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EPIDEMIOLOGICAL RELATIONSHIPS AMONG STRAINS OF *Salmonella enterica* subsp. *enterica*, ISOLATED FROM HUMANS, POULTRY AND FOOD

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ABSTRACT

Human gastro-enteritis caused by *Salmonella enterica* is a major health problem in developing countries such as Colombia. In some parts of Colombia, the disease is endemic, and its incidence appears to be increasing, with outbreaks and sporadic cases of diarrhea becoming more frequent. At this time, it is not very clear if either poultry or food is responsible for human salmonellosis contamination in Colombia. The objectives of the present study were to analyze the Pulsed-field gel electrophoresis profiles (PFGEPs) of *Salmonella enterica* from human patients, poultry and food found in Colombia and to determine the epidemiologic associations between these strains. Twenty-nine isolates of *Salmonella enterica* subsp. *enterica* were isolated from: 10 pediatric patients in Bogotá, D.C., 10 different types of food and 9 chickens. All isolates were analyzed by means of the molecular technique *Xba*I PFGE. Eleven different patterns were observed. These patterns consisted of 12-17 restriction fragments, each with a molecular size of 30-800 kb. The results suggested that *Salmonella enterica* was transmitted from poultry and food to humans. Surprisingly, among the strains investigated it was impossible to find a direct linkage between poultry and food, indicating, either that *Salmonella* was incorporated into the food during food processing by handlers, or that foods other than poultry products were the source of human infection. This study about the molecular epidemiology of *Salmonella enterica* in Colombia provided new information about possible means of human contamination, and should permit health institutions to take adequate measures to avoid sporadic cases and outbreaks of salmonellosis.

Key words: *Salmonella enterica* subsp. *enterica*, PFGE, epidemiology.

RESUMEN

La gastroenteritis humana causada por *Salmonella enterica* es el mayor problema de salud en los países en desarrollo como Colombia. En algunos lugares de Colombia, la enfermedad es endémica, y su incidencia parece ir en aumento, con brotes y casos esporádicos de diarrea cada vez más frecuentes. En la actualidad no está totalmente claro si algunos alimentos y el pollo son responsables de la salmonelosis humana en Colombia. Los objetivos del presente estudio fueron analizar los patrones de Electrofóresis en Gel de Campo Pulsado (PFGE) de *Salmonella enterica*, aislada de pacientes humanos, pollos y otros alimentos colombianos y determinar la asociación epidemiológica entre estas cepas. Veintinueve aislamientos de *Salmonella enterica* subsp. *enterica* fueron obtenidos de 10 pacientes pediátricos en Bogotá, D.C., 10 a partir de diferentes tipos de alimentos y 9 a partir de pollos. Todos los aislamientos fueron analizados a través de la técnica molecular PFGE- *Xba*I. Se observaron once patrones diferentes. Los patrones presentaron entre 12 a 17 fragmentos de restricción, con rangos de tallas moleculares entre 30-800kb. Los resultados sugieren que *Salmonella enterica* subsp. *enterica* es transmitida de pollos y alimentos a los

humanos. Sorpresivamente entre las cepas de *Salmonella* investigadas fue imposible encontrar una relación directa entre derivados del pollo y el resto de alimentos, indicando que *Salmonella* es incorporada en los alimentos a través de los manipuladores durante el procesamiento, o que otros alimentos diferentes a los derivados del pollo son la fuente de la infección humana. Este estudio sobre epidemiología molecular de *Salmonella enterica* en Colombia provee nueva información acerca de las posibles vías de contaminación humana y deberá permitir a las instituciones de salud tomar las medidas adecuadas para evitar los casos esporádicos y los brotes de salmonelosis.

Palabras clave: *Salmonella enterica* subsp. *enterica*, PFGE, epidemiología.

