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Characterization of Plasmid-Mediated SHV-11 β-lactamase Gene of *Klebsiella pneumoniae* Isolated from the Clinical Specimens

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In this study, we characterized extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae isolated from clinical specimens in Korea and found two strains harboring plasmid-mediated bla_{SHV-11}, *Klebsiella pneumoniae*. First, the isolates were detected using the Vitek system and confirmed by the double-disk synergy test. The classification of gene coding for ESBL was also performed by polymerase chain reactions and followed by DNA sequencing. The transmission of genes was confirmed by transconjugation and transformation. Resistant expression of transformants was determined by broth microdilution minimal inhibitory concentration test. Genotypic analysis revealed that one strain harbored the bla_{TEM-1}, bla_{SHV-11} and $bla_{CTX-M-15}$ and the other strain harbored the bla_{SHV-11} and $bla_{CTX-M-15}$. They showed high resistance to oxyiminocephalosphorins (3rd-generation cephalosporins), while the transformant containing only bla_{SHV-11} did not show any resistance to the antibiotics.

Key words : Extended-spectrum β-lactamase, bla_{SHV-11}, $bla_{CTX-M-15}$, oxyiminocephalosphorins, Klebsiella pneumoniae

Introduction

Escherichia coli, *Klebsiella pneumoniae* and *Serratia marcescens* are prevalent pathogens that cause nosocomial infection such as urinary tract infection, sepsis, and wound infection [8]. Since ampicillin having clinical efficacy against gram negative bacilli was introduced, β-lactam antibiotics have been widely used to heal these species [6]. Extended spectrum β-lactamases (ESBLs) are enzymes responsible for many failures of antimicrobial therapy because they hydrolyze β-lactam antibiotics to become inert and ineffective [3,4]. Infections caused by ESBL-producing *K. pneumoniae* resistant to β-lactam antibiotics have been shown to be associated with significantly longer hospital stay and higher hospital charges [23]. ESBL-producing strains that first emerged in 1980s have constantly increased to 2000s and many clinicians have several difficulties in treating against infections of the stains. Occurrence of ESBL production in *K. pneumoniae* isolated from intensive care unit (ICU) ranges from 20 to 25% in Europe and it in isolates of *Enterobacteriaceae* 9% in the United States [25]. Interestingly, in Netherland, a survey of 11 hospital laboratories showed that the strains less than 1% of *E. coli* and *K. pneumoniae* possessed an ESBL [22]. In Korea, it has been reported that ESBL production in *K. pneumoniae* and in *E. coli* isolated from 12 university-affiliated hospitals was 29.2% and 9.1%, respectively [10]. Sulfhydryl variable (SHV) β-lactamase gene is one type of the ESBLs gene. And the SHV-11 β-lactamase gene is known to be chromosomally encoded in the majority of isolates of *K. pneumoniae* [20].

In this study, we performed phenotypic and genotypic analyses of two strains harboring plasmid-mediated one transferred from chromosomal β-lactamase gene, bla_{SHV-11}.

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Materials and Methods

Bacterial strains

Forty-seven ESBL producing strains out of 188 clinical isolates of *Enterobacteriaceae* strains (23 *E. coli*, 23 *K. pneumoniae*, 1 *S. marcescens*) were collected from 4 general hospitals located in Busan, Korea, from January 2007, till January, 2008. The isolates were identified by using the Vitek GNI card (BioMerieux Inc., Hazelwood, MO, USA). And two SHV-11 β-lactamase genes harboring *K. pneumoniae* out of forty-seven ESBL producing strains were selected and analyzed.

Double-disk synergy test for detecting ESBL producing strains

Detection of extended-spectrum β-lactamase was performed with the double-disk synergy test (DDST) as the previous method [7].

PCR and sequence analysis

Plasmid DNA was purified from bacterial cells by using a AccupreP^{R} plasmid extraction kit (Bioneer, Seoul, Korea). *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes were amplified with specific primers (Table 1). Purified PCR products were sequenced directly using the primers used in PCR reactions, on a fully automatic ABI PRISM 3130 DNA analyzer (Applied Biosystems, Foster city, CA, USA). Sequence alignment and analysis were performed online using Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Transconjugation and transformation

Transconjugation experiments were performed as described previously [14] with *E. coli* RG176Na^{r} as the recipient. Transformation experiments were also performed with plasmid DNA prepared from *K. pneumoniae* (strain No. 60036) which was electroporated into *E. coli* DH5-α as described previously [13].

