Original Article

Nontyphoid *Salmonella* gastroenteritis in pediatric patients from urban areas in the city of Mérida, Venezuela

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Abstract

Background: Nontyphoid *Salmonella* (NTS) infections are a frequent cause of self-limited diarrhoeal illness in healthy children that do not usually require antibiotic treatment. This study was conducted by analyzing the phenotypic and genotypic traits of NTS strains from pediatric patients with acute gastroenteritis living in urban areas in the city of Mérida, Venezuela.

Methodology: Thirty-seven *Salmonella* strains (18 *S.* Enteritidis; 14 *S.* Typhimurium; 2 *S*. Java; 2 *S*. Saintpaul; 1 *S*. Infantis) were isolated from 243 stool specimens. These strains were biochemically identified and serotyped. Antimicrobial susceptibility was determined by the disc-diffusion assay. Genetic characterization included plasmid profiling, PCR detection of the *spv* region and *inv* genes, and IS*200* typing.

Results: Thirty (81.0%) of the *Salmonella* isolates were resistant to the antimicrobial tested. Of these strains, 17 (56.7%) were resistant to at least one antibiotic. Five resistance patterns were observed, of which the most frequently found was the single type (tetracycline, streptomycin or ampicillin). All the *S*. Typhimurium harbored plasmids, but only three large plasmids (60, 72 and 84 kb) yielded amplicons with a *spv*R specific primers. All the *Salmonella* serotypes showed the presence of an *inv* region. Eight distinct IS*200* profiles could be detected among the 37 *Salmonella* strains studied.

Conclusions: Predominant Enteritidis and Typhimurium serotypes, as well as serotypes Java, Saintpaul and Infantis, are circulating in the city of Mérida, Venezuela. Most of these strains are susceptible to first-line antibiotics but active monitoring of isolates for antimicrobial resistance is necessary. IS*200* typing, applied in association with conventional methods, allowed the characterization of all isolates and suggested the presence of different infection sources.

Key Words: Nontyphoid *Salmonella;* gastroenteritis; pediatric; *spv*R; *inv*; IS*200*

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Introduction

Nontyphoid *Salmonella* (NTS) is a common cause of gastroenteritis, which is usually a self-limited illness in healthy children that does not require antimicrobial treatment [1]. However, in invasive infections such as meningitis or septicemia, effective antibiotic therapy can be lifesaving [1,2]. As happens with most enteropathogens, there has been an alarming increase of resistance to different antimicrobial agents, and especially to those most commonly used in developing countries, such as ampicillin, trimethoprim/ sulfamethoxazole, chloramphenicol or tetracycline [2,3]. The spread of resistant *Salmonella* strains is a relevant issue for pediatricians, because few therapeutic options are available to treat infants and children [2,4]. More rapid identification of the pathogen and its antibiotic sensitivity pattern could potentially guide antibiotic choice, reducing unnecessary use of expensive second-line antibiotics for susceptible isolates and improving the outcome for multiresistant isolates [3,5,6]. Unfortunately, in Venezuela, limited financial and laboratory resources generally preclude the use of routine stool cultures and antimicrobial drug susceptibility testing to guide therapy. Hence, an empiric therapy is often employed without supporting data and is usually based on the clinical experience of the physician.

Moreover, epidemiological studies of NTS infections are hampered by the lack of adequate procedures. Standard methods, including phenotyping procedures, may not be discriminatory [7]. In recent years, molecular-based techniques, such as plasmid profile analysis, ribotyping, random amplified polymorphic DNA analysis, pulsed-field gel electrophoresis (PFGE), Rep-PCR and IS*200* profiling, have shown to be useful methods for discriminating

among isolates of *Salmonella* species [8-10]. IS*200* is currently considered to be one of the most reliable typing procedures and a suitable epidemiological tool, because it is able to discriminate among strains from different geographic origins and also to relate strains isolated from a given area [8].

