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Molecular Characterization of Extraintestinal *Escherichia coli* Isolates in Japan: Relationship between Sequence Types and Mutation Patterns of Quinolone Resistance-Determining Regions Analyzed by Pyrosequencing

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Infection from fluoroquinolone-resistant *Enterobacteriaceae* is an increasing health problem worldwide. In the present study, we developed a pyrosequencing-based high-throughput method for analyzing the nucleotide sequence of the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC*. By using this method, we successfully determined the QRDR sequences of 139 out of 140 clinical *Escherichia coli* isolates, 28% of which were nonsusceptible to ciprofloxacin. Sequence results obtained by the pyrosequencing method were in complete agreement with those obtained by the Sanger method. All fluoroquinolone-resistant isolates ($n = 35$; 25%) contained mutations leading to three or four amino acid substitutions in the QRDRs. In contrast, all isolates lacking a mutation in the QRDR ($n = 81$; 57%) were susceptible to ciprofloxacin, levofloxacin, and nalidixic acid. The *qnr* determinants, namely, the *qnrA*, *qnrB*, and *qnrS* genes, were not detected in the isolates, and the *aac(6′)-Ib-cr* gene was detected in 2 (1.4%) of the isolates. Multilocus sequence typing of 34 randomly selected isolates revealed that sequence type 131 (ST131) ($n = 7$; 20%) is the most prevalent lineage and is significantly resistant to quinolones ($P < 0.01$). The genetic background of quinolone-susceptible isolates seemed more diverse, and interestingly, neighboring STs of ST131 in the phylogenetic tree were all susceptible to ciprofloxacin. In conclusion, our investigation reveals the relationship between fluoroquinolone resistance caused by mutations of QRDRs and the population structure of clinical extraintestinal *E. coli* isolates. This high-throughput method for analyzing QRDR mutations by pyrosequencing is a powerful tool for epidemiological studies of fluoroquinolone resistance in bacteria.

Fluoroquinolones are powerful broad-spectrum antimicrobial agents used for the treatment of a wide variety of community-acquired and nosocomial infections (1). However, following the introduction of fluoroquinolones in the 1980s, the population of fluoroquinolone-resistant bacteria has increased markedly over the years (2, 3). The Asia-Pacific region in particular is an area where fluoroquinolone resistance is endemic among clinical isolates of *Escherichia coli* (4, 5).

As quinolones inhibit bacterial DNA gyrase and topoisomerase IV, amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of these enzymes might lead to changes that decrease the binding of quinolone (6). In fact, the accumulation of mutations in the QRDRs of *gyrA* and *parC* is recognized as the most common and important mechanism of quinolone resistance in *Enterobacteriaceae* (7). However, the relationship of quinolone resistance with the genetic background has not been clearly established.

The Clinical and Laboratory Standards Institute (CLSI) continues to reevaluate the breakpoints of fluoroquinolones for *Enterobacteriaceae* (8). Following previous reports (3, 9, 10), the CLSI recently released a new lowered breakpoint of ciprofloxacin for *Salmonella enterica* serotype Typhi and extraintestinal *Salmonella* spp. (susceptible at ≤0.06 μg/ml). Furthermore, revising the breakpoint of levofloxacin for this species is under discussion by a CLSI working group. Possible changes in the breakpoints for other *Enterobacteriaceae* were also discussed by this working group. Sometimes, however, the clinical response to an infection caused by an isolate considered "susceptible" according to the present CLSI breakpoint appears suboptimal (11, 12). As our current understanding of the genetic basis for the development of quinolone resistance is not sufficient, further understanding of the relationship between quinolone susceptibility and QRDR mutations in *Enterobacteriaceae* should provide some fundamental information for making rational decisions.

Pyrosequencing is a real-time sequence analysis method based on the detection of pyrophosphate that is released during the synthesis of DNA (13). Because pyrosequencing is less labor- and time-intensive than the conventional Sanger method for nucleotide sequence analysis, this method has already been used successfully to identify the resistance-conferring genes of several bacterial species (14–17). This method appears to be especially suitable as a tool for identifying "hot spot" mutations of QRDRs, namely, at amino acid positions 83 and 87 in GyrA and at positions 80 and 84

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in ParC of *E. coli*. To the best of our knowledge, pyrosequencing has yet to be used as a tool for analyzing the QRDRs in *E. coli*.

