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Mutations in *gyrA* Gene of Quinolone-Resistant Salmonella Serotypes Isolated from Humans and Animals

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The quinolone resistance-determining regions (QRDRs) of the *gyrA* genes of quinolone-resistant clinical and veterinary salmonella isolates were sequenced. Substitutions analogous to a substitution of a Ser to a Phe at position 83 (Ser83→Phe) and Asp87→Gly or Tyr in *Escherichia coli* were found, as was a single novel mutation outside of the QRDR resulting in Ala119→Glu. The data suggest that *gyrA* mutations are associated with quinolone resistance in veterinary and clinical salmonella isolates and that the limits of the QRDR may require revision.

Since the 1940s there has been a dramatic increase in the incidence of human salmonella infections caused by nontyphoidal serotypes in industrialized countries (28); the global increase in the number of cases of food poisoning caused by *Salmonella enteritidis* is thought to be related to the widespread infection of poultry flocks with *S. enteritidis* (1, 22). Fluoroquinolones have been used successfully to treat salmonellosis, including infections caused by multiresistant strains (2, 23, 25), although failure of ciprofloxacin therapy because of the development of resistance in clinical isolates of salmonella and/or the isolation of ciprofloxacin-resistant strains has been reported in several countries (3, 4, 8, 10, 12, 14, 20). Recently, Heisig et al. (11) described three multiply antibiotic-resistant isolates of *S. typhimurium* serovar Copenhagen from cattle which were highly resistant to ciprofloxacin (MIC, 32 μg/ml). Typing methods had demonstrated that these strains were related to three clinical isolates of the same serovar, and it was suggested that one multiply antibiotic-resistant clone had spread among humans and animals in Germany. Modification of DNA gyrase because of mutations in the *gyrA* and *gyrB* genes was the proposed mechanism of quinolone resistance in one of the clinical isolates, and presumably, the veterinary clones possessed the same mechanism of resistance (11). The use of antibiotics in veterinary medicine may promote the emergence of resistance in bacteria pathogenic to humans, thus presenting a potential risk to public health from zoonotic infections, e.g., with *Salmonella* and *Campylobacter* spp. Although the cost of administration of fluoroquinolones to farm animals is supposedly prohibitive (30), they are undoubtedly in widespread use in parts of Europe (11a, 17). There is pressure to limit the use of these agents in animals to preserve their value in the treatment of human infections (6, 15, 18, 21).

The clinical isolates of *S. typhimurium* used in the present study have been described previously and originated from two patients who failed ciprofloxacin therapy (19). Isolates L1 and L2 were obtained from the urine of patient A before and after ciprofloxacin therapy. Strain L3 was isolated from a hematoma in patient B prior to a course of ciprofloxacin therapy. Eleven resistant posttherapy (isolates L4 to L6, L10 to L16, L18) were obtained from patient B over a period of 19 weeks. Biochemical data and *gyrA* dominance tests suggested that the posttherapy isolate from patient A possessed a change in DNA gyrase that conferred resistance, whereas the posttherapy isolates from patient B were of several phenotypes: (i) alterations in DNA gyrase and/or (ii) reduced permeability independent of OmpF expression. Ninety-eight nalidixic acid-resistant salmonellae isolated from veterinary sources (including chickens, turkeys, eggs, litter and nest material, and imported exotic birds) in the United Kingdom and Europe between 1988 and 1991 were obtained from the Central Veterinary Laboratory (Ministry of Agriculture, Fisheries, and Food, Weybridge, United Kingdom). Twenty-seven of the isolates (isolates L20 to L25, L33 to L49, L89 to L91, and L112) have been described previously (9). *S. typhimurium* NCTC 74 was obtained from the Public Health Laboratory Service, and *S. typhimurium* KS313 (*nalA* mutant) was obtained from K. Sanderson at the Canadian Salmonella Genetic Stock Centre. Ciprofloxacin and enrofloxacin were gifts from Bayer AG and were made up and used according to the manufacturer's instructions. The MIC of each agent for each strain was determined by a routine agar plate doubling dilution method.

