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Increasing Prevalence of Quinolone Resistance in Human Nontyphoid *Salmonella enterica* Isolates Obtained in Spain from 1981 to 2003

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From January 1981 to December 2003, susceptibility to nalidixic acid was tested in 10,504 nontyphoid *Salmonella enterica* isolates from patients with acute enteric disease in Gipuzkoa, Spain. The prevalence of nalidixic acid resistance steadily increased from less than 0.5% before 1991 to 38.5% in 2003, mainly due to the increase in resistance among isolates of the most prevalent serovar, *S. enterica* serovar Enteritidis. For nalidixic acid-resistant isolates, the ciprofloxacin MIC was eightfold higher than that for susceptible isolates, and the nalidixic acid-resistant isolates contained a single point mutation in the *gyrA* gene (at codons for Ser83 or Asp87). The same mutations were found in a sample of nalidixic acid-resistant nontyphoid *Salmonella* strains isolated between 1999 and 2003 from retail food for human consumption. In 2003, we identified five *S. enterica* serovar Typhimurium clinical isolates with high-level fluoroquinolone resistance (ciprofloxacin MIC, 16 μg/ml) with two point mutations in the *gyrA* gene (coding for Ser83→Phe and Asp87→Asn) and one point mutation in the *parC* gene (coding for Ser80→Arg). Strict sanitary controls are needed to avoid the spread of ciprofloxacin-resistant serovar Typhimurium isolates, and a more efficient veterinary policy must be adopted to decrease the large burden of *Salmonella* serovar Enteritidis infections in humans in our region.

Antibiotic resistance in zoonotic bacteria is a worldwide problem involving both animal and human isolates. In the last few years in Spain (21, 23) and other European countries (2, 8, 14, 25), as well as in the United States of America (10), the number of nontyphoid *Salmonella* isolates resistant to nalidixic acid has increased with a concomitant decrease in the level of susceptibility to ciprofloxacin and even occasional resistance to that drug. A strong relationship between specific *Salmonella* serovars and phage types and quinolone resistance has been observed. The types most frequently described as resistant to quinolones (8, 21) are *S. enterica* serovars Hadar, Virchow, and Enteritidis and serovar Typhimurium definitive phage type 104 (DT104). Nowadays, fluoroquinolones are routinely used for the treatment of invasive gastrointestinal infections in adults, and nontyphoid *Salmonella* is one of the main causes of bacterial gastroenteritis in Spain (21).

The present study analyzes the changes in the prevalence of quinolone susceptibility in nontyphoid *Salmonella* clinical isolates obtained from 1981 to 2003 and the mutations in the quinolone resistance-determining region (QRDR) of nalidixic acid-resistant isolates. A sample of isolates from retail food for human consumption obtained between 1999 and 2003 was also analyzed. Finally, the mechanisms of resistance of the first fluoroquinolone-resistant nontyphoid *Salmonella* clinical isolates in Gipuzkoa, Spain, were investigated.

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MATERIALS AND METHODS

Isolate collection and isolation. Quinolone resistance in clinical isolates of nontyphoid *S. enterica* obtained between January 1981 and December 2003 in patients from a region of northern Spain was studied at the Microbiology Department of the Hospital Donostia, San Sebastián, Gipuzkoa, Spain, which covers a currently estimated population of 395,000 inhabitants. Only the first isolate from each episode of *Salmonella* gastroenteritis from inpatients and outpatients who sought medical care for acute gastroenteritis was included in the study. Therefore, all *Salmonella* isolates from control stool cultures of the same patient showing the same serovar and antibiotic susceptibility pattern as the first isolate were excluded. The study population was divided into children (≤14 years old) and adults (>14 years old).

Stool cultures were obtained by standard selective and enrichment culture techniques (24). All *Salmonella*-like colonies were identified to the genus level by their biochemical characteristics by use of the commercially available API 20 E identification system (BioMerieux, Marcy-l'Etoile, France) in accordance with the manufacturer's instructions. *Salmonella* serovars were established by slide agglutination tests using both polyvalent and specific rabbit sera (Pasteur Diagnostics, Paris, France) against somatic (O) and flagellar (H) antigens according to the Kauffmann-White scheme (20). Phage typing of ciprofloxacin-resistant *Salmonella* serovar Typhimurium isolates was performed at the Enterobacteria Service, National Center for Microbiology, Instituto Carlos III, Majadahonda, Madrid, Spain.

