The Journal of Microbiology, October 2008, p. 478-481 Vol. 46, No. 5
DOI 10.1007/s12275-008-0150-y

The Identification of CTX-M-14, TEM-52, and CMY-1 Enzymes in *Escherichia coli* Isolated from the Han River in Korea

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(Received June 17, 2008 / Accepted July 17, 2008)

From water samples collected monthly between 2000 and 2001 from the Han River in Seoul, sixteen strains of *Escherichia coli* which confer resistance to at least 10 kinds of antimicrobial agents were isolated. From these isolates, 2 kinds of extended-spectrum β-lactamases (ESBLs) and one plasmid-mediated AmpC β-lactamase were detected; CTX-M-14 from 10 isolates, TEM-52 from 5 isolates, and CMY-1 from one isolate. Class 1 integron gene cassettes, such as *aadA1*, *dfr12-orfF-aadA2*, and *dfr17-aadA5*, were also detected and the integrons are the same as those found in *E. coli* isolated from swine, poultry, and humans in Korea. The result of this study indicated the importance of river water as a reservoir for antimicrobial resistance genes and resistant bacteria.

***Keywords*:** CTX-M-14, TEM-52, CMY-1, *E. coli* from river water

In an environment, the presence and persistence of antimicrobial resistant bacteria is a growing public health concern. The expansive application of antibiotics in humans, veterinary medicine, and agricultural practices has led to a large-scale dissemination of bacteria which are resistant to antibiotics (Kümmerer, 2003). River water receives the sewage of urban effluents which are known to contain high levels of antibiotics and antibiotic-resistant bacteria that belong to the human and animal commensal flora, mainly *Enterobacteriaceae* (Smith *et al.*, 1994; Perrentin *et al.*, 1997), therefore river is the main receptacle for these pollutants. As rivers are one of the major sources of water, directly or indirectly, for human and animal consumption, this pollution may contribute to the maintenance and even the spread of bacterial antibiotic resistance.

The Han River passes through the middle of Seoul (Korea) and it is the major source for drinking and irrigation water for the city. In this study, we examined the presence of antibiotic-resistant bacteria, especially extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, in the Han River. The *E. coli* isolates that produce ESBLs were further characterized by the ESBL type and harboring Class 1 integrons and their integrated gene cassettes.

Materials and Methods

The characterization of ESBL-producing *E. coli* isolated from the Han River

Between 2000 and 2001, water samples were collected monthly from Chungrang-chun, a branch of the Han River. Water samples were serially diluted and then, inoculated on a MacConkey agar plate (MAP) containing 16 μg/ml of cephalothin. During three months from May to July on 2000, 219 colonies were grown on the MAP and considered as β-lactam-resistant bacteria. Among the pink colonies that appeared on the MAP, 22 isolates were identified as *E. coli* by the API 20E Kit (bioMerieux, France) and the isolates were screened for ESBL production by a double disk synergy test (DDST), as described previously (Kim *et al.*, 1998). Sixteen of the 22 isolates were confirmed as ESBL-producers and they were characterized according to antimicrobial susceptibility and the type of ESBL produced. Antimicrobial susceptibility testing was performed by an agar dilution method, which was described in the guidelines of the CLSI (NCCLS, 2003). Isoelectric focusing and inhibition assay, with 0.3 mM of clavulanic acid or cloxacillin, were performed as described previously (Matthew *et al.*, 1975). The identification of ESBL genes was carried out by a PCR and subsequent sequencing described previously (Kim *et al.*, 2005).

The characterization of Class I integrons

The presence of Class I integrons in the *E. coli* isolates was examined by a PCR (White *et al.*, 2000) and the integrated gene cassettes were identified by a sequencing analysis of the amplicons obtained from the PCR.

The transfer of resistance determinant by conjugation

The transfer of $bla_{\text{CTX-M-14}}$ was tested by a broth culture conjugation experiment using *E. coli* J53 Azide^{R} (Coetzee *et al.*, 1972) as recipient. Mating was performed at 37°C for 20 h, and the transconjugants were selected on MacConkey agar plates supplemented with sodium azide (150 μg/ml) and cefotaxime (4 μg/ml).

