Comparison of 3 culture methods and PCR assays for *Salmonella gallinarum* and *Salmonella pullorum* detection in poultry feed

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ABSTRACT To detect *Salmonella gallinarum* or *Salmonella pullorum* in artificially contaminated poultry feed, 9 culture combinations were compared, including 3 preenrichment/enrichment methods (tryptic soy broth plus ferrous sulfate/tetrathionate Hajna, tryptic soy broth plus ferrous sulfate/selenite cystine broth, and Salmosyst) in combination with 3 selective agars (xylose lysine desoxicholate agar added with tergitol 4, EF-18, and Önöz), respectively. Additionally, a single PCR technique was applied combined with 2 different preenrichment media (tryptic soy broth plus ferrous sulfate and Salmosyst). The specificity and positive predictive value were 1 for all methods. There were some differences among *Salmonella* strains for sensitivity and accuracy in the culture and Salmosyst-PCR methods. The sensitivity and accuracy values were less than 0.60 and 0.64, respectively, whereas the negative predictive values were between 0.12 and 0.23. Two PCR methods did not show any difference in the parameters of performance evaluated. Kappa coefficients showed good agreement between both methods. None of the culture combinations was able to detect *S. gallinarum* or *S. pullorum* when the inoculum was less than 3×10^2 cfu/25 g, except the Salmosyst broth method, which could recover *S. gallinarum* from 3×10^1 cfu/25 g onward. Overall, there were differences in the detection limits among the strains and methods used. In general, the 3 selective plating media did not show any significant difference in the parameters of performance studied for each strain. On the other hand, the agreements were slight to fair when culture methods were compared among them and with both PCR methods. The differences in the detection levels that were obtained using these methods and the difficulty in detecting *S. gallinarum* or *S. pullorum* in feed represent a potential problem when a poultry feed sample is considered to be negative. It is highly recommended to use at least 2 methods to increase the chances of detecting *S. gallinarum* or *S. pullorum* in poultry feed.

Key words: *Salmonella gallinarum*, *Salmonella pullorum*, poultry feed, culture method, PCR

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INTRODUCTION

Avian *Salmonella* infections are important as both a cause of clinical disease in poultry and as a source of foodborne transmission of disease to humans. Host-adapted salmonellae are responsible for pullorum disease (*Salmonella pullorum*) and fowl typhoid (*Salmonella gallinarum*; Waltman and Gast, 2008). These avian-adapted serotypes (nonmotile) lack flagella and associated motility (Guard-Petter, 2001).

Fowl typhoid is an acute or chronic septicemic disease that usually affects adult birds, although birds of all ages may be susceptible. Pullorum disease is an acute systemic disease more common in young birds (Barrow and Freitas Neto, 2011). Horizontal and vertical transmissions are both important in the epidemiology of fowl typhoid and pullorum disease. Birds can become chronic carriers for both organisms, passing them to their offspring through eggs. Horizontal transmission occurs via the respiratory and oral routes. Birds may ingest bacteria after environmental contamination or during cannibalism. Many poultry feeds carry *Salmonella* that are consumed in large numbers by birds eating these feeds. These organisms multiply rapidly in the intestinal tract, and high populations become established in carrier animals. Birds can remain carriers for long

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periods of time, which poses a most important problem in poultry production (Williams, 1981).

In general, the susceptibility of *S. pullorum* and *S. gallinarum* is about the same as that of members of the paratyphoid groups. Both biovars may survive for several years in a favorable environment, but they are less resistant than paratyphoid salmonellae to heat, chemicals, and adverse environmental factors (Shivaprasad, 2003). *Salmonella* control efforts are complicated by the sporadic and uneven distribution of these *Salmonella* in feed, as well as the fact that conventional detection methods do not allow quantification (Jones, 2011). Besides, the few bacterial cells that are present in the feed are often damaged, making the detection even more difficult (Jones and Richardson, 2004). Killing *Salmonella* may involve thermal processing (pelleting) or chemical addition. Pelleting reduces contamination, but may not completely eliminate *Salmonella* due to limitations of the process or recontamination after thermal processing. Although pelleting systems have been reported to reduce *Salmonella* isolation rates, such systems largely rely on steam addition for pathogen destruction, and steam adds moisture to pelleted feeds. Although properly functioning pelleting systems remove most added moisture via pellet coolers, contamination in coolers may be elevated because condensation in the interior surfaces increases moisture, which encourages *Salmonella* growth (Jones, 2011).

It was reported that nonmotile isolates represent <1% of the isolates from animal feeds (Poppe et al., 2004), and transmission through feed contamination by *S. gallinarum/pullorum* appears to be of minor importance (Shivaprasad, 2003). A study showed that *S. gallinarum* could survive for 29 d in broiler mash, long enough to be of great significance in the recurrence and transmission of fowl typhoid (Orr and Moore, 1953). In general, different studies (Williams, 1981; Cox et al., 1982; De Franceschi et al., 1989; del Pozo Saenz et al., 2001) only reported the isolation of motile *Salmonella* from poultry feeds, although the lack of isolation of *S. gallinarum/S. pullorum* may be due to the application of culture methods unable to detect nonmotile salmonellae. Furthermore, recently, it was described that the difference between the detection levels obtained with the methods designed for the isolation of motile and *S. gallinarum/S. pullorum* may be caused by the difficulty in detecting nonmotile strains, which represents a potential problem when a poultry feed sample is diagnosed as *Salmonella* false-negative (Soria et al., 2011). To know the ability to detect *Salmonella gallinarum* or *Salmonella pullorum* in artificially contaminated poultry feed samples, 9 culture combinations were compared including, 3 preenrichment/enrichment methods [tryptic soy broth (**TSB**) plus ferrous sulfate (**TSBF**)/ tetrathionate Hajna (**TTH**), TSBF/selenite cystine (**SC**) broth, and Salmosyst (**Sst**)] in combination with 3 selective agars (xylose lysine desoxicholate agar added with tergitol 4, EF-18, and Önöz), respectively. Additionally, a single PCR technique was applied combined with 2 different preenrichment media (TSBF and Sst). Furthermore, the accuracy, sensitivity, specificity, positive predictive value, and negative predictive value of each method and the agreement among these methods were investigated.

MATERIALS AND METHODS

Feed Material

Feed samples were provided by egg-laying hen farms from the state of Entre Rios, Argentina. Before artificial contamination, each sample was cultured into the tetrathionate method (Soria et al., 2011) to ensure the absence of *Salmonella* spp. This method was used in this case because it is routinely used in our laboratory. Furthermore, total bacteria and *Enterobacteriaceae* counts of feed samples were respectively determined onto tryptic soy agar (**TSA**; Acumedia, Lansing, MI) and MacConkey agar (**MC**, Acumedia) using the surface viable count method (Miles et al., 1938).

