A comparative study of culture methods and PCR assay for *Salmonella* detection in poultry drinking water

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ABSTRACT The present work compared 2 culture methods and PCR assays for motile and nonmotile *Salmonella* detection using artificially contaminated poultry drinking water. The specificity was 1 for all methods studied. The accuracy and sensitivity were 1 for all motile strains, whereas these parameters were between 0 and 0.7 for nonmotile *Salmonella* strains. The positive predictive value and negative predictive value were 1 for all motile *Salmonella* strains in the 3 methods used. Nonmotile S*almonella* strains showed a positive predictive value of 1 in the PCR method. However, the positive predictive value was indeterminate in the tetrathionate (TT) methods for both strains tested and in the modified semisolid Rappaport-Vassiliadis (MSRV) method for S*almonella* Pullorum. On the other hand, the negative predictive value was between 0.20 and 0.43 for the 3 methods. The detection level of motile strains was 4 to 7 cfu/25 mL for all methods. Nonmotile *Salmonella* strains could not be detected in the TT method, whereas only *Salmonella* Gallinarum could be recovered from 1.1×10^1 cfu/25 mL in the MSRV method. In relation to the molecular methods, PCR could detect these strains from 1.1×10^4 cfu/25 mL. Extending incubation time of the enrichment medium to 6 d in the TT method did not improve the isolation rates. In general, all selective plating media did not show any statistical differences in the parameters of performance studied. The kappa coefficient showed that there was an excellent agreement between the 3 methods for motile strains. For nonmotile strains, the agreement was poor between the MSRV and the PCR; there was no agreement when the TT method was compared with the MSRV and the PCR methods. The difference in detection levels obtained with the methods used for motile and nonmotile *Salmonella* strains and the difficulty in detecting these last strains represents a potential problem when a poultry water sample is considered negative for the presence of *Salmonella*.

Key words: *Salmonella*, water, culture method, polymerase chain reaction

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INTRODUCTION

Salmonella enterica is a diverse bacterial species that is currently divided into 6 subspecies and more than 2,400 serotypes. Avian *Salmonella* infections are important as both a cause of clinical disease in poultry and as a source of foodborne transmission of disease to humans. Host-adapted salmonellae are responsible for pullorum disease (*Salmonella enterica* serovar Gallinarum biovar Pullorum) and fowl typhoid (*Salmonella enterica* serovar Gallinarum biovar Gallinarum; Waltman and Gast, 2008). This avian-adapted serotype (nonmotile) lack flagella and associated motility (Guard-Petter, 2001). Other serotypes with no specific host, such as Typhimurium and Enteritidis, may infect chickens and persist in the final poultry product, inducing or not inducing clinical disease during rearing. Thus, the control of *Salmonella* in poultry flocks is crucial for the success of the poultry industry (Gama et al., 2003).

Salmonella is introduced in poultry farms by several ways, including day-old infected chicks, domestic animals, human, equipment, water, and feed (Shivaprasad, 2003; Gast, 2008). Once the farm is contaminated, it is very difficult to eliminate *Salmonella* from the environment (Gama et al., 2003). At normal temperatures, poultry consume at least twice the amount of water to feed. When heat stress occurs, water consumption will double or quadruple (Amaral, 2005). Although the survival of *Salmonella* in water is a function of interacting biological and physical factors (Rhodes and Kator, 1988), these bacteria do not seem to multiply significantly in the natural environment. However, they can survive several weeks in water and in soil if conditions of temperature, humidity, and pH are favorable (Cabral, 2010). Because of that, the use of consumption

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water with high microbiological qualities is fundamental in poultry production, considering that many birds have continuous access to the water source (Carter and Sneed, 1996). Furthermore, control measures must be considered a priority to prevent the occurrence of diseases that are spread through water and would certainly result in great economic losses (Amaral, 2005).