The aim of the present study was to determine serotype distribution and resistance patterns among NTS strains isolated from pediatric patients with acute gastroenteritis from urban areas in the city of Mérida, Venezuela, during November 2004 to November 2005. The study further aimed to evaluate the genotypic characteristics of NTS strains causing this infection.

Materials and Methods

Patients and bacterial strains

From November 2004 to November 2005, thirty-seven *Salmonella* strains (18 *S.* Enteritidis; 14 *S.* Typhimurium; 2 *S.* Java; 2 *S.* Saintpaul; 1 *S.* Infantis) were isolated from stool specimens obtained from pediatric patients with acute gastroenteritis from urban areas in the city of Mérida and admitted to the Instituto Autónomo Hospital Universitario de Los Andes (Mérida, Venezuela). The average age of the children studied was three years; 66% were female. The typical duration of illness was six days and cultures were usually obtained within 48 hours of onset. Freshly excreted whole stool specimens were collected from each patient, inoculated into Cary Blair medium (Oxoid Ltd., Basingstoke, UK), and transported to the laboratory within three hours after inoculation. Slides of fecal specimens were stained with Gram and methylene blue and were examined for fecal leukocytes by using light microscopy; specimens with > 5 leukocytes per high-power field were considered positive for inflammatory diarrhoea.

Isolation and biochemical identification of *Salmonella* species were performed using standard procedures. *Salmonella* strains were grouped with polyvalent antisera and serotyped based on somatic O and phases I and II flagellar antigens, by agglutination test with antisera (Bio-Rad and WHO Collaborative Centre for Reference and Research on *Salmonella*) as specified by the White-Kauffman-Le Minor scheme [11].

Antimicrobial susceptibility testing

All isolates were screened for resistance to 16 antibiotics by the disc diffusion method on Mueller-Hinton agar (Oxoid), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [12]. Discs with the following antibiotics (Oxoid) were tested: ampicillin, amoxicillin/clavulanic acid, cephalothin, cefuroxime, ceftazidime, nalidixic acid, ciprofloxacin, chloramphenicol, tetracycline, streptomycin and trimethoprim/sulfamethoxazole.

Plasmid analysis

Plasmid DNA was extracted by the alkaline denaturation method of Birnboim and Doly [13]. Plasmids were electrophoretically separated in 0.8% horizontal agarose gels at 100 V for three hours in Tris-borate-EDTA buffer. Gels were stained with ethidium bromide and photographed under UV illumination. Reference plasmids (RP4, 54 kb; pIPI73, 126 kb) and the supercoiled DNA ladder (Invitrogen Life Technologies, Karlsruhe, Germany) served as size standards.

Detection by PCR of the spv *region and* inv *genes*

PCR was performed with the following sets of primer pairs: PG48 (CCCCGGGAATTCGCTGCATAAGGTCAGAAGG) and PG49 (CCCCGGGATCCAT-GGATTTCTTGATTAATAAA) for the *spv*R gene [14], and INVA-1 (ACAGTGCTCGTTTACGACCTGAAT) and INVA-2 (AGACGACTGGTACTGATCGAT-AAT) for the invasion gene *inv*AE [15]. The reaction mixtures and cycling conditions of amplification were conducted as previously described [14,15]. The amplified products were separated by electrophoreses on 1.2% agarose gels and stained with ethidium bromide (0.5 mg/l); amplicons were visualized and photographed under UV illumination.

Extraction and digestion of cellular DNA

Genomic DNA was extracted as previously described [16] from exponential cultures. DNA was digested with the *Pst*I (Promega, Madison, WI) restriction enzyme known to have no recognition site within the IS*200* sequence.

IS200 profiling

Southern blotting of *Pst*I-cleaved genomic DNA was performed as previously described [17]. The probe was generated by PCR amplification of a 557-bp internal fragment of the IS*200* element, using the IS200-F CCTAACAGGCGCATACGATC and IS200-R ACATCTTGCGGTCTGGCAAC primers [18]. Preparation of the dUTP-11 fluorescein-labeled PCR-generated IS*200* probe, prehybridization, and hybridization were carried out using the ECL random

prime labeling and detection systems (version II; Amersham Biosciences) in accordance with the manufacturers. All hybridizations were performed under highly stringent conditions. Membranes were exposed to X-ray film (ECL Hyperfilm; Amersham Biosciences).

