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Experimental and Clinical Studies on Fluoroquinolone-insusceptible Escherichia coli Isolated from Patients with Urinary Tract Infections from 1994 to 2007

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Abstract

Urinary tract infections (UTIs) due to fluoroquinolone-insusceptible Escherichia coli have become increasingly common in recent years. We investigated the potential relationships between clinical measures to combat fluoroquinolone-insusceptible E. coli and experimental analyses on E. coli isolates. Over a 14-year period from 1994 through 2007, a total of 828 E. coli isolates were collected from patients (one isolate per patient) with UTI at the urology ward of Okayama University Hospital. We analyzed the mutations in quinolone resistance-determining regions of DNA gyrase (gyrA) and topoisomerase IV (parC). The production of biofilm by these isolates was also examined and the associated medical records were retrospectively reviewed. Seven of 189 (3.7%) strains from uncomplicated UTIs and 82 of 639 (12.8%) strains from complicated UTIs were insusceptible to fluoroquinolones. Amino acid replacements of type II topoisomerases were frequently observed at positions 83 and 87 in GyrA and at positions 80 and 84 in ParC. No significant difference in the biofilm-forming capabilities was observed between fluoroquinolone-susceptible and -insusceptible E. coli. Our study suggests that biofilm formation of fluoroquinolone-insusceptible E. coli isolates is not a major mechanism of resistance in patients with UTI.

KEYWORDS: fluoroquinolone, Escherichia coli, biofilm, MICs, QRDRs

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Original Article

Experimental and Clinical Studies on Fluoroquinolone-insusceptible *Escherichia coli* Isolated from Patients with Urinary Tract Infections from 1994 to 2007

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Urinary tract infections (UTIs) due to fluoroquinolone-insusceptible *Escherichia coli* have become increasingly common in recent years. We investigated the potential relationships between clinical measures to combat fluoroquinolone-insusceptible *E. coli* and experimental analyses on *E. coli* isolates. Over a 14-year period from 1994 through 2007, a total of 828 *E. coli* isolates were collected from patients (one isolate per patient) with UTI at the urology ward of Okayama University Hospital. We analyzed the mutations in quinolone resistance-determining regions of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*). The production of biofilm by these isolates was also examined and the associated medical records were retrospectively reviewed. Seven of 189 (3.7%) strains from uncomplicated UTIs and 82 of 639 (12.8%) strains from complicated UTIs were insusceptible to fluoroquinolones. Amino acid replacements of type II topoisomerases were frequently observed at positions 83 and 87 in GyrA and at positions 80 and 84 in ParC. No significant difference in the biofilm-forming capabilities was observed between fluoroquinolone-susceptible and -insusceptible *E. coli*. Our study suggests that biofilm formation of fluoroquinolone-insusceptible *E. coli* isolates is not a major mechanism of resistance in patients with UTI.

Key words: fluoroquinolone, *Escherichia coli*, biofilm, MICs, QRDRs

Escherichia coli is one of the major causes of urinary tract infections (UTIs). Fluoroquinolones are often used as potent antimicrobial agents against *E. coli* and other pathogens in Japan as well as in other Asian countries and Europe. As a result, there have been reports in Japan, Korea [1], and Europe [2] on the emergence of *E. coli* strains with increasing resistance to fluoroquinolones. Resistance to fluoroquinolones has also been observed in other bacteria, such as *Neisseria gonorrhoeae*. Thus the widespread use of fluoroquinolones has resulted in fluoroquinolone-resistant *N. gonorrhoeae*, which accounts for 80% to 90% of *N. gonorrhoeae* infections, particularly in Asia [3]. Therefore, cefotriaxone, cefodizime, and spectinomycin are recommended instead of fluoroquinolones for the treatment of gonorrheal infections in Japan [4].

There have been numerous reports on the mecha-

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nism of fluoroquinolone resistance in *E. coli* [5, 6]. There is an urgent need to stop the spread of resistance by prompt implementation of clinical measures. In the present study, we investigated the potential relationships between clinical measures to combat fluoroquinolone-insusceptible (intermediate or resistant) *E. coli* and experimental analyses on *E. coli* isolates. Over a 14-year period from 1994 through 2007, a total of 828 *E. coli* isolates were collected from patients with UTI at the urology ward of Okayama University Hospital. We analyzed the prevalence and the mutations in quinolone resistance-determining regions (QRDRs) of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) and antimicrobial resistance. In addition, the production of biofilm by these isolates was examined and the associated medical records were retrospectively reviewed.

Materials and Methods

Clinical samples and bacterial isolates. The subjects were UTI patients who were seen at the outpatient and inpatient departments (wards) of the Urology Clinic of Okayama University Hospital between 1994 and 2007. The patients with pyuria (WBC $\geq$ 5/HPF) and bacteriuria (bacteria $\geq 1.0 \times 10^4$ CFU/ml) were included in this study. There were 828 strains of *E. coli* which were isolated from the urine of these patients. If multiple strains were isolated in 1 patient, the bacterial strain with the lower susceptibility was selected and established as the isolated strain. Then the strains, from which *E. coli* was isolated again in the same patient for follow-up, were excluded.

