Salmonella spp. contamination in commercial layer hen farms using different types of samples and detection methods

M. C. Soria,[*,1] M. A. Soria,[*] D. J. Bueno,[*,3] E. I. Godano,[†] S. C. Gómez,[‡,2] I. A. ViaButron,[*] V. M. Padin,[§] and A. D. Rogé[§]

[*]*Instituto Nacional de Tecnología Agropecuaria EEA Concepción del Uruguay, Ruta Provincial 39 Km 143,5, 3260, Concepción del Uruguay, Entre Ríos, Argentina;* [†]*Tecnovo S.A., Parque Industrial C.C. 17, 3116, Crespo, Entre Ríos, Argentina.;* [‡]*Fundación ArgenINTA, Ruta 11, Km 12.5, 3101, Oro Verde, Paraná, Entre Ríos, Argentina; and* [§]*Servicio de Antígenos y Antisueros, Instituto Nacional de Producción de Biológicos (INPB) - ANLIS "Dr Carlos G. Malbrán", Av. Vélez Sarsfield 563, C1282AFF, Ciudad de Buenos Aires, Argentina*

ABSTRACT The performance of detection methods (culture methods and polymerase chain reaction assay) and plating media used in the same type of samples were determined as well as the specificity of PCR primers to detected *Salmonella* spp. contamination in layer hen farms. Also, the association of farm characteristics with *Salmonella* presence was evaluated. Environmental samples (feces, feed, drinking water, air, boot-swabs) and eggs were taken from 40 layer hen houses. *Salmonella* spp. was most detected in boot-swabs taken around the houses (30% and 35% by isolation and PCR, respectively) follow by fecal samples (15.2% and 13.6% by isolation and PCR, respectively). Eggs, drinking water, and air samples were negative for *Salmonella* detection. *Salmonella* Schwarzengrund and *S.* Enteritidis were the most isolated serotypes. For plating media, relative specificity was 1, and the relative sensitivity was greater for EF-18 agar than XLDT agar in feed and fecal samples. However, relative sensitivity was greater in XLDT agar than EF-18 agar for boot-swab samples. Agreement was between fair to good depending on the sample, and it was good between isolation and PCR (feces and boot-swabs), without agreement for feed samples. *Salmonella* spp. PCR was positive for all strains, while *S.* Typhimurium PCR was negative. *Salmonella* Enteritidis PCR used was not specific. Based in the multiple logistic regression analyses, categorization by counties was significant for *Salmonella* spp. presence (P-value = 0.010). This study shows the importance of considering different types of samples, plating media and detection methods during a *Salmonella* spp. monitoring study. In addition, it is important to incorporate the sampling of floors around the layer hen houses to learn if biosecurity measures should be strengthened to minimize the entry and spread of *Salmonella* in the houses. Also, the performance of some PCR methods and *S.* Enteritidis PCR should be improved, and biosecurity measures in hen farms must be reinforced in the region of more concentrated layer hen houses to reduce the probability of *Salmonella* spp. presence.

Key words: *Salmonella*, detection, sampling, layer hen farm

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INTRODUCTION

The intense international trade of animals and animal products facilitates the spread of *Salmonella* spp., making salmonellosis an international public-health subject, responsible for serious economic losses to the poultry industry and governments. Although humans can become infected by *Salmonella* spp. through a wide range of food products, poultry meat and eggs are among the most frequently implicated sources of human *Salmonella* outbreaks (Gast, 2013). Therefore, knowledge of the actual prevalence of *Salmonella* spp. in laying hens and the factors that influence the presence and persistence of *Salmonella* on a layer hen farm are of the utmost importance (Van Hoorebecke et al., 2010b).

Because *Salmonella* Enteritidis **(SE)** and other important serotypes do not typically cause mortality in layer flocks, samples collected from the environment tend to more readily indicate the presence of them in the flock (Holt et al., 2011). Environmental monitoring is a useful, effective and less invasive method to predict potential infection or colonization of the poultry flocks (Waltman and Gast, 2008). Furthermore, a high level of environmental contamination of the house is also

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[1]Present address: INIQUI-CONICET, Universidad Nacional de Salta, Avda. Bolivia 5150, C.P. 4400, Salta, Argentina.
[2]Present address: Facultad de Ciencia y Tecnología, Universidad Autónoma de Entre Ríos, 25 de Mayo 353, 3260, Concepción del Uruguay, Entre Ríos, Argentina.
[3]Corresponding author: dantejb@yahoo.com.ar

associated with a higher risk of producing contaminated eggs in laying houses (Henzler et al., 1998). Even after cleaning and disinfection among laying cycles, SE can appear in a variety of environmental samples (Dewaele et al., 2012).

A wide range of culture methods and polymerase chain reaction (**PCR**) assays are available, and several studies had been developed to test their ability to detect *Salmonella* in different matrices (Rybolt et al., 2004; Myint et al., 2006; Eriksson and Aspan, 2007; Love and Rostagno, 2008; Singer et al., 2009). Routinely used methods for isolating *Salmonella* rely on pre-enrichment in nonselective media, selective enrichment, and plating on selective and differential media. Subsequently, the pathogens are identified by biochemical and serological tests. According to Rybolt et al. (2004), the sensitivity and specificity of the method depend on the sample type as well as the isolation conditions. Also, the process of isolating *Salmonella* spp. for environmental monitoring is determinate by different factors: sampling, level of contamination by the pathogen, or presence of competitors. Depending on the type of competitive bacteria, detection of occasional colonies of *Salmonella* may be easier if the appropriate plating medium has been used (Busse, 1995). In addition, processing a greater number of samples with a single effective method is probably more efficient than testing a small number of samples using complex laboratory protocols (Carrique-Mas and Davies, 2008).

