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Evolution of Multiresistance in Nontyphoid *Salmonella* Serovars from 1984 to 1998 in Argentina

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Molecular evolution of multiresistance in nontyphoid *Salmonella* spp. was investigated with 155 isolates obtained in Argentina from 1984 to 1998. In 74 isolates obtained from 1984 to 1988 resistance was associated with the presence of Tn*3*, Tn*9*, class I (In0) and II (Tn*7*) integrons, and the *aac*(*3*)-*IIa* gene. Extended-spectrum cephalosporin (ESC) resistance in *Salmonella* spp. emerged in 1989, and 81 isolates resistant to at least one ESC and one aminoglycoside were collected thereafter. Among these, two patterns of antimicrobial resistance mechanisms were found: from 1989 to 1992, resistance was related to the spreading of Tn*1331* and $bla_{CTX\text{-}M\text{-}2}$, in addition to the persistence of In0 and Tn*7*. From 1993 to 1998, several integrons were added to the first pattern and three integron groups (IG), namely, IG1 (38% of the isolates), IG2 (51%), and IG3 (11%), were identified. At least two β-lactamase genes were detected in 65% of the isolates (after 1989) by PCR analysis. Furthermore, five β-lactamase genes, $bla_{CTX\text{-}M\text{-}2}$, $bla_{OXA\text{-}9}$, $bla_{OXA\text{-}2}$, $bla_{TEM\text{-}1}$, and $bla_{PER\text{-}2}$, were found in two isolates. The $bla_{CTX\text{-}M\text{-}2}$ gene was found in several complex *sulI*-type integrons with different rearrays within the variable region of class I integrons, suggesting evolution of these integrons in nontyphoid *Salmonella*. In conclusion, progressive acquisition and accumulation of plasmid-mediated resistance determinants occurred from 1984 to 1998 in nontyphoid *Salmonella* isolates of the most prevalent serovars from Argentina. It is suggested that antimicrobial resistance mechanisms in these bacteria may have been the consequence of plasmid exchange between *Salmonella enterica* serovar Typhimurium and *Escherichia coli* or *Shigella flexneri* and/or spreading of mobile elements from the nosocomial environment.

Nosocomial nontyphoid *Salmonella* infections have been reported in recent years from many geographic areas, including countries with high public health and hygiene standards (13, 19, 26). Multiresistant *Salmonella* isolates of different serovars are increasingly common, appear with variable geographical incidence, and have become an issue of worldwide concern (10, 30). Currently, several industrialized countries have ongoing programs on surveillance of multiresistance in zoonotic bacteria, including *Salmonella*. Serovars of this genus, other than *Salmonella enterica* serovar Typhi, are major agents of gastroenteritis and can also cause systemic infections in animals and humans (39, 41). Despite the fact that *Salmonella* spp. are not typical members of the hospital microflora, several outbreaks due to multiresistant isolates have been reported in hospitals from Argentina, as well as in other countries (25, 26, 38). Although antibiotic therapy is not usually recommended for treatment of patients with *Salmonella* gastroenteritis, invasive complications such as meningitis, sepsis, and bacteremia require it. Combinations of β-lactams and an aminoglycoside are widely used in Argentina in the treatment of neonatal and pediatric nosocomial infections due to *Salmonella* spp. It has been suggested that extended-spectrum β-lactamases (ESBL) associated with plasmids are responsible for the extended-spectrum cephalosporin (ESC) resistance. In a national surveillance study for ceftriaxone-resistant *Salmonella* infections in the United States, $bla_{CMY\text{-}2}$ has been found to be the most prevalent β-lactamase gene causing ESC resistance (17). Although a few ESBL in *Salmonella* spp. causing short-term nosocomial outbreaks have been reported (5, 6, 18, 27, 39), only PER-1 was recognized in multiple-antibiotic-resistant *S. enterica* serovar Typhimurium strains isolated over a 28-month period from the nosocomial environment in Turkey (38).

From 1969 to 1985 the most prevalent *Salmonella* serovar in hospitals from different cities of Argentina was *Salmonella* serovar Typhimurium, followed by *S. enterica* serovar Oranienburg. *S. enterica* serovar Enteritidis emerged in 1986 and since 1987 has been the most frequent serovar, with the exception of 1991 and 1992, when the most prevalent serovars were, respectively, *Salmonella* serovar Typhimurium and *S. enterica* serovar Infantis. *Salmonella* serovar Enteritidis has been mainly involved in food-borne outbreaks. Before 1989, resistant nosocomial *Salmonella* isolates exhibited resistance to aminoglycosides, ampicillin, chloramphenicol, and/or sulfonamides. In 1989 resistance to ESC emerged in pediatric hospitals of Argentina, reaching levels of combined aminoglycoside and ESC resistance of over 40% (26, 33). Resistance to ESC was detected first in *Salmonella* serovar Typhimurium and later in *Salmonella* serovar Infantis and *Salmonella* serovar Agona; it was rarely detected in *Salmonella* serovar Enteritidis. No resistance to ciprofloxacin was detected in *Salmonella* isolates from Argentina.

