Prevalence, antimicrobial resistance profile and comparison of selective plating media for the isolation of *Salmonella* spp. in free-ranging waterfowl from Entre Rios, Argentina

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ABSTRACT The present study was conducted to estimate the apparent prevalence of *Salmonella* spp. in free-ranging waterfowl that inhabitant Entre Ríos, Argentina, determine the antimicrobial resistance of the isolated, and compare the performance of two selective plating media used for *Salmonella* isolation. Five hundred ninety nine free-living waterfowl were sampled one time by cloacal swab from April 2014 to July 2016. Only 6 samples from waterfowl belonged to all counties sampled were positive to *Salmonella* spp., so the apparent prevalence was 1%. Four serovars were isolated (*Salmonella* ser. Typhimurium, *S.* ser. Schwarzengrund, *S. enterica* subsp. I [4,12: i: -], *S. enterica* subsp. IIIb [60: r: e, n, x, z_{15}]), which were susceptible to 15 antibiotics tested and resistant to erythromycin. Furthermore, some strains showed an intermediate resistant to neomycin, ciprofloxacin and/or streptomycin. The multiple antibiotic resistances index was 0.05. For Hektoen enteric agar and Salmonella Shigella agar, the relative accuracy, sensitivity, specificity, positive predictive value, and negative predictive value did not show any difference between them. The agreement was good between these two plating-media and the difference between these plating-media was not statistically significant. The low prevalence of *Salmonella* spp. in waterfowl in Entre Rios should not be discounted, since *Salmonella* ser. Typhimurium was the most prevalent serovar and some free-ranging waterfowl species studied can migrate from/to different countries, increasing the possibility to cross-contaminated *Salmonella* to resident or other migrant birds.

Key words: *Salmonella;* plating media, waterfowl, antibiotic, Argentina

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INTRODUCTION

Argentina is the second country in Neotropical extension and it has great wetland varieties like seacoasts, estuaries, andean-patagonian and pampas lakes, flooded depressions, marshes, plain rivers and streams, and saline lakes (Cabrera, 1976; Canevari et al., 1998). This permits the development of an important aquatic environmental variety, which shelter near 250 resident and migrant waterfowl species. That represents 25% from the world waterfowl (Delany & Scott, 2006) and a similar percentage from total bird species in Argentina (Narosky & Izurieta, 2003).

Entre Rios is a northeastern province of Argentina, located in the Mesopotamia region. It is limited and traversed by many rivers, which transform the province into an idyllic green island. The weather varies from subtropical in the north to temperate in the south and the annual rainfall is about 1150 mm in average. These conditions permit that many waterfowl and other wild birds live in this province (De Chemin et al., 1992; Raffo et al., 2009). Furthermore, Entre Ríos is a state in Argentina where poultry production is so concentrated

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that, from an epidemiological point of view, it is similar to a very large multiage farm. It has almost 3355 poultry houses, which correspond to 43% of the poultry houses from Argentina (Livestock, Pigs and Poultry Division, 2014).

Waterfowl and other migratory birds are unique among wildlife in their potential to carry zoonotic pathogens across a widespread geographic area (Acha & Szyfres, 2001). *Salmonella enterica* is a diverse bacterial species, currently divided into 6 subspecies and more than 2400 serotypes; certain serotypes can be important pathogens in humans and animals, with varying levels of host specificity (Singer, Mayer, Hanson & Isaacson, 2009; Issenhuth-Jeanjean et al., 2014). Although different antibacterial agents can be used in wild birds to threat bacterial infections (Flinchum, 2006), there are few studies of *Salmonella* susceptibility to different anti-microbial agents in these birds (Fresno et al., 2013; Grigar, et al., 2017).

Cloacal swabs or samples of voided feces have been used to provide evidence of persistent intestinal colonization by salmonellae in individual birds (Gast, 2013). Although numerous agar media are available for the isolation of salmonellae, the process of isolating *Salmonella* spp. 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 (Busse, 1995; Gast, 2013; Soria and Bueno, 2016).

