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CTX-M and SHV-12 β-lactamases are the most common extended-spectrum enzymes in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* collected from 3 university hospitals within Korea

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Abstract

Among the 443 clinical isolates of *Escherichia coli* and *Klebsiella* spp. collected between June and November 2003 from 3 university hospitals in Korea, 62 isolates were confirmed as extended-spectrum β-lactamase (ESBL)- or plasmid-mediated AmpC β-lactamase-producers by double disk synergy test, PCR and sequencing for β-lactamase genes. The most frequently identified ESBL gene among *E. coli* and *K. pneumoniae* isolates was $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{CTX-M-15}}$). Four kinds of plasmid-mediated AmpC β-lactamases, ACT-1, CMY-1, CMY-2, and DHA-1, were detected. ESBL production was associated with high levels of resistance to tetracycline, sulfisoxazole, streptomycin, kanamycin, gentamicin and tobramycin when compared to non-ESBL producing isolates. Conclusively, this study suggests that the CTX-M β-lactamases are prevalent and various kinds of plasmid-mediated AmpC enzymes are distributed in clinical isolates of *E. coli* and *Klebsiella* spp. in Korea.

Keywords: CTX-M ESBL; SHV-12; *Escherichia coli*; *Klebsiella pneumoniae*; Korea

1. Introduction

The evolution and dissemination of extended-spectrum β-lactamases (ESBL) have compromised the clinical use of third-generation cephalosporins worldwide and caused the crisis of antimicrobial drug resistance. ESBLs are usually described as acquired β-lactamases that are encoded mostly by plasmid-located genes, leading to the complex and dynamic evolution and epidemiology of ESBLs. The number of ESBL variants identified has been constantly growing and more than 100 different ESBL variants are known at present (http://www.lahey.org/studies/webt.htm). Most ESBLs are derivatives of TEM-1, TEM-2, or SHV-1 enzymes; however, reports describing the emergence of β-lactamases

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belong to other families, such as PER, VEB, CTX-M and/or OXA derivatives, are increasing worldwide [1–5].

Among gram-negative pathogens in Korea, the incidence of resistance to extended-spectrum β-lactam antibiotics is becoming an ever-increasing problem and rapid increase in ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* has been reported [6–9]. Although, the most commonly identified ESBLs in Korea are TEM-52, SHV-2a and SHV-12 [6–9], recent report suggests dissemination of CTX-M-14 in Korea [10]. Indeed, we here report the dissemination of CTX-M enzymes, such as CTX-M-3, CTX-M-9, CTX-M-15, and CTX-M-14, among the isolates of *E. coli* and *Klebsiella* spp. collected in 2003 from 3 university hospitals located in different cities of Korea. We also report on the first identification of ACT-1 and CMY-2, plasmid-mediated AmpC β-lactamases, from the isolates of *E. coli* in Korea.

2. Materials and methods

2.1. Clinical isolates

Consecutive non-duplicate nosocomial isolates of *E. coli*, *K. oxytoca*, and *K. pneumoniae* were collected between June and November 2003 from 3 university hospitals located in 3 different cities in Korea. Species identification was carried out with VITEK-GNI CARDS (bioMérieux Vitek Inc., Hazelwood, Mo.) by standard methods [11]. For selection of ESBL-producing isolates, strains for which the cefotaxime MIC was $\geqslant 2\ \mathrm{mg\,l^{-1}}$ were subjected to the double-disk synergy test with cefotaxime, ceftazidime, aztreonam, cefepime, and amoxicillin-clavulanic acid disks (Oxoid, Basingstoke, United Kingdom) as described previously [8]. The disks were placed 20 mm apart (from center to center); for selected isolates the test was repeated with the distance reduced to 15 mm.

2.2. Antimicrobial susceptibility tests

Minimal inhibitory concentrations (MICs) were measured using a standard agar dilution method according to the approved method of the National Committee for Clinical Laboratory Standards [12]. *E. coli* ATCC 25922 was used as a quality reference strain. The antimicrobial agents included were ampicillin, cefoxitin, cefotaxime, kanamycin, tobramycin, gentamicin, amikacin, sulfisoxazole, chloramphenicol, streptomycin, tetracycline, and trimethoprim (all from Sigma).

