A Systematic Approach to the Selection of the Appropriate Avibactam Concentration for Use with Ceftazidime in Broth Microdilution Susceptibility Testing

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ABSTRACT Selection of the avibactam concentration to combine with ceftazidime in susceptibility testing was determined using Gram-negative isolates with characterized β -lactamases predefined as susceptible or resistant, based on the known inhibition spectrum of avibactam. MIC values were determined by broth microdilution of ceftazidime with fixed concentrations and ratios of avibactam. A constant concentration of 4 $\mu\text{g/ml}$ of avibactam was selected for susceptibility testing with ceftazidime because of its ability to correctly categorize susceptible and resistant isolates while minimizing categorical errors.

KEYWORDS ceftazidime-avibactam, MIC testing, beta-lactamase inhibitor combination

Ceftazidime, an oxyimino cephalosporin, has potent *in vitro* activity against a broad-spectrum of Gram-negative bacteria, including *Enterobacteriaceae* species and *Pseudomonas aeruginosa*. Production of β -lactamases is a major mechanism of widespread resistance among Gram-negative bacteria. Like most cephalosporins and penicillins, ceftazidime is susceptible to hydrolytic inactivation by various β -lactamases, which results in its decreased activity against many clinically important Gram-negative bacteria. Avibactam is a non- β -lactam β -lactamase inhibitor that reacts with the active site serine β -lactamases, including Class A extended spectrum β -lactamases (ESBLs) and carbapenemases (KPCs). It also inhibits both plasmid-mediated and chromosomally mediated Class C enzymes, and some Class D OXA-type enzymes, but not class B metallo- β -lactamases (1–4). When combined with ceftazidime, avibactam restores susceptibility to isolates resistant to ceftazidime because of the production of one or more serine β -lactamases (3, 5). Avibactam alone has shown a low level of intrinsic antibacterial activity and inhibits some *Enterobacteriaceae* (*Escherichia coli* and *Klebsiella pneumoniae*) at concentrations of $\geq 8 \mu\text{g/ml}$, which is likely due to avibactam binding directly to penicillin-binding proteins (6, 7).

For most antibacterials, including β -lactams, the MIC measurement is relatively simple, in that it measures the bacterial response to various drug concentrations at a defined endpoint in time. Understanding relationships between MIC, pharmacokinetics (PK), and pharmacodynamics (PD) within any one antimicrobial class, such as the cephalosporins, has developed and is fairly straightforward (8–12). However, both MIC testing and PK/PD are more complicated for β -lactam- β -lactamase inhibitor combinations. Two methods can be used for varying the concentrations of the components of β -lactam- β -lactamase inhibitor combinations when measuring *in vitro* susceptibilities of bacteria by MIC. One method employs the dilution of both compounds simultaneously in fixed ratio and is utilized with amoxicillin-clavulanate and ampicillin-sulbactam, both of which are tested at a 2:1 ratio of β -lactam to inhibitor. This ratio reflects the ratio of drug substance given to patients in a pharmacologic dose and is also technically easy to perform in the microbiology laboratory. For ampicillin-sulbactam, there

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was very little differentiation between various ratios or concentrations used in MIC testing (13–15). Other combinations, including ticarcillin-clavulanate and piperacillin-tazobactam, used fixed inhibitor concentrations of 2 and 4 $\mu\text{g/ml}$, respectively (16–18). By utilizing a predictor panel of isolates that produce well-characterized β -lactamases to examine MIC tests with piperacillin-tazobactam and an undeveloped combination, piperacillin-BLI489, it was shown that ratios of drug to inhibitor had a tendency to overpredict susceptibility, and that a constant concentration of 4 $\mu\text{g/ml}$ most accurately separated susceptible from resistant isolates (16, 19). The β -lactamase inhibition spectrum and potency of avibactam has been thoroughly studied; therefore, one can reasonably predict whether an isolate will test with a low or high MIC of ceftazidime-avibactam based on the β -lactamases expressed by any given strain (5, 6, 20–22). The objective of this study was to select test methodology for MIC testing of ceftazidime-avibactam that most accurately identified and separated susceptible from resistant isolates, based on the β -lactamase genes present in the bacterial isolates. Throughout the study, a ceftazidime-avibactam susceptible breakpoint of $\leq 8 \mu\text{g/ml}$ was used to interpret the MIC tests, based on PK/PD predictions (23).

