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Characterization of Quinolone-Resistant Clinical Isolates of *Escherichia coli* in Korea

Yoojung Oh, Seohyung Park, Misun Ha, and Yeonhee Lee*

Department of Biology, Seoul Women's University, Seoul 139-774, Korea

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Twenty-eight clinical isolates of *Escherichia coli*, composed of thirteen norfloxacin resistant isolates (MIC of >16 μg/ml), one intermediately resistant isolate (MIC of 8 μg/ml), and fourteen susceptible isolates (MIC of <4 μg/ml), were randomly selected to study the norfloxacin resistance mechanism and phylogeny in clinical isolates in Korea. Eleven norfloxacin resistant isolates and one susceptible isolate were multi-drug resistant (MDR). Every norfloxacin resistant isolate with MIC higher than 32 μg/ml had the same three mutations: Ser83→Leu and Asp87→Asn or Tyr in GyrA and Ser80→Ile in ParC. Whereas a resistant isolate with MIC of 16 μg/ml had three mutations but Asp87 in GyrA was replaced with Gly instead of Asn. The intermediately resistant isolate had the same two mutations in GyrA but a different mutation in ParC, Glu84→Lys. Among the susceptible isolates, two isolates with MIC of 4 μg/ml had one mutation: Ser83→Leu in GyrA, and no mutation was found in the susceptible isolates. Resistant isolates showed higher efflux activity than the susceptible ones, with random amplification of polymorphic DNA (RAPD), six susceptible isolates form a separate group from the rest of the isolates.

Key words: clinical isolate, *Escherichia coli*, norfloxacin, QRDR, RAPD, resistance

Quinolone is a specific inhibitor of DNA gyrase and topoisomerase IV (Bauernfeind, 1971; Chen *et al.*, 1996; O'Dea *et al.*, 1996). DNA gyrase is encoded by *gyrA* and *gyrB* and unwinds the supercoiled DNA helix prior to replication and transcription while topoisomerase IV is encoded by *parC* and *parE* and involved in decatenation. Interaction of quinolone with a complex of DNA with DNA gyrase or topoisomerase VI results in conformational changes in both enzyme-bound DNA (Krueger *et al.*, 1990; Marians, *et al.*, 1997) and the enzyme itself (Kampranis and Maxwell, 1998) and the quinolone-enzyme-DNA complex blocks the progression of the replication fork (Hiasa *et al.*, 1996).

Since quinolone resistance is not transferred via plasmid, quinolone was assumed to be free from the resistant problem. However, the emergence of infection due to fluoroquinolone-resistant *Esherichia coli* has been observed all around the world (Cometta *et al.*, 1994; Kern *et al.*, 1994; Carratala *et al.*, 1995). As the clinical use of quinolone increases, the occurrence of the quinolone-resistant strain has increased. Investigations for the frequency of the resistance manifest and the resistant mechanism have been progressing actively (Aoyama *et al.*, 1987; Hirai *et al.*, 1987; Cullen *et al.*, 1989; Watanabe *et al.*, 1990; Kern *et al.*, 1994; Park *et al.*, 1996; Schmitz *et al.*, 1999; Brisse *et al.*, 2000; White *et al.*, 2000).

Mutations responsible for the fluoroquinolone resistance are found in the chromosomal DNA and mechanisms of bacterial resistance to fluoroquinolone fall into three principal categories: 1, alteration in the target enzymes (Barnard and Maxwell, 2001; Friedman *et al.*, 2001); 2, alteration that limits the drug permeation (Yamano *et al.*, 1990); 3, existence of an efflux system (Cohen *et al.*, 1988; Maseda, 2000)

In this study, norfloxacin-resistant, intermediately resistant, and susceptible clinical isolates of *E. coli* in Korea were selected randomly and their norfloxacin resistance mechanism and phylogeny was studied.

Materials and Methods

Bacterial strains

Twenty-eight clinical isolates of *E. coli* in CCARM (Culture Collection of Antibiotics Resistant Microbes) were used for this study. These were originally isolated in a clinical environment and composed of thirteen norfloxacin-resistant, one intermediately resistant, and fourteen susceptible isolates.

Assay of minimal inhibitory concentration

Assay of minimal inhibitory concentration (MIC) was performed following the procedure recommended by the

* To whom correspondence should be addressed.
(Tel) 82-2-970-5664; (Fax) 82-2-970-5669
(E-mail) yhlee@swu.ac.kr

National Committee for Clinical Laboratory Standards (NCCLS, 2000).

DNA sequencing of QRDR in gyrA and parC

DNA was extracted from a loopful of bacterial cells by the cetyltrimetyl ammonium bromide (CTAB) method. Amplification of quinolone resistant determining region (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. After PCR products were confirmed on a 1% agarose gel and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany), DNA sequencing was performed following the manufacturer's guide and analyzed in an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). The DNA sequence of each sample was compared with the sequences of GyrA (Accession No. X57174) and ParC (Accession No. X58408) at GenBank.