Midstream urine and catheter urine were collected from male and female subjects. The samples were inoculated onto CLED (cysteine-, lactose-, and electrolyte- deficient) agar (Becton Dickinson, Franklin Lakes, NJ, USA) and cultured for 48 h at 37℃. In the strains which formed colonies, bacteria were confirmed to be *E. coli* using the rapid ID32 Strep system (bioMérieux, Marcy l'Etoile, France). The strains were stored in Casitone-based complex medium at room temperature. Then the following methods were used to examine their characteristics.

Antimicrobial susceptibility testing. The minimal inhibitory concentrations (MICs) of antimicrobial agents were measured for 79 of 89 strains of fluoroquinolone-insusceptible *E. coli*. A broth microdilution method was used according to the guidelines of the Clinical Laboratory Standards Institute [7]. The antimicrobial agents for which the MICs were measured were ampicillin, cefazolin, cefozopran, minocycline, imipenem, gentamycin, and fluoroquinolones (ofloxacin, norfloxacin, levofloxacin, sparfloxacin, ciprofloxacin, tosufloxacin, and sitafloxacin). Fluoroquinolone-insusceptible *E. coli* strains were defined as those with ofloxacin MICs of $\geq 4\mu$g/ml according to the standards of the Clinical Laboratory Standards Institute [8].

Review of clinical backgrounds of patients. A retrospective examination was performed on the clinical backgrounds of patients in whom *E. coli* was isolated by using their medical records. The following were examined: age, sex, underlying diseases of the urinary tract, whether or not catheterization was performed, history of UTI, use of antimicrobial agents before bacterial isolation, and therapeutic course. The cases were classified into complicated UTI and uncomplicated UTI depending on the presence or absence of underlying diseases. The isolation rates of fluoroquinolone-insusceptible *E. coli* were compared by the χ^2-test.

DNA sequencing (amino acid replacements in type II topoisomerases). In the isolated fluoroquinolone-insusceptible *E. coli*, direct sequencing was performed to analyze the mutations in QRDRs of DNA gyrase and topoisomerase IV. Then the amino acid replacements were determined at those sites. Nine strains of fluoroquinolone-susceptible *E. coli* were randomly selected and used as the control strains.

The samples were cultured in a CLED medium for 24 h. The resulting *E. coli* colonies were picked with sterilized toothpicks. They were shaken in microtubes (volume, 1.5 ml) with 50 μl of 7.5% Chelex-100 solution (Bio-Rad Laboratories, Hercules, CA, USA) in distilled water, and were heated at 100℃ for 10 min to prepare crude genomic DNA lysates. The mixture was held for approximately 10 sec at room temperature and then centrifuged at 10,000 $\times$ g for 1 min. The supernatant (5 μl) was collected, and the first PCR was performed (Table 1). The reaction solution was purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA). The DNA concentration of the purified product was measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop

Table 1 PCR primers and conditions used in the direct sequence method

Primer specificity		Primer sequences	Product length [bp]	PCR conditions			
				Initial denaturation	Cycling	Cycle	Final extension
DNA gyrase A gene (*gyrA*)	1st PCR	F: 5'-GAGGAAGAGCTGAAGAGCTCC-3' R: 5'-CGAGATCGGCCATCAGTTC-3'	376	5 min, 94℃	1 min, 94℃; 1 min, 55℃; 1 min, 72℃	25	7 min, 72℃
	2nd PCR	F: 5'-GAGGAAGAGCTGAAGAGCTCC-3'	–		30 sec, 95℃; 4 min, 60℃	30	–
Topoisomerase IV gene (*parC*)	1st PCR	F: 5'-AAACCTGTTCAGCGCCGCATT-3' R: 5'-GTGGTGCCGTTAAGCAAA-3'	395	5 min, 95℃	1 min, 94℃; 1 min, 55℃; 1 min, 72℃	25	7 min, 72℃
	2nd PCR	F: 5'-AAACCTGTTCAGCGCCGCATT-3'	–		30 sec, 95℃; 4 min, 60℃	30	–

Technologies, Wilmington, DE, USA). The purified product with 8 μg of DNA was used in the second PCR for direct sequencing (Table 1), and a Big Dye Terminator Kit (Applied Biosystems Japan, Tokyo, Japan) was used to perform fluorescent labeling. The second reaction solution was ethanol precipitated, and ethanol was evaporated at 80℃. The resulting product was dehydrated and stored at 4℃. The dried PCR product was dissolved in 10 μl of Hi-Di formamide, and then sequencing was performed. An ABI PRISM® 3100 sequencer (Biosystems) at the Central Research Laboratory, Okayama University Medical School was used to decode the base sequences. The obtained data were analyzed using BLAST databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The standard strain, *E. coli* K12, was used as the control, and amino acid replacements of type II topoisomerases were examined.

