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Molecular Characteristics of Extended Spectrum β-Lactamases in *Escherichia coli* and *Klebsiella pneumoniae* and the Prevalence of *qnr* in Extended Spectrum β-Lactamase Isolates in a Tertiary Care Hospital in Korea

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Purpose: Extended spectrum β-lactamases (ESBLs) are cephalosporinases that confer resistance to a wide variety of oxyimino cephalosporins and create serious therapeutic problems. In addition, the quinolone resistance *qnr* genes are becoming increasingly prevalent in clinical isolates, some of which also produce ESBL. This study was designed to evaluate the occurrence and genotypic distribution of ESBL producing *Escherichia coli (E. coli)* and *Klebsiella pneumoniae* (*K. pneumoniae)* as well as the prevalence and distribution of *qnr* genes in ESBL-producing isolates in a tertiary care hospital in Korea. **Materials and Methods:** We tested a total of 111 ESBL-producing isolates of *E. coli* and *K. pneumoniae*, which were collected at Kyung Hee Medical Center from November 2006 to June 2008. ESBL production was determined by the Clinical and Laboratory Standards Institute (CLSI) ESBL confirmatory test. The cefotaxime and ceftazidime resistance of the ESBL-producers were transferred to azide-resistant *E. coli* J53 by conjugation. The presence and identity of ESBL and *qnr* genes were determined by polymerase chain reaction (PCR) and nucleotide sequencing. **Results:** The prevalence of ESBLs was 17.7% (297/1,680) of *E. coli* and 26.5% (240/904) of *K. pneumoniae* in our hospital during the study periods. Of the 111 collected isolates, 69 isolates were *E. coli* and 42 isolates were *K. pneumoniae*. The most prevalent ESBL genotype was CTX-M15. Among the ESBL-producing isolates, 4 *E. coli* (5.8%) and 17 *K. pneumoniae* (40.5%) contained *qnr* genes. *qnr*B4 was the most frequent type in both *E. coli* and *K. pneumoniae*. **Conclusion:** CTX-M15 was the most frequently encountered ESBL. In addition, a high prevalence of *qnr* genes among ESBL-producing *K. pneumoniae* was identified in this study.

Key Words: *E. coli, K. pneumoniae, Antimicrobial resistance,* ESBL, *qnr*

INTRODUCTION

Escherichia coli (E. coli) and *Klebsiella pneumoniae* (*K. pneumoniae)* are classified in the family *Enterobacteriaceae*. Species in these two genera are gram negative rods and frequently cause nosocomial and community-associated infections such as urinary tract infections, wound infections, and bacteremia. As narrow and

extended-spectrum cephalosphorins are frequently used for treatment of infections by these species, resistance rates to these antimicrobial agents have also been increasing.[1] Extended-spectrum β-lactamase (ESBL) producers are resistant to penicillins, narrow- and extended-spectrum cephalosporins, and aztreonam. These organisms are also frequently resistant to aminoglycosides, trimethoprim-sulfamethoxasole (TMP-SMZ), and quinolones. Therefore, the choice of antimicrobial agent to properly treat ESBL producers requires accurate identification. The ESBLs were first reported in 1983 and the prevalence and genotypes of ESBL-producing isolates have been reported in Korea since then.[2-7]

The first plasmid-mediated quinolone resistance-conferring gene (*qnr*) was reported in a *K. pneumoniae* clinical isolate from the USA in 1998.[8] The *qnr* gene protects DNA gyrase against the effect of the quinolone.[9] Recently, many studies have reported that the *qnr* gene is frequently encountered in ESBL-producing isolates.[10-15]

The distribution of ESBL genotypes has been found to vary according to the antimicrobial agents used in each hospital or local community.[16,17] However, the distribution of genotypes of ESBL has not yet been reported in this hospital. Therefore, this study was designed to assess the prevalence and genotypes of ESBL-producing clinical isolates of *E. coli* and *K. pneumoniae* in Kyung Hee Medical Center as well as to evaluate the prevalence and genotypes of *qnr* genes in ESBL-producing isolates.

MATERIALS AND METHODS

Bacterial strains

Nonduplicate ESBL-producing clinical isolates of *E. coli* and *K. pneumoniae* were randomly collected at Kyung Hee Medical Center from November 2006 to June 2008. All isolates were collected from inpatients. Conventional biochemical tests and MicroScan WalkAway 96 (Dade Behring, Sacramento, CA, USA) were used for bacterial identification.

