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Research Note

Molecular Characterization of *Salmonella* Paratyphi B dT+ and *Salmonella* Heidelberg from Poultry and Retail Chicken Meat in Colombia by Pulsed-Field Gel Electrophoresis

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ABSTRACT

Salmonella Paratyphi B dT+ variant (also termed *Salmonella* Java) and *Salmonella* Heidelberg are pathogens of public health importance that are frequently isolated from poultry. As a step toward implementing the Colombian Integrated Program for Antimicrobial Resistant Surveillance, this study characterized molecular patterns of *Salmonella* Paratyphi B dT+ and *Salmonella* Heidelberg isolated from poultry farms, fecal samples, and retail chicken meat using pulsed-field gel electrophoresis (PFGE). The objective of this study was to determine the genetic relationship among isolates and to determine potential geographically predominant genotypes. Based on PFGE analysis, both serovars exhibited high heterogeneity: the chromosomal DNA fingerprints of 82 *Salmonella* Paratyphi B dT+ isolates revealed 42 PFGE patterns, whereas the 21 isolates of *Salmonella* Heidelberg revealed 10 patterns. Similar genotypes of both serovars were demonstrated to be present on farms and in retail outlets. For *Salmonella* Paratyphi B dT+, closely genetically related strains were found among isolates coming from different farms and different integrated poultry companies within two departments (Santander and Cundinamarca) and also from farms located in the two geographically distant departments. For *Salmonella* Heidelberg, there were fewer farms with genetically related isolates than for *Salmonella* Paratyphi B dT+. A possible dissemination of similar genotypes of both serovars along the poultry production chain is hypothesized, and some facilitating factors existing in Colombia are reviewed.

Salmonella Paratyphi B *d*-tartrate-fermenting (dT+) variant (also called *Salmonella* Java) and *Salmonella* Heidelberg are foodborne pathogens associated worldwide with human salmonellosis *(21, 26)*. Risks associated with these two human pathogens are twofold: their specific pathogenicity in humans and their resistance to two or more antimicrobial agents, namely multidrug resistance. Both of these factors have generated interest in implementing longitudinal surveillance programs.

Salmonella Paratyphi B dT+ is an emerging public health problem that causes enteric fever and self-limiting gastroenteritis in humans *(9, 41)*. *Salmonella* Paratyphi B dT+ has animal reservoirs and has caused significant outbreaks in several countries as a result of food product contamination *(41)*. The organism has been reported in poultry worldwide *(4, 22, 38)* and is thought to be the most important human pathogen at the end of the broiler growing period *(38)*. Strains of *Salmonella* Paratyphi B dT+ are often multidrug resistant to antimicrobials such as ampicillin, chloramphenicol, streptomycin, spectomycin, sulphonamides, and tetracyclines *(12)*.

Salmonella Heidelberg was among the top four serotypes isolated in 2006 in the United States from human salmonellosis *(8)* and was associated with approximately 7% of *Salmonella*-related deaths. *Salmonella* Heidelberg is mainly derived from poultry, is notable for multidrug resistance, and is of great concern because it can cause septicemia and myocarditis *(28)*. Antimicrobial-resistant *Salmonella* Paratyphi B, as well as antimicrobial-resistant *Salmonella* Heidelberg, can spread through the food chain from primary production to retail chicken meat, posing significant risks to human health.

To monitor foodborne pathogens and to conduct antimicrobial resistance surveillance, the World Health Organization has recommended a threefold approach, to

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include human clinical cases, food animals, and retail meats *(42)*. Countries such as Canada *(5),* the United States *(27),* and Denmark *(1)* have established integrated and unified programs for the surveillance of antimicrobial resistance. These programs integrate data along the food chain to monitor foodborne pathogens and to prevent the spread of antimicrobial-resistant bacteria from animals to humans.

