Introduction

Extended-spectrum β-lactamases (ESBLs), such as the plasmid-mediated class A TEM- and SHV-type enzymes, have developed by stepwise mutations in their structural genes, resulting in either single or multiple amino acid changes in the encoded enzymes, and these changes sufficiently remodel the active site to allow attack on aminothiazolyl compounds.\(^1\)

Since they were first identified at the beginning of the 1980s, ESBL-producing microorganisms, belonging mostly to the Enterobacteriaceae, have spread by nosocomial routes throughout the world. The incidence of ESBL producers in Korean isolates of \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} were in the range 4.8–22.5% and 13.2–22.4%, respectively.\(^2\)

In contrast to the USA and Europe, where SHV-2, SHV-4 and SHV-5 are the prevalent extended-spectrum SHV enzymes, in Korea SHV-2a and SHV-12 are the most frequently identified extended-spectrum SHV enzymes. A 6.6 kb \textit{BamHI} fragment containing the \textit{bla}_{SHV-12} gene of strain K7746 isolated from one university hospital in Korea was cloned into the pCRScript-CAM vector. Sequencing of the constructed recombinant plasmid pK7746-C1 revealed that the immediate upstream sequence of the \textit{bla}_{SHV-12} gene showed little similarity to the part of the prototype \textit{bla}_{SHV-1} gene due to the insertion of an IS26 element next to the –10 region. Instead, the upstream sequences of \textit{bla}_{SHV-12} retained 100% DNA identity with the part of plasmid pMPA2a from \textit{Klebsiella pneumoniae} KPZU-3 carrying \textit{bla}_{SHV-2a}. The restriction map of the inserted 6.6 kb DNA fragment of plasmid pK7746-C1 was also homologous to that of plasmid pMPA2a, suggesting a common lineage of \textit{bla}_{SHV-12} and \textit{bla}_{SHV-2a}. We also studied, using PCR, the upstream non-coding region of several SHV β-lactamase genes for the presence of IS26 sequence. The flanking IS26 sequence in the immediate upstream region of the \textit{bla}_{SHV} gene was not detected in five standard strains producing SHV-1, SHV-2, SHV-3, SHV-4 or SHV-5. However, IS26 was detected in all 69 clinical strains producing SHV-2a or SHV-12 isolated from three university hospitals in Korea during 1993–1999. The above findings suggest a direct evolution of SHV-12 from SHV-2a, not from SHV-2 to -5, and it is considered to be one of the reasons for the absolute predominance of SHV-2a and SHV-12 in Korea.

Relationship between \textit{bla}_{SHV-12} and \textit{bla}_{SHV-2a} in Korea

Jungmin Kim\(^a\), Haeng-Seop Shin\(^b\), Sung-Yong Seol\(^b\) and Dong-Taek Cho\(^b*\)

\(^a\)Department of Microbiology, College of Medicine, Dankook University, Cheonan; \(^b\)Department of Microbiology, Kyungpook National University School of Medicine, 101, DongIn-2Ga, Taegu 700-422, South Korea

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*Corresponding author. Tel: +82-53-420-6951; Fax: +82-53-427-5664; E-mail: dtcho@knu.ac.kr

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Materials and methods

Bacterial strains and plasmids

*K. pneumoniae* K7746 was isolated from a urine specimen of a patient hospitalized in Kyungpook National University Hospital of Taegu, Korea, in 1997. Strain K7746 was resistant to ampicillin and cephalosporins, including extended-spectrum cephalosporins, but susceptible to cefotixin and imipenem. It also conferred resistance to chloramphenicol, sulfisoxazole, trimethoprim, kanamycin, gentamicin and tobramycin. The MIC of ceftazidime was 256 mg/L, the MICs of ceftriaxone and cefotaxime were 128 and 512 mg/L, respectively, and the MIC of aztreonam was 64 mg/L. *E. coli* strain XL1-Blue was per-

