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Development of a Rapid Assay for Detecting *gyrA* Mutations in *Escherichia coli* and Determination of Incidence of *gyrA* Mutations in Clinical Strains Isolated from Patients with Complicated Urinary Tract Infections

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The MICs of ofloxacin for 743 strains of *Escherichia coli* isolated from 1988 to 1994 were determined by testing. The strains were from patients with urinary tract infections complicated by functional or anatomical disorders of the urinary tract. Those determined to be ofloxacin resistant (MIC, ≥12.5 μg/ml) comprised 3 of 395 strains (1.3%) from the 1988 to 1990 group, 2 of 166 strains (1.2%) from the 1991 to 1992 group, and 7 of 182 strains (3.8%) from the 1993 to 1994 group. The incidence of resistant strains increased significantly during this period. The percentage of isolates with moderately decreased susceptibilities to ofloxacin (MIC, 0.39 to 3.13 μg/ml) also rose during the same period. To determine the incidence of *gyrA* mutations in urinary-tract-derived strains of *E. coli*, we developed a simple and rapid assay based on PCR amplification of the region of the *gyrA* gene containing the mutation sites followed by digestion of the PCR product with a restriction enzyme. Using this assay, we examined all 182 strains isolated in 1993 and 1994 for the presence of mutations at Ser-83 and Asp-87 in the *gyrA* gene. Of these strains, 33 (18.1%) had mutations in the *gyrA* gene. The incidences of mutations at Ser-83, at Asp-87, and at both codons were 10.4 (19 strains), 4.4 (8 strains), and 3.3% (6 strains), respectively. To determine the correlation of the mutations in the *gyrA* gene with susceptibilities to quinolones (nalidixic acid, ofloxacin, norfloxacin, and ciprofloxacin), we further examined 116 strains for which the MICs of ofloxacin were ≥0.2 μg/ml that were chosen from the isolates in the 1988 to 1992 group. The MICs of nalidixic acid for the strains without mutations at either Ser-83 or Asp-87 were ≤25 μg/ml, whereas those for the strains with single mutations or double mutations were from 50 to >800 μg/ml. For the fluoroquinolones, significant differences in the distributions of the MICs were observed among the strains without mutations, with single mutations, and with double mutations. The accumulation of mutations in the *gyrA* gene was associated with an increase in fluoroquinolone resistance. Ofloxacin MICs for the majority of the strains with single and double mutations were 0.39 to 3.13 and 6.25 to 100 μg/ml, respectively. This study demonstrates a chronological increase in the percentage of not only highly fluoroquinolone-resistant strains, corresponding to those with double mutations in the *gyrA* gene, but also strains with moderately decreased susceptibilities to fluoroquinolones, corresponding to those with single mutations. This increase in the incidence of strains with a single mutation in the *gyrA* gene portends a further increase in the incidence of strains with clinically significant resistance to fluoroquinolones.

Fluoroquinolones are quite active against gram-positive and gram-negative bacteria. Although *Escherichia coli* strains are commonly susceptible to fluoroquinolones, we have observed the emergence of urinary-tract-derived strains with decreased susceptibilities to fluoroquinolones and fluoroquinolone treatment failures associated with quinolone resistance of *E. coli* in urinary tract infections.

In *E. coli*, quinolone resistance has been reported to correlate with mutations located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene (6, 13, 20). Amino acid changes at Ser-83 and/or Asp-87 in GyrA have most frequently been found in the laboratory *E. coli* mutant strains and in clinical isolates exhibiting high levels of resistance to nalidixic acid and decreased susceptibilities to fluoroquinolones (2, 5, 6, 18, 19).

In this study, we initially tested all 734 *E. coli* strains isolated from 1988 through 1994 in the urology departments of Gifu University Hospital and affiliated hospitals. The strains, which were from patients with urinary tract infections complicated by functional or anatomical disorders of the urinary tract, were analyzed for susceptibility to ofloxacin in order to assess whether there were chronological changes in the incidence of quinolone resistance in urinary-tract-derived *E. coli*. Next, we attempted to determine the incidence of quinolone resistance-associated *gyrA* mutations in the *E. coli* isolates. Several methods have been used to identify mutations in the *gyrA* gene. Although present technology allows reliable direct DNA sequencing of the *gyrA* gene QRDR (3, 12), this procedure is still labor intensive and time consuming. Thus, it is not suitable for the analysis of a large number of clinical strains. Therefore, we developed a rapid and simple assay for detecting mutations at Ser-83 and Asp-87 (1).

