

ORIGINAL ARTICLE

Prevalence and molecular identification of *Salmonella* spp. isolated from commercialized eggs at Ibagué, Colombia

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ABSTRACT: *Salmonella* is a Gram-negative bacterium responsible for salmonellosis, a disease transmitted by the consumption of contaminated poultry products such as eggs and raw chicken meat. The *Salmonella* serovars present on the surface of eggs marketed in Ibagué city are currently unknown. To address this issue, an observational cross-sectional study was designed to estimate *Salmonella* spp. prevalence on the surface of eggs sold in Ibagué city from January - August 2014. A total of 1,705 eggs were collected from stores and supermarkets, and 341 samples (pools of five eggs each) were processed for *Salmonella* spp. isolation, followed by serotyping and typing by multiple PCR. *Salmonella* spp. prevalence in egg surface was 2.93%, and *S. Enteritidis* and *S. Paratyphi B* were the main serotypes present. *S. Enteritidis* and *S. Paratyphi B* were correctly identified by three multiplex PCR directed to amplify the *rjB* and *wzX*, *fliC* and *fljB* genes for serogroup and serovar identification, respectively. The frequency of cleaning and disinfection (OR= 19,8), the presence of long nails (OR=5,46) and storage temperature (OR=10,05) were identified as potential risk factors for *Salmonella* spp. contamination at the market place. It is concluded that *S. Enteritidis* and *S. Paratyphi B*, two potential human pathogens, were isolated from the surface of eggs marketed in Ibagué city, and multiplex PCR-based typing correlated well with the conventional serotyping in identifying those serovars.

Key words: Poultry, eggshell, serovars, transmission.

Prevalencia e identificación molecular de *Salmonella* spp. aislada de huevos comercializados en Ibagué, Colombia

RESUMEN: *Salmonella* es una bacteria Gram-negativa responsable de salmonelosis, enfermedad transmitida por el consumo de productos avícolas contaminados, como huevos y carne de pollo cruda. Los serotipos de *Salmonella* presentes en la superficie de huevos comercializados en la ciudad de Ibagué son actualmente desconocidos. Para abordar este problema se diseñó un estudio observacional de tipo transversal con el fin de estimar la prevalencia de *Salmonella* spp. en la superficie de huevos comercializados en tiendas y supermercados de la ciudad Ibagué, durante el periodo de enero a agosto de 2014. Para el aislamiento de *Salmonella*. Se colectaron 1.705 huevos y se procesaron 341 muestras (pools de cinco huevos cada una), seguido de serotipificación y tipificación por PCR múltiple. En la superficie del huevo, la prevalencia de *Salmonella* spp. fue 2,93%; los serotipos *S. Enteritidis* y *S. Paratyphi B* fueron los más prevalentes, los cuales también se identificaron correctamente a través de tres PCR múltiples diseñados para la amplificación de los genes *rjB* y *wzX*, *fliC* y *fljB*, que permiten la identificación de serogrupo y serotipo, respectivamente. Como potenciales factores de riesgo de contaminación por *Salmonella* spp. en el lugar de expendio, se identificaron la frecuencia de la limpieza y la desinfección (OR = 19,8), la presencia de las uñas largas en operarios (OR = 5,46) y la temperatura de almacenamiento (OR = 10,05). Se concluye que *S. Enteritidis* y *S. Paratyphi B*, dos patógenos de humano, se aislaron de la superficie de los huevos comercializados en la ciudad Ibagué y que la tipificación basada en PCR múltiple se correlaciona con la serotipificación convencional en la identificación de dichos serotipos.

Palabras clave: Aves de corral, cáscara de huevo, serotipos, transmisión.

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INTRODUCTION

Salmonellosis is a zoonotic disease caused by species of the genus *Salmonella*. They are Gram-negative microorganisms that include the species *S. enterica* and *S. bongori*, of which only the first one is of clinical relevance to humans and animals (1, 2). *S. enterica* includes over 2,610 serovars (3, 4). It is transmitted by direct contact with contaminated food from animal origin such as eggs and chicken meat (5). *S. Enteritidis* is considered the principal cause of human infection, and poultry products constitute the main source of the bacteria (6, 7, 8). The presence of *Salmonella* spp. in eggs is largely due to its ability to colonize the ovarian tissue and the surface of eggs during formation (9).

