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Isolation of Norfloxacin Resistant *Escherichia coli* from the Han River and Characterization of Resistance Mechanism

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A total of twenty-five norfloxacin resistant *Escherichia coli* were isolated from Joongrang-chun stream, a branch of the Han River in Seoul, Korea from May to July in 2000 and their norfloxacin resistance mechanism was characterized for target site mutation, permeability, and efflux pump. Fourteen isolates contained the same three mutations, Ser83→Leu and Asp87→Asn in GyrA and Ser90→Ile in ParC. Six isolates had Ser83→Leu and Asp87→Tyr in GyrA and Ser80→Ile in ParC while one isolate had Ser83→Leu and Val103→Ala in GyrA and Ser80→Ile in ParC. Two isolates had mutation(s) in GyrA without any mutation in ParC. Two isolates had Ser80→Arg in ParC instead of the commonly found Ser80→Ile. Every norfloxacin resistant isolate had an efflux system but the correlation between the efflux activity and MIC was not observed. The amount of OmpF for norfloxacin permeability decreased in resistant isolates compared to the susceptible strains. When amplified polymorphic DNA (RAPD) and pulse field gel electrophoresis (PFGE) were performed, these isolates showed no similarity to each other or clinical isolates.

Key words : antibiotic environmental isolate, *Escherichia coli*, MDR, norfloxacin, quinolone, resistance

Antibiotic-resistant bacteria and antibiotics are discharged in various amounts in the environment as a result of the increasing and often indiscriminate use of antibiotics in medical, veterinary, and agricultural practices. River water is the main receptacle for these pollutants, since rivers receive the sewage of urban effluents (Al-Jebouri *et al.*, 1985). 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. It has been reported that antibiotic resistance of environmental isolates can be transferred to clinical isolates (Kariuki *et al.*, 1999; Oppegaard *et al.*, 2001). Since the Han River is used for the municipal water system as well as for recreation, the existence of antibiotic resistant zoonotic pathogens in the water can present a big health hazard.

In this study, norfloxacin resistant *Escherichia coli* was chosen as an indicator for the antibiotic resistant pathogens in the environment and the resistant mechanism of the environmental isolate was studied to see whether it has the same mechanism of the clinical isolate. *E. coli* was selected because it is zoonotic and was used as the indicator for contamination by other pathogens. Norfloxain is a synthetic drug that does not exist in nature and mainly targeting gram-negative bacteria. Therefore fluoroquinolone resistant bacteria cannot develop in nature by itself but can only be produced by contamination from humans or animals or induced by the chemicals present in the environment.

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Materials and Methods

Isolation and identification of E. coli

An aliquot of a sample taken from Chungrang-chun, a branch of the Han River was inoculated on MacConkey agar plates containing norfloxacin (32 μg/ml). Colonies appearing on this medium were randomly selected and identified with an API 20E kit.

Assay of minimal inhibitory concentration (MIC)

An MIC of each isolate was assayed with the agar dilution method following the NCCLS (National Committee for Clinical Laboratory Standards, 2000). *E. coli* ATCC25922 was used as the control.

DNA sequencing of QRDR of GyrA and ParC

Amplification of QRDR of *gyrA* (nucleotides 372 to 850) and *parC* (nucleotides 90 to 481) was achieved by PCR with 5-GAGGGATAGCGGTTAGATGAG-3' and 5'-TTTTTCCGTGCCGTCATAG-3' and 5'-GTTGCCGTTTATTGGTGATGG-3' and 5'-GACGGGCAGCTAGCATTTTC-3', respectively. Reactions were composed of a total 30

cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C and a final extension step at 72°C. After PCR products were confirmed on 1% agarose gel and purified using the QIAquick Gel extraction Kit (Qiagen, Valencia, CA), DNA sequencing was performed with an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) following the manufacturer's guide. DNA sequences were compared with those of *gyrA* (Accession No. X57174) and *parC* (Accession No. X58408) at GenBank.

RAPD

RAPD was performed with 20 ng gemomic DNA, 3 mM $MgCl_2$, 10X TaKaRa buffer, 240 μM dNTP, 20 pmol random primer (5'-AAGAGCCCGT-3'), and Taq polymerase. PCR constituted 4 cycles of denaturing, annealing, and extending for 5 min each at 94°C, 34°C, and 72°C; 30 cycles for 1 min at 94°C, 1 min at 34°C, and 2 min at 72°C, and a final extension for 10 min at 72°C. The final products were electrophoresed on a 1% agarose gel and analyzed with a Bioprofile analyzer (Viber Lourmat, Marne la Vallee, France).

Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) was performed with a CHEF DRIII system (Bio-Rad, Hercules, CA) following the procedure in a previously published report (Health *et al.*, 1992). Macrorestriction of bacterial genomic DNA was obtained by digestion with *Xba*I of the genomic DNA and separated at 14°C for 21 h with pulse times of 10 to 40 s. The restricted products were stained with ethidium bromide and analyzed with a Bioprofile image analysis system (Viber Lourmat, Marne la Vallee, France).

Assay of the intracellular norfloxacin concentration

The intracellular norfloxacin concentration was assayed as described in a previous paper (Kim *et al.*, 1996). Bacterial cells in log phase grown in MH broth were harvested with centrifugation and suspended in new MH broth making 3×10^9 cfu/ml. Norfloxacin (final concentration, 50 μg/ml) was added to the cells and kept at 37°C. After 5 min, 100 μl of cells were layered on the top of 1 ml cold silicon oil in a microcentrifuge tube and centrifuged to collect cells while eliminating norfloxacin bound outside of the cells. The end of the tube containing bacterial cells was cut with a tube cutter and transferred into 1 ml of 10 mM potassium phosphate buffer (pH 7.4). The intracellular norfloxacin was extracted from cells by boiling for 10 min and measured at 288 nm after excitation at 456 nm in a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan).

Isolation of outer membrane proteins

Outer membrane proteins were isolated as described in a previous paper (Kim, 1996). Cells grown in MH were harvested and suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM PMSF. Cells were broken with 20 sec pulses and 20 sec rests for 30 min by a sonicator. After unbroken cells were removed with centrifugation, membrane parts were collected by centrifugation at 50,000×g for 1 h at 4°C. After inner membrane parts were dissolved in 1%(v/v) N-lauryl sarcosine (Sigma, St. Louis, MO), the outer membrane parts were collected by centrifugation at 100,000×g for 1 h. Proteins were dissolved out from the outer membrane parts by boiling in 2% (w/v) SDS. The extracted outer membrane proteins were electrophoresed in a 15% SDS-PAGE.

Results

Isolation of norfloxacin resistant E. coli and MIC to various antimicrobial agents

During a three month period, twenty-five isolates of *E. coli* appeared on MacConkey media containing 32 μg/ml norfloxacin and MICs of each isolate to various antimicrobial agents are shown in Table 1. One isolate (No. 1126) was resistant to all four antibiotics and ten isolates (Nos. 11008, 1109, 1111, 1114, 1118, 1123, 1125, 127, 1129, 1121) were MDR, resistant to more than three antibiotics. Two isolates

Table 1. MICs of norfloxacin resistant environmental isolates.

CCARM No.	MIC (μg/ml)			
	Norfloxacin	Gentamycin	Ampicillin	Cephalothin
1108	32	16	64	16
1109	>128	1	>128	64
1110	>128	<1	>128	16
1111	>128	<1	>128	32
1112	>128	<1	>128	8
1113	>128	<1	>128	16
1114	>128	<1	>128	32
1115	>128	<1	>128	16
1116	>128	8	>128	16
1117	128	>64	4	8
1118	32	64	>128	8
1119	32	<1	>128	16
1120	>128	<1	>128	16
1121	>128	<1	>128	8
1122	>128	4	>128	16
1123	64	64	>128	8
1124	64	64	16	16
1125	>128	64	>128	8
1126	>128	32	>128	32
1127	32	1	>128	64
1128	128	<1	>128	16
1129	>128	64	>128	8
1130	>128	<1	>128	8
1131	>128	<1	>128	32
1132	>128	<1	>128	8

Table 2. Amino acid change in QRDR in GyrA and ParC.

