A comparative study of culture methods and polymerase chain reaction assay for *Salmonella* detection in poultry feed

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ABSTRACT The present work compared 2 culture methods and PCR assay for the detection of motile and non-motile *Salmonella* strains using artificially contaminated poultry feed. The specificity was 1 in all methods. The accuracy and sensitivity were between 0.5 and 1 for motile *Salmonella* strains, whereas these parameters were between 0 and 0.6 for non-motile *Salmonella* strains. The positive predictive value was 1 for tetrathionate (TT), PCR, and modified semisolid Rappaport-Vassiliadis (MSRV) methods in most of the strains studied. The negative predictive value of each method was very low for non-motile *Salmonella* strains. The detection level of motile strains was 8 to 20 cfu/25 g for all methods, whereas it was $\geq 10^4$ cfu/25 g in culture methods for non-motile *Salmonella* strains. In general, the PCR method detected lower non-motile *Salmonella* contamination levels in feed than did culture methods. Extending incubation time of the enrichment medium to 6 d in the TT method did not improve the isolation rates. All selective plating media did not show any statistical differences in the parameters of performance studied. Kappa coefficients showed that there was good agreement between TT and MSRV methods, and MSRV and PCR methods for motile *Salmonella* strains in poultry feed samples. The agreement was fair between TT and PCR methods for these strains. For non-motile *Salmonella* strains, there was poor (TT and MSRV methods), slight (PCR and TT methods), and fair (MSRV and PCR methods) agreement. The TT, MSRV, and PCR methods are similar in terms of accuracy, sensitivity, specificity, positive predictive value, and negative predictive value for different motile *Salmonella* strains in poultry feed. For non-motile *Salmonella* strains, the use of the PCR method improves the same parameters, described before, in this matrix. The difference in detection levels obtained with the methods used for motile and nonmotile *Salmonella* strains and the difficulty to detect these last strains represent a potential problem, when a poultry feed sample is considered negative for the presence of *Salmonella*.

Key words: *Salmonella*, poultry feed, 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. Certain serotypes of *Salmonella* can be important bacterial pathogens in humans and animals, with different serotypes having varying levels of host specificity (**Sp**; Singer et al., 2009). Chickens can be infected with many different serovars of *Salmonella* (Betancor et al., 2010). Fowl are the specific host of *Salmonella* enterica serovar Gallinarum biovar Pullorum and Gallinarum, which cause pullorum disease and fowl typhoid, respectively.These avian-adapted serotypes (non-motile) 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 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, humans, equipment, water, and feed (Barrow, 2000). Many lots of poultry feeds carry *Salmonella* and are consumed in large numbers by birds eating the feeds. These organisms multiply rapidly in the intestinal tract and large populations become established in carriers. Birds can remain carriers for long periods of time, which pose a most important problem in poultry production. *Salmonella*-contaminated processed poultry are also an important problem in human public health (Williams, 1981).

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The presence of *Salmonella* in animal feed and feed ingredients is not unusual (Koyunku and Haggblom, 2009). Most ingredients of both animal and plant origin used as ingredients in compound feed seem to be prone to *Salmonella* contamination. However, prevalence data for *Salmonella* in feed ingredients or compound feed are usually very difficult to compare between different studies due to differences in sampling and analitycal methods applied. In most studies, no information is available about the probability of correctly identifying a *Salmonella*-positive consignment (EFSA, 2008). Because feed materials are usually dry products with low water activity, isolation methods for *Salmonella* in feed must be able to regenerate the multiplication of dehydrated and stressed bacterial cells (Koyunku and Haggblom, 2009). Besides, contamination is not uniform, making detection even more difficult (Jones and Richardson, 2004).

To help decrease the problem of *Salmonella*, feedstuffs delivered in bulk for layer, broiler, duck, or turkey parent/grandparent stock are sometimes heated and chemically treated (Matlho et al., 1997). Apart from significantly increasing the cost of the feed in the case of heat-treated feed, it can damage vitamins/nutrients and may not kill all pathogens. *Salmonella* can be in the injured state in both cases. In fact, that heat treatment has no residual effect also means that unless other measures are implemented, re-contamination can occur in the mill during transport or on farms (EFSA, 2008).

The standard culture methods are primarily developed for food materials and only very few feed materials have been included in the validation studies (Koyunku and Haggblom, 2009). Routinely used methods for isolating and identifying *Salmonella* rely on preenrichment in non-selective media, selective enrichment, plating in selective and differential media, and biochemical and serological identification. Some selective enrichment media are modified semisolid Rappaport-Vassiliadis (**MSRV**) and tetrathionate (**TT**) broth. The isolation on MSRV is based on motility of the strain, meaning that non-motile variants of *Salmonella* may be more difficult to detect on this medium. Non-spreading MSRV is often taken to be non-*Salmonella*, so may not be routinely subcultured to plating agars as an economy measure (EFSA, 2010).

Shivaprasad (2003) informed that contaminated feed can also be sources of both *S.* Pullorum and *S.* Gallinarum, but feed contamination by *S.* Pullorum appears to be of minor importance. Several studies of culture methods for *Salmonella* detection in feed/food and feed ingredients only used motile *Salmonella* strains (Edel and Kampelmacher, 1974; Juven et al., 1984; Koyuncu and Haggblom, 2009; Miyahara et al., 2010). In principle, non-motile *Salmonella* strains might be expected to be more readily detected in the selective broths, which must always be plated, but there have been no formal studies to compare isolation of non-motile strains (EFSA, 2010). On the other hand, PCR has been found to be a highly specific molecular diagnostic tool (Löfström et al., 2004). Therefore, the present work was conducted to compare 2 culture methods and PCR assay, to know their relative ability to detect low levels of injured motile and non-motile *Salmonella* strains in artificially contaminated poultry feed. Furthermore, the accuracy (**Ac**), sensitivity (**Se**), Sp, positive predictive value (**PPV**), and negative predicitve value (**NPV**) of each method and the agreement among methods were investigated.

MATERIALS AND METHODS

Feed Material

Feed samples were provided by egg-laying hen farms from the state of Entre Rios, Argentina. Each sample was analyzed by the TT method, described below, before carrying out assays to ensure the absence of *Salmonella* spp. This method was used in this case because it is commonly used in our laboratory. Furthermore, total bacteria, *Enterobacteriaceae*, and fungi counts of feed samples were determined in tryptic soy agar (TSA; Acumedia, Lansing, MI), MacConkey agar (**MC**; Acumedia), and Dichloran Rose Bengal Chloramphenicol Agar (DRBC; Oxoid Ltd., Basingtoke, UK), respectively. Chloramphenicol was purchased from Anedra (San Fernando, Argentina).

Salmonella Strains and Culture

As summarized in Table 1, a total of 10 *Salmonella* strains were selected to assay; 4 of them were motile *Salmonella* (*S.* Enteritidis, *S.* Typhimurium, *Salmonella* Kentucky, and *Salmonella* Livingstone) and the others were non-motile *Salmonella* (*S.* Gallinarum and *S.* Pullorum). The strains belong to the collections from the Laboratory of Bacteriology of the Estación Experimental Agropecuaria (EEA) Instituto Nacional de Tecnología Agropecuaria (INTA) Balcarce (Buenos Aires, Argentina) and the Poultry Health Laboratory of EEA INTA Concepción del Uruguay (Entre Ríos, Argentina). Eight of them were isolated from chickens and 2 were isolated from feed. Each *Salmonella* strain was activated from nutrient agar (Acumedia) and was grown for 24 h in tryptic soy broth (**TSB**; Merck, Darmstadt, Germany) at 37°C. The purity of the cultures was confirmed by streaking onto MC and TSA. The number of viable microorganisms was estimated by the method of Miles and Misra (1938) and was expressed as cfu/mL. Cells were pelleted by centrifugation in a tabletop centrifuge at 302 × *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).

