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Mechanism of Resistance to Several Antimicrobial Agents in *Salmonella* Clinical Isolates Causing Traveler's Diarrhea

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The evolution of antimicrobial resistance in *Salmonella* isolates causing traveler's diarrhea (TD) and their mechanisms of resistance to several antimicrobial agents were analyzed. From 1995 to 2002, a total of 62 *Salmonella* strains were isolated from stools of patients with TD. The antimicrobial susceptibility to 12 antibiotics was determined, and the molecular mechanisms of resistance to several of them were detected as well. The highest levels of resistance were found against tetracycline and ampicillin (21 and 19%, respectively), followed by resistance to nalidixic acid (16%), which was mainly detected from 2000 onward. Molecular mechanisms of resistance were analyzed in 16 isolates. In these isolates, which were resistant to ampicillin, two genes encoding β-lactamases were detected: *oxa-1* (one isolate) and *tem*-like (seven isolates [in one strain concomitantly with a *carb-2*]). Resistance to tetracycline was mainly related to *tetA* (five cases) and to *tetB* and *tetG* (one case each). Resistance to chloramphenicol was related to the presence of the *floR* and *cmlA* genes and to chloramphenicol acetyltransferase activity in one case each. Different genes encoding dihydrofolate-reductases (*dfrA1*, *dfrA12*, *dfrA14*, and *dfrA17*) were detected in trimethoprim-resistant isolates. Resistance to nalidixic acid was related to the presence of mutations in the amino acid codons 83 or 87 of the *gyrA* gene. Further surveillance of the *Salmonella* spp. causing TD is needed to detect trends in their resistance to antimicrobial agents, as we have shown in our study with nalidixic acid. Moreover, such studies will lead to better treatment and strategies to prevent and limit their spread.

Traveler's diarrhea (TD) is the most frequent infection acquired by travelers to developing countries, affecting 20 to 50% of the 35 million travelers from industrialized countries each year. The most important risk factors are the destination of the traveler, host-associated factors, and exposure to contaminated food and water (19). Farm animals often carry salmonellas, affecting meat, dairy products and eggs. Thus, salmonella is one of the main etiological agents causing TD (7, 19, 36).

Salmonella usually produces a self-limited illness, although the duration or the severity of the symptoms may require antibiotic treatment. *Salmonella* spp. display high natural susceptibility levels to the most commonly used antibacterial agents (34). However, the occurrences of isolated *Salmonella* strains showing resistance to one or more antibacterial agent have steadily increased, probably due to continuous antibiotic pressure (3, 6, 18, 27, 35). This is an important public health problem that may be related to therapeutic failure (43).

This problem is especially relevant in developing areas, where the lack of economic resources does not allow a wide antibacterial armamentarium. Moreover, in some of these areas, both the social situation and the presence of other basal illnesses, such as malaria, favor the acquisition of systemic *Salmonella* infections (9). To date, only a few studies have extensively analyzed the levels of resistance to antimicrobial agents in *Salmonella* spp. isolated in developing areas (9, 15, 16, 21, 31). Moreover, none of them have analyzed in depth the mechanisms of resistance underlying the resistant phenotypes.

Analysis of bacterial infections in international travelers is an indirect source of information about these developing countries, providing information on both the characteristics of these particular infections and the current situation in some less-developed areas.

Thus, resistance to some antibiotics, such as β-lactam, tetracycline, chloramphenicol, or trimethoprim-sulfamethoxazole is being reported with increasing frequency (3, 12). Moreover, the development of quinolone resistance has been described not only in some clones of the widespread *Salmonella enterica* serovar Typhimurium definitive phage type 104 (8) but also in some *Salmonella* strains isolated from travelers returning from India and other areas due to the introduction of nalidixic acid for the treatment of some infections (4, 12).

The aim of our study was to analyze the evolution of antimicrobial resistance in *Salmonella* isolates causing TD and to characterize the mechanisms of resistance to several antimicrobial agents.

