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Broth Microdilution Method To Detect Extended-Spectrum β-Lactamases and AmpC β-Lactamases in *Enterobacteriaceae* Isolates by Use of Clavulanic Acid and Boronic Acid as Inhibitors[▽]

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This study was designed to evaluate the performance of the broth microdilution (BMD) method to detect production of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases in *Enterobacteriaceae* by using clavulanic acid (CA) and boronic acid (BA) as ESBL and AmpC β-lactamase inhibitors, respectively. A total of 100 clinical isolates of *Enterobacteriaceae* were analyzed. Mueller-Hinton broth containing serial twofold dilutions of cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM), or cefepime (FEP) with or without either or both CA and BA was prepared. An eightfold or greater decrease in the MIC of CTX, CAZ, ATM, or FEP in the presence of CA and BA was considered a positive result for ESBL and plasmid-mediated AmpC β-lactamase (PABL), respectively. In tests with CA, expanded-spectrum β-lactams containing BA (CTX-BA, CAZ-BA, ATM-BA, and FEP-BA) showed higher positive rates in detecting ESBL producers than those without BA. The combination of CTX- and CAZ-based BMD tests with CA and BA showed sensitivity and specificity of 100% for the detection of ESBLs and PABLs. The BMD testing could be applicable for routine use in commercially available semiautomated systems for the detection of ESBLs and PABLs in *Enterobacteriaceae*.

The rapid global dissemination of *Enterobacteriaceae* harboring plasmid-borne extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (PABLs) represents a significant clinical threat (2, 11). ESBLs can confer resistance to penicillins, oxyimino-cephalosporins, and monobactams to bacterial hosts, and their hydrolytic activities are usually inhibited by clavulanic acid (CA) (15). PABLs also confer resistance to expanded-spectrum β-lactams, but not carbapenems, but their hydrolytic activities are poorly inhibited by CA (16). The rapid and accurate detection of ESBLs and PABLs in *Enterobacteriaceae* is important to guide proper antimicrobial therapy and appropriate infection control measures. Many methods for the detection of ESBLs and PABLs have been proposed, but some procedures are difficult to perform in practice, time-consuming, and hard to interpret (7, 9). Dissemination of class A carbapenemases, including KPC enzymes, makes the detection more difficult (3, 17).

The Clinical and Laboratory Standards Institute (CLSI) has described standard broth microdilution (BMD) and disk susceptibility test methods to screen and confirm ESBL production in *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Proteus mirabilis*, but there are currently no available guidelines for other genera (5). Several semiautomated antimicrobial susceptibility testing systems, which infer ESBL production by inhibitory effects of CA, are now commercially available. However, they are available only for *K. pneumoniae*, *K. oxytoca*, and *E. coli*.

No standardized method is recognized for screening and confirmation of the presence of AmpC enzymes. Resistance to a cephamycin is suggestive of the presence of an AmpC enzyme, but this can be mimicked by porin loss (8, 12). Recently, a test for AmpC β-lactamases that involves augmentation of the inhibition zones around cefotaxime (CTX), ceftazidime (CAZ), and cefotetan disks by an AmpC inhibitor, a boronic acid (BA) compound, has been proposed (6, 24). BA testing cannot distinguish between plasmid-borne and chromosome-borne AmpC β-lactamases. We recently evaluated the diagnostic utility of the BA disk test for the detection of strains producing ESBLs and PABLs, which is similar to the CLSI disk confirmatory test for ESBLs. We found that the BA disk test was an accurate and simple tool for the detection of ESBLs and PABLs in *Enterobacteriaceae* (10, 20, 21).

The aim of the present study was to evaluate the performance of the BMD method, which is appropriate for routine use in commercially available semiautomated systems, for the detection of ESBLs and PABLs, using CA and BA to inhibit ESBLs and PABLs, respectively.

MATERIALS AND METHODS

Bacterial strains. A total of 100 clinical isolates of *E. coli* ($n = 14$), *K. oxytoca* ($n = 3$), *K. pneumoniae* ($n = 28$), *P. mirabilis* ($n = 5$), *Salmonella* spp. ($n = 1$), *Enterobacter cloacae* ($n = 20$), *Enterobacter aerogenes* ($n = 8$), *Serratia marcescens* ($n = 13$), and *Citrobacter freundii* ($n = 8$) were used in this study; 53 were ESBL producers, 11 were PABL and ESBL coproducers, 15 were PABL producers, and 21 were chromosomal AmpC hyperproducers (Table 1). Ninety-eight isolates had been previously characterized by appropriate biochemical, phenotypic, and molecular procedures to determine their types of β-lactamase production (10, 20, 21). Two *P. mirabilis* isolates, harboring TEM-52 and CTX-M-14 β-lactamase, respectively, were obtained from Kyungwon Lee (Yonsei University College of Medicine, Seoul, Republic of Korea).