Although the National Poultry Health Plan in Argentina includes some *Salmonella* serovars in the control plan for breeders, broilers and hens (National Agrifood Health and Quality Service, 2015, 2016), and it uses culture methods in the laboratory procedure, waterfowl are not usually monitored for diseases or vaccinated. These birds are useful tools as indicators of the conservation status and health of wetland habitats (Morrison, 1986; Kushlan 1993) and also an important part of our natural heritage and a renewable resource utilized for research, education, and recreation as well as a food resource (Blanco et al., 2001). Therefore, the present study was conducted to: (1) estimate the apparent prevalence of *Salmonella* in cloacal swab samples of free-ranging waterfowl in Entre Rios, Argentina, (2) determinate the antibiotic resistance profile of the isolated, and (3) compare the performance of two differential plating media used in a sample for *Salmonella* isolation.

MATERIALS AND METHODS

Study design

A total of 599 free-ranging waterfowl were sampled one time by cloacal swab from hunter-harvested birds, collected from hunting lodges, during hunting season (May-November and February-April) from April 2014 to July 2016, in accordance with local hunting laws (Entre Ríos Government, 1969; Flora and Fauna Directorate, 1991). These lodges were located in four counties (Gualeguaychu, Gualeguay, Uruguay and Victoria) of Entre Rios, Argentina. Species, sex and age group were recorded for each bird sampled. The species belong to four families (Table 1): Anatidae (594 birds), Ardeidae (2 birds), Threskiornithidae (2 birds), and Recurvidostridae (1 bird). Among all sampled waterfowl, 298 (49.75%) were male and 301 (50.25%) were female. A total of 368 (61.44%) were recorded as adults and 231 (38.56%) as juveniles.

One cloacal swab was taken from each bird within the same day of harvest and collocated in a sterile tube. Samples were labeled and transferred to the INTA Laboratory of Poultry Health (Concepción del Uruguay, Entre Ríos) in ice chests for *Salmonella* isolation. The number of cloacal swabs was based on the following equation (Mateu and Casal, 2003), with an expected prevalence of 50% and a precision of 5.2% with 99% confidence limit:

$n = Z^2\ p\ q/B^2$, where:
n = sample size
Z = 2.56 for 99% confidence limit
p = disease expected frequency (0.5)
q = 1 – p
B = precision

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; 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 h at 35 ± 2°C, a loopful of broth was streaked on Hektoen enteric agar (HEA, Britania, Buenos Aires, Argentina) and Salmonella Shigella agar (SSA, Merck, Darmstadt, Germany), and incubated at 35 ± 2°C for 18–24 h. 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, phenylalanine agar (Hi-Media, Mumbai, India), and urea agar (Britania, Buenos Aires, Argentina). If there were no bacterial colonies compatible with *Salmonella* sp. in a selective-differential agar plate, two atypical *Salmonella* sp. colonies were also taken and the same biochemical tests, as mentioned before, were done. All *Salmonella* isolations were preserved on nutritive (Merck) slants agar until serotyping, which was carried out according to the White-Kauffmann-Le Minor Scheme, using

Table 1. Number and species of free-ranging waterfowl studied in different counties from Entre Ríos, Argentina, from April 2014 to July 2016.

Waterfowl		N° of bird sampled/county of Entre Rios				Total
Family	Name	Gualeguaychu	Uruguay	Gualeguay	Victoria	of birds
Anatidae	Rosy-billed Pochard (*Netta peposaca*)	0	0	10	227	237
	Brazilian Duck (*Amazonetta brasiliensis*)	24	60	15	123	222
	Speckled Teal (*Anas flavirostris*)	11	33	3	11	58
	Fulvous Whistling-Duck (*Dendrocygna bicolor*)	0	0	0	27	27
	Silver Teal (*Anas versicolor*)	1	0	3	11	15
	White-faced Whistling-Duck (*Dendrocygna viduata*)	8	5	0	0	13
	Ringed Teal (*Callonetta leucophrys*)	3	0	3	2	8
	Yellow-billed Pintail (*Anas georgica*)	0	0	2	5	7
	Muscovy Duck (*Cairina moschata*)	5	0	0	0	5
	Domestic goose (*Anseranser domesticus*)	2	0	0	0	2
Ardeidae	Great Egret (*Egretta alba*)	2	0	0	0	2
Threskiornithidae	Buff-necked Ibis (*Theristicus caudatus*)	2	0	0	0	2
Recurvidostridae	South American Stilt (*Himantopus melanurus*)	1	0	0	0	1
Total		59	98	36	406	599

