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Development of Broth Microdilution MIC and Disk Diffusion Antimicrobial Susceptibility Test Quality Control Ranges for the Combination of Cefepime and the Novel β-Lactamase Inhibitor Enmetazobactam

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ABSTRACT Third-generation cephalosporin resistance among *Enterobacteriaceae*, mediated by the spread of extended-spectrum β-lactamases (ESBLs), is a very serious medical concern with limited therapeutic options. Enmetazobactam (formerly AAI101) is a novel penicillanic sulfone β-lactamase inhibitor active against a wide range of ESBLs. The combination of enmetazobactam and cefepime has entered phase 3 development in patients with complicated urinary tract infections. Using the Clinical and Laboratory Standards Institute (CLSI) M23 tier 2 study design, broth microdilution MIC and disk diffusion quality control (QC) ranges were determined for cefepime-enmetazobactam. Enmetazobactam was tested at a fixed concentration of 8 μg/ml in the MIC assay, and a cefepime-enmetazobactam disk mass of 30/20 μg was used in the disk diffusion assay. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *E. coli* NCTC 13353, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853 were chosen as reference strains. The CTX-M-15-producing *E. coli* NCTC 13353 isolate is recommended for routine testing to control for inhibition of ESBL activity by enmetazobactam. Broth microdilution MIC QC ranges spanned 3 to 4 doubling dilutions and contained 99.6% to 100.0% of obtained MIC values for the five reference strains. Disk diffusion yielded inhibition zone diameter QC ranges that spanned 7 mm and encompassed 97.1% to 100.0% of the obtained values. Quality control ranges were approved by the CLSI in 2017 (broth microdilution MIC) and 2019 (disk diffusion). The established QC ranges will ensure that appropriate assay performance criteria are attained using CLSI reference methodology when determining the susceptibility of clinical isolates to cefepime-enmetazobactam.

KEYWORDS AAI101, ESBL, *Enterobacteriaceae*, carbapenem, cefepime, enmetazobactam, extended-spectrum beta-lactamase, quality control

Extended-spectrum β-lactamases (ESBLs) are a diversified group of enzymes that confer resistance to third- and fourth-generation cephalosporins (1). The prevalence of ESBL-producing *Enterobacteriaceae* has risen globally (2–5), prompting the World Health Organization to list these pathogens as a priority for development of new therapies (6). Using carbapenems, a "last resort" class of β-lactams, to treat serious infections caused by ESBL-producing *Enterobacteriaceae* (7) promotes the emergence and dissemination of carbapenem-resistant pathogens (2, 8, 9). Efforts to limit resistance development in Gram-negative pathogens recognize the importance of developing new "carbapenem-sparing" options as empirical therapy for ESBL-producing *Enterobacteriaceae* (10, 11).

Although piperacillin-tazobactam has been a β-lactam/β-lactamase inhibitor (BL/BLI) mainstay for treating serious infections caused by ESBL-producing *Enterobacteria-*

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ceae, its continued appropriateness has been questioned due to microbiological concerns and inconsistent clinical efficacy (10, 12–14). Outcomes in a recent randomized clinical trial did not support using piperacillin-tazobactam rather than meropenem for treatment of bloodstream infections caused by ceftriaxone-resistant isolates of *Escherichia coli* and *Klebsiella pneumoniae* (15). These results, considered along with the precepts of antibiotic stewardship, underscore the need for carbapenem-sparing therapies for empirical treatment of infections caused by microorganisms expressing contemporary ESBLs (11).

Enmetazobactam (formerly AAI101) is an investigational penicillanic acid sulfone BLI active against a wide range of β-lactamases, particularly ESBLs (TEMs, SHVs, and CTX-Ms) (16). The combination of cefepime with enmetazobactam has *in vitro* activity comparable to that of meropenem and is more potent than piperacillin-tazobactam against clinical isolates of ESBL-producing *Enterobacteriaceae* collected in surveillance programs (17). Cefepime-enmetazobactam has entered phase 3 pivotal studies for complicated urinary tract infections, including acute pyelonephritis, attributed to *Enterobacteriaceae* (ClinicalTrials registration number NCT03687255). In the present study, quality control (QC) ranges for cefepime-enmetazobactam antimicrobial susceptibility testing by broth microdilution and disk diffusion (18–20) were determined.

(Data in this study were presented at the 28th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Madrid, Spain, 21 to 24 April 2018 [21], and the 29th ECCMID, Amsterdam, Netherlands, 13 to 16 April 2019 [22].)

MATERIALS AND METHODS

Bacterial isolates and culture media. Bacterial strains were subcultured on Mueller-Hinton agar (MHA) overnight at 35°C. Isolates used to select the appropriate cefepime-enmetazobactam disk mass included a challenge panel of 58 recent geographically diverse clinical isolates consisting of 20 *E. coli* isolates, 36 *K. pneumoniae* isolates, and 2 *Proteus mirabilis* isolates expressing defined β-lactamases including ESBLs (CTX-M-15, SHV-11, SHV-12, SHV, and TEM-1), OXA-1/30, OXA-48, and KPC-2, of which 37.9% (22 of 56) were resistant to meropenem. An additional test panel of 518 clinical isolates (all obtained during 2016 from a worldwide surveillance program) was included consisting of 21 *Citrobacter freundii* isolates, 21 *Citrobacter koseri* isolates, 28 *Klebsiella aerogenes* isolates, 77 *Enterobacter cloacae* isolates, 103 *E. coli* isolates, 27 *Klebsiella oxytoca* isolates, 101 *K. pneumoniae* isolates, 27 *Morganella morganii* isolates, 25 *P. mirabilis* isolates, 21 *Proteus vulgaris* isolates, 21 *Providencia rettgeri* isolates, 21 *Providencia stuartii* isolates, and 25 *Serratia marcescens* isolates and expressing a diversity of ESBLs (CTX-M-14, CTX-M-15, CTX-M-27, CTX-M-55, CTX-M-91, SHV-2a, SHV-12, VEB-1), carbapenemases (KPC-2, KPC-3, OXA-48), metallo-β-lactamases (IMP-27, NDM-1, VIM-1), AmpC (ACT-16, ACT-17, ACT-18, ACT-47, CMY-2, CMY-86), and other β-lactamases, such as DHA-1, SHV-11, SHV-28, TEM-1, OXA1/30, OXA-9, and OXA-10.

