A comparative study of culture methods and polymerase chain reaction for *Salmonella* detection in egg content

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ABSTRACT The present work compared 2 culture methods and a PCR assay applied with 2 enrichment methods for the detection of motile and nonmotile *Salmonella* strains using artificially contaminated egg content. The specificity (Sp) was 1 in all methods. The sensitivity (Se), accuracy (Ac), positive predictive value (PPV), and negative predictive value (NPV) were 1 in both culture methods for motile and nonmotile strains. In reference to the PCR methods, Se and PPV were between 0 and 1, whereas Ac and NPV were between 0.14 and 1. The detection level of motile and nonmotile strains was 5 to 54 cfu per 25 mL for both culture methods, but some strains could not be detected by the PCR methods. Extending incubation time of the enrichment medium to 5 d in the tetrathionate broth (TT), and Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) methods 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 an excellent agreement between the bacteriological methods for all *Salmonella* strains. The agreement was very good and good between the PCR methods, for motile and nonmotile strains, respectively. However, there was a poor agreement when the PCR and bacteriological methods were compared for motile and nonmotile *Salmonella* strains. The TT and MKTTn methods are similar in terms of Ac, Se, Sp, PPV, and NPV for different *Salmonella* strains in egg content. The use of the PCR method cannot improve the same parameters, described before, in this matrix. So, further studies are needed to improve the performance parameters and limit of detection in egg content for the PCR methods, so that test can be used in poultry and food industry.

Key words: *Salmonella*, egg content, culture method, PCR

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INTRODUCTION

Infections with bacteria of the genus *Salmonella* are responsible for a variety of acute and chronic disease in poultry and human beings. Poultry and poultry products have been implicated as a major source of *Salmonella* infections in human (Zahraei Salehi et al., 2005; Singer et al., 2009). Poultry producers are faced with intensifying pressures from public health authorities, elected officials, and consumers regarding food safety issues (Gast, 2003).

Salmonella enterica biovars Pullorum and Gallinarum are host-specific and represent a major concern to the poultry industry. These avian-adapted biovars (nonmotile) lack flagella and associated motility. They cause a serious systemic disease of poultry (fowl typhoid and pullorum disease) with large-scale economic losses through mortality, morbidity, and reduction in egg production (Barrow and Freitas Neto, 2011). These biovars can be transmitted to an egg through transovarial infection; they cause rare cases of diseases in humans from massive exposure following the ingestion of contaminated foods or experimental challenges (Shivaprasad, 2003). On the other hand, there are typically no clinical signs in birds infected with other *Salmonella* to suggest to the farmer that the eggs they are producing might pose a public health threat (Guard-Petter, 2001).

Although the relative contribution of food-animal sources to human *Salmonella* infection varies between regions and countries, eggs are the major vehicle of these bacteria (Braden, 2006; Pires et al., 2011). Eggs can be contaminated on the outer shell surface and internally. Internal contamination can be the result of penetration through the eggshell or by direct contamination of egg contents before oviposition, originating from infection of the reproductive organs. Once inside the egg, the bacteria need to cope with antimicrobial

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factors in the albumen and vitelline membranes before migration to the yolk can occur (Gantois et al., 2009).

The prevalence of eggs with *Salmonella*-positive contents can be variable. There are several factors that could explain this variability, such as sample size, timing of sampling, site(s) within the eggs that were tested, techniques used, investigations of eggs laid by artificially or naturally infected hens, and so on (Humphrey, 1994). During several decades, standardized methods for detection of *Salmonella* in food and food ingredients have been independently developed in both the United State and Europe. Although the basic procedures are similar, differences exist in the specified media and incubation conditions (Feldsine et al., 2003). The conventional culture methods include nonselective preenrichment followed by selective enrichment, plating on selective and differential agars, biochemical tests, and serological tests (World Organization for Animal Health, 2008). A wide range of culture methods and PCR assays are available, and several studies have been developed to test their ability to detect *Salmonella* in eggs (Gast and Holt, 2003; Mancera Martinez et al., 2005; Pérez et al., 2008; Loongyai et al., 2010; Wallace and Hammack, 2011). However, no one method has superiority over another, and the sensitivity and specificity of the method depends on the sample type as well as the isolation conditions (Rybolt et al., 2004).