INTRODUCTION

Studies in Colombia have shown that enteropathogenic *Escherichia coli*, *Salmonella* and *Shigella* are the most common bacterial causes of diarrhea in children (Máttar *et al.*, 1997b; Máttar and Vásquez, 1998). According to the Ministry of Health of Colombia, Local Sanitation and Census Information Office, morbidity among infants during 1993 in Bogotá, D.C., was 483.000 cases of diarrhea (Máttar *et al.*, 1997a). *Salmonella enterica* serotypes cause a food- borne human infection that results in gastroenteritis (Agasan *et al.*, 2002). Symptoms appear within 6-24h after ingestion of contaminated food or water, and last for as long as 1 week. Even if diarrhea disappears, the infected person will continue to excrete bacteria for up to 3 months, but in a small percentage of cases an infected person can continue to shed the bacteria for more than 1 year. Outbreaks have involved many different types of food, but the most commonly implicated foods are milk and poultry products (Hong *et al.*, 2003). Because many farm animals carry *Salmonella enterica* in their intestinal tracts, slaughterhouse by-products are heavily contaminated (Salyers and Whitt, 1994). In Colombia, poultry farms are located primarily in four regions; one of them is adjacent to Bogotá, D.C., the capital of Colombia. *Salmonella* typing from different sources has been conducted using several molecular methods to improve the identification of diarrhea infection and to differentiate strains beyond the level of precision available through serotyping. In all cases, PFGE has been useful in discriminating among isolates of various species of *Salmonella* (Olsen *et al.*, 1994; Puohiniemi *et al.*, 1997; Ruiz *et al.*, 1997; Ridley *et al.*, 1998). A previous report from Thailand using *Xba*I PFGE to analyze 302 *Salmonella* strains from human patients, food, and chickens showed that some sporadic human *Salmonella* infections were due to the consumption of contaminated broiler chicken meat (Boonmar *et al.*, 1998). Furthermore, PFGE with *Xba*I and *Spe*I supported an epidemiologic association and also suggested a transmission pathway between food, food handlers, and patrons in a restaurant in suburban Boston, USA (Lee *et al.*, 1998). Here we report the use of PFGE in Colombia to establish a possible chain of transmission for *Salmonella enterica* from poultry or food to humans.

MATERIALS AND METHODS

Bacterial strains. Human strains (n=10) used in this study were previously isolated in Bogotá, D.C., Colombia, from fecal samples of pediatric patients with sporadic diarrhea; table 1 (Díaz *et al.*, 1998). Poultry strains (n=9) were obtained from chicken farms and food strains (n=10) were taken from different sources and geographic areas in Bogotá, D.C. (north, downtown, south). All isolates were formally identified as *Salmonella enterica* subsp. *enterica* by biochemical and serological procedures (Díaz *et al.*, 1998).

PFGE. Genomic DNA was prepared in agarose plugs and treated by lysis and ESP (EDTA, deoxycholate and proteinase K) solution, as described previously (Díaz *et al.*, 1998). Slices of agarose blocks containing DNA were digested for 4h with 30U of *Xba*I (Promega, Co., Madison, WI, USA) according to the manufacturer's instructions. Resultant DNA fragments were separated in an agarose 1% (w/v) gel (Pharmacia, Biotech, Uppsala, Sweden) that was prepared and run in 0.5X TBE. Pulsed-field gel electrophoresis was performing using a Pharmacia electrophoresis system (Gene Navigator, Pharmacia, Biotech, and Uppsala, Sweden). The operating conditions were 180V at 12°C for 22h. Pulse times were increased stepwise, as follows: 5s for 5h, 15s for 5h, 25s for 6h and 45s for 6h. Gels were stained with 0.5µg/ml ethidium bromide for 30m, followed by 30m distaining in water, and photographed under UV-light (Díaz *et al.*, 1998). To interpret the PFGE profiles and to transform these patterns into epidemiologically useful information, we followed the guidelines suggested by Tenover *et al.*, (1995).

Dendrogram analysis. To establish the degree of relationship between strains, we conducted a hierarchical cluster analysis using SPSS for MS Windows release 6.1 (1997, SPSS, Inc, Chicago Illinois, USA). Each *Xba*I fragment was assigned a value of one (Present) or zero (absent).

RESULTS

All strains used in this study were classified according to their *Xba*I PFGE profile (table 1). The 10 isolates from pediatric patients

Table 1
XbaI* PFGE classification of 29 strains of *Salmonella enterica

Profile	No. of bands	No. of isolates/ Total number per group	Frequency %	Source	Location[a]
H1	14	2/10	20	Human feces	All[b]
H2	13	4/10	40	Human feces	All
H3	16	3/10	30	Human feces	South
H4	17	1/10	10	Human feces	South
P1	13	5/9	55	Poultry feces	Suburb[c]
P2	12	1/9	11	Poultry feces	Suburb
P3	12	1/9	11	Poultry feces	Suburb
P4	12	1/9	11	Poultry feces	Suburb
P5	14	1/9	11	Poultry feces	Suburb
F1	16	5/10	50	Beef	South
				Pork sausage	North
				Pork ham	
				Cheese pancake	
				Chicken pie	Downtown
F2	16	3/10	30	Egg & chicken pie	Downtown
				Chicken liver	
F3	15	1/10	10	Pork sausage	South
F4	14	1/10	10	Cheese pancake	North