The presence of *Salmonella* in water is very variable. There are several procedures for *Salmonella* isolation in that source (Rice et al., 2012), but the standard methods for detecting *Salmonella* generally analyze food or fecal samples (Hsu et al., 2011). Usually, the techniques for isolating and identifying *Salmonella* rely on preenrichment in nonselective media, selective enrichment, plating in selective and diferential media, and biochemical and serological identification. The sensitivity and specificity of the method depends on the sample type as well as the isolation conditions (Rybolt et al., 2004). On the other hand, a wide range of culture methods and 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). Furthermore, Knight et al. (1990) indicated that *Salmonella* spp. were not often detected in water samples by culture methods, even when they are present in significant numbers. So, molecular study can be an interesting tool to improve the detection of this bacteria. Based on the usual procedures for the detection of *Salmonella* and in view of the lacked standardized methods for it detection in water, the present work was conducted to compare 2 culture methods and combinations of PCR with them, to know their ability to detect motile and nonmotile *Salmonella* strains in artificially contaminated poultry water samples. Furthermore, the accuracy (**Ac**), sensitivity (**Sen**), specificity (**Sp**), positive predictive value (**PPV**), and negative predictive value (**NPV**) of each method and the agreement among methods were investigated.

MATERIALS AND METHODS

Poultry Drinking Water Samples

Poultry drinking water samples were provided by a broiler farm from the state of Entre Ríos, Argentina. To take the samples, water runs for about 3 to 5 min before sampling. The outside part of the faucet was cleaned under a flame, and water was taken in a sterile bottle. The samples were labeled and transferred to the Poultry Health Laboratory of the Agricultural Experimental Station (**EEA**) of National Institute of Agricultural Technology (**INTA**) Concepción del Uruguay (Entre Ríos, Argentina) within 1 h of being taken; they were immediately processed for the assays. Water from this farm was checked previously for the absence of *Salmonella* spp. by the 2 bacteriological methods described below. Furthermore, free and total chlorine was measured in each sample of water with a chlorine test (Aquamerck, Merck, Darmstadt, Germany).

Salmonella Strains and Culture

A total of 6 *Salmonella* strains were selected for the assay. The strains belong to American Type Culture Collection (**ATCC**) and the collection from the Poultry Health Laboratory of the EEA INTA Concepción del Uruguay. Four of them were motile *Salmonella*: *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium ATCC 13311, *Salmonella* Kentucky CUB 19/08 (soy expeller), and *Salmonella* Infantis CUB 08/08 (chicken). The others were nonmotile *Salmonella*: *Salmonella* Gallinarum CUB 55/10 (chicken) and *Salmonella* Pullorum ATCC 13036. Each *Salmonella* strain was activated from Nutrient Agar (Acumedia, Lansing, MI) and was grown for 24 h in tryptic soy broth (Merck) at 37°C. Purity of the cultures was confirmed by streaking onto MacConkey agar (Acumedia) and tryptic soy agar (Acumedia). The number of viable microorganisms was estimated by the method of Miles et al. (1938) and was expressed as cfu/mL. Cells were pelleted by centrifugation in a tabletop centrifuge at $302 \times g$ for 15 min at room temperature (25 ± 2°C). The supernatant was discarded and the pellet cell was resuspended to the original volume (5 mL) with PBS (pH 7.4).

Preparation of Salmonella spp. Inocula in Poultry Drinking Water Samples

Twenty-five mL of *Salmonella*-free poultry drinking water sample were introduced into a sterile plastic bag. Each *Salmonella* strain was grown as described above. After that, serial dilutions were made in peptone water (0.1%) to inoculate 4 bacterial concentrations, between 4.0×10^0 and 6.6×10^3 cfu/25 mL for motile *Salmonella* strains, and between 1.1×10^1 and 1.2×10^4 cfu/25 mL for nonmotile *Salmonella* strains. All treatments were performed in triplicate, so 3 samples of each dose for each *Salmonella* strain were considered in the assays. Altogether 72 spiked samples were constructed in the study. For each trial set, 3 nonseeded samples were analyzed as the negative control.

Recovery of Salmonella spp. Strains from Poultry Drinking Water Samples

Figure 1 shows a flowchart diagram for detection of *Salmonella* in water by the tetrathionate (**TT**) and modified semisolid Rappaport-Vassiliadis (**MSRV**) methods. *Salmonella*-free poultry water contaminated with different concentration of *Salmonella* strains was preenriched in 225 mL of buffered peptone water (Merck) in a double concentration (**BPWdc**). The mixture was incubated at 35 ± 2°C for 1 d. One milliliter of incubated broth was transferred to 10 mL of TT broth

