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2 Molecular characterization of extra-intestinal *Escherichia coli* isolates in Japan: the relationship
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19 **RUNNING TITLE (51 characters, with spaces):**

QRDR mutations and STs in clinical *E. coli* isolates

KEY WORDS:

pyrosequencing, quinolone resistance determining region (QRDR), multilocus sequence typing (MLST), plasmid-mediated quinolone resistance (PMQR), *Escherichia coli*

4,368 words

Three tables, two figures, and one supplemental table

28 **ABSTRACT (244 words)**

29 Fluoroquinolone-resistant *Enterobacteriaceae* is one of the increasing health problems worldwide.

30 In the present study, we developed a pyrosequencing-based high-throughput method for analyzing

31 the nucleotide sequence of the quinolone resistance determining regions (QRDRs) of *gyrA* and *parC*.

32 By using this method, we successfully determined the QRDR sequences of 139 out of 140 clinical

33 *Escherichia coli* isolates, twenty-eight percent of which were non-susceptible to ciprofloxacin.

34 Sequence results obtained by the pyrosequencing method were in complete agreement with those

35 obtained by the Sanger method. All fluoroquinolone-resistant isolates (n = 35; 25%) contained

36 mutations leading to three or four amino acid substitutions in the QRDRs. In contrast, all isolates

37 lacking mutation in the QRDR (n = 81; 57%) were susceptible to ciprofloxacin, levofloxacin, and

38 nalidixic acid. The *qnr* determinants, namely *qnrA*, *qnrB*, and *qnrS* genes, were not detected and

39 *aac(6')-Ib-cr* gene was detected in 2 (1.4%) isolates. Multilocus sequence typing of 34 randomly

40 selected isolates revealed sequence type (ST) 131 (n = 7; 20%) as the most prevalent lineage and

41 significantly resistant to quinolones ($p < 0.01$). The genetic background of quinolone susceptible

42 isolates seemed more diverse and, interestingly, neighbor STs of ST131 in the phylogenetic tree

43 were all susceptible to ciprofloxacin. In conclusion, we could reveal the relationship between

44 fluoroquinolone resistance caused by mutations of QRDRs and population structure in the clinical

45 extra-intestinal *E. coli* isolates. This high-throughput method for analyzing QRDR mutations by

46 pyrosequencing is a powerful tool for epidemiological studies of fluoroquinolone resistance in

bacteria.

INTRODUCTION

Fluoroquinolones are powerful broad-spectrum antimicrobial agents used for the treatment of a wide variety of community-acquired and nosocomial infections (1). However, following their introduction in 1980s, the population of fluoroquinolone-resistant bacteria has increased markedly over the years (2, 3). Especially in the Asia-Pacific region, 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 could lead to changes decreasing the binding of quinolone (6). In fact, 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, its relationship with the genetic background is not 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), CLSI has recently released a new lowered breakpoint of ciprofloxacin for *Salmonella* Typhi and extraintestinal *Salmonella* spp. (susceptible at ≤ 0.06 μg/ml). Furthermore, revising the breakpoint of levofloxacin for this species is under discussion by the CLSI working group. Possible changes in

66 the breakpoints for other *Enterobacteriaceae* were also discussed by this working group. Sometimes,

67 however, the clinical response to an infection caused by an isolate considered "susceptible"

68 according to the present CLSI breakpoint appears suboptimal (11, 12). As our current understanding

69 of the genetic basis for the development of quinolone resistance is not sufficient, further

70 understanding of the relationship between quinolone susceptibility and QRDR mutations in

71 *Enterobacteriaceae* should provide some fundamental information for making rational decisions.

72 Pyrosequencing is a real-time sequence analysis method based on the detection of pyrophosphate

73 that is released during the synthesis of DNA (13). Because pyrosequencing is less labor and time

74 intensive than the conventional Sanger method for nucleotide sequence analysis, this method has

75 already been used successfully to identify the resistance-conferring genes of several bacterial

76 species (14-17). This method appears especially suitable as a tool for identifying "hot spot"

77 mutations of QRDRs, namely at amino acid positions 83 and 87 in GyrA and at positions 80 and 84

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

79 analyzing the QRDRs in *E. coli*.