H3-F1 (HF group), H2-P1 (HP group).
[a] Referred to Bogotá, D.C.
[b] South, Downtown and North.
[c] From chicken farms.

with sporadic diarrhea showed 4 different PFGE types (figure 1), with 13 to 17 bands each and molecular sizes between 30-800kb. Strains from poultry showed 5 *Xba*I PFGE profiles (figure 2), with fewer *Xba*I bands (12 to 14) but with sizes still between 30 and 800kb. The ten strains from food were grouped into 4 electrophoretic types (figure 3) consisting of 14-16 fragments each, ranging from 30 to 800kb in size. Two clusters with the same *Xba*I PFGE profile were observed and corresponded to H2 - P1 (HP-13 bands) and H3 - F1 (HF-16 bands) (figure 4). Other profiles (F2, P2, P3 and P4) presented different band patterns (12 fragments and 16 fragments) H1, H4, P5, F3, y F4.

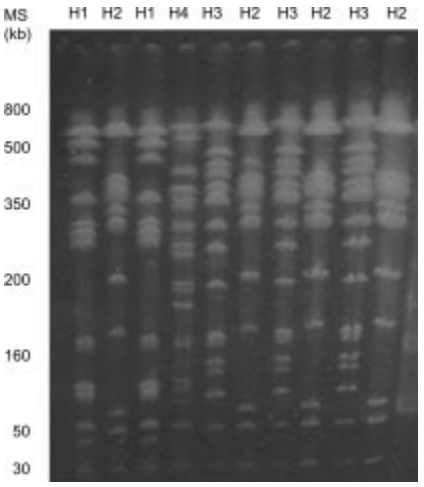

FIGURE 1. PFGE patterns from human isolates.

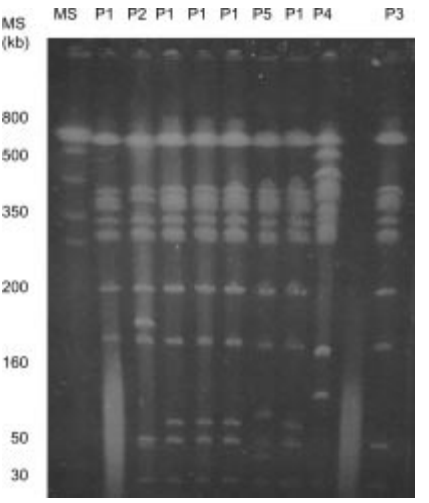

FIGURE 2. Poultry isolates.

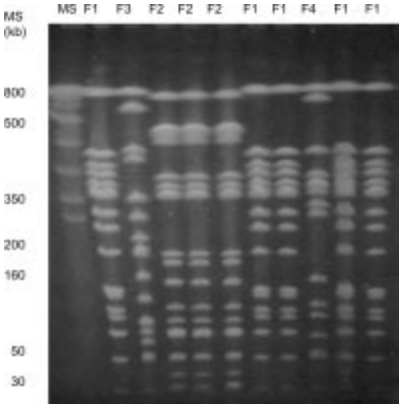

FIGURE 3. PFGE patterns from food isolates.

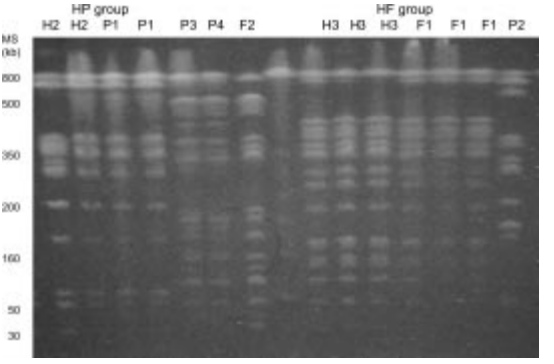

FIGURE 4. Human, poultry and food isolates.