Heat-Injured Bacteria

Tubes containing 4.5 mL of PBS (pH 7.4) were immersed in a water bath. Once the temperature had at-

Table 1. *Salmonella* strains used in the comparison of different methods to detect this bacteria

Salmonella strain	Bacterial collection[1]	Source
Salmonella Enteritidis PT1	EEA INTA Balcarce	Chicken
Salmonella Typhimurium 06/11	EEA INTA Balcarce	Chicken
Salmonella Kentucky CBU19/08	EEA INTA C. del Uruguay	Soy expeller
Salmonella Livingstone CBU 52/10	EEA INTA C. del Uruguay	Meat expeller
Salmonella Pullorum 90/142	EEA INTA Balcarce	Chicken
Salmonella Gallinarum 93/110	EEA INTA Balcarce	Chicken
S. Gallinarum 88/322	EEA INTA Balcarce	Chicken
S. Gallinarum 80/111	EEA INTA Balcarce	Chicken
S. Gallinarum 81/86	EEA INTA Balcarce	Chicken
S. Gallinarum 03/121	EEA INTA Balcarce	Chicken

[1]EEA INTA = Estación Experimental Agropecuaria Instituto Nacional de Tecnología Agropecuaria.

tained stability at 60°C, the tubes were inoculated with 0.5 mL of *Samonella* culture. Each strain was treated at different times (Table 2). Heat injury was determined by plating appropriately diluted suspensions on non-selective and selective plates. Tryptic soy agar (Acumedia) was used as the non-selective plate and brilliant green agar (**BG**; Oxoid Ltd.) and MC (Acumedia) as the selective plate. Tryptic soy agar was used to enumerate both injured and non-injured cells; BG and MC were used for enumeration of non-injured cells. Heat injury (%) was expressed as the proportion of injured cells in the total population (Liao and Fett, 2003):

$$\text{Heat injury (\%)} = \frac{\text{cfu/mL on TSA} - \text{cfu/mL on BG or MC}}{\text{cfu/mL on TSA}} \times 100.$$

Preparation of Salmonella spp. Inocula in Poultry Feed Samples

Twenty-five grams of *Salmonella*-free poultry feed material was introduced into a sterile plastic bag. Each *Salmonella* strain was grown and heat injured as described above. After that, serial dilutions were made in peptone water (0.1%) to inoculate from 8×10^0 to 8.3×10^4 cfu/25 g, or 7.4×10^0 to 2.3×10^7 cfu/25 g for motile *Salmonella* and non-motile *Salmonella* strains, respectively. All treatments were performed in duplicate and replicated twice, so 4 samples of each dose for each *Salmonella* strain were considered in the assays. Controls were included as *Salmonella* cultures in TSB with ferrous sulfate (**TSBF**, 35 mg of ferrous sulfate added to 1,000 mL of TSB) or *Salmonella*-free poultry feed.

Recovery of Salmonella spp. Strains from Poultry Feed

Figure 1 shows a flowchart diagram for detection of *Salmonella* in feed by the TT and MRSV methods. *Salmonella*-free poultry feeds contaminated with different concentrations of *Salmonella* strains were pre-enriched in 225 mL of TSBF. The mixture was incubated at 35 ± 2°C for 24 h. 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-Aldrich Chemie GmbH, Steinheim, Germany), and 40 mg/mL of novobiocin (Sigma-Aldrich Chemie GmbH), and incubated at 35 ± 2°C for 6 d (TT method). At 1 (**TT first**) and 6 (**TT sixth**) d, a loopful of TT broth was streaked on xylose lysine desoxicholate agar (XLD; Oxoid Ltd.) with tergitol 4 (**XLDT**; 4.6 mL/L; Sigma, St. Louis, MO), EF-18 (Acumedia), MC, and BG agar,

Table 2. Heat injury (%) of each *Salmonella* strain in MacConkey agar (MC) and brilliant green agar (BG)

Strain	Exposure time (min) at 60°C	Heat injury (%)	
		MC	BG
Salmonella Enteritidis PT1	2	79.9	79.9
Salmonella Typhimurium 06/11	2	88.3	85.2
Salmonella Kentuky CUB 19/08	6	78.0[a]	61.4[b]
Salmonella Livingstone CUB 52/10	4	83.3[a]	45.1[b]
Salmonella Pullorum 90/142	2	76.8[a]	41.7[b]
Salmonella Gallinarum 93/110	2	90.2	76.8
S. Gallinarum 88/322	2	65.5[a]	27.8[b]
S. Gallinarum 80/111	2	89.5	83.1
S. Gallinarum 81/86	2	51.8[a]	14.4[b]
S. Gallinarum 03/121	2	78.2[a]	56.2[b]

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

Table 3. Sensitivity (Se) and accuracy (Ac) of the tetrathionate (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods for each motile and non-motile *Salmonella* strain in artificially contaminated poultry feed[1]

	Se			Ac		
Strain	TT	MSRV	PCR	TT	MSRV	PCR
Salmonella Enteritidis PT 1	$1^{a,A}$ (0.92–0.99)	$1^{a,A}$ (0.92–0.99)	$0.58^{b,A}$ (0.26–0.82)	$1^{a,A}$ (0.92–0.99)	$1^{a,A}$ (0.92–0.99)	$0.64^{b,A}$ (0.34–0.84)
Salmonella Typhimurium 06/11	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$0.95^{a,B}$ (0.80–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$0.95^{a,B}$ (0.81–0.99)
Salmonella Kentucky CUB 19/08	$0.93^{a,A}$ (0.76–0.99)	$1^{a,A}$ (0.93–0.99)	$0.81^{a,B}$ (0.61–0.96)	$0.94^{a,A}$ (0.78–0.99)	$1^{a,A}$ (0.94–0.99)	$0.83^{a,B}$ (0.57–0.95)
Salmonella Livingstone CUB 52/10	$0.55^{b,B}$ (0.40–0.84)	$1^{a,A}$ (0.93–0.99)	$0.93^{a,B}$ (0.76–0.99)	$0.60^{b,B}$ (0.40–0.84)	$1^{a,A}$ (0.94–0.99)	$0.94^{a,B}$ (0.78–0.99)
Salmonella Pullorum 90/142	$0.10^{a,C}$ (0.06–0.36)	$0^{a,B}$	$0.35^{b,A}$ (0.17–0.56)	$0.22^{a,C}$ (0.06–0.36)	$0.09^{b,B}$ (0.01–0.25)	$0.40^{a,A}$ (0.22–0.60)
Salmonella Gallinarum 93/110	$0.10^{a,C}$ (0.10–0.27)	$0.15^{a,BC}$ (0.04–0.34)	$0.55^{b,A}$ (0.34–0.74)	$0.22^{a,C}$ (0.06–0.36)	$0.22^{a,B}$ (0.09–0.42)	$0.59^{b,A}$ (0.39–0.77)
S. Gallinarum 88/322	$0.25^{a,C}$ (0.10–0.45)	$0.20^{a,C}$ (0.06–0.39)	$0.55^{b,A}$ (0.34–0.74)	$0.31^{a,C}$ (0.15–0.51)	$0.27^{a,B}$ (0.12–0.47)	$0.59^{b,A}$ (0.39–0.77)
S. Gallinarum 80/111	$0.15^{ab,C}$ (0.04–0.34)	$0^{a,B}$	$0.20^{b,C}$ (0.06–0.39)	$0.22^{a,C}$ (0.09–0.42)	$0.09^{b,B}$ (0.01–0.25)	$0.27^{a,C}$ (0.12–0.47)
S. Gallinarum 81/86	$0.40^{a,B}$ (0.21–0.60)	$0.04^{b,B}$ (0–0.20)	$0.45^{a,A}$ (0.25–0.65)	$0.45^{a,C}$ (0.26–0.65)	$0.13^{b,B}$ (0.03–0.31)	$0.50^{a,A}$ (0.30–0.69)
S. Gallinarum 03/121	$0.15^{a,C}$ (0.04–0.34)	$0^{a,B}$	$0.50^{b,A}$ (0.29–0.70)	$0.22^{a,C}$ (0.09–0.42)	$0.09^{b,B}$ (0.01–0.25)	$0.54^{c,A}$ (0.34–0.73)

