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Prevalence and Characterization of Extended-Spectrum β-Lactamase among *Escherichia coli* Isolated from Geumho River in Korea

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In this study, prevalence and characteristics of extended-spectrum β-lactamase (ESBL)-producing coliform from the Geumho River in Korea were assessed. A total of 342 coliforms were isolated from seven different sites of the Geumho River from April to November 2008. The mean coliform and fecal colifrom population in the Geumho River was 62 CFU/mL and 22 CFU/mL, respectively. Among 342 isolates, 21 for which the MIC of ceftazidime was ≥2 ug/mL were confirmed to be positive for ESBLs, and all of them were identified as *Escherichia (E.) coli*. Twenty-one ESBL-producing *E. coli* showed high resistance of 81~100% to ampicillin, cefaclor, cephalexin, ceftriaxone, cefprozil, cefotaxime, ciprofloxacin, nalidixic acid, and tetracycline, followed by trimethoprim, kanamycin, gentamycin, streptomycin, and ceftazidime with resistance rate of 38.1~61.9%, respectively. The resistance rate to chloramphenicol was as low as 14.3%, and all isolates were susceptible to amikacin. All 21 isolates showed resistance to eight or more drugs, and 14 isolates (66.7%) transferred their antimicrobial resistance to recipient strains by conjugation. Among the 21 isolates, the TEM and CTX-M genes were detected in all of them by PCR, whereas no SHV gene was recognized, and the 14 transconjugants from these isolates also contained identical ESBL gene. The most frequent β-lactamase type was TEM-116 and CTX-M-15 with the ratio of 57.1%, followed by TEM-1 and CTX-M-15 (23.8%), TEM-1 and CTX-M-14 (9.5%), TEM-1 and CTX-M-27 (4.8%), and TEM-116 and CTX-M-14 (4.8%). MICs of cefotaxime were more than eight-fold higher compared to those of ceftazidime in the 21 isolates.

Key Words: Coliform, *Escherichia coli*, ESBL, TEM, CTX-M.

Escherichia (E.) coli is an intestinal flora that inhabits the intestinal tract of healthy humans and animals, and is widely distributed in the natural world including the aquatic environment and soil. In addition, *E. coli* can survive in water for weeks or months and thus if the water contaminated with *E. coli* is used as drinking water, it can function as an important medium of spread of diseases and antibiotic-resistant bacteria in humans and livestock[1].

β-lactams are among the most clinically important antimicrobials in both human and veterinary medicine. Bacterial resistance of β-lactams had been increasingly observed in bacteria[2]. The predominant mechanism of β-lactam resistance is the production of β-lactamases, which

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is an inactivation enzyme that hydrolyzes the four membered β-lactam rings[3].

Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporin and aztreonam but are inhibited by clavulanic acid and sulbactam[4]. Since these enzymes were first identified as early as 1983 in Germany[5], ESBLs spread rapidly to Europe, US and Asia and are now found all over the world[6]. Most ESBLs can be divided into three groups: TEM, SHV, and CTX-M types. ESBLs such as plasmid-encoded class A TEM- and SHV-type were derived from SHV-1, TEM-1, or TEM-2 β-lactamase by substitution of single or multiple amino acid residues at critical positions[7]. TEM-52, SHV-2a and SHV-12 are most commonly reported in Korea[8-12].

In recent years, CTX-M that preferentially hydrolyzes cefotaxime has arisen in many parts of the world[13]. CTX-M enzymes are also supplanting TEM and SHV variants in the Far East[14]. After first detection in 1989[15], CTX-M enzymes more than 50 different types can be divided into five groups based on their amino acid identities such as CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group[16]. In Korea, Pai *et al.,*[17] reported the first identification of CTX-M-14 in clinical isolates of *Klebsiella (K.) pneumonia*, *E. coli*, and *Shigella sonnei* in 2001. Since CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-15, and CTX-M-27 have been reported in clinical isolates, the frequency of identification of CTX-M is on the rise[8, 10, 18, 19].

Many studies have been carried out on the prevalence and characteristics of ESBL genes of *E. coli* and *K. pneumonia* from various clinical specimens of hospitalized patients[2, 6, 13], but very little has been done in bacterial isolates from living environment, sewage, and rivers[11, 20]. In particular, ESBL genes are not limited to clinical specimens of hospitalized patients, and their potential for spread beyond the environment via human and animal excreta from disposal into the sewage may serve to exacerbate public-health concerns. This study was conducted to investigate the status of coliform and fecal coliform contamination in the Geumho River and determine the prevalence and characteristics of ESBL genes of the isolates.