DISCUSSION

PFGE allowed the resolution of *Xba*I fragments from 29 strains isolated in Colombia into 11 distinct types (table 1, figure 5) revealing the extraordinary genetic variability found in *Salmonella enterica*. For the several PFGE types, the differences in band number could not be explained by a single genetic mutation, deletion or insertion. It could possibly involve, however, additional genetic events or a different clonal origin. H2, one of the most prevalent human profiles, was previously reported as A (Díaz *et al.,* 1998); A1 and A2 profiles were not included in our study, but we enhanced the PFGE resolution and assigned a greater number of *Xba*I bands for each electrophoretic type. According to Tenover *et al.*, (1995), PFGE types that differ by more than 7 bands, as did H1, H2, H3 and H4 H1, H2, H3 and H4, must be considered as unrelated. The differences within poultry and food profiles observed here indicated that these profiles could either be related or unrelated. When we made a cross analysis between all PFGE types, we could cluster the H3 and F1 patterns (HF group) and the H2 and P1 patterns (HP group) since they had the same *Xba*I fragments that belonged to the same clone (figure 4). In the HF group, a

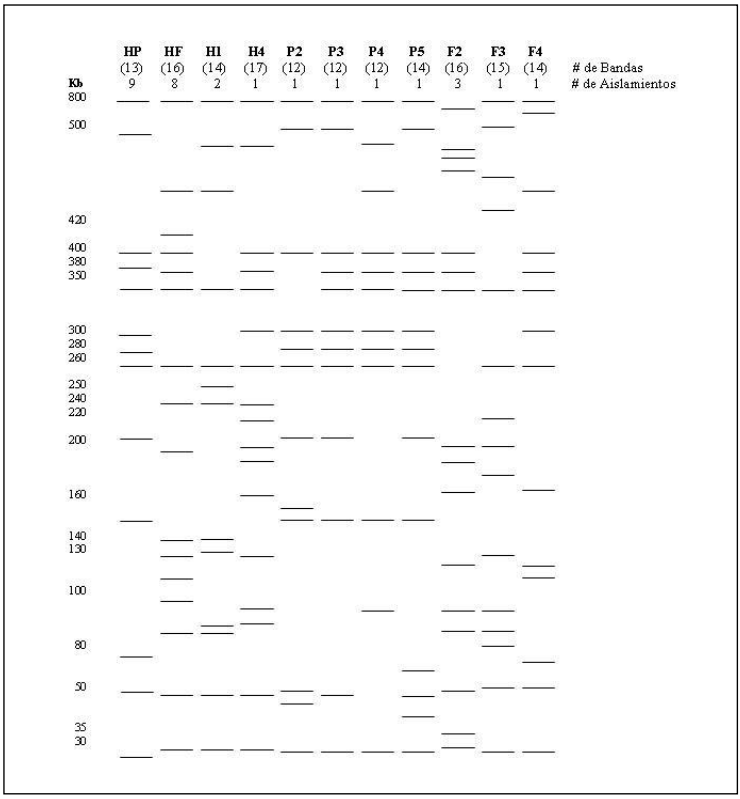

FIGURE 5. General representation of 11 distinct types of *Xba*I PFGE fragments from Colombian strains.

geographic correlation was found because both F1 and H3 were collected from the southern part of the city. Children's diarrhea in Bogotá, D.C., was caused by at least four different strains of *Salmonella enterica* subsp. *enterica*, but H2 and H3 were more prevalent. According to standard guidelines (Tenover *et al.*, 1995) the human strains from Bogotá, D.C., were not clonally related. For the food collection category, strains were isolated from other sources besides poultry products, such as pork and cheese products, but when we searched for a relationship between poultry and poultry products, we found that these profiles were quite different (F1 and F2 from poultry products were unrelated to all P profiles). Three out of 4 isolates from poultry products were clustered in F2. The P and F profiles probably reflected different clonal origins. It is interesting to note that different types of food (pork, poultry products, beef, and cheese) presented the same macrodigestion pattern (HF group) suggesting a possible transmission from a common source, such as food handlers to humans. P2, P3 and P4 (12 bands) with the same *Xba*I fragment number, differed in the molecular size of several bands, and must be interpreted as possibly related to P2-P3 and P3-P4, and unrelated to P2-P4. Poultry profiles differed from the most prevalent PFGE human type H2 by five (P2), one (P3), seven (P4) and three (P5) fragments. All of these could either be related or unrelated, and the differences could have been caused by one or two independent genetic events, such as a point mutation, deletion or insertion and very probably indicate a different clonal origin (Tenover *et al.*, 1995).