Salmonella spp. is prevalent in many developed countries including USA, Canada, UK, Norway, and Denmark (10, 11, 12), where the bacterium is responsible for high morbidity and mortality ratios, particularly in children, aged people and immunocompromised patients (13). Nontyphoidal *Salmonella* might be responsible for about 1 million illnesses, 100,000 hospitalizations and 731 deaths in the United States annually (14). Salmonellosis in developing countries may also have a high prevalence; however, the disease is not properly notified-reported to the health-care centers, and the impact of the bacteria on public health is not accurately known. In Colombia, *Salmonella* was isolated in 31,7% (32 outbreaks) of foodborne diseases reported between January 2008 - August 2010 (15). The National Health Institute of Colombia reported 7,219 *Salmonella* isolates from clinical cases. *S. Typhimurium* (33,7%), *S. Enteritidis* (28,6%) and *S. Typhi* (9,2%) were the most prevalent serotypes (16). In the Tolima region, the epidemiological studies on the presence of *Salmonella* were very limited in different segments of the poultry industry. *S. Enteritidis* y *S. Shannon* were found as the main serotypes present in laying hen farms in this region (17), and subsequently a number of *Salmonella* serovars were also identified in chicken carcasses sold at stores and supermarkets in Ibagué city (18). This study was designed to estimate the prevalence of *Salmonella* in the surface of eggs marketed at stores of Ibagué city for the period January – August, 2014, and to compare the conventional serotyping and genoserotyping using multiplex PCR-based typing.

MATERIAL AND METHODS

Study design

An observational cross-sectional study was conducted (between January - August, 2014) to establish the prevalence of *Salmonella* spp., in the surface of eggs marketed at stores and supermarkets in Ibagué, Tolima. The sample size was calculated by the formula described by Thrusfield (2007) (19), with a 95% confidence level, 5% error, and an expected prevalence of 50%, given that no systematic studies were found on *Salmonella* in commercial eggs. The expected prevalence used in this study was based on the data from commercial egg laying hens, where the bacterium was more prevalent on eggshells than in feed or environmental samples (17).

Sample collection

The sampling included all the 13 communes belonging to Ibagué city. The number of samples per commune was proportional to the number of stores registered at the authority (Chamber of Commerce in Ibagué). Each sample consisted on a pool of five eggs collected at each store, and a total of 341 samples was processed for *Salmonella* isolation. Thus, 1.705 eggs were sampled from the randomly selected stores or supermarkets across the city, where eggs were usually stored at room temperature (>25°C) or under cooling condition (<20°C). The eggs were packaged in sterile airtight plastic bags and transported to the Veterinary Diagnosis Laboratory for being processed within 12 hours. In addition, three *S. Enteritidis* isolates obtained from the egg surface in a pilot study (n=30) and six *S. Enteritidis* that were previously isolated from crushed eggshells (17) were also included in the study.

Isolation of *Salmonella* from egg surface

Salmonella spp. isolation followed the the international guidelines ISO 6579:2002/Amd1:2007. Briefly, eggs were immersed into a peptonated water solution and gently rubbed on the shell's surface for about five minutes to obtain a surface wash sample. A wash aliquot was inoculated into fresh peptone-buffered water for pre-enrichment and incubated at 37°C for 24 hr. Then, 1 ml of the pre-enrichment peptone water was seen in 9 ml tetrathionate broth (Müller-Kauffmann) and incubated at 37°C. A second aliquot was inoculated in 9 ml of Rappaport Vassiliadis (MERCK®) broth and incubated at 42°C for a selective

enrichment. Later, bacterial colonies were seen on SS (*Salmonella-Shigella* - MERCK®), XLD (Xylose Lysine Desoxycholate - MERCK®) and XLT4 (Xylose Lysine Tergitol 4 - MERCK®) agar. The compatible colonies were seen in McConkey (MERCK®) and Rambach (MERCK®) agar and confirmed as *Salmonella* spp by an agglutination test with Poli AI + Vi (Difco® 222641) antibodies. The positive colonies were also confirmed biochemically by using API® 20E gallery (Biomereux, France).