This study was designed to (i) determine the resistance genes involved in five multiresistant serovars of *Salmonella* spp. strains isolated from nosocomial infections in Argentina, (ii) evaluate the evolution of the mechanisms involved in the

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TABLE 1. Characteristics of nontyphoid *Salmonella* sp. isolates

Salmonella serovar	No. of isolates	Hospital[a]	Yr of isolation	Type of infection[b]
Typhimurium	65	H1	1984	O
Typhimurium	5	H1	1985	NI
Typhimurium	4	H2	1987	NI
Typhimurium	8	H2	1990	O
Agona	26	H2	1990	O
Infantis	4	H2	1990	O
Oranienburg	12	H2	1991	O
Enteritidis	10	H2	1991	O
Typhimurium	5	H3	1991	NI
Typhimurium	4	H3	1993	NI
Infantis	2	H4	1994	NI
Typhimurium	3	H4	1995	NI
Agona	2	H4	1995	NI
Infantis	4	H5	1996	NI
Typhimurium	1	H6	1998	NI

[a] H1, hospital in La Plata, 70 km from Buenos Aires; H2 to H6, hospitals in Buenos Aires.
[b] O, outbreak; NI, sporadic nosocomial infection (seemingly epidemiologically unrelated nontyphoidal sp. isolates).

spreading of antimicrobial resistance in a significant number of multiresistant nontyphoid *Salmonella* spp. isolated between 1984 and 1998, and (iii) scrutinize those resistance mechanisms found in *Salmonella* spp. in the gram-negative population of the same hospital environment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 155 isolates were utilized in this study (Table 1). Isolates were obtained from stools (97%), blood (2%), and urine (1%). All isolates were biochemically (API 20E strip; BioMérieux) and serologically identified according to the standard international scheme for *Salmonella* serotyping (32) at the reference laboratory INEI-ANLIS "Dr. Carlos G. Malbrán." Criteria for strain selection were based upon the emergence of antimicrobial resistance, and two resistance phenotypes were selected. Group R1, from the period 1984 to 1988, included 74 isolates resistant to ampicillin and gentamicin. Group R2 (81 isolates) included *Salmonella* spp. resistant to at least one ESC and at least one aminoglycoside; these were obtained from 1989, when ESC resistance in the genus *Salmonella* emerged in Argentina. To determine the putative transference of resistance determinants into *Salmonella* from other isolates in the same nosocomial environment, the presence of plasmids and antimicrobial resistance mechanisms in *Enterobacteriaceae* isolates were investigated. *Enterobacteriaceae* isolates were obtained from the same hospitals and within the same periods as the *Salmonella* isolates, and a number of them were obtained from patients that were coinfected with *Salmonella* spp. During the course of the outbreak in H1 (Table 1), 15 patients were concomitantly infected with *Shigella flexneri* and/or *Escherichia coli* resistant to at least two antimicrobial agents. These two species were recovered mainly from stools and urine, respectively. We investigated one *E. coli* isolate and one *S. flexneri* isolate from the outbreak. From 1989 to 1998, 55 seemingly epidemiologically unrelated *E. coli* ($n = 5$), *Klebsiella pneumoniae* ($n = 17$), *Serratia marcescens* ($n = 3$), *Citrobacter freundii* ($n = 4$), *Enterobacter cloacae* ($n = 6$), *Enterobacter aerogenes* ($n = 5$), and *Proteus mirabilis* ($n = 20$) isolates were selected. These isolates were resistant to ESC and to at least one aminoglycoside, and they were isolated from nosocomial infections from the same hospitals where the *Salmonella* sp. strains had been collected. Cultures were routinely performed at 37°C in brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with ampicillin (50 μg/ml) or cefotaxime (16 μg/ml), as required.

Antimicrobial susceptibility testing. Tests for susceptibility to ampicillin, cefotaxime, ceftazidime, amikacin, gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, imipenem, and cefoxitin were performed by using the agar diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (29). Antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.). MICs for the *Salmonella* sp. strains from the R2 group were determined on Mueller-Hinton agar (Difco) plates containing serial twofold dilutions of the following antimicrobial agents: cefotaxime, ceftazidime, amikacin, and gentamicin (29). An inoculum of 10^4 bacteria per spot was deposited with a Steer inoculator.

Conjugation and plasmid analysis. Nalidixic acid-resistant *E. coli* C600 was used as the recipient in conjugation experiments. Conjugation was performed in brain heart infusion media for 4 h at 37°C, and transconjugants were selected on Mueller-Hinton agar containing ampicillin (50 μg/ml) or cefotaxime (4 μg/ml) plus nalidixic acid (100 μg/ml).