Antibiotic susceptibility testing and analytical isoelectric focusing

MICs were determined by the agar dilution method according to NCCLS guidelines.9 The drugs tested were chloramphenicol, sulfisoxazole, trimethoprim, kanamycin, gentamicin, tobramycin, ampicillin, ticarcillin, ceftriaxone, cefotaxime, cefoxitin, ceftazidime, aztreonam and imipenem. Isoelectric focusing of \( \beta \)-lactamase was performed as described previously.10

Transfer of resistance and plasmid analysis

To test the transmissibility of the ceftazidime and aztreonam resistance of the isolate K7746, a conjugation experiment was performed with *E. coli* strain RG488 as the recipient. Logarithmic phase cells of K7746 were mated with similar cultures of *E. coli* strain XL1-Blue were used as recipients in conjugation and transformation experiments, respectively.

Cloning and sequencing of the \( \beta \)-lactamase gene

Plasmid DNA was prepared with the Qiagen plasmid kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer’s instructions and then digested with *BamHI* (Boehringer Mannheim, Mannheim, Germany). T4 DNA ligase, ligation buffer and calf intestinal phosphatase were purchased from Gibco-BRL, Tsuen Wan, Hong Kong, and cloning of the *BamHI* fragments into pCRScriptCAM SK+ cloning vector (Stratagene, La Jolla, CA, USA) followed by transformation of *E. coli* strain XL1-Blue was performed according to Maniatis et al.12 Clones were initially selected on Luria–Bertani agar plates containing 100 mg/L of chloramphenicol, X-Gal and IPTG. The \( \beta \)-lactamases of the selected clones were tested by isoelectric focusing. A clone showing the \( \beta \)-lactamase with a pI of 8.2 was finally selected for further studies. DNA sequencing was performed by the dye cideoxy chain-termination method13 using the OmniBase sequencing kit (Promega, Madison, WI, USA) and \([\s^{32}P]\)dATP (DuPont, North Billerica, MA, USA) according to the manufacturer’s instructions. DNA sequence homology search was carried out with the Gen-Bank BLAST program.

Restriction endonuclease mapping of recombinant clone and Southern blot hybridization

Restriction enzymes were purchased from Boehringer Mannheim. Restriction enzyme digests, 1% agarose gel electrophoresis and Southern blotting by vacuum on to nylon membranes (Boehringer Mannheim) were carried out using conventional methods.12 The location of the *blaSHV* gene was studied by Southern blot hybridization with the *blaSHV* gene probe. The probe was prepared by PCR with the primers S1 (5’-CTACTCGCCGGTCAGCG-3’) and S2 (5’-GACCGATGTCCTCACAT-3’), corresponding to nucleotides 486–502 and 805–822 of the *blaSHV*-2 gene, respectively.14 The probe hybridized to the IS26 element was prepared by PCR with the primers IS26-1 (5’-TTACATTTCAAAAAACTCTGTG-3’) and IS26-2 (5’-ATGAACCATTCAAAGGCCGGG-3’), corresponding to nucleotides 681–700 and 1365–1385 of pMPA2a clone, respectively.15 Probe labelling and hybridization were performed with the digoxigenin labelling and detection kit (Boehringer Mannheim), according to the manufacturer’s instructions.

PCR mapping of IS26-*blaSHV* region

The presence of IS26 in the promoter region of seven SHV \( \beta \)-lactamase genes, including *blaSHV*-1, *blaSHV*-2, *blaSHV*-3, *blaSHV*-4, *blaSHV*-5, *blaSHV*-2a and *blaSHV*-12, was examined using a PCR mapping method. Five strains (kindly provided by G. A. Jacoby), each carrying one of *blaSHV*-1 (R1010), *blaSHV*-2 (pMG229), *blaSHV*-3 (pUD18), *blaSHV*-4 (pUD21) or *blaSHV*-5 (pAFF2), and 69 clinical isolates carrying *blaSHV*-2a or *blaSHV*-12 (55 strains of *K. pneumoniae*, four strains of *E. coli* and 10 strains of *E. cloacae*) were used. Primers used in the PCR mapping experiment were IS (5’-GGCGTATCAGATGTTGC-3’) and S3 (5’-GGCCAGATCATTCTTATCA-3’), corresponding to nucleotides 1272–1287 and 1646–1659 of the pMPA2a clone, respectively. The primer set was designed to detect both sequences of IS26 and *blaSHV*. PCR amplification was performed in 25 μL reaction mixtures containing 1 μL of crude cellular lysate, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1 mM MgCl2, 0.1 μM oligonucleotide primers, 200 μM

