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Prevalence, antimicrobial resistance profile and comparison of selective plating media for the isolation of *Salmonella* in backyard chickens from Entre Rios, Argentina

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Summary

This study was conducted to estimate the apparent prevalence of *Salmonella* spp. in birds kept under backyard system in Entre Ríos, Argentina, and determine the performance of two selective plating media used for *Salmonella* isolation, and the antimicrobial resistance of the isolated. Also, the association of farms characteristics with *Salmonella* presence was evaluated. A total of 657 backyard chickens and 15 gooses were sampled one time by cloacal swab, belonging to 51 and one family farms, respectively, and four counties in Entre Rios state from April 2014 to May 2015. Only four samples from backyard chickens belonged to three family farms from Uruguay County were positive to *Salmonella* spp., so the apparent prevalence was 0.6% for this kind of chicken. Four serovars were isolated (*Salmonella* ser. Lille, *S*. ser. Newport, *S*. ser. Enteritidis and *S*. ser. Rissen), which were susceptible to all antibiotics tested with the exception of erythromycin. For Hektoen enteric agar and brilliant green agar, relative specificity and positive predictive value were 1, and the relative sensitivity and negative predictive value did not show any difference between them. The agreement was very good between these two plating media. None of the variables studied could be selected to calculate the risk factors associated with *Salmonella* isolation because *p* > .15. Although the prevalence of *Salmonella* spp. is low in backyard birds in Entre Rios, the presence of *S*. ser. Enteritidis should not be discounted, because it is found in the county that concentrates a large population of intensive poultry production in the state.

KEYWORDS
Argentina, backyard chicken, plating media, *Salmonella*

1 | INTRODUCTION

In Argentina, as in other developing countries, there is a sizable backyard poultry industry that, together with large-scale commercial producers, supplies domestic markets. The governments have adopted a system to breed backyard chickens and give them to low-income people so that they can have high-quality protein in their diets (Bonino & Canet, 1999). Backyard or free range evokes a positive image of chickens living outdoors with plenty of fresh air, sunshine and open space to roam in, and they have contact with other animals (Freire, Melo, dos Santos Silva, & da Silva, 2005; Kperegbeyi, Meye, & Ogboi, 2009; SA PPLPP, 2009). Furthermore, small poultry keepers have limited contact with veterinarians and their biosecurity practices are poor compared with enterprises. Thus, backyard chickens become a possible reservoir for bacteria like *Salmonella* and sometimes could be

involved in avian disease outbreaks with negative influence in commercial poultry production (Beam, Garber, Sakugawa, & Kopral, 2013; Kelly et al., 1994; Manning, Gole, & Chousalkar, 2015). Infected poultry flocks are also among the most frequently implicated reservoirs of salmonellae that can be transmitted through the food chain to humans. Poultry producers are faced with intensifying pressure from public health authorities, elected officials and consumers regarding food safety issues (Gast, 2003).

The genus *Salmonella* consist of only two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided into six subspecies and more than 2,500 serovars; some of them can be important pathogens in humans and animals, with varying levels of host specificity (Issenhuth-Jeanjean et al., 2014; Singer, Mayer, Hanson, & Isaacson, 2009). Fowls are the specific host of *Salmonella enterica* ser. Gallinarum biovar Pullorum and Gallinarum, which cause pullorum disease and fowl typhoid, respectively. Other serovars with no specific host, such as *S.* ser. Typhimurium and *S.* ser Enteritidis (SE), may infect humans, chickens and other animals (Guard-Petter, 2001).