2.3. PCR amplification and nucleotide sequencing

PCR was performed to detect β-lactamase genes with primers shown in Table 1. PCRs were performed with 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 50 °C for TEM, CTX-M-9 and DHA-1, and 55 °C for SHV, CTX-M-3 and OXA, respectively, and 1 min at 72 °C for polymerization. Final products were

Table 1
Oligonucleotide primers used for detection of β-lactamase genes

Primers	T_m[a] (°C)	Nucleotide sequences	References (GenBank No.)	Expected amplicon size (bp)
CTX-M-9-S	50	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-3-S	55	5′-CGT CAC GCT GTT GTT AGG AA-3′	AJ632119.1; 180–209	780
CTX-M-3-AS		5′-ACG GCT TTC TGC CTT AGG TT-3′	AJ632119.1; 941–960	
TEM-S	50	5′-ATA AAA TTC TTG AAG ACG AAA-3′	AB103506; 166–186	1080
TEM-AS		5′-GAC AGT TAC CAA TGC TTA ATC-3′	AB103506; 1225–1245	
SHV-S	55	5′-TGG TTA TGC 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	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	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	
CMY-2-S	60	5′-TGGCCAGAACTGACAGGCAAA-3′	X78117; 478–498	462
CMY-2-AS		5′-TTTCTCCTGAACGTGGCTGGC-3′	X78117; 919–939	
ACT-1-S	50	5′-AAACCTGTCACTCCACAAAC-3′	U58495; 244–264	887
ACT-1-AS		5′-GGGTTCGGATAGCTTTTATT-3′	U58495; 1111–1130	
DHA-1-S	50	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.

extended by incubation for 10 min at 72 °C. Amplified PCR products were sequenced on both strands. Sequencing was carried out with the *Taq* DyeDeoxyTerminal cycle-sequencing kit using primers used for PCR and the sequence was analyzed in an automatic DNA sequencer (377 ABI Prism, Perkin Elmer). DNA sequence analysis was performed with DNASIS for Windows (Hitachi Software Engineering America Ltd., San Bruno, CA). Database similarity searches for both the nucleotide sequences and deduced protein sequences were performed with BLAST at the National Center for Biotechnology Information website.

3. Results

Between June and November 2003, 443 isolates including 272 of *E. coli*, 13 of *K. oxytoca*, and 158 of *K. pneumoniae* were collected from 3 university hospitals in Korea. Among them, 87 isolates revealed $\geqslant 2\ \mathrm{mg\,l^{-1}}$ of MIC against cefotaxime and they were subjected to the double disk synergy test (DDST), PCR of β-lactamase genes, and subsequent sequencing of amplified β-lactamase genes. Of 87 isolates revealed $\geqslant 2\ \mathrm{mg\,l^{-1}}$ of MIC against cefotaxime, 45 isolates (35 of *E. coli* and 10 of *K. pneumoniae*) showed resistance to cefoxitin. They were screened for plasmid-mediated AmpC β-lactamases with the primers shown in Table 1 and amplified PCR products were analyzed by nucleotide sequencing.