Because it is important that egg processors obtain evidence to show that *Salmonella* is not present in their product, the method and media employed must permit the detection of very small numbers of pathogens (Busse, 1995). Furthermore, detecting internal contamination of eggs with *Salmonella* is an important aspect of efforts to identify infected laying flocks (Gama et al., 2003). On the other hand, it is reported that the detection methods do not offer every *Salmonella* serotype an equal chance of isolation, because certain *Salmonella* serotypes are more competitive than others (Jones, 2011). Therefore, the present work was conducted comparing 2 culture methods and a PCR assay to learn their ability to detect low levels of motile and nonmotile *Salmonella* strains in artificially contaminated egg content. Furthermore, the accuracy (**Ac**), sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**), and negative predictive value (**NPV**) of each method and the agreement among methods were investigated.

MATERIALS AND METHODS

Egg Samples

Eggs samples were purchased from supermarkets in the state of Entre Rios, Argentina. The egg contents were collected after sterilizing the egg surface by immersion in 70% ethyl alcohol for 10 min, and then by immersion in boiling water for 5 s (Gast, 1993; Himathongkham et al., 1999). Each egg was aseptically broken and the egg contents (mixture of yolk and albumen, YA) were stomached (Stomacher 400 circulator, Seward, UK) 2 min at 2,300 rpm at room temperature (25 ± 2°C) in groups of 6 eggs. Each sample was analyzed by the tetrathionate (**TT**) method, described below before carrying out assays to ensure the absence of *Salmonella* spp. Furthermore, total bacteria, *Enterobacteriaceae*, and fungi counts of egg contents were determined in tryptic soy agar (**TSA**, Acumedia, Lansing, MI); MacConkey agar (**MC**, Acumedia), and Dichloran Rose Bengal Chloramphenicol agar (**DRBC**, Oxoid, Basingstoke, Hampshire, UK), respectively. Chloramphenicol was purchased from Anedra (San Fernando, Argentina). The detection limit was 4×10^2 cfu/mL for total bacteria and *Enterobacteriacea*, and 1×10^2 cfu/mL for fungi counts.

Salmonella Strains and Culture

As summarized in Table 1, a total of 8 *Salmonella* strains were selected to assay. These strains belong to the collections from the Balcarce Laboratory of Bacteriology (Buenos Aires, Argentina) of the Agricultural Experimental Station (**EEA**), National Institute of Agricultural Technology (**INTA**), the Poultry Health Laboratory of EEA INTA Concepcion del Uruguay (Entre Rios, Argentina), and the American Type Culture Collection (**ATCC**). Two of them were isolated from chickens, 1 was isolated from the poultry meat, 1 was isolated from the eggshell, and 1 was isolated from the pool yolk-albumen. Each *Salmonella* strain was activated from Nutrient Agar, NA (Acumedia) and was grown for 24 h in tryptic soy broth (**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 by the method of Miles and Misra (1938) and expressed as cfu/mL. Cells were pelleted by centrifugation in a tabletop centrifuge at $302 \times g$ for 15 min at room temperature. Supernatant was discarded and the pellet cell was resuspended to the original volume (5 mL) with PBS (pH 7.4).

Preparation of Salmonella Inocula in Yolk—Albumen Samples

Twenty-five mL of *Salmonella*-free YA material was introduced into a sterile plastic bag. *Salmonella* strains were grown and serial dilutions were made in peptone water (0.1%) to inoculate from 5×10^0 to 6.2×10^5 cfu/25 mL, and 5.2×10^0 to 1.3×10^6 cfu/25 mL for motile *Salmonella* and nonmotile *Salmonella* strains, respectively. Five serial dilutions were used for *S.* Enteritidis and *S.* Typhimurium, whereas 6 serial dilutions were used for all other serovars. All treatments were performed in triplicate, so 3 samples of each dose for each *Salmonella* strain were considered in the assays. Altogether 276 spiked samples were constructed in the study. For each trial set, 3 nonseeded samples were analyzed as negative control.

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

Salmonella strain	Source
Salmonella Enteritidis ATCC 13076	American Type Culture Collection
Salmonella Typhimurium ATCC 13311	Human feces, American Type Culture Collection
Salmonella Infantis CUB 05/08	Poultry meat, EEA INTA C. del Uruguay[1]
Salmonella Hadar CUB 13/08	Eggshell, EEA INTA C. del Uruguay
Salmonella Pullorum ATCC 13036	Egg, American Type Culture Collection
Salmonella Pullorum INTA 90/142	Chicken, EEA INTA Balcarce
Salmonella Gallinarum INTA 03/121	Chicken, EEA INTA Balcarce
Salmonella Gallinarum CUB 05/10	Pool yolk-albumen, EEA INTA C. del Uruguay

[1]EEA INTA: Agricultural Experimental Station, National Institute of Agricultural Technology.

