Detection of *Salmonella* spp, *Salmonella* Enteritidis and Typhimurium in naturally infected broiler chickens by a multiplex PCR-based assay

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**Abstract**

The presence of *Salmonella* in the intestinal tract, on the chickens skin and among their feathers, may cause carcasses contamination during slaughtering and processing and possibly it is responsible by the introduction of this microorganism in the slaughterhouses. A rapid method to identify and monitor *Salmonella* and their serovars in farm is becoming necessary. A pre-enriched multiplex polymerase chain reaction (m-PCR) assay employing specific primers was developed and used to detect *Salmonella* at the genus level and to identify the *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) in broiler chicken swab samples. The method was validated by testing DNA extract from 90 fresh culture cloacal swab samples from poultry chicken cultured in phosphate buffer peptone water at 37 °C for 18 h. The final results showed the presence of *Salmonella* spp. in 25% of samples, *S.* Enteritidis was present in 12% of the *Salmonella*-positive samples and *S.* Typhimurium in 3% of the samples. The m-PCR assay developed in this study is a specific and rapid alternative method for the identification of *Salmonella* spp and allowed the observation of specific serovar contamination in the field conditions within the locations where these chickens are typically raised.

**Key words:** aviculture, bacteria, detection.

**Introduction**

*Salmonella enterica* is the representative pathogen causing salmonellosis in humans and animals worldwide and is sub-classified into more than 2500 serovars. *Salmonella* serovars Enteritidis and Typhimurium are the most important agents of food-borne salmonellosis in humans (Popoff et al., 2003). *S.* Enteritidis was the most prevalent serovar isolated from patients and food preparations in a survey conducted in southern Brazil from 1999 to 2008 (Kottwitz et al., 2010). It was estimated that approximately 75% of human *Salmonella* infection cases were due to contaminated food products derived from beef, pork, poultry and eggs (Hald et al., 2004) Poultry often become infected through the consumption of contaminated feed, cross-contamination in brooding houses, or during slaughter and processing (Doyle et al., 2006; Fratamico, 2003). With increasing regulatory pressure placed on poultry and livestock processors to reduce pathogen contamination in processed meats, more emphasis is likely to be focused on reducing pathogen contamination on farms (Rasschaert et al., 2008). Therefore, development of a rapid and sensitive method to *Salmonella* spp and their serovars is desirable. Several techniques for improving the detection of *Salmonella* serovars in fecal material such as the use of a selective culture medium and enzyme-linked immunosorbent assay have been developed (Araj and Chugh, 1987, Aspinall et al., 1992). However, problems remain with sensitivity and specificity that have limited routine use of these procedures. In general, these methods are laborious and
time-consuming, in contrast with molecular methods that reduce the time of diagnostic with the same efficiency (Aabo et al., 1993, Malorny et al., 2009). We herein report a simple, accurate and fast PCR assay to detect and identify Salmonella at the genus level and the serovars Enteritidis and Typhimurium, that utilizes the sequences of the Inv-A, IE-1 and Flic-C respectively (Fratamico and Strobaugh 1998, Rahn et al., 1992; Wang and Yeh, 2002) as targets for amplification of Salmonella bacteria in broiler swabs.

**Materials and Methods**

**Bacterial strains**

*S. Enteritidis* (*ATCC 13076*) and Typhimurium (*ATCC 14028*) were used as reference strains in the m-PCR assays. The *Salmonella* strains and the non-*Salmonella* bacterial strains used in this study are listed in Table 1. The isolates were grown in brain and heart infusion broth (Hi-media) at 37 °C for 24 h.

**DNA extraction**

Pure culture from the different bacteria genera and swabs collected from broiler chickens were cultured in 10 mL of phosphate buffer peptone water (Acumedia) for 18 h. One milliliter of these cultures was then centrifuged at 14,000 × g and re-suspended in 1 mL 0.9% (m/v) saline solution by vortexing. The tubes were centrifuged at 14,000 × g for 5 min, and the supernatants were carefully discarded. The pellets were re-suspended in 0.5 mL 0.1% Triton X-100 (Nuclear) by vortexing. The cell suspensions were held in a boiling water-bath for 10 min to lyse the cells and immediately placed on ice bath. The tubes were then centrifuged for 5 min at 14,000 × g. The supernatants were carefully transferred into new microcentrifuge tubes, and a 4 μL aliquot of the supernatant was used as the template DNA for the m-PCR.

**m-PCR experiments**

Reactions were carried out in a total volume of 20 μL containing 1 U Taq Polymerase (Invitrogen), 1 × Taq buffer (5 mM KCl, 10 mM Tris-HCl, pH 8.5), 1.5 mM MgCl₂, 0.1 mM dNTPs (Promega), 0.9 μM Inv-A primers, and 0.4 μM IE1 and Flic-C primers (Invitrogen). Amplifications were performed in a DNA thermocycler (MJ Research, PTC 100) The m-PCR protocol consisted of an initial denaturation step for 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 58 °C, and 30 s at 72 °C) and by a final extension step for 7 min at 72 °C. The PCR products were analyzed by electrophoresis using 1.5% agarose gels (Rozen et al., 2000) with TBE (45 mmol L⁻¹ Tris, pH 8.3, 45 mmol L⁻¹ borate, and 2 mmol L⁻¹ EDTA) as the running buffer. The gels were stained with SYBR® Safe.
(Invitrogen) and photographed with UV illumination by a photodocumentation system (Loccus, L-PIX HE).

