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SALMONELLA PREVALENCE IN POULTRY FARMS OF ECUADOR AND SEROTYPE IDENTIFICATION BASED ON MULTIPLEX PCR SYSTEMS

Prevalencia de *Salmonella* en Granjas Avícolas de Ecuador y Serotipificación Basada en Sistemas de PCR múltiple

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ABSTRACT

Salmonellosis control is in part based on the reduction of *Salmonella* presence in primary poultry production. The aim of this study was to determine the prevalence of *Salmonella* in poultry farms of Ecuador. In 2014, the first national survey of industrialized poultry farms was done. *Salmonella* spp. was isolated from manure samples and serotypes were determined by conventional serotyping. Additionally, the ability of multiplex Polymerase Chain Reaction (PCR) systems to identify the *Salmonella* serotype of the isolates was evaluated and results were confirmed with serological methods. One hundred forty five farms were sampled and a total of 26 isolates of *Salmonella* spp. were recovered from 16 of them, resulting in a national apparent prevalence at the farm level of 11%; 12.7% in broiler chickens farms, 11.5% in breeder flocks and 8.9% in egg-laying hens farms. The isolated serotypes of *Salmonella enterica* subsp. *enterica* were: Enteritidis, Infantis, Saintpaul and Serogroup O:8:Z$_4$ Complex. A flow diagram for the use of the evaluated multiplex PCR systems is proposed and the potential significances of the identified serotypes for public health are discussed.

Key words: Broilers; infantis; molecular typing; salmonellosis; serotyping.

RESUMEN

Una parte del control de la Salmonelosis se basa en reducir la presencia de *Salmonella* a nivel de la producción avícola primaria. El objetivo de este estudio fue determinar la prevalencia de *Salmonella* en las granjas avícolas ecuatorianas. En 2014, se realizó el primer muestreo nacional de granjas avícolas industriales en Ecuador. A partir de las muestras ambientales recolectadas, se aislaron e identificaron bacterias del género *Salmonella* para establecer su prevalencia e identificar los serotipos circulantes. Adicionalmente, se evalúo la capacidad de unos sistemas de Reacción en Cadena de la Polimerasa (PCR) múltiple para determinar el serotipo de *Salmonell*a spp. de estos aislados y los resultados fueron verificados por ensayos serológicos. Se muestrearon 145 granjas, recuperando 26 aislados de *Salmonella* spp. a partir de 16 de las mismas. La prevalencia aparente de *Salmonella enterica* subs. *enterica* en granjas avícolas fue de 11%, con una prevalencia por estratos de: 12,7% para pollos de engorde, 11,5% en gallinas reproductoras y 8,9% en granjas de ponedoras. Los aislados fueron identificados como *Salmonella enterica* subsp. *enterica* ser. Enteritidis, *Salmonella* ser. Infantis, *Salmonella* ser. Saintpaul y miembros del O:8:Complejo Z$_4$, tanto por tipificación molecular como por ensayos serológicos. Se propone un diagrama de flujo para el uso de los sistemas de PCR múltiple y se discute el impacto potencial de estos serotipos en la salud pública.

Palabras clave: Infantis; pollos de engorde; salmonelosis; serotipo; tipificación molecular.

INTRODUCTION

Salmonellosis is a major cause of bacterial enteric illness in both humans and animals. Human salmonellosis is directly linked to contaminated poultry and poultry products, like eggs [21]. Poultry and egg consumption is an important part of Ecuadorian diet due to its nutritional value and lower prices compared to other sources of animal protein. In 2010, an intake of 32 kg/person/year of chicken (_Gallus gallus domesticus_) and 140 eggs/person/year were reported [6]. Ecuador produces approximately 490,000 metric tons of chicken and 2,093 millions of eggs per year that supply the national market [6]. Due to the importance of poultry production in Ecuador, in 2013 a National Poultry Surveillance Program was established, that included a project for _Salmonella_ monitoring [22].