80 In the present study, we have used pyrosequencing to determine the QRDR mutations of *gyrA*

81 and *parC* in clinical *E. coli* isolates obtained from a university hospital in Japan, where quinolone

82 resistant *E. coli* is endemic and plasmid-mediated quinolone resistance (PMQR) seems to be

83 relatively uncommon (18). Additionally, we have used multilocus sequence typing (MLST) for

84 genotyping analysis and interpreted the results to delineate an evolutionary pathway of quinolone

resistance.

MATERIALS AND METHODS

Bacterial strains.

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

Antimicrobial susceptibility test, detection of extended-spectrum beta-lactamase (ESBL) producers, and effect of efflux pump inhibitor.

MICs were determined by a broth microdilution method according to the CLSI testing standards (19). MICs of cefepime, cefpodoxime, cefpodoxime/clavulanic acid, and meropenem were measured with frozen plates for antimicrobial susceptibility testing (Eiken Chemical Co., Ltd., Tokyo, Japan). MICs of levofloxacin (Daiichi Sankyo Co., Ltd., Tokyo, Japan), ciprofloxacin (MP Biomedicals, LLC, Southern California, CA) and nalidixic acid (Sigma, St Louis, MO) were measured separately. The results were interpreted according to the criteria recommended by CLSI (8).

An ESBL producer was determined following the CLSI recommendation. Thus, when the MIC of cefpodoxime for an isolate was ≥ 4 μg/ml and clavulanic acid reduced the MIC by 2-fold,

104 the isolate was considered to be an ESBL producer.

105 To assess the contribution of efflux pumps to quinolone resistance, MICs of ciprofloxacin

106 and nalidixic acid were compared in the absence and presence of 20 μg/ml of Phe-Arg-β-

107 naphthylamide (Sigma), an inhibitor of efflux pumps (20).

108 **DNA extraction, PCR amplification and Sanger DNA sequencing.**

109 DNA templates for PCR amplification were prepared by re-suspending fresh bacterial colonies in

110 500 μl of Tris-EDTA, heating the re-suspended cells for 15 min at 100°C and then centrifuging the

111 mixture for 5 min at 10000 rpm. Unless mentioned otherwise, all PCR reactions were performed

112 using ExTaq (Takara Bio, Shiga, Japan). Nucleotide sequences of PCR amplified DNAs of the

113 region corresponding to amino acid positions from 81 to 87 in GyrA and from 78 to 84 in ParC were

114 determined by the conventional Sanger method to confirm the reliability of the pyrosequencing

115 results. Primers used in PCR amplification and nucleotide sequencing are listed in **Table 1**.

116 **Pyrosequencing of QRDR.**

117 The PCR primers used in the pyrosequencing method were designed using the PyroMark Assay

118 Design Software 2.0 (Qiagen, Hilden, Germany) on the basis of sequence information available for

119 the QRDRs of *gyrA* and *parC* genes, respectively (GenBank accession number U00096), with

120 manual modifications to avoid possible template loops, self-annealing duplex, or alternate annealing

121 sites, and the sequences of these primers are listed in **Table 1**. The forward primers were labeled

122 with “-F”, and the reverse primers labeled with “-R” were covalently coupled to biotin at the 5’ end

123 to obtain a pyrosequencing template from the PCR product.

124 The PCR reaction for pyrosequencing was performed in a reaction volume of 25 μl with

125 amplification primers and PyroMark PCR kit (Qiagen) and following the manufacturer’s

126 instructions. PCR reaction began with a 15 min hot start step at 95°C followed by 45 cycles of

127 amplification consisting of 30 sec at 95°C, 1 min at 54°C, and 30 sec at 72°C.

128 Pyrosequencing was carried out using a PyroMark Q24 system (Qiagen) based on 4-enzyme

129 solution system according to the manufacturer’s instructions. Briefly, PCR products were captured

130 and separated by using Streptavidin-Sepharose beads (GE healthcare, Pittsburgh, PA), and the

131 resulting single-stranded DNA was used as a template for pyrosequencing. A sequence primer,

132 labeled with “-S” at the end in the **Table 1**, was annealed to a single-stranded PCR product. Single

133 nucleotides were dispensed individually in a predetermined order to the reaction mixture as follows:

134 TGTGACT6(CTGA) for *gyrA* and GCGA8(CTGA) for *parC* shown on the x-axis in the **Figure 1**.

135 The analysis range was the QRDRs of GyrA and ParC, corresponding to amino acid positions from

136 81 to 87 in GyrA and from 78 to 84 in ParC. The signal strength, reflecting light emitted

137 enzymatically from pyrophosphate, is proportional to the number of nucleotides incorporated in a

138 single nucleotide flow. The pyrograms were analyzed using PyroMark Q24 Software (Qiagen).