**Primer design**

For multiplex PCR analysis, two primers pairs were used: Inv-A, which is specific for *Salmonella* spp. described by Fratamico and Strobaugh (1998) and IE 1, specific for *S. Enteritidis* described by designed by Wang and Yeh (2002), amplifying fragments of 796 bp and 316 bp. A third primer pair specific for *Salmonella Typhimurium* was designed in this work to amplify a fragment between 796 and 316 bp (Table 2). Primers were selected from a gene sequence involved in flagellin synthesis from complete sequence from *Salmonella Typhimurium* strain AY649720, recovered from the GenBank database. Primers were designed using the software Primer 3.0 (Rozen et al., 2000). The specificity of pair of primers was evaluated by nucleotide similarity searched with the BLAST algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov). Additionally, the PCR amplification was evaluated with DNA samples from the bacterial species listed in Table 2.

**PCR sensitivity**

PCR sensitivity was determined using suspensions of *S. Enteritidis* and *S. Typhimurium* in BHI broth incubated at 37 °C overnight without and with introduction of chicken swab. A serial of 10-fold dilutions was prepared in phosphate-buffered peptone water to obtain suspensions containing 10^3–10^8 CFU of each *Salmonella* serovar per ml. The cell bacterial concentrations were estimated by plating 0.1 mL of each dilution onto BHI agar. The plates were incubated at 37°C overnight, and colonies were counted. Experiments were conducted in triplicate. One milliliter of each dilution was centrifuged at 12,000 x g for 10min and the pellets washed twice with 900 μL of 0.85% saline solution. The pellets were resuspended in 500 μL of 1% Triton X-100, heated in a water bath for 10 min, cooled in ice, and centrifuged at 8000 x g for 5 min. The supernatants containing DNA of each dilution were used in m-PCR, adding five microliters of each serovar *Enteritidis* and *Typhimurium* DNA separately and both mixed in order to test the triplex specificity and sensitivity.

**Sampling**

Newly hatched Cobb chicks were obtained from a commercial broiler hatchery. Feed and drinking water were provided ad libitum. Thirty swabs from cloacae were assessed for the presence of *Salmonella* spp *Enteritidis* and *Typhimurium* on the dietary treatment at 10 and 20 days of chicken age and during the first day of the chick’s housing period.

**Results and Discussion**

**Specificity and sensitivity of multiplex PCR**

In the present study, two primer sets were selected to detect *Salmonella* spp and the serovar *Enteritidis* and a pair of primers was designed to detect *Typhimurium* serovar simultaneously using a m-PCR. The pair of primers used to detect *Salmonella* at genus level codifies the invasion protein A gene (InvA), widely distributed in *Salmonella* spp (Rahn et al., 1992; Fratamico 2003; Germini et al., 2009). The pair of *Enteritidis*-specific primers, using the IE-1 DNA sequence is an Insertion Element, found only in this serovar (Wang and Yeah, 2002; Silva et al., 2011). The pair of primers used to detect *S. Typhimurium* was designed in this work using the gene sequence involved in flagellin synthesis, ie, the Flic-C gene (Soumet et al., 1999) gives an amplicon size of 432 bp located in the central region of the gene (from nucleotides 794 to 1226) based on DNA sequence from AY649720.1. To assess and evaluate the specificity of the three primers sets, the m-PCR was first optimized and then tested with DNA templates prepared from the 38 bacterial strains, including positive control strains, as shown in Table 1. All bacteria from *Salmonella* genera showed a 796 bp amplicon, yielded from Inv-A pair of primer amplification. *S. Enteritidis* showed two amplicons, a 796 bp and a 316 bp amplicon, specific to this serovar, yielded from IE-1 amplification. *S. Typhimurium* also showed a 796 bp amplicon and a 432 bp specific amplicon, resulted from Flic-C pair of primers amplification. These results confirmed that the Inv-A, IE1 and Flic-C genes were appropriate candidates for the specific detection of *Salmonella* spp. and serovars Enteritidis and Typhimurium. In recent years, many studies have been published using specific primers complementary to virulence genes in combination.

| Primers | Length | Primer sequence 5' → 3' | Amplification product (bp) | Source               |
|---------|--------|------------------------|---------------------------|----------------------|
| Inv-A forward | 22 | CGG TGG TTT TAA GCG TAC TCT T | 796 | Fratamico (2003) |
| Inv-A reverse | 21 | CGA ATA TGC TCC ACA AGG TTA | |                     |
| IE-1 forward | 20 | AGT GCC ATA CTT TTA ATG AC | 316 | Wang and Yeh (2002) |
| IE-1 reverse | 19 | ACT ATG TCG ATA CGG TGG G | |                     |
| Flic-C forward | 20 | CCCGCTTACAGGTGACTAC | 432 | This work |
| Flic-C reverse | 20 | AGCGGTTTTGCGTGGTGT | |                     |

Table 2 - Primers used for identification of *Salmonella* spp. *Salmonella Enteritidis* and *Typhimurium*. 

