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Detection of Extended-Spectrum β-Lactamases by Using Boronic Acid as an AmpC β-Lactamase Inhibitor in Clinical Isolates of *Klebsiella* spp. and *Escherichia coli*[▽]

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We evaluated highly sensitive methods using boronic acid (BA) to detect extended-spectrum β-lactamase (ESBL) production. A total of 182 clinical isolates of *Klebsiella* spp. ($n = 118$) and *Escherichia coli* ($n = 64$) were analyzed: 62 harbored only ESBLs, 80 harbored both ESBLs and plasmid-mediated AmpC β-lactamases (pAmpCs), and 40 harbored only pAmpCs. The CLSI confirmatory test detected all isolates that produce only ESBLs but detected 85% of isolates that produce both enzymes. When a ≥5-mm increase in the zone diameter of either the cefotaxime (CTX) or the ceftazidime (CAZ) disk in the presence of both clavulanic acid (CA) and BA was considered to be a positive result, the test detected all isolates that harbor ESBLs (± pAmpCs) but showed frequent false-positive results (50%) for isolates that produce only pAmpCs. Meanwhile, when a ≥3-mm increase in the zone diameter of either the CTX/BA or the CAZ/BA disk in the presence of CA was considered to be a positive result, the test also detected all isolates that harbor ESBLs (± pAmpCs) and showed less frequent false-positive results (5%) in isolates that produce only pAmpCs. The latter new interpretive guideline has enhanced detection of ESBLs in clinical isolates of *Klebsiella* spp. and *Escherichia coli* and allowed detection of an ESBL even when potentially masked by a pAmpC.

Since extended-spectrum β-lactamases (ESBLs) were initially reported to be in *Klebsiella ozaenae* in the first half of the 1980s in Germany, ESBLs have been spreading globally (2). The CLSI interpretive guidelines stated that strains of *Klebsiella* spp. and *Escherichia coli* that produce ESBLs may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparent in vitro susceptibility to some of these agents (4). Therefore, detection of ESBLs in *Klebsiella* spp. and *E. coli* isolates is crucial for optimal treatment of patients and to control the spread of resistance. The CLSI recommends a phenotypic confirmatory test for ESBL production that consists of measuring the zone diameters of cefotaxime (CTX; 30 μg) or ceftazidime (CAZ; 30 μg) disks with or without clavulanic acid (CA; 10 μg) in *Klebsiella* spp. and *E. coli*. An increase in the zone diameter of ≥5 mm in the presence of CA is taken to be a phenotypic confirmation of ESBL production.

There are numerous reports in which *Klebsiella pneumoniae* and *E. coli* isolates have been found to produce both plasmid-mediated AmpC β-lactamases (pAmpCs) and ESBLs (6, 8). In Korea, 8.7% of pAmpC producers also produced ESBLs (8). AmpCs resist inhibition by CA, and hence, the presence of an ESBL can be masked by the expression of an AmpC. Thus, the coexistence of both pAmpCs and ESBLs in the same strain may result in false-negative tests for the detection of ESBLs (5). There is a need, therefore, for an alternative method that can detect ESBLs in *Klebsiella* spp. and *E. coli* isolates with a high sensitivity, even though the isolates simultaneously harbor pAmpCs.

Boronic acids (BAs) were reported as reversible inhibitors of AmpCs (1). There are a few reports of studies designed to detect pAmpC-producing isolates of *Klebsiella* spp. and *E. coli* by a method using BA as an inhibitor that closely resembles the phenotypic confirmatory tests for ESBLs (3, 5). And Coudron (5) recently described that a BA disk also enhances detection of isolates that harbor both ESBLs and pAmpCs.

The aim of this work was to modify the phenotypic confirmatory test for ESBL production by using BA disks to improve its sensitivity for the detection of ESBLs in *Klebsiella* spp. and *E. coli* isolates even though the isolates simultaneously harbor pAmpCs.