MATERIALS AND METHODS

Bacterial strains and antibiotics. Seven hundred forty-three strains of *E. coli* were isolated from patients with urinary tract infections complicated by functional or anatomical disorders of the urinary tract who visited several independent hospitals, including the Department of Urology in Gifu University Hospital. To avoid testing multiple isolates from a single patient, *E. coli* that was isolated

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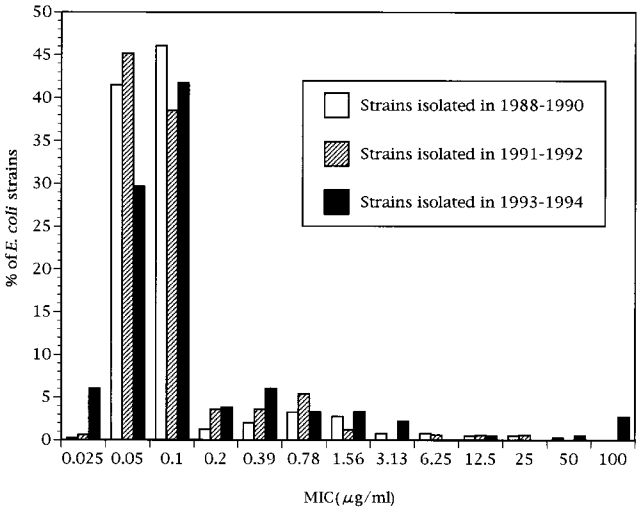

FIG. 1. Chronological change in distribution of ofloxacin MICs for clinical isolates of *E. coli.*

in only one urinary culture from each patient during the infection period was used for this study. The following quinolones were kindly provided by the manufacturers: nalidixic acid and ofloxacin from Dai-ichi Pharmaceutical Co. Ltd., Tokyo, Japan; norfloxacin from Kyorin Pharmaceutical Co. Ltd., Tokyo, Japan; and ciprofloxacin from Bayer Yakuhin, Osaka, Japan.

Susceptibility test. All 743 isolates were examined for susceptibility to ofloxacin. In addition, all 182 strains isolated in 1993 and 1994 and 116 strains for which the MICs of ofloxacin were ≥0.2 μg/ml, which were chosen from the strains isolated from 1988 through 1992, were examined for susceptibilities to nalidixic acid, norfloxacin, and ciprofloxacin. The susceptibilities of the strains to the quinolones were determined by the agar dilution method (8). The strains were cultured in a Mueller-Hinton broth overnight at 37°C, and an inoculum of 10^4 CFU per spot was applied with an inoculator (Microplanter; Sakuma Seisakusho, Tokyo, Japan) to Mueller-Hinton II agar plates (Becton Dickinson, Cockeysville, Md.) containing serial twofold dilutions of each drug. The MIC was defined as the lowest concentration of the drug that completely inhibited visible growth of the inoculum after incubation for 18 h at 37°C.

Assay for detecting mutations at Ser-83 and Asp-87 in the *gyrA* gene. A rapid and simple assay, involving the introduction of an artificial restriction enzyme cleavage site into the PCR product by using a primer-specified restriction site modification method (4) and restriction enzyme digestion of the PCR product, was developed to detect mutations at Ser-83 and Asp-87 in the *E. coli gyrA* gene. DNA fragments including the QRDR of the *gyrA* gene were amplified from chromosomal DNAs of the strains by PCR with the primers EC-GYRA-A and EC-GYRA-*Hin*fI. The sense primer, EC-GYRA-A, was identical to nucleotide positions 139 to 162 (5′-CGCGTACTTTACGCCATGAACGTA-3′) of the *E. coli gyrA* gene (17). The antisense primer, EC-GYRA-*Hin*fI (5′-ATATAACGCAGCGAGAATGGCTGCGCCATGCGGACAATCGAG-3′ [nucleotides 261 to 302]), was located in the vicinity of the mutation sites at Asp-87 with one mismatched nucleotide (underlined) to create a new *Hin*fI cleavage site in the region including Asp-87. These primers were expected to produce a 164-bp DNA fragment. Preparation of DNA from the strains, PCR amplification, and restriction enzyme digestion were performed as reported previously (1). The digested fragments were electrophoresed on a 3% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and visualized with a UV transilluminator. *E. coli* GU-EC87, GU-EC88, GU-EC152, and GU-EC153, which had been examined for mutations in the QRDR of the *gyrA* gene by sequencing amplified PCR products, were used as control strains for the assay. *E. coli* GU-EC87 had a single C-to-T transition at nucleotide position 248 (Ser-83→Leu), GU-EC88 had a single G-to-A transition at position 259 (Asp-87→Asn), GU-EC152 had no mutations in the QRDR of the *gyrA* gene, and GU-EC153 had a double mutation of a C-to-T transition at position 248 (Ser-83→Leu) and a G-to-A transition at position 259 (Asp-87→Asn) (unpublished data). A total of 298 strains, consisting of all 182 strains isolated in 1993 and 1994 and 116 strains chosen from the strains isolated from 1988 through 1992, were analyzed with this assay.

