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Detection of CTX-M and Clonal Analyses using MLST in Cefotaxime Resistant *Escherichia coli* Isolated from the Han-River, Korea

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Abstract − Bacteria resistant to various antibiotics have recently become an issue of the utmost importance. Resistant strains are not uncommon, even in municipal drinking water sources. The health threat posed by resistant, pathogenic bacteria has serious ramifications for both public health and agriculture. In this study, we isolated antibiotic resistant bacteria from water samples from the Han River, Korea, which is contaminated by the wastewater from many industrial complexes, hospitals, agricultural and animal husbandry estates, and from wastewater treatment facilities. We determined the degrees of resistance to various antibiotics exhibited by the isolated strains. The similarities between the isolated *E. coli* strains were examined, using the pulsed field gel electrophoresis and multilocus sequence typing, in order to trace their origins and to explore the syntechnic adaptations and pathogenicity of the various strains and relate these to their genetic sequence. A total of 25 *E. coli* strains were isolated from six stations along the Han River. All the 25 strains exhibited resistance to ampicillin. We also investigated resistance to amoxicillin, clavulanic acid, cefazolin, cofoxitin, cefotaxime, cefpodoxime, ceftriaxone, cefepime, nalidixic acid, aztreonam, ciprofloxacin, streptomycin, gentamicin, chloramphenicol and imipenem. Based on the ESBL detection, 14 strains belonged to the ESBL producing strains. The number of the clonal complex producing strains was 5 among the 14 isolated strains. The 5 strains were included in the 168, 23, 38, 469, 156 clonal complex, respectively. The rest 9 strains were not included in the clonal complex, but showed independent STs.

Key words : antibiotic, *Escherichia coli*, CTX-M, cefotaxime, MLST

INTRODUCTION

Since the discovery of ampicillin, β-lactam type antibiotics have frequently been used for the treatment of infectious diseases caused by gram negative, rod shaped bacteria. Recently, however, there has been an increase in the presence of *Escherichia coli* producing CTX-M type Extended-Spectrum β-lactamase (ESBL), an enzyme group that appears to induce resistance to β-lactam type antibiotics. This rise in prevalence has resulted in many of the bacteria resident in hospitals developing resistance to these antibiotics (Clermont *et al.* 2009; Qi *et al.* 2010; Mora *et al.* 2011; Peirano *et al.* 2011). The enzyme group ESBL allows enterobacteria to acquire resistance to β-lactam type antibiotics (Hsieh *et al.* 2010) as these enzymes hydrolyze oxymino-cephalosporins (i.e., cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefepime) and monobactams (i.e., aztreonam) (Paterson *et al.* 2005). The expression of the ESBL gene, which is encoded in a plasmid, induces the hydrolysis of the amide bonds of β-lactam ring compounds, and this leads to the inactivation of the β-lactam ring and resistance to β-lactam type antibiotics (Bonnet 2004; Tomanicek *et al.* 2010; Cindy *et al.* 2010).

Since the initial detection of ESBL in *Klebsiella pneumonia*, in Germany, 1983, it has been found in a wide variety of

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bacteria, such as *Salmonella* spp., *Pseudomonas* spp., *Acinetobacter* spp. and *Klebsiella* spp. (Eckert *et al.* 2004; Dierikx *et al.* 2010; Horii *et al.* 2011; Wannaprasat *et al.* 2011; Zou *et al.* 2011). Recently, many gram negative, rod-shaped bacteria have also been reported to produce ESBL (Lewis *et al.* 2007). ESBLs can be separated into four classes: classes A, B, C and D, where the A, C and D are serine types and B is a zinc type. Among the ESBLs, SHV, exhibiting a high homogeny between the amino acid sequences, TEM, and CTX-M type exist in the class A ESBL (Pitout *et al.* 2008; Tomanicek *et al.* 2010). Class A ESBLs exhibit hydrolytic activity against oxymino-cephalosporins and aztreonam type antibiotics, but not against 7-α-substituted β-lactams, and as a result bacteria producing class A ESBLs are generally susceptible to clavulanate, sulbactam and tazobactam, which are inhibitors of β-lactamase type antibiotic resistance (Bonnet 2004).

