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ORIGINAL ARTICLE

Molecular tracking of *Salmonella* spp. in chicken meat chain: from slaughterhouse reception to end cuts

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Abstract Due to the importance of *Salmonella* spp. in poultry products, this study aimed to track its main contamination routes since slaughtering reception to processing of chicken end cuts. Samples from different steps of slaughtering and processing ($n = 277$) were collected from two chicken slaughterhouses (Sl1 and Sl2) located in Minas Gerais state, Brazil, and subjected to *Salmonella* spp. detection. The obtained isolates were subjected to serological identification and tested by PCR for specific *Salmonella* spp. genes (*omp*C and *sif*B). Also, *Salmonella* spp. isolates were subjected to *Xba*I macrorestriction and pulsed-field gel electrophoresis (PFGE). Sixty-eight samples were positive for *Salmonella* spp. and 172 isolates were obtained. Sl1 and Sl2 presented similar frequencies of *Salmonella* spp. positive samples during reception, slaughtering and processing ($p > 0.05$), except for higher frequencies in Sl1 for chicken carcasses after de-feathering and evisceration ($p < 0.05$). PFGE allowed the identification of cross contamination and persistence of *Salmonella* spp. strains in Sl1. The results highlighted the relevance of the initial steps of chicken slaughtering for *Salmonella* spp. contamination, and the pre-chilling of carcasses as an important controlling tool. In addition, the presence of *Salmonella* spp. in chicken end cuts samples represents a public health concern.

Keywords *Salmonella* spp · PFGE · Chicken · Contamination sources

Highlights • Detection of *Salmonella* during chicken slaughtering and processing
• Initial slaughter steps as contamination sources, and the pre-chilling as control.
• *Salmonella* spp. tracking by PFGE and identification of contamination routes

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Introduction

The Centers for Disease Control and Prevention (CDC) estimates that every year around 48 million North Americans are sick, 128 thousand are hospitalized, and 3000 die due to foodborne diseases (CDC 2014). In Brazil, the Ministry of Health reported 795 foodborne disease outbreaks in 2011, resulting in approximately 20,000 illnesses (Brasil 2013). Salmonellosis is highlighted as one of the most important foodborne diseases, responsible for millions of sick animals and humans and economic losses for the food industry (Nogrady et al. 2008). In the United States, approximately 42,000 salmonellosis cases are reported annually (CDC 2014). and in Brazil, *Salmonella* spp. is the most common etiological agent in notified and investigated foodborne diseases (Brasil 2013). Poultry products are considered important vehicles of *Salmonella* spp. for humans, and this commodity is associated with the majority of salmonellosis cases and outbreaks (Carrasco et al. 2012; Finstad et al. 2012).

Different steps of poultry slaughtering can contribute to carcass contamination, such as shipping, de-feathering, evisceration, and carcasses pre-chilling (Corry et al. 2002; Goksoy et al. 2004; Heyndrickx et al. 2002; Nogrady et al. 2008; Olsen et al. 2003; Rasschaert et al. 2007; Rasschaert et al. 2008; Rodrigues et al. 2008; Von Rückert et al. 2009). Thus, investigation of the occurrence and distribution of *Salmonella* spp. at different slaughtering steps is essential to adopt

preventive and corrective procedures to minimize the contamination (Wang et al. 2013).

Conventional culture methods are widely employed for *Salmonella* spp. detection in foods, being considered as reference for monitoring this foodborne pathogen despite their limitations as laborious and time-consuming (Lee et al. 2015). As alternative, molecular methodologies are used for *Salmonella* spp. identification and tracking in foods, associated or not to conventional protocols, enhancing their discriminatory power (Akiba et al. 2011; Alvarez et al. 2004; Liu et al. 2011; Steve Yan et al. 2003). Some of these methodologies are particularly valuable for epidemiological studies, allowing the characterization of subtypes and identification of contamination routes in the food processing environment (Barco et al. 2013). Among these methods, pulsed-field gel electrophoresis (PFGE) is the golden standard for characterizing foodborne pathogens, including *Salmonella* spp. (Barco et al. 2013; Goering 2010).

