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DNA Profiles of Salmonella Spp. Isolated from Chicken Products and From Broiler and Human Feces

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ABSTRACT

Salmonella species have been isolated from various kinds of food and are accountable for outbreaks of foodborne diseases in humans. This study aimed at identifying the similarities between the DNA profiles of *Salmonella* isolated from chicken feces, chicken products, and human feces in southern Brazil. Six hundred samples were collected (200 from chicken products, 200 from broiler chicken feces, and 200 from human feces) and tested for the presence of *Salmonella*. Isolates proven to be *Salmonella* compatible by biochemical and serological tests were tested by the Polymerase Chain Reaction. Their DNA profiles were then analyzed by PFGE and rep-PCR. *Salmonella* was isolated from 16 out of 600 analyzed samples, with Schwarzengrund serotype presenting the highest incidence, followed by Mbandaka in chicken meat and fecal samples, and Panama in human fecal samples. Some strains isolated from chicken fecal and product samples were indistinguishable by the molecular methods used in the study, suggesting that that the contamination of the broilers on the farm can be transmitted the processed products.

INTRODUCTION

Food safety professionals try hard to prevent the growth of deteriorating and pathogenic microorganisms in food. *Salmonella* species have been isolated from various kinds of food and are accountable for outbreaks of foodborne diseases (FBD) in humans. The Centers for Disease Control and Prevention (CDC) estimate that FBD cause about 1.2 million cases of salmonellosis and about 450 deaths each year in the United States (CDC, 2015). In Brazil, between 2007 and 2014, 450 FBD outbreaks caused by *Salmonella* were reported, affecting 13,165 people (Portal Brasil, 2015).

Among the different sources of contamination and transmission of *Salmonella* to humans, food products of poultry origin are the most frequently reported, and are mainly associated with consumption of undercooked poultry meat and/or products that were contaminated with raw poultry meat (Grant *et al*., 2016). The main source of contamination of chicken products is often the farm, where the occurrence of *Salmonella* is also high (Kanashiro *et al*., 2005).

The identification of *Salmonella* clones from animal, food, and human samples is important to understand the epidemiological dynamics of salmonellosis in the food chain, and it is essential for the development of outline food safety programs ineffective disease control plans.

Pulsed-field gel electrophoresis (PFGE), which is currently the gold standard technique by molecular epidemiologists (CDC, 2003b), has been an important tool for the investigation of FBD outbreaks. It has been used to identify correlations between reported cases and implicated food items (Yin *et al*., 2016) by estimating the genetic distances

between strains. In addition, the discrimination ability of this technique makes it a relevant tool to trace the contamination source within a food chain, thereby enabling the isolation of strains of the same species from different sites involved in food processing (Ribot *et al*., 2006).

Another molecular method used in the genetic distance characterization of bacterial species is the amplification of repetitive extragenic regions (repetitive extragenic palindromic sequence-based polymerase chain reaction [rep-PCR]) dispersed in their genomes, which provides distinct patterns of amplified bands. Rep-PCR is a simple and fast method that, despite its good reproducibility, has moderate discriminatory power as compared to PFGE (Tyler *et al*., 1997).

This study aimed at identifying the similarities between the DNA profiles of *Salmonella* strains isolated from chicken stool samples, chicken products, and human stool samples in southern Brazil.

MATERIAL AND METHODS

Sample

Six hundred samples were collected: 200 chicken product samples, 200 broiler chicken fecal samples, and 200 human fecal samples.

The chicken product samples (40 whole legs, 40 whole wings, 40 backs, 40 ground meat, and 40 liver samples) were obtained at retail stores in southern Brazil. The originally chilled samples were kept in their packages, stored in cool boxes, and immediately submitted to the Laboratory of Animal Product inspection of the Federal University of Pelotas for further processing. Products of 16 different brands were collected. Brand A products (80 samples) were produced in a processing plant located in the studied region. The other 120 samples belonged to15 different brands (B to P) were marketed in the same region.

The chicken fecal samples were collected in a processing plant with official inspection services from broilers reared on 40 different farms. At the time of slaughter, the large intestine was longitudinally sectioned immediately after the cecal region using sterile surgical scissors, and the contents were collected using swabs. Five random batch samples were simultaneously collected from each flock. Swabs were placedin tubes containing 10 mL buffered peptone water (BPW, Acumedia, Lansing, MI, USA) and submitted to laboratory.

