We analyzed an outbreak of *Escherichia coli* bacteremia in eight patients with leukemia in a hematology-oncology unit from July to September 1994. The antibiograms and genotypic patterns of the isolates were different, thus suggesting that the outbreak did not originate from a single clone. However, all the isolates were resistant to quinolones, which led us to examine the microbiological records from 1992 to 1994. The incidence of quinolone-resistant *E. coli* bacteremia in the hematology-oncology unit ranged from 81.8% to 94.6% during this period. We then analyzed 36 more isolates recovered from late 1994 to 1995. Field inversion gel electrophoresis patterns of these isolates were also different. Analysis of the quinolone resistance determining region in *gyrA* revealed that all the isolates had a double mutation in *gyrA*. In conclusion, quinolone-resistant *E. coli* could be an emerging threat to neutropenic patients with leukemia who receive a quinolone prophylactically, and attention must be paid to this trend of resistance.

Quinolones are potent antimicrobial agents used for treatment of infection caused by gram-negative organisms [1]. These agents are also useful for prophylactic gut decontamination in patients with neutropenia [2]. Along with the increased use of quinolones, there have been many reports on the increasing incidence of quinolone resistance in *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but resistance in members of the family Enterobacteriaceae, especially *Escherichia coli*, has been extremely rare until recently [1]. However, some investigators have reported the emergence of quinolone resistance in *E. coli* isolated from patients with malignancies who received fluoroquinolone prophylaxis [3–5].

Between July and September 1994, we recognized an unusually frequent occurrence of *E. coli* bacteremia in eight patients with leukemia in a hematology-oncology unit. To discriminate the strain-to-strain relatedness, we examined the genotypic patterns of these eight isolates. We also found that all of these isolates were resistant to ciprofloxacin; this finding led us to review the data on the antibiotic susceptibility patterns of *E. coli* isolated from 1992 to 1995 to investigate whether there were more cases of bacteremia due to quinolone-resistant *E. coli*. Since this outbreak, we have collected more isolates of *E. coli* and analyzed their genotypic patterns and mechanisms of resistance.

Materials and Methods

Clinical Setting

St. Mary’s Hospital is a tertiary care teaching hospital in Seoul, Korea, that serves a diverse patient population. It also has an eight-bed bone marrow transplantation center and a hematology-oncology unit with eight isolation rooms and 32 inpatient rooms. Of the eight cases of *E. coli* bacteremia, four (cases 1, 3, 7, and 8) occurred in the same room, and two (cases 2 and 6) were in adjacent rooms. Surveillance cultures were done at that time, and samples were obtained from water sources, patients’ stools, and health care workers’ hands.

Patients and Bacteria

Eight cases of *E. coli* bacteremia were identified from July to September 1994. All the *E. coli* isolates from blood were used for genotyping. From October 1994 to September 1995, we collected 36 more blood isolates of *E. coli* to analyze the clonal relationship and quinolone resistance.

Plasmid Typing

Plasmid from *E. coli* isolates was extracted by an alkaline lysis method [6], and electrophoresis through 0.9% agarose gels was performed. The gels were stained with ethidium bromide and inspected by ultraviolet transillumination (320 nm).

Field Inversion Gel Electrophoresis (FIGE)

Isolates of *E. coli* were suspended on Mueller-Hinton broth at 37°C. After incubation overnight, the bacterial cells were centrifuged and washed twice. The pellets were resuspended in 1 mL of TEEN (10 mM Tris-Cl [pH 7.6], 10 mM EDTA [pH 8.0], 10 mM ethylene glycol bis[β-aminoethyl ether] N,N′-tetraacetic acid [EGTA] [pH 7.5], 1 M NaCl); 100 μL of each suspension was mixed with 100 μL of 1% low-melting-point agarose (FMC Bioproducts, Rockland, ME). The mixtures were cast into a mold the size of 0.2 × 0.6 × 1.6 cm (the mold was made in our laboratory). The plugs were solidified at
4°C for 20 minutes. The plugs were then incubated at 37°C for 18 hours in 1 mL of each of the following buffer solutions: 6 mM Tris-HCl (pH, 7.6), 100 mM EDTA (pH, 8.0), 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% lauryl sarcosine–sodium, 1 mg of lysozyme/mL, and 10 g of RNase A/mL. After incubation, the plugs were reacted with 1 mL of proteinase buffer solution (250 mM EDTA [pH, 9.0], 20 mM EGTA [pH, 9.0], 1% [w/v] lauryl sarcosine) at 50°C for 20 hours with gentle shaking.

