J. Microbiol. Biotechnol. (2006), **16**(6), 889–895

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of Extended Spectrum β-Lactamase Genotype TEM, SHV, and CTX-M Producing *Klebsiella pneumoniae* Isolated from Clinical Specimens in Korea

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Received: October 15, 2005
Accepted: January 12, 2006

Abstract To investigate the antibiotic-resistant patterns and the gene types of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*, we collected 226 *Klebsiella pneumoniae* strains from three general hospitals with more than 500 beds in Busan, Korea from September 2004 to October 2005. The minimum inhibitory concentration (MIC) of antibiotics was measured using the Gram-negative susceptibility (GNS) cards of Vitek (Vitek system, Hazelwood Inc., MO, U.S.A.). Of the 226 *K. pneumoniae* isolates, 65 ESBL-producing *K. pneumoniae* strains were detected by the Vitek system and confirmed by the double-disk synergy test. TEM (Temoniera) type, SHV (sulfhydryl variable) type, and CTX-M (cefotaxime) type genes were detected by polymerase chain reaction. All 65 *K. pneumoniae* strains were resistant to ampicillin, cefazolin, cefepime, ceftriaxone, and aztreonam, and 83.0% of the organisms were resistant to ampicillin/sulbactam, 66.1% to tobramycin, 67.6% to piperacillin/tazobactam, 61.5% to ciprofloxacin, and 47.6% to trimethoprim/sulfamethoxazole, and 43.0% to gentamicin. TEM-type ESBLs (TEM-1 type, -52 type) were found in 64.6% (42 of 65) of the isolates, SHV-type ESBLs (SHV-2a type, -12 type, -28 type) in 70.7% (46 of 65) of isolates, and CTX-M-type ESBLs (CTX-M-15 type) in 45% (29 of 65) of isolates. Of the 65 ESBL-producing *K. pneumoniae* strains, two strains were found to harbor *bla*SHV-28, which were detected in Korea for the first time. Therefore, more investigation and research on SHV-28 are needed in order to prevent the ESBL type-producing *K. pneumoniae* from spreading resistance to oxyimino cephalosporin antibiotics.

Key words: ESBL, *bla*TEM, *bla*SHV, *bla*CTX-M, *Klebsiella pneumoniae*, antibiotic susceptibility

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Klebsiella pneumoniae is widespread throughout the environment, inhabiting the oral cavity, respiratory tract, and intestinal tract [1]. ESBL (extended spectrum β-lactamase)-producing *Klebsiella pneumoniae* has recently been isolated from clinical specimens and it has become one of the most prevalent pathogens that cause nosocomial infection [18]. ESBLs are enzymes responsible for many failures of antimicrobial therapy, because they hydrolyze β-lactam antibiotics to become inert and ineffective [10, 20]. ESBL-producing *K. pneumoniae* infection has been shown to be associated with significantly longer hospital stay and higher hospital charges, indicating that these infections have an important impact on clinical outcomes [8].

Bacterial strains that produce new β-lactamase have begun to be isolated. The most successful plasmid-encoded β-lactamase in terms of clinical significance are members of the TEM and SHV families.

The wide geographic spread of ESBL variants has been a known epidemiological phenomenon since the second half of the 1980s [10]. After the sequence was known, a TEM number was then assigned. Many genera of Gram-negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase. The first plasmid, TEM-1, which mediates β-lactamase in Gram-negative, bacteria was described in the early 1960s [8]. The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, and hence the designation TEM [8]. Another common plasmid-mediated β-lactamase found in *K. pneumoniae* and *E. coli* is SHV-1 (sulfhydryl variable). The SHV-1 β-lactamase is chromosomally encoded in the majority of isolates of *K. pneumoniae*, but is usually plasmid mediated in *E. coli* [8].

