Comparison of 7 culture methods for *Salmonella* serovar Enteritidis and *Salmonella* serovar Typhimurium isolation in poultry feces

Francisco I. Rodríguez,[*,†] Francisco Procura,[*,†] and Dante J. Bueno[*,‡,1]

[*]*Instituto Nacional de Tecnología Agropecuaria EEA Concepción del Uruguay, 3260, Concepción del Uruguay, Entre Ríos, Argentina;* [†]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Laboratorio de Sanidad Aviar, Instituto Nacional de Tecnología Agropecuaria EEA Concepción del Uruguay, 3260, Concepción del Uruguay, Entre Ríos, Argentina; and* [‡]*Facultad de Ciencia y Tecnología, Sede Basavilbaso, Universidad Autónoma de Entre Ríos, 3170 Basavilbaso, Entre Ríos, Argentina*

ABSTRACT The present work compared 7 different culture methods and 3 selective-differential plating media for *Salmonella* ser. Enteritidis (SE) and *S.* ser. Typhimurium (ST) isolation using artificially contaminated poultry feces. The sensitivity (Se) and accuracy (AC) values increased when Modified Semisolid Rappaport Vassiliadis (MSRV) methods were used in place of the Tetrathionate (TT) or Tetrathionate Hajna broth (TTH) method in the enrichment step. However, there was no significant difference between the pre-enrichment incubation at 4 to 6 and 18 to 24 h for MSRV5 and MSRV24 methods, respectively. All *Salmonella* strains were recovered in the lowest dilutions tested for MSRV24 and 3 out of 4 for MSRV5 methods (2 to 10 cfu/25 g). The TT and TTH methods showed a detection limit between 2.2×10^1 and 1.0×10^6 cfu/25 g of fecal sample. The agreement was variable between the methods. However, there was a very good agreement between the MSRV5 and MSRV24 methods, and between tetrathionate direct (TTD, no pre-enrichment media used) and buffered peptone water 18 to 24 h Tetrathionate broth combination (TT24 method) for *Salmonella* strains. The 3 selective-differential plating media showed an agreement between fair and excellent. They performed a high Se and AC in the MSRV methods for *Salmonella* strains. There was a significant difference between center and periphery for MSRV methods, and there was a fair agreement between them for all strains. The MSRV methods are better than TT/TTH methods for the isolation of different strains of SE and ST in poultry fecal samples. The MSRV5 method can be used to reduce the time for the detection of SE and ST in these samples. Furthermore, a loopful of the periphery of the growth should be streaked onto differential-selective plating media, even in the absence of halo, to decrease the number of false negative results.

Key words: culture method, poultry feces, *Salmonella*

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INTRODUCTION

The genus *Salmonella* is divided into 2 species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into 6 subspecies, the most important being *enterica*, and greater than 2,400 serovars (Grimont and Weill, 2007). *Salmonella* has adapted and can survive in a wide range of different environments, such that a large number of human infections are associated not only with animal food sources, but also with pets, reptiles, fruits, vegetable, legumes, and other humans (Centers for Disease Control and Prevention, 1997; Batz et al., 2011). Poultry and poultry-associated products are widely recognized as being among the most important vehicles for human *Salmonella* infections (Batz et al., 2011, 2012).

In poultry, paratyphoid salmonellae are numerous, motile, and non-host-adapted *Salmonella* serotypes. The most commonly associated with human infections include *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ST), *Salmonella enterica* subsp. *enterica* serovar Newport, and *Salmonella enterica* subsp. *enterica* serovar Heidelberg (Foley et al., 2008). Most often paratyphoid salmonellae infections in chickens are characterized as asymptomatic with an occasional persistent colonization of the intestinal tract and internal organs, which can potentially lead to contamination of the finished carcass. The principal *Salmonella* serovar associated with infections linked to eggs and egg products in the UK, a large number of European countries, and North America is SE. However, other serovars have been implicated in a number of egg-associated

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[1]Corresponding author: dantejb@yahoo.com.ar

outbreaks, most notable ST, which exhibits a range of phage types (Threlfall et al., 2014). Consequently, control of *Salmonella* in poultry flocks is crucial for the success of the poultry industry (Gama et al., 2003).

Salmonella can be introduced in poultry farms by several means, for example, day-old infected chicks, domestic animals, human, equipment, water, and feed (Shivaprasad, 2003; Gast, 2013). Birds can remain carriers for long periods of time, which poses a significant problem in poultry production (Williams, 1981). Vertical transmission of this bacteria may result in internal or external contamination of eggs. Eggshells are often contaminated with paratyphoid salmonellae by fecal contamination during oviposition. Horizontal transmission can be mediated by mechanisms including direct bird-to-bird contact, ingestion of contaminated feces or litter, contaminated water, or by personnel and equipment. Therefore, feces from infected birds are a source of bacteria, both in horizontal and vertical transmission (Shivaprasad, 2003; Gast et al., 2005; Gast, 2013).

The National Poultry Health Plan for *Salmonella* in various countries is based on sampling in production and rearing periods by using feces samples and culture methods in the laboratory procedure, as the reference in layers (Kyprianou, 2006; Ministério da Agricultura, Pecuária e Abastecimiento, 2010; National Agrifood Health and Quality Service, 2016; United States Department of Agriculture, 2017). In general, these directives focus on the reduction of mainly SE and ST found in poultry since because these are most frequently associated with human illness. Routinely used methods for isolating and identifying *Salmonella* rely on pre-enrichment in non-selective media, selective enrichment, plating in selective and differential media, and biochemical and serological identification. Numerous agar media are available for the isolation of salmonellae; therefore, at least 2 different media, preferably with dissimilar indicator systems for differentiating salmonellae from other organisms, should be used (Gast, 2013; Waltman and Gast, 2016).

