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NOTE

Isolation of Quinolone-Resistant *Escherichia coli* Found in Major Rivers in Korea

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Culture Collection of Antimicrobial Resistant Microbes, Department of Biology, Seoul 139-774, Seoul Women's University
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Twenty isolates resistant to seven quinolones were isolated from major rivers in Korea. All isolates had three mutations, Ser83→Leu and Asp87→Asn in GyrA and Ser80→Ile or Ser80→Arg in ParC and three isolates had an additional mutation Glu84→Gly or Glu84→Val in ParC. In addition, a clonal spread was not found in these isolates.

Keywords: fluoroquinolone, antimicrobial resistance, *Escherichia coli*

Since nalidixic acid was used to treat respiratory and urinary tract infections in 1960 (Lesher *et al.*, 1962), various quinolones have been widely used in humans and animals, even as growth promoters. The initial assumption was that quinolone-resistance is much less frequent than resistance to other antimicrobial agents because it contains a chromosomal mutation responsible for resistance. However, quinolone-resistance developed so rapidly that its resistance problem prevails in a clinical environment as well as in the animal industry (Bazile-Pham-Khac *et al.*, 1996; Lindgren *et al.*, 2003; Yang *et al.*, 2004; Zhao, *et al.*, 2005). Quinolone-resistance primarily results from mutations in quinolone resistance determining regions (QRDRs) in DNA gyrase and topoisomerase II (Katie *et al.*, 2005).

Since quinolones are synthetic antimicrobial agents that cannot be synthesized naturally and *Escherichia coli* is the indicator of fecal contamination, the presence of quinolone-resistant *E. coli* will reveal the contamination from humans and animals treated with quinolone as well as the prevalence of antimicrobial resistance in our environment. In this study, *E. coli* showing extreme resistance to quinolones was isolated from three major rivers and their branches in Korea and the resistance mechanisms and relationships among these isolates were studied.

During a one-year period from February 2004 to January 2005, samples were obtained from the Han River, Joongrang-chun (a branch of the Han River), Nakdong River, Kumho River (a branch of the Nakdong River), the Sumjin River, and upstream of the Sumjin River. The number of total bacteria was assayed using Standard methods agar (Difco, USA) and the number of enterobacteria was assayed using MacConkey media (Difco). Samples were inoculated by spreading on MacConkey solid media containing 16 μg/ml norfloxacin. A total of 645 colonies suspected to be *E. coli* were subjected to an indole test, methyl red test, Voges-Proskawer test, and citrate test (IMVIC test) and were identified using an API20E kit (bioMérieux, France). Minimal inhibitory concentrations (MICs) were assayed using the agar dilution method following the guideline of the Clinical and Laboratory Standards Institute (NCCLS, 2005). Twenty norfloxacin-resistant *E. coli* isolates with an MIC higher than 128 μg/ml were selected and the resistance of each was studied. Each colony was suspended in saline making McFarland nephelometer No. 0.5 and an aliquot (10^4 CFU) was inoculated on Muller-Hinton media (MH, Difco) containing 0.25-128 μg/ml of each quinolone. After 18 h incubation at 37°C, the concentration at which less than 10 colonies appeared was defined as the MIC. *E. coli* ATCC 25922 was used as the control. Norfloxacin and nalidixic acid were purchased from Sigma (USA) and ciprofloxacin, moxifloxacin, levofloxacin, gemifloxacin, and gatifloxacin were obtained from Dongwha Pharmaceutical Co. (Korea). The breakpoint MICs for nalidixic acid, ciprofloxacin, gatifloxacin, gemifloxacin,

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levofloxacin, moxifloxacin, and norfloxacin are 32 μg/ml, 4 μg/ml, 4 μg/ml, 8 μg/ml, 1 μg/ml, and 8 μg/ml, and 4 μg/ml, respectively as set by CLSI (2006). The MIC interpretation standard of *Streptococcus penumoniae* was used as a breakpoint for moxifloxacin.

The QRDRs of *gyrA* and *parC* were amplified via PCR and sequenced as previously described (Jung *et al.*, 2002). The outer membrane proteins were obtained according to the method described by Oh *et al.* (2002). The proteins were assayed with bicinchoninic acid (Sigma) using bovine serum albumin as a control. The proteins were separated on a 15% gel using SDS-polyacrylamide gel electrophoresis (PAGE).

