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Bacteriology

Reproducibility of broth microdilution MICs for the novel siderophore cephalosporin, cefiderocol, determined using iron-depleted cation-adjusted Mueller-Hinton broth

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ABSTRACT

In 2017, the Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing approved the use of iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB) prepared with Chelex® 100 resin (Bio-Rad Laboratories, Hercules, CA) to determine MICs for cefiderocol. The current study examined the reproducibility of cefiderocol MICs generated for 19 clinical isolates of Gram-negative bacilli, with CAMHB produced by three manufacturers; each of the 19 isolates was tested for 10 replicates in ID-CAMHB from each manufacturer. When analyzed by individual media lot, greater than 95% of MIC results were within ± one doubling-dilution of the mode for each of the 19 isolates tested. The remaining 5.0% of MIC results were within ± two doubling-dilutions of the modal MIC. For all media lots combined, 92.2% of MIC results were within ± one doubling-dilution of the modal MIC for each isolate, 99.8% were within ± two doubling-dilutions and 100% were within three doubling-dilutions.

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1. Introduction

Cefiderocol, formerly S-649226, is a parenteral siderophore cephalosporin in late-stage clinical development with Shionogi & Co., Ltd. (Osaka, Japan). The United States Food and Drug Administration (FDA) has designated cefiderocol a Qualified Infectious Disease Product (QIDP), which permits priority review and eligibility for fast-track approval status. Cefiderocol has completed an international multicenter, double-blind, randomized, non-inferiority trial designed to evaluate its efficacy, safety, and tolerability in hospitalized adult patients with serious complicated urinary tract infections with Gram-negative bacteria, with or without pyelonephritis, where it was shown to be superior to imipenem/cilastatin (www.clinicaltrials.gov NCT02321800). Additional trials in patients infected with carbapenem-resistant pathogens (www.clinicaltrials.gov NCT02714595) and a trial studying the treatment of patients with nosocomial pneumonia caused by Gram-negative pathogens (www.clinicaltrials.gov NCT03032380) are ongoing.

The chemical structure of cefiderocol includes a catechol moiety (siderophore) at the 3-position side chain of the cephalosporin that facilitates formation of a chelated complex with ferric iron and promotes its transport across the outer membrane of Gram-negative bacilli to the periplasmic space using receptor-mediated iron transport systems. Once present in the periplasmic space, the cephalosporin moiety of cefiderocol, like other β-lactams, binds to penicillin binding proteins (PBP). In the case of cefiderocol, binding is preferentially to PBP3 (Ito et al., 2015a, 2016a).Cefiderocol inhibits the growth of all ESBL-producing *Enterobacteriaceae* (e.g. CTX, SHV, TEM) as well as most carbapenem-resistant and carbapenemase-producing (class A [KPC], class B [VIM, IMP, NDM, GIM-1, SPM-1], class D [OXA]) Gram-negative bacilli (*Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*) at a concentration of ≤4 μg/mL (Ito et al., 2016b; Ito-Horiyama et al., 2016; Kohira et al., 2016; Tsuji et al., 2014).

Accurate *in vitro* susceptibility testing of cefiderocol requires the use of iron-depleted conditions to insure the induction of ferric iron transporters (Huband et al., 2017; Ito et al., 2016b; Kohira et al., 2016; Otto et al., 1992). Iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB; iron concentration <0.03 μg/mL) mimics the *in vivo* condition encountered

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by bacteria infecting human tissues and fluids. In 2017, the Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing approved broth microdilution and disk diffusion methods, and quality control MIC ranges, for the *in vitro* testing of cefiderocol (CLSI, 2016a, 2016b, 2017; Huband et al., 2017).The results of the CLSI M23-A4 eight-laboratory Tier 2 study used to approve the quality control ranges for cefiderocol broth microdilution for *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 tested in ID-CAMHB were published in 2017 (Huband et al., 2017).The method of preparation of ID-CAMHB approved by the CLSI Subcommittee on Antimicrobial Susceptibility Testing (CLSI, 2016a, 2016b, 2017; Tsuji et al., 2016) supplanted all previous media preparation methods including those using 20 μM human apotransferrin, which resulted in MIC reproducibility issues, and Chelex-treated Iso-Sensitest broth because of the limited number of manufacturers of that media (Ito et al., 2015b, 2016b; Ito-Horiyama et al., 2016; Kohira et al., 2016; Tsuji et al., 2016). In 2019, the CLSI published investigational MIC interpretative breakpoints for cefiderocol tested against *Enterobacteriaceae*, *P. aeruginosa*, *Acinetobacter* species, and *Stenotrophomonas maltophilia* (CLSI, 2019).