Some culture methods demonstrated that SE and ST can be recovered from very low initial concentrations (2 to 5 $\times$ 10^0 cfu/25 g of feces) in artificially contaminated poultry fecal samples (Soria et al., 2012). However, there is a strong interest to reduce the time for the detection of *Salmonella* spp. from animal feces without affecting the different performance parameters of a method. Therefore, the present study was conducted to compare 7 culture methods and 3 differential plating media to know their relative ability to detect SE and ST in artificially contaminated poultry fecal samples. Furthermore, the accuracy (**AC**), sensitivity (**Se**), specificity (**SP**), positive predictive value (**PPV**), and negative predictive value (**NPV**) of each method and plating media and the agreement between them were investigated.

MATERIAL AND METHODS

Fecal Samples

Poultry fecal samples were provided by laying hen farms from the state of Entre Rios, Argentina. Four samples of each lot of feces used were analyzed by Modified Semisolid Rappaport Vassiliadis (**MSRV**) method (Soria et al., 2012) before carrying out assays to ensure the absence of *Salmonella* spp. Furthermore, total bacteria, Enterobacteriaceae, and fungi counts of fecal samples were determinated in tryptic soy agar (Acumedia, MI), MacConkey agar (Acumedia), and Dichloran Rose-Bengal Chloramphenicol agar (Oxoid, Hampshire, England), with the addition of Chloramphenicol (Anedra, China) at a concentration of 0.25 g/L, respectively.

Salmonella Strains and Culture

Four *Salmonella* strains were selected to assay. Two SE and 2 ST: SE CUB 08/12, SE ATCC 13076, ST ATTC 13311, and ST CUB 59/10 were used. The strains belong to the American Type Culture Collection (**ATTC**) and the collection from the Poultry Health Laboratory (**CUB**) of the Estación Experimental Agropecuaria, Instituto Nacional de Tecnología Agropecuaria, in Concepcion del Uruguay, Entre Rios, Argentina.

Each *Salmonella* strain was activated from nutrient agar and was grown for 18 to 24 h in tryptic soy broth (Acumedia) at 37°C. Purity of 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 per milliliter. Cells were pelleted by centrifugation in a tabletop centrifuge at 302 $\times$ g for 15 min at room temperature(25 $\pm$ 2°C).The supernatant was discarded and the pellet cell was washed twice in sterile 0.85% NaCl solution, and then resuspended in the original volume (5 mL) with the same solution.

Natural Injured Bacteria

To know the true concentration of the strains, which was inoculated for the assays, natural injury of the bacteria was determinated by plating appropriately diluted suspensions on non-selective and selective plates. Tryptic soy agar (Acumedia) was used as the non-selective plate to enumerate both injured and non-injured cells. MacConkey agar (Acumedia), as the selective plate, was used for enumeration of non-injured cells. Injury (%) was expressed as the proportion of injured cells in the total population (Liao and Fett, 2003):

$$\text{Injury}(\%) = \frac{\text{cfu/mL on tryptic soy agar} - \text{cfu/mL on MacConkey agar}}{\text{cfu/mL on tryptic soy agar}} \times 100$$

Preparation of Salmonella Inocula in Poultry Fecal Samples

Twenty-five grams of a *Salmonella*-free poultry fecal sample was introduced into a sterile plastic bag. Each *Salmonella* strain was grown as described above. Serial dilutions were then made in ClNa 0.85% solution to inoculate between 2.2×10^0 and 1.0×10^8 cfu/25 g of different *Salmonella* strains. All treatments were performed in triplicate (3 samples of each dose for each *Salmonella* strain). A total of 192 spiked samples (6 samples/strain × 8 dilutions/strain × 4 strains) were studied. For each trial set, 3 non-seeded samples were analyzed as negative controls.

Recovery of Salmonella Strains from Poultry Fecal Samples

Figure 1 shows a flowchart diagram for the detection of *Salmonella* in poultry fecal samples by different culture methods. *Salmonella*-free poultry fecal samples contaminated with different concentrations of *Salmonella* strains were pre-enriched in 225 mL of buffered peptone water (**BPW**; Merck, Darmstadt, Germany). The mixture was incubated at 35 ± 2°C for 18 to 24 h. At 4 to 6 and 18 to 24 h, 1 mL of incubated broth was transferred to 10 mL of tetrathionate broth (Acumedia) plus supplements (20 mL/L of iodine potassium iodide solution (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 18 to 24 h (TT5 and TT24 methods). Also, 1 mL of incubated broth was transferred to 10 mL of tetrathionate broth base, Hajna (Acumedia), in addition to 40 mL/L of iodine potassium iodide solution (8 g of iodine; 5 g of potassium iodide; 40 mL of demineralized water) and 40 mg/mL of novobiocin (Sigma) and incubated at 35 ± 2°C for 18 to 24 h (TTH5 and TTH24 methods). Furthermore, incubated BPW cultures were inoculated at 3 separate spots (30 μL each) in MSRV medium (Acumedia) agar plates supplemented with 1 mL/L of a 2% novobiocin solution, which were incubated at 41.5 ± 1°C for 18 to 24 h (MSRV5 and MSRV24 methods).

Additionally, *Salmonella*-free poultry fecal samples contaminated with various concentrations of *Salmonella* strains were selectively enriched in 225 mL of tetrathionate 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), and 40 mg/mL of novobiocin (Sigma). They were incubated at 35 ± 2°C for 18 to 24 h (TTD method).

