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Types of Extended-Spectrum β-Lactamase Produced in Enteric Bacteria Isolated from Sewage Plant Drain Water

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This study focused on typing of the extended-spectrum β-lactamase (ESBL) produced in organisms isolated from a natural environment, rather than a clinical setting. Samples were collected from drain water issuing from a sewage plant in Kwanganri (Busan, Korea). Following double disk synergy testing, 29 strains were selected as potential ESBL positive strains. Of these, 15 strains were transconjugants of the sodium azide resistant recipient strain *Escherichia coli* J53 and analyzed biochemically including indole, methyl-red, Voges-Proskauer, Simmon's citrate, decarboxylase-dihydrolase and sugar-fermentation tests. The tests classified the 15 strains as *Klebsiella pneumoniae* (n=13) and *Escherichia coli* (n=2). The type of ESBL from each strain was deduced by isoelectric focusing point analysis and DNA sequencing. The results indicated that the types of ESBL were SHV-12 (n=4) and SHV-12/TEM-1 (n=9) from *K. pneumoniae* and TEM-1 (n=2) from *E. coli* strains.

Key words : Extended-spectrum β-lactamase (ESBL), SHV-12, TEM-1, *Klebsiella pneumoniae*, *Escherichia coli*

Introduction

β-lactamase (EC 3.5.2.6) is an enzyme that hydrolyses amide, amidine, and other C-N bonds, especially ring-associated amides [3,5]. The hydrolysis of amide connection in penicillin, cephalosporin or other related β-lactam antibiotics hydrolyzing the β-lactam ring, inactivating the antibiotic [3]. β-lactamases are classified based on either the difference in amino acid sequence [1] or, more commonly, DNA molecular structure [3]. DNA-based classification further divides β-lactamases into TEM and SHV types. These types are mutants made by a point mutation involving transposition of 1 - 4 amino acids [4]. In terms of substrates utilized and antibiotic specificity, β-lactamases can be classified in four groups [4,5,8,9,13].

Since plasmid-mediated extended-spectrum β-lactamase (ESBL) producing strains were isolated first in 1983, they have spread globally and increasingly become the source of clinical infections [16,17,21]. Bacteria capable of producing several types of β-lactamase have been identified clinically and in non-clinical environments including slaughter houses, drain waters and rivers [11,12]. The *Enterobacteriaceae* genera *Klebsiella* and *Escherichia* constitute the most commonly isolated ESBL producers from the clinical and more natural environment [11,12]. Their antibiotic resistance is both plasmid- and chromosome- mediated [21].

In Korea, the strains possessing a plasmid-mediated cephalosporinase type of β-lactamase (group 1) are suspected to be widely spread type [14,15]. Our studies have focused on slaughter houses, waste water, and plant drain water in Busan. Presently, we report the isolation and characterization of plasmid-mediated ESBL-producing strains from the overflow water draining from a sewage treatment plant at Kwanganri in Busan.

Materials and Methods

Isolation of strains

Samples were collected once a month immediately following a day of precipitation from the overflow drain of a sewage plant at Kwanganri in Busan from June - August, 2007. Aliquots (0.1 ml) of drain water were spread onto MacConkey agar (Difco, Detroit, MI, USA) containing 4 μg/ml of the third generation antibiotic ceftazidime (Young-Jin Pharmaceuticals, Seoul, Korea) [20]. After 17 hr of incubation at 37°C, representative colonies were isolated and used for two cycles of pure culture on Brain Heart Infusion (BHI) agar (Difco).

Tests of biochemical and antibiotic susceptibility

The isolated pure colonies were analyzed by biochemical

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tests and antimicrobial susceptibility. Biochemical tests included indole, methyl-red, Simmon's citrate, decarboxylase-dihydrolase activity, and fermentation of 10 selected sugars. Preparation of culture media and strain verification followed previously described protocols [6,7]. Antimicrobial susceptibility tests were performed against mother strains which formed conjugants in a transconjugation test [14,15]. Antibiotic disk diffusion testing utilized commercially available antibiotic disks (BBL, Cokeysville, MD, USA). Results for the eight tested antibiotics (ampicillin, amikacin, cefalothin, chloramphenicol, kanamycin, tetracyclin, gentamycin and nalidixic acid) were determined using National Committee for Clinical Laboratory Standards protocol [18].