The diversity of sources from which salmonellae can be introduced into flocks or houses complicates efforts to establish specific critical control points for preventing zoonotic *Salmonella* infections in poultry (Gast, 2013). In addition, every poultry farm has its own risk profile for the introduction of pathogens, subsequent development of disease, and the spread of pathogens to other farms. This risk profile is determined by a complex interaction between the levels of infection in an area, the measures implemented on the farm to prevent disease, and other factors including the density of farms in the area and linkages with other farms and markets (Sims, 2007). Different countries have different regulations to control *Salmonella* spp. in laying flocks. Most of them are focused on *Salmonella* Typhimurium (**ST**) and SE (Barroso, 2011; USDA, 2011).

Minimizing *Salmonella* contamination in commercial layer hen houses required a continuous improvement of the environmental monitoring process (Im et al., 2015), methods used for pathogen detection, and the management measures taken in poultry production. For this reason, the aims of this work were to 1) estimate the contamination of *Salmonella* spp. in layer hens farms using different types of samples, 2) evaluate the performance of detection methods, plating media used and the specificity of PCR primers, and 3) study the association of farm characteristics with *Salmonella* presence in a layer hen house.

MATERIALS AND METHODS

Sampling

Forty layer hen houses belonging to 30 hen farms from 6 counties (Colon, Diamante, Gualeguay, Nogoyá, Paraná, and Uruguay) of Entre Rios, Argentina were sampled one time, from August 2011 to April 2012. At the moment of sampling, 39 layer hen flocks, housed in battery cage systems, were in egg-laying phase. One flock was 4 months old. Without this flock, the average age of the hens was 18 months, with 7 and 36 months as minimum and maximum, respectively. Flock sizes were between 450 and 30,500 hens. Most of the layer hens were white egg layers, and they had not received any *Salmonella* vaccine.

Automatic and manual battery cage systems for layer hens were considered in the sampling. Automatic systems included manure belts, mobile feed chutes, mechanized egg collections, and advanced climate systems for cooling and ventilation. On the other hand, the manual battery cage farms did not have automated infrastructure and most of the activities were performed manually inside the house.

When the farm had 4 or fewer houses, one commercial layer hen house was sampled. If the farm had more than 4 houses, 2 houses were sampled. When there were both manual battery cage and automatic systems on the same farm, one of each type of house was sampled. So, 33 and 7 manual and automatic battery cage houses were sampled, respectively. If the farm had animals with different ages, the houses that had older layer hens were selected for the study because they are more likely to be contaminated with *Salmonella* spp. (Van Hoorebeke et al., 2010a).

Number and Sample Types Taken on Layer Hen Farms

Feed. Samples were taken from each mobile feed chute in manual battery cage houses. In houses without any kind of chute, samples were collected from layer feeders or from the external silo. In automatic house systems, samples were taken from mobile feed chutes. In all cases, 3 ± 1 kg of feed were taken and put in plastic bags, using disinfected plastic spoons.

Eggs. Two fiber cartons of 30 eggs each one were collected from each house. Mainly, dirty eggs were selected to be sampled. It was not possible to analyze eggs from 2 layer houses from different farms. In one of them, the hens were not in laying phase. From the other house, the farm owner did not authorize egg sampling.

Feces. Pooled feces (300 ± 50 g) were taken from all belts in each line of the house after running the manure removal system (automatic). In step cage houses without scrapers or belts (manual battery cage), 300 ± 50 g of mixed fresh feces were collected from 20 to 30

different places beneath the cages in the dropping pits. In all cases, samples were taken using sterilized spoons.

Water. From each farm, 1 L of water was taken from the external water tank. Furthermore, 1 L was taken from taps located inside the houses. Water was allowed to run for about 3 to 5 min before sampling. The outside part of the faucet was cleaned under a flame, and water was collected into sterile bottles. The samples were labeled and transferred to the laboratory.

Air Contamination. The settle plate method was used to isolate *Salmonella* spp. at different places along the central corridors of the hen houses. MacConkey agar (**MC;** Acumedia, Lansing, MI) plates (9 cm diameter) were put on plastic stools to isolate the bacteria. Six to 10 Petri dishes were used in houses with a single corridor. In those houses with 2 or 3 central corridors, 5 Petri dishes were used for each. Therefore, 5 to 15 Petri dishes were used and exposed to the air of the house during 10 min.

Floor Around the Houses. Considering that external environment of the layer hen houses can represent a potential source of *Salmonella* contamination, 2 pairs of boot-swabs were used for sampling. Boot-swabs (moistened in 0.8% sterile saline solution) were worn over the boots, which had been disinfected previously. The sample was taken by walking on the floor around the periphery outside the poultry house. Each pair of boot-swabs covered about 50% of the floor around the house. Swabs from one poultry house were pooled into one sample in a sterile plastic bag.

Samples were transferred to the Poultry Health Laboratory (Concepción del Uruguay, Entre Ríos, Agriculture Experimental Station of the National Institute of Agricultural Technology [**INTA**]).

Salmonella *spp. Isolation*

Feed. Two subsamples (25 g each one) were taken from each plastic bag of 3 kg. *Salmonella* isolation was done according to the tetrathionate (**TT**) method, proposed by Soria et al. (2011), with a pre-enrichment in 225 mL of tryptic soy broth (**TSB**; Merck, Darmstadt, Germany) with ferrous sulphate (**TSBF**, 35 mg of ferrous sulphate added to 1,000 mL of TSB) incubated at 35 ± 2°C for 18 to 24 h, followed by an enrichment step in TT broth base (Acumedia, Lansing, MI) plus supplements (20 mL/L of iodine potassium iodide solution −6 g of iodine; 5 g of potassium iodide; 20 mL of demineralized water-, brilliant green 0.1% [Sigma, Steinheim, Germany], and 40 mg/mL of novobiocin [Sigma]) incubated at 35 ± 2°C for 18 to 24 h. After that, samples were streaked on xylose lysine desoxicholate agar (Oxoid) with tergitol 4 (4.6 mL/L, Sigma) (XLDT) and EF-18 agar (Acumedia) and incubated at 35 ± 2°C for 18 to 24 h.