The plugs were then washed in 5 mL of each of the following washing solutions at 37°C for 16 hours with gentle shaking: 10 mM Tris-HCl (pH, 8.0), 1 mM EDTA (pH, 8.0), and 1 mM phenylmethylsulfonyl fluoride. For restriction endonuclease digestion, the plugs were cut into 0.2-cm slices and equilibrated with 100 µL of restriction buffer at 4°C for 30 minutes, and then the slices were supplemented with 8 U of XbaI (Boehringer Mannheim, Mannheim, Germany) and incubated at 37°C for 6 hours. After digestion, the slices were loaded into the gel, and FIGE was performed. The conditions of FIGE were as follows: the run-in time, 10 minutes; switch interval, 1–25 seconds; forward-to-reverse ratio, 3:1; temperature of running solution, performed in 1 TAE at 4.2 V/cm for 1 hour. The RFLP pattern was visualized under ultraviolet light after staining with ethidium bromide, and the band patterns were observed under ultraviolet transillumination. If there was more than a seven-band difference, the strains were regarded as clonally nonrelated [8].

Determination of MICs

The susceptibility of the eight E. coli isolates to ciprofloxacin (Pfizer Korea, Seoul, Korea) was examined by an agar dilution method. A strain was regarded as quinolone resistant if the MIC of ciprofloxacin was >4 µg/mL [9].

Retrospective Review of Data from 1992 to 1995

We investigated and searched all the microbiological literature on E. coli bacteremia from 1992 to 1995, when the data on microbiological records were computerized. All the data were grouped into leukemia and nonleukemia cases, and the incidence of quinolone resistance determined by the disk diffusion method was calculated for each group.

Amplification of the Quinolone Resistance Determining Region (QRDR) in gyrA and HinfI Restriction Fragment Length Polymorphism (RFLP) Analysis

A 684-bp gyrA fragment from an E. coli strain was amplified. DNA was subjected to PCR analysis with use of 20-mer and 23-mer oligonucleotide primers (5’-TAC ACC GGT CAA CAT TGA GG-3’ and 5’-CCG GTA CGG TAA GCT TCT TCA AT-3’, respectively), 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 [10]. The chromosomal DNA was extracted with use of the QIAamp tissue kit (QIAGEN, Hilden, Germany). One microliter of DNA was added to a total volume of 100 µL of the PCR mixture containing 50 pmol of each primer, 0.2 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM TTP, 0.2 mM UTP, and 2.5 U of Taq DNA polymerase with use of the reaction buffer (Boehringer Mannheim). The mixture was then overlaid with mineral oil. Amplification was initiated in the DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA) with an initial denaturing 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.

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

Single-Stranded Confirmation Polymorphism (SSCP) Analysis

The SSCP analysis was done by a heat denaturation method [11]. Aliquots of 1 µL of PCR products were mixed with 1 µL of denaturant 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 × tris-borate EDTA 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 staining procedure was then done with use of the silver stain plus kit (Bio-Rad, Richmond, CA). The gel was rinsed two times for 10 minutes and was stained in 100 mL of staining solution containing 5 mL of silver complex solution, 5 mL of reduction modulator solution, 5 mL of 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 in gyrA