The clinical isolates for this study were comprised of 60 *Enterobacteriaceae* and 27 *P. aeruginosa* isolates (Table 1). The isolates were geographically diverse and recently obtained, with characterized β -lactamases representing a broad range of ceftazidime-avibactam MIC values. Identification of β -lactamase genes and porin mutations was confirmed by whole-genome sequence analysis (Illumina). Using foreknowledge of the inhibitory activity of avibactam, these isolates were predefined for the susceptibility category of ceftazidime-avibactam, based on the β -lactamases produced and application of the known spectrum of β -lactamase inhibition of avibactam. Organisms with Class A or Class C enzymes, as well as several Class D enzymes known to be efficiently inhibited by avibactam (e.g., OXA-48) were designated susceptible for this study. Organisms that expressed Class B enzymes, which are not inhibited by avibactam, and one isolate of *P. aeruginosa* that had a mutated form of AmpC were designated resistant. Isolates that expressed OXA-10 or another Class D enzyme that is weakly or partially inhibited by avibactam were assessed separately and were designated neither susceptible or resistant (Table 1).

MIC values were determined using CLSI broth microdilution methodology (24). At least one quality control reference organism and control antibacterial was used to validate the susceptibility testing each day (25). Ceftazidime was obtained from the U.S. Pharmacopeia (Rockville, MD), and avibactam powder was obtained from Forest Laboratories (Cerexa, Oakland, CA). Avibactam was added to doubling dilutions of ceftazidime either at constant concentrations of 1, 2, 4, or 8 $\mu\text{g/ml}$ or in ratios of 1:1, 2:1, 4:1, 8:1, or 16:1 ceftazidime-avibactam.

MIC distributions for *Enterobacteriaceae* spp. and *P. aeruginosa* against ceftazidime-avibactam determined with the various combinations were grouped by organisms that expressed β -lactamases that are all inhibited by avibactam, those that express at least one β -lactamase that is only partially inhibited by avibactam, and those that contain at least one β -lactamase that is not inhibited by avibactam, and representative graphs are shown in Fig. 1. Using the predefined categorization of isolates, discrepancies in susceptibility results were determined (Table 2). Four of the five testing conditions correctly identified all of the isolates that expressed a β -lactamase that was not inhibited by avibactam as resistant (Fig. 1A, B, D, and E). Susceptible results were obtained with two isolates (one *E. coli* and one *K. pneumoniae*, both expressing the metallo- β -lactamase [MBL] NDM-1) using the constant concentration of 8 $\mu\text{g/ml}$ of avibactam. These results were due to the intrinsic activity of avibactam alone against some strains of *Enterobacteriaceae* at concentrations of $\geq 8 \mu\text{g/ml}$ (Fig. 1C). Because MBLs are not within the spectrum of inhibition for avibactam, this low-level intrinsic activity was categorized as falsely susceptible, as such a result risks recommendation of ineffective therapy for these kinds of isolates. In addition to the categorization errors noted in Table 2, the constant concentration of 8 $\mu\text{g/ml}$ of avibactam exhibited intrinsic activity against 27% of the *Enterobacteriaceae* isolates tested, an attribute that has been