The use of TEM numbers is now preferred, because the assignment of phenotypic names can be subjective [11, 12]. Most commonly, ESBLs are classified by genes for

narrower spectrum TEM-1, TEM-2, or SHV-1 β-lactamases by mutations that alter the amino acid configuration around the active site of these enzymes [15]. More than 100 genetically distinct TEM-type and SHV-type ESBLs have now been characterized [16]. CTX-M β-lactamases have been frequently recognized and described in literature [7, 14]. A new family of plasmid-mediated ESBLs, which is called CTX-M and preferentially hydrolyzes cefotaxime, has recently arisen. They have mainly been found in strains of *Salmonella enterica* serovar *typhimurium* and *E. coli*, but have also been observed in other species of *Enterobacteriaceae* [8].

This resistance mechanism appears to be widespread throughout the world, evidenced by reports of clinical isolates producing these β-lactamases from Europe, Africa, Asia, South America, and most recently North America [14]. TEM and SHV type ESBLs are most often found in *Klebsiella pneumoniae* and *Escherichia coli* [19]. The proportion of ESBL producers among hospital isolates varies, depending on geographical areas [2, 3]. In the United States, ESBLs are produced by more than 25% of intensive care unit *K. pneumoniae* isolates, and they are much more common in some parts of Europe, Asia, and South America [8, 9]. TEM, SHV, and CTX-M type ESBL-producing clinical isolates from general hospitals need to be assessed for prevalence of various types of derivatives, possible existence of new enzymes, and for phenotypical and genotypical characteristics.

The present study was conducted to determine antibiotic resistance patterns and isoelectric points of enzymes and sequence DNA of ESBL-producing strains using clinical isolates of *K. pneumoniae* isolated from general hospitals, but not from the university hospital, in Busan, Korea, from September 2004 to October 2005.

MATERIALS AND METHODS

Bacterial Strains

Sixty-five ESBL-producing strains out of 226 clinical isolates of *K. pneumoniae* were collected between 2004 and 2005 from 3 general hospitals located in Busan, Korea. The isolates were identified by using the Vitek GNI Card (bio Merieux Vitek Inc., Hazelwood, MO, U.S.A.).

Antibiotic Susceptibility and Double-Disk Synergy Test

Antibiotic susceptibility was determined by the Vitek GNS Card (bio Merieux Vitek Inc., Hazelwood, MO, U.S.A.), performed according to the recommendations of the National Committee for Clinical Laboratory Standards [27]. The isolates were tested for ESBL production by the double-disk synergy (DDS) test with disks containing cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), and ticarcillin/clavulanate (75/10 μg, Becton Dickinson, U.S.A.) (Fig. 1).

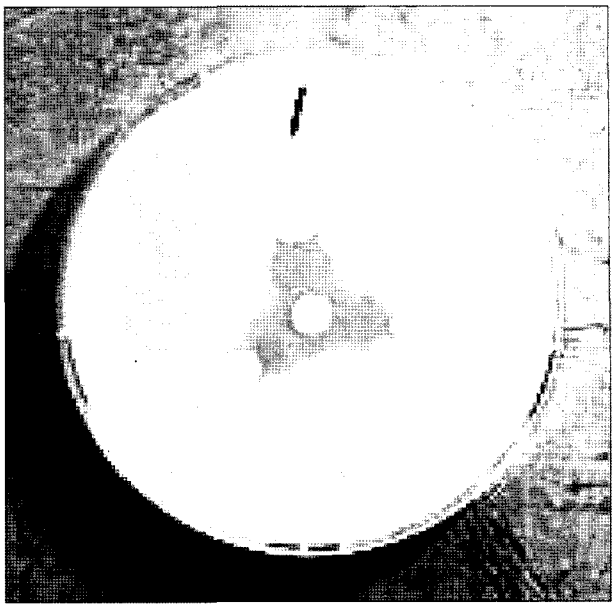

Fig. 1. Double-disk synergy test.
Center: Clavulanic acid disk; Around: 3rd generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone).

The disks were placed 15 mm apart (from center to center); for certain isolates, the test was repeated with the distance reduced to 10 mm and interpreted by NCCLS [27].