Recovery of Salmonella spp. Strains from Egg Contents

Figure 1 shows a flowchart diagram for detection of *Salmonella* in YA by the TT and Muller-Kauffmann tetrathionate-novobiocin (**MKTTn**) methods. For both bacteriological methods, the *Salmonella*-free YA were contaminated with different concentrations of the *Salmonella* strains. The TT and MKTTn methods were based on chapter 5 (*Salmonella*) of the Bacteriological Analytical Manual (Wallace and Hammack, 2011) and on the Microbiology of Food and Animal Feeding Stuffs (ISO, 2002), respectively. For the TT method, samples were preenriched in 225 mL of TSB with ferrous sulfate (**TSBF**, 35 mg of ferrous sulfate added to 1,000 mL of TSB). 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, Steinheim, Germany), and 40 mg/mL of novobiocin (Sigma), and incubated at 35 ± 2°C for 5 d. On the other hand, for the MKTTn method, the sample was preenriched in 225 mL of buffered peptone water (**BPW**, Merck) and incubated at 35 ± 2°C for 24 h. One milliliter of incubated broth was transferred to 10 mL of MKTTn broth (Oxoid) and incubated at 35 ± 2°C for 5 d. For both methods, at d 1 (TT or MKTTn first) and d 5 (TT or MKTTn fifth), a loopful of each selective enrichment broth was streaked on xylose lysine desoxicholate agar (**XLD**, Oxoid) and Hektoen enteric agar (**H**, Acumedia) and incubated at 35 ± 2°C for 24 h. 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 (Britania, Buenos Aires, Argentina) and agglutination reaction with somatic (O) polivalent antisera (Difco, Becton Dickinson, Sparks, MD).

DNA Extraction

For detection of *Salmonella* from YA samples, bacterial cells were recovered from 1 mL of TSBF and BPW preenrichment broths (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 the 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

Deoxyribonucleic acid samples (5 μL) were amplified in an optimized 25-μL reaction mixture consisting of 0.25 μL of each primer 0.1 m*M*, 2.5 μL of buffer 1× (Fermentas), 1.5 μL of $MgCl_2$ 1.5 m*M* (Fermentas), 0.5 μL of each dNTP 0.2 m*M* (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, Germany). *Salmonella* genus-specific primers 139 and 141 (Operon Biotechnologies GmbH, Germany), which were based on the invA gene of *Salmonella,* were used in the PCR assay. They have the following nucleotide sequences: (5′→3′) GTGAAATTATCGCCACGTTCGGGCAA and TCATCGCACCGTCAAAGGAACC, respectively. A reagent blank containing all the components of the reaction mixture with the exception of template DNA (which was replaced by sterile distilled water) was included with every PCR assay. Furthermore, negative and positive DNA controls were included, which were prepared from *Citrobacter* sp. (isolated from egg content) 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, the PCR tubes were held at 4°C.

Detection of PCR Products

The PCR products were analyzed by gel electrophoresis. Ten microliters of each sample was loaded onto 2.0% of agarose gel in 0.5× 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