MATERIALS AND METHODS

Bacterial strains. A total of 182 nonrepeat ESBL- and/or pAmpC-producing clinical isolates of *Klebsiella* spp. ($n = 118$) and *E. coli* ($n = 64$) collected from 12 tertiary-care hospitals in Korea during 2004 and 2005 were included in this study (Table 1): 62 harbored ESBLs but not pAmpCs, 80 harbored both ESBLs and pAmpCs, and 40 harbored pAmpCs but not ESBLs. The isolates were identified with API-20 E systems (bioMérieux, Marcy l'Etoile, France). Searches for genes coding for the class A ESBLs and pAmpCs were performed by PCR amplification as described previously (7, 8). The PCR products were subjected to direct sequencing. Both strands of each PCR product were sequenced twice with

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TABLE 1. Bacterial strains used for the validation of test methods

Strain group and genotype(s)	No. of strains		
	Klebsiella spp.	*E. coli*	Total
Producing ESBLs but not pAmpCs			
$bla_{CTX\text{-}M\text{-}3}$	1	2	3
$bla_{CTX\text{-}M\text{-}3}$ plus $bla_{CTX\text{-}M\text{-}14}$	1		1
$bla_{CTX\text{-}M\text{-}9}$		1	1
$bla_{CTX\text{-}M\text{-}14}$		8	8
$bla_{CTX\text{-}M\text{-}15}$		11	11
$bla_{CTX\text{-}M\text{-}15}$ plus $bla_{SHV\text{-}2a}$ or $bla_{SHV\text{-}12}$		5	5
$bla_{SHV\text{-}2a}$	1		1
$bla_{SHV\text{-}12}$	28	2	30
$bla_{TEM\text{-}52}$		2	2
Subtotal	31	31	62
Producing both ESBLs and pAmpCs			
$bla_{CTX\text{-}M\text{-}3}$ plus $bla_{CMY\text{-}10}$ or $bla_{DHA\text{-}1}$	1	2	3
$bla_{CTX\text{-}M\text{-}3}$ plus $bla_{SHV\text{-}12}$ plus $bla_{DHA\text{-}1}$	4		4
$bla_{CTX\text{-}M\text{-}9}$ plus $bla_{CMY\text{-}2}$ or $bla_{DHA\text{-}1}$		3	3
$bla_{CTX\text{-}M\text{-}9}$ plus $bla_{SHV\text{-}2a}$ or $bla_{SHV\text{-}12}$ plus $bla_{DHA\text{-}1}$	1	1	2
$bla_{CTX\text{-}M\text{-}12}$ plus $bla_{SHV\text{-}12}$ plus $bla_{DHA\text{-}1}$	1		1
$bla_{CTX\text{-}M\text{-}14}$ plus $bla_{CMY\text{-}2}$ or $bla_{DHA\text{-}1}$	1	7	8
$bla_{CTX\text{-}M\text{-}15}$ plus $bla_{CMY\text{-}2}$		2	2
$bla_{CTX\text{-}M\text{-}15}$ plus $bla_{SHV\text{-}12}$ plus $bla_{DHA\text{-}1}$		1	1
$bla_{SHV\text{-}12}$ plus $bla_{CMY\text{-}2}$ or $bla_{DHA\text{-}1}$	56		56
Subtotal	64	16	80
Producing pAmpCs but not ESBLs			
$bla_{CMY\text{-}1}$	3	4	7
$bla_{CMY\text{-}2}$	1	6	7
$bla_{CMY\text{-}10}$	1	1	2
$bla_{DHA\text{-}1}$	18	6	24
Subtotal	23	17	40
Total	118	64	182

an automatic sequencer (model 3730*xl*; Applied Biosystems, Weiterstadt, Germany).

ESBL detection by the CLSI confirmatory test and two different tests using BA. Disks containing BA were produced in-house by using commercially available antibiotic-containing disks (BBL, Cockeysville, MD) as described previously (5). Briefly, 20 μl of the 3-aminophenyl BA stock solution (20 g/liter; Sigma, St. Louis, MO) was dispensed onto each disk containing CTX (30 μg) or CAZ (30 μg) with or without CA (10 μg). The final amount of BA on the disks was 400 μg. The disks were allowed to dry for 60 min and used immediately or stored in airtight vials with desiccant at 4°C. The CLSI confirmatory test for ESBL production was carried out on Mueller-Hinton agar (Difco Laboratories, Detroit, MI) by using antibiotic-containing disks with or without CA. In two new different tests using BA (400 μg), (i) a ≥5-mm increase in the zone diameter of the disk containing CTX (and/or CAZ) and CA tested in combination with BA (CTX/CA/BA and/or CAZ/CA/BA) versus CTX and/or CAZ alone was considered positive for ESBL, and (ii) a ≥3-mm increase in the zone diameter of the disk containing CTX (and/or CAZ) and CA tested in combination with BA (CTX/CA/BA and/or CAZ/CA/BA) versus CTX and/or CAZ containing BA (CTX/BA and/or CAZ/BA) was considered positive for ESBL. CTX, CTX/CA, CAZ, and CAZ/CA disks with or without BA were spaced over the agar surface. After overnight incubation at 35°C in ambient air, the inhibitory-zone diameters of the antibiotic-containing disks were measured.