Serotyping of *Salmonella* spp. isolates

Salmonella spp. isolates were serotyped by using the White-Kauffman-Le Minor scheme (20) that identified the presence of specific somatic (O) and flagellar (H) antigens with the commercial antisera (Difco, Becton, Dickinson and Company Sparks, MD). Serotyping was performed based on the antigenic description established by Grimont and Weill (1) and the nomenclature described by Tindall *et al.* (21), and the Judicial Commission of the International Committee on Systematics of Prokaryotes (22). The procedure was carried out at the Colombian Agriculture Institute (ICA), following the methodology ISO 6579:2002/Amd1:2007.

Multiplex PCR-based typing of *Salmonella* spp.

The genomic DNA was extracted from each *Salmonella* isolate by the phenol chloroform isoamyl alcohol (25:24:1) method (23). All *Salmonella* samples isolated from the egg surface were subjected to 3 multiplex PCRs according to the previously described method (24, 25, 26). Three *S. Enteritidis* isolates from the egg surface in a pilot study (n=30) and six *S. Enteritidis* previously isolated from crushed eggshells (17) were also included in this analysis. The procedure included a primary PCR reaction using 10 primers to identify *Salmonella* spp. serogroups based on the amplification of the genes involved in sugar biosynthesis pathways and transferases (*rflB*, *wzX*). *Salmonella* serovars were identified by two additional multiplex PCR reactions targeting *fliC* and *fliB* genes encoding the phase I (H1, 13 primers) and phase II (H2, 10 primers) flagellar antigens. The PCR reaction was performed in 25 µl total volume using the AccuPrime™ Taq DNA polymerase System (Invitrogen Life Technologies), containing 2.5 µl MgCl₂ (50 mM), 2.5 µl 10 × buffer, 0.8 µl of each primer (10 µM), 8 mM of dNTPs, 0.5 µl Taq DNA polymerase and 1 µl of DNA template. Amplification was carried out in a T-100 (Bio-Rad) thermal cycler with the following program: an initial denaturation step at 95°C for 5 minutes, followed by 35 denaturation cycles at 94°C for 1 min, annealing at 55°C for 30s and extension at 68°C for 30s, and a

final extension step at 68 °C for 7 minutes. *Salmonella* Typhimurium ATCC 14028 and *Salmonella* Enteritidis ATCC 13076 were used as positive controls and the negative control did not contain DNA template. PCR products were mixed with 2µl of 10× loading buffer and then resolved by electrophoresis on 2% agarose gel with 100 bp DNA ladder (Invitrogen®). The gel was stained with ethidium bromide and visualized under the UV light by using an ENDURO™ GDS (Labnet International, Inc.) gel documentation system.

Epidemiologic survey

To identify the potential risk factors for *Salmonella* spp. contamination, a questionnaire was administered to the shop owner/administrators during an interview at the sampling time. The variables collected followed those previously described (27), and included the presence of pets in the store, cleaning conditions and frequency of cleaning and disinfection of the store (one vs. more than once per week), presence of sanitary unit or sink, presence of flies, presence of dirty (stool or dust) eggs, type of surface (smooth or rough), presence of long nails in the staff (long vs. short nails), storage temperature of eggs (20-25°C vs. >25 °C), and staff personal clothing. The variables were optimized in the pilot study conducted by our research group at the University of Tolima.

Statistical Analysis

A descriptive analysis of the epidemiologic variables evaluated at each store was conducted by using IBM SPSS Statistics® 20 version software and GraphPad Prism® 5.03 version software. The independence between the epidemiologic variable and the positivity to *Salmonella* spp. was determined by cross tabulation analysis. The association strength was calculated by the odds ratio. Prevalence was determined as the proportion of positive samples over the total samples, expressed as a percentage.