MATERIALS AND METHODS

Bacterial isolates. From 1995 to 2002, a total of 2,216 travelers with TD presented at the Tropical Medicine Unit; feces samples were processed at the laboratory of clinical microbiology of the Hospital Clinic, Barcelona, Spain. In all cases, diarrhea commenced during the trip or no more than 3 days after return. Diarrheagenic pathogens were isolated and identified by conventional methods (20). All patients filled out a clinical and epidemiological protocol form.

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TABLE 1. Sequences of primers for amplification

Antibiotic	Gene	Primer sequence	Amplicon size (bp)	Annealing temp (°C)	Source or reference
Nalidixic acid	*gyrA*	5′-AAATCTGCCCGTGTCGTTGGT-3′ 5′-GCCATACCTACG GCGATACC-3′	343	55	30
Tetracycline	*tetA*	5′-GATATTCTGAGCACTGTCGC-3′ 5′-CTGCCTGGACAACATTGCTT-3′	950	55	10
	tetB	5′-TTGGTTAGGGGCAAGTTTTG-3′ 5′-GTAATGGGCCAATAACACCG-3′	600	55	26
	tetG	5′-GCTCGGTGGTATCTCTGC-3′ 5′-AGCAACAGAATCGGGAAC-3′	500	55	26
Trimethoprim	*dfrA1*	5′-GTGAAACTATCACTAATGG-3′ 5′-TTAACCCTTTTGCCAGATTT-3′	474	55	25
	dfrA14	5′-GAGCAGCTICTITTIAAAGC-3′ 5′-TTAGCCCTTTIICCAATTTT-3′	393	60	25
	dfrA12	5′-GGTGSGCAGAAGATTTTTCGC-3′ 5′-TGGGAAGAAGGCGTCACCCTC-3′	319	60	25
	dfrA7	5′-TTGAAAATTTCATTGATTG-3′ 5′-TTAGCCTTTTTTCCAAATCT-3′	474	55	25
	dfrB	5′-GATCACGTGCGCAAGAAATC-3′ 5′-AAGCGCAGCCACAGGATAAAT-3′	141	60	25
Chloramphenicol	*floR*	5′-CACGTTGAGCCTCTATAT-3′ 5′-ATGCAGAAGTAGAACGCG-3′	868	55	26
	cmlA	5′-TGTCATTTACGGCATACTCG-3′ 5′-ATCAGGCATCCCATTCCCAT-3′	435	55	This study
Ampicillin	*tem*	5′-TTGGGTGCACGAGTGGGTTA-3′ 5′-GACAGTTACCAATGCTTAATCA-3′	503	55	6
	carb	5′-AATGGCAATCAGCGCTTCCC-3′ 5′-GGGGCTTGATGCTCACTCCA-3′	586	55	6
	oxa-1-like	5′-ACCAGATTCAACTTTCAA-3′ 5′-TCTTGGCTTTTATGCTTG-3′	598	55	6
	shv	5′-ATGCGTTATATTCGCCTGTG-3′ 5′-TTAGCGTTGCCAGTGCTCG-3′	841	55	This study

Serotyping and phage typing. Serotyping was performed with somatic and flagella antiserum. The flagellum phase was determined by the inversion of phase method as described by Kauffman and White and according to the recommendations of Edward and Ewing (20). We also determined the phage types of *Salmonella enterica* serovars Enteritidis, Typhimurium, Hadar, and Virchow (20).

Antimicrobial susceptibility testing. The antimicrobial susceptibility test to 12 antimicrobial agents: ampicillin, amoxicillin-clavulanic acid, nalidixic acid, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, gentamicin, amikacin, imipenem, norfloxacin, ciprofloxacin, and ceftazidime were performed by using the Kirby-Bauer method (2). Interpretation of results was performed according to NCCLS guidelines.

The MICs of ampicillin, chloramphenicol, nalidixic acid, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, and amoxicillin-clavulanic acid to the strains showing resistance was also determined by the agar dilution method according to NCCLS guidelines. *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls (23).

