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Investigation of *Klebsiella pneumoniae* Isolates Producing SHV-12 and SHV-11 β-Lactamases in Korean Hospitals

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Of 143 clinical isolates of *Klebsiella pneumoniae* collected from Korean non-tertiary hospitals, 24 (16.8%) showed an extended-spectrum β-lactamase-positive phenotype. PCR and sequence analysis revealed the presence of TEM-116 (n=13), CTX-M-3 (n=5), CTX-M-14 (n=2), CTX-M-15 (n=3), and SHV-12 (n=16). Each of the 24 isolates encoded more than one β-lactamase, and seven isolates (29%) harbored two different SHV-type β-lactamase genes (bla_{SHV-11} and bla_{SHV-12}) bounded by insertion sequence IS26 in a single transferable plasmid.

Keywords: *Klebsiella pneumoniae*, SHV-11, SHV-12, extended-spectrum β-lactamase

Infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* are difficult to treat owing to the organism's ability to hydrolyze penicillin and cephalosporins, which contain a β-lactam ring. Numerous ESBL variants have been reported, most of which contain more than one amino acid substitution and are resistant to the extended-spectrum cephalosporins derived from known non-ESBL β-lactamase genes, such as bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. ESBL-producing *Klebsiella pneumoniae* isolates are critical nosocomial pathogens that produce ESBLs belonging to the SHV family, which are prevalent in Gram-negative bacteria and are encoded by SHV-type β-lactamase genes (bla_{SHV}). In the many SHV β-lactamases reported, substitutions at amino acid positions 238 and 240 are the most important for acquiring ESBL activity.

Several reports have documented the prevalence of ESBLs in Korea and have identified TEM-116 and SHV-12 as the most common variants [7, 8]. However, these surveys were based on sequencing of a primary PCR product, enabling identification of only one gene; the question remains whether different alleles, that is, multiple bla_{SHV}s, exist in *cis*. The coexistence of multiple *bla* genes within individual isolates is of considerable practical significance, because isolates that express two or more *bla* alleles may not be accurately identified by the current typing system. In addition, within-strain shuffling of ESBL gene regions facilitates insertion sequence (IS) acquisition, by which dissemination of ESBL genes could be accelerated. Such a case was recently described in which different bla_{SHV} variants, containing different promoters, existed in *cis* [5]. However, no studies have addressed the distribution of bla_{SHV} variants in ESBL-producing organisms.

Upon investigating the prevalence and genotypes of ESBL-producing *K. pneumoniae* isolates in Korea, we found ambiguities among the sequences of several SHV-positive isolates. Therefore, we carried out the present study to examine the possible existence and prevalence of different *bla* alleles and their ISs in *K. pneumoniae* at non-tertiary Korean hospitals.

Materials and Methods

Clinical Samples

A total of 143 clinical isolates of *K. pneumoniae* were collected from three commercial laboratories at 83 non-tertiary hospitals in all provinces of Korea, from January to June 2006. The major sources of isolates were sputum (44.8%; n=64) and urinary tract specimens (37.8%; n=54). The isolates were identified as *K. pneumoniae* either by automated (GNI+; bioMerieux Vitek, Hazelwood, MO, U.S.A.) or manual biochemical analyses.

Identification of ESBL-Producing Strains

Antibiotic susceptibility was determined by disk diffusion tests that were performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, U.S.A.) guidelines [1, 2]. Putative ESBL-producing isolates were tested by both the clavulanate double-disk synergy test of Jarlier *et al.* [6] and the confirmatory test for ESBL production according to CLSI guidelines.