The nalidixic acid resistance of 164 nontyphoid *Salmonella* isolates obtained from a food source between 1999 and 2003 was also studied. All these isolates were obtained from retail food products from our region at the Public Health Laboratory of the Health Department, also located in the city of Donostia-San Sebastián. In 1999, 24 nontyphoid *Salmonella* isolates were collected from a sample of 65 uncooked chickens bought from retail outlets. Between January 2000 and May 2001 and during the first half of 2003, another 140 nontyphoid *Salmonella* isolates were obtained from poultry products used for human consumption (chicken meat, eggs, or egg-derived products).

Antibiotic susceptibility testing. Nalidixic acid resistance was routinely studied for all isolates by using the disk diffusion method with Mueller-Hinton agar in accordance with the NCCLS guidelines (16); this method was also used as a screening test of quinolone resistance. Records of the resistance results were retrospectively obtained for 8,802 of the 10,504 nontyphoid *Salmonella* human isolates and for all food isolates. Between 1981 and 1991, the resistance of 3,049 (67.3%) of the 4,528 isolates was recorded, and between 1992 and 2003, the resistance of 5,753 (96.3%) of the 5,976 isolates was recorded (Table 1).

TABLE 1. Number and percentage of nalidixic acid-resistant nontyphoid *Salmonella* isolates from human stools, Gipuzkoa, Spain, 1981 to 2003

Yr	Serovar Enteritidis			Serovar Typhimurium			Other serovars			Total		
	R[a]	Tested[b]	%R[c]	R	Tested	%R	R	Tested	%R	R	Tested	%R
1981–1991	7	2,530	0.3	0	303	0	2	216	0.9	9	3,049	0.3
1992	1	265	0.4	2	51	3.9	0	15	0.0	3	331	0.9
1993	1	285	0.4	1	45	2.2	2	21	9.5	4	351	1.1
1994	2	161	1.2	0	39	0	7	34	20.6	9	234	3.8
1995	6	211	2.8	2	29	6.9	8	25	32.0	16	265	6.0
1996	23	223	10.3	2	56	3.6	4	35	11.4	29	314	9.2
1997	47	249	18.9	1	64	1.6	8	33	24.2	56	346	16.2
1998	59	413	14.3	0	69	0	12	34	35.3	71	516	13.8
1999	94	320	29.4	2	60	3.3	11	31	35.5	107	411	26.0
2000	129	344	37.5	7	64	10.9	3	18	16.7	139	426	32.6
2001	388	785	49.4	1	53	1.9	8	22	36.4	397	860	46.2
2002	267	799	33.4	6	60	10.0	10	32	31.3	283	891	31.8
2003	297	705	42.1	5	71	7.0	9	32	28.1	311	808	38.5
Total	1,314	7,290	18.1	29	964	3.0	84	548	15.3	1,434	8,802	16.3

[a] R, number of nalidixic acid-resistant isolates.
[b] Tested, number of isolates tested.
[c] %R, percent of nalidixic acid-resistant isolates.

MICs of nalidixic acid and other quinolones were determined for arbitrarily selected isolates by the agar dilution method according to the NCCLS guidelines (16). The antibiotics tested were obtained as pure powder for nalidixic acid (Sigma, Madrid, Spain), norfloxacin (Sigma), and ciprofloxacin (Bayer, Leverkusen, Germany). The enrofloxacin used was a commercial product (Baytril; Bayer) (100 mg/ml). To assess the enrofloxacin MICs obtained, the enrofloxacin MICs for 15 arbitrarily selected isolates were also determined by using Etest strips (AB Biodisk, Solna, Sweden) in accordance with the manufacturer's instructions. The MIC ranges evaluated were 2 to 1,024 μg/ml for nalidixic acid and 0.008 to 16 μg/ml for norfloxacin, enrofloxacin, and ciprofloxacin. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were included as controls. Nalidixic acid-resistant isolates that produced a zone diameter of ≥21 mm with a 5-μg ciprofloxacin disk and/or for which the ciprofloxacin MIC was ≤1 μg/ml are referred to in the text as ciprofloxacin susceptible, although these strains may be associated with clinical failure in patients with extraintestinal salmonellosis who were treated with ciprofloxacin.

