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ANALYSIS OF THE CLONAL RELATIONSHIP AMONG CLINICAL ISOLATES OF *Salmonella enterica* SEROVAR INFANTIS BY DIFFERENT TYPING METHODS

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SUMMARY

Salmonella Infantis has been the second most common serovar in Argentina in the last two years, being isolated mostly from paediatric hospitalised patients. In order to determine the clonal relationship among *Salmonella* Infantis strains, we examined 15 isolates from paediatric patient faeces in Argentina (12 geographically related and 3 geographically non-related) by using antimicrobial susceptibility, plasmid profiling, repetitive extragenic palindromic (REP) PCR, enterobacterial repetitive intergenic consensus (ERIC) PCR, and low-frequency restriction analysis of chromosomal DNA by pulsed field gel electrophoresis (PFGE). Four Spanish strains were included as controls of clonal diversity in molecular techniques. Antibiotype and plasmid profile was not useful as epidemiological tools. PFGE and REP-PCR were able to discriminate between Argentinean and Spanish isolates of *Salmonella* Infantis allowing to detect genetically related strains in three different cities. This finding indicates that a possible spread of a clone of this serovar in the North-eastern Region of Argentina has taken place in 1998.

KEYWORDS: *Salmonella* Infantis; Typing; Pulse Field Gel Electrophoresis, Polymerase Chain Reaction; Argentina.

INTRODUCTION

Infections caused by *Salmonella* strains can produce symptoms ranging in severity from intestinal disturbances to death, especially in neonates and immunocompromised patients[15]. *Salmonella* Infantis has been the second most common serovar in Argentina in the last two years, being isolated mostly from paediatric hospitalised patients[25]. In order to have an effective surveillance and to develop rational control strategies for this important human disease, the availability of detailed and accurate data related to the epidemiology of *Salmonella* is crucial.

Various typing techniques have been used in epidemiological studies to differentiate isolates of *Salmonella* serovars, but only a few of them have been used to discriminate *Salmonella* Infantis strains, since this serovar is infrequently encountered causing human disease in developed countries[8,17,23]. The applied epidemiological tools include biotyping, phage typing, colicine typing, antimicrobial susceptibility testing, plasmid profiling, restriction endonuclease analysis of whole chromosomal DNA by pulsed field gel electrophoresis (PFGE), repetitive extragenic palindromic (REP) sequences analysis by PCR, enterobacterial repetitive intergenic consensus (ERIC) analysis by PCR, restriction fragment length polymorphism (RFLP) of 16S rRNA and insertion sequence IS200[13,14,22].

In the present study, we examined strains of *Salmonella* Infantis isolated in the Northeast of Argentina from paediatric patients by several typing methods and evidence for the clonal spread of genetically related strains is presented.

Table 1
Dates and cities of isolation of *Salmonella* Infantis strains

Strain	City of origin of isolates	Date of isolation (mm/dd/yy)
SP30	Pcia. R. Sáenz Peña	02/04/98
SP31	Pcia. R. Sáenz Peña	02/11/98
SP32	Pcia. R. Sáenz Peña	02/17/98
SP33	Pcia. R. Sáenz Peña	02/19/98
SP34	Pcia. R. Sáenz Peña	02/23/98
SP94	Pcia. R. Sáenz Peña	08/10/98
SP97	Pcia. R. Sáenz Peña	08/26/98
SP98	Pcia. R. Sáenz Peña	08/30/98
SP99	Pcia. R. Sáenz Peña	09/01/98
SP100	Pcia. R. Sáenz Peña	09/03/98
SP102	Pcia. R. Sáenz Peña	09/04/98
SP103	Pcia. R. Sáenz Peña	09/04/98
HV141	Corrientes	03/08/98
HV2	Corrientes	02/17/98
HG1	Juan José Castelli	12/15/97

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MATERIALS AND METHODS

Bacterial strains: Among 29 isolates of *Salmonella* Infantis obtained from paediatric patients faeces during 1998 in the Northeast of Argentina, 15 strains were examined in this study. Twelve strains (SP30 to SP103) were isolated in Presidencia Roque Sáenz Peña, Province of Chaco. Three epidemiologically unrelated strains were included in this study for comparison: one strain (HG1) was isolated in Juan José Castelli, Province of Chaco, and two strains (HV2 and HV141) were collected in Corrientes, Province of Corrientes. Additionally, four Spanish clinical strains provided by the Spanish Collection of Type Cultures (University of Valencia, Spain) were included as controls of clonal diversity. Bacteria were identified to specie level by conventional methods[2]. The Argentinean strains were serotyped in the Service of Enterobacterias of National Reference Laboratory in Buenos Aires (Argentina).

Patients: All patients from which the isolates were obtained were hospitalised and their ages ranged from 0 to 2.25 years (Average: 8.2 months). Only four of them had potable water provision in their houses.