Statistical analysis. The Wilcoxon rank sum test was used for statistical comparisons. All statistical comparisons were two tailed and were performed with the significance set at a P of <0.05.

RESULTS

To analyze the chronological changes in MICs of ofloxacin for urinary-tract-derived strains of *E. coli*, the 743 isolates were divided into three groups: the strains isolated from 1988 to 1990, those isolated from 1991 to 1992, and those isolated from 1993 to 1994. Figure 1 presents the distribution of MICs of ofloxacin for the isolates in each group. According to the proposed criteria for interpretation of the susceptibility of *E. coli* to ofloxacin (9), when the breakpoint for resistance to ofloxacin corresponded to 12.5 μg/ml, the incidence of ofloxacin-resistant strains was 1.3% (5 of 395 isolates) in 1988 and 1990, 1.2% (2 of 166 isolates) in 1991 and 1992, and 3.8% (7 of 182 isolates) in 1993 and 1994. The incidence of ofloxacin-resistant *E. coli* isolates was significantly higher in the 1993 to 1994 group than in the 1988 to 1990 group or in the 1991 to 1992 group ($P < 0.05$). The incidence of isolates with moderately decreased susceptibilities to ofloxacin (MIC, 0.39 to 3.13 μg/ml), which were assigned to the ofloxacin-susceptible category according to the criteria (9), was 9.1% (35 of 395 isolates) from 1988 through 1990, 10.2% (17 of 166 isolates) in 1991 and 1992, and 14.8% (27 of 182 isolates) in 1993 and 1994. This also represents a significant chronological rise, with the incidence in 1993 and 1994 significantly higher than that in the other periods ($P < 0.01$).

To determine the incidence of quinolone resistance-associated mutations in the *gyrA* gene, all 182 strains isolated in 1993 and 1994 were examined for mutations at Ser-83 and Asp-87 in the *gyrA* gene by the assay developed in this study. When a wild type of the *gyrA* gene is amplified with the primers EC-GYRA-A and EC-GYRA-*Hin*fI, the PCR product will have a naturally occurring *Hin*fI cleavage site in the region containing Ser-83 and an artificially created *Hin*fI cleavage site in the region containing Asp-87 (Fig. 2). Digestion of the amplified 164-bp DNA fragment with *Hin*fI will produce fragments of 109, 15, and 40 bp. In the case of PCR products amplified from DNAs having mutations at Ser-83 and/or Asp-87, the enzyme

