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Mutations in Topoisomerase Genes of Fluoroquinolone-Resistant Salmonellae in Hong Kong

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A total of 88 salmonella isolates (72 clinical isolates for which the ciprofloxacin MIC was >0.06 μg/ml, 15 isolates for which the ciprofloxacin MIC was ≤0.06 μg/ml, and *Salmonella enterica* serotype Typhimurium ATCC 13311) were studied for the presence of genetic alterations in four quinolone resistance genes, *gyrA*, *gyrB*, *parC*, and *parE*, by multiplex PCR amplimer conformation analysis. The genetic alterations were confirmed by direct nucleotide sequencing. A considerable number of strains had a mutation in *parC*, the first to be reported in salmonellae. Seven of the isolates sensitive to 0.06 μg of ciprofloxacin per ml had a novel mutation at codon 57 of *parC* (Tyr57→Ser) which was also found in 29 isolates for which ciprofloxacin MICs were >0.06 μg/ml. Thirty-two isolates had a single *gyrA* mutation (Ser83→Phe, Ser83→Tyr, Asp87→Asn, Asp87→Tyr, or Asp87→Gly), 34 had both a *gyrA* mutation and a *parC* mutation (29 isolates with a *parC* mutation of Tyr57→Ser and 5 isolates with a *parC* mutation of Ser80→Arg). Six isolates which were isolated recently (from 1998 to 2001) were resistant to 4 μg of ciprofloxacin per ml. Two of these isolates had double *gyrA* mutations (Ser83→Phe and Asp87→Asn) and a *parC* mutation (Ser80→Arg) (MICs, 8 to 32 μg/ml), and four of these isolates had double *gyrA* mutations (Ser83→Phe and Asp87→Gly), one *parC* mutation (Ser80→Arg), and one *parE* mutation (Ser458→Pro) (MICs, 16 to 64 μg/ml). All six of these isolates and those with a Ser80→Arg *parC* mutation were *S. enterica* serotype Typhimurium. One *S. enterica* serotype Typhi isolate harbored a single *gyrA* mutation (Ser83→Phe), and an *S. enterica* serotype Paratyphi A isolate harbored a *gyrA* mutation (Ser83→Tyr) and a *parC* mutation (Tyr57→Ser); both of these isolates had decreased susceptibilities to the fluoroquinolones. The MICs of ciprofloxacin, levofloxacin, and sparfloxacin were in general the lowest of those of the six fluoroquinolones tested. Isolates with a single *gyrA* mutation were less resistant to fluoroquinolones than those with an additional *parC* mutation (Tyr57→Ser or Ser80→Arg), while those with double *gyrA* mutations were more resistant.

Salmonellosis remains a major public health problem worldwide. In contrast to gastroenteritis caused by salmonellae, invasive salmonellosis requires prompt antibiotic therapy. The antibiotics used for the treatment of salmonellosis have traditionally been chloramphenicol, sulfamethoxazole-trimethoprim, and ampicillin. However, with the development of resistance to these drugs, fluoroquinolones have been used and have been successful in treating many clinical cases. Unfortunately, fluoroquinolone-resistant strains have rapidly developed in recent years, and treatment failures have been reported (2, 8, 13, 20, 37, 44, 47).

Resistance to the fluoroquinolones in salmonellae has mainly been attributed to mutations in the *gyrA* gene. Mutations have rarely been reported in the *gyrB* gene, and none have been reported in the *parC* gene (7, 12, 36, 48, 51). Other resistance mechanisms such as efflux and reduced uptake of drugs have also been demonstrated (9, 10, 22).