Antimicrobial susceptibility tests: were performed by an agar diffusion disk method according to the standards outlined by the National Committee for Clinical Laboratory Standards[20]. The commercial disks (Britania Laboratories, Buenos Aires, Argentina) used were: ampicillin 10 µg (AMP), cephalothin 30 µg (CEP), cefotaxime 30 µg (CTX), neomycine 30 µg (NEO), gentamicin 10 µg (GEN), tetracycline 30 µg (TET), furazolidone 300 µg (FUR), chloramphenicol 30 µg (CMP), trimethoprim/sulfamethoxazole 1.25/23.75 µg (TMS), nalidixic acid 30 µg (NAL), ciprofloxacin 5 µg (CIP), colistin 10 µg (COL), and fosfomycin 50 µg (FOS). *Escherichia coli* 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212 were tested as quality control organisms.

DNA extraction: Plasmids were extracted by the alkaline lysis described previously by KADO & LIU[7] and modified by NAKAMURA *et al*.[19]. For each PCR-based technique, 25 µl of boiled bacterial suspension were used according to GALLARDO *et al.*[4]. The DNA for low-frequency restriction analysis by pulsed field gel electrophoresis was prepared as described previously by MATUSHEK *et al.*[12].

Plasmid profiling: Extracted plasmid DNA was electrophoresed on 0.7% horizontal agarose gels (BioRad) in Tris-Acetate-EDTA buffer (Sigma) for 16 h to 20 V, and stained with ethidium bromide solution (0.5 µg/ml). The molecular sizes of the plasmids were assessed by comparison with plasmids from *Escherichia coli* V517.

Repetitive extragenic palindromic (REP) PCR: REP-PCR fingerprinting was carried out following the method previously described by GALLARDO *et al*.[4] using the primer 5'-GCG CCG ICA TGC GGC ATT-3' (MWG-Biotech). Samples of each PCR end-product were analysed on agarose 1.5% gels containing ethidium bromide 0.5 µg/ml. Four Spanish strains were included for comparison.

Enterobacterial repetitive intergenic consensus (ERIC) PCR: ERIC-PCR was carried out by the method described by BEYER *et al*.[1]: 1 initial cycle at 94 ºC for 1 min, 30 cycles of denaturalization at 95 ºC for 1 min, annealing at 52 ºC for 1 min, and extension at 65 ºC for 8 min, with a single final extension step at 65 ºC for 16 min. We used the following primers (MWG-Biotech): ERIC1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3'). Samples of each PCR end-product were analysed on agarose 1.5% gels containing ethidium bromide 0.5 µg/ml. Two Spanish strains were also included for comparison.

Low-frequency restriction analysis of chromosomal DNA by pulsed field gel electrophoresis (PFGE): Total genomic DNA was digested with *Xba*I (Promega) and separated in 1% agarose gels with a contour-clamped homogeneous-field apparatus (CHEF-DRIII, Bio-Rad). It was run under 200 V, with the pulse time increasing from 5 to 8 for 20 h. This study was repeated using *Xho*I (Pharmacia Biotech) as restriction enzyme, under the conditions described above. Two strains of Spanish *Salmonella* Infantis were included for comparison.

Fingerprints interpretation: Analysis of the patterns was performed by visual inspection. Two isolates were said to have the same electrophoretic profile when their band patterns were identical. Minor differences in band intensity were not considered. PFGE patterns were interpreted according to the criteria suggested by TENOVER *et al.*[27]. Isolates were considered genetically indistinguishable if they possessed PFGE patterns with the same number and same size of bands. Closely related strains differed by changes consistent with a single event (2 or 3 bands differences) or two independent events (4 to 6 band differences), respectively. Unrelated strains differed by three or more independent genetic events (≥ 7 band differences).

RESULTS

Resistance to more than one of the antimicrobial agents tested was detected in all of the isolates studied. All strains were resistant to AMP and CEP and were susceptible to NAL, CIP, TET, CMP, NEO, FOS and COL. Since the isolates presented variable susceptibilities to CTX, GEN, and FUR, these drugs were used for comparison between strains.

Three different plasmid profiles, each including one or two plasmids with approximate molecular sizes ranging from 54 to 130 Kb were found in the 15 strains analysed. 130 Kb plasmid was the more frequent. The relationship between plasmid profiles and antimicrobial resistance patterns to selected drugs is shown in Table 2.

Fingerprinting with REP1 primer generated identical patterns between Argentinean strains but different to those of Spanish isolates (Fig. 1).

Patterns obtained with ERIC-PCR methods showed few clearly visible bands and other faint bands among both Argentinean and Spanish strains, which are not enough to differentiate them (Fig. 2).