Calculation of discriminatory power

The discriminatory power of the IS*200* profiling was determined by calculating the indices of discrimination (*D*) according to Hunter and Gaston [19].

Statistical analysis

Variables in NTS-susceptible and NTS-resistant isolates were compared using chi-squared test. A value of $p < 0.05$ was considered significant.

Results

A total of 243 pediatric patients with acute gastroenteritis from urban areas in the city of Mérida, Venezuela, were included in the study. Cultures of fecal specimens from 37 children (15.2%) yielded *Salmonella* organisms. *S.* Enteritidis and *S.* Typhimurium were the most frequently isolated serotypes, accounting for 18 (48.7%) and 14 (37.8%) isolates, respectively. The remainder was a distribution of the other serotypes, in which *S.* Java and *S.* Saintpaul accounted for two (5.4%) cases each, and *S.* Infantis was isolated in one case (2.7%). The remaining patients were excluded from microbiologic analysis because a different enteropathogen was identified in the fecal specimens (*Shigella* spp. 25.1%, *Aeromonas* spp. 21.1%, *Campylobacter* spp. 14.5%, enterotoxigenic *Escherichia coli* 9.2% and others 4.6%) or because specimens yielded more than one pathogen (10.3%). The relationship between clinical findings and susceptible or resistant *Salmonella* strains isolated from pediatric patients with acute gastroenteritis are shown in Table 1. Of the 37 strains of *Salmonella* isolates, 30 (81.0%) were resistant to at least one of the antimicrobial agents tested, while seven (18.9%) were susceptible to all antibiotics tested. Diarrhoea and presence of blood and leukocytes in feces were the clinical and laboratory characteristics most frequently observed in the patients. Also, the nutritional conditions of most patients were within normal values and approximately one third of them consumed some type of antibiotic at least two weeks before this study. There was no significant difference in clinical characteristics between the NTS-susceptible and NTS-resistant isolates.

Table 1. Clinical characteristics associated with susceptible or resistant nontyphoid *Salmonella* isolated from pediatric patients with acute gastroenteritis.

	Nontyphoid *Salmonella* N(%)		
Clinical characteristics	**Susceptible 7 (18.9)**	**Resistant* 30 (81.0)**	***p*-value**
Temperature > 38.5^0 C	3(42.9)	19(63.5)	.320
Stool frequency > 5 in 24 hours	7(100.0)	28(93.3)	.601
Liquid stool (without blood or mucus)	0(0.0)	1(3.3)	.624
Presence of blood in stool	5(71.4)	24(80.0)	.620
Presence of mucus in stool	2(28.6)	5(16.7)	.429
> 5 leukocytes/high-power field	5(71.4)	29(96.7)	.028
Nutritional state			
Normal	7(100.0)	25(83.3)	.245
First-degree malnutrition†	0(0.0)	5(16.7)	.245
Receiving antibiotic therapy for any reason in the last two weeks.	2(28.6)	11(36.7)	.686
Reporting diarrhoea in any family member in the last two weeks	1(14.3)	7(23.3)	.601

* Resistant to at least one antimicrobial agent tested.
† Height/weight deficit between 5 and 10% of the normal values.

The findings obtained in the antimicrobial susceptibility assays (Table 2) showed the following results: two (11.1%) of the *S.* Enteritidis strains and all *S.* Java, *S.* Saintpaul and *S.* Infantis strains were susceptible to the antibiotics tested, while all of the *S.* Typhimurium strains were resistant to at least one antibiotic. Five resistance patterns were observed, but the most frequent was the single-type resistance (Tet, Str or Amp). Tetracycline was the resistance marker most frequently observed, both in strains resistant to one or two antibiotics (Tet+Str or Tet+Amp). Regardless of serotypes of *Salmonella* isolated, these strains were susceptible to amoxicillin/clavulanic acid, cephalothin, cefuroxime, ceftazidime, nalidixic acid, ciprofloxacin, chloramphenicol and trimethoprim/ sulfamethoxazole (data not shown).