TABLE 2. Test results for the 182 ESBL- and/or pAmpC-producing clinical isolates of *Klebsiella* spp. (n = 118) and *E. coli* (n = 64)

Strain group and species	No. (%) of isolates that showed positive results for indicated test[a]:		
	CLSI confirmatory[b]	CTX/CA/BA vs CTX and/or CAZ/CA/BA vs CAZ[c]	CTX/CA/BA vs CTX/BA and/or CAZ/CA/BA vs CAZ/BA[d]
Producing ESBLs but not pAmpCs			
Klebsiella spp. (n = 31)	31 (100)	31 (100)	31 (100)
E. coli (n = 31)	31 (100)	31 (100)	31 (100)
Subtotal (n = 62)	62 (100)	62 (100)	62 (100)
Producing both ESBLs and pAmpCs			
Klebsiella spp. (n = 64)	55 (86)	64 (100)	64 (100)
E. coli (n = 16)	13 (81)	16 (100)	16 (100)
Subtotal (n = 80)	68 (85)	80 (100)	80 (100)
Producing pAmpCs but not ESBLs			
Klebsiella spp. (n = 23)	3 (13)	9 (39)	1 (4)
E. coli (n = 17)	3 (18)	11 (65)	1 (6)
Subtotal (n = 40)	6 (15)	20 (50)	2 (5)

[a] Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; CA, clavulanic acid; BA: boronic acid.
[b] The zone diameter of the disk containing CTX (or CAZ) and CA was ≥5 mm greater than that of the disk containing CTX (or CAZ).
[c] The zone diameter of the disk containing CTX (or CAZ), CA, and BA was ≥5 mm greater than that of the disk containing CTX (or CAZ).
[d] The zone diameter of the disk containing CTX (or CAZ), CA, and BA was ≥3 mm greater than that of the disk containing CTX (or CAZ) and BA.

RESULTS AND DISCUSSION

All 62 clinical isolates that produce ESBLs but not pAmpCs showed ≥5-mm increases in the zone diameters of the combined disks (CTX [or CAZ]/CA or CTX [or CAZ]/CA/BA) compared with those for CTX or CAZ alone (Table 2). The average increases in the zone diameters of the CTX and CAZ disks in the presence of both CA and BA (13.1 mm and 12.6 mm, respectively) were higher than those for the CLSI confirmatory test (11.3 mm and 10.9 mm, respectively). In tests with CA, the CTX and CAZ disks yielded positive tests with 60 (96.8%) and 58 (93.5%) of 62 isolates, respectively (Fig. 1A and B). In tests with both CA and BA, the CTX and CAZ disks yielded positive tests with 61 (98.4%) and 62 (100%) of 62 isolates, respectively (Fig. 1C and D). Meanwhile, all 62 clinical isolates showed ≥3-mm increases in the zone diameters of both the CTX/BA and the CAZ/BA disks in the presence of CA, with the exception of a *K. pneumoniae* isolate that produces SHV-2a, which showed a 2-mm increase in the zone diameter of a CAZ/BA disk in the presence of CA (Fig. 1E and F). The average increases in the zone diameters of the CTX/BA and CAZ/BA disks in the presence of CA were 11.8 mm and 11.1 mm, respectively.