The current study tested the reproducibility of cefiderocol broth microdilution MICs, for 19 clinical isolates of Gram-negative bacilli with various phenotypic and genotypic antimicrobial resistance profiles, determined by the standard CLSI method (CLSI, 2019, 2018) with ID-CAMHB prepared using the approved CLSI method for CAMHB purchased from three manufacturers. Cefiderocol MICs were determined 10-times for each isolate in each of the three media.

2. Materials and methods

2.1. Bacterial isolates

The 19 isolates of Gram-negative bacilli tested in this study were selected from the International Health Management Associates, Inc. (IHMA, Schaumburg, IL, USA) surveillance study frozen stock culture collection based on their previously determined antimicrobial susceptibility testing phenotypes and/or their PCR-confirmed β-lactamase genetic content. Four *A. baumannii* strains were chosen based their propensity to demonstrate a trailing endpoint when tested against cefiderocol (Table 1). The identities of all isolates were confirmed by IHMA using MALDI-TOF spectrometry (Bruker Daltonics, Billerica, MA, USA).

2.2. Antimicrobial susceptibility testing

CLSI standard methods were used to generate broth microdilution panels as well as to perform panel inoculation, incubation, and MIC reading and MIC interpretation of cefepime, which served as a control agent, and cefiderocol (CLSI, 2016a, 2017, 2018, 2019).All aspects of antimicrobial susceptibility testing were performed on-site at IHMA. Broth microdilution panels included cefepime (doubling dilution range tested, 0.06–64 μg/mL) and cefiderocol (0.004–8 μg/mL). Cefiderocol was provided to IHMA by Shionogi & Co., Ltd. and cefepime was purchased from the U.S. Pharmacopeia (Rockville, MD). Cefiderocol was dissolved and diluted in sterile normal saline (CLSI, 2016a).

Cation-adjusted Mueller-Hinton broth (CAMHB) from three manufacturers was tested: BD-BBL (Becton-Dickinson, Sparks, MD; catalog number: 212322; lot number: 2000007846); Oxoid (ThermoFisher Scientific, Lenexa, KS; catalog number: CM0405; lot number: 1583507); and BD-Difco (Becton-Dickinson, Sparks, MD; catalog number: 275730; lot number: 4045151). CAMHB was prepared following each manufacturer's instructions. Iron-depleted CAMHB (ID-MHB) was prepared as follows, and was tested in experiments together with standard (non-iron-depleted) CAMHB.

ID-CAMHB was prepared by adding 100 g of Chelex® 100 resin (Bio-Rad Laboratories, Hercules, CA) to 1 L of autoclaved CAMHB and the suspension stirred for 2 h at room temperature (23 °C) to remove cations in the medium. The iron-depleted broth was then filtered using a 0.2 μm filter to remove the resin and the pH of the broth adjusted to 7.3 using 0.1 M hydrochloric acid. The ID-CAMHB was then supplemented with calcium ($CaCl_2$), magnesium ($MgCl_2$), and zinc ($ZnSO_4$) to final concentrations of 22.5 μg/mL (range, 20–25 μg/mL), 11.25 μg/mL (range, 10–12.5 μg/mL), and 10 μM (0.56 μg/mL; range 0.5–1.0 μg/mL), respectively, and again passed through a 0.2 μm filter.

Table 2
Cation concentrations in the three manufacturer lots of CAMHB tested.

Manufacturer[a]	Status	Concentration (μg/mL)			
		Ca	Fe	Mg	Zn
BD	Untreated[b]	25.06	0.24	12.61	1.12
Difco	Untreated	NA[c]	NA	NA	NA
Oxoid	Untreated	26.35	0.83	12.84	0.97
BD	Chelex-treated and unsupplemented	0.04	BDC[d]	0.04	BDC
Difco	Chelex-treated and unsupplemented	NA	NA	NA	NA
Oxoid	Chelex-treated and unsupplemented	0.02	0.02	0.18	BDC
BD	Chelex-treated and supplemented	23.93	BDC	11.37	0.59
Difco	Chelex-treated and supplemented	29.47	BDC	11.87	0.96
Oxoid	Chelex-treated and supplemented	28.07	0.02	12.84	0.70

[a] The lot numbers of the three manufacturer lots were: BD (lot no. 2000007846); Difco (lot no. 4045151); and Oxoid (lot no. 1583507).
[b] Untreated was defined as not Chelex-treated and not supplemented.
[c] NA, not available; sample lost by testing facility.
[d] BDC, below detectable concentration, defined as <0.0001 μg/mL.