The finding of HP and HF groups, but not an HFP group, indicated that there was a direct transmission route from poultry and food to humans. However, is very probable that any food could be involved in transmission from poultry to humans. In this study, though, the reduced number of poultry product strains that we analyzed could not reliably confirm a direct transmission between poultry, poultry products and humans, as was suggested for different *Salmonella enterica* serotypes (Newport, Enteritidis and Typhimurium) in different studies in Spain, Peru, Bolivia, Chile, Thailand and Iceland (Boonmar *et al.*, 1998; Echeita *et al.*, 2001; Fernandez *et al.*, 2003; Gudmundsdottir *et al.*, 2003; Zhao *et al.*, 2003). It would be very important to extend the food collection beyond poultry products, but another possibility would be to search for other *Salmonella* reservoirs, such as pigs and cattle (Liebisch and Schwarz 1996; Heurtin-lecorre *et al.*, 1999; McDonough *et al.*, 2000; Tamada *et al.*, 2001; Duijkeren *et al.*, 2003), for all *Xba*I PFGE types found in food and humans. PFGEPs revealed that several DNA chromosomal regions (fragments of 800, 400, 380, 350, 260, 50 and 30kb) were conserved in the greatest number of strains. This suggested that they could contain housekeeping and chromosomal virulence genes. Cluster analysis (Figure 6) supported our conclusion that P and F strains had different clonal origins and that poultry products (the F2 profile) were not related to poultry (Squared Euclidean Distance SED >100% or <85% of similarity), suggesting that poultry products were probably contaminated from sources other than poultry, perhaps from food handlers. P profiles were closely related, revealing clonal similarity (SED <77.5%), although P2-P4 differed by more than seven fragments, we hypothesized that the variations in PFGEPs from poultry reflected chromosomal rearrangement possibly due to point mutations in the chromosomal DNA of the bacterium. On the other hand, statistical analysis supported the assumption of diversity between human strains (SED>100%). According to Tenover *et al.* (1995), F1/F2 (16 bands), F3 (15

bands) and F4 (14 bands) also were unrelated because there were more than seven fragment differences. The dendrogram (figure 6) confirmed that these profiles had different clonal origins (SED >100%). The food profiles F2, F3, F4 versus the HP group (H3-F1) were shown to be unrelated. The comparison among human, poultry and food strains, except for the HP and HF strains, demonstrated clonal diversity among those strains (SED >100%). It would be important nevertheless, to verify the genetic origin of human, food and poultry strains by means of other molecular techniques, such as IS200, RFLPs and plasmid analysis. Liebisch and Schawarz (Liebisch and Schwarz 1996), using cluster analysis of the *Avr*II macro restriction patterns, showed that 33 isolates of *Salmonella dublin* were 86.7% similar, confirming the close relationship among the *S. dublin* isolates. A recent study in Ille-et-Vilaine (France) with *Salmonella enterica* subsp. *enterica* isolates from humans and animals using PFGE and cluster analysis concluded that human isolates were quite close to those of the bovine isolates and that there was a clonal diversity between human and poultry or pig strains (Heurtin-lecorre *et al.*, 1999). Based on these studies, cluster analysis perhaps would be a better way to verify the epidemiologic relatedness and clonal similarity or diversity between strains. This analysis could include the number of bands and their molecular size. The present study demonstrated that there were several strains of *Salmonella enterica* with different clonal origins circulating in our population. It also showed that, there was a way of transmission from poultry and food to humans. For these reasons, it is clear that appropriate food handling and an ongoing continuous study of the presence of *Salmonella* in humans, food and poultry at the molecular and epidemiologic level could prevent human infections in Colombia.

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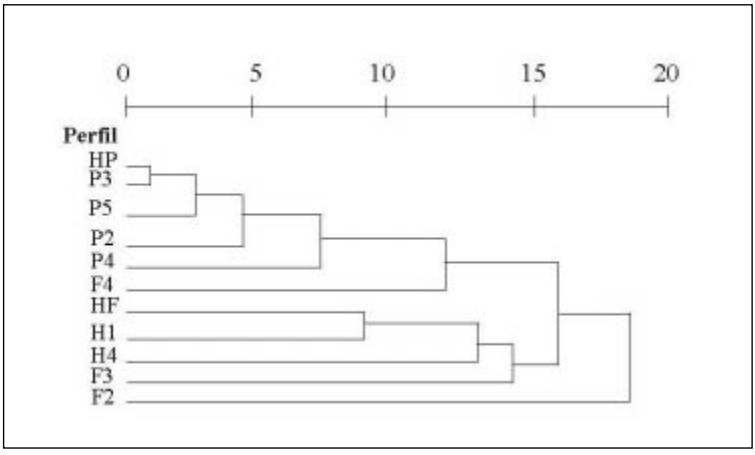

FIGURE 6. Hierarchical cluster analysis of PFGE fingerprint. Coefficients belong to Squared Euclidean Distance.

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