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Prevalence of Mutations within the Quinolone Resistance-Determining Region of *gyrA*, *gyrB*, *parC*, and *parE* and Association with Antibiotic Resistance in Quinolone-Resistant *Salmonella enterica*

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***Salmonella enterica* isolates ($n = 182$) were examined for mutations in the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE*. The frequency, location, and type of GyrA substitution varied with the serovar. Mutations were found in *parC* that encoded Thr57-Ser, Thr66-Ile, and Ser80-Arg substitutions. Mutations in the *gyrB* quinolone resistance-determining region were located at codon Tyr420-Cys or Arg437-Leu. Novel mutations were also found in *parE* encoding Glu453-Gly, His461-Tyr, Ala498-Thr, Val512-Gly, and Ser518-Cys. Although it is counterintuitive, isolates with a mutation in both *gyrA* and *parC* were more susceptible to ciprofloxacin than were isolates with a mutation in *gyrA* alone.**

The four most common nontyphoidal *Salmonella enterica* serovars isolated from humans in England and Wales in 1999 were Enteritidis (57%), Typhimurium (18%), Virchow, and Hadar (14). In the last decade, the numbers of isolates of these serovars with decreased susceptibility to ciprofloxacin (MIC, ≥1 μg/ml) have increased (12). In 2000, 52% of the *S. enterica* serovar Virchow isolates tested exhibited decreased susceptibility to ciprofloxacin (12). It is postulated that many of these isolates have arisen in animals after fluoroquinolone exposure and have been transferred to humans via the food chain (11).

In salmonellae, where DNA gyrase is the primary target of quinolone action, a single point mutation in the quinolone resistance-determining region (QRDR) of *gyrA* can mediate resistance to the nonfluorinated quinolone nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin, e.g., an MIC of 0.25 μg/ml (10). Mutations in the *gyrB* and topoisomerase IV genes *parC* and *parE* are considered rare in salmonellae (6, 7, 9, 11). It was hypothesized that those isolates with decreased susceptibility harbored a single mutation in *gyrA*, whereas resistant isolates would contain two or more mutations in *gyrA* and/or *gyrB* and/or *parC* and/or *parE*.

S. enterica isolates ($n = 182$; 156 from animals, 18 from humans, and 18 from the environment) representative of the 25 serotypes typically isolated between 1997 and 2000 were obtained from the Enteric Bacteria Reference Laboratory of the Veterinary Laboratories Agency. Because of the recent debate about the breakpoint concentration of ciprofloxacin (1, 8), isolates resistant to ≥0.12 μg of ciprofloxacin per ml were investigated. Isolates were serotyped by a microagglutination method (13) and, where appropriate, were phage typed (15). All isolates were from geographically and temporally distinct samples. Isolates were also analyzed by pulsed-field gel electrophoresis, which confirmed that no clones were examined.

The MIC of each agent was determined on solid medium by the agar doubling-dilution procedure of the British Society of Antimicrobial Chemotherapy (3). Control strains included the National Collection of Type Cultures type strain of each serovar and *Escherichia coli* NCTC 10418. Of the 182 isolates studied, 159 were inhibited by ≤0.5 μg of ciprofloxacin per ml. The isolates were divided into three groups (Table 1): Group A comprised 11 isolates sensitive to nalidixic acid (MICs of 4 to 8 μg/ml) and ciprofloxacin (MICs of 1 to 2 μg/ml). Thirty-nine isolates were multiply drug resistant, i.e., resistant to four or more antibiotics, detergents, disinfectants, and/or dyes (data not shown).

The QRDR of *gyrA*, *gyrB*, *parC*, and *parE* was amplified by PCR from the DNA of all isolates. *gyrA* was amplified as described previously (5). A 181-bp region covering the QRDR of *gyrB* (Ala415 to Ile470) was amplified with primers stmgyrB1 (5′-GCGCTGTCCGAACTGTACCT-3′) and stmgyrB2 (5′-TGATCAGCGTCGCCACTTCC-3′) (accession no: AE008878). The QRDR from *parC* was amplified in a 270-bp fragment (Tyr47 to Leu133) with primers stmparC1 (5′-CTATGCGATG TCAGAGCTGG-3′) and stmparC2 (5′-TAACAGCAGCTCGGCGTATT-3′) (accession no: AE008846). A 240-bp region covering Ser450 to Arg528 was amplified from *parE* with primers stmparE1 (5′TCTCTTCCGATGAAGTGCTG-3′) and stmparE2 (5′-ATACGGTATAGCGGCGGTAG-3′) (accession no: AE008846). Primers were synthesized by MWG Biotech, Milton Keynes, United Kingdom. Mutations in *gyrA* were identified at partial denaturation temperatures of 61 and 63°C (5), and those in *gyrB*, *parC*, and *parE* were identified at partial denaturation temperatures of 63, 62, and 64°C, respectively. DNA sequencing was done exactly as described previously (5).