TABLE 1 Organisms tested and their β -lactamase content

Organism	Strain name	β -Lactamase(s) detected ^a	β -Lactamase class(es)	Expected inhibition by AVI ^b
<i>Escherichia coli</i>	ATCC 25922	None		NA
	ATCC 35218	TEM-1	A	Y
<i>Klebsiella pneumoniae</i>	ATCC 700603	SHV-18, OXA-2	A, C	Y
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Inducible AmpC	C	Y
<i>Citrobacter freundii</i>	ARC3522	Derepressed AmpC, TEM-1, CMY-65	A, C	Y
	ARC3523	Derepressed AmpC	C	Y
<i>Enterobacter cloacae</i>	ARC3519	Derepressed AmpC, TEM-1, KPC-2, LAP-2, KLUC-2	A, C	Y
	ARC3525	Inducible AmpC (P99)	C	Y
	ARC3528	Chromosomal AmpC (with loop deletion)	C	N
	ARC3532	Chromosomal AmpC, KPC-3, TEM-1, OXA-9	A, C, D	Y
	ARC3601	NDM-1, TEM-1, CTX-M-15, OXA-1, OXA-9, LAP-2	A, B, C, D	N
	ARC3662	TEM-1, CTX-M-15, OXA-1, OXA-9	A, D	Y
<i>Escherichia coli</i>	ARC3467	OXA-48	D	Y
	ARC3518	KPC-2, TEM-1, OXA-1	A, D	Y
	ARC3520	TEM-16	A	Y
	ARC3531	VEB-5, TEM-1	A	Y
	ARC3534	TEM-1, CTX-M-14, CMY-2	A, C	Y
	ARC3600	NDM-1, CMY-6, OXA-1	B, C, D	N
	ARC3627	CTX-M-15, TEM-1, OXA-1	A, D	Y
	ARC3663	CTX-M-15, OXA-1	A, D	Y
	ARC3665	CTX-M-15, TEM-1, OXA-1	A, D	Y
	ARC3666	TEM-1, CTX-M-15, SHV-31, OXA-1/30	A, D	Y
	ARC3667	CTX-M-15, TEM-1, OXA-1, ACC-4	A, C, D	Y
	ARC3672	TEM-1, CTX-M-15, OXA-1	A, D	Y
	ARC3677	OXA-1, CMY-42	C, D	Y
	ARC3680	NDM-1, TEM-1, CTX-M-15, OXA-1, CMY-2	A, B, C, D	N
	ARC3689	CTX-M-15	A	Y
	ARC3690	CTX-M-15	A	Y
	ARC3691	CTX-M-15, OXA-1, CMY-42	A	Y
	ARC3692	SHV-5	A	Y
	ARC3695	CTX-M-15, OXA-1, CMY-42	A, C, D	Y
	ARC3701	CTX-M-15, OXA-1	A, D	Y
	ARC3704	TEM-1, CTX-M-15	A	Y
	ARC3707	CTX-M-15, OXA-1, CMY-42	A, C, D	Y
	ARC3709	CMY-6	C	Y
	ARC3799	NDM-3, TEM-1, CTX-M-15, OXA-2, CMY-6	A, B, C, D	N
	ARC3805	NDM-1, TEM-208, OXA-1, OXA-2, CTX-M-15, CMY-4	A, B, C, D	N
	ARC3807	NDM-1, TEM-1, SHV-12, CMY-42, OXA-9	A, B, C, D	N
<i>Klebsiella oxytoca</i>	ARC1920	OXA-2	D	Y
	ARC2879	K1	A	Y
	ARC2882	OXA-1, OXA-2	D	Y
<i>Klebsiella pneumoniae</i>	ARC2708	TEM-1, SHV-11, KPC-2, OXA-9	A, D	Y
	ARC2710	TEM-1, SHV-11, KPC-2, OXA-1	A, D	Y
	ARC2711	TEM-1, SHV-11, SHV-12, KPC-2	A	Y
	ARC2712	TEM-1, SHV-11, SHV-12, KPC-2A (OmpK35 ⁻)	A	Y
	ARC2929	KPC-3, TEM-1, SHV-11	A	Y
	ARC3536	CTX-M-15, TEM-1, OXA-1, SHV-11, SHV-12	A	Y
	ARC3592	SHV-5	A	Y
	ARC3602	NDM-1, TEM-1, CTX-M-15, SHV-11, CMY-6	A, B, C	N
	ARC3712	CTX-M-15, SHV-11, TEM-1	A	Y
	ARC3713	CTX-M-15, OXA-1, SHV-5, TEM-1	A, D	Y
	ARC3715	NDM-1, TEM-1, CTX-M-15, SHV-1	A, B	N
	ARC3716	NDM-1, TEM-1, CTX-M-15, SHV-11, DHA	A, B, C	N
	ARC3717	NDM-1, CTX-M-15, SHV-11, TEM-1	A, B	N
	ARC3720	CTX-M-15, SHV-11, OXA-1	A, D	Y
	ARC3797	NDM-1, TEM-1, CTX-M-15, SHV-11, CMY-6, OXA-1 (OmpK35 ⁻)	A, B, C, D	N
	ARC3800	TEM-1, CTX-M-15, SHV-1, OXA-1, OXA-9	A, D	Y
	ARC3804	NDM-1, TEM-1, CTX-M-15, SHV-5 (OKP-A)	A, B	N
	ARC3816	NDM-1, TEM-1, CTX-M-15, OXA-1	A, B, D	N

(Continued on next page)

TABLE 1 (Continued)