Cloning of *bla*$_{SHV-11}$

Total cellular DNA was isolated from *K. pneumoniae* 60036 using a plasmid midi-prep kit (Qiagen GmbH, Hilden, Germany) and partially digested with *Bam*HI and *Hin*dIII (Gibco-BRL, Gaithersburg, MD, USA) to select SHV-11 gene only. The PCR primers for inserted clone are shown in Table 1. T-easy Vector (Promega, Madison, Wi, USA.) was constructed and used as the vector in this study. The partially digested DNA was ligated into the corresponding sites of the vector by using T4 DNA ligase (Gibco-BRL, Gaithersburg, MD, USA) and electroporated into *E. coli* DH5-α. Clones were selected on Luria-Bertani agar plates containing 100 μg of ampicillin/ml.

Minimal inhibitory concentration (MIC) test

Resistant expression of transformants was determined by the agar dilution MIC test according to the Clinical and Laboratory Standards Institute guidelines [18].

Results

Detection of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, sequence analysis and transconjugation

Two strains (No. of strain: 60036, 60041) producing *bla*$_{SHV-11}$ detected in this study were confirmed to be with other two resistant ESBL genes, *bla*$_{TEM}$, and *bla*$_{CTX-M}$. The strain 60036 harboring *bla*$_{SHV-11}$ was revealed to have *bla*$_{TEM}$ and *bla*$_{CTX-M}$, simultaneously, while the other strain, 60041 harboring *bla*$_{SHV-11}$ was revealed to have only *bla*$_{CTX-M}$. In other words, based on the sequence analysis, the strain 60036 was confirmed to have TEM-1, SHV-11 and CTX-M-15, while the strain 60041 to have SHV-11 and CTX-M-15, simultaneously (Table 2).

It has been known that ESBL producing strains could transfer plasmid-mediated resistance to other strains [2]. When the two strains producing *bla*$_{SHV-11}$ were transconjugated the strain 60036 only was successfully transmitted but the other strain 60041 was not.

Transformation and cloning of *bla*$_{SHV-11}$

The three genes TEM, SHV and CTX-M amplified by PCR with plasmid DNA extracted from transformant were con-

Table 1. Nucleotide sequences of the primers

Primers	Sequence (5 to 3, synthesized)	Product size (bp)	GenBank accession no.
TEM F[a]	5′-ATAAAATTCTTGAAGACGAAA	1080	A13194682
TEM R[b]	5′-GACAGTTACCAATGCTTAATC		
SHV F	5′-TCGTTATGCGTTATATTCGCC	861	AY826416
SHV R	5′-GGTTAGCGTTGCCAGTGCT		
CTX-M F	5′-CGCTTTGCGATGTGCAG	551	X92506
CTX-M R	5′-ACCGCGATATCGTTGGT		
SHV-11[c] (*Hin*dIII)	5′-AGAAGCTTATGCGTTATATTCGCCTGTG	870	In this study
SHV-11[d] (*Bam*HI)	5′-AGGGATCCTTAGCGTTGCCAGTGCTC		

[a]Forwar, [b]Revers, [c]Forward primer for cloning PC, [d]Reverse primer for cloning PCR.
Abbreviations: TEM, Temoneira; SHV, Sulfhydryl reagent variable; CTX-M, Cefotaxime-Munich

Table 2. Identification and source of strains harboring SHV-11 gene

No. of strains	ID of Strains	Source		ESBL gene		
		Specimen	Department	TEM	SHV	CTX-M
60036	*K. pneumoniae*	bile	medical ward	TEM-1	SHV-11	CTX-M-15
60041	*K. pneumoniae*	sputum	medical ward	-	SHV-11	CTX-M-15

Abbreviations: TEM, Temoneira; SHV, Sulfhydryl reagent variable; CTX-M, Cefotaxime-Munich

firmed for transformation, and the expression of ESBL gene in the transformant was also confirmed through phenotypic analyses such as high resistance to oxyiminocephalosporins and positive result by DDS test.

As the results of DDST, PCR and sequence analysis for recombinant strains cloned with only SHV-11 gene, the recombinants were negative to DDST and showed SHV-11 gene on PCR and sequence analysis (Fig. 1).