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Molecular Subtyping by Pulsed-Field Gel Electrophoresis (PFGE)

Genomic DNA was digested with XbaI (New England Biolabs, Ipswich, MA, U.S.A.) and analyzed by PFGE using the contour-clamped homogeneous electric field (CHEF) technique [4]. DNA fragments were separated by electrophoresis in 1% SeaKem gold agarose (Cambrex, Rockland, ME, U.S.A.) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) in a CHEF Mapper XA apparatus (Bio-Rad, Hercules, CA, U.S.A.) at 14°C and 6 V/cm with alternating pulses at a 120° angle in a 2–40 s pulse time gradient for 22.5 h. The Lambda Ladder PFG marker (New England Biolabs) was used as the molecular size standard. Restriction patterns were interpreted by the criteria proposed by Maslow *et al.* [10].

Conjugation Experiments

Conjugation was performed with *Escherichia coli* J53 Azide^R as the recipient strain. Transconjugants were selected on Mueller-Hinton agar supplemented with 150 mg/l sodium azide (Sigma, St. Louis, MO, U.S.A.) to inhibit the growth of the donor strain, and with 2.5 mg/l ceftazidime (Sigma) to inhibit the growth of the recipient strain.

PCR Amplification, DNA Sequencing, and NheI Digestion Analysis

Primers for PCR amplification were designed by selecting consensus sequences in a multiple sequence alignment of reported *bla* genes (data not shown). Five type-specific primer sets were used to detect the genes bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, and bla_{GES} in phenotypically confirmed ESBL-producing isolates. The distribution of β-lactamases was determined by sequence analysis using the following primers, comprising 10 sets: TEM-F (AGTCACAGAAAAGCATCTTA), TEM-R (ATCTCAGCGATCTGTCTAT), TEM-T1-F (TCAAATATG TATCCGCTC), SHV-F (AAGATCCACTATCGCCAGCAG), SHV-R (TAGCGTTGCCAGTGCTCG), SHV-S1-F (GGGTTATTCTTATTT GTCGCTTCT), CTX-M-U-F (ATGTGCAGYACCAGTAARGTKAT), CTX-M-U-R (TGGGTRAARTARGTSACCAGAA), CTX-C2-R(3), (GACAGCACTTTTGCCGTCTA); CTX-C1-F(14), (GAATACTGA TGTAACACGGATTG), CTX-C1-R(14) (GTTGTCGGGAAGATAC GTG), CTX-C1-F(15), (AGGGACTATTCATGTTGTTGCTA), GES-F (AAGTTAGACGGGCGTACA), GES-R (GAAGCTTAGCGTAGC ACG); VEB-F (GTTAGCGGTAATTTAACCAG), and VEB-R (TAT TCAAATAGTAATTCCACG).

Following PCR, bla_{SHV} amplicons were digested with NheI (New England Biolabs), which can identify mutations at codon 238 in bla_{SHV} by recognizing the sequence AGC (encoding Ser). An NheI-insensitive band and two NheI-sensitive bands were excised from an agarose gel and extracted using a QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The NheI-insensitive DNA fragment was cloned into pGEM-T easy vector (Promega, Madison, WI, U.S.A.) and transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's guidelines. The two NheI-sensitive DNA bands were ligated using T4 DNA ligase (New England Biolabs), and then cloned and transformed using the methods described for the undigested band. These bands were subjected to sequencing analysis.

Southern Blotting and Gene Walking

Plasmid DNA was prepared from transconjugant *E. coli* strains with a plasmid extraction kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was digested with BamHI (New England Biolabs), and then electrophoresed on 0.8% agarose gels and visualized with ethidium bromide under UV light. Gels were subjected to Southern blotting using nylon membranes (GE Healthcare, Little Chalfont, England). To detect the presence of an SHV gene, we hybridized the digested plasmid DNA of conjugants with a 593-bp PCR product of an internal fragment of bla_{SHV} generated using primers SHV-F and SHV-R and end-labeled using an ECL nonradioactive labeling kit (GE Healthcare). To obtain the unknown sequences adjacent to the bla_{SHV} genes, the detected bands were extracted from the gel and subjected to DNA walking, which consisted of serial amplifications of the unknown target region followed by nested PCR (Seegene, Seoul, Korea).