One hundred thirty-nine of 182 isolates contained a mutation that encoded an amino acid substitution within the QRDR

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of *gyrA*. Of these 139 isolates, 69 (49.6%) contained mutations only in *gyrA* and the remaining 70 (55.4%) possessed one or more mutations within the QRDR of *gyrB* ($n = 2$), *parC* ($n = 65$), and/or *parE* ($n = 10$). All of the types of *gyrA* mutation observed have been described previously (5). Of those with mutations in *gyrA* (Table 2), 80 isolates (57%) contained a change at codon Ser83 to either phenylalanine ($n = 49$) or tyrosine ($n = 31$); 39.6% of the isolates contained a change at codon Asp87 to either asparagine ($n = 42$), tyrosine ($n = 9$), or glycine ($n = 4$). Four isolates contained an Asp82-Asn substitution. Four isolates contained a silent mutation at Asp144; these were all *S. enterica* serovar Senftenberg. Other substitutions were observed, and some were limited to specific serovars (Table 2).

The positions of GyrA substitutions varied with the serovar (Table 2). For example, serovar Typhimurium only contained mutations at codons Ser83 and Asp87, whereas all isolates of *S. enterica* serovar Senftenberg contained mutations at codon Ser83 (alone or combined with additional mutations at codon Asp72, Val73, Leu98, or Ala139). In *S. enterica* serovar Montevideo, the predominant mutations were at Asp87 (90.9%) and likewise for *S. enterica* serovar Hadar (70%). The majority of the *S. enterica* serovar Dublin isolates (87.5%) did not contain a mutation within the QRDR of *gyrA*. Conversely, all *S. enterica* serovar Hadar isolates ($n = 10$) contained a mutation within the QRDR of *gyrA*.

The type of amino acid substitution also varied with the serovar. All mutations at the Ser83 codon in *S. enterica* serovar Typhimurium encoded a phenylalanine residue. All mutations at the Ser83 codon in *S. enterica* serovars Montevideo, Newport, and Hadar encoded a tyrosine residue (Table 2). Mutations at the Ser83 codon in *S. enterica* serovars Enteritidis and Senftenberg and other serovars encoded either phenylalanine or tyrosine substitutions.

Mutations within the Asp87 codon resulted in substitutions to asparagine, tyrosine, or glycine residues. *S. enterica* serovars Montevideo and Hadar contained only asparagine substitutions. *S. enterica* serovar Typhimurium contained both asparagine and tyrosine substitutions, and *S. enterica* serovar Newport contained both asparagine and glycine substitutions. *S. enterica* serovar Enteritidis was the only serotype that harbored mutations that encoded all three amino acid substitution types (Table 2).

Eighty-four of 182 isolates contained a mutation encoding an amino acid substitution at Thr57-Ser within the QRDR of ParC. Sixty-five of 84 also contained a substitution in GyrA. Three isolates contained a second but novel mutation in ParC at Thr66-Ile (two of *S. enterica* serovar Mbandaka and one of *S. enterica* serovar Heidelberg). One isolate (*S. enterica* serovar Fischerkietz) contained a second substitution at Ser80-Arg. *S. enterica* serovars Typhimurium, Enteritidis, and Dublin contained no mutations within the QRDR of *parC*.

Five of 182 isolates contained novel substitutions within GyrB. One isolate of *S. enterica* serovar Senftenberg contained a substitution at codon Tyr420-Cys, and one isolate of *S. enterica* serovar Newport contained a substitution at codon Arg437-Leu. Both mutation types were found in *S. enterica* serovars Enteritidis and Mbandaka. The close proximity of these substitutions to the quinolone-binding pocket may contribute to the decreased binding affinity of the quinolone.