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deoxynucleoside triphosphate mix and 2.5 U of Taq DNA polymerase (Promega). PCR assay was performed in a Gene Cycler thermal cycler (Bio-Rad, Hercules, CA, USA) with the following cycling parameters: denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s; and a final extension period of 72°C for 10 min.

Nucleotide sequence accession number

The nucleotide sequence of upstream non-coding and coding region of *bla*<sub>SHV-12</sub> reported in this study will appear under the GenBank accession number AY008838.

Results and discussion

β-Lactam resistance of the strain K7746 was transferred to *E. coli* RG488 in conjugation experiments. Resistance to chloramphenicol, sulisoxazole, trimethoprim, kanamycin and tobramycin was not cotransferred with ceftazidime resistance; however, gentamicin resistance was transferred. Isoelectric focusing of crude lysates of pre-selected clones, suggested a common lineage. The *E. coli* RG488 transconjugant T7746 revealed a single β-lactamase with a pI of 8.2, presumed to be SHV-12 or SHV-5. Plasmid DNA from K7746 and transconjugant T7746 was extracted and analysed by gel electrophoresis. A 126 kb plasmid was detected in both strains.

Plasmid DNA (126 kb) from the transconjugant T7746 was digested with *Bam*HI and ligated to the *Bam*HI-digested vector plasmid pCRScriptCAM, a chloramphenicol resistance conferring cloning vector. By further isoelectric focusing of crude lysates of pre-selected clones, one clone of the β-lactamase with a pI of 8.2, K7746-C1, was selected and analysed. Its plasmid, pK7746-C1 (10 kb), consisted of pCRScriptCAM (3.4 kb) and a 6.6 kb *Bam*HI fragment containing the *bla*<sub>SHV</sub> gene. Locations of sites for restriction endonucleases and the location of the *bla*<sub>SHV</sub> gene on pK7746-C1 were determined by several digestions and subsequent Southern blot hybridization with the *bla*<sub>SHV</sub> gene probe (Figure 1). The restriction map of pK7746-C1 obtained is shown in Figure 2.

The 1134 bp nucleotide sequence, including the *bla*<sub>SHV</sub> gene, within pK7746-C1 was determined (GenBank accession number AY008838). It revealed that *bla*<sub>SHV-12</sub> was the gene responsible for extended-spectrum cephalosporin resistance of strain K7746. Notably, the nucleotide sequence upstream of the –10 region of *bla*<sub>SHV-12</sub> showed little similarity to that of the prototype *bla*<sub>SHV-1</sub> gene, but it corresponded exactly to the sequence from IS26, including the 14 bp terminal inverted repeat sequence (IRS).<sup>16</sup> In addition, sequences homologous to *bla*<sub>SHV-1</sub> begin immediately past the outside base of the IS26 IRS, suggesting an insertion of IS26 next to the –10 sequence of *bla*<sub>SHV-12</sub>. The IS26-insertion generated a hybrid promoter in which the TTGTGA—35 region of the *bla*<sub>SHV-12</sub> promoter was replaced by the –35 sequence TTGCAA provided by the left inverted repeat of IS26.