The aim of this study was to track the origins of contamination by *Salmonella* spp. at different steps in the slaughtering and processing of chicken by using conventional and molecular methodologies, and then establishing the possible contamination routes.

Material and methods

Slaughterhouses and sampling

Two slaughterhouses located in Minas Gerais state, Brazil, were included in this study after the consent of the owners. Both facilities develop their activities under official inspection services and present the following characteristics:

- Slaughterhouse 1 (Sl1): large industry with an average slaughter of 180,000 chickens per day, mechanized evisceration line, supervised by the Federal Inspection Service from the Brazilian Ministry of Agriculture, able to export; farms animals produced by an integrated system (Sl1 provides full support to farmers, from birds to technical assistance);
- Slaughterhouse 2 (Sl2): small industry with an average slaughter from 3500 to 4000 chickens per day, manual evisceration line, supervised by the City Inspection Service from the City Agriculture Secretariat.

The study was conducted in a 9-month period (2014 January to September), being the facilities visited five times each. During the visits, samples from reception, slaughtering, processing, and end products were obtained (Table 1). Nine poultry lots in Sl1 (numbered from 1 to 9) and five poultry lots in Sl2 (numbered from 10 to 14) were sampled during reception and slaughtering. The sampled carcasses were tagged for proper identification of lots after pre-chilling. Carcasses and end cuts (leg, breast, and wings) samples were collected, transferred to a sterile plastic bags and added with 400 mL of phosphate buffer, pH 7.2 (PB, Oxoid Ltd., Basingstoke, England) with further agitation; then, the homogenate was transferred to a sterile container and kept cooled until analysis (USDA/FSIS 2014). Surface samples (shipping boxes, cutting boards, employees hands, and knives) were collected by swabbing sterile sponges pre-moistened with 40 mL of PB in four 100-cm^2 regions of the sample; then, the sponges set were transferred to sterile bags with 160 mL of PB to yield a final volume of 200 mL, corresponding to 400 cm^2. Samples were cooled until analysis. All samples were homogenized in a peristaltic homogenizer (Stomacher 400 circulator, Seward, Worthing, England) before laboratory analysis.

Table 1 Number of samples and sampling procedure at different steps of the production chain of chicken meat in two slaughterhouses located in Minas Gerais state, Brazil

Step	Sample	Specification	Code	Sl1	Sl2	Sampling procedure
Reception	Boxes	Shipping boxes	B1	17	13	Swab[1]
Slaughter	Carcasses	After de-feathering	C1	27	24	Rinsing[2]
		After evisceration	C2	28	24	Rinsing[2]
		After pre-chilling	C3	25	24	Rinsing[2]
Processing	Knife	-	K	11	7	Swab[1]
	Cutting board	-	M	11	7	Swab[1]
	Hands	Hands of employees	H	11	7	Swab[1]
Cuts	Leg	-	L	11	2	Rinsing[2]
	Wing	-	W	12	2	Rinsing[2]
	Breast	-	B	12	2	Rinsing[2]
Total	-	-	-	165	112	-

[1] sampling of 400cm^2, [2] USDA/FSIS 2014

Salmonella spp. detection

The collected samples were subjected to *Salmonella* spp. detection according to USDA/FSIS (2014) and ISO (2002). First, 30 mL of carcasses and end cuts homogenates were transferred to 30 mL of buffered peptone water at 4 % (*w*/*v*) (Oxoid) and incubated at 37 °C for 18 h (USDA/FSIS 2014). For environment samples, 25 mL of the obtained homogenates were transferred to 225 mL of buffered peptone water at 1 % (*w*/*v*) (Oxoid) and incubated at 37 °C for 18 h. From this step on, only the methodology ISO 6579 was considered (ISO 2002). aliquots of 1 mL and 0.1 mL of cultures were transferred to Muller-Kauffmann tetrathionate broth supplemented with novobiocin (Oxoid) and Rappaport Vassiliadis