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

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

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

and incubated at 35 ± 2°C for 24 h. On the other hand, 30 μL of incubated TSBF was inoculated in MSRV medium (Acumedia) agar plates, which were incubated at 41.5 ± 1°C for 24 h and subsequently streaked on the same selective media listed above (MSRV method). Colonies of presumptive *Salmonella* were inoculated onto triple-sugar iron agar (TSI; Acumedia) and lysine iron agar (LIA; Merck). Further confirmation was done based on the ortho-nitrophenyl-β-galactoside (ONPG) test and agglutination reaction with somatic (O) polivalent antisera (Difco; Becton, Dickinson and Co., Sparks, MD).

Table 4. Positive predictive value (PPV) and negative predictive value (NPV) for each motile and non-motile *Salmonella* strain in artificially contaminated poultry feed, according to tetrathionate (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods[1]

	PPV in different methods for *Salmonella* detection			NPV in different methods for *Salmonella* detection		
Strain	TT	MSRV	PCR	TT	MSRV	PCR
Salmonella Enteritidis PT1	$1^{a,A}$ (0.91–0.99)	$1^{a,A}$ (0.91–0.99)	$1^{a,A}$ (0.86–0.99)	$1^{a,A}$ (0.51–0.98)	$1^{a,A}$ (0.51–0.98)	$0.27^{b,A}$ (0.06–0.62)
Salmonella Typhimurium 06/11	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.94–0.99)	$1^{a,A}$ (0.51–0.98)	$1^{a,A}$ (0.51–0.98)	$0.67^{a,A}$ (0.09–0.90)
Salmonella Kentucky CUB 19/08	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.92–0.99)	$0.67^{ab,A}$ (0.22–0.95)	$1^{b,A}$ (0.51–0.98)	$0.40^{a,A}$ (0.10–0.76)
Salmonella Livingstone CUB 52/10	$1^{a,A}$ (0.90–0.99)	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.93–0.99)	$0.25^{a,A}$ (0.05–0.56)	$1^{b,A}$ (0.51–0.98)	$0.67^{ab,A}$ (0.22–0.95)
Salmonella Pullorum 90/142	$1^{a,A}$ (0.51–0.98)	IND[2]	$1^{a,A}$ (0.86–0.99)	$0.10^{a,A}$ (0.01–0.27)	$0.09^{a,B}$ (0.01–0.25)	$0.13^{a,A}$ (0.02–0.35)
Salmonella Gallinarum 93/110	$1^{a,A}$ (0.51–0.98)	$1^{a,A}$ (0.67–0.99)	$1^{a,A}$ (0.91–0.99)	$0.10^{a,A}$ (0.01–0.29)	$0.11^{a,B}$ (0.01–0.28)	$0.18^{a,A}$ (0.03–0.45)
S. Gallinarum 88/322	$1^{a,A}$ (0.80–0.99)	$1^{a,A}$ (0.75–0.99)	$1^{a,A}$ (0.93–0.99)	$0.12^{a,A}$ (0.02–0.31)	$0.11^{a,B}$ (0.01–0.30)	$0.18^{a,A}$ (0.03–0.45)
S. Gallinarum 80/111	$1^{a,A}$ (0.67–0.99)	IND	$1^{a,A}$ (0.75–0.99)	$0.11^{a,A}$ (0.01–0.28)	$0.09^{a,B}$ (0.01–0.25)	$0.11^{a,A}$ (0.01–0.28)
S. Gallinarum 81/86	$1^{a,A}$ (0.87–0.99)	$1^{a,A}$ (0.02–0.97)	$1^{a,A}$ (0.89–0.99)	$0.14^{a,A}$ (0.02–0.37)	$0.11^{a,B}$ (0.01–0.26)	$0.15^{a,A}$ (0.02–0.39)
S. Gallinarum 03/121	$1^{a,A}$ (0.67–0.99)	IND	$1^{a,A}$ (0.02–0.39)	$0.11^{a,A}$ (0.01–0.28)	$0.09^{a,B}$ (0.01–0.25)	$0.17^{a,A}$ (0.03–0.42)

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

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

Table 5. Results obtained when motile *Salmonella* strains were inoculated in poultry feed and were isolated following tetrathionate (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods[1]

Strain	Range of inoculation (cfu/25 g)	Methodology to detect *Salmonella* from poultry feed			
		TT first	TT sixth	MSRV	PCR
Salmonella Enteritidis PT1	0	0/2	0/2	0/2	0/2
	8	3/4	3/4	4/4	1/4
	79	4/4	4/4	4/4	3/4
	7.9×10^2	4/4	4/4	4/4	3/4
Salmonella Typhimurium 06/11	0	0/2	0/2	0/2	0/2
	8	4/4	4/4	4/4	3/4
	83	4/4	4/4	4/4	4/4
	8.3×10^2	4/4	4/4	4/4	4/4
	8.3×10^3	4/4	4/4	4/4	4/4
	8.3×10^4	4/4	4/4	4/4	4/4
Salmonella Kentucky CUB 19/08	0	0/2	0/2	0/2	0/2
	18	3/4	3/4	4/4	1/4
	1.8×10^2	4/4	4/4	4/4	4/4
	1.8×10^3	4/4	4/4	4/4	4/4
	1.8×10^4	4/4	3/4	4/4	4/4
Salmonella Livingstone CUB 52/10	0	0/2	0/2	0/2	0/2
	20	0/4	0/4	4/4	4/4
	2.0×10^2	3/4	0/4	4/4	4/4
	2.0×10^3	3/4	1/4	4/4	4/4
	2.0×10^4	4/4	0/4	4/4	3/4

[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.

Table 6. Results obtained when non-motile *Salmonella* strains were inoculated in poultry feed and were isolated following tetrathionate (TT), modified semisolid Rappaport-Vassiliadis (MSRV), and PCR methods[1]