The similarity of the isolates was studied using pulse field gel electrophoresis (PFGE). Each isolate was inoculated in brain heart infusion medium (BHI, Difco) and incubated with shaking for 18 h at 37°C. One milliliter of the culture was centrifuged in order to collect bacterial cells. The cells were suspended in suspension buffer (10 mM Tris-Cl, 1 M NaCl, pH 7.6) and mixed with the same volume of 1% Incert agar (Cambrex, USA) in order to make a plug. After the cells were lysed, the genomic DNA was treated with *Xba*I (MBI Fermentas, USA) and electrophoresed in a CHEF DRIII system (Bio-Rad, USA) with 5 sec to 40 sec pulse for 20 h at 14°C. The separated DNA fragments were subjected to data processing in a Gel Compar II (Applied Maths, Belgium) using the unweighted-pair group method using arithmetic averages, the Dice coefficient, a position tolerance of 1% and an optimization of 1%. The isolates were determined to be the same strains (being indistinguishable) if they possessed 100% similarity. The isolates were defined as having a clonal relationship if they possessed 85% similarity to the PFGE profiles as previously suggested (Ejrnaes *et al.*, 2006).

A total of 645 norfloxacin-resistant isolates (MIC≥ 16 μg/ml) were obtained during a one-year period from February 2004 through January 2005 (Table 1). Each river and branch showed various concentrations of enterobacteria and quinolone-resistant isolates of *E. coli*. The Jungrang-chun branch of the Han River had the highest concentrations of total bacteria, enterobacteria, and quinolone-resistant *E. coli*. Since the region around

Table 1. Numbers of total bacteria, enterobacteria and norfloxacin-resistant *Escherichia coli*

Location (Index No.)		CFU/ml												
		2004.2	2004.3	2004.4	2004.5	2004.6	2004.7	2004.8	2004.9	2004.1	2004.11	2004.12	2005.1	Ave.
Jungrang-chun (1)	total bacteria	50000	5630	1390	19650	107700	29700	198600	1111000	18070	60400	63200	9500	139570
	Enterobacteria	2500	174	97	5252	7510	1100	3060	515400	3300	2287	4840	260	45482
	nor[a]-resistant *E. coli*	3	1.33	2.76	1.2	2.24	3.6	2.7	20	8	13.3	41	1.1	8.35
Han River (2)	total bacteria	840	254	675	696	11750	840	20880	35470	588	6089	710	9400	7349
	Enterobacteria	60	8	6	99	587	49	1016	2490	28	138	28	250	397
	nor-resistant *E. coli*	0.09	0.1	0.006	0.04	0.15	0.02	0.41	0.12	0.22	2.4	0.64	0.18	0.36
Kumho River (3)	total bacteria	510	486	425	2660	4903	2050	14160	3300	1300	1328	3430	258	2901
	Enterobacteria	39	77	35	127	652	350	615	389	69	53	41	5	204
	nor-resistant *E. coli*	0	0.004	0.002	0.022	0	0	0.66	0.13	0.21	1.69	0.1	0.1	0.24
Nak-dong River (4)	total bacteria	1760	4110	266	629	2410	18600	6750	3060	931	17630	2370	1030	4962
	Enterobacteria	52	68	29	81	527	12	368	108	53	59	328	62	146
	nor-resistant *E. coli*	0.01	0.052	0	0.044	0.128	0.1	0.03	0.26	0.24	0.32	0.04	0	0.10
Upstream of Sumjin River (5)	total bacteria	276	167	311	923	1603	-	-	380	461	885	1342	154	542
	Enterobacteria	17	114	11	179	587	-	-	36	76	73	32	5	94
	nor-resistant *E. coli*	0	0.002	0.01	0.082	0.11	-	-	0	0	0.2	0	0.4	0.07
Sumjin River (6)	total bacteria	2300	265	255	1450	10330	-	-	16330	1130	2900	770	1690	3118
	Enterobacteria	34	38	8	128	277	-	-	67	40	62	76	20	63
	nor-resistant *E. coli*	0	0	0.03	0.07	0.08	-	-	0.126	0	0.04	0	0	0.03

[a], norfloxacin-resistant *E.coli* (-, sample could not be obtained due to a flood)

Jungrang-chun is densely populated, the incomplete sewage treatment must be responsible for the heavy contamination. In the case of the Sumjin River, no big difference was observed among the concentrations of enterobacteria and norfloxacin-resistant *E. coli* in the upstream and the downstream regions. The most plausible explanation for this finding is that the region around the Sumjin-River is primarily an agricultural region and is less populated than other regions.