TABLE 1. MICs of nalidixic acid, ciprofloxacin, tetracycline, and chloramphenicol for each group of isolates[b]

Class	Genotype or phenotype	No. of isolates	MIC (μg/ml)															
			Nalidixic acid					Ciprofloxacin				Tetracycline				Chloramphenicol		
			Range	GMM	Mode	MIC_{50}	MIC_{90}[c]	Range	GMM	Mode	MIC_{50}	MIC_{90}	Range	GMM	Mode	MIC_{50}	MIC_{90}	Range	GMM	Mode	MIC_{50}	MIC_{90}
A	Nal^s Cip^s	8[a] (11)	4–8	7.5	8	8	8	0.03–0.25	0.06	0.03	0.03	0.06	1–256	49.2	2	2	128	4–256	42	16	16	16
B	Nal^r Cip^s	125[a] (148)	16–256	223.2	256	256	256	0.03–0.5	0.25	0.25	0.25	0.5	1–256	28.5	1	2	128	2–256	12.7	4	4	32
C	Nal^r Cip^r	14[a] (23)	256	256	256	256	256	1–2	1.21	1	1	2	1–32	5.29	4	4	8	4–32	15.14	16	16	32
1	WT *gyrA* WT *parC*	20[a] (24)	4–256	90.6	256	32	256	0.03–1	0.17	0.12	0.12	0.25	1–256	24.6	8	4	32	1–256	31.4	32	16	32
2	WT *gyrA parC*	19	8–256	116.6	256	16	256	0.03–0.25	0.11	0.12	0.12	0.25	1–256	33.6	4	4	64	4–32	17.9	16	16	32
3	*gyrA* WT *parC*	43[a] (74)	128–256	253	256	256	256	0.03–2	0.49	0.25, 0.5	0.5	1	1–256	35.2	1	2	128	4–128	19.1	4	4	16
4	*gyrA parC*	65	256	256	256	256	256	0.12–2	0.34	0.25	0.25	0.5	1–256	21.3	1	1	128	2–32	5.3	4	4	8
All isolates		147[a] (182)	4–256	241.6	256	256	256	0.015–2	0.33	0.25	0.25	0.5	1–256	27.4	1	2	128	2–256	14.5	4	4	32

[a] Excludes serovar Typhimurium isolates DT104 and DT193. Numbers in parentheses include these isolates.

[b] Nal^r, MIC of nalidixic acid ≤8 μg/ml; Nal^r, MIC of nalidixic acid, ≥16 μg/ml; Cip^s, MIC of ciprofloxacin, ≤0.5 μg/ml; Cip^r, MIC of ciprofloxacin, ≥1 μg/ml; *gyrA*, mutation within QRDR of *gyrA*; *parC*, mutation within QRDR of *parC*; WT, wild type (no mutation within QRDR); GMM, geometric mean.

[c] MIC_{90}, MIC for 90% of strains tested.

TABLE 2. Distribution of substitutions within the QRDR of GyrA

Serovar(s)	No. of isolates			% of isolates with following amino acid substitution:											
	Total	WT[b]	*gyrA*	Asp72→Gly	Val73→Ile	Asp82→Asn	Ser83→Phe	Ser83→Tyr	Asp87→Asn	Asp87→Tyr	Asp87→Gly	Leu98→Val	Ala119→Ser	Ala131→Gly	Ala139→Ser
All	182	43	139	0.7	0.7	2.9	35.2	22.3	30.2	6.5	2.9	0.7	0.7	0.7	14.4
Typhimurium[a]	40	5	35				60.0		34.3	5.7					
Montevideo	25	2	23			9.1			90.9						
Enteritidis	11	4	7				28.6	14.3	14.3	28.6	14.3				
Senftenberg	22	7	15	6.2	6.2		43.8	56.2				6.2			6.2
Newport	17	1	16			6.2		68.8		18.8	6.2		6.2		
Dublin	11	9	2				50.0			50.0					
Hadar	10		10			10.0		20	70						
Other[b]	46	16	30				56.7	26.7	6.7	3.3	6.7				

[a] Includes serovar Typhimurium isolates DT104 and DT193.
[b] Other serovars include *S. enterica* serovars Livingstone (n = 9), Binza (n = 5), Fisherkietz (n = 5), Indiana (n = 5), Mbandaka (n = 3), Saint Paul (n = 3), Virchow (n = 3), Heidelberg (n = 2), Kedougou (n = 2), Albany (n = 1), Blockley (n = 1), Emek (n = 1), Haardt (n = 1), Haifa (n = 1), Muenchen (n = 1), Reading (n = 1), Stanley (n = 1), and Thompson (n = 1).
[c] WT, wild type.