RESULTS

A total of 341 samples was cultured for *Salmonella* isolation, and 10 different isolates were obtained from egg surface. Thus a 2.93% prevalence of *Salmonella* in the surface of eggs marketed at Ibaguè, Colombia, was estimated for the period January - August 2014. *Salmonella* isolates were identified by conventional serotyping as *S. Enteritidis* (n=8), *S. Paratyphi B* (n=1) and *S. bongori* (n=1) (Table 1).

The multiplex PCR targeting the genes encoding the O-antigen showed two amplification products, one of 615 bp and the second of 230 bp (Fig. 1). All *Salmonella* Enteritidis (serogroup D1) amplified a single

TABLE 1. Serotyping and PCR-based typing of *Salmonella* isolated from eggs marketed in Ibagué, Colombia, between January - August, 2014./ *Serotificación y tipificación por PCR de Salmonella aislada de huevos comercializados en Ibagué, Colombia, entre enero y agosto de 2014.*

<i>Salmonella</i> code	Sample type	Origin/ commune or farm	Serogroup/ Serotype	Multiplex PCR (bp) Serogroup	Multiplex PCR (bp) Phase I	Multiplex PCR (bp) Phase II
UT-SEH001	Surface wash	7	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH002	Surface wash	7	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH003	Surface wash	7	Serogroup D1, <i>S. Enteritidis</i> (9,1:g.m)	615	500, 333	
UT-SBH004	Egg' surface	6	Serogroup G, <i>Salmonella</i> <i>bongori</i> V (1,13,22,i)	615,230	250	400,100
UT-SPH005	Surface wash	6	Serogroup B, <i>S. Paratyphi</i> B (1,4,5,12 b, 2)	230	150	400
UT-SEH006	Surface wash	4	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH007	Surface wash	4	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH008	Surface wash	3	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH009	Surface wash	3	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH010	Surface wash	3	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH011	Surface wash (pilot study)	3	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH012	Surface wash (pilot study)	3	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UT-SEH013	Surface wash (pilot study)	3	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	
UTSE130001	Crushed Eggshell	Farm	Serogroup D1, <i>S. Enteritidis</i> (9,12:g.m)	615	500, 333	

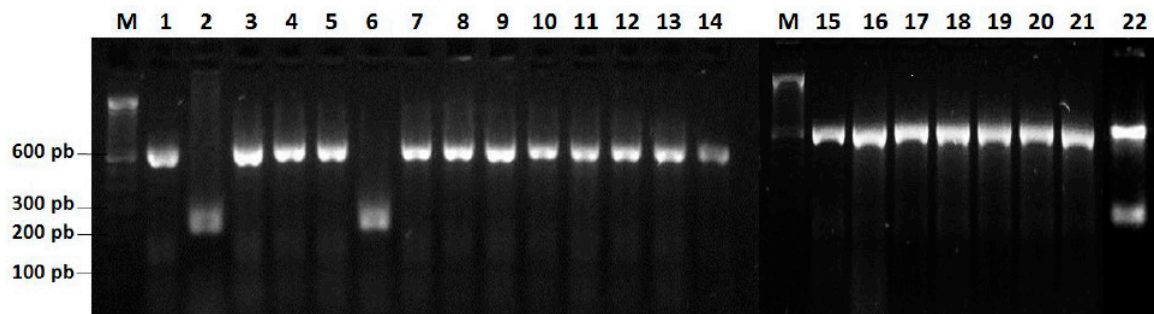


FIGURE 1. Genoserotyping of *Salmonella* serogroups isolated from eggs marketed at Ibagué, Colombia, by multiplex PCR. Lanes M, 100 bp ladder; Lanes 1, 15, positive control *Salmonella* Enteritidis ATCC 13076; Lane 2, positive control *S. Typhimurium* ATCC 14028; Lanes 3-5 and 7-11 *Salmonella* Enteritidis from egg surface, Lane 6, *S. Paratyphi* B from egg surface; Lanes 12-14, *S. Enteritidis* from egg' surface (pilot study); Lanes 16-21, *S. Enteritidis* from crushed eggshells (17); Lane 22, *S. bongori*./ *Genoserotipaje de serogrupos de Salmonella aislada de huevos comercializados en Ibagué, Colombia por PCR Múltiple. Línea M: Marcador de peso de 100 pb; Líneas 1, 15, control positivo Salmonella Enteritidis ATCC 13076; Línea 2, control positivo S. Typhimurium ATCC 14028; Líneas 3-5 y 7-11, Salmonella Enteritidis aislada de superficie de huevo, Línea 6: S. Paratyphi B aislada de superficie de huevo; Líneas 12-14, S. Enteritidis aislada de superficie de huevo (estudio piloto); Líneas 16-21, S. Enteritidis aislada de cáscaras de huevo trituradas (17), línea 22, S. bongori.*