CCARM No.	Norfloxacin MIC (μg/ml)	GyrA	ParC
1108	32	Ser83→Leu, Asp87→Asn	Ser80→Ile
1109	>128	Ser83→Leu	Ser80→Arg
1110	>128	Ser83→Leu	
1111	>128	Ser83→Leu, Val103→Ala	Ser80→Ile
1112	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1113	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1114	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1115	>128	Ser83→Leu, Asp87→Asn	
1116	>128	Ser83→Leu, Asp87→Tyr	Ser80→Ile
1117	128	Ser83→Leu, Asp87→Asn	Ser80→Ile, Glu84→Gly
1118	32	Ser83→Leu, Asp87→Asn	Ser80→ Ile
1119	32	Ser83→Leu, Asp87 →Asn	Ser80→Ile
1120	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1121	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1122	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1123	64	Ser83→Leu, Asp87→Asn	Ser80→Ile
1124	64	Ser83→Leu, Asp87→Asn	Ser80→Ile
1125	>128	Ser83→Leu	Ser80→Ile, Arg119→Pro
1126	>128	Ser83→Leu, Asp87→Tyr	Ser80→Ile
1127	32	Ser83 →Leu, Asp87→Tyr	Ser80→Ile
1128	128	Ser83→Leu, Asp87→Tyr	Ser80→Ile
1129	>128	Ser83→Leu, Asp87→Tyr	Ser80→Ile
1130	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile
1131	>128	Ser83→Leu, Asp87→Tyr	Ser80→Arg
1132	>128	Ser83→Leu, Asp87→Asn	Ser80→Ile

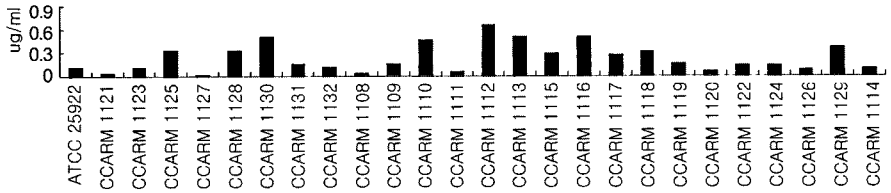

Fig. 1. The amount of effluxed norfloxacin. Intracellular norfloxacin concentration was assayed in the presence of and absence of CCCP and the difference was considered as the amount of the effluxed norfloxacin.

(Nos. 1109, 1127) were resistant to cephalothin.

Mutation in QRDR of GyrA and ParC

Fourteen isolates contained the same three mutations, Ser83→Leu and Asp87→Asn in GyrA and Ser90→Ile in ParC. Six isolates had Ser83→Leu and Asp87→Tyr in GyrA and Ser80→Ile in ParC while one isolate had Ser83 →Leu and Val103→Ala in GyrA and Ser80→Ile in ParC. Two isolates (Nos. 1110, 1115) had mutation(s) only in GyrA without any mutation in ParC. One isolate (No. 1109) had Ser80→Arg instead of the commonly found Ser80→Ile in ParC (Table 2). There was no correlation between mutations and MIC.

Intracellular norfloxacin concentration

Intracellular norfloxacin concentration in the presence of CCCP in every isolate including the susceptible one was higher than that in the absence of CCCP suggesting the existence of an efflux system in every isolate. When the difference between the intracellular norfloxacin concentration in the presence and absence of CCCP was calculated to obtain the amount of effluxed norfloxacin, no relationship was found between MICs and the amount of effluxed norfloxacin concentration (Fig. 1).

The amount of outer membrane proteins

Hydrophilic quinolones such as norfloxacin are transported via outer membrane proteins and norfloxacin is permeabilized via OmpF (Mortimer and Piddock, 1993). When the amount of outer membrane proteins extracted from each isolate were compared, each isolate had less OmpF compared to the susceptible one (Fig. 2 and Table 3).