Transconjugation Experiments

Transconjugation experiments were performed as described previously [24] with nalidixic acid-resistant *E. coli* $RG176^{Na^{\oplus}}$ as the recipient. Transconjugants were selected on MacConkey agar supplemented with nalidixic acid (Sigma, St. Louis. MO, U.S.A.) (16 μg/ml), in order to inhibit the growth of the donor and recipient strains with ceftazidime (30 μg/ml).

Detection of ESBL Gene by Polymerase Chain Reaction (PCR)

Plasmid DNA was purified from bacterial cells by using a AccuPreP^{R} Plasmid Extraction Kit (BIONEER, Korea). A pair of primers (TEM/F: 5'-ATAAAATTCTTGAAGACGAAA-3', reverse 5'-GACAGTTACCAATGCTTAATC-3' and TEM/R: 5'-GACAGTTACCAATGCTTAATC-3') was used for the amplification of a 1,080-bp fragment that covers the entire *bla*TEM genes. A pair of primers (SHV/F: 5'-TCG-TTATGCGTTATATTCGCC-3' and SHV/R: 5' GGTTAG-CGTTGCCAGTGCT-3') was used for the amplification of a 861-bp fragment that covers the entire *bla*SHV genes. A pair of primers (CTX-M/F: 5'-CGCTTTGCGATGTGCAG-3' and CTX-M/R: 5'-ACCGCGATATCGTTGGT-3') was used for the amplification of a 551-bp fragment that covers the entire *bla*CTX-M genes. As for the creation of oligonucleotides, we followed Kim and Lee's method [24] for the TEM type and obtained the DNA sequence of the β-lactamase gene from GenBank (http://www.nlm.nih.gov)

Table 1. Antimicrobial resistance of ESBL-producing *K. pneumoniae* using the Vitex system.

Antimicrobial agents (resistance breakpoint, μg/ml)	Antimicrobial resistance rates (%, n=65)					
	R		I		S	
	No.	%	No.	%	No.	%
Amikacin (≥64)	15	23.0	9	13.8	41	63.0
Ampicillin (≥32)	65	100				
Ampicillin/sulbactam (≥32/16)	54	83.0	11	16.9		
Aztreonam (≥32)	65	100				
Cefazolin (≥32)	65	100				
Cefepime (≥32)	65	100				
Cefoxitin (≥32)	11	16.9	2	3.1	52	80.0
Ceftriaxone (≥64)	65	100				
Ciprofloxacin (≥4)	40	61.5			25	38.4
Gentamicin (≥16)	28	43.0			37	56.9
Imipenem (≥16)					65	100
Piperacillin/tazobactam(≥128/4)	44	67.6	2	3.0	19	29.2
Tobramycin (≥16)	43	66.1	3	4.6	19	29.2
SXT (≥4/76)	31	47.6			34	52.3

*Abbreviations: R, resistant; I, intermediate; S, susceptible; SXT, trimethoprim/sulfamethoxazole.

for the SHV type, and referred to David's method [14] for the CTX-M type. Using an AccuPower©PCR Premix Kit (BIONEER Co. Ltd. Seoul, Korea), 8 μl of plasmid DNA extract, 1 μl of 10 pmol of each primer, and 10 μl of distilled water were added in a tube. An Perkin-Elmer 9600 apparatus (Perkin-Elmer, Norwalk, CT, U.S.A.) was used, and the reactions were run under the following conditions: 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 1 min 30 sec at 45°C, and 1 min at 72°C, and finally, 10 min at 72°C for the *bla*TEM amplification; 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C, and finally, 10 min at 72°C for the *bla*SHV amplification; 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 58°C, and 1 min at 72°C, and finally, 10 min at 72°C for the *bla*SHV amplification. The resulting PCR products were run in 1.5% agarose gels.