Random Amplification of Polymorphic DNA

PCR was performed with random primers 5'-GTAGACCCGT-3' and 5'-AAGAGCCCGT-3'. PCR was constituted with 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 34°C, and 2 min at 72°C, and final extension for 10 min at 72°C. The amplified products were electrophoresed in a 2% agarose gel 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 previously (Kim *et al.*, 1996; Cho *et al.*, 2001). Bacterial cells in log phase grown in Muller-Hinton (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 was 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 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).

Results

MICs of norfloxacin-resistant isolates of E. coli

Among twenty-eight isolates, eleven isolates are multi-drug resistant (MDR) to more than three antibiotics and these are all norfloxacin-resistant. And one isolate (CCARM 1052) was MDR even though it was susceptible to norfloxacin (Table 1).

Table 1. MICs of norfloxacin-resistant clinical isolates

CCARM No.	Ampicillin	Cephalothin	Gentamycin	Norfloxacin
1001	>128	16	2	64
1002	>128	8	128	64
1003	>128	32	>128	32
1004	>128	16	64	<0.25
1005	>128	64	8	32
1006	>128	16	0.5	1
1007	>128	4	64	32
1008	>128	16	64	>128
1009	>128	32	64	32
1010	>128	32	64	64
1011	>128	32	128	>128
1012	128	4	0.25	>128
1013	8	16	8	16
1014	>128	>128	32	32
1015	>128	64	128	>128
1046	>128	16	2	<0.25
1047	>128	16	8	<0.25
1048	16	32	>128	4
1049	16	32	>128	4
1050	16	8	1	<0.25
1051	>128	16	128	4
1052	>128	32	>128	4
1053	8	8	0.5	<0.25
1054	16	32	>128	4
1058	>128	8	4	8
1061	8	32	>128	4
1062	>128	16	0.5	<0.25
1105	8	32	>128	4

DNA sequence of QRDR of gyrA and parC

Twelve norfloxacin-susceptible isolates with MIC to norfloxacin less than 2 μg/ml did not have any mutation in either GyrA or ParC. Two isolates with MIC=4 μg/ml had the same mutation in GyrA, Ser83→Leu but no mutation in ParC. An intermediately resistant isolate (MIC=8 μg/ml) had three mutations, two mutations (Ser→83Leu, Asp87→Asn) in GyrA and one mutation (Glu84→Lys) in ParC. Among thirteen resistant isolates, one isolate (MIC =16 μg/ml) had three mutations, Ser83→Leu and Asp87 →Gly in GyrA and Glu84→Lys in ParC. Eight resistant isolates had three mutations, Ser83→Leu and Asp87→ Asn in GyrA and Ser80→Ile in ParC and four resistant isolates had Ser83→Leu and Asp87→Tyr in GyrA and Ser80→Ile in ParC. In the case of CCARM 1011, it had an additional mutation, Ala108→Thr in ParC (Table 2).

Efflux system

When the ratio between the intracellular norfloxacin con-

Table 2. Mutations in QRDR in GyrA and ParC

CCARM No.	MIC (μg/ml)	GyrA	ParC
1008	128	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1010	128	Ser83-->Leu, Asp87-->Tyr	Ser80-->Ile
1011	128	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile, Ala108-->Thr
1012	128	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1015	128	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1001	64	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1002	64	Ser83-->Leu, Asp87-->Tyr	Ser80-->Ile
1005	64	Ser83-->Leu, Asp87-->Tyr	Ser80-->Ile
1003	32	Ser83-->Leu, Asp87-->Tyr	Ser80-->Ile
1007	32	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1009	32	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1014	32	Ser83-->Leu, Asp87-->Asn	Ser80-->Ile
1013	16	Ser83-->Leu, Asp87-->Gly	Ser80-->Ile
1058	8	Ser83-->Leu, Asp87-->Asn	Glu84-->Lys
1051	4	Ser83-->Leu	None
1052	4	Ser83-->Leu	None
1054	2	None	None
1004	<0.25	None	None
1006	<0.25	None	None
1046	<0.25	None	None
1047	<0.25	None	None
1048	<0.25	None	None
1049	<0.25	None	None
1050	<0.25	None	None
1053	<0.25	None	None
1061	<0.25	None	None
1062	<0.25	None	None
1105	<0.25	None	None