In all cases, a loopful of different tetrathionate/tetrathionate Hajna broths was streaked onto Hektoen enteric agar (HE; Acumedia), Salmonella-Shigella agar (SS; Merck, Darmstadt, Germany), and Bismuth sulfite agar (SB; Acumedia) and incubated at 35 ± 2°C for 18 to 24 h. The HE and BS agars were incorporated in the study because they are included in the *Salmonella* isolation chapter in the Bacteriological Analytical Manual (Andrews et al., 2018). SS agar is a plating media that we use in the laboratory for *Salmonella* isolation from poultry organs. For MSRV medium agar plates supplemented with novobiocin solution, a loopful from the inoculated drop (center), and, if applicable, from the turbid zone extending out from the inoculated drop (periphery), was subsequently streaked onto the same selective media listed above. Two presumed *Salmonella* colonies on each selective-differential agar plate were biochemically confirmed using triple-sugar iron agar (Britania, Buenos Aires, Argentina), lysine iron agar (Acumedia), Simmons citrate (Merck), sulfide 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* sp. in a selective-differential agar plate, 2 atypical *Salmonella* sp. colonies were also taken and the same biochemical tests, as mentioned before, were done. If a *Salmonella* strain did not exhibit a periphery zone ("halo" effect) on the MSRV medium, one of the initial concentrations of the strain would be chosen, and *Salmonella* detection was done following the MSRV method. This was performed on 6 different *Salmonella*-free poultry fecal samples.

Analysis of Performance Criteria

The detection limit of the methods was considered and it was defined as the lowest concentration (cfu/25 g) of the *Salmonella* strain inoculum that could be recovered. The AC, Se, SP, PPV, and NPV were calculated for each method according to Soria et al. (2012). The assumption was that all non-spiked samples were negative for *Salmonella* and all samples spiked with *Salmonella* were considered true positives. Samples positive on at least 1 selective-differential agar plate (HE, SS, and SB) were considered positive.

Agreement between the different techniques and differential plating media used in samples for the detection of *Salmonella* was evaluated by the use of the kappa coefficient (Martin, 1977) and McNemar's test (GraphPad Software, 2017). These were calculated to test how well the techniques agreed in classifying the samples as positive or negative. Kappa coefficients (Dawson and Trapp, 2004) 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 bacterial counts in MacConkey agar and tryptic soy agar were transformed to log counts and subjected

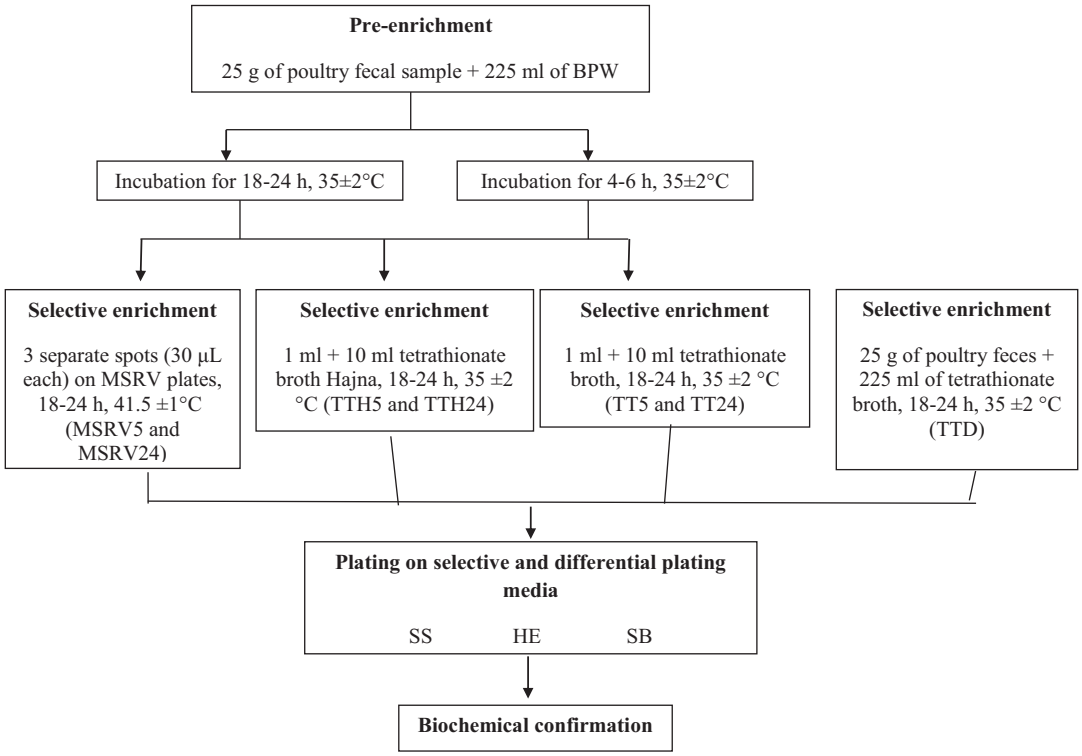

Figure 1. Flowchart diagram for the detection of *Salmonella* in poultry fecal samples by 7 culture methods. BPW = buffered peptone water; MSRV = modified semisolid Rappaport Vassiliadis medium; SS = Salmonella-Shigella agar; HE = Hektoen enteric agar; SB = Bismuth sulfite agar. Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively), tetrathionate broth methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TT5 and TT24, respectively) and without pre-enrichment (TTD), and tetrathionate broth Hajna methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TTH5 and TTH24, respectively).

to a 1-way analysis of variance test to determine the effect of media on bacterial populations. To compare the results of all assays, a hypothesis test for a difference of proportions was made using INFOSTAT Software (Di Rienzo et al., 2014). The AC, Se, PPV, and NPV of the test were reported at the shortest confident intervals, under the assumption that all values are equally probable. Also, Z test was used in order to test the statistical significance of kappa coefficients. McNemar's test was calculated using a chi-square approximation at $P \leq 0.05$ (GraphPad Software, 2017). The values reported define the boundaries of an interval that, with 95% certainty, contains the true value of AC, Se, PPV, or NPV. The results were considered to be statistically different if $P < 0.05$.