broth (Oxoid) and incubated at 37 °C and 42.5 °C, respectively, for 24 h. Then, the obtained cultures were streaked onto Xylose Lysine Deoxycholate agar (Oxoid) and Bright Green agar (BD, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37 °C for 24 h. *Salmonella* spp. typical colonies were transferred to Triple Sugar Iron agar (Oxoid) and Lysine Iron agar (Oxoid) and incubated at 37 °C for 24 h. The cultures that presented typical *Salmonella* spp. reactions were subjected to serological tests with somatic and flagellar polyvalent anti-serum (Probac, Brazil, São Paulo, SP, Brazil).

The cultures that presented positive reactions for *Salmonella* spp. on serological identification were subjected to DNA extraction using the Wizard Genomic DNA Purification kit (Promega Corp. Madison, WI, USA) and PCR reactions were conducted according to Alvarez et al. (2004) for *sif*B, and to Almeida et al. (2014) for *omp*C, both genes specific for *Salmonella* spp.

Pulsed field gel electrophoresis (PFGE)

The isolates identified as *Salmonella* spp. by PCR were subjected to DNA macro-restriction with the enzyme *Xba*I and PFGE as indicated by PulseNet (Centers for Disease Control and Prevention, Atlanta, GA, USA), following the protocol described by Ribot et al. (2006). Briefly, cultures plugs were subjected to macro-restriction with 50 U of *Xba*I enzyme (Promega) at 37 °C for 2 h, and the products from macro-restriction were separated by agarose gel electrophoresis (Agarose Seakem Gold 1 % in Buffer TE 0.5X) using a CHEF-DR II (Bio-Rad Lab., Hercules, CA, USA) with the following parameters: initial switch time of 2.2 s, final switch time of 63.8 s, angle of 120°, 6 V/cm, run time of 18 h. The gels were strained with GelRed (Biotium Inc., Hayward, CA, USA) bath and digestion standards were visualized under ultra-violet light.

Data analysis

The frequency of positive results for *Salmonella* spp. from Sl1 and Sl2 were compared by the Chi-square test ($p < 0.05$) and by the Marascuilo procedure for paired comparison ($p < 0.05$) using the software XLSat 2010.2.03 (AddinSoft, New York, NY, USA). PFGE pulsotypes were analyzed by using BioNumerics 6.6 (Applied Maths, Gand, Belgium), considering an optimization of 1 % and a Dice coefficient of 5 %.

Results and discussion

Salmonella spp. frequencies recorded in this study are presented in Table 2. Among 277 samples, 68 (24.5 %) were positive for *Salmonella* spp. and 172 isolates were obtained, being identified by serological tests and by presenting PCR products typical for *omp*C (204 bp) and *sif*B (498 bp), as respectively described by Alvarez et al. (2004) and Almeida et al. (2014). The frequencies of *Salmonella* spp. positive samples in shipping boxes were not significantly different among Sl1 and Sl2 (Table 2). Even not being significant, Sl1 presented higher frequency of *Salmonella* spp. positive shipping boxes than Sl2, indicating the relevance of the reception as possible entrance of this foodborne pathogen in this slaughterhouse. Similar findings were described by other studies, highlighting that the intrinsic contamination of poultry favors the entrance

Table 2 Frequency of positive results for *Salmonella* spp. obtained from different steps of slaughtering and processing of chicken in two slaughterhouses located in Minas Gerais state, Brazil