somatic (AgO) and flagellar (AgH) antigens (Grimont and Weill, 2007).

Antibiotic susceptibility test

The antibiotic susceptibility test was performed by the standard disk diffusion method in Mueller-Hinton agar (Difco™, Sparks, USA) and the results were interpreted in accordance with the criteria of the Clinical and Laboratory Standards Institute (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); suphamethoxazole/trimethoprim (25 μg); doxycycline (30 μg); neomycin (30 μg); cephalothin (30 μg); norfloxacin (10 μg); ampicillin (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 disks, 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.

Multiple antibiotic resistances (MARs) index for each resistance pattern was calculated by employing the formula given below. Isolates classified as intermediate on the basis of inhibition zone were considered as sensitive for MAR index (Singh et al., 2010).

MAR index = Number of resistance antibiotics/total number of antibiotics tested

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 SSA, used for *Salmonella* spp. isolation from waterfowl cloacal swab samples, were analyzed according to Soria et al. (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 & 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. On the other hand, McNemar's test was calculated using a chi-square approximation at $P \leq 0.05$ (GraphPad Software, 2018).

RESULTS

Apparent prevalence of Salmonella spp. in free-ranging waterfowl in Entre Ríos, Argentina, and antibiotic resistance profile of the isolated

Out of 599 samples of free waterfowl, only 6 samples, belonged to 3 families (Anatidae, Ardeidae, and Threskiornithidae) and 5 specie birds, were positive to *Salmonella* spp. So the apparent prevalence was 1.0%. This bacteria was isolated from one Red-billed Pochards (*Netta peposaca*), two Brazilian duck (*Amazonetta brasiliensis*), one Speckled teal (*Anas flavirostris*), one Great white heron (*Egretta alba*) and one Buff-necked ibis (*Theristicus caudatus*), which was sampled in Gualeguaychu (Great Egret and Brazilian duck), Gualeguay (Brazilian duck), Uruguay (Buff-necked ibis), and Victoria (Speckled Teal, and Red-billed Pochards). Nineteen *Salmonella* strains were isolated from those positive samples and were typified into 4 serovars: *Salmonella* ser. Typhimurium (16 strains), *S.* ser. Schwarzengrund (1 strain), *S. enterica* subsp. I (4,5,12: i: -; 1 strain), *S. enterica* subsp. IIIb

Table 2. Antimicrobial susceptibility patterns to neomycin, ciprofloxacin and streptomycin of *Salmonella* spp. isolated from free-ranging waterfowl of Entre Rios, Argentina. Susceptible (S), Intermediate (I) and Resistant (R).

Waterfowl	*Salmonella* serovars (N° strains)	Antimicrobial susceptibility patterns		
		Neomycin	Ciprofloxacin	Streptomycin
Great Egret (*Egretta alba*)	Typhimurium (4)	S	S	S
Speckled Teal (*Anas flavirostris*)	Typhimurium (4)	S	S	I
Buff-necked ibis (*Theristicus caudatus*)	Typhimurium (3)	I	I	I
	S. enterica subsp. I (4,5,12:i:-)(1)	S	S	S
Brazilian duck (*Amazonetta brasiliensis*) 1	Typhimurium (4)	S	S	S
Brazilian duck (*Amazonetta brasiliensis*) 2	*S. enterica* subsp. IIIb(60:r:e,n,x,z_{15}) (1)	S	I	I
Red-billed Pochards (*Netta peposaca*)	Typhimurium (1)	S	S	S
	Schwarzengrund (1)	S	S	S

(60: r: e, n, x, z_{15}; 1 strain). Furthermore, two different serovars were isolated from one bird in two cases: *S.* ser Typhimurium-*S. enterica* subsp. I (4,5,12: i: -), and *S.* ser. Typhimurium- *S.* ser. Schwarzengrund (Table 2).