Of the 37 *Salmonella* strains studied, 30 carried 9.5 to 84 kb plasmids, and of these strains, eight carried other smaller plasmids (2 to 6.5 kb) (Table 3). None of the susceptible *Salmonella* strains were found to have plasmids. All the *S.* Typhimurium harbored plasmids, but only three large plasmids (60, 72 and 84 kb) yielded amplicons with the *spv*R-specific primers and were then assigned to the *Salmonella* virulence plasmid group. All the serotypes of *Salmonella* isolated showed a 457 bp band of amplification confirming the presence of the *inv* region (Table 3).

Table 2. Resistance patterns of *Salmonella* serotypes isolated from pediatric patients with acute gastroenteritis.

Salmonella Serotypes	Total N (%)	Strains S *n* (%)	R *n* (%)	Resistance patterns	*n* (%)
Enteritidis	18 (48.7)	2 (11.1)	16 (88.9)	Tet+Str Tet+Amp Tet	6(37.5) 2(12.5) 8(50.0)
Typhimurium	14 (37.8)	0 (0.0)	14 (100)	Tet+Str Tet+Amp Tet Str Amp	3(21.4) 2(14.3) 7(50.0) 1(7.1) 1(7.1)
Java	2 (5.4)	2 (100)	0 (0.0)	S	_
Saintpaul	2 (5.4)	2 (100)	0 (0.0)	S	_
Infantis	1 (2.7)	1 (100)	0 (0.0)	S	_

Str: streptomycin; Tet: tetracycline; Amp: ampicillin; S: susceptible; R: resistant.

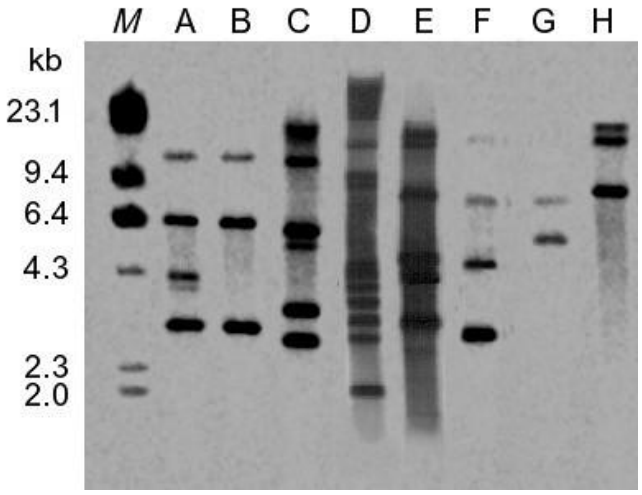
Eight distinct profiles could be detected within the 37 *Salmonella* strains studied (Table 3 and Fig. 1).

Table 3. Characterization of *Salmonella* serotypes isolated from pediatric patients with acute gastroenteritis.