Step	Source	Sl1		Sl2		Statistic
		n	*Salmonella* spp.	n	*Salmonella* spp.	
Reception	Box	17	7^a	13	1^a	χ^2: 4.22, DF: 1, *p*: 0.040
Slaughter	C1	27	26^b	24	0^a	χ^2: 131.3, DF: 5, $p < 0.001$*
	C2	28	26^b	24	1^a	
	C3	25	1^a	24	0^a	
Processing	Cutting board	11	0	7	1	χ^2: 1.66, DF: 1, *p*: 0197
	Knife	11	1	7	0	χ^2: 0.67, DF: 1, *p*: 0412
	Hand	11	0	7	0	-
Cuts	Breast	12	0	2	0	-
	Wing	12	3	2	0	χ^2: 0.64, DF: 1, *p*: 0425
	Leg	11	1	2	0	χ^2: 0.20, DF: 1, *p*: 0.657

n number of samples, χ^2, chi-square, *DF*, degree of freedom, p level of significance ($p < 0,05$), C1 after de-feathering;,C2 after evisceration;,C3 after pre-chilling. * Obs paired test by Marascuilo procedure ($p < 0.05$). In the reception step, the same superscript letters indicate that there were no significant differences among mean values presented in the respective lines. In the slaughtering step, values in the same line and/or column with different superscript letters represent a statistic difference

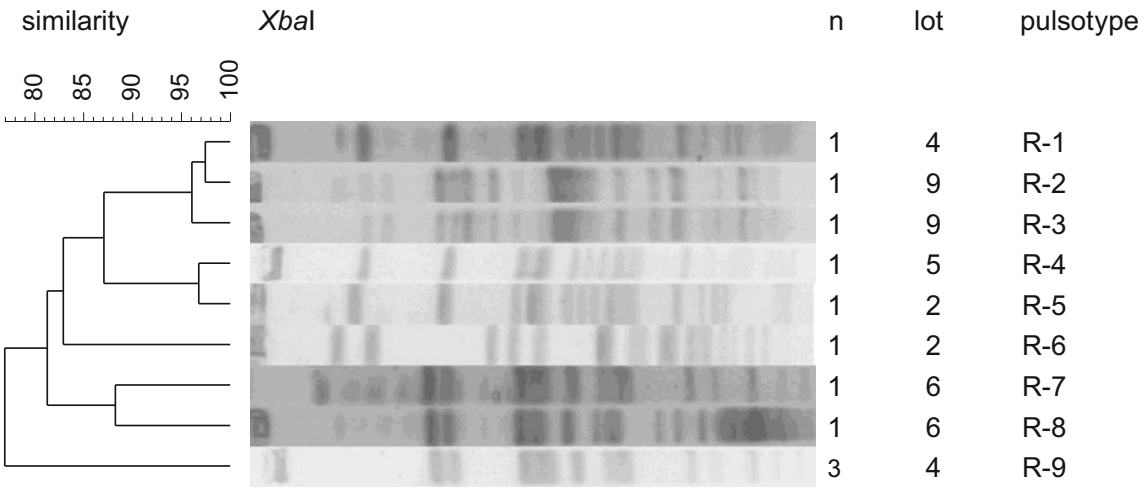

Fig. 1 Schematic representation of genetic profiles (pulsotypes) obtained by PFGE after macro-restriction of DNA using *Xba*I from isolates of *Salmonella* spp. that were obtained in the reception step (shipping boxes). Similarities among pulsotypes was estimated by using the Dice coefficient (5 % of tolerance). n: number of isolates

of pathogens into slaughterhouses (Corry et al. 2002; Olsen et al. 2003; Rasschaert et al. 2007). However, Slader et al. (2002) demonstrated that *Salmonella* spp. free poultry, raised under contamination-controlled conditions, are not relevant for the entrance of this pathogen in the production chain.