From the results, 62 strains were identified as ESBL- or plasmid-mediated AmpCs-producers, including 32 isolates of *E. coli*, 2 isolates of *K. oxytoca*, and 28 isolates of *K. pneumoniae* (Table 2). The most frequently identified ESBL gene among *E. coli* and *K. pneumoniae* isolates was $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M}}$ which identified from 18 (29%) and 18 (29%) isolates, respectively. Among 32 isolates of *E. coli*, 8 isolates carried bla_{TEM}- and $_{\text{SHV}}$-derived genes ($bla_{\text{TEM-52}}$ in 2 isolates and $bla_{\text{SHV-12}}$ 6 isolates); 15 isolates carried $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-9}}$ in 1 isolate, $bla_{\text{CTX-M-14}}$ in 4, $bla_{\text{CTX-M-3}}$ in 2, and $bla_{\text{CTX-M-15}}$ in 8); $bla_{\text{CMY-2}}$ and $bla_{\text{ACT-1}}$, PABL-coding genes, were identified in one and six isolates, respectively; and remaining 2 isolates carried several bla_{ESBL} genes ($bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-9}}$ in 1 isolate and $bla_{\text{CTX-M-3}}$, $bla_{\text{OXA-30}}$, and $bla_{\text{CMY-1}}$ in 1 isolate). Among 28 isolates of *K. pneumoniae*, 10 isolates carried $bla_{\text{SHV-12}}$ and 3 isolates carried $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-14}}$ in one and $bla_{\text{CTX-M-3}}$ in two); $bla_{\text{CMY-1}}$ and $bla_{\text{DHA-1}}$ were identified in one and one isolate, respectively; and remaining 13 isolates carried several bla_{ESBL} genes. Seven of eight isolates carrying $bla_{\text{DHA-1}}$ also carried $bla_{\text{SHV-12}}$, bla_{TEM}, $bla_{\text{CTX-M-3}}$, and/or $bla_{\text{OXA-30}}$. One *K. pneumoniae* isolate carried five different kinds of *bla* genes, such as $bla_{\text{TEM-52}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{DHA-1}}$, and $bla_{\text{OXA-30}}$.

Table 2
Distribution of ESBLs among the clinical isolates of *E. coli* and *Klebsiella* spp. collected from 3 university hospitals in Korea

Species	Number of isolates	Type of ESBL (number of isolates)				
		TEM	SHV	CTX-M	AmpC	Mixed ESBLs
E. coli	32	TEM-52 (2)	TEM-1/SHV-12 (3)	CTX-M-9 (1)	ACT-1 (1)	SHV-12/CTX-M-9 (1)
			SHV-12 (1)	CTX-M-14 (1)	TEM-1/ CMY-2 (6)	CTX-M-3/OXA-30/CMY-1(1)
			TEM-1/SHV-12/OXA-30 (2)	TEM-1/CTX-M-14 (2)		
				TEM-1/CTX-M-14/OXA-30 (1)		
				TEM-1/CTX-M-3 (1)		
				TEM-1/CTX-M-3/OXA-30 (1)		
				TEM-54/CTX-M-15/OXA-30 (3)		
				TEM-1/CTX-M-15/OXA-30 (5)		
K. oxytoca	2	TEM-1/SHV-12 (1)				
		TEM-54/SHV-12 (1)				
K. pneumoniae	28		TEM-1/SHV-12/OXA-30 (1)	SHV-11/CTX-M-3 (2)	TEM-1/SHV-1/OXA-30/CMY-1(1)	SHV-12/CTX-M-3/DHA-1/OXA-30 (1)
			SHV-12 (3)	SHV-11/CTX-M-14(1)	TEM-1/DHA-1(1)	TEM-54/SHV-12/DHA-1(2)
			TEM-54/SHV-12 (2)			TEM-52/SHV-12 (1)
			TEM-1/SHV-12 (4)			SHV-12/DHA-1(2)
						SHV-12/CTX-M-14 (4)
						TEM-52/SHV-12 (1)
						TEM-52/SHV-12/OXA-30 (1)
						TEM-1/SHV-12/CTX-M-3/DHA-1/OXA-30 (1)
Number of strains (%)	62	2 (3.3)	18 (29.0)	18 (29.0)	9 (14.5)	15 (24.2)

Table 3
Antimicrobial susceptibilities (%) of ESBL-producing isolates of *E. coli* and *Klebsiella* spp.