Salmonellosis refers to a group of infections caused by members of the genus _Salmonella_; being _Salmonella enterica_ subsp. _enterica_ the most frequently isolated from humans and warm blooded animals. There are 1,531 serotypes of this subspecies [13], some of them are able to cause disease in a wide range of hosts and others are host-specific or have a greatly reduced host range. _Salmonella enterica_ subsp. _enterica_ ser. Enteritidis is a major concern for most egg industries around the world. This serotype is associated with the majority of egg-related outbreaks of human salmonellosis (77.2%) occurring in the European Union (EU) [9]. The non-motile host-specific avian pathogens, biotypes Gallinarum and Pullorum, are not normally capable of causing disease in mammalian hosts, but are still able to cause important losses in poultry industry [20]. Consequently, the circulating serotypes of _Salmonella_ spp. must be monitored in poultry farms.

The investigation of _Salmonella_ in poultry at the primary production stage may involve the collection of material from the environment in the poultry house (e.g. faeces, litter, dust), or the collection of samples from birds themselves (e.g. blood, cloacal swabs, post-mortem samples of the small intestine and caecum) [4]. Single swabs are currently used in the Food and Drug Administration (FDA) official method for sampling the environment of commercial laying hens for the detection of _Salmonella_ ser. Enteritidis [15]. Once the presence of viable _Salmonella_ spp. in these samples is detected, the serotype must be determined. Although the conventional serotyping method based on the Kauffmann-White scheme [13] is widely used, there are some limitations; for example, the method is time-consuming and tedious, as well as subjective in interpretation. Furthermore, it requires good trained technicians and high-quality anti-sera, both of which could be difficult to obtain consistently and are very costly in resource-limited settings. Such limitations have encouraged development of several molecular methods based on the amplification of DNA for identification of _Salmonella_ serotypes. These molecular methods, such as multiplex Polymerase Chain Reaction (mPCR), are very specific, fast and reproducible. [1, 17] This pioneer study contributes to epidemiological data focused on the prevalence of _Salmonella_ spp. and serotype diversity in Ecuadorian poultry farms. Additionally, the mPCR systems designed by Akiba _et al._ [1] for serotype identification were evaluated and results were confirmed by conventional serotyping.

MATERIALS AND METHODS

Study population and area

This work focused on industrialized conventional poultry farms (>1,000 birds), registered in the Poultry Farms Census [6]. A total of 1,558 poultry farms were registered: 1,223 dedicated to broilers production, 51 breading hens farms and 284 laying hens farms. Study area was continental Ecuador, specifically the Provinces of: Azuay, Bolívar, Cañar, Carchi, Cotopaxi, Chimborazo, El Oro, Esmeraldas, Guayas, Imbabura, Loja, Los Ríos, Manabí, Morona Santiago, Napo, Orellana, Pastaza, Pichincha, Santa Elena, Santo Domingo de los Tsáchilas, Sucumbíos, Tungurahua y Zamora Chinchipe.

Survey design and sampling

This was a descriptive cross-sectional study. Samples were taken from January to March, 2014. The number of samples was calculated using the software WIN EPISCOPE 2.0 [28]. Due to the lack of data on _Salmonella_ prevalence in poultry farms of Ecuador, the error and confidence level were established arbitrarily. Data from the Poultry Farms Census [18] was used to estimate total population and population per stratum. A sample size of 159 farms was determined with an absolute error of 5 and 95% levels of confidence and an expected prevalence of 13.2%, based on data from other Latin American Countries [29]. Subsequently, a random cluster sampling procedure was used. Total poultry population was divided in three clusters: broilers, breeding hens and laying hens. The sample size for each cluster was calculated with an absolute error of 5 and 95% levels of confidence and expected prevalence of 5% for each cluster. After the sample size of each cluster was determined, the number of poultry farms to be sampled per Province was chosen at random, taking into account the size of each cluster. The number of sampled farms per Province is shown in TABLE I. Two poultry houses were sampled at each farm, selecting those that hosted the older chickens.