Organism	Strain name	β -Lactamase(s) detected ^a	β -Lactamase class(es)	Expected inhibition by AVI ^b
<i>Proteus mirabilis</i>	ARC3725	TEM-1, ACC-4	A, C	Y
<i>Providencia stuartii</i>	ARC3726	ACC-1, SCO-1	A, C	Y
<i>Pseudomonas aeruginosa</i>	ARC2764	Inducible AmpC	C	Y
	ARC3471	Inducible AmpC, VIM-2	B, C	N
	ARC3497	Derepressed AmpC	C	Y
	ARC3501	Inducible AmpC, GES-2, OXA-5 (OprD)	A, C, D	Y
	ARC3502	Inducible AmpC, VIM-1 (OprD)	B, C	N
	ARC3504	Inducible AmpC, SHV-2	A, C	Y
	ARC3505	Inducible AmpC, PER-1, OXA-10	A, C, D	Partial
	ARC3506	Inducible AmpC, VEB-1, OXA-10 (OprD)	A, C, D	Partial
	ARC3507	Derepressed AmpC (OprD)	C	Y
	ARC3508	Derepressed AmpC (OprD)	C	Y
	ARC3509	Derepressed AmpC (OprD)	C	Y
	ARC3511	Inducible AmpC, KPC-2	A, C	Y
	ARC3514	Inducible AmpC, KPC-2, OprD	A, C	Y
	ARC3604	Inducible AmpC (OprD)	C	Y
	ARC3605	Inducible AmpC, TEM-24 (OprD)	A, C	Y
	ARC3606	Inducible AmpC, TEM-24 (OprD)	A, C	Y
	ARC3607	Inducible AmpC, OXA-2 (OprD)	C, D	Y
	ARC3608	Inducible AmpC (OprD)	C	Y
	ARC3609	Inducible AmpC (OprD)	C	Y
	ARC3610	Inducible AmpC (OprD)	C	Y
	ARC3611	Inducible AmpC	C	Y
	ARC3612	Inducible AmpC (OprD)	C	Y
	ARC3727	Inducible AmpC, VIM-2, OXA-4 (OprD)	B, C, D	N
	ARC3729	Inducible AmpC, OXA-10, VEB-1a (OprD)	A, C, D	Partial
	ARC3730	Derepressed AmpC, VEB-1a, OXA-10	A, C, D	Partial
	ARC3731	Inducible AmpC, OXA-10, VEB-1a (OprD)		Partial

^aOprD, downregulation or deletion of the OprD outer membrane porin protein.

^bAlso includes β -lactamases such as OXA-1 that are not well inhibited by avibactam (AVI), but which do not hydrolyze ceftazidime and therefore should have no impact on susceptibility to ceftazidime-avibactam. NA, not applicable; Y, yes; N, no.

documented previously (6). This constraint limits the concentration of avibactam that could be used for *in vitro* susceptibility testing because it could increase the risk of false-susceptible results for isolates expressing enzymes that are not inhibited by avibactam testing. Therefore, this concentration is not recommended for use in combination with ceftazidime.

The constant concentration of 4 μ g/ml of avibactam gave only one probable false-resistant result that was common to all of the combinations (Table 1 and Fig. 1B). This isolate, *E. coli* ARC3531 (ceftazidime-avibactam MIC, 32 μ g/ml) expressed TEM-1 and VEB-5, both of which are inhibited well by avibactam (26). *E. coli* ARC3531 has tested resistant to ceftazidime-avibactam in every condition and with multiple replicates, and therefore these results do not represent a testing error. Whole-genome sequencing did not reveal any alterations in the genes encoding penicillin-binding proteins, outer membrane porin proteins (OMPs), or efflux; therefore, the resistance of this isolate to ceftazidime-avibactam was unexpected and remains unexplained (data not shown).

Early studies in the development path for ceftazidime-avibactam also used the method of testing with a constant concentration of 4 μ g/ml of avibactam (3, 27). This study builds upon previous work by providing insights with a larger panel of organisms that had been fully characterized by whole-genome sequencing. Given the known spectrum of β -lactamase hydrolysis of ceftazidime and inhibition by avibactam, the combination of ceftazidime with 4 μ g/ml of avibactam was the testing condition that correctly separated susceptible from resistant isolates of *Enterobacteriaceae* and was more reliable at avoiding categorical errors than the other constant concentration combinations or ratios. As a result, performing MIC testing with doubling dilutions of ceftazidime in the presence of a constant concentration of 4 μ g/ml of avibactam was

FIG 1 MIC distributions of ceftazidime combined with avibactam at various ratios or concentrations. Isolates were predefined as having β -lactamases that should be inhibited, partially inhibited, or not inhibited by avibactam, based on the results of whole-genome sequencing and the spectrum of inhibition of avibactam against these enzymes (2, 3, 5, 6). (A) Ceftazidime tested with a constant concentration of 2 μ g/ml of avibactam; (B) ceftazidime tested with a constant concentration of 4 μ g/ml of avibactam; (C) ceftazidime tested with a constant concentration of 8 μ g/ml of avibactam; (D) ceftazidime and avibactam tested in a 2:1 ratio; and (E) ceftazidime and avibactam tested in a 4:1 ratio. The dotted vertical line represents the susceptible MIC breakpoint for ceftazidime-avibactam.