Significant differences were observed among the frequencies of positive samples during slaughtering (Table 2). Sl1 presented higher frequencies of positive carcasses after defeathering (C1) and after evisceration (C2) when compared to the same steps in Sl2, and when compared to the frequencies obtained after pre-chilling (C3) in Sl1 and Sl2 ($p < 0.05$, Table 2). These results indicate the relevance of *Salmonella* spp. contamination in the initial stages of slaughtering in Sl1, probably explained by the large number of processed chicken, high speed of slaughtering and automated evisceration process, leading to an inherent difficult in controlling the microbiological contamination. However, the pre-chilling step (C3) in Sl1 was efficient in reducing the contamination by *Salmonella* spp. in carcasses (Table 2, $p < 0.05$), which is an evidence of the importance of this step in controlling the carcasses contamination. Sl2 presented only one sample contaminated by *Salmonella* spp. (C2, Table 2), indicating a relative control of the contamination by this foodborne pathogen in the initial steps of slaughtering. This finding can be explained by the small size of the facility and small volume of processed chicken, resulting in a slower slaughtering process when compared to Sl1, and allowing some control of the procedures and easiness in correcting eventual failures. Similar results were found by Von Rückert et al. (2009). who reported high contamination by *Salmonella* spp. in the initial slaughtering steps of chicken and the pre-chilling as an efficient procedure to control carcass contamination. Wang et al. (2013) found that 35 % of the samples from broiler carcasses were positive for *Salmonella* spp. in the evisceration step, with a significant reduction to 19 % after pre-chilling ($p < 0.05$). The variation in the frequencies of contamination by *Salmonella* spp. in several slaughtering steps depends on specific conditions in the researched establishments. Goksoy et al. (2004) demonstrated the importance of the steps after de-feathering and evisceration, such as pre-chilling, in contamination by *Salmonella* spp. in broilers carcasses. Lillard (1990) also observed an increased contamination by *Salmonella* spp. in broilers carcasses after the pre-chilling step in comparison to the initial slaughtering steps, and attributed this result to possible cross-contamination in the pre-chilling tanks.

Considering these evidences, the pre-chilling step might be characterized as critical since it can contribute positively and negatively for *Salmonella* spp. contamination in chicken carcasses. This dual effect of pre-chilling can be credited to different processes and equipment that can be adopted by the slaughtering industries, such as immersion tanks, aspersion, or cold air, which demand different control procedures and monitoring. The use of immersion tanks, for example, can determine reduction in contamination when the conditions of chloride content, temperature, flow, drinkability, volume, and water renovation, are efficiently controlled; otherwise, the contact among carcasses allows for cross contamination (Allen et al. 2000; James et al. 2006; Rasschaert et al. 2008).

Frequencies of *Salmonella* spp. from processing environment and end cuts were not significantly different among Sl1 and Sl2 (Table 2, $P > 0.05$). Sl1 presented some end cuts contaminated by *Salmonella* spp., which can be explained by the positive results of carcasses during the slaughtering steps. In the processing environment, only one knife sampled in Sl1 (9 %) and one cutting board sample from Sl2 (14.2 %) were positive for *Salmonella* spp. Despite the low frequencies of *Salmonella* spp. that were observed in the processing