Eggs. Two samples of eggshell and 2 of egg content were obtained from each layer hen houses (n = 152; pool of 76 egg content and 76 eggshells) according to the following procedure. One out of 2 egg cartons was used to eggshell culture, while the other was utilized for culturing egg contents. Each carton of 30 eggs was divided in 2 groups of 15 eggs and eggshells were processed in sterile plastic bags as 2 pools of 15 eggshells each one. In the same way, 2 pools of 15 egg contents were processed. The egg contents were collected after sterilizing the egg surface by immersion in 70% ethyl alcohol for 10 min., and then by immersion in boiling water for 5 s (Soria et al., 2012a). Each egg was aseptically broken and the egg contents (mixture of yolk and albumen, **YA**) were stomached (Stomacher 400 circulator, Seward, England) 2 min at 2,300 rpm. Shell and YA samples (25 g) were processed according to Soria et al. (2012a) with a pre-enrichment in 225 mL of TSBF. The enrichment step was done in TT broth (Acumedia) plus supplements described above, incubated at 35 ± 2°C for 5 days. A loopful of the incubated selective enrichment broth was streaked on Hektoen (Acumedia) and XLD (Oxoid) agar on d 1 (for egg content and shell samples) and d 5 (for eggshell samples).

Feces. Two subsamples of 25 g were taken from each of feces sampled (300 ± 50 g). Samples were processed following the modified semisolid Rappaport-Vassiliadis (**MSRV**)-feces method described by Soria et al. (2012b), with a pre-enrichment in 225 mL Buffered Peptone Water (**BPW**, Merck, Darmstadt, Germany). After 18 to 24 h incubation at 35 ± 2°C, 30 μL of incubated BPW culture was inoculated in MSRV medium (Acumedia, Lansing, Michigan), which was supplemented with 1 mL/L of a 2% novobiocin solution, and then incubated at 41.5 ± 1°C for 24 h, and subsequently streaked on XLDT and EF-18 (Acumedia) agars.

Water. Two subsamples of 25 mL were taken from each 1 L sample and were cultured according to Soria et al. (2013) with MSRV-water method with a pre-enrichment in 225 mL BPW (Merck, Darmstadt, Germany) in a double concentration. After 18 to 24 h incubation at 35 ± 2°C, 30 μL of incubated BPW culture was inoculated in MSRV (Acumedia, Lansing, Michigan) and subsequently streaked on XLDT, and EF-18 agar (Acumedia).

Air. Immediately after the arrival at the laboratory, the MC agar plates exposed to air house were incubated during 48 h at 35 ± 2°C.

Boot Swabs. Each plastic bag containing 2 pairs of boot-swabs were pre-enriched (1/10) on BPW (Merck) and subsequently processed with the MSRV-feces method described by Soria et al. (2012b). XLDT, and EF-18 agar (Acumedia) were used as differential-selective agars.

Two presumed *Salmonella* colonies on each selective-differential agar plate were biochemically confirmed using triple-sugar iron agar (Acumedia), lysine iron agar (Merck), Simmons citrate (Merck), sulfide indole motility medium (Merck), Jordan's tartrate agar and phenylalanine agar (Hi-Media). All *Salmonella* spp. isolations were preserved on nutritive (Merck) slant agar until

serotyping confirmation and identification of the *invA* gene by PCR, as described below. The serotyping was carried out according to the White-Kauffmann-Le Minor scheme, with somatic (**AgO**) and flagellar (**AgH**) antigens (Grimont and Weill, 2007). A farm was considered positive to *Salmonella* spp. when this bacterium was detected by isolation from culture or PCR detection from pre-enrichment or enrichment broth (see below) at least on one processed sampled from the layer hen house.

DNA Extraction

A suspension in saline solution (0.85% NaCl) was prepared for each *Salmonella* strain isolated. This suspension was centrifuged at 4,000 × *g* for 15 min at 4°C and washed twice with sterile demineralized water. The pellet was suspended in 500 μL of sterile demineralized water, and the DNA was released by heating at 100°C for 10 min on a hot block (Labnet D1100; Labnet International Inc., Edison, NJ). The cellular debris was pelleted by centrifugation at 9,300 × *g* for 1 min at 4°C, and the supernatant containing nucleic acids was fractionated in Eppendorf tubes and stored at –70°C until it was used in subsequent PCR assays. The same procedure was performed for 1 mL of pre-enrichment broth from poultry feed, eggs and water samples. For feces and boot-swabs, a loopful of enrichment cultures on MSRV was re-suspended in 1 mL of sterile demineralized water and DNA extraction was carried out according to Soria et al. (2012b).