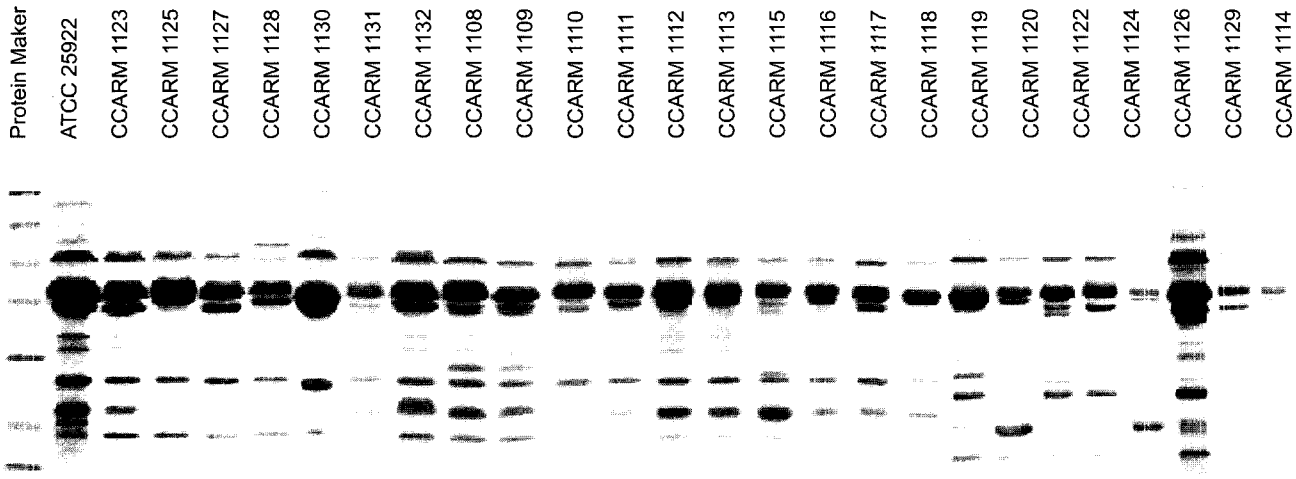

Fig. 2. Outer membrane proteins of norfloxacin-resistant environmental isolates of *E. coli*. Outer membrane proteins were extracted from outer membrane parts of the resistant isolates and electrophoresed in a 10% SDS denaturing gel. The amounts of OmpA, OmpC, and OmpF in the susceptible strain was considered as 100.

Table 3. Amount of OmpA, OmpC, and OmpF.

	ATCC 25922	CCARM 1121	CCARM 1123	CCARM 1125	CCARM 1127	CCARM 1128	CCARM 1130	CCARM 1131	CCARM 1132	CCARM 1108	CCARM 1109	CCARM 1110	CCARM 1111	CCARM 1112	CCARM 1113	CCARM 1115	CCARM 1116	CCARM 1117	CCARM 1118	CCARM 1119	CCARM 1120	CCARM 1122	CCARM 1124	CCARM 1126	CCARM 1129	CCARM 1114
Omp C	100[a]	83	75	56	28	88	37	139	88	41	33	22	48	37	26	23	35	10	44	20	28	30	<10	96	27	<10
Omp F	100	64	93	62	43	89	38	68	73	53	41	37	53	40	31	29	19	25	47	26	39	39	15	79	25	11
Omp A	100	110	11	88	80	125	25	123	51	66	14	43	43	31	18	28	32	27	38	132	22	43	20	142	37	14

[a]The amount of each OMP in each norfloxacin resistant isolate was calculated when the amount of OMP in the susceptible strain was considered as 100%.

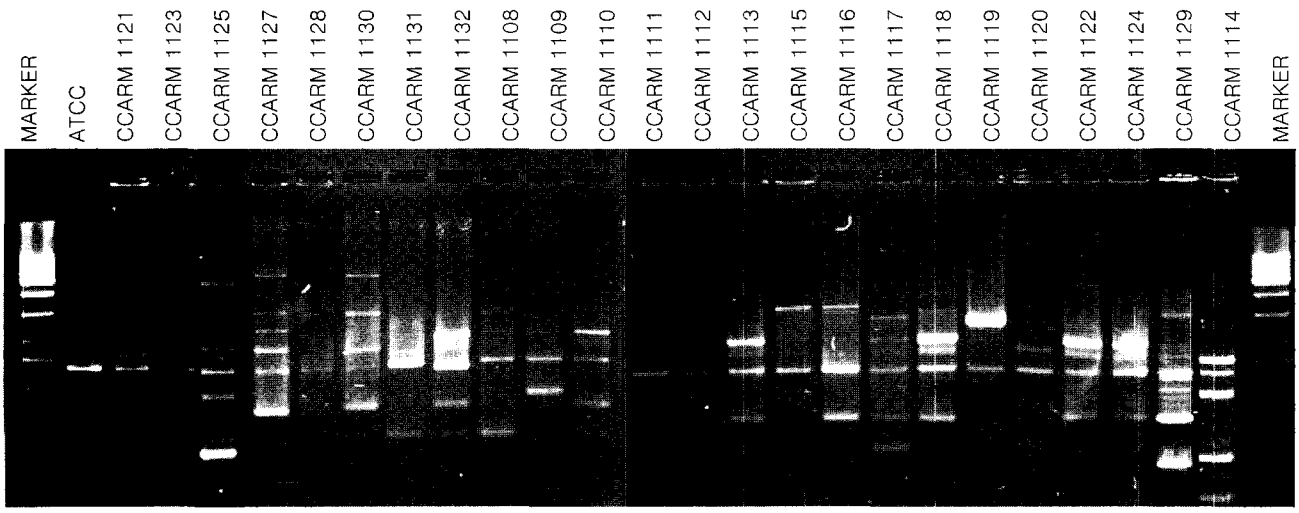

Fig. 3. RAPD of norfloxacin-resistant environmental isolates of *E. coli*. RAPD was performed with each resistant isolate and the susceptible one and the products were electrophoresed in 0.8% agarose gels.