Salmonella Strains and Culture

As summarized in Table 1, a total of 6 nonmotile *Salmonella* strains were selected for the assay; 4 strains were *S. gallinarum* and 2 were *S. pullorum*. All *Salmonella* strains were cultured from nutrient agar (Acumedia) grown for 24 h in TSB (Merck, Darmstadt, Germany) at 37°C. Purity of cultures was confirmed by streaking onto MC and TSA. The number of viable microorganisms was estimated using the surface viable count method (Miles et al., 1938) and was expressed as cfu per milliliter. 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 attained stability at 60°C, the tubes were inoculated with 0.5 mL of each *S. gallinarum* or *S. pullorum* culture (10^8 cfu/mL) and incubated for 2 min (Table 2). After incubation, heat injury was determined by plating ap-

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

Salmonella strain	Source
Salmonella gallinarum 93/110	Chicken, EEA INTA Balcarce[1]
S. gallinarum 80/111	Chicken, EEA INTA Balcarce
S. gallinarum 81/86	Chicken, EEA INTA Balcarce
S. gallinarum 88/322	Chicken, EEA INTA Balcarce
S. pullorum ATCC 13036	American Type Culture Collection
S. pullorum 90/142	Chicken, EEA INTA Balcarce

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Table 2. Heat injury (%) of each nonmotile *Salmonella* strain in MacConkey agar after incubation at 60° for 2 min

Salmonella strain	Heat injury (%)
Salmonella gallinarum 93/110	95.0 ± 3.3^{b}
S. gallinarum 80/111	88.1 ± 3.8^{ab}
S. gallinarum 81/86	70.3 ± 4.2^{a}
S. gallinarum 88/322	67.0 ± 9.3^{a}
S. pullorum ATCC 13036	87.6 ± 0.8^{ab}
S. pullorum 90/142	70.8 ± 2.5^{a}

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

propriately diluted suspensions onto nonselective and selective plates. Other tubes from the original resuspended pellet cells of each strain were used as unheated controls. Tryptic soy agar (Acumedia) was used as the nonselective plate to enumerate both injured and noninjured cells, whereas MC (Acumedia) was used as the selective plate for enumeration of noninjured cells. The heat injury (%) was expressed as the proportion of injured cells in the total population (Liao and Fett, 2003):

$$\text{heat injury (\%)} = \left(\frac{\text{cfu/mL on TSA} - \text{cfu/mL on MC}}{\text{cfu/mL on TSA}}\right) \times 100.$$

Preparation of S. gallinarum and S. pullorum Inocula in Poultry Feed Samples

Twenty-five grams of *Salmonella*-free poultry feed material was introduced into each sterile plastic bag (total 291). Each nonmotile *Salmonella* strain was grown and heat-injured as described above. After that, serial dilutions were made in peptone water (0.1%) to inoculate 8 bacterial dilutions, from 3×10^0 to 9×10^7 cfu/25 g of each *Salmonella* strain. All treatments were performed in triplicate, so 3 samples of each dilution dose (3 bags per dilution) for each nonmotile *Salmonella* strain were carried out in every assay. Altogether, 288 spiked samples (6 *Salmonella* strains × 8 dilutions × 3 repetitions × 2 preenrichments) were constructed in the study. For each trial set, 3 nonseeded samples were added as negative controls.

Recovery of S. gallinarum/S. pullorum Strains from Poultry Feed

Figure 1 shows a flowchart diagram for detection of *S. gallinarum/S. pullorum* in feed using TTH, SC, or Sst methods. *Salmonella*-free poultry feeds contaminated with different concentrations of *S. gallinarum* or *S. pullorum* strains were preenriched in 225 mL of TSB (Merck) supplemented with ferrous sulfate (TSBF, 35 mg of ferrous sulfate added to 1,000 mL of TSB) or Salmosyst broth (**Sb**, Merck). The mixture was incubated at 35 ± 2°C for 24 h. One milliliter of each sample, previously incubated in TSBF broth, was transferred into 10 mL of tetrathionate Hajna broth base (Difco Laboratories, Detroit, MI) added to novobiocin (Sigma, Steinheim, Germany) 40 mg/mL, brilliant green (Sigma) 0.1%, and iodine solution 2% (5 g of iodine, 8 g of potassium iodide, 40 mL of distilled water; TTH method), or SC broth (Acumedia, SC method) added with brilliant green (Sigma) according to Stokes and Osborne (1955) and incubated at 35 ± 2°C for 24 h. On the other hand, 10 mL from Sb cultures was transferred to sterile tubes and added to a Salmosyst selective supplement tablet (Merck, Sst method). After that, samples were incubated at 35 ± 2°C for 24 h. Then, 1 loopful of each broth was streaked onto xylose lysine desoxicholate agar (Oxoid, Basingtoke, Hampshire, UK) added with tergitol 4 (Sigma, 4.6 mL/L, **XLDT**), EF-18 (Acumedia), and Önöz agar (Merck) and incubated at 35 ± 2°C for 24 h. Colonies of presumptive *Salmonella* were inoculated onto triple-sugar iron agar (Acumedia) and lysine iron agar (Merck). Further confirmation was done based on an *ortho*-nitrophenyl-β-galactoside test and agglutination reaction with somatic (O) polivalent antisera (Becton Dickinson and Company, Sparks, MD).

Pre-PCR Sample Preparation

For detection of *Salmonella* from poultry feed samples, bacterial cells were recovered from 1 mL of TSBF or Sb preenrichment broth (Figure 1) by centrifugation at 4,000 × *g* for 15 min at 4°C and washed twice with sterile demineralized water. The pellet was suspended in 500 μL of sterile demineralized water, and DNA was released by heating at 100°C for 10 min on a hot block (Labnet D1100, Labnet International Inc., Edison, NJ). The cellular debris was pelleted by centrifugation at 9,300 × *g* for 1 min, and the clear supernatant fluid containing nucleic acids was fractionated in Eppendorf tubes and conserved at −70°C until it was used in subsequent PCR assays.