All salmonellae in Hong Kong had remained susceptible to the fluoroquinolones (26–29). However, we saw a trend of increasing MICs (from 0.03 to 2 μg/ml) of fluoroquinolones for salmonellae isolated from 1993 to 1998 (unpublished observation). In 1998, we isolated the first fluoroquinolone-resistant salmonella strain in the Prince of Wales Hospital in Hong Kong. Clinical isolates of salmonellae with significant fluoroquinolone resistance (MICs, ≥8 μg/ml) remain extremely rare worldwide (36). It is important to understand the underlying mechanisms that cause high-level resistance in salmonellae and how they differ from those of the low-level resistance phenotypes in order to elucidate factors associated with increases in resistance to fluoroquinolones among these organisms. We therefore studied mutations in the gyrase and topoisomerase IV genes that lead to fluoroquinolone resistance in clinical salmonella isolates and the association of such mutations with resistance phenotypes and serotypes.

MATERIALS AND METHODS

Bacterial strains. A total of 2,348 salmonella isolates (71% of all salmonella isolates recovered) recovered from patients in the Prince of Wales Hospital from 1990 to 2001 were tested for their susceptibilities to the fluoroquinolones. Of these, 72 (3%) that were resistant to 0.06 μg of ciprofloxacin per ml were studied. Fifteen isolates for which ciprofloxacin MICs were ≤0.06 μg/ml and which were isolated throughout the study period and randomly selected and a standard strain of *Salmonella enterica* serotype Typhimurium (ATCC 13311) were included as sensitive controls.

Antimicrobial susceptibility testing. Screening for resistance to 0.06 μg of ciprofloxacin per ml was performed by an agar dilution method (32) by using a breakpoint concentration of 0.06 μg/ml. The MICs of six fluoroquinolones, ciprofloxacin, ofloxacin, sparfloxacin, levofloxacin, moxifloxacin, and gemifloxacin, for isolates resistant to 0.06 μg of ciprofloxacin per ml and the 15 isolates for which the ciprofloxacin MICs were ≤0.06 μg/ml were then determined by the

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TABLE 1. Primers used for PCR and their nucleotide positions and sizes of amplified products

Gene	Primer (nucleotide position)		Product size (bp)
	Forward	Backward	
gyrA[a]	5′-TGTCCGAGATGGCCTGAAGC-3′ (108–127)	5′-TACCGTCATAGTTATCCACG-3′ (454–435)	347
gyrA[a,b]	5′-TACACCGTCGCGTACTTTACGCCA-3′ (131–154)	5′-CCATCAGTTCGTGGGCGATTTTCG-3′ (406–383)	275
gyrB[c]	5′-CAAACTGGCGGACTGTCAGG-3′ (1215–1234)	5′-TTCCGGCATCTGACGATAGA-3′ (1560–1541)	345
parC[d]	5′-ATGAGCGATATGGCAGAGCG-3′ (14–33)	5′-TGACCGAGTTCGCTTAACAG-3′ (426–407)	412
parE[e]	5′-GACCGAGCTGTTCCTTGTGG-3′ (1285–1304)	5′-AGCAGAGTAGCGATATGCAA-3′ (1557–1538)	272

[a] From Griggs et al. (12).
[b] Two pairs of primers were used in MPAC analysis.
[c] According to GenBank accession no. Y07916.
[d] According to GenBank accession no. M68936.
[e] According to GenBank accession no. L05544.

agar dilution method (32). The geometric mean MICs (GMMs) were calculated by the formula

$$\sqrt[n]{y_1 y_2 y_3 \ldots y_n}$$

where y represents the individual MIC and n is the number of MICs used in the calculation.

MPAC analysis. Total DNA was extracted by suspending a few overnight colonies in 0.5 ml of double-distilled water and heating the mixture at 100°C for 10 min. The extracted DNA was subjected to amplification by PCR with primers specific for the quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC*, and *parE* (Table 1). Amplification was performed in a thermal cycler (PTC-100; MJ Research, Watertown, Mass.) by using the following protocol: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 52°C, and 1 min of extension at 72°C, with a final extension of 5 min at 72°C.

