Salmonella enterica *diversity and antimicrobial resistance profile in broiler slaughterhouse by-products*

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Environmental contamination, Poultry by-products, Poultry farming, Salmonellosis.

Summary
The aim of this study was to investigate the presence of *Salmonella enterica* in by-products (feathers, spleen, cecum, and crop) from broiler slaughterhouses as well as to determine the antimicrobial resistance profile of the identified serovars. Forty-four lots of broilers in nine slaughterhouses located in the central-west region of Brazil were evaluated. Samples of spleen, feathers, cecum, and crop were collected in a pool and a total of 1,232 samples were evaluated. These were processed for conventional bacterial isolation and subjected to biochemical and serological tests to identify serovars. The identified serovars were subjected to the antimicrobial susceptibility test, where nine different antimycotics were investigated. *Salmonella enterica* was identified in 7.1% (87/1,232) of all evaluated samples, mostly in feathers (12.3%) and spleen (8.1%). The most frequent serovars were Schwarzengrund (29.9%), Agona (25.4%), Mbandaka (12.7%) and Anatum (8.1%). Nine serovars showed resistance to at least one antimicrobial, especially serovars Mbandaka, Infantis and Typhimurium. Amoxicillin and tetracycline were not effective in inhibiting at least five and four serovars, respectively.

Introduction

Over the years, despite the adoption of different control and prevention measures, infections by non-typhoid *Salmonella enterica* serovars such as Enteritidis, Typhimurium, Agona and Derby are considered one of the main causes of foodborne diseases in humans (Kirk *et al.* 2015). These infections have been reported as a serious problem for human and animal health, mainly due to the diversity of isolates in poultry products as well as antimicrobial multidrug resistance (Alvarez *et al.* 2019).

Salmonella serovars can survive for long periods in several reservoirs such as facilities, poultry litter, insects and water and become a source of infection for birds during the production flow (Afshin *et al.* 2014). Infected birds are potential disseminators of *Salmonella*, mainly during the pre-slaughter, transport, and slaughter management, as the stress caused during these processes, along with possible environmental contamination, is directly related to an increase in the number of infected animals in the lot (Gonçalves *et al.* 2014).

In this respect, by-products deriving from the chicken slaughter process, such as feathers and offal, can become sources of contamination. These by-products are usually intended for the manufacture of meals. If not properly treated, they cross-contaminate the surroundings of slaughterhouses, soil, water, and vegetation (Cardoso and Tessari 2008). They can even reach not only animal production systems, but the human food chain (Hsieh *et al.* 2016). Despite the scarse research on this topic, its investigation will make it possible to assess the level of infection of slaughtered animals as well as the level of contamination in slaughterhouses and of the products that will be generated (Djeffal *et al.* 2018). Therefore, studies in this regard enable the adoption of control and prophylaxis measures to ensure the quality of the final product.

In addition to examination for the presence of *Salmonella*, epidemiological investigation of the

antimicrobial resistance of this pathogen is also an important tool that can be used to understand its impact on human health, since resistant bacteria can spread from an ecosystem to another in bacterial populations across the food chain (Anderson *et al.* 2003). Thus, based on this assumption, the present study was conducted to investigate the presence of *Salmonella enterica* in by-products (feathers, spleen, cecum, and crop) from broiler slaughterhouses as well as to obtain the antimicrobial resistance profile of the identified serovars.

Materials and methods

The study was carried out with 44 broiler lots in nine slaughterhouses located in the central-west region of Brazil. Six of these nine slaughterhouses were small (up to 50,000 birds slaughtered per day) and the other three were large (over 51,000 birds slaughtered per day).

Twenty-one samples of spleen, cecum, crop, and feathers were collected from each lot and subjected to ante- and postmortem inspection. These were processed in pools, each of which was composed of three organs and feather. Seven pools were made for each sample type and for each lot, totaling 1,232 samples. The organs were collected in the evisceration room, directly from the overhead conveyor, whereas the feathers were collected in the scalding and plucking room, directly from the plucking machine while the birds passed through it.

The samples were packed individually in labeled polystyrene bags, which were then placed in polystyrene boxes with ice and transported to the Bacteriology Laboratory of the Veterinary and Animal Science School of the Federal University of Goiás.