Both SHV and TEM type β-lactamases have already been distributed worldwide through plasmid transfer. Various point mutations in the $bla_{\text{TEM-1}}$, $bla_{\text{TEM-2}}$, $bla_{\text{SHV-1}}$ genes, which encode TEM β-lactamase, have resulted in the evolution of the CTX-M, VEB, GES/IBC, PER, TLA, BES, OXA and SFO types of β-lactamase (Philippon *et al.* 1994; Kim *et al.* 2005; Rossolini *et al.* 2008; Lee *et al.* 2009; Reinthaler *et al.* 2010). Since the CTX-M-type ESBL was first identified in Japan, Europe and Argentina in the 1980's, it has been a well-known mediator of resistance similar to TEM or SHV (Bonnet *et al.* 2004; Kim *et al.* 2008). Currently, the CTX-M type ESBL family can be classified into six groups, epitomized by CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and CTX-M-45; each group includes more than 60 different β-lactamases (Rossolini *et al.* 2008; Pitout *et al.* 2008; Achour *et al.* 2009). CTX-M type ESBLs exhibit stronger hydrolytic activity against the 3rd generation, cephalosphorin type antibiotics than against the 2nd generation, cephalosporin type antibiotics (Walther-Rasmussen *et al.* 2004; Corvec *et al.* 2010); as a result of this, combined with the rapid spread of ESBL *E. coli* strains exhibiting resistant to various β-lactam antibiotics, many bacterial infections globally cannot be treated with the 3rd generation cephalosporin type antibiotics and incur high mortality rates (Tenover *et al.* 2003; Qi *et al.* 2010; Peirano *et al.* 2010).

The ESBL producing genes commonly found in *E. coli* can be transferred to other bacterial species through horizontal plasmid transfer. This gene has become a serious matter of concern from a clinical view point, because it is being detected in many other enterobacteria, such as *Kluyvera ascorbata*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Bonnet *et al.* 1999).

River water, which may often be used as a municipal drinking-water source, is commonly polluted by wastewater from residential and industrial districts, which may contain high concentrations of antibiotics (Perrentin *et al.* 1997). Moreover, it may also be contaminated by *Enterobacteriaceae* that may cause infectious disease among both humans and animals, and which may evolve resistance against the antibiotics polluting the habitat (Smith *et al.* 1994). Most of the antibiotic pollutants originate from those used in agriculture and medicine, 30 to 90% of which is excreted as urine. Continuous exposure to antibiotics allows bacteria to develop resistance (Armstrong *et al.* 1982), and wastewater treatment facilities provide ample opportunities for this process (Thomas *et al.* 2003). Genetic conferring resistance can effectively spread from these areas via the conjugal transfer of plasmids (Rossolini *et al.* 2008). Smalla *et al.* (2002) discovered mobile genetic elements from many different habitats within isolates from a wastewater treatment system. Furthermore, the antibiotic resistance exhibited by many clinical bacterial strains may have originated from the natural environment (Oppegaard *et al.* 2001).

The Han River flows across the center of Seoul, supplying domestic and industrial estates with water (Kim *et al.* 2008). The existence of the antibiotic resistant, pathogenic bacteria in the Han River could impose a large cost to public health and agriculture (Qureshi *et al.* 1992; Jung *et al.* 2002). However, there have been no scientific reports of antibiotic resistant bacteria isolated from the lifeline of the capital city of Korea. In this regard, we isolated bacteria from surface water samples collected at different locations along the Han River, which is polluted by the wastewater from industrial complexes, hospitals, agriculture and animal husbandry estates, and wastewater treatment facilities. We determined the degree of resistance to various the 2nd and 3rd generation cephalosphorin type antibiotics, which are frequently used for the prevention and cure of diseases in clinics as well as for animal husbandry, exhibited by the isolated *E. coli* strains. For the detection of the CTX-M type ESBL encoding gene, $bla_{\text{CTX-M}}$, *E. coli* exhibiting resistance against the 3rd generation cephalosporin type antibiotics were sequenced. The similarities between the isolated *E. coli* strains were examined

using the pulsed field gel electrophoresis and multilocus sequence typing to trace the source of the isolated strains and to explore the syntechnic adaptations and pathogenicity of the strains, which were related to their genetic sequence.

MATERIALS AND METHODS

1. Sample collection

The river water samples were collected in November, 2009 from the surface water of a station located on the upper Han-River, Paldangdaegyo (Gyeonggi, Korea), and five other stations located at the lower reaches of the Han-River: Wangsukchon (Gyeonggi), Tanchon, Seongsudaegyo, Seongsandaegyo and Anyangchon (Seoul, Korea). As shown in Fig. 1 and Table 1.