Double disk synergy test

Bacteria obtained from a representative pure colony were resuspended in a McFarland turbidity No. 0.5, and aliquots (0.1 ml) were spread evenly on Mueller-Hinton agar (MHA) (Difco). Double disk synergy test was performed by positioning antibiotic-containing disks 25 mm apart at the center of each plate as previously described [14,15]. The third-generation of cephalosporins [cefotaxime (30 μg), ceftazidime (30 μg) and ceftriaxone (30 μg)] and ticarcillin/clavulanate (75/10 μg) were applied for the examination. After 18 hr of incubation at 37°C, the inhibitory zone formed between each cephalosporin containing disk and the ticarcillin/clavulanate disk.

Analysis of isoelectric focusing (IEF)

Bacteria were inoculated into 30 ml of BHI broth and incubated for 24 hr at 37°C. The bacteria were recovered by centrifugation for 10 min at 5,000× *g* and the cell pellet was resuspended in 1 ml of triple distilled water. The bacteria were ultrasonically homogenized using a model 4710 apparatus (Cole-Palmer, Chicago IL, USA). The homogenized cells were centrifuged for 3 min at 14,000 rpm and then the supernatant was transferred to an Eppendorf tube and stored at -20°C until required for IEF [12,15]. For IEF, a drop of distilled water was dispensed onto a hydrophilic support film on a glass slide. Polyacrylamide (3 - 4 ml) was dispensed onto the support film with the pipette. Polymerization occurred during 1 hr exposure to fluorescent light. After polymerization, 2 μl of sample was loaded with the guidance of a sample template, which was removed after 5 min at room temperature. A graphite electrode was kept wet with distilled water in the chamber of the Mine IEF Cell III apparatus (Bio-Rad, Hercules, CA, USA). The electrode maintained contact with the gel layer on the support slide. Electrophoresis was sequentially carried out for 30 min at 100 V, 30 min at 200 V, 60 min at 450 V [15]. After electrophoresis, the gel support film was separated from the glass gel plate. Nitrocefin (GlaxoSmithKline, Brentford, UK; 1 - 2 ml of a 500 μg/ml solution in phosphate buffer, pH 7.0) was spread onto the surface of the gel. When a red band appeared, the gel was covered by Whatman No. 2 filter paper. IEF point was analyzed using an IEF 3 - 10 marker (Serva Electrophoresis, Heidelberg, Germany) as previously described [12]. Staining and destaining of marker proteins followed a previously described protocol [22].

Plasmid isolation and classification of ESBL gene

One milliliter of a BHI culture was centrifuged at 3,000× *g*, and then the pellet was resuspended and washed three times using 1 ml of triple distilled water. DNA was extracted from the final sample by plasmid Minipreps kit and DNA purification kits following the manufacturer's protocol (Injae Science, Seoul, Korea). ESBL typing was done by polymerase chain reaction (PCR) using the following primers: TEM type - F-primer: 5' - ATA AAA TTC TTG AAG ACG AAA - 3' and R-primer: 5' - GAC AGT TAC CAA TGC TTA ATC - 3'; SHV type - F-primer: 5' - CAC TCA AGG ATG TAT TGT G - 3' and R-primer: 5' - TTA GCG TTG CCA GTG CTC G - 3'. PCR was performed with pre-mix (Biosesang, Seoul. Korea) in the presence of each primer (10 pmol, 1 μl), template (1 μl), and distilled water (7 μl) in a total reaction volume of 20 μl. PCR for the TEM type was performed in 30 cycles, consisting of denaturation at 94°C for 30 sec, annealing at 45°C for 90 sec, and extension at 72°C for 60 sec. PCR for the SHV type consisted of 35 cycles of denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec, and extension at 72°C for 120 sec. PCR was done using a GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT, USA). PCR products were stained with 3 μl of a 1:100 dilution of DMF gel staining reagent (Komabio-Technology, Seoul, Korea), loaded onto the wells, and 1% agarose gel electrophoresis was performed using TBE buffer at 100 V for 20 min. Each gel was photographed under ultraviolet illumination [12,15].

