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Occurrence of extended-spectrum β-lactamases among chromosomal AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens* in Korea and investigation of screening criteria

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Abstract

We assessed the occurrence and screening criterion for extended-spectrum β-lactamases (ESBLs) among AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens*. The 413 isolates (158 *E. cloacae*, 126 *C. freundii*, and 129 *S. marcescens*) isolated from 11 clinical laboratories in Korea were investigated. ESBL production was confirmed by double-disk synergy test and inhibitor-potentiated diffusion test using ceftazidime (CAZ), cefotaxime (CTX), aztreonam (AZT), and cefepime (FEP) with or without clavulanic acid. One hundred seven isolates (25.9%) were as ESBL producers. Of them, resistance was transferred by conjugation in 82 isolates. In transconjugants, structural genes for CTX-M (53.7%), TEM (46.3%), SHV (29.3%) were found. To evaluate the ESBL screening minimum inhibitory concentration (MIC) criteria, MICs for cefuroxime, CAZ, CTX, AZT, and FEP were determined and cutoff value was selected using receiver operator characteristic curve. The FEP MIC $\geq$ 1 μg/mL had the highest sensitivity (95.3%), specificity (82.7%), and positive (65.8%) and negative predictive values (98.3%).

Keywords: Extended-spectrum β-lactamase; *Enterobacter cloacae*; *Citrobacter freundii*; *Serratia marcescens*; Screening criteria

1. Introduction

Since the first report in 1983 (Knothe et al., 1983), extended-spectrum β-lactamases (ESBLs) have contributed to the dramatic increase in resistance to β-lactam agents among members of the family Enterobacteriaceae (Gianneli et al., 1994; Cantón et al., 2002). Although ESBLs have been reported at a much lower frequency among chromosomal AmpC β-lactamase-producing members of the family Enterobacteriaceae, such as *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. (Luzzaro et al., 1998; De Champ et al., 2000), the prevalence of ESBL in these species is increasing (Bell et al., 2003). These AmpC-producing organisms have proven to be one of the most important causes of nosocomial infections during the last few years (Sanders and Sanders, 1997), and these organisms can act as hidden reservoirs for ESBLs. Therefore, it is important for clinical microbiology laboratories to be able to detect ESBL production in these organisms on a routine basis.

The National Committee for Clinical Laboratory Standards (NCCLS, 2004) has issued guidelines for ESBL screening criteria and confirmation tests that apply to *Escherichia coli*, *Klebsiella pneumoniae*, and *K. oxytoca*. The screening criteria include an elevated minimum inhibitory concentration (MIC) of any one of a few cephalosporins and/or aztreonam (AZT). However, elevated MICs of broad-spectrum cephalosporins in AmpC producers usually imply stable derepression of AmpC enzymes (Bush et al., 1995). The AmpC enzymes are not inhibited by clavulanic acid (CLA), and it can rather be induced by CLA (Thomson et al., 1999). Hence, CLA effect in these species is highly suggestive of the presence of ESBL, and there is a need for specified screening criteria for ESBL detection in these AmpC producers.

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In the present study, we aimed to examine (a) the occurrence of ESBLs among AmpC-producing *E. cloacae*, *C. freundii*, and *S. marcescens* collected from 11 nationwide clinical laboratories in Korea and (b) to evaluate the screening MIC criteria for ESBL in these species.

2. Materials and methods

2.1. Bacterial isolates and susceptibility testing

A total of 413 consecutive, nonduplicate isolates, including *E. cloacae* (158), *C. freundii* (126), and *S. marcescens* (129), were collected were collected from clinical specimens at 11 clinical laboratories.

They were collected between March and July 2003 and identified by the Vitek GNI card (bioMérieux, Marcy-l'Etoile, France) or the Microscan GN combo card (Dade Behring, West Sacramento, CA). The MICs of cefuroxime (CXM, 2–128 μg/mL), ceftazidime (CAZ, 0.5–256 μg/mL), cefotaxime (CTX, 0.5–256 μg/mL), AZT (0.5–256 μg/mL), cefepime (FEP, 0.25–32 μg/mL), and cefoxitin (0.5–256 μg/mL) were determined by an agar dilution method in accordance with the NCCLS guideline M7-A6 (NCCLS, 2003). The respective manufacturers provided all antibiotics as powders. Quality control was performed using *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853. The isolates were stored at -76 °C in 20% skim milk until used in this study.