band of 615 bp, whereas *S. Paratyphi B* (serogroup B) amplified a band of 230 bp. The species *S. bongori* amplified 2 bands, one of 615 bp and one of 230 bp. The positive controls *S. Enteritidis* ATCC 13076 and *S. typhimurium* ATCC 14028 (serogroup B) also amplified a single band of 615 bp and 230 bp, respectively.

The multiplex PCR for phase I flagellar antigen H1 showed amplification products of 500 and 333 bp that correspond to the flagellar antigens H: 9, 12 and H: b: 1, 2. All *Salmonella* Enteritidis showed 2 bands, one of 500 bp and a second one of 333 bp. *S. Paratyphi B* showed a band of 150 bp. The positive controls *Salmonella* Enteritidis ATCC 13076 and *Salmonella* Typhimurium ATCC 14028 showed the corresponding 500 bp, 333 bp and 250 bp, respectively (Fig. 2). The third multiplex PCR directed to detect phase II flagellar antigens showed amplification products of 400 bp corresponding to the H:b:1,2 flagellar antigens described by Herrera-León *et al.* (25) and Echeita *et al.* (24). All *S. Enteritidis* showed no clear amplification products, whereas *S. Paratyphi B* showed a single amplification product of 400 bp. The positive control *S. Enteritidis* ATCC 13076 showed no amplification products, whereas *S. Typhimurium* ATCC 14028 showed a single band of about 400 bp (Fig. 3). The species *S. bongori* showed two bands, one of 400 bp and a second one of 100 bp.

Salmonella spp. was more frequently isolated (42.85%) from eggs at stores having a frequency of cleaning and disinfection once a week. A reduced

frequency of cleaning at the store was identified as a potential risk factor for the presence of *Salmonella* (OR: 19.84, $p < 0.05$) on eggs. The lifestyle of staff may also contribute to the contamination of eggs by *Salmonella*. The store staff with long nails were identified as a risk factor (OR: 5.46, $p < 0.05$) for the presence of *Salmonella* on marketed eggs. Finally, in the present study, eggs kept at temperatures less than 25°C were identified as a risk factors for being contaminated with *Salmonella* (OR: 10.05, $p < 0.05$) than eggs stored at temperatures over than 25°C.

DISCUSSION

This study established a prevalence of 2.93% (10/341) *Salmonella* in the surface of eggs marketed in Ibagué, Colombia, for the period January – August, 2014. The prevalent serovars of *Salmonella enterica* on egg surface were *S. Enteritidis* and *S. Paratyphi B*, which were appropriately typed by a panel of 3 multiplex PCRs that allowed a correct identification of serogroups and serovars. Although the multiplex-PCRs were not designed to discriminate the species *S. bongori* (26), the results obtained in this study indicated that *S. bongori* was also present on the surface of eggs and that it could be differentiated from some subspecies of *S. enterica* by the presence of additional PCR products (Table 1, Figures 1,2,3).