The current study is part of a pilot initiative to implement an integrated system in Colombia, namely, the Colombian Integrated Program for Antimicrobial Resistance Surveillance. In the first phase, the prevalence of *Salmonella* isolates and *Salmonella* serovars, along with their antimicrobial resistance patterns, were defined in chickens on 70 farms, including 350 houses from the two main poultry production departments of Colombia, Santander and Cundinamarca *(15)*. That study was followed by a second study of retail poultry meat from 100 independent stores and the main chain distributor center in Bogota District Capital (DC) *(14)*. Results from these preliminary studies showed a prevalence of 41% of *Salmonella* isolates on farms (65% at house level) and 26% in retail meat samples. *Salmonella* Paratyphi B dT+ was the most prevalent serovar on farms (76%) and in retail meat samples (49%), followed by *Salmonella* Heidelberg with 23 and 16% prevalence on farms and in retail meat samples, respectively. Both serovars exhibited multidrug resistant profiles to antibiotics.

The objectives of this study were to assess the genetic relatedness of isolates and to determine whether there were geographically predominant clones of *Salmonella* Paratyphi B dT+ and *Salmonella* Heidelberg in the Colombian poultry and poultry products tested. Isolates were obtained from poultry farms from Santander and Cundinamarca, as well as from independent stores and the main retailer distribution center of Bogota DC. Pulsed-field gel electrophoresis (PFGE) was used for molecular characterization because it is considered a highly discriminatory and reproducible typing method to detect high degrees of DNA polymorphism for epidemiological purposes *(16, 31, 44)*. Our hypothesis was that more than 50% of the isolates from farms from the two departments, Santander and Cundinamarca, shared the same PFGE pattern.

MATERIALS AND METHODS

***Salmonella* strain sources.** A total of 114 serotyped (Kauffman-White scheme) *Salmonella* isolates were included in this study. *Salmonella* serovars were isolated from samples collected during 2008 and 2009 on poultry farms from the departments of Santander and Cundinamarca *(15)* and from independent retail stores and a main chain meat distributor center in Bogota *(13)*. The isolation of *Salmonella* followed World Organization of Animal Health recommendations *(43)*. Briefly, following preenrichment in brain heart infusion broth (all media from Difco, BD, Sparks, MD, unless otherwise indicated), drag swabs and fecal samples were subcultured in tetrathionate broth and Rappaport-Vassiliadis broth. Then *Salmonella* was cultured on MacConkey agar and on the selective medium xylose lactose Tergitol 4. Three presumptive *Salmonella* colonies per plate were screened using biochemical tests: lysine iron agar, triple sugar iron agar, citrate agar, sulfur indole motility medium, and urea agar test. Identification was confirmed by the use of direct slide agglutination with *Salmonella* O antiserum Poly A-I & Vi. Three to five suspect colonies from each *Salmonella*-positive plate were streaked on nutrient agar and incubated for 18 to 24 h at 37°C, with reconfirmation of *Salmonella* done on one of these colonies by the automated Phoenix system *(7)*.