In the present study, we used pyrosequencing to determine the QRDR mutations of *gyrA* and *parC* in clinical *E. coli* isolates obtained from a university hospital in Japan, a location where quinolone-resistant *E. coli* is endemic and plasmid-mediated quinolone resistance (PMQR) seems to be relatively uncommon (18). Additionally, we used multilocus sequence typing (MLST) for genotyping analysis and interpreted the results to delineate an evolutionary pathway of quinolone resistance.

MATERIALS AND METHODS

Bacterial strains. We investigated 140 nonrepetitive consecutive clinical *E. coli* isolates, including 20 isolates from blood, 59 from sputum, and 61 from urine samples, all isolated in 2009 in the Toho University Omori Medical Center, which is a 972-bed university hospital located in Tokyo, Japan. All of the isolates were identified as *E. coli* by using the Vitek 2 system (bioMérieux, Lyon, France). *E. coli* ATCC 25922 was used as a control for MIC measurements.

Antimicrobial susceptibility test, detection of extended-spectrum beta-lactamase producers, and effect of efflux pump inhibitor. MICs were determined by a broth microdilution method according to the CLSI testing standards (19). The MICs of cefepime, cefpodoxime, cefpodoxime-clavulanic acid, and meropenem were measured with frozen plates for antimicrobial susceptibility testing (Eiken Chemical Co., Ltd., Tokyo, Japan). The MICs of levofloxacin (Daiichi Sankyo Co., Ltd., Tokyo, Japan), ciprofloxacin (MP Biomedicals, LLC, Santa Ana, CA), and nalidixic acid (Sigma, St. Louis, MO) were measured separately. The results were interpreted according to the criteria recommended by the CLSI (8).

An extended-spectrum beta-lactamase (ESBL) producer was determined following the CLSI recommendations. Thus, when the MIC of cefpodoxime for an isolate was ≥4 μg/ml and clavulanic acid reduced the MIC by ≥2-fold, the isolate was considered to be an ESBL producer.

To assess the contribution of efflux pumps to quinolone resistance, MICs of ciprofloxacin and nalidixic acid were compared in the absence and presence of 20 μg/ml of Phe-Arg-β-naphthylamide (Sigma), an inhibitor of efflux pumps (20).

DNA extraction, PCR amplification, and Sanger DNA sequencing. DNA templates for PCR amplification were prepared by resuspending fresh bacterial colonies in 500 μl of Tris-EDTA, heating the resuspended cells for 15 min at 100°C, and then centrifuging the mixture for 5 min at 10,000 rpm. Unless mentioned otherwise, all PCRs were performed using *Ex Taq* (TaKaRa Bio, Shiga, Japan). Nucleotide sequences of PCR-amplified DNAs of the region corresponding to amino acid positions from 81 to 87 in GyrA and from 78 to 84 in ParC were determined by the conventional Sanger method to confirm the reliability of the pyrosequencing results. Primers used in PCR amplification and nucleotide sequencing are listed in Table 1.

Pyrosequencing of QRDR. The PCR primers used in the pyrosequencing method were designed using the PyroMark assay design software 2.0 (Qiagen, Hilden, Germany) on the basis of sequence information available for the QRDRs of the *gyrA* and *parC* genes (GenBank accession number U00096), with manual modifications to avoid possible template loops, self-annealing duplexes, or alternate annealing sites, and the sequences of these primers are listed in Table 1. The forward primers, labeled with "-F," and the reverse primers, labeled with "-R," were covalently coupled to biotin at the 5′ end to obtain a pyrosequencing template from the PCR product.

The PCR for pyrosequencing was performed in a reaction volume of 25 μl with amplification primers and a PyroMark PCR kit (Qiagen) and following the manufacturer's instructions. PCR began with a 15-min hot-start step at 95°C and was followed by 45 cycles of amplification consisting of 30 s at 95°C, 1 min at 54°C, and 30 s at 72°C.