The rate of detection of ESBLs by the CLSI confirmatory

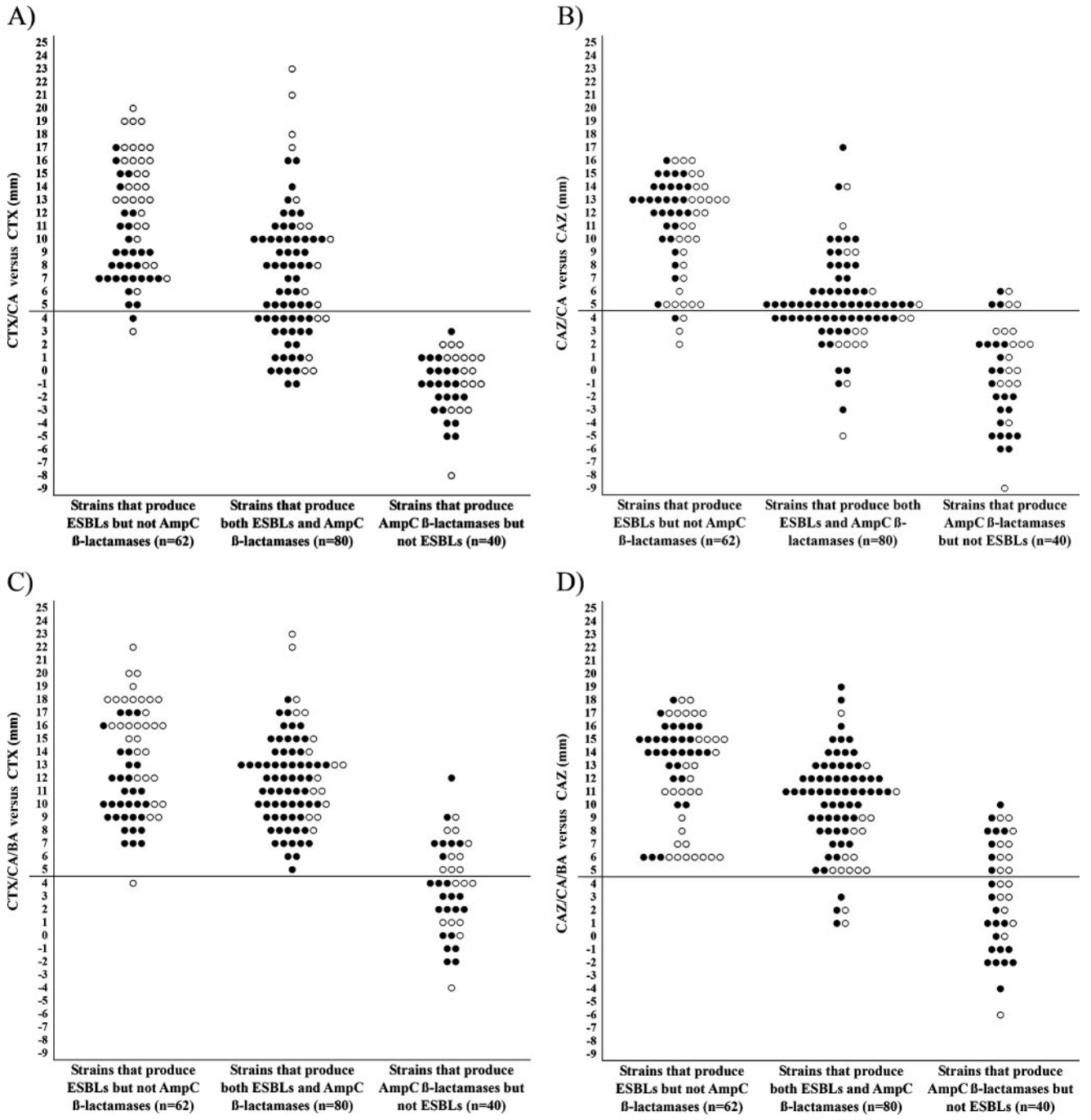

FIG. 1. Comparison of the results for the CLSI confirmatory test (A and B) and two new tests (C to F). The abbreviations (CTX/CA versus CTX, CAZ/CA versus CAZ, CTX/CA/BA versus CTX, CAZ/CA/BA versus CAZ, CTX/CA/BA versus CTX/BA, and CAZ/CA/BA versus CAZ/BA) are explained in footnote *a* to Table 2. Black circle, *Klebsiella* spp.; white circle, *E. coli*.