Fourteen of 182 isolates contained a substitution within ParE. Novel mutations were found at five different codons and comprised Glu453-Gly (n = 4), His461-Tyr (n = 1), Ala498-Thr (n = 6), Val512-Gly (n = 3), and Ser518-Cys (n = 4). Only serovars Typhimurium, Enteritidis, Newport, and Dublin had no substitutions in ParE. Ling et al. (9) found no mutations in *gyrB* (although this may have been because single-strand conformational polymorphism was used to identify those isolates with a mutation and denaturing high-performance liquid chromatography [5] is more accurate) and only one isolate with a mutation in *parE*. Data from both studies indicate that mutations in *gyrB* and *parE* are much less common than those in *gyrA*.

This is the largest analysis of the QRDR of *gyrA*, *gyrB*, *parC*, and *parE* and included the most commonly isolated serovars of *S. enterica* in the United Kingdom. This study revealed that the frequency and distribution of each mutation type for any particular serovar were not typical for all isolates. Substitutions within GyrA at Ser83 or Asp87 were most frequently observed, but the substituting amino acid differed between serovars. The codon most commonly containing a mutation also varied with the serovar; this is an important observation, as it suggests that no generalization for *S. enterica* can be made, despite previous publications suggesting otherwise. Mutations within the other topoisomerase genes were rare, suggesting that other mechanisms of resistance are more important in this species.

In the present study, mutations in *parC* were most frequently observed at Thr57 (not a tyrosine as described by Ling et al. [9]), all of which were converted to a serine codon. Although *E. coli* also possesses a threonine at codon 57 of *parC*, no mutations at this locus have been described. Occasional second substitutions in ParC (Thr66-Ile and Ser80-Arg) were also seen. Unlike the *gyrA* mutations, Thr57-Ser does not change the nucleophilic functional group of the residue although the replacement residue is smaller. This could result in a change in binding affinity for the quinolone.

The isolates were classed together on the basis of the presence or absence of a mutation in a topoisomerase gene (Table 1, classes 1 to 4). To enable statistical analyses of MIC data without bias due to the presence of multiple mechanisms of antibiotic resistance and their effect on the MICs of chloramphenicol and tetracycline, *S. enterica* serovar Typhimurium isolates DT104 and DT193 were excluded. The distribution of the MICs of nalidixic acid and ciprofloxacin differed for each group and class (Table 1). A two-tailed Mann-Whitney analysis revealed that there was a significant difference in the shape of the distribution of the MICs. This is most clearly seen in the geometric mean (GMM) and median MICs (MIC for 50% of the strains tested [MIC_{50}]). Surprisingly, those isolates that had a mutation in *parC* (Table 1, class 2) were more susceptible to ciprofloxacin, tetracycline, and chloramphenicol than those isolates with no mutation in *gyrA* or *parC* (class 1). Those with a mutation in both *gyrA* and *parC* (class 4) were also more susceptible to ciprofloxacin than those with a mutation in *gyrA* alone (class 3) (for ciprofloxacin, $P = 0.0046$ [two-tailed Mann-Whitney test]). These data suggest that a Thr57-Ser substitution in ParC makes isolates more sensitive to ciprofloxacin but not to nalidixic acid. It has been previously observed that compensatory mutations reversing resistance mapped to *gyrA* in nalidixic acid-resistant laboratory mutants of *S. enterica*,

although the precise nature of the mutation was not determined (2, 4; D. Hughes, personal communication). It is hypothesized that the mutation at codon 57 of *parC* could be a naturally occurring compensatory mutation. Ling et al. (9) also noted a mutation at codon 57 in 36 human isolates of *S. enterica*, 29 of which also contained a mutation in *gyrA*. However these authors did not associate the mutation in *parC* with increased susceptibility to ciprofloxacin but suggested that the mutation in *parC* resulted in higher MICs. The discrepancy between the two studies may be due to the number of isolates investigated and the presence of multiple mutations in most of the isolates described by Ling et al. (9), making analysis difficult. However, in agreement with Ling et al. (9), none of the *S. enterica* serovar Typhimurium isolates contained a mutation in *parC*.