DNA techniques. Total and plasmidic DNA was prepared as described before (16, 34). Plasmidic DNA was digested with different restriction enzymes, and the fragments were separated in horizontal gels of 0.8 to 1.0% (wt/vol) agarose dissolved in 0.4 M Tris-acetate–0.01 M EDTA. PCR amplifications were carried out in 100-μl volumes containing 10 ng of DNA, 10 μl of 10× PCR buffer, 10 μl of 10× deoxynucleoside triphosphate mixture (2 mM [each] dATP, dCTP, dGTP, and dTTP), 10 μl of each primer stock solution (2.5 pmol of each primer per μl), and 60 μl of sterile distilled water. Each reaction mixture was covered with 75 μl of mineral oil. *Taq* DNA polymerase (Promega) was added (1 μl of the 3-U/μl diluted solution) after 12 min at 94°C (hot-start method). To amplify the DNA in the thermocycler (Perkin-Elmer Cetus, Emeryville, Calif.), a three-step profile was utilized (23). Primers for PCRs were specific for the nucleotide sequences exhibited in Table 2. PCR mapping of integrons, transposons, and unusual class I integrons was done as follows (see also Table 2). (i) Class I and II integron mapping was performed with primers for the 5′ and 3′ conserved segments. To determine the gene order in the variable region, we used primers for sequences located at the ends of the inserted resistance gene cassettes in combination with those specific for other gene cassettes, as well as with those for the conserved segments. (ii) Tn*3* mapping was performed with specific primers for the transposase and the bla_{TEM} gene. Tn*1331* mapping was performed with primers for the transposase (which is identical to that of Tn*3*) and for *aac*(*6′*)-*Ib*. Tn*9* mapping was performed with specific primers for the *cat* gene. (iii) The mapping of the unusual class I integrons was performed with specific primers for ORF513 and the $bla_{CTX\text{-}M\text{-}2}$ gene. To identify the class I integron variable region, we used primers located at the ends of the last inserted cassette in the variable region and a primer for the $bla_{CTX\text{-}M\text{-}2}$ gene which rendered amplicons of approximately 4,000 bp. *E. coli* and *S. flexneri* plasmid DNA digested with *Eco*RI, *Bam*HI, and *Hin*dIII was transferred to nylon filters by Southern blotting and hybridized with plasmid DNA of *Salmonella* serovar Typhimurium from the R1 group digested with *Hin*dIII as described elsewhere (34). Colony hybridization was performed as described previously (34). Kodak X-Omat AR film was used for autoradiography. Several PCR products, amplified with cloned *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), were sequenced after purifying the DNA by using the QIAquick kit according to the manufacturer's directions (Qiagen Inc., Studio City, Calif.). Sequencing was performed on both DNA strands with an ABI 373 sequencer. Internal oligonucleotide primers were used where necessary to ensure that both strands were sequenced. Several $bla_{CTX\text{-}M\text{-}2}$-like, $bla_{PER\text{-}2}$-like, and $bla_{TEM\text{-}1}$-like PCR products that were sequenced corresponded to $bla_{CTX\text{-}M\text{-}2}$, $bla_{PER\text{-}2}$, and $bla_{TEM\text{-}1}$ genes, respectively; the remaining β-lactamase-encoding PCR products were subsequently named $bla_{CTX\text{-}M\text{-}2}$-like, $bla_{PER\text{-}2}$-like, and $bla_{TEM\text{-}1}$-like, respectively. The nucleotide sequences were analyzed with the Genetics Computer Group software.

RESULTS

Antimicrobial resistance of *Salmonella* spp. According to their susceptibility profiles, four phenotypes of group R1 and five phenotypes of group R2 were identified in 155 *Salmonella* sp. isolates (Tables 3 and 4). All isolates were susceptible to cefoxitin and imipenem, whereas 95% of the isolates were resistant to gentamicin. As shown in Tables 3 to 5, emergence of cefotaxime resistance was observed in the R2 group.

Determination of antimicrobial resistance mechanisms other than *bla* genes. All isolates from the R1 and R2 groups were analyzed by PCR for the presence of Tn*7*, Tn*9*, Tn*3*, Tn*1331*, class I and II integrons, *aac*(*3*)-*IIa*, and ORF513, previously known as ORF341 (GenBank accession no. L06418). From 1984 to 1988 (group R1) *Salmonella* serovar Typhimurium isolates harbored Tn*3* ($bla_{TEM\text{-}1}$, which confers resistance to ampicillin), Tn*9* (*cat*, which confers resistance to chloramphenicol), *aac*(*3*)-*IIa* (which confers resistance to gentamicin), Tn*7* (*dfrA1*, which confers resistance to tri-