base (Acumedia) in addition to 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), and incubated at 35 ± 2°C for 6 d (TT method). At d 1 (TT first) and 6 (TT sixth), a loopful of TT broth was streaked on xylose lysine desoxicholate agar (Oxoid, Basingtoke, Hampshire, UK) with or without tergitol 4 (4.6 mL/L, Sigma, St. Louis, MO), and EF-18 (Acumedia) agar, and incubated at 35 ± 2°C for 1 d. On the other hand, 30 μL of incubated BPW culture were inoculated in MSRV medium (Acumedia) agar plates supplemented with 1mL/L of a 2% novobiocin solution, which were incubated at 42 ± 1°C for 1 d and subsequently streaked on the same selective media listed above (MSRV method). This last method was based on Draft Amendment 1 Annex D: detection of *Salmonella* spp. in animal feces and in samples from the primary production stage, which is suggested as a new addendum to ISO 6579 (ISO, 2002). Colonies of presumptive *Salmonella* were inoculated onto triple-sugar iron agar (Acumedia) and lysine iron agar (Merck). Further confirmation was done based on ortho-nitrophenyl-β-galactoside tests and agglutination reaction with somatic (**O**) polivalent antisera (Difco, Becton Dickinson, Sparks, MD).

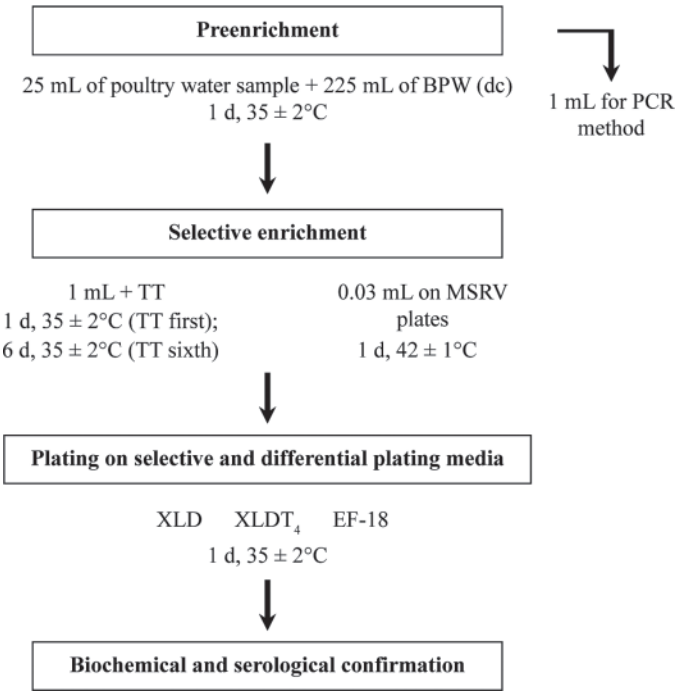

Figure 1. Flowchart diagram for detection of *Salmonella* in poultry water samples by tetrathionate broth (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods. BPW (dc) = buffered peptone water double concentration; TT first = d 1 of incubation in the TT method; TT sixth: d 6 of incubation in the TT method; XLD = xylose lysine desoxicholate agar; $XLDT_4$ = xylose lysine desoxicholate agar with tergitol 4; EF18 agar is from Acumedia (Lansing, MI).

Pre-PCR Sample Preparation

For detection of *Salmonella* from poultry drinking water samples, bacterial cells were recovered from 1 mL of BPWdc preenrichment broth (Figure 1) by centrifugation 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 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 fluid containing nucleic acids was fractionated in Eppendorf tubes and conservated at −70°C until it was used in subsequent PCR assays.

