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Molecular Analysis of Fluoroquinolone-Resistance in *Escherichia coli* on the Aspect of Gyrase and Multiple Antibiotic Resistance (*mar*) Genes

Yoon-Hee Park[1], Jin-Hong Yoo[1], Dong-Ho Huh[2], Yoon-Kyung Cho[2], Jung-Hyun Choi[1], and Wan-Shik Shin[1]

We analyzed the fluoroquinolone resistance mechanism of 28 isolates of ciprofloxacin-resistant E. coli from patients who received ciprofloxacin as a regimen of a selective gut decontamination. Isolates distinctive by infrequent restriction site polymerase chain reaction (IRS-PCR) were subjected to Hinf I restriction fragment length polymorphism analysis, single-stranded conformation polymorphism (SSCP), and nucleotide sequencing of the quinolone resistance determining region (QRDR) in gyrA. Double mutations in QRDR of gyrA (Ser83 Leu and Asp87Asn) were found from most of the strains. Nucleotide sequencing of the marR locus showed that 18 out of 28 (64%) ciprofloxacin-resistant E. coli strains had three types of base change in marR loci: a double-base change at nucleotides 1628 and 1751, or 1629 and 1751: and a single-base change at 1751. However, all the mutated strains showed no tolerance to cyclohexane test, suggesting the mutation in the marR region had no influence on overexpression of the MarA protein. In conclusion, mutation in gyrA was the main mechanism of ciporfloxacin resistance in E. coli from patients with selective gut decontamination. Therefore, mutation in the mar region did not influence the levels of ciprofloxacin resistance in our isolates.

Key Words: Fluoroquinolone-resistant E. coli, marR, mar mutation, gyrA

The incidence of the fluoroquinolone resistance of *Escherichia coli* has been increasing with worldwide use of the drug since 1989 (Pérez-Trallero *et al.* 1993). Several investigators have reported that the incidence of fluoroquinolone-resistant *E. coli* was especially high among those isolates from neutropenic cancer patients owing to the use of fluoroquinolone as a prophylactic regimen (Kern *et al.* 1994). Point mutations in *gyrA* are known to be the main mechanism of quinolone resistance. In addition, point mutation in the *marRAB* operon has been suggested as a primary mechanism of fluoroquinolone resistance (Alekshun and Levy, 1997). The *mar* mutants which show cross-resistance to fluoroquinolone can be selected on agar plates containing tetracycline, chloramphenicol, salicylates, and other unrelated substances (Geoge and Levy, 1983; Cohen *et al.* 1993a; Cohen *et al.* 1993b; Maneewannakul and Levy, 1996). It has been suggested that previously selected *mar* mutants should offer an easy opportunity to gain fluoroquinolone resistance, which in turn is followed by any mutation on the chromosome.

At the Catholic Hemopoietic Stem Cell Transplantation Center (CHSTC) in Seoul, Korea, a prophylactic regimen including fluoroquinolone for patients with leukemia has been useful for the prevention of gram-negative bacterial infections. However, the emergence of fluoroquinolone-resistant *E. coli* has recently become a troublesome issue, as we have already reported a cluster of infections (Yoo *et al.* 1995).

To investigate the possible role of mutation in gyrase and the *mar* gene on fluoroquinolone resistance, we analyzed the QRDR of *gyrA* and the *mar* locus of *E. coli* from patients with leukemia who received fluoroquinolone as a regimen of selective gut decontamination.

MATERIALS AND METHODS

Bacterial strains and determination of MICs

Twenty-eight *E. coli* strains were isolated from blood of leukemic patients with ciprofloxacin prophylaxis. *E. coli* K-12 strain (AG100) and its *marR1* mutants derivative (AG102) (Geoge *et al.* 1983) were kindly provided by Stuart B. Levy. The susceptibility of *E. coli* isolates to ciprofloxacin (Bayer Korea, Seoul) was tested by an agar dilution method (NCCLS, 1998). A strain was interpreted as ciprofloxacin resistant if the MIC was ≥ 4 μg/mL.