Salmonella prevalence in eggs and laying-hen farms may vary considerable between different countries and geographical regions (27). In Europe,

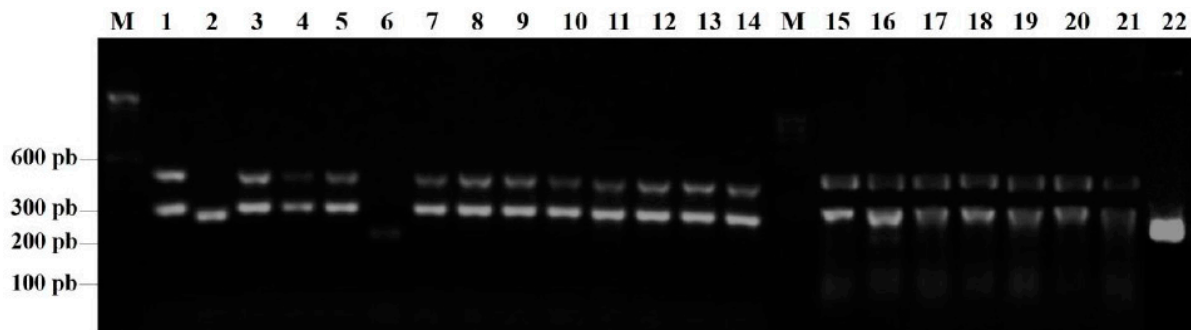


FIGURE 2. Genosero-typing of *Salmonella* serotypes isolated from eggs marketed at Ibagué, Colombia, by multiplex PCR to phase I flagellar antigen. Lanes M, 100 bp ladder; Lanes 1, 15, positive control *Salmonella* Enteritidis ATCC 13076; Lane 2, positive control *S. typhimurium* ATCC 14028; Lanes 3-5 and 7-11 *Salmonella* Enteritidis from egg surface; Lane 6, *S. Paratyphi B* from egg surface; Lanes 12-14, *S. Enteritidis* from egg surface (pilot study); Lanes 16-21, *S. Enteritidis* from crushed eggshells (17); Lane 22, *S. bongori*./ Genoserotipaje de serotipos *Salmonella* aislada de huevo comercializado en Ibagué, Colombia por PCR Múltiple para la fase I del antígeno flagelar. Línea M, Marcador de peso de 100 pb; Líneas 1, 15, control positivo *Salmonella* Enteritidis ATCC 13076; Línea 2, control positivo *S. typhimurium* ATCC 14028; Líneas 3-5 y 7-11, *Salmonella* Enteritidis aislada de superficie de huevo, Línea 6, *S. Paratyphi B* aislada de superficie de huevo; Líneas 12-14, *S. Enteritidis* aislada de superficie de huevo (estudio piloto); Líneas 16-21, *S. Enteritidis* aislada de cáscaras de huevo trituradas (17), línea 22, *S. bongori*.