PCR Assay

The extracted DNA samples (5 μL) were amplified in an optimized 25-μL reaction mixture consisting of 0.25 μL of each 0.1 m*M* primer, 2.5 μL of buffer 1× (Fermentas Inc., Hanover, MD), 1.5 μL of 1.5 m*M* $MgCl_2$ (Fermentas), 0.5 μL of each 0.2 m*M* dNTP (Fermentas), 0.2 μL of 5 U/μL *Taq* DNA polymerase (Fermentas), and double-distilled water to 25 μL. The reaction mixture was incubated in a programmable DNA thermal cycler (model Mastercycler Gradient, Eppendorf, Hamburg, Germany). *Salmonella* genus-specific primers, 139 and 141 (Operon Biotechnologies GmbH, Köln, Germany), based on the *inv*A gene of *Salmonella* (Rahn et al., 1992), were used in the PCR assay. They have the following nucleotide sequences: (5′→3′) GTGAAATTATCGCCACGTTCGGGCAA (139) and TCATCGCACCGTCAAAGGAACC (141), respectively. A reagent blank containing all the components of the reaction mixture with the exception of template DNA (which was replaced by sterile distilled water) was included with every PCR assay. Negative and positive DNA controls were also included, which were prepared from *Citrobacter* sp. and *Salmonella* sp., respectively. Futhermore, 2 μL of an internal amplification control (**IAC**) was included, according to Malorny et al. (2003), and coamplified with each sample to indicate possible PCR inhibitors derived from the DNA sample. The cycling parameters used 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 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 was loaded onto 2.0% of agarose gel in 0.5 × Tris-borate-EDTA buffer at 120 V/cm for 1 h. The gel was stained with 0.5 μg/mL of ethidium bromide, and electrophoresed products were visualized with UV transilluminator (model M-20, UVP Inc., Upland, CA). A 100-bp ladder (PB-L Productos Bio-Lógicos, Buenos Aires, Argentina) was used as molecular weight marker.

Analysis of Performance Criteria

The detection limit of the methods was considered, and it was defined as the lowest concentration (cfu/25 mL) of the *Salmonella* strain inoculum that could be recovered. The Ac, Sen, Sp, PPV, and NPV were calculated for each method (Soria et al., 2011) The assumption was that all nonspiked samples were negative for *Salmonella* and only those samples spiked with *Salmonella* were true positive (**TP**). Samples being positive on at least 1 selective agar plate (xylose lysine desoxicholate, xylose lysine desoxicholate plus tergitol, or EF-18) were considered positive. Based on this, the Ac, Sen, Sp, PPV, and NPV rates were obtained by using the following definitions: a sample was defined as TP when *Salmonella* was detected in a sample where *Salmonella* had been added; a sample was defined as true negative (**TN**) when *Salmonella* was not detected in a sample where *Salmonella* had not been added; a sample was defined as false positive (**FP**) when *Salmonella* was detected in a sample where *Salmonella* had not been added; and a sample was defined as false negative (**FN**) when *Salmonella* was not detected in a sample where *Salmonella* had been added.

On the other hand, agreement between culture- and PCR-based methods for detection of *Salmonella* was evaluated by the use of the kappa coefficient (Martin, 1977). This was calculated to test how well the methods agreed in classifying the samples as positive or negative. The kappa statistic measured agreement between 2 tests that is beyond chance (Dawson and Trapp, 2004). Kappa coefficients were summarized as excellent agreement (0.93 to 1.00), very good agreement (0.81 to 0.92), good agreement (0.61 to 0.80), fair agreement (0.41 to 0.60), slight agreement (0.21 to 0.40), poor agreement (0.01 to 0.20), and no agreement (<0.01).

Statistical Analysis

The number of 15 samples for each assay was determinate according to Flahault et al. (2005). On the other hand, to compare the results of all assays, a hypothesis test for a difference of proportions was made. The Sen, Ac, PPV, and NPV of the test were reported at the shortest confident intervals, under the assumption that all values are equally probable. The calculations were performed using Octave Program, developed by the Group of Numerical Method, from the National Technological University of Concepcion del Uruguay (Entre Rios, Argentina, Projects 25D041). The values reported defines the boundaries of an interval that, with 95% certainty, contains the true value of Ac, Sen, PPV, or NPV. The results were only considered to be statistically different at $P < 0.05$. In relation to agreement, the 3 methods were treated as raters, and the Z test was used to test the statistical significance of kappa coefficients.

RESULTS

When the water was checked prior to assays, bacteria different from *Salmonella* sp. were isolated, such as coliforms, *Citrobacter* sp., and *Pseudomonas* sp. All samples showed free and total chlorine values less than 0.1 and 0.1 mg/L, respectively. In relation to the performance of the methods, the Sp was 1 for all methods studied (data not shown). The rest of the parameters showed significant differences between motile and nonmotile *Salmonella* strains. The Sen, Ac, PPV, and NPV were 1 for all motile strains in the methods tested (data not shown).