PCR Assays

Salmonella spp. PCR assay for *invA* gene (Malorny et al., 2003) was performed both for each strain isolated and environmental samples. Besides, SE PCR assay was conducted only for isolated strains, according to Agron et al. (2001). Subsequently, a multiplex PCR for ST (Lim et al., 2003) was carried out on isolated strains negative for SE PCR. All DNA extractions (5 μL) were amplified in an optimized 25 μL reaction mixture containing: 0.1 μM of each primer, 1X Taq buffer (Fermentas Inc., Hanover, MD), 1.5 mM $MgCl_2$ (Fermentas), 0.2 mM of each dNTP (Fermentas), 0.04 U/μL (*Salmonella* spp. and SE assays) or 0.02 U/μL (ST assay) of Taq DNA polymerase and double distilled water up to 25 μL. *Salmonella* genus-specific primers 139 and 141 (Operon Biotechnologies GmbH, Cologne, Germany) based on the *invA* gene of *Salmonella* was used in the PCR assay. The primers have the following nucleotide sequences: (5′→3′) GTGAAATTATCGCCACGTTCGGGCAA and TCATCGCACCGTCAAAGGAACC, respectively. The SE primers (Ruralex, Argentina) have the following nucleotide sequences: *Sdf* 1 Forward (5′-TGTGTTTTATCTGATGCAAGAGG-3′) y *Sdf* 1 Reverse (5′-CGTTCTTCTGGTACTTACGATGAC-3′). Negative SE PCR samples were used as templates for the following genes amplifications: *rfbj* (primers 5′-CCAGCACCAGTTCCAACTTGATAC and 5′-GGCTTCCGGCTTTATTGGTAAGCA); *flicC* (5′-ATAGCCATCTTTACCAGTTCCCCC and 5′-GCTGCAACTGTTACAGGATATGCC) and *fljb* (primers 5′-ACGAATGGTACGGCTTCTGTAACC and 5′-TACCGTCGATAGTAACGACTTCGG). For each assay, positive (*Salmonella* spp., SE ATCC 13076, ST 06/11 for *Salmonella* spp., SE and ST detection, respectively) and negative (*Citrobacter* spp. isolated from poultry feed for *Salmonella* spp. detection, and *E. coli* ATCC 25922 for SE and ST detection) controls were included as well as reagent blank containing all of the components of the reaction mixture, with the exception of template DNA (which was replaced by sterile distilled water). The reaction mixtures were incubated in a programmable DNA thermal cycler (model T18; Ivema, Argentina). The cycling parameters used for *Salmonella* spp. and SE detection were initial denaturation at 95°C for 1 min, followed by 38 cycles of amplification of 30 s at 95°C, 30 s at 64°C, and 30 s at 72°C. The reaction was completed by a final 3 min at 72°C. The cycling parameters used for ST detection were initial denaturation at 95°C for 3 min, followed by 30 cycles of amplification of 30 s at 95°C, 1 min at 65°C, and 30 s at 72°C with a final extension at 72°C during 1 min. The reaction was completed by a final 3-min extension at 72°C. Then, PCR tubes were held at 4°C.

Detection of PCR Products

The PCR products were analyzed by gel electrophoresis. Ten microliters of each sample were loaded onto 2.0% agarose gel in 0.5 × Tris-borate-EDTA (**TBE**) buffer at 120 V/cm for 1 h. The gel was stained with 0.5 μg/mL of ethidium bromide and the electrophoresis products were visualized with a UV transilluminator (model M-20; UVP Inc., Upland, CA). A 100-bp ladder (PB-L Productos Bio-Logicos, Buenos Aires, Argentina) was used as molecular weight marker.

Analysis of Performance Criteria for Feed, Fecal, and Boot-Swab Samples and Statistical Analysis

Agreement (Kappa coefficient) was calculated between culture and PCR methods for the same type of sample and between different types of samples according to Soria et al. (2011). The performance of the differential-selective agars was evaluated by Relative sensitivity (RSe), specificity (RSp), and agreement (Kappa coefficient) in samples of feed, feces, and boot-swabs.

For isolation methodology, relative true positive was defined when a sample was positive to *Salmonella* spp. in at least one differential-selective agar. Relative

true negative was defined as samples where *Salmonella* spp. was not detected in any differential-selective agar. Kappa coefficients were summarized, according to Dawson and Trap (2004), as an excellent agreement (0.93 to 1.00), a very good agreement (0.81 to 0.92), a good agreement (0.61 to 0.80), a fair agreement (0.41 to 0.60), a slight agreement (0.21 to 0.40), a poor agreement (0.01 to 0.20), and no agreement (<0.01). Z test was used in order to test the statistical significance of kappa coefficients.

Relationships Between Different Variables in a Layer Hen House and Salmonella Presence

A questionnaire was designed and completed in layer hen farms that had different sections related to farm identification data, animal vaccination plan, biosecurity measures applied on the farm, feed characteristics, water supply, egg destination, and type and management of egg cartons. The survey was filled out during an on-farm interview on the same day of the sample collection. The questions were posed to the owner or the person in charge of the farm by the primary author or by the veterinarian that connected us with the farm. Closed-ended questions (dichotomous or multiple choice) were used based on biosecurity measures for poultry farms, according to the Argentinean National Agrifood Health and Quality Service regulation 542/2010 (SENASA, 2010). Information from the questionnaires was coded and put in a database (Excel, Microsoft Corp., Redmond, WA). Data were analyzed using Infostat Software (Di Rienzo et al., 2014).

Taking into account that farms have different type of commercial layer hen houses (manual and automatic battery cage), each house was considered a unit. A 2-step statistical procedure was used to assess the relationship between the different variables (taken from the survey) and the *Salmonella* status in the house sampled. The hen age variable, number of laying hens/house, and counties were all divided into 2 categories. Hen age was based on the average age of the first molting in laying hens, ≥18 months old and <18 months old. The 2 categories for the number of laying hens were <10,000 and ≥10,000, based on the average number of hens per poultry house. In reference to counties, 2 categories were considered: Paraná (18 hen houses; 13 and 5 manual and automatic battery cage systems, respectively) and the other counties (22 hen houses; 20 and 2 manual and automatic battery cage systems, respectively), since Paraná has a high concentration of laying hen farms (Schell et al., 2010). All potential risk factors were tested by univariate analysis and only variables with a $P < 0.15$ were selected (Fisher or χ^2 test). To eliminate correlated independent variables, significant variables of the first step were assessed for collinearity by Phi test. Variables were considered collinear when Phi was higher than 0.5. When a pair of variables was found to be collinear, only the more biologically plausible variable was kept for further analysis by means of logistic multiple-regression model. Hosmer-Lemeshow $\chi^2$was used as a goodness of fit test. The test used for the *P*-value criterion was the Wald chi-square test. The significant level was $P < 0.05$ with results expressed as odds ratio (**OR**), including a 95% confidence interval (**CI**) for all significant variables.