Microplate assay for assessment of biofilm-forming capabilities. The biofilm-forming capabilities were tested in 81 of 89 strains of fluoroquinolone-insusceptible *E. coli* and 40 randomly selected strains of fluoroquinolone-susceptible *E. coli*. Each *E. coli* strain was cultured at 37℃ for 48 h in a 96-well microplate (MBEC BioProducts, Edmonton, Alberta, Canada) which was filled with artificial urine as in the report by Minuth *et al.* [9]. Biofilm formed on the peg surface in each well was stained with 2% crystal violet and eluted in 95% ethanol. Then absorbance was measured at 570 nm using a model 680 microplate reader (Bio-Rad). This procedure was repeated 3 times for each strain, and the arithmetic mean of the absorbance was used as an indicator of the biofilm-forming capabilities. The Mann-Whitney *U* test was used to compare the biofilm-forming capabilities between these 2 groups. All results were considered statistically significant at the $p < 0.05$ level.

Results

E. coli isolates and antimicrobial susceptibility testing. In the period between 1994 and 2007, a total of 828 strains of *E. coli* were isolated from midstream urine and catheter urine. According to the MICs of ofloxacin (MIC $\geq$ 4 μg/ml), 89 *E. coli* strains (10.7%) were classified as fluoroquinolone-insusceptible. Fig. 1 shows the annual changes of isolation frequency of fluoroquinolone-insusceptible *E. coli* and the percentage of fluoroquinolone-insusceptible *E. coli* among the total isolated *E. coli*. In our study, fluoroquinolone-insusceptible *E. coli* was first isolated in 1994, and its isolation frequency and its percentage

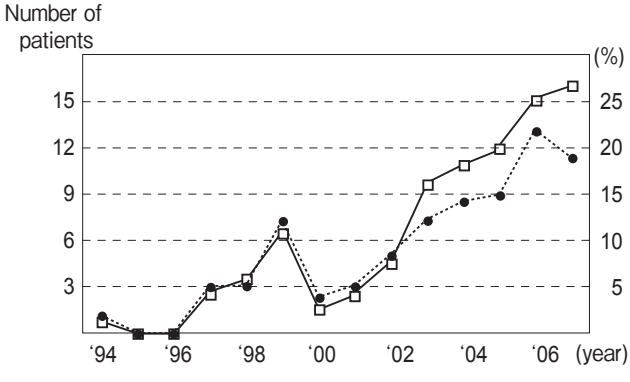

Fig. 1 Annual changes in the incidence of UTI due to fluoroquinolone-insusceptible *E. coli*.
lines: —□— Number of patients with UTI due to fluoroquinolone-insusceptible *E. coli*. --●-- Percentage of fluoroquinolone-insusceptible *E. coli* among the total isolated *E. coli*.

tended to increase annually beginning in 2000. In 2006 and 2007, approximately 20% of the total *E. coli* strains isolated from urine were fluoroquinolone-insusceptible *E. coli*.

The measurement of MICs of other antimicrobial agents showed that fluoroquinolone-insusceptible *E. coli* was highly susceptible to cefozopran and imipenem. Their MIC_{90}s were both 0.5 μg/ml (Fig. 2). The MICs of ofloxacin showed wide variabilities among strains. There tended to be correlations between ofloxacin MICs and the MICs of other fluoroquinolones (Fig. 3). For sitafloxacin, the MICs of all 79 strains tested were $\leq 2\,\mu$g/ml. Among these strains, the MICs of 64 strains (81.0%) were $\leq 0.5\,\mu$g/ml, and those of 13 strains (16.5%) and 2 strains (2.5%) were 1 μg/ml and 2 μg/ml, respectively. When these MICs were compared with those of other fluoroquinolones, the high effectiveness was confirmed *in vitro*.

Clinical backgrounds. Of 189 strains of *E. coli* isolated from uncomplicated UTIs, 7 strains

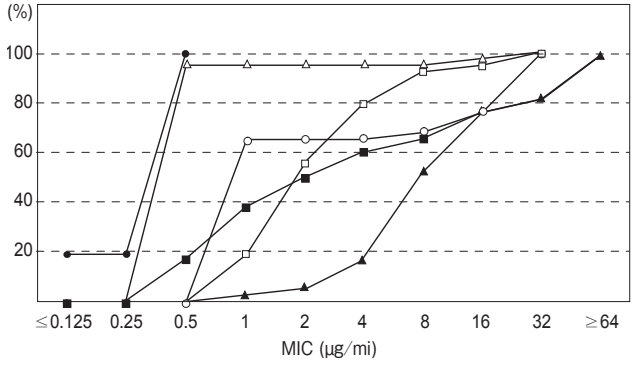

Fig. 2 Cumulative percentages of fluoroquinolone-insusceptible *E. coli* strains at MICs (μg/ml) of several antibiotics except fluoroquinolones. The MIC_{90} values of ampicillin, cefazoline, cefozopran, imipenem, minocycline and gentamycin were 64, 8, 0.5, 0.5, 32 and 32 μg/ml, respectively.
lines: ampicillin, cefazolin, cefozopran, imipenem, minocycline, gentamycin