Transmission of ESBL plasmids by transconjugation

To identify transmission of ESBL plasmids, a transconjugation test was carried out with ESBL-producing

strains. *Escherichia coli* J53, which is resistant to sodium azide [14,15], was used as the recipient. Transconjugant and co-transconjugant in BHI (0.1 and 1.0 ml, respectively) were inoculated in 10 ml of BHI. After 17 hr of incubation at 37°C, 0.1 ml of culture was obtained and spread onto MacConkey agar containing 30 µg/ml ceftazidime and 50 µg/ml sodium azide. After 18 hr incubation at 37°C, colonies were checked and examined biochemically. The biochemical tests were double-checked using *E. coli* J53. ESBL plasmid-mediated transmission was confirmed by PCR, electrophoresis, and IEF. PCR product was purified with a gel extraction kit (Dyne Bio, Seoul, Korea) and analyzed by DNA sequencing [15]. The initiation codon was identified and its amino acid sequence compared using Multiple Sequence Alignment and Sequence Utility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and BCM Search Launcher (http://searcherlaumcher.bcm.tmc.edu/). ESBL typing was done by the Lahey classification scheme (http://www.lahey.org/temtable.asp) [3-5], and the final classification was determined by analysis of the amino acid sequence.

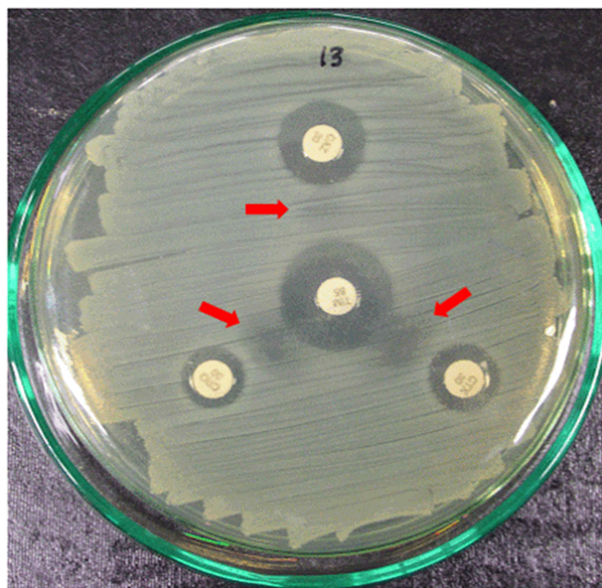

Fig. 1. Detection of the double-disk synergy test between the third generation of cephalosporins (ceftazidime, ceftriaxson, and cefotaxime) and ticacillin/clavulanic acid. The distance of each of the third-generation antibiotics from ticacillin/clavulanic acid (center) is 25 mm. The arrows indicate synergic effects (clear zone) between ticarcillin/clavulanic acid and the selected third-generation cephalosporin antibiotics.

Results

Isolation and classification of ESBL-producing *Enterobacteriaceae*

Colonies that developed on ceftazidime containing MacConkey agar were recovered and their purity was determined by two further growth cycles on BHI agar. Twenty-nine strains shown synergic effects (clear zone) between ticarcillin/clavulanic acid and the selected the third-generation cephalosporin antibiotics in the double disk synergy test (Fig. 1). Biochemical analyses of these 29 strains classified 16 strains as *E. coli* and three strains as *K. pneumoniae*. They were all Gram-negative, oxidase-negative, and catalase positive.

E. coli formed red plateaus on MacConkey agar and produced acid in both slant and butt (A/A) on Kligler iron agar (KIA). All isolated *E. coli* strains were motile in BHI containing 0.4% agar and were positive in both indole and methyl-red tests. Furthermore, all strains produced acids from glucose, mannitol, sorbitol, and trehalose but did not ferment dulcitol. The majority of the strains fermented lactose (88% of strains), sucrose (75%), adonitol (56%), arabinose (94%) rhamnose (88%), salicin (88%), and raffinose (63%) (Table 1). They could not use citrate as the sole carbon source in Simmon's citrate agar and all strains were Voges-Proskauer negative. Most strains (94%) produced gas from glucose in Durham tubes. All strains were positive for lysine decarboxylase, while only 66% of strains were positive for ornithine decarboxylase and none of them were positive for arginine dihydrolase.

K. pneumoniae colonies on MacConkey agar were sticky, smoothly convex, and red centered with a clear border. Acid was produced in both slant and butt (A/A) on KIA. There was no motility in 0.4% agar medium, and both indole and methyl-red tests were negative. All of *K. pneumoniae* strains used citrate as carbon source in Simmon's citrate agar and were Voges-Proskauer positive.

