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*Escherichia coli*의 Extended-Spectrum β-Lactamase 및 *qnr* 유전자 보유 현황

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Prevalence of the Extended-Spectrum β-Lactamase and qnr Genes in Clinical Isolates of Escherichia coli

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Background : This study was performed to investigate the prevalence of *qnr* genes in clinical isolates of *Escherichia coli* from Korea that produce extended-spectrum β-lactamases (ESBLs).

Methods : During the period of May to June 2005, we collected clinical isolates of *E. coli* that were intermediate or resistant to ceftazidime and/or cefotaxime from 11 Korean hospitals. Antimicrobial susceptibility was determined by the disk diffusion and agar dilution methods. ESBL production was confirmed phenotypically by the double-disk synergy test. ESBL and *qnr* genes were searched for by PCR amplification, and the PCR products were then subjected to direct sequencing.

Results : Double-disk synergy tests were positive in 84.3% (118/140) of ceftazidime- and/or cefotaxime-nonsusceptible *E. coli* isolates. The most prevalent types of ESBL in *E. coli* isolates were CTX-M-14 (N=41) and CTX-M-15 (N=58). Other ESBLs were also identified, including CTX-M-3 (N=7), CTX-M-9 (N=8), CTX-M-12 (N=1), CTX-M-57 (N=1), SHV-2a (N=2), SHV-12 (N=17) and TEM-52 (N=4). The *qnrA1* and *qnrB4* genes were identified in 4 and 7 ESBL-producing isolates, respectively.

Conclusions : CTX-M-type enzymes were the most common type of ESBL in *E. coli* isolates from Korea, and the *qnr* genes were not uncommon in ESBL-producing *E. coli* isolates. Dissemination of *E. coli* containing both ESBL and *qnr* genes could compromise the future usefulness of the expanded-spectrum antibiotics for the treatment of infections. *(Korean J Lab Med 2009;29:218-23)*

Key Words : *Escherichia coli, CTX-M ESBL, qnrA1, qnrB4*

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INTRODUCTION

The increasing tendency of oxyimino-cephalosporin resistance in *Escherichia coli* represents a clinical threat. Clavulanic acid-inhibitory extended-spectrum β-lactamases (ESBLs) represent the main mechanism of resistance in *E. coli*. The

rapid dissemination of CTX-M ESBLs has repeatedly been reported in many parts of the world [1], although classical TEM and SHV ESBLs are still dominant in the USA [2]. Past reports have shown that the most common ESBL in *E. coli* isolates from Korea is TEM-52 [3], and a nationwide survey in 2003 reported that 23 of 246 clinical isolates (9.3%) of *E. coli* produced ESBLs, while only 8 of these harbored CTX-M enzymes [4].

Fluoroquinolones have widely been used to treat various infections caused by gram-negative bacilli. However, more than 10% of *Enterobacteriaceae* isolated from patients hospitalized at intensive care units in the United States exhibited resistance to these drugs [5]. Furthermore, about 20% and 15% of *E. coli* isolates from Taiwan and Spain, respectively, were reported to be resistant to fluoroquinolones [6, 7]. In Korea, a notable increase in the fluoroquinolone-resistance rate of *E. coli* isolates from a university hospital was reported as 5% in 1994 to 38% in 2005 [8].

In the past, the resistance mechanisms of *Enterobacteriaceae* to fluoroquinolones were restricted to mutations in the chromosomal DNA gyrase (topoisomerase II) and topoisomerase IV genes and to changes in the efflux pumps or porins that decrease intracellular drug concentration [9]. However, the plasmid-mediated fluoroquinolone-resistance protein, Qnr, was detected in a clinical isolate of *Klebsiella pneumoniae* from the United States in 1994, exhibiting that horizontal transfer of fluoroquinolone-resistance is achievable [10]. In addition, another plasmid-mediated fluoroquinolone-resistance gene, *aac(6′)-Ib-cr*, was first detected in *E. coli* isolates from China in 2003 and was reported to be disseminating [11]. A recent survey detected the *qnr* genes in 33/59 (56%) and 8/143 (6%) clinical isolates of *K. pneumoniae* and *E. coli*, respectively, from urinary tract infections in Korea [12]. The present study was performed to investigate the prevalence of *qnr* genes in clinical isolates of *E. coli* producing ESBLs in Korea.

MATERIALS AND METHODS

1. Bacterial strains

Ceftazidime- and/or cefotaxime-nonsusceptible isolates of *E. coli* were collected during May to July 2005 from 11 hospitals in Korea. The isolates were identified with API-20E systems (bioMérieux, Marcy l'Etoile, France). *E. coli* J53 Azide^R was used as a recipient strain for conjugative transfer. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as MIC reference strains.