Twenty of the norfloxacin-resistant isolates were

Table 2. MICs, QRDR mutation, the presence of outer membrane proteins of quinolone-resistant environmental isolates of *E. coli*

Location	CCARM No.	MIC (μg/ml)							Amino Acid Change in		OMP	
		NOR[a]	CIP[b]	NAL[c]	GAT[d]	GEM[e]	LEV[f]	MOX[g]	*gyrA*	*parC*	OmpF[h]	OmpC[i]
1	15531	512	128	128	8	16	16	16	Ser83Leu, Asp87Asn	Ser80Ile	-[j]	+[k]
6	15545	512	128	128	8	32	8	16	Ser83Leu, Asp87Asn	Ser80Ile, Glu84Gly	-	+
1	15553	1024	128	128	32	128	32	32	Ser83Leu, Asp87Asn	Ser80Ile	-	+
2	15580	512	128	128	8	8	8	16	Ser83Leu, Asp87Asn	Ser80Ile	-	+
1	15618	512	128	128	16	32	16	32	Ser83Leu, Asp87Asn	Ser80Ile	-	+
6	15624	512	128	128	8	16	16	16	Ser83Leu, Asp87Asn	Ser80Ile, Glu84Gly	-	+
6	15643	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile	+	+
1	15645	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile	+	+
1	15651	512	128	128	32	128	32	64	Ser83Leu, Asp87Asn	Ser80Ile	+	+
4	15657	1024	128	128	8	16	16	16	Ser83Leu, Asp87Asn	Ser80Ile	+	+
1	15666	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile	-	+
6	15697	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile	-	+
2	15719	512	128	128	8	16	16	16	Ser83Leu, Asp87Asn	Ser80Ile	-	+
1	15721	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile	+	+
1	15722	1024	128	128	32	32	32	32	Ser83Leu, Asp87Asn	Ser80Arg, Glu84Val	+	+
1	15724	1024	128	128	16	128	32	64	Ser83Leu, Asp87Asn	Ser80Ile	+	+
5	15747	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile	+	+
2	15780	512	128	128	16	32	32	64	Ser83Leu, Asp87Asn	Ser80Ile	+	+
2	15819	512	128	128	4	8	8	8	Ser83Leu, Asp87Asn	Ser80Ile	+	+
3	15836	512	128	128	8	16	8	16	Ser83Leu, Asp87Asn	Ser80Ile		

[a], Norfloxacin; [b], Ciprofloxacin; [c], Nalidixic acid; [d], Gatifloxacin; [e], Gemifloxacin; [f], Levofloxacin; [g], Moxifloxacin; [h], Outer membrane protein F; [i], Outer membrane protein C; [j], Not detected; [k], Detected

Location: 1, Jungrang-chun; 2, Han River; 3, Upstream of Sumjin River; 4, Sumjin River; 5, Kumho River; 6, Nakdong River

extremely resistant to norfloxacin (MIC≥128 μg/ml) and were subjected to further studies. Only one isolate was selected when isolates from the same sample showed the same antimicrobial resistance profile. These were nine isolates from Jungrang-chun, four isolates from the Nakdong River, three isolates from the Han River, two isolates from the Kumho River, one isolate from the Sumjin River, and one isolate from the region upstream of the Sumjin River. These isolates were resistant to various quinolones, including

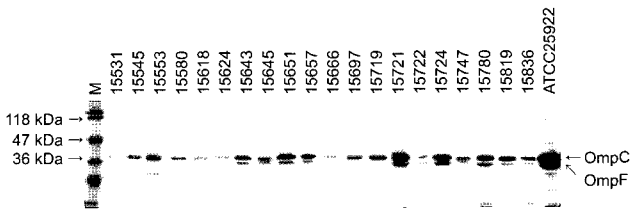

Fig. 1. SDS-PAGE of outer membrane proteins of quinolone-resistant environmental isolates of *E. coli*.
The outer membrane was prepared as described in the Materials and Methods. Proteins extracted from the outer membrane were separated on a 15% acrylamide gel using SDS-PFGE. M, Prestained protein molecular weight marker (Sigma).