Conjugative transmission of plasmid-mediated ESBL

To identify plasmid-mediated ESBL transmission, 29 strai ns resistant to ceftazidime (sodium azide^S) and *E. coli* J53 (sodium azide^R, ceftazidime^S) were used as the donors and the recipient, respectively. Aliquots (0.1 ml) of a 1:10 BHI dilution of each were spread on MacConkey agar containing 30 µg/ml ceftazidime and 50 µg/ml sodium azide, and incub ated at 37°C for 18 hr. Thirteen strains of *K. pneu-*

Table 1. Boichemical characteristics of the strains isolated from sewerage plant drain water at Kwanganri in Busan, Korea

Tests \ Strain No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
KIA	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole Production	-	+	-	+	-	+	+	+	+	+	+	+	-	-	+
Methyl-Red	-	+	-	+	-	+	+	+	+	+	+	+	-	-	+
Voges-Proskauer	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-
Citrate, Simmons	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-
Lysine, Moeller's	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+
Arginine, Moeller's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine, Moeller's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Motility	-	+	-	+	-	+	+	+	+	+	+	+	-	-	+
Gas from Glucose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Acid production from:															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	+	+	-	-	+	-	-	-	-	-	-	-	+	-	-
Adonitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Inositol	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	-	+	-	+	-	-	-	-	-	-	-	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Species	*K. pneumoniae*	*E. coli*	*K. pneumoniae*	*E. coli*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*E. coli*

Tests \ Strain No.	16	17	18	19	20	21	22	23	24	25	26	27	28	29
KIA	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole Production	+	+	-	+	+	+	-	-	-	-	-	-	+	-
Methyl-Red	+	+	-	+	+	+	-	-	-	-	-	-	+	-
Voges-Proskauer	-	-	+	-	-	-	+	+	+	+	+	+	-	+
Citrate, Simmons	-	-	+	-	-	-	+	+	+	+	+	+	-	+
Lysine, Moeller's	+	+	+	+	+	+	+	+	+	+	+	+	+	+
arginine, Moeller's	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Ornithine, Moeller's	+	+	-	+	+	+	-	-	-	-	-	-	-	-
Motility	+	+	-	+	+	+	-	-	-	-	-	-	+	-
Gas from Glucose	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Acid production from:														
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	+	-	+	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	+	-	-	-	+	+	+	+	+	+	-	+
Inositol	-	-	+	-	-	+	+	+	+	+	+	+	-	+
Sorbitol	+	+	+	+	+	+	+	-	-	-	-	-	+	+
Arabinose	+	+	+	+	+	+	+	-	-	-	-	-	-	+
Raffinose	+	+	+	+	+	+	+	+	-	-	+	-	-	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Trehalose	+	+	+	+	+	+	+	-	-	-	-	-	+	+
Species	*E. coli*	*E. coli*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	*E. coli*	*K. pneumoniae*

moniae and two strains of *E. coli* formed conjugants. Biochemical tests clearly proved the identity of the conjugants and the mother strain, *E. coli* J53 are the same (Table 2).

Antimicrobial susceptibility test

Fifteen of transconjugant-forming strains were tested using eight kinds of antibiotics. One of two strains formed a transconjugant with *E. coli* and displayed resistance to seven antibiotics. The other strains were resistant to one or two antibiotics (Table 3). *K. pneumoniae* strains were also resistant to 2 - 7 antibiotics. Seven of the 13 strains were resistant to nalidixic acid, kanamycin, cephalothin, ampicillin, and amikacin (Table 3).

IEF points of transconjugant β-lactamase

Fifteen strains that formed transconjugants were tested for IEF point and the results were described as below. Two strains of *E. coli* (strain Nos. 6 and 17) formed a pI 5.4 product (Fig. 2). Thirteen of *K. pneumoniae* transconjugants were divided into two groups. One group contained three strains (Nos. 1, 3, and 14) having a pI 8.3 product and the other group contained 10 strains with products of pI 8.3, 6.0, and 5.4.