For optimization of the PCR technique, well-characterized ESBL and *qnr*-positive strains were used as positive controls: *K. pneumoniae* SHV-12+,[12] *Shigella sonnei (S. sonnei)* TEM-19+ (Korea Centers for Disease Control and Prevention, unpublished results), *K. pneumoniae* CTX-M3+,[12] and *S. sonnei* CTX-M14+ (Korea Centers for Disease Control and Prevention, unpublished results) ; *Enterobacter cloacae (E. cloacae) qnr*A+, *Citrobacter freundii qnr*B+, and *E. cloacae qnr*S+.[13]

ESBL confirmatory test

The phenotypic confirmatory test for ESBL production was performed by the disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[18]

Antimicrobial susceptibility test

Antimicrobial susceptibilities were determined by agar dilution methods according to the recommendations of the CLSI.[18] The tested antibiotics were amikacin (Boryung, Seoul, Korea), cefoxitin (Shinpoong, Seoul, Korea), cefepime (Boryung, Seoul, Korea), ceftazidime (LG Life Sciences, Seoul, Korea), cefotaxime (Handok, Seoul, Korea), aztreonam (Bristol-Myers Squibb, Princeton, NJ, USA), and ciprofloxacin (Bayer Vital GmbH, Berlin, Germany).

β-lactam resistance transfer assays

Conjugation experiments were carried out using the azide-resistant recipient strain *E. coli* J53 according to the method of Jacoby and Han.[19] Each clinical and recipient strain was inoculated into brain heart infusion broth and incubated for 3 hours. 0.2 mL of donor culture solutions and 2.2 mL of recipient culture solutions were mixed in a tube and incubated at 37˚C for 1 hour. These mixed cultures were then inoculated on MacConkey agar supplemented with ceftazidime or cefotaxime (8 μg/mL, respectively) and 100 μg/mL of sodium azide (Sigma chemical Co., St. Louis, MO, USA). Transconjugants were selected after overnight incubation at 35˚C in ambient air. To confirm transference of resistance, transconjugants were subjected to the ESBL confirmation test and polymerase chain reaction (PCR) with primers listed in Table 1.

Molecular analysis

The plasmid deoxyribonucleic acid (DNA) was prepared using the Plasmid Purification Kit (SolGent Co., Daejeon, Korea) according to the manufacturer's recommendation. PCR amplification of the genes of ESBL and *qnr* genes were performed with primers listed in Table 1, as described previously.[10,17,20] PCR products were analyzed by electrophoresis in a 2% agarose gel containing 0.5 μg/mL ethidium bromide at 120V for 40 min. The PCR products were purified with Agencourt Ampure (Beckman Coulter, Brea, CA, USA) and Biomek FX (Beckman Coulter, Brea, CA, USA) and sequenced on a 3730xl DNA analyzer (PE Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were analyzed with software available from the National Center for Biotechnology Information BLAST service (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Study sample

A total of 111 isolates, including 69 *E. coli* isolates and 42

K. pneumoniae isolates, were collected from the Kyung Hee Medical Center for analysis. These isolates were obtained from urine (64 isolates), sputum (27 isolates), blood (8 isolates), and other patient samples (12 isolates).

During the study period, the overall incidence of ESBL-producing isolates identified in the Kyung Hee Medical Center was 17.7% (297/1,680) of *E. coli* and 26.5% (240/904) of *K. pneumoniae* cases.

Susceptibility testing

The resistance rates of *E. coli* and *K. pneumoniae* for amikacin, cefoxitin, cefepime, ceftazidime, cefotaxime, aztreonam, and ciprofloxacin are shown Table 2. Isolates containing *qnr* genes were highly resistant to ciprofloxacin (MIC ≥ 16 μg/mL) except for two *K. pneumoniae* isolates that were intermediately resistant (MIC 2 μg/mL). These isolates encoded *qnr*B4 and *qnr*S1, respectively.