MATERIALS AND METHODS

Sample collection

The Geumho River, located in south-eastern Korea, receives the wastewater discharge from the sewage of the city of Yeongchon and Daegu. The six sampling sites (A~F) in the Geumho River and one sampling site (G) in the Nakdong River, joined in the Geumho River, were chosen along the pollution gradient in the river (Fig. 1). Water samples (500 mL) were collected separately at seven sites at different seven times from April to November 2008, using sterile screw-capped glass bottles, and stored in cold bags at 4 °C until analysis in the laboratory within 4 hr after collection.

Isolation of coliform and fecal coliform

For isolation of coliform and fecal coliform, 0.1 mL portions of undiluted or serially diluted water samples were spread evenly across the surface of the duplicate MacConkey agar (Difco, USA) with an L-shaped spreader. After incubation at 37 °C and 44.5 °C for 20 to 24 hr, lactose-fermenting colonies were counted and 3~4 lactose-fermenting colonies were randomly selected from each plate and used for this study.

Detection of ESBL and identification of ESBL-producing isolates

Colifrom isolates with a minimum inhibitory concentration (MIC) greater than or equal to 2 ug/mL against cefotaxime (Boryung, Korea) among 342 isolates were considered possible ESBL producers. The confirmatory testing of which strains were ESBL producers was performed with the double-disc synergy test (DDST) according to the method of Sanders *et al.,*[21]. DDST was carried out on Mueller-Hinton agar (Difco Laboratories, USA) with discs of ceftazidime, cefotaxime, aztreonam and cefepime (Becton Dickinson, USA), each containing 30 µg of the drug, placed 20 mm (center to center) away from a disc containing amoxicillin-clavulanic acid (20 µg/10 µg, Becton Dickinson, USA) in the center of the plate. Inoculated plates were incubated overnight at 37°C for 24 hr, enhancement of the zone of inhibition between the clavulanic acid disc and any one of the β-lactam discs indicated the presence of ESBL. The identification of isolates that were screened positive for the confirmation of ESBL production

were carried out using conventional methods[22] and Vitek GNI card (BioMérieux, France).

Antimicrobial susceptibility test

Antimicrobial susceptibility was determined by the agar dilution method according to the Clinical and Laboratory Standards Institutes[23] recommendation. The following 16 antimicrobial agents were used in this study: ampicillin from USB, USA; amikacin, ciprofloxacin, gentamicin, and nalidixic acid from Fluka, Switzerland; cefotaxime, ceftazidime from Boryung, Korea; cefaclor, ceftriaxone, cephalexin, chloramphenicol, kanamycin, and tetracycline from Sigma Chemical, USA; cefprozil from Ranbaxy, USA; streptomycin and trimethoprim from ICN Biomedicals, USA. The interpretive criteria for susceptibility used in this study were also those established by the CLSI[23]. *E. coli* ATCC 25922 was used as control strains.

Transferability of antibiotic resistance

Transferability of the antibiotic resistance was examined by conjugation assay using the liquid mating method. The cultures of donor and recipient (sodium azide-resistant *E. coli* J53) cells were incubated via shaking at 37°C in logarithmic phase, and then 0.5 mL of both cultures was added to 4 mL of fresh Triptic soy broth (Difco, USA) and incubated overnight without shaking. The transconjugants were selected on Trypticase soy agar (Difco, USA) plates with sodium azide (100 µg/mL, Sigma Chemical Co., St. Louis, MO) to inhibit donor and ciprofloxacin (8 ug/mL), gentamicin, streptomycin, tetracycline, trimethoprim (32 ug/mL), ampicillin, ceftazidime, cefaclor, cephalexin, chloramphenicol, cefprozil, nalidixic acid (64 ug/mL), amikacin, cefotaxime, ceftriaxone or kanamycin (128 ug/mL) to inhibit recipient. The transconjugants were assayed for susceptibility to the antimicrobial agents by agar dilution method.

PCR detection of TEM, SHV and CTX-M genes

Screening for ESBL genes was carried out by PCR amplification of TEM, SHV, and CTX-M genes using specific primers previously described[24, 25, 26]. All of the primers used in this study were synthesized by Bioneer (Korea). Plasmid DNA was isolated from the ESBL-producing isolates and transconjugants by the alkaline lysis method with GeneAll^{R} Exprep^{TM} Plasmid SV (GeneAll, Korea), according to the manufacturer's procedure. Target fragments were amplified using a Tprofessional Thermal Cycler (Biometra, Germany) as follows: initial denaturation step of 15 min at 95 °C, 30 cycles consisting of 94 °C for 30 s, 50 °C for 60 s, and 72°C for 2 min for TEM as well as 94 °C for 30 s, 58°C for 30 s, and 72 °C for 1 min for CTX-M, and a final extension step of 5 min at 72 °C was employed. Also, cycling parameters for SHV included 15 s of denaturation at 96 °C, 24 cycles consisting of 96 °C for 15 s, 50 °C for 15 s, and 72°C for 2 min, ending with a final extension period of 72 °C for 10 min. Amplification products were provisionally identified by their size in ethidium bromide-stained agarose gels. The positive amplified PCR product was analyzed with an automated DNA sequencing system.