139 **MLST.**

140 Sequence types (STs) of 34 randomly selected *E. coli* isolates were determined according to the

141 MLST scheme (21). These *E. coli* isolates included 14 from urine, 15 from sputum, and 5 from

142 blood samples. Clustering of each sequence type (ST) was determined using the program eBURST

143 version 3, and single locus variants were used to define the clonal complexes (CCs) (22).

144 Phylogenetic analysis was performed using the maximum likelihood method by MEGA5 (23).

145 **Identification of PMQR genes.**

146 *qnrA, qnrB,* and *qnrS* genes were detected using a multiplex PCR method and the primers are listed

147 in **Table 1** (24). *aac(6')-Ib-cr* gene, which differed from *aac(6')-Ib* by two single nucleotide

148 polymorphisms, namely T304C/A and G535C, was detected by using pyrosequencing method and

149 listed primers in **Table 1 (16)**.

150 **Statistical analysis.**

151 Two-sided Fisher's exact test was used for analyzing categorical data. A *P* value of less than 0.05

152 was considered statistically significant.

153

154 **RESULTS**

155 **Prevalence of fluoroquinolone-resistant and ESBL-producing clinical *E. coli* isolates.**

156 **Table 2** summarizes the distribution of MIC of each antimicrobial agent tested against 140 *E. coli*

157 isolates used in this study. According to the breakpoints by CLSI in 2012, 35 (25%) isolates were

158 resistant (MIC ≥4 μg/ml) and 40 (28%) isolates were non-susceptible (MIC ≥2 μg/ml) to

159 ciprofloxacin (8). Significantly, more isolates from sputum samples were resistant to ciprofloxacin

160 than those from urine and blood samples (42% from sputum vs. 13% and 10% from urine and blood,

161 respectively; $p < 0.01$). Fifty-nine isolates (42.1%) were resistant to nalidixic acid (MIC ≥32 μg/ml).

162 Eighteen isolates (12.8%), which included both fluoroquinolone resistant and susceptible

163 isolates, were identified as ESBL producers. Among them, 12 (66%) were from sputum, while one

164 and five were from blood and urine samples, respectively. All 11 cefepime resistant isolates were

165 ESBL producers. None of the isolates was meropenem resistant.

166 **Accumulations of mutations in QRDRs of *gyrA* and *parC* in relation to quinolone resistance.**

167 Using the sequencing primers gyrA-Pyro-S and parC-Pyro-S (**Table 1**), we obtained sequence

168 information on the QRDRs of *gyrA* and *parC* genes, respectively, in 139 by pyrosequencing (**Figure**

169 **1**). While the gyrA-Pyro-S primer also yielded sequence information on the *gyrA* QRDR of the

170 remaining isolate, the parC-Pyro-S primer failed to provide any sequence information on the *parC*

171 QRDR due to a silent mutation in the primer region. Another sequencing primer with one base

172 change (parC-Pyro-S-alt; **Table 1**), however, yielded sequence information on the *parC* QRDR of

173 the remaining isolate. The sequencing results by the pyrosequencing method were found to be in

174 complete agreement with those obtained by the Sanger sequencing as a conventional methodology

175 throughout this study.