Salmonella serotypes isolate No.	Resistance patterns	Plasmid content (kb)	*invAE* gene	*spvR* gene	IS*200* profile (No. copies)
***S.* Enteritidis**					
SEula 239	Str+Tet	40	+	-	C (6)
SEula 241	Str+Tet	26	+	-	A (4)
SEula 244	Str+Tet	11	+	-	C (6)
SEula 245	Str+Tet	54	+	-	C (6)
SEula 256	Str+Tet	20	+	-	C (6)
SEula 240	Str+Tet	43	+	-	B (3)
SEula 251	Amp+Tet	54/6.5	+	-	A (4)
SEula 259	Amp+Tet	30	+	-	A (4)
SEula 232	Tet	20	+	-	A (4)
SEula 257	Tet	9.5	+	-	B (3)
SEula 261	Tet	20	+	-	B (3)
SEula 249	Tet	11/4.6	+	-	B (3)
SEula 247	Tet	32	+	-	B (3)
SEula 243	Tet	25	+	-	C (6)
SEula 255	Tet	20	+	-	B (3)
SEula 258	Tet	9.5/4.6	+	-	B (3)
SEula 260	S	-	+	-	A (4)
SEula 246	S	-	+	-	B (3)
S. Typhimurium					
STula127	Str+Tet	15	+	-	D (11)
STula235	Str+Tet	20/2.5	+	-	D (11)
STula238	Str+Tet	72	+	+	D (11)
STula252	Amp+Tet	60	+	+	D (11)
STula254	Amp+Tet	84	+	+	E (6)
STula257	Tet	11	+	-	D (11)
STula234	Tet	25/4.6	+	-	D (11)
STula236	Tet	9.5/2.0	+	-	D (11)
STula237	Tet	40	+	-	D (11)
STula262	Tet	25	+	-	D (11)
STula266	Tet	43	+	-	E (6)
STula278	Tet	40/6.5	+	-	E (6)
STula233	Str	10	+	-	E (6)
STula231	Amp	11/2.0	+	-	E (6)
S. Java					
SJula273	S	-	+	-	F (4)
SJula275	S	-	+	-	F (4)
S. Saintpaul					
SSula270	S	-	+	-	G (2)
SSula271	S	-	+	-	G (2)
S. Infantis					
SIula121	S	-	+	-	H (3)

Str: streptomycin; Tet: tetracycline; Amp: ampicillin, S: susceptible, +: positive detection, -: negative detection.

Figure 1. IS*200* profiles of representative *Salmonella* serotypes isolated from pediatric patients with acute gastroenteritis. Lanes **A** to **C**, *S.* Enteritidis (SEula 241, SEula 258, and SEula 245, respectively); lanes **D** and **E**, *S.* Typhimurium (STula238 and STula254, respectively); lane **F**, *S.* Java (SJula273); lane **G**, *S.* Saintpaul (SSula271) and lane **H**, *S.* Infantis (SIula121). ***M***, molecular weight marker λ*Hin*dIII.



The number of copies of the IS*200* element was 11 in profile D and 2 in G. Profiles A, B and C belonged to *S.* Enteritidis strains. Of these profiles, B was the most frequent, occurring in eight of the 18 isolates of *S.* Enteritidis, whereas profiles A and C were observed in each of five strains of this serotype. The IS*200* element was found in restriction fragments of approximately 10 kb and 6.4 kb and were a common feature in these three profiles (A, B and C). Profiles D and E belonged to *S.* Typhimurium strains. Nine of the 14 serotype Typhimurium strains were included in profile D. *S.* Java, *S.* Saintpaul and *S.* Infantis displayed 3 profiles (F, G and H), and these strains had 4, 2 and 3 copies of IS*200*, respectively.

The discrimination index, determined by combining serotyping and IS*200* typing of the 37 NTS isolated, was 0.856.

Discussion

In this study, the most frequent serotypes of *Salmonella* causing acute gastroenteritis in children were *S.* Enteritidis (48.7%) and *S.* Typhimurium (37.8%). These results are in line with those described by Hohmann [1] and de Jong and Ekdahl [20], in which the dominant serotypes causing food-borne gastroenteritis in humans are *S.* Enteritidis and *S.* Typhimurium. Bloody dysentery, the most pathognomonic feature of salmonellosis, was observed in the majority of children studied. None of the patients developed extraintestinal complications.