Cloacal swabs or samples of voided faeces have been used to provide evidence of persistent intestinal colonization by salmonellae in individual birds (Gast, 2013). Culture methods are used in laboratory procedure recommended for the bacteriological examination of *Salmonella* in the Poultry Improvement Plan of different countries (Barroso, 2010, 2011, 2012; National Agrifood Health and Quality Service, 2003, 2016, United States Department of Agriculture, 2017). However, the process of isolating *Salmonella* is to some extend prone to failure. Depending on the type of competitive bacteria, detection of occasional colonies of *Salmonella* may be easier if the appropriate plating medium has been used. Unfortunately, the composition of the flora is never known in advance. Therefore, the appropriate plating medium may not be used for culture. Numerous agar media are available for the isolation of salmonellae (Busse, 1995; Gast, 2013; Soria & Bueno, 2016). Furthermore, Antibiotics are used as veterinary medicine for treatment and prevention of diseases, as prophylactic agents in the water of healthy birds and also as growth promoters in low concentrations in food (Apata, 2009; Phillips et al., 2004). The efficacy and wisdom of medication with these drugs to prevent or treat *Salmonella* infections in poultry are topics of considerable debate because of the inconsistent history of these drugs in eliminating *Salmonella* colonization and because indiscriminate veterinary and agricultural uses may imperil their medical usefulness by promoting microbial resistance (Gast, 2013; Shivaprasad & Barrow, 2008). The potential risk of genetic exchange that exists in the intestine is one of the reasons that explain a zoonotic risk of transfer of resistance (Errecalde, 2004).

There are few studies of *Salmonella* on backyard poultry in Argentina. Although some *Salmonella* serovars control plan is included in the National Poultry Health Plan in Argentina for breeders, broilers and hens (National Agrifood Health and Quality Service, 2003, 2016), backyard chickens play a significant socio-economic role in poor communities, and their flocks are not usually monitored for diseases or vaccinated. Xavier et al. (2011) found that the seropositive family farms were between 0% and 66.7% for *Salmonella* in Entre Rios, Argentina, during the period 2003–2007. However, these authors did not try to

isolate this bacteria. It is well recognized that some animals or poultry with a positive serological response may no longer be infected with *Salmonella* organisms. Likewise, animals or poultry that are actively excreting salmonellae may be serologically negative (World Organization for Animal Health, 2008).

Entre Ríos is a state in Argentina where poultry production is so concentrated that, from an epidemiological point of view, it is similar to a very large multi-age farm. This state is divided into 17 counties (Uruguay County concentrated 31% of the poultry farm and hatcheries) and has almost 3,355 poultry houses, which correspond to 43% of the poultry houses from Argentina. It also accounts for almost 48.7% of the poultry production and 25.2% of the egg production in Argentina (Livestock, Pigs and Poultry Division, 2014, Ministry of Agrindustry, 2016). Therefore, the present study was conducted to (i) estimate the apparent prevalence of *Salmonella* in birds kept under backyard system in Entre Ríos, Argentina, (ii) determine the performance of two differential plating media used in a sample for *Salmonella* isolation, and the antibiotic resistance profile of the isolated and (iii) study the association of farm characteristics with *Salmonella* presence in these types of farms.

2 | MATERIAL AND METHODS

2.1 | Study design

A total of 657 backyard chickens were sampled one time by cloacal swab from April 2014 to May 2015. These animals belonged to 51 family farms and four counties in Entre Rios state (Table 1). The number of cloacal swabs from backyard chickens was based on the following equation (Mateu & Casal, 2003), with an expected prevalence of 50% and a precision of 5% with 99% confidence limit:

$$n = Z^2 pq/B^2,$$

where: n = sample size, Z = 2.56 for 99% confidence limit, p = disease expected frequency (0.5), q = 1 − p, B = precision.

Five to 15 samples were taken from each backyard chicken farm. Furthermore, 15 gooses from another family farm were sampling by cloacal swab. A sterile cotton swab, pre-moistened with sterile 0.85% ClNa solution, was inserted into the cloaca of each bird and rotated gently against the lining of the cloaca to collect a sample. Swab from each bird was transferred to an independent sterile tube.

Impacts

- The prevalence of *Salmonella* spp. is low in backyard chickens in Entre Rios, Argentina.
- *Salmonella* strains isolated are resistant to erythromycin.
- Hektoen enteric agar is very similar to Brilliant green agar for *Salmonella* sp. isolation from cloacal swab samples in backyard chickens.