BMD testing. Mueller-Hinton broth media containing serial twofold dilutions of CTX, CAZ, aztreonam (ATM), and cefepime (FEP) at concentrations ranging from 0.25 to 512 μg/ml, with or without CA at a fixed concentration of 4

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TABLE 1. Summary of the 100 clinical isolates of *Enterobacteriaceae* used in the study

Species	No. of isolates	β-Lactamase(s)	Reference or source
E. coli	2	CTX-M-3	17
	5	CTX-M-14	17
	2	CTX-M-15	17
	1	DHA-1	17
	2	CMY-1	17
	2	CMY-2	17
K. oxytoca	1	SHV-12, DHA-1	18
	2	DHA-1	18
K. pneumoniae	3	SHV-2a	18
	1	SHV-5	18
	5	SHV-12	18
	1	TEM-52	18
	1	CTX-M-9	18
	3	CTX-M-14	18
	3	SHV-2a, DHA-1	18
	5	SHV-12, DHA-1	18
	1	SHV-12, ACT-1	18
	1	SHV-12, CTX-M-14, DHA-1	18
	3	DHA-1	18
	1	CMY-1	18
P. mirabilis	1	TEM-52	K. Lee
	1	CTX-M-14	K. Lee
	3	CMY-2	18
Salmonella	1	DHA-1	18
E. cloacae	4	SHV-12	9
	1	CTX-M-3	9
	2	CTX-M-9	9
	5	SHV-12, CTX-M-9	9
	8	Derepressed AmpC	9
E. aerogenes	1	SHV-12	9
	2	TEM-52	9
	1	CTX-M-14	9
	4	Derepressed AmpC	9
S. marcescens	2	SHV-12	9
	5	TEM-52	9
	1	CTX-M-3	9
	5	Derepressed AmpC	9
C. freundii	3	SHV-12	9
	1	TEM-52	9
	4	Derepressed AmpC	9

μg/ml and/or BA at a fixed concentration of 200 μg/ml, were prepared and placed in a 96-well microplate. A bacterial suspension was inoculated into each well, according to the recommendations of CLSI in document M7-A8 (4). The plate was incubated at 37°C overnight. An eightfold or greater decrease in the MICs of (i) expanded-spectrum β-lactams in combination with CA (CTX-CA, CAZ-CA, ATM-CA, or FEP-CA) versus CTX, CAZ, ATM, or FEP or (ii) expanded-spectrum β-lactams containing BA in combination with CA (CTX-CA-BA, CAZ-CA-BA, ATM-CA-BA, or FEP-CA-BA) versus expanded-spectrum β-lactams containing BA (CTX-BA, CAZ-BA, ATM-BA, or FEP-BA) was considered a positive result for ESBL production. An eightfold or greater decrease in the MICs of (i) expanded-spectrum β-lactams in combination with BA (CTX-BA, CAZ-BA, or ATM-BA) versus CTX, CAZ, or ATM or (ii) expanded-spectrum β-lactams containing CA in combination with BA (CTX-CA-BA, CAZ-CA-BA, or ATM-CA-BA) versus expanded-spectrum β-lactams containing CA (CTX-CA, CAZ-CA, or ATM-CA) was considered a positive result for AmpC β-lactamase production in *E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, and *Salmonella* spp. *E. coli* ATCC 25922 was used as a negative control.

RESULTS

BMD testing for ESBL detection. The ESBL detection tests with expanded-spectrum β-lactams containing BA exhibited a higher sensitivity than those without BA. While CTX, CAZ, ATM, and FEP yielded positive tests with 46 (72%), 44 (69%), 54 (83%), and 60 (94%) of 64 ESBL-producing isolates, respectively, CTX-BA, CAZ-BA, ATM-BA, and FEP-BA yielded positive tests with 63 (98%), 58 (91%), 63 (98%), and 63 (98%) of 64 ESBL-producing isolates, respectively. FEP-based BMD testing with or without BA yielded false-positive results with 4 (11%; 3 DHA-1-producing *K. pneumoniae* isolates and 1 CMY-1-producing *E. coli* isolate) and 1 (3%; a CMY-1-producing *E. coli* isolate) of 36 ESBL-nonproducing isolates, respectively. The test also showed false-negative results with 1 (a CTX-M-3-producing *S. marcescens* isolate) and 4 (1 SHV-12-producing *K. pneumoniae* isolate, 1 SHV-12-producing *E. cloacae* isolate, 1 CTX-M-9-producing *E. cloacae* isolate, and 1 CTX-M-3-producing *S. marcescens* isolate) of 64 ESBL-producing isolates, respectively. Sensitivities and specificities for ESBL detection reached 100% when the results with CTX-BA and CAZ-BA or CTX-BA and ATM-BA were combined (Table 2).