Isoelectric Focusing

Crude bacterial extracts were obtained from isolated strains after centrifugation of sonicated culture as previously described [12, 24]. Sonic extracts were used for the determination of isoelectric points by isoelectric focusing (IEF) in Ready Gel precast IEF polyacrylamide gels (BIO-Rad, Hercules, Calif.) as previously described [12, 24].

DNA Sequencing

DNA sequencing was performed to identify the TEM, SHV, and CTX-M derived ESBLs from selected samples.

Table 2. The presence of ESBL genes in this study.

ESBL gene type	No. of isolates	Gene-detected isolates	ESBL DDS		
			CAZ	CTX	CRO
TEM	4	YT-9, 19, 46, 53	+	+	+
SHV	7	YT-26, 50, 61, 62, 63, 64, 65	+	+	+
CTX-M	8	YT-3, 36, 11, 12, 55, 58, 59, 60	+	+	+
TEM+SHV	22	YT-1, 4, 5, 7, 8, 21, 22, 25, 29, 30, 38, 39, 41, 42, 43, 44, 45, 47, 48, 49, 54	+	+	+
		YT-20	+	-	-
TEM+CTX-M	4	YT-13, 23, 57	+	+	+
SHV+CTX-M	5	YT-10, 16, 24, 28, 33, 56	+	+	+
TEM+SHV+CTX-M	12	YT-14, 15, 17, 18, 31, 32, 34, 36, 37, 40	+	+	+
		YT-27, 35	+	-	-
Not detected	3	YT-51, 52	+	+	+
		YT-2	+	-	-
Total		65			

*Abbreviations: ESBL DDS, Extended spectrum β-lactamase double disk synergy.
CAZ. ceftazidime: CTX. cefotaxime: CRO. ceftriaxone.

TEM and SHV PCR products were purified with a PCR purification kit (Qiagen, Hilden, Germany) and were subjected to direct sequencing of both strands by Macrogen Company (Seoul, Korea), using primers used in PCR reactions, on a fully automatic ABI PRISM 3730 DNA analyzer (Perkin-Elmer, Norwalk, CT, U.S.A.).

RESULTS AND DISCUSSION

Antibiotic Susceptibility Test

All the clinical isolates (100%) of *K. pneumoniae* were resistant to ampicillin, cefazolin, cefepime, ceftriaxone and aztreonam: 83.0% of the organisms were resistant to ampicillin/sulbactam, 66.1% to tobramycin, 67.6% to piperacillin/tazobactam, 61.5% to ciprofloxacin, 47.6% to trimethoprim/sulfamethoxazole, 43.0% to gentamicin, 23.0% to amikacin, and 16.9% to cefoxitin, and all strains were susceptible to imipenem (Table 1). *K. pneumoniae* isolates harboring ESBLs are significantly more frequently found to be resistant to antibiotics than non-ESBL-producing strains [33].

Detection of ESBL-Producing Strains

On the basis of positive results by the DDS test with ceftazidime (30 μg) disk, all 65 isolates were identified as putative ESBL producers. However, when the test was done using cefotaxime (30 μg) or ceftriaxone (30 μg), the remaining 3 isolates were either negative or difficult to interpret, in spite of decreased distance between the disks. The result of the double-disk synergy test for the detection of ESBL-producing strains, using ceftazidime, was the same (100%) as that of the Vitek system ESBL card [13]. The disk synergy test was the most effective for detecting ESBL-producing strains.

Transconjugation

Eleven strains of 65 ESBL-producing *K. pneumoniae* isolates transferred oxyimino β-lactam antibiotic resistance to *E. coli* RG176$^{Na^{R}}$. The 54 remaining strains did not transfer the resistance gene, and this is most probably due to degeneration of outer membrane protein of the bacteria