Table 1. Sequences of the Primers Used in This Study

Primers	Target	Primer sequence (5'-3')	Product size (bp)	Position*	Reference
SHV	bla_{SHV}	GGTTATGCGTTATATTAATC	867	121 - 140	38
		TTAGCGTTGCCAGTGCTC		971 - 988	
TEM	bla_{TEM}	ATAAAATTCTTGAAGACGAAA	1080	1 - 21	38
		GACAGTTACCAATGCTTAATC		1061 - 1081	
CTX-M1	bla_{CTX-M} (CTX-M1 group)	AGTTCACGCTGATGGCGACG	676	85 - 104	17
		AACCCAGGAAGCAGGCAGTCC		741 - 761	
CTX-M9	bla_{CTX-M} (CTX-M9 group)	GATTGACCGTATTGGGAGTTT	947	108 - 128	6
		CGGCTGGGTAAAATAGGTCA		919 - 938	
*Qnr*A	*qnr*A1- *qnr*A6	AGAGGATTTCTCACGCCAGG	580	30 - 49	10
		TGCCAGGCACAGATCTTGAC		589 - 608	
*Qnr*B	*qnr*B1- *qnr*B6	GGMATHGAAATTCGCCACTG†	264	283 - 302	10
		TTTGCYGYYCGCCAGTCGAA†		526 - 545	
*Qnr*S	*qnr*S1- *qnr*S2	GCAAGTTCATTGAACAGGGT	428	137 - 156	10
		TCTAAACCGTCGAGTTCGGCG		543 - 563	

*nucleotide numbering begins at the initiation codon.
†M = A or C; H = A or C or T; Y = C or T.

Table 2. Susceptibility of ESBL-Producing *E. coli* and *K. pneumoniae* Isolates to 7 Antimicrobial Agents

Isolates (No)	MIC (μg/mL)			%		
	50	90	Range	S	I	R
E. coli (69)						
Amikacin	16	32	≤ 2 to ≥ 64	87.1	8.6	4.3
Cefoxitin	8	8	1 to ≥ 64	94.3	1.4	4.3
Cefepime	≥ 64	≥ 64	≤ 1 to ≥ 64	38.6	10.0	51.4
Ceftazidime	≥ 64	≥ 64	≤ 1 to ≥ 64	21.4	8.6	70.0
Cefotaxime	≥ 64	≥ 64	≤ 1 to ≥ 64	2.9	7.1	90.0
Aztreonam	≥ 64	≥ 64	≤ 1 to ≥ 64	2.8	8.6	88.6
Ciprofloxacin	≥ 16	≥ 16	≤ 0.25 to ≥ 16	11.4	1.4	87.2
K. pneumoniae (42)						
Amikacin	16	≥ 64	≤ 2 to ≥ 64	59.5	7.1	33.3
Cefoxitin	≥ 64	≥ 64	2 to ≥ 64	45.2	9.6	45.2
Cefepime	32	≥ 64	4 to ≥ 64	42.9	0.0	57.1
Ceftazidime	≥ 64	≥ 64	≥ 64	0.0	9.5	90.5
Cefotaxime	≥ 64	≥ 64	8 to ≥ 64	7.1	16.7	76.2
Aztreonam	≥ 64	≥ 64	≥ 64	0.0	2.4	97.6
Ciprofloxacin	≥ 16	≥ 16	2 to ≥ 16	14.3	4.8	80.9

ESBL, extended-spectrum β-lactamase; *E. coli, Escherichia coli*; *K. pneumoniae, Klebsiella pneumoniae*; MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant.

β-lactam resistance transfer assays

Transconjugants were obtained for 20 strains (29.0%) of *E. coli* and 24 strains (57.1%) of *K. pneumoniae* using *E. coli* J53 Az^r as the recipient strain, and tested positive in an ESBL confirmatory test and PCR test for *bla*$_{SHV}$ or *bla*$_{CTX-M}$. One transconjugant displayed different results compared with the original isolate, which contained SHV5 and CTX-M15 while the transconjugant contained only CTX-M.

Table 3. ESBL Genotypes of *E. coli* and *K. pneumoniae* Isolates

Isolates	Type of β-lactamase	No. of isolates
E. coli		
	CTX-M15	23
	CTX-M14	13
	CTX-M15 + TEM-1	12
	CTX-M14 + TEM-1	5
	CTX-M14 + CTX-M15	3
	CTX-M14 + CTX-M15 + TEM-1	3
	CTX-M14 + SHV-12	1
	CTX-M15 + SHV-5 + TEM-1	1
	CTX-M15 + SHV-5	1
	CTX-M15 + SHV-12	1
	SHV-12	4
	SHV-12 + TEM-1	1
	SHV-5	1
K. pneumoniae		
	CTX-M15	14
	CTX-M15 + TEM-1	2
	CTX-M57	6
	CTX-M14	1
	CTX-M14 + TEM-1	1
	CTX-M14 + CTX-M15 + TEM-1	1
	CTX-M14 + SHV-12	1
	CTX-M14 + SHV-2a	1
	SHV-12	11
	SHV-5	4

ESBL, extended-spectrum β-lactamase; *E. coli, Escherichia coli*; *K. pneumoniae, Klebsiella pneumoniae*.

