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Occurrence of CTX-M-3, CTX-M-15, CTX-M-14, and CTX-M-9 Extended-Spectrum β-Lactamases in *Enterobacteriaceae* Clinical Isolates in Korea

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Among 603 isolates of *Enterobacteriaceae* collected between June and November 2003 from three university hospitals within Korea, $bla_{CTX-M-3}$, $bla_{CTX-M-15}$, $bla_{CTX-M-14}$, and $bla_{CTX-M-9}$ were detected in 41 isolates of species from five different genera of *Enterobacteriaceae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter* spp., and *Serratia marcescens*.

Although most extended-spectrum β-lactamases (ESBL) belong to the TEM- and SHV-type ESBL families, the members of a novel ESBL family, CTX-M, are increasingly being reported in gram-negative bacilli (1). Here, we examined the presence of CTX-M enzymes and the predominant type of CTX-M enzyme in Korea.

Between June and November 2003, 603 consecutive nonduplicate nosocomial isolates of *Enterobacteriaceae* were collected from three university hospitals located in three different cities—Daegu, Daejun, and Cheonan—in Korea. Among the 603 isolates collected, 163 (27%) were grown on Mueller-Hinton agar plates containing 2 μg of cefotaxime (Sigma)/ml, and they were subjected to PCR for detecting bla_{CTX-M} with primers listed in Table 1, designed for detection of enzymes from the CTX-M-1, CTX-M-2, and CTX-M-9 groups. As a result of the PCR experiment, 41 of 163 isolates (25.2%) have been shown to carry bla_{CTX-M}: 28 strains were positive for the PCR of the CTX-M-1 group, and 13 strains were positive for the PCR of the CTX-M-9 group. Further determination of bla_{CTX-M} alleles was performed by nucleotide sequencing of PCR products on both strands with primers used for PCR. Sequencing was carried out with the *Taq* DyeDeoxyTerminal

TABLE 1. Oligonucleotide primers used for detection of β-lactamase genes

Primer	Temp[a]	Nucleotide sequence	GenBank accession no.	Nucleotide position	Expected amplicon size (bp)
CTX-M-2-S	58°C	5′-TTAATGATGACTCAGAGCATTC-3′	X92507	3–24	901
CTX-M-2-AS		5′-GATACCTCGCTCCATTTATTG-3′	X92507	884–904	
CTX-M-9-S	50°C	5′-TAT TGG GAG TTT GAG ATG GT-3′	AF4546633.2	742–761	932
CTX-M-9-AS		5′-TCC TTC AAC TCA GCA AAA GT-3′	AF4546633.2	1655–1674	
CTX-M-1-S	55°C	5′-CGT CAC GCT GTT GTT AGG AA-3′	AJ632119.1	180–209	780
CTX-M-1-AS		5′-ACG GCT TTC TGC CTT AGG TT-3′	AJ632119.1	941–960	
TEM-S	50°C	5′-ATA AAA TTC TTG AAG ACG AAA-3′	AB103506	166–186	1,080
TEM-AS		5′-GAC AGT TAC CAA TGC TTA ATC-3′	AB103506	1225–1245	
SHV-S	55°C	5′-TGG TTA TGG GTT ATA TTC GCC-3′	AY223863	166–186	865
SHV-AS		5′-GGT TAG CGT TGC CAG TGC T-3′	AY223863	1015–1031	
OXA-1-S	55°C	5′-AGC CGT TAA AAT TAA GCC C-3′	AV162283.2	1052–1070	908
OXA-1-AS		5′-CTT GAT TGA AGG GTT GGG CG-3′	AV162283.2	1941–1960	
CMY-1-S	60°C	5′-GAG CAG ACC CTG TTC GAG AT-3′	X92508	570–589	846
CMY-1-AS		5′-GAT TGG CCA GCA TGA CGA TG-3′	X92508	1397–1416	
DHA-1-S	50°C	5′-GTT ACT CAC ACA CGG AAG GT-3′	AY205600	75–94	869
DHA-1-AS		5′-TTT TAT AGT AGC GGG TCT GG-3′	AY205600	925–944	

[a] Annealing temperature used for PCR.