Detection of the mechanism of resistance. To determine the quinolone resistance mechanisms, mutations in the *gyrA* and *parC* genes were detected by PCR with the primers and conditions presented in Table 1. The presence of the *cmlA* and *floR* genes associated with chloramphenicol resistance was determined by PCR (Table 1) and electrophoresis in 2% agarose gels. A previously described colorimetric assay was performed to determine the presence of chloramphenicol acetyltransferase (CAT) activity (6, 11). The detection of genes encoding β-lactamases (*tem*-like, *carb*-like, *shv*-like, and *oxa-1*-like (24) was carried out by PCR (Table 1). The PCR products were detected by electrophoresis in 2% agarose gels. To detect the mechanism of tetracycline resistance, the presence of *tetA*, *tetB*, and *tetG* genes was determined by PCR and electrophoresis in 2% agarose gels (Table 1) (10, 11, 26). To determine the mechanism of trimethoprim resistance, the presence of dihydrofolate reductases was detected by PCR with generic primers (Table 1), and posterior restriction fragment length polymorphism with the appropriate restriction enzyme as described previously (25).

DNA sequencing of the PCR products. The purified PCR products visualized in gels were processed for DNA sequencing and analyzed in an automatic DNA sequencer (ABI 377; Perkin-Elmer, Emeryville, Calif.) by using the BigDye terminator cycle sequencing kit (v3.1; Perkin-Elmer).

RESULTS AND DISCUSSION

From 1995 to 2002, 2,216 patients with TD were treated in the Tropical Medicine Unit of our hospital. *Salmonella* spp. were identified in 62 patients (3.8%) in as described in previous studies developed for Spanish travelers (37), showing that the relevance of this pathogen remains unaltered as a cause of TD. The distribution of the *Salmonella* spp. clinical isolates according to serovar and geographical origin is shown in Table 2. Overall, 20 different serovars were identified, with serovar Enteritidis being the most prevalent, followed by serovar Typhimurium. Similar results were reported by Hakanen et al. (12) for *Salmonella* spp. isolated from travelers to Southeast Asia. The remaining isolates belonged to a wide variety of serovars (Table 2).

According to the geographical origins, 16 isolates (25.8%) were from Western Africa and 8 (12.9%) were from the Indian subcontinent. Meanwhile, other geographical areas showed <10% of the total isolates (Table 2), and six isolates were of

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TABLE 2. Distribution of *Salmonella* clinical isolates according to serovar and geographical origin

No. of strains	Serovar	Phage type	Country(ies) visited (*n*)[b]
4	Enteritidis	4	Senegal, Ecuador, Thailand, ND
10	Enteritidis	1	Kenya, Ecuador, Australia, India, Mexico/Guatemala, Peru, Morocco, ND (3)
1	Enteritidis	20	Tanzania
4	Enteritidis		Turkey, Nepal, Dominican Republic, SEA
5	Typhimurium		Maldivas, Mali, Cuba, India/Nepal, Gambia
1	Typhimurium	104B	Ivory Coast
1	Braenderup		Nicaragua
1	Tenesse		Egypt
1	Newport		Mexico/El Salvador
1	London		ND
1	Vinohrady		Mali
1	Paratyphi A		India
1	Carno		Ivory Coast
1	Typhi		Philippines
1	Kiambu		Mali/Burkina-Faso
1	Muenchen		Cameroon
1	Hadar	NRP[a]	Bolivia
1	Agona		India
1	Infantis		Equatorial Guinea
2	Kentucky		Mali/Burkina-Faso, Senegal
1	Cholerasuis		Mexico
1	Haifa		Egypt
1	Wangata		Equatorial Guinea
1	Lexington		Uganda
1	Montevideo		Senegal
1	Goldcoast		Senegal
1	Risseu		Mali/Burkina-Faso
1	Virchow	31	India
14	*Salmonella* spp.		India (3), Mali (2), SEA, Morocco, South Africa, Peru, Cuba, Vietnam, Nepal, Indonesia (2)

[a] NPR, nonrecognized pattern.
[b] ND, not determined; SEA, Southeast Asia. *n* = number of isolates.

unknown origin. No relationship was observed between the serotypes found and the geographical origin of the samples.

