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NOTES

DNA Sequence Analysis of DNA Gyrase and DNA Topoisomerase IV Quinolone Resistance-Determining Regions of *Salmonella enterica* Serovar Typhi and Serovar Paratyphi A

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The mutations that are responsible for fluoroquinolone resistance in the *gyrA*, *gyrB*, *parC*, and *parE* genes of *Salmonella enterica* serovar Typhi and serovar Paratyphi A were investigated. The sequences of the quinolone resistance-determining region of the *gyrA* gene in clinical isolates which showed decreased susceptibilities to fluoroquinolones had a single mutation at either the Ser-83 or the Asp-87 codon, and no mutations were found in the *gyrB*, *parC*, and *parE* genes.

Fluoroquinolones have become the first-line drugs for the treatment of typhoid fever (3, 12, 18, 23). However, some *Salmonella enterica* serovar Typhi strains that exhibit decreased susceptibilities to fluoroquinolones have been already reported (2, 7, 13, 21). Furthermore, several clinical treatment failures after the administration of ciprofloxacin and other fluoroquinolones to patients with typhoid fever due to strains with decreased susceptibilities to fluoroquinolones have also been reported (17, 21). The emergence and spread of these organisms have been reported in developing countries. There is evidence that the incidence of strains that are resistant to nalidixic acid and that exhibit decreased susceptibilities to the most recent fluoroquinolones used for the treatment of typhoid fever is increasing. In most strains, the acquired fluoroquinolone resistance was attributed to mutations in the genes encoding DNA gyrase (GyrA, GyrB) (10, 24–26) or DNA topoisomerase IV (ParC, ParE) (8, 9). The purpose of this study was to investigate the association of quinolone resistance with mutations in the genes coding for gyrase and topoisomerase IV of *S. enterica* serovar Typhi and serovar Paratyphi A, which are especially clinically important serotypes of *Salmonella* spp.

The bacterial strains used in this study were collected from regional public health offices in Japan between 1995 and 2001, and all isolates were obtained from a culture of either blood or stool from individual patients and identified by biochemical tests and serological tests on the basis of standard criteria. *S. enterica* serovar Typhi Ty2 and ATCC 19430 and *S. enterica* serovar Paratyphi A NCTC13, NCTC5702, and RIMD 1015 were used as reference and control strains (Tables 1 and 2). The MICs of several fluoroquinolones, including norfloxacin, levofloxacin, ofloxacin, sparfloxa cin, and ciprofloxacin, and nalidixic acid were determined by the Etest (AB Biodisk, Solna, Sweden), according to the instructions of the manufacturer. The quality control strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were included in each test. The criterion for ciprofloxacin resistance was an MIC of $\geq$4 μg/ml, according to the NCCLS breakpoint criteria for members of the family *Enterobacteriaceae*. The criterion for decreased susceptibility to ciprofloxacin that we used in this study was an MIC between $\geq$0.25 and $<$4 μg/ml, and that for ciprofloxacin susceptibility was an MIC $<$0.25 μg/ml (19, 20). An attempt to increase the level of fluoroquinolone resistance was done by culturing the fluoroquinolone-susceptible strains and the strains with decreased susceptibilities to fluoroquinolones in medium supplemented with ciprofloxacin, as described by Giraud et al. (4). The sequences of the primers used for PCR amplification and determination of the nucleotide sequences of the *gyrA*, *gyrB*, *parC*, and *parE* genes were previously described by Giraud et al. (4). The PCR products were directly used as templates for sequencing. DNA sequencing was performed with an ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) with an automated sequencer (311A; Perkin-Elmer, Applied Biosystems). The amino acid sequences between positions 54 and 171 of GyrA, positions 397 and 520 of GyrB, positions 12 and 130 of ParC, and positions 421 and 524 of ParE, which contain the quinolone resistance-determining regions (QRDRs), were determined from the DNA sequences.