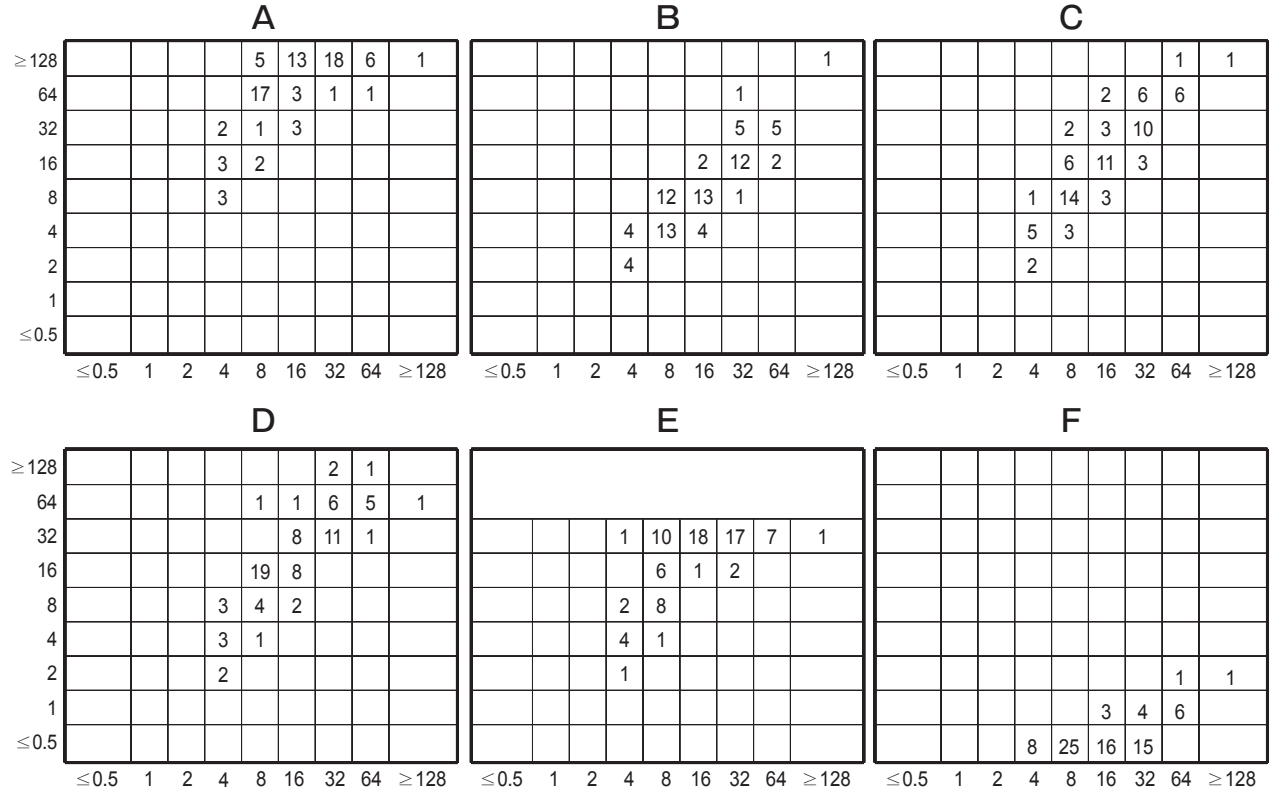

Fig. 3 Correlations between MICs (μg/ml) of ofloxacin (horizontal) and other fluoroquinolones (vertical) for fluoroquinolone-insusceptible *E. coli* strains (n = 79). **A**, norfloxacin; **B**, levofloxacin; **C**, sparfloxacin; **D**, ciprofloxacin; **E**, tosufloxacin; **F**, sitafloxacin. MICs of tosufloxacin ($\geq 64\,\mu$g/ml) could not be measured due to insolubility.

(3.7%) were fluoroquinolone-insusceptible *E. coli*. Of the 639 strains of *E. coli* isolated from complicated UTIs, 82 strains (12.8%) were fluoroquinolone-insusceptible *E. coli*. The analysis by the χ^2-test indicated that the isolation rate of fluoroquinolone-insusceptible *E. coli* from complicated UTIs was significantly higher than that from uncomplicated UTIs.