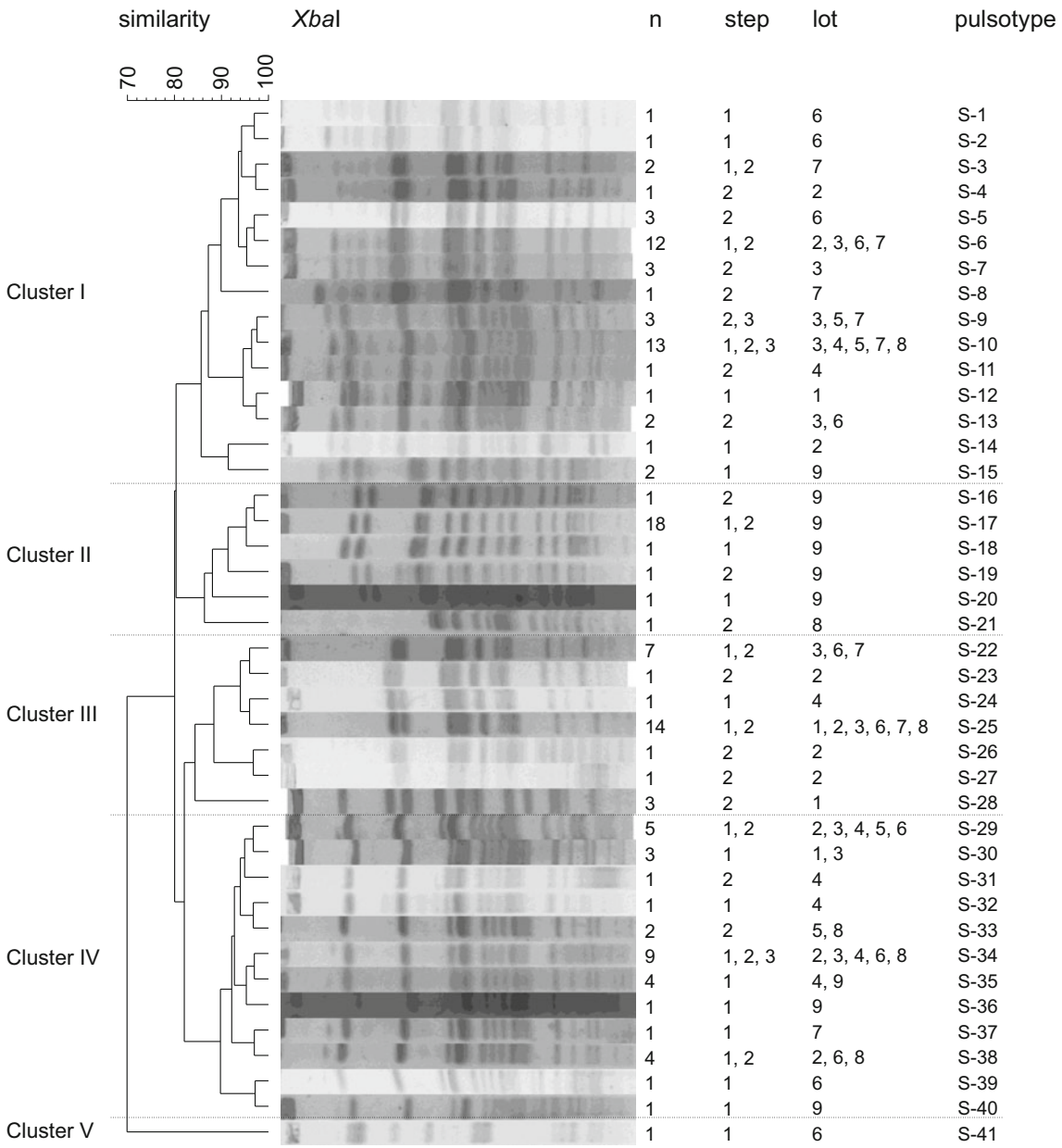

Fig. 2 Schematic representation of genetic profiles (pulsotypes) obtained by PFGE after macro-restriction of DNA using *Xba*I from isolates of *Salmonella* spp. that were obtained in different steps of slaughtering (step 1: after de-feathering, step 2: after evisceration, and step 3: after pre-chilling). Similarities among pulsotypes was estimated by using the Dice coefficient (5 % of tolerance). n: number of isolates

environment of Sl1 and Sl2, the presence of the pathogen in these sites can represent a risk for cross contamination, indicating the need for adequate hygienic procedures, as described by Wang et al. (2013).

Macro-restriction was not possible for only 24 isolates. Figures 1, 2, and 3 present macro-restriction profiles from isolates grouped by different steps (reception, slaughtering, and processing, respectively). Macro-restriction was possible for only one isolate that was obtained from a cutting board in Sl2 and it presented a different pulsotype from all other isolates, preventing a comparison of it with those isolates obtained in Sl1.