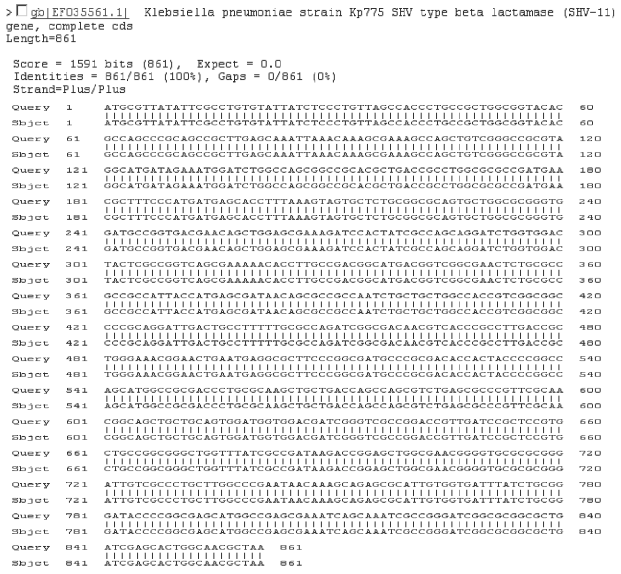

Fig. 1. The DNA sequence of the gene of *K. pneumoniae* plasmid β-lactamase bla_{SHV-11} in GenBank showed 100% of homology compared with that of the gene detected from *K. pneumoniae* isolated from clinical specimens.

Minimal inhibitory concentration (MIC) test

MIC of the strain 60036 harboring the three genes, TEM-1, SHV-11 and CTX-M-15 were ≥256 μg/ml, 128 μg/ml and ≥256 μg/ml to ceftazidime (30 μg/ml), cefotaxime (30 μg/ml) and ceftriaxone (30 μg/ml), respectively and MIC of the strain 60041 harboring the two genes, SHV-11 and CTX-M-15 were 128 μg/ml, 128 μg/ml, 64 μg/ml respectively. MIC of recipient *E. coli* DH5-α before transformation were 0.25 μg/ml, 0.5 μg/ml and 0.5 μg/ml to ceftazidime, cefotaxime and ceftriaxone, respectively (Table 3). Also, MIC of transformant (*E. coli* DH5-α) harboring the three genes TEM-1, SHV-11 and CTX-M-15 were ≥256 μg/ml, 128 μg/ml and 128 μg/ml to ceftazidime, cefotaxime and ceftriaxone, respectively and that harboring only SHV-11 gene were 0.25 μg/ml, 0.5 μg/ml and 0.5 μg/ml, respectively (Table 3).

Discussion

It is not a common phenomenon that chromosomal β-lactamase gene, SHV-11, can be transferred to other strains by plasmid DNA. Since the SHV-1 discovery, many SHV types have detected up to the present. Among them, bla_{SHV-1} and bla_{SHV-11} exist inherently in chromosome of *K. pneumoniae* and bla_{SHV-12} which is antibiotics-resistance gene carriying a high pathogen has been detected from clinical isolates in Korea [16]. However, bla_{SHV-11} alone does not reveal resistance against antibiotics.

Interestingly, it has been reported that chromosomal

Table 3. Results of MIC (Minimum Inhibitory Concentration) by agar dilution method for *Klebsiella pneumoniae* from clinical isolates, uncloned strain (*E. coli* DH5-α), transformed strain and cloned strains with SHV-11 gene

Antibiotics	MIC (μg/ml)					
	60036[a]	60041[b]	*E. coli* DH5-α[c]	*E. coli* DH5-α[d]	*E. coli* DH5-α[e]	*E. coli* DH5-α[f]
ceftazidime	≥256	128	0.25	≥256	0.25	0.50
cefotaxime	128	128	0.50	128	0.50	0.25
ceftriaxone	≥256	64	0.25	128	0.50	0.25

[a]harboring TEM-1 SHV-1 an CTX-M-15 genes *K. pneumoniae*from clinical isolates
[b]harboring TEM-1 SHV-1 an CTX-M-15 genes *K. pneumoniae*from clinical isolates
[c]Competent cell of uncloned strain, [d]Transformants with Plasmid DNA from 60036 strain
[e]Cloned strain with SHV-11 gene from 60036 strain, [f]Cloned strain with SHV-11 gene from 60041 strain

bla_{SHV-11} in the strains carrying bla_{SHV-12} predominantly occurs. Exactly in other words, *Klebsiella pneumoniae* strains carrying the chromosomal SHV-11 β-lactamase gene produce the plasmid mediated SHV-12 extended spectrum β-lactamase more frequently than those carrying the chromosomal SHV-1 β-lactamase gene [15]. In this study, we detected *K. pneumoniae* carrying bla_{SHV-11} in plasmid and tried to investigate a role of bla_{SHV-11}.