On the other hand, *Salmonella* isolated strains revealed different resistance pattern. However, all strains were sensible to fosfomycin, colistin, tetracycline, florfenicol, enrofloxacin, gentamicin, suphamethoxazole/trimethoprim, doxycycline, cephalothin, norfloxacin, ampicillin, kanamycin, chloramphenicol, cefotaxime, and fosfomycin/tylosin and they were resistant to erythromycin. Three *S.* ser. Typhimurium strains from Buff-necked ibis showed an intermediate resistant to neomycin, ciprofloxacin and streptomycin, and four *S.* ser. Typhimurium strains from Speckled Teal were only intermediate to streptomycin. On the other hand, *S. enterica* subsp. IIIb (60: r: e, n, x, z_{15}) strain showed an intermediate resistance to streptomycin and ciprofloxacin (Table 2). The MAR index of all *Salmonella* spp. strains was 0.053.

Performance of two selective-differential plating media used in a sample for Salmonella isolation

Overall, 6 and 4 samples yield *Salmonella* spp. on HEA and SSA, respectively. Because of the absence of false positive samples, the RSp, and RPPV were 1 for both selective differential agar media. Furthermore, there were not any statistical difference between HEA and SSA in the RAc, RNPV, and RSe. The RAc and RNPV were 1 for both selective agar plating. The RSe was 1 (0.59–1.00) and 0.67 (0.32–0.92) for HEA and SSA, respectively. The agreement (Kappa coefficient) was good (0.75) between HEA and SSA, and Mc Nemar's test showed that the difference between the two plating-media was not statistically significant, with the two-tailed P value of 0.4795.

DISCUSSION

In the present study, the apparent prevalence of *Salmonella* in free-ranging waterfowl was 1%. Reports of the frequency of isolation of *Salmonella* serovars from waterfowl sources around the world have yielded a wide range of results. Isolation rates for *Salmonella* spp., using the same sample, was 8.7% (23/264) in ring-billed gulls taken at four sites near Montreal, Canada (Quessy and Messier, 1992), 8% (16/198) in Poland's waterfowl (great cormorant and mallard duck) (Krawiec et al., 2015), 6% (46/758) in Chile's waterfowl along 2000 km of the Chilean coast (Fresno et al., 2013), 6% in water captive wildlife birds at the Emperor Valley Zoo, Trinidad (Gopee et al., 2000), 0.5% (2/375) in waterfowl along the Texas Gulf coast (Grigar et al., 2017), 0.2% in waterfowl from metropolitan parks in central Ohio (Fallacara et al., 2001), and 0% (n = 318) during the 2008–2010 waterfowl hunting seasons in Spain (Antilles et al., 2015). On the other hand, investigators were unable to culture *Salmonella* from 331 samples collected from ducks in Oklahoma, United States (Waldrup and Kocan, 1985). However, 1.5% and 3.0% of serum samples collected were reactive to *Salmonella* serogroup B and D antisera, respectively. 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 feces of infected birds (Gast, 2013).