Table 2 shows the clinical backgrounds of patients with UTI whose causative agent was fluoroquinolone-insusceptible *E. coli*. The male to female ratio was 42:47, and thus the difference between the number of male and female subjects was not significant. The ratio of outpatients to inpatients was 58:31, indicating that fluoroquinolone-insusceptible *E. coli* was isolated more often in outpatients than inpatients. Urethral catheters were placed in 26 patients (29.2%), and clean intermittent self-catheterization was performed in 14 patients (15.7%). There were 66 patients (74.2%) who had a previous history of UTI before fluoroquinolone-insusceptible *E. coli* was isolated. With respect to underlying diseases, neurogenic bladder (39.0%) was seen most often, followed by malignant diseases (26.8%) (such as prostate cancer and bladder cancer), and vesico-vaginal or vesico-rectal fistula (13.4%). There were 21 patients (23.6%) who had not been administered any antimicrobial agents in the two-year period prior to the isolation of fluoroquinolone-insusceptible *E. coli*. There were 68 patients (76.4%) who had been administered some type of antimicrobial agent. Among the 68 patients, 48 (70.6%) had been administered fluoroquinolones. When the selection was limited to a two-week period prior to the bacterial isolation, there were 26 patients (29.2%) who had a history of being administered antimicrobial agents. Of these patients, 20 (76.9%) had been administered fluoroquinolones.

Fig. 4 shows the efficacy of treatments and outcomes among these patients. A total of 49 patients were administered cephems, and 43 of these patients (87.8%) recovered from their UTIs. Administration of penicillins, penems, carbapenems, minocycline, sulfamethoxazole-trimethoprim, and aminoglycosides led to complete recovery or a lessening of symptoms, and all urine cultures were negative after these treatments, in addition, most of urinalysis results improved after these treatments. Among the patients administered fluoroquinolones, many had urine cultures and urinalysis results that did not improve, and follow-ups were not possible in others. There were patients who selected urethral catheterization or clean intermittent self-catheterization rather than administrations of antimicrobial agents among complicated UTI patients in whom fluoroquinolone-insusceptible *E. coli* was isolated and in whom clinical symptoms (such as fever or pain) were very limited. There were 16 patients (18.0%) in whom antimicrobial agents were not used, and spontaneous resolution was observed in

Table 2 Backgrounds of the patients with UTI due to fluoroquinolone-insusceptible *E. coli* (n = 89)

Sex: male/female	42/47
Age: median ± SD (range)	63 ± 19 (8 to 86)
Outpatient/Inpatient	58/31
Catheterization	26/89 (29.2%)
Self-catheterization	14/89 (15.7%)
Repeated UTI: yes/no	66/22 (unclear: 1)
Underlying diseases of the patients with complicated UTI (n = 82)	
Neurogenic bladder	32 (39.0%)
Malignancy	22 (26.8%)
Fistula (vagina, rectum)	11 (13.4%)
Others	17 (20.7%)
Administration of the antimicrobial agent before bacterial isolation	
Prescribed for the last 2 years	68/89 (76.4%)
Prescription of fluoroquinolones	48/68 (70.6%)
Just before isolation (for last 2 weeks)	26/89 (29.2%)
Prescription of fluoroquinolones	20/26 (76.9%)
No prescription	21/89 (23.6%)

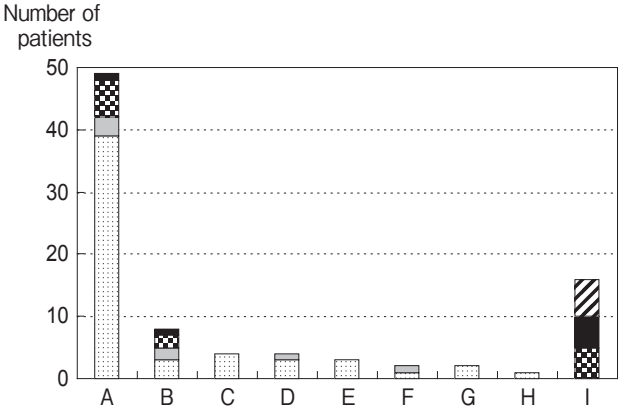

Fig. 4 Decisive treatments and outcomes. **A**, cephems; **B**, fluoroquinolones; **C**, penicillins; **D**, penems; **E**, carbapenems; **F**, minocycline; **G**, sulfamethoxazole-trimethoprim; **H**, aminoglycosides; **I**; none.
bars: cure, light symptom, no change, naturally disappeared, unclear.

5 (31.3%) of these patients.

There were 2 strains of fluoroquinolone-insusceptible *E. coli* for which the sitafloxacin MICs were 2 μg/ml (Table 3). One of these strains was isolated from a patient with uncomplicated UTI in 2007, and the other strain was isolated from a patient with complicated UTI in 2002. The underlying disease of the complicated UTI case was neurogenic bladder, and urethral catheterization or clean intermittent self-catheterization was not performed. The uncomplicated UTI case had not been administered any antimicrobial agents, including fluoroquinolones, prior to the bacterial isolation. However, the complicated UTI case had been administered levofloxacin within 1 week prior to the bacterial isolation. These 2 patients were both treated with cefcapene pivoxil HCl and were cured.