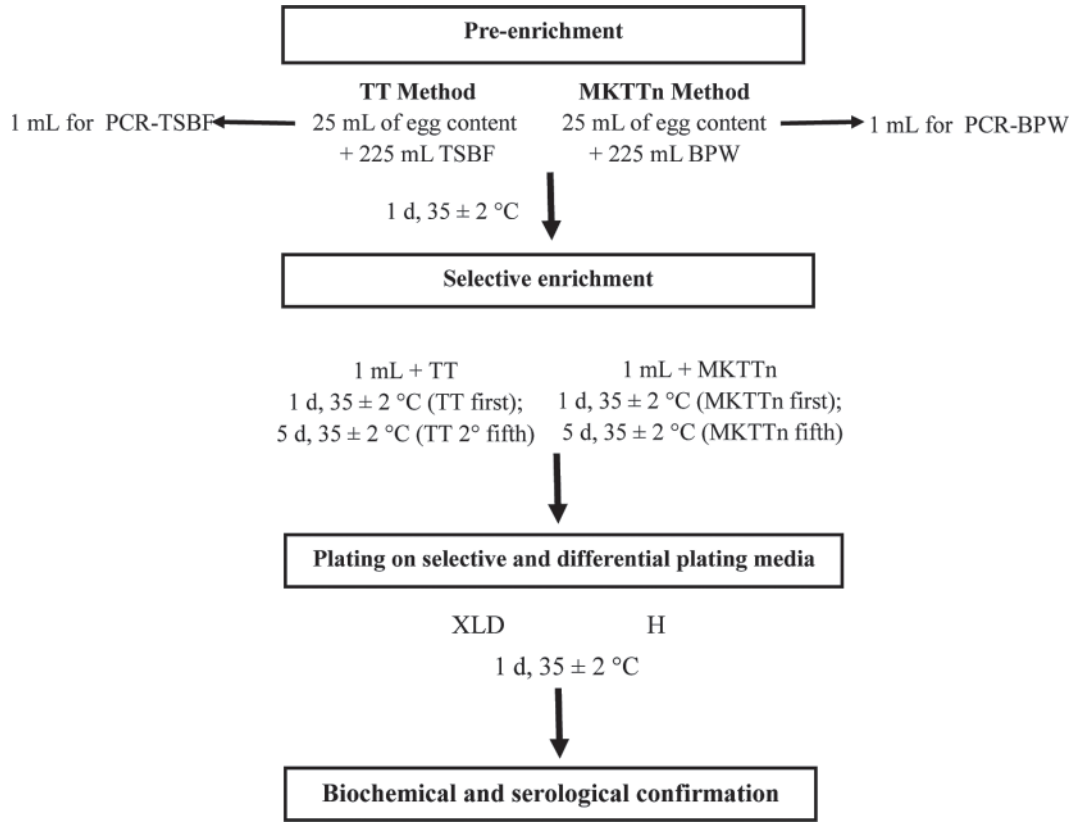

Figure 1. Flowchart diagram for detection of *Salmonella* in egg content by tetrathionate broth (TT), Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn), and PCR methods. TSBF = tryptic soy broth with ferrous sulfate. BPW = buffered peptone water. TT first = d 1 of incubation in the TT broth. TT fifth = d 5 of incubation in the TT broth. MKTTn first = d 1 of incubation in the MKTTn broth. MKTTn fifth = d 5 of incubation in the MKTTn broth. XLD = xylose lysine desoxicholate agar. H = Hektoen enteric agar.

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 a molecular weight marker.

Analysis of Performance Criteria

The detection limit of the methods was considered and defined as the lowest concentration (cfu/25 mL) of the *Salmonella* strain inoculum that could be recovered. The Ac, Se, Sp, PPV, and NPV were calculated for each method (Soria et al., 2011). The assumption was that all nonspiked samples were negative for *Salmonella* and only those samples spiked with *Salmonella* were true positive (**TP**). Samples being positives on at least one selective agar plate (XLD or H) 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 for the ability of a method to correctly classify samples containing *Salmonella* as positive for *Salmonella*, and samples not containing *Salmonella* as negative for *Salmonella*.

$$\mathrm{Ac} = (\mathrm{TP} + \mathrm{TN})/(\mathrm{TP} + \mathrm{TN} + \mathrm{FP} + \mathrm{FN}).$$

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

$$\mathrm{Se} = \mathrm{TP}/(\mathrm{TP} + \mathrm{FN}).$$

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

$$\mathrm{Sp} = \mathrm{TN}/(\mathrm{TN} + \mathrm{FP}).$$

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

$$\mathrm{PPV} = \mathrm{TP}/(\mathrm{TP} + \mathrm{FP}).$$

Negative predictive value is a measure for the probability of the samples with negative test results for *Salmonella* that are correctly determined:

$$NPV = TN/(TN + FN).$$

Statistical Analysis

To compare the results of all 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 confident intervals, under the assumption that all values are equally probable. The calculations were performed using Octave Program, developed by the Group of Numerical Method, from the National Technological University of Concepcion del Uruguay, Entre Rios, Argentina, Projects 25D041. The reported values define the boundaries of an interval that, with 95% certainty, contain the true value of Ac, Se, PPV, or NPV. The results were only considered to be statistically different at $P < 0.05$.