RESULTS AND DISCUSSION

Antimicrobial agents are widely used to treat the bacterial infectious diseases of humans and animals, and in particular they are added to feed for the growth promotion of animals and prevention of diseases in livestock industry[27, 28]. In addition, antimicrobial agents that were used for clinical specimens and treatment of animals are flowing into river water through various routes such as sewage and livestock wastewater[29, 30, 31], and resistant bacteria inside river water can spread to humans and animals that use river water as drinking water[1].

Coliform and fecal coliform contamination of the Geumho River by region and by season is presented in Table 1. Coliform level in downstream stations (E, F and G) was high, measuring average 113~83 CFU/mL, whereas in upstream and midstream stations (A, C and D) except for B stations was low, measuring average 34~16 CFU/mL. Seasonally, coliform contamination in September and November was as high as 103 CFU/mL and 136 CFU/mL, respectively, whereas in May and June was as low as 24 CFU/mL and 13 CFU/mL, respectively. While the fecal coliform contamination was much lower than coliform contamination, the distribution of fecal coliform contamination showed similar tendencies with that of coliform contamination by region and by season. In particular, the vicinity of Paldal Bridge (station E) had the highest level of contamination of coliform

and fecal coliform due to the confluence of effluent discharged from the sewage disposal plant of the densely populated Daegu city.

In the double-disc synergy test of 21 isolates for which the MIC of ceftazidime was ≥2 ug/mL, all isolates produced ESBLs. All 21 isolates were identified as *E. coli*. The frequency of ESBL-producing strains reported for each country showed a big difference. *Enterobacter cloacae* and *Salmonella spp.* isolated from clinical specimens of hospitals in China and the Republic of South Africa showed a high frequency of ESBL genes of 30.5% and 39%, respectively[32, 33]. On the other hand, *E. coli* and *K. pneumonia*e isolated from clinical specimens in New Zealand showed a low frequency of ESBL genes of 0.7% and 4.2%, respectively[34]. In addition, in Korea *E. coli* and *K. pneumoniae* isolated from clinical specimens by Jeong *et al.,*[9] showed a frequency of ESBL genes of 9.2% and 30%, respectively, and Lee *et al.,*[19] also detected ESBL genes of 8.7% and 11.3% from *E. coli* and *K. pneumoniae* isolated from clinical specimens in domestic hospitals since 2005 through 2007, respectively and it was higher than the frequency of ESBL of 6.1% from coliforms isolated from Geumho River in this study.

ESBL-producing isolates have a high resistance to most cephalosporins[35, 36]. Padmini *et al.,*[36] reported that all ESBL-producing *E. coli* and *K. pneumoniae* are resistant to cefotaxime and ceftazidime, and Munday *et al.,*[35] also described that Enterobacteriaceae isolated from clinical specimens have a high resistance rate of 100% to cefotaxime and 69% to ceftazidime. In addition, most ESBL-producing strains are known to have multidrug resistance to antimicrobial agents[36, 37]. According to the study of Padmini *et al.,*[36], ESBL-producing isolates showed a high resistance rate of 91.0% to gentamicin and 86.2% to ciprofloxacin, and Liu *et al.,*[37] also reported that ESBL-producing *E. coli* isolated from animals have high resistance rate of 82~100% to ciprofloxacin, gentamycin, kanamycin and tetracycline, respectively. In the present study, ESBL-producing *E. coli* has a high resistance rate to β-lactams and non-β-lactam antimicrobial agents. All isolates were resistant to ampicillin, cefaclor, cefprozil, ceftriaxone, cephalexin, ciprofloxacin and nalidixic acid. They were highly resistant to tetracycline (95.2%) and cefotaxime (81.0%), followed by trimethoprim, gentamicin, kanamycin, streptomycin and ceftazidime with resistance rate of 38.1~61.9%,