Table 2. Type and IEF of transconjugant ESBL

Strain no.	Species	Transconjugant pI value	Transconjugant ESBL type
1	*K. pneumoniae*	8.3	SHV-12
2	*E. coli*		
3	*K. pneumoniae*	8.3	SHV-12
4	*E. coli*		
5	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
6	*E. coli*	5.4	TEM-1
7	*E. coli*		
8	*E. coli*		
9	*E. coli*		
10	*E. coli*		
11	*E. coli*		
12	*E. coli*		
13	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
14	*K. pneumoniae*	8.3	SHV-12
15	E. coli		
16	*E. coli*		
17	*E. coli*	5.4	TEM-1
18	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
19	*E. coli*		
20	*E. coli*		
21	*E. coli*		
22	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
23	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
24	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
25	*K. pneumoniae*	8.3, 6.0, 5.4	SHV-12
26	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
27	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12
28	*E. coli*		
29	*K. pneumoniae*	8.3, 6.0, 5.4	TEM-1, SHV-12

Table 3. The pattern of multidrug resistance

Species	Resistance pattern	Strain No.
K. pneumoniae	Na, K, Te, Gm, Cf, Ap, An	29
	Na,K,Cf, Ap, An	26, 24, 22, 5, 23, 25, 27
	Na, Te, Gm, Cf, Ap	17
	Na, K, Cf, Ap	13, 18
	K, Cf, Ap	14
	Cf, Ap	3
E. coli	Na, K, Te, Gm, Cf, Ap, An	6
	Cf, Ap	1

Abbreviations: An (amikacin 10 μg/ml), Ap (ampicillin 10 μg/ml), C (chloramphenicol 30 μg/ml), Cf (cephalothin 30 μg/ml), Gm (gentamicin 10 μg/ml), K (kanamycin 30 μg/ml), Na (nalidixic acid 30 μg/ml), Te (tetracycline 30 μg/ml).

Isolation of plasmid and classification of ESBL genes by PCR

Two strains of *E. coli* (Nos. 6 and 17) displayed the same size of product (1,080 bp) as the TEM type. DNA sequence and protein analyses clarified that type of all *E. coli* transconjugants were TEM-1. On the other hand, electrophoresis of four *K. pneumoniae* strains (Nos. 1, 3, 14, and 25) detected products of approximately 780 bp indicative of SHV-12. The remaining nine strains produced both TEM and SHV types, in particular TEM-1 and SHV-12 (Figs. 3 and 4, Table 2).

Discussion

While ESBL-producing *Enterobacteriaceae* have been extensively studied and characterized in clinically-relevant settings [21], only a few studies concerning environmental isolates have been published [2,24]. However, presence of ESBL strains in non-clinical environments is expected, since plasmid-mediated ESBL bacteria and their ESBL types have been identified in slaughter houses, waste water, and river water [14,15]. The present study was performed to isolate plasmid-mediated ESBL-producing strains and to classify the ESBL types from sewage plant drain water at Kwanganri in Busan. The particular sewage plant was built to pump out sewage to the sea during periods of rainfall. Hence, we collected water samples on the day following rain.

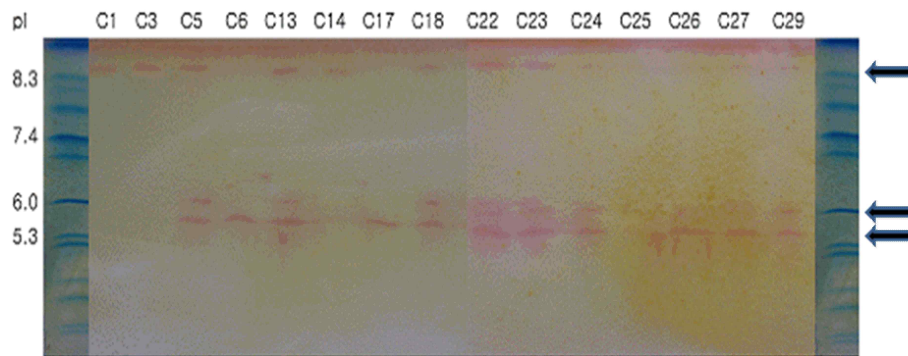

Fig. 2. IEF points of ESBL-producing transconjugants. *E. coli* (strain Nos. C6 and C17) formed a pI 5.4 product. *K. pneumoniae* (Nos. C1, C3, and C14) having a pI 8.3 product and the rest of 10 strains with products of pI 8.3, 6.0, and 5.4.