Fingerprinting with *Xba*I digestion produced 14 fragments (Fig. 3) and with *Xho*I digestion produced 15 fragments, all of them clearly distinguishable. The patterns generated by PFGE *Xba*I digestion were identical for all the Argentinean strains despite their geographical origin or date of isolation. However, the Spanish controls did produce two different patterns (Results not shown). Similar results were obtained after *Xho*I digestion. The patterns obtained with *Xba*I and *Xho*I were stable and reproducible when repeated analysis of these strains was performed under identically conditions of digestion and electrophoresis.

Table 2
Relationship between antibiotypes and plasmid profiles of *Salmonella* Infantis strains

Antibiotypes	Approx. plasmid sizes (Kb)	Strains
(CTX^R GEN^R FUR^R)	130	SP30, SP31, SP32, SP33, HV2, SP102
	130, 70	SP97, SP98, SP100
(CTX^S GEN^S FUR^S)	130	HV141
(CTX^S GEN^R FUR^S)	130, 70	SP99
(CTX^S GEN^R FUR^R)	130, 70	HG1
	54	SP34, SP94, SP103

CTX: cefotaxime, GEN: gentamicin, FUR: furazolidone, S: susceptible, R: resistant.

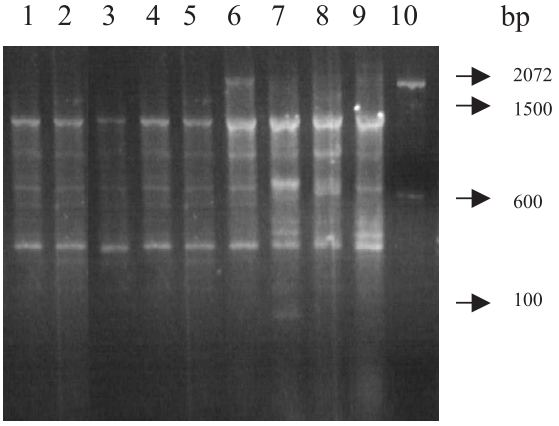

Fig. 1 - REP PCR-based molecular typing. Lanes 1-5: Selected Argentinean strains; lanes 4-7: Spanish strains; lane 8: 100 bp DNA Ladder

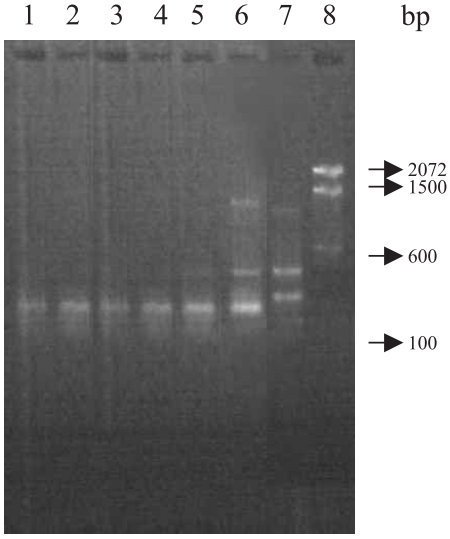

Fig. 2 - ERIC PCR-based molecular typing. Lanes 1-5: Argentinean strains; lanes 6-7: Spanish strains; lane 8: 100 bp DNA Ladder

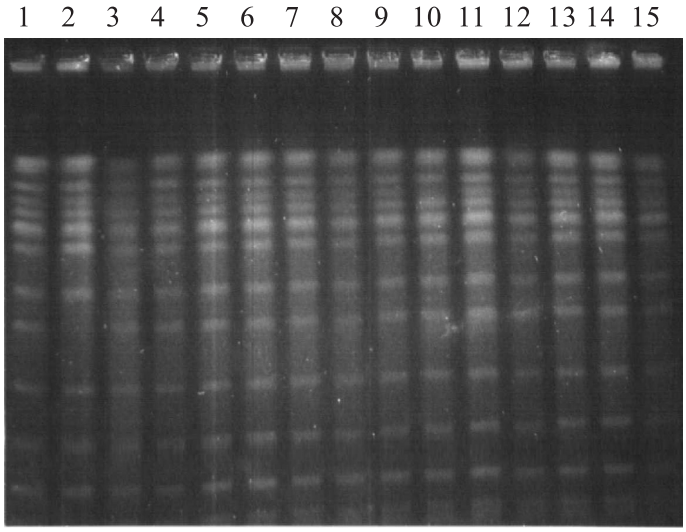

Fig. 3 - PFGE patterns of *Xba*I-digested chromosomal DNA from Argentinean strains. Lanes 1 to 3: strains HV141, HV2, and HG1. Lanes 4 to 15: strains SP30, SP31, SP32, SP33, SP34, SP94, SP97, SP98, SP99, SP100, SP102, and SP103.