RESULTS

Contamination of Salmonella *spp. Using Different Types of Samples*

From the total of 30 commercial layer hen farms sampled, 13 and 14 farms were positive for *Salmonella* spp. by culture and PCR test, respectively. Of the 40 poultry houses examined, 14 (35%) and 17 (42.5%) houses were positive for *Salmonella* culture and PCR detection, respectively.

A total of 1,167 samples were analyzed by isolation methods with 56 samples positive to *Salmonella* spp. (4.8%). All samples of eggs (n = 152, 76 pools of eggshells and 76 of eggs contents) and water (n = 84) were negative to *Salmonella* by isolation and PCR. The same result was obtained for air samples (n = 389), analyzed only by bacteriological method. Table 1 shows the results from the 30 farms studied for feed, feces and boot swabs samples processed by isolation and PCR methods. Only 4.6% of feed samples were positive by isolation method without detection for *Salmonella* by PCR. In this sense, 13.3% of the farms were positive taking feed samples. On the other hand, from the total fecal samples, 15.2 and 13.6% were positive to *Salmonella* by isolation and PCR methods, respectively. Considering this results, farms with *Salmonella* contamination represent 33.3% with both methods applied (isolation and PCR). In relation to the total boot-swab samples (floor around the house) processed, *Salmonella* was detected both by isolation and PCR, with 30 and 35% of positives samples, respectively. One farm was only positive by PCR method, without presence of *Salmonella* by isolation method (farm number 13). Similar to fecal samples results, the numbers of *Salmonella* positives farms considering boot-swab samples were 33.3% and 36.6% by isolation and PCR, respectively.

Ninety-eight *Salmonella* strains were isolated from 14 commercial layer hen houses and they were typified into 17 serotypes. The most frequently *Salmonella* serotypes observed were *S.* Schwarzengrund (17.5%), *S.* Enteritidis (15%), *S.* Mbandaka (7.5%) and *S.* Newport (7.5%). *S.* Schwarzengrund was isolated mainly in samples of feces, followed by boot-swabs, and feed. On the other hand, *S.* Enteritidis was presented primarily in boot-swabs, followed by feces and feed.

The distribution of multiple *Salmonella* serotypes in the commercial layer hen houses (tested positive for bacterial contamination) is shown in Table 2. Nine hen

Table 1. Detail overview of *Salmonella* spp. detection by isolation (I) and polymerase chain reaction (PCR) detection in feed, feces, and boot-swab samples from 40 houses of the 30 laying hen farms of Entre Ríos, Argentina. Positive samples are in bold font.

		N° positive samples for *Salmonella* spp./total number of samples processed (%)[1]					
		Feed		Feces		Boot swab (floor around house)	
Farm number	Layer hen-house	I	PCR	I	PCR	I	PCR
1	1	0/6	0/6	0/6	0/6	0/1	0/1
2	1	0/2	0/2	0/8	0/8	**1**/1	1/1
3	1	0/8	0/4	0/4	0/4	0/1	0/1
	2	0/8	0/8	0/4	0/4	0/1	0/1
4	1	0/8	0/8	0/4	0/4	0/1	0/1
5	1	0/4	0/4	0/4	0/4	0/1	0/1
	2	0/4	0/4	0/4	0/4	0/1	0/1
6	1	0/2	0/2	0/4	0/4	0/1	0/1
7	1	0/8	0/8	0/4	0/4	0/1	0/1
8	1	0/2	0/2	0/2	0/2	0/1	0/1
9	1	0/6	0/6	0/6	0/6	0/1	0/1
10	1	0/12	0/12	0/6	0/6	0/1	0/1
	2	0/12	0/12	0/6	0/6	0/1	0/1
11	1	0/12	0/12	0/6	0/6	0/1	0/1
	2	0/4	0/4	0/4	0/4	0/1	0/1
12	1	0/6	0/6	0/6	0/6	0/1	0/1
13	1	0/6	0/6	0/6	0/6	0/1	0/1
	2	0/6	0/6	0/6	0/6	0/1	**1**/1
14	1	0/4	0/4	**1**/4	**1**/4	**1**/1	**1**/1
15	1	0/8	0/8	**1**/4	**1**/4	0/1	0/1
16	1	0/12	0/12	**2**/6	**2**/6	**1**/1	**1**/1
	2	0/4	0/4	0/4	1/4	0/1	0/1
17	1	0/8	0/8	0/4	0/4	0/1	0/1
	2	**3**/4	0/4	**4**/4	**4**/4	**1**/1	**1**/1
18	2	0/12	0/12	0/6	0/6	**1**/1	**1**/1
19	1	**1**/16	0/16	**1**/8	**1**/8	**1**/1	**1**/1
20	1	0/8	0/8	**3**/4	**3**/4	**1**/1	**1**/1
	2	0/4	0/4	0/4	2/4	**1**/1	1/1
21	1	0/8	0/8	0/4	0/4	0/1	0/1
22	1	0/16	0/16	0/8	0/8	0/1	0/1
23	1	0/4	0/4	0/4	0/4	0/1	0/1
24	1	0/8	0/8	0/4	0/4	0/1	0/1
	2	0/8	0/8	0/4	0/4	0/1	0/1
25	2	0/8	0/8	0/6	0/6	0/1	0/1
26	1	**2**/12	0/12	**4**/6	**2**/6	0/1	0/1
27	1	0/12	0/12	**3**/6	**1**/6	**1**/1	**1**/1
28	1	0/8	0/8	**3**/4	**1**/4	**1**/1	**1**/1
	2	0/8	0/8	**2**/4	**2**/4	**1**/1	**1**/1
29	1	0/4	0/4	0/4	0/4	**1**/1	**1**/1
30	1	**8**/12	0/12	**6**/6	**6**/6	0/1	0/1
Total samples analyzed	40	14/304 (4.6)	0/304 (0)	30/198 (15.2)	27/198 (13.6)	12/40 (30)	14/40 (35)
Number of *Salmonella*-positive farms/total farms sampled (%)		4/30 (13.3)	0/30 (0)	10/30 (33.3)	10/30 (33.3)	10/30 (33.3)	11/30 (36.6)