2.2. ESBL detection

The ESBL detection was based on the double-disk synergy test (DDST) (Tzelepi et al., 2000) and inhibitor-potentiated diffusion test (IPDT) (NCCLS, 2004). DDST was performed as follows: the surface of a Mueller-Hinton (MH) agar plate was inoculated evenly using a cotton swab, with an overnight culture suspension of clinical isolate, which was adjusted to the McFarland 0.5. After inoculation, disks (BBL, Cockeysville, MD) containing 30 μg of CAZ, CTX, AZT, FEP, and amoxicillin–clavulanic acid (20/10 μg, BBL) were placed at distances of 20 mm (center to center). For the IPDT, the zones of inhibition of each isolate were determined using disks containing 30 μg of CAZ, CTX, AZT, FEP, either alone or in combination with 10 μg of CLA (GlaxoSmithKline). An organism was classified as an ESBL producer if there is a presence of an enlarged zone by DDST or the zone of inhibition differed by ≥5 mm between at least one of the combination disks and its corresponding standard antibiotic disk by IPDT. AmpC derepressant was defined as follows: for *E. cloacae* and *C. freundii*, an organism resistant to CTX, CAZ, and cefoxitin, for *S. marcescens*, an organism resistant to CTX (Livermore et al., 2001). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls for ESBL production, respectively.

Table 1
Primer pairs used in this study

Primer	Sequence	Product size (bp)
bla_{TEM}	5′-ATAAAATTCTTGAAGACGAAA-3′ 5′-GACAGTTACCAATGCTTAATC-3′	1079
bla_{SHV}	5′-TGGTTATGCGTTATATTCGCC-3′ 5′-GGTTAGCGTTGCCAGTGCT-3′	868
$bla_{CTX\text{-}M\text{-}1}$	5′-GGTTAAAAAATCACTGCGTC-3′ 5′-TTGGTGACGATTTTAGCCGC-3′	863
$bla_{CTX\text{-}M\text{-}2}$	5′-ATGATGACTCAGAGCATTCG-3′ 5′-TGGGTTACGATTTTCGCCGC-3′	865
$bla_{CTX\text{-}M\text{-}9}$	5′-ATGGTGACAAAGAGAGTGCA-3′ 5′-CCCTTCGGCGATGATTCTC-3′	863
$bla_{PER\text{-}1}$	5′-AATTTGGGCTTAGGGCAGAA-3′ 5′-ATGAATGTCATTATAAAAGC-3′	924

Table 2
Distribution of ESBLs in *E. cloacae*, *C. freundii*, and *S. marcescens* as detected by PCR from transconjugants

Type of ESBLs	No. (%) of transconjugants harboring			Total (%)
	E. cloacae (45)	*C. freundii* (22)	*S. marcescens* (15)	
$bla_{TEM\text{-}like}$	19 (42.2)	16 (72.7)	3 (20.0)	38 (46.3)
$bla_{SHV\text{-}like}$	17 (37.8)	5 (22.7)	2 (13.3)	24 (29.3)
$bla_{CTX\text{-}M\text{-}like}$	23 (51.1)	8 (36.4)	11 (78.3)	44 (53.7)
$bla_{CTX\text{-}M\text{-}1\text{-}like}$	14 (31.1)	6 (27.3)	11 (78.3)	31 (37.8)
$bla_{CTX\text{-}M\text{-}2\text{-}like}$	0 (0)	0 (0)	0 (0)	0 (0)
$bla_{CTX\text{-}M\text{-}9\text{-}like}$	11 (24.4)	4 (18.2)	0 (0)	15 (18.3)
$bla_{PER\text{-}1}$	0 (0)	0 (0)	0 (0)	0 (0)
$bla_{TEM} + bla_{SHV}$	6 (13.3)	3 (13.6)	1 (6.7)	10 (12.2)
$bla_{TEM} + bla_{CTX}$	1 (2.2)	3 (13.6)	0 (0)	4 (4.9)
$bla_{SHV} + bla_{CTX}$	10 (22.2)	1 (4.5)	0 (0)	11 (13.4)
$bla_{TEM} + bla_{SHV} + bla_{CTX\text{-}M}$	1 (2.2)	0 (0)	0 (0)	1 (2.2)

2.3. Conjugation and PCR amplification of ESBL from transconjugants

Mating experiments were performed with sodium azide-resistant *E. coli* J53 as the recipient. Overnight cultures of recipient and donor strains grown on Luria-Bertani broth (Difco), with shaking at 37 °C, were inoculated at a 1:10 ratio (donor to recipient) into fresh Luria-Bertani broth and were then incubated overnight at 37 °C. Samples (0.1 mL) of this mixture were spread onto the surfaces of MH agar plates with 150 μg of sodium azide plus 2 μg/mL of CAZ or CTX. Samples from donors and recipients were used as controls. The transconjugants growing on the selection plates were subjected to DDST to confirm the presence of ESBL in transconjugants. PCR experiments were performed with the crude lysates of the transconjugants. The pairs of primers specific for bla_{TEM} (Mabilat and Goussard, 1993), bla_{SHV} (Kim et al., 1998), $bla_{CTX\text{-}M\text{-}1,\ M\text{-}2,\ M\text{-}9}$ (Saladin et al., 2002), and $bla_{PER\text{-}1}$(Danel et al., 1995) that were used are listed in Table 1. Strains encoding bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and $bla_{PER\text{-}1}$ genes were used as positive controls for PCR amplification.