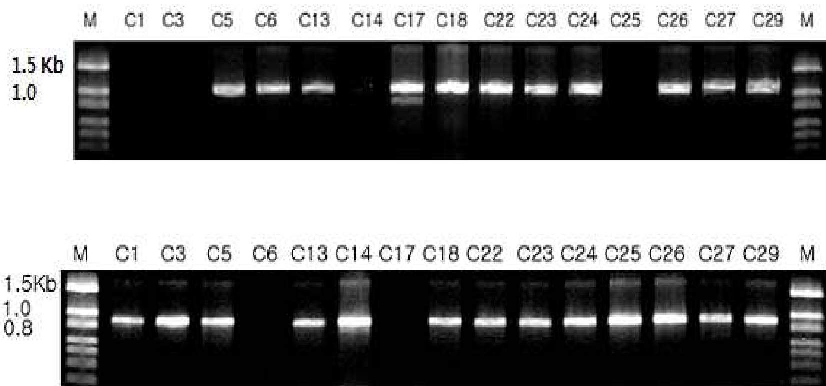

Fig. 3. PCR products (1,080 bp) of transconjugant TEM-type. M: molecular standard DNA ladder, C designates the conjugant strains which are transmitted by ESBL plasmid of parental strains.

Fig. 4. PCR products of transconjugant SHV-type. Detected products of approximately 780 bp indicate the SHV-12 type of ESBL. M is the abbreviation of molecular standard DNA ladder.

We focused on *E. coli* and *K. pneumoniae*, because both are representative plasmid-mediated ESBL-producing strains and also very important as opportunistic (*E. coli*) and nosocomial (*K. pneumoniae*) pathogens [9,11,23].

In Korea, TEM-52 is the dominant TEM type [10,20] and SHV-12 has been reported in clinical samples [11]. We previously reported the isolation of TEM-52 and SHV-12 from slaughter house and river [14,15]. SHV-12 has a high probability of being the dominant SHV type in Korea [15]. SHV-12 has a pI 8.2 and originated from SHV-1 with transpositions such as 35Leu→Gln, 238Gly →Ser, and 240Glu→Lys [19]. It is a nocosomial pathogen [10].

In the present study, 29 strains isolated by the double disk diffusion test were classified as strains of *E. coli* (n=16) and *K. pneumoniae* (n=13). All of *K. pneumoniae* isolates transferred ESBL gene to transconjugant *E. coli* J53 in a plasmid-mediated fashion. For *E. coli*, however, only two strains transferred ESBL gene(s) in lower number than *K. pneumoniae* suggesting that the latter may more efficiently transfer plasmid-mediated ESBL genes than *E. coli*.

The study adds to the list of non-clinical sources of ESBL-producing strains. Their environmental presence may pose a risk to humans and may spur the development of greater genetic diversity, especially concerning plasmid-mediated transmission.

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초록 : 하수처리수에서 분리된 장내세균의 광범위 베타락탐분해효소의 유형

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본 연구는 임상검체만이 아닌 주변 생활환경에도 광범위 베타 락탐분해효소를 생성하는 균주(extended-spectrum β-lactamase, ESBL)가 존재하는지를 확인하고 만약 존재할 경우 그 균주를 분리하고 ESBL유형을 알아보기 위하여 실시되었다. 부산 광안리 하수처리 방류수에서 이중 디스크 확산 검사 결과 양성반응을 나타낸 29균주를 선별하였다. 이중 sodium azide에 내성을 가진 피전달 균주인 *Escherichia coli* J5에 교차접합이 이루어진 15균주를 대상으로 indole, methyl-red, Voges-Proskauer, Simmon's citrate 시험과 decarboxylase-dihydrolase 및 여러 종류의 당 발효 시험 등 생화학 검사를 실시한 결과 *Klebsiella pneumoniae*(13균주)와 *Escherichia coli*(2균주)가 동정되었다. 등전점, PCR, 유전자서열 분석을 실시하여 ESBL 유형을 결정하였다. *Klebsiella pneumoniae*의 ESBL 생성유형은 SHV-12(4균주)와 SHV-12/TEM-1(9균주)의 2종류로 구분되었고, *Escherichia coli*의 ESBL 생성유형은 TEM-1(2균주)로 판명되었다.