Collection of manure drag swabs from poultry houses

The swabs consisted of an assembly of sterile gauze pads moistened in buffered peptone water (BPW), attached to a string stapled to a wooden stick [4]. Each chicken house was divided in 3 imaginary areas of 33 meters (m) length per 12 m wide. The sampling procedure consisted on dragging a swab over manure along the length of an area of the chicken house or along one of three selected pits (when present) in laying houses. The three drag swabs taken from the same poultry house were pooled in one sterile reclose-able plastic bag. BPW was used as transport media and bags were placed in an ice box with gel packs and

transported to the laboratory the same day.

Isolation and biochemical characterization

Upon arrival to the laboratory, 30 mL of BPW were added to the pooled swabs in the same reclose-able bags and these were incubated at 37°C, for 24 hours (h). Then, 1 mL of pre-enriched sample was inoculated into Tetrathionate broth (9 mL) and 1 mL was inoculated into Selenite cysteine broth (9 mL. Both tubes were incubated at 37±1 °C, for 24 h. After incubation, broth cultures were streak plated onto: Xylose-lysine desoxycholate agar, Hektoen agar and Bismuth sulfite agar [20]. A presumptive colony of *S. enterica* from each selective agar was grown on Motility-Indole-Ornithine agar and on slants of: Triple Sugar Iron agar, Lysine Iron agar, Simmons citrate agar and Urea agar. Isolates identified as *S. enterica* were purified and bacterial glycerol stocks were stored in liquid nitrogen until further characterization by mPCR and serotyping. All culture media used were from Difco™ (Becton Dickinson and Co., USA).

Multiplex PCR assay

DNA extraction was performed according to Akiba *et al.* [1] with minor modifications. A single colony of each isolate on Nutritive agar plate was picked up and suspended in 50 µL of 25 mM NaOH. The suspension was boiled for 5 minutes (min) and 8 µL 0.5 M Tris-HCl (pH 8.0) were added. Supernatant was collected after centrifuging at 13,000 x g for 10 min and 5 µL used as DNA template.

Three mPCR systems, designed to detect *Salmonella* ser. Typhimurium, *Salmonella* ser. Enteritidis and *Salmonella* ser. Infantis, were performed using the primers published by Akiba *et al.* [1]. The amplicon sizes expected were: a *Salmonella* specific fragment of 605 base pairs (bp) from *invA* gene [24] and fragments of approximately 100, 200 and 300 bp from the serovar-specific genomic regions (SSGRs), [1]. The mPCR assay was carried out in a 25 µL reaction solution containing: 1X PCR Buffer, 2.5 mM $MgCl_2$ 1.6 mM deoxiynucleoside triphosphates, 1 U *Taq* DNA polymerase, recombinant (Invitrogen, Brasil) and 0.3 µM of each primer For the *Salmonella* ser. Typhimurium and *Salmonella* ser. Infantis mPCR systems, the primers InvAF, InvAR, TMP2F, TMP2R or IMP3F, IMP3R, respectively, were used at 0.5 µM (each), as recommended by Gole *et al.* [12]. The samples were amplified in a T100™ thermal cycler (BIORAD), with an initial denaturation step at 95°C for 2 min, followed by 35 cycles of amplification (denaturation at 95°C for 15 seconds (s), annealing at 60 °C for 30 s and extension at 72 °C for 30 s), and a final extension step at 72 °C for 10 min. The PCR products were separated by 2% agarose gel electrophoresis in Tris-Borate-EDTA (TBE) buffer 0.5X, stained with SYBR® Safe (Invitrogen, USA) and visualized under ultraviolet light on a Mini Lumi (Bio-Imaging, Major Science).

Serotyping

Salmonella isolates were serotyped following the Kauffman-White-Le Minor scheme [13] for O and H antigens and by using commercial antisera (Difco™, Becton Dickinson and Co., USA) observing the instructions of the antisera manufacturer. Some recommendations of Caffer *et al.* [3] for slide and tube test procedures were followed.