For the M23 tier 2 QC studies, the QC reference strains used were *E. coli* ATCC 25922 (constitutive low-level EC-5 narrow-spectrum AmpC expression) (23, 24), *E. coli* ATCC 35218 (non-ESBL, TEM-1 β-lactamase-producing), *E. coli* NCTC 13353 (CTX-M-15, ESBL-producing), *K. pneumoniae* ATCC 700603 (SHV-18, OXA-2 genotype, ESBL-producing), and *P. aeruginosa* ATCC 27853 (inducible AmpC [PDC-5] β-lactamase-producing) (25). For broth microdilution MIC studies, the three lots of cation-adjusted Mueller-Hinton broth (CAMHB) used were from Difco (lot number 5181782; Becton, Dickinson, Franklin Lakes, NJ), BD/BBL (lot number 5257869; Becton, Dickinson), and Oxoid (lot number 1433705; Thermo Fisher Scientific, Waltham, MA). For disk diffusion studies, the three lots of MHA were from Remel (lot number 348358; Thermo Fisher Scientific), BD/BBL (lot number 8123531; Becton, Dickinson), and Hardy Diagnostics (lot number 417498; Hardy Diagnostics, Santa Maria, CA).

Broth microdilution susceptibility testing. Bacterial inocula were quantified by serial dilution plating. Broth microdilution MIC testing was performed according to CLSI M07 (18) and M100 (19) guidelines in CAMHB for both cefepime and the combination of cefepime-enmetazobactam. Use of enmetazobactam at a fixed concentration of 8 μg/ml was derived from the exposure-response relationship described in an *in vivo* infection model (26). MIC panels were manufactured by Thermo Fisher Scientific (Oakwood Village, OH) and shipped frozen to participating laboratories.

Disk diffusion testing and selection of cefepime-enmetazobactam disk mass. Disk diffusion testing on MHA was performed according to CLSI M02 (20) and M100 (19) guidelines. Bacterial inocula were quantified by serial dilution plating. A CLSI M23 tier 1 study (27) was performed to identify the appropriate cefepime-enmetazobactam disk mass from disks impregnated with either 30/10 μg, 30/15 μg, 30/20 μg, or 30/30 μg of the combination. Disks of cefepime-enmetazobactam were prepared by spotting 20 μl of 50× drug stock solution (cefepime or enmetazobactam) onto sterile Taxo blank disks (lot number 231039; Becton, Dickinson), which were air-dried in a laminar flow hood in the dark before adding the second drug. Control disks containing solvent only were prepared and tested for inhibitory activity. Pending use, impregnated disks were stored at −20°C for up to 2 weeks. Reference broth microdilution MIC values and inhibition zone diameters (determined in duplicate) were obtained concurrently, using identical inocula, for the challenge panel of 58 *Enterobacteriaceae* isolates spanning

a wide range of cefepime-enmetazobactam MIC values (enmetazobactam fixed at 8 μg/ml). Data were examined in scatterplot format, and the error rate-bounded method was used to identify the disk mass minimizing the number of very major (false susceptible), major (false resistant), and minor (misclassification in the range of 1 or 2 doubling dilutions above or below the intermediate MIC [i.e., I + 1, I + 2, I − 1, or I − 2]) discrepancy errors.

Further analysis of cefepime-enmetazobactam disk masses was performed using the test panel of 518 *Enterobacteriaceae* clinical isolates. For each isolate, broth microdilution MIC and inhibition zone diameters (tested in duplicate) were obtained concurrently, and the error rate-bounded method was used to select the final disk mass. QC strains *E. coli* ATCC 25922, *E. coli* ATCC 35218, *E. coli* NCTC 13353, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853 were used to ensure appropriate assay performance according to CLSI MIC and disk diffusion QC ranges for cefepime (lot number 6307919; Becton, Dickinson, Sparks, MD), meropenem (lot number 7019661; Becton, Dickinson), and piperacillin-tazobactam (lot number 7019661; Becton, Dickinson).

CLSI M23 tier 2 QC range study design. The CLSI M23 tier 2 guidelines (27) were followed to establish QC ranges for broth microdilution MIC and disk diffusion assays. For broth microdilution MIC QC ranges, eight participating laboratories (exceeding the recommended testing at seven sites and allowing for exclusion of a data set from one laboratory if meeting statistical outlier criteria as described in reference 28) performed 10 MIC replicates in the three different media lots from three manufacturers for the five QC strains, totaling 240 MIC determinations per strain (a minimum of 210 MIC determinations are required). Each MIC replicate utilized an individually prepared inoculum suspension. Susceptibility testing was performed over a minimum of 3 days, with up to four replicates tested per day. Appropriate assay performance was verified by comparing cefepime MIC values for *E. coli* ATCC 25922, *E. coli* NCTC 13353, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853 to the established QC ranges reported in CLSI M100 (29). For disk diffusion QC ranges, 10 replicates of two lots of cefepime-enmetazobactam 30/20-μg disks (lot numbers 3044 and 3045; Oxoid Ltd, Basingstoke, UK) were tested on MHA from three different sources ($2 \times 3 \times 10 = 60$ inhibition zone diameters per laboratory) in each of the 8 participating laboratories, for a total of 480 inhibition zone diameters per QC strain (a minimum of 420 inhibition zone diameters are required). Two lots of cefepime 30-μg disks (lot number 8030809; Becton, Dickinson and lot number 2288845; Oxoid) were used. One lot of piperacillin-tazobactam 100/10-μg disks (lot number 8052746, Becton, Dickinson) also was tested ($1 \times 3 \times 10 = 30$ inhibition zone diameters per laboratory $\times$ 8 sites = 240 total inhibition zone diameter values per QC strain). Appropriate assay performance was assessed by comparing inhibition zone diameters obtained for cefepime and piperacillin-tazobactam disks to established QC ranges. Testing was performed over a minimum of 3 days, with no more than four replicates tested per day. Each replicate utilized an individually prepared inoculum suspension (minimum of five inoculum verifications per organism per participating laboratory). Three laboratories participated in both broth microdilution and disk diffusion M23 QC testing.