Inappropriate candidates for the specific detection of *Salmonella* spp Enteritidis and Typhimurium. In recent years, many studies have been published using specific primers complementary to virulence genes in combination.
with different pre-enrichment strategies (Cardona-Castro et al., 2009, Kumar et al., 2009, Lim et al., 2003). Soumet et al. (1999) developed an m-PCR assay, but they were not able to detect *Salmonella* without the isolation of suspicious colonies. In this study we were able to detect *Salmonella* using pre-enrichment culture and it was not necessary to submit the samples to the enrichment step and isolation of suspicious colonies thus reducing the time of analysis at least 1.5 days. Another advantage is the simplicity of DNA extraction (by boiling), even though the most part of pathogens detection using feces, the DNA is extracted after a treatment with proteinase K and phenol-chloroform to eliminate the PCR inhibitors (Frankel et al., 1990, Stone et al., 1999). These techniques, however, greatly increase the detection cost and time and certain chemicals as phenol-chloroform also are able to inhibit PCR.

The m-PCR efficiency was not affected in the detection of target microorganisms when DNA of *Salmonella* Enteritidis and Typhimurium were mixed as observed by comparing PCR product intensity on agarose gel after staining. The m-PCR detection sensitivity was approximately $10^2$ CFU/mL of *S.* Enteritidis mL$^{-1}$ and *S.* Typhimurium in phosphate-buffered peptone water (Figure 1). While these serovars were cultured with swab material this sensitivity was reduced to $10^1$ CFU/mL, showing matrix effect in amplification results. This was possible because swab as fecal samples might contain PCR inhibitors.

**Analysis of cloacal chicken swabs**

Ninety chicken cloacal swabs from poultry collected on the first, tenth, and twentieth days of age were cultured in peptone water for 18 h and used to extract DNA for m-PCR assays. It was verified whether the m-PCR assay could be applied in samples from field conditions (Figure 2). The amplification of *Salmonella* spp and serovars Enteritidis and Typhimurium in chicken swab samples naturally infected presented a reliable sensitivity of the method.

*Salmonella* spp., *S.* Enteritidis and Typhimurium were not detected in any of the first day collected chicken samples suggesting that the birds were originally *Salmonella*-free at the hatchery. However, on the tenth day, 12 samples were positive for *Salmonella* spp., five of them belonging to the serovar Enteritidis and 2 of them to the Typhimurium serovar (Table 3). Finally, on the twentieth day, ten samples were positive for *Salmonella* spp., and six of them belonging to the serovar Enteritidis and none Typhimurium serovar was detected. This tendency in reduction of contamination throughout 20 days was possible because the newly hatched chicks are highly susceptible to infection with *Salmonella* than the older because the acquisition of microorganisms that constitute the normal chickens intestinal microbiota as pointed is gradual (Sterzo et al., 1994). Infected young birds excrete *Salmonella* in the feces at greater rates (higher concentration of bacteria and higher number of birds) when compared to excretion by older infected chickens (Smith et al., 1995). Therefore it is possible to conclude that the m-PCR assay described herein was found to be a rapid and simple

**Figure 1** - Multiplex PCR assay with the primer sets Inv-A, IE-1 and Flec-C, respectively. Lane M: 1 Kb ladder (Invitrogen); Lane 1: Negative control; Lane 2: *S.* Enteritidis (*ATCC* 14028); Lane 3: *S.* Typhimurium; Lanes 4 to 7: PCR results from a serial of 10-fold dilutions prepared in phosphate-buffered peptone water containing $10^3$ to $10^2$ CFU/mL of *S.* Enteritidis and *S.* Typhimurium.

**Table 3** - Number of samples positive for *Salmonella* spp., *S.* Enteritidis and Typhimurium from swabs cloacae of poultry.

| Number of contaminated chicken samples per day of life | first day | 10 days | 20 days | Total |
|--------------------------------------------------------|----------|---------|---------|-------|
| *S.* Enteritidis                                       | 0/30     | 5/30    | 6/30    | 11/90 |
| *S.* Typhimurium                                       | 0/30     | 2/30    | 0/30    | 3/90  |
| Other (s) *Salmonella* *                                | 0/30     | 5/30    | 4/30    | 9/90  |
| *Salmonella* spp                                        | 0/30     | 12/30   | 10/30   | 22/90 |

*Represents *Salmonella* spp serovars of *Salmonella* enterica except the serovars Enteritidis and Typhimurium.
method to detect the *Salmonella* genus and for the identification of *S. Enteritidis* and *Typhimurium* in chicken swab samples.

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