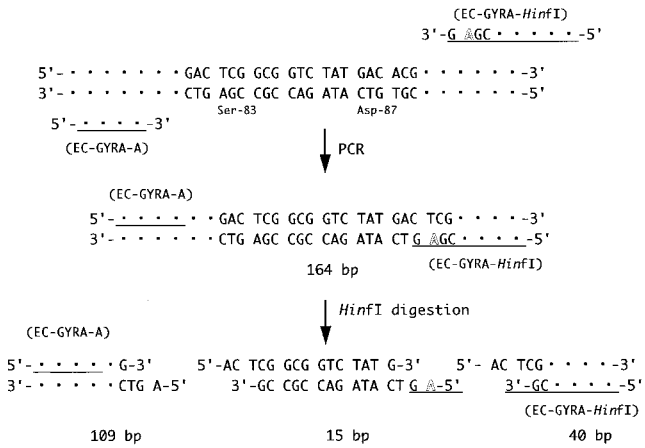

FIG. 2. Schematic diagram of the assay involving introduction of an artificial restriction enzyme cleavage site into the PCR product by using the primer-specified restriction site modification method and restriction enzyme digestion of the PCR product. The modified primer, EC-GYRA-*Hin*fI, is complementary, apart from the incorporation of adenine in place of thymine at the position indicated by the outlined letter. During PCR, the modified primer introduces a thymine into the PCR product. When a DNA fragment is amplified from a wild-type *gyrA* gene by PCR, introduction of thymine creates a *Hin*fI restriction site. Consequently, the PCR product (164 bp) has both naturally occurring and artificial *Hin*fI restriction sites (arrows) in the regions containing Ser-83 and Asp-87 and produces fragments of 109, 15, and 40 bp upon *Hin*fI restriction enzyme digestion.

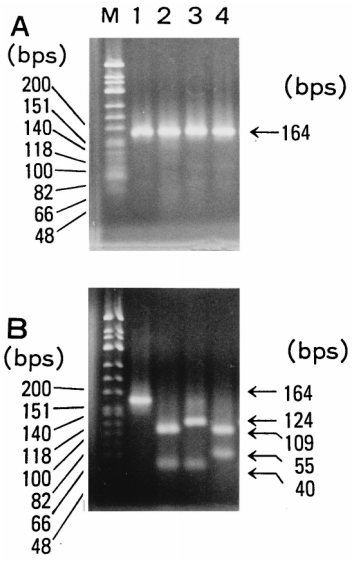

FIG. 3. *Hin*fI restriction fragment length polymorphism. (A) The 164-bp products were amplified from the chromosomal DNAs of *E. coli* GU-EC153, with a double mutation of a C-to-T transition at position 248 (Ser-83→Leu) and a G-to-A transition at position 259 (Asp-87→Asn) in the *gyrA* gene (lane 1); GU-EC152, with no mutations in the QRDR of the *gyrA* gene (lane 2); GU-EC87, with a single C-to-T transition at nucleotide position 248 (Ser-83→Leu) (lane 3); and GU-EC88, with a single G-to-A transition at position 259 (Asp-87→Asn) (lane 4). (B) The *Hin*fI restriction enzyme digestion gave rise to restriction fragment length polymorphism of the PCR products. When there was a 164-bp DNA fragment identical in size to that of the nondigested PCR product after the digestion, as observed for GU-EC153 (lane 1), the strains were assumed to have mutations at both Ser-83 and Asp-87. When two DNA fragments of 109 bp and 40 bp were produced from a PCR product, as observed for GU-EC152 (lane 2), the strains were assumed to have no mutations at either Ser-83 or Asp-87 in the *gyrA* gene. When two DNA fragments of 124 bp and 40 bp were produced, as observed for GU-EC87 (lane 3), the strains were assumed to have a mutation only at Ser-83. When two DNA fragments of 109 bp and 55 bp were produced by *Hin*fI digestion, as observed for GU-EC88 (lane 4), the strains were assumed to have a mutation only at Asp-87. Lanes M, *Hin*fI-digested ϕ X174 DNA molecular size standards.

will not be able to cleave the sites containing Ser-83 and/or Asp-87 to give rise to restriction fragment length polymorphisms. Figure 3 presents representative agarose gel electrophoresis profiles obtained in the assay with the isolates GU-EC87, GU-EC88, GU-EC152, and GU-EC153. When two DNA fragments of 109 bp and 40 bp were produced from a PCR product, as observed for GU-EC152, the strains were assumed to have no mutations at either Ser-83 or Asp-87. The 15-bp fragment theoretically produced was not observed on the gel because it ran off the gel. When two DNA fragments of 124 bp and 40 bp were produced, as observed for GU-EC87, we assumed that *Hin*fI failed to digest the PCR products at the site containing Ser-83 and only digested them at the site containing Asp-87. Therefore, these strains were assumed to have a mutation only at Ser-83. When two DNA fragments of 109 bp and 55 bp were produced by *Hin*fI digestion, as observed for GU-EC88, the site containing codon 87 was destroyed but the site containing codon 83 was digested by the enzyme. These strains were assumed to have a mutation only at Asp-87. When there was a 164-bp DNA fragment identical in size to that of the nondigested PCR product after the digestion, as observed for GU-EC153, *Hin*fI cleavage sites at both Ser-83 and Asp-87 were destroyed; thus, the strains were assumed to have mutations at both Ser-83 and Asp-87.