Bacteriological analysis

The analytical methodology of conventional bacteriological tests followed the procedures described in ISO6579. Three to five colony-forming units (CFU) with morphological characteristics of *Salmonella* were selected and transferred to tubes containing triple iron-sugar agar (TSI), which were incubated at 37 °C for 18-24 h. The TSI with suggestive growth of *Salmonella* were subjected to biochemical tests, namely urease, indole, methyl red, motility, glucose, lactose, sucrose, lysine decarboxylase, malonate, and Simmons citrate. Samples with compatible biochemical tests for *Salmonella* were subjected to serological testing using the polyvalent somatic anti-*Salmonella* serum.

The samples were sent on nutrient agar to the Oswaldo Cruz Foundation (FIOCRUZ-RJ) for antigenic characterization of serovars. This was performed by the detection of somatic and flagellar antigens, using polyvalent and monovalent antiserum, with or without induction of the adopted flagellar phases, adopting the Kauffmann-White scheme. Results were subjected to descriptive statistical analysis and the percentages of relative frequency for the detection of *Salmonella enterica* were calculated and the serovar identified.

Antimicrobial susceptibility test

The antimicrobial susceptibility profile was determined by using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2019). The antibiotics tested were ampicillin (10 µg), amoxicillin (3 µg), ceftiofur (30 µg), ciprofloxacin (10 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), fosfomycin (200 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg). The reference strain for *Salmonella typhimurium* (ATCC 14028) was used as a standard.

Results

According to the microbiological tests, 50.0% (22/44) of the evaluated lots were positive for *Salmonella enterica*, regardless of the sample category evaluated. Considering the sample type, 12.30% (38/308) of the evaluated feathers were positive for *Salmonella enterica*. Feather was the sample type found most frequently positive in the evaluated lots (34.10%, 15/44). Regarding the examination of the bacterium in the organs, 8.1% (25/308) of the spleen samples were positive, with *Salmonella* detected in 31.8% (14/44) of the bird lots. As such, the spleen was the organ with the highest frequency of isolates, when compared with the cecum and the crop (Table I).

After the identification of *Salmonella* sp., the 8 isolates were characterized antigenically and 15 different serovars were identified. The most frequent among those identified (Table II) were Schwarzengrund 29.9% (26/87), Agona 25.4% (22/87), Mbandaka 12.7% (11/87), Anatum 8.1% (7/87), Infantis (4.6%) and Rissen 3.4% (3/87). Other serovars, e.g., Typhimurium, Livingstone, Cerro,

Table I. *Frequency of* Salmonella enterica *in broiler by-products obtained from slaughterhouses in the central region of Brazil.*

By-product	Lots		Samples/Lot	
	n/N	%	n/N	%
Feather	15/44	34.1	38/308	12.3
Spleen	14/44	31.8	25/308	8.1
Cecum	8/44	18.1	12/308	3.9
Crop	7/44	16.0	12/308	3.9
Total	-	100	87/1232	7,1

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Montevideo and Panama, were identified in 2.2% (2/87) of the samples, whereas Senftenberg, Derby, Lexington and Braenderup were detected in 1.1% (1/88) of the samples.

The Schwarzengrund, Agona and Mbandaka serovars were present in all sample categories, with greater frequency occurring in the samples of feathers and spleens. The Cerro, Montevideo, Panama, Derby and Lexigton serovars were identified only in the spleen samples, Livingstone, only in the crop. Infantis was detected in feathers and spleen and Braenderup was found only in the cecum.

Table III illustrates the results of the antimicrobial susceptibility test. Nine serovars showed resistance to at least one antimicrobial used in this study. Mbandaka, Infantis and Typhimurium are stood out, with resistance to five, four and three antimicrobials, respectively. Amoxicillin and tetracycline were the antimicrobials that were not effective in inhibiting at least five and four serovars, respectively.