The PCR products were subjected to multiplex PCR amplimer conformation (MPAC) analysis by the method of McIlhatton et al. (31), with slight modifications. Briefly, the method used standard single-stranded conformational polymorphism analysis procedures, except that a reference PCR product, such as that of the ATCC 13311 control strain, was mixed with the test sample in equal quantities prior to analysis. Alternatively, two PCR products with an overlapping region amplified from the same test sample product were mixed prior to heat denaturation. MPAC analysis was found to greatly enhance the sensitivity of mutation detection. A denaturing solution containing 94% formamide, 0.05% xylene cyanol, and 0.04% bromophenol blue was added to the mixture, which was then heated at 95°C for 5 min, immediately placed on ice, and loaded onto 12.5% ExcelGel (Amersham Biosciences, Uppsala, Sweden). The samples were electrophoresed in a Multiphor II electrophoresis system (Amersham) at 15°C and 600 V for 80 min. The MPAC patterns were detected by silver staining (PlusOne silver staining kit; Amersham).

Direct nucleotide sequencing. The amplified products were purified by using GFX columns (Amersham) and were then sequenced by using the Silver Sequence DNA sequencing system (Promega, Madison, Wis.). Approximately 120 fmol of purified PCR products and 4.5 pmol of sequencing primer were included in each reaction mixture. The sequencing primer was either the forward or the reverse primer used in the PCR. The sequencing reaction conditions were the same as those used for the PCR, except that a total of 50 amplification cycles were used. The sequencing reaction products were analyzed on a 6% polyacrylamide gel (acrylamide-bisacrylamide [19:1], 7 M urea) at 1,700 V with an S2 sequencing apparatus (Life Technologies, Rockville, Md.). DNA bands were detected by silver staining (Promega).

Pulsed-field gel electrophoresis. Isolates of the same serotypes were typed by pulsed-field gel electrophoresis, as described previously (28).

RESULTS

Of the 72 isolates that were resistant to 0.06 μg of ciprofloxacin per ml, 14 were *S. enterica* serotype Typhimurium, 12 were *S. enterica* serotype Enteritidis, 12 were *S. enterica* serotype Blockley, 10 were *S. enterica* serotype Virchow, and 1 to 3 isolates each were of 15 other serotypes (Table 2). Six isolates were shown to have similar or identical pulsed-field gel electrophoresis patterns. These comprised three *S. enterica* serotype Blockley isolates, two *S. enterica* serotype Enteritidis isolates, and one *S. enterica* serotype Typhimurium isolate. Thus, there were a total of 66 nonidentical isolates. Since the numbers were small, the total numbers of isolates are provided and the numbers of nonidentical isolates are indicated in parentheses in Table 2. The MIC ranges and GMMs of the six fluoroquinolones tested for these isolates are shown in Table 3. Only six isolates showed high-level resistance to the drugs (MICs, ≥8 μg/ml). Fifteen isolates for which ciprofloxacin MICs were 0.015 to 0.06 μg/ml and *S. enterica* serotype Typhimurium ATCC 13311 were included as sensitive controls.

A total of 122 mutations in *gyrA*, *parC*, and *parE* were found in these 72 isolates. No *gyrB* mutations were detected. Seven of the 16 fluoroquinolone-sensitive isolates also harbored a single mutation each. The most common mutation found in 36 isolates (28% of mutations) was at codon 57 of *parC*, where a C→G transversion led to substitution of serine for tyrosine (Tyr57→Ser) (Table 2). Twenty-nine of these had an additional *gyrA* mutation. The next most common mutation was at codon 87 of *gyrA*, in which a G→A transversion led to substitution of asparagine for aspartate (Asp87→Asn) and which was found in 29 isolates (22% of mutations), with 19 isolates having an additional *parC* mutation (Tyr57→Ser) and 2 isolates having two additional mutations, one at *gyrA* (Ser83→Phe) and another at *parC* (Ser80→Arg). A mutation at the same codon involving a G→T transversion leading to a tyrosine substitution was found in 17 isolates. The mutation at codon 83 (C→T transversion) resulting in Ser83→Phe was found in 22 isolates, with 11 isolates having one to three additional mutations. Asp87→Gly and Ser83→Tyr changes in *gyrA* were found in eight and two isolates, respectively. Thirty-four isolates had double mutations, 32 isolates had one mutation, and 2 isolates had three mutations. Four isolates had four mutations, two in *gyrA* (Ser83→Phe, Asp87→Gly) and one each in *parC* (Ser80→Arg) and *parE* (Ser458→Pro).