Both class 1 and class 2 isolates (both lack mutations in *gyrA*) contained greater numbers of isolates that were resistant to tetracycline and chloramphenicol than did classes 3 and 4 isolates (Table 1). However, the most striking observation was that for those isolates with a mutation in *parC* the distribution of MICs indicated a preponderance of isolates inhibited by low MICs of tetracycline and chloramphenicol. This distribution is clearly reflected in the GMM values and is statistically significant for chloramphenicol ($P = 0.022$ [two-tailed Mann-Whitney test]). In fact, there were no highly chloramphenicol-resistant isolates in class 2 or 4. For the isolates in class 3, the distribution of MICs was evenly spread around the mode MIC. This association between isolates with mutation in *parC* and lower MICs of ciprofloxacin, chloramphenicol, and tetracycline suggests that the isolates with mutations in *gyrA* may also overexpress an efflux pump.

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REFERENCES

1. **Aarestrup, F. M., C. Wiuff, K. Mølbak, and E. J. Threlfall.** 2003. Is it time to change fluoroquinolone breakpoints for *Salmonella* spp.? Antimicrob. Agents Chemother. **47:**827–829.
2. **Anderrsson, D. I., Björkman and J., D. Hughes.** 1998. Antibiotic resistance here to stay? Compensatory mutations restore virulence of resistant bacteria. Lakartidningen **95:**3940, 3943–3944.
3. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48**(S1)**:**5–16.
4. **Björkman, J., D. Hughes, and D. I. Andersson.** 1998. Virulence of antibiotic-resistant *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **95:**3949–3953.
5. **Eaves, D. J., E. Liebana, M. J. Woodward, and L. J. V. Piddock.** 2002. Detection of *gyrA* mutations in quinolone-resistant *Salmonella enterica* by denaturing high-performance liquid chromatography. J. Clin. Microbiol. **40:** 4121–4125.
6. **Guerra, B., B. Malorny, A. Schroeter, and R. Helmuth.** 2003. Multiple resistance mechanisms in fluoroquinolone-resistant *Salmonella* isolates from Germany. Antimicrob. Agents Chemother. **47:**2059.
7. **Hansen, H., and P. Heisig.** 2003. Topoisomerse IV mutations in quinolone-resistant salmonellae selected *in vitro*. Microb. Drug Resist. **9:**25–32.
8. **Kahlmeter, G., D. F. J. Brown, F. W. Goldstein, A. P. MacGowan, J. W. Mouton, A. Österlund, A. Rodloff, M. Steinbakk, P. Urbaskova, and A. Vatopoulos.** 2003. European harmonisation of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J. Antimicrob. Chemother. **52:**145–148.
9. **Ling, J. M., E. W. Chan, A.W. Lam, and A. F. Cheng.** 2003. Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. Antimicrob. Agents Chemother. **47:**3567–3573.
10. **Piddock, L. J. V.** 1999. Mechanisms of fluoroquinolone resistance: an update 1994–1998. Drugs **58**(S2)**:**11–18.
11. **Piddock, L. J. V.** 2002. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. FEMS Microbiol. Rev. **26:**3–16.
12. **Public Health Laboratory Service.** 2002. Antimicrobial resistance in 2000: England and Wales. London: Public Health Laboratory Service.
13. **Shipp, C. R., and B. Rowe.** 1980. A mechanical microtechnique for *Salmonella* serotyping. J. Clin. Pathol. **33:**595–597.
14. **Threlfall, E. J.** 2002. Antimicrobial drug resistance in *Salmonella*: problems and perspective in food- and water-borne infections. FEMS Microbiol. Rev. **26:**141–148.
15. **Ward, L. D., J. D. H. De Sa, and B. Rowe.** 1987. A phage typing scheme for *Salmonella enteritidis*. Epidemiol. Infect. **99:**291–304.