Table 1. Coliform population of Geumho River

Sampling site[a]	Culture temp.(°C)	Month(2008)							Mean
		April	May	June	July	August	September	November	
A	37	10[b]	0	0	40	0	10	10	10
	44.5	0	0	0	0	0	0	0	0
B	37	20	0	0	50	50	10	430	80
	44.5	0	0	10	20	30	10	70	20
C	37	50	0	0	60	0	0	0	16
	44.5	0	0	0	0	0	0	0	0
D	37	70	20	30	40	30	20	30	34
	44.5	10	0	0	50	30	30	0	17
E	37	120	90	30	60	210	250	30	113
	44.5	10	0	10	50	80	160	50	51
F	37	10	30	0	60	50	400	30	83
	44.5	0	0	10	30	50	10	0	14
G	37	10	30	30	70	120	30	420	101
	44.5	0	20	30	10	90	30	180	51
Mean	37	41	24	13	54	66	103	136	62
	44.5	3	3	9	23	40	34	43	22

[a] A: Area around Yanghang bridge B: Area around Bongjuk bridge C: Area around Daebujamsu bridge
D: Area around Gangchon E: Area around Paldal bridge F: Area around Gangchang bridge
G: Area around Samunjin bridge
[b] Coliform and fecal coliform number in 1.0 mL of river water

respectively. The resistance rate to chloramphenicol was as low as 14.3%, and all isolates were susceptible to amikacin (Table 2). All isolates were resistant to eight or more antimicrobial agents. The isolates showed 15 different antimicrobial resistance patterns and the common resistance pattern was ApCfCtCpCdCr ChCiGmKmNaSmTcTp and ApCfCtCpCdCrCh CiKmNaTc seen in each 3 of the resistant strains, followed by ApCfCtCpCrChCiGmKmNaSmTcTp and ApCfCpCrChCiGmNaSmTcTp observed in each 2 of them (Table 3). In addition, the MIC ranges of β-lactams for the 21 ESBL-producing were 64~>512 ug/mL except for ceftazidime (≤1~64 ug/mL). MICs of the first-generation quinolone, nalidixic acid, of isolates were 256~>512 ug/mL, which was higher than those of the third-generation quinolone, ciprofloxacin (16~128 ug/mL). They had broad MICs of aminoglycosides (≤1~512 ug/mL) and chloramphenicol (4~>512 ug/mL), tetracycline (≤1~512 ug/mL), and trimethoprim (≤1~>512 ug/mL) except for low MIC ranges of amikacin (2~32 ug/mL) (Table 4).

As the results of detection of the TEM, SHV and CTX-M genes among 21 ESBL-producing isolates and their 14 transconjugants, all of them carried both TEM and CTX-M genes (Fig. 2 and 3), but none of them contained SHV gene (Data not shown). In genotypes of TEM genes, thirteen (61.9%) among 21 isolates produced TEM-166 and the remainders (38.1%) produced TEM-1 (Table 4). The TEM-116 was derived from TEM-1 by the Valine to Isoleucine substitution at position 84, and the Alanine to Valine substitution at position 184. In Korea, Jeong *et al.,*[9] first reported in *E. coli* and *K. pneumoniae* isolated from large domestic hospitals in 2002, and Lee *et al.,*[38] detected TEM-116 in *K. pneumoniae* isolated from domestic third general hospital and it was reported that it is isolated from only clinical isolates. In this study, TEM-116 was first detected in *E. coli* isolated from river water in Korea. The ESBL type of *E. coli* and *K. pneumoniae* isolated from clinical specimens in the region of Daegu city that discharged sewage to the Geumho River in 2000 is TEM-52, SHV-2a and SHV-12[39], and it was different from the result of this study that examined the ESBL type of *E. coli* isolated from the river water of Geumho River in 2008. It is supposed that this is due to the difference of antimicrobial agents of cephalosporins mainly used in 2000 and 2008, and it is also thought that it is needed to conduct an examination about the ESBL type that is prevalent in hospitals in Daegu city at present and a epidemiologic surveillance about the route in which TEM-116 type ESBL gene flowed into the Geumho River.

Table 2. Transferability of individual antimicrobial drug resistance of 21 ESBL-producing *E. coli* isolates

Classification	Antimicrobial drug	No.(%) of resistant strains	No.(%) of strains transferred resistance
β-lactams	Ampicillin	21(100)	14(66.7)
	Cefaclor	21(100)	12(57.1)
	Cefotaxime	17(81.0)	10(58.8)
	Cefprozil	21(100)	12(57.1)
	Ceftazidime	8(38.1)	1(12.5)
	Ceftriaxone	21(100)	12(57.1)
	Cephalexin	21(100)	12(57.1)
Quinolones	Ciprofloxacin	21(100)	0
	Nalidixic acid	21(100)	0
Aminoglycosides	Amikacin	0	0
	Gentamicin	12(57.1)	10(83.3)
	Kanamycin	12(57.1)	1(8.3)
	Streptomycin	10(47.6)	1(10)
Others	Chloramphenicol	3(14.3)	0
	Tetracycline	20(95.2)	10(50)
	Trimethoprim	13(61.9)	10(76.9)