Agreement between cultural and the 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 was 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). The Z test was used to test the statistical significance of kappa coefficients.

RESULTS

Total bacteria, *Enterobacteriaceae*, and fungi could not be detected in yolk-albumen samples, and the XLD and H agar plates did not show any growth in the negative control. In relation to the performance of the methods, the Sp values were 1 for all methods studied (data not shown). The Se and Ac were different from the culture and the PCR methods. They were 1 in both bacteriological methods for motile and nonmotile *Salmonella* strains (Table 2). In reference to the PCR methods, Se was from 0 to 1, whereas Ac was between 0.14 and 1. On the other hand, there was only a statistical difference between the PCR methods for 2 strains, *S.* Hadar CUB 13/08 and *S.* Gallinarum INTA 03/121.

Table 3 shows the PPV and NPV for all strains tested. These parameters were 1 for both culture methods in all strains, whereas they depended on the strains for the PCR methods. The PPV was indeterminate (0/0) or 1 in both the PCR methods, whereas the NPV was between 0.14 and 1 in those methods.

When the detection limit of each technique was studied, all motile *Salmonella* strains were recovered in the lowest dilutions tested for both culture methods (5 to 54 cfu/25 mL). The detection limit of the PCR methods was similar to the culture methods for *S.* Enteritidis and *S.* Hadar. However, the PCR methods could not detect *S.* Typhimurium and *S.* Infantis in any concentration tested (Table 4). Regarding to nonmotile *Salmonella* strains, all of them were recovered in the lowest dilutions tested for both culture methods (5 to 13 cfu/25 mL), except *S.* Pullorum ATCC 13036, which showed a detection limit of 5.8×10^1 cfu per 25 mL of an egg content in the bacteriological methods (Table 5). The BPW-PCR method could detect both *S.* Gallinarum strains in the lowest dilutions tested, whereas

Table 2. Sensitivity (Se) and accuracy (Ac) of the tetrathionate (TT), Muller-Kauffmann tetrathionate-novobiocin (MKTTn), and PCR [from tryptic soy broth with ferrous sulfate (TSBF) or buffered peptone water (BPW)] methods for each motile and nonmotile *Salmonella* strain in artificially contaminated egg content[1]

	Se				Ac			
Strain	TT	TSBF-PCR	MKTTn	BPW-PCR	TT	TSBF-PCR	MKTTn	BPW-PCR
S. Enteritidis ATCC 13076	$1^{a,A}$ (0.95–1.00)	$0.83^{a,B}$ (0.60–0.94)	$1^{a,A}$ (0.95–1.00)	$0.83^{a,B}$ (0.60–0.94)	$1^{a,A}$ (0.95–1.00)	$0.86^{a,B}$ (0.65–0.94)	$1^{a,A}$ (0.95–1.00)	$0.86^{a,B}$ (0.65–0.94)
S. Typhimurium ATCC 13311	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)
S. Infantis CUB 05/08	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)
S. Hadar CUB 13/08	$1^{a,A}$ (0.95–1.00)	$1^{a,C}$ (0.82–0.10)	$1^{a,A}$ (0.95–1.00)	$0.72^{b,C}$ (0.49–0.87)	$1^{a,A}$ (0.95–1.00)	$1^{a,C}$ (0.85–0.10)	$1^{a,A}$ (0.95–1.00)	$0.76^{b,C}$ (0.54–0.89)
S. Pullorum ATCC 13036	$1^{a,A}$ (0.95–1.00)	$0.06^{b,A}$ (0.01–0.26)	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0.19^{b,A}$ (0.07–0.40)	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)
S. Pullorum INTA 90/142	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0^{b,B}$	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)
S. Gallinarum INTA 03/121	$1^{a,A}$ (0.95–1.00)	$0.67^{b,B}$ (0.43–0.84)	$1^{a,A}$ (0.95–1.00)	$1^{a,B}$ (0.82–0.1)	$1^{a,A}$ (0.95–1.00)	$0.71^{b,B}$ (0.50–0.86)	$1^{a,A}$ (0.95–1.00)	$1^{a,B}$ (0.85–0.10)
S. Gallinarum CUB 05/10	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0^{b,A}$	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)	$1^{a,A}$ (0.95–1.00)	$0.14^{b,A}$ (0.05–0.34)

[a,b]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.