Strain	Range of inoculation (cfu/25 g)	Methodology to detect *Salmonella* from poultry feed			
		TT first	TT sixth	MSRV	PCR
Salmonella Pullorum 90/142	0	0/2	0/2	0/2	0/2
	1.4×10^2	0/4	0/4	0/4	0/4
	1.4×10^3	0/4	0/4	0/4	0/4
	1.4×10^4	0/4	0/4	0/4	0/4
	1.4×10^5	0/4	0/4	0/4	3/4
	1.4×10^6	1/4	0/4	0/4	4/4
Salmonella Gallinarum 93/110	0	0/2	0/2	0/2	0/2
	74	0/4	0/4	0/4	0/4
	7.4×10^2	0/4	0/4	0/4	1/4
	7.4×10^3	0/4	0/4	0/4	3/4
	7.4×10^4	1/4	0/4	0/4	3/4
	7.4×10^5	2/4	0/4	3/4	4/4
S. Gallinarum 88/322	0	0/2	0/2	0/2	0/2
	25	0/4	0/4	0/4	0/4
	2.5×10^2	0/4	0/4	0/4	2/4
	2.5×10^3	0/4	0/4	0/4	2/4
	2.5×10^4	1/4	0/4	0/4	3/4
	2.5×10^5	3/4	0/4	3/4	4/4
S. Gallinarum 80/111	0	0/2	0/2	0/2	0/2
	6.2×10^2	0/4	0/4	0/4	0/4
	6.2×10^3	0/4	0/4	0/4	0/4
	6.2×10^4	0/4	0/4	0/4	0/4
	6.2×10^5	0/4	0/4	0/4	0/4
	6.2×10^6	3/4	0/4	0/4	4/4
S. Gallinarum 81/86	0	0/2	0/2	0/2	0/2
	2.3×10^3	0/4	0/4	0/4	0/4
	2.3×10^4	0/4	0/4	0/4	0/4
	2.3×10^5	1/4	0/4	0/4	1/4
	2.3×10^6	3/4	0/4	0/4	4/4
	2.3×10^7	4/4	0/4	0/4	4/4
S. Gallinarum 03/121	0	0/2	0/2	0/2	0/2
	1.4×10^2	0/4	0/4	0/4	0/4
	1.4×10^3	0/4	0/4	0/4	0/4
	1.4×10^4	0/4	0/4	0/4	3/4
	1.4×10^5	0/4	0/4	0/4	3/4
	1.4×10^6	3/4	2/4	0/4	4/4

[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.

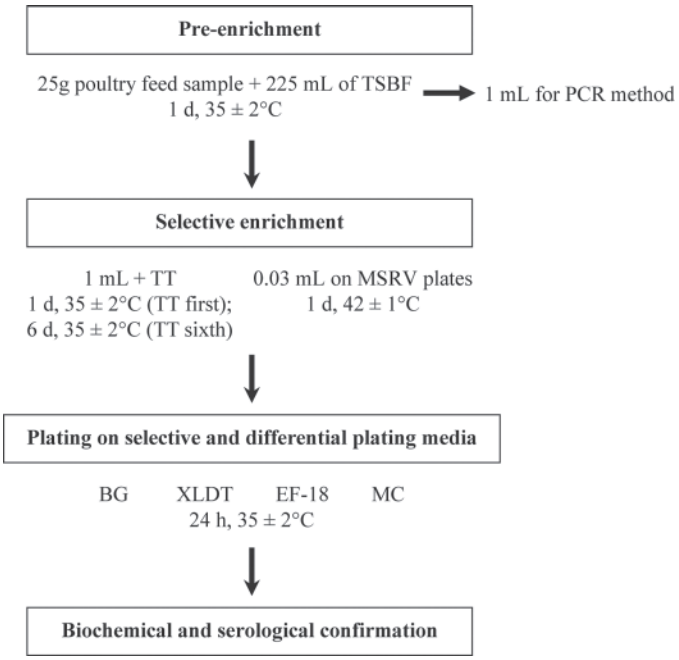

Figure 1. Flowchart diagram for detection of *Salmonella* in poultry feed by tetrathionate broth (TT), modified semisolid Rappaport-Vassiliadis (MSRV) medium, and PCR methods. TSBF = tryptic soy broth with ferrous sulfate; TT first = d 1 of incubation in the TT method; TT sixth = d 6 of incubation in the TT method; BG = brillant green agar; XLDT = xylose lysine desoxicholate agar with tergitol 4; MC = MacConkey agar; EF-18 agar is from Acumedia (Lansing, MI).

DNA Extraction

For detection of *Salmonella* from poultry feed samples, bacterial cells were recovered from 1 mL of TSBF pre-enrichment 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 clear supernatant fluid containing nucleic acids was fractionated in Eppendorf tubes and stored at −70°C until it was used in subsequent PCR assays.

PCR Assay

Deoxyribonucleic acid 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* deoxyribonucleotide triphosphate (dNTP; Fermentas), 0.2 μL of Taq DNA polymerase (5 U/μL; Fermentas), and double-distilled water to 25 μL. The reaction mixture was incubated in a programmable DNA thermal cycler (model Mastercycler Gradient; Eppendorf AG, Hamburg, Germany). *Salmonella* genus-specific primers 139 and 141 (Operon Biotechnologies GmbH, Cologne, Germany) based on the *invA* gene of *Salmonella* was used in the PCR assay. The primers have the following nucleotide sequences: (5′→3′) GTGAAATTATCGCCACGTTCGGGCAA and TCATCGCACCGTCAAAGGAACC, respectively. A reagent blank containing all of 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. Furthermore, negative and positive DNA controls were included, which were prepared from *Citrobacter* spp. (isolated from poultry feed) and *Salmonella* spp., respectively. 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% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer at 120 V/cm for 1 h. The gel was stained with 0.5 μg/mL of ethidium bromide and electrophoresed products were visualized with a 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 a low dilution where each *Salmonella* strain was recovered. The Ac, Se, Sp, PPV, and NPV were calculated for each method. The assumption was that all non-spiked 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 (XLDT, EF-18, BG, or MC) were considered positive for the bacteriological methods used. Based on this, the Ac, Se, Sp, PPV, and NPV rates were obtained by using the following definitions and equations: 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.

Accuracy is a measure of the ability of a method to correctly classify samples containing *Salmonella* as positive for *Salmonell*a, and samples not containing *Salmonella* as negative for *Salmonella*.

$$\text{Ac} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{TN} + \text{FP} + \text{FN}}.$$

Sensitivity is a measure of the ability of a method to classify a sample containing *Salmonella* as positive for *Salmonella*.

$$\text{Se} = \frac{\text{TP}}{\text{TP} + \text{FN}}.$$

Specificity is a measure of the ability of a method to classify a sample not containing *Salmonella* as negative for *Salmonella*.

$$\text{Sp} = \frac{\text{TN}}{\text{TN} + \text{FP}}.$$

Positive predictive value is a measure of the probability of the samples with positive test results for *Salmonella* to be correctly determined.

$$\text{PPV} = \frac{\text{TP}}{\text{TP} + \text{FP}}.$$

Negative predictive value is a measure of the probability of the samples with negative test results for *Salmonella* to be correctly determined.

$$\text{NPV} = \frac{\text{TN}}{\text{TN} + \text{FN}}.$$

Statistical Analysis

The differences in the mean values of heat injury (%) in MC and BG were evaluated by ANOVA. To compare the results of all the rest of the assays, a hypothesis test for a difference of proportions was made. The Se, Ac, PPV, and NPV of the test were reported at the shortest CI, under the assumption that all values are equally probable. The calculations were performed using the Octave program, developed by the Group of Numerical Methods (GMN), 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, Se, PPV or NPV. The results were only considered to be statistically different at $P < 0.05$.

Agreement between culture- and PCR-based methods for detection of *Salmonella* was evaluated by the use of the kappa statistic (Martin, 1977). The 3 methods were treated as raters and the simple kappa statistic 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, 2005). 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). The Z test was used to test the statistical significance of kappa coefficients.

RESULTS

Feed samples had an average of 2.0×10^5 cfu/g of total bacteria; 6.4×10^3 cfu/g of *Enterobacteriaceae*, and 4.5×10^4 cfu/g of fungi. On the other hand, the heat injury (%) depended on non-selective plates and *Salmonella* strains used; it was higher in MC than in BG in 6 of 10 *Salmonella* strains used (Table 2).

In relation to the performance of the methods, the Sp was 1 for all methods studied (data not shown). The Se and Ac were 1 in the MSRV method for motile *Salmonella* strains. The same parameters were 0.55 to 1 and 0.58 to 0.93 in the TT and PCR methods, respectively. The 3 methods showed Se and Ac values less than 0.6 for non-motile *Salmonella* strains (Table 3).