PCR analysis was performed again with use of two 20-mer oligonucleotide primers, 5’-AGG AAG AGC TGA AGA GCT
CC-3' and 5'-TTT CCG TAT AAC TTG CC-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* [10]; the QRDR (nucleotides 166 to 355; total size, 190 bp) is included in these positions. The whole procedure was performed with use of the Sequenase PCR product sequencing kit (USB, Amersham Life Science, Cleveland). Two microliters of a 333-bp PCR product was pretreated by reacting it with 10 U of exonuclease I/mL and 2.0 U of shrimp alkaline phosphatase/mL 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 was denatured by heating for 3 minutes at 100°C.

Ten microliters of the annealed mixture was added to a labeling solution containing 0.1 M dithiothreitol, [35S]dATP, Sequenase DNA polymerase, and reaction buffer. The labeling mixture was incubated at room temperature for 5 minutes. It was then transferred to the 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 of the gel was performed.

Results

Patients

All the patients had acute myelocytic leukemia and were neutropenic at the onset of bacteremia. Therapy with ciprofloxacin (500 mg/d) and fluconazole (50 mg twice daily) was given to all patients for selective decontamination of the gastrointestinal tract. Only one patient died of intractable pneumonia (case 8). Surveillance cultures were negative for *E. coli* (data not shown).

Genotypic Analysis

When the switch interval was set to the range of 1–25 seconds, the patterns in lanes 1, 3, and 5 and lanes 2, 4, and 6 were similar in the 100- to 500-kbp area (figure 1A). To enhance the migration distance in this area, we shortened the switch interval to 15 seconds and performed FIGE again [7]. Compared with the pattern in lane 1, those in lanes 3 and 5 had a difference of five to six fragments. The pattern in lane 5 also had a difference of four fragments compared with that in lane 2. Because all the strains had a low rate of resistance to the empirical antimicrobial agents (e.g., amikacin, sulbactam, and aztreonam) that were used as therapy for neutropenic patients with hematologic diseases in our hospital (table 2).

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To complement and clarify the epidemiological relationship, we also performed plasmid typing. The plasmid profiles were obviously different from each other (figure 2). Extensive surveillance cultures revealed no further isolates from any human or environmental source. The results of chromosomal and extrachromosomal profile analysis led us to conclude that each strain was not clonally related.

Retrospective Review of Data from 1992 to 1995

Although the antibiograms were different from each other, all the isolates were resistant to ofloxacin and ciprofloxacin. The MICs for all the isolates were >128 µg/mL. Therefore, we retrospectively investigated data on *E. coli* bacteremia from 1992 to 1995. Surprisingly, there had been a high incidence of bacteremia due to quinolone-resistant *E. coli* since 1992. In 1992, the incidence of bacteremia due to ciprofloxacin-resistant *E. coli* in patients with and without leukemia was 81.8% and 5.9%, respectively. Since then, the incidence steadily increased among patients with leukemia: 89.4% in 1993, 94.6% in 1994, and 92.9% in 1995 (table 1). However, most of the quinolone-resistant *E. coli* strains had a low rate of resistance to the empirical antimicrobial agents (e.g., amikacin, sulbactam, and aztreonam) that were used as therapy for neutropenic patients with hematologic diseases in our hospital (table 2).

Further Analysis of Clinical Isolates of *E. coli* Since the Pseudoutbreak

Because all the *E. coli* strains causing this outbreak were uniformly resistant to ciprofloxacin, we investigated the cause and pattern of quinolone resistance in more clinical isolates of...
Table 2. Rate of resistance to empirical antimicrobials among quinolone-resistant Escherichia coli isolated from 130 patients with hematologic malignancy from 1992 to 1995.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. (%) of resistant organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>5 (3.8)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>3 (2.3)</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>7 (5.4)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>5 (3.8)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0</td>
</tr>
</tbody>
</table>

In addition to this mutational change, there was one more mutation at deduced amino acid position 87. Most strains had replacement of aspartic acid by asparagine (Asp87Asn; guanosine to adenosine at nucleotide 259). Other replacements were as follows: aspartic acid for tyrosine (Asp87Tyr; guanosine to thymidine at nucleotide 259), glycine (Asp87Gly; adenosine to guanosine at nucleotide 260), and histidine (Asp87His; guanosine to cytidine at nucleotide 259). It is interesting that the MICs for the strains with the same mutation pattern were different from each other (table 3).