2. Antimicrobial susceptibility testing

Antibiotic-containing disks (BBL, Cockeysville, MD, USA) were used for routine antibiograms in a disk diffusion assay according to the CLSI guidelines [13]. The double-disk synergy test (DDST) was carried out on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) with disks of ceftazidime, cefotaxime, aztreonam and cefepime, each containing 30 μg of the drug, placed 20 mm (center to center) away from a disk containing amoxicillin-clavulanic acid (20 μg/10 μg) in the center of the plate. MICs were determined by the CLSI agar dilution method [13]. MICs of cefotaxime and ceftazidime were determined alone or in combination with a fixed concentration of clavulanic acid (4 μg/mL).

3. Mating-out assays

Conjugation experiments were carried out between donors and the azide-resistant recipient strain *E. coli* J53 on Mueller-Hinton agar plates. Transconjugants were selected on Mueller-Hinton agar plates supplemented with ceftazidime or cefotaxime (2 μg/mL, respectively) and sodium azide (100 μg/mL).

4. Characterization of antimicrobial resistance genes

Searches for genes coding for plasmid-mediated ESBLs and Qnrs were performed by PCR amplification with the primers listed in Table 1 as described previously [4, 14]. The templates for PCR amplification from the clinical isolates and the transconjugants were a plasmid preparation. The PCR products were then subjected to direct sequencing. Both strands of all PCR products were sequenced twice with an

Table 1. Sequences of the PCR primers

Name	Nucleotide Sequence	Product size (bp)	GenBank Accession No.
TEM F	5′-ctt gaa gac gaa agg gcc tc-3′	997	M36543
TEM R	5′-tga ctc ccc gtc gtg tag at-3′		
SHV F	5′-cgc cgg att ctt att tg-3′	1,071	X98100
SHV R	5′-cca cgt tta tgg cgt tac ct-3′		
CTX-M-1F	5′-gga cgt aca gca aaa act tgc-3′	891	X92506
CTX-M-1R	5′-cgg ttc gct ttc act ttt ctt-3′		
CTX-M-2F	5′-cgg tgc tta aac aga gcg ag-3′	624	X92507
CTX-M-2R	5′-cca tga ata agc agc tga ttg ccc-3′		
CTX-M-8F	5′-acg ctc aac acc gcg atc-3′	490	AF189721
CTX-M-8R	5′-cgt ggg ttc tcg ggg ata a-3′		
CTX-M-9F	5′-gat tga ccg tat tgg gag ttt-3′	947	AJ416345
CTX-M-9R	5′-cgg ctg ggt aaa ata ggt ca-3′		
PER-1 F	5′-gtt aat ttg ggc tta ggg cag-3′	855	Z21957
PER-1 R	5′-cag cgc aat ccc cac tgt-3′		
VEB F	5′-acc aga tag gag tac aga cat atg a-3′	727	AF220758
VEB R	5′-ttc atc acc gcg ata aag cac-3′		
GES F	5′-gtt aga cgg gcg tac aaa gat aat-3′	903	AY260546
GES R	5′-tgt ccg tgc tca gga tga gt-3′		
TLA F	5′-cgc gaa aat tct gaa atg ac-3′	992	AF148067
TLA R	5′-agg aaa ttg tac cga gac cct-3′		
qnrA-F	5′-tca gca aga gga ttt ctc acg -3′	606	DQ989302
qnrA-R	5′-ggt tcc agc agt tgc tcc t-3′		
qnrB1-F	5′-acc tga gcg gca ctg aat tta t-3′	424	DQ351241
qnrB1-R	5′-tcg caa tgt gtg aag ttt gc-3′		
qnrB4-F	5′-gat gac tct ggc gtt agt tgg-3′	641	DQ303921
qnrB4-R	5′-cca tga cag cga tac caa ga-3′		
qnrS-F	5′-gac gtg cta act tgc gtg at-3′	380	DQ449578
qnrS-R	5′-act taa gtc tga ctc ttt cag tga tgc-3′		

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

RESULTS

A total of 140 ceftazidime- and/or cefotaxime-nonsusceptible *E. coli* isolates were recovered from specimens of urine (N=60, 42.9%), sputum (N=29, 20.7%), wound (N=16, 11.4%), body fluids (N=9, 6.4%), blood (N=9, 6.4%) and others (N=17, 12.1%). Among these isolates, 118 (84.3%) showed positive results in DDST, and ESBL genes were detected by PCR in 112 (80.0%) isolates. Transfer of the ESBL genes to the azide-resistant *E. coli* J53 by conjugation was successful in 59/118 (50%) isolates.