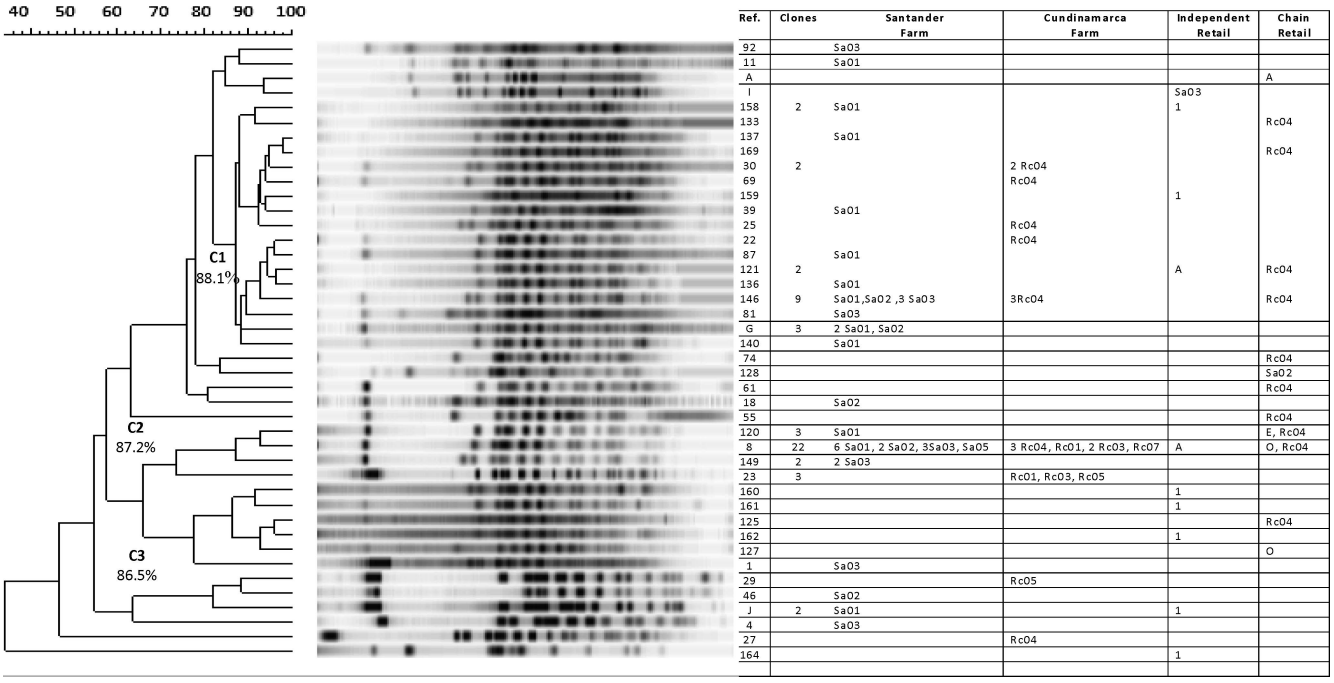
Salmonella Paratyphi B dT+ isolates ($n = 85$) were obtained from three different sources: 38 drag swab samples and 16 fecal samples from 29 poultry farms, 6 cecal content samples from processing plants, and 25 retail chicken meat samples from 10 independent stores and one main chain center in Bogota DC. *Salmonella* Heidelberg isolates ($n = 29$) came from 16 drag swabs, 5 fecal samples from poultry farms, and 8 retail store samples.

PFGE. PFGE was used to assess the genetic relatedness among isolates following the protocol of the Centers for Disease Control and Prevention *(33)* with minor modifications. Cell suspensions were made using the procedure of the National Health Institute of Colombia, which consisted of suspending the cells in 2 ml of cell suspension buffer (100 mM Tris–100 mM EDTA, pH 8.0) and then a dilution of 1:10 of the initial suspension (1,900 μl of cell suspension buffer plus 100 μl of the initial suspension) was made. Next, the concentration of the cell suspension was adjusted to an optical density of 1.35 at 610 nm in the spectrophotometer. Finally, the concentration of the cell suspension was adjusted using the formula 200/(OD × 10), where 200 is the final volume in microliters, OD is the optical density, and 10 is the dilution factor. Ten microliters of proteinase K solution (20 mg/ml; Sigma-Aldrich, Baltimore, MD) was added to the suspension. Casting plugs were made by adding 200 μl of 1% Seakem Gold agarose (Cambrex, Cambridge, MD) to 200 μl of the cell suspension. Restriction endonuclease digestion of DNA was performed by incubating a quarter of each casting plug in *Xba*I (30 U per sample) (Fermentas, Burlington, Ontario, Canada). Gel electrophoresis was carried out using a CHEF DR-III unit (Bio-Rad Laboratories, Hercules, CA) with initial and final switch times of 2.2 and 63.8 s, respectively. The included angle was 120° at 14°C, and the run time was 18 h. *Salmonella* Braenderup H9812 was used as the reference strain on each gel. Gels were stained with ethidium bromide (10 mg/ml) and were photographed in Universal Hood II (BioRad, Milan, Italy).