Cefepime-enmetazobactam 30/20-μg disks from a second supplier (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) became available for testing after completing the M23 tier 2 studies. Performance of the Liofilchem disks was assessed for the five QC strains following CLSI tier 1 guidelines, in which 10 replicates of a single disk lot on three different sources of MHA ($1 \times 3 \times 10 = 30$ inhibition zone diameters per QC strain) were tested in a single laboratory over 3 days and with three inoculum preparations. Cefepime-enmetazobactam 30/20-μg disks from Oxoid and cefepime 30-μg disks (Becton, Dickinson; used for QC purposes) were tested concurrently to assess appropriate assay performance.

Analysis of QC reference ranges. Inhibition zone diameter ranges for each QC reference strain were determined using the Gavan statistic (30) and RangeFinder (28) statistical program. RangeFinder also determines if the central tendencies (mean, median, and mode) of data sets obtained from individual laboratories are statistical outliers (28) and should be excluded from analysis. Only the CLSI-accepted QC ranges are presented in the text.

RESULTS

Determination of broth microdilution MIC QC ranges. Cefepime-enmetazobactam (fixed enmetazobactam concentration of 8 μg/ml) broth microdilution MIC QC ranges for the five QC reference strains were established following a CLSI M23 tier 2 study design that included eight participating laboratories (Table 1). Overall, cefepime-enmetazobactam MIC determinations demonstrated acceptable intra- and interlaboratory reproducibility, as 99.2 to 100% of all reported values for the five QC strains were within a span of $\leq$3 doubling dilutions (Tables 2 to 6). The commercial source of CAMHB had no meaningful impact on MIC determinations for cefepime or cefepime-enmetazobactam, as the median and modal values for all strains varied by no more than a single doubling dilution. For *E. coli* ATCC 25922, cefepime-enmetazobactam MIC values were within a three $\log_2$ dilution range (0.03 to 0.12 μg/ml) (Table 2). All cefepime MIC values for this isolate were within the CLSI-approved four doubling dilution QC range of 0.016 to 0.12 μg/ml (data not shown), confirming appropriate assay performance. For TEM-1-producing strain *E. coli* ATCC 35218, which is highly susceptible to cefepime, MIC values obtained for cefepime with or without enmetazo-

TABLE 1 CLSI-approved broth microdilution MIC QC ranges determined for cefepime and cefepime-enmetazobactam (fixed enmetazobactam concentration of 8 μg/ml) against selected reference strains

	Cefepime-enmetazobactam			Cefepime		
Reference strain	MIC QC range[a]	No. of doubling dilutions in range	% Of values in range[b]	MIC QC range	No. of doubling dilutions in range	% Of values in range
E. coli ATCC 25922	0.03/8–0.12/8	3	100.0	0.016–0.12[c]	4	100.0
E. coli ATCC 35218	0.008/8–0.06/8	4	100.0	0.008–0.06	4	100.0
E. coli NCTC 13353	0.03/8–0.12/8[d]	3	100.0	≥64[c]		100.0
K. pneumoniae ATCC 700603	0.12/8–0.5/8	3	100.0	0.25–2[c]	4	100.0
P. aeruginosa ATCC 27853	0.5/8–2/8	3	99.6	0.5–4[c]	4	100.0

[a]QC ranges were approved at the June 2017 meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing.
[b]Percentage of values in range determined from 240 replicates performed in 8 laboratories.
[c]Current CLSI QC range (35).
[d]Excluding data from one laboratory (statistical outlier).

bactam were within the established cefepime CLSI four doubling dilution range of 0.008 to 0.06 μg/ml (Table 3). For CTX-M-15-producing strain *E. coli* NCTC 13353, which is highly resistant to cefepime, cefepime-enmetazobactam MIC values were within a three doubling dilution range (0.03 to 0.12 μg/ml) (Table 4). All cefepime MICs determined against *E. coli* NCTC 13353 were ≥64 μg/ml, affirming ESBL expression in this strain. The MIC data set collected in one participating laboratory for this isolate was determined to be a statistical outlier for the mean, median, and modal MIC values based on the RangeFinder program and was excluded from the analysis. For SHV-18-producing strain *K. pneumoniae* ATCC 700603, which encodes an ESBL that inefficiently hydrolyzes cefepime (31), cefepime-enmetazobactam MIC values were within a three doubling dilution range (0.12 to 0.5 μg/ml) (Table 5) that overlapped the four doubling dilution range of MICs obtained for cefepime (0.25 to 2 μg/ml; data not shown); all cefepime MICs were within the CLSI-approved range. For *P. aeruginosa* ATCC 27853, including enmetazobactam had no effect on the QC range relative to that of cefepime alone, as cefepime MICs with or without enmetazobactam were within the CLSI-approved cefepime QC range of 0.5 to 4 μg/ml (Table 6; data not shown).