Table 1
Phenotypic/genotypic descriptions of the 19 isolates of Gram-negative bacilli tested for cefiderocol MIC reproducibility.

Bacterial species	Isolate number	Isolate phenotypic/genotypic trait	Isolates demonstrates trailing
Escherichia coli	1,068,226	Cephem-susceptible	No
Escherichia coli	1,104,276	NDM-positive	No
Escherichia coli	1,172,735	KPC-positive	No
Klebsiella pneumoniae	1,143,131	NDM-positive	No
Klebsiella pneumoniae	1,144,285	KPC-positive	No
Klebsiella pneumoniae	1,217,642	Cephem-susceptible	No
Klebsiella pneumoniae	1,217,643	Cephem-susceptible	No
Pseudomonas aeruginosa	1,106,428	VIM-positive	No
Pseudomonas aeruginosa	1,221,056	Carbapenem-resistant	No
Pseudomonas aeruginosa	1,261,964	OprD-deficient	No
Acinetobacter baumannii	924,283	Carbapenem-resistant	Yes
Acinetobacter baumannii	942,428	Carbapenem-resistant	Yes
Acinetobacter baumannii	1,073,418	Carbapenem-resistant	No
Acinetobacter baumannii	1,073,445	Carbapenem-resistant	No
Acinetobacter baumannii	1,065,051	Carbapenem-resistant	Yes
Acinetobacter baumannii	1,070,594	Carbapenem-resistant	No
Acinetobacter baumannii	1,103,193	Carbapenem-resistant	No
Acinetobacter baumannii	1,145,223	Carbapenem-resistant	No
Acinetobacter baumannii	1,179,626	Carbapenem-resistant	Yes

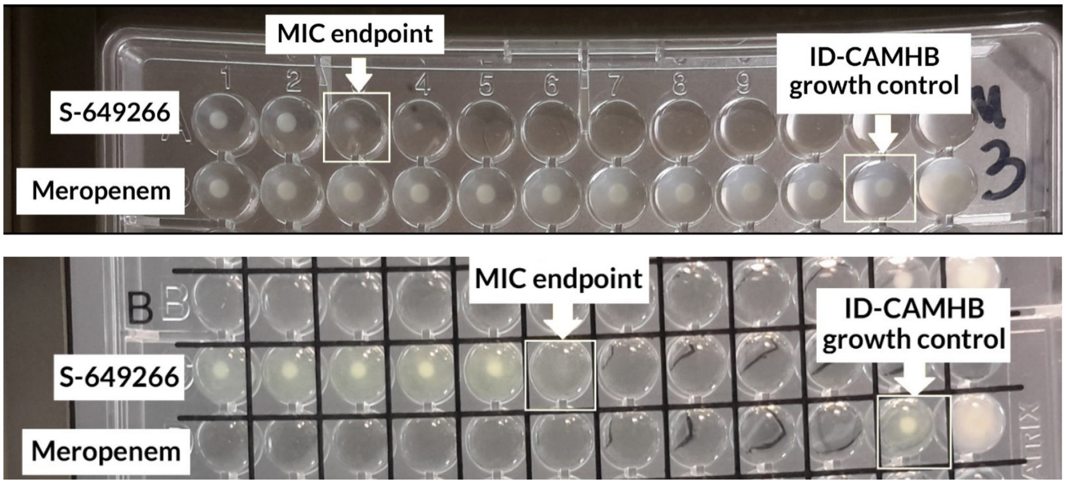

Fig. 1. Examples of two challenging types of cefiderocol (S-649266) MIC endpoints that may be encountered when reading broth microdilution MICs for cefiderocol tested against Gram-negative bacilli. The upper figure shows a trailing endpoint with a button of <1 mm. The lower figure shows an endpoint determination when light/faint turbidity was present.