Results

A total of 143 *K. pneumoniae* isolates were screened for their ability to produce ESBLs. Confirmatory testing yielded 24 ESBL-positive isolates, representing 16.8% of the total. According to ESBL type-specific PCR results, all of these isolates contain bla_{TEM} (n=24), 95.8% (n=23) contain bla_{SHV}, and 41.7% (n=10) contain bla_{CTX-M}. No isolates contain bla_{GES} or bla_{VEB}. More than one β-lactamase gene was identified in all isolates, and nine (37.5%) isolates harbor three genes, namely bla_{TEM}, bla_{SHV}, and bla_{CTX-M} (Table 1). Sequence analysis revealed that the 24 isolates containing bla_{TEM} carry either TEM-116 (n=13) or TEM-1 (n=11), and the 10 isolates harboring bla_{CTX-M} carry CTX-M-3 (n=5), CTX-M-14 (n=2), or CTX-M-15 (n=3).

Table 1. Prevalence of multiple β-lactamases in ESBL-producing *K. pneumoniae*.

β-Lactamase type			% (No.) of isolates
TEM	SHV[a]	CTX-M	
1		15	4.0 (1)
	1-n	14	4.0 (1)
	11-n		4.0 (1)
		3	4.0 (1)
		14	4.0 (1)
		15	8.0 (2)
	12-t	3	12.5 (3)
	12-t, 11-t		4.0 (1)
116	1-n		4.0 (1)
	12-t		25.0 (6)
	12-t, 11-t		20.8 (5)
		3	4.0 (1)
Total			100 (24)

[a]For SHV, "n" and "t" indicate that the lactamase is encoded by a non-transferable or transferable plasmid, respectively.