TABLE 2. Primers used for PCRs

Amplified DNA[e]	Primer	Oligonucleotide sequence (5′–3′)	Accession no. and reference
Class I integrons			
intiI	IntiIf	TTC GAA TGT CGT AAC CGC	M73819[g]
	IntiIr	CGA GGC ATA GAC TGT AC	M73819[g]
	Sulpro3	GCC TGA CGA TGC GTG GA	M73819 (23)
3′ conserved segment	3′-CS	AAG CAG ACT TGA CCT GA	M73819 (23)
Class II integrons			
intiII	IntiIIf	GCA AAT GAA GTG CAA CGC	AF318072[g]
	IntiIIr	ACA CGC TTG CTA ACG ATG	AF318072[g]
Class III integrons			
intiIII	IntiIIIf	AGG TGC CTC CGG CAG CG	AF4162297[g]
	IntiIIIr	TGT CTG TGG ACC CAC AA	AF4162297[g]
Tn*1331*			
tnpR	TnpR	AAG TTC ATC GGG TTC GC	AF479774[g]
aac(6′)-Ib	aac6-Ibr	TGT GAC GGA ATC GTT GC	AF227505 (23)
Tn*3* and bla_{TEM}-like			
tnpR	TnpR	AAG TTC ATC GGG TTC GC	AF479774[g]
bla_{TEM}	201r	TCG CCG CAT ACA CTA TTC T	AF516720[g]
ORF513	Orf513f[d]	GCG AAC ACT GCG GCG GTC AC	L06418 (2)
	Orf513r[d]	CTG AGG GTG TGA GCG AG	L06418[g]
Chloramphenicol R[f] gene (Tn*9*)	Catf	GGT GAG CTG GTG ATA TGG	V00622[g]
	Catr	GGG ATT GGC TGA GAC GA	V00622[g]
β-Lactam R genes			
bla_{OXA-2} (OXA-15, -32, -34)	Oxa-2f[b]	GAA GAA ACG CTA CTC GC	AF315351[g]
	Oxa-2r[a]	TAC CCA CCA ACC CAT AC	AF315351[g]
bla_{OXA-9}	Oxa-9fb[b]	GAA CAC CAA CAT ATG CA	AF034958[g]
	Oxa9r[a]	GGG ACA ATA ACG GCA AG	AF034958[g]
bla_{OXA-11} (OXA-13, -14, -16, -17, -19, -28, -35)	Oxa 11f	ACT CAG TTC CCA CAC CA	AF300984[g]
	Oxa11r	TCC CCA ACG CAA TTA TC	AF300984[g]
$bla_{CTX-M-2}$ (KLUA-1, KLUA-2, CTX-M-4, TOHO-1)	Ctx-M2f	ATG ACT CAG AGC ATT CGC	AF286192[g]
	Ctx-m2r[d]	TCA CTT TAT CGG GAC CAC	AF286192[g]
bla_{PER-2}	Per-2f	CGC TTC TGC TCT GCT GAT	X93314[g]
	Per2r	GGC AGC TTC TTT AAC GCC	X93314[g]
bla_{FOX-2}	Foxf	AGT TCC CTG ATG AGG TG	Y10282[g]
	Foxr	GAA TAG CCG TAG GCA TAG	Y10282[g]
bla_{SHV} (SHV-1, -2, -5–8, -11, -12, -14, -18, -26–29, -33–35, -38)	Shvf	ATT ACC ATG AGC GAT AAC A	AF462394[g]
	shvr	GTA TCC CGC AGA TAA ATC A	AF462394[g]
Aminoglycoside R genes			
aac(6′)-Ib	Aac6Ibr[a]	TGT GAC GGA ATC GTT GC	AF227505 (23)
	Aac6Ibf[b,c]	AAA CAC GCC AGG CAT TC	AF227505[g]
aac(6′)-Iq	Aac6Iq[a]	GAC TTT CCC AAT ACC CC	AF047556[g]
aac(3)-IIa	AacC2f	CGC TAA ACT CCG TTA CC	M62833[g]
	AacC2r	TAG CAC TGA GCA AAG CC	M62833[g]
ant(2′′)-Ia	AadBf[a]	CTA TGC CGA TGA AGT ACC	AF453998 (23)
	AadBr[b,c]	AGA CCT CAA CCT TTT CC	AF453998[g]
ant(3′′)-Ia	AadA1r[a]	TGC ATG ACG CCA ACT AC	AF327064 (23)
	AadA1f[b,c]	CGC AGA TCA GTT GGA AG	AF327064[g]
Trimethoprim R gene *dfrAI*	dfrAIr[a]	AGC TGT TCA CCT TTG GC	AF455254 (23)
	dfrAIf[b]	CCT GAA ATC CCC AGC AA	AF455254[g]
orfD	OrfDr[a]	CAT TCT GCG GTC GGC TT	U13880[g]
	OrfDf[b,c]	CAT TCT GCG GTC GGC TT	U13880[g]

[a] Primer used to perform PCR mapping of class I integrons in combination with the Sulpro3 primer from the *intiI* gene. The PCR amplicons differ in expected size depending on their locations in the class I integron variable region.

[b] Primer used to perform PCR mapping of class I integrons in combination with the 3′-CS primer from the 3′ conserved segment. The PCR amplicons differ in expected size depending on their locations in the class I integron variable region.

[c] Primer used to perform PCR mapping in combination with the Orf513r primer (PCR amplicons with an average length of approximately 3,000 bp) and with the Ctx-m2r primer (PCR amplicons with an average length of approximately 4,000 bp) to identify the unusual class I integron rearrangements.

[d] Primer used to detect the $bla_{CTX-M-2}$ gene in the unusual class I integrons.

[e] Designations in parentheses in subscripts of *bla* genes represent the genes also detected with the corresponding primer combination. The remaining sets of *bla* primers are specific for the gene or mechanism detected.

[f] R, resistance.

[g] Accession number of the sequence of a primer designed for this study.

TABLE 3. Antibiotic resistance phenotypes of nontyphoidal *Salmonella* serovar Typhimurium isolates in resistance group R1

Hospital[a]	Yr of isolation	No. of isolates in resistance subgroup[b]:			
		R1a	R1b	R1c	R1d
H1	1984	10	19	5	27
H1	1985		2		3
H2	1987			4	4

[a] Hospitals are identified in Table 1.
[b] Resistance subgroups are defined as follows: R1a, resistance to ampicillin (AP), gentamicin (GN), and trimethoprim-sulfamethoxazole (SXT); R1b, resistance to AP, chloramphenicol (CM), and GN; R1c, resistance to AP and GN; R1d, resistance to AP, GN, CM, and SXT.