The ciprofloxacin MICs for all isolates without any mutations and those with a single *parC* mutation (Tyr57→Ser) were ≤0.06 μg/ml, although the GMMs of the six fluoroquinolones tested and the overall GMMs were two- to more than fourfold higher for isolates with the *parC* mutation (Table 3). The MICs for isolates with a single mutation at codon 87 of *gyrA* were about twofold higher than those for isolates with a single *parC* mutation (Tyr57→Ser). The MICs for the isolate with Ser83→Tyr ranged from 0.25 to 1 μg/ml, and the GMM was 0.5 μg/ml, which was similar to that for isolates with

TABLE 2. Distribution of mutations in *gyrA*, *parC*, and *parE* genes in salmonellae in Hong Kong

Gene, mutation[a]	Yr	No. of isolates											
		Group B		Group C					Group D		Others[d]	Total
		Typhimurium	Other[b]	Blockley	Braenderup	Haardt	Virchow	Other[c]	Enteritidis	Typhi		
parC, Tyr57→Ser (AC<u>C</u>→AG<u>C</u>)	2000		2									7
	2001		5									
gyrA, Ser83→Phe (TC<u>C</u>→TT<u>C</u>)	1992	1										8
	1996								1			
	1998						1					
	2000								1			
	2001		1				2			1		
gyrA, Ser83→Phe *parC*, Tyr57→Ser (AC<u>C</u>→AG<u>C</u>)	2001		1 (Agona)					2 (Hadar)				3
gyrA, Ser83→Tyr (TC<u>C</u>→TA<u>C</u>)	1998							1				1
gyrA, Ser83→Tyr *parC*, Tyr57→Ser	2000										1 (Paratyphi A)	1
gyrA, Asp87→Asn (G<u>A</u>C→A<u>A</u>C)	1998	3 (2)[e]							1			8 (7)
	2000					1						
	2001								3			
gyrA, Asp87→Asn	1997			4 (3)			1					19 (16)
parC, Tyr57→Ser	1998			1							1	
	1999			6 (4)				1 (Rissen)				
	2000			1		1						
	2001		1 (Derby)		1			1 (Newport)				
gyrA, Asp87→Tyr (G<u>A</u>C→T<u>A</u>C)	1994		1									11
	1995		1									
	1996						1					
	1998						2	1				
	1999						1		1			
	2000						1					
	2001		1				1					
gyrA, Asp87→Tyr	2000					1						6
parC, Tyr57→Ser	2001		2 (Indiana)		2				1			
gyrA, Asp87→Gly (G<u>A</u>C→G<u>G</u>C)	2001								4 (2)			4 (2)
gyrA, Ser83→Phe	1990	1										5
parC, Ser80→Arg (<u>A</u>GC→<u>C</u>GC)	1992	1										
	1993	2										
	1995	1										
gyrA, Ser83→Phe, Asp87→Asn	1998	1										2
parC, Ser80→Arg	2001	1										
gyrA, Ser83→Phe, Asp87→Gly	1998	1										4
parC, Ser80→Arg	1999		1									
parE, Ser458→Pro (T<u>C</u>G→<u>C</u>CG)	2000	2										
Total		14 (13)	16	12 (9)	3	3	10	6	12 (10)	1	2	79 (73)

[a] Underscored nucleotides represent the mutated nucleotide.
[b] Includes serotypes Agona, Derby, Indiana, Panama, Reading, and Stanley.
[c] Includes serotypes Emek, Hadar, Newport, Rissen, and Thompson.
[d] Includes serotypes Paratyphi A and Weltevreden.
[e] The numbers of strains with different pulsed-field gel electrophoresis types are given in parentheses.