test for clinical isolates that produce both ESBLs and pAmpCs was lower than that for clinical isolates that produce ESBLs but not pAmpCs (Table 2). Among 80 clinical isolates that produce both ESBLs and pAmpCs, 68 (85%) showed ≥5-mm increases in the zone diameters of either the CTX or the CAZ disks in the presence of CA. In this test, the CTX and CAZ disks yielded positive tests with 51 (63.8%) and 45 (56.3%) of 80 isolates, respectively (Fig. 1A and B). All 80 clinical isolates, however, showed ≥5-mm increases in the zone diameters of either the CTX or the CAZ disks in the presence of both CA and BA (Table 2). In this test, CTX and CAZ disks yielded positive tests with 80 (100%) and 75 (93.8%) of 80 isolates, respectively (Fig. 1C and D). The average increases in the zone diameters of the CTX and CAZ disks in the presence of both CA and BA (11.9 mm and 9.9 mm, respectively) were higher than those for the CLSI confirmatory test (7.1 mm and 5.1 mm,

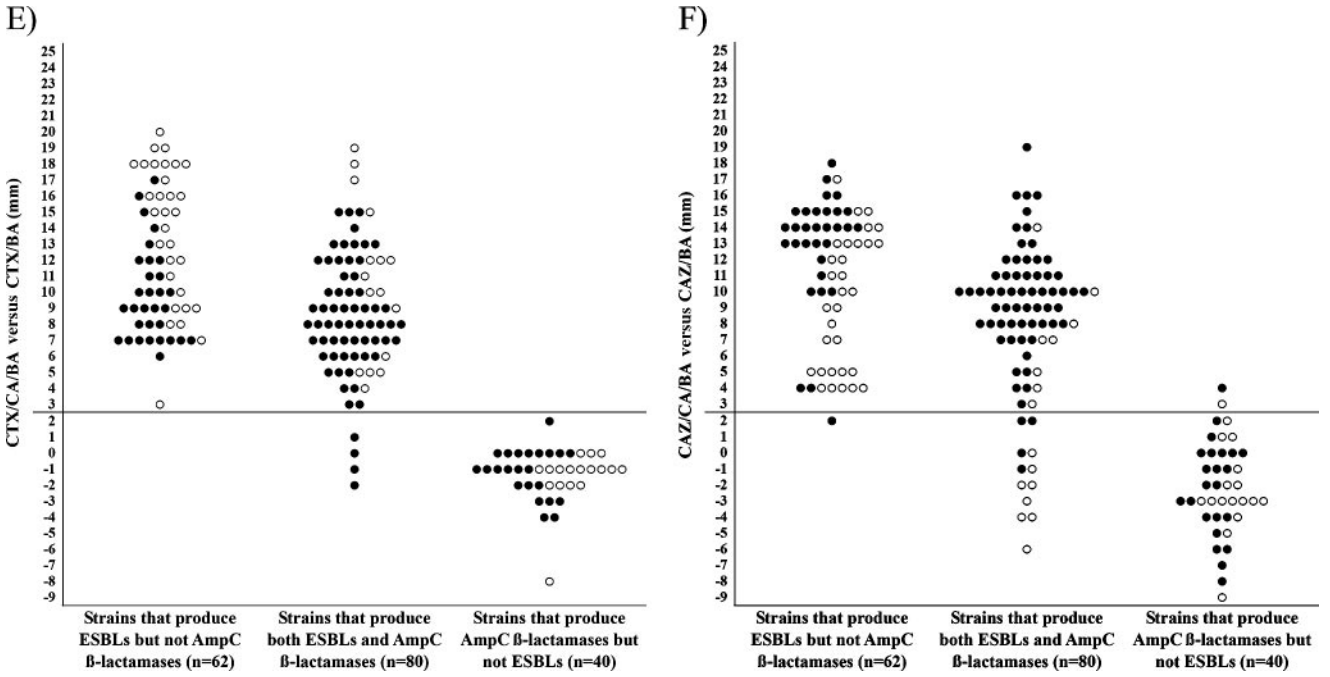

FIG. 1—*Continued.*

respectively). All 80 clinical isolates also showed ≥3-mm increases in the zone diameters of either the CTX/BA or the CAZ/BA disks in the presence of CA (Table 2). In this test, 76 (95%) and 68 (85%) of 80 isolates showed ≥3-mm increases in the zone diameters of the CTX/BA and CAZ/BA disks in the presence of CA, respectively (Fig. 1E and F). The average increases in the zone diameters of the CTX/BA and CAZ/BA disks in the presence of CA were 8.7 mm and 7.9 mm, respectively.