To rule out the presence of an active efflux mechanism of quinolone resistance in ciprofloxacin-resistant *Salmonella* serovar Typhimurium isolates, the MICs of nalidixic acid and ciprofloxacin were also determined by broth microdilution according to the NCCLS guidelines (16) in combination with final concentrations of 0.5 and 1 μg of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) per ml (Sigma).

Molecular characterization of gyrases and topoisomerases. An assay combining allele-specific PCR and restriction fragment length polymorphism (AS-PCR-RFLP) was performed to detect mutations at codons 81, 83, and 87 of the *gyrA* gene (5). The sequences of the QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes were determined after amplification of fragments with the primers and using conditions previously described (5). The PCR products were sequenced in an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.), and the sequences were analyzed using the BLAST 2.0 (Basic Local Alignment Search Tool) software available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Human clinical nontyphoid *Salmonella* isolates. From January 1981 to December 2003, nontyphoid *Salmonella* was isolated in the stools of 10,504 patients with acute enteric infections. Of the patients with *Salmonella* enteric infection episodes, 53% (5,564) were children and 47% (4,940) were adults. Although the incidence varied over the years, in each year the serovar most frequently isolated was serovar Enteritidis, accounting for 81.9% (8,598 of 10,504) of all human nontyphoid *Salmonella* stool isolates. The other most frequently isolated serovars were serovar Typhimurium, serovar Infantis, serovar Hadar, serovar Newport, and serovar Ohio, which were responsible for 11.4% (1,198 episodes), 1.8% (190), 0.8% (88), 0.4% (45), and 0.4% (42) of all the *Salmonella* gastroenteric episodes, respectively.

Nalidixic acid resistance in human nontyphoid *Salmonella* isolates. Between 1981 and 1991, resistance to nalidixic acid was nearly null. The first nalidixic acid-resistant isolate appeared in 1984 in a 6-year-old boy, who had not received previous treatment, attending the emergency service of the hospital for acute gastroenteritis with a *Salmonella* serovar Enteritidis stool isolate. Only 9 of the 3,049 (0.3%) *Salmonella* isolates studied in this period showed resistance to nalidixic acid (Table 1).

Between 1992 and 2003, 1,425 (24.8%) of the 5,753 isolates tested were resistant to nalidixic acid. The prevalence of resistance in this period was 21.8% (705 of 3,228 isolates) in children and 28.5% (720 of 2,525 isolates) in adults.

Until 1993, the annual prevalence of nalidixic acid resistance was below 1.2%, but in 1994 this prevalence rose to 3.8%. From 1994, the prevalence of nalidixic acid resistance of *Salmonella* isolates from patients with gastroenteritis steadily increased, peaking at 46.2% in 2001 and reaching 38.5% in 2003. The overall prevalence of nalidixic acid resistance in serovar Enteritidis isolates, the most frequently isolated serovar, was 18.1% (1,321 of 7,290 isolates). Before 1994, the prevalence of nalidixic acid-resistant serovar Enteritidis isolates was below 1%, but it increased from 1.2% in 1994 to 10.3% in 1996. The prevalence of nalidixic acid-resistant serovar Enteritidis continued to increase, reaching 42.1% in 2003 (Table 1).