Selection of cefepime-enmetazobactam disk mass. A two-step approach was taken to select a suitable cefepime-enmetazobactam disk mass. Initially, four different cefepime-enmetazobactam disk masses were evaluated in a pilot study against a challenge panel of 58 *Enterobacteriaceae* isolates that express a range of β-lactamases including ESBLs (CTX-M, SHV, and TEM), AmpC, OXA, and KPC and with MIC values bracketing the projected susceptibility breakpoint for cefepime-enmetazobactam (susceptible-dose-dependent MIC of ≤8 μg/ml). When this evaluation was completed, two of the four cefepime-enmetazobactam disk masses from the pilot study were selected to assess their performance against a test panel of 518 contemporary *Enterobacteriaceae* clinical isolates. All cefepime-enmetazobactam disks contained a cefepime mass of 30 μg to match the cefepime mass used in commercially available disks approved by the CLSI and EUCAST. Inhibition zone diameters and MICs were correlated in scatterplots and error rates determined as described in CLSI document M23-04 (27).

Cefepime-enmetazobactam (fixed enmetazobactam concentration of 8 μg/ml) broth microdilution MIC values covered a range from 0.03 μg/ml to 32 μg/ml for the challenge panel of 58 isolates. Disk diffusion was performed concurrently, and results were presented in scatterplots for cefepime-enmetazobactam disk masses of 30/10 μg (see Fig. S1 in the supplemental material), 30/15 μg (see Fig. S2 in the supplemental material), 30/20 μg (see Fig. S3 in the supplemental material), and 30/30 μg (see Fig. S4 in the supplemental material).

When the CLSI cefepime breakpoint interpretive criteria (susceptible, ≤2 μg/ml; susceptible dose dependent, 4 to 8 μg/ml; resistant, ≥16 μg/ml) for *Enterobacteriaceae* were applied to the scatterplot of cefepime-enmetazobactam 30/20-μg disks, the proposed inhibition zone diameter breakpoints of ≥22 mm for susceptible and

TABLE 2 Inter- and intralaboratory comparisons of cefepime-enmetazobactam MICs (fixed enmetazobactam concentration of 8 μg/ml) and inhibition zone diameters with 30/20-μg disks for *E. coli* ATCC 25922 obtained in the CLSI M23 tier 2 study to establish QC ranges

Assay Measure[a]	No. of occurrences by medium lot[b]			No. of occurrences by disk lot		No. of occurrences by laboratory[c]								Total no. of occurrences
	A	B	C	A	B	A	B	C	D	E	F	G	H	
MIC (μg/ml)														
0.03	**41**	**23**	**3**			**19**	**9**	**3**	**7**	**3**	**10**	**3**	**13**	**67**
0.06	**39**	**57**	**75**			**11**	**21**	**27**	**22**	**26**	**20**	**27**	**17**	**171**
0.12			**2**						**1**	**1**				**2**
IZD (mm)														
31			1	1					1					1
32														
33	16	8	4	14	14	4		1	4		10		9	28
34	27	28	29	39	45	8	10	15	10	2	17		22	84
35	50	69	48	86	81	23	19	19	28	32	17	3	26	167
36	49	36	48	72	61	20	31	14	14	25	10	16	3	133
37	10	13	20	17	26	5		11	3	1	37	17		43
38	6	2	3	4	7							11		11
39	2	4	5	6	5							11		2
40			2	1	1							2		
Total	**80**/160	**80**/160	**80**/160	240	240	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**240**/480
Mean	**0.04**/35.2	**0.05**/35.3	**0.06**/35.5	35.3	35.4	**0.04**/35.2	**0.05**/35.4	**0.06**/35.3	**0.05**/35.0	**0.06**/35.4	**0.05**/34.8	**0.06**/37.3	**0.05**/34.4	**0.05**/35.3
Median	**0.03**/35	**0.06**/35	**0.06**/35	35	35	**0.03**/35	**0.06**/36	**0.06**/35	**0.06**/35	**0.06**/35	**0.06**/35	**0.06**/37	**0.06**/34	**0.06**/35
Mode	**0.03**/35	**0.06**/35	**0.06**/35,36	35	35	**0.03**/35	**0.06**/36	**0.06**/35	**0.06**/35	**0.06**/35	**0.06**/34,35	**0.06**/37	**0.06**/35	**0.06**/35
Geometric mean	**0.04**/35.2	**0.05**/35.2	**0.06**/35.5	35.3	35.3	**0.04**/35.2	**0.05**/35.3	**0.06**/35.3	**0.05**/35.0	**0.06**/35.4	**0.05**/34.7	**0.06**/37.3	**0.04**/34.4	**0.05**/35.3
Range	**2**/7	**2**/7	**3**/10	10	8	**2**/5	**2**/3	**2**/5	**3**/7	**3**/4	**2**/5	**2**/6	**2**/4	**3**/10

[a]Values in bold are results from the broth microdilution MIC assay. Enmetazobactam was included in the combination at a fixed concentration of 8 μg/ml. IZD, inhibition zone diameter.

[b]The medium used for broth microdilution MIC determinations was cation-adjusted Mueller-Hinton broth from (A) Difco (lot number 5181782), (B) BD (lot number 5257869), and (C) Oxoid (lot number 1433705). The medium used in the disk diffusion assay was Mueller-Hinton agar from (A) Remel (lot number 348358), (B) BBL (lot number 8123531), and (C) Hardy Diagnostics (lot number 417498).

[c]The eight qualified participating laboratories are coded A through H. Only three of the laboratories participated in both the broth microdilution MIC and disk diffusion M23 QC studies.