Discussion

Most studies on the presence of *Salmonella*

Table II. *Frequency of* Salmonella enterica *serovars isolated from feathers, spleen, crop and cecum of broilers slaughtered in slaughterhouses in the central region of Brazil.*

Serovar	Feather	Spleen	Crop	Cecum	Total	Frequency (%)
Schwarzengrund	14	05	05	02	26	29.9
Agona	14	04	02	02	22	25.4
Mbandaka	02	02	03	04	11	12.7
Anatum	05	-	-	02	07	8.1
Infantis	01	03	-	-	04	4.6
Rissen	-	02	-	01	03	3.4
Typhimurium	01	01	-	-	02	2.3
Livingstone	-	-	02	-	02	2.3
Cerro	-	02	-	-	02	2.3
Montevideo	-	02	-	-	02	2.3
Panama	-	02	-	-	02	2.3
Senftenberg	01	-	-	-	01	1.1
Derby	-	01	-	-	01	1.1
Lexington	-	01	-	-	01	1.1
Braenderup	-	-	-	01	01	1.1
Total	38	25	12	12	87	100.00

Table III. *Antimicrobial susceptibility of* Salmonella enterica *serovars isolated from by-products from broiler slaughterhouses in the central region of Brazil.*

Serovar	Amo	Amp	Cef	Cip	Clo	Enr	Fos	Tet	Sut
Schwarzengrund	-	-	-	-	-	-	-	-	-
Agona	R	-	R	-	-	-	-	-	-
Mbandaka	R	-	-	R	-	R	-	R	R
Anatum	-	-	-	-	-	-	-	R	-
Infantis	R	-	R	-	-	-	-	R	R
Rissen	-	-	-	-	-	-	-	-	-
Typhimurium	R	-	-	R	-	-	R	-	-
Livingstone	-	-	-	-	-	-	-	-	-
Cerro	-	-	-	-	-	-	-	-	-
Montevideo	-	-	-	R	-	R	-	-	-
Panama	R	-	R	-	-	-	-	-	-
Senftenberg	-	-	-	-	-	-	-	-	-
Derby	-	-	-	-	-	-	-	R	R
Lexington	-	-	-	-	-	-	-	-	-
Braenderup	-	-	-	-	-	-	-	-	-
ATCC 14028	-	-	-	-	-	-	-	-	-

R = Resistant; - = Sensitive or intermediate; Amo = Amoxicillin; Amp = Ampicillin; Cef = Ceftiofur; Cip = Ciprofloxacin; Clo = Chloramphenicol; Enr = Enrofloxacin; Fos = Fosfomycin; Tet = Tetracycline; Sut = Trimethoprim-sulfamethoxazole).

enterica in slaughterhouses consist basically of the investigation of the agent in carcasses (Cunha-Neto *et al.* 2018). Also, in the central region of Brazil, Cunha-Neto and colleagues (Cunha-Neto *et al.* 2018) found different *Salmonella enterica* serovars in broiler carcasses and in their slaughterhouses. However, it is important to stress that the occurrence of these serovars varies according to the geographic region, mainly due to varying animal management techniques, host immune response, intestinal microbiota and genetic characteristics of the pathogen (Andino and Hanning 2015).

The presence of the agent in by-products has a high sanitary impact in terms of environmental contamination and on the probable infection of other animals, which can potentially influence the indirect infection of slaughterhouse workers (Ullah *et al.* 2017). A fact that emphasizes this reality is the high frequency of *Salmonella* found in the feathers of the slaughtered birds. Along with excreta, feathers are considered important sources of contamination and infection (Miskiewicz *et al.* 2018). In an immunohistochemical study, Rimet and colleagues (Rimet *et al.* 2019) recently demonstrated that *Salmonella enterica* cells accumulate in the lumen of feather follicles, mainly in the neck region, which protects them during the washing procedures and chemical treatments, making their elimination difficult (Lee *et al.* 2014).

The frequency of feather contamination observed in this study is lower than that described by Lee and colleagues (Lee *et al.* 2019), who investigated the sequential transmission of *Salmonella enterica* in the chicken slaughter and found a frequency rate close to 68%. Therefore, the scalding and plucking processes constitute the two most important moments of slaughter (Waghamare *et al.* 2019), since the contact of infected birds with these initial stages results in the contamination of machinery and the tank water, potentially enabling cross-contamination (Borges *et al.* 2019).

In addition to the feathers, organs such as the spleen, liver, cecum and small intestine are the most affected in infected chickens and have the highest bacterial load (Zeng *et al.* 2018). After invading the intestinal tract, *Salmonella* sp. survives in the macrophage cytoplasm then reaches the lymphoid clusters and circulation. The spleen is considered one of the main target organs of the bacterium, as it is a reservoir of lymphocytes and macrophages (Mittrucker *et al.* 2002), which is also corroborated by the observed increase in splenic CD^{3+} cells (Audia *et al.* 2001).