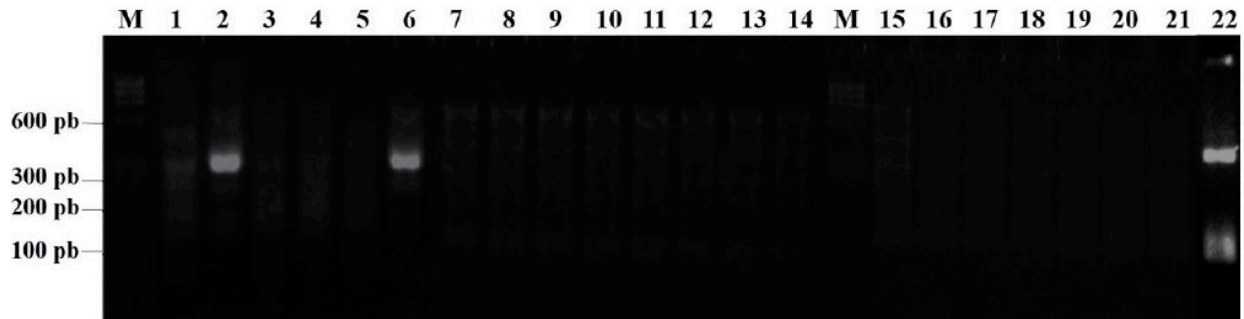


FIGURE 3. Genoserotyping of *Salmonella* serotypes isolated from eggs marketed at Ibagué, Colombia, by multiplex PCR to phase II Flagellar antigen. Lanes M, 100 bp ladder; Lanes 1, 15, positive control *Salmonella* Enteritidis ATCC 13076; Lane 2, positive control *S. Typhimurium* ATCC 14028; Lanes 3-5 and 7-11 *Salmonella* Enteritidis from egg surface; Lane 6, *S. Paratyphi B* from egg surface; Lanes 12-14, *S. Enteritidis* from egg surface (pilot study); Lanes 16-21, *S. Enteritidis* from crushed eggshells (17); Lane 22, *S. bongori*./ *Genoserotipaje de serotipos Salmonella aislada de huevo comercializado en Ibagué, Colombia por PCR Múltiple para la fase II del antígeno flagelar. Línea M, Marcador de peso de 100 pb; Líneas 1, 15, control positivo Salmonella Enteritidis ATCC 13076; Línea 2, control positivo S. Typhimurium ATCC 14028; Líneas 3-5 y 7-11, Salmonella Enteritidis aislada de superficie de huevo, Línea 6, S. Paratyphi B aislada de superficie de huevo; Líneas 12-14, S. Enteritidis aislada de superficie de huevo (estudio piloto); Líneas 16-21, S. Enteritidis aislada de cáscaras de huevo trituradas (17), Línea 22, S. bongori.*

Salmonella prevalence in laying-hen farms ranged from 0% to 79.5% in 2007, with an average of 30.8%. In UK, a prevalence of 18% *Salmonella* in laying hens was reported in 2010 (28), and the frequency of *Salmonella* in eggs at the market place in Europe may vary between 7 - 24% (29). In Argelia, a prevalence range of 7.14% - 41.17% *Salmonella* in laying hens was reported in 2012 (30). In Colombia, a prevalence of 45.71% *Salmonella* in laying hen farms was reported in a study that included Cundinamarca, Santander, Bolivar and San Andres regions in 2013 (31), and recently our group reported a prevalence of 33.33% *Salmonella* in egg-laying-farms in the Tolima region (17). In this study, *Salmonella* prevalence in egg surface was slightly lower than that determined in crushed eggshells (5.16%) from eggs (8/155) in laying-hen farms in the Tolima region (17). The difference might be due to the methodology used to isolate *Salmonella* from eggs. The authors used eggshells and therefore incubated them into peptone water, whereas, in this study, we wanted to explore the possibility to isolate the bacteria from the egg surface without the need of breaking the eggs and by using a surface rinse obtained by 5 minute-incubation and rubbing the egg surface in peptone water. The prevalence of *Salmonella* estimated in this study is within the range (0 - 22.6%) reported in eggs for human consumption in the European Union and the United Kingdom (32); and it was very similar to the prevalence (3%) of *Salmonella enterica* reported in eggs from laying hen

farms in other regions of Colombia (7). The use of an egg surface rinse to establish the *Salmonella* load in the egg surface may constitute a practical procedure to screen large volumes of eggs from laying-hen farms during the process of egg disinfection.

This study identified *S. Enteritidis* (80%) and *S. Paratyphi B* (10%) as the main serovars of *Salmonella* enterica present on the surface of eggs marketed in stores and supermarkets of Ibagué city (Table 1). These results are in partial agreement with the results of Rodríguez *et al.* 015) (17), who found *S. Enteritidis* (42.85%) and *S. Shannon* (57.15%) in laying hen farms in this region. The authors isolated most frequently *Salmonella* from crushed eggshells (57.15%) than from feed (28.57%) or environmental (14.29%) samples (17). *S. Enteritidis* was the most prevalent serovar found on the egg surface (80%), and this is the predominant serovar associated to *Salmonella* outbreaks in several countries. In 2012, *S. Enteritidis* accounted for 179 outbreaks and 2,177 human cases of salmonellosis in the European Union, most of which were attributed to eggs and egg products (168 outbreaks, 22%) (33). Given the lack of epidemiologic data on *Salmonella* in the poultry industry of Tolima, the finding of *S. Enteritidis* in the surface of eggs commercialized in Ibagué city may raise concern on the potential transmission to egg consumers and may indicate the need to assess its relationship with clinical cases of salmonellosis in this region.