methoprim), and/or In0 (*sulI*, which confers resistance to sulfamethoxazole) (Fig. 1a). In the R2 group (1989 to 1998), two patterns of antimicrobial resistance mechanisms were found and all isolates harbored at least one class I integron. The first pattern, from 1989 to 1992, was associated with the emergence of ESC and amikacin resistance by acquisition of $bla_{\text{CTX-M-2}}$ (100% of isolates) and Tn*1331* (40% of isolates), respectively. At least one of the integrons, either In0 or In4 [with *ant*(*3″*)-*Ia* within the variable region], was harbored by every isolate from this period (1989 to 1992). The second pattern, found between 1993 and 1998, involved $bla_{\text{CTX-M-2}}$, at least two of the same antimicrobial determinants which were found in the R1 group, Tn*1331* in 9.5% of the isolates, and several class I integrons conferring resistance to a wide variety of antimicrobial agents. Three integron groups (IG) were harbored by this second pattern. IG1 (38% of isolates) harbored two class I integrons with *ant*(*2″*)-*Ia*-*ant*(*3″*)-*Ia* and *orfD* cassettes within the variable region; IG2 (51%) harbored three class I integrons with *dfrA1*-*ant*(*3″*)-*Ia*, *aac*(*6′*)-*Ib*-*orfD*, and *ant*(*3″*)-*Ia* cassettes; IG3 (11%) harbored two class I integrons with *aac*(*6′*)-*Ib*-$bla_{\text{OXA-2}}$-*orfD* and *orfD* cassettes.

Tn*7* and Tn*9* were found in both R1 and R2 group isolates as well as *aac*(*3*)-*IIa*, which was found in 95% of *Salmonella* sp. isolates. Thirty-eight percent of *Salmonella* sp. isolates from the R2 group harbored two genes coding for gentamicin resistance, *aac*(*3*-*IIa*) and *ant*(*2″*)-*Ia*. Tn*1331* (49.5% of R2 isolates), *aac*(*3*)-*IIa* and $bla_{\text{CTX-M-2}}$ genes were found in isolates belonging to all five *Salmonella* serovars (Table 6). *Salmonella* serovar Typhimurium, *Salmonella* serovar Infantis, and *Salmonella* serovar Agona harbored the three IGs, whereas *Salmonella* serovar Typhimurium harbored both class I and class II (Tn*7*) integrons.

TABLE 4. Antibiotic resistance phenotypes of nontyphoidal *Salmonella* sp. isolates in resistance group R2

Salmonella serovar	No. of isolates in resistance subgroup[a]:					Hospital[b]	Yr of isolation
	R2a	R2b	R2c	R2d	R2e		
Typhimurium	4	4				H2	1990
Agona	7	10				H2	1990
Infantis		2	1	1		H2	1990
Oranienburg	3	9				H2	1991
Enteriditis	6	3		1		H2	1991
Typhimurium	4			1		H3	1991
Typhimurium		4				H3	1993
Infantis		2				H4	1994
Typhimurium			3			H4	1995
Agona		1			1	H4	1995
Infantis		4				H5	1996
Typhimurium					1	H6	1998

[a] Resistance subgroups are defined as follows: R2a, resistance to cefotaxime (CTX) and gentamicin (GN); R2b, resistance to CTX, amikacin (AK), and GN; R2c, resistance to CTX, ceftazidime (CAZ), AK, and trimethoprim-sulfamethoxazole (SXT); R2d, resistance to CTX, AK, and chloramphenicol (CM); R2e, resistance to CTX, CAZ, GN, CM, and SXT.
[b] Hospitals are identified in Table 1.

TABLE 5. Levels of susceptibility of *Salmonella* isolates to β-lactams and aminoglycoside antibiotics

Antibiotic	MIC (μg/ml)	
	Range	90[c]
R1 group TEM-1[a]		
Ampicillin	64–>256	128
Cefotaxime	0.03–0.5	0.06
Ceftazidime	0.06–0.25	0.06
Amikacin	0.5–2	1
Gentamicin	32–128	32
R2 group CTX-M-2[b]		
Ampicillin	>1,024	>1,024
Cefotaxime	32–512	64
Cefotaxime-clavulanic acid	0.015–0.03	0.03
Ceftazidime	1–16	1
Amikacin	1–64	32
Gentamicin	32–256	64

[a] All 74 *Salmonella* sp. isolates from the R1 group harbored $bla_{\text{TEM-1}}$ according to PCR analysis.
[b] All 81 *Salmonella* sp. isolates from the R2 group harbored $bla_{\text{CTX-M-2}}$. The $bla_{\text{OXA-9}}$, $bla_{\text{OXA-2}}$, $bla_{\text{TEM-1}}$, and/or $bla_{\text{PER-2}}$-like genes were also tested by PCR analysis.
[c] 90, MIC at which 90% of the isolates were inhibited.

Characterization of β-lactamase genes. $bla_{\text{TEM-1}}$ was found in all *Salmonella* sp. isolates from group R1 and 65% of group R2 isolates, as shown by PCR and sequencing studies. The $bla_{\text{CTX-M-2}}$-like gene was found in all *Salmonella* sp. isolates belonging to group R2. The $bla_{\text{PER-2}}$-like gene was found in 2.5% of the isolates, whereas the $bla_{\text{FOX-1}}$-like and the bla_{SHV}-like genes were never found.