In reference to nonmotile strains (Table 1), Sen was 0 in TT method and less than 0.68 in the other methods. The Ac was less than 0.74 in these strains. The Sen and Ac were only higher in the MSRV method than in the TT and the PCR methods for *Salmonella* Gallinarum strain. On the other hand, nonmotile S*almonella* strains showed a PPV of 1 in the PCR method. It could not be calculated (0/0 = indeterminate) in the TT methods for both strains tested and in the MSRV method for *Salmonella* Pullorum because of the absence of TP and FP samples. NPV was between 0.20 and 0.43 for the 3 methods.

When the detection limit of each technique was studied, all motile *Salmonella* strains were recovered in the lowest dilutions tested for all methods, from 4 to 7 cfu/25 mL (Table 2). In reference to nonmotile *Salmonella* strains, they could not be detected in TT method, whereas *Salmonella* Gallinarum could only be recovered from 1.1×10^1 cfu/25 mL in the MSRV method. In relation to the molecular methods, PCR could detect these strains from 1.1×10^4 cfu/25 mL.

The 3 selective plating media did not show any significant differences among them in terms of Sen, Ac, PPV, and NPV in both bacteriological methods and the TT1 and the TT6 for motile *Salmonella* strains (data not shown). The plating media used in this study showed Sen and Ac values from 0.83 to 1, and 0.87 to 1, respectively. The PPV and NPV were 1, and 0.60 to 1, respectively. In reference to nonmotile *Salmonella* strains, the 3 selective plating media were only significant different among them for Sen and Ac in *S.* Gallinarum in the case of MSRV method (Table 3). For *Salmonella* Pullorum, plating media had Sen and Ac values from 0 to 0.20, respectively, in both bacteriological methods. However, these parameters were higher in the MSRV method than in the TT method for *Salmonella* Gallinarum. In relation to the 2 times of incubation in the TT method (TT1 and TT6), Sen and Ac were similar in the TT1 and the TT6 for nonmotile *Salmonella* strains studied. The Sen and Ac were from 0 to 0.20, with a Sen of 0 in the 3 selective plating media used. On the other hand, PPV was indeterminate (0/0) in the nonmotile *Salmonella* strains, except for *Salmonella* Gallinarum in MSRV method. The NPV was between 0.20 and 0.43 for these strains.

Table 1. Sensitivity (Sen), Accuracy (Ac), positive predictive value (PPV), and negative predictive value (NPV) for each nonmotile *Salmonella* strain in artificially contaminated poultry drinking water, according to tetrathionate (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods[1]

		Nonmotile *Salmonella* strain	
Performance parameter	Method	*Salmonella* Gallinarum CUB 55/10	*Salmonella* Pullorum ATCC 13036
Sen	TT	$0.00^{a,A}$ (0.00–0.60)	$0.00^{a,A}$ (0.00–0.60)
	MSRV	$0.67^{b,A}$ (0.38–0.86)	$0.00^{a,B}$ (0.00–0.60)
	PCR	$0.17^{a,A}$ (0.05–0.45)	$0.17^{a,A}$ (0.05–0.45)
Ac	TT	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
	MSRV	$0.73^{b,A}$ (0.47–0.88)	$0.20^{a,B}$ (0.07–0.45)
	PCR	$0.33^{a,A}$ (0.15–0.58)	$0.33^{a,A}$ (0.15–0.58)
NPV	TT	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
	MSRV	$0.43^{a,A}$ (0.15–0.75)	$0.20^{a,A}$ (0.07–0.45)
	PCR	$0.23^{a,A}$ (0.08–0.50)	$0.23^{a,A}$ (0.08–0.50)
PPV	TT	IND[2]	IND
	MSRV	1.00^{a} (0.66–0.99)	IND
	PCR	$1.00^{a,A}$ (0.29–0.99)	1.00^{A} (0.29–0.99)

^{a,b}Values followed by different lowercase letters in the same column are significantly different ($P < 0.05$).

^{A,B}Values followed by different uppercase letters in the same row are significantly different ($P < 0.05$).

[1]Values in parentheses indicate a 95% CI for the respective parameter.

[2]IND: indeterminate (0/0).

There was excellent agreement among the 3 methods for motile strains (kappa coefficient = 1). For nonmotile strains, the agreement was poor between MSRV and PCR (kappa coefficient = 0.17); there was no agreement (kappa coefficient = 0) when the TT method was compared with the MSRV and PCR.