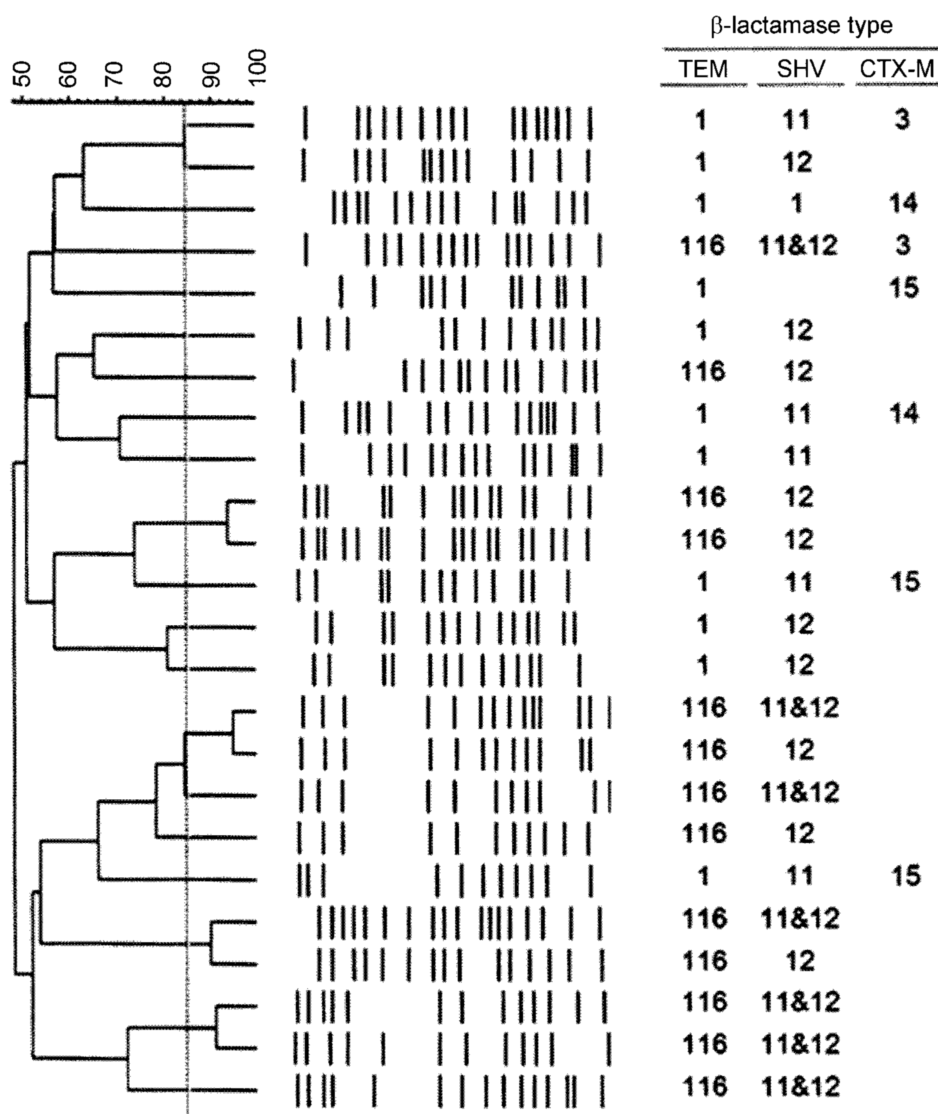

Fig. 1. PFGE patterns and dendrogram of 24 ESBL-producing *K. pneumoniae* isolates generated by digestion with XbaI.
A 1.0% tolerance and a 1.5% optimization were used during cluster analysis with the unweighted pair-group method, and DNA relatedness was calculated based on the Dice coefficient of correlation. Isolates were considered to be genetically related if the Dice coefficient was 85% or greater.

Conjugation and genotyping of isolates producing SHV-type ESBLs demonstrated that 16 isolates harbor transferable bla_{SHV}: 29.2% (n=7) carry two bla_{SHV} alleles (bla_{SHV-11} and bla_{SHV-12}) in a single transferable plasmid, whereas 37.5% (n=9) harbor bla_{SHV-12} only (Table 1). Of the 16 SHV-12-producing isolates, 43.8% (n=7) also encode SHV-11 in a transferable plasmid, but no isolates producing ESBLs encode SHV-11 alone in a transferable plasmid. Every plasmid containing these two genes is >150 kb in size (data not shown). Additionally, six isolates harbor nontransferable bla_{SHV}: two isolates contain bla_{SHV-1} and four isolates contain bla_{SHV-11}. A dendrogram of the different molecular subtypes is shown in Fig. 1. Twenty unique subtypes were identified from the 24 ESBL-producing isolates. The nine isolates carrying bla_{SHV-11} and bla_{SHV-12} represent multiple genotypes, among which one cluster of two related isolates (>85% similarity) could be observed. There was no close relatedness between SHV-11- and SHV-12-producing isolates.

To investigate bla_{SHV-11} and bla_{SHV-12} insertion sites, we identified these two genes in isolate 06K-006. Southern blot analysis of the BamHI-digested plasmid of the corresponding transconjugant revealed that both SHV enzymes are encoded on the same plasmid but at two distinct sites. The detected restriction digest fragments were 3.5 kb and 7.2 kb in size (Fig. 2).

DNA walking performed on these two fragments showed that both types of bla_{SHV} are bracketed by IS26 (Fig. 3); however, each IS26 element is inserted at a different site in the plasmid. The IS26 elements for bla_{SHV-12} are inserted upstream of the methyl-accepting chemotaxis protein gene