PCR experiments detected the bla_{CTX-M} genes in 100/118 (84.7%) of *E. coli* isolates with an ESBL phenotype (Table 2). The most common types of CTX-M identified in *E. coli* were CTX-M-15 (N=58) and CTX-M-14 (N=41). Genes encoding CTX-M-3 (N=7), CTX-M-9 (N=8), CTX-M-12 (N=1), and CTX-M-57 (N=1) were also detected. The bla_{SHV-12} and the bla_{SHV-2a} genes were detected in 17 and 2 isolates, respectively, and the bla_{TEM-52} gene was detected in only 4 isolates. Multiple ESBL genes were identified in 13 *E. coli* isolates. Genes encoding PER, VEB, GES, TLA, and CTX-M-2, and CTX-M-8 cluster ESBLs were not detected in this study. The

Table 2. Characteristics of Ambler class A ESBL and *qnr*-producing *E. coli* isolates

Type of Ambler class A ESBLs (N)	Type of *qnr* genes (N)	MIC range (MIC_{50}) (μg/mL)					
		FOX	CAZ	CAZ-CLA	CTX	CTX-CLA	CIP
TEM-52 (4)		2-4	16-32	0.25-1	16-32	0.06-0.25	4->256
SHV-2a+CTX-M-14+CTX-M-15 (1)	*qnrA1* (1)	8	>256	32	>256	128	>256
SHV-2a+CTX-M-15 (1)		4	64	1	256	1	>256
SHV-12 (8)	*qnrB4* (1)	2-128(8)	32-128 (64)	0.5-32 (2)	2-16 (8)	0.06-8 (0.12)	4->256 (>256)
SHV-12+CTX-M-9 (1)	*qnrB4* (1)	32	16	0.5	8	0.25	>256
SHV-12+CTX-M-14 (3)	*qnrA1* (1)	4-256	16-128	0.5-128	16-64	4-128	64->256
SHV-12+CTX-M-15 (5)	*qnrA1* (1)	2-128	64->256	0.5-32	256->256	0.25-32	>256
CTX-M-3 (6)	*qnrB4* (1)	2-64	4-32	0.5-32	128-256	1-128	>256
CTX-M-9 (2)	*qnrB4* (2)	64->256	16-64	8-64	32->256	32-256	4->256
CTX-M-12 (1)		4	1	0.5	32	2	4
CTX-M-14 (31)	*qnrA1* (1) *qnrB4* (1)	1->256 (16)	0.5-64 (4)	0.12-64 (0.5)	4-256 (32)	0.25-128 (2)	2->256 (>256)
CTX-M-14+CTX-M-15 (1)		16	128	4	>256	8	>256
CTX-M-15 (47)		2-32 (8)	16-256 (64)	0.25-128 (2)	128->256 (>256)	0.25-256 (2)	4->256 (>256)
CTX-M-57 (1)		8	32	0.5	256	1	>256

Abbreviations: FOX, cefoxitin; CAZ, ceftazidime; CLA, clavulanic acid; CTX, cefotaxime; CIP, ciprofloxacin.

qnrA1 and *qnrB4* genes were detected in 4 and 7 ESBL-producing isolates, respectively, but *qnrB1* and *qnrS* cluster genes were not detected (Table 2).

In the isolates harboring CTX-M-3, CTX-M-9, CTX-M-12, and CTX-M-14, MICs of cefotaxime were more than eight-fold higher compared to those of ceftazidime. The isolates producing CTX-M-15 and CTX-M-57 exhibited a high level of resistance to ceftazidime. All isolates with an ESBL phenotype except one (MICs, 2 μg/mL) exhibited resistance to ciprofloxacin. MIC_{50} of ciprofloxacin for these isolates were >256 μg/mL. Six isolates harboring Qnrs (4 QnrB4 and 2 QnrA1) exhibited a high level resistance (MICs >256 μg/mL) to ciprofloxacin.

DISCUSSION

DDST showed positive results in 84.3% (118/140) of *E. coli* isolates that were non-susceptible to cefotaxime and/or ceftazidime, and ESBL genes were detected in 112 of the 118 DDST-positive isolates. These results imply that most of the *E. coli* strains acquired oxyimino-cephalosporin resistance through ESBL production. Six isolates that did not harbor ESBL genes might have other resistance mechanisms, which were not identified in this study.

In the present study, the most common type of ESBL in *E. coli* isolates was CTX-M-15 and some of the isolates harboring CTX-M-15 also produced SHV or other CTX-M type ESBLs. Although CTX-M ESBLs are generally known to be inactive against ceftazidime, CTX-M-15 has an expanded activity against ceftazidime, similar to classical ESBLs including TEM-52 and SHV-12. However, TEM-52 and SHV-12, which had previously been regarded as the most common types of ESBL in Korea, were detected in only 4 and 17 isolates, respectively. Thus, it appears CTX-M-15 may be replacing the classical ceftazidimases, SHV-12 and TEM-52, in *E. coli* isolates from Korea [15, 16]. Dissemination of strains harboring CTX-M-15 would make antimicrobial therapies more difficult because of the strong hydrolytic activity against both cefotaxime and ceftazidime.