Table 3. Drug resistant patterns of 21 ESBL-producing *E. coli* isolates and transconjugants

Multiplicity of resistance	Resistance patterns of donor	No. of donor	No. of donor with R plasmid	Resistance patterns of transconjugant	No. of transconjugant
14	ApCfCtCpCdCrChCiGmKmNaSmTcTp[a]	3	3	ApCfCtCpCdCrChGmKmTcTp	1
				ApCfCtCpCrChGmTcTp	1
				ApCfCtCpCrCh	1
13	ApCfCtCpCdCrChCiGmKmNaTcTp	1	1	ApCfCtCpCrChGmTcTp	1
13	ApCfCtCpCrChCiGmKmNaSmTcTp	2	2	ApCfCtCpCrChGmTcTp	1
				ApCfCtCpCrChGmTcTp	1
12	ApCfCpCrChCmCiKmNaSmTcTp	1	1	ApCfCpCrChTp	1
12	ApCfCtCpCrChCiGmKmNaTcTp	1	1	ApCfCtCpCrChGmTcTp	1
11	ApCfCtCpCdCrChCiKmNaTc	3			
11	ApCfCtCpCrChCmCiNaTcTp	1			
11	ApCfCtCpCrChCiGmKmNaTc	1	1	ApCfCtCpCrChGmTc	1
11	ApCfCtCpCrChCiGmNaSmTc	1	1	ApGm	1
11	ApCfCtCpCrChCiGmNaTcTp	1	1	ApCfCtCpCrChGmTcTp	1
11	ApCfCtCpCrChCiNaSmTcTp	1	1	ApCfCtCpCrChTcTp	1
11	ApCfCpCrChCiGmNaSmTcTp	2	1	ApCfCpCrCh	1
			1	ApGmSmTcTp	1
10	ApCfCpCrChCmCiNaTcTp	1			
10	ApCfCtCpCdCrChCiNaTc	1			
8	ApCfCtCpCrChCiNa	1			
Total		21	14		14

[a]Ap, ampicillin; Ct, cefotaxime; Cd, ceftazidime; Cr, ceftriaxone; Cf, cefaclor; Cp, cefprozil; Ch, cephalexin; Na, nalidixic acid; Ci, ciprofloxacin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Cm, chloramphenicol; Tc, tetracycline; Tp, trimethoprim

Table 4. Characterization of 21 ESBL-producing *E. coli* isolates

Sampling site[a]	Isolate No.	DDST	β-lactamase types		MIC(ug/mL) of antimicrobials[b]														
					Ap	Ct	Cd	Cr	Cf	Cp	Ch	Na	Ci	Ak	Gm	Km	Sm	Cm	Tc	Tp
A	D14	+	TEM-1	CTX-M-15	>512	256	8	64	512	>512	128	>512	64	8	64	64	8	4	256	>512
B	D1	+	TEM-116	CTX-M-15	>512	256	16	512	>512	>512	512	>512	32	2	64	8	256	4	256	>512
B	D2	+	TEM-1	CTX-M-14	>512	16	≤1	64	>512	>512	128	>512	64	8	2	64	8	4	128	≤1
B	D9	+	TEM-116	CTX-M-15	>512	512	32	512	>512	>512	512	256	128	4	≤1	≤1	4	8	128	≤1
B	D10	+	TEM-116	CTX-M-15	>512	256	32	256	>512	>512	512	>512	32	2	≤1	4	8	4	≤1	≤1
B	D16	+	TEM-1	CTX-M-15	>512	256	16	64	>512	>512	512	>512	64	4	32	32	8	4	256	>512
B	D17	+	TEM-116	CTX-M-15	>512	512	16	512	>512	>512	512	>512	64	32	256	256	32	4	256	>512
B	D20	+	TEM-116	CTX-M-14	>512	16	2	64	512	>512	128	>512	64	4	≤1	32	256	4	128	>512
C	D3	+	TEM-1	CTX-M-15	>512	>512	64	512	>512	>512	>512	>512	64	8	128	64	16	4	256	>512
D	D15	+	TEM-1	CTX-M-15	>512	512	32	512	>512	>512	512	>512	64	4	64	64	8	4	256	>512
D	D21	+	TEM-116	CTX-M-15	>512	256	64	512	>512	>512	512	512	128	4	≤1	64	4	4	128	≤1
E	D6	+	TEM-1	CTX-M-15	>512	256	16	128	>512	>512	512	>512	64	4	32	64	128	4	256	>512
E	D7	+	TEM-116	CTX-M-15	>512	>512	64	>512	>512	>512	>512	>512	32	2	64	16	128	4	128	>512
E	D12	+	TEM-116	CTX-M-15	>512	256	16	128	>512	>512	256	>512	64	2	256	32	64	4	256	≤1
E	D18	+	TEM-1	CTX-M-27	>512	16	2	64	64	512	128	>512	128	4	64	64	128	16	512	>512
F	D13	+	TEM-1	CTX-M-14	>512	16	≤1	64	512	>512	64	>512	32	4	2	32	4	256	256	>512
G	D4	+	TEM-116	CTX-M-15	>512	>512	64	512	>512	>512	512	>512	128	8	64	64	8	4	128	≤1
G	D5	+	TEM-116	CTX-M-15	>512	512	16	512	>512	>512	512	256	16	2	≤1	4	8	512	32	>512
G	D8	+	TEM-116	CTX-M-15	>512	256	16	512	>512	>512	512	>512	64	4	32	64	128	4	256	>512
G	D11	+	TEM-116	CTX-M-15	>512	>512	64	512	>512	>512	512	512	16	2	≤1	>512	512	256	128	>512
G	D19	+	TEM-116	CTX-M-15	>512	256	16	128	>512	>512	512	512	128	4	≤1	64	4	4	128	≤1