Molecular analysis

The distribution of the genotypes of ESBLs in this study is reported in Table 3. The most common types of ESBLs identified were CTX-M15 (n = 44, 63.8%) and CTX-M14 (n = 25, 36.2%) among *E. coli* isolates and CTX-M15 (n = 17, 40.5%) and SHV-12 (n = 12, 28.6%) among *K. pneumoniae* isolates. Genes encoding CTX-M57 (n = 6, 14.3%) were detected in *K. pneumoniae* isolates. Genes encoding TEM-type β-lactamases were detected in 22 of 69 *E. coli* isolates and 4 of 42 *K. pneumoniae* isolates, but all of them were the broad-spectrum TEM-1.

Among the ESBL producers, 5.8% (4/69) of *E. coli* and 40.5% (17/42) of *K. pneumoniae* contained *qnr* genes. Among the known *qnr* subtypes, only *qnr*B4 (n = 18, 85.7%) and *qnr*S1 (n = 3, 14.3%) were detected. The distribution of ESBL types in *qnr* containing isolates is shown in Table 4.

DISCUSSION

Although β-lactam antimicrobial agents are frequently used for the treatment of *E. coli* and *K. pneumoniae* infections, ESBL-producing isolates are becoming increasingly common. ESBL-producing isolates are of particular concern because these traits can be horizontally-transmitted to other isolates by plasmids and often cause nosocomial infections.[21] The prevalence and genotype of ESBLs from clinical isolates vary according to the country and even

Table 4. Distribution of ESBL Genotypes of *qnr* Gene Positive Isolates

Isolates	*qnr* gene	Type of β-lactamase	No. of isolates
E. coli	*qnr*B4	CTX-M15	1
		CTX-M14 + CTX-M15 + TEM-1	1
		SHV-12	1
		SHV-5	1
K. pneumoniae	*qnr*B4	CTX-M15	3
		CTX-M14 + SHV-12	1
		CTX-M14 + SHV-2a	1
		SHV-12	7
		SHV-5	2
	*qnr*S1	CTX-M15	1
		CTX-M14	1
		SHV-12	1

ESBL, extended-spectrum β-lactamase; *E. coli, Escherichia coli*; *K. pneumoniae, Klebsiella pneumoniae*.

hospital at which they are isolated from. For example, 3% (0-25%) of *Enterobacteriaceae* isolates from the USA were reported to be ESBL-producing strains[22,23] while 0.1% of *E. coli* and 0.3% of *K. pneumoniae* isolates in Japan were ESBLs.[24] Additionally, there have been several reports regarding the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in Korea, and previous reports presented that approximately 10 % of *E. coli* and 25-30% of *K. pneumoniae* isolates produced ESBLs.[5,6] ESBL-producing *E. coli* and *K. pneumoniae* isolates from Kyung Hee Medical Center were investigated, and among the 1,680 *E. coli* and 904 *K. pneumoniae* cases, a total of 297 *E. coli* isolates (17.7%) and 240 *K. pneumoniae* isolates (26.5%) were found to be ESBL-producing during the study periods. These results are similar to the ESBL incidence previously reported within Korea, although a slight increase in prevalence of ESBL-producing *E. coli* was detected.

Regarding the prevalence of the genotype of ESBLs in Korea, Pai[25] reported that TEM-12 ESBL-producing *E. coli* and SHV-12 producing *K. pneumoniae* were the most common genotypes in the late 1990s. More recently, Hong, et al.[26] reported that TEM-52 was the most prevalent in both *E. coli* and *K. pneumoniae* in 2001. In contrast, Kim, et al.,[27] Rhoo, et al.,[28] and Kang, et al.[4] reported that CTX-M was most frequently produced in *E. coli* in 2005. Similarly, CTX-M14- and CTX-M15- producing *E. coli* were the most frequently encountered genotypes in this study. Surprisingly, TEM-52, the most frequently encountered genotype from previous reports, was not detected in this study. In the case of *K. pneumoniae*, SHV-12 accounted for 69% (27/39) of ESBLs in 1998[29] and 86% (31/36) of ESBLs in 2006[28] in Korea. However, in this study, CTX-M15 was the most prevalent (40.5%, 17/42), followed by SHV-12 (28.6%, 12/42). CTX-M3 or 15 types in *E. coli* and SHV-12 type in *K. pneumoniae* were predominant in most parts of Korea, but CTX-M3 and CTX-M14 types were most common in *E. coli* and *K. pneumoniae*, respectively, in a hospital in Gyeonggi in 2005.[4] Our results were of a similar distribution to other hospitals in the different locations in Korea.