Discussion

The antibiograms and genotypic patterns revealed little evidence of clonal spread, which disproved our initial assumption that the outbreak might have originated from a single clone. Although some isolates’ genotypic patterns revealed by FIGE showed a possible relation with the outbreak pattern, those isolates turned out to be different according to the plasmid profiles. As a result, we concluded that the outbreak of E. coli bacteremia was at least not due to person-to-person transmission.

Although surveillance cultures of specimens from patients were negative, we thought that the strains might have been part of a patient’s own flora, such as that of the gastrointestinal tract. More important, we found that all the isolates had a common feature of quinolone resistance. All the patients with bacteremia received ciprofloxacin prophylactically for selective decontamination of the gastrointestinal tract, which suggested the possible cause of this unique pattern of quinolone resistance. Although there was no obvious proof of this postulation because of the E. coli—negative stool cultures, these quinolone-resistant isolates could have persisted despite selective decontamination and ultimately invaded the bloodstream via translocation. All of the isolates were susceptible to empirically used therapeutic agents such as sulfactam, amikacin, aztreonam, and imipenem, and the prognosis for all but one patient was good.

Quinolone-resistant strains of E. coli have been very rare [1]. However, the result of this genotyping study suggested that there must have been more cases of bacteremia due to quinolone-resistant E. coli because every neutropenic patient with leukemia had routinely received ciprofloxacin prophylactically for selective gut decontamination since the end of 1991.

Table 1. Incidence of bacteremia due to quinolone-resistant Escherichia coli among patients with and without hematologic malignancy from 1992 to 1995.

<table>
<thead>
<tr>
<th>Year</th>
<th>With hematologic malignancy (%)</th>
<th>Without hematologic malignancy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>9/11 (81.8)</td>
<td>1/17 (5.9)</td>
</tr>
<tr>
<td>1993</td>
<td>42/47 (89.4)</td>
<td>7/107 (6.5)</td>
</tr>
<tr>
<td>1994</td>
<td>53/56 (94.6)</td>
<td>10/97 (10.3)</td>
</tr>
<tr>
<td>1995</td>
<td>26/28 (92.9)</td>
<td>10/69 (14.5)</td>
</tr>
</tbody>
</table>

E. coli. From October 1994 to September 1995, we collected 36 more E. coli isolates from blood. All but one of the isolates were resistant to ciprofloxacin. FGE patterns of all the resistant isolates had a difference of more than seven bands. Cluster analysis with a dendrogram of these strains revealed that each strain was not clonally related (figure 3). Amplification and restriction analysis with use of HinI of the QRDR in these isolates revealed two bands, while analysis of a wild-type strain showed three bands (figure 4), which indicated that there were alterations of the HinI restriction site on the QRDR. SSCP analysis revealed various mobility patterns (figure 5).

According to cluster analysis, there were 10 types of mobility patterns (figure 6), which suggested that there had been various conformational changes in the QRDR in gyrA that were possibly induced by at least one point mutation in that area. After selecting representative strains on the basis of SSCP patterns, we performed sequencing on the QRDR in gyrA. Nucleotide sequencing on the QRDR in gyrA revealed a transition (cytidine to thymidine) at nucleotide 248 in all strains, thus leading to the substitution of leucine for serine at position 83 (Ser83Leu).