[a] A: Area around Yanghang bridge , B: Area around Bongjuk bridge , C: Area around Daebujamsu bridge, D: Area around Gangchon , E: Area around Paldal bridge , F: Area around Gangchang bridge , G: Area around Samunjin bridge.

[b]Ap, ampicillin; Ct, cefotaxime; Cd, ceftazidime; Cr, ceftriaxone; Cf, cefaclor; Cp, cefprozil; Ch, cephalexin; Na, nalidixic acid; Ci, ciprofloxacin; Ak, amikacin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Cm, chloramphenicol; Tc, tetracycline; Tp, trimethoprim

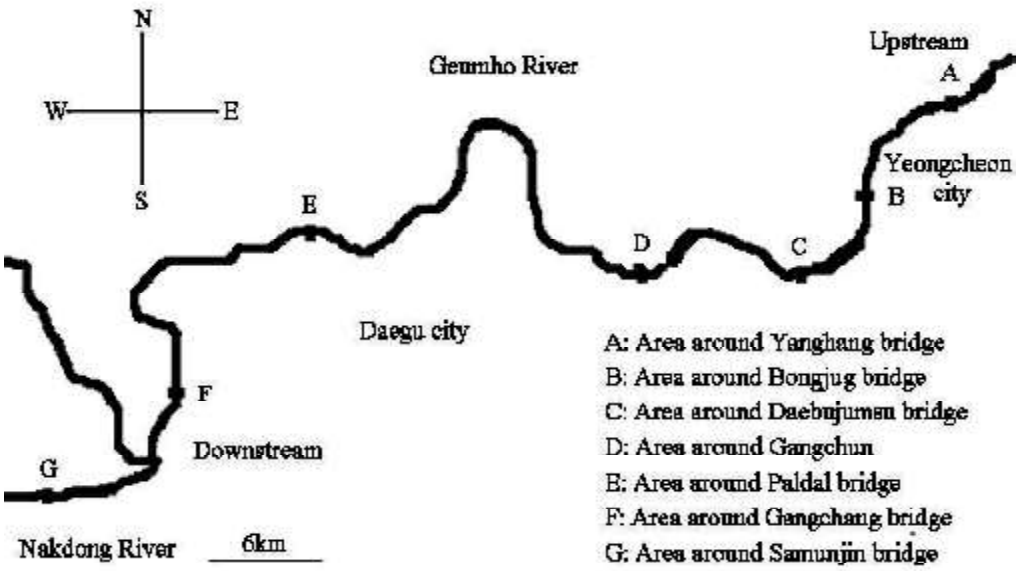
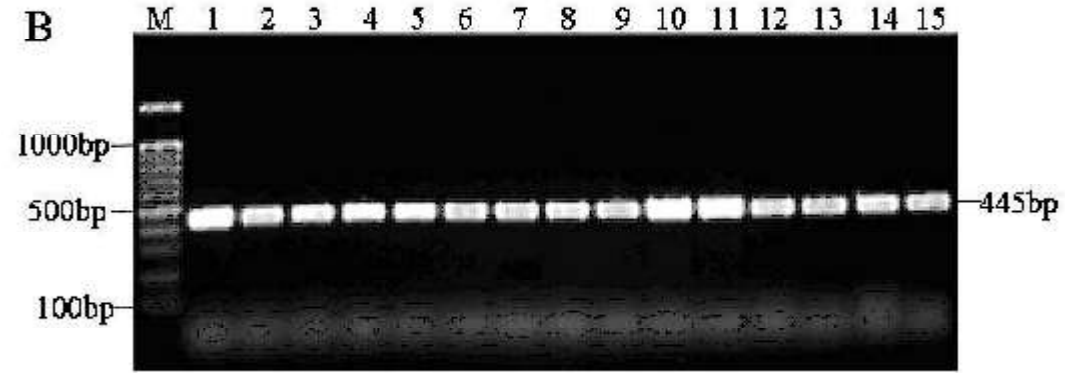