The immediate upstream sequences of *bla*<sub>SHV-12</sub> (Figure 3) retained 100% DNA identity with the part of plasmid pMPA2a from *K. pneumoniae* KPZU-3 producing SHV-2a<sup>15</sup> and pMK105 from *Shigella dysenteriae* PB-10 producing SHV-11.<sup>17</sup> Comparison of the sequence of the coding region also revealed that the *bla* genes shared the same substitution of glutamine for leucine at position 35, and the same silent mutations in the coding triplets for Leu-138 (CTG) and for Thr-268 (ACG). The restriction map of the inserted 6.6 kb DNA fragment of pK7746-C1 was also homologous to plasmid pMPA2a<sub>15</sub>. This high degree of identity between *bla*<sub>SHV-12</sub>, *bla*<sub>SHV-2a</sub>, and *bla*<sub>SHV-11</sub> in both non-coding and coding regions of the genes suggests a common lineage.

SHV-11 (or SHV-1a), SHV-2a and SHV-12 (or SHV-5-2a) differ from SHV-1, SHV-2 and SHV-5, respectively, by one amino acid change from leucine to glutamine at position 35, which is far from the active site and known not to alter the isoelectric point. They are thus considered as variants of SHV-1, SHV-2 and SHV-5, respectively. How-

![Figure 1](image-url)
ever, the above data indicate that the upstream non-coding region of SHV-11, SHV-2a and SHV-12 might be different from that of SHV-1, SHV-2 and SHV-5 by the presence of the IS26 sequence inserted next to the –10 sequence. To confirm this possibility, we studied the presence of the IS26 element in the immediate upstream regions of the following SHV β-lactamase genes: *bla*<sub>SHV-1</sub>, *bla*<sub>SHV-2</sub>, *bla*<sub>SHV-3</sub>, *bla*<sub>SHV-4</sub>, *bla*<sub>SHV-5</sub>, *bla*<sub>SHV-2a</sub> and *bla*<sub>SHV-12</sub> using PCR mapping. First, we performed PCRs on 69 clinical isolates carrying *bla*<sub>SHV-2a</sub> or *bla*<sub>SHV-12</sub> isolated from three university hospitals during 1993–1999 (55 strains of *K. pneumoniae*, four *E. coli* and 10 *E. cloacae*). The flanking IS26 sequence was detected in all of the 69 strains. However, we could not find the flanking IS26 sequence in the five standard strains, each producing one of SHV-1, SHV-2, SHV-3, SHV-4 or SHV-5. This suggests the separate evolutionary development of SHV-2a and SHV-2. In a further plasmid analysis and Southern blot hybridization study with a *bla*<sub>SHV</sub> or IS26 probe among 39 strains of *K. pneumoniae* carrying *bla*<sub>SHV-2a</sub> or *bla*<sub>SHV-12</sub> isolated from three university hospitals during 1993–1999 (55 strains of *K. pneumoniae*, four *E. coli* and 10 *E. cloacae*). The flanking IS26 sequence was detected in all of the 69 strains. However, we could not find the flanking IS26 sequence in the five standard strains, each producing one of SHV-1, SHV-2, SHV-3, SHV-4 or SHV-5. This suggests the separate evolutionary development of SHV-2a and SHV-2. In a further plasmid analysis and Southern blot hybridization study with a *bla*<sub>SHV</sub> or IS26 probe among 39 strains of *K. pneumoniae* carrying *bla*<sub>SHV-2a</sub> or *bla*<sub>SHV-12</sub>, both *bla*<sub>SHV</sub> and IS26 probes were hybridized to the same plasmid and the sizes of the plasmids carrying both genes were in the range 30–121 kb.