In some of these patients other microorganisms, such as *Shigella* sp. (one case), *Campylobacter* spp. (two cases), *Aeromonas* sp. (one case), diarrheagenic *Escherichia coli* (four cases), and *Giardia lamblia* (one case) were isolated.

Twenty-eight strains showed resistance to at least one of the antibacterial agents analyzed. Seven of these strains showed resistance to three or more unrelated antibacterial agents being considered as multiresistant strains. The antimicrobial resistance observed mainly affected five antibacterial agents: ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, and nalidixic acid. Similar results have been observed in analyses of clinical isolates in developing areas (9, 16, 21, 31, 39). The highest levels of resistance, 21 and 19%, were found against tetracycline and ampicillin, respectively, followed by nalidixic acid (16%). Resistance to the other antimicrobial agents tested was <10% (Table 3). The incidence of nalidixic acid-resistant *Salmonella* isolates causing TD has been steadily rising since 2000 (Fig. 1) and has mainly been observed in isolates belonging to serotype Enteritidis. The wide use of quinolones such as nalidixic acid and ciprofloxacin for the treatment of infections in regions such as India and Central America has been correlated with the increase in resistance to these antimicrobial agents (4, 39, 40). In our study, two nalidixic acid-resistant strains were isolated from feces of travelers to India, and two strains were from Central America. The remaining nalidixic acid-resistant isolates were from Egypt, Morocco, Peru, and Kenya, with two strains of unknown origin. These results suggest that quinolone resistance is not limited to aforementioned areas but is widespread.

Antibiotic treatment (mainly ciprofloxacin) was required in 73% of the patients. In all cases the treatment was done empirically prior to obtaining laboratory results. The analysis of the outcome of these patients showed that treatment with

TABLE 3. Antimicrobial susceptibility of *Salmonella* clinical isolates[a]

Antimicrobial agent	Susceptible		Intermediate		Resistant	
	n	%	*n*	%	*n*	%
Ampicillin	49	79	1	2	12	19
Amoxicillin-clavulanic acid	57	92	5	8	0	0
Imipenem	62	100	0	0	0	0
Ceftazidime	62	100	0	0	0	0
Norfloxacin	62	100	0	0	0	0
Nalidixic acid	51	82	1	2	10	16
Ciprofloxacin	62	100	0	0	0	0
Gentamicin	59	95	0	0	3	5
Amikacin	62	100	0	0	0	0
Trimethoprim-sulfamethoxazole	56	91	0	0	6	9
Tetracycline	47	76	2	3	13	21
Chloramphenicol	57	92	0	0	5	8

[a] *n*, number of strains.

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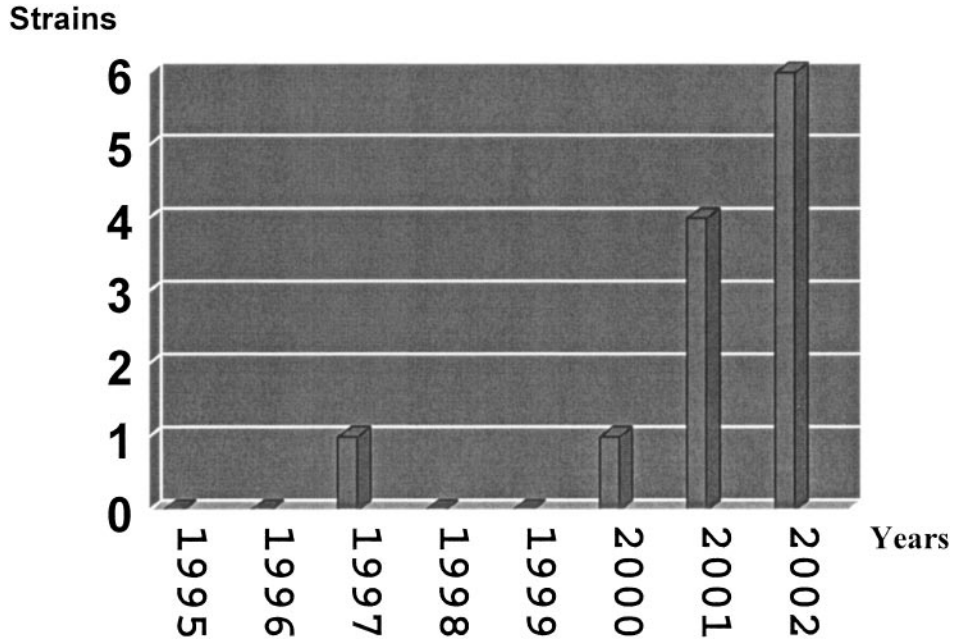