[1]The total number of samples processed includes the duplicate samples.

houses from Paraná (50% of houses sampled) were positive to *Salmonella* spp. Two poultry houses were contaminated by only one *Salmonella* serotype. However, 12 poultry houses displayed contamination by multiple serotypes, with the isolation of 2 to 6 serotypes. *S.* Enteritidis, *S.* Schwarzengrund and *S.* Mbandaka were isolated from manual and automatic battery cage system.

Performance of Salmonella *Detection Methods*

The agreement between MSRV-feces method and MSRV-PCR method for fecal and boot-swab samples were 0.75 (CI 95% 0.62–0.88; SE 0.06) and 0.76 (CI 95% 0.60–0.93; SE 0.07), respectively. Agreement between the methods for feed samples could not be calculated because of the absence of positive samples by PCR. On the other hand, the agreement was from slight to good for *Salmonella* spp. culture analysis in the layer hen houses sampled; and from no agreement to good for *Salmonella* spp. PCR analysis (Table 3).

Because of the absence of false positive samples, the RSp for all plating media was 1. The results of RSe and the agreement (Kappa coefficient) calculation for the selective-differential media used are shown in Table 4. In relation to XLDT agar and EF-18 agar, the RSe was 0.46–1 and 0.76–1, respectively, with a significance difference in feed, fecal, and boot-swab

Table 2. Multi-serotype contamination in 14 *Salmonella*-positive poultry houses.

Farm number code (County, type of poultry house)	No. serotypes isolated	Serotypes isolate per sample type		
		Feed	Feces	Boot-swabs
2 (Colón, manual)	1	-	-	Infantis
14 (Diamante, automatic)	2	-	Mbandaka	Mbandaka, Sandiego
15 (Diamante, manual)	1	-	Schwarzengrund	-
16 (Nogoyá, manual)	2	-	Mbandaka	Schwarzengrund
17 (Paraná, automatic)	3	Schwarzengrund, Newport, Stanley	Schwarzengrund	Schwarzengrund, Stanley
18 (Paraná, manual)	2	-	-	Enteritidis, Schwarzengrund
19 (Paraná, manual)	2	Livingstone	Enteritidis	Enteritidis
20 (Paraná, manual)	2	-	Schwarzengrund	Kingston, Schwarzengrund
26 (Gualeguay, manual)	6	Enteritidis, Rissen, Montevideo	Enteritidis, Rissen. Newport	Agona, Derby
27(Paraná, manual)	5	-	Newport, Senftenberg, Schwarzengrund, Westhampton	Anatum
28 (Paraná, manual)	2	-	Kentucky	Enteritidis
28 (Paraná, automatic)	2	-	Enteritidis, Kentucky	Enteritidis
29 (Paraná, manual)	2	-	-	Enteritidis, Montevideo
30 (Paraná, manual)	5	Infantis, Mbandaka, Schwarzengrund, Stanley	Infantis, Mbandaka, Schwarzengrund, Sandiego	-

Table 3. Kappa coefficient values showing agreement between feces, feed, and boot-swab for culture and polymerase chain reaction (PCR) samples in the layer hen houses sampled.

Type of analysis in a poultry house	Comparison between samples	Kappa coefficient (Standard error; confidence interval)
Culture	Feces/feed	0.45* (0.15; 0.14–0.76)
	Feces/boot-swab	0.70* (0.12; 0.44–0.94)
	Boot-swab/feed	0.26* (0.15; 0.01–0.51)
PCR	Feces/feed	0.00
	Feces/boot-swab	0.61* (0.13; 0.34–0.86)
	Boot-swab/feed	0.00

*Indicates that kappa is significantly nonzero ($P < 0.05$).

Table 4. Relative sensitivity (RSe) and agreement (kappa coefficient) for selective-differential plating media used in feed, feces and boot-swabs for *Salmonella* isolation from laying hen farms of Entre Ríos, Argentina.

Type of sample	Media	RSe	Kappa coefficient (Standard error; confidence interval)
Feed	XLDT	0.46[a] (0.24–0.69)	0.48* (0.13; 0.21–0.75)
	EF-18	0.86[b] (0.61–0.95)	
Feces	XLDT	0.70[a] (0.51–0.83)	0.80* (0.64; 0.67–0.92)
	EF-18	1.00[b] (0.88–1)	
Boot swabs	XLDT	1.00[a] (0.76–1)	0.81* (0.09; 0.62–1.00)
	EF-18	0.76[b] (0.49–0.91)	

[a,b]Values followed by different letters in the same column for each type of sample are significantly different (P < 0.05).

*Indicates that kappa is significantly nonzero ($P < 0.05$).