BMD testing for PABL detection. The PABL detection tests with expanded-spectrum β-lactams containing CA exhibited a higher sensitivity than those without CA. While CTX, CAZ, and ATM yielded positive tests with 16 (62%), 14 (54%), and 10 (38%) of 26 PABL-producing isolates, respectively, CTX-CA, CAZ-CA, and ATM-CA yielded positive tests with 25 (96%), 25 (96%), and 22 (85%) isolates, respectively. While the tests with CTX or CAZ yielded false-positive results with 4 (16%) and 3 (12%) of 25 PABL-nonproducing isolates, respectively, the tests with CTX-CA, CAZ-CA, and ATM-CA did not yield false-positive results. Sensitivities and specificities for PABL detection reached 100% when the results with CTX-CA and CAZ-CA were combined (Table 3).

DISCUSSION

A concern with *E. coli* and *K. pneumoniae* isolates exhibiting positive ESBL screening results but negative ESBL confirmation results was recently raised based on SENTRY Asia-Pacific data. The phenotype was observed in 8.9% and 20.3% of *E. coli* and *K. pneumoniae* isolates, respectively, and up to 75% of the isolates carried PABL genes (1). The coexistence of both PABL and ESBL in the same strain is the most important cause of false-negative results in ESBL confirmatory tests (6). We found that the CLSI disk confirmatory tests yielded false-negative results in 19% and 14%, respectively, of *E. coli* and *K. pneumoniae* isolates coproducing ESBLs and PABLs (20). Detection of ESBLs in *Enterobacter* spp., *S. marcescens*, and *C. freundii* harboring chromosome-borne AmpC enzymes is also not easy. Our previous data showed that the CLSI disk confirmatory tests yielded false-negative results with 28% of ESBL-producing isolates of these species (10). The present study showed that the CLSI BMD confirmatory test using CTX and CAZ with or without CA yielded false-negative results with 16

TABLE 2. Results for the BMD method for detecting ESBL production among 100 *Enterobacteriaceae* isolates

Antimicrobial agents used in BMD test	No. of confirmed ESBL producers identified as[a]:		No. of confirmed ESBL nonproducers identified as[b]:		Sensitivity (%)	Specificity (%)
	ESBL positive	ESBL negative	ESBL positive	ESBL negative		
CTX vs CTX-CA	46 (25/18/3)	18 (0/10/8)	0	36 (20/16)	72	100
CAZ vs CAZ-CA	44 (25/19/0)	20 (0/9/11)	0	36 (20/16)	69	100
ATM vs ATM-CA	53 (24/19/10)	11 (1/9/1)	0	36 (20/16)	84	100
FEP vs FEP-CA	60 (24/25/11)	4 (1/3/0)	1 (0/1)	35 (20/15)	94	97
CTX-BA vs CTX-CA-BA	63 (25/27/11)	1 (0/1/0)	0	36 (20/16)	98	100
CAZ-BA vs CAZ-CA-BA	58 (21/26/11)	6 (4/2/0)	0	36 (20/16)	91	100
ATM-BA vs ATM-CA-BA	63 (24/28/11)	1 (1/0/0)	0	36 (20/16)	98	100
FEP-BA vs FEP-CA-BA	63 (25/27/11)	1 (0/1/0)	4 (0/4)	32 (20/12)	98	89
CTX vs CTX-CA and/or CAZ vs CAZ-CA	48	16	0	36	75	100
CTX vs CTX-CA and/or ATM vs ATM-CA	55	9	0	36	86	100
CTX vs CTX-CA and/or FEP vs FEP-CA	61	3	1	35	95	97
CAZ vs CAZ-CA and/or ATM vs ATM-CA	55	9	0	36	86	100
CAZ vs CAZ-CA and/or FEP vs FEP-CA	61	3	1	35	95	97
ATM vs ATM-CA and/or FEP vs FEP-CA	61	3	1	35	95	97
CTX-BA vs CTX-CA-BA and/or CAZ-BA vs CAZ-CA-BA	64	0	0	36	100	100
CTX-BA vs CTX-CA-BA and/or ATM-BA vs ATM-CA-BA	64	0	0	36	100	100
CTX-BA vs CTX-CA-BA and/or FEP-BA vs FEP-CA-BA	64	0	4	32	100	89
CAZ-BA vs CAZ-CA-BA and/or ATM-BA vs ATM-CA-BA	63	1	0	36	98	100
CAZ-BA vs CAZ-CA-BA and/or FEP-BA vs FEP-CA-BA	63	1	4	32	100	89
ATM-BA vs ATM-CA-BA and/or FEP-BA vs FEP-CA-BA	64	0	4	32	100	89