Human fecal samples were obtained from human clinical analysis laboratories located in the studied region, which kindly provided the material for analysis. Swab samples were collected from collection containers submitted to the human laboratories, placedin tubes containing 10 mL BPW, and submitted to the laboratory. All patients whose fecal samples were included in this study reported abdominal discomfort and had been instructed by their personal physician to collect fecal samples for analysis. The human fecal samples were obtained with written consent from the subjects whose stool samples were forwarded for laboratory analysis.

Isolation and identification

The chicken product samples, depending on each case, either 25 g or the whole sample, were placed in sterile plastic bags containing 100 mL BPW and massaged for 5 min. The resulting suspension was drained and used as pre-enrichment step for the detection of *Salmonella* species, in compliance with the recommendations of the US Food and Drug Administration (FDA) (Andrews *et al*., 2014). The chicken and human fecal samples were incubated in test tubes to which 10 mL BPW was added as pre-enrichment, as well as for other *Salmonella*-related research procedures, as mentioned in Andrews *et al*. (2014).

Isolates proven to be positive for *Salmonella* according to standard biochemical and serological tests results, as described by Andrews *et al*. (2014), were tested by PCR as suggested by Malorny *et al*. (2003) to confirm their identity. The DNA was extracted according to Sambrook & Russel (2001). The primers used were

5’-GTGAAATTATCGCCACGTTCGGGCAA-3’

and

5’-TCATCGCACCGTCAAAGGAACC-3’,

targeting *invA*, using the following PCR cycling parameters: initial denaturation at 95°C for 1 min; 38 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s; final extension at 72°C for 4 min. The reactions were performed in a TC-3000 thermal cycler (Techne, Staffordshire, UK).

After PCR confirmation, the strains were referred to the Department of Bacteriology of the Enterobacteria Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ, Manguinhos, Rio de Janeiro, Brazil) for serotype identification.

Bacteria strains were stored in Brain-Heart Infusion broth (BHI, Acumedia) with the addition of 20% glycerolat 37°C for24h and then frozen at -70°C. The strains were incubated in BHI at 37°C for recovery.

Molecular profiles

Isolates were analyzed using PFGE to compare DNA profiles following the suggested protocol from Centers for Disease Control and Preventions (CDC, 2013c). Briefly, the DNA isolated from each sample was digested with *Xba*I restriction endonuclease (New England Biolabs™ Inc., Beverly, MA, USA), and analyzed on a 1% agarose gel using Pulsed Field Gel Electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) apparatus. The gel was then stained with ethidium bromide, and visualized with ultraviolet light.

Rep-PCR was performed according to Versalovic *et al*. (1994), using the 5'-GTGGTGGTGGTGGTG-3' primer and the following PCR cycling parameters: initial denaturation at 94°C for 1 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 60 s, and extension at 60°C for 5 min; final extension at 60°C for 16 min. The reactions were carried out in a TC-3000 thermal cycler.

The PFGE and rep-PCR patterns were interpreted in accordance with the criteria suggested by Tenover *et al*. (1995) using the following classifications: indistinguishable (no different bands), closely related (2 to 3 distinct bands), possibly related (4 to 6 distinct bands), and different (over 7 distinct bands).

RESULTS AND DISCUSSION

Salmonella was isolated from 16 out of 600 analyzed samples, 8 (8/200; 4%) from chicken products, 4 (4/200; 2%) from chicken fecal samples, and 4 (4/200; 2%) from human fecal samples. All strains phenotypically characterized as *Salmonella* contained *invA*, a highly preserved DNA region of this genus, which can be used to confirm its identity at the molecular level.

Among the 200 chicken product samples analyzed from 16 different brands (A to P), 8 samples from 3 brands, A (5/80); L (2/18), and N (1/3), were contaminated with *Salmonella*: 3 from the liver (brands A, L, and N), 3 from whole legs(brands A [2 samples] and L), 1 from whole wings (brand A),and 1 from the back (brand A).This low prevalence was also reported by other authors. Panzenhagen *et al*. (2016) analyzed 60 chicken carcasses from six processing plants in Rio de Janeiro, and found 6.67% and 8.33% *Salmonella* contamination, using the conventional method and PCR, respectively. Duarte *et al*. (2009) analyzed 260 chicken carcasses bought from five different processing plants, and found 9.6% carcasses positive for *Salmonella*.

Among the 200 analyzed fecal samples of broilers derived from 40 farms, *Salmonella* was isolated from four chicken fecal samples from three different farms that supplied brand A at time of this study.

Four distinct serotypes were identified in chicken fecal samples (Table 1). One broiler harbored two distinct serotypes simultaneously. These two strains (FF02 and FF03), confirmed as *Salmonella* by serology and PCR, showed phenotypically distinct colonies (one of the colonies showed typical biochemical characteristics, and the other grew on completely acidified TSI agar). The serotypes of these isolates were identified as Schwarzengrund (FF02) and Mbandaka (FF03).

