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Serotypes of Salmonella in Broiler Carcasses Marketed at Ibague, Colombia.

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ABSTRACT

Salmonella enterica is a large group of Gram-negative bacteria responsible for a number of foodborne infections associated with the consumption of contaminated poultry products. The hygienic status of raw chicken meat marketed at Ibague, Tolima, Colombia, is currently unknown. To address this issue, a cross-sectional study was conducted to estimate the prevalence of *Salmonella* spp., in raw chicken marketed at different outlets in this city. *Salmonella* spp. was isolated by standard microbiological methods, followed by biochemical, serological, and molecular confirmation. Additionally, risk factors associated with the presence of the bacteria were identified. The prevalence of *Salmonella* in raw chicken was 17.41% (47/270), and 14 different serotypes were found, out of which *S*. Paratyphi B (36.17%), *S.* Hvittingfoss (19.15%) and *S.* Muenster (10.64%) were the most prevalent and represented 65.95% of all serotypes. Amplification of 284 bp of the *inv*A gene was achieved by PCR in a number of randomly selected isolates. Raw chicken as the only type of meat sold at stores (odds ratio: 2,157, $p<0.05$), and stainless steel as a contact surface of chicken meat (odds ratio: 13,29, $p<0.05$), were found to be potential risk factors for the presence of *Salmonella* in chicken meat. This work serves as a reference about the current status of *Salmonella* in chicken meat marketed in Ibague, Tolima, Colombia, and indicates the need to establish appropriate control and contingency measures to minimize the presence of the bacteria in raw chicken to prevent its transmission to humans.

INTRODUCTION

The genus *Salmonella* is a group of Gram-negative bacteria belonging to the Enterobacteriaceae family that cause food poisoning in humans worldwide. Two species are recognized within this genus; *S. enterica* and *S. Bongori* and today more than 2,600 serotypes have been described. Importantly, about 93.8 million illnesses, of which 80.3 million are foodborne and 115,000 deaths each year are caused by non typhoidal *Salmonella* (Majowicz *et al*. 2010), where raw chicken meat has been recognized as a significant source of human salmonellosis (Mercado *et al*. 2012; Yang *et al*. 2010). *Salmonella* can infect birds in different steps of the production chain including live production (*e.g*. breeding and broiler farms), where it can induce clinical symptoms or an asymptomatic infection (Barua *et al*. 2013; Carrasco *et al*. 2012; Ibrahim *et al*. 2014). *Salmonella* may also be present in processing plants and in further-processing plants, where cross-contamination between carcasses by contact with feces or by sharing materials used in processing may occur (Carrasco *et al*. 2012). Additionally, the bacterium has been reported in end products, such as meat and eggs sold to consumers (El-Aziz, 2013; Tammakritsada & Todhanakasem, 2012; Zhu *et al*. 2014).

Salmonellosis in humans is characterized by fever, diarrhea and acute abdominal pain that maybe a self-limiting gastrointestinal infection, such as those caused by serotypes Typhimurium and Enteritidis (Mercado *et al*. 2012; Santos *et al*. 2001);however, when the bacteria enter the bloodstream it can be life-threatening (Gómez & Zuñiga, 2005; Jiménez *et al*. 2011). Nevertheless, the virulence factors expressed by the bacteria and the immune status of the patient may be critical in determining the clinical form of the disease. The serotypes *S*. Infantis, *S*. Newport, and *S*. Hadar are commonly isolated from poultry and represent potential risks to human health since they have recently been associated with *Salmonella* outbreaks by direct contact with live birds (Centers of Disease Control and Prevention, 2014). In addition, strains of *S.* Java and Schwarzengrund have been isolated from chicken meat, presenting pulsed field gel electrophoresis patterns identical to those isolates from humans, strongly suggesting that the poultry carcasses maybe the source of infection (Brown *et al*. 2003; Chen *et al*. 2012).