176 These results were concordant with the established framework that accumulation of QRDR

177 mutations increases resistant level to fluoroquinolones (25). The MIC levels of ciprofloxacin and

178 levofloxacin demonstrated a trimodal distribution (**Table 3** and **Supplemental table**). Eighty-one

179 out of the 140 *E. coli* isolates (58%) had no mutations in the QRDRs of *gyrA* and *parC*, and MIC_{50}

180 of ciprofloxacin for these isolates was ≤0.008 μg/ml. Pyrosequencing identified nine different

181 combinations of amino acid substitutions in the QRDRs. Isolates with single mutation (found only

182 in *gyrA* QRDR) and double mutations (one in *gyrA* and one in *parC* QRDR) were 22 and 2,

183 respectively, and MIC_{50} of ciprofloxacin for these isolates was 0.5 μg/ml. Thirty-five isolates (25%)

184 contained either three mutations (4 isolates containing 2 amino acid substitutions in GyrA QRDR

185 and 1 amino acid substitution in ParC QRDR) or four mutations (31 isolates containing 2 amino acid

186 substitutions in GyrA QRDR and 2 amino acid substitutions in ParC QRDR), and MIC_{50} of

187 ciprofloxacin for these isolates was 32 μg/ml.

188 None of the isolates, however, revealed any other mutation in the QRDRs of *gyrB* or *parE*,

189 as well as in the remaining regions of *gyrA* and *parC*, as determined by the Sanger sequencing

190 method. The *qnr* determinants, namely *qnrA*, *qnrB*, and *qnrS* genes were not detected in these

191 isolates. However, *aac(6')-Ib-cr* gene was detected in 2 (1.4%) isolates: one of which harbored no

192 QRDR mutation and the other harbored four QRDR mutations. The efflux pump inhibitor Phe-Arg-

193 β-naphthylamide affected the susceptibility of nalidixic acid but not that of ciprofloxacin or

194 levofloxacin (data not shown).

195 **Diverse lineage of quinolone susceptible isolates and predominance of ST131 among the**

196 **quinolone resistant isolates.**

197 The 34 clinical *E. coli* isolates, selected in an unbiased manner, were assigned to 19 STs by MLST

198 (**Figure 2**). According to the result of phylogenetic analysis, we divided the isolates into two groups,
199 the cluster A and B. Quinolone susceptible isolates seemed more diverse than quinolone resistant
200 isolates: seventeen isolates with no QRDR mutation belonged to 13 STs, 10 of which consisted of
201 single isolate. Similarly, seven isolates with a single QRDR mutation belonged to 6 different STs. In
202 contrast, seven isolates with four QRDR mutations consisted of only two STs.

203 ST131 was outstanding with respect to both frequency and resistance. ST131 was the most
204 common ST (n = 7; 20% of isolates) followed by ST95 (n = 6; 17%), ST127 (n = 3; 8%), ST38 (n =
205 2; 5%), and ST69 (n = 2; 5%). In addition, most of the highly quinolone-resistant isolates with four
206 QRDR mutations (6/7) were the ST131 isolates, and ST131 isolates were more likely to exhibit
207 ciprofloxacin resistance than other genotypes ($P < 0.01$). Moreover, the ST131 isolates included two
208 ESBL producers and one isolate with *aac(6')-Ib-cr* gene. Interestingly, isolates of the cluster A
209 except ST131 had significantly fewer QRDR mutations than isolates of the cluster B isolates
210 including ST38 and ST354 ($p < 0.05$) (**Figure 2**). Epidemiologically, seven ST131 isolates were
211 recovered from patients in different wards, implying that nosocomial transmission appeared
212 unlikely.

213 Besides the number of mutations in QRDR, mutation content further discriminated the
214 isolates. For example, two ST95 isolates harbored different single mutations in QRDRs. Similarly,
215 two ST38 isolates had mutation at position 87 in GyrA but did not share the same amino acid
216 residue at that position. Although ST354 shared three QRDR mutations with ST131, the amino acid

217 at position 84 in ParC differed between these two STs.

218

219 **DISCUSSION**

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

227 Our data, obtained from 140 clinical *E. coli* isolates from a university hospital in Tokyo,
228 Japan in 2009, shows a robust relationship between the quinolone resistance and the number of
229 mutations in the QRDRs of *gyrA* and *parC*, as was reported previously (25). The prevalence of
230 ciprofloxacin resistance (25%) was, however, higher than that was previously reported in a Japanese
231 study, where only 15% of *E. coli* isolates from urine samples was resistant to fluoroquinolone (26).
232 Besides the difference of the study time, this discrepancy might be due to the fact that in this study
233 we also included sputum samples, which was previously reported to contain more fluoroquinolone
234 resistant isolates than all other samples including blood and urine (27, 28). Meanwhile, compared
235 with previous reports (18, 29, 30), roles of PMQRs and efflux pumps in fluoroquinolone resistance

236 were less apparent in our study, although the PCR detection in this study might not cover all

237 currently known PMQR determinants.