Table 2. Results obtained when *Salmonella* strains were inoculated in poultry drinking water and were isolated following tetrathionate (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods[1]

		Methodology to detect *Salmonella* from poultry drinking water			
Strain	cfu/25 mL	TT first	TT sixth	MSRV	PCR
Salmonella Typhimurium ATCC 13311	0	0/3	0/3	0/3	0/3
	7.0×10^0	3/3	3/3	3/3	3/3
	6.6×10^1	3/3	3/3	3/3	3/3
	6.6×10^2	3/3	3/3	3/3	3/3
	6.6×10^3	3/3	3/3	3/3	3/3
Salmonella Enteritidis ATCC 13076	0	0/3	0/3	0/3	0/3
	4.0×10^0	3/3	3/3	3/3	3/3
	4.2×10^1	3/3	3/3	3/3	3/3
	4.2×10^2	3/3	3/3	3/3	3/3
	4.2×10^3	3/3	3/3	3/3	3/3
Salmonella Infantis CUB 08/08	0	0/3	0/3	0/3	0/3
	5.0×10^0	3/3	3/3	3/3	3/3
	5.2×10^1	3/3	3/3	3/3	3/3
	5.2×10^2	3/3	3/3	3/3	3/3
	5.2×10^3	3/3	3/3	3/3	3/3
Salmonella Kentucky CUB 19/08	0	0/3	0/3	0/3	0/3
	7.0×10^0	3/3	3/3	3/3	3/3
	6.6×10^1	3/3	3/3	3/3	3/3
	6.6×10^2	3/3	3/3	3/3	3/3
	6.6×10^3	3/3	3/3	3/3	3/3
Salmonella Pullorum ATCC 13036	0	0/3	0/3	0/3	0/3
	1.2×10^1	0/3	0/3	0/3	0/3
	1.2×10^2	0/3	0/3	0/3	0/3
	1.2×10^3	0/3	0/3	0/3	0/3
	1.2×10^4	0/3	0/3	0/3	2/3
Salmonella Gallinarum CUB 55/10	0	0/3	0/3	0/3	0/3
	1.1×10^1	0/3	0/3	1/3	0/3
	1.1×10^2	0/3	0/3	1/3	0/3
	1.1×10^3	0/3	0/3	3/3	0/3
	1.1×10^4	0/3	0/3	3/3	2/3

[1]The TT method was separated, considering the different time of streaking in the selective plating media at 1 (TT first) or 6 (TT sixth) d of incubation of the selective broth. Data represent number of positive samples/number of total samples.

Table 3. Sensitivity (Sen), accuracy (Ac), negative predictive values (NPV), and positive predictive value (PPV) of selective plating media in tetrathionate (TT) and modified semisolid Rappaport-Vassiliadis (MSRV) methods for nonmotile *Salmonella* strains[1,2]

Performance parameter	Medium[3]	*Salmonella* Gallinarum CUB 55/10			*Salmonella* Pullorum ATCC 13036		
		TT first	TT sixth	MSRV	TT first	TT sixth	MSRV
Sen	XLD	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)	$0.42^{b,AB}$ (0.19–0.68)	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)
	EF18	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)	$0.67^{b,A}$ (0.38–0.86)	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)
	XLDT	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)	$0.25^{b,B}$ (0.09–0.53)	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)	$0.00^{a,A}$ (0.00–0.24)
Ac	XLD	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.53^{b,AB}$ (0.29–0.75)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
	EF18	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.73^{b,A}$ (0.47–0.88)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
	XLDT	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.40^{a,B}$ (0.19–0.64)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
NPV	XLD	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.30^{a,A}$ (0.10–0.60)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
	EF18	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.43^{a,A}$ (0.15–0.75)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
	XLDT	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.25^{a,A}$ (0.09–0.53)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)	$0.20^{a,A}$ (0.07–0.45)
PPV	XLD	IND[4]	IND	1.00^{A} (0.54–0.99)	IND	IND	IND
	EF18	IND	IND	1.00^{A} (0.66–0.99)	IND	IND	IND
	XLDT	IND	IND	1.00^{A} (0.39–0.99)	IND	IND	IND

[a,b]Values followed by different lowercase letters in the same row are significantly different ($P < 0.05$).

[A,B]Values followed by different uppercase letters in the same column are significantly different ($P < 0.05$).

[1]The TT method was separated considering the different time of streaking in the selective plating media at 1 (TT first) or 6 (TT sixth) d of incubation of the selective broth.

[2]Values in parentheses indicate a 95% CI for the respective parameter.