Poultry products are well-known sources of *Salmonella* infection to humans and preventive measures on the farm need to be established to begin its control. Control programs for *Salmonella* have proven to be efficient to reduce economic losses (INFOSAN, 2005). In Colombia, limited information is available on the status of *Salmonella* species circulating in poultry products and those responsible for human infections. Recently, *Salmonella* serotype Typhimurium variant 5 was isolated from human cases of salmonellosis in Paz del Rio, Boyacá (Díaz *et al*., 2014). However, most of the cases are not reported to the medical centers and underreporting of cases of bacterial gastroenteritis predominates. In addition, people do not always go to a health centers and usually they are treated as outpatients without any clinical analysis and laboratory isolation. This situation is worsened by reports of emerging multidrug resistant enterobacteria causing huge economic losses to the health system and compromising the patient's life (Rivera *et al*. 2012). The chicken markets in Colombia, in most of the cases, do not meet the standards of good manufacturing practices (Flórez *et al*. 2008).

The aim of this study was to estimate the prevalence of *Salmonella* spp., circulating in chicken carcasses marketed at the Ibague city, during the period February to May 2014. The study also provides the main serotypes and identifies potential risk factors associated with *Salmonella* contamination.

MATERIALS AND METHODS

Study design and sample collection

Across-sectional study was conducted (between February and May 2014) to establish the prevalence of *Salmonella* spp. in broiler carcasses marketed at Ibague, department of Tolima, Colombia. The sample size was calculated by the formula described by Thrusfield (2007), with a 95% confidence level, 5% error, and an expected prevalence of 22.2%, based on a pilot study conducted by our research group at the University of Tolima (unpublished data). For the purposes of this study, 270 samples were taken. The sampling included all the 13 communes that make up the Ibague city, and the number of samples per commune was proportional to the number of stores registered at the authority (Cámara de Comercio, Ibague). Each sample consisted of one drumstick of chicken weighing about 200 g that was randomly taken and immediately packaged in sterile airtight plastic bags, refrigerated on ice, and transported to the Laboratory of Veterinary diagnosis for processing within 3 hr.

Epidemiological survey

A questionnaire was designed and applied to shop owners during an interview at the time of sampling. The variables included in the survey followed those described in other studies (Carrasco *et al*. 2012; Donado-Godoy *et al*. 2012; Nguyen *et al*. 2014), and perfected in the pilot study conducted by our research group at the University of Tolima. The assessed variables are shown in Table 1. An epidemiological map was constructed to indicate the location and number of positive samples per commune using ArcGIS 10.1 version software.

Salmonella isolation and serotyping.

All samples were processed according to the standard international guidelines ISO 6579:2002; ISO 6579:2002/Amd1: 2007 (Reid, 2009). Briefly, samples were incubated in peptone-buffered water for pre-enrichment, with an incubation time of 24 hours at 37°C, which were further seeded in tetrathionate broth (Müller-Kauffmann) and incubated at 37°C and in Rappaport Vassiliadis and incubated at 42°C for selective enrichment. Later cells were seeded on McConkey and XLT4 (Xylose Lysine Tergitol 4) agar. Compatible colonies were seeded in Rambach agar and confirmed as *Salmonella* spp, by challenge with antibodies Poli AI + Vi (Difco® 222641). Positive colonies were confirmed biochemically by using the API® 20E gallery (Biomereux, France). The isolates

Table 1 – Variables and categories assessed to owner/administers of meat shops of Ibague, Tolima in an epidemiological survey.

VARIABLE	CATEGORIES	DESCRIPTION
Storage type	Frozen	Temperature at time of collection of -5 ° C
	Cooled	Temperature at the time of collection of 4-10 ° C
Source	Integrated Company	All production cycle is handled by one company
	Non integrated company	Production cycle is handled by several companies
Market type	Supermarkets and neighbor hoodstores	Facility where a variety of food products are marketed
	Outlets	Retail stores that sell only meat and byproducts
Number of workers	<2	Presence of 1 to 2 persons at the time of sampling
	>2	Presence of 3 or more people at the time of sampling
Selling other meats	Yes	Retail store that sell different types of meat
	No	Retail store that only sell poultry meat and poultry byproducts
Storage surface	Stainless Steel	At sampling time, the meat is disposed on stainless steel
	Plastic	At sampling time, the meat is disposed on plastic
Production system	Conventional	Poultry characterized by white skin after slaughter
	Free range	Poultry characterized by yellow skin after slaughter
Handling	Gloves	Meat is handled with gloves
	Skin	Meat is handled without protection