PCR amplification and DNA sequencing of the *gyrA* fragments was performed as follows. A 347-nucleotide *gyrA* gene fragment was amplified from a genomic DNA template by using the 20-mer oligonucleotide primer P1 (5′-TGTCCGA GATGGCCTGAAGC-3′) and the biotinylated 20-mer oligonucleotide primer P2BIO (5′-TACCGTCATAGTTATCCA CG-3′), corresponding to nucleotides 108 to 127 and nucleotides 435 to 454 for forward and reverse reactions, respectively. These salmonella-specific primers were constructed with reference to partial sequence data for a 422-nucleotide *S. typhimurium* PCR product generated with degenerate primers (5′-GAT/CGGNT/CTNAAA/GCCNGTNCA-3′ and 5′-GCCAT NCCNACNGCA/G/TATNCC-3′; where N is G, A, T, or C), based on the *Escherichia coli gyrA* sequence data (26) (GenBank accession number X06373). These primers bound at sites corresponding to nucleotides 115 to 134 and nucleotides 517 to 536 for forward and reverse reactions, respectively, on *E. coli gyrA*. DNA amplified with the biotinylated primer P2BIO was purified with Dynabeads (Dynal), and the bound DNA was sequenced by using Sequenase (version 2.0; United States Biochemicals) and was radiolabelled with ^{35}S-deoxyadenosine α-thiotriphosphate. Sequencing reactions were electrophoresed on denaturing 6% acrylamide gels, and the DNA ladder was visualized by autoradiography. The DNA sequences of

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     D  G  L  K  P  V  H  R  R  V  L  Y  A  M  N  V
    GAGATGGCCTGAAGCCGGTACACCGTCGCGTACTTTACGCCATGAACGTA
  1 --------------------------------------------------

    L  G  N  D  W  N  K  A  Y  K  K  S  A  R  V  V  G
    TTGGGCAATGACTGGAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGG
 51 C-A-----------------------------------------------

     D  V  I  G  K  Y  H  P  H  G  D  S  A  V  Y  D  T
    TGACGTAATCGGTAAATACCATCCCCACGGCGATTCCGCAGTGTATGACA
101 ---------------------------T--T--C--G--G--C-------

      I  V  R  M  A  Q  P  F  S  L  R  Y  M  L  V  D
    CCATCGTTCGTATGGCGCAGCCATTCTCGCTGCGTTACATGCTGGTGGAT
151 -G--T--C--C--------------------------T--------A--C

    G  Q  G  N  F  G  S  I  D  G  D  S  A  A  A  M  R
    GGTCAGGGTAACTTCGGTTCTATTGACGGCGACTCCGCGGCGGCAATGCG
201 -----------------------C-----------T--------------

     Y  T  E  I  R  L  A  K  I  A  H  E  L  M  A  D  L
    TTATACGGAGATCCGTCTGGCGAAAATCGCCCACGAACTGATGGCCGATC
251 ---------A-----------------T-----T----------------

      E  K  E  T  V  D  F  V  D  N  Y  D  G  T  E  K
    TCGAAAAAGAGACGGTGGATTTCGTGGATAACTATGACGGTACGGAAAAA
301 ----------------C--------T--------------C---------

    I  P  D  V  M
    ATTCCGGACGTCATGCC
351 -----------------
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of a 367-bp fragment of *S. typhimurium* NCTC 74 (L19) *gyrA*. The sequence of *E. coli gyrA* is shown below if it is different. The codon equivalent to Ser83 of *E. coli gyrA* is shown in boldface type. *Hin*fI binding sites are underlined with double lines. The P2BIO binding site is underlined with a single line. The deduced amino acid sequence of *S. typhimurium gyrA* is identical to that of *E. coli gyrA* over this region. A 261-bp section of this sequence is lodged in the EMBL GenBank database (accession number X78977).

both the 347- and 422-bp *gyrA* fragments (generated with both sets of primers) were combined, and the sequence was submitted to GenBank, Bethesda, Md., in June 1994 (Fig. 1). This *S. typhimurium* NCTC 74 (L19) DNA fragment was homologous to nucleotides 113 to 479 (codons 39 to 159) of the *E. coli gyrA* gene (26). The sequence showed 23 nucleotide differences compared with the *E. coli* sequence over this region (94% identity), but the deduced amino acid sequence was identical to that of *E. coli*. The fragment sequenced included the 120-bp region analogous to the quinolone resistance-determining region (QRDR) of *E. coli gyrA* (codons 67 to 106 [32]) and the catalytic site of DNA gyrase, the tyrosine residue at position 122 (Tyr122). There was 89% identity with the *E. coli* gene at the nucleotide level over the QRDR.