The serotypes Enteritidis and Typhimurium have been reported in other research studies (Abd-Elghany *et al*., 2015; Suresh *et al*., 2011; Thakur *et al*., 2013) as the most common in chickens. This study, however, did not detect any isolates of these serotypes in chicken product or fecal samples. The most frequent isolate was serotype Schwarzengrund, followed by Mbandaka. Other studies have also reported a higher prevalence of serotypes other than Enteritidis and Typhimurium, e.g., that of Aslam *et al*. (2012) in Canada, in ground beef samples, and Le Bouquin *et al*. (2010) who found a higher prevalence of serotype Hadar in France in chicken samples, differently from the present study.

In a study conducted by the Department of Bacteriology of the Oswaldo Cruz Foundation, Hofer *et al*. (1997) reported that the serotype Mbandaka belongs to a common *Salmonella* group; serotype Schwarzengrund, on the other hand, is thought to belong to a rare, or accidental, *Salmonella* group, according to occurrence levels recorded during 1962–1991. Kanashiro *et al*. (2005) also investigated the

Table 1 – *Salmonella* serotypes isolated from 600 analyzed samples

Serotype	Chicken meat isolates (200 samples)	Chicken fecal isolates (200 samples)	Human fecal isolates (200 samples)
Mbandaka	CF02	FF03*; FF05	-
Schwarzengrund	CF03; CF04; CF05; CF06; CF07; CF08	FF01; FF02*;FF04	-
Panama	-	-	FH02; FH03; FH04
Typhimurium	-	-	FH01
Auto-agglutinable	CF01	-	-

* Strains isolated from the same chicken.

presence of *Salmonella* spp. in samples received from diverse chicken processing plants located in different regions in Brazil between July 1997 and December 2004, and did not report the serotype Schwarzengrund as one of the main isolates. Nevertheless, as previously mentioned, Schwarzengrund was the predominant serotype identified both in chicken fecal samples (3 isolates from 2 aviaries) and in chicken products (6 isolates) in the present study. Boni *et al*. (2011) also reported this serotype as the most frequently isolated in broiler carcasses obtained between August 2005 and December 2006 from a processing plant located in Mato Grosso do Sul State, Brazil. However, the serotype Schwarzengrund was not isolated on the farms supplying this plant, which suggests that carcasses were contaminated in the processing plant rather than on the chicken farm. Chen *et al*. (2010) reported a high prevalence of this serotype in raw chicken meat (30.5%) in Taiwan.

The nine *Salmonella* Schwarzengrund strains isolated were submitted to genotyping by PFGE with the *Xba*I restriction enzyme, and no differences were observed between the band patterns, except for the FF04 strain (Figure 1A). However, the rep-PCR results of the other identified showed further differences (Figure 1B). The strains CF03, CF04, CF06, CF07, and CF08 isolated from chicken products were indistinguishable from each other, and were closely related with CF05. Notwithstanding, they are possibly related with the FF01 and FF02 strains, which were isolated from chicken fecal samples, and were indistinguishable from each other, but different from FF04, which was also isolated in chicken fecal samples.

The strains CF03 and CF06 were isolated from products of a specific commercial brand (brand L), which suggests the same source of contamination, possibly at the processing plant that supplied brand L. In the case of the CF04, CF07, and CF08 strains, isolated from brand A products, the results indicate a common contamination source. Moreover, the correlation found between the strains isolated in brand A products with the strains FF01 and FF02 from chicken fecal samples obtained in the processing plant that supplied this brand suggests that the source contamination was the chicken farms.

Aarestrup *et al*. (2007), using PFGE for the detection of *Salmonella* Schwarzengrund clones in chicken products and human isolates, demonstrated the transmission of this microorganism from food to man, as opposed to our study, where chicken isolates were not found to be related with those obtained from humans.