Table 4 shows PPV and NPV for all strains tested. The PPV was 1 for all *Salmonella* strains in the 3 methods, except for *S.* Pullorum 90/142, *S.* Gallinarum 80/111, and *S.* Gallinarum 03/121 in the MSRV method. On the other hand, NPV values were between 0.25 and 1 and 0.09 and 0.18 for motile and non-motile *Salmonella* strains, respectively.

When the detection limit of each technique was studied, all motile *Salmonella* strains were recovered in the lowest dilutions tested for all methods, except *S.* Livingstone, which showed a detection limit of 2×10^2 cfu/25 g of feed material in the TT method (Table 5). Detection limits were between 2.5×10^4 and 6.2×10^6 cfu/25g for non-motile *Salmonella* strains in the TT and MSRV methods (Table 6). However, PCR was able to detect some strains from 2.5 to 7.4×10^2 cfu/25 g of poultry feed.

The Se and Ac for combinations of each method with the 4 selective plating media did not show any significant differences among them in both bacteriological methods for motile and non-motile *Salmonella* strains. The 4 media had a high Se and Ac in the MSRV method for motile strains, with values of greater than 0.87 (Table 7). However, these parameteres had a value less than 0.30 for non-motile *Salmonella* strains (Table 8). Regarding the 2 times of incubation in the TT method (TT first and TT sixth), Se and Ac were better in TT first than in TT sixth for *S.* Enteritidis and *S.* Livingstone. Furthermore, the performance changed after 6 d compared to the first day, with low values for BG and MC in *S.* Enteritidis. The XLDT and EF-18 had the same performance in TT first and TT sixth in that strain. Regarding the non-motile *Salmonella* strains, Se was 0 or near 0 and Ac was near 0 in most of the strains and selective plating media used (Table 8).

Table 9 shows the PPV and NPV for each agar medium studied for the bacteriological methods in motile *Salmonella* strains, with details of the TT method. The

Table 7. Sensitivity (Se) and accuracy (Ac) values of selective plating media in tetrathionate (TT) and modified semisolid Rappaport-Vassiliadis (MSRV) methods for motile *Salmonella* strains[1]

	Se			Ac		
Strain and medium[2]	TT first	TT sixth	MSRV	TT first	TT sixth	MSRV
Salmonella Enteritidis PT1						
BG	$0.83^{a,A}$ (0.55–0.98)	$0.41^{b,A}$ (0.1–0.65)	$1^{a,A}$ (0.91–1)	$0.85^{a,A}$ (0.60–0.98)	$0.50^{b,A}$ (0.20–0.72)	$1^{a,A}$ (0.93–1)
XLDT	$0.91^{a,A}$ (0.68–1)	$0.83^{a,B}$ (0.55–0.98)	$1^{a,A}$ (0.91–1)	$0.92^{a,A}$ (0.72–1)	$0.85^{a,B}$ (0.60–0.98)	$1^{a,A}$ (0.93–1)
EF18	$0.91^{a,A}$ (0.68–1)	$0.75^{a,B}$ (0.43–0.93)	$1^{a,A}$ (0.91–1)	$0.92^{a,A}$ (0.72–1)	$0.78^{a,B}$ (0.51–0.94)	$1^{a,A}$ (0.93–1)
MC	$0.91^{a,A}$ (0.68–1)	$0.41^{b,A}$ (0.1–0.65)	$1^{a,A}$ (0.91–1)	$0.92^{a,A}$ (0.72–1)	$0.50^{b,A}$ (0.20–0.72)	$1^{a,A}$ (0.93–1)
Salmonella Typhimurium 06/11						
BG	$0.95^{a,A}$ (0.80–0.99)	$0.85^{a,A}$ (0.68–0.97)	$1^{a,A}$ (0.91–1)	$0.95^{a,A}$ (0.82–0.99)	$0.86^{a,A}$ (0.65–0.96)	$1^{a,A}$ (0.93–1)
XLDT	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.91–1)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.93–1)
EF18	$1^{a,A}$ (0.95–0.99)	$0.90^{a,A}$ (0.74–0.99)	$1^{a,A}$ (0.91–1)	$1^{a,A}$ (0.95–0.99)	$0.90^{a,A}$ (0.72–0.98)	$1^{a,A}$ (0.93–1)
MC	$1^{a,A}$ (0.95–0.99)	$0.70^{a,A}$ (0.50–0.88)	$1^{a,A}$ (0.91–1)	$1^{a,A}$ (0.95–0.99)	$0.72^{a,A}$ (0.46–0.86)	$1^{a,A}$ (0.93–1)
Salmonella Kentucky CUB 19/08						
BG	$0.62^{a,A}$ (0.34–0.82)	$0.56^{a,A}$ (0.28–0.77)	$0.93^{b,A}$ (0.78–0.99)	$0.66^{a,A}$ (0.40–0.84)	$0.61^{a,A}$ (0.34–0.80)	$0.94^{b,A}$ (0.76–0.99)
XLDT	$0.93^{a,B}$ (0.76–0.99)	$0.87^{a,B}$ (0.65–0.98)	$1^{a,A}$ (0.93–0.99)	$0.94^{a,B}$ (0.78–0.99)	$0.88^{a,B}$ (0.69–0.98)	$1^{a,A}$ (0.94–0.99)
EF18	$0.93^{a,B}$ (0.76–0.99)	$0.87^{a,B}$ (0.65–0.98)	$0.93^{a,A}$ (0.78–0.99)	$0.94^{a,B}$ (0.78–0.99)	$0.88^{a,B}$ (0.69–0.98)	$0.94^{a,A}$ (0.76–0.99)
MC	$0.87^{a,B}$ (0.65–0.98)	$0.62^{a,B}$ (0.34–0.82)	$0.93^{a,A}$ (0.78–0.99)	$0.88^{a,B}$ (0.69–0.98)	$0.66^{b,B}$ (0.40–0.84)	$0.94^{a,A}$ (0.76–0.99)
Salmonella Livingstone CUB 52/10						
BG	$0.18^{a,A}$ (0.06–0.43)	$0.06^{b,A}$ (0.01–0.28)	$0.88^{c,A}$ (0.76–0.99)	$0.27^{a,A}$ (0.05–0.47)	$0.16^{a,A}$ (0.06–0.39)	$0.94^{b,A}$ (0.78–0.99)
XLDT	$0.37^{a,A}$ (0.11–0.59)	$0^{b,A}$	$0.88^{c,A}$ (0.76–0.99)	$0.44^{a,A}$ (0.18–0.65)	$0^{b,A}$	$0.94^{c,A}$ (0.78–0.99)
EF18	$0.37^{a,A}$ (0.11–0.59)	$0^{b,A}$	$0.88^{c,A}$ (0.76–0.99)	$0.44^{a,A}$ (0.18–0.65)	$0^{b,A}$	$0.94^{c,A}$ (0.78–0.99)
MC	$0.18^{a,A}$ (0.06–0.43)	$0^{b,A}$	$1^{c,A}$ (0.93–0.99)	$0.27^{a,A}$ (0.05–0.47)	$0^{b,A}$	$1^{c,A}$ (0.94–0.99)

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

[2]BG = brilliant green agar; XLDT = xylose lysine desoxicholate agar with tergitol 4; EF18 agar is from Acumedia (Lansing, MI); MC = MacConkey agar.

PPV was 1 for all motile *Salmonella* strains, except in *S.* Livingstone on TT sixth. On the other hand, PPV was 1 in most of the non-motile *Salmonella* strains for TT first, especially in XLDT and EF-18 agar (Table 10). In general, this value changed to indeterminate (0/0) for TT sixth and the MSRV method. The NPV was between 0.11 and 1 for motile *Salmonella* strains and it was very low for non-motile *Salmonella* strains (Tables 9 and 10).