Infrequent restriction site PCR (IRS-PCR)

IRS-PCR was performed as previously described (Mazurek *et al.* 1996). In brief, AH and AX adaptors were constructed using oligonucleotide AH1, AH2, AX1 and AX2, respectively. Chromosomal DNA of *E. coli* was isolated by using a QIAamp tissue kit (QIAGEN, Hilden, Germany) and digested with *Hha* I and *Xba* I (Boehringer Mannheim, Mannheim, Germany). Then they were ligated to AH and AX by using a Rapid DNA ligation kit (Boehringer Mannheim) and digested again with the same restriction endonuclease in order to cleave any restriction sites reformed by ligation. Amplification was performed in a DNA thermal cycler (Gene Cycler; Bio-Rad, Richimond, CA, U.S.A.), using AH1 and PX primer. The PCR products were separated on a polyacrylamide gel in 0.5 $\times$ Tris borate EDTA (TBE) buffer and then photographed.

Amplification of the quinolone resistance determining region (QRDR) and analysis of restriction fragment length polymorphism (RFLP)

A 684-bp *gyrA* fragment from an *E. coli* strain was amplified. The primers used were 20-mer and 23-mer oligonucleotide: 5'-TACACCGGTCAACAT-TGAGG-3' and 5'-CCGGATCGGTAAGCTTCTT-CAAT-3'. The sequences of which are identical to nucleotide positions 24 to 43 and complementary to positions 707 to 685, respectively, of the KL-16 *gyrA* gene of *E. coli* (Ausubel *et al.* 1987). The chromosomal DNA was extracted with the use of the QIAamp kit. One microliter of DNA was added to a total volume of 100 μL of PCR mixture containing 50 pmol of each primer, 0.2 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM ITP, 0.2 mM TTP, 0.2 mM UTP, and 2.5 U of Taq DNA polymerase with the use of the reaction buffer (Boehringer Mannheim, Mannheim, Germany).

The mixture was then overlaid with mineral oil. Amplification was initiated in the DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA, U.S.A.) with an initial denaturating step at 95°C for 7 minutes; thereafter, the denaturing step was 30 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The final extension step was done at 72°C for 5 minutes.

PCR products were purified by the addition of 100 μL of chloroform:isoamyl alcohol (24 : 1) and centrifugation at 10,000 $\times$ *g* for 5 minutes. After centrification, 70 μL of the upper aqueous layer was transferred to a 0.5-mL microcentrifuge tube and stored at -20°C. Two microliters of purified PCR product were digested with 8 U of *Hinf* I at 37°C for 2 hours. The reaction mixture was loaded on 3% multipurpose agarose (Boehringer Mannheim, Mannheim, Germany), and gel electrophoresis was performed in 1 $\times$ tris acetate EDTA (TAE) buffer at 4.2 V/cm for 1 hour. The RFLP pattern was visualized under ultraviolet light after staining with ethidium bromide for 30 minutes. As a size marker, 0.4 μg of a 100-bp DNA ladder (GIBCO BRL, Burlington, Ontario, Canada) was used.