TABLE 3 Inter- and intralaboratory comparisons of cefepime-enmetazobactam MICs (enmetazobactam concentrations fixed at 8 μg/ml) and inhibition zone diameters with 30/20-μg disks for *E. coli* ATCC 35218 obtained in the CLSI M23 tier 2 study to establish QC ranges

Assay measure[a]	No. of occurrences by medium lot[b]			No. of occurrences by disk lot		No. of occurrences by laboratory[c]								Total no. of occurrences
	A	B	C	A	B	A	B	C	D	E	F	G	H	
MIC (μg/ml)														
0.008		**49**				**9**	**5**	**8**	**4**	**6**	**6**	**4**	**7**	**49**
0.016	**59**	**5**	**41**			**20**	**11**	**9**	**12**	**9**	**12**	**9**	**23**	**105**
0.03	**21**	**26**	**37**			**1**	**12**	**13**	**14**	**15**	**12**	**17**		**84**
0.06			**2**				**2**							**2**
IZD (mm)														
32	3	3	2	3	5	2		1					5	8
33	11	12	4	12	15	7		5	3		2		10	27
34	31	40	29	50	50	27	8	9	12	2	19		23	100
35	38	51	44	67	66	22	23	13	14	23	16	6	16	133
36	61	39	60	79	81	2	25	28	25	35	14	25	6	160
37	15	15	17	26	21		4	3	6		9	25		47
38	1		4	3	2			1				4		5
Total	**80**/160	**80**/160	**80**/160	240	240	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**240**/480
Mean	**0.02**/35.2	**0.016**/35.0	**0.024**/35.4	35.2	35.1	**0.013**/34.3	**0.020**/35.4	**0.018**/35.3	**0.020**/35.6	**0.019**/35.6	**0.018**/35.2	**0.021**/36.5	**0.013**/34.1	**0.017**/35.2
Median	**0.016**/35	**0.008**/35	**0.016**/36	35	35	**0.016**/34	**0.016**/35	**0.016**/36	**0.016**/36	**0.02**/36	**0.016**/35	**0.03**/36	**0.016**/34	**0.016**/35
Mode	**0.016**/36	**0.008**/35	**0.016**/36	36	36	**0.016**/34	**0.03**/36	**0.03**/36	**0.03**/36	**0.03**/36	**0.016, 0.03**/34	**0.03**/36, 37	**0.016**/34	**0.016**/36
Geometric mean	**0.019**/35.2	**0.012**/35.0	**0.022**/35.4	35.2	35.1	**0.013**/34.2	**0.020**/35.4	**0.017**/35.3	**0.020**/35.3	**0.019**/35.5	**0.018**/35.1	**0.021**/36.4	**0.014**/34.1	**0.017**/35.2
Range	**2**/7	**3**/6	**3**/7	7	7	**3**/5	**4**/4	**3**/7	**3**/5	**3**/3	**3**/5	**3**/4	**2**/5	**4**/7

[a]Values in bold are results from the broth microdilution MIC assay. Enmetazobactam was included in the combination at a fixed concentration of 8 μg/ml.

[b]The medium used for broth microdilution MIC determinations was cation-adjusted Mueller-Hinton broth from (A) Difco (lot number 5181782), (B) BD (lot number 5257869), and (C) Oxoid (lot number 1433705). The medium used in the disk diffusion assay was Mueller-Hinton agar from (A) Remel (lot number 348358), (B) BBL (lot number 8123531), and (C) Hardy Diagnostics (lot number 417498).

[c]The eight qualified participating laboratories are coded A through H. Only three laboratories participated in both broth microdilution MIC and disk diffusion M23 QC studies.

TABLE 4 Inter- and intralaboratory comparisons of cefepime-enmetazobactam MICs (fixed enmetazobactam concentration of 8 μg/ml) and inhibition zone diameters with 30/20-μg disks for *E. coli* ATCC 13353 obtained in the CLSI M23 tier 2 study to establish QC ranges

Assay measure[a]	No. of occurrences by medium lot[b]			No. of occurrences by disk lot		No. of occurrences by laboratory[c]								Total no. of occurrences
	A	B	C	A	B	A	B	C	D	E	F	G	H	
MIC (μg/ml)														
0.03	**2**					**1**		**1**						**2**
0.06	**67**	**66**	**64**			**29**	**24**	**29**	**29**	**30**	***1***	**28**	**28**	**197**
0.12	**1**	**4**	**6**				**6**		**1**			**2**	**2**	**11**
0.25											***18***			
0.5											***11***			
IZD (mm)														
27				4	6	2							8	10
28				14	11	9				2			14	25
29				47	46	30	1	11	1	14	11		25	93
30				70	73	10	17	23	21	27	20	16	9	143
31				67	77	9	32	22	21	17	21	18	4	144
32				31	23		10	4	13		8	19		54
33				7	4				4			7		11
Total	**80**/160	**80**/160	**80**/160	240	240	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	***30***/60	**30**/60	**30**/60	**240**/480
Mean	**0.06**/30.4	**0.06**/29.8	**0.07**/30.4	30.3	30.2	**0.06**/29.3	**0.07**/30.9	**0.06**/30.3	**0.06**/31.0	**0.06**/30.0	***0.34***/30.4	**0.07**/31.3	**0.07**/28.8	**0.06**/30.2
Median	**0.06**/31	**0.06**/30	**0.06**/30.5	30	30	**0.06**/29	**0.06**/31	**0.06**/30	**0.06**/31	**0.06**/30	***0.25***/30	**0.06**/31	**0.06**/29	**0.06**/30
Mode	**0.06**/31	**0.06**/29	**0.06**/31	30	31	**0.06**/29	**0.06**/31	**0.06**/30	**0.06**/30, 31	**0.06**/30	***0.25***/31	**0.06**/32	**0.06**/29	**0.06**/31
Geometric mean	**0.06**/30.4	**0.06**/29.8	**0.06**/30.4	30.2	30.2	**0.06**/29.2	**0.07**/30.8	**0.06**/30.3	**0.06**/31.0	**0.06**/30.0	***0.31***/30.4	**0.06**/31.3	**0.06**/28.8	**0.06**/30.2
Range	**3**/7	**2**/6	**2**/7	7	7	**2**/5	**2**/4	**2**/4	**2**/5	**1**/4	**4**/4	**2**/4	**2**/5	**3**/7

[a]Values in bold are results determined from the broth microdilution MIC assay. Enmetazobactam was included in the combination at a fixed concentration of 8 μg/ml. Values in bold italics are MIC results that have been excluded from the analysis as the data set determined in the participating laboratory (F) was deemed to be a statistical outlier.