In general, BG and MC plates showed considerably more competitive flora than XLDT and EF-18, with some plates without typical colonies of *Salmonella*. Despite this, triple-sugar iron, lysine iron agar, and serological agglutination confirmed the presence of added serotypes.

Analysis of data using kappa coefficients showed that there was good agreement between the TT and MSRV methods, and MSRV and PCR methods for motile *Salmonella* strains in poultry feed samples (Table 11). The agreement was fair between the TT and PCR methods for these strains. For non-motile *Salmonella* strains, there was poor (TT and MSRV methods), slight (PCR and TT methods), and fair (MSRV and PCR methods) agreement.

DISCUSSION

We studied the performance of 2 culture and PCR methods for motile and non-motile *Salmonella* detection in poultry feed, using artificially contaminated samples. The heat stressing of *Salmonella* before isolation was done to simulate more natural conditions prevailing in poultry feed. This is important because animal feed is exposed to different treatments to decrease the presence of a bacterial load. Sherry et al. (2004) studied the heat resistance of 40 *Salmonella enterica* serovars, and they concluded that each *Salmonella* isolated was unique in its inherent ability to withstand the

Table 8. Sensitivity (Se) and accuracy (Ac) values of selective plating media in tetrathionate (TT) and modified semisolid Rappaport-Vassiliadis (MSRV) methods for *Salmonella* Pullorum and *Salmonella* Gallinarum strains[1]

	Se			Ac		
Strain and medium[2]	TT first	TT sixth	MSRV	TT first	TT sixth	MRSV
S. Pullorum 90/142						
BG	$0^{a,A}$	0^{a}	$0^{a,A}$	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
XLDT	$0.05^{a,A}$ (0–0.20)	$0^{a,A}$	$0^{a,A}$	$0.13^{a,A}$ (0.03–0.31)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
EF18	$0.05^{a,A}$ (0–0.20)	$0^{a,A}$	$0^{a,A}$	$0.13^{a,A}$ (0.03–0.31)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
MC	$0^{a,A}$	$0^{a,A}$	$0^{a,A}$	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
S. Gallinarum 93/110						
BG	$0^{a,A}$	$0^{a,A}$	$0.05^{a,A}$ (0–0.20)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)	$0.13^{a,A}$ (0.03–0.31)
XLDT	$0.10^{a,A}$ (0.01–0.27)	$0^{a,A}$	$0.05^{a,A}$ (0–0.20)	$0.18^{a,A}$ (0.06–0.36)	$0.09^{a,A}$ (0.01–0.25)	$0.13^{a,A}$ (0.03–0.31)
EF18	$0.10^{a,A}$ (0.01–0.27)	$0^{a,A}$	$0.15^{a,A}$ (0.04–0.34)	$0.18^{a,A}$ (0.06–0.36)	$0.09^{a,A}$ (0.01–0.25)	$0.22^{a,A}$ (0–0.42)
MC	$0^{a,A}$	$0^{a,A}$	$0.05^{a,A}$ (0–0.20)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)	$0.13^{a,A}$ (0.03–0.31)
S. Gallinarum 88/322						
BG	$0.10^{a,A}$ (0.01–0.27)	$0^{a,A}$	$0^{a,A}$	$0.18^{a,A}$ (0.06–0.36)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
XLDT	$0.15^{a,A}$ (0.04–0.34)	$0^{a,A}$	$0.15^{a,A}$ (0.04–0.34)	$0.22^{a,A}$ (0.03–0.31)	$0.09^{a,A}$ (0.01–0.25)	$0.22^{a,A}$ (0.03–0.341)
EF18	$0.20^{a,B}$ (0.06–0.39)	$0^{b,A}$	$0.20^{a,B}$ (0.06–0.39)	$0.27^{a,A}$ (0.12–0.47)	$0.09^{a,A}$ (0.01–0.25)	$0.27^{a,A}$ (0.12–0.47)
MC	$0.10^{a,A}$ (0.01–0.27)	$0^{a,A}$	$0^{a,A}$	$0.18^{a,A}$ (0.06–0.36)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
S. Gallinarum 80/111						
BG	$0.05^{a,A}$ (0–0.20)	$0^{a,A}$	$0^{a,A}$	$0.13^{a,A}$ (0.03–0.31)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
XLDT	$0.10^{a,A}$ (0.01–0.27)	$0^{a,A}$	$0^{a,A}$	$0.18^{a,A}$ (0.06–0.36)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
EF18	$0.05^{a,A}$ (0.01–0.20)	$0^{a,A}$	$0^{a,A}$	$0.13^{a,A}$ (0.03–0.31)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
MC	$0.15^{a,A}$ (0.04–0.34)[a]	$0^{a,A}$	$0^{a,A}$	$0.22^{a,A}$ (0.09–0.42)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
S. Gallinarum 81/86						
BG	$0.40^{a,A}$ (0.16–0.59)	$0^{b,A}$	$0^{b,A}$	$0.45^{a,A}$ (0.22–0.64)	$0.09^{b,A}$ (0.01–0.25)	$0.09^{b,A}$ (0.01–0.25)
XLDT	$0.35^{a,A}$ (0.12–0.54)	$0^{b,A}$	$0^{b,A}$	$0.40^{a,A}$ (0.18–0.59)	$0.09^{b,A}$ (0.01–0.25)	$0.09^{b,A}$ (0.01–0.25)
EF18	$0.35^{a,A}$ (0.12–0.54)	$0^{b,A}$	$0^{b,A}$	$0.40^{a,A}$ (0.18–0.59)	$0.09^{b,A}$ (0.01–0.25)	$0.09^{b,A}$ (0.01–0.25)
MC	$0.35^{a,A}$ (0.12–0.54)	$0^{b,A}$	$0.05^{b,A}$ (0–0.20)	$0.40^{a,A}$ (0.18–0.59)	$0.09^{b,A}$ (0.01–0.25)	$0.13^{b,A}$ (0.03–0.31)
S. Gallinarum 03/121						
BG	$0.15^{a,A}$ (0.04–0.34)	$0^{a,A}$	$0^{a,A}$	$0.22^{a,A}$ (0.09–0.42)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
XLDT	$0.15^{a,A}$ (0.04–0.34)	$0^{a,A}$	$0^{a,A}$	$0.22^{a,A}$ (0.09–0.42)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)
EF18	$0.15^{a,A}$ (0.04–0.34)	$0.10^{a,A}$ (0.06–0.36)	$0^{a,A}$	$0.22^{a,A}$ (0.09–0.42)	$0.18^{a,A}$ (0.01–0.27)	$0.09^{a,A}$ (0.01–0.25)
MC	$0.15^{a,A}$ (0.04–0.34)	$0^{a,A}$	$0^{a,A}$	$0.22^{a,A}$ (0.09–0.42)	$0.09^{a,A}$ (0.01–0.25)	$0.09^{a,A}$ (0.01–0.25)

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

[2]BG = brilliant green agar; XLDT = xylose lysine desoxicholate agar with tergitol 4; EF18 agar is from Acumedia (Lansing, MI); MC = MacConkey agar.

heat. In our study, each *Salmonella* strain was subject to 60°C at different times, and we could recover all motile strains. On the other hand, non-motile *Salmonella* strains, although belonging to the same biovar, showed different heat injury (from 52 to 90%), when they were subject to 60°C for 2 min, and it was very difficult to isolate them. However, *Salmonella* detection was better by PCR and many PCR positives could not be confirmed by *Salmonella* isolation in that case.