Pyrosequencing was carried out using a PyroMark Q24 system (Qiagen) based on a 4-enzyme solution system, according to the manufacturer's instructions. Briefly, PCR products were captured and separated by using streptavidin-Sepharose beads (GE health care, Pittsburgh, PA), and the resulting single-stranded DNA was used as a template for pyrosequencing. A sequence primer, labeled with "-S" at the end in Table 1, was annealed to a single-stranded PCR product. Single nucleotides were dispensed individually in a predetermined order to the reaction mixture: TGTGACT6(CTGA) for *gyrA* and GCGA8(CTGA) for *parC* (shown on the *x* axis in Fig. 1). The analysis range was the QRDRs of GyrA and ParC, corresponding to the amino acid positions from 81 to 87 in GyrA and from 78 to 84 in ParC. The signal strength, reflecting light emitted enzymatically from pyrophosphate, is proportional to the number of nucleotides incorporated in a single nucleotide flow. The pyrograms were analyzed using PyroMark Q24 software (Qiagen).

MLST. Sequence types (STs) of 34 randomly selected *E. coli* isolates were determined according to the MLST scheme (21). These *E. coli* isolates included 14 from urine, 15 from sputum, and 5 from blood samples. Clustering of each sequence type (ST) was determined using the program eBURST version 3, and single-locus variants were used to define the clonal complexes (CCs) (22). Phylogenetic analysis was performed using the maximum-likelihood method by MEGA5 (23).

Identification of PMQR genes. *qnrA*, *qnrB*, and *qnrS* genes were detected using a multiplex PCR method and the primers listed in Table 1 (24). The *aac(6′)-Ib-cr* gene, which differed from *aac(6′)-Ib* by two single-nucleotide polymorphisms, namely, T304C/A and G535T, was detected by using a pyrosequencing method and the primers listed in Table 1 (16).

Statistical analysis. A two-sided Fisher's exact test was used for analyzing categorical data. Differences at *P* values of <0.05 were considered statistically significant.

RESULTS

Prevalence of fluoroquinolone-resistant and ESBL-producing clinical *E. coli* isolates. Table 2 summarizes the distribution of the MICs of each antimicrobial agent tested against the 140 *E. coli* isolates used in this study. According to the breakpoints reported by the CLSI in 2012, 35 (25%) of the isolates were resistant (MIC ≥ 4 μg/ml) and 40 (28%) of the isolates were nonsusceptible (MIC ≥ 2 μg/ml) to ciprofloxacin (8). Significantly, more isolates from sputum samples were resistant to ciprofloxacin than those from urine and blood samples (42% from sputum versus 13% and 10% from urine and blood, respectively; $P < 0.01$). Fifty-nine isolates (42.1%) were resistant to nalidixic acid (MIC ≥ 32 μg/ml).

Eighteen isolates (12.8%), which included both fluoroquinolone-resistant and -susceptible isolates, were identified as ESBL producers. Among them, 12 (66%) were from sputum samples, while 1 and 5 were from blood and urine samples, respectively. All 11 cefepime-resistant isolates were ESBL producers. None of the isolates was meropenem resistant.

Accumulations of mutations in QRDRs of *gyrA* and *parC* in relation to quinolone resistance. Using the sequencing primers gyrA-Pyro-S and parC-Pyro-S (Table 1), we obtained sequence information on the QRDRs of the *gyrA* and *parC* genes, respectively, in 139 *E. coli* isolates by pyrosequencing (Fig. 1). While the gyrA-Pyro-S primer also yielded sequence information on the *gyrA* QRDR of the remaining isolate, the parC-Pyro-S primer failed to provide any sequence information on the *parC* QRDR due to a silent mutation in the primer region. However, another sequencing primer with one base change (parC-Pyro-S-alt) (Table 1) yielded sequence information on the *parC* QRDR of the remaining isolate. The sequencing results by the pyrosequencing method were found to be in complete agreement with those ob-