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TABLE 2. Phenotypic and genotypic characterization of 41 isolates carrying the bla_{CTX-M} gene

Species	Strain	Hospital[a]	CTX-M group	pI(s)[b]	Other β-lactamase gene product(s)	MIC[c] (μg/ml)					Antimicrobial resistance pattern[d]
						CTX	CAZ	ATM	FEP	FOX	
C. freundii	03K892	K	CTX-M-3	5.4, 7.4, 8.4, 8.7	TEM-1, OXA-30, AmpC	256	8	32	64	512	AMP AMK GEN KAN CHL TET STR SXT TMP
E. coli	EC25	D	CTX-M-3	5.4, 8.4	TEM-1	128	2	16	16	8	AMP AMK GEN KAN TET STR SXT TMP
E. coli	03K681	K	CTX-M-3	5.4, 8.4	TEM-1	512	8	32	128	256	AMP AMK GEN KAN CHL TET STR SXT TMP
E. coli	03K203	K	CTX-M-3	7.4, 8.0, 8.4	OXA-30, CMY-1	128	128	256	256	≥512	AMP AMK GEN KAN STR SXT
K. pneumoniae[e]	KP2	D	CTX-M-3	7.4, 7.8, 8.4	OXA-30, DHA-1	256	128	256	32	256	AMP AMK KAN CHL STR SXT
K. pneumoniae	KP7	D	CTX-M-3	8.4		256	8	32	128	16	AMP AMK GEN KAN SXT TMP
K. pneumoniae	KP9	D	CTX-M-3	8.4		256	8	32	128	8	AMP AMK GEN KAN SXT TMP
K. pneumoniae	03K839	K	CTX-M-3	5.4, 7.4, 7.8, 8.4	TEM-1, OXA-30, DHA-1	≥512	256	512	256	≥512	AMP AMK GEN KAN CHL STR SXT TMP
S. marcescens	03K188	K	CTX-M-3	7.4, 8.4, 8.7	OXA-30, AmpC	≥512	128	256	512	≥512	AMP AMK GEN KAN STR SXT
S. marcescens	03K196	K	CTX-M-3	7.4, 8.4, 8.7	OXA-30, AmpC	≥512	128	128	512	≥512	AMP AMK GEN KAN STR SXT
S. marcescens	03K201	K	CTX-M-3	7.4, 8.4, 8.7	OXA-30, AmpC	≥512	64	128	512	≥512	AMP AMK GEN KAN STR SXT
S. marcescens	03K205	K	CTX-M-3	7.4, 8.4, 8.7	OXA-30, AmpC	≥512	128	128	512	≥512	AMP AMK GEN KAN STR SXT
S. marcescens	03K168	K	CTX-M-3	7.4, 8.4, 8.7	OXA-30, AmpC	≥512	64	128	>512	≥512	AMP AMK GEN KAN STR SXT
S. marcescens	03K921	K	CTX-M-3	8.4, 8.7	AmpC	≥512	32	128	512	256	AMP AMK GEN KAN STR SXT
S. marcescens	03K980	K	CTX-M-3	8.4, 8.7	AmpC	≥512	64	256	512	≥512	AMP AMK GEN KAN STR SXT
S. marcescens	SM 16	D	CTX-M-3	8.4		64	≥1	32	32	8	AMP AMK GEN KAN STR SXT TMP
S. marcescens	SM 3	D	CTX-M-3	8.4		512	4	64	64	16	AMP AMK GEN KAN STR SXT TMP
Enterobacter aerogenes	EA11	D	CTX-M-15	5.4, 7.4, 7.6, 8.6	TEM-1, OXA-30, SHV-1	512	128	128	256	4	AMP STR SXT TMP