Andrews (1986) recommends that any method to recover damaged organisms should include a resuscitative, or a repair, process that will restore the injured

Table 9. Positive predictive value (PPV) and negative predicitve value (NPV) of selective plating media in tetrathionate (TT) and modified semisolid Rappaport-Vassiliadis (MSRV) methods for motile *Salmonella* strains[1]

	PPV			NPV		
Strain and medium[2]	TT first	TT sixth	MSRV	TT first	TT sixth	MSRV
Salmonella Enteritidis PT1						
BG	$1^{a,A}$ (0.90–0.99)	$1^{a,A}$ (0.80–0.99)	$1^{a,A}$ (0.91–0.99)	$0.50^{a,A}$ (0.14–0.85)	$0.22^{a,A}$ (0.04–0.52)	$1^{b,A}$ (0.51–0.98)
XLDT	$1^{a,A}$ (0.91–0.99)	$1^{a,A}$ (0.90–0.99)	$1^{a,A}$ (0.91–0.99)	$0.67^{ab,A}$ (0.22–0.95)	$0.50^{a,A}$ (0.14–0.85)	$1^{b,A}$ (0.51–0.98)
EF18	$1^{a,A}$ (0.91–0.99)	$1^{a,A}$ (0.89–0.99)	$1^{a,A}$ (0.91–0.99)	$0.67^{ab,A}$ (0.22–0.95)	$0.40^{a,A}$ (0.10–0.76)	$1^{b,A}$ (0.51–0.98)
MC	$1^{a,A}$ (0.91–0.99)	$1^{a,A}$ (0.80–0.99)	$1^{a,A}$ (0.91–0.99)	$0.67^{ab,A}$ (0.22–0.95)	$0.22^{a,A}$ (0.04–0.52)	$1^{b,A}$ (0.51–0.98)
Salmonella Typhimurium 06/11						
BG	$1^{a,A}$ (0.94–0.99)	$1^{a,A}$ (0.94–0.99)	$1^{a,A}$ (0.95–0.99)	$0.66^{a,A}$ (0.22–0.95)	$0.40^{a,A}$ (0.10–0.76)	$1^{a,A}$ (0.95–0.99)
XLDT	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.29–0.99)	$1^{a,B}$ (0.29–0.99)	$1^{a,A}$ (0.95–0.99)
EF18	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.94–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.29–0.99)	$0.50^{b,C}$ (0.14–0.85)	$1^{a,A}$ (0.95–0.99)
MC	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.95–0.99)	$1^{a,A}$ (0.29–0.99)	$0.25^{b,D}$ (0.05–0.56)	$1^{a,A}$ (0.95–0.99)
Salmonella Kentuky CUB 19/08						
BG	$1^{a,A}$ (0.90–0.99)	$1^{a,A}$ (0.89–0.99)	$1^{a,A}$ (0.93–0.99)	$0.25^{a,A}$ (0.07–0.60)	$0.22^{a,A}$ (0.04–0.52)	$0.66^{a,A}$ (0.22–0.95)
XLDT	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.93–0.99)	$0.66^{a,A}$ (0.22–0.95)	$0.50^{a,A}$ (0.14–0.85)	$1^{a,A}$ (0.29–0.99)
EF18	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.93–0.99)	$0.66^{a,A}$ (0.22–0.95)	$0.50^{a,A}$ (0.14–0.85)	$0.66^{a,A}$ (0.22–0.95)
MC	$1^{a,A}$ (0.93–0.99)	$1^{a,A}$ (0.90–0.99)	$1^{a,A}$ (0.93–0.99)	$0.50^{a,A}$ (0.14–0.85)	$0.25^{a,A}$ (0.07–0.60)	$0.66^{a,A}$ (0.22–0.95)
Salmonella Livingstone CUB 52/10						
BG	$1^{a,A}$ (0.67–0.99)	$1^{a,A}$ (0.02–0.97)	$1^{a,A}$ (0.93–0.99)	$0.13^{a,A}$ (0.02–0.35)	$0.11^{a,A}$ (0.02–0.31)	$0.66^{a,A}$ (0.22–0.95)
XLDT	$1^{a,A}$ (0.83–0.99)	IND[3]	$1^{a,A}$ (0.93–0.99)	$0.16^{a,A}$ (0.03–0.42)	$0.11^{a,A}$ (0.02–0.31)	$0.66^{a,A}$ (0.22–0.95)
EF18	$1^{a,A}$ (0.83–0.99)	IND	$1^{a,A}$ (0.93–0.99)	$0.16^{a,A}$ (0.03–0.42)	$0.11^{a,A}$ (0.02–0.31)	$0.66^{a,A}$ (0.22–0.95)
MC	$1^{a,A}$ (0.67–0.99)	IND	$1^{a,A}$ (0.93–0.99)	$0.13^{a,A}$ (0.02–0.35)	$0.11^{a,A}$ (0.02–0.31)	$1^{b,A}$ (0.29–0.99)

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

[A–D]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. Values in parentheses indicate a 95% CI for the respective parameter.

[2]BG = brilliant green agar; XLDT = xylose lysine desoxicholate agar with tergitol 4; EF18 agar is from Acumedia (Lansing, MI); MC = MacConkey agar.

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

cells to sound physiological condition before subjecting them to the severity of selective enrichment media. Wilson and Davies (1976) demonstrated that for both M-9 medium and lactose broth, less repair was observed with cells subjected to heat injury in the stationary phase than with cells injured in the exponential phase. Poultry feed samples were pre-enriched in TSBF medium in our study and *Salmonella* strains were in a stationary phase when they were heat injured. Suggested broth media for the pre-enrichment of samples for *Salmonellae* include buffered peptone water (**BPW**) and TSB (Gast, 2003). Cogan et al. (2001) reported that iron in the form of ferrous sulfate promotes the growth of gram-negative bacteria in eggs, and supplementation at levels of 35 mg/L in nonselective broth effectively promotes *Salmonella* isolation in raw eggs. The TSBF medium was not reported to be used in *Salmonella* isolation from poultry feed and for non-motile *Salmonella* strains before, although there are some substances present in poultry feed, like phytic acid, which binds iron, making it insoluble and, thus, unavailable as a nutritional factor (Bohn et al., 2008).

Because of the low levels of *Salmonella* potentially present in animal feed (Williams, 1981), low levels of *Salmonella* were added to the feed samples and the calculated Ac and Se data were lower in non-motile than motile *Salmonella* strains. Non-motile *Salmonella* strains could be recovered from TSBF, and most of them were detected only in high concentrations (10^4–10^6 cfu/25 g) by the MSRV or TT method, even though most of the strains had a heat injury of 78% or less, measured in MC.