2. Isolation and identification of the bacterial strains

The river water samples (1,000 mL each) harvested from the 6 stations were filtered through a 0.45 μm pore sized membrane filter (Advantec. Japan). The diffusion culture was performed in EC broth (Difco, USA) at 37˚C for 24 hours, followed by incubation at 37˚C for 24 hours in Eosin Methylene Blue (EMB) Agar (Difco) containing cefotaxime (Sigma, USA). The colonies manifesting a metallic gloss, which typically represents *E. coli*, were selected and a pure culture was obtained in Luria-Bertani (LB) broth (Difco) at 37˚C for 24 hours.

The biochemical identification of the pure culture was carried out using the Vitek system (BioMérieux, France). The strains exhibiting a positive response on the oxidase test were inoculated by means of the Gram Negative Identification (GNI+) card (bioMérieux) and then identified using the Vitek system. Each selected strain was incubated in the LB broth. Total genomic DNA was extracted using the DNeasy® blood & tissue kit (Qiagen, Netherland). Molecular biological identification was performed according to the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov) based on the analyses of PCR products produced through 16S rDNA sequencing of extracted total genomic DNA.

3. The minimum inhibitory concentration

MIC values were determined according to the method suggested by the Clinical Laboratory Standard Institute (CLSI, 2009). The isolated *E. coli* strains were inoculated into Mueller Hinton (MH) Broth (Difco) and incubated at 37˚C for 24 hours, followed by dilution to a concentration of 0.5 McFarland standard to perform the antibiotics susceptibility tests.

Disk diffusion tests were conducted with the diluted suspensions of the isolated strains, which were smeared on MH Agar (Difco) plates. For the antibiotic disks, the following compounds (concentrations) were used: streptomycin (10 μg), ampicillin (10 μg), aztreonam (30 μg), amoxicillin with clavulanic acid (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), cefazolin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), cefpodoxime (10 μg), ceftriaxone (30 μg), cefepime (30 μg), gentamicin (120 μg), imipenem (10 μg), ciprofloxacin (5 μg). All the antibiotics were purchased from Becton Dickinson (BBL, USA).

The MIC tests were performed according to the agar dilution method. Each solution of cephalothin, cefoxitin, cefotaxime and ceftazidime in MH Agar was diluted to the predetermined concentration, to which the *E. coli* pure culture suspension was inoculated after incubation at 37˚C for 24 hours in MH Broth followed by dilution to the McFarland 0.5 concentration. *E. coli* ATCC 25922 was used as the control strain. Both the disk diffusion test results and the MIC were interpreted after incubating the final cell suspension at 37˚C for 18 hours.

4. Detection of ESBL producing *E. coli*

The detection of ESBL was determined according to the method suggested by the CLSI. The antibiotics used were cefoxitin (30 μg), cefoxitin/clavulanic acid (30/10 μg), cefotaxime (30 μg) and cefotaxime/clavulanic acid (30/10 μg). The production of ESBL was recorded as positive when the difference in the diameter of the growth inhibition zone in the disk diffusion tests with or without clavulanic acid was larger than 5 mm (CLSI, 2009).

5. Detection of CTX-M type ESBL

For the ESBL producing cefotaxime resistant strains, the presence of CTX-M type ESBL was detected by performing PCR using primers as suggested Johann *et al.* (2004). The PCR conditions comprised of 30 cycles of predenaturation at 94˚C for 2 min, denaturation at 94˚C for 15 sec, annealing at 55～65˚C for 15 sec, extension at 72˚C for 30 sec, and a final extension at 72˚C for 3 min. The PCR products were

subjected to nucleotide sequence analyses to confirm the presence of CTX-M type ESBL based on the NCBI BLAST database.

6. Pulsed field gel electrophoresis (PFGE)

The genomic homogeny between the isolated *E. coli* strains was analyzed using PFGE (Gautom, 1997). The isolated strains were incubated in LB broth at 37°C for 24 hours, following which plugs were made using 1% agarose gel. The genomic DNA was treated with *Xba* I restriction enzyme (Enzynomics, Korea) for 18 hours. Electrophoresis was performed with 1% seakem golden agarose at 14°C, 6 V cm^{-1} for 20 hours in CHEF-DR III (Bio-Rad, USA) containing 0.5× TBE buffer (0.09 M of Tris, 2 mM of disodium EDTA: pH 8.5, 0.09 M of boric acid).