238 In the present study, we have shown that at their currently recommended breakpoint

239 concentrations fluoroquinolone (ciprofloxacin MIC ≥4 μg/ml), we were able to identify *E. coli*

240 isolates harboring three or four QRDR mutations. In addition, lower concentration of ciprofloxacin

241 (MIC ≤0.06 μg/ml), which was the recommended breakpoint for *S.* Typhi and *Salmonella* spp., was

242 useful to detect *E. coli* isolates with no or single QRDR mutation.

243 Among the previous phylogenetic studies on clinical *E. coli* isolates, most applied different

244 criteria in terms of isolate selection, drug resistance and severity of infections (31, 32). This could

245 potentially lead to a biased representation of certain STs. Therefore, in the present study, we

246 analyzed the population structure of clinical *E. coli* strains by randomly selecting 34 isolates, which

247 included both quinolone-susceptible and -resistant isolates, for MLST analysis.

248 ST131 has been reported as a globally disseminated multidrug-resistant clone, including

249 resistance to cephalosporins and fluoroquinolones (33). Interestingly, fluoroquinolone-resistance and

250 ESBL producers are often epidemiologically related in *Enterobacteriaceae* (4). In our data, most of

251 the ST131 isolates showed high-level resistance to fluoroquinolones, including 2 ESBL producers

252 and one isolate positive for *aac(6')-Ib-cr* gene. The other ESBL-producing and *aac(6')-Ib-cr* gene

253 harboring isolates were distributed among 4 different STs, suggesting the role of horizontal gene

254 transfer in these cases. Though the reason why ST131 was extremely resistant to fluoroquinolones

255 than other isolates in the same cluster A is not clear, further investigations, such as comparison of
256 whole genomes of wider range of isolates may reveal the actual pathway for developing the
257 quinolone resistance in the clinical population of *E. coli*.

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

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

272

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277

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394

395

396 **TABLES**

397 **Table 1.** Primers used in the present study.

Primers	Primer sequence (5'-3')	PCR Annelaing Temperature (°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-R-biotin[a]	AGCCACTTCGCGCAGGTTAT		This study
parC-Pyro-S	TGGGTAAATACCATCCGCAC		This study
parC-Pyro-S-alt	TGGGTAAATACCATCCGCAT		This study
QRDRs 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]

398

399 [a] 5'-biotinated

Table 2. Drug susceptibility of clinical *Escherichia coli* isolates (n = 140) used in the present study.

Antimicrobials	No. of isolates of indicated MIC (µg/ml)[a]										ranges	MIC_{50}	MIC_{90}	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 - >64	≤0.5	32	35	25
Levofloxacin		104[c]	1		5	30[e]					≤1 - >8	≤1	>8	35	25
Nalidixic acid	3	26	39	9	4		1	3	2	53	0.5 - >128	4	>128	59	42.1
Cefepime		122[c]	3	3	1		5	6[g]			≤0.5 - >32	≤1	4	11	7.8
Cefpodoxime	109	9	1	1		2	18[f]				≤0.5 - >16	≤0.5	>16	20	14.2
Cefpodoxime/clavulanic acid	126	7		5	2						≤0.5 - 8	≤0.5	0.5	NA	NA
Meropenem			140[d]								≤2	≤2	≤2	0	0

[a] Vertical lines are between resistant and non-resistant isolates.

[b] Breakpoint adopted from CLSI recommendation, 2012.

[c] ≤ 1

[d] ≤ 2

[e] > 8

[f] > 16

[g] > 32

[h] > 64

Table 3. Relationship between quinolone resistance determining region (QRDR) sequences and ciprofloxacin susceptibility of clinical *Escherichia coli* isolates.

Amino acid substitution				No. of isolates	Ciprofloxacin MIC (µg/ml) [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] No. of ESBL producing isolates is shown within brackets.