A total of 31 clinical isolates of *S. enterica* serovar Typhi and 13 clinical isolates of *S. enterica* serovar Paratyphi A were examined in this study. The data are shown in Tables 1 and 2.

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TABLE 1. MICs for strains and nucleotide changes in the *gyrA* gene of *Salmonella* serovar Typhi

Classification[a] and strain no.[b]	MIC (μg/ml)[c]						Nucleotide change at GyrA position:	
	NOR	LVX	OFX	SPX	CIP	NAL	83 (TCC [Ser])	87 (GAC [Asp])
A								
NIHP3-4 (990102)	64	>32	>32	>32	>32	>256	TTC (Phe)	TAC (Tyr)
NIHP3-43 (010063)	16	>32	>32	>32	16	>256	TTC (Phe)	TAC (Tyr)
NIHP3-39 (010053)	16	16	>32	>32	8	>256	TTC (Phe)	TAC (Tyr)
NIHP3-9 (990113)	16	4	8	2	4	>256	TTC (Phe)	None
B								
990018	1	0.25	4	0.25	0.25	>256	TTC (Phe)	None[d]
000006	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000007	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000008	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000009	2	0.25	0.5	0.25	0.5	>256	TAC (Tyr)	None
000012	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000015	1	0.5	0.5	0.25	0.5	>256	None	GGC (Gly)
000016	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000019	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
990069	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
990026	2	0.25	1	0.25	0.5	>256	TAC (Tyr)	None
980083	2	0.5	1	0.25	0.5	>256	TAC (Tyr)	None
990106	2	0.5	1	0.25	0.5	>256	TTC (Phe)	None
990097	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
990120	1	0.25	0.5	0.25	0.25	>256	TTC (Phe)	None
990102	1	0.25	1	0.25	0.25	>256	TTC (Phe)	None
000022	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000023	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
000025	2	0.5	0.5	0.25	0.5	>256	TAC (Tyr)	None
970004	1	0.25	0.5	0.25	0.25	>256	TTC (Phe)	None
990020	1	0.25	0.5	0.25	0.25	>256	None	GGC (Gly)
990104	2	0.25	0.25	0.25	0.25	64	TTC (Phe)	None
000015	1	0.25	0.25	0.125	0.25	64	None	TAC (Tyr)
000027	1	0.25	0.5	0.125	0.25	64	None	TAC (Tyr)
000037	1	0.25	0.25	0.125	0.25	64	None	TAC (Tyr)
C								
990053	0.064	0.032	0.064	0.016	0.032	4	None	None
980118	0.064	0.016	0.064	0.016	0.032	4	None	None
990113	0.032	0.032	0.064	0.016	0.016	2	None	None
990100	0.25	0.032	0.064	0.064	0.032	2	None	None
010053	0.25	0.032	0.125	0.008	0.016	2	None	None
010063	0.25	0.032	0.125	0.008	0.016	2	None	None
Reference strains Ty2 (ATCC 19430)	0.064	0.032	0.032	0.008	0.006	2	None	None
	0.125	0.032	0.064	0.008	0.032	2	None	None

[a] A, ciprofloxacin-resistant strains (in vitro-selected strains); B, ciprofloxacin-strains with decreased susceptibilities to fluoroquinolones (clinical isolates); C, ciprofloxacin-susceptible strains (clinical isolates).
[b] The strains with the NIHP3 designation are ciprofloxacin-resistant mutants obtained by in vitro selection. The designations in parentheses are those for the original laboratory mutants.
[c] NOR, norfloxacin; LVX, lerofloxacin; OFX, ofloxacin; SPX, sparfloxacin, CIP, ciprofloxacin; NAL, nalidixic acid.
[d] None, no alterations were found in the genes.