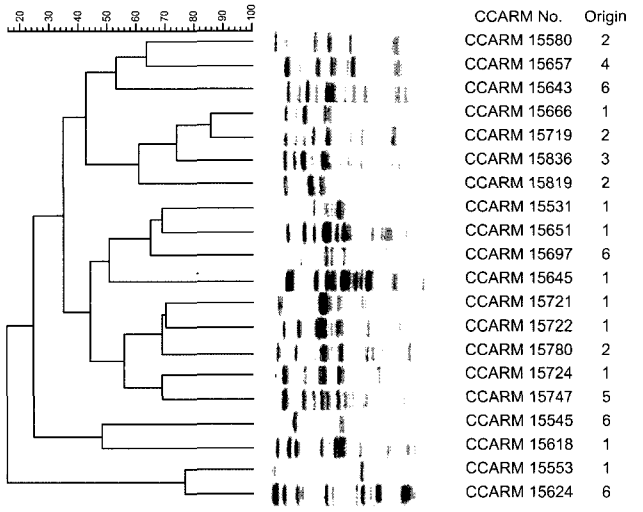

Fig. 2. Dendrogram of quinolone-resistant environmental isolates of *E. coli* based on PFGE.
The genomic DNA was treated with *Xba*I and the resulting DNA fragments were separated with PFGE. The DNA profiles were subjected to data processing in a Gel Compar II (Applied Maths, Belgium), using the unweighted-pair group method using arithmetic averages, the Dice coefficient, a position tolerance of 1% and an optimization of 1%. Isolates were defined as representing the same strain (being indistinguishable) if they possessed 100% similarity to the restriction fragment patterns of the DNA. Isolates were defined as having a clonal relationship if they possessed 85% similarity to the PFGE profiles. (1, Jungrang-chun; 2, Han River; 3, Upstream of Sumjin River; 4, Sumjin River; 5, Kumho River; 6, Nakdong River)

the third generation quinolones, such as moxifloxacin, levofloxacin, and gemifloxacin (Table 2). Every quinolone-resistant *E. coli* isolate had the same three mutations-Ser83→Leu and Asp87→Asn in the QRDR of GyrA and Ser80→Ile in the QRDR of ParC, with the exception of isolate No. 15722 which had Ser80 →Arg instead of Ser80→Ile in ParC. Ser80→Arg was reported to be related to a high MIC (Zao *et al.*, 2005), but not very often. Two isolates (Nos. 15545 and 15624) from the Sumjin River had an additional mutation of Glu84→Gly in ParC and one isolate (No. 15722) from Jungrang-chun had a Glu84→Val mutation in ParC. However, the presence of these mutations was not related to an increase in MIC.

Quinolone resistance can be rendered by decreased expression of an outer membrane protein responsible for quinolone import (Dechene-M *et al.*, 1990). OmpF, in particular, has been reported to be related to fluoroquinolone resistance (Hooper, 1989; Xia *et al.*, 2002). However, the results obtained from this study (Fig. 1.) showed no relationship between the amount of OmpF and susceptibility. Eight out of twenty isolates did not express OmpF. This result supports the previous finding that there is no relationship between a loss of OmpF and ciproflorxacin-resistance in clinical isolates (Lehn *et al.*, 1996).

Isolates with higher MICs to gatifloxacin, levofloxacin, and moxacillin belonged to the same group (Fig. 2) in PFGE. However, clonal spread was not found in these isolates.

High quinolone-resistance rates (40%) in both a clinical environment and the animal industry in Korea (www.kfda.or.kr) provoked a concern about the direct infection of these resistant *E. coli* in humans as well as the transfer of resistance to human microbiota. This study showed that the prevalence of antimicrobial resistance in our environment is due to human activity. Since the rivers tested in this study are being used for agriculture, antimicrobial resistance can accumulate in humans. Without a thorough sewage treatment system, antimicrobial resistant bacteria induced by human activity will continue to contaminate our environment and finally end in humans.

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