Reference strains

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC® 14028™, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC® 13076™ were used as reference strains for serotyping and positive control for the mPCR. The strain 11-111, isolated from one of the sampled poultry farm, serotyped as *Salmonella enterica* subsp. *enterica* serovar Infantis, was used as positive control for the Infantis mPCR system.

RESULTS AND DISCUSSION

This study was conducted to establish the prevalence of *Salmonella* spp. at industrialized poultry farms in Ecuador. Samples were taken from 145 poultry farms, instead of the planned 159, due to non-existence of birds in some of them or access restrictions from farm's owner. Sixteen of the 145 farms were positive for *Salmonella* spp.; thus the national apparent prevalence at the farm level was 11.0%. TABLE I shows the numbers of registered and sampled poultry farms per Province and the number of farms that had positive results for *Salmonella* spp. When compared to other regions of the world, the apparent prevalence of *Salmonella* spp. at poultry farms in Ecuador was high. In the EU, 18 member States reached the EU *Salmonella* reduction target of 1% or less for breeding or broilers flocks, by 2009 [8]. Meanwhile, higher prevalence levels have been reported at commercial laying hen farms regions in Colombia (33.3%) and Argentina (60%), [25, 27]. However, these studies are based on more than one environmental sample type and on samples taken directly from the birds, a methodology that increases the detection capacity for *Salmonella* [27]. The Provinces with higher numbers of positive farms were Tungurahua (4) and Imbabura (3); despite not being the provinces with higher number of sampled farms. However, these Provinces registered the highest production during 2014. At the time of sampling, the use of Antibiotics (enrofloxacin, colistin, bromhexine) was reported in some poultry farms at Cotopaxi Province, which could explain the absence of positive isolations.

For samples with presumptive *Salmonella* colonies in more than one selective agar media, a colony from each culture media plate was analyzed independently. For that reason, 26 colonies with typical morphology and biochemistry for mobile, non-typhoid *Salmonella* spp. were isolated from the 16 positive poultry farms. The results of the mPCR confirmed that all 26 isolates where members of the genera Salmonella and allowed the assignment

TABLE I

REGISTERED AND SAMPLED POULTRY FARMS PER PROVINCE AND APPARENT PREVALENCE OF *SALMONELLA* SPP

Province	Registered Farms[a]	Sampled Farms	Farms positive for *Salmonella*(%)[b]
Azuay	74	10	0 (0)
Bolívar	5	1	0 (0)
Cañar	44	4	0 (0)
Carchi	13	2	0 (0)
Chimborazo	47	5	0 (0)
Cotopaxi	42	9	0 (0)
El Oro	207	6	1 (16.7)
Esmeraldas	4	2	0 (0)
Guayas	80	9	1 (11.1)
Imbabura	72	7	3 (42.9)
Loja	61	2	1 (50.0)
Los Ríos	35	4	0 (0)
Manabí	211	18	0 (0)
Morona Santiago	16	1	0 (0)
Napo	18	6	2 (33.3)
Orellana	31	3	0 (0)
Pastaza	28	4	0 (0)
Pichincha	243	20	2 (10.0)
Santa Elena	22	2	0 (0)
Santo Domingo	41	6	2 (33.3)
Sucumbíos	7	1	0 (0)
Tungurahua	193	22	4 (18.2)
Zamora Chinchipe	56	1	0 (0)
TOTAL	1,558	145	16 (11.0)

[a] Based on Poultry Farms Census [18]
[b] % of *Salmonella* spp. positive farms in relation to sampled farms.

TABLE II

SEROTYPES OR SEROGROUP OF *SALMONELLA ENTERICA* SUBSP. *ENTERICA* ISOLATED FROM MANURE DRAG SWABS AT INDUSTRIALIZED POULTRY FARMS IN ECUADOR