PCR Assay

The extracted DNA samples (5 μL) were amplified in an optimized 25-μL reaction mixture consisting of 0.25 μL of each primer 0.1 *M* mol/L, 2.5 μL of buffer 1x (Fermentas Inc., Hanover, MD), 1.5 μL of $MgCl_2$ 1.5 mmol/L (Fermentas Inc.), 0.5 μL of each dNTP 0.2 mmol/L (Fermentas Inc.), 0.2 μL of *Taq* DNA polymerase (Fermentas Inc.) 5 U/μL, and double-distilled water to 25 μL. The reaction mixture was incubated in a programmable DNA thermal cycler (Mastercycler Gradient, Eppendorf, Germany). *Salmonella* genus-specific primers 139 and 141 (Operon Biotechnologies GmbH, Cologne, Germany) based on the *invA* gene of *Salmonella* were used in the PCR assay. The primers had the following nucleotide sequences: (5′→3′) GTGAAATTATCGCCACGTTCGGGCAA and TCATCGCACCGTCAAAGGAACC, respectively. A reagent blank containing all the components of the reaction

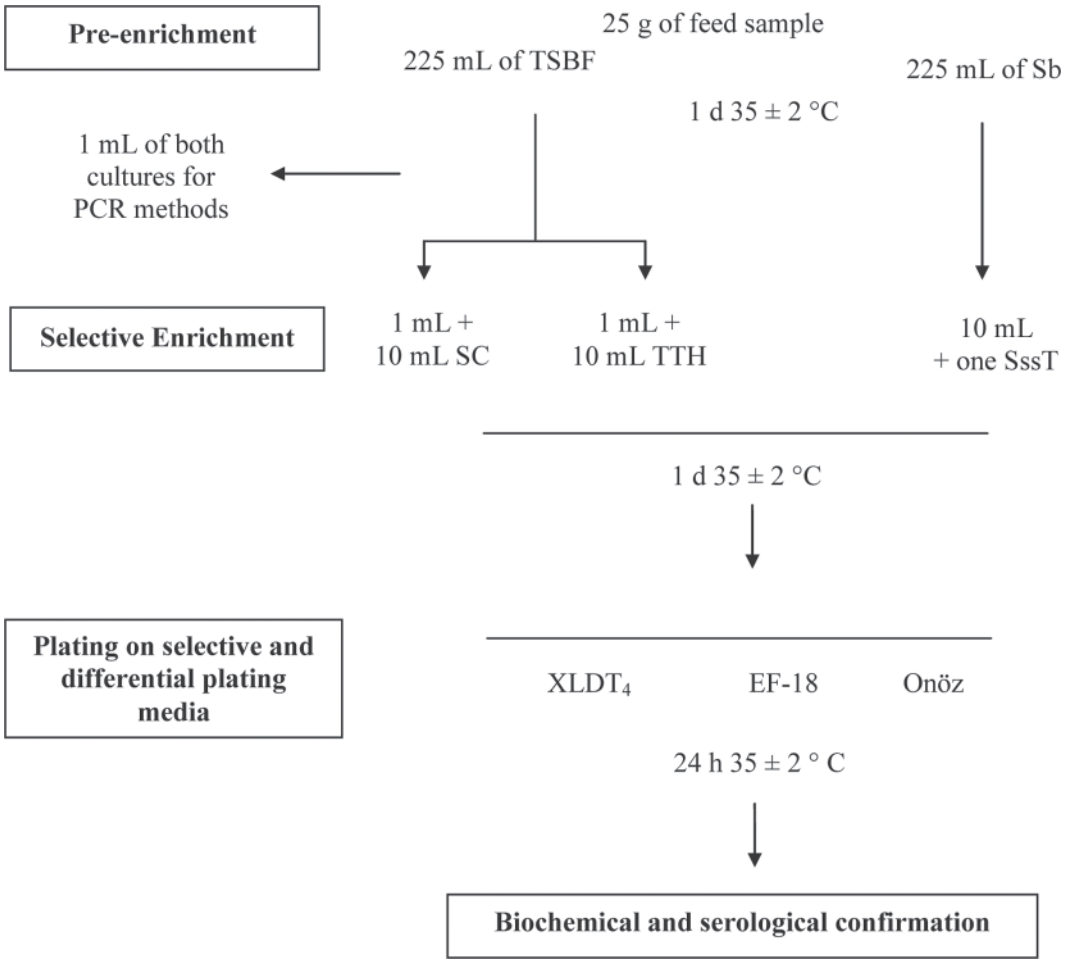

Figure 1. Flowchart for the detection of nonmotile *Salmonella* in poultry feed samples by tetrathionate Hajna broth (TTH), selenite cystine broth (SC), Salmosyst, and PCR methods. TSBF = tryptic soy broth with ferrous sulfate; Sb = Salmosyst broth; SssT = Salmosyst selective supplement tablet; $XLDT_4$ = xylose lysine desoxicholate agar with tergitol 4; EF-18 agar is from Acumedia (Lansing, MI).

mixture with the exception of template DNA (which was replaced by sterile distilled water) was included with every PCR assay. Negative and positive DNA controls were also included, which were prepared from *Citrobacter* sp. and *Salmonella* sp., respectively. 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 were loaded onto 2.0% of agarose gel in 0.5 × TBE buffer at 120 V/cm for 1 h. The gel was stained with GelRedTM Nucleic Acid Gel Stain (Bioutium Inc., Hayward, CA), and electrophoresed products were visualized with UV transilluminator (model M-20, UVP Inc., Upland, CA). A 100-bp ladder (PB-L Productos Bio-Lógicos, Buenos Aires, Argentina) was used as molecular weight marker.

Analysis of Performance Criteria

The detection limit of the methods was considered and defined as the lowest concentration (cfu/25 g) of the *Salmonella* strain inoculum that could be recovered. The accuracy, specificity, sensitivity, positive predictive value, and negative predictive value were calculated for each method (Soria et al., 2011). Assumptions were that all nonspiked samples were negative for *S. gallinarum* or *S. pullorum* and only those samples spiked with nonmotile *Salmonella* were true positive (**TP**). Samples that were positive on at least one selective agar plate (XLDT, EF-18, Önöz agar) were considered positive. Based on this, the accuracy, sensitivity, specificity, positive predictive value, and negative predictive value rates were obtained by using the following definitions: a sample was defined as TP when *Salmonella* was detected in a sample where *Salmonella* had been added; a sample was defined as true negative when *Salmonella* was not detected in a sample where *Salmonella* had not been added; a sample was defined as false positive when *Salmonella* was detected in a sample where *Salmonella* had not been added; and a sample was defined as false

Table 3. Sensitivity (Se), accuracy (Ac), and negative predictive value (NPV) of tetrathionate Hajna (TTH), selenite cystine (SC), and tryptic soy broth with ferrous sulfate–PCR (TSBF-PCR) methods for each *Salmonella* strain in artificially contaminated poultry feed samples[1]