Single-stranded conformation polymorphism (SSCP) analysis

The SSCP analysis was done by a heat denaturation method (Birren and Lai, 1993). Aliquots of 1 μL of PCR products were mixed with 1 μL of denaturation solution (90% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The mixture was heated at 95°C for 5 minutes and then immediately cooled on ice. The denatured mixture was briefly centrifuged before use. The single-stranded PCR products were then separated by polyacrylamide gel (6.5% thymidine; 2.7% cytidine) electrophoresis at 15°C in 0.5 × TBE buffer at a constant voltage of 100 V for 16 hours. After electrophoresis, the gel was placed in a fixative enhancer solution containing 200 mL of methanol (50% v/v), 40 mL of acetic acid (10% v/v), 40 mL of fixative enhancer concentrate (10% v/v), and 120 mL of deionized distilled water for 20 minutes with gentle agitation. The gel was stained using the silver-stain-plus kit (Bio-Rad). The gel was rinsed twice for 10 minutes and then stained in 100 mL of staining solution containing 5 mL each of silver complex solution, reduction modulator solution and image development reagent, and 50 mL of room-temperature development accelerator solution for 20 minutes. The staining reaction was stopped by placing the gel in a 5% acetic acid solution. After rinsing for 5 minutes and drying, DNA bands were visualized.

Sequencing of the QRDR of *gyrA*

PCR analysis was performed again using two 20-mer oligonucleotide primers, 5'-AGGAAGAGC-TGAAGAGCTCC-3' and 5'-TTTCCGTATAACGC-ATTGCC-3', respectively. The sequences of these primers are identical to nucleotide positions 41 to 60 and complementary to positions 373 to 354, respectively, of the KL-16 *gyrA* gene of *E. coli* (Ausubel *et al.* 1987). The QRDR (nucleotide 166 to 355; total size, 190 bp) is included in these positions. The whole procedure was performed using the Sequenase PCR product sequencing kit (Amersham Life Science, Cleveland, Ohio, U.S.A.). Two microliters of a 333-bp PCR product were pretreated with 10 U of exonuclease I and 2.0 U of shrimp

alkaline phosphatase at 37°C for 15 minutes and then at 80°C for 15 minutes. The treated PCR product was annealed with the first primer and denatured by heating for 3 minutes at 100°C.

Ten microliters of annealed mixture was added to a labeling solution containing 0.1 M dithiothreitol, [^{35}S]dATP, sequenase DNA polymerase, and reaction buffer. The labeling mixture was incubated at room temperature for 5 minutes. It was then transferred to a tube and maintained at room temperature for 5 minutes. The reaction was stopped by adding 4 μL of stop solution. The mixture was heated at 75°C for 2 minutes immediately before loading onto the sequencing gel, and electrophoresis was performed. After running, the gel was soaked in 10% acetic acid and 15% methanol to remove urea. The gel was dried at 80°C to preserve resolution and autoradiography was performed.

Amplification and nucleotide sequencing of *marRAB* operon

The 568 base pair fragment including *marR* locus was amplified by PCR. Oligonucleotide primers were corresponding to the positions at *marO* (5'-3', 1311-1328) and *marR* (5'-3', 1879-1858). The PCR reaction mixture contained 1 μL genomic DNA, 0.5 μL (5 units/μL) of Taq DNA polymerase (Boehringer Mannheim), 50 pmol each of primer, 8 μL of 2.5 mM dNTP, 10 μL of 10 × PCR buffer in a final volume of 100 μL.

The conditions for amplification of *marR* were denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes with 30 cycles.

Sequencing of PCR product of 568 bp was performed by chain termination method using a Sequenase PCR sequencing kit. 2',3'-dideoxy nucleotide 5'-triphosphate (ddNTP), was mixed with dNTPs and nucleotide synthesis with DNA polymerase was performed.

The PCR product, 5 μL in amount, was mixed with 1 μL (10 U) of exonuclease I and 1 μL (2 U) of shrimp alkaline phosphatase. The mixture was treated at 37°C for 15 minutes to remove excessive single-stranded primers and nucleotides, and then heated at 80°C for 15 minutes. The pretreated PCR product (0.5 pmol) was mixed with 1 μL of primer

(10 pmol/μL) and 10 μL of water. This mixture was denatured by heating at 100°C for 3 minutes and annealed for 5 minutes on ice.

Ten microliters of annealed DNA was mixed with 1 μL of DTT, 2 μL of labeling mixture, 2 μL of reaction buffer, 0.5 μL of [^{35}S]-dATP and 2 μL of Sequenase DNA polymerase. The mixture was incubated at room temperature for 5 minutes.