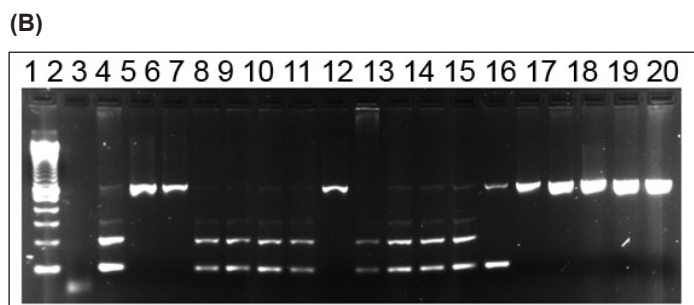| Serotype/Serogroup	No. of isolates (%)	Positive farms (%)
Enteritidis/Blegdam	3 (11.5)	2 (12.5)
Saintpaul	5 (19.2)	3 (18.7)
Infantis	9 (34.6)	7 (43.8)
O:8; Z_4 Complex	9 (34.6)	4 (25.0)
TOTAL	26	16

of a serotype for 17 (65.4%) of them. These *Salmonella* isolates belonged to serotypes: Enteritidis (3), Saintpaul (5), and Infantis (9). See FIG. 1 and TABLE II. However, the mPCR systems failed to produce a serotype assignment for nine (34.6%) of the 26 isolates. Results of the molecular typing were confirmed by conventional serotyping. Additionally, serotyping indicated that the nine isolates, non-typeable by the mPCR systems, belonged to serogroup O:8. All these isolates expressed phage determined factor O:20 and partial analysis of flagellar antigens indicated that they belong to Z4 Complex. Therefore, according to the Kauffmann-White-Le Minor scheme [13] the antigens identified in this study for nine isolates suggested that *Salmonella* serotype

could be: Davou, Corvallis or Albany. A study about *Salmonella* in commercial reared broiler batches at slaughter in the Province of Pichincha, Ecuador, with samples taken from June 2013 to July 2014, found that the most common serotype of *Salmonella* was Infantis followed by Enteritidis and Corvallis [31]. The presence of those *Salmonella* serotypes in broilers at slaughter age is in concordance with the results found at poultry farms shown here.

(A)
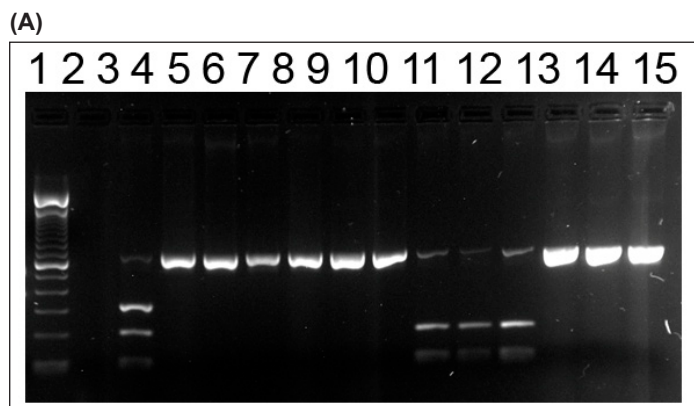
1: 100 pb DNA ladder TrackIt (Invitrogen), 2: Negative control, 3: *Salmonella enterica* serovar Typhimurium ATCC® 14028™, Lanes 10-12 positives for ser. Saintpaul Lanes 4-9 and 13-15 negative for ser. Typhimurium and ser. Saintpaul. 2% Agarose.

(B)

1: 100 bp DNA ladder TrackIt (Invitrogen), 2: Negative control, 3: *Salmonella enterica serovar* Infantis, Lanes 6-9, 11-14 positive samples to ser. Infantis. Lanes 4, 5, 10 and 16-20 negative samples to ser. Infantis. 2% Agarose.

FIGURE 1. RESULTS OF THE mPCR SYSTEMS USED TO IDENTIFY THE SEROTYPES OF *SALMONELLA ENTERICA* ISOLATED FROM MANURE SAMPLES OF POULTRY FARMS. A) mPCR System for Typhimurium; B) mPCR SYSTEM FOR INFANTIS.