The $bla_{\text{OXA-2}}$ and the $bla_{\text{OXA-9}}$ genes were found in 11 and 49.5% of R2 group isolates, respectively, whereas $bla_{\text{OXA-11}}$ was never found. At least two β-lactamases were found in 73% of the R2 group isolates by PCR analysis. Twenty-seven percent possessed the $bla_{\text{CTX-M-2}}$-like gene alone; 47% possessed $bla_{\text{CTX-M-2}}$-like, $bla_{\text{OXA-9}}$, and $bla_{\text{TEM-1}}$-like genes; 15% possessed $bla_{\text{CTX-M-2}}$-like and $bla_{\text{TEM-1}}$-like genes; 8.5% possessed $bla_{\text{CTX-M-2}}$-like and $bla_{\text{OXA-2}}$ genes; and 2.5% possessed $bla_{\text{CTX-M-2}}$-like, $bla_{\text{OXA-9}}$, $bla_{\text{OXA-2}}$, $bla_{\text{TEM-1}}$-like, and $bla_{\text{PER-2}}$-like genes. The $bla_{\text{CTX-M-2}}$ gene was previously found in the unusual class I integrons (2, 15), and in this study it was located at the same position in the genetic structures which characterize this group, involving different arrays of cassettes in the variable regions of the isolates studied [In0 (n = 40), In4 (n = 28), In35 (n = 3), *ant*(*2″*)-*Ia*-*ant*(*3″*)-*Ia* (n = 8) and *dfrA1*-*ant*(*3″*)-*Ia* (n = 2) within the variable region] by PCR cartography (Fig. 1b). Colony hybridization of the R1 and R2 group isolates was performed by using as a probe ORF513 from the unusual class I integrons, where $bla_{\text{CTX-M-2}}$ and other resistance genes (31, 35, 40) are located. ORF513-related sequences were detected in all R2 group isolates, whereas they

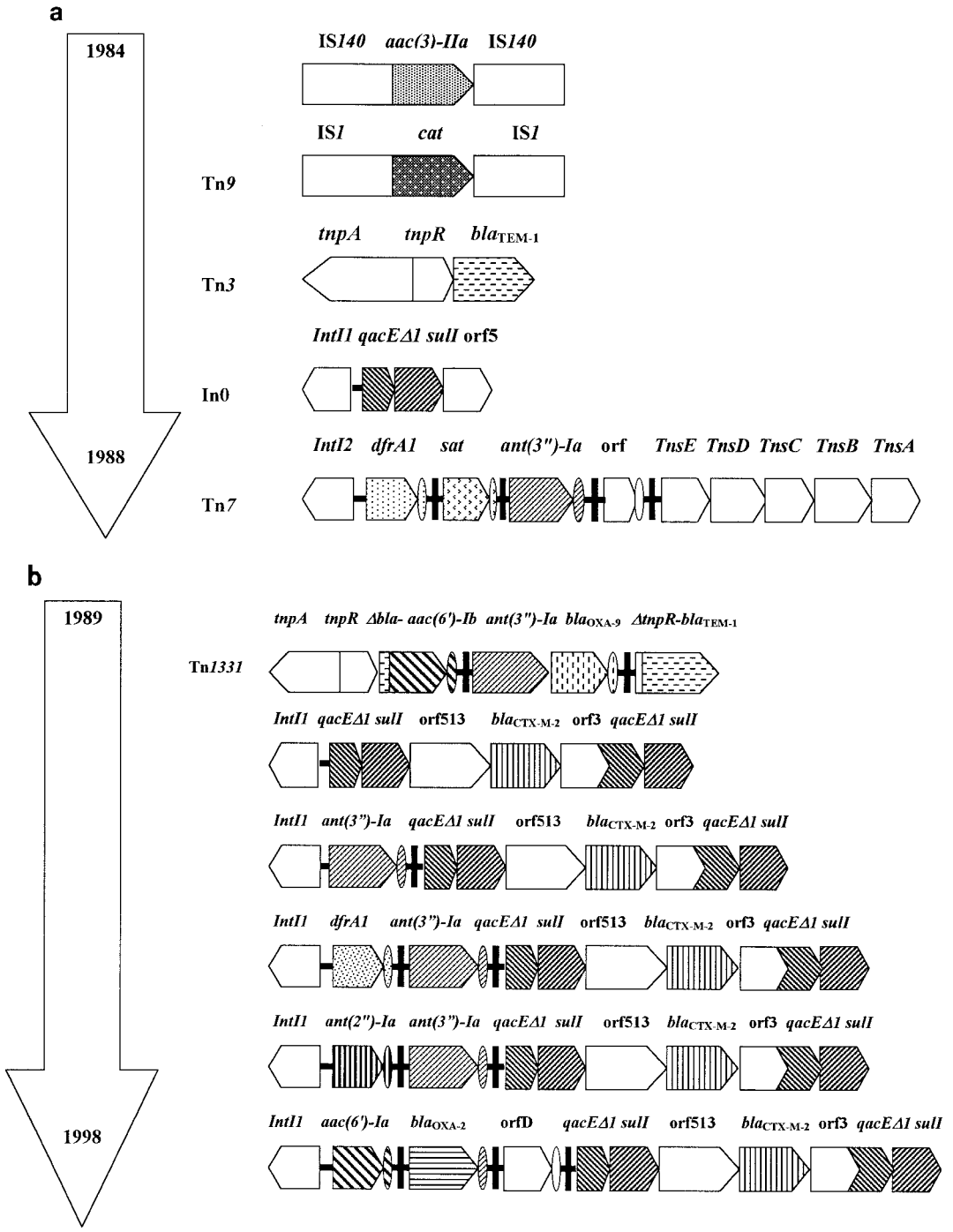

FIG. 1. Physical maps of the antimicrobial resistance determinants from 1984 to 1998, showing the evolution and accumulation of antimicrobial resistant determinants in the R1 group (1984 to 1988) (a) and the R2 group (1989 to 1998) (b). Only class I integrons harboring ORF513 are shown. *orf3* and *qacEΔ1* have been found in In35 as a gene fusion (2).

were not found in isolates from the R1 group, which did not harbor $bla_{\text{CTX-M-2}}$.