The final concentration of iron in ID-CAMHB prepared using the above method is ≤0.03 μg/mL (CLSI, 2016a).The cation concentrations present in CAMHB (untreated), ID-MHB (unsupplemented), and ID-CAMHB were measured by inductively coupled plasma-mass spectrometry (ICP-MS) at Becton-Dickinson (Franklin Lakes, NJ, USA), and are shown in Table 2.

The broth microdilution panels included growth control wells for both CAMHB and ID-CAMHB. The panels were incubated at 35 °C for 20 h in ambient air before MIC endpoints were read. ID-CAMHB did not significantly affect the growth of any test isolate. Technologists were directed to follow specific instructions for reading the cefiderocol wells. Reading the MIC of cefiderocol was contingent on the presence of strong growth in the ID-CAMHB growth control well (i.e. a button of approximately 2 mm or greater). If strong growth was not obtained in the growth control well, the reading of the cefiderocol MIC did not proceed, and the test was repeated. If strong growth was still not observed, the MIC was recorded as not available. In this study, sufficient growth was obtained in the growth control well for all isolates tested. The cefiderocol MIC was read as the first panel well in which isolate growth was significantly reduced (i.e. a button of <1 mm or light/faint turbidity) relative to the growth observed in the ID-CAMHB growth control well (Fig. 1). The method described above for reading MIC endpoints for cefiderocol was the method approved by the CLSI Subcommittee on Antimicrobial Susceptibility Testing (CLSI, 2016a, 2016b, 2017; Huband et al., 2017; Tsuji et al., 2016).Ten replicates (individual inocula) of each isolate were tested in each different broth over 2 days with five replicates set up on each day. A different technologist read all panels on each day. Ten MIC values were obtained in each of the media for each isolate, for a total of 30 MIC values for each isolate. The percentage essential agreement (± one doubling-dilution from the mode) for each of the 19 isolates in each of the media was calculated for ID-CAMHB as well as the overall percent essential agreement. Where there was an off-scale result (i.e. the MIC was greater than or less than the MIC range tested), the test value was not included in the calculation of essential agreement or categorical agreement.

3. Results

The percentage essential agreement for each of the 19 isolates tested in ID-CAMHB is shown in Tables 3-6. Five replicates of *E. coli* 1,068,226 had MIC values less than the lowest concentration tested (≤0.004 μg/mL) (three in Oxoid broth, and two in BD broth); one replicate of

Table 3
Variation of cefiderocol MIC values compared to the mode for each of the 19 isolates of Gram-negative bacilli tested 10 times each in Oxoid CAMHB.

Bacterial species	Isolate number	Modal MIC (μg/mL)	Change in doubling dilution of cefiderocol MIC values compared to the modal MIC (Oxoid)					
			−2	−1	0	1	2	3
Escherichia coli	1,068,226	0.008		3[a]	5	2		
Escherichia coli	1,172,735	0.06			5	4	1	
Escherichia coli	1,104,276	8			10			
Klebsiella pneumoniae	1,217,642	1	1	1	5	3		
Klebsiella pneumoniae	1,217,643	8			9	1[a]		
Klebsiella pneumoniae	1,144,285	0.12		1	9			
Klebsiella pneumoniae	1,143,131	1			8	2		
Pseudomonas aeruginosa	1,221,056	0.12			6	4		
Pseudomonas aeruginosa	1,261,964	1			9	1		
Pseudomonas aeruginosa	1,106,428	0.5			9	1		
Acinetobacter baumannii	924,283	0.25			9	1		
Acinetobacter baumannii	942,428	0.12		1	5	4		
Acinetobacter baumannii	1,065,051	1		3	7			
Acinetobacter baumannii	1,070,594	0.25			5	4	1	
Acinetobacter baumannii	1,073,418	0.06			5	2	3	
Acinetobacter baumannii	1,073,445	0.06			5	3	2	
Acinetobacter baumannii	1,103,193	0.25			9	1		
Acinetobacter baumannii	1,145,223	0.25			8	2		
Acinetobacter baumannii	1,179,626	0.12		1	7	2		

[a] Underlined numbers indicate off-scale MIC results.

Table 4
Variation of cefiderocol MIC values compared to the mode for each of the 19 isolates of Gram-negative bacilli tested 10 times each in BD CAMHB.