This study implemented a series of 3 multiplex PCRs designed for typing commonly isolated *Salmonella* spp. from clinical samples that targeted the serogroups D (615 bp), C1 (483 bp), E (345 bp), B (230 bp) and C2 (154 bp) (24, 25, 26). This PCR-based method identified O:D1 and O:B as the serogroups of *Salmonella* present in the surface of eggs marketed in Ibagu  Tolima; and these results correlated with the results obtained by the conventional serotyping (Table 1). The multiplex PCR also identified correctly a number of *S. Enteritidis* previously isolated from crushed eggshells (17). Thus, the use of multiplex PCR for typing *Salmonella* spp. present in eggs might be advantageous by the limited number of *Salmonella* spp. subspecies that survived in this product and might be a useful tool to speed up the identification of *Salmonella* spp. in poultry products.

The second multiplex PCR for phase 1 flagellar antigens allowed the identification of serovar *S. Enteritidis* with two amplification products, one of 500 bp that corresponded to the G-complex and a second band of 333 bp specific for this serovar (Fig. 2). The serovar *S. Paratyphi B* showed the specific 150 bp band described by Herrera-Leon *et al.* (25). The isolate corresponding to the species *S. bongori* showed a single band of about 250 bp, similar to the single band amplified from the positive control *S. Typhimurium* ATCC 14028.

The third multiplex PCR for phase 2 flagellar antigens showed no bands in all *S. Enteritidis*, according to the findings of Echeita *et al.* (24). This multiplex also showed a correct identification of *S. Paratyphi B* with an amplification product of 400 bp and *S. Enteritidis* ATCC 13076 with no amplification products as described by others (24), whereas *S. bongori* showed 2 bands (400 bp and 100 bp) that could differentiate this species from *S. enterica*. However, the multiplex PCRs were developed for the identification of serogroups and serovars mostly present in the subspecies I, and they did not take into account subspecies of *S. bongori*. The results of PCR-typing indicated that this methodology could identify *S. Enteritidis*, *S. Typhimurium* and *S. Paratyphi B* isolated from eggs commercialized at stores in Ibagu  city, and the banding pattern of those serovars could differentiate them from the members of the species *S. bongori*, another *Salmonella* that could be recovered from the egg surface but lacking association to clinical diseases in animals and human, they are usually present in the environment (reptiles, amphibians and fish) (34).

The epidemiological survey in each store and supermarket revealed that a low frequency (once per

week) of cleaning and disinfection of the stores (OR: 19.84, $p < 0.05$), the staff with long nails (OR: 5.468, $p < 0.05$) and the storage of eggs at temperatures less than 25 C (OR 10.05, $p < 0.05$) may be potential risk factors for the presence of *Salmonella* spp. in eggs marketed in the city of Ibagu , Tolima (35). The finding also reflected the importance of strengthening the cleaning and disinfection procedures along the chain of egg production, as it has been observed in the egg packing plants in Europe, where significant differences were found between the prevalence of *Salmonella* in plants with and without disinfection plans (36). It has also been observed that a high frequency (21.6%) of meat plant operators with long nails was positive to *Salmonella* in Nari o, Colombia (37). Finally, at low temperatures, *Salmonella* may slow down its metabolism and could survive longer on the outer shell surface (38). Other possibility that could explain this finding is that *Salmonella* may be susceptible to desiccation and dryness of the egg surface at temperatures higher than 25 C (39).

Concluding, this study estimated for the first time a prevalence of 2.93% *Salmonella* in the surface of eggs marketed at shops and supermarkets of Ibagu  city. *S. Enteritidis* and *S. Paratyphi B* were the main serotypes of *Salmonella* circulating in eggs, and the use of 3 independent multiplex PCRs for the typing of *Salmonella* showed a good correlation with serotyping and then constituted a useful tool to speed up the identification of this pathogen at the species level upon isolation. The results may support the need to conduct studies with a larger coverage. In addition, they reveal the need to increase awareness of poultry producers, vendors and health authorities on the health risk imposed by *Salmonella*, and the need to implement appropriate diagnosis and epidemiological surveillance tools to detect and identify *Salmonella* species potentially contaminating food products in a short time.

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