FIG. 1. Evolution of nalidixic acid resistance in *Salmonella* spp. causing TD from 1995 to 2002. The resistance to nalidixic acid represented in this figure was established according to the number of strains isolated in each year.

ciprofloxacin was effective in all patients with nalidixic acid-resistant isolates except one, in whom a change in the treatment to amoxicillin-clavulanic acid was required.

Reports of different microorganisms, such as *Shigella flexneri* or *Salmonella enterica* serovar Typhi, showed high levels of therapeutic failure in treatments with fluoroquinolones in patients infected with isolates showing nalidixic acid resistance but fluoroquinolone susceptibility (17, 41). Thus, although fluoroquinolones might be used as treatment in diarrhea by nalidixic acid-resistant *Salmonella* strains, this option should be carefully chosen, as shown by the therapeutic failure reported in the present study.

Only 44 of the 62 *Salmonella* strains were able to grow from the frozen stock, and these strains were therefore serotyped again and phage typed in a reference laboratory (Table 2). Of these 44 isolates, 16 isolates showed resistance to any antibiotic and were further investigated to determine the mechanisms of antibacterial resistance.

Single amino acid substitutions in both positions 83 and 87 of GyrA have been described as a cause of nalidixic acid resistance in *Salmonella* spp., whereas substitutions at both positions plus substitutions in ParC have been detected in fluoroquinolone-resistant isolates (1, 11, 13, 22, 30, 32). In the present study 9 of these 16 isolates showed resistance to nali-

TABLE 4. Mechanisms of resistance of *Salmonella* spp. causing TD

Strain	Serovar	Origin	Resistance[b] (MIC [μg/ml])	Mechanism of resistance				
				Q (GyrA)	CHL	AMP	TET	SXT
30922	Virchow	India	NAL (≥256), SXT (≥32)	Tyr-87				*dfrA14*
62155	Enteritidis	Mexico	AMP (≥256), NAL (≥256)	Tyr-87		*tem*		
57360	Haifa	Egypt	TET (≥256), NAL (≥256)	Tyr-83			*tetA*	
37758	Enteritidis	Morocco	NAL (≥256)	Tyr-87				
13472	Enteritidis	Peru	NAL (≥256)	Tyr-87				
14089	Enteritidis	ND[a]	NAL (≥256)	Tyr-87				
49297	Enteritidis	ND	NAL (≥256)	Tyr-87				
33568	Enteritidis	India	NAL (≥256)	Tyr-87				
86 DV	Enteritidis	Kenya	NAL (≥256)	Tyr-87				
36452	Typhimurium	Ivory Coast	TET (128), AMP (≥256)			*tem*	*tetA*	
21340	Typhimurium	Gambia	TET (≥256), AMP(≥256), SXT (≥32), CHL (128)		*cmlA*	*tem*	*tetA*	*dfrA12*
27976	Goldcoast	Senegal	TET (128), AMP (≥256), SXT (≥32), CHL (32)			*tem*	*tetA*	*dfrA17*
29839	Risseu	Mali	TET (128), AMP (≥256)			*tem*	*tetA*	
36498	Paratyphi	India	TET (≥258), AMP (≥256), SXT (≥32), CHL (≥128)		*cat*	*oxa-1*	*tetB*	*dfrA1*
13805	Kiambu	Mali	TET (64), AMP (≥256), CHL (≥256)		*floR*	*tem, carb*	*tetG*	
15095	Hadar	Bolivia	TET (128), AMP (≥256)			*tem*	*tetA*	

[a] ND, not determined.
[b] NAL, nalidixic acid; SXT, trimethoprim-sulfamethoxazole; AMP, ampicillin; TET, tetracycline; CHL, chloramphenicol; Q, quinolones.