415 **FIGURE LEGENDS**

416 **Figure 1.**

417 Analysis of QRDRs in *gyrA* **(a, b)** and *parC* **(c, d)** by pyrosequencing in quinolone susceptible **(a**
418 and **c)** and quinolone resistant **(b** and **d)** isolates. The shaded region shows polymorphism at
419 positions 83 and 87 in *gyrA* **(a, b)** and at positions 80 and 84 in *parC* **(c, d)**. Single nucleotides were
420 dispensed individually in a predetermined order shown on the x-axis of the trace. The signal strength,
421 reflecting light emitted enzymatically from pyrophosphate, is proportional to the number of
422 nucleotides incorporated in a single nucleotide flow. The additions of enzymes and substrates to the
423 reaction mix are indicated by E and S, respectively.

424

425 **Figure 2.**

426 Molecular phylogenetic analysis of 34 clinical *E. coli* isolates, including 9 ciprofloxacin resistant
427 isolates, according to the MLST scheme. The evolutionary history was estimated using the
428 maximum likelihood method and the isolates were divided into two groups, namely the cluster A
429 and B, according to the result of phylogenetic analysis. Isolates in the cluster A except ST131 had
430 significantly fewer mutation in QRDRs than ones in cluster B ($p < 0.05$). Underlined numbers
431 indicate the number of mutations in QRDRs. Closed square, an isolate harboring *aac(6')-Ib-cr*; open
432 circle, an ESBL-producing isolate; closed circle, an isolate not harboring *aac(6')-Ib-cr* or producing
433 ESBL. ST, sequence type; CC, clonal complex.

434 [a] S83L in GyrA.

435 [b] all isolates with S83L and D87N in GyrA and S80I and E84V in ParC.

436 [c] one isolate with S83L in GyrA and one with D87N in GyrA.

437 [d] S83L and D87N in GyrA and S80I and E84R in ParC.

438 [e] D87G in GyrA.

439 [f] S83L and D87N in GyrA and S80I in ParC.

440 [g] S83L in GyrA and S80I in ParC.

Figure 1

(Aoike N et al.)

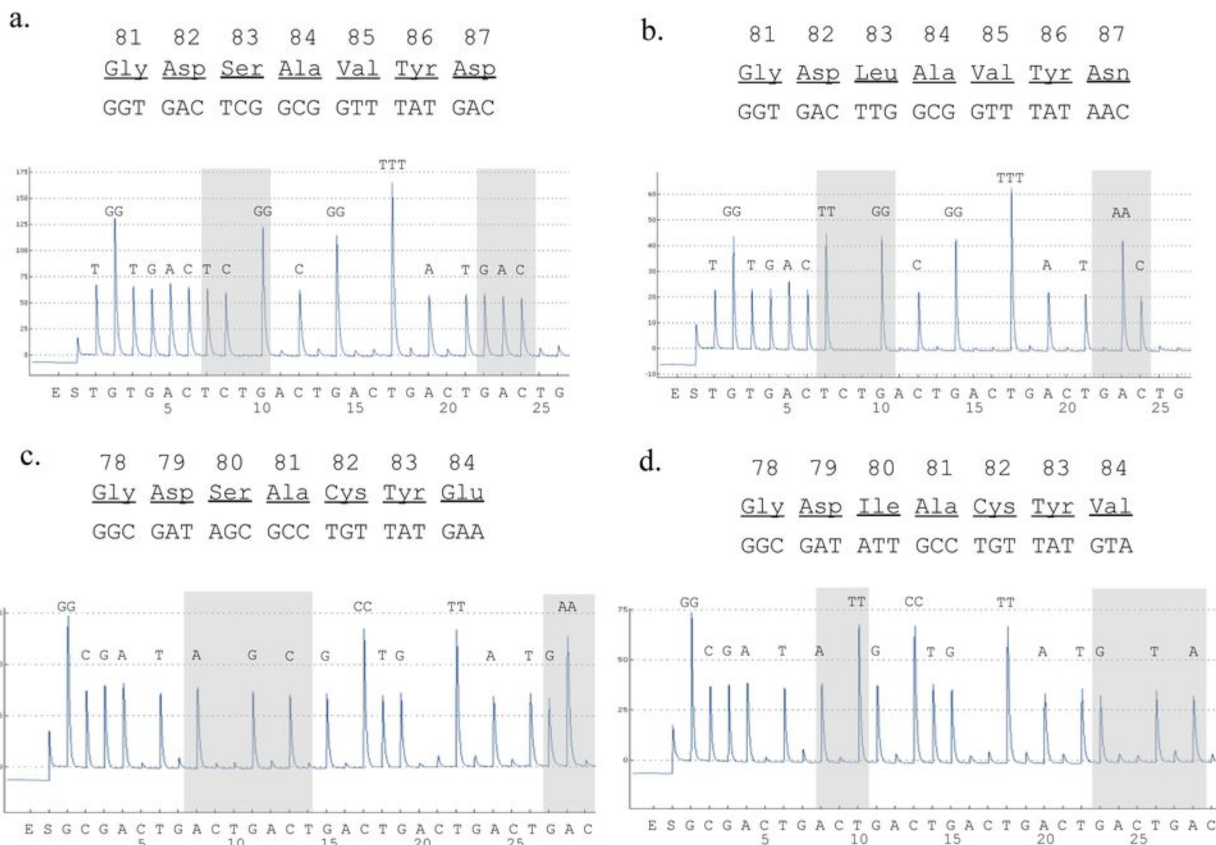
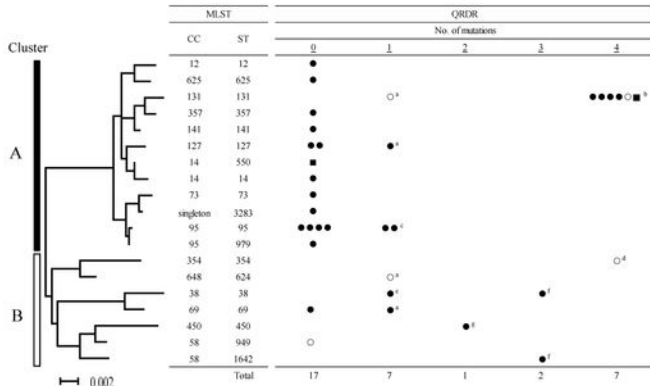