7. Analyses of multilocus sequence typing (MLST)

The MLST of the isolated *E. coli* strains was conducted according to the method suggested in the website (http://mlst.ucc.ie/mlst/dbs/Ecoli). The primers for the seven housekeeping genes were *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *recA* and *purA* (Wirth *et al.* 2006). The Sequence Type (ST) was identified by analyzing the nucleotide sequence of the PCR products of the seven housekeeping genes and, based on the assayed sequences, the ST was finally determined through MLST database analyses. The ST was compared to that of the MLST database to confirm the clonal complex through the eBURST v3 program (http://eburst.mlst.net).

RESULTS AND DISCUSSION

1. Isolation and identification

E. coli strains were isolated from surface water samples harvested from six stations along the Han River as shown in Fig. 1. The isolation of bacterial strains was achieved using EMB agar with added cefotaxime. The isolated strains were identified through 16S rDNA nucleotide sequence analyses and according to their biochemical characteristics by the Vitek system. As summarized in Table 1, a total of 25 different *E. coli* strains were isolated: 6 strains from the station A, 4 from the station B, 3 from the station C, 2 from the station D, 4 from the station E and 6 from the station F. It is interesting that six strains were isolated from station A, even though it was located at upstream, while only 2～4 strains were isolated from stations B-E, which were situated in densely populated areas.

Table 1. The isolated strains from the Han River, Korea in Nov., 2009

Station	A	B	C	D	E	F
Isolated strains	EC 1-1	EC 2-1	EC 3-3	EC 4-4	EC 5-1	EC 6-1
	EC 1-2	EC 2-2	EC 3-4	EC 4-5	EC 5-2	EC 6-3
	EC 1-3	EC 2-3	EC 3-7		EC 5-3	EC 6-4
	EC 1-4	EC 2-4			EC 5-6	EC 6-5
	EC 1-5					EC 6-6
	EC 1-7					EC 6-7

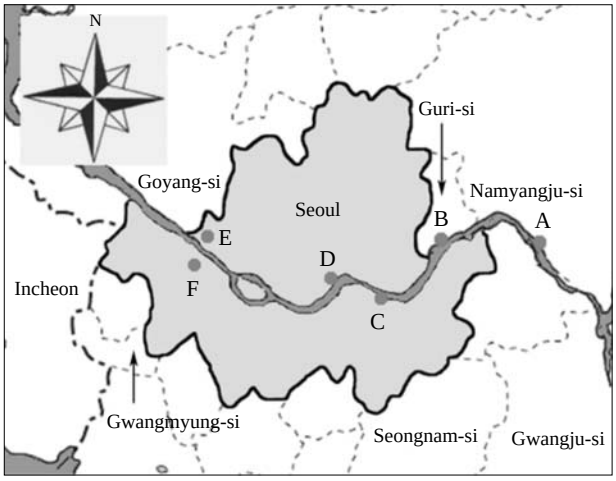

Fig. 1. The location of the sampling stations along the Han-River, Korea.

Kim *et al.* (2008) isolated and identified 22 ESBL producing *E. coli* strains using MacConkey agar with added 16 μg mL^{-1} cephalothin and the API 20E kit from the Han River basin. Jung *et al.* (2002) isolated 25 *E. coli* strains exhibiting resistance to norfloxacin from the Han River.

2. Susceptibility of the isolated strains to antibiotics

The antibiotic resistances of the 25 isolated *E. coli* strains were investigated using the disk diffusion test method (CLSI, 2009), as summarized in Table 2. All 25 strains exhibited the resistance to ampicillin, while only one strain was resistant to amoxicillin with added clavulanic acid. Lim *et al.* (2007) isolated 334 and 410 *E. coli* strains from cow and pig feces, respectively, from which they observed that 40 out of the 334 *E. coli* strains isolated from cow feces and 271 out of the 410 *E. coli* strains isolated from pig feces exhibited resi-

Table 2. The antibiotics resistance of the isolated strains

Antibiotics		Resistant (n=25)	Intermediate (n=25)	Susceptible (n=25)
Penicillins (β-lactam/β-lactactamase combination)	AM*	25 (100%)	0 (0%)	0 (0%)
	AMC*	1 (4%)	7 (28%)	17 (68%)
Cephalosporins	CZ*	25 (100%)	0 (0%)	0 (0%)
	FOX*	1 (4%)	0 (0%)	24 (96%)
	CTX*	25 (100%)	0 (0%)	0 (0%)
	CPD*	25 (100%)	0 (0%)	0 (0%)
	CRO*	25 (100%)	0 (0%)	0 (0%)
	FEP*	15 (60%)	8 (32%)	2 (8%)
Quinolone	NA*	19 (76%)	1 (4%)	5 (20%)
Monobactam	ATM*	18 (72%)	7 (28%)	0 (0%)
Fluoroquinolone	CIP*	11 (44%)	1 (4%)	13 (52%)
Aminoglycosides	S*	7 (28%)	14 (56%)	4 (16%)
	GM*	5 (20%)	0 (0%)	20 (80%)
Phenicol	C*	5 (20%)	1 (4%)	19 (76%)
Carbapenem	IPM*	0 (0%)	0 (0%)	25 (100%)