CTX-M-15 has expanded its hydrolytic activity against ceftazidime by a single amino-acid substitution (Asp240Gly) in CTX-M-3 [17]. The MIC_{50} of ceftazidime for the strains producing CTX-M-15 ESBL was 64 μg/mL, which was remarkably higher than that for strains producing other CTX-M ESBLs. The plasmid-borne $bla_{CTX-M-15}$ gene is known to be associated with the IS*Ecp1* or IS*Ecp1*-like insertion sequences [17]. Further investigations into the genetic environment and transfer mechanism of ESBL genes including the $bla_{CTX-M-15}$ gene may provide clues about the dissemination mechanism of CTX-M-15 ESBL.

CTX-M-14, a typical cefotaximase, was the second most common ESBL found in this study. It has a single amino acid substitution (Ala231Val) in CTX-M-9, being much more active against cefotaxime than ceftazidime. This enzyme was first identified in *K. pneumoniae*, *E. coli* and *Shigella sonnei* isolates from Korea in 2001 [18]. In this study, the MIC_{50} of cefotaxime for the isolates harboring only CTX-M-14 was 32 μg/mL, which was higher than that of ceftazidime (4 μg/mL).

CTX-M-57 ESBL was identified in an *E. coli* isolate recovered from a blood specimen of a patient admitted to the nephrology department of a university hospital in Busan, Korea. CTX-M-57, which has an alanine to valine substitution in the 80th amino acid of CTX-M-15, was first detected in *Salmonella enterica* serovar Typhimurium isolated from feces of an inpatient admitted to a hospital during a visit to Thailand in 2006 [19]. CTX-M-57 has never been identified in Korea before. MICs of ceftazidime and cefotaxime for the CTX-M-57 producing strains were 32 μg/mL and 256 μg/mL, respectively.

It was noteworthy that CTX-M-12 was identified in an *E. coli* isolate. Outbreak of a *K. pneumoniae* strain producing this enzyme was first reported in Kenya in 2000, and *K. pneumoniae* producing this enzyme was then identified in Colombia [20, 21]. In Korea, three *E. coli* and one *K. pneumoniae* isolates producing CTX-M-12 ESBL were first identified in a nationwide survey on antimicrobial resistance in 2004, and CTX-M-12 producers were isolated again in 2005, indicating that CTX-M-12 enzyme had already spread in Korea [15, 16].

The plasmid-mediated resistance to nalidixic acid was first detected from a *Shigella dysenteriae* strain, which

caused an epidemic of shigellosis in southern Bangladesh in 1987 [22]. In 1998, the quinolone resistance from a transferable plasmid was identified in a *K. pneumoniae* isolate from the United States [10], but the clinical significance of *qnr* genes remained unknown until 2003, when the prevalence of plasmids containing *qnr* genes was revealed to be 7.7% among 78 ciprofloxacin-resistant clinical isolates of *E. coli* from Shanghai hospitals in China [11]. Since then, *qnr* genes have been identified from various gram-negative bacilli in many parts of the world. In Korea, *qnr* genes were first detected from 2 (0.8%) of 260 *E. coli* isolates collected during the period of 2001 to 2003 [23]. But, the *qnr* genes were not detected in another survey performed in 2005 to characterize the mechanisms of acquiring quinolone-resistance in *Salmonella enterica* [24]. However, a recent study reported that the prevalence of *qnr* genes, including the *qnr*A1, *qnrB1*, *qnrB2*, *qnrB4*, *qnrB6*, and *qnrS1*, was high in *Citrobacter freundii* (53/138, 38.4%) and *Enterobacter cloacae* (53/186, 28.5%) isolates, but low in *Enterobacter aerogenes* (5/154, 3.2%) and *Serratia marcescens* (4/166, 2.4%) isolates [25]. And another study detected the *qnrB2* and *qnrB4* genes in 3 (5.1%) and 29 (49.2%), respectively, of 59 *K. pneumoniae* isolates, and the *qnrB4* gene in 8/143 (5.6%) *E. coli* isolates [12].

Most of our *E. coli* isolates harboring ESBLs exhibited a high level of resistance to fluoroquinolones, while the *qnrA1* and the *qnrB4* genes were identified in only 4 and 7 isolates, respectively. These results indicate that the accumulation of mutations in chromosomal DNA gyrase and topoisomerase IV genes is still the main mechanism of acquiring fluoroquinolone-resistance in *E. coli*.

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