Genotypes were compared (Molecular Analysis Fingerprinting Software, version 1.6, Bio-Rad Laboratories) using the Dice coefficient of similarity with the unweighted pair group technique using arithmetic averages to prepare the dendrograms. To evaluate banding patterns, a demarcation system based on the concepts of Tenover et al. *(37)* was used: ≥93% band pattern similarity was classified as ''probably the same isolate''; 85 to 92% similarity was considered ''very similar''; 80 to 84% was ''similar''; 75 to 79% was ''somewhat similar''; and ≤74% was ''not similar.'' These criteria state that if an isolate varies from a main type by three or fewer bands, it will be considered a subtype.

RESULTS

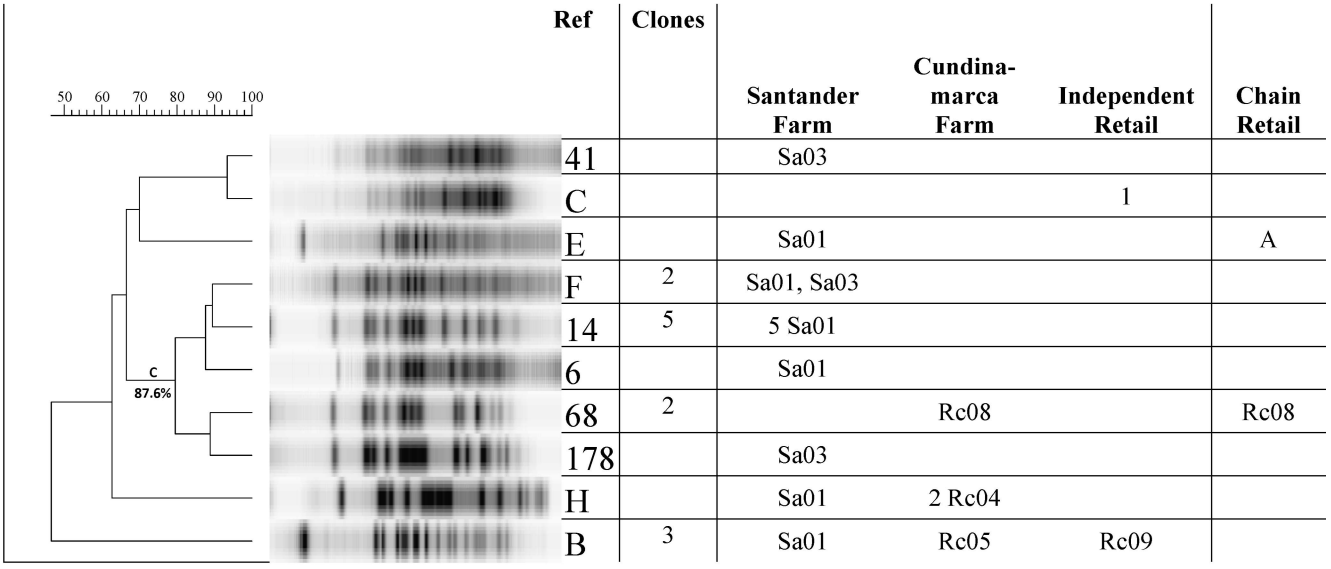
The PFGE fingerprints were analyzed separately by *Salmonella* serovar category, and dendrograms were constructed for *Salmonella* Paratyphi B dT+ (Fig. 1) and *Salmonella* Heidelberg (Fig. 2). Both serovars exhibited extensive heterogeneity. Of the 114 isolates, 103 were characterized with PFGE, and 11 isolates were not included in the analysis, either because they were untypeable due to DNA degradation *(35)* or because they had insufficient DNA loading in repeated trials.



Ref.	Clones	Santander Farm	Cundinamarca Farm	Independent Retail	Chain Retail
92		Sa03			
11		Sa01			
A					A
I				Sa03	
158	2	Sa01		1	
133					Rc04
137		Sa01			
169					Rc04
30	2		2 Rc04		
69			Rc04		
159				1	
39		Sa01			
25			Rc04		
22			Rc04		
87		Sa01			
121	2			A	Rc04
136		Sa01			
146	9	Sa01,Sa02 ,3 Sa03	3Rc04		Rc04
81		Sa03			
G	3	2 Sa01, Sa02			
140		Sa01			
74					Rc04
128					Sa02
61					Rc04
18		Sa02			
55					Rc04
120	3	Sa01			E, Rc04
8	22	6 Sa01, 2 Sa02, 3Sa03, Sa05	3 Rc04, Rc01, 2 Rc03, Rc07	A	O, Rc04
149	2	2 Sa03			
23	3		Rc01, Rc03, Rc05		
160				1	
161				1	
125					Rc04
162				1	
127					O
1		Sa03			
29			Rc05		
46		Sa02			
J	2	Sa01		1	
4		Sa03			
27			Rc04		
164				1	