TABLE 1 Number of family farms and cloacal swab tested in the different counties from Entre Ríos, Argentina, from April 2014 to May 2015

County of Entre Ríos	No. of farms	No. of cloacal swab tested
Colon	1	15
Gualeguaychu	1	5
Tala	3	45
Uruguay	46	592
Total	51	657

Samples were labelled and transferred to the INTA Laboratory of Poultry Health (Concepción del Uruguay, Entre Ríos) in ice chests for *Salmonella* isolation.

2.2 | *Salmonella* spp. isolation and identification

At the laboratory, 5 ml of tetrathionate broth (Acumedia, Michigan, USA) plus supplements (20 ml/L of iodine–potassium iodide solution—6 g of iodine and 5 g of potassium iodide; 20 ml of demineralized water—brilliant green 0.1 %, Sigma, Steinheim, Germany; and 40 mg/ml of novobiocin, Sigma) was added to each tube with cloacal swab. After incubation during 18–24 hr at 35 ± 2°C, a loopful of broth was streaked on Hektoen enteric (HE) agar (Acumedia, HEA) and brilliant green (BG) agar (Oxoid, Basingstoke, UK) for chicken samples, and HE agar (Britania, Buenos Aires, Argentina) and *Salmonella Shigella* agar (Merck, Darmstadt, Germany) for goose samples, and incubated at 35 ± 2°C for 18–24 hr. 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), sulphide indole motility medium (Merck), Jordan's tartrate agar, phenylalanine agar (Hi-Media, Mumbai, India) and urea agar (Britania). If there were no bacterial colonies compatible with *Salmonella* spp. in a selective–differential agar plate, two atypical *Salmonella* spp. colonies were also taken and the same biochemical tests, as mentioned before, were carried out. All *Salmonella* isolations were preserved on nutritive (Merck) slants agar until serotyping, which was carried out according to the White–Kauffmann–Le Minor scheme, with somatic (AgO) and flagellar (AgH) antigens (Grimont & Weill, 2007).

2.3 | Antibiotic susceptibility test

The antibiotic susceptibility test was performed by the standard disc diffusion method in Mueller-Hinton agar (Difco™, Sparks, USA) and the results were interpreted in accordance with the criteria of the National Committee for Clinical Laboratory Standards (2013, 2015). The isolates were screened for resistance to the following antibiotics: fosfomycin (50 μg); colistin (10 μg); tetracycline (30 μg); florfenicol

(30 μg); enrofloxacin (10 μg); gentamicin (10 μg); erythromycin (15 μg); sulphamethoxazole/trimethoprim (25 μg); doxycycline (30 μg); neomycin (30 μg); cephalothin (30 μg); norfloxacin (10 μg); amoxicillin (10 μg); kanamycin (30 μg); ciprofloxacin (5 μg); chloramphenicol (30 μg); cefotaxime (30 μg); streptomycin (10 μg); and fosfomycin/tylosin (160 μg/40 μg). All the antibiotic discs, except fosfomycin/tylosin (FOSBAC PLUS T-BEDSON™, Britania), were purchased from Oxoid. The zone diameter breakpoint used for fosfomycin/tylosin was the same as fosfomycin.

2.4 | Analysis of the performance criteria for selective–differential culture media and statistical analysis

Relative accuracy (RAc), sensitivity (RSe), specificity (RSp), positive predictive value (RPPV) and negative predictive value (RNPV) and agreement (kappa coefficient and McNemar's test) of HEA and BG agar, used for *Salmonella* spp. isolation from chicken cloacal swab samples, were analysed according to Soria, Soria, Bueno, and Colazo (2011). 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 Trapp (2004), as an excellent agreement (.93–1.00), a very good agreement (.81–.92), a good agreement (.61–.80), a fair agreement (.41–.60), a slight agreement (.21–.40), a poor agreement (.01–.20) and no agreement (<.01). Z test was used in order to test the statistical significance of kappa coefficients. On the other hand, McNemar's test was calculated using a chi-square approximation at $p \leq .05$ (GraphPad Software, 2017).