Enterobacter aerogenes	EA6	D	CTX-M-15	5.4, 7.4, 7.6, 8.6	TEM-1, OXA-30, SHV-1	512	128	128	256	4	AMP TET STR SXT TMP
Enterobacter aerogenes	EA7	D	CTX-M-15	5.4, 7.4, 7.6, 8.6	TEM-1, OXA-30, SHV-1	512	64	128	256	4	AMP TET STR SXT TMP
E. coli	03K969	K	CTX-M-15	5.4, 7.4, 8.6	TEM-1, OXA-30	256	16	32	16	4	AMP GEN TET STR SXT TMP
E. coli	03K865	K	CTX-M-15	5.4, 7.4, 8.6	TEM-54, OXA-30	256	8	16	4	2	AMP GEN KAN TET
E. coli	03K582	K	CTX-M-15	5.4, 7.4, 8.6	TEM-54, OXA-30	256	32	64	128	8	TET
E. coli	J133	E	CTX-M-15	5.4, 7.4, 8.6	TEM-54, OXA-30	256	32	64	128	8	AMP GEN KAN STR SXT TMP
E. coli	J144	E	CTX-M-15	5.4, 7.4, 8.6	TEM-1, OXA-30	512	128	256	256	128	AMP GEN KAN TET SXT TMP
E. coli	J158	E	CTX-M-15	5.4, 7.4, 8.6	TEM-1, OXA-30	512	128	256	256	16	AMP GEN KAN TET
E. coli	J159	E	CTX-M-15	5.4, 8.6	TEM-1	512	64	256	256	32	AMP GEN TET SXT TMP
E. coli	EC35	D	CTX-M-15	5.4, 7.4, 8.6	TEM-1, OXA-30	≥512	128	256	128	16	AMP GEN KAN TET
E. cloacae	J187	E	CTX-M-9	8.0, 8.5	AmpC	32	4	4	4	≥512	AMP KAN CHL TET STR SXT TMP
E. cloacae	03K42	K	CTX-M-9	8.0, 8.2, 8.5	SHV-12, AmpC	32	64	128	4	512	AMP KAN CHL TET SXT TMP
E. coli	03K380	K	CTX-M-9	8.0, 8.2, 8.5	SHV-12, AmpC	8	32	32	<1	128	AMP KAN CHL TET STR SXT TMP
E. coli	03K776	K	CTX-M-9	8.0, 8.5	AmpC	8	≤1	1	<1	256	AMP KAN TET STR SXT TMP
E. coli	EC27	D	CTX-M-14	8.0		128	2	8	64	8	AMPTET
E. coli	ECS	D	CTX-M-14	5.4, 8.0	TEM-1	512	8	32	64	32	AMP GEN KAN CHL TET STR SXT
E. coli	J167	E	CTX-M-14	5.4, 8.0	TEM-1	128	2	16	8	8	AMP GEN TET STR SXT TMP
E. coli	03K319	K	CTX-M-14	5.4, 8.0, 8.5	TEM-1	32	≤1	8	4	4	TET STR SXT
K. pneumoniae	03K930	K	CTX-M-14	8.0		512	8	32	128	32	AMP KAN STR SXT
K. pneumoniae	J22	E	CTX-M-14	6.2,[f] 8.0, 8.2	OXA, SHV-12	256	8	32	64	32	AMP KAN STR
K. pneumoniae	J64	E	CTX-M-14	6.2,[f] 8.0, 8.2	OXA, SHV-12	256	8	512	64	8	AMP KAN STR
K. pneumoniae	J1	E	CTX-M-14	6.2,[f] 8.0, 8.2	OXA, SHV-12	128	256	512	16	8	AMP KAN STR
K. pneumoniae	J112	E	CTX-M-14	6.2,[f] 8.0, 8.2	OXA, SHV-12	256	512	512	32	8	AMP KAN STR