Twenty-five and six strains of serovar Typhi had decreased susceptibility to ciprofloxacin and were ciprofloxacin susceptible, respectively; and seven and six strains of serovar Paratyphi A had decreased susceptibility to ciprofloxacin and were ciprofloxacin susceptible, respectively. Typical resistant strains were never found among the strains tested. The serovar Typhi and serovar Paratyphi A clinical isolates with decreased susceptibility to fluoroquinolone had only a single mutation in the *gyrA* gene, at either position 83 or 87 of GyrA. Strains with high-level resistance to fluoroquinolones induced by in vitro selection with ciprofloxacin had double mutations in the *gyrA* gene at both position 83 and position 87 of GyrA (Tables 1 and 2). Only one serovar Paratyphi A strain (strain NIHP3-1, Table 2) had a mutation in the *parC* gene, at Glu-84 of ParC, in addition to double mutations in the *gyrA* gene. For the *parC* gene, the mutation was a change of GAA (Glu) to AAA (Lys) at codon 84. Alterations in the QRDRs of the *gyrB* and *parE* genes were not found in any of the strains tested.

In this study, three groups could be distinguished among the clinical isolates and the strains in which resistance was experimentally selected in vitro on the basis of the ciprofloxacin MICs and *gyrA* mutations (Tables 1 and 2). The first group consisted of strains which were susceptible to fluoroquinolones and which had no mutations in the QRDR of the *gyrA* gene (group C in Tables 1 and 2). The second group consisted of strains which exhibited slightly reduced susceptibilities to fluo-

TABLE 2. MICs for strains and nucleotide changes in the *gyrA* gene of *Salmonella* serovar Parayphi A

Classification[a] and strain no.[b]	MIC (μg/ml)[c]						Nucleotide change at GyrA position:	
	NOR	LVX	OFX	SPX	CIP	NA	83 (TCC [Ser])	87 (GAC [Asp])
A								
NIHP3-1 (950040)	>256	>32	>32	>32	>32	>256	TTC (Phe)	AAC (Asn)
NIHP3-44 (990118)	16	>32	>32	>32	8	>256	TTC (Phe)	TAC (Tyr)
NIHP3-3 (990021)	16	24	>32	>32	8	>256	TTC (Phe)	TAC (Tyr)
NIHP3-41 (010044)	16	12	>32	24	8	>256	TTC (Phe)	TAC (Tyr)
NIHP3-6 (980043)	32	8	>32	12	8	>256	TTC (Phe)	None[d]
NIHP3-10 (990110)	32	8	>32	4	8	>256	TTC (Phe)	None
NIHP3-16 (970087)	16	4	>32	4	4	>256	TTC (Phe)	None
NIHP3-40 (000066)	16	8	16	8	4	>256	TTC (Phe)	None
NIHP3-45 (000056)	16	8	8	4	4	>256	TTC (Phe)	None
B								
950040	8	4	16	4	2	>256	TTC (Phe)	None
990112	2	1	2	0.5	0.5	>256	TTC (Phe)	None
990110	2	1	2	1	0.5	>256	TTC (Phe)	None
000040	2	1	2	1	0.5	>256	TTC (Phe)	None
000055	2	1	2	1	0.5	>256	TTC (Phe)	None
990021	2	1	2	1	0.5	>256	TTC (Phe)	None
980043	2	1	2	0.5	0.5	>256	TTC (Phe)	None
C								
970087	0.25	0.125	0.25	0.064	0.064	4	None	None
970131	0.25	0.125	0.125	0.064	0.032	4	None	None
000056	0.25	0.125	0.25	0.064	0.064	4	None	None
000066	0.125	0.064	0.125	0.032	0.016	4	None	None
990118	0.25	0.064	0.125	0.032	0.064	2	None	None
010044	0.25	0.064	0.125	0.064	0.032	1	None	None
Reference strains								
NCTCI3	0.032	0.032	0.064	0.032	0.016	2	None	None
NCTC5702	0.064	0.032	0.064	0.032	0.016	2	None	None
RIMD1015	0.064	0.064	0.125	0.032	0.032	2	None	None