The overall resistance to nalidixic acid in serovar Typhimurium isolates was 3.0% (29 of 964 isolates). The annual percentages of resistant isolates of this serovar fluctuated between 0 and 10.9% throughout the study. The prevalence of nalidixic acid resistance in other frequent serovars was 6.2% (8 resistant isolates of 130 tested) for serovar Infantis, 65.8% (50 of 76) for serovar Hadar, 28.9% (11 of 38) for serovar New-

TABLE 2. Quinolone MICs for nalidixic acid-susceptible, nalidixic acid-resistant, and ciprofloxacin-resistant *Salmonella* isolates obtained by the agar dilution method

Antibiotic	MIC (μg/ml) for:						
	Nalidixic acid-susceptible isolates (n = 62)			Nalidixic acid-resistant isolates (n = 124)			Ciprofloxacin-resistant isolates[a] (n = 5)
	50%	90%	Range	50%	90%	Range	
Nalidixic acid	4	8	4–8	512	>1,024	128–>1,024	>1,024
Norfloxacin	0.12	0.12	0.12	1	2	0.25–4	>16
Enrofloxacin	0.06	0.06	0.06	0.5	1	0.25–2	>32[b]
Ciprofloxacin	0.03	0.06	0.03–0.06	0.25	0.5	0.25–2.0	16

[a] The five isolates were *Salmonella* serovar Typhimurium DT104.
[b] Determined by Etest.

port, 0% (0 of 35) for serovar Ohio, and 5.9% (1 of 17) for serovar Virchow.

The *Salmonella* serovar with the highest percentage of nalidixic acid resistance observed in our region was serovar Hadar. The first nalidixic acid-resistant serovar Hadar isolate was obtained in 1993 from a 9-month-old girl; since then, 75.8% (50 of 66) of the serovar Hadar isolates were resistant to nalidixic acid.

One hundred twenty-four nontyphoid *Salmonella* isolates identified as nalidixic acid resistant by the disk diffusion method were retested by the agar dilution method. The nalidixic acid MIC for all of these isolates was ≥128 μg/ml, at least 64-fold higher than the MIC of nalidixic acid-susceptible isolates (Table 2). The norfloxacin, enrofloxacin, and ciprofloxacin MICs for nalidixic acid-resistant isolates were at least eightfold higher than those of nalidixic acid-susceptible isolates. Enrofloxacin MICs obtained by Etest were twice as high as MICs obtained by agar dilution.

These 124 nalidixic acid-resistant human nontyphoid *Salmonella* isolates with decreased susceptibility to ciprofloxacin (ciprofloxacin MIC range, 0.25 to 2 μg/ml) were studied by AS-PCR-RFLP. All of these isolates had one point mutation in the QRDR of the *gyrA* gene. A mutation in the codon for Asp87 was found in 108 of 124 isolates studied (83 of 86 serovar Enteritidis isolates, 20 of 20 serovar Hadar isolates, 3 of 5 serovar Newport isolates, 1 of 2 serovar Typhimurium isolates, 1 of 6 nontypeable *Salmonella* isolates, 0 of 4 serovar Infantis isolates, and 0 of 1 serovar Virchow isolate). A mutation in the codon for Ser83 was found in the 16 remaining nalidixic acid-resistant isolates studied (5 of 6 nontypeable *Salmonella* isolates, 3 of 86 serovar Enteritidis isolates, 4 of 4 serovar Infantis isolates, 2 of 5 serovar Newport isolates, 1 of 2 serovar Typhimurium isolates, and 1 of 1 serovar Virchow isolate).

With the AS-PCR-RFLP assay, no mutations were found among 62 nalidixic acid-susceptible isolates studied (49 serovar Enteritidis isolates, 5 serovar Typhimurium isolates, 4 serovar Hadar isolates, and 1 isolate each of serovar Infantis, serovar Newport, serovar Ohio, and nontypeable *Salmonella*).

To confirm the AS-PCR-RFLP assay results, the QRDRs of the *gyrA* genes of nine nalidixic acid-resistant *Salmonella* isolates were sequenced, five with a mutation in the codon for Asp87 (three serovar Enteritidis isolates, two serovar Hadar isolates, and one serovar Typhimurium isolate) and four with a mutation in the codon for Ser83 (two serovar Enteritidis isolates and one isolate each of serovar Typhimurium and serovar Infantis). As expected, all of these isolates had the corresponding point mutations. As a control, the QRDRs of the *gyrA* genes of two nalidixic acid-susceptible serovar Enteritidis isolates were sequenced, and no mutations were found. The QRDRs of the *parC* genes of these nine nalidixic acid-resistant and two nalidixic acid-susceptible *Salmonella* isolates were sequenced, and no mutations were found.