RESULTS

Fecal samples had a range of 1 to 9 $\times$ 10^{10} cfu/g of total bacteria, 6 $\times$ 10^{7} to 4.4 $\times$ 10^{8} cfu/g of Enterobacteriaceae, and 3.6 $\times$ 10^{3} to 2.2 $\times$ 10^{6} cfu/g of fungi. Natural injured bacteria was 8.26, 8.82, 11.11, and 11.54% for SE ATCC 13076, SE CUB 08/12, ST CUB 59/10, and ST ATTC 13311, respectively. However, *Salmonella* enumeration (log cfu/mL) did not show a significant difference for all strains studied using tryptic soy agar and McConkey agar (data not shown).

In relation to the performance of the methods, there were not any significant differences in the parameters studied among *Salmonella* strains for the same isolation method. The SP and PPV values were both 1 for the 4 strains studied in the 7 bacteriological method assays. The Se and AC values for all methods were between 0.33 and 0.92, and 0.41 and 0.93, respectively (Table 1). The Se and AC values were higher in MSRV than in TT/TTH methods. However, there was not any significant difference between MSRV5 and MSRV24 methods. The TTH5 method presented the lowest values of Se and AC for the *Salmonella* strains. In reference to NPV, *Salmonella* strains showed values from 0.16 to 0.60 for all the methods (Table 2). There was only a significant difference among the various isolation methods when this parameter was calculated for 4 strains together. The highest value of this parameter was observed for MSRV5, MSRV24, and TTH24 methods.

When the detection limit of the different methods was studied (Tables 3 and 4), all *Salmonella* strains were only recovered in the lowest dilutions tested for MSRV24 method (2.2 to 10 cfu/25 g), whereas 3 strains were recovered in the lowest dilutions tested for MSRV5 method (2.2 to 10 cfu/25 g). The TT/TTH methods showed a detection limit between 2.2 $\times$ 10^{1} and 1.0 $\times$ 10^{6} cfu/25 g of fecal sample. In general, TT24 and TTH24 methods showed a better detection limit than TT5 and TTH5 methods, respectively. However,

Table 1. Sensitivity (Se) and accuracy (AC) of the Modified Semisolid Rappaport Vassiliadis (MSRV5, MSRV24), tetrathionate broth (TTD, TT5, TT24), and tetrathionate broth Hajna (TTH5 and TTH24) methods for each *Salmonella* ser. Enteritidis (SE) and *S.* ser. Typhimurium (ST) strain in artificially contaminated poultry fecal samples.

		Methodology to detect SE and ST from poultry fecal samples[2]						
Salmonella strains[1]	Performance parameter	MSRV5	TT5	TTH5	TTD	MSRV24	TT24	TTH24
SE ATCC 13076	Se	0.88^{a}	0.38^{b}	0.38^{b}	$0.58^{b,c}$	0.92^{a}	$0.58^{b,c}$	$0.75^{a,c}$
	AC	0.89^{a}	0.44^{b}	0.44^{b}	$0.63^{b,c}$	0.93^{a}	$0.63^{b,c}$	$0.78^{a,c}$
SE CUB 08/12	Se	0.92^{a}	$0.54^{b,c}$	0.33^{b}	$0.50^{b,c}$	0.92^{a}	$0.54^{b,c}$	0.67^{c}
	AC	0.93^{a}	$0.59^{b,c}$	0.41^{b}	$0.56^{b,c}$	0.93^{a}	$0.59^{b,c}$	0.70^{c}
ST CUB 59/10	Se	$0.71^{a,b}$	$0.46^{b,c}$	0.38^{c}	$0.54^{b,c}$	0.83^{a}	$0.50^{b,c}$	0.58^{a-c}
	AC	$0.74^{a,c}$	$0.52^{b,c}$	0.44^{b}	$0.59^{b,c}$	0.85^{a}	$0.56^{b,c}$	0.63^{a-c}
ST ATCC 13311	Se	$0.88^{a,c}$	$0.63^{b,c}$	0.42^{b}	0.50^{b}	$0.88^{a,c}$	$0.67^{b,c}$	$0.83^{a,c}$
	AC	0.89^{a}	$0.67^{a,b}$	0.48^{b}	0.56^{b}	0.89^{a}	$0.70^{a,b}$	0.85^{a}
Four *Salmonella* strains	Se	0.84^{a}	$0.50^{c,d}$	0.38^{d}	0.53^{c}	0.89^{a}	$0.57^{b,c}$	0.71^{b}
	AC	0.86^{a}	$0.56^{c,d}$	0.44^{d}	0.58^{c}	0.90^{a}	$0.62^{b,c}$	0.74^{b}

[a–d]Values followed by different lowercase letters in the same row are significantly different ($P < 0.05$).
[1]ATCC = American type culture collection; CUB = Concepcion del Uruguay-Bacteriology.
[2]Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively), tetrathionate broth methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TT5 and TT24, respectively) and without pre-enrichment (TTD), and tetrathionate broth Hajna methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TTH5 and TTH24, respectively).

Table 2. Negative predictive value of the Modified Semisolid Rappaport Vassiliadis (MSRV5, MSRV24), tetrathionate broth (TTD, TT5, TT24), and tetrathionate broth Hajna (TTH5 and TTH24) methods for each *Salmonella* ser. Enteritidis (SE) and *S.* ser. Typhimurium (ST) strain in artificially contaminated poultry fecal samples.