XLDT = xylose lysine desoxicholate agar plus 4.6 mL/L of tergitol 4.

samples. This parameter was greater for EF-18 agar than XLDT agar in feed and fecal samples. However, RSe was greater in XLDT agar than EF-18 agar for boot-swab samples. On the other hand, the agreement was fair between XLDT and EF-18 agar for feed, whereas it was good and very good for feces and boot-swabs, respectively.

Specificity of PCR Primers to Detect Salmonella *spp.*

All *Salmonella* strains were positive to *invA* gene. None of the isolated strains were positive to ST by PCR. On the other hand, 14 isolations identified as *S.* Enteritidis by serotyping were positive to

Table 5. Results from univariate analysis for risk factors identification of *Salmonella* spp. The analysis describes the data from the 40 houses of the 30 laying hen farms sampled from Entre Rios, Argentina.

Variable	n	% of positives houses for *Salmonella* spp.	*P*-value
Number of hens			0.059
≥10,000	10	43.8	
<10,000	30	56.3	
Hen ages			0.441
≥ 12 months	31	31.2	
<12 months	9	68.8	
Number of hens per cage			0.042
3 to 4 hens	32	62.5	
More than 5 hens	8	37.5	
County			0.002
Paraná	18	75.0	
Other counties	22	25.0	
Biosecurity level according to National Agrifood Health and Quality Service			0.197
A (Very good)	2	13.3	
B (Good)	33	80.0	
C (Bad)	3	6.7	
Fenced			1.000
absent	3	6.3	
present	37	93.8	
Disinfection equipment			0.50
absent	25	56.3	
present	15	43.8	
Other species on the farm			0.104
absent	14	50.0	
present	26	50.0	
Fly control			1.000
absent	7	18.8	
present	32	81.3	
Rodent control			1,000
absent	1	0	
present	39	100	
Previous diseases			0.709
yes	16	37.5	
no	23	62.5	
Feed sources			0.010
Own production	25	87.5	
Purchases to third parties	15	12.5	
Feces removal system			0.029
absent	34	68.8	
present	6	31.3	

S. Enteritidis PCR. However, 10 serotypes different from *S.* Enteritidis (17 strains) were positive to the *sdf* PCR reaction: *S.* Essen (1 strain), *S.* Newport (4 strains), *S.* Rissen (1 strain), *S.* Agona (1 strain), *S.* Derby (1 strain), *S.* Westhampton (1 strain), S. Schwarzengrund (2 strains), *S.* Kentucky (2 strains), *S.* Montevideo (1 strain) and *S.* Infantis (3 strains).

Association of Farm Characteristics with Salmonella *spp. in a Layer Hen House*

Table 5 shows the results of univariate analysis for risk factors identification of *Salmonella* spp. isolation with house as the sampling unit. Six variables were significant for this step of analysis ($P < 0.15$): number of hens, number of hens per cage, categorization by county (Paraná, other counties sampled), other species in the farm, feed sources and feces removal system. As a result of collinearity test, the variables number of hens per cage, and feces removal system were associated with number of hens. Also, the variable other species in the farm was associated with county. For this reason, the multiple logistic regression analysis included only 3 variables (Table 6). The categorization by counties was significant ($P = 0.010$) and turned out to be a risk factor for *Salmonella* spp. presence. The OR for this variable was 9.40. Therefore, the layer hen houses from Paraná have 9.4-fold greater odds for the *Salmonella* spp. presence than the house farms from the other counties. Also, the Hosmer-Lemeshow test was not significant ($P = 0.43$), indicating that the model fit the data.

DISCUSSION

Environmental monitoring or surveillance has become a useful tool for predicting potential infection or colonization of flocks with the paratyphoid salmonellae, which are numerous motile and non-host-adapted *Salmonella* serotypes principally of concern as a cause of foodborne disease in humans (Waltman and Gast, 2008). Although sampling fresh feces themselves likely provides the most sensitive test for the shedding of salmonellae, drag-swab samples, obtained by dragging moistened gauze pads or by cotton swabs across the floor of poultry houses, also provide sensitive results (Dewaele et al., 2012; Gast, 2013; Hulaj et al., 2016). Im et al. (2015) found that the collection of both dust and fecal samples was necessary to improve the detection of *Salmonella* in layer farms. These facts agree with the present work, which indicated that fecal samples were that showed the highest number of hen houses positive to *Salmonella* spp. Furthermore, although we did not study the floor inside the layer hen house, boot-swab samples around the floor houses were the samples with the greatest percentage of *Salmonella* spp. isolations. Feces and these types of samples showed a good agreement for *Salmonella* detection. Schulz et al. (2011) suggested that the presence of *Salmonella* during a laying-period is affected by the relatively low within-flock prevalence of shedding hens even in a *Salmonella*-contaminated environment. Therefore, the collection of pooled fecal material (i.e., originated from a large number of birds) increases the chance of inclusion of feces from infected birds which may contain high numbers of organisms (Carrique-Mas and Davies, 2008).

The collection of eggs during the sampling is a direct epidemiological measure of the farm health status. In contrast, other environmental samples only represent the potential of flocks to produce contaminated eggs (Holt et al., 2011). Different experimental or sampling studies about SE isolation showed that this bacterium was found in environmental and/or organ samples, but the prevalence in shell or content eggs was low (García et al., 2011; Dewaele et al., 2012; Gast et al., 2013). Furthermore, usually, the contents of 10 to 30 eggs are pooled for culturing, because of the low incidence and level of *Salmonella* contamination (Waltman and Gast,

Table 6. Multiple logistic-regression model of risk factors for *Salmonella* isolation. The analysis describes the data from the 40 houses of the 30 laying hen farms sampled from Entre Rios, Argentina.*

Category level		OR[1]	95% Confidence interval	P-value
County		9.40	0.01–0.57	0.010
	Paraná			
	Other counties sampled (ref.)[2]			
Number of hens		13.9	0.02–1.01	0.051
	≥10,000			
	<10,000 (ref.)			
Feed sources		597.9	0.81–44.14	0.080
	own production			
	purchases from third parties (ref.)			