[a] Hospital K is located in the city of Daegu, hospital D is in Cheonan, and hospital E is in Daejun in Korea.
[b] The pI of β-lactamase as determined by IEF; underlined pIs correspond to the CTX-M enzymes.
[c] MIC as determined by the agar dilution method. Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime; FOX, cefoxitin.
[d] Abbreviations: AMP, ampicillin; CHL, chloramphenicol; TET, tetracycline; STR, streptomycin; SXT, sulfisoxazole; TMP, trimethoprim; KAN, kanamycin; GEN, gentamicin; AMK, amikacin.
[e] All *K. pneumoniae* isolates were positive for the PCR with the SHV primer sets, and further sequence determination revealed that these isolates have SHV-11, the β-lactamase located on the chromosome of *K. pneumoniae*, although the β-lactamase with a pI of 7.6 corresponding to the SHV-11 was not detected by the IEF analysis.
[f] The β-lactamase with a pI of 6.2 was not inhibited by either 0.3 mM clavulanic acid or 0.3 mM cloxacillin, indicating an OXA-type β-lactamase. We did not perform further characterization of this β-lactamase.

TABLE 3. Transfer of resistance for cefotaxime and other antimicrobial agents of clinical isolates carrying $bla_{CTX\text{-}M}$

Transconjugant	Donor strain	Transferred *bla* gene(s)	MIC[a] (μg/ml)					Antimicrobial resistance pattern[b]
			CTX	CAZ	ATM	FEP	FOX	
P1-1J	03K892	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	32	<1	2	<1	2	AMP AMK GEN KAN SXT
11-1J	03K681	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	128	4	16	16	8	AMP AMK GEN KAN SXT
15-1J	EC25	$bla_{CTX\text{-}M\text{-}3}$	64	<1	16	8	2	AMP AMK GEN KAN SXT TMP
35-1J	KP7	$bla_{CTX\text{-}M\text{-}3}$	32	<1	2	<1	4	AMP AMK GEN KAN SXT TMP
36-1J	KP9	$bla_{CTX\text{-}M\text{-}3}$	32	<1	2	<1	4	AMP AMK GEN KAN SXT TMP
34-2J	KP2	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	32	<1	2	<1	2	AMP AMK GEN KAN SXT
34-1J	KP 2	$bla_{CTX\text{-}M\text{-}3}$, $bla_{SHV\text{-}12}$, $bla_{DHA\text{-}1}$	32	16	32	<1	32	AMP AMK GEN KAN CHL STR SXT
24-1J	03K839	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$, $bla_{TEM\text{-}1}$	128	16	32	8	4	AMP GEN KAN SXT TMP
38-1J	03K188	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	32	<1	2	<1	4	AMP AMK GEN KAN SXT
39-1J	03K196	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	64	<1	8	4	4	AMP AMK GEN KAN SXT
40-1J	03K201	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	32	<1	2	<1	4	AMP AMK GEN KAN SXT
41-1J	03K205	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	32	2	16	8	8	AMP AMK GEN KAN SXT
37-1J	03K168	$bla_{CTX\text{-}M\text{-}3}$, $bla_{OXA\text{-}30}$	32	<1	2	<1	4	AMP AMK GEN KAN SXT
43-1J	03K921	$bla_{CTX\text{-}M\text{-}3}$	32	2	8	32	2	AMP KAN STR
44-1J	03K980	$bla_{CTX\text{-}M\text{-}3}$	4	2	8	32	4	AMP AMK KAN STR
45-1J	SM 16	$bla_{CTX\text{-}M\text{-}3}$	4	2	16	32	8	AMP AMK GEN KAN STR SXT TMP
46-1J	SM 3	$bla_{CTX\text{-}M\text{-}3}$	8	2	16	16	8	AMP AMK GEN KAN STR SXT TMP
13-1J	03K865	$bla_{CTX\text{-}M\text{-}15}$	128	<1	8	2	4	AMP
14-1J	03K969	$bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}30}$, $bla_{TEM\text{-}1}$	512	8	16	8	4	AMP SXT TMP
20-1J	J144	$bla_{CTX\text{-}M\text{-}15}$	256	8	16	8	4	AMP SXT
22-1J	J159	$bla_{CTX\text{-}M\text{-}15}$	128	<1	4	8	32	AMP
19-1J	J133	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}54}$	256	4	16	4	4	AMP
06-1J	J187	$bla_{CTX\text{-}M\text{-}9}$	8	<1	2	2	32	AMP CHL TET SXT TMP
05-1J	03K42	$bla_{CTX\text{-}M\text{-}9}$, $bla_{SHV\text{-}12}$	4	4	8	<1	2	AMP CHL SXT TMP
09-2J	03K380	$bla_{CTX\text{-}M\text{-}9}$, $bla_{SHV\text{-}12}$	8	64	64	<1	2	AMP CHL SXT TMP
18-1J	18-1J	$bla_{CTX\text{-}M\text{-}14}$, $bla_{OXA\text{-}30}$, $bla_{TEM\text{-}1}$	512	2	8	16	4	AMP TET STR SXT
16-1J	EC27	$bla_{CTX\text{-}M\text{-}14}$	128	2	16	16	8	AMP
23-1J	J167	$bla_{CTX\text{-}M\text{-}14}$	64	<1	8	8	8	AMP STR SXT
25-1J	03K930	$bla_{CTX\text{-}M\text{-}14}$	64	<1	2	4	4	AMP STR SXT
	E. coli J53 Azide^R recipient		<1	<1	<1	<1	4	