Antimicrobials	Total (87)[a]				*E. coli* (51)				*Klebsiella* spp. (36)			
	ESBL (62)		Non-ESBL (25)		ESBL (32)		Non-ESBL (19)		ESBL (30)		Non-ESBL (6)	
	R	S	R	S	R	S	R	S	R	S	R	S
Ampicillin	89	11	96	4	84	16	95	5	85	15	100	0
Chloramphenicol	32	68	32	68	41	59	37	63	21	79	17	83
Tetracycline	40	60	28	72	69	31	37	63	9	91	0	100
Sulfisoxazole	73	27	56	44	81	19	58	42	58	42	50	50
Trimethoprim	47	53	52	48	59	41	53	47	30	70	50	50
Streptomycin	63	37	40	60	69	31	47	53	52	48	17	83
Kanamycin	81	19	52	48	75	25	53	47	79	21	50	50
Gentamicin	60	40	48	52	66	34	47	53	48	52	50	50
Amikacin	29	71	28	72	25	75	26	74	30	70	33	67
Tobtramycin	92	8	48	52	91	9	47	53	85	15	50	50

[a] Parenthesis indicates the number of isolates.

Resistance rates of the ESBL- or plasmid-mediated AmpCs-producing *E. coli* and *Klebsiella* spp. against non-β-lactam antibiotics are given in Table 3. Production of ESBL or plasmid-mediated AmpCs among *E. coli* and *Klebsiella* spp. was associated with high levels of resistance to tetracycline, sulfisoxazole, streptomycin, kanamycin, gentamicin and tobramycin when compared to non-ESBL producing isolates. Especially, resistance rates of ESBL-producers to aminoglycosides except amikacin were very high as 63% to streptomycin, 81% to kanamycin, 60% to gentamicin, and 92% to tobramycin. But the resistance rates to ampicillin, chloramphenicol, trimethoprim, and amikacin were the same or lower in ESBL-producing isolates than in non-ESBL producing isolates.

4. Discussion

In this study, the overall prevalence rates of ESBL-producers in *E. coli* and *K. pneumoniae* isolates were 11.8% (32 of 272 isolates) and 17.7% (28 of 158 isolates), respectively. The result was very similar to the ESBL survey in 1999 in 28 hospitals in Korea which revealed a frequency of 8.3% and 18.1% ESBL producers among *E. coli* and *K. pneumoniae*, respectively [13]. Data from the SENTRY Antimicrobial Surveillance Program, performed on *K. pneumoniae*, *E. coli*, *P. mirabilis* and *Salmonella* spp. isolates collected in 1997–1999 from all over the world, showed that ESBL frequency in *K. pneumoniae* may account for about 45% in Latin America, 25% in the Western Pacific, 23% in Europe and 8% in the USA [14]. Therefore, it seems that the frequency of ESBL among *K. pneumoniae* in Korea may be higher than in USA but lower than in Latin America and Europe.

So far, TEM-52 and SHV-12 are the most common types of ESBL in Korea and CTX-M enzyme has been rarely found in Korea [6–10]. Although CTX-M-14 has been identified from only 4 isolates in Korea [10], identification of this enzyme in three different genera, i.e., *Shigella sonnei*, *E. coli*, and *K. pneumoniae*, and from different parts of Korea suggests dissemination of this enzyme in Korea. Indeed, CTX-M ESBL, with SHV-12, was the most frequently found ESBL among *E. coli* and *K. pneumoniae* isolates in this study. Moreover, four kinds of CTX-M ESBLs, CTX-M-3, CTX-M-9, CTX-M-15, and CTX-M-14, were found, suggesting that CTX-M enzymes have been persisted for longer periods and evolved in Korean hospital environments. Therefore, more attention and evaluation for CTX-M enzymes are needed to catch the precise situation of ESBLs in Korea.

Since first CTX-M-β-lactamase, FEC-1 was discovered in a cefotaxime-resistant *E. coli* strain in 1986 [14], CTX-M β-lactamases become the most widespread non-TEM, non-SHV plasmid-mediated class A ESBLs. In Far East, there have been reports of CTX-M-2, CTX-M-3, CTX-M-15, and CTX-M-14 in Japan [15–17] and of CTX-M-3, CTX-M-9, CTX-M-13, and CTX-M-14 from isolates of *E. coli*, *K. pneumoniae* and *S. marcescens* in China and Taiwan [18–21]. CTX-M-14 and CTX-M-17 have commonly been observed in *E. coli* and *K. pneumoniae* strains in Vietnam [22,23] and CTX-M-15 was reported in India [24]. Therefore, it seems that CTX-M-3 and CTX-M-14 types are the most prevalent type of CTX-M enzymes in Far East.