Table 3. Positive predictive value (PPV) and negative predictive value (NPV) for each motile and nonmotile *Salmonella* strain in artificially contaminated egg content (pool of yolk-albumen), according to tetrathionate (TT), Muller-Kauffmann tetrathionate-novobiocin (MKTTn), and PCR [from tryptic soy broth with ferrous sulfate (TSBF) or buffered peptone water (BPW)] methods[1]

	PPV in different methods for *Salmonella* detection				NPV in different methods for *Salmonella* detection			
Strain	TT	TSBF-PCR	MKTTn	BPW-PCR	TT	TSBF-PCR	MKTTn	BPW-PCR
S. Enteritidis ATCC 13076	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.79–0.98)	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.79–0.98)	$1^{a,A}$ (0.40–1.00)	$0.50^{b,A}$ (0.18–0.81)	$1^{a,A}$ (0.40–1.00)	$0.50^{b,B}$ (0.18–0.81)
S. Typhimurium ATCC 13311	$1^{a,A}$ (0.82–1.00)	$IND^{b,B,2}$	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.40–1.00)	$0.14^{b,A}$ (0.05–0.35)	$1^{a,A}$ (0.40–1.00)	$0.14^{b,B}$ (0.05–0.34)
S. Infantis CUB 05/08	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.40–1.00)	$0.14^{b,A}$ (0.05–0.34)	$1^{a,A}$ (0.40–1.00)	$0.14^{b,B}$ (0.05–0.34)
S. Hadar CUB 13/08	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.77–1.00)	$1^{a,A}$ (0.40–1.00)	$1^{a,B}$ (0.40–1.00)	$1^{a,A}$ (0.40–1.00)	$0.38^{b,B}$ (0.14–0.70)
S. Pullorum ATCC 13036	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.16–0.98)	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.40–1.00)	$0.15^{b,A}$ (0.05–0.36)	$1^{a,A}$ (0.40–1.00)	$0.14^{b,B}$ (0.05–0.34)
S. Pullorum INTA 90/142	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.40–1.00)	$0.14^{b,A}$ (0.05–0.34)	$1^{a,A}$ (0.40–1.00)	$0.14^{b,B}$ (0.05–0.34)
S. Gallinarum INTA 03/121	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.75–1.00)	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.82–1.00)	$1^{a,A}$ (0.40–1.00)	$0.30^{b,A}$ (0.12–0.65)	$1^{a,A}$ (0.40–1.00)	$1^{a,A}$ (0.40–1.00)
S. Gallinarum CUB 05/10	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.82–1.00)	$IND^{b,B}$	$1^{a,A}$ (0.40–1.00)	$0.14^{b,B}$ (0.05–0.34)	$1^{a,A}$ (0.40–1.00)	$0.14^{b,B}$ 0.05–0.34)

^{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).

the TSBF-PCR detected *S.* Gallinarum 03/121 from the level of 1×10^3 cfu/25 mL. On the other hand, neither PCR method could detect *S.* Pullorum 90/142 in an egg content; whereas *S.* Pullorum ATCC 13036 was recovered from 5.8×10^4 and from 5.8×10^5 cfu per 25 mL in the TSBF-PCR and the BPW-PCR methods, respectively.

For all *Salmonella* strains, the 2 selective plating media did not show any significant differences between them in terms of the parameters studied in both bacte-

Table 4. Results obtained when motile *Salmonella* strains were inoculated in egg content (pool of yolk-albumen) and were isolated following tetrathionate (TT), Muller-Kauffmann tetrathionate-novobiocin (MKTTn), and PCR [from tryptic soy broth with ferrous sulfate (TSBF) or buffered peptone water (BPW)] methods[1]