[a] MIC as determined by the agar dilution method. Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime; FOX, cefoxitin.
[b] Abbreviations: AMP, ampicillin; CHL, chloramphenicol; TET, tetracycline; STR, streptomycin; SXT, sulfisoxazole; TMP, trimethoprim; KAN, kanamycin; GEN, gentamicin; AMK, amikacin.

cycle-sequencing kit using primers used for PCR, and the sequence was analyzed by using an automatic DNA sequencer (377 ABI Prism; Perkin Elmer). Of the 28 strains positive for the CTX-M-1 group, 17 were confirmed to carry $bla_{CTX\text{-}M\text{-}3}$, and the remaining 11 strains carried $bla_{CTX\text{-}M\text{-}15}$. Of the 13 strains positive for the CTX-M-9 group, 9 were confirmed to carry $bla_{CTX\text{-}M\text{-}14}$, and the remaining 4 strains carried $bla_{CTX\text{-}M\text{-}9}$ (Table 2). In *Escherichia coli* isolates, all four kinds of $bla_{CTX\text{-}M}$ were demonstrated. $bla_{CTX\text{-}M\text{-}3}$ was identified in species from four different genera of *Enterobacteriaceae*, *Citrobacter freundii* (one isolate), *E. coli* (three isolates), *Klebsiella pneumoniae* (four isolates), and *Serratia marcescens* (nine isolates), indicating horizontal transfer and wide dissemination of $bla_{CTX\text{-}M\text{-}3}$ among the family *Enterobacteriaceae*. $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$ were detected in all three university hospitals located in three different cities. $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}9}$ were not detected in the hospital located in Daejun and in the hospital located in Cheonan, respectively.

Characterization of 41 isolates carrying $bla_{CTX\text{-}M}$ was performed via antimicrobial susceptibility testing, an isoelectric focusing (IEF) assay (2), PCR, and nucleotide sequencing for β-lactamase genes. MICs were measured using a standard agar dilution method according to the approved method of the National Committee for Clinical Laboratory Standards (3). *E. coli* ATCC 25922 was used as a quality reference strain. Isoelectric focusing and inhibition assays with 0.3 mM clavulanic acid or cloxacillin were performed as described previously (2, 4).

As shown in Table 2, most isolates expressing CTX-M enzyme were found to produce additional β-lactamases. The β-lactamase with a pI of 5.4 was confirmed as TEM-1 or TEM-54, inhibitor-resistant TEM, by TEM-specific PCR and sequencing. The β-lactamases with pIs of 7.6 and 8.2 and whose activity was inhibited by 0.3 mM clavulanic acid were SHV-1 and SHV-12, respectively. The β-lactamase with a pI of 7.4 whose activity was not inhibited by either 0.3 mM clavulanic acid or 0.3 mM cloxacillin was OXA-30, confirmed by OXA-1-specific PCR and subsequent sequencing. The β-lactamases with pIs of 8.0 and 7.8 whose activity was inhibited by 0.3 mM cloxacillin were CMY-1 and DHA-1, respectively.