It was possible to perform the subtyping of 11 out of 16 isolates obtained from shipping boxes, which were grouped in 9 pulsotypes numbered from R-1 to R-9 (Fig. 1). All of these pulsotypes, except for R-9, were composed by only one isolate. R-9 was composed by three isolates, all from the same chicken lot. The lots that presented positive results for *Salmonella* spp. presented isolates with different genetic profiles, indicating a continuous introduction of new strains into the slaughterhouse from chicken production farms. Moreover, it was possible to observe that a same lot can present isolates with different genetic profiles; for example, lot 2 showed

pulsotypes R-5 and R-6, an evidence of the isolates diversity from a same farm. The genetic diversity of isolates obtained from farms and their spreading in slaughterhouses were also observed by Kim et al. (2007).

Figure 2 presents the enzymatic restriction analysis of 132 *Salmonella* spp. isolates from chicken carcasses, distributed in 41 pulsotypes (S-1 to S-41) and grouped in 5 clusters. Cluster I grouped 47 isolates that presented a similarity greater or equal to 85.7 % (pulsotypes S-1 to S-15). Isolates from cluster I obtained from different slaughtering steps and/or from different chicken lots presented identical genetic profiles (pulsotypes S-3, S-6, S-9, S-10, and S-13). In cluster II, 23 isolates shared a minimum of 86.4 % similarity (S-16 to S-21) and almost all of these isolates were from lot 9, with only one from lot 8; still, pulsotype S-17 included isolates from the same chicken lot, which were obtained from different slaughtering steps (C1 and C2). In cluster III, 28 isolates presented a minimum of 84.3 % similarity (S-22 to S-28); in this cluster, it was observed that isolates with identical profiles were obtained from different slaughtering steps and/or from different lots, more specifically pulsotypes S-22 and S-25. In cluster IV, 33 isolates shared a minimum of 89.8 % similarity (S-29 to S-40), and isolates obtained from different slaughtering steps and/or from different lots presented identical genetic profiles (S-29, S-30, S-33, S-34, S-35, and S-38). Only one isolate was included in cluster V, which shared a 69.9 % similarity with the other clusters. Pulsotypes S-10 (Cluster I) and S-34 (Cluster IV) were recorded in the three slaughtering steps, indicating their cross-contamination among chicken carcasses (Fig. 2).

Figure 3 presents the pulsotypes of the isolates obtained in the processing environment and end cuts; the single isolate obtained from the processing environment (pulsotype P-1) was not genetically identical to any others. Regarding the end cuts, it was possible to subtype four isolates, all obtained from chicken wings samples: each of these isolates was grouped in a different pulsotype (C-1 to C-4), evidencing the genetic diversity of them even being obtained from a same processing environment.

Considering the subtyping results, it is interesting to highlight that isolates obtained from different chicken lots presented identical genetic profiles, which is an evidence of the persistence of these isolates among animals that were obtained from different farms. Moreover, it was possible to identify isolates from the reception step (shipping boxes) with identical genetic profiles to isolates from the slaughtering steps (C1, C2, and C3); by analyzing Figs. 1 and 2, the following pulsotypes are identical: R-1 and S-10, R-3 and S-19, R-7 and S-6, and R-9 and S-25. Also, isolates with identical genetic profiles were obtained from shipping boxes, from carcasses after de-feathering and evisceration, and from end cuts (Figs. 1, 2, and 3: R-4, S-29, and C-4). These results indicate possible cross-contamination routes, persistence, or reintroduction of isolates with identical profiles in the slaughterhouse where the samples were obtained.