	Negative predictive value[2]						
Salmonella strains[1]	MSRV5	TT5	TTH5	TTD	MSRV24	TT24	TTH24
SE ATCC 13076	0.50^{a}	0.17^{a}	0.17^{a}	0.23^{a}	0.60^{a}	0.23^{a}	0.33^{a}
SE CUB 08/12	0.60^{a}	0.21^{a}	0.16^{a}	0.20^{a}	0.60^{a}	0.21^{a}	0.27^{a}
ST CUB 59/10	0.30^{a}	0.19^{a}	0.17^{a}	0.21^{a}	0.43^{a}	0.20^{a}	0.23^{a}
ST ATCC 13311	0.50^{a}	0.25^{a}	0.18^{a}	0.20^{a}	0.50^{a}	0.27^{a}	0.43^{a}
Four *Salmonella* strains	$0.44^{a,b}$	0.20^{c}	0.17^{c}	0.21^{c}	0.52^{a}	$0.23^{b,c}$	$0.30^{a,b}$

[a–c]Values followed by different lowercase letters in the same row are significantly different ($P < 0.05$).
[1]ATCC = American type culture collection; CUB = Concepcion del Uruguay-Bacteriology.
[2]Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively), tetrathionate broth methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TT5 and TT24, respectively) and without pre-enrichment (TTD), and tetrathionate broth Hajna methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TTH5 and TTH24, respectively).

TTH24 showed the same or a better detection limit than TT24. The TTD methods showed similar or a worst detection limit than TT24 and TTH24 methods.

Analysis of the data using kappa coefficients showed that the agreement was variable between the methods (0.24 to 0.90; Table 5). There was very good agreement between the MSRV5 and MSRV24 methods and TTD and TT24 methods for *Salmonella* strains, and there was not any significant difference ($P > 0.05$) between these methods. On the other hand, there was a slight agreement between MSRV24 and TTH5/TTD/TT5, and MSRV5 and TTH5, and there was a significant difference ($P < 0.05$) between these methods.

The results of Se, AC, and NPV calculation for the selective-differential media used are shown in Table 6. There were significant differences among the isolation methods for the same selective-differential media. For all *Salmonella* strains, the 3 selective plating media did not show any significant differences among them in terms of NPV. This parameter was between 0.14 and 0.50. The 3 media performed high Se and AC in the MSRV methods for *Salmonella* strains, with values of greater than 0.68. There was not any significant difference between MSRV5 and MSRV24 for HE and SS agar. However, the Se and AC were higher in MSRV24 than in MSRV5 for SB agar. These parameters had values less than 0.57 and 0.73 in TT and TTH methods for *Salmonella* strains, respectively. For Se, the 3 selective-differential plating media had only a different value in TTH5, TT24, and TTH24 methods. On the other hand, in reference to AC, the values of the plating media were different in MSRV5, TT24, and TTH24 methods. The SB agar showed the lowest values in those cases.

Analysis of the data using kappa coefficients and McNemar's test for the selective-differential plating media for strains is shown in Table 7. Kappa coefficients showed that the agreement was fair to excellent (0.59 to 1.00). There was good to excellent agreement between the H and SS in all methods. In relation to agreement between the H and SB and SS and SB, it was between good and very good, and fair and very good in all the methods, respectively. The agreement was

Table 3. Results obtained when *Salmonella* ser. Typhimurium (ST) strains were inoculated in poultry fecal samples and were isolated following Modified Semisolid Rappaport Vassiliadis (MSRV5, MSRV24), tetrathionate broth (TTD, TT5, TT24), and tetrathionate broth Hajna (TTH5 and TTH24) methods.[1]

		Methodology to detect *Salmonella* from poultry fecal samples[2]						
ST strain	cfu/25 g	MSRV5	MSRV24	TTD	TT5	TT24	TTH5	TTH24
ST CUB 59/10	2.7×10^7	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	2.7×10^6	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	2.7×10^5	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	2.7×10^4	3/3	3/3	2/3	2/3	2/3	0/3	3/3
	2.7×10^3	3/3	3/3	2/3	0/3	1/3	0/3	2/3
	2.7×10^2	1/3	2/3	0/3	0/3	0/3	0/3	0/3
	2.7×10^1	1/3	2/3	0/3	0/3	0/3	0/3	0/3
	2.7×10^0	0/3	1/3	0/3	0/3	0/3	0/3	0/3
	0	0/3	0/3	0/3	0/3	0/3	0/3	0/3
ST ATCC 13311	7.8×10^7	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	7.8×10^6	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	7.8×10^5	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	7.8×10^4	3/3	3/3	2/3	3/3	3/3	1/3	3/3
	7.8×10^3	3/3	3/3	1/3	2/3	2/3	0/3	3/3
	7.8×10^2	3/3	3/3	0/3	1/3	2/3	0/3	3/3
	7.8×10^1	2/3	2/3	0/3	0/3	0/3	0/3	2/3
	7.8×10^0	1/3	1/3	0/3	0/3	0/3	0/3	0/3
	0	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[1] Data represent the number of positive samples per number of total samples.
[2]Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively), tetrathionate broth methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TT5 and TT24, respectively) and without pre-enrichment (TTD), and tetrathionate broth Hajna methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TTH5 and TTH24, respectively).

Table 4. Results obtained when *Salmonella* ser. Enteritidis (SE) strains were inoculated in poultry fecal samples and were isolated following Modified Semisolid Rappaport Vassiliadis (MSRV5, MSRV24), tetrathionate broth (TTD, TT5, TT24), and tetrathionate broth Hajna (TTH5 and TTH24) methods.[1]