TEM-1 is known to be the most predominant β-lactamase gene in gram-negative bacilli [4] and resistance to ampicillin to the extent of 90% in *E. coli* is attributed to the gene TEM-1 [9]. The SHV-1 β-lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species [24]. The SHV-12 was first identified in *E. coli* and *K. pneumoniae* from Switzerland [20] and the deduced amino acid sequence of the gene revealed three amino acid substitutions (Leu35Gln, Gly238Ser and Glu240Lys) compared with that of SHV-1. The gene SHV-12 has frequently occurred in Korea [12,16]. The SHV-11 was first detected in *E. coli* and *K. pneumoniae* from Switzerland [20] and the deduced amino acid sequence of the gene revealed only one amino acid substitution (Leu35Gln) compared with that of SHV-1 (Table 4). Then Nuesch-Inderbinen et al. reported that two new SHV variants, SHV-11 and SHV-12 were non-ESBL and ESBL, respectively. In other words, the SHV-11 did not confer high resistance to β-lactam antibiotics, whereas the SHV-12 did. Also, it was reported that the SHV-11 detected in *Shigella dysenteriae* in India could hydrolyze oxacillin, cloxacillin and oxyiminocephalosporins like cefotaxime [1]. However, the SHV-11 detected in our study did not show high resistance to oxyiminocephalosporins such as cefotaxime, ceftriaxone and ceftazidime, indicating non-ESBL of the gene.

Since CTX-M ESBL was first isolated from *E. coli* in 1989 in Germany, it has been reported in the worldwide [5,17]. CTX-M β-lactamases are not very closely to TEM or SHV β-lactamases in that they show only approximately 40% identity with the two commonly isolated β-lactamases [17].

Table 4. Comparison of amino acid substitutions of SHV ESBL genes

ESBL gene	Positions at amino acid			
	7	35	238	240
SHV-1	Tyr	Leu	Gly	Glu
SHV-11	Tyr	Gln	Gly	Glu

Abbreviations: SHV, Sulfhydryl reagent variable

The deduced amino acid sequence of CTX-M-15 revealed 5 amino acid substitutions, compared with that of CTX-M-1 (Asp114Asn, Ser140Ala, Val177Ala, Asn240Gly, Asn288Asp. Since CTX-M-15 ESBL was first isolated in 2001 in India, the enzyme has been reported in Asia and Europe [19,21]. In Korea, Kim and Kim also reported that CTX-M-15 ESBL was isolated in totally 32 strains of *E. coli* and *K. pneumoniae* [11]. In our study, the CTX-M-15 ESBL was detected with SHV-11 and showed high resistance to oxyiminocephalosporins.

In summary, MIC of transformant (*E. coli* DH5-α) harboring the three genes TEM-1, SHV-11 and CTX-M-15 were remarkably higher than that harboring only SHV-11 gene. High resistance in ESBL producing strains is likely to be attributed to transmission of ESBL gene to other strain by plasmid.

In conclusion, it seems that two strains (60036, 60041) identified in this study have high resistance to oxyiminocephalosporins due to the presence of CTX-M-15 gene in plasmid rather than SHV-11. These results are similar to those of Nüesch-Inderbinen group [20] where SHV-11 was non-ESBL gene, but was not to those of Ahamed group [1]. In this respect, more detailed study on SHV-11 and its associated genes will be needed in order to prevent wide-spreading resistance to oxyiminocephalosphorin antibiotics, because the gene have a possibility to cause high resistance to the antibiotics by new mutation.

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초록 : 임상검체로부터 분리한 플라스미드 매개성 SHV-11 β-lactamase 유전자의 특성

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Chromosomal 인 SHV-11 β-lactamase가 plasmid를 매개로 다른 균주로 전달 되는 현상은 흔하지 않다. 본 연구에서는 플라스미드성 SHV-11 β-lactamase를 동시에 가지고 있는 ESBL생성 두 균주를 검출하였다. 따라서 이들 균주에 대한 유전적 특성과 임상적 의의에 대해 알아보고자 하였다. Vitek system과 이중디스크확산법을 이용하여 ESBL생성균주를 검출하였고, PCR과 DNA 염기서열분석을 이용하여 SHV-11 β-lactamase를 가지고 있는 ESBL생성균주를 확인 하였다. 이들 균주를 교차접합실험과 형질전환실험을 이용하여 유전자전이를 확인하고 액체배지 희석법으로 3세대 cephalosphorin 항생제에 대한 최소억제농도를 측정하였다. 이들 균주의 유전형 분석결과는 SHV-11 β-lactamase 유전자와 CTX-M-15 ESBL 유전자를 동시에 가지고 있었다. 3세대 cephalosphorin 항생제에 대한 최소억제농도는 SHV-11 β-lactamase와 CTX-M-15 ESBL 유전자를 동시에 가지고 있는 균주에서 64 μg/ml 이상이었고, SHV-11 β-lactamase 만을 가지고 재조합 한 균주에서 0.5 μg/ml 이하로 나타났다.