[a] The numbers of isolates producing ESBL only/ESBL with chromosomal AmpC/ESBL with plasmid-mediated AmpC are given in parentheses.
[b] The numbers of isolates producing chromosomal AmpC only/plasmid-mediated AmpC only are given in parentheses.

(25%) of 64 ESBL-producing *Enterobacteriaceae* isolates. All the isolates exhibiting false-negative results harbored both ESBL and AmpC enzymes (data not shown). However, our modified CLSI BMD confirmatory test using CTX-BA and CAZ-BA with or without CA exhibited perfect performance in detecting ESBLs without reference to the presence of PABLs. These results suggest that BMD testing with CTX and CAZ with or without BA and/or CA may be useful in detecting ESBL- and/or AmpC-producing *Enterobacteriaceae* in clinical microbiology laboratories without reference to species.

The BMD method is one of the most familiar methods for the determination of MICs in clinical laboratories, due to the recent introduction of semiautomated antimicrobial susceptibility test systems (24). However, the performance of Vitek 2, MicroScan, and BD Phoenix for the detection of ESBL-positive *Enterobacteriaceae* is variable, particularly for organisms such as AmpC-producing *Enterobacter* and *Citrobacter* species (23). The BD Phoenix results indicated poor performance of currently employed ESBL confirmatory methods in the settings of concomitant PABL (18). Our BMD method with CTX and CAZ with or without BA and/or CA may be applicable for routine use in commercially available semiautomated systems for the detection of ESBLs and PABLs.

Our study has some limitations. First, a positive response to BA indicates the production of an AmpC but not necessarily a plasmid-mediated enzyme, since in *E. coli* strains this pheno-

TABLE 3. Results for the BMD method for detecting PABL production among 51 *E. coli*, *Klebsiella*, *P. mirabilis*, and *Salmonella* isolates

Antimicrobial agents used in BMD test	No. of confirmed PABL producers identified as[a]:		No. of confirmed PABL nonproducers identified as:		Sensitivity (%)	Specificity (%)
	PABL positive	PABL negative	PABL positive	PABL negative		
CTX vs CTX-BA	16 (15/1)	10 (0/10)	4	21	61	84
CAZ vs CAZ-BA	14 (14/0)	12 (1/11)	3	22	54	88
ATM vs ATM-BA	10 (10/0)	16 (5/11)	0	25	38	100
CTX-CA vs CTX-CA-BA	25 (15/10)	1 (0/1)	0	25	96	100
CAZ-CA vs CAZ-CA-BA	25 (14/11)	1 (1/0)	0	25	96	100
ATM-CA vs ATM-CA-BA	22 (12/10)	4 (3/1)	0	25	85	100
CTX vs CTX-BA and/or CAZ vs CAZ-BA	16	10	5	20	62	80
CTX vs CTX-BA and/or ATM vs ATM-BA	16	10	4	21	62	84
CAZ vs CAZ-BA and/or ATM vs ATM-BA	15	11	3	22	58	88
CTX-CA vs CTX-CA-BA and/or CAZ-CA vs CAZ-CA-BA	26	0	0	25	100	100
CTX-CA vs CTX-CA-BA and/or ATM-CA vs ATM-CA-BA	25	1	0	25	96	100
CAZ-CA vs CAZ-CA-BA and/or ATM-CA vs ATM-CA-BA	25	1	0	25	96	100

[a] The numbers of isolates producing plasmid-mediated AmpC only/ESBL with plasmid-mediated AmpC are given in parentheses.

type may also result from overexpression of the chromosomal *ampC* gene (13). Second, we could not include class A carbapenemase-producing isolates in this study. A strain producing KPC enzyme would have a positive response to CA or BA and thus could be falsely categorized as producing an ESBL or an AmpC if a reduction in carbapenem susceptibility is not evident (14, 19, 22). Further studies are needed to establish the reliability of commercially available semiautomated testing systems by using our method for the detection of ESBL and PABL enzymes in *Enterobacteriaceae*.

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