Asp87→Asn plus Tyr57→Ser or Ser83→Phe mutations. A *parC* mutation in addition to a *gyrA* mutation caused isolates to be more resistant, with the effect of Tyr57→Ser being slightly more pronounced than that of Ser80→Arg (Table 3). An additional Tyr57→Ser mutation caused the MICs for the isolate with the Ser83→Tyr mutation to be twofold higher (GMMs, 0.5 versus 1.0 μg/ml).

Other than the ciprofloxacin GMMs for isolates with a single *parC* mutation (Tyr57→Ser), the ciprofloxacin GMMs for isolates with a single *gyrA* mutation at either codon 83 or codon 87 were the lowest (0.14 to 0.35 μg/ml), with the MIC range being 0.12 to 1 μg/ml (Table 3). The GMMs of sparfloxacin and levofloxacin for these isolates were 0.13 to 0.55 μg/ml, with the GMMs of ofloxacin being the highest (0.53 to 1 μg/ml). For isolates with more than two mutations ($n = 6$), the MICs of ciprofloxacin were 8 to 32 μg/ml, the levofloxacin MICs were up to 16 μg/ml, and the MICs of the other four fluoroquinolones tested were up to 64 μg/ml.

TABLE 3. Correlation of gene mutations and antimicrobial susceptibilities

Mutation	No. of strains	MIC (μg/ml)												GMM for all isolates
		Ciprofloxacin		Ofloxacin		Sparfloxacin		Levofloxacin		Moxifloxacin		Gemifloxacin		
		Range	GMM	Range	GMM	Range	GMM	Range	GMM	Range	GMM	Range	GMM	
None	9	0.015–0.12	0.03	0.06–0.25	0.10	0.0075–0.12	0.03	0.03–0.25	0.06	0.06–0.25	0.11	0.03–0.25	0.07	0.06
Thr57→Ser (*parC*)	7	0.06	0.06	0.25–0.5	0.28	0.06–0.25	0.13	0.06–0.25	0.12	0.12–0.25	0.23	0.03–0.25	0.10	0.13
Asp87→Gly (*gyrA*)	4	0.12–0.25	0.14	1	1.00	0.12–0.25	0.21	0.12–0.25	0.21	0.5–1	0.84	0.12	0.12	0.29
Asp87→Asn (*gyrA*)	8	0.12–0.5	0.21	0.5–1	0.65	0.0075–0.5	0.13	0.25–0.5	0.30	0.5–1	0.71	0.12–1	0.23	0.31
Asp87→Tyr (*gyrA*)	11	0.12–1	0.22	0.25–2	0.53	0.12–2	0.26	0.12–2	0.32	0.5–2	0.57	0.12–1	0.53	0.38
Ser83→Tyr (*gyrA*)	1	0.25		1		0.25		0.5		0.5		1		0.50
Asp87→Asn (*gyrA*), Tyr57→Ser (*parC*), Ser83→Phe (*gyrA*)	19	0.12–0.5	0.36	0.25–2	0.96	0.0075–2	0.36	0.12–1	0.52	0.5–2	1.08	0.12–2	0.67	0.60
	8	0.25–1	0.35	0.5–2	0.92	0.25–1	0.55	0.25–1	0.55	0.25–2	0.65	0.12–4	0.99	0.63
Ser83→Phe (*gyrA*) Ser80→Arg(*parC*)	5	0.5	0.50	1	1.00	0.5–1	0.66	0.5	0.50	1	1.00	0.5–1	0.76	0.71
Ser83→Phe (*gyrA*) Tyr57→Ser (*parC*)	3	0.25–2	0.50	1–2	1.26	0.5–1	0.79	0.5–1	0.63	1	1.00	0.25–4	0.79	0.79
Asp87→Tyr (*gyrA*) Tyr57→Ser (*parC*)	6	0.25–1	0.50	0.5–2	1.26	0.5–1	0.63	0.25–2	0.63	0.5–2	1.12	0.25–4	0.89	0.79
Ser83→Tyr (*gyrA*) Tyr57→Ser (*parC*)	1	0.5		2		1		1		2		0.5		1.00
Ser83→Phe and Asp87→Asn (*gyrA*), Ser80→Arg (*parC*)	2	8–16	11.30	16	16.00	8–16	11.31	8	8.00	16	16.00	16–32	22.63	13.45
Ser83→Phe and Asp87→Gly (*gyrA*), Ser80→Arg (*parC*), Ser458→Pro (*parE*)	4	16–32	19.03	32–64	38.05	32–64	45.25	16	16.00	32–64	45.25	32–64	45.25	32.55