The nucleotide sequence of *gyrA* from the pretherapy isolate from patient A (L1) was identical to that determined for *S. typhimurium* NCTC 74; however, in posttherapy isolate L2, a C→T transition was identified at the position equivalent to nucleotide 248 of *E. coli gyrA*, resulting in a Ser83→Phe substitution (Fig. 2); this was also found in the veterinary isolates *S. enteritidis* L34 and L45. All four other veterinary isolates sequenced (*S. virchow* L21, *S. senftenberg* L24, *S. heidelberg* L35, and *S. saint-paul* L90) had a mutation at Asp87, with three isolates (isolates L21, L24, and L35) possessing an A→G mutation at nucleotide 260 of *gyrA* (Asp→Gly) and a G→T mutation at nucleotide 259 in *S. saint-paul* L90 (Asp→Tyr) (Fig. 2). A single mutation at this site in *E. coli gyrA* (Ser83→Trp) was shown to be solely responsible for clinical resistance in an isolate arising during enoxacin therapy (5). Gyrase A complementation experiments with pNJR3-2 ($GyrA^S$) suggested that an alteration in *gyrA* was solely responsible for quinolone resistance in strain L2 (19) and also the veterinary isolates L34, L45, L21, L24, L35, and L90 (9), and although there may be mutations in the gene outside of the region sequenced, the Ser83→Phe substitution is probably associated with quinolone resistance in this isolate. It is of interest that the same Ser83→Phe substitution arose independently in clinical and veterinary situations and indicates that such a mutation is important in conferring quinolone resistance in salmonellae. The substitution of Ser83→Phe is similar to the Ser83→Leu or Trp mutations identified in *E. coli gyrA* at the equivalent codon, in that a small polar amino acid is replaced with a larger hydrophobic residue (5, 16), which is not unexpected, given the genetic similarity between the two species. The substitution of Ser83 with a hydrophobic amino acid has been shown to reduce the level of binding of fluoroquinolones to the gyrase-DNA complex (31). The loss of the hydroxyl group of serine and the consequent loss of the ability to form hydrogen bonds following the replacement of Ser83 with a hydrophobic residue may result in a reduced level of fluoroquinolone binding (31, 33). Both of the veterinary strains shown to have Ser83→Phe substitutions were *S. enteritidis*, the serotype most commonly implicated in human salmonellosis (1). They were isolated from the same flock of chickens at a farm where enrofloxacin was known to be in use, although the two strains were of different phage types (15a).

Four veterinary isolates had substitutions at Asp87; in three, Asp87→Gly was found, resulting in the exchange of a negatively charged residue with a small polar molecule, and *S. saint-paul* L90 had an Asp87→Tyr substitution, substituting the negatively charged residue with a larger polar amino acid. Substitutions in *E. coli* of Asp87→Asn, Val, or Gly all lead to the loss of a negative charge, which suggests that the charge at this residue is important in the quinolone-gyrase interaction (11, 16, 32). Similar mutations at analogous sites have been described for *Staphylococcus aureus* (Glu88 [13, 29]) and *Mycobacterium tuberculosis* (Asp94 [27]). Although tyrosine is much larger than glycine, the MICs of quinolones associated with the two different substitutions in salmonellae are the same.

DNA sequencing of *S. typhimurium* L30 (*nalA* mutant) and the 11 posttherapy isolates from patient B revealed no nucleotide differences over the QRDR (codons 67 to 106) when compared with the sequence of the pretherapy isolate (isolate L3) or *S. typhimurium* NCTC 74 (L19) (Fig. 2). However, L30 and five isolates (isolates L4, L5, L11, L13, and L14) did possess a novel nucleotide substitution at the position equivalent to nucleotide 356 of *E. coli gyrA*: L30 had a substitution of C→T resulting in the predicted Ala119→Val substitution, while the C→A substitution in clinical isolates L4, L5, L11, L13, and L14 resulted in a predicted Ala119→Glu substitution. Although this codon is close to Tyr122, no mutation at this site has so far been described in other species. Previous experiments with pNJR3-2 suggested that the presence of a mutation in *gyrA* in L30 and these five isolates from patient B was solely responsible for quinolone resistance (19). It is possible that other mutations are present in the *gyrA* genes of these isolates, outside of the region of the gene sequenced, and that the Ala119 mutation is not involved in quinolone resistance. Further work to determine the role of mutations at this site in quinolone resistance is under way.