2.5 | Relationships between different variables in a family farm and *Salmonella* presence

Data concerning the general characteristics of the family farm, the feeding, watering and sanitary practices and the measures taken for *Salmonella* control were also collected. The questionnaire (related to 10 topics), consisting of questions of which most of them were close-ended (dichotomous or multiple choice), was filled out by the investigator during an interview with the farmer. Closed-ended questions were designed based on biosecurity measures for poultry farms, according to the Argentinean National Agrifood Health and Quality Service regulation 542/2010 (2010) and the resolution No. 52/2005 from Entre Ríos General Directorate of Animal Production (2005). Information from the questionnaires was coded and put in a database (Excel, Microsoft Cooperation). Data were analysed using INFOSTAT Software (Di Rienzo et al., 2014).

Each farm was considered a unit. A two-step statistical procedure was used to assess the relationship between the different variables (taken from the survey) and the *Salmonella* status in the farm sampled. All potential risk factors were tested by univariate analysis and only variables with a $p < .15$ were selected (Fisher or chi-square test).

3 | RESULTS

3.1 | Apparent prevalence of *Salmonella* in backyard poultry in Entre Ríos, Argentina, and antibiotic resistance profile of the isolated

Of 657 samples of backyard chickens, only four samples, belonged to three family farms from Uruguay County, were positive to *Salmonella* spp. So the apparent prevalence was 0.6%. Twelve *Salmonella* strains were isolated from those positive samples and were typified into four serovars (Table 2): *Salmonella* ser. Lille (4), *S*. ser. Newport (4), SE (3) and *S*. ser. Rissen (1). *S*. ser. Lille was isolated from two backyard chickens from the same family farm, while *S*. ser. Newport was isolated from two birds from different family farm. Furthermore, two different serotypes were isolated from one chicken in two cases (*S*. Lille-*S*. Rissen and *S*. Newport-SE). In reference to geese samples, *Salmonella* spp. was not isolated from them. On the other hand, *Salmonella* isolated strains revealed the same resistance pattern. All strains were susceptible to all antibiotics tested with the exception of erythromycin.

3.2 | Performance of two selective–differential plating media used in a sample for *Salmonella* isolation

Overall, four and three samples yield *Salmonella* spp. on HEA and BG agar, respectively. Because of the absence of false-positive samples, the RSp and RPPV were 1 for HEA and BG agar. There were not any statistical difference between HEA and BG agar in the RSe and the RNPV. The RAc was 1 for both selective agar plating. The RSe and RNPV was 1 (.52–1.00) for HEA. On the other hand, the RSe and RNPV were .71 (.26–.95) and 1.00 (.98–1.00) for BG agar, respectively. The agreement (kappa coefficient) was very good (.83) between HEA and BG agar, and McNemar's test showed that the difference between the two plating media was not statistically significant, with the two-tailed *p*-value of .4795.

3.3 | Association of farm characteristics with *Salmonella* spp. in backyard chicken in Entre Ríos, Argentina

In relation to 51 family farms studied, 23.5% (12/51) of them use mains water. The remaining 76.5% (39/51) uses ground water and from this per cent, 15% (6/39) usually disinfects it and makes microbiological analysis, 8% (3/39) does not disinfect it, but makes analysis and 77% (30/39) neither disinfects or makes analysis. Backyard chicken feeding includes feeds (44/51), kitchen waste (4/51) and homemade feeds (3/51). The disposal of mortalities makes by incineration (28/51), using as food for animals (3/51), laying far from the farm (6/51), using in a compost system (9/51) and buried (5/51). In regard to the proximity of a commercial poultry enterprise, 52.9% (27/51) of backyard farms complies the national minimum required distance (1,000 m), while 47.1% (24/51) does not, and within this per cent, 33.3% (8/24) has both production systems in the same place. In respect of the proximity of a pig farm, 68.6% (35/51) of the farms complies the minimum state required distance (1,000 m), while the remaining 31.4% (16/51) does not, and within this per cent, 60%

