0.06	0.25	0.12	0.25	0.25	0.12	0.25	0.25	0.12	0.25	0.25
Geometric Mean	0.22	0.20	0.08	0.23	0.13	0.19	0.17	0.16	0.21	0.22	0.12	0.20	0.17
Range	3	3	3	3	3	3	3	3	4	4	4	3	5

a. A = Difco (lot 4045148), B = Difco (lot 3120127), C = BD (lot 4044343), D = Oxoid (lot 1433705).
b. 99.7% of the qualified results are in the CLSI approved QC range of (0.06 - 0.5 µg/mL).

Table 3. Media lot and inter- and intra-laboratory comparisons of S-649266 MIC results versus *P. aeruginosa* ATCC 27853 using iron depleted cation-adjusted Mueller Hinton broth in an eight medical center protocol meeting the study design guidelines found in CLSI M23-A4 (2016).

MIC (µg/mL)	Occurrences by lot[a]:				Laboratory code (occurrences):								Total
	A	B	C	D	A	B	C	D	E	F	G	H	
0.015													
0.03			4		4								4
0.06	1	1	33		6	7	3	8	1	7	3		35
0.12	37	32	28	1	16	3	13	5	12	18	26	6	99
0.25	32	30	13	35	13	19	14	11	16	14	10	22	119
0.5	9	7	1	34	1	8	8	14	9	1	1	9	51
1	1		1	10		3	2	2	2			3	12
Total	80	80	80	80	80	80	80	80	80	80	80	80	320
Median	0.25	0.25	0.12	0.5	0.12	0.25	0.25	0.25	0.25	0.12	0.12	0.25	0.12
Geometric Mean	0.20	0.18	0.10	0.40	0.12	0.23	0.22	0.23	0.24	0.14	0.14	0.29	0.20
Range	5	4	6	4	5	5	5	5	5	4	4	4	6

a. A = Difco (lot 4045148), B = Difco (lot 3120127), C = BD (lot 4044343), D = Oxoid (lot 1433705).
b. 95.0% of the qualified results are in the CLSI approved QC range of (0.06 - 0.5 µg/mL).

Table 4. CLSI broth microdilution quality control ranges for S-649266.

QC reference strain	S-649266 CLSI QC ranges (µg/mL)	
	Proposed Range (% in range)	CLSI Approved Range (% in range)
E. coli ATCC 25922	0.06 - 0.5 (97.5%)	0.06 - 0.5 (97.5%)
P. aeruginosa ATCC 27853	0.06 - 0.5 (95.0%)	0.06 - 0.5 (95.0%)

Figure 1. Chemical structure of S-649266.

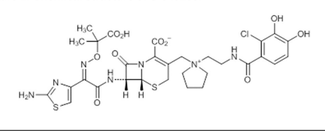


Figure 2. S-649266 MIC distributions for *E. coli* ATCC 25922 for an eight laboratory CLSI M23 (Tier 2) study using iron depleted cation-adjusted Mueller Hinton Broth.

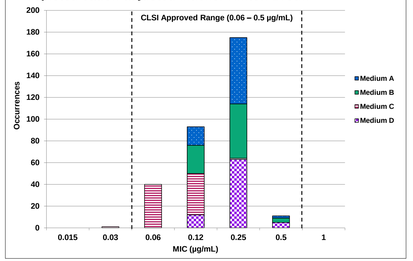


Figure 3. S-649266 MIC distributions for *P. aeruginosa* ATCC 27853 in an eight laboratory CLSI M23 (Tier 2) study using iron depleted cation-adjusted Mueller Hinton Broth.

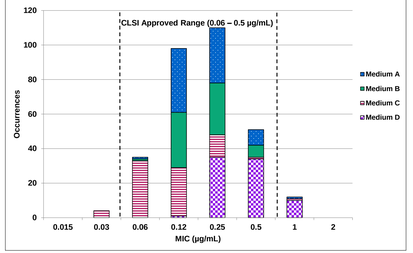


Figure 4. Cefepime MIC distributions for *E. coli* ATCC 25922 in cation-adjusted Mueller Hinton Broth.

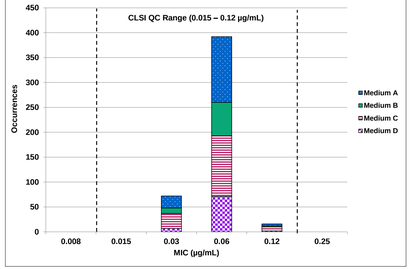
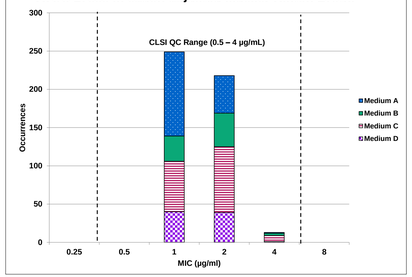


Figure 5. Cefepime MIC distributions for *P. aeruginosa* ATCC 27853 in cation-adjusted Mueller Hinton Broth.



Conclusions

- This study established MIC QC ranges for S-649266 against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 reference strains when performing CLSI broth microdilution susceptibility testing in iron depleted cation-adjusted MHB.
- The CLSI subcommittee on Antimicrobial Susceptibility Testing approved S-649266 QC ranges for both *E. coli* ATCC 25922 (0.06 – 0.5 µg/mL) and *P. aeruginosa* ATCC 27853 (0.06 – 0.5 µg/mL) reference strains at the January 2016 meeting (Tempe, AZ) based on the acceptable inter- and intra-laboratory reproducibility observed in this eight laboratory study,
- The recently established CLSI QC ranges for S-649266 can now be utilized to support accurate antimicrobial susceptibility testing of Gram-negative bacterial isolates using iron depleted cation-adjusted MHB.

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