were serotyped using the Kauffman-White scheme (Brenner, 1998), for O and H antigens with commercial antisera (Difco, Becton, Dickinson and Company Sparks, MD). Serotyping was performed based on the antigenic description by Grimont & Weill (2007) and the nomenclature described by Tindall *et al*. (2005), and the Judicial Commission of the International Committee on Systematics of Prokaryotes (2005), and was carried out at the Colombian Institute of Agriculture (ICA).

Polymerase chain reaction

Salmonella isolates were seeded in tryptone soy broth (TSB), and incubated for 24h at 37 °C. Crude DNA was prepared by boiling a culture broth of bacteria for 10 minutes, incubated on ice for a few minutes and then centrifuged at 12,500 rpm for 5 minutes to pellet the particulate matter. The supernatant was collected as crude DNA and 4µl were used as template in the PCR mixture to amplify the *invA* gene by using the forward 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' and reverse 5'-TCA TCG CAC CGT CAA AGG AAC C-3' primers (Invitrogen™,Thermo Fisher Scientific Inc.) with and expected amplicon size of 284 bp.

PCR was carried out in a total volume of 25 µL containing 4µL of template DNA, 1µL of forward primer, 1µL of reverse primer, 0,2 µL of Taq polymerase, 2,5 µL of buffer 10 X, 2,5 µL of $MgCl_2$, 13,8 µL of nuclease free water was also added. PCR was performed in a DNA thermal cycler BIO-RADT100™, after initial denaturation of 1 minute at 94°C, 35 cycles of amplification were performed. Each cycle consisted of the following steps: 60 seconds at 94°C (denaturation), 30 seconds at 64°C (primer annealing), and 30 seconds at 72°C (extension), followed by 7 minutes at 72°C for final extension. *Salmonella Typhimurium* (ATCC 14028) was used as a positive control, whereas the negative control did not contain DNA template. The reaction mixture was mixed with 10 X gel loading buffer and then resolved by electrophoresis on 2% agarose gel with 100 bp DNA ladder. The reaction products were stained with ethidium bromide and visualized under the UV light by using an ENDURO™ GDS (Labnet International, Inc.), GEL documentation system.

Statistical Analysis

Data were analyzed in the IBM SPSS Statistics® 20 version software, and GraphPad Prism® 5.03 version software. Independence between the variables and the presence of *Salmonella* was determined by using 2x2 contingency tables. The strength of association was calculated by the odds ratio. Prevalence was determined as the proportion of positive samples over the total samples, expressed as a percentage.

RESULTS

The prevalence of *Salmonella* spp., in poultry carcasses marketed at Ibague, Tolima was 17.41% (47/270). Isolation of *Salmonella* was slightly higher from supermarkets and small neighborhood stores (42.5%; 20/47), than outlets (57.5%; 27/47), however, there were no statistically significant differences. 57.5% (27/47) of *Salmonella* were isolated from stores with more than two workers, and from stores where different types of meats are sold. *Salmonella* was also isolated with more frequency from non-integrated companies 65,96% (31/47) than integrated ones.

Regarding the source of the poultry contaminated carcasses, 78.7% (37/47) originated from free-range production systems, 51.1% (24/47) were handled without gloves and 91.5% (43/47) were kept in refrigeration. Finally, the majority of isolates (97.9%, 46/47) were obtained from chickens that had been in contact with stainless steel surfaces. The number of isolates per commune is shown in Figure 1, where communes 12 and 13 were *Salmonella* free.

Figure 1. Number of positive samples per commune of the Ibague city.