We can infer that, even if infected, the birds slaughtered in the slaughterhouses evaluated in this study indicate low excretion. Similar results were described by Elmonir and colleagues (Elmonir *et al.* 2017), in which *Salmonella enterica* was more frequently detected in the spleen than in the organs of the gastrointestinal tract. In addition, we observed that the majority of isolates in cecum and crop were also found in the spleen, suggesting that these animals are asymptomatic carriers.

Our results disagree with those described by Moraes and colleagues (Moraes *et al.* 2014), who found a higher frequency of isolation in the crop, although the serovars found in the organ were similar. The pre-slaughter fast can promote changes in the pH of the crop, modifying the local microbiota and, consequently, favoring the growth of *Salmonella* in this organ (Hinton *et al.* 2000). However, in the face of stressful situations, the isolation of the bacteria in these two organs is considered significant, becoming a problem during slaughter, as they are prone to rupture during evisceration (Buh *et al.* 2017).

In our study, a wide variety of serovars were isolated, some of which are very important from the public-health perspective. Among these are Schwarzengrund, Infantis, Anatum, Braenderup and Typhimurium, which have been identified as highly important circulating serovars in the food production chain (Monte *et al.* 2019, Tegegne 2019). These are most often characterized as multidrug-resistant serovars capable of carrying a wide diversity of virulence genes (Monte *et al.* 2019).

Many of the identified serovars are associated with outbreaks of infections in human beings linked to poultry farming, characterizing an emerging public health problem. Among them, those with the greatest impact include Typhimurium (Anderson *et al.* 2016), Infantis, Agona and Montivideo (Basler *et al.* 2016). The Schwarzengrund and Mbandaka serovars have been identified as important environmental contaminants due to their high ability to adapt to *ex vivo* survival (Hayward *et al.* 2016) and have also been reported in cases of infections in humans (Lindsay *et al.* 2018).

Increasing antimicrobial resistance rates have been reported in several serovars of *Salmonella enterica* isolated during the slaughter process in slaughterhouses (Lee *et al.* 2019). Similar to the results of this study, higher rates of resistance to tetracyclines and amoxicillin were also observed by Alvarez and colleagues (Alvarez *et al.* 2019) and Dantas and colleagues (Dantas *et al.* 2020). Along with quinolones, resistance to these antimicrobials is worrying since they are applied in the treatment of infectious diseases in humans and in production animals intended for food (Miskiewicz *et al.* 2018).

Although not identified, resistance to ampicillin (Baptista *et al.* 2018) and chloramphenicol (Dantas *et al.* 2020) is often observed in isolates of *Salmonella enterica* in birds in Brazil. In addition, despite being the most frequent serovar, none of the

S. Schwarzengrund isolates showed resistance to the tested drugs. This finding disagrees with the reports of Monte and colleagues (Monte *et al.* 2019), who identified fluoroquinolone and β-lactam resistance genes in several field isolates in Brazil. However, it is important to consider that there is a trend in the resistance pattern depending on the region where the strain is isolated, which explains the differences between national studies.

The multidrug resistance of some serovars, such as Typhimurium, Mbandaka and Infantis was recently described by Monte and colleagues (Monte *et al.* 2019) and Lee and colleagues (Lee *et al.* 2019). Thus, they are identified as important contaminants in the processing line and of chicken carcasses. A fact that reinforces these results is well demonstrated by Mendonça and colleagues (Mendonça *et al.* 2019), who stated that the greatest antimicrobial resistance is found in isolates from slaughterhouses. Several of these multidrug-resistant serovars circulating in poultry production have varied genetic elements, which contributes to the permanence of strains along the food chain (Monte *et al.* 2019). Therefore, based on these results, contamination of these by-products by *Salmonella enterica* becomes a concern for the food industry, especially when these raw materials are used in animal feed.

Conclusions

Salmonella enterica is a microorganism present in the by-products from broiler slaughterhouses, especially feathers and spleen. A wide variety of serovars were identified, the most frequent being Schwarzengrund, Agona and Mbandaka. These results demonstrate potential risks of environmental contamination during processing in slaughterhouses as well as negative impacts from the public-health point of view, as these microorganisms are resistant to antimicrobials such as tetracycline and amoxicillin, drugs commonly used in veterinary and human medicine.

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