For almost all strains expressing CTX-M enzyme, except five strains which coexpressed SHV-12 or CMY-1, the MICs of cefotaxime were higher than those of ceftazidime (Table 2). The cefotaxime MICs for such strains were two- to sevenfold

higher dilutions than those of ceftazidime. Ratios of cefotaxime MIC to ceftazidime MIC for isolates expressing CTX-M-15 were lower than those for isolates expressing CTX-M-3, as demonstrated by other reports (6, 7). Although there is only one amino acid difference between CTX-M-3 and CTX-M-15 (Asp^{240}→Gly), Poirel et al. (6) demonstrated that the amino acid difference in the omega loop region of CTX-M-15 results in increased ceftazidime hydrolysis and antibiotic resistance compared to those for CTX-M-3. For four strains expressing CTX-M-9 (two *E. coli* strains and two *Enterobacter cloacae* strains), the MICs of cefotaxime were lower, ranging from 8 to 32 μg/ml, than those for strains expressing CTX-M-3, CTX-M-15, or CTX-M-14.

Some strains demonstrated high levels of resistance to cefoxitin, and these strains were found to produce additional chromosomal AmpC enzyme or plasmid-mediated AmpC enzymes, such as CMY-1 and DHA-1.

Transferability of cefotaxime resistance was determined by conjugation experimentation using *E. coli* J53 $Azide^R$ (confers resistance to sodium azide) as a recipient. Donor and recipient strains at logarithmic phase were grown in 4 ml of Trypticase soy broth (Difco Laboratories) and were mixed at a ratio of 4 (recipient) to 1 (donor) at 37°C for 20 h. Transconjugants were selected on Mueller-Hinton agar plates (Difco Laboratories) supplemented with sodium azide (150 μg/ml) and cefotaxime (4 μg/ml). By conjugation, cefotaxime resistance was transferred in 29 isolates, and the bla_{CTX-M} gene was confirmed in all 29 transconjugants by PCR (Table 3). Some other *bla* genes, such as bla_{OXA-30}, bla_{TEM}, bla_{DHA-1}, and bla_{SHV-12}, were cotransferred with bla_{CTX-M} to transconjugants. Especially, bla_{OXA-30} was cotransferred with $bla_{CTX-M-3}$ in almost all strains, indicating that $bla_{CTX-M-3}$ and bla_{OXA-30} might be located on the same transferable plasmid.

Resistance to chloramphenicol, tetracycline, aminoglycosides, and co-trimoxazole was found in most strains carrying bla_{CTX-M}, and the resistance was also found in most transconjugants (Table 3). Interestingly, a high level of amikacin resistance (MIC, ≥512 μg/ml) was demonstrated in all 17 isolates carrying $bla_{CTX-M-3}$ but not in isolates carrying another subtype of bla_{CTX-M}, and the amikacin resistance was transferred to transconjugants.

In conclusion, the occurrence of CTX-M-3, CTX-M-15, CTX-M-9, and CTX-M-14 in species from five different genera of *Enterobacteriaceae*, *C. freundii*, *E. coli*, *Enterobacter* spp., *K. pneumoniae*, and *S. marcescens* was demonstrated. This finding indicates horizontal transfer and wide dissemination of these enzymes in Korea and would suggest that CTX-M enzymes have existed for several years and have evolved in Korean hospital environments. Although CTX-M-14 was identified in one isolate of *Shigella sonnei*, two of *K. pneumoniae*, and one of *E. coli* in Korea in 2001 (5), to our knowledge this study represents the first identification of CTX-M-3, CTX-9, and CTX-M-15 in Korea.

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