TABLE 1 Primers used in the study

Primer	Primer sequence (5′–3′)	PCR annealing temp (°C)	Reference
gyrA QRDR for pyrosequencing			
gyrA-Pyro-F	CCTCTGGATTATGCGATGTCGGTCAT	54	This study
gyrA-Pyro-Rbiotin[a]	TCAGCCCTTCAATGCTGATGTCT		This study
gyrA-Pyro-S	TAATCGGTAAATACCATCCCCA		This study
parC QRDR for pyrosequencing			
parC-Pyro-F	ACTACTCCATGTACGTCATCATGGAC	54	This study
parC-Pyro-Rbiotin[a]	AGCCACTTCGCGCAGGTTAT		This study
parC-Pyro-S	TGGGTAAATACCATCCGCAC		This study
parC-Pyro-S-alt	TGGGTAAATACCATCCGCAT		This study
QRDR for Sanger sequencing			
gyrA-QRDR-F	TCTGGATTATGCGATGTCGGTCAT	54	This study
gyrA-QRDR-R	TCAGCCCTTCAATGCTGATGTCT		This study
parC-QRDR-F	ACTACTCCATGTACGTCATCATGGAC	54	This study
parC-QRDR-R	CGCCACTTCGCGCAGGTTAT		This study
gyrB-QRDR-F	GCTGAGCGAATACCTGCTGG	54	This study
gyrB-QRDR-R	TCGGTCATGATGATGATGCTGTGAT		This study
parE-QRDR-F	GCGGAAGATATCTGGGATCGCT	54	This study
parE-QRDR-R	CTGGCTCAGATCGTCGCTGT		This study
qnr multiplex PCR detection			
QnrAm-F	AGAGGATTTCTCACGCCAGG	56	24
QnrAm-R	TGCCAGGCACAGATCTTGAC		24
QnrBm-F	GGMATHGAAATTCGCCACTG	56	24
QnrBm-R	TTTGCYGYYCGCCAGTCGAA		24
QnrSm-F	GCAAGTTCATTGAACAGGGT	56	24
QnrSm-R	TCTAAACCGTCGAGTTCGGCG		24
MLST			
E. coli MLST adkF	ATTCTGCTTGGCGCTCCGGG	58	21
E. coli MLST adkR	CCGTCAACTTTCGCGTATTT		21
E. coli MLST fumCF	TCACAGGTCGCCAGCGCTTC	58	21
E. coli MLST fumCR	GTACGCAGCGAAAAAGATTC		21
E. coli MLST gyrBF	TCGGCGACACGGATGACGGC	65	21
E. coli MLST gyrBR	ATCAGGCCTTCACGCGCATC		21
E. coli MLST icdF	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	58	21
E. coli MLST icdR	GGACGCAGCAGGATCTGTT		21
E. coli MLST mdhF	CCAGGCGCTTGCACTACTGTTAA	58	21
E. coli MLST mdhR	GCGATATCTTTCTTCAGCGTATC		21
E. coli MLST purAF	CGCGCTGATGAAAGAGATGA	65	21
E. coli MLST purAR	CATACGGTAAGCCACGCAGA		21
E. coli MLST recAF	CGGCAAACTCAACGTTCC	58	21
E. coli MLST recAR	CTGACGCTGCAGGTGAT		21
aac(6′)-Ib-cr: T304C/A for pyrosequencing			
T304C-Fbiotin[a]	GGAGAGCCGATTGGGTATG	58	16
T304C-R	TAACTGGTCTATTCCGCGTACTC		16
T304C-So	CGGTTTCTTCTTCCCAC		16
aac(6′)-Ib-cr: G535T for pyrosequencing			
G535T-Fbiotin[a]	CGATCCGATGCTACGAGAAA	58	16
G535T-R	CATGTACACGGCTGGACCA		16
G535T-So	TGTACACGGCTGGAC		16

[a] 5′-biotinylated.

tained by the Sanger sequencing used as a conventional methodology throughout this study.

These results were concordant with the established theory that the accumulation of QRDR mutations increases the level of resistance to fluoroquinolones (25). The MIC levels of ciprofloxacin and levofloxacin demonstrated a trimodal distribution (Table 3; also see Table S1 in the supplemental material). Eighty-one out of the 140 *E. coli* isolates (58%) had no mutations in the QRDRs of *gyrA* and *parC*, and the MIC_{50} of ciprofloxacin for these isolates was ≤0.008 μg/ml. Pyrosequencing identified nine different com-