Human ciprofloxacin-resistant isolates. In March 2003, we identified the first human nontyphoid *Salmonella* isolates with a high level of ciprofloxacin resistance (MIC, 16 μg/ml) in our region. They were isolated from three apparently unrelated outpatients, although all of them were children (1, 3, and 6 years old) with a *Salmonella* serovar Typhimurium isolate in the stool culture. None of these children had received quinolone, and there was no record of antibiotic treatment in the previous year.

In May 2003, two more ciprofloxacin-resistant serovar Typhimurium isolates were identified in two adult inpatients admitted to different rooms in the oncology unit of our hospital due to tumor complications.

The first isolate was obtained on 8 May 2003 in the blood and stool cultures of a 49-year-old man with septic shock caused by serovar Typhimurium. The isolates were obtained 28 days after admission, and the patient had received 2 days of ciprofloxacin therapy at the time of isolation. The second isolate was obtained on 22 May 2003 in the stool culture of a 47-year-old woman, 24 days after admission. At the time of isolation, this patient had received levofloxacin treatment for 6 days due to a respiratory infection.

These five ciprofloxacin-resistant serovar Typhimurium isolates were additionally resistant to amoxicillin, amoxicillin-clavulanate, tetracycline, chloramphenicol, streptomycin, and sulfonamides but were susceptible to cefotaxime, gentamicin, and trimethoprim. One of the isolates from children was mislaid when frozen; the nalidixic acid and ciprofloxacin MICs for the four remaining isolates were unaffected by the presence of CCCP.

Sequencing of the *gyrA, gyrB, parC* and *parE* genes was performed for the four ciprofloxacin-resistant isolates available. All of these isolates had the same two point mutations in the *gyrA* gene, TCC→TTC (coding for Ser83→Phe) and GAC→AAC (coding for Asp87→Asn), and one point mutation in the *parC* gene, AGC→CGC (coding for Ser80→Arg). No mutations were found in the *gyrB* or *parE* genes of any of the four isolates. These isolates were phage typed as serovar Typhimurium DT104.

Nontyphoid isolates from retail food. Of the 164 nontyphoid *Salmonella* isolates of food origin, 65 (39.6%) were nalidixic acid resistant, but there was no isolate for which the ciprofloxacin MIC was >1 μg/ml. The *gyrA* genes of nine of these resistant isolates were sequenced, and all showed a point mutation; six at the codon for Asp87 (three serovar Hadar isolates, two serovar Enteritidis isolates, and one nontypeable *Salmonella* isolate) and three at the codon for Ser83 (one isolate each of *S. enterica* serovar Anatum and serovar Enteritidis and one nontypeable *Salmonella* isolate).

DISCUSSION

Increasing nalidixic acid resistance in nontyphoid *Salmonella* is a problem which affects several countries, including Spain, and which mainly began in the mid-1990s (2, 8, 21, 23, 25). In the present study, resistance to nalidixic acid steadily increased from 1991, with the highest percentages of resistance observed from 2000, affecting 38.5% of all *Salmonella* stool isolates in 2003. This percentage of resistance is more than twice that in other surrounding European countries (26). The similar percentages of resistance reported by other authors in other regions of Spain and the figures of resistance reported for travelers returning from Spain (14) prove that this problem is affecting the entire country (3, 17, 21).

Nalidixic acid resistance in *Salmonella* serovar Enteritidis was more frequent than that in *Salmonella* serovar Typhimurium (18.1% versus 3.0%), as reported in other studies of human cases performed in Spain (3, 17) and in an international multicenter surveillance study in Europe (26). In Spain, the high proportion of *Salmonella* serovar Enteritidis isolates (12) and the high prevalence of nalidixic acid resistance in this specific serovar are the causes of the overall high prevalence of nalidixic acid resistance in nontyphoid *Salmonella*. This leads us to think that, apparently, a clone or several clones of nalidixic acid-resistant *Salmonella* serovar Enteritidis isolates appeared in our region in the mid-1990s and that far from disappearing, they continue to spread. Other authors have confirmed the dissemination of a single clone of *Salmonella* serovar Enteritidis from human and animal sources throughout Europe (11).