Two *Hin*fI restriction sites were located in the salmonella *gyrA* fragment, as shown in Fig. 1. A mutation at the codon analogous to Ser83 of *E. coli gyrA* leads to the loss of one of the *Hin*fI recognition sequences (GANTC [7]), and so mutations at Ser83 could be detected by digestion with *Hin*fI and agarose gel electrophoresis of the *gyrA* fragments. This method may be used to screen large numbers of isolates for a mutation at Ser83. By this method, only one of the posttherapy clinical

	Strain	gyrA 82 83 84 85 86 87 88	119	MIC (μg/ml) NAL	CIP	ENR
		AspSerAlaValTyrAspThr	Ala	NAL	CIP	ENR
L19	*S. typhimurium* NCTC 74	GATTCCGCAGTGTATGACACC	GCA	2	0.008	0.015
L30	*S. typhimurium* (*nalA*)	---------------------	-T-	32	0.03	0.25
			Val			
L53	*S. enteritidis* NCTC 5188	---------------------		8	0.03	0.06
L51	*S. virchow* NCTC 5742	---------------------		8	0.06	0.12
L50	*S. senftenberg* NCTC 3158	---------------------		4	0.03	0.06
L54	*S. heidelberg* NCTC 5171	---------------------		8	0.03	0.06
L94	*S. saint-paul* NCTC 6022	---------------------		4	0.03	0.06
Clinical isolates:						
L1	*S. typhimurium*	---------------------	---	8	0.03	nd
L2	*S. typhimurium*	----T----------------	---	256	2	nd
		Phe				
L3	*S. typhimurium*	---------------------	---	8	0.03	nd
L4	*S. typhimurium*	---------------------	-A-	>256	0.12	nd
			Glu			
L5	*S. typhimurium*	---------------------	-A-	256	0.25	nd
			Glu			
L6	*S. typhimurium*	---------------------	---	64	0.25	nd
L10	*S. typhimurium*	---------------------	---	32	0.06	nd
L11	*S. typhimurium*	---------------------	-A-	256	0.5	nd
			Glu			
L12	*S. typhimurium*	---------------------	---	32	0.25	nd
L13	*S. typhimurium*	---------------------	-A-	32	0.03	nd
			Glu			
L14	*S. typhimurium*	---------------------	-A-	>256	0.25	nd
			Glu			
L15	*S. typhimurium*	---------------------	---	32	0.25	nd
L16	*S. typhimurium*	---------------------	---	32	0.12	nd
L18	*S. typhimurium*	---------------------	---	32	0.5	nd
Veterinary isolates:						
L34	*S. enteritidis*	----T----------------	---	>512	0.5	2
		Phe				
L45	*S. enteritidis*	----T----------------	---	>512	1	2
		Phe				
L21	*S. virchow*	----------------G----	---	512	0.12	0.5
		Gly				
L24	*S. senftenberg*	----------------G----	---	256	0.12	0.25
		Gly				
L35	*S. heidelberg*	----------------G----	---	256	0.25	2
		Gly				
L90	*S. saint-paul*	---------------T-----	---	512	0.25	1
		Tyr				

FIG. 2. Nucleotide sequence and deduced amino acid sequence of *gyrA* from clinical and veterinary isolates of salmonellae. Isolates L1 (pretherapy) and L2 (posttherapy) were obtained from patient A. Isolates L3 (pretherapy) and L4 to L6, L10 to L16 and L18 (posttherapy) were obtained from patient B. When compared with *S. typhimurium* NCTC 74 (L19), nucleotide changes were identified at codons equivalent to codons 83, 87, or 119 of *E. coli gyrA*. The predicted amino acid substitution at these codons for each isolate are given below. The MICs of nalidixic acid, ciprofloxacin, and enrofloxacin for each isolate are given alongside the sequence (NAL, nalidixic acid; CIP, ciprofloxacin; ENR, enrofloxacin; nd, not done).