Bacterial species	Isolate number	Modal MIC (μg/mL)	Change in doubling dilution of cefiderocol MIC values compared to the modal MIC (BD)					
			−2	−1	0	1	2	3
Escherichia coli	1,068,226	0.008		2[a]	8			
Escherichia coli	1,172,735	0.12		1	5	4		
Escherichia coli	1,104,276	2			10			
Klebsiella pneumoniae	1,217,642	1	1	2	7			
Klebsiella pneumoniae	1,217,643	4		3	7			
Klebsiella pneumoniae	1,144,285	0.25		4	6			
Klebsiella pneumoniae	1,143,131	2		2	8			
Pseudomonas aeruginosa	1,221,056	0.06			10			
Pseudomonas aeruginosa	1,261,964	0.25			7	3		
Pseudomonas aeruginosa	1,106,428	0.12			8	2		
Acinetobacter baumannii	924,283	0.12		1	8	1		
Acinetobacter baumannii	942,428	0.25		4	6			
Acinetobacter baumannii	1,065,051	0.5			8	2		
Acinetobacter baumannii	1,070,594	0.12		1	7	2		
Acinetobacter baumannii	1,073,418	0.06			4	3	3	
Acinetobacter baumannii	1,073,445	0.06		4	6			
Acinetobacter baumannii	1,103,193	0.12			9	1		
Acinetobacter baumannii	1,145,223	0.12		1	9			
Acinetobacter baumannii	1,179,626	0.06			4	4	2	

[a] Underlined numbers indicate off-scale MIC results.

K. pneumoniae 1,217,643 had an MIC value greater than the highest concentration tested (>8 μg/mL) in Oxoid broth. When analyzed by each medium, 95.7% (178/186 of on-scale results) of MIC results in Oxoid broth (Table 3), 96.8% (182/188 of on-scale results) in BD broth (Table 4), and 97.9% (186/190 of on-scale results) in Difco broth (Table 5) were within ± one doubling-dilution of the modal MIC for each isolate. For all media lots combined, 92.2% (520/564 of on-scale results) of MIC results were within ± one doubling-dilution of the modal MIC for each isolate, 99.8% (563/564 of on-scale results) were within two doubling-dilutions of the modal MIC, and 100% were within three doubling-dilutions of the modal MIC (Table 6).

CLSI investigational MIC interpretative breakpoints for cefiderocol tested against clinical isolates of *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* species are ≤4 μg/mL (susceptible), 8 μg/mL (intermediate), and ≥16 μg/mL (resistant) (CLSI, 2019).Cefiderocol MICs generated in all three CAMHB media were susceptible (≤4 μg/mL) for two of the three *E. coli* isolates tested (1,068,226, 1,172,735) (Tables 3-6). For the third isolate of *E. coli* (1104276), the 20 replicates tested with BD CAMHB and Difco CAMHB were cefiderocol-susceptible (MIC, 2–4 μg/mL) while the 10 replicates tested in Oxoid CAMHB were intermediate (MIC, 8 μg/mL). Cefiderocol MICs generated in all three media were susceptible (≤4 μg/mL) for three of the four *K. pneumoniae* isolates tested (1,217,642, 1,144,285, 1,143,131). For the fourth isolate of *K. pneumoniae* (1217643), the 10 replicates in BD CAMHB were susceptible (MICs, 2–4 μg/mL) while the 10 replicates in Difco CAMHB were either susceptible or intermediate (MIC, 4–8 μg/mL) and the 10 replicates in Oxoid CAMHB were either intermediate or resistant (MIC, 8–>256 μg/mL). For five of the *A. baumannii* and one *P. aeruginosa*, trailing was reported for at least one of the replicates. All cefiderocol MICs generated in the three media were susceptible (≤4 μg/mL) for all isolates of *P. aeruginosa* and *A. baumannii* tested.

4. Discussion

MIC testing of cefiderocol in ID-CAMHB was reproducible (essential agreement ≥90%) using three different manufacturers of CAMHB media.

Table 5
Variation of cefiderocol MIC values compared to the mode for each of the 19 isolates of Gram-negative bacilli tested 10 times each in Difco CAMHB.