TABLE 2 Distribution of serovars of *Salmonella* isolated in *Salmonella*-positive backyard chicken farms in Entre Rios, Argentina

Salmonella serovars	No. of *Salmonella*-positive cloacal swab samples[a]	No. of *Salmonella*-positive family farms
Lille	2	1
Newport	2	2
Rissen	1	1
Enteritidis	1	1

[a]Two different serotypes were isolated from the same bird in two backyard chickens.

(9/15) has backyard poultry and pigs in the same area. None of the farmers has a written biosecurity plan, and only 9.8% (5/51) has veterinary assistance. The 74.5% (38/51) of farmers makes rodent control, whereas none of them makes fly control. Finally, none of the farmers had reported *Salmonella* outbreaks. The results of the univariate analysis revealed that none of the variables studied could be selected to calculate the risk factors associated with *Salmonella* isolation because *p* > .15 (Table 3).

4 | DISCUSSION

In the present study, the apparent prevalence of *Salmonella* in backyard chickens was 0.6%. The application of rodent control, the use of industrial food (with some microbiological control) and the distance (more than 1,000 m) from a pig and intensive poultry farms in most of backyard chicken farms sampled could help to decrease *Salmonella* sp. contamination in these animals. Although it was observed that the care and health of the birds was limited, coinciding with extensive breeding systems in Vietnam (Burgos, Hong Hanh, Roland-Holst, & Burgos, 2007), the low prevalence of our study made it difficult to find *Salmonella* spp. risk factors. Isolation rates for *Salmonella* sp., using the same sample, were 5.8% in Iran (Jafari, Ghorbanpour, & Jaideri, 2007), 3.5% in Paraguay (Leotta et al., 2010), 4% in Morocco (Bouzoubaa, Lemainguer, & Bell, 1992), 0.02% in Australia (Manning et al., 2015) and 15% in West Bengal, India (Samanta et al., 2014). The difference between data could be attributed to the presence of different dominant serovars of *Salmonella* in each of the countries, and hence different rates of infection and shedding, differences in the general standard of health of the chickens in each country and biosecurity practices (Manning et al., 2015). It is known that cloacal swabs can provide sensitive indicators of persistent intestinal colonization in individual bird, but their diagnostic reliability is diminished by the intermittent shedding of salmonellae in the faeces of infected birds (Gast, 2013).

Reports of the frequency of isolation of *Salmonella* serovars from poultry sources around the world have yielded a wide range of results, although several serovars appear to be of continuing international significance. The unique epidemiologic association of *S*. ser Enteritidis with disease transmission via contaminated eggs has made the prevalence of this serovar a topic of particular interest (Gast, Guraya, &

TABLE 3 Results from univariate analysis for risk factors identification of *Salmonella* sp. The analysis describes the data from the 51 family farms of backyard chickens in Entre Rios, Argentina

Variable	n	% of positive family farm for *Salmonella* sp.	*p*-Value
Source of water			
Well	39	66.7	.68
Mains	12	33.3	
Feeding			
Commercial balanced feed	44	66.7	.31
Leftovers from human consumption	4	0	
Homemade feed	3	33.3	
Carcass disposal			
Incinerates dead birds	28	66.7	.67
Uses as food for other animals	3	0	
Throws it away from the farm area	6	0	
Compost system	9	33.3	
Buries	5	0	
Distance to poultry house (enterprise)			
>1,000 m	27	66.7	.57
≤1,000 m	24	33.3	
Distance to pig farm			
>1,000 m	35	66.7	.94
≤1,000 m	16	33.3	
Veterinary assistance			
Yes	5	0	.56
No	46	100	
Other animal species (not poultry)			
Yes	51	100	1.00
No	0	0	
Rodent control			
Yes	38	66.7	.81
No	13	33.3	
Fly control			
Yes	0	0	1.00
No	51	100	
Salmonella background			
Yes	0	0	1.00
No	51	100	