Relationship among norfloxacin resistant isolates

Environmental norfloxacin resistant isolates could originate from one isolate or developed separately. Among various random primers tested, PCR with one specific primer produced variously sized fragments. When their patterns were analyzed, there was no similarity among isolates except two pairs (Fig. 3 and Fig. 4). When PFGE was performed with DNA fragments digested with *Xma*I,

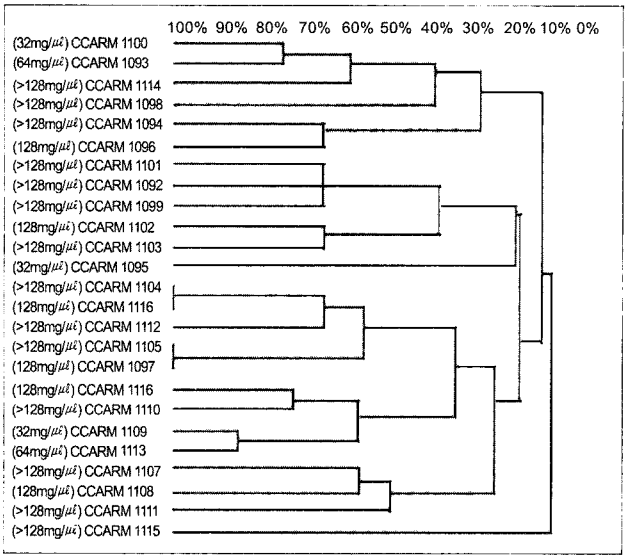

Fig. 4. Pylogeny analysis of the norfloxacin-resistant isolates with RAPD.

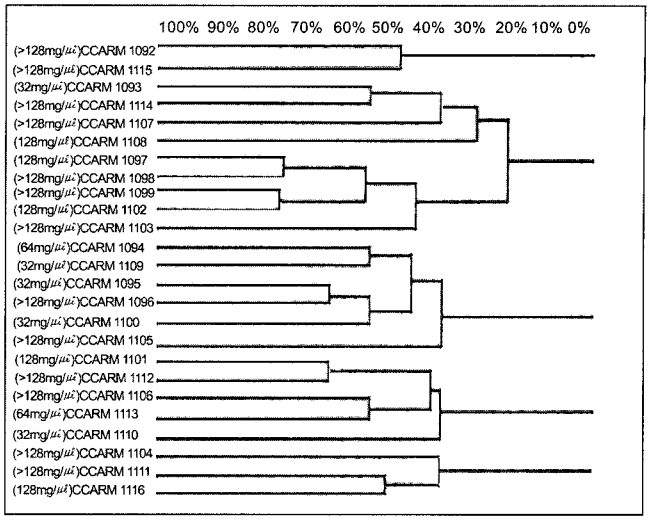

Fig. 6. Pylogeny of norfloxacin-resistant environmental isolates of *E. coli*. Genomic DNA fragments digested with *Xma*I were analyzed with PFGE and their relation was analyzed as described in Materials and Methods.

each isolate including these two pairs showed different patterns (Fig. 5 and 6).

Discussion

Many reports have been published recently stating that an environmental isolate can transfer antibiotic resistance to human isolates (Kariuki *et al.*, 1999; Oppegaard *et al.*, 2001). The environment contains naturally developed antibiotic resistant bacteria originating from antibiotic producing bacteria, for example β-lactam-resistant bacteria. However, quinolone-resistant bacteria can originate