Figure 2. Plasmid profiles of Escherichia coli. Lanes 1–8, E. coli strains isolated from eight neutropenic patients with leukemia and bacteremia on a hematology unit; lane 9, E. coli strain isolated on another ward during the same period as an epidemiologically unrelated strain. chr = chromosome; M = molecular size marker; kb = kilobase.
Figure 4. *Hin* II restriction fragment length polymorphism analysis of *Escherichia coli* strains revealed two fragments (364 and 320 bp) in the quinolone-resistant strains (lanes 1–5) and three bands 364, 221, and 99 bp in size in a wild-type strain (W; lane 6). M = molecular weight marker.

that some mutation had developed in *gyrA*. Further testing by SSCP analysis revealed that the mobility patterns varied and were different than that of the wild-type strain.

As a final step, we performed nucleotide sequencing of the QRDR in *gyrA*. There were four types of double mutation. Most strains had a Ser83Leu and Asp87Asn mutation, while others had different changes at deduced amino acid position 87 (Asp87Tyr, Asp87Gly, and Asp87His). The focus of interest was that the MICs for the strains with the same mutation pattern were different from each other. Because it was reported that one single mutation in *gyrA* could lead to a change of 0.25 μg/mL in the MIC [12], we thought that another resistance mechanism, such as active efflux or a mutation in *parC* [13–15], had contributed to the increase in the MIC in addition to the mutation in *gyrA*. In summary, there was no cross-transmission of a quinolone-resistant clone, and a double mutation on the QRDR in *gyrA* was the primary mechanism of resistance.

Although we could not elucidate the reason for the selection effect on and the predominance of these strains of quinolone-
resistant *E. coli* in the hematology unit, we assumed that routine prophylactic use of ciprofloxacin by all the neutropenic patients with leukemia might play a role. On the basis of this assumption, there is a dilemma: should ciprofloxacin continue to be used as prophylaxis for selective decontamination? While the routine use of quinolones can be a precipitating cause of an increased rate of quinolone resistance, quinolones have many advantages as prophylaxis: they are the most palatable antimicrobials, and they do not significantly affect anaerobes (only causing little derangement of anaerobic bowel flora [2]). We believe that it is tentatively acceptable to continue prophylaxis with this drug; there are two reasons for our opinion. First, most of the strains of quinolone-resistant *E. coli* in our hospital were susceptible to empirical antimicrobial agents (such as third-generation cephalosporins, carbapenem, and monobactam). As the resistance patterns showed, the management of infectious diseases in granulocytopenic patients with hematologic malignancy was not impeded. Second, because most gram-negative bacilli other than *E. coli* are usually susceptible to quinolones, quinolone prophylaxis may still be effective for the eradication of potential gram-negative pathogens from the gut flora.

Since strains of quinolone-resistant *E. coli* are a potential threat in the future, however [4, 5], we must pay attention to this new trend of resistance and restrict the use of quinolones to more strictly selected cases. In addition, we should weigh and reappraise the benefit of a selective decontamination regimen including quinolones through further prospective study.

References


### Table 3. Summary of nucleotide sequencing of the quinolone resistance determining region in the gyrA gene of *Escherichia coli*.

<table>
<thead>
<tr>
<th>Deduced amino acid changes by mutation, strain number(s)</th>
<th>MIC (µg/mL) of ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser83Leu and Asp87Asn 4139, 4309, 4151</td>
<td>32</td>
</tr>
<tr>
<td>4206, 4578, 4648</td>
<td>16</td>
</tr>
<tr>
<td>4225, 4328, 4291</td>
<td>8</td>
</tr>
<tr>
<td>4388, 4431</td>
<td>4</td>
</tr>
<tr>
<td>Ser83Leu and Asp87Tyr 4075, 4077</td>
<td>16</td>
</tr>
<tr>
<td>Ser83Leu and Asp87Gly 4092</td>
<td>4</td>
</tr>
<tr>
<td>Ser83Leu and Asp87His 4138</td>
<td>8</td>
</tr>
</tbody>
</table>

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