Mutations appeared as early as 1990. A double mutation (Ser83→Phe in *gyrA* and Ser80→Arg in *parC*) was found only in strains isolated from 1990 to 1995. Instead, the *gyrA* mutation (Ser83→Phe) was found in strains isolated from 1992 to 2001, whereas Asp87→Tyr was first detected in 1994 and Asp87→Asn was first detected in 1997. Triple mutations (Ser83→Phe and Asp87→Asn of *gyrA*, Ser80→Arg of *parC*) and quadruple mutations (Ser83→Phe and Asp87→Gly of *gyrA*, Ser80→Arg of *parC*, Ser458→Pro of *parE*) appeared only in recent years (1998 to 2001). Similarly, the *parC* mutation (Tyr57→Ser) was detected only after 1997.

S. enterica serotypes Typhimurium, Enteritidis, Blockley, and Virchow were the serotypes in which mutations were most commonly found (14 to 19%). The *parC* mutation Tyr57→Ser was not detected in *S. enterica* serotype Typhimurium but was detected in the rarer serotypes (Table 2). In contrast, all except one of the isolates resistant to 4 μg of the six fluoroquinolones tested per ml were *S. enterica* serotype Typhimurium; the exception was an *S. enterica* serotype Agona strain. *S. enterica* serotypes Typhi and Paratyphi A had decreased susceptibilities to the fluoroquinolones, with MICs ranging from 0.12 μg/ml for gemifloxacin to 0.5 μg/ml for ofloxacin and from 0.5 μg/ml for ciprofloxacin and gemifloxacin to 2 μg/ml for ofloxacin and moxifloxacin, respectively.

DISCUSSION

Mutations in *gyrA*. An increase incidence of clinical salmonella isolates with reduced susceptibilities to the fluoroquinolones (i.e., ciprofloxacin MICs ranging from 0.12 to 2 μg/ml) has been seen in recent years (16, 30, 42). Although they are susceptible to the NCCLS breakpoint for susceptible strains (32), treatment failures have been encountered when fluoroquinolones are used to treat infections caused by these strains (5, 15, 46). A number of mechanisms of fluoroquinolone resistance in different bacterial species have been suggested, and the most common ones are mutations in the *gyrA* gene, usually at either codon 83 or 87, and/or the *parC* gene (6, 12, 34, 36, 39, 49). The mutations that we found in the *gyrA* region of our isolates (Ser83→Phe; Ser83→Tyr; Asp87→Gly, Asn, or Tyr) have been reported previously (12, 14, 36). Ser83 is suggested to be an important site for fluoroquinolone resistance (12, 23, 33, 39, 49). This is supported by our results, in that of the five single *gyrA* mutations, the presence of Ser83→Phe was associated with the highest level of resistance (GMMs, 0.63 versus ≤0.5 μg/ml) (Table 3). Other mutations in *gyrA* that have been reported include Ser83→Leu (6, 14); Gly81→Asp, Gly81→Cys, and Asp82→Gly (43, 49); Asp87→His (6); Asp87→Tyr (6, 12, 14); and Ala119→Glu (12). All of these mutations conferred low-level resistance to fluoroquinolones. It appears that a *gyrA* mutation alone, regardless of the mutation type, may not contribute to a resistance level greater than 2 μg of ciprofloxacin per ml. Instead, strains with double amino acid changes in GyrA were more resistant than those with single amino acid changes in GyrA (4, 49).