Bacterial species	Isolate number	Modal MIC (μg/mL)	Change in doubling dilution of cefiderocol MIC values compared to the modal MIC (Difco)					
			−2	−1	0	1	2	3
Escherichia coli	1,068,226	0.03		3	6	1		
Escherichia coli	1,172,735	0.25			6	4		
Escherichia coli	1,104,276	4			10			
Klebsiella pneumoniae	1,217,642	1	2	1	6	1		
Klebsiella pneumoniae	1,217,643	4			7	3		
Klebsiella pneumoniae	1,144,285	0.25			6	4		
Klebsiella pneumoniae	1,143,131	4		3	7			
Pseudomonas aeruginosa	1,221,056	0.25		5	5			
Pseudomonas aeruginosa	1,261,964	0.5			10			
Pseudomonas aeruginosa	1,106,428	0.25			9	1		
Acinetobacter baumannii	924,283	0.25		1	9			
Acinetobacter baumannii	942,428	0.25			9	1		
Acinetobacter baumannii	1,065,051	2		3	7			
Acinetobacter baumannii	1,070,594	0.25		2	8			
Acinetobacter baumannii	1,073,418	0.12			5	4	1	
Acinetobacter baumannii	1,073,445	0.12		1	9			
Acinetobacter baumannii	1,103,193	0.25		1	9			
Acinetobacter baumannii	1,145,223	0.25		2	6	1	1	
Acinetobacter baumannii	1,179,626	0.12			10			

Table 6
Variation of cefiderocol MIC values compared to the mode for each of the 19 isolates of Gram-negative bacilli tested 10 times each in three manufacturer lots of CAMHB.

Bacterial species	Isolate number	Modal MIC (μg/mL)	Change in doubling dilution of cefiderocol MIC values compared to the modal MIC					
			−2	−1	0	1	2	3
Escherichia coli	1,068,226	0.008		5[a]	13	5	6	1
Escherichia coli	1,172,735	0.25	6	9	11	4		
Escherichia coli	1,104,276	2, 4, 8	10	10	10			
Klebsiella pneumoniae	1,217,642	1	4	4	18	4		
Klebsiella pneumoniae	1,217,643	4		3	14	12	1[a]	
Klebsiella pneumoniae	1,144,285	0.12		1	13	12	4	
Klebsiella pneumoniae	1,143,131	2		10	13	7		
Pseudomonas aeruginosa	1,221,056	0.12		10	11	9		
Pseudomonas aeruginosa	1,261,964	0.5		7	13	9	1	
Pseudomonas aeruginosa	1,106,428	0.25		8	11	10	1	
Acinetobacter baumannii	924,283	0.25	1	9	19	1		
Acinetobacter baumannii	942,428	0.25	1	9	19	1		
Acinetobacter baumannii	1,065,051	1		11	12	7		
Acinetobacter baumannii	1,070,594	0.25	1	9	15	4	1	
Acinetobacter baumannii	1,073,418	0.12		9	10	10	1	
Acinetobacter baumannii	1,073,445	0.12	4	12	12	2		
Acinetobacter baumannii	1,103,193	0.25		10	19	1		
Acinetobacter baumannii	1,145,223	0.25	1	11	14	3	1	
Acinetobacter baumannii	1,179,626	0.12		5	21	4		

[a] Underlined numbers indicate off-scale MIC results.

When analyzed by media, >95% of MIC results were within ± one doubling-dilution of the modal MIC for each isolate. The remaining 5% were within two doubling-dilutions of the modal MIC. For all media lots combined, 92.2% of MIC results were within ± one doubling-dilution of the modal MIC for each isolate, 99.8% were within two doubling-dilutions of the modal MIC and 100% were within three doubling-dilutions of the modal MIC (Table 6).

The reading of cefiderocol MIC endpoints may present a challenge for some isolates. The key point to accurately reading cefiderocol MICs in ID-CAMHB is that trailing should be ignored when a button of <1 mm or light/faint turbidity relative to the growth control (*i.e.*, >2 mm button) is observed. The MIC is the first well with a button of <1 mm or light/faint turbidity. It is also important to recognize that rare clinical isolates may not grow well in iron-depleted conditions and testing in ID-CAMHB will not generate a MIC. It is important to establish an *in vitro* method that accurately predicts *in vivo* activity. MICs determined using ID-CAMHB have been reported to correlate with *in vivo* efficacy in animal models (Tsuji et al., 2015).

Cefiderocol represents a potentially significant advance in the treatment options available to clinicians to care for patients infected with antimicrobial-resistant Gram-negative bacilli. The current study determined that cefiderocol tested using the recently approved CLSI method (CLSI, 2016a, 2016b, 2017) generated reproducible MICs when clinical isolates of Gram-negative bacilli were tested as previously demonstrated for the quality control strains (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) in the Tier 2 study (Huband et al., 2017).

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