Guard, 2013). Different authors reported that cloacal swabs were not the best sample for detection of *S.* ser Enteritidis carriers in commercial layer farms (García, Soriano, Benítez, & Catalá-Gregori, 2011; Mutalib, McDonough, Shin, Patten, & Lein, 1992). In our study, this serovar was only isolated from one sample, indicating that this serovar is present, but with low incidence.

In our study, *Salmonella* strains were sensitive to 18 antibiotics tested, and only resistant to erythromycin, a macrolide antibiotic. Other authors reported a high resistant of *Salmonella* strains, isolated from poultry farms or clinical and environmental samples, to this antibiotic (El-Tayeb, Ibrahim, Al-Salamah, Almaary, & Elbadawi, 2017; Phagoo & Neetoo, 2015; Thung et al., 2016). Although the number of *Salmonella* strains isolated was low in our study and the resistant to this antibiotic could be due to improper usage or overuse, it is known that Gram-negative bacilli, as *Salmonella* sp., are usually intrinsically resistant to macrolide antibiotics (Chambers, 2006; Nakajima, 1999).

The objective of *Salmonella* spp. isolation in selective and differential plating media is to differentiate and separate the selected or target microorganism from the competitive microflora. It is recommended that samples should always be streaked onto two different media, preferably with dissimilar indicator systems for differentiating salmonellae from other organisms. This strategy decreases the number of the false-negative results, although with a little extra cost (Gast, 2013; Petersen, 1997). In our study, we used two selective and differential plating media: BG agar and HEA. Velma, Goo, Ching, and Gooch (1973) indicated that BG agar is more effective than HE agar in isolating salmonellae from food products. The selectivity of the first medium derives from the presence of brilliant green dye, lactose and sucrose, which are the basis for the differential capabilities of the media. Almost all *Salmonella* fail to ferment either lactose or sucrose and their colonies appear either pink to red, with reddening of the media (Waltman, 2000). On the other hand, the HE agar uses bile salts for selective inhibition and two indicator systems: (i) bromothymol blue and acid fuchsine as indicators of carbohydrate dissimilation (involve the sugars lactose, sucrose and salicin), and (ii) ferric iron as an indicator of the formation of hydrogen sulphide from thiosulphate. Most *Salmonella* do not ferment the three sugars, but produce H_2S, which results in bluish-green colonies with black centre (Van der Zee, 2003; Waltman, 2000). Although BG agar and HE agar use dissimilar indicator systems for differentiating salmonellae from other organisms, we did not find any difference in *Salmonella* isolation in both media and the agreement was very good between them without any statistical difference.

Temelli, Eyigor, and Carli (2010) evaluated the *Salmonella* serogroup detection in poultry meat samples by examining multiple colonies from selective plates following the FDA/BAM and ISO 6579 methods. They founded multiple serogroups (D and E4; B and C2; C1; G; and E1 and F) and concluded that a selective plate for *Salmonella* culture method can harbour more than one serogroup. In our study, two different serovars were isolated from one chicken in two cases (*S.* ser. Lille-*S.* ser. Rissen and *S.* ser. Newport-SE).

Although the prevalence of *Salmonella* spp. is low in backyard birds in Entre Rios with the isolation technique used, it should not be discounted, as *S.* ser. Enteritidis isolates were found only in the Uruguay County, which concentrates a large population of intensive poultry production in the state. On the other hand, because all *Salmonella* strains were only resistant to erythromycin, it is inadvisable to use this antibiotic to treat salmonellosis. Finally, the performance of HEA and BG agar is very similar for cloacal swab samples, so the combination of these two media for this kind of sample does not decrease the number of the false-negative results.

CONFLICTS OF INTEREST

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