AM* : ampicillin, AMC* : amoxicillin with clavulanic acid, CZ* : cefazolin, FOX* : cefoxitin; CTX* : cefotaxime, CPD* : cefpodoxime, CRO* : ceftriaxone, FEP* : cefepime, NA* : nalidixic acid, ATM* : aztreonam, CIP* : ciprofloxacin, S* : streptomycin, GM* : gentamicin, C* : chloramphenicol, IPM* : imipenem

stance against ampicillin; however, only one *E. coli* strain isolated from pig feces was resistant to amoxicillin with added clavulanic acid.

The resistance of the isolated strains towards cephalosporin antibiotics such as cefazolin, cofoxitin, cefotaxime, cefpodoxime, ceftriaxone and cefepime was examined; among the 25 isolated strains, 25, 1, 25, 25, 25 and 15 strains displayed resistance against the respective antibiotics. With respect to nalidixic acid, aztreonam and ciprofloxacin, 19, 18 and 11 strains were resistant, respectively. Seven strains were resistant to streptomycin, five to gentamicin, and five strains were resistant to chloramphenicol. However, none of the 25 strains were resistant to imipenem, which is concurrent with Edelstein *et al.* (2003), in which none of the 78 strains isolated from the hospital wastewater demonstrated imipenem resistance.

3. The analyses of the PFGE pattern

PFGE was performed for the 25 isolated strains as shown in Fig. 2. The nucleotide pattern distributions of the isolated strains, which had been digested by *Xba* I restriction enzyme, were expressed as subtypes. The PFGE results are illustrated in Fig. 2 for the 14 strains that had different subtypes. When the genomes of the 25 isolated *E. coli* strains were treated with *Xba* I, the nucleotide fragments appeared as 10∼20 band patterns as displayed in Fig. 2. The strains isolated from the station F, i.e., EC 6-1, EC 6-4 and EC 6-5, exhibited nucleotide fragment patterns with a similar but not completely coincident homogeny.

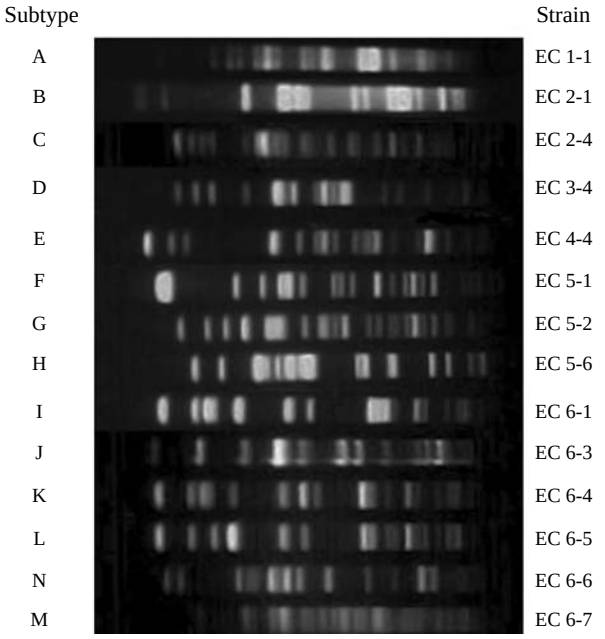

Fig. 2. The PFGE of the antibiotics resistant strains. Genomic DNA of the antibiotics resistant strains was digested with *Xba* I.

4. Confirmation of ESBL producing *E. coli* and detection of CTX-M type ESBL

Since CTX-M type ESBL plays a crucial role in the evo-

lution of resistance against the 3rd generation, cephalosporin type antibiotics and the loss of resistance to the 2nd generation, cephalosporin type antibiotics (Corvec *et al.* 2010), we tested 14 of the isolated strains for ESBL production, the results of which are shown in Fig. 2. The 14 isolated strains, each with a different PFGE subtype, were incubated in medium in the presence of the 2nd or 3rd generation, cephalosporin type antibiotics, i.e., cefoxitin and cefotaxime with or without clavulanic acid (CLSI 2009). Those strains producing a growth inhibition zone larger than 5 mm in diameter were classified as positive with respect to the production of ESBL. Based on this classification criterion, all the 14 strains were proved to be capable of producing ESBL.