Salmonella Mbandaka was found in 2 chicken fecal samples from 2 different origins and in 1 chicken product sample (whole wing). This serotype accounted for 17% (3/17) of the isolated samples. Suresh *et al*. (2011) and Hue *et al*. (2011) reported low occurrence of this serotype in chicken carcasses in India and France, respectively. Oliveira (2012) analyzed samples from four broiler processing plants in Goiás State, Brazil, and found a 3.45% prevalence of the Mbandaka serotype. Upon analyzing different chicken carcass parts, Suresh *et al*. (2011) observed that this was one of the least common serotypes in southern India; yet, it was present in different chicken parts, in addition to having been found in environmental sample collected from a cage. In France, Hue *et al*. (2011) also recorded

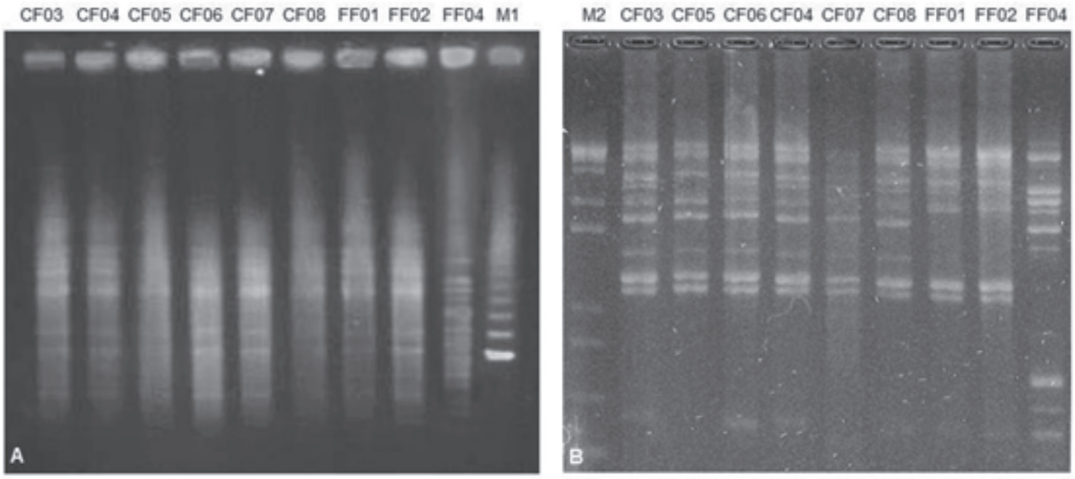

Figure 1 – Photographs of pulsed-field gel electrophoresis (A) and repetitive extragenic palindromic sequence-based polymerase chain reaction (B) electrophoresis gels of band profiles of 9 *Salmonella* Schwarzengrund isolates. CF03: isolate from brand A whole leg; CF04: isolate from brand L whole leg; CF05: isolate from brand N liver; CF06: isolate from brand A liver; CF07: isolate from brand L liver; CF08: isolate from brand A back; FF01: isolate from fecal sample of chicken from farm G02; FF02 and FF04: isolates from fecal sample of chicken from farm G15; M1: DNA Size Standards – Lambda ladder; M2: GeneRuler™ 1 kb DNA ladder.

low occurrence of this serotype, having identified only 1 isolate from 425 chicken carcasses in a processing plant. On the other hand, Murgia *et al*. (2015) reported that, out of 94 *Salmonella* strains isolated from food in Morocco, 36% were *Salmonella* Mbandaka. Despite its usually low occurrence, the serotype Mbandaka has been implicated in FBD (CDC, 2013a), and its presence in foods represents a hazard to consumers.

PFGE (Figure 2A) and rep-PCR (Figure 2B) results show that the strains CF02 and FF03 are indistinguishable from each other, suggesting that the sources of contamination to these samples are related. The FF05 strain, which is not related to the strains CF02 and CF03, probably had a different origin. Hoszowski & Wasyl (2001) reported that biotyping and antimicrobial susceptibility and plasmid profiles were not sufficient to differentiate the analyzed *Salmonella* Mbandaka strains, and only genomic macrorestriction proved to be an efficient method for epidemiological studies of this serotype. However, in the present study, the discriminatory power of rep-PCR was similar to that of PFGE for this serotype, with the advantage that the former is less expensive and faster than PFGE.

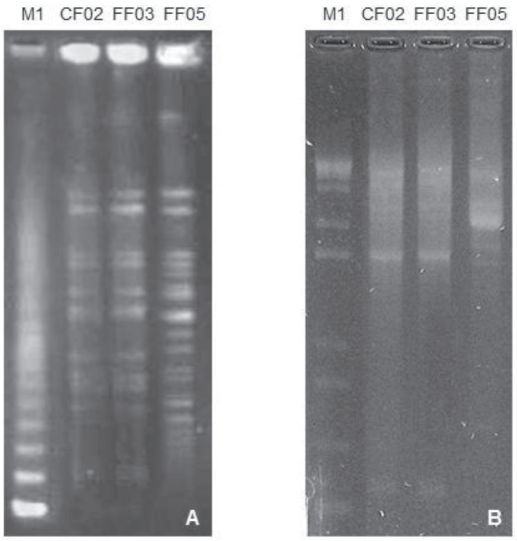

Figure 2 – Photographs of pulsed-field gel electrophoresis (A) and repetitive extragenic palindromic sequence-based polymerase chain reaction (B) electrophoresis gels of band profiles of 3 *Salmonella* Mbandaka isolates. M1(A): DNA Size Standards – Lambda ladder; M1(B): GeneRuler™ 1 kb DNA ladder; CF02: isolate from brand A whole wing; FF03 and FF05: isolates from G15 farm and G28 chicken fecal samples, respectively.