isolates, isolate L2 from patient A, appeared to possess a mutation at Ser83; this mutation was verified by DNA sequencing. In contrast, 82 of 98 veterinary isolates appeared to possess a mutation at Ser83, demonstrating that mutations at this codon are common in nalidixic acid-resistant salmonellae. Of the 82 isolates, 68 were *S. newport*, 3 were *S. typhimurium* (isolates L22, L89, and L284), 1 was *S. bredeney* (isolate L25), 8 were *S. enteritidis* (isolates L34, L45, and L175 to L180), and 2 were *S. heidelberg* (isolates L181 and L182). Two of the isolates (*S. enteritidis* L34 and L45) had the Ser83→Phe substitution, as determined by DNA sequencing. The DNA sequence was not determined for any *S. newport* strains. Of the 16 veterinary isolates which did not possess a Ser83 mutation (*S. oranienberg* L20, *S. virchow* L21, L185, and L186, *S. senftenberg* L24 and L39, *S. heidelberg* L35 and L182, *S. haardt* L37, *S. saint-paul* L90, *S. typhimurium* L91 and L285 to L287, and *S. enteritidis* L173 and L174), 4 were found to have a substitution at Asp87→Gly (L21, L24, and L35) or Tyr (L90). Clearly, the limitation of this restriction fragment length polymorphism screen is that it will only identify a mutation at one site, and so the technique of single-stranded conformational polymorphism is now routinely used in this laboratory.

Although it has not been shown that quinolone resistance in salmonellae is caused solely by the observed mutations in *gyrA*, the identity between the *gyrA* sequences of *E. coli* and salmonellae over the QRDR of this gene and the similar substitutions observed in resistant isolates strongly suggest that the mechanisms of quinolone resistance for the two species are identical. The present study has shown that the same substitution at Ser83 of *gyrA* can arise independently in salmonellae of human and animal origin and suggests that this substitution may be important in conferring quinolone resistance in salmonellae, regardless of their source. Recently, the Ser83→Phe substitution has been reported for a laboratory mutant (24), but no biochemical tests were performed to confirm that this mutation conferred quinolone resistance. The data from the present study and those of Reyna et al. (24) suggest that, like *E. coli*, Ser83 is the most important site for fluoroquinolone resistance and is the mutation found most frequently.

It is interesting that although only 15 of 98 of the veterinary isolates were thought to have originated on farms where enrofloxacin was in use (15a), the restriction fragment length polymorphism analysis data suggested that most isolates had a substitution at Ser83. It is not possible to establish how quinolone resistance arose in isolates from those poultry flocks which were not thought to have been treated with quinolones, although it has been suggested that resistant bacteria were transferred from a contaminated flock which had been exposed to quinolones.

The emergence of quinolone-resistant isolates of salmonellae from poultry since 1988 is a potential public health risk and may compromise effective antibiotic therapy. Moreover, some of the strains studied were isolated from poultry meat intended for human consumption. However, the numbers of nalidixic acid-resistant salmonellae isolated by the Central Veterinary Laboratory are small compared with the total numbers of isolates of this species examined per annum (approximately 15,000 isolates) and have remained steady at approximately 0.5% of the total number of isolates since 1991 (15a). The risk to humans of illness from such strains is likely to be minimal for the following reasons. (i) The nalidixic acid-resistant salmonella serotypes described in this report are not common in human infections; more than 60% of cases of salmonellosis in humans are caused by *S. enteritidis* (1), while only 7% (10 strains) of the isolates studied were *S. enteritidis*, with most being *S. newport* (78%). (ii) Although all of the veterinary isolates were highly resistant to nalidixic acid, the MICs of ciprofloxacin were below the recommended breakpoint concentration for this agent in most countries for most of the strains. Therefore, uncomplicated infections with such strains could still be treated effectively with ciprofloxacin. However, it is feasible that treatment failure could result if such strains were to cause complicated salmonella infections in humans, because the MICs of ciprofloxacin for the veterinary isolates were similar to those for the clinical isolates of *S. typhimurium* from patient B, who failed ciprofloxacin therapy. It is also possible that under conditions of prolonged fluoroquinolone therapy, further mutations in *gyrA*, *gyrB*, or another locus may arise, resulting in a highly fluoroquinolone-resistant isolate not amenable to fluoroquinolone therapy, such as that described by Heisig (10). It appears that the risk to humans of acquiring nalidixic acid-resistant salmonellae from animals in the United Kingdom is small, but it will be necessary to closely monitor the emergence of quinolone resistance in salmonellae as the use of enrofloxacin in veterinary medicine increases.

Nucleotide sequence accession number. The DNA sequences of both the 347- and 422-bp *gyrA* fragments were combined, and the sequence was submitted to GenBank and was assigned the accession number X78977.

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