		Methodology to detect *Salmonella* from poultry fecal samples[2]						
SE strains	cfu/25 g	MSRV5	MSRV24	TTD	TT5	TT24	TTH5	TTH24
SE ATCC 13076	2.2×10^7	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	2.2×10^6	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	2.2×10^5	3/3	3/3	3/3	2/3	3/3	2/3	3/3
	2.2×10^4	3/3	3/3	2/3	1/3	2/3	1/3	2/3
	2.2×10^3	3/3	3/3	2/3	0/3	2/3	0/3	3/3
	2.2×10^2	3/3	3/3	1/3	0/3	1/3	0/3	2/3
	2.2×10^1	2/3	3/3	0/3	0/3	0/3	0/3	2/3
	2.2×10^0	1/3	1/3	0/3	0/3	0/3	0/3	0/3
	0	0/3	0/3	0/3	0/3	0/3	0/3	0/3
SE CUB 08/12	1.0×10^8	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	1.0×10^7	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	1.0×10^6	3/3	3/3	3/3	2/3	3/3	2/3	3/3
	1.0×10^5	3/3	3/3	2/3	3/3	2/3	0/3	3/3
	1.0×10^4	3/3	3/3	1/3	2/3	1/3	0/3	3/3
	1.0×10^3	3/3	3/3	0/3	0/3	1/3	0/3	1/3
	1.0×10^2	3/3	3/3	0/3	0/3	0/3	0/3	0/3
	1.0×10^1	1/3	1/3	0/3	0/3	0/3	0/3	0/3
	0	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[1] Data represent the number of positive samples per number of total samples.
[2]Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively), tetrathionate broth methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TT5 and TT24, respectively) and without pre-enrichment (TTD), and tetrathionate broth Hajna methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TTH5 and TTH24, respectively).

between very good and excellent among the 3 selective-differential plating media in MSRV24 method without any significant difference ($P > 0.05$). However, this parameter was between good and very good among the 3 selective-differential plating media in MSRV5 method. No significant difference ($P > 0.05$) was observed in the comparison between H and SS.

Three *Salmonella* strains showed a turbid zone extending out from the inoculated drop (periphery) in MSRV medium. However, *Salmonella* Typhimurium CUB 59/10 only displayed a halo in a few initial concentrations of this strain. There was a periphery zone when the initial concentration of this strain was 2.7×10^7 cfu/25 g and 2.7×10^0 to 2.7×10^7

Table 5. Kappa coefficient values and McNemar's test showing agreement between different culture methods for poultry fecal samples.

Isolation methods	Kappa coefficient[1] (P value[2]) between culture methods					
	MSRV24	TTD	TT5	TT24	TTH5	TTH24
MSRV5	0.90 (0.1336)	0.46 (0.0001)	0.42 (0.0001)	0.51 (0.0001)	0.29 (0.0001)	0.72 (0.0009)
MSRV24	–	0.39 (0.0001)	0.36 (0.0001)	0.44 (0.0001)	0.24 (0.0001)	0.63 (0.0001)
TTD	–	–	0.76 (0.5791)	0.91 (0.3711)	0.72 (0.0003)	0.69 (0.0001)
TT5	–	–	–	0.80 (0.0704)	0.77 (0.0015)	0.64 (0.0001)
TT24	–	–	–	–	0.69 (0.0001)	0.76 (0.0009)
TTH5	–	–	–	–	–	0.46 (0.0001)

[1] Kappa is significantly non-zero ($P < 0.05$)
[2] Determined with McNemar´s chi-square test for paired samples.

Table 6. Sensitivity, accuracy and negative predictive value of selective-differential plating media in Modified Semisolid Rappaport Vassiliadis (MSRV5, MSRV24), tetrathionate broth (TTD, TT5, TT24), and tetrathionate broth Hajna (TTH5 and TTH24) methods for 4 *Salmonella* strains in artificially contaminated poultry fecal samples.

Methods to isolate *Salmonella* from poultry fecal samples[1]	Selective-differential plating media[2]								
	Sensitivity			Accuracy			Negative predictive value		
	HE	SS	SB	HE	SS	SB	HE	SS	SB
MSRV5	$0.80^{A,B,a}$	$0.77^{A,a}$	$0.68^{A,a}$	$0.82^{A,B,a}$	$0.80^{A,a,b}$	$0.69^{A,b}$	$0.39^{A,B,a}$	$0.35^{A,B,a}$	$0.28^{A,B,a}$
TT5	$0.45^{C,D,a}$	$0.42^{C,D,a}$	$0.31^{C,D,a}$	$0.51^{C,a}$	$0.48^{C,D,a}$	$0.39^{D,E,a}$	$0.18^{C,a}$	$0.18^{B,C,a}$	$0.15^{B,a}$
TTH5	$0.34^{D,a}$	$0.30^{D,a,b}$	$0.21^{D,b}$	$0.42^{C,a}$	$0.38^{D,a}$	$0.30^{E,a}$	$0.16^{C,a}$	$0.15^{C,a}$	$0.14^{B,a}$
TTD	$0.49^{C,a}$	$0.45^{C,a}$	$0.38^{C,a}$	$0.55^{C,a}$	$0.51^{B,C,D,a}$	$0.44^{C,D,a}$	$0.20^{B,C,a}$	$0.18^{B,C,a}$	$0.17^{B,a}$
MSRV24	$0.88^{A,a}$	$0.88^{A,a}$	$0.84^{B,a}$	$0.89^{A,a}$	$0.89^{A,a}$	$0.86^{B,a}$	$0.50^{A,a}$	$0.50^{A,a}$	$0.44^{A,a}$
TT24	$0.45^{C,D,a}$	$0.50^{B,C,a}$	$0.30^{C,D,b}$	$0.51^{C,a}$	$0.56^{B,C,a,b}$	$0.38^{D,E,b}$	$0.18^{C,a}$	$0.19^{B,C,a}$	$0.15^{B,a}$
TTH24	$0.69^{B,a}$	$0.59^{B,a,b}$	$0.54^{A,b}$	$0.72^{B,a}$	$0.64^{B,a,b}$	$0.59^{A,C,b}$	$0.29^{A-C,a}$	$0.24^{B,C,a}$	$0.21^{B,a}$

$^{A-E}$Values followed by different uppercase letters in the same column are significantly different ($P < 0.05$).
^{a,b}Values followed by different lowercase letters in the same row are significantly different ($P < 0.05$).
[1]Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively), tetrathionate broth methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TT5 and TT24, respectively) and without pre-enrichment (TTD), and tetrathionate broth Hajna methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (TTH5 and TTH24, respectively).
[2]SS = Salmonella-Shigella agar; HE = Hektoen enteric agar; SB = Bismuth sulfite agar.