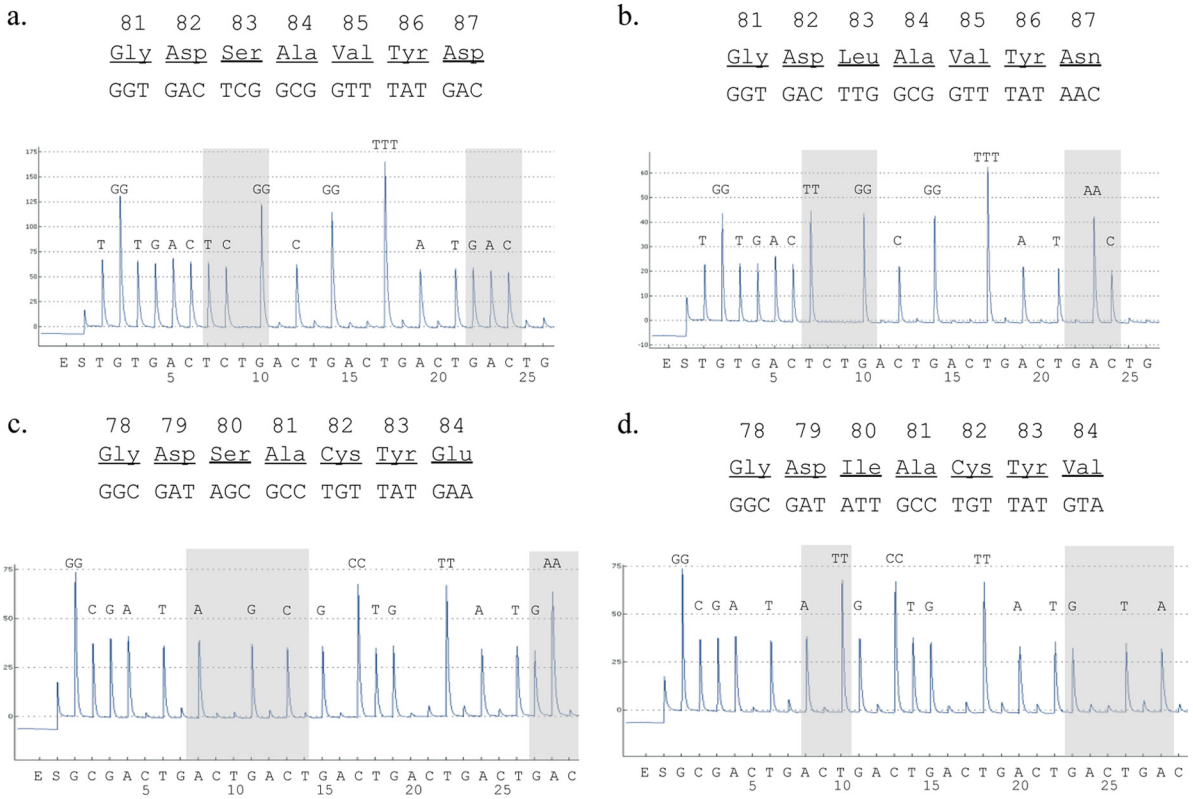

FIG 1 Analysis of QRDRs in *gyrA* (a and b) and *parC* (c and d) by pyrosequencing in quinolone-susceptible (a and c) and quinolone-resistant (b and d) isolates. The shaded regions show polymorphisms at positions 83 and 87 in GyrA (a and b) and at positions 80 and 84 in ParC (c and d). Single nucleotides were dispensed individually in a predetermined order as shown on the *x* axis of the trace. The signal strength, reflecting light emitted enzymatically from pyrophosphate, is proportional to the number of nucleotides incorporated in a single nucleotide flow. The additions of enzymes and substrates to the reaction mix are indicated by E and S, respectively.

binations of amino acid substitutions in the QRDRs. The numbers of isolates with a single mutation (found only in the *gyrA* QRDR) and double mutations (one in *gyrA* and one in *parC* QRDR) were 22 and 2, respectively, and the MIC_{50} of ciprofloxacin for these isolates was 0.5 μg/ml. Thirty-five isolates (25%) contained either three mutations (4 isolates containing 2 amino acid substitutions in GyrA QRDR and 1 amino acid substitution in ParC QRDR) or four mutations (31 isolates containing 2 amino acid substitutions in GyrA QRDR and 2 amino acid substitutions in ParC QRDR), and the MIC_{50} of ciprofloxacin for these isolates was 32 μg/ml.