Fig. 1. Sampling site of Geumho River (A, B, C, D, E, F and G: tested)

A; Lanes: M, 100 bp DNA ladder; 1, positive control for TEM gene; donor isolate number: 2, D1; 3, D2; 4, D3; 5, D4; 6, D5; 7, D6; 8, D7; 9, D8; 10, D9; 11, D10; 12, D11; 13, D12; 14, D13; 15, D14; 16, D15; 17, D16; 18, D17; 19, D18; 20, D19; 21, D20; 22, D21.

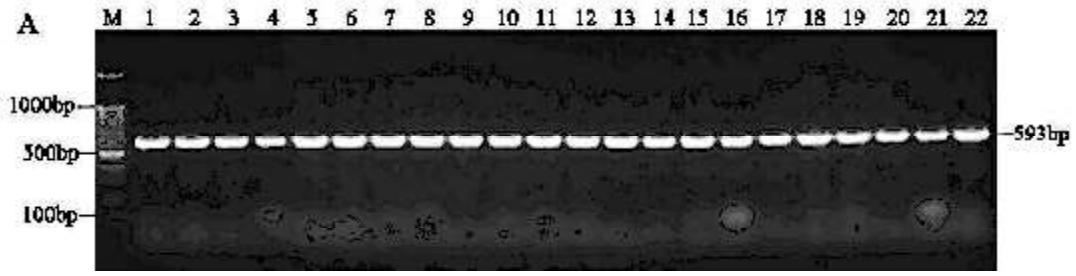


B; Lanes: M, 100 bp DNA ladder; 1, positive control for TEM gene; transconjugant number: 2, T1; 3, T2; 4, T6; 5, T7; 6, T8; 7, T9; 8, T10; 9, T12; 10, T13; 11, T14; 12, T15; 13, T17; 14, T19; 15, T20.

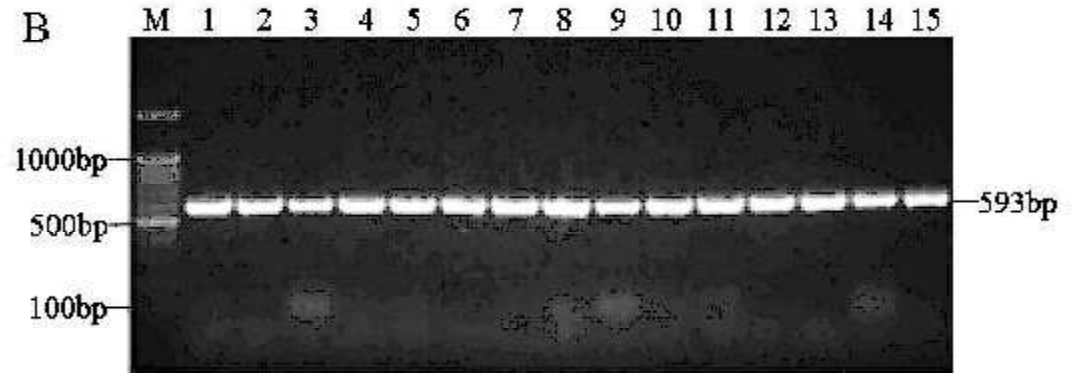
Fig. 2. PCR for detecting TEM gene in donor isolates (A) and transconjugants (B)

ESBL gene types of TEM-52 and SHV-12 were detected from the river water of the region of Busan city[11]. They are different from the ESBL gene type of TEM-116 that were detected in this study, and it is thought that this is due to the difference of antimicrobial agents of cephalosporin that are used in each region.