	Se			Ac			NPV		
Strain	TTH	SC	TSBF-PCR	TTH	SC	TSBF-PCR	TTH	SC	TSBF-PCR
Salmonella gallinarum 93/110	$0.17^{a,A}$ (0.06–0.36)	$0.59^{b,AB}$ (0.24–0.61)	$0.38^{ab,A}$ (0.16–0.55)	$0.26^{a,AB}$ (0.08–0.41)	$0.48^{a,A}$ (0.27–0.65)	$0.44^{a,A}$ (0.20–0.57)	$0.13^{a,A}$ (0.03–0.30)	$0.18^{a,A}$ (0.04–0.38)	$0.17^{a,A}$ (0.04–0.37)
S. gallinarum 80/111	$0.33^{a,AB}$ (0.17–0.53)	$0.54^{a,A}$ (0.34–0.72)	$0.31^{a,A}$ (0.13–0.50)	$0.41^{a,AC}$ (0.20–0.57)	$0.59^{a,A}$ (0.38–0.75)	$0.38^{a,A}$ (0.20–0.57)	$0.16^{a,A}$ (0.04–0.35)	$0.21^{a,A}$ (0.06–0.45)	$0.14^{a,A}$ (0.03–0.32)
S. gallinarum 81/86	$0.04^{a,A}$ (0.00–0.20)	$0.13^{a,CD}$ (0.04–0.31)	$0.38^{b,A}$ (0.16–0.55)	$0.15^{a,B}$ (0.00–0.27)	$0.22^{b,B}$ (0.05–0.36)	$0.44^{b,A}$ (0.23–0.61)	$0.12^{a,A}$ (0.03–0.27)	$0.13^{a,A}$ (0.03–0.29)	$0.17^{a,A}$ (0.04–0.37)
S. gallinarum 88/322	$0.54^{a,B}$ (0.34–0.72)	$0.58^{a,A}$ (0.38–0.75)	$0.42^{a,A}$ (0.20–0.59)	$0.59^{a,C}$ (0.38–0.75)	$0.63^{a,A}$ (0.42–0.79)	$0.48^{a,A}$ (0.27–0.65)	$0.21^{a,A}$ (0.06–0.45)	$0.23^{a,A}$ (0.06–0.48)	$0.18^{a,A}$ (0.04–0.38)
Salmonella pullorum 90/142	$0.04^{a,A}$ (0.00–0.20)	$0.25^{b,BC}$ (0.12–0.45)	$0.21^{ab,A}$ (0.03–0.36)	$0.15^{a,B}$ (0.00–0.27)	$0.33^{a,B}$ (0.14–0.50)	$0.30^{a,A}$ (0.11–0.45)	$0.12^{a,A}$ (0.03–0.27)	$0.14^{a,A}$ (0.03–0.32)	$0.14^{a,A}$ (0.03–0.31)
S. pullorum ATCC 13036	$0.04^{a,A}$ (0.00–0.20)	$0.08^{a,D}$ (0.02–0.26)	$0.38^{b,A}$ (0.16–0.55)	$0.15^{a,B}$ (0.00–0.27)	$0.19^{a,B}$ (0.03–0.32)	$0.44^{b,A}$ (0.20–0.57)	$0.12^{a,A}$ (0.03–0.27)	$0.12^{a,A}$ (0.03–0.27)	$0.17^{a,A}$ (0.04–0.37)

[a,b]Values with different lowercase superscripts in the same row are significantly different ($P < 0.05$).
[A–D]Values followed by different uppercase superscripts in the same column are significantly different ($P < 0.05$).
[1]Values in parentheses indicate a 95% CI for the respective parameter. ATCC = American Type Culture Collection.

negative when *Salmonella* was not detected in a sample where *Salmonella* had been added.

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

Statistical Analysis

The differences in the mean values of heat injury (%) were evaluated by ANOVA. To compare the results of all assays, a hypothesis test for a difference of proportions was made. The accuracy, specificity, sensitivity, positive predictive value, and negative predictive value 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 Method from the National Technological University of Concepcion del Uruguay (Entre Ríos, Argentina, Project 25D041). The values reported defines the boundaries of an interval that, with 95% certainty, contains the true value of accuracy, specificity, sensitivity, positive predictive value, and negative predictive value. The results were only considered to be statistically different at $P < 0.05$. In relation to agreement, the 3 methods were treated as raters and the Z test was used to test the statistical significance of kappa coefficients.

RESULTS

Feed samples had an average of 3.8×10^5 cfu/g of total bacteria and 5.1×10^4 cfu/g of *Enterobacteriaceae*. On the other hand, all strains tested showed a heat injury rate between 0.67 and 0.95 (Table 2).

In relation to the performance of the methods, the specificity and positive predictive value were 1 for all methods studied (data not shown). Table 3 shows the sensitivity, accuracy, and negative predictive value values for the TTH, SC, and TSBF-PCR methods and the same parameters for Sst and Sst-PCR are shown in Table 4. There were some differences among *Salmonella* strains for sensitivity and accuracy in the culture and Salmosyst-PCR methods. The sensitivity and accuracy values for the methods were below than 0.60 and 0.64, respectively. The SC and Sst methods only showed higher sensitivity values than the TTH method for 2 strains (*S. gallinarum* 93/110 and *S. pullorum* 90/142). The negative predictive value showed values from 0.12 to 0.23 without any statistical difference among the

Table 4. Sensitivity (Se), accuracy (Ac), and negative predictive value (NPV) of Salmosyst and Salmosyst-PCR methods for each *Salmonella* strain in artificially contaminated poultry feed samples[1]

	Se		Ac		NPV	
Strain	Salmosyst	Salmosyst-PCR	Salmosyst	Salmosyt-PCR	Salmosyst	Salmosyst-PCR
Salmonella gallinarum 93/110	0.42[a,AB] (0.17–0.53)	0.58[a,A] (0.36–0.75)	0.40[a,AB] (0.20–0.57)	0.63[a,A] (0.42–0.79)	0.16[a,A] (0.04–0.35)	0.23[a,A] (0.06–0.48)
S. gallinarum 80/111	0.58[a,A] (0.38–0.75)	0.46[a,A] (0.31–0.71)	0.62[a,A] (0.42–0.79)	0.52[a,AB] (0.30–0.68)	0.13[a,A] (0.03–0.30)	0.19[a,A] (0.03–0.32)
S. gallinarum 81/86	0.25[a,B] (0.12–0.45)	0.33[a,AB] (0.13–0.50)	0.29[a,BC] (0.15–0.48)	0.41[a,AB] (0.20–0.57)	0.14[a,A] (0.03–0.32)	0.16[a,A] (0.04–0.35)
S. gallinarum 88/322	0.54[a,A] (0.34–0.72)	0.21[b,B] (0.03–0.36)	0.59[a,A] (0.38–0.75)	0.30[b,B] (0.11–0.45)	0.21[a,A] (0.06–0.45)	0.14[a,A] (0.03–0.31)
Salmonella pullorum 90/142	0.25[b,B] (0.12–0.45)	0.21[a,B] (0.03–0.36)	0.33[a,BC] (0.14–0.50)	0.30[a,B] (0.11–0.45)	0.14[a,A] (0.03–0.32)	0.14[a,A] (0.03–0.31)
S. pullorum ATCC 13036	0.04[a,C] (0.00–0.20)	0.21[a,B] (0.03–0.36)	0.15[a,C] (0.00–0.27)	0.30[a,B] (0.11–0.45)	0.12[a,A] (0.03–0.27)	0.14[a,A] (0.03–0.31)

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

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

[1]Values in parentheses indicate a 95% CI for the respective parameter. Salmosyst-PCR = PCR from Salmosyst broth culture. ATCC = American Type Culture Collection.

methods tested. In reference to PCR methods, there was no difference between the 2 methods assayed (data not shown). However, the sensitivity and accuracy were only better in TSBF-PCR than in TTH and SC for 2 and 1 *Salmonella* strains, respectively (Table 3).