DISCUSSION

Numerous papers about the clonal relationship between endemic *Salmonella* strains or between isolates involved in outbreaks can be encountered in scientific publications[5,6] but to our knowledge, this represents the first study in Argentina in which a wide variety of epidemiological tools are applied to study *Salmonella* Infantis human isolates.

Several investigators have used the antimicrobial susceptibility typing of *Salmonella* strains for an epidemiological purpose[3,9]. However, we found that antimicrobial resistance was not very specific as an epidemiological marker due to the variability between the resistance profiles obtained.

Plasmid pattern analysis has been widely applied for the characterisation of epidemic strains and several authors[13,16,24]. Nevertheless, in our study the same plasmid profile was related with different antimicrobial resistance profile and vice versa and this could be due to the plasmids can be gained or loosed along a wide period of time[10].

PCR-mediated genome fingerprinting based on ERIC or REP has been found useful for the typing of outbreak and sporadic *Salmonella* isolates. VERSALOVIC *et al.*[28] used consensus PCR primers to amplify the REP and ERIC sequences in several bacterial species which were later applied by other investigators on several *Salmonella* serovars[1,21]. In our work, REP amplification data revealed the genetic homogeneity of epidemiologically related and geographically unrelated Argentinean strains, while Spanish strains showed four different banding patterns.

ERIC-PCR method was applied by several authors with good results among other serovars of *Salmonella* but not among *Salmonella* Infantis strains[1,5]. We found that this method, as it has been described previously, was not able to differentiate the strains studied, because the patterns obtained presented few clearly visible bands. May be this technique must

be modified to find the most adequate conditions for *Salmonella* Infantis typing, but it is not the focus of this work.

The most powerful tool for discrimination of even closely related bacterial isolates has been reported to be the macrorestriction analysis of whole DNA by pulsed field gel electrophoresis (PFGE)[21]. Several restriction enzymes have been used for *Salmonella* strains typing, but not all are convenient for the analysis of *Salmonella* Infantis serovar[11,18]. *Xba*I is the most commonly used because it produces few and easily interpretable fragments.

In this work, all Argentinean strains, without regard to their origin, displayed identical PFGE patterns although different to those shown by the Spanish strains. MURAKAMI *et al*.[18] studied the genetic diversity among human and environmental *Salmonella* Infantis strains by PFGE, obtaining 35 distinct profiles and WEGENER & BAGGESEN[29] obtained 21 different PFGE profiles among *Salmonella* Infantis strains when studying 135 isolates from various sources. These findings support the fact of the clonal variability of *Salmonella* Infantis isolates.

At the start of this study, we expected that strains HG1, HV2 and HV141 would be epidemiologically different from the rest of the strains (SP30 to SP103), given their temporal and geographical diversity. However, their close genetic relatedness to the *Salmonella* Infantis strains isolated in Chaco and Corrientes is clearly demonstrated by the molecular methods applied. The cities of Juan José Castelli and Corrientes are distant 125 and 195 km, respectively, of Presidencia Roque Sáenz Peña. That is why it seems as though a single strain has been spread throughout a region of Argentina during a long lapse of time. A similar finding was reported by SULAKVELIDZE *et al.*[26] when they studying two apparently distinct outbreaks in the Republic of Georgia.

In conclusion, antibiotype and plasmid profile was not useful as epidemiological tools. PFGE and REP-PCR were able to discriminate between Argentinean and Spanish isolates of *Salmonella* Infantis allowing to detect genetically related strains in three different cities. This finding indicates that a possible spread of a clone of this serovar in the North-eastern region of Argentina has taken place in 1998.

RESUMEN

Análisis de la relación clonal entre aislamientos clínicos de *Salmonella enterica* serovar Infantis mediante diferentes métodos de tipificación

Salmonella Infantis ha sido el segundo serovar más común en la Argentina en los últimos dos años, siendo aislada principalmente, a partir de pacientes pediátricos hospitalizados. La relación clonal entre 15 aislamientos de *Salmonella* Infantis obtenidos de heces de pacientes pediátricos en Argentina se estudió mediante la susceptibilidad antimicrobiana, el perfil plasmídico, amplificación por reacción en cadena de la polimerasa (PCR) de las secuencias repetitivas REP y ERIC, y electroforesis de ADN total en campo pulsátil (PFGE). Cuatro cepas españolas fueron incluidas como control de diversidad clonal. El antibiotipo y el perfil plasmídico no fueron herramientas útiles en la tipificación. PFGE y REP-PCR fueron capaces de discriminar entre las cepas argentinas y españolas de *Salmonella* Infantis, permitiendo detectar cepas genéticamente relacionadas en tres ciudades diferentes. Este hallazgo indica que una posible diseminación clonal de este serovar ha tenido lugar en la región nordeste de Argentina en 1998.

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