CTX-M enzymes hydrolysis and confer resistance to cefotaxime preferentially over ceftazidime, which is reflected in substantially higher MICs to cefotaxime than to ceftazidime[15]. In this study, all isolates demonstrated cefotaxime MICs at least eight-fold greater than those of ceftazidime. In genotypes of CTX-M genes, the most common type of CTX-M was CTX-M-15 (n=17, 81.0%). Genes encoding CTX-M-14 (n=3, 14.2%) and CTX-M-27 (n=1, 4.8%) were also detected (Table 4). Since the CTX-M-15 was first detected in India in 2001[40], it is prevalent in Europe, Oceania, the United States and Asia[13]. Since Bae *et al.,*[18] reported CTX-M-15 in 2 isolates of *E. coli* in Korea, it is increasing in clinical strains isolated from large hospitals[8, 19, 41]. Sung *et al.,*[42] and Lim *et al.,*[43] also detected CTX-M-15 from domestic avian-pathogenic *E. coli* and *E. coli* originating in pigs. CTX-M-14 and CTX-M-27 belong to the CTX-M-9 group. CTX-M-14 was derived from CTX-M-9 by Alanine to Valine substitution at position 231. This enzyme was first detected in *K. pneumonia*, *E. coli* and *Shigella sonnei* isolates from Korea in 2001[17]. CTX-M-27 was originally identified in an *E. coli* strain from France in 2000[44], and in Korea was first detected in human clinical isolates collected in 2007[45]. CTX-M-27 differs from CTX-M-14 only by Aspartic acid to Glycine substitution at position 240 conferring higher levels of resistance to ceftazidime than CTX-M-14[44]. In this study, the MICs of ceftazidime in the one CTX-M-27-producing isolates were 2 ug/mL, higher than or equal that of the three CTX-M-14-producing isolates (≤1~2 ug/mL). In addition, the MICs of cefotaxime (16 ug/mL) and ceftazidime (≤1~2 ug/



A; Lanes: M, 100 bp DNA ladder; 1, positive control for CTX-M gene; donor isolate number: 2, D1; 3, D2; 4, D3; 5, D4; 6, D5; 7, D6; 8, D7; 9, D8; 10, D9; 11, D10; 12, D11; 13, D12; 14, D13; 15, D14; 16, D15; 17, D16; 18, D17; 19, D18; 20, D19; 21, D20; 22, D21.



B; Lanes: M, 100 bp DNA ladder; 1, positive control for CTX-M gene; transconjugant number: 2, T1; 3, T2; 4, T6; 5, T7; 6, T8; 7, T9; 8, T10; 9, T12; 10, T13; 11, T14; 12, T15; 13, T17; 14, T19; 15, T20.

Fig. 3. PCR for detecting CTX-M gene in donor isolates (A) and transconjugants (B)

mL) for *E. coli* harboring CTX-M-14 and CTX-M-27 are lower than those of *E. coli* harboring CTX-M-15. As above mentioned, the CTX-M-14, CTX-M-15, and CTX-M-27 genes had been previously detected in human and veterinary clinical isolates but never before in *E. coli* isolates in river water in Korea[17,18,42,43,45].

In our conjugation study, 14 (66.7%) among 21 isolates transferred their antimicrobial resistance to recipient strains. Transferability of each antimicrobial agents was as high as 83.3~66.7% to gentamicin, trimethoprim, or ampicillin, respectively, followed by cefaclor, cefotaxime, cefprozil, ceftriaxone, cephalexin, or tetracycline with transferability of 50.0~58.8%, respectively. Meanwhile, transferability to ceftazidime, streptomycin, or kanamycin was as low as 8.3~12.5%, and all isolates with resistance to ciprofloxacin, nalidixic acid, or chloramphenicol did not transferred these resistances to recipient strain. Most of the transconjugants showed the multidrug resistance phenotypes similar to donors. In addition, the β-lactam resistance genes of ESBL-producing strains are carried on plasmids. It was known that the CTX-M-encoding genes are located at the plasmids, and some TEM-1 genes are also located at the same plasmids[17]. The ESBL gene that is identical with the donor strain was detected in 14 transconjugants (66.7%) and this result showed higher transferability of ESBL genes than 52.0% of Jeong *et al.,*[9] and 56.5% of Padmini *et al.,*[36]. It was thought that ESBL gene detected in river water and clinical isolates in human and veterinary spread among microorganisms via horizontal transfer of plasmid.

In conclusion, this study illuminates that the isolates containing not only TEM-type ESBL and but also CTX-M-type ESBL are not limited to clinical isolates and their habitats are being diversified to even the hydrosphere through various routes such as sewage and livestock waste. It is also thought that it will be possible to spread the ESBL-producing strains to humans and animals that took in river water. Therefore we need to consider the wide-ranging epidemiologic surveillance about the occurrence of ESBL-producing strains in the hydrosphere and measures for prevention of spread in the near future.

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