To terminate the DNA synthesis, 3.5 μL of labeling reaction mixture was transferred to a termination tube containing 10 μL of mixture of 20 μmol of ddGTP, ddATP, ddTTP, ddCTP and then incubated at room temperature for 5 minutes. The reaction was stopped by adding 4 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol).

It was denatured by incubating at 75°C for 2 minutes and placed for 5 minutes on ice. A 3 μL amount of this mixture was loaded onto each lane of polyacrylamide gel and electrophoresis was performed at 1500 V for 3 hours (short run) and 6 hours (long run). After running, the gel was soaked in 10% acetic acid and 10% methanol for 30 minutes to remove the urea and dried at 80°C. Finally, the dried gel was autoradiographed for 24 hours.

Cyclohexane tolerance test

Cyclohexane tolerance test was performed as described previously (Aono *et al.* 1994). In brief, one drop of overnight growth in Luria broth (LB) was spotted on to agar plate, and overlaid with 5 ml of cyclohexane and the plates were sealed with paraffin film to avoid evaporation. Then they were incubated for 24 hours. If there was confluent growth of cells after 24-hour incubation, it was interpreted as tolerant to cyclohexane. AG102 which is tolerant to cyclohexane was used as a control.

RESULTS

Genotypic analysis

IRS-PCR of chromosomal DNA of *E. coli* from leukemic patients showed distinct DNA patterns with each other (Fig. 1) in all isolates, indicating that these isolates did not originate from cross-infection.

Analysis of QRDR in *gyrA*

We tested ciprofloxacin susceptibility of 28 clinical isolates of *E. coli* and found that all except one isolate were resistant to ciprofloxacin. Amplification and restriction analysis with the use of *Hinf* I of the QRDR in these isolates revealed two bands, while analysis of the wild-type strain showed three
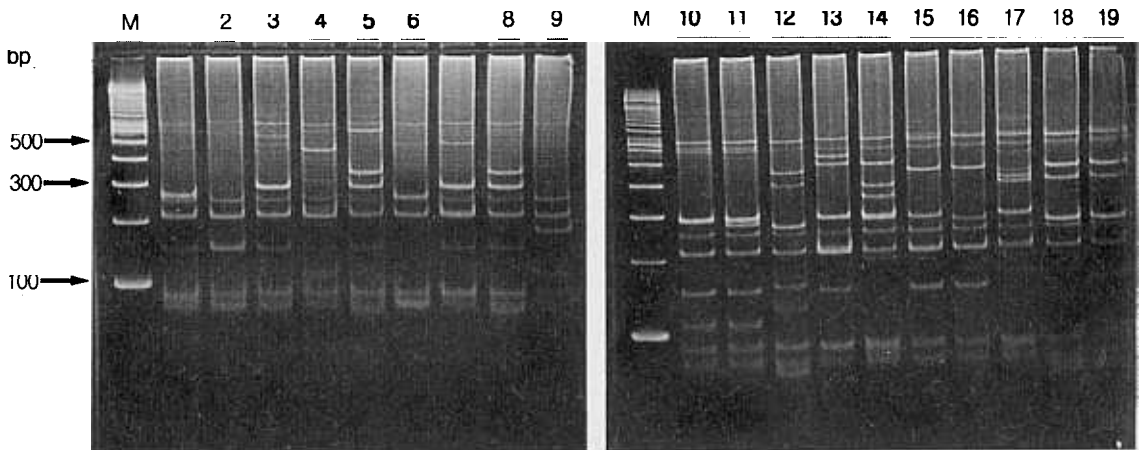

Fig. 1. The results of IRS-PCR on chromosomal DNA of E. coli from leukemic patients with fluoroquinolone prophylaxis. Lane M is the molecular weight marker. Strains were: lane 1, strain 4077; 2, 4092; 3, 4138; 4, 4139; 5, 4181; 6, 4206; 7, 4207; 8, 4309; 9, 4328; 10, 4339; 11, 4429; 12, 4431; 13, 4459; 14, 4578; 15, 4648; 16, 4693; 17, 4698; 18, 5009; 19, 5012.