Table 7. Kappa coefficient values and McNemar's test showing agreement between different selective-differential plating media for poultry fecal samples.

Methodology to detect *Salmonella* from poultry fecal samples	Comparison between selective-differential plating media[1]		
	HE vs. SS	HE vs. SB	SS vs. SB
	Kappa coefficient[2] (P value[3])	Kappa coefficient (P value[2])	Kappa coefficient (P value[2])
MSRV5	0.89 (0.3711)	0.72 (0.0033)	0.70 (0.0389)
TT5	0.86 (0.1306)	0.70 (0.0019)	0.71 (0.0162)
TTH5	0.82 (0.2888)	0.68 (0.0009)	0.66 (0.0265)
TTD	0.77 (0.3865)	0.71 (0.0098)	0.78 (0.0704)
MSRV24	1.00 (> 1.0000)	0.92 (0.2482)	0.92 (0.2482)
TT24	0.72 (0.3017)	0.67 (0.0012)	0.59 (0.0001)
TTH24	0.83 (0.0077)	0.71 (0.0012)	0.76 (0.2673)

[1]SS = Salmonella-Shigella agar; HE = Hektoen enteric agar; SB = Bismuth sulfite agar.
[2] Kappa is significantly non-zero ($P < 0.05$).
[3] Determined with McNemar´s chi-square test for paired samples.

cfu/25 g in MSRV5 and MSRV24, respectively. In the last case, when the initial concentration of *Salmonella* Typhimurium CUB 59/10 was between 2.7×10^0 and 2.7×10^2 cfu/25 g the presentation of a turbid zone extending out from the inoculated drop was variable. However, the rate of isolation of this strain was high even though there was no periphery zone in MSRV medium (data not shown). When the MSRV5 method was repeated using ST CUB 59/10 in an initial inoculum of $1.5–4.4 \times 10^5$ cfu/25 g in 6 *Salmonella*-free poultry fecal samples, there were a turbid zone extending out from the inoculated drop in all samples.

The performance parameters in MSRV methods and selective-differential plating media comparing center and periphery for all strains, in the case of the presentation of a halo, are shown in Table 8. There was a significant difference between center and periphery for MSRV methods, and there was a fair agreement between them

Table 8. Sensitivity (Se), accuracy (AC), negative predictive value (NPV), and agreement (Kappa coefficient and McNemar's test) for center and periphery zones of modified semisolid Rappaport-Vassiliadis (MSRV) methods and selective-differential plating media for 4 *Salmonella* strains in artificially contaminated poultry fecal samples.

		Performance parameters of the method and agreement between MSRV5 and MSRV24				Selective-differential plating media[4]								
						Se			AC			NPV		
Methods to isolate *Salmonella* sp.[1]		Se	AC	NPV	Kappa coefficient[2] (P value[3])	HE	SS	SB	HE	SS	SB	HE	SS	SB
MSRV5	Center	0.67^{a}	0.71^{a}	0.32^{a}	0.57	$0.64^{a,A}$	$0.59^{a,A}$	$0.50^{a,A}$	$0.68^{a,A}$	$0.64^{a,A}$	$0.56^{a,A}$	$0.26^{a,A}$	$0.24^{a,A}$	$0.20^{a,A}$
	Periphery	0.89^{b}	0.91^{b}	0.60^{a}	(0.0001)	$0.88^{b,A}$	$0.88^{b,A}$	$0.80^{b,A}$	$0.90^{b,A}$	$0.90^{b,A}$	$0.83^{b,A}$	$0.57^{b,A}$	$0.57^{b,A}$	$0.44^{b,A}$
MSRV24	Center	0.64^{a}	0.68^{a}	0.48^{a}	0.45	$0.64^{a,A}$	$0.45^{a,B}$	$0.36^{a,B}$	$0.68^{a,A}$	$0.51^{a,B}$	$0.44^{a,B}$	$0.26^{a,A}$	$0.18^{a,A}$	$0.16^{a,A}$
	Periphery	0.92^{b}	0.93^{b}	0.63^{b}	(0.0001)	$0.88^{b,A}$	$0.88^{b,A}$	$0.84^{b,A}$	$0.89^{b,A}$	$0.89^{b,A}$	$0.86^{b,A}$	$0.50^{a,A}$	$0.50^{b,A}$	$0.44^{b,A}$

[a,b]Values followed by different lowercase letters in the same column are significantly different ($P < 0.05$) for each parameter and method.

[A,B]Values followed by different uppercase letters in the same row are significantly different ($P < 0.05$) for each selective plating media.

[1]Modified Semisolid Rappaport Vassiliadis (MSRV) methods with 4 to 6 or 18 to 24 h buffered peptone water incubation (MSRV5 and MSRV24, respectively). Colonies taking from the inoculated drops (center), and from turbid zone extending out from the inoculated drop (periphery).

[2]Kappa is significantly non-zero ($P < 0.05$).

[3]Determined with McNemar's chi-square test for paired samples.

[4]SS = Salmonella-Shigella agar; HE = Hektoen enteric agar; SB = Bismuth sulfite agar.

independent of the selective plating media used. The Se, AC, and NPV were higher when *Salmonella* strains were isolated from periphery than from center zone. Overall, there were not any statistical differences in these parameters among the 3 selective plating media.