FIGURE 1. *Dendrogram of* Salmonella *Paratyphi B dT+ macrorestriction patterns generated using* Xba*I. The clones column indicates the number of indistinguishable isolates presenting the same PFGE pattern. The clusters are reported as Cx (percent similarity coefficient). The origins of the isolates are reported as Santander farms, Cundinamarca farms, independent retail shops, or one retail chain in Bogota DC. The different integrated company identifiers begin with Sa0 or Rc0. The scale bar indicates percent similarity coefficient.*

***Salmonella* Paratyphi B dT+.** The chromosomal DNA fingerprinting by PFGE of the 82 isolates revealed 42 patterns: 19 patterns among the 37 isolates from farms in the department of Santander, 9 patterns among the 20 isolates from farms in Cundinamarca, and 20 patterns among the 25 isolates from retail stores in Bogota DC. The similarity dendrogram of *Salmonella* Paratyphi B dT+ genotypes showed three clusters: C1 ($n = 30$), C2 ($n = 27$), and C3 ($n = 5$), with a percent similarity coefficient of >85% within each cluster. Within the C1 cluster, 50% of isolates were from farms of three different integrated companies operating in Santander department, 27% were isolates from farms of one integrated company operating in Cundinamarca, and 23% of the isolates were from retail stores and the main chain center in Bogota DC. Within the C2 cluster, 56, 26, and 18% of isolates were from farms in Santander



Ref	Clones	Santander Farm	Cundinamarca Farm	Independent Retail	Chain Retail
41		Sa03			
C				1	
E		Sa01			A
F	2	Sa01, Sa03			
14	5	5 Sa01			
6		Sa01			
68	2		Rc08		Rc08
178		Sa03			
H		Sa01	2 Rc04		
B	3	Sa01	Rc05	Rc09	

FIGURE 2. *Similarity dendrogram of* Salmonella *Heidelberg macrorestriction patterns generated using* Xba*I. The clones column indicates the number of indistinguishable isolates presenting the same PFGE pattern. Clusters are labeled as C, with clusters defined as those groups for which the percentage of coefficient is >87.6%. The origins of the isolates are reported as Santander farms, Cundinamarca farms, independent retail shops, or one retail chain in Bogota, DC. The different integrated company identifiers begin with Sa0 or Rc0. The scale bar indicates percent similarity.*

department (belonging to four different integrated companies), farms in Cundinamarca (belonging to three integrated companies), and retail stores and the main chain center of Bogota DC, respectively. Within the C3 cluster, all isolates were from retail market chicken collected in Bogota DC. Groups of 9 and 22 indistinguishable isolates were identified within C1 and C2 clusters, respectively. In both groups, isolates were from farms in Santander and in Cundinamarca, as well as from retail chicken meat from Bogota.

***Salmonella* Heidelberg.** The DNA fingerprints of 21 *Salmonella* Heidelberg isolates revealed 10 patterns: 8 patterns among the 13 isolates from farms in the department of Santander, 3 patterns from farms in Cundinamarca, and 4 patterns among the retail isolates from Bogota DC (Fig. 2). The similarity dendrogram of *Salmonella* Heidelberg genotypes showed one cluster of eight isolates with a percent similarity coefficient of >85%. All the isolates were from farms in Santander belonging to two integrated companies. Within the cluster, five isolates coming from farms of one integrated company were indistinguishable.