Although it was reported that non-motile *Salmonella* represent <1% of the isolates from animal feeds (Poppe et al., 2004), different studies (Williams, 1981; Cox et al., 1983; De Franceschi et al., 1989; del Pozo Saenz et

Table 10. Positive predictive value (PPV) and negative predictive value (NPV) of selective plating media in tetrathionate (TT) and modified semisolid Rappaport-Vassiliadis (MSRV) methods for *Salmonella* Pullorum and *Salmonella* Gallinarum strains[1]

	PPV			NPV		
Strain and medium[2]	TT first	TT sixth	MSRV	TT first	TT sixth	MSRV
S. Pullorum 90/142						
BG	IND[3]	IND	IND	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
XLDT	1[A] (0.02–0.97)	IND	IND	0.09[a,A] (0.01–0.26)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
EF18	1[A] (0.02–0.97)	IND	IND	0.09[a,A] (0.01–0.26)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
MC	IND	IND	IND	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
S. Gallinarum 93/110						
BG	IND	IND	1[A] (0.02–0.97)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.26)
XLDT	1[a,A] (0.67–0.99)	IND	1[a,A] (0.02–0.97)	0.09[a,A] (0.01–0.26)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.26)
EF18	1[a,A] (0.67–0.99)	IND	1[a,A] (0.67–0.99)	0.09[a,A] (0.01–0.26)	0.09[a,A] (0.01–0.25)	0.10[a,A] (0.01–0.28)
MC	IND	IND	1[A] (0.02–0.97)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.26)
S. Gallinarum 88/322						
BG	1[A] (0.51–0.98)	IND	IND	0.11[a,A] (0.01–0.27)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
XLDT	1[a,A] (0.67–0.99)	IND	1[a,A] (0.67–0.99)	0.11[a,A] (0.01–0.28)	0.09[a,A] (0.01–0.25)	0.10[a,A] (0.01–0.28)
EF18	1[a,A] (0.75–0.99)	IND	1[a,A] (0.75–0.99)	0.11[a,A] (0.01–0.30)	0.09[a,A] (0.01–0.25)	0.11[a,A] (0.01–0.30)
MC	1[A] (0.51–0.98)	IND	IND	0.11[a,A] (0.01–0.27)	0.09[a,A] (0.01–0.25)	IND
S. Gallinarum 80/111						
BG	1[A] (0.02–0.97)	IND	IND	0.09[a,A] (0.01–0.26)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
XLDT	1[A] (0.51–0.98)	IND	IND	0.10[a,A] (0.01–0.27)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
EF18	1[A] (0.02–0.97)	IND	IND	0.09[a,A] (0.01–0.26)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
MC	1[A] (0.02–0.97)	IND	IND	0.10[a,A] (0.01–0.28)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
S. Gallinarum 81/86						
BG	1[A] (0.87–0.99)	IND	IND	0.14[a,A] (0.02–0.37)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
XLDT	1[A] (0.86–0.99)	IND	IND	0.13[a,A] (0.02–0.35)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
EF18	1[A] (0.86–0.99)	IND	IND	0.13[a,A] (0.02–0.35)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
MC	1[a,A] (0.86–0.99)	IND	1[a,A] (0.02–0.97)	0.13[a,A] (0.02–0.35)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.26)
S. Gallinarum 03/121						
BG	1[a,A] (0.67–0.99)	IND	IND	0.10[a,A] (0.01–0.28)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
XLDT	1[A] (0.67–0.99)	IND	IND	0.10[a,A] (0.01–0.28)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)
EF18	1[a,A] (0.67–0.99)	1[a] (0.51–0.98)	IND	0.10[a,A] (0.01–0.28)	0.10[a,A] (0.01–0.27)	0.09[a,A] (0.01–0.25)
MC	1[A] (0.67–0.99)	IND	IND	0.10[a,A] (0.01–0.28)	0.09[a,A] (0.01–0.25)	0.09[a,A] (0.01–0.25)

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

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

[2]BG = brilliant green agar; XLDT = xylose lysine desoxicholate agar with tergitol 4; EF18 agar is from Acumedia (Lansing, MI); MC = MacConkey agar.

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

al., 2001) reported only the isolation of motile *Salmonella* from poultry feeds. The NPV for the non-motile *Salmonella* strains were very low for the bacteriological methods studied in our assays. This could be explained because it is possible that those strains could be less competitive against microflora present in the sample.

Kuijpers et al. (2010) reported that the number of positive isolations is more influenced by the choice of

Table 11. Kappa coefficient values showing agreement between modified semisolid Rappaport-Vassiliadis (MSRV), tetrathionate (TT), and PCR methods for poultry feed samples

Salmonella strains	Comparison between methods	Kappa coefficient
Motile	TT-MSRV	0.65*
	MSRV-PCR	0.61*
	PCR-TT	0.57*
Non-motile	TT-MSRV	0.20*
	MSRV-PCR	0.56*
	PCR-TT	0.37*

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

the selective enrichment medium than by the choice of the plating-out medium. van Schothorst et al. (1977) showed that growth of competitors in TT broth may decrease the inhibitory effect of the medium for *Salmonella* isolation. Delayed secondary enrichment, in which selective enrichment broth cultures are held for an additional 5 d at room temperature to allow *Salmonellae* an extended opportunity to grow to detectable levels, has been found to improve the recovery of *Salmonellae* from poultry diagnostic and environmental samples (Waltman et al., 1991) Furthermore, it was reported that a longer incubation time than 24 h was more important (more positive results after 48 h) for selective enrichment medium (Edel and Kampelmacher, 1974; Kuijpers et al., 2008). We incubated TT broth at 35 ± 2°C for 6 d (TT method) and we found better results for *Salmonella* isolation with TT first than TT sixth. In the case of non-motile *Salmonella* strains, they were only recovered in TT first in high-spiking samples. Therefore, 1-d incubation of this broth was enough to isolate *Salmonella*. On the other hand, it is known that MSRV is unable to detect non-motile *Salmonella* bacteria (Poppe et al., 2004), but 2 *S.* Gallinarum strains could only be isolated from high-spiking samples in the MSRV method in our study.

The type of sample, and especially the composition of the background flora, is of considerable importance for the efficiency of a specific plating media. Growth of non-*Salmonella* may disturb the reading of plates, because well-isolated colonies of *Salmonella* may not be obtained (Busse, 1995). Petersen (1997) reported that the combination of the 2 media clearly would decrease the number of FN results, although with a little extra cost. We used 4 selective plating media in our assay. Although no significant differences were found among selective agars used in our study, MC and BG agar showed higher numbers of FN than did XLDT and EF-18 in the MSRV and TT methods. Some samples resulted in unusual colors and appearance of colonies in MC and BG media.

Comparing results between studies for the evaluation of PCR is also made difficult by the lack of the standard protocols for sample handling and enrichment and varying quality of reagents and equipment (Myint et al., 2006). Löfström et al. (2004) showed that various animals feeds tested were found to be highly inhibitory to PCR in samples with pre-enrichment in BPW and they concluded that the biological composition of the PCR mixture, including the sample to be analyzed, should be considered when optimizing the PCR protocol. Furthermore, they found a worse detection level for *Salmonella* than we reported in our study. Bansal et al. (2006) investigated the reliability and application of a PCR-based assay that can be used after BPW culture enrichment for the routine examination of naturally contaminated food for *Salmonella*. They found that PCR results were in perfect agreement with the results of the standard culture methods. However, the PCR assay was extremely rapid, and results could be obtained within 4 h of testing of enrichment broths. We had PCR positives for all minimum-load bacteria tested, except for *S.* Livingstone, and non-motile serotypes. We used the TSBF medium as a pre-enrichment step and, in general, similar to those of Bansal et al. (2006), PCR results were in agreement with the results of the culture methods used for most motile *Salmonella* strains tested. However, PCR had better results than the MSRV and TT methods for non-motile *Salmonella* strains. On the other hand, Koyuncu et al. (2010) found that PCR-based methods performed similarly to culture-based methods, with respect to Se and Sp, but they only used motile *Salmonella* strains. Nevertheless, PCR-based methods cannot presently replace culture-based methods when typing information is required for tracing studies or epidemiological investigations.

The TT, MSRV, and PCR methods are similar in terms of Ac, Se, Sp, PPV, and NPV for different motile *Salmonella* strains in poultry feed. For non-motile *Salmonella* strains, the use of the PCR method improves the same parameters, described before, in this matrix. The difference in detection levels obtained with the methods used for motile and non-motile *Salmonella* strains and the difficulty of detecting these last strains represents a potential problem when a poultry feed sample is considered negative for the presence of *Salmonella*.

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