Some findings suggest a link between resistance to nalidixic acid in human nontyphoid *Salmonella* isolates and the food chain. In Spain, enrofloxacin was authorized for veterinary use in 1990. Although the levels and spread of quinolone resistance among nontyphoid *Salmonella* are lower than those of other enteric pathogens such as *Campylobacter* (18), the prevalence of nalidixic acid resistance has steadily increased while susceptibility to fluoroquinolones in *Salmonella* isolates has decreased since that date. Half of the nalidixic acid-resistant nontyphoid *Salmonella* isolates in our study were obtained from children, a group of patients for which quinolones are not recommended and therefore are not routinely used in our country, suggesting that nalidixic acid resistance was present in these isolates before disease onset. Finally, the same point mutations were found in the QRDRs of the *gyrA* genes in isolates from human and retail food sources.

Only five isolates with resistance to ciprofloxacin according to current NCCLS breakpoints (MIC ≥ 4 μg/ml) were found, although if we had considered the limit of ≥0.125 μg/ml as indicating resistance (1), all nalidixic acid-resistant isolates would also have been ciprofloxacin resistant.

Salmonella resistance to quinolones is usually a consequence of a single point mutation in the QRDR of the *gyrA* gene (nucleotides 67 to 122), which encodes the A subunit of DNA gyrase (5). Point mutations in this gene, especially in the codons for Gly81, Ser83, or Asp87, have been described previously (5, 6). Mutations in the *gyrB* gene (coding for Ser464) and *parC* gene (coding for Gly78 and Ser80) and evidence of active efflux as well as decreased permeability of the outer membrane to these antibiotic agents have also been described for mutants with high-level fluoroquinolone resistance (4, 6, 7, 9, 15).

In the present study, all nalidixic acid-resistant isolates showed decreased susceptibility to ciprofloxacin (ciprofloxacin MIC range, 0.25 to 2 μg/ml) and had a unique point mutation in the *gyrA* gene. Only five isolates with a high level of ciprofloxacin resistance were found (ciprofloxacin MIC, 16 μg/ml). They belonged to the serovar Typhimurium phage type DT104 and appeared in 2003. These isolates had two mutations in the *gyrA* gene and one mutation in the *parC* gene; these mutations are known to confer a high level of fluoroquinolone resistance (9). No mutations were found in the *gyrB* and *parE* genes, and there was no evidence of an active efflux mechanism of resistance. Therefore, mutations in *gyrA* conferred low-level ciprofloxacin resistance, while the addition of another *gyrA* mutation together with a *parC* mutation increased this resistance to a high level.

Nontyphoid-*Salmonella* symptomatic infections usually cause self-limiting enteric disease not requiring antibiotic treatment unless other factors such as age (newborn or elderly), suspected bacteremia or extraintestinal infection, or immunosuppression are present (13). Fluoroquinolones are one of the best choices for treating these kinds of infections in adults (13, 22). However, treatment failures related to reduced ciprofloxacin susceptibility that developed during quinolone treatment have been described (19, 27). These treatment failures and the finding of mutations in the *gyrA* gene of all the nalidixic acid-resistant isolates studied suggest that a reduction in fluoroquinolone MIC breakpoints for *Salmonella* should be advised (1).

Continuous monitoring of antimicrobial resistance of zoonotic enteric bacteria at the animal, food, and human levels will help to identify changes in the antimicrobial resistance profile of zoonotic bacteria, which would enable informed decision-making on the use of antimicrobials to maintain their efficacy as therapeutic agents for the future. The development of *Salmonella* serovar Typhimurium with *gyrA* and *parC* mutations which confer a high level of fluoroquinolone resistance and the high prevalence of nalidixic acid-resistant *Salmonella* serovar Enteritidis, the most common of all *Salmonella* serovars in Spain, are a serious concern requiring an integrated response from both the medical and veterinary professions.

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