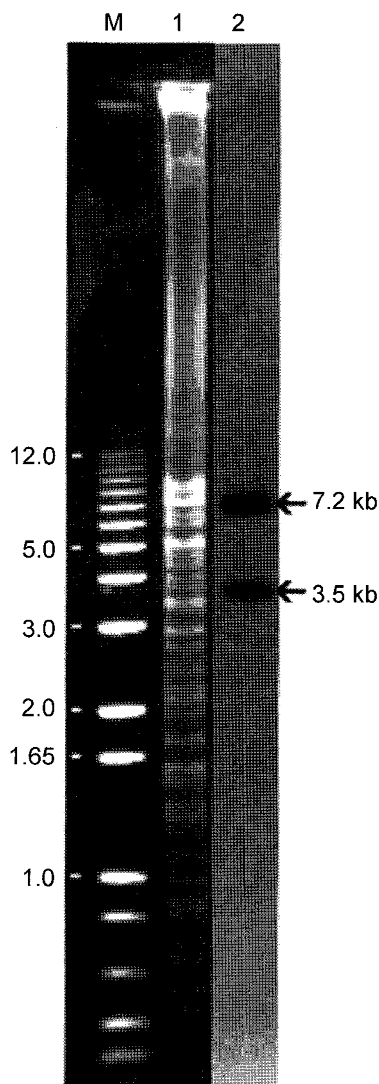

Fig. 2. Electrophoresis (lane 1) and Southern hybridization (lane 2) of BamHI digests of plasmid DNA from *E. coli* transconjugant 06K-006 with a general SHV probe.

The sizes (in kilobases) of molecular markers (M) are indicated to the left. The two bands detected (7.2 and 3.5 kb) were subsequently subjected to gene walking analysis.

in the 7.2-kb fragment, whereas the IS26 elements for $bla_{\text{SHV-11}}$ are inserted downstream of the class II aldolase/adducin domain protein gene in the 3.5-kb fragment (Fig. 3).

Discussion

The prevalence of ESBL-producing *K. pneumoniae* isolates in Korean tertiary hospitals was about 9.3% in 1997 and 23% in 2003 [7, 12], and the most common ESBL type was SHV-12 in both studies. In the present study, which was conducted on *K. pneumoniae* isolates from non-tertiary hospitals, the prevalence of ESBL-producing isolates was 16.8%, and the most common ESBL type was SHV-12. Considering that non-tertiary hospitals at least partly reflect the Korean community, the relatively low frequency of SHV-12 was expected. However, the fact that all isolates simultaneously produced two or even three different β-lactamases indicates significant dissemination of ESBLs.

Dissemination of SHV ESBLs may be largely mediated by plasmids or ISs. Owing to IS-mediated spread of ESBLs, the prevalence of *K. pneumoniae* isolates with more than one β-lactamase has increased in recent years [3]. In the present study, all ESBL-producing isolates harbor at least two different types of β-lactamases, and nine isolates (37.5%) harbor three types, namely bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$. The experimental approach of direct sequencing of PCR-amplified genes cannot reliably detect multiple TEM- or SHV-encoding genes with high sequence similarity. The possibility of multiple alleles in a single isolate has previously been reported [5, 11], and Hammond *et al.* [5] identified coexisting bla_{SHV} alleles within individual isolates and determined their copy number using a real-time PCR assay. When we genotyped the isolates producing SHV-type β-lactamases using direct sequencing, isolate 06K-006 showed mixed sequences from $bla_{\text{SHV-31}}$ (Leu35→Gln, Glu240→Lys), $bla_{\text{SHV-11}}$ (Leu35→Gln), and $bla_{\text{SHV-12}}$ (Leu35→Gln, Gly238→Ser, Glu240→Lys) (data not shown); however, further molecular analysis using NheI digestion showed that this isolate encodes both SHV-11 and SHV-12.

Lee *et al.* [9] showed that *K. pneumoniae* strains carrying $bla_{\text{SHV-11}}$ on the chromosome abundantly express plasmid-derived SHV-12, suggesting that $bla_{\text{SHV-11}}$ was transferred from the chromosome by an IS26 originating from a $bla_{\text{SHV-12}}$ element. This conclusion is supported by the exact sequence match between the IS26-$bla_{\text{SHV-11}}$ element, including IS26-IRL and IS26-IRR, and the IS26-$bla_{\text{SHV-12}}$ element. Similar results have been obtained in studies on the distribution and genetics of SHV-2a and SHV-12 in Korea [9] and the detection of $bla_{\text{SHV-11}}$ in *cis* with the IS26 promoter [5], which showed a direct relationship between IS26 and bla_{SHV}.