The mPCR system evaluated in this work, allowed rapid and accurate assignment of a serotype for the majority of *Salmonella* isolates in this study. In FIG. 2, a flow diagram followed to use the mPCR systems designed by Akiba *et al.* [1] is presented. This sequence is the one we considered to be more appropriated to analyze *Salmonella* isolates from poultry farms, based on the most frequently reported serotypes at this premises worldwide [12, 16, 23, 26] and was the sequence used to analyze the samples in this study.
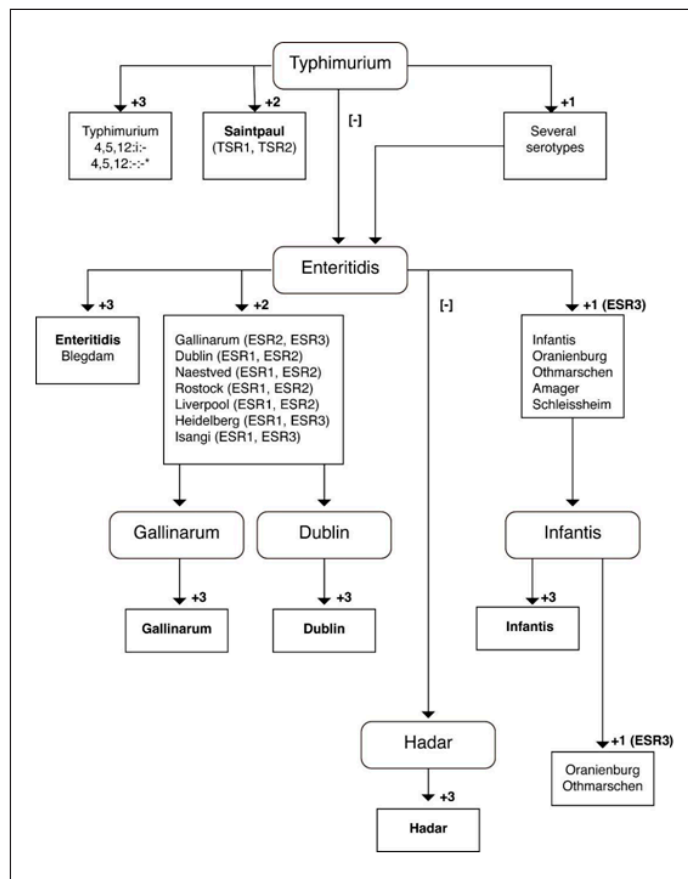

FIGURE 2. FLOW DIAGRAM TO EVALUATE *SALMONELLA* ISOLATES WITH THE mPCR SYSTEMS REPRESENTED HERE WITH ROUNDED RECTANGLES. IN THE BOXES, THE NAME OF THE IDENTIFIED SEROTYPE IS INDICATED IN BOLD OR THE NAME(S) OF SUSPECTED SEROTYPE(S) ARE FOLLOWED BY THE SSGRs THAT ARE AMPLIFIED BETWEEN PARENTHESIS. [-]: NO SSGRs AMPLICONS DETECTED. +N: WHERE N IS THE NUMBER OF SSGR AMPLIFIED.

None of the *Salmonella* isolates corresponded to *Salmonella* ser. Gallinarum, biotypes Gallinarum or Pullorum. These biotypes are of great concern for poultry industry in many parts of the world for being the causing agents of Typhoid and Pullorum disease, respectively [20]. Probably, this result obeys to the fact that the samples come from the farm environment and not from bird's organs, important differences have being reported regarding sampling methods and isolated serotypes. Soria [27] indicates that when evaluating *Salmonella* prevalence in laying hens at Argentina, positive farms for *Salmonella* ser. Gallinarum were detected only by culturing the organs from birds. If this type of sample is not included, presence of serotype Gallinarum could be underestimated in asymptomatic carriers. Therefore, results of this study did not rule out the presence of *Salmonella* ser. Gallinarum in the evaluated farms.