The most frequently isolated *Salmonella* serotypes were *S*. Paratyphi B (36.17%; 17/47), Hvittingfoss (19.15%;9/47), and Muenster (10.64%; 5/47). The serotypes Typhimurium, Newport, Heidelberg, Braenderup, and Kalina were found at a frequency of 4.26% (2/47) each, while Bovismorbificans, Budapest, Manhattan, Othmarschen, Schwarzengrund, and Skansen were found at a lower frequency (2.13%;1/47) for each serotype.

A number of *Salmonella* isolates were selected for detection of the invasion A gene (*Inv*A) by using polymerase chain reaction. Figure 2 shows a representative image of the PCR results where the expected 284 bp band of the *inv*A gene of *Salmonella* was present in all selected isolates.

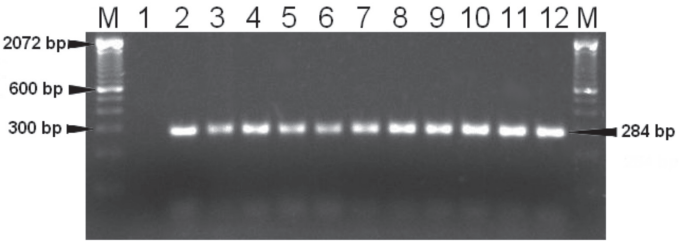

Figure 2 – PCR amplification of *inv*A gene in selected isolates. Lane M represents 100bp molecular weight marker, lane 1 represent negative control, lane 2 represent positive control (*Salmonella* Typhimurium), lanes 3 – 12 represent selected isolates.

Among the variables evaluated, the outlets that sold only meat and byproducts (OR: 2.157, $p<0.05$) and the presence of stainless steel as the contact surface (OR: 13.29, $p<0.05$) were identified as risk factors for the presence of *Salmonella* spp., in the chicken carcasses marketed in Ibague, Tolima. Likewise, keeping the chicken meat refrigerated (OR:1.7) and the source of chicken meat from nonintegrated companies (OR:1.5) may also affect the presence of *Salmonella*; however, those differences were not significant ($p> 0.05$). On the other hand and contrary to what was expected, handling of carcasses without any protection by workers was not associated ($p> 0.05$) with the presence of *Salmonella*. Table 2 shows the distribution of isolates and potential risk factors for the presence of *Salmonella* in chicken meat marketed at Ibague, Tolima.

Table 2 – Risk factors and frequency distribution of *Salmonella* in chicken meat marketed at Ibague Tolima.

VARIABLE	No. (%) OF POSITIVE SAMPLES	OR*	CI** 95%	*P*
Storage type				
Cooled	43 (18.3)	1.736	0.5819 - 5.177	0.4728
Frozen	4(11.43)			
Source				
Nonintegrated Company	31 (20)	1.547	0.8005 - 2.989	0.2557
Integrated Company	16 (13.91)			
Market type				
Outlet	27 (17.76)	1.058	0.5603 - 1.999	1
Supermarket	20 (16.95)			
Number of workers				
<,= 2	27 (15.43)	0.6841	0.3601 - 1.300	0.2452
> 2	20 (21.05)			
Selling other meats				
no	20 (25.97)	2.157	1.124 - 4.141	0.0317
yes	27 (13.99)			
Storage surface				
Stainless steel	46 (21)	13.29	1.788 - 98.88	0.0004
Plastic	1 (1.96)			
Production system				
Free range	37 (17.62)	1.069	0.4969 - 2.301	1
Conventional	10 (16.67)			
Handling				
Skin	24 (14.37)	0.5838	0.3096 - 1.101	0.1009
Gloves	23 (22.33)			

*OR: Odds ratio; **CI: Confidence interval

DISCUSSION

Salmonella spp., was isolated from 17.41% of chicken samples (47/270) marketed in Ibague, Tolima, and a number of serotypes were identified and confirmed by amplification of a fragment of the *invA* gene using PCR (Galan, Curtiss, 1991; Li *et al*., 2012; O'Regan *et al*., 2008; Shanmugasamy *et al*., 2011), a rapid and powerful technique used for *Salmonella* identification (Cardona-castro *et al*., 2007; Ibrahim *et al*., 2014; Molina *et al*., 2010; Tafida *et al*., 2013). Thus, this data is a representative estimation of the occurrence of *Salmonella* in a region with traditional poultry industry and it may indicate poor hygienic and disinfection practices, which have been associated with cross-contamination and recontamination of poultry carcasses (Carrasco *et al*. 2012).