Since 1989, over 20 plasmid-mediated AmpC β-lactamases have been reported worldwide [25,26]. Whereas ESBLs confer resistance to the oxyimino-cephalosporins such as ceftazidime, cefotaxime, and aztreonam, plasmid-mediated AmpCs confer resistance to cephamycins such as cefoxitin and cefotetan. From this study, 8 of 272 (2.9%) *E. coli* isolates and 8 of 158 (5.1%) *K. pneumoniae* isolates demonstrated plasmid-mediated AmpCs-producers. Similarly, a survey performed in 2002 in 12 university hospitals in Korea showed that the prevalence rate of plasmid-mediated AmpCs-producers was

1.5% (8 of 544 isolates) in *E. coli* and 5.4% (20 of 367 isolates) in *K. pneumoniae* [27].

To date in Korea, CMY-1, CMY-11, and DHA-1 plasmid-mediated AmpCs has been identified from cefoxitin-resistant *E. coli* and *K. pneumoniae* isolates [8,27–29]. However, from this study, CMY-2 and ACT-1, in addition to CMY-1 and DHA-1, were firstly identified in six *E. coli* isolates and one *E. coli* isolate, respectively. Although this may be a first report on the $bla_{\text{CMY-2}}$-carrying *E. coli* isolates in Korea, the spread of $bla_{\text{CMY-2}}$ among *E. coli* and *Salmonella* isolates from food animals has recently been reported in North America and has raised a public health concern [15,30–34]. Moreover, a connection between CMY-2-producing *E. coli* and *Salmonella* isolates from humans and those from food animals has been established in the United States [32–35] and $bla_{\text{CMY-2}}$-carrying *Salmonella* and *E. coli* isolates associated with community-acquired infections in Taiwan [36]. To prevent further spread of $bla_{\text{CMY-2}}$-carrying isolates in Korea, constant and consistent surveillance is needed.

Since ESBL producers express their β-lactamase genes from plasmids, these organisms may also have genes coding for resistance to additional classes of antibiotics. This study showed association of ESBL production with high levels of resistance to tetracycline, sulfisoxazole, and aminoglycosides such as streptomycin, kanamycin, gentamicin and tobramycin when compared to non-ESBL producing isolates. This finding suggests that genes coding for ESBLs and genes coding for resistance to these antibiotics may reside within the same plasmids and therefore be spread together. This means that resistance to two different kinds of drugs may be co-selected by the use of either one.

It should be also emphasized that most of ESBL-producers from this study carried more than two kinds of *bla* genes. Of 62 ESBL-producers, 30 isolates (48.4%) and 18 isolates (29.0%) carried $bla_{\text{TEM-1}}$ or $bla_{\text{Oxa-30}}$, respectively, besides to bla_{ESBL}. Even more, 15 (24.2%) of 62 ESBL-producers carried more than two kinds of bla_{ESBL}. Surprisingly, one isolate of *K. pneumoniae* carried three kinds of bla_{ESBL}, such as $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M}}$, and $bla_{\text{DHA-1}}$, in addition to $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-30}}$. These findings indicate that very complex and dynamic evolution and dissemination of *bla* genes have been progressed in hospital environments for long periods.

In conclusion, the overall prevalence rates of ESBL-producers were relatively high as 11.8% and 17.7% in *E. coli* and *K. pneumoniae*, respectively, and the most common types of ESBLs were CTX-M and SHV-12. To our knowledge, CTX-M-3, CTX-M-9, CTX-M-15, ACT-1, and CMY-2 were firstly identified in *E. coli* and *K. pneumoniae* isolates from Korea. The relatively high prevalence of CTX-M enzyme and the presence of diverse plasmid-mediated AmpCs underline the need for routine surveillance for these β-lactamases.

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