Table 1. Summary of nucleotide sequencing of the quinolone resistance determining region in the *gyrA* gene of *E. coli*

MIC (μg/mL) of ciprofloxacin	Strains	Deduced amino acid change
32	4139, 4309, 4151	Ser83Leu and Asp87Asn
16	4206, 4578, 4648	Ser83Leu and Asp87Asn
8	4225, 4328, 4291	Ser83Leu and Asp87Asn
4	4388, 4431, 4730	Ser83Leu and Asp87Asn
16	4075, 4077	Ser83Leu and Asp87Tyr
4	4092	Ser83Leu and Asp87Gly
8	4138	Ser83Leu and Asp87His

Asn, asparagine; Asp, aspartic acid; Gly, glycine; His, histidine; Leu, leucine; Ser, serine; Tyr, tyrosine.

Table 2. Mutation in the *marR* gene of quinolone-resistant clinical isolates of *E. coli*

Nucleotide change at:			Strains
1628	1629	1751	
−	A→G	G→A	4077, 4092
A→G	−	G→A	4459, 4181, 4648, 5009
−	−	G→A	4138, 4139, 4206, 4207, 4328, 4339, 4429, 4431, 4578, 4693, 4698, 5012

bands, which indicated that there were alterations of the *Hinf* I restriction site. SSCP analysis revealed various mobility patterns, which suggested that there were strains with various conformational changes in the QRDR of *gyrA* that were possibly the result of at least one point mutation in that area. We selected 16 strains with different SSCP patterns and performed nucleotide sequencing on the QRDR of *gyrA*. The result revealed a transition (cystein to thymidine) at nucleotide 248 in all strains, thus deduced an amino acid change of serine to leucine at position 83 (Ser83Leu). In addition to this mutation, there was another mutation guanosine to adenosine at nucleotide 259, with deduced an amino acid change from aspartic acid to asparagine at position 87 (Asp87Asn). Other replacements and the MICs of ciprofloxacin are shown in Table 1.

Mutation of *marR* locus

Overall, 18 of 28 (64%) of fluoroquinolone-resistant *E. coli* isolates showed point mutation in *marR* (Table 2). All of the 18 strains had a base change (G→A) at position 1751, indicating an amino

acid change from glycine (Gly) to serine (Ser). Among the mutant strains, 6 had another base change: 4 had base changes (A→G) at position 1628, indicating a change from lysine (Lys) to glutamine (Glu), and 2 had changes (A→G) at position 1629, indicating a change from lysine (Lys) to arginine (Arg).

Cyclohexane tolerance

All of the 18 *marR* mutant strains showed no growth after 24-hour incubation in Luria broth containing cyclohexane, while control *E. coli* AG102 (*mar* mutant) showed growth.

DISCUSSION

Bacteremia due to quinolone-resistant strains of *E. coli* has increased in the CHSTC since the end of 1991. Until the end of 1995, the rate of quinolone resistance in *E. coli* from CHSTC was over 90%, whereas that of the non-hematology unit in another

country was less than 10% (Pérez-Trallero *et al.* 1993). We had assumed that the routine prophylactic use of ciprofloxacin by all neutropenic patients may have played a role in the emergence of quinolone-resistant strains of *E. coli* in the Hematology unit.

Because some *E. coli* strains were highly resistant to ciprofloxacin, we investigated the relation of genotypic and fluoroquinolone-resistant patterns in our strains cultured from 1992 to 1995 (Yoo *et al.* 1997). The antibiogram and genotypic patterns revealed little evidence of clonal spread, due to person-to-person transmission. Therefore, we thought the strains might have been part of a patient's own flora, such as that of the gastrointestinal tract.