Figure 1.

Analysis of QRDRs in *gyrA* **(a, b)** and *parC* **(c, d)** by pyrosequencing in quinolone susceptible **(a** and **c)** and quinolone resistant **(b** and **d)** isolates. The shaded region shows polymorphism at positions 83 and 87 in *gyrA* **(a, b)** and at positions 80 and 84 in *parC* **(c, d)**. Single nucleotides were dispensed individually in a predetermined order 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.

Figure 2 (Aoike N et al)



MLST		QRDR				
		No. of mutations				
CC	ST	0	1	2	3	4
12	12	●				
625	625	●				
131	131		○[a]			●●●●○■[b]
357	357	●				
141	141	●				
127	127	●●	●[a]			
14	550	■				
14	14	●				
73	73	●				
singleton	3283	●				
95	95	●●●●	●●[c]			
95	979	●				
354	354					○[d]
648	624		○[a]			
38	38		●[e]		●[f]	
69	69	●	●[a]			
450	450			●[g]		
58	949	○				
58	1642				●[f]	
	Total	17	7	1	2	7

Figure 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 two groups, namely the cluster A and B, according to the result of phylogenetic analysis. Isolates in the cluster A except ST131 had significantly fewer mutation in QRDRs than ones in cluster B ($p < 0.05$). Underlined numbers indicate the number of mutations in QRDRs. Closed square, an isolate harboring *aac(6')-Ib-cr*; open circle, an ESBL-producing isolate; closed circle, an isolate 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.

Supplemental table. Relationship between quinolone resistance determining region (QRDR) sequences and levofloxacin susceptibility of clinical *Escherichia coli* isolates.

Amino acid substitutions				No. of isolates	Levofloxacin MIC (μg/ml) [a]										
GyrA		ParC			≤0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	>8
Ser83	Asp87	Ser80	Glu84												
				81	54 (3)	21	6								
Leu				20				3	2	11 (2)	4				
	Gly			1			1								
	Asn			1				1							
Leu		Ile		1								1			
Leu			Gly	1							1				
Leu	Asn	Ile		4										2	2 (1)
Leu	Asn	Ile	Gly	1											1
Leu	Asn	Ile	Arg	3											3 (3)
Leu	Asn	Ile	Val	27										3 (1)	24 (8)
Total				140	54	21	7	4	2	11	5	1	0	5	30

[a] No. of ESBL producing isolates is shown within brackets.