[b]The medium used for broth microdilution MIC determinations was cation-adjusted Mueller-Hinton broth from (A) Difco (lot number 5181782), (B) BD (lot number 5257869), and (C) Oxoid (lot number 1433705). The medium used in the disk diffusion assay was Mueller-Hinton agar from (A) Remel (lot number 348358), (B) BBL (lot number 8123531), and (C) Hardy Diagnostics (lot number 417498).

[c]The eight qualified participating laboratories are coded A through H. Only three laboratories participated in both broth microdilution MIC and disk diffusion M23 QC studies.

TABLE 5 Inter- and intralaboratory comparisons of cefepime-enmetazobactam MICs (enmetazobactam concentration fixed at 8 μg/ml) and inhibition zone diameters with 30/20-μg disks for *K. pneumoniae* ATCC 700603 obtained in the CLSI M23 tier 2 study to establish QC ranges

Assay measure[a]	No. of occurrences by medium lot[b]			No. of occurrences by disk lot		No. of occurrences by laboratory[c]								Total no. of occurrences
	A	B	C	A	B	A	B	C	D	E	F	G	H	
MIC (μg/ml)														
0.12	**10**	**3**	**4**			**12**		**2**			**1**		**2**	**17**
0.25	**62**	**68**	**69**			**17**	**23**	**27**	**26**	**27**	**24**	**27**	**28**	**199**
0.5	**8**	**9**	**7**			**1**	**7**	**1**	**4**	**3**	**5**	**3**		**24**
IZD (mm)														
23	1				1								1	1
24														
25														
26	1	4	1	5	1	6								6
27	8	19	4	18	13	14	7			6			4	31
28	36	53	31	57	63	26	35	12		21	8	1	17	120
29	46	39	46	66	65	12	18	20	10	22	23	9	17	131
30	37	32	41	51	59	2		22	25	10	15	16	20	110
31	17	9	26	28	24			6	16	1	14	15		52
32	12	4	6	11	11				9			12	1	22
33	2		5	4	3							7		7
Total	**80**/160	**80**/160	**80**/160	240	240	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**240**/480
Mean	**0.26**/30.4	**0.27**/29.8	**0.27**/30.4	30.3	30.2	**0.19**/27.8	**0.29**/28.2	**0.24**/29.4	**0.27**/30.4	**0.27**/28.7	**0.27**/29.6	**0.27**/30.8	**0.24**/28.9	**0.26**/29.2
Median	**0.25**/31	**0.25**/30	**0.25**/30.5	30	30	**0.25**/28	**0.25**/28	**0.25**/29	**0.25**/30	**0.25**/29	**0.25**/29	**0.25**/31	**0.25**/29	**0.25**/29
Mode	**0.25**/31	**0.25**/29	**0.25**/31	30	31	**0.25**/28	**0.25**/28	**0.25**/30	**0.25**/30	**0.25**/29	**0.25**/29	**0.25**/30	**0.25**/30	**0.25**/29
Geometric mean	**0.24**/30.4	**0.25**/29.8	**0.26**/30.4	30.2	30.2	**0.19**/27.8	**0.29**/28.2	**0.24**/29.4	**0.27**/30.4	**0.27**/28.6	**0.27**/29.6	**0.27**/30.8	**0.24**/28.8	**0.25**/29.2
Range	**3**/7	**3**/6	**3**/7	7	7	**3**/5	**2**/3	**3**/4	**2**/4	**2**/5	**3**/4	**2**/6	**2**/10	**3**/11

[a]Values in bold are results from the broth microdilution MIC assay. Enmetazobactam was included in the combination at a fixed concentration of 8 μg/ml.

[b]The medium used for broth microdilution MIC determinations was cation-adjusted Mueller-Hinton broth from (A) Difco (lot number 5181782), (B) BD (lot number 5257869), and (C) Oxoid (lot number 1433705). The medium used in the disk diffusion assay was Mueller-Hinton agar from (A) Remel (lot number 348358), (B) BBL (lot number 8123531), and (C) Hardy Diagnostics (lot number 417498).

[c]The eight qualified participating laboratories are coded A through H. Only three laboratories participated in both broth microdilution MIC and disk diffusion M23 QC studies.