This prevalence is higher than that reported in raw chicken (5.26%; n = 209) sold in Bangkok, Thailand (Akbar, Kumar, 2013), and in chicken at slaughter plants (7.52%; n = 425) in France (Hue *et al*., 2011). Those differences may be due to differences in market conditions (*e.g*., production volume, cold chain) and regulations in each country. Previously in Colombia, Donado-Godoy *et al*. (2012) estimated a *Salmonella* prevalence of 27% (n = 1003) in chicken carcasses marketed at different stores across the country; however, the number of samples taken at the chicken market in Ibague (n=27) was considerably low, suggesting that this prevalence may not be representative of this city. The prevalence of *Salmonella* in this study is very close to that reported in raw chicken (20%; n=45) marketed in Mérida, Venezuela (Molina *et al*., 2010), and significantly lower than the prevalence (41.6%) reported in chicken meat marketed in 6 provinces of China (Zhu *et al*., 2014), where other studies also have documented a prevalence of *Salmonella* up to 54% (n=515) by using molecular techniques such as PCR (Yang *et al*., 2010). These studies reveal that the prevalence of *Salmonella* in chicken meat may vary dramatically between distinct geographical regions based on the use of more sensitive diagnostic techniques.

Salmonella serovar Enteritidis and Typhimurium are the main serotypes isolated from poultry (Ibrahim *et al*., 2014; Kim, 2010). In the present study, *Salmonella* ser. Paratyphi B was the most prevalent in chicken meat, consistently with the report of Boscán *et al*. (2005), who isolated the bacteria from chicken viscera in two slaughter plants in the state of Zulia, Venezuela, and the report by Barua *et al*. (2013), who isolated this serotype from breeders and broiler farms in Bangladesh. *S.* Paratyphi B is known to be adapted to commercial poultry (Toboldt *et al*., 2013; van de Giessen *et al*., 2006; Van Immerseel *et al*., 2004), and it can be isolated from farm to store (Egervärn *et al*., 2014). *S*. Muenster has been isolated from pork sausages (Torres *et al*., 2013), ground beef (Bosilevac*et al*., 2009), cheese from goat milk (Van Cauteren *et al*., 2009), pork and poultry (Meneses, 2010). In our study, *S*. Muenster represented 10.64% (5/47) of isolates, which is similar to the study of Khallaf *et al*. (2014), who found 13% (5/38) S. Muenster in chicken meat samples. The serotypes found in our study differ from those reported in egg-laying hen farms in the Tolima region of Colombia by Rodriguez *et al*. (2015), who identified *S*. Enteritidis and *S*. Shannon. Taken together, these studies show diverse geographical distribution of *Salmonella* serovars in poultry and byproducts as well as in other kind of meats, and highlight the importance of contamination from farm and the cross-contamination in the stores and slaughter plants.

The serotypes *Salmonella* Paratyphi, *S*. Typhimurium, *S*. Newport, *S*. Heidelberg, *S*. Braenderup, and *S*. Schwarzengrund found in our study have previously been associated with outbreaks of disease in humans (CDC, 2014). Likewise, *Salmonella* Typhimurium, *S*. Branderup, and *S*. Muenster have been associated with foodborne outbreaks in Colombia (National Institute of Health, 2011), suggesting a potential link between poultry and salmonellosis in this region. However, the impact of *Salmonella* in the Tolima region has not been currently addressed.