DISCUSSION

We studied the performance of 7 culture methods for SE and ST detection in poultry feces using artificially contaminated samples. We found that MSRV methods were better than TT methods to recover SE and ST strains from this sample type. Eriksson and Aspan (2007) found that a method similar to the MSRV24 method, using Xylose Lysine Desoxicholate agar and Brillant Green agar as selective-differential platting media, was the most sensitive and specific method, when compared to NMKL71 method, 3 commercial ELISA-based systems, and 4 PCR-based methods in artificially contaminated fecal specimens from cattle, pigs, and poultry with different *Salmonella* serovars, including SE and ST. Fujihara et al. (2016) reported that *S. enterica* was more successfully isolated from artificially contaminated fecal samples after enrichment in Hajna tetrathionate broth or modified semisolid Rappaport agar than in Rappaport broth. However, Soria et al. (2012), using artificially contaminated poultry feces, found that the Se and AC of a method similar than TT24 method, but with xylose lysine desoxicholate agar with or without tergitol 4, and EF-18 agar as selective-differential platting media, were equally high as a method similar than MSRV24, with the same platting media, for SE and ST strains. They used 2 *Salmonella* strains that we used in our study (ST ATCC 13311 and SE ATCC 13076) and the detection limit for SE ATCC 13076 was better in that report than in our assay. The different in the initial concentration of total bacteria and Enterobacteriaceae in feces and/or selective-differential plating media used could explain these results.

We did not induce an injured bacteria in the study. *Salmonella* enumeration in tryptic soy agar and MacConkey agar was not significantly different amongst the strains and the natural injured bacteria were very low (8.26 to 11.54%). We used a non-selective pre-enrichment (BPW) to encourage the growth of very small numbers of salmonellae and to allow for the recovery of injured *Salmonella* cells (Gast, 2013; Soria and Bueno, 2016). It is known that pre-enrichment is not advisable when testing samples, such as intestinal contents or feces, with large numbers of competing organisms that might overgrow salmonellae in the non-selective broth (Gast, 2013). The agreement between TTD and TT5/TT24 was good and very good in our assay without any significant difference ($P > 0.05$) between the methods. Therefore, there was no advantage in using a selective enrichment without pre-enrichment.

Approaches to decrease detection time include reducing the length of non-selective and/or selective enrichments, changing broth formulations, and altering incubation temperatures. Different authors reported that *Salmonella* spp. was able to resuscitate sufficiently after 5 to 6 h in a non-selective pre-enrichment broth and could overcome the toxic effects of selective enrichment (Chen et al., 1993; Daquigan et al., 2016). A lesser incubation time failed to recover this bacteria (Mohr et al., 1974; van Schothorst and van Leusden, 1975; D'Aoust and Maishment, 1979; Chen et al., 1993). In our assay, the effect of the incubation time in pre-enrichment broth depended on selective enrichment used to detect *Salmonella*. The agreement between TT5 and TT24 and MSRV5 and MSRV24 was good and very good, respectively, without any significant difference between them. However, the agreement between TTH5 and TTH24 was fair, with a significant difference between them. The Se, Ac, and detection limit were higher in TTH24 than in TTH5.

The ISO 6579:2002/Amd 1:2007 Standard Method (International Organization for Standardization, 2002), for the detection of *Salmonella* spp. in animal feces from, for example, poultry, pigs, and cattle, is included in many National Poultry Health Plan. It uses MSRV medium in the selective enrichment step for the detection of motile *Salmonella*. The efficiency of this medium is based on the ability of *Salmonella* to migrate through the selective medium ahead of competing motile microorganisms, thus producing opaque halos of growth (Oxoid, 2006). Gelinski et al. (2002) considered a white halo with a radius greater than 10 mm as a positive migration around the spot. However, Fujihara et al. (2016) reported that Se of MSRV was not restricted by inoculum volume and the diameter of the migration zone in this medium depended on *Salmonella* serovars and strains. In our assay, 1 ST strain (CUB 59/10) showed only a periphery zone in some samples taken from the initial concentration. This strain could be isolated from the center zone, when there was not a periphery zone. Wu et al. (2012) found that another ST strain (LB5010) did not exhibit a "halo" effect on the MSRV medium and the medium remained blue around the inoculated drop. However, when we repeated the assay, using ST CUB 59/10 feces samples, the opaque halos of growth appeared in MSRV medium. This indicated that the diameter of the migration zone also depended on the sample.

Sample type, especially the composition of the background flora, is of considerable importance for the efficiency of a specific plating media. Growth of non-*Salmonella* makes it more difficult to isolate *Salmonella* colonies, because well-isolated colonies of *Salmonella* may not be obtained (Busse, 1995). Petersen (1997) reported that the combination of the 2 media clearly decreases the number of false negative results; however, this results in a slight cost increase. Three selective plating media were used in the present study—HE, SS, and SB agar; there were found to be significant differences among the isolation methods for the same selective-differential media, strengthening the use

of pre-enrichment and enrichment steps. Furthermore, although kappa coefficients showed that the agreement was fair to excellent, the 3 selective plating media showed different Se and AC values in some methods. The SB agar showed less than or equal to values than H and SS agar in these parameters. Similar results were reported by Cox et al. (1972), who compared various plating media for the isolation of *Salmonella* from poultry feces and poultry food products. They found that the highest percentage of positive isolations was seen in SS agar (41.6%), whereas HE and SB agar had 22.2 and 13.8% positive recovery, respectively.

The MSRV methods are better than TT methods for the isolation of different strains of SE and ST in poultry fecal samples. The MSRV5 and MSRV24 are similar in terms of AC, Se, SP, PPV, and NPV for these strains. Therefore, MSRV5 method can be used to reduce the time for the detection of SE and ST from naturally contaminated poultry feces. Furthermore, due to the fact that the diameter of the migration zone in MSRV medium depends on many factors, a loopful of the periphery of the growth should be streaked onto selective-differential plating media, even in the absence of halo. The combination of HE and SS agar can be used to decrease the number of false negative results.

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