Among the 182 strains isolated in 1993 and 1994, 33 strains (18.1%) had mutations in the *gyrA* gene. The incidences of mutations at Ser-83, at Asp-87, and at both codons were 19 strains (10.4%), 8 strains (4.4%), and 6 strains (3.3%), respectively. Figure 4 presents the distributions of the MICs of nalidixic acid and ofloxacin for all 182 strains and the associations of the mutations in the *gyrA* gene detected by this assay with these MICs. All the strains for which the MIC of nalidixic acid was ≤25 μl/ml showed no mutations at either Ser-83 or Asp-87, whereas those for which the MIC was ≥50 μl/ml had mutations at Ser-83 and/or Asp-87. For ofloxacin, all the strains for which the MIC was ≤0.2 μl/ml had no mutations, while 33 of 34 strains (97.0%) for which MIC of ofloxacin was ≥0.39 μl/ml had the mutations.

In order to determine the correlation of mutations in the *gyrA* gene with susceptibilities to quinolones, we further examined 116 strains for which the MIC of ofloxacin was ≥0.2 μg/ml, which were chosen from the strains isolated from 1988 to 1992, for the presence of mutations in the *gyrA* gene. The results of analyzing a total of 298 strains, consisting of the 182 strains isolated in 1993 and 1994 and the 116 strains isolated from 1988 through 1992, are summarized in Table 1. Of these 298 strains, 66, 20, and 13 strains had mutations at Ser-83, Asp-87, and both codons, respectively. The strains with mutations at Ser-83 and/or Asp-87, for which the MIC of nalidixic acid was ≥50 μg/ml, were significantly more resistant to nalidixic acid than those without mutations, for which the MIC was ≤25 μg/ml ($P < 0.01$). In relation to ofloxacin, norfloxacin, and ciprofloxacin, the strains with mutations at Ser-83 and/or Asp-87 were significantly more resistant than those without mutations ($P < 0.01$). MICs of the fluoroquinolones for the strains with a double mutation at Ser-83 and Asp-87 were significantly higher than those for the strains with a single mutation at one or the other position ($P < 0.01$). In addition, the strains with a single mutation at Ser-83 were significantly more resistant to fluoroquinolones than those with a single mutation at Asp-87 ($P < 0.01$).

According to the proposed criteria for interpretation of *E. coli* susceptibility to ofloxacin (9), 10 (78.6%) of 13 isolates with the double mutation were assigned to the ofloxacin-resistant category (MIC of ofloxacin, ≥12.5 μg/ml). Almost all the ofloxacin-resistant isolates also exhibited cross-resistance to nalidixic acid, norfloxacin, and ciprofloxacin and belonged to the resistant category for these quinolones (9). In contrast, the majority (96.5%) of 86 isolates with a single mutation were ofloxacin susceptible.

DISCUSSION

To analyze the chronological change in MICs of ofloxacin, we examined 743 strains isolated from 1988 through 1994. The incidence of resistant strains was significantly higher in 1993 and 1994 than in the other periods. In studies prior to 1990, the incidence of fluoroquinolone-resistant *E. coli* isolates in a variety of clinical infections was reported to be ≤1% (9, 16). Lehn et al. (12) reported that fluoroquinolone resistance significantly increased (from <1 to 2.4%) among clinical strains of *E. coli* isolated between 1992 and 1994. For urinary-tract-derived isolates of *E. coli*, the incidence of quinolone resistance was higher than 10% in 1994 (12). Pérez-Trallero et al. (16) also reported a rapid increase from 0.8% in 1989 to 7.1% in 1993 in the incidence of quinolone-resistant strains isolated from urinary tract infections. Peña et al. (15) reported an increase from 0% in 1988 to 7.5% in 1992 in the incidence of quinolone-resistant *E. coli* strains found in patients with bacteremia. These increases are chronologically similar to the trend demonstrated here. In addition, the incidence of isolates showing moderately decreased susceptibility to ofloxacin