The detection limits for the 5 methods are shown in Table 5. There were differences in the detection limit among the strains and methods used. None of the methods was able to detect these 6 nonmotile *Salmonella* strains when the inoculum concentration was less than 3×10^2 cfu/25 g, except the Sst method, which could recover *S. gallinarum* strain 80/111 from 3×10^1 cfu/25 g onward. For this method, the detection limit was registered between 3×10^1 and 3×10^7 cfu/25 g. On the other hand, this parameter was from 8×10^3 to 3×10^8 cfu/25 g for the TTH method and from 3×10^2 to 3×10^7 cfu/25 g for the SC method. In reference to PCR methods, the detection limit was between 9×10^3 and 3×10^6 cfu/25 g for PCR-TSBF and between 5×10^3 and 9×10^6 cfu/25 g for PCR-Sb.

For all *Salmonella* strains, in general, the 3 selective plating media did not show any significant difference among them in terms of sensitivity, accuracy, and negative and positive predictive value in any of the 3 culture methods studied (Table 6). Sensitivity and accuracy were less than 0.60 for XLDT, EF18, and Önöz agar. On the other hand, negative predictive value was from 0.11 to 0.21. Hence, the positive predictive value was 1 for 3 agar plates, except for some media in *S. pullorum* strains, where this parameter could not be calculated (indeterminate) in some cases due to the absence of TP samples (Table 7).

The analysis of data using kappa coefficients showed that there were slight to fair agreements among culture methods (Table 8). In relation to PCR methods, when TSBF-PCR was compared with Sst-PCR, agreement was good for all *Salmonella* strains tested. On the other hand, kappa coefficients showed slight to fair agreement between PCR and culture methods.

DISCUSSION

Cultivation media clearly impose selection pressure on *Salmonella* (Singer et al., 2009). Because of that, it is known that a detection method does not offer every *Salmonella* serotype an equal chance of isolation (Jones, 2011). Our previous study on poultry feed (Soria et al., 2011) showed that *S. gallinarum* and *S. pullorum* strains were difficult to detect in poultry feed. Because of that, in the present study we evaluated the performance of 3 enrichment procedures, different from the ones we have published before, and 2 combinations of PCR with preenrichment cultures for detection of 4 *S. gallinarum* and 2 *S. pullorum* strains.

To reduce bacterial load, poultry feed is usually treated by a heating process. For this reason if *Salmonella* is present, probably it would be injured by high temperatures. The purpose of preenrichment is to revive injured salmonellae that may be present in some samples (Waltman and Gast, 2008). Therefore, in this work all *Salmonella* strains tested were exposed to 60°C to simulate the natural conditions prevailing in poultry feed. Under these conditions, 2 different preenrichment media were compared. In our study, poultry feed samples were preenriched in TSBF or Sst medium and the *Salmonella* strains were tested in a stationary phase after they had been injured by heat. The TSBF was used before (Soria et al., 2011) due to the need to add iron in the form of ferrous sulfate to counteract some iron-binding substances that may be present in poultry feed, such as phytic acid, a compound able to bind iron and make it insoluble and, thus, unavailable as a nutritional factor for bacteria (Bohn et al., 2008). On the other hand, Pignato et al. (1995) worked with

Table 5. Results obtained when *Salmonella* strains were inoculated in poultry feed samples and afterward detected following tetrathionate Hajna (TTH), selenite cystine (SC), Salmosyst, and PCR (TSBF-PCR, Salmosyst-PCR) methods[1]

		Methodology to detect *Salmonella* from poultry feed samples				
Strain	cfu/25 g	TTH	SC	Salmosyst	TSBF-PCR	Salmosyst-PCR
Salmonella gallinarum 93/110	0	0/3	0/3	0/3	0/3	0/3
	5×10^0	0/3	0/3	0/3	0/3	0/3
	5×10^1	0/3	0/3	0/3	0/3	0/3
	5×10^2	0/3	0/3	0/3	0/3	0/3
	5×10^3	0/3	2/3	0/3	0/3	2/3
	5×10^4	1/3	2/3	0/3	2/3	3/3
	5×10^5	0/3	1/3	1/3	2/3	3/3
	5×10^6	1/3	2/3	2/3	3/3	3/3
	5×10^7	1/3	3/3	3/3	3/3	3/3
S. gallinarum 80/111	0	0/3	0/3	0/3	0/3	0/3
	3×10^0	0/3	0/3	0/3	0/3	0/3
	3×10^1	0/3	0/3	1/3	0/3	0/3
	3×10^2	0/3	1/3	1/3	0/3	0/3
	3×10^3	0/3	2/3	1/3	0/3	0/3
	3×10^4	1/3	2/3	2/3	1/3	2/3
	3×10^5	1/3	3/3	3/3	2/3	3/3
	3×10^6	3/3	3/3	3/3	3/3	3/3
	3×10^7	3/3	2/3	3/3	3/3	3/3
S. gallinarum 81/86	0	0/3	0/3	0/3	0/3	0/3
	3×10^1	0/3	0/3	0/3	0/3	0/3
	3×10^2	0/3	0/3	0/3	0/3	0/3
	3×10^3	0/3	0/3	0/3	0/3	0/3
	3×10^4	0/3	0/3	0/3	0/3	0/3
	3×10^5	0/3	0/3	0/3	0/3	0/3
	3×10^6	0/3	0/3	1/3	3/3	2/3
	3×10^7	0/3	2/3	2/3	3/3	3/3
	3×10^8	1/3	1/3	3/3	3/3	3/3
S. gallinarum 88/322	0	0/3	0/3	0/3	0/3	0/3
	9×10^0	0/3	0/3	0/3	0/3	0/3
	9×10^1	0/3	0/3	0/3	0/3	0/3
	9×10^2	0/3	1/3	0/3	0/3	0/3
	9×10^3	0/3	3/3	2/3	1/3	0/3
	9×10^4	3/3	2/3	3/3	1/3	0/3
	9×10^5	2/3	2/3	3/3	3/3	0/3
	9×10^6	3/3	3/3	3/3	3/3	2/3
	9×10^7	2/3	3/3	2/3	2/3	3/3
Salmonella pullorum 90/142	0	0/3	0/3	0/3	0/3	0/3
	8×10^0	0/3	0/3	0/3	0/3	0/3
	8×10^1	0/3	0/3	0/3	0/3	0/3
	8×10^2	0/3	0/3	0/3	0/3	0/3
	8×10^3	3/3	1/3	3/3	1/3	0/3
	8×10^4	3/3	3/3	2/3	1/3	0/3
	8×10^5	2/3	2/3	3/3	3/3	0/3
	8×10^6	3/3	2/3	3/3	3/3	2/3
	8×10^7	2/3	3/3	2/3	2/3	3/3
S. pullorum ATCC 13036	0	0/3	0/3	0/3	0/3	0/3
	3.4×10^0	0/3	0/3	0/3	0/3	0/3
	3.4×10^1	0/3	0/3	0/3	0/3	0/3
	3.4×10^2	0/3	0/3	0/3	0/3	0/3
	3.4×10^3	0/3	0/3	0/3	0/3	0/3
	3.4×10^4	0/3	0/3	0/3	0/3	0/3
	3.4×10^5	0/3	0/3	0/3	3/3	0/3
	3.4×10^6	0/3	2/3	0/3	3/3	2/3
	3.4×10^7	1/3	0/3	1/3	3/3	3/3