Scrutiny of resistance determinants in other gram-negative *Enterobacteriaceae*. To establish the chromosomal or plasmidic location of the genetic determinants for gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin resistance, conjugation analysis was performed on isolates of the R1 group. Plasmids of 40 kbp, which transferred resistance to ampicillin, gentamicin, and chloramphenicol, were found in five *Salmonella* serovar Typhimurium isolates, one *S. flexneri* isolate, and one *E. coli* isolate. These plasmids were identified as similar, if not identical, by digestion with three DNA endonucleases (*Eco*RI, *Hin*dIII, and *Bam*HI) and by DNA-DNA hybridization analysis using as a probe the 40-kbp *Salmonella*

TABLE 6. Detection of antimicrobial resistance determinants in five serovars of genus *Salmonella*

Salmonella serovar	Presence of determinant:						
	Tn*3*	Tn*9*	Tn*1331*	*aac*(*3*)-*IIa*	$bla_{CTX\text{-}M\text{-}2}$	Class I[a]	Class II[b]
Typhimurium	+	+	+	+	+	+	+
Agona	+	+	+	+	+	+	
Enteritidis		+	+	+	+	+	
Oranienburg		+	+	+	+	+	
Infantis		+	+	+	+	+	

[a] Class I integrons.
[b] Class II integrons.

serovar Typhimurium plasmid DNA digested with *Hin*dIII. The antimicrobial determinants $bla_{TEM\text{-}1}$, *aac*(*3*)-*IIa*, and Tn*9* were found in the conjugative plasmids of the three species. The same antimicrobial determinants [$bla_{TEM\text{-}1}$, *aac*(*3*)-*IIa*, Tn*7*, In0, and Tn*9*] were also found in nine seemingly epidemiologically unrelated *Salmonella* serovar Typhimurium isolates obtained from H1 and H2 in 1985 and 1987, respectively.

From 1989 to 1998, a total of 55 multiresistant *Enterobacteriaceae* isolates including *E. coli*, *K. pneumoniae*, *S. marcescens*, *C. freundii*, *E. cloacae*, *E. aerogenes*, and *P. mirabilis* were analyzed for the presence of $bla_{CTX\text{-}M\text{-}2}$, *aac*(*3*)-*IIa*, and class I and II integrons by PCR cartography. The same class I integrons that were described for IG1, IG2, and IG3, as well as Tn*7*, *aac*(*3*)-*IIa*, and $bla_{CTX\text{-}M\text{-}2}$, were found in the abovementioned bacterial population. However, several other rearrangements in class I and II integrons were also found (data not shown). Conjugation experiments with 10 *Salmonella* sp. isolates (two of each serovar), 12 *P. mirabilis* isolates, and 2 *K. pneumoniae* isolates demonstrated that cefotaxime, gentamicin, and amikacin resistance was carried by conjugative plasmids (data not shown).

DISCUSSION

The findings of the present study on frequency and spreading of class I and II integrons among nontyphoidal *Salmonella* serotypes permit the following conclusions. (i) Evolution of class I integrons is presumed, since In0 was detected in early isolates dating from 1984, whereas several cassettes inserted within the variable region were found in contemporary isolates. (ii) Accumulation of class I integrons was the mechanism involved in the expression of multiresistance in the five serovars. (iii) Class II integrons were detected in our collection from 1984, while their spreading was limited to one serovar (*Salmonella* serovar Typhimurium). (iv) The spreading of $bla_{CTX\text{-}M\text{-}2}$ was linked to several class I integrons. (v) Although certain class I integrons found in the bacterial population under scrutiny were consistent with others found previously in this genus elsewhere (9, 21, 28, 37), In35 was found only in isolates from Argentina.

Our results clearly showed that, in Argentina, the emergence of resistance to ESC in 1989 in the genus *Salmonella* was due to acquisition of the $bla_{CTX\text{-}M\text{-}2}$ gene. Although several CTX-M-like enzymes from all over the world have been described (http//www.lahey.org/studies/webt/htm), this is the first report that describes a CTX-M-like enzyme in several serovars of nontyphoid *Salmonella* spp. involved in outbreaks. In addition to our findings on $bla_{CTX\text{-}M\text{-}2}$, only very recently $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}10}$ were found to be responsible for the prevalence over 10 years as a mechanism for ESC resistance in other gram-negative organisms (8, 12). The $bla_{CTX\text{-}M\text{-}2}$-like gene spreading is mediated by conjugative plasmids. In our *Enterobacteriaceae* population, several other ESBL have been described, such as PER-2, FOX-1, SHV-5, and SHV-2a (4, 20; J. M. Casellas, personal communication). All four enzymes may be considered ceftazidimases rather than cefotaximases (7, 24). PER-1, which possesses 86.4% homology with PER-2, has been found to be responsible for ESC resistance in *Salmonella* serovar Typhimurium isolates from Turkey (38). Our results, however, demonstrated the high prevalence of CTX-M-2-like enzymes in our *Salmonella* sp. population, even though PER-like enzymes were also widespread in our geographical area. This event could be related to, among other factors, the widespread use of cefotaxime and ceftriaxone rather than ceftazidime in our hospitals. This is the first report in which ESBL spreading and the epidemiological evolution of class I and II integrons in *Salmonella* spp. are described. The location of $bla_{CTX\text{-}M\text{-}2}$ in the unusual class I integrons and its presence in conjugative plasmids may explain the high dissemination of this ESBL among *Salmonella* sp. isolates from Argentina.