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TABLE III
**APPARENT PREVALENCE AND SEROTYPE/SEROGROUP OF _SALMONELLA ENTERICA_ SUBSP.
ENTERICA IN THE DIFFERENT TYPES OF POULTRY FARMS**

</div>

Poultry farms	No. of positive	Serotype/Serogroup
	Farms/No. of farms (%)	
Breeding hens	3/26 (11.5)	Enteritidis, Saintpaul, Serogroup O:8; Z_4 Complex
Laying hens	5/56 (8.9)	Infantis, Serogroup O:8;Z_4 Complex
Broilers	8/63 (12.7)	Enteritidis, Saintpaul, Infantis, Serogroup O:8; Z_4 Complex
TOTAL	16/145 (11.0)	

The profile of serotypes isolated from poultry farms is presented in TABLE II. _Salmonella_ ser. Infantis and Serogroup O:8,20:Z_4 Complex were the more frequently isolated. Serotypes Saintpaul and Enteritidis were also isolated. These results are in concordance with results from _Salmonella_ isolated from poultry farms in other countries [9, 10, 23, 25, 29]. Most of the _Salmonella_ serovars, including _Salmonella_ ser. Enteritidis and _Salmonella_ ser. Infantis are not serious pathogens in the chicken but they pose a potential threat to public health [16]. The serotypes Enteritidis and Infantis are carried within the gut of the birds and are shed from infected birds through faeces, feather dust and secretions from the eyes and nose contaminating the environment [30]. Serotypes Enteritidis, Saintpaul and Infantis are implicated in human salmonellosis outbreaks linked to contaminated food [5, 26, 32]. The foodborne outbreaks caused by _Salmonella_ ser. Saintpaul are related to raw produces, like vegetables or fruits [5]. Up to this date, there are no reports associating Saintpaul serotype with eating poultry or poultry products, like eggs. However, the contamination of water or agricultural soil with poultry manure could cause the contamination of fruits and vegetable with pathogenic bacteria like _Salmonella_ [2].

As shown in TABLE III, _Salmonella_ was isolated from all the poultry farms types evaluated: 12.7% in broilers farms, 11.5% in breeding hen flocks and 8.9% in laying hen flocks. Therefore, actions should be taken at all levels of primary production to reduce the presence of this foodborne pathogen at chicken meat and eggs. _Salmonella_ ser. Enteritidis was isolated from breeding hens and broilers. However, this serotype was not isolated from environmental samples of laying hens flocks. _Salmonella_ ser. Enteritidis presence in fecal samples of laying hens flocks is highly correlated with contamination of egg and eggshells [7, 14]. In Australia, a study showed that _Salmonella_ ser. Infantis was the most frequently reported serovar from the eggshell and wash of eggs collected from 31 flocks [11]. The presence of _Salmonella_ in poultry population is considered as a risk factor for the presence of _Salmonella_ in meat and eggs. Therefore, the EU had set targets for the reduction of certain _Salmonella_ serotypes in different poultry populations within a frame regulation for zoonoses. The serotype Infantis is one of the five _Salmonella_ serotypes considered for reduction during the actual control programme of the EU, together with serotypes Enteritidis, Typhimurium, Hadar and Virchow. Therefore, plans have been established to reduce their prevalence to less than 1% in laying hens flocks with more than 250 adult birds [19].

The human salmonellosis is directly linked to contaminated poultry and poultry products, like eggs [22]. Therefore, the results of this study should reinforce the importance to keep evaluating the _Salmonella_ prevalence of industrialized poultry farms in Ecuador and to promote strategies to improve biosecurity measures needed to control this bacteria during primary production, in order to prevent the potential impact of foodborne contamination with _Salmonella_ in public health.

CONCLUSIONS

Salmonella apparent prevalence and the serotypes found in the poultry farms evaluated in this study indicate that primary production needs specific interventions in order to control the presence of this foodborne pathogen in the Ecuadorian food chain.

Regarding _Salmonella_ serotypes, Enteritidis, Infantis and Saintpaul were present at the poultry farm environment. This could pose a risk for poultry meat and eggs contamination with potentially human pathogens.

The mPCR systems tested resulted a useful and suitable alternative, to conventional serotyping, for serotype identification of _Salmonella enterica_ subs. _enterica_ isolates from poultry farms samples.

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