Interestingly, six isolates of *K. pneumoniae* expressed CTX-M57. CTX-M57 was first identified from *Salmonella enterica* in 2008.[30] CTX-M57 shares 99% amino acid identity with CTX-M15 and differs from CTX-M15 by an A80V substitution. Though CTX-M occurred in 1.7% (9/520) of ESBLs in 2002,[2] its prevalence within ESBLs increased to 4.3% (25/585) in 2005.[28] This increase in prevalence of CTX-M is likely because gene acquisition of CTX-M or SHV is more effective than TEM in *Enterobacteriaceae*.[17] In order to test for transference of β-lactam resistance, transconjugants were obtained from 29.0% (20/69) of *E. coli* and 57.1% (24/42) of *K. pneumoniae* tested using *E. coli* J53 Az^r as the recipients strain, and represented positive results for the ESBL confirmatory test and tested positive in SHV or CTX-M by PCR. However, one transconjugant displayed different results compared with the original strain, which contained SHV5 and CTX-M15 while the transconjugant contained only CTX-M. This result suggests that SHV and CTX-M were not present on the same plasmid in the original isolate. There are several reports of isolates which contain both SHV and CTX-M, and these isolates express high resistance to not only ceftazidime but also ceftaxime. Therefore, a further investigation into whether SHV and CTX-M were present on same plasmid is required.

Quinolone resistance of *Enterobactericeae* is usually caused by various chromosomal mutations that alter the target enzymes, such as DNA gyrase, type IV topoisomerase, or active efflux systems. However, since the first plasmid-mediated quinolone resistance-conferring gene (*qnr*) was discovered in a *K. pneumoniae* isolate from USA, this gene has been reported worldwide,[15,31,32] and is frequently encountered in ESBL-producing isolates.[10,12,14] Recently, there have been some reports regarding the distribution of the *qnr* gene in Korea. Kim, et al.[12] identified 44 isolates of ESBL-producing *K. pneumoniae* that tested positive for the *qnr*B gene, and 2 of these also tested positive for the *qnr*S gene. Shin, et al.[33] reported that 5.6% (8/143) of *E. coli* and 55.9% (33/59) of *K. pneumoniae* isolates contained the *qnr*B gene among ciprofloxacin-resistant isolates. In this study, 5.8% (4/69) of *E. coli* and 33.3% (14/42) of *K. pneumoniae* had the *qnr*B4 gene and 7.1% (3/42) of *K. pneumoniae* had the *qnr*S1 gene. These results confirm previous reports regarding the high prevalence of the *qnr* gene, especially *qnr*B4, in *K. pneumoniae*. Interestingly, only one study[13] has previously reported the identification of *qnr*S1 in Korea, which was found in a non-ESBL *E. cloacae* isolate. However, in this study, three isolates of *K. pneumoniae* contained the *qnr*S1. This could be an indication that the *qnr* gene has spread to several *Enterobacteriaceae* species. Two isolates of *qnr*-containing strains expressed intermediate susceptibility to ciprofloxacin. Previous analyses have reported that despite the cases that reported susceptibility to quinolone in the MIC test according to the CLSI guidelines, some isolates contained the *qnr* gene.[33-36] The *qnr*-positive strain have been associated with only low-level resistance to fluoroquinolones in transconjugants,[33] and most donor strains showed high levels of quinolone resistance with additional chromosomal mutations.[9] Therefore, the presence of *qnr* may be contributed to the intensity and facilitation of quinolone resistance.[9,33] In particular, because fluoroquinolones were widely used in Korea,[37] the emergence and spread of *qnr* genes would contribute to the rapid development and spread

of fluoroquinolone resistance.[33] Therefore, we considered that early detection of *qnr* gene was very important for the prevention of the development and spread of this resistance.

In summary, CTX-M15 was the most frequently encountered in *E. coli* and *K. pneumoniae* isolated in the Kyung Hee Medical Center. In addition, new CTX-M57 was frequently detected in *K. pneumoniae*. Finally, a high prevalence of *qnr* genes on ESBL-producing strains was detected. β-lactam agents and quinolones should therefore be used cautiously in these species, and continuously monitored for resistance patterns.

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