TABLE 2 Drug susceptibility of clinical *Escherichia coli* isolates ($n = 140$) used in the study

Antimicrobial	No. of isolates at the indicated MIC (μg/ml)[a]										Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Resistant isolates[b]	
	≤0.5	1	2	4	8	16	32	64	128	>128				No.	Rate (%)
Ciprofloxacin	92	8	5		3	5	15	7	5[h]		≤0.5 to >64	≤0.5	32	35	25
Levofloxacin	99	5	1		5	30[e]					≤1 to >8	≤1	>8	35	25
Nalidixic acid	3	26	39	9	4		1	3	2	53	0.5 to >128	4	>128	59	42
Cefepime		122[c]	3	3	1		5	6[g]			≤0.5 to >32	≤1	4	11	7.9
Cefpodoxime	109	9	1	1		2	18[f]				≤0.5 to >16	≤0.5	>16	20	14
Cefpodoxime-clavulanic acid	126	7		5	2						≤0.5 to 8	≤0.5	0.5	NA[i]	NA[i]
Meropenem			140[d]								≤2	≤2	≤2	0	0

[a] Vertical lines are shown between resistant and nonresistant isolates.
[b] Breakpoint adopted from CLSI recommendations (8).
[c] This number represents isolates for which the MIC was ≤1 μg/ml.
[d] This number represents isolates for which the MIC was ≤2 μg/ml.
[e] This number represents isolates for which the MIC was >8 μg/ml.
[f] This number represents isolates for which the MIC was >16 μg/ml.
[g] This number represents isolates for which the MIC was >32 μg/ml.
[h] This number represents isolates for which the MIC was >64 μg/ml.
[i] NA, not applicable.

TABLE 3 Relationship between quinolone resistance-determining region sequences and ciprofloxacin susceptibility of clinical *Escherichia coli* isolates

Amino acid substitution				No. of isolates	No. of isolates at a ciprofloxacin MIC (μg/ml) of[a]:													
GyrA		ParC																
Ser83	Asp87	Ser80	Glu84		≤0.008	0.015	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	>64
				81	51[b] (3)	27	3												
Leu				20					2	2 (1)	5	8 (1)	3						
	Gly			1						1									
	Asn			1						1									
Leu		Ile		1									1						
Leu			Gly	1									1						
Leu	Asn	Ile		4											1	1		1	1 (1)
Leu	Asn	Ile	Gly	1														1	
Leu	Asn	Ile	Arg	3															3 (3)
Leu	Asn	Ile	Val	27											2	4 (1)	15 (7)	5[b] (1)	1
Total				140	51	27	3	0	2	4	5	8	5	0	3	5	15	7	5

[a] Numbers of ESBL-producing isolates are shown in parentheses.
[b] One of the isolates harbored the *aac(6′)-Ib-cr* gene.

None of the isolates, however, revealed any other mutation in the QRDRs of *gyrB* or *parE*, or in the remaining regions of *gyrA* and *parC*, as determined by the Sanger sequencing method. The *qnr* determinants, namely, the *qnrA*, *qnrB*, and *qnrS* genes, were not detected in these isolates. However, the *aac(6′)-Ib-cr* gene was detected in 2 (1.4%) of the isolates, one of which harbored no QRDR mutation and the other which harbored four QRDR mutations. The efflux pump inhibitor Phe-Arg-β-naphthylamide affected the susceptibility of nalidixic acid but not that of ciprofloxacin or levofloxacin (data not shown).

Diverse lineage of quinolone-susceptible isolates and predominance of ST131 among the quinolone-resistant isolates. The 34 clinical *E. coli* isolates, selected in an unbiased manner, were assigned to 19 STs by MLST (Fig. 2). According to the results