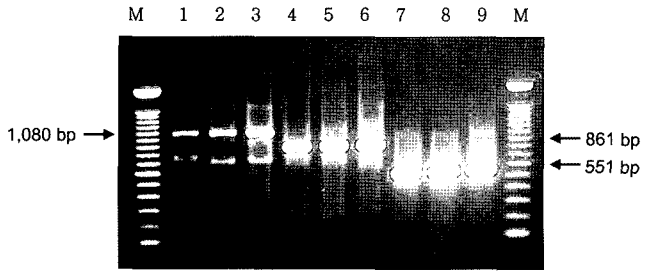

Fig. 2. Detection of amplified products of bla_{TEM} genes, bla_{SHV} genes, $bla_{CTX\text{-}M}$ genes after 1.5% agarose gel electrophoresis.
Lanes 1–3, bla_{TEM} genes; lanes 4–6, bla_{SHV} genes; lanes 7–9, $bla_{CTX\text{-}M}$ genes. M; markers.

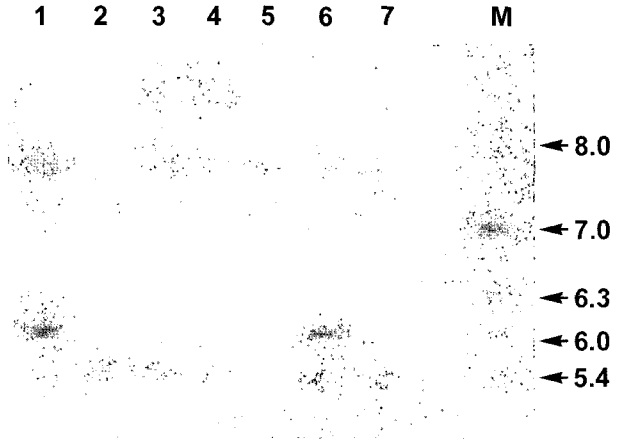

Fig. 3. Isoelectric point of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ type.
Lanes 1–2, $bla_{CTX\text{-}M}$, type; lanes 3–5, bla_{SHV} type; lanes 6–7 bla_{TEM} type. M; markers.

or to the activation of chromosomal β-lactamase [29, 31]. β-Lactamase originally confers the organism with resistance to oxyimino cephalosporin antibiotics. The enzyme was thought to be produced only in chromosome and not to transfer to other bacteria. Therefore, it was not an epidemiological problem. However, in the early 1980s, some new bacteria were shown to produce a new enzyme that hydrolyzes the oxyimino antibiotics. It was found that the β-lactamase was produced not only in chromosome, but also in plasmid, and the enzyme is transferred to other bacteria by plasmid. ESBL is a representative enzyme that is transferred to other bacteria by plasmid. A big problem is that ESBL-producing bacteria transfer their ESBL gene to other genus and species by plasmid and many new bacterial strains that are resistant to various antibiotics are expected to appear.

Detection of *bla*TEM, *bla*SHV, and *bla*CTX-M Genes by Polymerase Chain Reaction

The results on the detection of ESBL-encoding genes by PCR are shown in Table 2 and Fig. 2. All clinical isolates

Table 3. The presence of ESBL genes and pI in this study.

ESBL gene type	No. of isolates	pI
TEM only	4	5.4, 6.0,
SHV only	7	7.6, 8.2
CTX-M only	8	5.4, 8.6
TEM+SHV	22	5.4, 6.0, 7.0, 7.6, 8.2
TEM+CTX-M	4	5.4, 8.6
SHV+CTX-M	5	5.4, 8.2, 8.6
TEM+SHV+CTX-M	12	5.4, 7.0, 8.2, 8.4
Not detected	3	5.4, 8.2
Total	65	

*Abbreviations; pI, isoelectric point.

Table 4. Amino acid substitutions of TEM type β-lactamases in this study.

β-Lactamases	No. of strains	No. of isolates	Positions at amino acid			
			104	182	238	280
TEM-1	14,15, 20, 28, 30, 36, 47	7	Glu	Met	Gly	Ala
TEM-52	23, 26	2	Lys	Thr	Ser	Ala

*Abbreviation: Glu, glutamic acid; Met, methionine; Gly, glycine; Ala, alanine; Lys, lysine; Thr, threonine; Ser, serine; Val, valine.