DISCUSSION

The present study showed that *Salmonella* Paratyphi B dT+ and *Salmonella* Heidelberg isolates obtained from the two most important poultry producing departments, as well as from retail meat from the area with the greatest chicken consumption in Colombia, exhibited high genetic heterogeneity. This study is important because *Salmonella* Paratyphi B dT+ and *Salmonella* Heidelberg, with potential dissemination along the Colombian poultry production chain, may represent a public health risk for human outbreaks, as has been seen in Europe and the United States *(2, 8)*.

For *Salmonella* Paratyphi B dT+, this situation of high genetic heterogeneity appears to be different from the one reported in Germany, where one clonal lineage of *Salmonella* Paratyphi B dT+ successfully displaced the others after a period of time *(29)*. With respect to *Salmonella* Heidelberg, the genetic diversity found in this study is consistent with results reported by Lynne et al. *(26)* in a study of *Salmonella* Heidelberg isolates from food animals in the United States, which showed 30 patterns among 58 *Salmonella* Heidelberg isolates using the *Xba*I restriction enzyme.

The presence of closely genetically related *Salmonella* Paratyphi B dT+ isolates in this study, some of them with indistinguishable PFGE patterns, among poultry farms in the two departments and the retail stores, among farms within the same department, and among farms located in the two distant departments, led us to hypothesize about potential dissemination of clones along the poultry chain in Colombia. Possible causes of the dissemination of *Salmonella* Paratyphi B dT+ could be related to (i) fecal contamination and transmission through the environment *(11),* (ii) the findings that hygiene measures may be less effective for *Salmonella* Paratyphi B dT+ than for other *Salmonella* serovars (Enteritidis, Infantis, and Virchow) *(40),* and (iii) the ability of this serovar to colonize and rapidly spread within a group of chickens, with persistence until slaughter *(39)*.

Various poultry industry practices in Colombia could facilitate dissemination of *Salmonella* Paratyphi B dT+. For example, birds are transported to the abattoirs in open trucks, using the same plastic boxes for different farms, which could facilitate farm-to-farm contamination *(30)*. Additionally, using the same feed trucks for many farms of the same poultry producer could be a cause of cross-contamination among farms *(34)*. Also, most of the farms have soil floors that readily allow for the development and persistence of rodent and beetle populations that can transmit *Salmonella* among farms *(36)*. Similarly, farms belonging to different integrated companies are geographically mixed, and the short distances between farms and between sources of water, along with the use of poor isolation measures, make effective *Salmonella* control challenging *(3)*. Lastly, poultry manure is transported and commercialized across the country for use as fertilizer and as a feed component for other animal production *(32),* which could contribute to the movement of pathogens.

For *Salmonella* Heidelberg, the presence of closely genetically related isolates, some with indistinguishable PFGE patterns, has been shown to exist on poultry farms of the two departments and the retail stores, mainly among farms within Santander department. The possible dissemination within a department appears to be less than for *Salmonella* Paratyphi B dT+. Almost 90% of the isolates from the *Salmonella* Heidelberg cluster came from the same integrated company. Besides the possible horizontal transmission, this result could be correlated to the demonstrated vertical transmission of *Salmonella* Heidelberg from layers to eggs *(18–20),* making the hatchery of an integrated company a possible potential source of contamination *(24)*. Contamination of the integrated feed mill *(10, 25)* and the *Salmonella* status of the previous flock also could play a role *(6)*. The demonstrated dynamic evolution of a clonal population of these serovars, such as the reported case for *Salmonella* Paratyphi B dT+ in Europe *(29),* suggests the need for implementing longitudinal studies in the poultry chain in Colombia, including hatcheries, farms, slaughterhouses, and retail stores.

The ''gold standard'' of molecular typing techniques has been PFGE since its promotion by the Centers for Disease Control and Prevention in 1995 *(2, 17)*. This technique is used worldwide for outbreak investigations because of its reproducibility, its discriminatory power, and its relatively simple procedure (2 to 4 days). Some *Salmonella* serotypes such as Enteritidis are highly clonal, and the discriminatory power of PFGE is decreased because mutation events outside of the enzyme restriction areas may not be identified as different genotypes *(17, 23)*. Therefore, we recommend, for these longitudinal studies, the use of other complementary molecular typing tools such as MLST, MLVA, and rep PCR *(23)*.

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