It is known that waterfowl living in contaminated waters may become infected with *Salmonella*, especially if sewage contamination has occurred (Clegg and Hunt, 1975). Although we did not study the presence of this bacteria in the water where the waterfowl lived, several serovars appear to be of continuing international significance. *Salmonella* ser. Typhimurium identified in our study, is the top serovar isolated from human patients with laboratory-confirmed salmonellosis in Argentina (Torres et al., 2016). Furthermore, *S. enterica* subsp. I (4,5,12: i: -) is a serotype that appears to be antigenically similar and genetically closely related to *Salmonella* ser. Typhimurium (which has the antigenic formula 4,5,12: i:1,2), but lacks expression of the second phase flagellar antigen, which is 1,2 in *Salmonella* ser. Typhimurium. The prevalence of this serotype among human salmonellosis cases has increased considerably since the mid-1990s and Salmonella 4,5,12: i:—currently (i.e., the first decade of the 2000s) represents one of the most common serotypes among human cases in

many countries around the world (Moreno Switt et al., 2009). On the other hand, although Entre Rios has an important number of the poultry houses from Argentina, *Salmonella* ser. Typhimurium could not be isolated from environmental samples (feces, feed, drinking water, air, boot-swabs) and eggs, taken from 40 layer hen houses in this state (Soria et al., 2017). In addition, it is known that some of the free-ranging waterfowl species sampled in our study can migrate to different states of Argentina, and countries (Olrog, 1968; Capllonch et al., 2008; Olmos, 2015), so this increase the possibility to cross-contaminated *Salmonella* or other microorganisms to resident or other migrant birds.

In our study, *Salmonella* strains were sensitive to 15 antibiotics tested, and only resistant to erythromycin (MAR index very low). Furthermore, some strains showed an intermediate resistant to neomycin, ciprofloxacin and/or streptomycin. It is known that Gram-negative bacilli, as *Salmonella* sp., are usually intrinsically resistant to erythromycin, a macrolide antibiotic (Nakajima, 1999; Chambers, 2006). Krawiec et al. (2017) reported that, among the 36 examined *Salmonella* spp., isolated from free-living birds, twenty were resistant to more than one antimicrobial agent, while most strains (94.5%) were resistant to sulfamethoxazol, using commercial Sensititre™ *Salmonella.* In the present study, we used another technique to study the antimicrobial susceptibility of the strains and we found that no resistance was detected in relation to suphamethoxazole/trimethoprim. On the other hand, Fresno et al. (2013) revealed almost all isolates found from Chile's waterfowl were resistant to at least one antimicrobial, with most of them resistant to tetracycline. Notably, 21 strains, most of which belonged to the serovar Enteritidis, had multidrug-resistant. Although these authors and Grigar et al. (2017) did not isolate S. ser. Typhimurium from waterfowl and they did not use erythromycin to test antibiotic susceptibility, we were unable to culture *Salmonella* ser. Enteritidis from samples collected and all *Salmonella* isolated strains were sensitive to tetracycline in our study.

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 (Petersen, 1997; Gast, 2013). SSA and HEA belong to the group of bile salt media, but they use dissimilar indicator systems for differentiating salmonellae from other organisms (Busse, 1995; Soria and Bueno, 2016). However, the agreement was good between them without any statistical difference. Poisson et al. (1992) did not find statistically significant in the isolation of non-Typhi *Salmonella* strains, when compared HEA and SSA on stools of human origin submitted for routine isolation of *Salmonella* sp. after direct streaking and overnight enrichment in Mueller-Kauffmann broth. N**evertheless**, Ruiz et al. (1996) found that the number of *Salmonella* isolates was significant higher in SSA than in HEA from human stools after direct streaking.

Although the prevalence of *Salmonella* spp. is low in waterfowl from Entre Rios with the isolation technique used, it should not be discounted, since *S.* ser Typhimurium was the most prevalent serovars and some free-ranging waterfowl species studied can migrate from/to different countries, increasing the possibility to cross-contaminated *Salmonella* to resident or other migrant birds. This serovar is in the top from human patients with laboratory-confirmed in Argentina, and Entre Rios has an important population of intensive poultry production, which can be affected by waterfowl as reservoirs. On the other hand, due to all *Salmonella* strains were only resistant to erythromycin; multi-resistance is not a problem in these strains until now. Finally, the performance of HEA and SSA is similar for cloacal swab samples taken from waterfowl, so the combination of these 2 media for this kind of sample does not decrease statistically the number of the false negative results.

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