Amino acid replacements in type II topoisomerases. Amino acid replacements were examined in the randomly selected 9 strains of fluoroquinolone-susceptible *E. coli* and 78 strains of fluoroquinolone-insusceptible *E. coli*. In a GyrA subunit of DNA gyrase and a ParC subunit of topoisomerase IV, amino acid replacements tended to be localized at specific sites. Namely, replacement patterns were frequently seen in which there were substitutions of: serine (Ser) to leucine (Leu) at position 83 (Ser-83-Leu) in GyrA, aspartic acid (Asp) to asparagine (Asn) or glycine (Gly) at position 87 (Asp-87-Asn/Gly) in GyrA, serine (Ser) to isoleucine (Ile) or arginine (Arg) at position 80 (Ser-80-Ile/Arg) in ParC, and glutamic acid (Glu) to valine (Val) or other amino acids at position 84 (Glu-84-Val/Others) in ParC. In GyrA, Ser-83-Leu was observed in 78 of 78 strains (100%) and Asp-87-Asn/Gly in 77 of 78 strains (98.7%). In ParC, Ser-80-Ile/Arg was observed in 73 of 78 strains (93.6%) and Glu-84-Val/Others in 44 of 78 strains (56.4%). The replacement at position 84 in ParC was a substitution to: valine in 31 of 44 strains (70.5%), glycine in 5 strains (11.4%), alanine in 4 strains (9.1%), and lysine in 4 strains (9.1%). There was no clear correlation between the number of amino acid replacements at these 4 positions and ofloxacin MIC. Of 78 strains, 75 had amino acid replacements at 3 or more of the aforementioned 4 positions. In addition, 73 of 78 strains (93.6%) had replacements at 3 positions: positions 83 and 87 in GyrA and position 80 in ParC (Table 4).

Among the 9 control strains of fluoroquinolone-susceptible *E. coli*, 5 strains (55.6%) did not have replacements at any of the aforementioned 4 positions. There were 2 strains (22.2%) with a replacement of 1 out of 4 positions, another 2 strains (22.2%) with replacements of 2 positions, and no strain with replacement of 3 positions or more (Table 4). When the 2 *E. coli* strains in which the sitafloxacin MICs were 2 μg/ml were examined, both strains had amino acid replacements at 3 positions (Ser-83-Leu and Asp-87-Asn/Gly in GyrA, and Ser-80-Ile/Arg in ParC) (Table 3).

Biofilm-forming capabilities testing. The biofilm-forming capabilities of 81 strains of fluoroquinolone-insusceptible *E. coli* and 40 strains of fluoroquinolone-susceptible *E. coli* were examined using the Mann-Whitney *U* test. As shown in Fig. 5A, there was no statistically significant difference in the biofilm-forming capabilities between fluoroquinolone-insusceptible and susceptible *E. coli* ($p = 0.19$). It is noteworthy that there were 2 fluoroquinolone-insusceptible strains in which the biofilm-forming capabilities were markedly stronger. One of these strains was isolated from a 70-year-old male with benign prostatic

Table 3 Backgrounds of the patients with UTI due to fluoroquinolone-insusceptible *E. coli*

	Year	Sex	Age	Underlying diseases	Biofilm OD_{570}	Major amino acid replacements		MIC (μg/ml)		
						GyrA	ParC	Ofloxacin	Sitafloxacin	Levofloxacin
A	2002	F	77	Neurogenic bladder	0	S83L, D87G	S80R	128	2	128
	2007	F	71	None	0.073	S83L, D87N	S80I	64	2	16
B	1998	F	18	Urethral stricture	0.783	S83L		32	≤ 0.5	16
	2006	M	70	Bladder stone	2.673	S83L, D87N	S80I, E84V	8	≤ 0.5	4

A: Two strains of *E. coli* which MIC of sitafloxacin was 2 μg/ml.
B: Two strains of *E. coli* with strong capabilities of biofilm formation.

hyperplasia, bladder stone, and a history of urethral catheterization. The other strain was isolated from an 18-year-old female without an underlying disease but who had repeated bouts of UTI treated with antimicrobial agents (Table 3).

The 2 strains of *E. coli* with sitafloxacin MICs of 2 μg/ml had weak biofilm-forming capabilities. The 77 strains with sitafloxacin MICs of $\leq 1\,\mu$g/ml were divided into a group with MICs of $\leq 0.5\,\mu$g/ml (64 strains) and a group with a MIC of 1 μg/ml (13 strains). There was no statistically significant difference between these 2 groups ($p = 0.93$) (Fig. 5B).