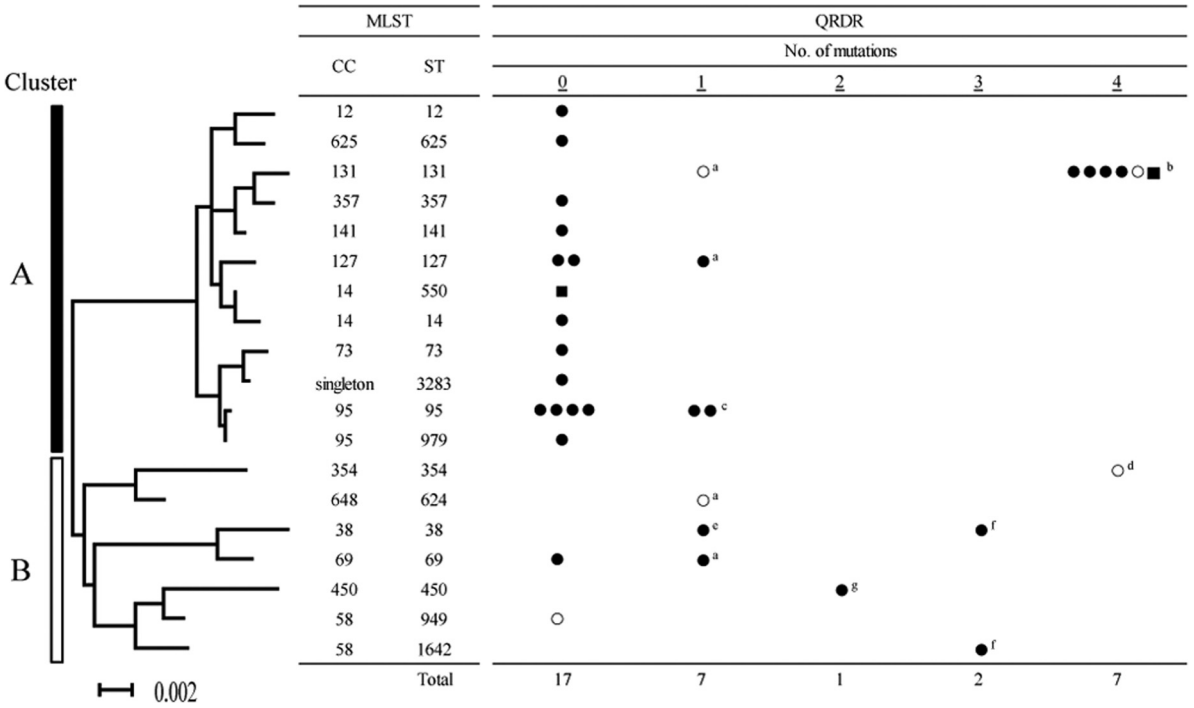

FIG 2 Molecular phylogenetic analysis of 34 clinical *E. coli* isolates, including 9 ciprofloxacin-resistant isolates, according to the MLST scheme. The evolutionary history was estimated using the maximum-likelihood method, and the isolates were divided into one of two groups, namely, clusters A and B, according to the results of phylogenetic analysis. Isolates in cluster A, except ST131, had significantly fewer mutations in the QRDRs than the isolates in cluster B ($P < 0.05$). Underlined numbers indicate the numbers of mutations in the QRDRs. Closed squares, isolates harboring *aac(6′)-Ib-cr*; open circles, ESBL-producing isolates; closed circles, isolates not harboring *aac(6′)-Ib-cr* or producing ESBL. ST, sequence type; CC, clonal complex. [a], S83L in GyrA; [b], all isolates with S83L and D87N in GyrA and S80I and E84V in ParC; [c], one isolate with S83L in GyrA and one with D87N in GyrA; [d], S83L and D87N in GyrA and S80I and E84R in ParC; [e], D87G in GyrA; [f], S83L and D87N in GyrA and S80I in ParC; [g], S83L in GyrA and S80I in ParC.

of phylogenetic analysis, we divided the isolates into two groups, clusters A and B. Quinolone-susceptible isolates seemed more diverse than quinolone-resistant isolates: seventeen isolates with no QRDR mutations belonged to 13 STs, 10 of which consisted of a single isolate. Similarly, seven isolates with a single QRDR mutation belonged to 6 different STs. In contrast, seven isolates with four QRDR mutations belonged to only 2 STs.

ST131 was outstanding with respect to both frequency and resistance. ST131 was the most common ST ($n = 7$; 21% of isolates) followed by ST95 ($n = 6$; 18%), ST127 ($n = 3$; 9%), ST38 ($n = 2$; 6%), and ST69 ($n = 2$; 6%). In addition, most of the highly quinolone-resistant isolates with four QRDR mutations (6/7) were the ST131 isolates, and ST131 isolates were more likely to exhibit ciprofloxacin resistance than were the isolates of other genotypes ($P < 0.01$). Moreover, the ST131 isolates included two ESBL producers and one isolate with the *aac(6′)-Ib-cr* gene. Interestingly, isolates of cluster A, except ST131, had significantly fewer QRDR mutations than isolates of cluster B, including ST38 and ST354 ($P < 0.05$) (Fig. 2). In an epidemiological investigation, seven ST131 isolates were recovered from patients in different wards, implying that nosocomial transmission was unlikely.

Besides the number of mutations in QRDR, the mutation content enabled us to further discriminate the isolates. For example, two ST95 isolates harbored different single mutations in the QRDRs. Similarly, two ST38 isolates had a mutation at position 87 in GyrA but did not share the same amino acid residue at that position. Although ST354 shared three QRDR mutations with ST131, the amino acid at position 84 in ParC differed from the amino acid at that position in these two STs.