Auto-agglutinable *Salmonella enterica* was isolated from 1 chicken product sample. Alcocer *et al*. (2006), evaluating 25 *Salmonella* strains obtained from chicken carcasses from four processing plants located in Paraná State, Brazil, found only one auto-agglutinable strain. Other authors have reported the isolation of auto-agglutinable strains in chicken fecal samples in Brazil (Salles *et al*., 2008; Andreatti Filho *et al*., 2009). Nevertheless, in the present study, auto-agglutinable strains were isolated from chicken products rather than chicken fecal samples. Although not investigated, the possibility that the chicken product samples were previously contaminated with chicken feces cannot be ruled out.

Children up to five years of age are more affected by salmonellosis, as well as the elderly and people with weakened immune systems (CDC, 2015). However, in a study reporting an outbreak in São Paulo, Brazil, Matsuoka *et al*. (2004) observed that the average age of the affected people was 36.5 years. In the present study, *Salmonella* was isolated from fecal samples of patients whose age ranged from nine months to 40 years, with no gender predominance.

In human fecal samples, four strains (three Panama and one Typhimurium serotypes) were isolated. These serotypes have been shown to cause gastroenteritis in humans, both in Brazil (Fernandes *et al*., 2006; Antunes *et al*., 2016) and in other countries (Soto *et al*., 2001; Tsai *et al*., 2007). However, during the period 2006-2015, there was no report by the CDC (2015) of Panama *Salmonella* outbreaks in humans related with the consumption of animal products. In our study, Panama serotype band profiles were indistinguishable (Figure 3) from each other, which is suggestive of an outbreak, insofar as the three samples were collected from patients at the same location on the same day.

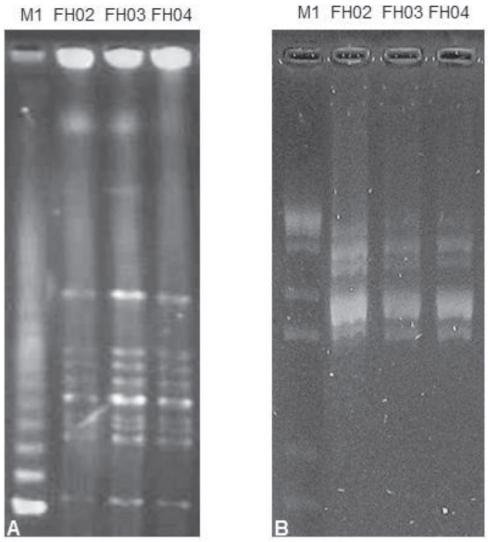

Figure 3 – Photographs of pulsed-field gel electrophoresis (A) and repetitive extragenic palindromic sequence-based polymerase chain reaction (B) electrophoresis gels of band profiles of three *Salmonella* Panama isolates. M1(A): DNA Size Standards – Lambda ladder; M1(B): GeneRuler™ 1 kb DNA ladder; FH02, FH03, and FH04: isolates from human fecal samples.

CONCLUSIONS

Salmonella is present in broiler chickens in southern Brazil, as well as in chicken products available for consumption, which represents a health risk for consumers. *Salmonella* Schwarzengrund is a common serotype in southern Brazil, followed by Mbandaka, both on farms and in chicken products. In this region, despite the few reports, Panama serotype occurs in humans. The strains whose genotypes were indistinguishable by the molecular methods used in the study were detected in chicken fecal and product samples. This finding suggests that the contamination of the broilers on the farm can reach the processed product. This emphasizes the need for better hygienic and health practices in the processing plants for the control of undesirable microorganisms and disease eradication in animals. In addition, stricter farm biosecurity measures are required in order to minimize the risk of contamination of the final product.

The serotypes of the strains isolated in humans were different from those found in chicken products. The fact that the human strains were indistinguishable among each other by the applied techniques used suggests the occurrence of an outbreak. In addition, other salmonellosis cases and outbreaks may not be reported to the authorities, contributing for the underestimation of the incidence of this disease in humans in Brazil.

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