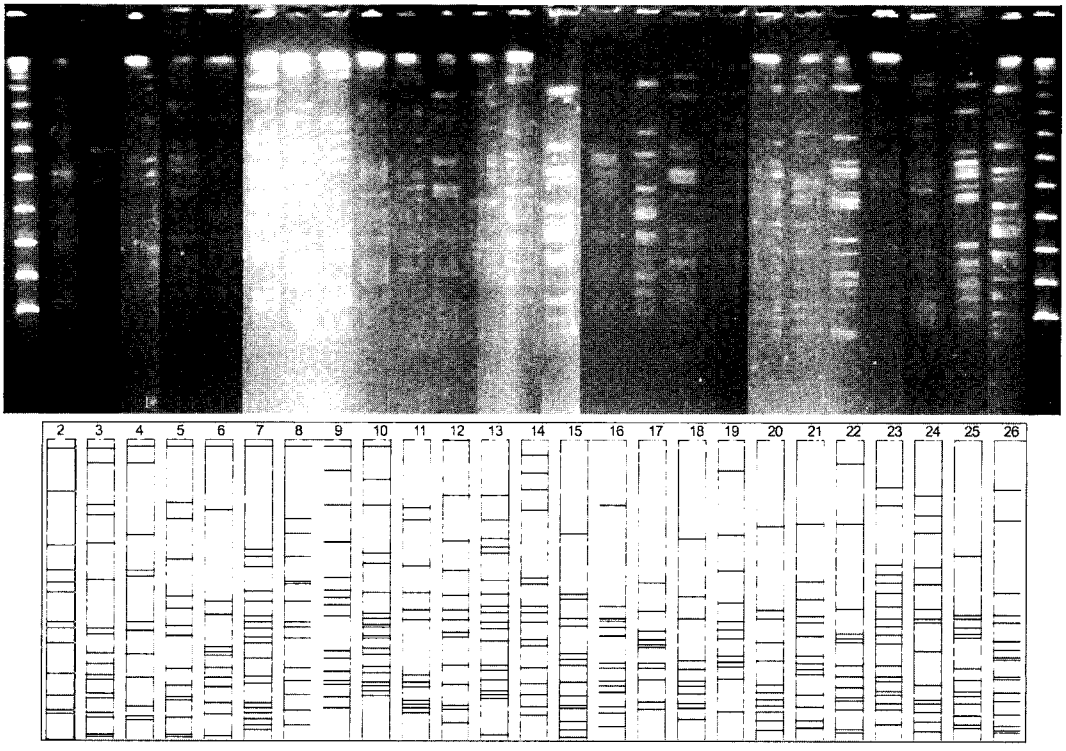

Fig. 5. PFGE of norfloxacin-resistant environmental isolates of *E. coli*. Genomic DNA of norfloxacin-resistant isolates was digested with *Xma*I and analyzed with PFGE.

only from animal or clinical isolates by misuse or overuse of quinolone, because quinolone is a synthetic antibiotic and thus cannot be produced naturally. Therefore norfloxacin resistant *E. coli* indicates contamination from human or animal origins, especially the possible contamination of other antibiotic resistant pathogens.

Bacteria resistant to synthetic antibiotics in the environment have been reported all around the world, indicating a major health problem (Al-Jebouri, 1985; Ogan and Nwiika, 1993). In Korea, Lee (2001) reported the presence of ESBL (extended spectrum of β-lactamase) producing *E. coli* in the Nakdong River in Pusan (southeast Korea) and Kim *et al.* (2001) reported animal isolates of VRE (vancomycin resistant enterococcus) near Mokpo (southwest Korea). In this study, we reported MDR *E. coli* in the Han River located in the middle part of Korea.

Norfloxacin resistant environmental isolates in this study showed no similarity to the clinical isolates previously isolated from hospital patients or to each other. This suggested that these isolates originated from animals and might develop resistance separately. When the resistance mechanism was studied, two isolates had a mutation only in GyrA even though their MICs were very high. Until now, it has been believed that two mutations, one at Ser83 in GyrA and the other at Ser80 in ParC, are necessary for quinolone resistance in *E. coli* while only one mutation at Ser83 in GyrA cannot render a high MIC (Suzuki, 1990; Piddock, 1999). The main factors responsible for high MIC in quinolone are mutations in Ser83 in GyrA and Ser79 in ParC at the same time (Hooper, 1995; Poole, 2000). Even though these two isolates showed little OmpF and the existence of an efflux system, this cannot explain the high MICs of these isolates (Nikaido, 1994 and 1998). We are looking for another factor to explain the high MIC of these two isolates.

There have been several reports that bacteria can contaminate vegetables and fruits through soil and survive inside of them (Besser *et al.*, 1993; Han *et al.*, 2000; Shoemaker *et al.*, 2001). Thus resistant bacteria in the river can contaminate the environment such as soil and irrigation water nearby and even humans provoking a serious health problem. As we reported in this study, MICs of most of these isolates are extremely high and originated from various sources and this suggested a possibility of transfer of resistant isolates to the environment and to humans. It is urgent to conduct a careful and extensive investigation to measure the extent of contamination from antibiotic resistant bacteria and prevent a health hazard.

Acknowledgment

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