DISCUSSION

We have successfully developed a method focusing on detecting hot-spot mutations of QRDRs in *gyrA* and *parC* using pyrosequencing as a real-time labor-saving sequencing technology. Although the read length is shorter than the read length for the Sanger method of DNA sequencing, the sequence information obtained by pyrosequencing is easy to interpret, and the target region in this study seems sufficient for estimating the level of quinolone resistance. As the measurement of the MICs of drugs with low concentration levels poses practical challenges for clinical microbiology laboratories, the availability of a uniform platform for genotyping seems to provide a considerable advantage.

Our data, obtained from 140 clinical *E. coli* isolates from a university hospital in Tokyo, Japan, in 2009, show a robust relationship between the quinolone resistance and the number of mutations in the QRDRs of *gyrA* and *parC*, as was reported previously (25). The prevalence of ciprofloxacin resistance (25%) was, however, higher than that previously reported in a Japanese study, where only 15% of *E. coli* isolates from urine samples were resistant to fluoroquinolone (26). Besides the difference of the study time, this discrepancy might be due to the fact that in this study, we also included sputum samples which were previously reported to contain more fluoroquinolone-resistant isolates than all other samples, including blood and urine (27, 28). In addition, the roles of PMQRs and efflux pumps in fluoroquinolone resistance were less apparent in our study than in previously reported studies (18, 29, 30), although the PCR detection in this study might not have covered all of the currently known PMQR determinants.

In the present study, at the currently recommended breakpoint concentration of fluoroquinolone (ciprofloxacin MIC $\geq$ 4 μg/ml), we were able to identify *E. coli* isolates harboring three or four QRDR mutations. In addition, a lower concentration of ciprofloxacin (MIC $\leq$ 0.06 μg/ml), which was the recommended breakpoint for *S.* Typhi and *Salmonella* spp., was useful for the detection of *E. coli* isolates with no QRDR mutations or with a single QRDR mutation.

Among the previous phylogenetic studies on clinical *E. coli* isolates, most applied different criteria in terms of isolate selection, drug resistance, and severity of infections (31, 32). This might lead to a biased representation of certain STs. Therefore, for MLST analysis in the present study, we analyzed the population structure of clinical *E. coli* strains by randomly selecting 34 isolates, a group which included both quinolone-susceptible and -resistant isolates.

ST131 has been reported as a globally disseminated clone with multidrug resistance, including resistance to cephalosporins and fluoroquinolones (33). Interestingly, fluoroquinolone resistance and ESBL production are often epidemiologically related in *Enterobacteriaceae* (4). In our data, most of the ST131 isolates showed high-level resistance to fluoroquinolones, including two ESBL producers and one isolate positive for the *aac(6′)-Ib-cr* gene. The other ESBL-producing and *aac(6′)-Ib-cr* gene-harboring isolates were distributed among 4 different STs, suggesting the role of horizontal gene transfer in these cases. Although the reason why the resistance of ST131 to fluoroquinolones was more extreme than that of the other isolates in the same cluster A is not clear, further investigations, such as a comparison of the whole genomes of a wider range of isolates, might reveal the actual pathway for the development of quinolone resistance in the clinical *E. coli* population.

According to the genotyping data, we hypothesize two possible pathways to the emergence of quinolone resistance among clinical *E. coli* isolates. The clonal spread of resistant strains such as ST131 might play a role, in part at least, although the epidemiological information did not provide robust evidence for nosocomial transmission. Alternatively, fluoroquinolone resistance might arise independently through target mutations of various strains. In fact, different *gyrA* mutations were observed in the same ST background, such as ST95 or ST38 isolates (Fig. 2). Further study is necessary to elucidate the true contribution of these two pathways.

In conclusion, in the present study, MLST analysis of these isolates provided their likely phylogeny with respect to drug resistance to fluoroquinolones. In addition, we successfully used pyrosequencing as a tool and unraveled the QRDR mutation pattern among the clinical *E. coli* isolates obtained from a university hospital in Japan. This methodology thus represents a powerful tool for epidemiological studies of fluoroquinolone-resistant isolates. Future investigations should be undertaken to examine wider ranges of isolates to elucidate their population structures and to understand the relationship between molecular evolution and the emergence of resistant clones in bacteria.

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