The strains positive for CTX-M type ESBL are listed in Table 3. All the ESBL producing strains, except EC 5-1, were susceptible to cefoxitin but were resistant to cefotaxime. CTX-M type ESBL detection indicated that the numbers of the strains positive for CTX-M-32, CTX-M-15, CTX-M-14 and CTX-M-64 were 5, 3, 2 and 1, respectively. Similarly, Hsieh *et al.* (2010) also reported that ESBL producing *E. coli* exhibited strong resistance to cephalosporin type antibiotics. Oh *et al.* (2005) reported that *E. coli* strains isolated from domestic hospitals commonly produced CTX-M type ESBL. Rossolini *et al.* (2008) revealed that CTX-M-15 and CTX-M-32 ESBLs are formed by the substitution of Asp240 of CTX-M-1 and of CTX-M-3 to Gly, respectively; this mutation takes place more commonly compared to that of CTX-M-1. Poirel *et al.* (2002) observed that these ESBLs (CTX-M-15 and CTX-M-32) exhibit strong resistance to the 3rd generation, cephalosporin type antibiotics such as cefotaxime, ceftriaxone, cefpodoxime and ceftazidime. CTX-M-14 has also been detected in *Shigella sonnei* isolated from gastroenteritis patients (Bonnet *et al.* 2004). CTX-M type ESBLs belong mostly to the CTX-M-1 and CTX-M-9 group ESBLs (Rosolini *et al.* 2008; Lee *et al.* 2009). The strains exhibiting CTX-M type ESBLs have developed resistance to a variety of antibiotics, and according to Gona *et al.* (2011), an increasing number of CTX-M type ESBL positive strains now exhibit multi drug resistant (MDR) characteristics. The *E. coli* strains isolated in the present study also showed the MDR characteristics, in that they were resistant to more than two types of antibiotics in addition to β-lactam type antibiotics. As demonstrated in Table 5, EC 6-4 exhibited resistance to 12 antibiotics and the other isolated strains were all resistant to more than six antibiotics.

5. MIC value of the ESBL producing *E. coli*

Table 4 summarizes the MIC values of the 14 strains shown in Fig. 2 as having different PFGE subtypes toward the 2nd/3rd generation cephalosporin type antibiotics. All the strains producing ESBL had MIC values higher than 512 μg mL^{-1} for cephalothin. The MIC values for cefotaxime were higher than 256 μg mL^{-1}, except in one strain with an MIC value of 128 μg mL^{-1}. However, the MIC values for cefotaxitin were far less than for cefotaxime for all isolated strains, except EC 5-6, which had an MIC value of 512 μg mL^{-1} for cefotaxitin. These results are in line with those reported by Achour *et al.* (2009), in that CTX-M type ESBL strains isolated from clinics had MIC values of 8 μg mL^{-1} for cefoxitin, while the MIC values against cefotaxime were

Table 3. The CTX-M type ESBLs and antibiotics resistant of the isolated *E. coli* strains

Isolated strain	ESBL production	CTX-M type	Antibiotics resistant by CLSI
EC 1-1	Yes	CTX-M-64	AM*, ATM*, NA*, CZ*, CTX*, FEP*, CPD*, CRO*
EC 2-1	Yes	ND*	AM, ATM, NA, CZ, CTX, FEP, CPD, CRO
EC 2-4	Yes	CTX-M-15	S*, AM, ATM, NA, CZ, CTX, FEP, CIP*, CPD, CRO
EC 3-4	Yes	CTX-M-15	AM, NA, CZ, CTX, CPD, CRO
EC 4-4	Yes	CTX-M-15	S, AM, ATM, NA, CZ, CTX, CIP, CPD, CRO
EC 5-1	Yes	ND	AM, ATM, AMC*, CZ, FOX*, CTX, FEP, CPD, CRO
EC 5-2	Yes	CTX-M-14	AM, C*, NA, CZ, CTX, CIP, CPD, CRO
EC 5-6	Yes	CTX-M-32	AM, ATM, CZ, CTX, FEP, CPD, CRO
EC 6-1	Yes	CTX-M-32	AM, ATM, C, NA, CZ, CTX, CIP, CPD, CRO
EC 6-3	Yes	CTX-M-32	AM, NA, CZ, CTX, CIP, CPD, CRO
EC 6-4	Yes	CTX-M-32	S, AM, ATM, C, NA, CZ, CTX, FEP, GM, CIP, CPD, CRO
EC 6-5	Yes	CTX-M-32	S, AM, ATM, C, NA, CZ, CTX, GM, CIP, CPD, CRO
EC 6-6	Yes	CTX-M-14	S, AM, NA, CZ, CTX, FEP, GM*, CIP, CPD, CRO
EC 6-7	Yes	ND	S, AM, ATM, C, NA, CZ, CTX, GM, CIP, CPD, CRO