[1]The data are presented as the number of positive samples/number of total samples. ATCC = American Type Culture Collection.

a pure culture of 4 motile serotypes of *Salmonella* that were exposed to heat injury. These authors found a limit detection between 10^0 and 10^2 cfu/mL, when the preenrichment was followed by an enrichment carried out with a Sst supplement tablet. Using other culture media, these results were similar to the ones described by us before (Soria et al., 2011) with motile *Salmonella* strains. However, in this work, we needed to provide the greatest *S. gallinarum* or *S. pullorum* concentration to be able to detect these strains by the Sst method.

Based on different selective properties, the different enrichments broths available in the market offer various possibilities for *Salmonella* recovery, depending on the type of food or feed to be tested, the type of competitive flora present in a given sample, and the time and temperature of incubation to which samples are subjected (Bailey et al., 1981). Blivet et al. (1997) used SC broth to recover pure *S. pullorum* and *S. gallinarum* cultures as well as strains from other serotypes. In that work, the authors could detect those strains from 1 to

Table 6. Sensitivity (Se) and accuracy (Ac) of selective plating media in tetrathionate Hajna (TTH), selenite cystine (SC), and Salmosyst methods for nonmotile *Salmonella* strains[1]

		Se			Ac		
Strain	Media	TTH	SC	Salmosyst	TTH	SC	Salmosyst
Salmonella gallinarum 93/110	$XLDT_4$	$0.13^{a,A}$ (0.03–0.00)	$0.21^{a,A}$ (0.08–0.39)	$0.25^{a,A}$ (0.11–0.43)	$0.22^{a,A}$ (0.09–0.39)	$0.30^{a,A}$ (0.15–0.47)	$0.33^{a,A}$ (0.17–0.51)
	EF-18	$0.04^{a,A}$ (0.00–0.17)	$0.38^{a,B}$ (0.20–0.56)	$0.21^{a,AB}$ (0.08–0.39)	$0.15^{a,A}$ (0.04–0.30)	$0.44^{a,B}$ (0.24–0.59)	$0.30^{a,AB}$ (0.15–0.47)
	Önöz	$0.13^{a,A}$ (0.03–0.29)	$0.21^{a,A}$ (0.08–0.39)	$0.25^{a,A}$ (0.11–0.43)	$0.22^{a,A}$ (0.09–0.39)	$0.30^{a,A}$ (0.15–0.47)	$0.33^{a,A}$ (0.17–0.51)
S. gallinarum 80/111	$XLDT_4$	$0.00^{a,A}$ (0.00–0.11)	$0.33^{a,B}$ (0.17–0.52)	$0.21^{a,B}$ (0.08–0.39)	$0.11^{a,A}$ (0.02–0.26)	$0.41^{a,B}$ (0.24–0.59)	$0.30^{a,B}$ (0.15–0.47)
	EF-18	$0.17^{b,A}$ (0.05–0.34)	$0.42^{a,B}$ (0.24–0.60)	$0.42^{a,B}$ (0.24–0.60)	$0.26^{a,A}$ (0.12–0.43)	$0.48^{a,B}$ (0.30–0.66)	$0.48^{a,B}$ (0.20–0.55)
	Önöz	$0.21^{b,A}$ (0.03–0.29)	$0.38^{a,A}$ (0.20–0.56)	$0.21^{a,A}$ (0.08–0.39)	$0.30^{a,A}$ (0.15–0.47)	$0.44^{a,A}$ (0.24–0.59)	$0.30^{a,A}$ (0.15–0.47)
S. gallinarum 81/86	$XLDT_4$	$0.04^{a,A}$ (0.00–0.17)	$0.04^{a,A}$ (0.00–0.17)	$0.04^{a,A}$ (0.00–0.17)	$0.15^{a,A}$ (0.04–0.30)	$0.15^{a,A}$ (0.04–0.30)	$0.15^{a,A}$ (0.04–0.30)
	EF-18	$0.04^{a,A}$ (0.00–0.17)	$0.04^{a,A}$ (0.00–0.17)	$0.21^{a,A}$ (0.08–0.39)	$0.15^{a,A}$ (0.04–0.30)	$0.15^{a,A}$ (0.04–0.30)	$0.30^{a,A}$ (0.15–0.47)
	Önöz	$0.04^{a,A}$ (0.00–0.17)	$0.08^{a,A}$ (0.01–0.23)	$0.04^{a,A}$ (0.00–0.17)	$0.15^{a,A}$ (0.04–0.30)	$0.19^{a,A}$ (0.07–0.35)	$0.15^{a,A}$ (0.04–0.30)
S. gallinarum 88/322	$XLDT_4$	$0.29^{ab,A}$ (0.14–0.48)	$0.42^{a,A}$ (0.24–0.60)	$0.29^{a,A}$ (0.14–0.48)	$0.37^{ab,A}$ (0.20–0.55)	$0.48^{a,A}$ (0.30–0.66)	$0.37^{a,A}$ (0.20–0.55)
	EF-18	$0.50^{b,A}$ (0.31–0.68)	$0.50^{a,A}$ (0.31–0.68)	$0.46^{a,A}$ (0.27–0.64)	$0.56^{b,A}$ (0.37–0.72)	$0.56^{a,A}$ (0.37–0.72)	$0.52^{a,A}$ (0.33–0.69)
	Önöz	$0.21^{a,A}$ (0.08–0.39)	$0.54^{a,B}$ (0.35–0.72)	$0.33^{a,AB}$ (0.17–0.52)	$0.30^{a,A}$ (0.15–0.47)	$0.59^{a,B}$ (0.40–0.75)	$0.41^{a,AB}$ (0.24–0.59)
Salmonella pullorum 90/142	$XLDT_4$	$0.04^{a,A}$ (0.00–0.17)	$0.17^{a,A}$ (0.05–0.34)	$0.21^{a,A}$ (0.08–0.39)	$0.15^{a,A}$ (0.04–0.30)	$0.26^{a,A}$ (0.12–0.43)	$0.30^{a,A}$ (0.15–0.47)
	EF-18	$0.00^{a,A}$ (0.00–0.11)	$0.08^{a,A}$ (0.01–0.23)	$0.04^{a,A}$ (0.00–0.17)	$0.11^{a,A}$ (0.02–0.26)	$0.19^{a,A}$ (0.07–0.35)	$0.15^{a,A}$ (0.04–0.30)
	Önöz	$0.04^{a,A}$ (0.00–0.17)	$0.08^{a,A}$ (0.01–0.23)	$0.08^{a,A}$ (0.01–0.23)	$0.15^{a,A}$ (0.07–0.35)	$0.19^{a,A}$ (0.07–0.35)	$0.19^{a,A}$ (0.07–0.35)
S. *pullorum* ATCC 13036	$XLDT_4$	$0.00^{a,A}$ (0.00–0.11)	$0.08^{a,A}$ (0.01–0.23)	$0.00^{a,A}$ (0.00–0.11)	$0.11^{a,A}$ (0.02–0.26)	$0.19^{a,A}$ (0.07–0.35)	$0.11^{a,A}$ (0.07–0.35)
	EF-18	$0.04^{a,A}$ (0.00–0.17)	$0.00^{a,A}$ (0.00–0.11)	$0.04^{a,A}$ (0.00–0.17)	$0.15^{a,A}$ (0.04–0.30)	$0.11^{a,A}$ (0.02–0.26)	$0.15^{a,A}$ (0.04–0.30)
	Önöz	$0.00^{a,A}$ (0.00–0.11)	$0.00^{a,A}$ (0.00–0.11)	$0.00^{a,A}$ (0.00–0.17)	$0.11^{a,A}$ (0.02–0.26)	$0.11^{a,A}$ (0.02–0.26)	$0.11^{a,A}$ (0.07–0.35)