chosen as the reference method for this β -lactam- β -lactamase inhibitor combination. This result correlates with previous studies that also showed that maintaining a constant concentration of the β -lactamase inhibitor while the β -lactam concentration is varied more clearly separated bacterial isolates into their susceptible and nonsusceptible groups (16, 19). A limitation to this study is that the genetic identification of β -lactamases does not provide insight into the expression level of each β -lactamase or the interplay between multiple β -lactamases. Therefore, the preassignment of these isolates as susceptible or resistant based on sequence may have been flawed. However, in this study, with the exception of one strain (*E. coli* ARC3531), all of the isolates behaved as predicted in the MIC tests.

The primary objective of *in vitro* susceptibility testing with antibacterial agents is to identify resistant isolates. A susceptibility test should accurately distinguish between isolates of a species that, when causing an infection, are either likely (susceptible) or unlikely (nonsusceptible) to respond to therapy with the tested antibacterial agent. Using PK/PD helps to relate the *in vitro* measurement of susceptibility with a predicted quantitative microbiological pharmacodynamic response in a patient upon systemic exposure to a given dose of the antibacterial agent (26, 28, 29). When considering a

TABLE 2 Discrepancies with various MIC conditions

Strain	β -Lactamase(s) detected ^a	False results for various avibactam-ceftazidime concentrations ^b									
		+2		+4		+8		2:1		4:1	
		S	R	S	R	S	R	S	R	S	R
<i>E. coli</i> ARC3531	VEB-5, TEM-1		X		X		X		X		X
<i>E. coli</i> ARC3691	CTX-M-15, OXA-1, CMY-42										X
<i>E. coli</i> ARC3707	CTX-M-15, OXA-1, CMY-42		X								
<i>E. coli</i> ARC3805	NDM-1, TEM-208, OXA-1, OXA-2, CTX-M-15, CMY-4					X					
<i>K. pneumoniae</i> ARC2711	TEM-1, SHV-12, KPC-2										X
<i>K. pneumoniae</i> ARC3804	NDM-1, TEM-1, CTX-M-15, SHV-5, OKP-A					X					
<i>P. aeruginosa</i> ARC3497	Derepressed AmpC										X
<i>P. aeruginosa</i> ARC3604	Inducible AmpC (OprD)		X					X			X
<i>P. aeruginosa</i> ARC3608	Inducible AmpC (OprD)										
<i>P. aeruginosa</i> ARC3610	Inducible AmpC (OprD)										X
<i>P. stuartii</i> ARC3726	ACC-1, SCO-1		X					X			X

^aOprD, downregulation or deletion of the OprD outer membrane porin protein.

^bFalse-susceptible (S) and false-resistant (R) results were based on MIC breakpoint of 8 μ g/ml of ceftazidime in the presence of avibactam under each condition and the expected inhibition by avibactam listed in Table 1. +2, constant concentration of 2 μ g/ml of avibactam; +4, constant concentration of 4 μ g/ml of avibactam; +8, constant concentration of 8 μ g/ml of avibactam; 2:1, 2:1 ratio of ceftazidime to avibactam; 4:1, 4:1 ratio of ceftazidime to avibactam; X, discrepant.

β -lactamase inhibitor, the concentration of inhibitor should be chosen to suppress β -lactamase activity in order to revert to the underlying PK/PD of the parent β -lactam antibacterial (28, 30, 31). It was shown previously that performing MIC tests with ceftazidime in the presence of a constant concentration of 4 μ g/ml of avibactam restored the MIC frequency distribution of ceftazidime against β -lactamase-producing isolates of *Enterobacteriaceae* and *P. aeruginosa* to mirror that of ceftazidime alone against wild-type isolates (32). Therefore, adding a constant concentration of 4 μ g/ml of avibactam to this testing paradigm also fulfils this requirement.

With these concepts in mind, the data demonstrated that the best method for determining ceftazidime-avibactam MIC values was to use doubling dilutions of ceftazidime in the presence of a constant concentration of 4 μ g/ml of avibactam. These methods have been adopted by the U.S. FDA, EUCAST, and CLSI, and were used to set the breakpoints for ceftazidime-avibactam (25, 33, 34). Having a reliable susceptibility test will assist laboratories in providing guidance to physicians treating patients with serious infections that might benefit from ceftazidime-avibactam.

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