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Table 1. Characteristics of extended-spectrum β-lactamase-producing *E. coli* isolated from the Han River in Korea

Strains	Date of isolation[a]	Isoelectric point (pI)[b]	β-Lactamase	Class I integron	
				Amplicon size (kb)	Integrated gene cassette
10001	May	5.4, 5.9, 7.4	TEM-1, TEM-52, OXA-4	1.0	*aadA1*
10003	May	5.4, 5.9, 7.4	TEM-1, TEM-52, OXA-4	1.0	*aadA1*
10004	May	5.4, 5.9, 7.4	TEM-1, TEM-52, OXA-4	1.0	*aadA1*
10005	May	5.4, 5.9, 7.4	TEM-1, TEM-52, OXA-4	1.0	*aadA1*
10006	May	5.4, 5.9, 7.4	TEM-1, TEM-52, OXA-4	1.0	*aadA1*
10009	June	5.4, 8.0	TEM-1, CTX-M-14	1.4 & 2.1	ND[c]
10010	June	5.4, 8.0	TEM-1, CTX-M-14	1.6	*dfrA17-aadA5*
10011	June	5.4, 8.0	TEM-1, CTX-M-14	1.6	*dfrA17-aadA5*
10012	June	5.4, 8.0	TEM-1, CTX-M-14	1.6	*dfrA17-aadA5*
10013	June	5.4, 7.4, **8.0**	TEM-1, OXA-4, CMY-1	Not detected	Not detected
10014	June	5.4, 8.0	TEM-1, CTX-M-14	1.6	*dfrA17-aadA5*
10016	July	8.0	CTX-M-14	2.0	*dfrA12-orfF-aadA2*
10018	July	8.0	CTX-M-14	2.0	*dfrA12-orfF-aadA2*
10019	July	8.0	CTX-M-14	2.0	*dfrA12-orfF-aadA2*
10020	July	8.0	CTX-M-14	2.0	*dfrA12-orfF-aadA2*
10022	July	8.0	CTX-M-14	2.0	*dfrA12-orfF-aadA2*

[a] May to July on 2000
[b] Underlined, β-lactamases whose activity was inhibited by clavulanic acid; Bold, β-lactamases whose activity was inhibited by cloxacillin
[c] ND, not determined

Pulsed-field gel electrophoresis (PFGE)

The genetic diversity of the *E. coli* isolates was examined by the PFGE method, as described previously (Gautom, 1997). Briefly, after growth with shaking in a Luria broth (Invitrogen Ltd., UK) at 37°C overnight, genomic DNAs were digested with *Xba*I (Boehringer Mannheim, Germany) for 18 h and separated on a 1.0% agarose gel using a contour-clamped homogeneous-field apparatus (CHEF DRIII Systems, Bio-Rad Laboratories, USA) in a 0.5× TBE buffer (0.09 M of Tris, 2 mM of disodium EDTA; pH 8.5, 0.09 M

Table 2. The results of transferring antimicrobial resistance determinants by conjugation

Species	Strains	MICs (μg/ml)[a] to β-lactams					β-Lactamase[b]	Resistance pattern to other antimicrobial agents[c]
		CTX	CAZ	AZT	FEP	FOX		
E. coli	10010	64	2	8	2	8	CTX-M-14, TEM-1	ApCmTcSmKmGmSuTp
Conjugant	T10-1J	64	<1	2	2	4	CTX-M-14, TEM-1	Ap
E. coli	10011	64	2	8	4	8	CTX-M-14, TEM-1	ApCmTcSmKmGmSuTp
Conjugant	T-11-1J	64	<1	2	2	4	CTX-M-14, TEM-1	Ap
E. coli	10012	128	4	8	4	8	CTX-M-14, TEM-1	ApCmTcSmKmGmSuTp
Conjugant	T12-1J	64	<1	<1	<1	4	CTX-M-14, TEM-1	Ap
E. coli	10014	128	2	8	4	8	CTX-M-14, TEM-1	ApCmTcSmKmGmSuTp
Conjugant	T-14-1J	32	<1	<1	<1	4	CTX-M-14	Ap
E. coli	10018	32	2	8	8	4	CTX-M-14	ApTcSmKmGmSuTp
Conjugant	T18-1J	128	<1	<1	<1	4	CTX-M-14	ApKmSm
E. coli	10020	64	2	8	8	4	CTX-M-14	ApTcSmKmGmSuTp
Conjugant	T-20-1J	128	64	<1	<1	4	CTX-M-14	ApSmKm
E. coli	10022	128	2	8	8	4	CTX-M-14	ApTcSmKmGmSuTp
Conjugant	T-22-1J	64	64	<1	<1	4	CTX-M-14	Ap