Mutations in *parC*. ParC and ParE are two subunits of topoisomerase IV, with the former being homologous with GyrA and the latter being homologous with GyrB (21, 35, 40). High-level fluoroquinolone resistance was found to be due to both *parC* and *gyrA* mutations (13). Other workers (4, 14, 24) have reported on the *parC* mutation causing the substitution Ser80→Arg found in this study in organisms other than salmonellae. This mutation caused only slight decreases in the susceptibilities of the isolates when it was present together with one other *gyrA* mutation. Recently, it has been suggested that Glu84 of *parC* plays an important role in topoisomerase IV-DNA interactions and that a mutation at this site appears to have more deleterious effects than one at Ser80 (17). That the resistance levels of the isolates were elevated to >4 μg of ciprofloxacin per ml in the presence of two *gyrA* mutations (Ser83→Phe, Asp87→Asn) was probably due to the *gyrA* mutations rather than the *parC* mutation (4). Thus, Ser80→Arg did not appear to play an important role in conferring resistance.

The Thr57→Ser mutation in *parC* detected in many of our isolates in this study has not been reported previously. Besides being the first report of a *parC* mutation in salmonellae, it is also the first report of a single *parC* mutation in gram-negative

bacteria without a *gyrA* mutation and in isolates for which ciprofloxacin MICs are <0.12 μg/ml. In general, mutations in *parC* are rarer in gram-negative bacteria and usually arise later than *gyrA* mutations, probably because in these organisms, gyrase rather than topoisomerase IV is the primary target of fluoroquinolones (38, 41). Hence, changes to *gyrA* mostly precede those to *parC* (6), as there is probably less selective advantage to single mutations in *parC*. Other mutations in *parC* that have been reported in other organisms include Gly78→Asp (14); Asp79→Ala (3); Ser80→Ile (4, 6, 24, 45); and Glu84→Gly and Glu84→Lys (3, 4, 6, 14, 24). Further studies on other resistance mechanisms such as alterations in membrane permeability, changes in influx or efflux, etc., are required to evaluate the contribution of *parC* mutations to fluoroquinolone resistance in salmonellae.

Mutations in *gyrB*. We did not detect *gyrB* mutations in our salmonella isolates. This is not surprising, as *gyrB* mutations remain extremely rare in most bacterial species, even among highly resistant strains, although they have been reported in salmonellae (7, 13). It may be useful to investigate whether the GyrB protein, along with essential regions of the GyrA, ParC, and ParE proteins where amino acid changes are not tolerated, represents a good target for future drug designs. Alternatively, the importance of amino acids 83 and 87 of the GyrA protein in fluoroquinolone resistance may infer that drugs targeting the altered GyrA proteins can be helpful in eliminating fluoroquinolone-resistant strains.

Mutations in *parE*. While most workers detected mutations in *gyrA* in highly fluoroquinolone-resistant strains of *Escherichia coli*, salmonellae, and *Shigella* spp., mutations in *gyrB* and *parE* are rare (3, 6, 9, 12, 25). We detected a mutation in *parE* (Ser458→Pro) that has not been reported in salmonellae. It is likely that this mutation might not be directly responsible for high-level resistance but rather increased the resistance levels in isolates already harboring two *gyrA* mutations and one *parC* mutation. Gensberg and colleagues (7) detected a *gyrB* mutation in *S. enterica* serotype Typhimurium (Ser463→Tyr). Breines et al. (1) reported a *parE* mutation (Leu445→His) in *E. coli*, and González et al. (11) reported a Pro424→Gln mutation in viridans group streptococci. It could be postulated that amino acids in the region from positions 463 to 468 in *gyrB* and those in the region from positions 424 to 460 of *parE* played a role in conferring fluoroquinolone resistance.