The emergence of antimicrobial resistance within *Salmonella* species has been reported worldwide [2]. In the present study, a strong prevalence of antibiotic-resistant *Salmonella* was observed (81.0%). Similar to previous reports [21-23], *S.* typhimurium was the most frequent resistant serotype. Although *S.* Enteritidis is usually reported sensitive to antibiotics [2], we found that this serotype was resistant in 17 (88.9%) cases. Regardless of *Salmonella* serotype, resistance to tetracycline was the most commonly observed. This antibiotic is not routinely used in pediatric patients in our country. Tetracycline is one of the drugs largely used in animals, reinforcing the commonly accepted view that the use or abuse of veterinary antibiotics is the leading cause of drug resistance spreading among *S. enterica* strains [4,22]. It is noteworthy that the Enteritidis and Typhimurium serotypes remained susceptible to cephalosporins, chloramphenicol, and other antibiotics that are usually considered as first-line treatment for severe or invasive infections caused by *Salmonella*. In contrast with other studies [6,24-27], *S.* Java, *S.* Saintpaul and *S.* Infantis were fully susceptible to the antibiotics tested. Although different susceptibility patterns may arise from a number of factors, it is possible that differences in the susceptibility patterns among the studied serotypes of *Salmonella* can be ascribed both to different reservoirs and to the sources in which these strains were isolated (sporadic cases of gastroenteritis). Hence, a continuous monitoring of both antimicrobial resistance and deliberate use of antimicrobial agents in animals and humans are essential.

The virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors. The chromosomally located invasion gene *inv*A, involved in cellular invasion, was present in all studied serotypes of *Salmonella*. These results agree with those of other authors who have found that this gene is a good target for detecting *Salmonellae* [15,28,29]. Plasmid profiling is a traditional method used for epidemiological studies in *Salmonella* strains within specific serotypes [9]. However, in this study, all resistant serotypes carried plasmids of different sizes and the frequency of an additional small plasmid was low. None of the susceptible strains harbored plasmids. Nevertheless, further studies will be necessary to know if the resistance observed is due to these plasmids. Because the results from plasmid profiling did not represent a discriminatory method for subtyping these strains, the plasmids detected were of little, if any, epidemiological importance, as reported in previous studies [30,31].

The true prevalence of virulence plasmids among natural isolates of *Salmonella* is unknown [31]. In this study, only three resistant Typhimurium serotypes carried virulence plasmids (*spv*R positive). Some studies support that plasmid-encoded *spv* genes enhance the ability of certain serotypes of *Salmonella* to produce severe extraintestinal disease [33,34], but these were not observed in the group of patients studied. It is possible that one or more factors, related to the genetic background of these isolates, is required to obtain virulence in strains carrying the *spv*-encoding virulence plasmid.

Molecular typing has enhanced the understanding of the epidemiology of *Salmonella* infections by increasing our knowledge of the genetic relationships among strains [7-10]. The molecular approaches used in this work allowed a more sensitive characterization of these isolates. Determination of the number of copies and localization of IS*200* fragment insertion showed a variety of profiles in the studied strains, including those belonging to the same serotype (Enteritidis and Typhimurium). Enteritidis serotype strains showed two common bands in IS*200* profiles, indicating that these particular strains might be clonally related. However, resistance patterns and IS*200* profiles did not show a clear correspondence. The IS*200* typing seems to provide a more reliable molecular marker for epidemiological studies, because it clearly discriminated among strains of different serotypes isolated from pediatric patients with gastroenteritis.

In addition, the presence of various serotypes with different phenotypic and genotypic characteristics suggests that the infection originated from multiple sources. It is possible that the presence of Enteritidis and Typhimurium serotypes is due to human-to-human transmission, while Java, Saintpaul and Infantis serotypes could be introduced into the community by food.

In summary, this study showed that predominant Enteritidis and Typhimurium serotypes, as well as Java, Saintpaul and Infantis are circulating in urban areas in the city of Mérida, Venezuela, and also that most of these strains are susceptible to first-line antimicrobial agents used to treat severe salmonellosis in children. Nevertheless, active monitoring of serotypes isolated for antimicrobial resistance is necessary because of the public health implications of a potential spread of resistance clones. IS*200* typing, applied in association with conventional methods, allowed a better characterization of all isolates and suggested the presence of different infection sources. Furthermore,

our findings indicate that enteric infections by nontyphoidal salmonellae continue to pose a major threat to children in Mérida, Venezuela. Identification of sources of human infection, early clinical and microbiological diagnosis, appropriate treatment, education about basic food hygiene, and implementation of a strict sanitary control of the food industry are all necessary to reduce the prevalence of salmonellosis.

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