[a] CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; FOX, cefoxitin
[b] Identification of β-lactamase genes was performed by PCR and nucleotide sequencing
[c] Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Sm, streptomycin; Su, sulfixosazole; Tp, trimethoprim; Km, kanamycin; Gm, gentamicin; Ak, amikacin

of boric acid) at a constant temperature of 14°C at 6 V/cm for 20 h with an increasing pulse time between 5 and 40 sec.

Results and Discussion

All 16 ESBL-producing *E. coli* strains were resistant to cephalothin, ampicillin, tetracycline, streptomycin, sulfixosazole, trimethoprim, kanamycin, and gentamicin. Eleven isolates (69%) were resistant to cefotaxime, norfloxacin, and chloramphenicol and four isolates (25%) were resistant to amikacin. From the results of IEF analysis, five out of 16 strains showed three kinds of β-lactamases with pIs of 5.4, 5.9, and 7.4 and another five strains showed two kinds of β-lactamases with pIs of 5.4 and 8.0. A β-lactamase with a pI of 8.0 and three kinds of β-lactamases with pIs of 5.4, 7.4, and 8.0 were observed in 5 strains and 1 strain, respectively (Table 1). For those producing β-lactamases with a pI of 5.4 or 5.9, TEM-specific PCR and subsequent sequencing were performed and these enzymes were identified as TEM-1 and TEM-52, respectively. For isolates which produced β-lactamases with a pI of 8.0 and whose activity was inhibited by 0.3 mM clavulanic acid, CTX-M-specific PCR and subsequent sequencing were performed and this enzyme was identified as CTX-M-14. The β-lactamases with a pI of 8.0, whose activity was inhibited by 0.3 mM of cloxacillin was CMY-1, plasmid-mediated AmpC β-lactamase (pACBL), which was observed in one strain (No. 10013). To characterize the β-lactamases with a pI of 7.4, whose activity was not inhibited by either of 0.3 mM of clavulanic acid or cloxacillin, OXA-1-specific PCR and subsequent sequencing were performed and this enzyme was identified as OXA-4 (Table 1).

From the examination of Class I integron, gene cassettes that encode resistance to aminoglycosides (*aadA1*, *aadA2*, and *aadA5*) and to trimethoprim (*dfrA12* and *dfrA17*) were detected in three different integrons, *aadA1*, *dfrA17-aadA5*, and *dfrA12-orfF-aadA2*. Five isolates carrying $bla_{\text{TEM-1}}$, $bla_{\text{TEM-52}}$, and $bla_{\text{OXA-4}}$ were found to have a Class 1 integron inserted *aadA1* gene cassette and four of the five isolates carrying $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ were found to have a Class 1 integron inserted *dfrA17-aadA5*. Five isolates carrying $bla_{\text{CTX-M-14}}$ were found to have a Class 1 integron inserted *dfrA12-orfF-aadA2*, but there was no Class 1 integron found in the isolate carrying $bla_{\text{TEM-1}}$, $bla_{\text{CMY-1}}$, and $bla_{\text{OXA-4}}$ (Table 1).