Sequential development of mutations in topoisomerase genes. Our results are in agreement with the observations of other workers that the *gyrA* mutations Ser83→Phe and Asp87→Tyr were the first to develop in salmonellae (12, 39), since they were first detected in our isolates in 1990 and 1994, respectively. Subsequently, other mutations accumulated, including the *parE* mutation (Ser458→Pro), so that triple and quadruple mutations were observed after 1998. It is interesting that the *parC* mutation (Tyr57→Ser) was also a recent development.

Gene mutations and *Salmonella* serotypes. *S. enterica* serotype Typhimurium was the serotype in which mutations were first detected. It was also the serotype that had more than two mutations and that did not have the *parC* mutation (Tyr57→Ser). In contrast, other than the Tyr57→Ser (*parC*) mutation, mutations in both *parC* and *parE* were not found in salmonellae other than *S. enterica* serotype Typhimurium. This was probably due to the minimal effect that this mutation has on fluoroquinolone resistance, which was rarely seen in salmonellae other than *S. enterica* serotype Typhimurium. However, it would be difficult to explain the absence of this mutation in *S. enterica* serotype Typhimurium. The development of decreased fluoroquinolone susceptibilities in *S. enterica* serotypes Typhi and Paratyphi A is a cause for concern. Hirose and colleagues (18) also recently detected *gyrA* mutations in *S. enterica* serotype Typhi and Paratyphi strains that were less susceptible to the fluoroquinolones. Continuous surveillance must be carried out to monitor the development of fluoroquinolone resistance in these organisms.

Effects of mutations on fluoroquinolone susceptibility. All mutations had the least, although an important, effect on susceptibility to ciprofloxacin, as the GMMs of ciprofloxacin were usually the lowest of those of all drugs tested, followed by those of levofloxacin, while the effect on susceptibility to ofloxacin was most prominent, as the ofloxacin GMMs were frequently the highest of those among the six fluoroquinolones tested, closely followed by the GMMs of moxifloxacin. Since fluoroquinolones preferentially bind to different target regions of gyrase or topoisomerase IV (19, 41, 50, 52), it can be postulated that fluoroquinolones with binding properties similar to those of ciprofloxacin would be most effective even against organisms with mutated genes.

We have also tested the susceptibilities of the isolates to nalidixic acid. The nalidixic acid GMM for isolates without any mutation was 6 μg/ml and that for isolates with a single *parC* mutation (Tyr57→Ser) was 11 μg/ml, while the nalidixic acid MICs for all other isolates were >128 μg/ml (results not shown). Isolates with a novel *parC* mutation (Tyr57→Ser) were also less susceptible to the other fluoroquinolones than those without any mutations. The susceptibility to nalidixic acid and the other fluoroquinolones therefore might be a marker of low-level resistance to fluoroquinolones.

Conclusion. This study has provided evidence that isolates with reduced susceptibilities to fluoroquinolones might be important in the clinical development of resistance, as they could become highly resistant upon sequential accumulation of target gene mutations. Further studies involving analysis of mutations in clonally related organisms or in mutants developed in vitro would be required to confirm this observation. Mutations in *gyrA* conferred low-level fluoroquinolone resistance, while addition of another *gyrA* mutation together with a *parC* and/or a *parE* mutation increased the resistance to a high level. The presence of *gyrA*, *parC*, and *parE* mutations in *S. enterica* serotype Typhimurium, the most common salmonella serotype resistant to high concentrations of fluoroquinolones, and the presence of *gyrA* mutations in *S. enterica* serotypes Typhi and Paratyphi are a serious concern and call for continuous monitoring of fluoroquinolone-resistant salmonellae.

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