TABLE 6 Inter- and intralaboratory comparisons of cefepime-enmetazobactam MICs (fixed enmetazobactam concentration of 8 μg/ml) and inhibition zone diameters with 30/20-μg disks for *P. aeruginosa* ATCC 27853 obtained in the CLSI M23 tier 2 study to establish QC ranges

Assay measure[a]	No. of occurrences by medium lot[b]			No. of occurrences by disk lot		No. of occurrences by laboratory[c]								Total no. of occurrences
	A	B	C	A	B	A	B	C	D	E	F	G	H	
MIC (μg/ml)														
0.5	**3**	**1**	**1**						**2**				**3**	**5**
1	**63**	**68**	**64**			**22**	**25**	**29**	**26**	**20**	**30**	**23**	**20**	**195**
2	**14**	**11**	**14**			**8**	**5**	**1**	**2**	**9**		**7**	**7**	**39**
4			**1**							**1**				**1**
IZD (mm)														
26	1	2		1	2	3								
27	8	12	4	13	11	19	2			2			1	
28	32	40	38	55	55	30	29	1		5	20		25	
29	40	46	44	59	71	8	27	16	7	31	4	9	28	
30	52	37	46	68	67		2	39	24	21	20	23	6	
31	20	21	22	35	28			4	24	1	13	21		
32	5	1	6	7	5				3		3	6		
33	2	1		2	1				2			1		
Total	**80**/160	**80**/160	**80**/160	240	240	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**30**/60	**240**/480
Mean	**1.2**/30.4	**1.1**/29.8	**1.2**/30.4	29.3	29.2	**1.1**/27.8	**1.1**/28.2	**1**/29.4	**1**/30.4	**1.3**/28.7	**1**/29.6	**1.2**/30.8	**1.1**/28.9	**1.1**/29.2
Median	**1**/31	**1**/30	**1**/30.5	29	29	**1**/28	**1**/28	**1**/29	**1**/30	**1**/29	**1**/29	**1**/31	**1**/29	**1**/29
Mode	**1**/31	**1**/29	**1**/31	30	29	**1**/28	**1**/28	**1**/30	**1**/30	**1**/29	**1**/29	**1**/30	**1**/30	**1**/29
Geometric mean	**1.1**/30.4	**1.1**/29.8	**1.1**/30.4	29.3	29.2	**1.2**/27.8	**1.1**/28.2	**1**/29.4	**1**/30.4	**1.3**/28.6	**1**/29.6	**1.2**/30.8	**1.1**/28.8	**1.1**/29.2
Range	**3**/8	**3**/8	**4**/6	8	8	**2**/5	**2**/3	**2**/4	**3**/4	**3**/5	**1**/4	**2**/6	**3**/10	**4**/11

[a]Values in bold are results from the broth microdilution MIC assay. Enmetazobactam was included in the combination at a fixed concentration of 8 μg/ml.

[b]The medium used for broth microdilution MIC determinations was cation-adjusted Mueller-Hinton broth from (A) Difco (lot number 5181782), (B) BD (lot number 5257869), and (C) Oxoid (lot number 1433705). The medium used in the disk diffusion assay was Mueller-Hinton agar from (A) Remel (lot number 348358), (B) BBL (lot number 8123531), and (C) Hardy Diagnostics (lot number 417498).

[c]The eight qualified participating laboratories are coded A through H. Only three of the laboratories participated in both broth microdilution MIC and disk diffusion M23 QC studies.

TABLE 7 CLSI disk diffusion QC ranges determined for cefepime (30 μg) and cefepime-enmetazobactam (30/20 μg) against relevant reference strains

	Cefepime-enmetazobactam			Cefepime		
Reference strain	Inhibition zone diameter QC range (mm)[a]	No. of mm	% Of values in range[b]	Inhibition zone diameter QC range (mm)	No. of mm	% Of values in range
E. coli ATCC 25922	32–38	7	97.1	31–37[c]	7	97.1
E. coli ATCC 35218	32–38	7	100.0	31–37	7	100.0
E. coli NCTC 13353	27–33	7	100.0	6–15[c,d]	10	99.5
K. pneumoniae ATCC 700603	26–32	7	98.3	23–29[c]	7	99.8
P. aeruginosa ATCC 27853	26–32	7	99.4	25–31[c]	7	99.2

[a]QC ranges were approved at the January 2019 meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing.
[b]Percentage of values in range determined from 480 replicates performed in eight laboratories.
[c]Current CLSI QC range (35).
[d]Excluding data from one laboratory (statistical outlier).

≤18 mm for resistant resulted in no very major errors or major errors (Fig. S3). Adjusting the breakpoints for 30/10-μg disks to ≥20 mm for susceptible and ≤15 mm for resistant (Fig. S1), for 30/15-μg disks to ≥21 mm for susceptible and ≤16 mm for resistant (Fig. S2), and for 30/30-μg disks to ≥22 mm for susceptible and ≤17 mm for resistant (Fig. S4) yielded the lowest very major error rates attainable for each disk mass (1.7%, 1.7%, and 2.6%, respectively) yet still exceeded the limit of 1.5%. A single major error occurred with both the 30/10-μg and 30/15-μg disks. Results for all four cefepime-enmetazobactam disk masses exceeded the minor error rates for ≥I + 2 (50.0 to 100.0%) and I + 1 to I − 1 (43.3 to 59.4%), an observation likely due to the small sample size and clinically nonrepresentative composition of the challenge panel. Based on these results, 30/10-μg and 30/20-μg disk masses were selected for further analysis using a much larger and more clinically representative strain panel.

The cefepime-enmetazobactam (fixed enmetazobactam concentration of 8 μg/ml) MIC values determined for the clinically representative test set of 518 contemporary *Enterobacteriaceae* isolates ranged from 0.008 μg/ml to 128 μg/ml. From the scatterplot with 30/10-μg disks (see Fig. S5 in the supplemental material), a proposed breakpoint of ≥23 mm for susceptible and ≤18 mm for resistant resulted in no very major errors or major errors, whereas a high minor error rate of 68.8% (I + 1 to I − 1) exceeded the 40% limit. From the scatterplot with 30/20-μg disks (see Fig. S6 in the supplemental material), breakpoints of ≥25 mm for susceptible and ≤19 mm for resistant resulted in acceptable error rates, with no very major errors, no major errors, a minor error rate within the I + 1 to I − 1 range of 25.0% (<40% required), and a total minor error rate of 0.9%. Based on these results, the cefepime-enmetazobactam 30/20-μg disk mass was selected to establish QC ranges.