S* : streptomycin, AM* : ampicillin, ATM* : aztreonam, AMC* : amoxicillin with clavulanic acid, C* : chloramphenicol, NA* : nalidixic acid, CZ* : cefazolin, FOX* : cefoxitin, CTX* : cefotaxime, FEP* : cefepime, GM* : gentamicin, CIP* : ciprofloxacin, CPD* : cefpodoxime; CRO* : ceftriaxone; ND* : not detection

as high as 512 μg mL^{-1}.

Among the isolated strains, 12 were resistant to ceftazidime which is a 3rd generation, cephalosporin type antibiotics like cefotaxime. EC 2-4, which was positive for CTX-M-15, had an MIC value of 256 μg mL^{-1} for ceftazidime, indicating that this strain was more resistant to the 3rd generation, cephalosporin type antibiotics than the other isolated strains. The cefoxitin and cefotaxime resistant EC 5-6 was found to form CTX-M-32 ESBL. According to Lee *et al.* (2000), the resistancw against two types of antibiotics was due to the AmpC type β-lactamase gene plasmid.

Table 4. MIC values of the antibiotics resistant *E. coli* isolated from the Han-River

Strain	MIC values (μg mL^{-1})			
	Cephalothin	Cefoxitin	Cefotaxime	Ceftazidime
EC 1-1	>512	4	>512	32
EC 2-1	>512	4	256	32
EC 2-4	>512	16	>512	256
EC 3-4	>512	16	128	32
EC 4-4	>512	16	512	32
EC 5-1	>512	128	>512	128
EC 5-2	>512	32	128	8
EC 5-6	>512	512	256	64
EC 6-1	>512	8	256	32
EC 6-3	>512	4	256	32
EC 6-4	>512	16	256	32
EC 6-5	>512	16	256	32
EC 6-6	>512	32	256	8
EC 6-7	>512	8	512	64

6. Analyses of MLST

MLST was performed for homogeny analyses of the isolated strains via the comparison of the house keeping genes. The STs of EC 1-1, EC 2-1 and EC 2-4 were ST93, ST1420 and ST2000, respectively. The STs of EC 3-4, EC 4-4, EC 5-1, EC 5-2 and EC 5-6 were ST88, ST38, ST1485, ST648 and ST952, respectively. Among the strains isolated from station F, both EC 6-1, EC 6-4 and EC 6-5 were ST457, while the STs of EC 6-3, EC 6-6 and EC 6-7 were ST162, ST1611 and ST348, respectively. There were five clonal complex producing strains among the 14 isolated strains. These five strains were part of the 168, 23, 38, 469, 156 clonal complexes, respectively. The other nine strains were not included in the clonal complex and had independent STs.

EC 1-1 isolated from station A, the point of drinking water uptake for Seoul city, had the independent ST93, as shown in Fig. 3. According to the MLST data base, ST93 was first detected in Ghana, and is reported as an ESBL positive strain according to clinical specimens from Madrid, Spain. This