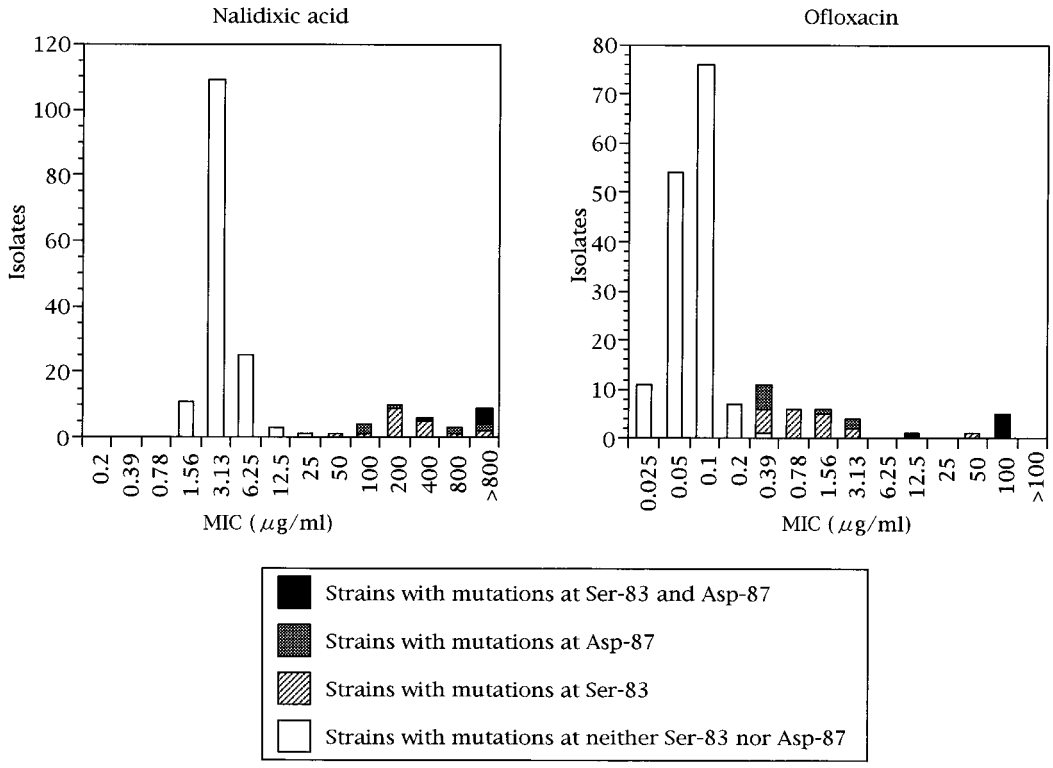

FIG. 4. Distribution of MICs of nalidixic acid and ofloxacin for strains with mutations at Ser-83 and/or Asp-87 and for strains without mutations.

(MIC, 0.39 to 3.13 μg/ml) also rose chronologically. In 1993 and 1994, this incidence was significantly greater than it was in the other periods.

Using a simple and rapid assay, we examined all 182 isolates from the 1993 to 1994 group for the presence of the *gyrA* mutation. Mutations at Ser-83 were most frequently found among these clinical isolates of *E. coli* (10.4%). Although mutations at Asp-87 have been thought to be uncommon in clin-

TABLE 1. Quinolone susceptibilities of 298 strains of *E. coli* classified by types of *gyrA* mutations

Quinolone	Location(s) of mutation(s)	No. of strains	MIC (μg/ml)[a]			No. of resistant[b] strains	Resistance rate (%)	Statistical difference (*P*)[c] in susceptibility to drug listed vs strains with mutations at:		
			Range	50%	90%			Ser-83	Asp-87	Ser-83 and Asp-87
Nalidixic acid	Neither Ser-83 nor Asp-87	199	1.56–25	3.13	6.25	0	0	<0.01	<0.01	<0.01
	Ser-83 alone	66	50–>800	200	>800	66	100		NS[d]	NS
	Asp-87 alone	20	100–>800	100	800	20	100			NS
	Ser-83 and Asp-87	13	400–>800	>800	>800	13	100			
Ofloxacin	Neither Ser-83 nor Asp-87	199	0.025–1.56	0.1	0.1	0	0	<0.01	<0.01	<0.01
	Ser-83 alone	66	0.2–50	0.78	3.13	3	4.5		<0.01	<0.01
	Asp-87 alone	20	0.2–3.13	0.39	1.56	0	0			<0.01
	Ser-83 and Asp-87	13	6.25–100	25	100	11	84.6			
Norfloxacin	Neither Ser-83 nor Asp-87	199	0.025–1.56	0.1	0.2	0	0	<0.01	<0.01	<0.01
	Ser-83 alone	66	0.2–100	0.78	6.25	4	6.1		<0.01	<0.01
	Asp-87 alone	20	0.39–6.25	0.78	6.25	0	0			<0.01
	Ser-83 and Asp-87	13	6.25–400	50	400	10	76.9			
Ciprofloxacin	Neither Ser-83 nor Asp-87	199	0.025–0.39	0.03	0.03	0	0	<0.01	<0.01	<0.01
	Ser-83 alone	66	0.05–50	0.2	1.56	3	4.5		<0.01	<0.01
	Asp-87 alone	20	0.05–0.78	0.1	0.78	0	0			<0.01
	Ser-83 and Asp-87	13	3.13–100	12.5	50	11	84.6			