[1]OR: odds ratio
[2]Ref.: Indicate the reference variable to OR calculation
*Hosmer-Lemeshow $\chi 2 = 3.81$; $P = 0.43$.

2008). This criterion was used in our study. However, although SE was isolated from feed, fecal, and boot-swab samples in our study, none of layer hen houses were positive to *Salmonella* spp. in eggshell or egg content either by isolation or PCR.

Historically, SE and ST have been the most important causes of non-typhoid salmonellosis. In our work, the 2 most frequently *Salmonella* serotypes observed were *S.* Schwarzengrund (17.5%), and *S.* Enteritidis (15%). However, Im et al. (2015) did not find these serotypes in commercial layer farms in Korea. *S.* Schwarzengrund is a less common cause of human salmonellosis worldwide, the relative incidence of this serotype increased in recent years (Chen et al., 2011). In the present study, *S.* Schwarzengrund had the highest percentage of isolation in the layer hen houses. This serotype is frequently associated to other types of poultry production such as chickens (Chen et al., 2011), although Poppe et al. (1991) also reported *S.* Schwarzengrund in laying hens.

Koyuncu et al. (2010) compared PCR and culture methods in artificially and naturally contaminated feed samples. For artificially contaminated feed, an excellent agreement was found between the methods, but it was low for feed naturally contaminated by *Salmonella.* On the other hand, Soria et al. (2011) reported that the agreement was fair between the TT and PCR methods in artificial contaminated avian feed samples with motile *Salmonella* strains. We found similar results in relation to feed samples, which were positive to the TT-method, but negative by PCR. Because of this result, the PCR method used for feed samples should be improved to be used as a tool that can help the bacteriological isolation in epidemiological studies.

Soria et al. (2011, 2012b) compared the sensitivity of XLDT agar and EF-18 agar in artificially contaminated fecal and feed samples for different *Salmonella* strains, utilized the same culture method as we used, and they did not find any difference in this parameter. However, we found that EF-18 agar had better RSe than XLDT agar in naturally contaminated feces and feed samples. Xylose-Lysine-Desoxycholate Tergitol 4 (**XLDT**) agar is an XLD modification, where the tergitol 4 (7-ethyl-2-methyl-4-undecanol hydrogensulphate sodium salt) is added in the XLD media as a selective inhibitor of *Proteus* species and other non-*Salmonella* organisms in the same way than it is used for Xylose-Lysine-Tergitol 4 agar (Miller et al., 1991, 1995). On the other hand, the EF18 medium is highly selective and contains bile salts, crystal violet, sulphapyridine and novobiocin. The differential properties are conferred by the presence of sucrose and lysine, and *Salmonella* colonies appear blue-green (Waltman, 2000). Although Warburton et al. (1994) encountered problems with the use of this medium for *Salmonella* isolation, due to the small colony size, abnormal colony coloration, and overgrowth by competitors; we found better result in this medium than in XLDT agar for feces and feed samples, but not for boot-swab samples.

In our work, 14 isolations were identified as SE by serotyping and PCR specific for this serotype. Although other authors tested the effectiveness of the primers we used for SE detection (Tennant et al, 2010; Maciel et al, 2011; Dewaele et al., 2012), our results are in disagreement with those showed by Agron et al. (2001), because other serotypes different from SE were also positive with the primers used. Furthermore, some strains from a same serotype different from SE were positive for SE primers.

In the present study, numerous variables in relation to biosecurity and features of houses were evaluated in order to determine possible risk factors associated with the presence of *Salmonella* spp. Although different descriptive researchers have identified potential sources and vectors for *Salmonella* contamination, there are few quantitative and epidemiological studies for laying hen farms (Van Hoorebecke et al, 2010b; Hulaj et al., 2016). The present study showed significant differences in relation to regional distribution of the houses respect to the presence of *Salmonella* spp. Houses from the Paraná county have a higher probability for the presence of *Salmonella* spp. respect to the other departments; 50%

of houses sampled from this county were positive to this bacteria. This result could be explained by the major concentration of farm in the region (Schell et al., 2010). It is known that areas with a high number of poultry production farms represent a challenge for the industry in reference to decrease the persistence of important diseases (Bermudez and Stewart-Brown, 2008).

This study shows the importance of considering different types of samples and detection methods during the monitoring or control of *Salmonella* spp. in layer hen farms. In addition, although *Salmonella* spp. can be found in feed and/or environmental samples, it may happen that egg samples are negative to *Salmonella* spp. isolation. Because of the concern of salmonellosis in poultry and human health, it is important to incorporate the sampling of floor around the layer hen houses in *Salmonella* spp. monitoring so biosecurity measures should be strengthened to minimize their spread and entry in the houses. The most prevalent serotypes, *S.* Schwarzengrund and *S.* Enteritidis, isolated from environmental samples, represent a serious problem both in an epidemiological and economical point of view. On the other hand, the difference in relative sensitivity and/or agreement of plating media indicates the importance of the inclusion of EF-18 agar in this kind of study, especially for feed samples. Furthermore, the effectiveness of primers that we used to detect SE and PCR method to detect *Salmonella* spp. in poultry feed should be improved to be used in epidemiological studies. Finally, the study points out some deficiencies in the hygiene program of hen houses from Paraná county, a region with more concentration of layer hen farms in Entre Rios. So biosecurity measures in hen farms must be reinforced in this region to reduce the probability of *Salmonella* spp. presence.

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