		Methodology to detect *Salmonella* from egg content			
		Culture		PCR	
Strain	Range of inoculation (cfu/25 mL)	TT	MKTTn	TSBF	BPW
S. Enteritidis ATCC 13076	0	0/3	0/3	0/3	0/3
	5.4×10^1	3/3	3/3	3/3	3/3
	5.4×10^2	3/3	3/3	3/3	3/3
	5.4×10^3	3/3	3/3	3/3	3/3
	5.4×10^4	3/3	3/3	3/3	3/3
	5.4×10^5	3/3	3/3	3/3	3/3
S. Typhimurium ATC 13311	0	0/3	0/3	0/3	0/3
	5.4×10^0	3/3	3/3	0/3	0/3
	5.4×10^1	3/3	3/3	0/3	0/3
	5.4×10^2	3/3	3/3	0/3	0/3
	5.4×10^3	3/3	3/3	0/3	0/3
	5.4×10^4	3/3	3/3	0/3	0/3
S. Infantis CUB 05/08	0	0/3	0/3	0/3	0/3
	5.0×10^0	3/3	3/3	0/3	0/3
	5.0×10^1	3/3	3/3	0/3	0/3
	5.0×10^2	3/3	3/3	0/3	0/3
	5.0×10^3	3/3	3/3	0/3	0/3
	5.0×10^4	3/3	3/3	0/3	0/3
	5.0×10^5	3/3	3/3	0/3	0/3
S. Hadar CUB 13/08	0	0/3	0/3	0/3	0/3
	6.2×10^0	3/3	3/3	3/3	3/3
	6.2×10^1	3/3	3/3	3/3	3/3
	6.2×10^2	3/3	3/3	3/3	3/3
	6.2×10^3	3/3	3/3	3/3	3/3
	6.2×10^4	3/3	3/3	3/3	3/3
	6.2×10^5	3/3	3/3	3/3	3/3

[1]Data represent number of positive samples per number of total samples.

Table 5. Results obtained when nonmotile *Salmonella* strains were inoculated in egg content (pool of yolk-albumen) and were isolated following tetrathionate (TT), Muller-Kauffmann tetrathionate-novobiocin (MKTTn), and PCR [from tryptic soy broth with ferrous sulfate (TSBF) or buffered peptone water (BPW)] methods[1]

		Methodology to detect *Salmonella* from egg content			
		Culture		PCR	
Strain	Range of inoculation (cfu/25 mL)	TT	TTMKn	TSBF	BPW
S. Pullorum ATCC 13036	0	0/3	0/3	0/3	0/3
	5.8×10^0	0/3	0/3	0/3	0/3
	5.8×10^1	3/3	3/3	0/3	0/3
	5.8×10^2	3/3	3/3	0/3	0/3
	5.8×10^3	3/3	3/3	0/3	0/3
	5.8×10^4	3/3	3/3	1/3	0/3
	5.8×10^5	3/3	3/3	3/3	3/3
S. Pullorum 90/142	0	0/3	0/3	0/3	0/3
	1.3×10^1	3/3	3/3	0/3	0/3
	1.3×10^2	3/3	3/3	0/3	0/3
	1.3×10^3	3/3	3/3	0/3	0/3
	1.3×10^4	3/3	3/3	0/3	0/3
	1.3×10^5	3/3	3/3	0/3	0/3
	1.3×10^6	3/3	3/3	0/3	0/3
S. Gallinarum 03/121	0	0/3	0/3	0/3	0/3
	1.0×10^1	3/3	3/3	0/3	3/3
	1.0×10^2	3/3	3/3	0/3	3/3
	1.0×10^3	3/3	3/3	3/3	3/3
	1.0×10^4	3/3	3/3	3/3	3/3
	1.0×10^5	3/3	3/3	3/3	3/3
	1.0×10^6	3/3	3/3	3/3	3/3
S. Gallinarum CUB 05/10	0	0/3	0/3	0/3	0/3
	5.2×10^0	3/3	3/3	3/3	3/3
	5.2×10^1	3/3	3/3	3/3	3/3
	5.2×10^2	3/3	3/3	3/3	3/3
	5.2×10^3	3/3	3/3	3/3	3/3
	5.2×10^4	3/3	3/3	3/3	3/3
	5.2×10^5	3/3	3/3	3/3	3/3

[1]Data represent the number of positive samples per number of total samples.

riological methods for motile and nonmotile *Salmonella* strains (data not shown). Furthermore, regarding the 2 times of incubation in the TT (TT1 and TT5) and the MKTTn (MKTTn1 and MKTTn5) method, there was not any significant difference in the parameters evaluated (data not shown).

Analysis of the data using kappa coefficients showed that there was an excellent agreement between bacteriological methods for all *Salmonella* strains (Table 6). The agreement was very good and good between the PCR methods for motile and nonmotile strains, respectively. However, it was poor, when the PCR and bacteriological methods were compared, for motile and nonmotile *Salmonella* strains.