Determination of disk diffusion QC ranges. A CLSI M23 tier 2 study with eight participating laboratories was used to establish cefepime-enmetazobactam 30/20-μg disk QC ranges for the five QC strains (Table 7). In each laboratory, 60 replicate inhibition zone diameters were determined for the QC strains and analyzed using RangeFinder and the Gavan statistics methods to determine appropriate QC ranges.

For *E. coli* ATCC 25922, the geometric means of cefepime-enmetazobactam 30/20-μg disk inhibition zone diameters ranged from 34.4 mm to 37.3 mm among the participating laboratories (Table 2). A QC range of 32 to 38 mm is proposed, with 97.1% of obtained inhibition zone diameters occurring within these limits (Table 7). For *E. coli* ATCC 35218, the geometric means ranged from 34.1 mm to 36.4 mm (Table 3). All inhibition zone diameters for this isolate were within the proposed QC range of 32 to 38 mm (Table 7). The geometric means for *E. coli* NCTC 13353 ranged from 28.8 mm to 31.1 mm (Table 4); the proposed QC range for this isolate is 27 to 33 mm, with 100% of reported values occurring within this boundary (Table 7). For *K. pneumoniae* ATCC 700603, geometric means varied from 27.8 mm to 29.6 mm (Table 5). A QC range of 26 to 32 mm is proposed, with 98.3% of reported inhibition zone diameters occurring within these limits (Table 7). The geometric means for *P. aeruginosa* ATCC 27853 ranged

from 27.7 mm to 30.5 mm (Table 6), and a QC range of 26 to 32 mm was calculated, encompassing 99.4% of replicate values obtained in the participating laboratories (Table 7). Across all participating laboratories, the source of commercially prepared MHA plates (from three different manufacturers) had minimal impact on inhibition zone diameters, with differences in geometric means of $<$1 mm for each of the five QC strains (Tables 2 to 6). Moreover, minimal variability was observed between the two disk lots from a single manufacturer (Oxoid, Thermo Fisher Scientific), as the geometric means for both lots differed by $\leq$0.1 mm for each QC strain (Tables 2 to 6).

All of the inhibition zone diameters determined with cefepime-enmetazobactam 30/20-μg disks from a second manufacturer (Liofilchem S.r.l.) in the tier 1 study were within the approved CLSI ranges for the five QC reference strains (see Table S1 in the supplemental material). Geometric means for these disks, though smaller by 1.1 mm to 2.6 mm for the QC strains, were still within acceptable limits.

DISCUSSION

As increased usage of carbapenems to treat ESBL-producing pathogens has helped drive carbapenem resistance, "carbapenem-sparing" therapeutic options are required to stem resistance development and dissemination among Gram-negative pathogens (2, 32, 33). Development of the combination of cefepime with the novel BLI enmetazobactam aims to provide empiric and definitive therapy for infections caused by *Enterobacteriaceae*, particularly those expressing ESBLs. Whereas cefepime exhibits intrinsic activity against isolates expressing β-lactamases, AmpCs, and many OXAs, including OXA-48 (34), this fourth-generation cephalosporin remains susceptible to most ESBLs. Enmetazobactam inhibits a broad array of class A β-lactamases, including SHV, TEM, and CTX-M ESBLs (16), and cefepime-enmetazobactam exhibited similar potencies against both a collection of 1,696 recent clinical isolates of *Enterobacteriaceae* and a subset of 211 ESBL-producing *Enterobacteriaceae*, with an MIC_{90} of 0.25 μg/ml for both groups (17). That MIC QC ranges of cefepime-enmetazobactam (fixed enmetazobactam concentration of 8 μg/ml) for CTX-M-15-producing *E. coli* NCTC 13353 and *E. coli* ATCC 25922 are identical indicates that ESBL activity was completely suppressed by enmetazobactam. Differences in the diffusion properties of cefepime and/or enmetazobactam through agar, or reduced enzyme-inhibitor interactions in semisolid medium, or differences in physiological status or amplitude of expression of $bla_{CTX\text{-}M\text{-}15}$ under liquid versus semisolid conditions may account for the smaller inhibition zone diameter range obtained for *E. coli* NCTC 13353 relative to that of *E. coli* ATCC 25922. An MIC QC range of $\geq$64 μg/ml was previously established for cefepime against *E. coli* NCTC 13353, and all cefepime MIC replicates in this study were determined to be $>$128 μg/ml. This strain has been recommended previously as a routine QC strain for testing cefepime-tazobactam (35, 36) and likewise serves as an appropriate control to assess the functionality of enmetazobactam in combination with cefepime. It is, therefore, recommended as a routine QC strain to assess cefepime-enmetazobactam performance in broth microdilution and disk diffusion assays.

The MIC QC ranges of cefepime-enmetazobactam (enmetazobactam concentration fixed at 8 μg/ml) for *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853 were nearly identical to those for cefepime alone (Table 1); similarly, cefepime-enmetazobactam QC ranges were marginally smaller but overlapped those for cefepime alone (Tables 2). Enmetazobactam provided little additional benefit to the antimicrobial activity of cefepime against these four strains, and they are not advised for monitoring BLI activity of enmetazobactam despite a recommendation that *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 are suitable for routine testing of most BL/BLI combinations (19).

In conclusion, the proposed broth microdilution and disk diffusion QC ranges for cefepime-enmetazobactam were approved by the CLSI Subcommittee on Antimicrobial Susceptibility Testing at the June 2017 and January 2019 meetings, respectively. The approved QC ranges ensure that clinical laboratories can reliably and reproducibly assess appropriate assay performance of cefepime-enmetazobactam against several reference

strains, most notably *E. coli* NCTC 13353. These results will help establish the susceptibility breakpoints for cefepime-enmetazobactam required to inform patient care.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00607-19.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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