(n=65) were tested for the presence of genes coding for the TEM, SHV, and CTX-M of ESBL, employing primers that are specific for each gene. It was found that 64.6% (42/65) of the isolates included TEM, 70.8% (46/65) included SHV, and 44.6% (29/65) included CTX-M. Four strains (6.2%) of 65 isolates had only *bla*TEM genes, 7 strains (10.8%) had only *bla*SHV, and 8 strains (12.3%) had only *bla*CTX-M. Twenty-two strains (33.8%) had both *bla*TEM and *bla*SHV, 4 strains (6.2%) had both *bla*TEM and *bla*CTX-M, and 5 strains (7.7%) had both *bla*SHV and *bla*CTX-M. Twelve strains (18.5%) had *bla*TEM, *bla*SHV, and *bla*CTX-M genes. Three strains (4.6%) did not have any of these three genes (Table 2, Fig. 2). Many strains of ESBL-producing *Enterobacteriaceae* isolated in Korea were of the SHV type [32]. Based on the results (42 strains of TEM type and 46 strains of SHV type), the SHV type appears to be on the increase.

Isoelectric Focusing (IEF)

The isoelectric point of β-lactamase was shown to vary, depending on genotype (Fig. 3). By using the nitrocefin procedure, two major bands of β-lactamase activity with pIs of 5.4 and 6.0 were detected in *K. pneumoniae* strain with TEM type gene, 7.6 and 8.2 were detected in *K. pneumoniae* strain with SHV type gene, and 5.4 and 8.6 were detected in *K. pneumoniae* strain with CTX-M type gene (Table 3).

In the early days of research on ESBLs, a determination of pI was usually sufficient to identify ESBL that was present [21]. More recently, however, this approach has failed to distinguish several ESBLs with identical isoelectric points. Therefore, this test is being used nowadays merely as a screening test of genotypes rather than giving conclusive identification.

DNA Sequencing

Nine TEM type genes, 16 SHV type, and 10 CTX-M type of 65 strains were detected completely. The sequencing of TEM genes revealed that 7 strains contained TEM-1, and 2 strains contained TEM-52 (Table 4). Twelve strains contained SHV-12, 2 strains contained SHV-2a and 2 strains contained SHV-28 (Table 5). The sequencing of CTX-M genes revealed that 10 stains contained CTX-M-15 (Table 6). The deduced amino acid sequence of TEM-52 revealed 3 amino acid substitutions, compared with that of TEM-1 (Glu-104→Lys, Met-182→Thr, Gly-238→Ser), SHV-2a had 2 amino acid substitutions, compared with that of SHV-1 (Leu-35→Gln, Gly-238→Ser), and SHV-12 had 2 amino acid substitutions, compared with that of SHV-1 (Leu-35→Gln, Gly-238→Ser, Glu-240→Lys). The deduced amino acid sequence of SHV-28 detected for the first time in Korea had one amino acid substitution, compared with that of SHV-1 (Tyr-7→Phe). The deduced amino acid sequence of CTX-M-15 revealed 5 amino acid substitutions, compared with that of CTX-M-1 (Asp-114→Asn, Ser-140→Ala, Val-177→Ala, Asn-240→Gly, Asn-288→Asp). TEM-3 seems to be the most common in France, whereas TEM-10, TEM-12, and TEM-26 are predominant in the United States [22]. SHV variants are also important worldwide: SHV-2 and SHV-5 enzymes each have been recorded in at least five countries, with the latter type widespread in Greece [25]. TEM-1, TEM-52, SHV-2a, SHV-5, and SHV-12 are predominant in Korea [4, 5, 17, 23]. SHV-12 was detected from *E. coli* and *K. pneumoniae* for the first time in Switzerland [28]. SHV-28 has not been detected in Korea, but the genes of two strains were detected in this study. The DNA sequence of the gene showed 100% homology to that of the gene detected from *K. pneumoniae*, which was isolated at Southwest Hospital of the Third

Table 5. Amino acid substitutions of SHV type β-lactamases in this study.