Nucleotide sequencing of the QRDR of *gyrA* revealed four types of double mutation. Most strains had Ser83Leu and Asp87Asn mutation, while others had different deduced amino acid changes at position 87 (Asp87Tyr, Asp87Gly, and Asp87His). We concluded that a double mutation on the QRDR of *gyrA* was a prime mechanism of resistance. It was interesting that the MICs of ciprofloxacin for strains with the same mutation pattern were different from each other. We assumed that another resistant mechanism, such as active efflux or a mutation in *parC* (Heisig and Tschormy, 1994), had contributed to the increase in the MICs in addition to the mutation in *gyrA*.

Various mutations of the *mar* locus, which is located at 34 min on the *E. coli* chromosome, have been reported and extensively investigated (Ariza *et al.* 1994; Maneewannakul and Levy, 1996; Alekshun and Levy, 1997). The *mar* locus has two transcriptional units, TU1 (*marC*) and TU2 (*marRAB*), divergently expressed from a central operator-promotor region, *marO* (Cohen *et al.* 1993a). Mutation in *marO* or *marR* leads to increased expression of *mar*-specific transcripts (Cohen *et al.* 1993a; Cohen *et al.* 1993b; Ariza *et al.* 1994). Activation of *marRAB* in laboratory mutants brings increased levels of resistance to fluoroquinolones as well as other antibiotics. The *mar* mutant is known to be selected on agar plates containing tetracycline, chloramphenicol, salycilates, or other unrelated substances (Cohen *et al.* 1993a; Cohen *et al.* 1993b; Maneewannakul and Levy, 1996), and then to show a cross-resistance to fluoroquinolone. As reported so far, the frequency of *mar* mutants with fluoro-

quinolone-resistance in clinical isolates is known to be 10% to 15% (Alekshun and Levy, 1997). Organisms with *mar* mutation are known to have an easy opportunity to gain higher levels of fluoroquinolone-resistance than those without it, which in turn is followed by a subsequent mutation of the genes on the chromosome (Alekshun and Levy, 1997).

On the other hand, it is presumed that *mar* mutants might be selected by fluoroquinolone prophylaxis. We examined the presence of *mar* mutants among 28 isolates of fluoroquinolone-resistant *E. coli* from leukemic patients who received prophylactic fluoroquinolone. Among the 28 isolates of FQRE, *mar* mutation was detected from 18 isolates (64.3%). Three types of *mar* mutation were identified; double mutations at two nucleotides 1628 and 1751, and 1629 and 1751, as well as a single mutation at nucleotide 1751.

However, all the mutated strains showed no tolerance to cyclohexane test, suggesting the mutation in the *marR* region did not influence the overexpression of the MarA protein.

In summary, gyrase A mutation and perhaps efflux mechanism may be the main mechanism of fluoroquinolone resistance in our study, while the possibility of a contribution by *marR* mutation to fluoroquinolone resistance appeared to be minor. The increasing current trend toward fluoroquinolone-resistant *E. coli* is an urgent problem in the clinical field which must be related to the increased usage of fluoroquinolones in hospitals. We felt that the "over-the counter" use of antibiotics could also partly account for the high rate of fluoroquinolone-resistant *E. coli*. Therefore, we felt the need to reconsider the effect of the prophylactic use of fluoroquinolone as a gut decontamination regimen as a consequnce of the high rate of fluoroquinolone-resistance. However, we have tentatively decided to continue the adminstration of fluoroquinolone for the following reasons: the incidence of *E. coli* bacteremia has decreased significantly with the prophylactic use of fluoroquinolone; and most isolates of gram-negative bacilli other than *E. coli*, such as *Pseudomonas aeruginosa*, in our center have remained susceptible to fluoroquinolone (Yoo *et al.* 1998).

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