[a] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.
[b] Isolates are defined as quinolone-resistant strains according to the proposed criteria for interpretation of *E. coli* susceptibility to nalidixic acid, ofloxacin, norfloxacin, and ciprofloxacin (9).
[c] Analyzed by the Wilcoxon rank sum test.
[d] NS, not significant.

ical isolates of *E. coli* (13, 14) and have been reported to usually be concomitant with mutations at Ser-83 (12), we found a single mutation at Asp-87 in 4.4% of the clinical *E. coli* isolates. Additionally, in 3.3% of the isolates, we found a double mutation at Ser-83 and Asp-87 in the *gyrA* gene. This is the first study to determine the incidence of the *gyrA* quinolone resistance-associated mutations in clinical strains isolated from complicated urinary tract infections.

In this study, because of the absence of *gyrA* mutations in strains for which the MIC of ofloxacin was $\leq$0.2 μg/ml, we further selected 116 strains for which the MIC of ofloxacin was $\geq$0.2 μg/ml from the strains isolated from 1988 through 1992 and examined them for mutations in the *gyrA* gene. In clinical isolates of *E. coli*, we confirmed that mutations in the *gyrA* gene, particularly those at Ser-83 and Asp-87, were associated with decreased susceptibilities to fluoroquinolones and that the accumulation of mutations at these codons correlated with an increase in fluoroquinolone resistance. The MIC of ofloxacin for 76 (88.4%) of 86 strains with a single mutation at Ser-83 or Asp-87 was 0.39 to 3.13 μg/ml, and that for 11 (84.6%) of 13 strains with a double mutation at both codons was 12.5 to 100 μg/ml. Conversely, we found that both the incidence of the *E. coli* isolates with clinically significant resistance (MIC of ofloxacin of 12.5 to 100 μg/ml) and that of isolates with moderately decreased resistance to fluoroquinolones (MICs of ofloxacin of 0.39 to 3.13 μg/ml) rose chronologically. Thus, these findings imply that both the strains with a single mutation and those with a double mutation in the *gyrA* gene are increasing.

The strains with a double mutation have been selected in vitro from quinolone-susceptible *E. coli* strains by multistep exposure to fluoroquinolones, and the exposure of strains with a single mutation to fluoroquinolones has selected those with a double mutation in vitro (7, 15). Although the strains with a single mutation in the *gyrA* gene are not clinically resistant to fluoroquinolones, repeated fluoroquinolone challenges against these single-mutation strains could cause in vivo selection of the strains having the double mutation in the *gyrA* gene with or without the simultaneous presence of mutations in the *parC* gene (10–12). These strains will be pathogens with clinically significant resistance to fluoroquinolones. This study implies a chronological increase in the percentage not only of highly fluoroquinolone-resistant strains, corresponding to those with a double mutation in the *gyrA* gene, but also of strains with moderately decreased susceptibility to fluoroquinolones, corresponding to those with a single mutation. The increase in the incidence of strains with a single mutation in the *gyrA* gene might be a portent of a further increase in the incidence of strains with clinically significant resistance to fluoroquinolones. Therefore, it is important to conduct periodic surveillance of the susceptibility of the clinical isolates of *E. coli* and to monitor the percentage of the isolates that harbor quinolone resistance-associated mutations in the *gyrA* gene. The rapid and simple assay described here will be useful in screening mutations of the *gyrA* genes in a large group of *E. coli* clinical isolates with decreased susceptibility to fluoroquinolones.

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