β-Lactamases	No. of strains	No. of isolates	Positions at amino acid			
			7	35	238	240
SHV-1		ND	Tyr	Leu	Gly	Glu
SHV-2a	23, 26	2	Tyr	Gln	Ser	Glu
SHV-12	1, 8, 15, 16, 17, 18, 19, 21, 22, 17, 38, 42	12	Tyr	Gln	Ser	Lys
SHV-28	5, 6	2	Phe	Leu	Gly	Glu

*Abbreviation: ND, Not detected; Tyr, tyrosine; Leu, leucine; Gly, glycine; Glu, glutamic acid; Gln, glutamine; Ser, serine; Lys, lysine; Phe, phenylalanine.

Table 6. Amino acid substitutions of CTX-M type β-lactamases in this study.

β-Lactamases	No. of strains	No. of isolates	Positions at amino acid				
			114	140	177	240	288
CTX-M-1		ND	Asp	Ser	Val	Asn	Asn
CTX-M-3		ND	Asn	Ala	Ala	Asn	Asp
CTX-M-15	7, 11, 12, 13, 14, 15, 24, 33, 34, 38	10	Asn	Ala	Ala	Gly	Asp

*Abbreviation: ND, Not detected; Asp, aspartic acid; Asn, asparagine; Gly, glycine; Ser, serine; Ala, alanine; Val, valine.

Military Medical College in China in 2002 (GenBank AF538324). The DNA sequence of the other gene showed 99% homology with that of the gene detected from *K. pneumoniae*, which was isolated at the Zhejiang Medical University in China in 2000 (GenBank AF299299). The CTX-M-1-producing *E. coli* strains were isolated in Germany in 1989 and has also been reported in Europe, South America, East Asia, and Africa [6, 26]. CTX-M-15, detected from Indian enterobacterial isolates that were resistant to both cefotaxime and ceftazidime, was detected in India and has also been reported in Asia and Europe [30]. The CTX-M-15 was detected from 2 strains of *E. coli* in Korea in 2003. All the CTX-M genes detected in this study were CTX-M-15, and the detection rate of CTX-M-15 has constantly been increasing in Korea [5].

ESBL-producing *K. pneumoniae* are widespread at all levels of Korean hospitals. The most common types of ESBLs in Korea are SHV-12, SHV-2a, and TEM-52 [17]. In this study, TEM-52, SHV-2a, SHV-12, SHV-28, and CTX-M-15 were found in clinical *K. pneumoniae*. Vandana *et al.* [34] examined the effect of antibiotics belonging to three different groups, including third generation cephalosporins, aminoglycosides, and quinolones on the outer membrane protein (OMP) profile of *K. pneumoniae*. They have identified two new proteins (porins), which are expressed on the surface of *K. pneumoniae*. Further research seem to be needed in order to find certain new OMPs, not only on *bla*SHV-28 type ESBL *K. pneumoniae* but also on other SHV, TEM, and CTX-M type organisms isolated in Korea.

Extended-spectrum β-lactamases, such as plasmid-mediated class A TEM and SHV type enzymes, have developed by stepwise mutations of their structural genes, resulting in either single or multiple amino acid changes in the encoded enzymes. These enzymes are often the cause for resistance to newer antibiotics such as cephalosporins and monobactams in members of the family *Enterobacteriaceae*.

The gene, encoding β-lactamase SHV-28, detected in this study is a genotype that differs from SHV-1 only by a single amino acid substitution (tyrosine to phenylalanine) at position 7 of SHV-1. There is no other published report on the finding of SHV-28 in Korea. Therefore, more investigation and research about SHV-28 are needed. It is very important to prevent resistant bacteria by correctly identifying ESBL-producing *K. pneumoniae* and treating infected patients with appropriate antibiotics.

Acknowledgment

This work was supported for two years by Pusan National University Research Grant.

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