The dissemination of ESBLs could be accelerated by acquisition of an IS by a single isolate, highlighting the importance of continuously monitoring resistance trends and vigorously pursuing measures to control potential or ongoing infections with this pathogen. Furthermore, our results demonstrate that molecular epidemiological analysis of ESBL-producing isolates should be confirmed by molecular genotyping, because different *bla* alleles can be acquired over time *via* IS-mediated transfer.

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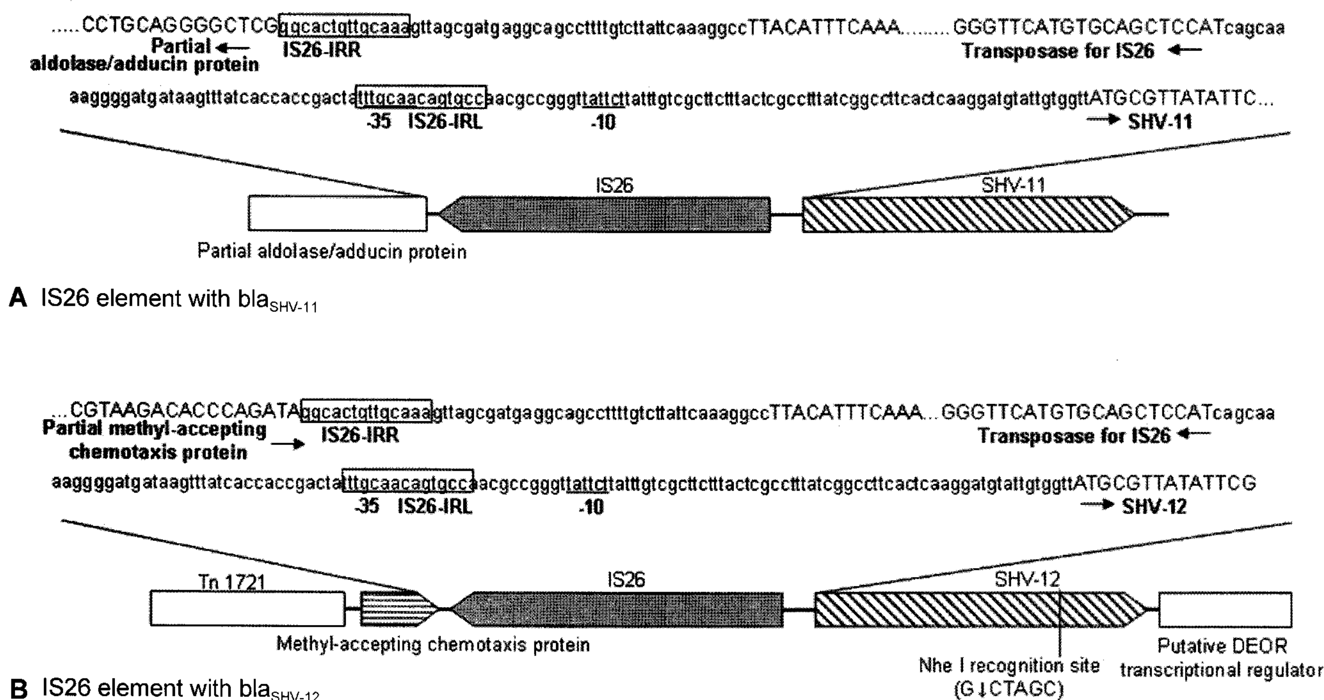

Fig. 3. Schematic map of the IS26 elements harboring (**A**) $bla_{\text{SHV-11}}$ and (**B**) $bla_{\text{SHV-12}}$.
The −35 and −10 promoter regions are underlined. The IRR (inverted repeat right) and IRL (inverted repeat left) of IS26 are boxed. Arrows indicate the direction of transcription. Adjacent genes are also labeled. The uppercase and lowercase nucleotides in the sequences indicate protein-coding regions and noncoding sequences, respectively.

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