A conjugation experiment showed that $bla_{\text{CTX-M-14}}$ was transferred in 7 of 10 isolates carrying $bla_{\text{CTX-M-14}}$, suggesting a plasmid location for this *bla* gene (Table 2). The $bla_{\text{TEM-1}}$ gene was transferred simultaneously with $bla_{\text{CTX-M-14}}$ but a Class 1 integron was not detected in transconjugants, suggesting different location for the *bla* gene and Class 1 integrons.

As shown in Fig. 1, the PFGE patterns generated from *Xba*I-digested genomic DNAs were typed into five groups. Isolates carrying the same *bla* genes and integron gene cassettes showed the same PFGE patterns, suggesting the same clonal origin.

The evolution and dissemination of extended-spectrum β-lactamases (ESBLs) have compromised the clinical use of third-generation cephalosporins worldwide. Most ESBLs have been reported from clinical isolates, especially nosocomial strains (Bradford, 2001). In the current study, however, ESBL or pACBL-producing *E. coli* strains were detected from river water: CTX-M-14, TEM-52, and CMY-1. These enzymes were frequently found in the clinical isolates of *E. coli* and *K. pneumoniae* and community isolates, such as *S. sonnei* and nontyphoidal *Salmonella* (Lee *et al.*, 2003; Kim *et al.*, 2004; Kim *et al.*, 2005). It is interesting that the gene cassettes inserted in the Class 1 integron carried by *E. coli* isolated from river water were very similar to those found in *E. coli* isolated from poultry, swine, and humans (Kang *et al.*, 2005). The high similarity of Class 1 integron gene cassettes among *E. coli* isolated from clinical specimens, poultry, swine, and river water strongly suggests that river water plays an important role in spreading antimicrobial re-

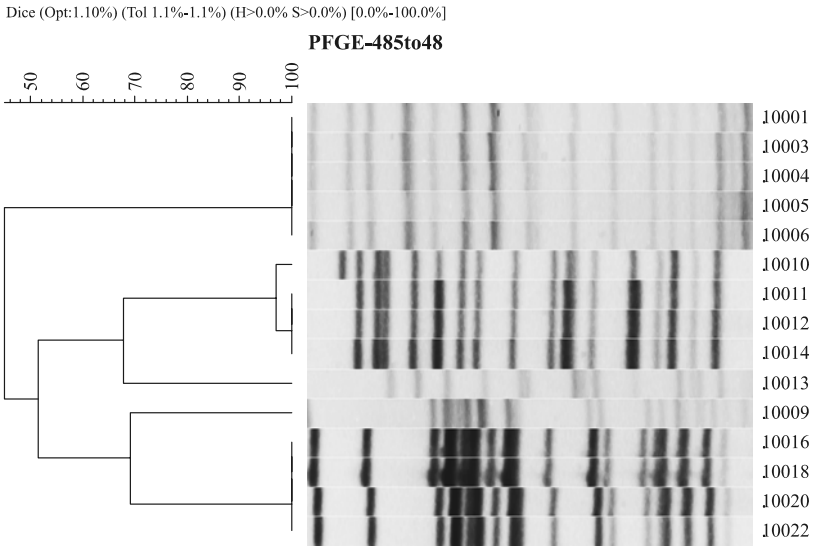

Fig. 1. The *Xba*I-digested PFGE pattern of *E. coli* isolated from the Han River in Korea. The PFGE patterns were analyzed with GelCompar software, using the Dice coefficient, and represented by the unweighted pair group method with arithmetic averages (UPGMA).

sistance determinants or antimicrobial resistant bacteria.

In summary, *E. coli* producing CTX-M-14, TEM-52, or CMY-1 were identified from river water and the Class 1 integron gene cassettes carried by these isolates were the same with those found in *E. coli* isolated from hospitalized or nonhopitalized humans, poultry, and swine in Korea. The results are indicative of the importance of river water as a reservoir for antimicrobial resistance genes and resistant bacteria.

Acknowledgements

This work was supported by a Grant from the Korean Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (03-PJ1-PG1-CH03-0002) and, in part, by the Brain Korea 21 Project (2006).

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