[a,b]Values with different lowercase superscripts in the same column are significantly different ($P < 0.05$).

[A,B]Values with different uppercase superscripts in the same row are significantly different ($P < 0.05$).

[1]Values in parentheses indicate a 95% CI for the respective parameter. $XLDT_4$ = xylose lysine desoxicholate agar (Oxoid, Basingtoke, Hampshire, UK) plus 4.6 mL/L of tergitol 4 (Sigma, Steinheim, Germany); EF-18, Acumedia, Lansing, MI; Önöz, Merck, Darmstadt, Germany. ATCC = American Type Culture Collection.

Table 7. Positive predictive values (PPV) and negative predictive values (NPV) of selective plating media on tetrathionate Hajna (TTH), selenite cystine (SC), and Salmosyst methods for nonmotile *Salmonella* strains[1]

		PPV			NPV		
Strain	Media	TTH	SC	Salmosyst	TTH	SC	Salmosyst
Salmonella gallinarum 93/110	$XLDT_4$	1.00[a] (0.39–0.99)	1.00[a] (0.54–0.99)	1.00[a] (0.15–0.98)	0.13[a] (0.03–0.29)	0.14[a] (0.03–0.32)	0.14[a] (0.03–0.32)
	EF-18	1.00[a] (0.15–0.98)	1.00[a] (0.69–0.99)	1.00[a] (0.15–0.98)	0.12[a] (0.03–0.27)	0.17[a] (0.04–0.37)	0.14[a] (0.03–0.32)
	Önöz	1.00[a] (0.39–0.99)	1.00[a] (0.54–0.99)	1.00[a] (0.15–0.98)	0.13[a] (0.03–0.29)	0.14[a] (0.03–0.32)	0.14[a] (0.03–0.32)
S. gallinarum 80/111	$XLDT_4$	IND	1.00[a] (0.66–0.99)	1.00[a] (0.54–0.99)	0.11[a] (0.02–0.26)	0.16[a] (0.04–0.35)	0.14[a] (0.03–0.32)
	EF-18	1.00[a] (0.47–0.99)	1.00[a] (0.71–0.99)	1.00[a] (0.71–0.99)	0.13[a] (0.03–0.30)	0.18[a] (0.04–0.38)	0.18[a] (0.04–0.38)
	Önöz	1.00[a] (0.54–0.99)	1.00[a] (0.69–0.99)	1.00[a] (0.54–0.99)	0.14[a] (0.03–0.32)	0.17[a] (0.04–0.37)	0.14[a] (0.03–0.32)
S. gallinarum 81/86	$XLDT_4$	1.00[a] (0.39–0.99)	1.00[a] (0.39–0.99)	1.00[a] (0.54–0.99)	0.12[a] (0.03–0.27)	0.12[a] (0.03–0.27)	0.12[a] (0.03–0.27)
	EF-18	1.00[a] (0.39–0.99)	1.00[a] (0.39–0.99)	1.00[a] (0.54–0.99)	0.12[a] (0.03–0.27)	0.12[a] (0.03–0.27)	0.14[a] (0.03–0.32)
	Önöz	1.00[a] (0.39–0.99)	1.00[a] (0.54–0.99)	1.00[a] (0.54–0.99)	0.12[a] (0.03–0.27)	0.12[a] (0.03–0.27)	0.12[a] (0.03–0.27)
S. gallinarum 88/322	$XLDT_4$	1.00[a] (0.63–0.99)	1.00[a] (0.71–0.99)	1.00[a] (0.63–0.99)	0.15[a] (0.04–0.34)	0.18[a] (0.04–0.38)	0.15[a] (0.04–0.34)
	EF-18	1.00[a] (0.75–0.99)	1.00[a] (0.69–0.99)	1.00[a] (0.73–0.99)	0.20[a] (0.05–0.43)	0.20[a] (0.05–0.43)	0.19[a] (0.05–0.40)
	Önöz	1.00[a] (0.54–0.99)	1.00[a] (0.76–0.99)	1.00[a] (0.66–0.99)	0.14[a] (0.03–0.32)	0.21[a] (0.06–0.45)	0.16[a] (0.04–0.35)
Salmonella pullorum 90/142	$XLDT_4$	1.00[a] (0.15–0.98)	1.00[a] (0.47–0.99)	1.00[a] (0.15–0.98)	0.12[a] (0.03–0.27)	0.13[a] (0.03–0.30)	0.14[a] (0.03–0.32)
	EF-18	IND	1.00[a] (0.29–0.99)	1.00[a] (0.15–0.98)	0.11[a] (0.02–0.26)	0.12[a] (0.03–0.28)	0.12[a] (0.03–0.27)
	Önöz	1.00[a] (0.15–0.98)	1.00[a] (0.29–0.99)	1.00[a] (0.15–0.98)	0.12[a] (0.03–0.27)	0.12[a] (0.03–0.28)	0.12[a] (0.03–0.28)
S. *pullorum* ATCC 13036	$XLDT_4$	IND	1.00 (0.69–0.99)	IND	0.11[a] (0.02–0.26)	0.12[a] (0.03–0.28)	0.11[a] (0.02–0.26)
	EF-18	1.00[a] (0.39–0.99)	IND	1.00[a] (0.39–0.99)	0.12[a] (0.03–0.27)	0.11[a] (0.02–0.26)	0.12[a] (0.03–0.27)
	Önöz	IND	IND	IND	0.11[a] (0.02–0.26)	0.12[a] (0.02–0.26)	0.11[a] (0.02–0.26)