[3]XLD = xylose lysine desoxicholate agar; XLDT = xylose lysine desoxicholate agar with tergitol 4; EF18 agar is from Acumedia (Lansing, MI).

[4]IND = indeterminate (0/0).

DISCUSSION

We studied the performance of 2 culture and PCR methods for motile and nonmotile *Salmonella* detection in poultry drinking water, using artificially contaminated samples. There is little information about the prevalence of *Salmonella* in drinking water for poultry. However, it is known that there is a low level of *Salmonella* potentially present in water (Murray, 1991). Therefore, low levels of *Salmonella* were added to the water samples in our study.

When performance of culture methods was compared between motile and nonmotile *Salmonella* strains, we found a significance difference. There is a lack of formal comparative research of *Salmonella* detection between motile and nonmotile strains in water. Furthermore, although Shivaprasad (2003) mentioned that water can be a source of *Salmonella* Gallinarum and *Salmonella* Pullorum, different studies (Alcaide et al., 1982; Moriñigo et al., 1986; Polo et al., 1999; Martinez-Urtaza et al., 2004; Arvanitidou et al., 2005) reported only the isolation of motile *Salmonella* from water. Our study showed the excellent recovery of *Salmonella* Enteritidis and *Salmonella* Kentucky with MSRV medium, which agrees with the results of Poppe et al. (1991). Apart from these 2 serotypes, we worked with water samples inoculated with other motile serotypes and MSRV method had an excellent performance. Moriñigo et al. (1993) compared selective enrichment broths for the detection of *Salmonella* spp. from water samples and suggested that tetrathionate broth could be inhibitory for microorganisms of this genus. However, we found high Sen and Ac values for TT method for motile *Salmonella* strains.

Our previous studies on poultry feed and fecal material (Soria et al., 2011, 2012) showed that *Salmonella* Gallinarum and *Salmonella* Pullorum were difficult to isolate from those kind of samples. In terms of the parameter values studied, we found similar low values in water samples. It is known that MSRV is unable to detect nonmotile *Salmonella* bacteria (Poppe et al., 2004). However, we could recover *Salmonella* Gallinarum from the MSRV method, despite Sen and Ac values being statistically different from motile strains. The low value of NPV in the bacteriological methods studied in our assays could be explained because nonmotile *Salmonella* strains were diluted by their competitors, composed by the microflora present in the sample. These results show that environmental sampling can underestimate the presence of nonmotile *Salmonella* strains. On the other hand, the low values of the parameters observed on TT and MSRV methods for nonmotile strains were also reflected in the plating media used.

Waltman et al. (1993) found that inoculation of the enrichment broths onto plating media after 24 h of incubation followed by a 5-d delayed secondary enrichment increased the detection of the *Salmonella*-positive environmental samples. However, we incubated TT broth at 35 ± 2°C for 6 d (TT method), and there was

no difference in *Salmonella* isolation between TT1 and TT6.

Polymerase chain reaction offers a more rapid and reliable method for the detection of *Salmonella* and has been used successfully to diagnose the presence of bacterial pathogens in aquatic environments, food products and clinical samples (Moganedi et al., 2007). On the other hand, an IAC is required to prevent FN results that might be caused by the PCR inhibitors (Oikonomou et al., 2008). With a preenrichment step before PCR assay, Moganedi et al. (2007) obtained a detection limit of 26 cfu/mL for a cell dilution of *Salmonella* Enteritidis ATCC 13076. We used IAC with each PCR assay and had a similar result for that strain and other motile strains. In reference to nonmotile biovars and PCR method, we could only detect these strains from 10^4 cfu/mL. Soria et al. (2011) and Oliveira et al. (2002) obtained similar results for *Salmonella* Pullorum and *Salmonella* Gallinarum in PCR assays, but using other matrices.

The TT, MSRV, and PCR methods are similar in terms of Ac, Sen, Sp, PPV, and NPV for different motile *Salmonella* strains in poultry water. For nonmotile *Salmonella* strains, the use of the PCR and MSRV methods can improve the detection limit in this matrix. The difference in detection levels obtained with the methods used for motile and nonmotile *Salmonella* strains and the difficulty of detecting these last strains represents a potential problem when a poultry water sample is considered negative for the presence of *Salmonella*. Finally, we present those methods as an alternative for water poultry samples considering the lack of standardized protocols.

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