Marketing of poultry carcasses as the only type of meat sold in a store was found as a potential risk factor for *Salmonella* contamination (Odds ratio: 2.157, $p < 0.05$), which is consistent with the report of Hue *et al*. (2011), who found that the slaughtering of the species *Gallus gallus* alone was a risk factor for *Salmonella* contamination in France (OR: 7.08, $p < 0.001$). The argument for this finding was that the sacrifice of various animal species demands stricter hygiene measures and better organization than processing a single species. Similarly, stores that sell meat from different species may involve more employees than those that sell meat from a single species. Nevertheless, opposite situations were reported by Acosta *et al*. (2013), who argued that a higher prevalence of *Salmonella* spp. in meat than other foods may be due to contamination of carcasses by handlers (Gomes-Neves *et al*., 2014). In our study, the number of workers at the chicken meat shop did not represent a statistically significant risk factor.

The serotype of *Salmonella* and the hydrophobicity of the contact surface may positively influence the adhesion process (Chia *et al*. 2009; Pérez-Rodríguez *et al*. 2008), and *Salmonella* has a greater adhesion capacity to surfaces made of stainless steel than plastic and acrylic (Chia *et al*., 2009; Nguyen et al., 2014), where it may be able to form biofilms (Tammakritsada & Todhanakasem, 2012; Giaouris *et al*. 2012). This study found that the contact of chicken meat with surfaces made of stainless steel was a risk factor (odds ratio: 13.29, p <0.05) for *Salmonella* contamination, suggesting that disinfection of equipment made of stainless steel may be insufficient, given that biofilms formed on stainless steel are more sensitive to disinfectants than those adhered to plastic (Joseph *et al*., 2001). Another possible reason for those findings could be the misuse of disinfection protocols and the use of disinfectant concentrations below the recommended level, inadequate exposure time, among other variables that may influence the effectiveness of disinfectants (Møretrø *et al*. 2012). Recently, Wang *et al*. (2015) reported biofilms of *Salmonella* on stainless steel surfaces that facilitated the transfer of the bacteria to meat products, and Arcos-Ávila *et al*. (2013) isolated *Salmonella* from fomites, such as knives, and counters made of stainless steel. This highlights the importance of implementing rigorous protocols for cleaning and disinfecting equipment and tools, as well as microbiological sampling to verify if these protocols fulfill their aims, in addition of creating awareness in food-handling staff.

Salmonella was isolated in 68.08% of samples from nonintegrated companies suggesting that contamination may involve different people during the marketing process. Nonintegrated companies was reported as a risk factor (OR: 2.0, p <0.001) for *Salmonella* contamination in Colombia (Donado-Godoy *et al*. 2012). However, this was not the case in the present study, and therefore, the impact of this variable needs to be evaluated to establish whether the quality and control measures at each step of the production chain are indeed reduced in nonintegrated companies. Finally, the majority of sampled stores sold chilled carcasses (87.41%), but this variable was not identified as a risk factor, contrary to previous studies (Donado-Godoy *et al*., 2012; Zhu *et al*.,2014). The reason for those results are currently unknown; however, attention and efforts should be focused on the time of refrigeration of carcasses, as well as in the rate of replacement of old carcasses by fresh ones, that may influence the presence of *Salmonella*.

In conclusion, this study estimated for the first time a prevalence of 17.4% S*almonella* in raw chicken meat marketed at Ibague, Tolima, where *S*. Paratyphi B, a well-known cause of human salmonellosis (Toboldt *et al*. 2013), was the most frequently isolated serotype, followed by Hvittingfoss and Muenster and by Typhimurium, Heidelberg, Braenderup, and Newport in terms of frequency. Selling raw chicken meat as the single meat type in the store and the use of stainless steel as a contact surface were found to be potential risk factors for *Salmonella* contamination, although they appear to be related to the hygienic measures and proper cleaning and disinfection, respectively. Thus, the information provided in this study may be used as a reference of the hygienic status of raw chicken marketed in this location and emphasizes the need to develop appropriate control and contingency measures to minimize the presence of *Salmonella* in chicken meat and its potential transmission to humans.

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