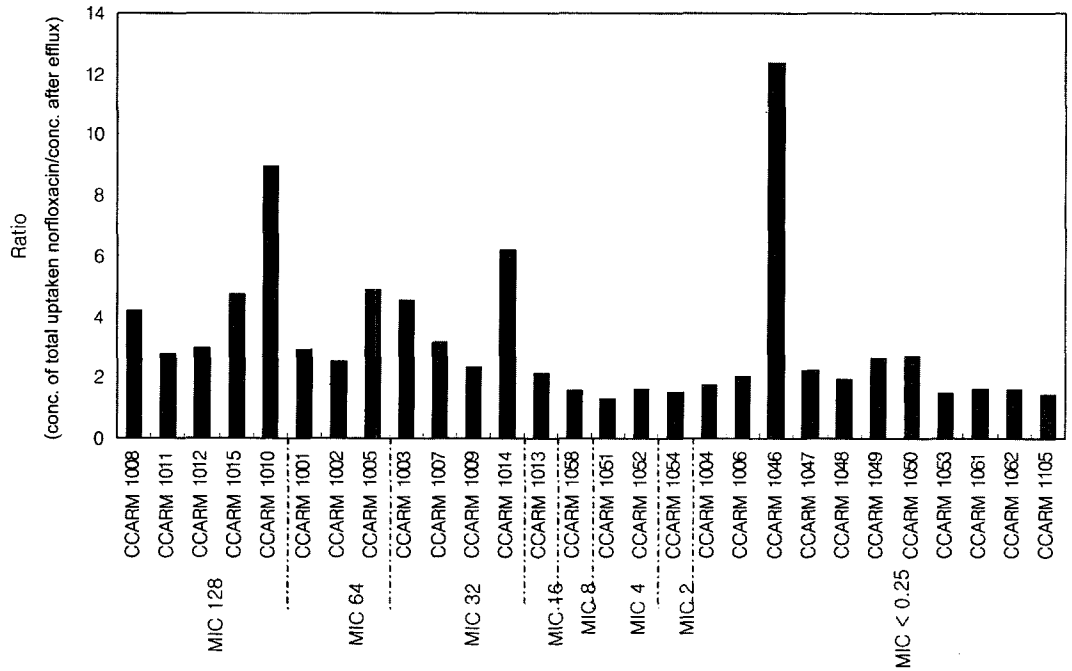

Fig. 1. Ratio between the concentration in the absence and presence of efflux. The ratio was calculated by dividing the intracellular norfloxacin concentration in the absence of CCCP (the concentration after efflux) by the concentration in the presence of CCCP (the total imported amount).

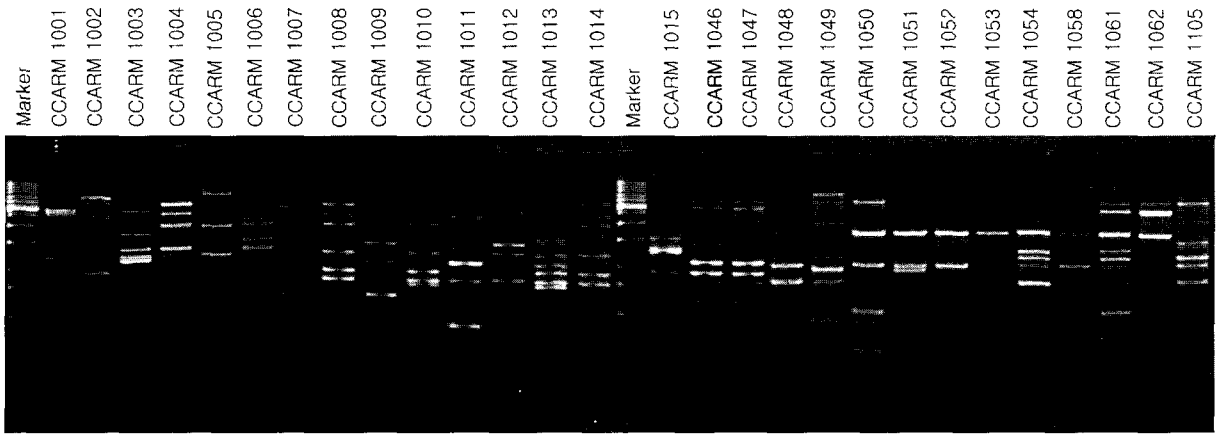

Fig 2. RAPD of norfloxacin resistant clinical isolates of *E. coli.* DNA fragments from RAPD was electrophoresed in a 1% agarose gel and visualized with ethidium bromide.