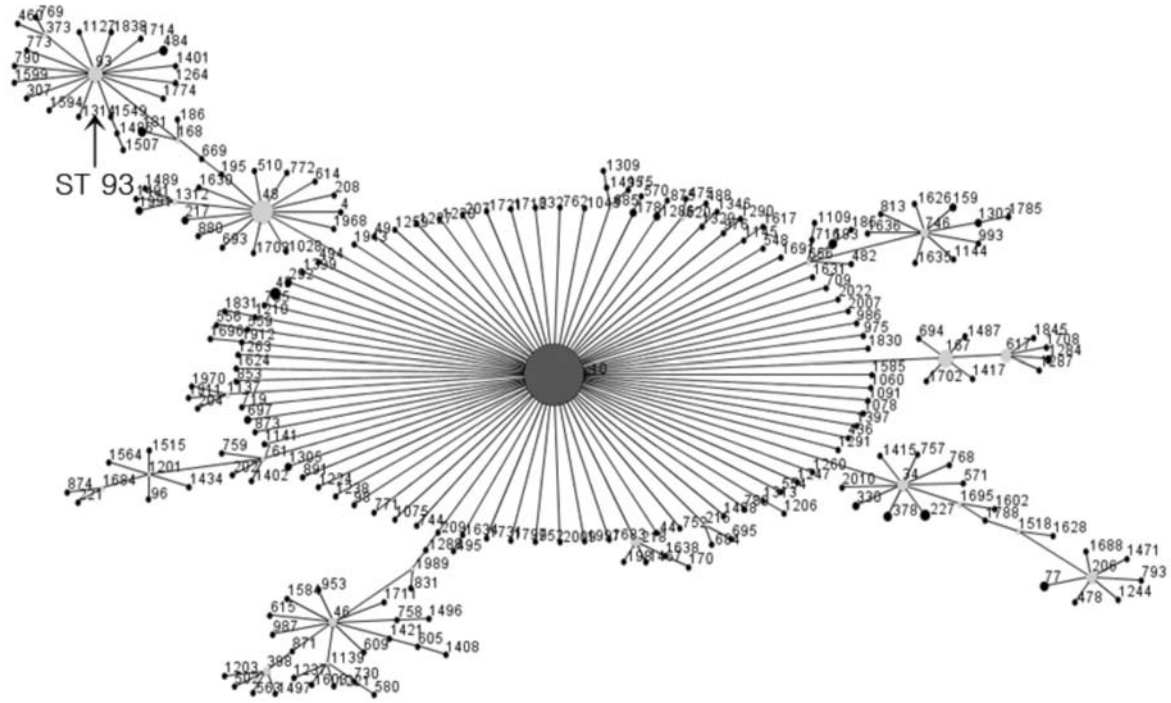

Fig. 3. The eBURST clustering of the MLST profiles indicated by arrows for the 14 isolated strains with the 2,028 MLST profiles representing 3,412 *E. coli* isolates from the database (http://eburst.mlst.net).

Table 5. The multilocus sequence typing of the isolated *E. coli* strains

Strain	Sequence type (ST)	Clonal complex
EC 1-1	93	168
EC 2-1	1420	–
EC 2-4	2000	–
EC 3-4	88	23
EC 4-4	38	38
EC 5-1	1485	–
EC 5-2	648	–
EC 5-6	952	–
EC 6-1	457	–
EC 6-3	162	469
EC 6-4	457	–
EC 6-5	457	–
EC 6-6	1611	–
EC 6-7	348	156

strain causes urinary tract infections and diarrhea. The MLST of strain EC 2-4, which exhibited strong resistance to the 3rd generation, cephalosporin type antibiotics, was classified as ST2000. This strain was first isolated in Japan from a clinical specimen and was reported to cause septicemia. Both of these strains were shown to be pathogenic and warrant special attention due to their presence at the point of drinking water uptake.

Among the strains exhibiting higher resistance to cefotaxime, ST457 was detected in EC 6-1, EC 6-4 and EC 6-5. Those strains with ST457 are pathogenic and cause urinary tract infections; strains with ST457 have also been isolated from urine samples (Lau *et al.* 2008A) and from hospitals (Lau *et al.* 2008B). CTX-M was not detected in ST457 *E. coli* isolated by Lau *et al.* (2008B); in contrast, CTX-M-32 belonging to the CTX-M-1 family was detected in both EC 6-1 and EC 6-5. The CTX-M-1 family is one of the most commonly detected ESBLs, and urinary tract infections caused by these strains may be hard to cure. Since several hospitals are located near station F, isolation of the ST457 strains from station F might have resulted from horizontal transfer via mobile elements in hospital wastewater. It should be noted that the strains with CTX-M type ESBL possessed similar and strong antibiotic resistance when the strains were isolated from the same place.

The subtypes and the ST of each strain were determined through the PFGE and MSLT, respectively. The pathogenicity and the antibiotic susceptibility of the strains by way of ST could be used to trace the origin of antibiotic resistance among strains exposed to antibiotics in their environment.

The development of MDR among ESBL producing bacterial strains will result in diseases that will be incredibly hard to treat. Therefore, we suggest that the distribution of antibiotics resistant bacterial strains in the Han-River should be continuously investigated and monitored for the well-being of the residents near the Han-River basin.

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