[a,b]Values with different superscripts in the same column are significantly different ($P < 0.05$).

[1]Values in parentheses indicate a 95% CI for the respective parameter. $XLDT_4$ = xylose lysine desoxicholate agar (Oxoid, Basingtoke, Hampshire, UK) plus 4.6 mL/L of tergitol 4 (Sigma, Steinheim, Germany); EF-18, Acumedia, Lansing, MI; Önöz, Merck, Darmstadt, Germany. IND = indeterminate (0/0). ATCC = American Type Culture Collection.

6×10^1 and 1 to 6×10^2 cfu/mL for biovars *pullorum* and *gallinarum*, respectively. Using the same culture media, we found similar results in terms of detection limit (3 to 5×10^2 cfu/mL). However, our results of detection limits were obtained from samples of feed that were artificially contaminated with *Salmonella*, meaning that the competitive flora or its possible inhibitory effect were not very strong, at least in 3 out of the 6 strains that were tested.

Hajna and Damon (1956) developed a tetrathionate broth containing yeast extract, peptone, carbon sources, and the selective agents, sodium desoxicholate and brilliant green (instead of bile salts). Since the formulation of the TTH broth, there were several other works that used this medium in different matrices and with different purposes (North, 1961; Cox et al., 1982; Tate et al., 1990; Carli et al., 2001). A comparison of direct enrichment using TTH or SC broths was made by inoculating pelleting feed samples with *Salmonella* Montevideo and *Salmonella* Heidelberg, and it was found that TTH broth was superior to SC (Cox et al., 1982). Nevertheless, in that study the authors did not use any kind of stress over the inoculated bacteria, and the media were immediately added after the addition of *Salmonella* cells. In contrast, we found that the sensitivity was only greater in the SC method than in the TTH method for 2 nonmotile *Salmonella* strains, although there were not any statistical differences in the other strains for both methods.

Selective plating media must be used to assist selective enrichment broths in inhibiting competing bacteria (Waltman and Gast, 2008). In general, we did not find any statistical difference between the plating media tested herein and the performance of XLDT and EF-18 agars described in our previous study (Soria et al., 2011). Furthermore, Önöz agar did not show any advantage for isolation of nonmotile *Salmonella* strains. This medium was originally published as enabling an immediate distinction of *Proteus* and *Citrobacter* and increasing positive *Salmonella* yield from feces specimens compared with Leifson agar and *Salmonella-Shigella* agar (Onöz and Hoffmann, 1978).

The PCR technique is very effective to detect pure solutions of nucleic acids. However, the sensitivity may be dramatically reduced when PCR is applied to biological matrices such as food or feed samples that contain salts, lipids, proteins, and other bacterial cells that are different from the target organism. The use of a preenrichment culture before performing PCR serves several different purposes, including dilution of inhibitory substances, multiplication of the target organism, and also the possibility of isolating this organism using a complementary culture procedure (Salomonsson et al., 2005). In this work, the detection limit was similar for both PCR methods used in our assay, and a concentration of at least 5×10^3 cfu/25 g was needed to be able to detect *S. gallinarum* or *S. pullorum* in any poultry feed sample. This value was similar to our previous report (Soria et al., 2011). Additionally, Soria et al. (2011) reported that PCR gave better results than the Modified Semisolid Rappaport Vassiliadis (**MSRV**) and tetrathionate methods to detect *S. gallinarum* and *S. pullorum* strains. However, we generally found that PCR methods did not show any advantage with respect to TTH, SC, and Sst methods for these *Salmonella* biovars.

Table 8. Kappa coefficient values showing agreement between all methods tested for poultry feed samples

Comparison between methods	Kappa coefficient
Selenite cystine/tetrathionate Hajna	0.51*
Salmosyst/tetrathionate Hajna	0.38*
Salmosyst/selenite cystine	0.46*
TSBF[1]-PCR/Salmosyst-PCR	0.68*
TSBF-PCR/tetrathionate Hajna	0.31*
TSBF-PCR/selenite cystine	0.34*
TSBF-PCR/Salmosyst	0.47*
Salmosyst-PCR/tetrathionate Hajna	0.25*
Salmosyst-PCR/selenite cystine	0.48*
Salmosyst-PCR/Salmosyst	0.42*

[1]TSBF: tryptic soy broth with ferrous sulfate.

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

Five of the strains used herein had been previously assayed, using MSRV, tetrathionate, and PCR methods (Soria et al., 2011). In general, sensitivity and accuracy were higher in TTH, SC, and SSt methods than in tetrathionate and MSRV methods. On the other hand, using the same *Salmonella* strains, the negative predictive value was similar among all the methods that were tested.

The TTH, SC, Sst, and PCR methods are different in terms of sensitivity, specificity, positive predictive value, and negative predictive value for the detection of *S. gallinarum* and *S. pullorum* strains that were tested in artificially contaminated poultry feed. The difference in the detection levels that were obtained with the methods tested, and particularly the difficulty of detecting *S. gallinarum* or *S. pullorum* strains, represent a potential problem when a poultry feed sample is considered to be negative for the presence of bacteria from the genus *Salmonella*. Therefore, to reduce the number of false negative results, even if it adds a low extra cost, the use of at least 2 methods to increase the chances of detection of nonmotile host-specific avian *Salmonella* is highly recommended.

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