Several studies track possible contamination routes of *Salmonella* spp. in the chicken production chain. Chen et al. (2011) demonstrated the persistence of *Salmonella* spp. for over six years in a chicken meat production chain through PFGE analysis. Lee et al. (2007) observed identical genetic profiles of *Salmonella* spp. isolated from farms, hatcheries, and carcasses obtained in slaughterhouses, demonstrating the relevance of breeding as a contamination source of the final product, as also demonstrated by Kim et al. (2007). In a study performed by Nogrady et al. (2008). different samples were collected from farms, slaughterhouses, and human feces, resulting in 145 isolates that were grouped into 3 clusters divided into 8 pulsotypes in total; in the same cluster, it was observed that samples from distinct origins had a high genetic similarity, thus indicating possible cross-contamination. Rasschaert et al. (2008) tracked the sources of contamination in chicken meat by *Salmonella* spp. since farms to slaughtering steps and described isolates with different genetic

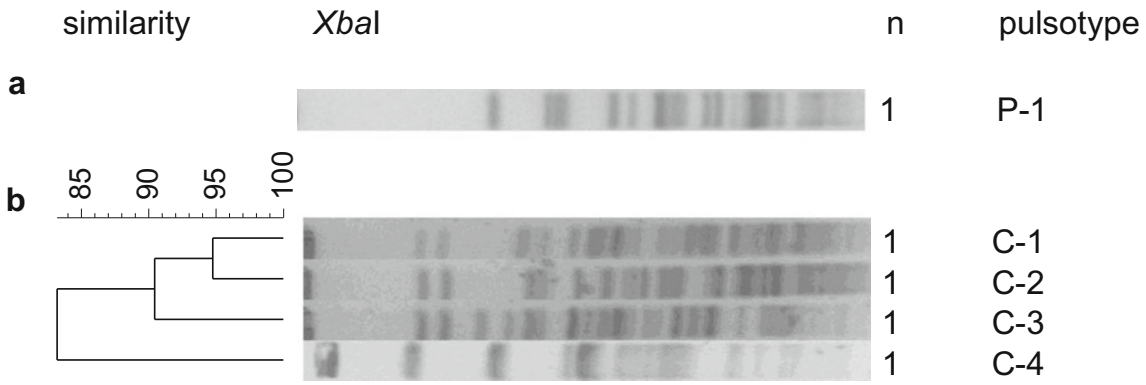

Fig. 3 Schematic representation of genetic profiles (pulsotypes) obtained by PFGE after macro-restriction of DNA using *Xba*I from isolates of *Salmonella* spp. that were obtained from processing ambient in Sl2 (A, cutting board) and cuts in Sl1 (B, wings). Similarities among pulsotypes was estimated by using the Dice coefficient (5 % of tolerance). n: number of isolates

profiles, highlighting the relevance of other sources than the poultry for contamination, such as the processing environment and the shipping boxes.

It was not possible to identify in the present study whether the isolates from the carcasses and boxes from Sl2 were identical to isolates obtained from Sl1. This similarity among isolates from different slaughterhouses was observed by Capita et al. (2007). who demonstrated by ribotyping a high genetic relationship among *Salmonella* spp. isolates obtained from different slaughterhouses. The obtained results allowed the identification of possible contamination routes of poultry carcasses during slaughtering, and revealed evidence that this contamination can be from the external environment, such as the breeding and shipping steps. Thus, it was demonstrated the relevance of adopting adequate control procedures in all the chicken meat production chain, since the initial stage of animals breeding up to the final processing steps.

In addition, the results also allowed the demonstration of the relevance of chicken meat as a source of *Salmonella* spp. for humans. Studies that compare the genetic profiles of isolates obtained from the chicken processing chain with the genetic profiles of isolates obtained from clinical samples must be hereafter performed, as has been described in some studies (Boonmar et al. 1998; Cardinale et al. 2005; Cheong et al. 2007).

It was possible to observe that contamination by *Salmonella* spp. is still a critical problem in the poultry slaughterhouses. Based on isolates obtained at different processing steps, it was observed that pre-chilling was important for reducing the frequencies of *Salmonella* spp. in chicken carcasses. Moreover, the macro-restriction analysis showed potential contamination routes by *Salmonella* spp. during chicken slaughtering and processing, revealing that the reception of contaminated animals is important due to the introduction of new strains into slaughterhouses as well as with regards to slaughtering steps that may favor carcass contamination. Finally, persistent *Salmonella* spp. strains were identified in Sl1, requiring proper control measures.

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