[a] A, ciprofloxacin-resistant strains (in vitro-selected strains); B, strains with decreased susceptibilities to fluoroquinolones (clinical isolates); C, ciprofloxacin-susceptible strains (clinical isolates).
[b] The strains with the NIHP3 designation are ciprofloxacin-resistant mutants obtained by in vitro selection. The designation in parentheses are those for the original laboratory mutants.
[c] NOR, norfloxacin; LVX, lerofloxacin; OFX, ofloxacin; SPX, sparfloxacin; CIP, ciprofloxacin; NA, nalidixic acid.
[d] None, no alterations were found in the genes.

roquinolones and which had only a single mutation in the QRDR of the *gyrA* gene (group B in Tables 1 and 2). The third and last group consisted of strains which were highly resistant to fluoroquinolones and which had a single mutation or double mutations in the QRDR of the *gyrA* gene (group A in Tables 1 and 2); all strains in the third group were experimentally selected. These findings indicate that *gyrA* mutations are of principal importance for the fluoroquinolone resistance of serovars Typhi and Paratyphi A. Alterations at position 83 or 87 of the GyrA amino acid sequence have been described previously for *Salmonella* strains (1, 4, 6, 14, 16, 22). Double mutations at positions 83 and 87 of the GyrA amino acid sequence were also reported in clinical isolates of serovar Schwarzengrund, which caused nosocomial infections in the United States and which exhibited ciprofloxacin resistance (11). Although strains with high-level fluoroquinolone resistance due to double mutations at codons 83 and 87 in the GyrA amino acid sequence have not been found in clinical isolates of serovars Typhi and Paratyphi A, several cases of the failure of treatment for typhoid fever due to strains with decreased susceptibilities to fluoroquinolones have been reported (21). Since we obtained isolates with double mutations in the *gyrA* gene by in vitro selection and a mutation in *parC* caused by a novel substitution in Lys-84, such mutations in clinical isolates of serovars Typhi and Paratyphi A may appear in the future. Establishment of a surveillance system for the detection of *gyrA* mutations will be important for the detection of fluoroquinolone resistance in *S. enterica* serovars Typhi and Paratyphi A.

We do not rule out the possibility that mutations which may contribute to fluoroquinolone resistance may be present outside of the regions sequenced. Although both NIHP3-4 and NIHP3-39 strains have double mutations in the *gyrA* gene, the MICs of ciprofloxacin for these two strains were very different. These results suggest that other mechanisms may also be responsible for high-level fluoroquinolone resistance in *S. enterica* serovars Typhi and Paratyphi A. Although high-level-resistant strains are commonly reported among clinical isolates of *E. coli*, resistant clinical isolates of *S. enterica* serovars Typhi and Paratyphi A were not found. The difference in fluoroquinolone resistance between two closely related species may be explained by differences in outer membrane permeabilities for fluoroquinolones and differences in active efflux activities (5, 15).

In conclusion, surveillance for antimicrobial resistance

among clinical isolates of *S. enterica* serovars Typhi and Paratyphi A should be continued, particularly to monitor the emergence of strains with double mutations in the *gyrA* genes.

Nucleotide sequence accession numbers. The partial DNA sequences of the *gyrA*, *gyrB*, *parC*, and *parE* genes of *S. enterica* serovar Typhi Ty2 and *S. enterica* serovar Paratyphi A NCTC13 reported here were registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: *S. enterica* serovar Typhi *gyrA*, accession no. AB071870; *S. enterica* serovar Typhi *gyrB*, accession no. AB072396; *S. enterica* serovar Typhi *parC*, accession no. AB071987; *S. enterica* serovar Typhi *parE*, accession no. AB072701; *S. enterica* serovar Paratyphi A *gyrA*, accession no. AB071871; *S. enterica* serovar Paratyphi A *gyrB*, accession no. AB072393; *S. enterica* serovar Paratyphi A *parC*, accession no. AB072700; and *S. enterica* serovar Paratyphi A *parE*, accession no. AB072702.

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