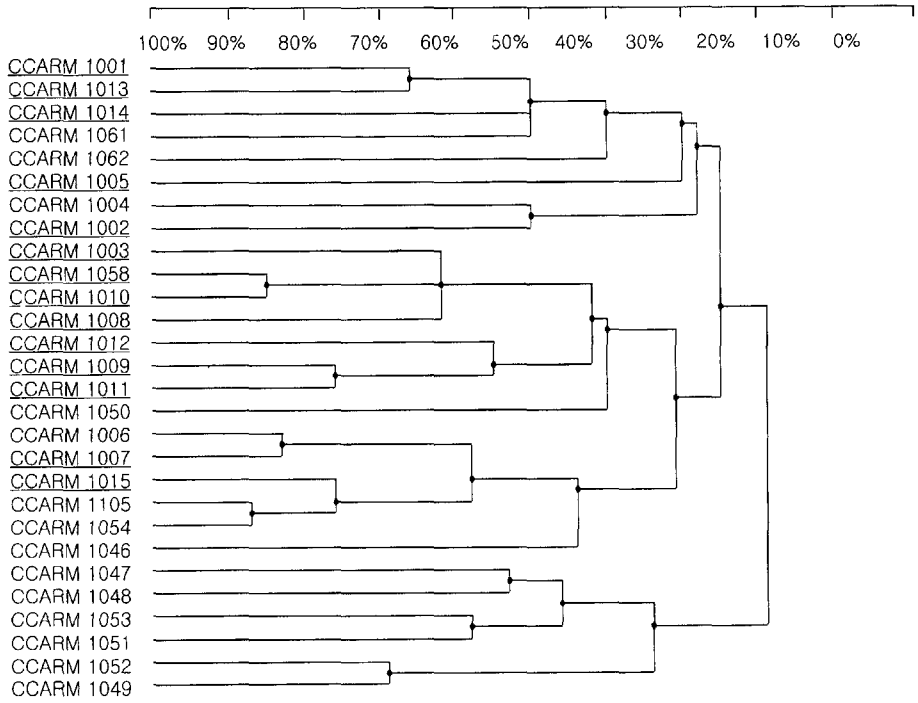

Fig 3. Phylogeny of clinical isolates of *E. coli.* Norfloxacin resistant isolates are underlined.

centration in the absence of CCCP (the concentration after efflux) and in the presence of CCCP (the total imported amount) was calculated, all resistant isolates showed an efflux system with the ratio larger than 1 and six isolates showed efflux activity with more than four-fold activity greater than the import (Fig. 1). In the case of norfloxacin-susceptible isolates, all except four showed less than two-fold activity of the efflux system.

RAPD

PCR was performed with twenty-eight isolates used in this study using ten different random primers purchased commercially. PCR with two primers showed a great variation and the isolates could be grouped into two (Fig. 2 and 3). Seven out of twelve norfloxacin-susceptible isolates showed similar RAPD patterns constituting one group while norfloxacin-resistant isolates could be grouped together but with remote relationship to each other.

Discussion

When quinolone started to be used in a clinical environment (Hooper and Wolfson, 1989; Hooper, 1995), it was assumed that it would have less resistance problems than other antibiotics. The basis for this hypothesis is that the quinolone-resistance mutation occurred in the main chromosome and not in the plasmid and transposon. Another

reason is that no quinolone-degrading or -modifying enzyme has been found, yet. On the contrary, the number of reports about the occurrence of quinolone-resistant bacteria has been increasing all over the world.

In the case of *E. coli,* all quinolone-resistant mutations have a mutation at the Ser80 in GyrA near the quinolone-binding site (Horowitz and Wang, 1987). Mutations at other sites without a mutation at Ser80 at the same time do not engender resistance. Highly quinolone-resistant *E. coli* always has three mutations, two at Ser83 and Asp87 in GyrA and one at Ser80 in ParC and all resistant isolates have the same amino acid changes, Ser80 in Gyr A replaced with Leu, Asp87 in GyrA replaced with Asn or Tyr, and Ser80 in ParC replaced with Ile (Heisig *et al.,* 1993; Willmott and Maxwell, 1993; Khodursky *et al.,* 1995; Heisig, 1996; Kumagai *et al.,* 1996; Khodursky *et al.,* 1998). In this study, the same three mutations were observed in the clinical norfloxacin resistant isolates in Korea, too. An isolate with Asp87 in GyrA replaced with Gly but not by Asn or Tyr showed low resistance with an MIC=16 μg/ml. An isolate, with the same two mutations in GyrA but Glu84→Lys instead of Ser80→Ile in ParC, had an MIC=8 μg/ml.

The efflux system has been reported to be involved in the resistance in *E. coli* (Cohen *et al.,* 1988), however the relation between the MIC and efflux activity was not as clear as in the case of *Pseudomonas aeruginosa* (Lee and Lee, 1998; Maseda *et al.,* 2000; Yoneyama *et al.,* 2000). In this study, resistant isolates showed better efflux activity than the susceptible ones with few exceptions. However, the correlation was not as clear as mutations in GyrA and ParC.

Clinical isolates in this study could be grouped into two groups. One of them is composed of six susceptible isolates and two of them with an MIC=4 μg/ml. The other group was composed of fourteen resistant isolates and seven susceptible isolates, however the similarity in this group was very low. Even though the sample size was not large enough to reach a definitive conclusion, it seemed that there are bacteria that are prone to develop resistance more easily and some are not.

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