DISCUSSION

We studied the performance of 2 culture methods and a PCR assay applied with 2 enrichment methods for motile and nonmotile *Salmonella* detection in the egg content, using artificially contaminated samples. Eyigor et al. (2010) studied 2 bacteriological methods (ISO 6579 and FDA/BAM) for detection of *Salmonella* in naturally contaminated poultry meat and red meat, and concluded that both methods had similar results. In our work, we studied the TT and MKTTn methods, which are based on the methods used in that study, but in another matrix (YA), and we obtained similar results.

When the performance of culture methods was applied to motile and nonmotile *Salmonella* strains, we did not find any significant differences. Works in other poultry samples showed that performance of culture methods was different between motile and nonmotile *Salmonella* (Soria et al., 2011, 2012). In fact, *S.* Enteritidis is the only human pathogen that contaminates eggs routinely, even though the on-farm environment of the chicken is a rich source of several *Salmonella* serovars. This determines its unique threat to food safety (Guard-Petter, 2001). The TT and MKTTn methods had a high value of Sp, Ac, Se, PPV, and NPV for motile, like *S.* Enteritidis, and nonmotile *Salmonella* strains in our study.

Kuijpers et al. (2010) reported that the number of positive isolations is more influenced by the choice of the selective enrichment medium than by the choice of the plating-out medium. Furthermore, the type of sample and especially the composition of the background flora are 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).

Table 6. Kappa coefficient values showing agreement between tetrathionate (TT), Muller-Kauffmann tetrathionate-novobiocin (MKTTn), and PCR [from tryptic soy broth with ferrous sulfate (TSBF) or buffered peptone water (BPW)] methods for egg content (pool of yolk-albumen)

Salmonella strain	Comparison between methods	Kappa coefficient
Motile	TT/TSBF-PCR	0.19*
	MKTTn/BPW-PCR	0.14*
	TT/MKTTn	1.00*
	TSBF-PCR/BPW-PCR	0.87*
Nonmotile	TT/TSBF-PCR	0.05
	MKTTn/BPW-PCR	0.09*
	TT/MKTTn	1.00*
	TSBF-PCR/BPW-PCR	0.72*

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

Although van Schothorst et al. (1977) showed that growth of competitors in the TT broth may decrease the inhibitory effect of the medium for *Salmonella* isolation, total bacteria, *Enterobacteriaceae*, and fungi could not be detected in yolk-albumen samples in our assay. Therefore, the inoculated *Salmonella* did not find any competitors. Kuijpers et al. (2008) demonstrated that MKTTn was not the optimal medium for selective enrichment of minced beef with much background flora. However, we found that this medium is good for *Salmonella* isolation in egg content, when there is not a background flora.

On the other hand, it was reported that an incubation time longer than 24 h was more important (more positive results after 48 h) for selective enrichment medium in chicken meat and in poultry and poultry environmental samples (Waltman et al., 1991; Kuijpers et al., 2008). We incubated the TT and MKTTn broth at 35 ± 2°C for 5 d, but we could not find any difference for *Salmonella* isolation between TT/MKTTn first and TT/MKTTn fifth. Although 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 could not find any significant differences between the 2 selective plating media used in our assay. Furthermore, the XLD and H agar plates did not show any growth in the negative control.

Bansal et al. (2006) investigated the reliability and application of a PCR-based assay that can be used after the 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. Piknová et al. (2002) found the PCR results positive from 1 to 10 cfu per 25 g in food samples artificially contaminated with *S.* Panama, which was consistent with the results obtained by the traditional culture method. Our PCR results were not as good as the culture method results for some *Salmonella* strains. Similar results were found by Pérez et al. (2008), who evaluated 2 PCR methods in eggs content (pool yolk and albumen) contaminated with 4 different *Salmonella* serovars. They found that all samples were positive to the culture method, whereas some of them were negative to the PCR. These authors attributed these results to the presence of inhibitors of the DNA amplification in the samples, e.g., excess of protein. However, they worked with different PCR conditions (DNA extraction, primers, and PCR cycling parameters) from our assay.

The TT and MKTTn methods are similar in terms of Ac, Se, Sp, PPV, and NPV for different *Salmonella* strains in egg content. The use of the PCR method cannot improve the same parameters, described before, in this matrix. So, further studies are needed to improve the different performance parameters and the limit of detection in egg content for the PCR methods, so the test can be used in the poultry and food industry.

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