The above findings indicate that SHV-12 may have evolved directly from SHV-2a, not from SHV-2 to -5. Therefore, we present a diagram of the possible evolutionary relationship of eight members of the SHV family (Figure 4). SHV-11 is a narrow-spectrum β-lactamase with activity against ampicillin, piperacillin and to some extent early cephalosporins (e.g. cefalothin), and it has been considered to be a variant of SHV-1. However, our recent finding implies that SHV-11 may be another chromosomal β-lactamase of *K. pneumoniae* carried by >90% of clinical isolates of *K. pneumoniae*. Our recent study determining the nucleotide sequences of the *bla*<sub>SHV</sub> genes of the strains shown by a ligase chain reaction<sup>3</sup> to have SHV-2a- or SHV-12-specific mutations confirmed the results from the ligase chain reaction and revealed that the majority of clinical isolates of *K. pneumoniae* with SHV-2a or SHV-12 sequence also had SHV-1 (three of 13 strains) or SHV-11 (five of 13 strains) sequence. The gene encoding SHV-2a or SHV-12 was transferred by conjugation, but the gene encoding SHV-11 was not transferred. From these results, we suspect that chromosomal SHV-11 might be a kind of chromosomal β-lactamase of *K. pneumoniae*, like SHV-1 or LEN-1. It is also possible that chromosomal SHV-11 has evolved from chromosomal SHV-1 by one amino acid substitution, L35Q. In any case, chromosomal SHV-11 is thought to be an ancestor of the plasmid-mediated SHV-11 found in an isolate of *S. dysenteriae*<sup>17</sup> and extended-spectrum β-lactamasases such as SHV-2a and SHV-12.

The presence of the IS26 element in the upstream non-coding regions of *bla*<sub>SHV-11</sub>, *bla*<sub>SHV-2a</sub> and *bla*<sub>SHV-12</sub> led to the question of the role of the sequence in the evolution of these genes. The close association of insertion sequences (ISs) with antibiotic resistance genes strongly suggests an active role for these sequences in the evolution or dissemination of antibiotic resistance genes. IS26 has been associated with several antibiotic resistance genes, including *aphA1* in Tn2689 and other transposons,<sup>18</sup> a *blaT–aac5* operon in plasmid pUZ3644,<sup>19</sup> an IAB operon in plasmid pBWH77,<sup>20</sup> *dhfrVIII* in plasmid pLMO226<sup>21</sup> and *dfr13, aadA4, bla<sub>TEM</sub>-1* and *sul2* gene in plasmid pUK2381.<sup>22</sup> It has been assumed from sequencing data that IS26 provides part of a hybrid promoter for *aacC* genes in plasmids pWP7b and pWP14a.<sup>23</sup> In addition, Prenki et al.<sup>24</sup> suggested that IS26 is a portable –35 promoter site. Insertion of the IS26 sequence immediately upstream of *bla*<sub>SHV-11</sub>, *bla*<sub>SHV-2a</sub> and *bla*<sub>SHV-12</sub> genes generates the hybrid promoter
**bla**<sub>SHV-12</sub> and **bla**<sub>SHV-2a</sub> in Korea

Consisting of the −35 region derived from IS26 and the −10 region derived from the **bla**<sub>SHV</sub> promoter itself. This hybrid promoter has been reported to increase β-lactam resistance when coupled to **bla**<sub>SHV-2</sub>, indicating that the IS26 insertion produces a more efficient promoter. This more efficient promoter could be adaptively significant, producing more gene product. It therefore appears that acquisition of both a strong promoter and point mutations in the structural genes, which contribute to the ability of the host to resist the lethal effect of third-generation cephalosporins, was selected for by extensive use of these drugs in clinical settings.

In conclusion, we suggest three possible explanations for the unusual predominance of SHV-2a and SHV-12 in Korea: the first is direct evolution of SHV-12 from SHV-2a; the second is a separate evolutionary development of SHV-2a and SHV-2; the third is the acquisition of a strong hybrid promoter of SHV-2a and SHV-12, created by IS26 insertion. The latter may contribute to the survival and dissemination of SHV-2a- and SHV-12-producing bacteria in the presence of third-generation cephalosporins. The presence of the IS26 sequence immediately upstream of SHV-2a and SHV-12 may also discriminate between strains producing these enzymes from the strains producing SHV-2 and SHV-5.

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