With regard to multiresistance propagation, on one hand, horizontal acquisition of resistance determinants is usually mediated by plasmids, transposons, or cassettes located in integrons. In fact, conjugative plasmids of the same Inc groups in *Enterobacteriaceae* isolates before and after medical use of antibiotics have been described (14, 22). Moreover, acquisition of transferable plasmids by *Salmonella* spp. from other enteric bacteria of the gut flora in the intestinal tract of individual patients has previously been reported (3). On the other hand, dissemination of a multiresistant clone over more than 10 years in *Salmonella* serovar Typhimurium DT104 has also been described (25). There are at least two hypotheses to explain the fact that the same antimicrobial resistance determinants, $bla_{CTX\text{-}M\text{-}2}$, Tn*1331*, Tn*9*, *aac*(*3*)-*IIa* and IG1, IG2, or IG3, have been found in the five *Salmonella* serovars from the present study: (i) the dissemination of a clone with identical resistance determinants for each *Salmonella* serovar and/or (ii) plasmid spreading and ulterior selection of certain resistance determinants and integrons by the *Salmonella* genus by independent events, possibly due to the close relationship between the cellular biology of *Salmonella* spp. and the antimicrobial pressure exerted in pediatric patients in every hospital environment. Although dissemination of resistance determinants through integrons within related plasmids over a long period of time in *Salmonella* serovar Typhimurium has been described (6), sequential acquisition of integrons, $bla_{CTX\text{-}M\text{-}2}$, Tn*1331*, and Tn*9* within plasmids and the ulterior spreading of these plasmids cannot be ruled out. Therefore, it is not unlikely that the strains under scrutiny in this study may have acquired resistance genes from a common nosocomial source. Most probably, the combination of hypotheses i and ii may be the most likely explanation for the emergence of multiresistant group R2.

Although the antimicrobial therapy for pediatric inpatients suffering nosocomial infections due to *Salmonella* spp. has not

been significantly modified from 1989 to the present day, evolution of the antibiotic mechanisms in the R2 group, leading to the accumulation of antimicrobial resistance mechanisms after 1993, has been observed. In isolates obtained from that year to 1998, several antimicrobial determinants conferring resistance to the same antimicrobial agent were observed. This feature was seen for ampicillin ($bla_{\text{CTX-M-2}}$, $bla_{\text{OXA-9}}$, $bla_{\text{OXA-2}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{PER-2}}$), ESC ($bla_{\text{CTX-M-2}}$ and $bla_{\text{PER-2}}$), gentamicin [*aac*(*3*)-*IIa* and *ant*(*2″*)-*Ia*], and amikacin [*aac*(*6′*)-*Ib* in class I integrons and in Tn*1331*] resistance in the *Salmonella* sp. strains isolated from nosocomial environments, where high antimicrobial pressure was exerted.

In a previous study (11), Tn*1331* (36) was detected in several *Salmonella* serovars from 1989 to 1991. In this work, we found that this mechanism still plays an important role in the spreading of amikacin resistance since it was detected in the five serovars of isolates dating from 1991 to 1998. In Argentina, as in other countries, antibiotics can be bought over the counter, and it is well known that the natural bacterial gastrointestinal flora of our human population could act as a reservoir for the dissemination of resistance-conferring plasmids (R plasmids). This event could also contribute to the high level of antimicrobial resistance in *Salmonella* spp. isolated from nosocomial infections. In contrast to other opportunistic pathogens, *Salmonella* spp. are causative agents of zoonotic rather than nosocomial infections. In this regard, widespread use of antimicrobial agents in domestic animals of economic importance may have also contributed to increased levels of resistance in *Salmonella* spp.

Although the genus *Salmonella* has been described as having very stable plasmid profiles (38), several studies by others and the results from our research concluded that *Salmonella* serovar Typhimurium isolates as well as the other four serovars acquire and exchange R plasmids in the hospital microflora (1, 37, 38). On one hand similar, if not identical, conjugative R plasmids have been found in *Salmonella* serovar Typhimurium, *E. coli*, and *S. flexneri* strains that were coinfecting the same patients. On the other hand, evolution of R plasmids has been detected, as supported by the pattern found in the first period (1984 to 1988), and the accumulation of resistance determinants found during the second period (1989 to 1998). These findings not only indicate the possible source of antimicrobial resistance mechanisms but also suggest that intergenus exchange of plasmids, as well as accumulation of antimicrobial resistance determinants, is a common event that plays an important role in the evolution of multiresistant nontyphoid *Salmonella* species in Argentina.

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