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dixic acid and decreased susceptibility to ciprofloxacin and norfloxacin. Seven were identified as serovar Enteritidis; the rest were identified as serovar Virchow and serovar Haifa. Eight of these nine nalidixic-resistant *Salmonella* strains, including all of the serovar Enteritidis strains, presented a mutation in codon 87 of the *gyrA* gene, resulting in the amino acid change Asp to Tyr, whereas the other strain (serovar Haifa) presented a mutation in amino acid codon 83 (Ser to Tyr). No mutation was found in the *parC* gene.

Eight isolates were resistant to ampicillin. This resistance was associated with the presence of β-lactamases. The *oxa-1* gene was detected in one strain, whereas a *tem*-like gene was found in the other seven strains, in one case concomitantly with a *carb-2* (*pse-1*) gene. This result is in agreement with previously developed studies (5, 6, 29, 42). Although in some of these studies, such as that performed by Roy et al. (29), the percentage of TEM-like β-lactamase-producing strains was higher than in ours, this may be explained by the diversity of the geographical origin of the strains collected in our study. In some of the strains producing a TEM-like β-lactamase an intermediate level of susceptibility to amoxicillin plus clavulanic acid was found. It has been shown that an overproduction of these enzymes may result in decreased susceptibility or resistance to amoxicillin plus clavulanic acid, which may explain the above-mentioned situation (33).

The main mechanism of resistance to trimethoprim is the presence of integron-borne dihydrofolate reductases. Only four isolates resistant to trimethoprim were studied, showing the presence of four different dihydrofolate reductase genes (*dfrA1*, *dfrA12*, *dfrA14*, and *dfrA17*) (Table 4). Despite the greater use of cotrimoxazole in developing countries, in our study only four strains showed resistance to this antimicrobial agent. This finding is in contrast to the high percentages of trimethoprim resistance in *Shigella* or diarrheagenic *E. coli* isolates causing TD (38, 39, 40). To our knowledge, this is the first time that *dfrA17* has been found in a *Salmonella* strain of African origin.

Resistance to chloramphenicol was determined in four cases and was associated with the presence of *floR* and *cmlA* genes and CAT activity in three different cases. These cases showed chloramphenicol MICs higher than 128 μg/ml, whereas no specific mechanism was identified in the remaining isolate (chloramphenicol MIC of 32 μg/ml) (Table 4).

Eight isolates were resistant to tetracycline; the *tetA* gene was detected in six cases and the *tetB* and *tetG* genes were detected in one case each (Table 4). The intestinal tract is a suitable habitat for the acquisition of the *tetA* and *tetB* genes by horizontal gene transfer, since these tetracycline determinants are common in *Enterobacteriaceae* (10, 14, 28). In a recent study developed in *Shigella* spp. and enteroinvasive *Escherichia coli* of different geographical origins, the *tetB* gene was more prevalent than the *tetA* gene (14). Our results show that the most prevalent mechanism of resistance to tetracycline among the strains we tested was *tetA*. This difference may be explained by the fact that *Shigella* and *Salmonella* spp. may have different ecological niches.

In summary, our results show the level of antimicrobial resistance of *Salmonella* spp. from different geographical origins and although, overall, this resistance is lower than that presented by *Shigella* spp. or diarrheagenic *Escherichia coli*, the increasing resistance to nalidixic acid in compared to the above-mentioned microorganisms is of special concern and may result in a loss of therapeutic usefulness of fluoroquinolones. Moreover, high heterogeneity of the mechanisms of resistance to ampicillin, trimethoprim, and tetracycline was observed. Surveillance of the *Salmonella* spp. causing TD is necessary in order to have current information on the resistance levels and the mechanism of resistance present in these strains, which will in turn help to provide better treatment and to develop strategies to prevent and limit their spread.

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