Discussion

Eighty-nine strains of fluoroquinolone-insusceptible *E. coli* were isolated at our department in a 14-year period between 1994 and 2007, and we examined these strains in the present study. A total of 82 of the 89 strains (92.1%) were isolated from complicated UTIs. There were 15 and 16 strains of fluoroquinolone-insusceptible *E. coli* isolated in 2006 and 2007, respectively. The percentages of fluoroquinolone-insusceptible *E. coli* strains relative to the total isolated *E. coli* strains from the respective years were

Table 4 Correlations between amino acid replacements and MICs of ofloxacin

GyrA (83, 87)	ParC (80, 84)	MICs of ofloxacin (μg/ml)								
		0.5	2	4	8	16	32	64	128	256
		Susceptible		Insusceptible						
(−, −)	(−, −)	4	1	0	0	0	0	0	0	0
(+, −)	(−, −)	0	2	0	0	0	1	0	0	0
(+, −)	(−, +)	0	2	0	0	0	0	0	0	0
(+, +)	(−, −)	0	0	0	1	1	0	0	0	0
(+, +)	(−, +)	0	0	0	1	0	1	0	0	0
(+, +)	(+, −)	0	0	3	5	9	8	5	0	1
(+, +)	(+, +)	0	0	0	14	17	7	4	0	0
Total number of strain		4	5	3	21	27	17	9	0	1
		9		78						

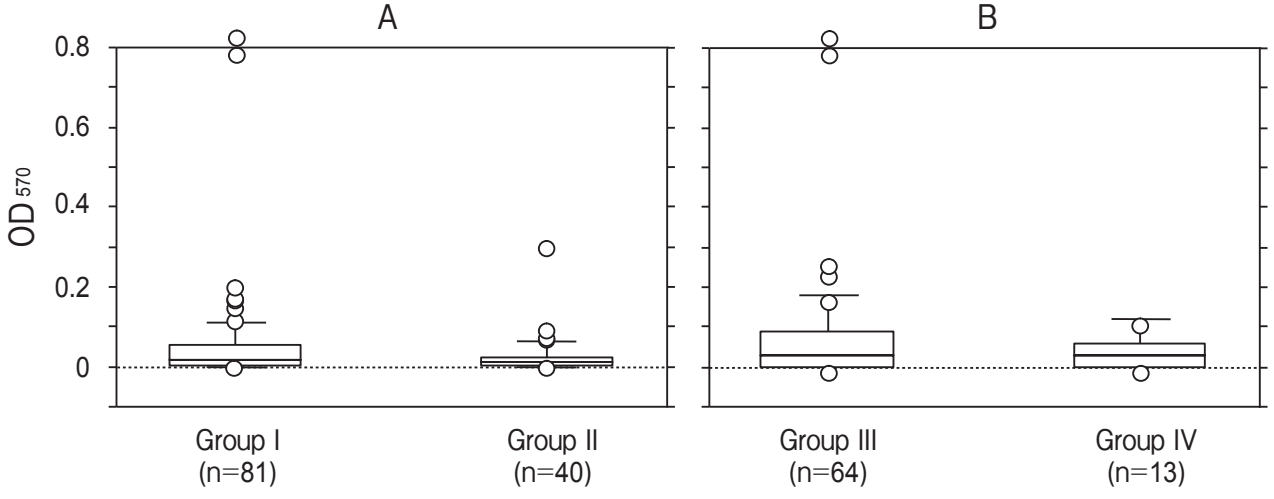

Fig. 5 Biofilm-forming capabilities of *E. coli* strains. **A**, Group I is fluoroquinolone-insusceptible *E. coli* (mean ± SD: 0.084 ± 0.314). Group II is fluoroquinolone-susceptible *E. coli* (mean ± SD: 0.029 ± 0.050). The *p*-value was 0.19; **B**, Group III is fluoroquinolone-insusceptible *E. coli* strains for which the MICs of sitafloxacin were 0.5 μg/ml (mean ± SD: 0.024 ± 0.345). Group IV is fluoroquinolone-insusceptible *E. coli* strains for which the MICs of sitafloxacin were over 1 μg/ml (mean ± SD: 0.029 ± 0.045). The *p*-value was 0.93.

22% and 19%, respectively. The isolation frequency of fluoroquinolone-insusceptible *E. coli* and its percentage of the total *E. coli* isolated tended to increase annually. The results reaffirmed that it is important to elucidate the mechanism of fluoroquinolone resistance and to select effective drugs for treatments.

Many reports have indicated that the widespread use of fluoroquinolones is contributing to the increasing percentages of fluoroquinolone-insusceptible bacterial strains, including *E. coli* [10, 11]. In our examination of the clinical backgrounds of patients, we found that there were a large number of patients who were administered fluoroquinolones in the past. We cannot rule out the possibility that the administration of fluoroquinolones had caused a selection for fluoroquinolone-insusceptible *E. coli*, which would have survived and been more frequently isolated. In recent years, fluoroquinolone-insusceptible *E. coli* accounted for approximately 10% of the total *E. coli* strains isolated in patients with acute uncomplicated UTI but without a history of fluoroquinolone administration. Thus the problem is no longer confined to within the bodies of individual patients who have taken fluoroquinolone, or, indeed, to within individual hospital systems, but rather is now of a wider scope. As the problem grows, we are confronted not only with the challenge of decreasing the rate of resistance by reducing the use of fluoroquinolones but also of treating the UTI caused by fluoroquinolone-insusceptible *E. coli*.