Among the 40 clinical isolates that produce pAmpCs but not ESBLs, 6 (15%) and 20 (50%) isolates showed ≥5-mm increases in the zone diameters of either the CTX or the CAZ disks in the presence of CA and both CA and BA, respectively (Table 2). Meanwhile, only 2 (5%) of the 40 isolates showed ≥3-mm increases in the zone diameters of either the CTX/BA or the CAZ/BA disks in the presence of CA (Table 2).

In summary, the CLSI confirmatory test detected ESBLs in all clinical isolates that produce ESBLs but not pAmpCs, but the detection rate (85%) was lowered in clinical isolates that produce both ESBLs and pAmpCs. The test showed false-positive results in 6 (15%) of 40 clinical isolates that produce pAmpCs but not ESBLs. When a ≥5-mm increase in the zone diameter of either the CTX or the CAZ disk in the presence of both CA and BA was considered to be a positive result for ESBL production, the new test detected all clinical isolates that harbor ESBLs (± pAmpCs) but showed frequent false-positive results (20/40, 50%) for clinical isolates that produce pAmpCs but not ESBLs. An inhibitory effect of BA on pAmpCs may have an influence on the high frequency of false-positive results. Meanwhile, when a ≥3-mm increase in the zone diameter of either the CTX/BA or the CAZ/BA disk in the presence of CA was considered to be a positive result for ESBL production, the test also detected all clinical isolates that harbor ESBLs (± pAmpCs) and the test showed less frequent false-positive results (2/40, 5%) for clinical isolates that produce pAmpCs but not ESBLs. In the case of the CTX/BA disk in the presence of CA, there were no false-positive results (Fig. 1E). The test missed none of the ESBL-producing isolates. Coudron (5) recently reported that a BA-based method using cefotetan enhanced detection of pAmpCs, but Coudron's method missed one of the pAmpC-producing *Proteus mirabilis* isolates. Although Coudron reported that the method can also detect isolates harboring pAmpCs and ESBLs, only three isolates were tested for the presence of both enzymes, and they did not include *E. coli* isolates.

In conclusion, we describe a newly modified method with a high sensitivity and a high specificity for the detection of ESBLs in clinical isolates of *Klebsiella* spp. and *E. coli*. A ≥3-mm increase in the zone diameter of either the CTX/BA or the CAZ/BA disk in the presence of CA is a new interpretive guideline that has enhanced detection of ESBLs in clinical isolates of *Klebsiella* spp. and *E. coli* and allowed detection of an ESBL even when potentially masked by a pAmpC. The exact detection of ESBLs in isolates that produce both ESBLs and pAmpCs is important for both treatment and epidemiology.

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REFERENCES

1. **Beesley, T., N. Gascoyne, V. Knott-Hunziker, S. Petursson, S. G. Waley, B. Jaurin, and T. Grundstrom.** 1983. The inhibition of class C beta-lactamases by boronic acids. Biochem. J. **209:**229–233.

2. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14:**933–951.
3. **Brenwald, N. P., G. Jevons, J. Andrews, L. Anq, and A. P. Fraise.** 2005. Disk methods for detecting AmpC β-lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. J. Antimicrob. Chemother. **56:**600–601.
4. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial disk susceptibility tests, 9th ed. Approved Standard M2–A9. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Coudron, P. E.** 2005. Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*. J. Clin. Microbiol. **43:**4163–4167.
6. **Goossens, H., and B. Grabein.** 2005. Prevalence and antimicrobial susceptibility data for extended-spectrum beta-lactamase- and AmpC-producing *Enterobacteriaceae* from the MYSTIC Program in Europe and the United States (1997–2004). Diagn. Microbiol. Infect. Dis. **53:**257–264.
7. **Ryoo, N. H., E. C. Kim, S. G. Hong, Y. J. Park, K. Lee, I. K. Bae, E. H. Song, and S. H. Jeong.** 2005. Dissemination of SHV-12 and CTX-M-type extended-spectrum β-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* and emergence of GES-3 in Korea. J. Antimicrob. Chemother. **56:**698–702.
8. **Song, W., J. S. Kim, H. S. Kim, D. Yong, S. H. Jeong, M. J. Park, and K. M. Lee.** 2006. Increasing trend in the prevalence of plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking chromosomal *ampC* gene at a Korean universitiy hospital from 2002 to 2004. Diagn. Microbiol. Infect. Dis. **55:**219–224.