The antimicrobial effect of fluoroquinolones results from the inhibition of type II topoisomerases [12]. The type II topoisomerases include DNA gyrase, which is composed of GyrA and GyrB subunits, and topoisomerase IV, which is composed of ParC and ParE subunits. DNA gyrase is an enzyme essential for the replication of DNA [13]. Topoisomerase IV is an enzyme which separates the DNA chains after replication [14]. In *E. coli*, GyrA is the primary site of action of fluoroquinolones and ParC is the secondary site [15]. One cause of quinolone resistance in *E. coli* is the mutations in the QRDRs of DNA gyrase and topoisomerase IV [5, 6]. It is known that double replacements in GyrA and one replacement in ParC result in a high resistance to fluoroquinolones [16]. In our study using clinically isolated strains from UTIs, we found amino acid replacements in 3 or more positions of the QRDRs of fluoroquinolone-insusceptible *E. coli*. This result was consistent with the findings of Lindgren *et al.* obtained using clinically isolated strains [17], and it reconfirmed that the mutations in the QRDRs were strongly involved in the quinolone resistance mechanism. In the present study, however, there was no apparent correlation between ofloxacin MIC and the number of amino acid replacements in the QRDRs. The mechanism of *E. coli* quinolone resistance could not be explained solely by amino acid replacements in the QRDRs of type II topoisomerases. Other mechanisms of resistance were thought to be involved, such as limited entrance of antimicrobial agents into the bacteria due to decreased cell envelope permeability and export of antimicrobial agents out of the bacteria by multi-drug efflux pumps [18–20]. In recent years, there have been many reports on other factors that may contribute to resistance such as plasmid transmission of resistance [21–23].

One of the fluoroquinolones, sitafloxacin, has stronger inhibitory activities against DNA gyrase and topoisomerase IV compared to conventional fluoroquinolones, and sitafloxacin has been shown to have a stronger bactericidal effect [24]. In a pharmacokinetic study of sitafloxacin, this fluoroquinolone reached a maximum drug concentration (C_{max}) at 1.2 to 2.0 h after administration. When a single dose of 100 mg was administered postprandially, it reached a C_{max} of $0.88 \pm 0.31\,\mu g/ml$ [25]. There have been no reports on tissue distribution in the prostate and epididymis. However, sitafloxacin can be said to be an effective antimicrobial agent for UTIs, particularly for UTIs whose causative agent is fluoroquinolone-insusceptible *E. coli*, and 70% to 80% of the administered sitafloxacin is excreted in urine. When we examined the *E. coli* strains which were isolated from clinical samples at our facility, the MICs of sitafloxacin tended to correlate with those of other fluoroquinolones. The sitafloxacin MICs were $\leq 2\,\mu g/ml$ for all strains, and were $\leq 1\,\mu g/ml$ in over 90% of the strains. These results indicated a very good susceptibility. In this present study, we did not observe any *E. coli* strains which showed strong resistance to sitafloxacin. This result suggests that amino acid replacements in the QRDRs do not necessarily contribute to the change in the sitafloxacin MIC for *E. coli*. In addition, we found that resistance mechanisms other than mutations in the QRDRs may play a role in increasing the MIC, although, if so, their contribu-

tion is likely to be small.

As in the case of other fluoroquinolones, the susceptibility to sitafloxacin could decrease due to its overuse. Under special conditions of complicated UTIs (for example, when a biofilm forms on a urethral catheter or on the surface of a bladder stone), *E. coli* in the deep layer of the biofilm is exposed to low concentrations of sitafloxacin. Thus, gene mutations could occur and some type of resistant bacteria could appear. In this present study, 29 of 89 strains (32.6%) of fluoroquinolone-insusceptible *E. coli* were isolated from UTI patients who had "foreign bodies" in their urinary tracts, such as urethral catheters and stones. We found that there was no statistically significant difference in the biofilm-forming capabilities between the fluoroquinolone-susceptible and -insusceptible strains. The 2 strains which had very strong biofilm-forming capabilities were fluoroquinolone-insusceptible strains. Although the relationship between fluoroquinolone susceptibility and biofilm-forming capabilities has not been clarified, the effectiveness of antimicrobial agents against bacteria within the biofilm is less than that against free bacteria or bacteria on the biofilm surface [26]. Although at this point it is only a supposition, *E. coli* strains with a strong biofilm-forming capability may gain quinolone resistance after exposure to fluoroquinolones. Further studies examining greater numbers of *E. coli* strains will be needed to clarify the relationship between the biofilm-forming capabilities and quinolone resistance, namely amino acid replacements in the QRDRs.

It is important to avoid the overuse of antimicrobial agents and to shorten the administration period of antimicrobial agents. Fluoroquinolones are suitable for UTIs because they have a broad antimicrobial spectrum and a strong antimicrobial activity, and because *E. coli* is isolated at the highest frequency in uncomplicated UTIs and is presumed to be present in complicated UTIs as well. Our present study has shown that sitafloxacin was more effective *in vitro* than other fluoroquinolones. Although its *in vivo* effects must be further examined in future studies, we must remember that overuse of fluoroquinolones accelerates the development of resistance to fluoroquinolones.

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