

Research Paper

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

F.G. Paião¹, L.G.A. Arisitides¹, L.S. Murate¹, G.T. Vilas-Bôas²,
L.A. Vilas-Boas², M. Shimokomaki¹

¹Universidade Estadual de Londrina, Centro de Ciências Agrárias,
Departamento de Ciência e Tecnologia de Alimentos, Londrina, PR, Brazil.

²Universidade Estadual de Londrina, Centro de Ciências Biológicas,
Departamento de Biologia Geral, Londrina, PR, Brazil.

Submitted: December 16, 2010; Approved: September 13, 2012.

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-con-

tamination in brooding houses, or during slaughter and processing (Doyle *et al.*, 2006; Fratamico, 2003). With increasing regulatory pressure placed on poultry and live-stock 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

Table 1 - Bacterial strains used to assay the specificity of the Multiplex PCR. *Salmonella* and related bacteria were grown in phosphate-buffered peptone water for 24 h at 37 °C prior DNA extraction to use in this m-PCR assay.

Strains	Number of strains	m-PCR positive results from amplified products		
		796 bp	316 bp	432 bp
<i>S. Enteritidis</i>	5	5	5	0
<i>S. Typhimurium</i>	3	3	0	3
<i>S. Agnona</i>	2	2	0	0
<i>S. Dublin</i>	2	2	0	0
<i>S. Infantis</i>	4	4	0	0
<i>S. Montevideo</i>	2	2	0	0
<i>S. Newport</i>	2	2	0	
<i>Bacillus cereus</i>	1	0	0	0
<i>Bacillus subtilis</i>	1	0	0	0
<i>Citrobacter freundii</i>	2	0	0	0
<i>Enterobacter aerogenes</i>	1	0	0	0
<i>Enterobacter cloacae</i>	1	0	0	0
<i>Escherichia coli</i>	2	0	0	0
<i>Klebsiella pneumonia</i>	2	0	0	0
<i>Morganella morganii</i>	2	0	0	0
<i>Proteus mirabilis</i>	3	0	0	0
<i>Shigella sonnei</i>	2	0	0	0
<i>Staphylococcus aureus</i>	1	0	0	0
<i>Staphylococcus saprophyticus</i>	1	0	0	0

(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 asses 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

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

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	CCCCTTACAGGTGGACTAC	432	This work
Flic-C reverse	20	AGCGGGTTTCGGTGGTTGT		

with different pre-enrichment strategies (Cardona-Castroa *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 in 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 phenolchloroform 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 phenolchloroform 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 10^3 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 cloacae 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).

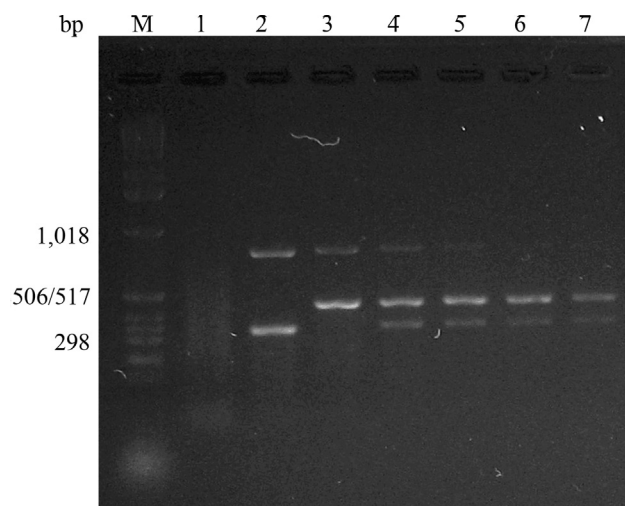


Figure 1 - Multiplex PCR assay with the primers sets Inv-A, IE-1 and Flic-C, respectively. Lane M: 1Kb ladder (Invitrogen); Lane 1: Negative control; Lane 2: *S. Enteritidis* (ATCC 14028); Lane 3: *S. Typhimurium*; Lanes 4 to 7: PCR results from a serial of 10-fold dilutions prepared in phosphate-buffered peptone water containing 10^5 to 10^2 CFU/mL of *S. Enteritidis* and *S. Typhimurium*.

re 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

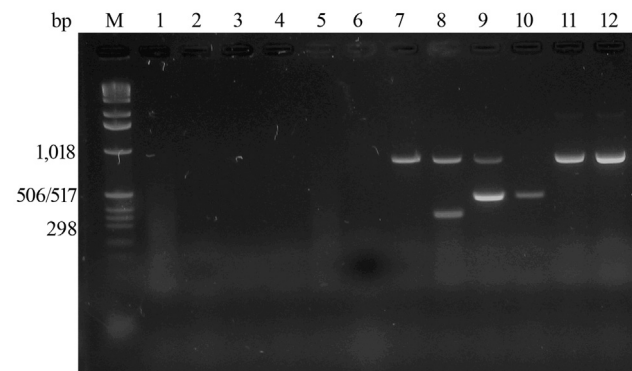


Figure 2 - Fragments resulting from m-PCR of chicken cloacal swabs. Lane M: 1 kb ladder (Invitrogen); Lane 1: negative control. Lanes 2 to 6: non contaminated chicken swabs samples. Lanes 7, 8 and 9: PCR positive controls: *S. Infantis*, *S. Enteritidis* and *S. Typhimurium*, respectively. Lanes 10 to 12: positive naturally contaminated swabs samples.

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
<i>S. Enteritidis</i>	0/30	5/30	6/30	11/90
<i>S. Typhimurium</i>	0/30	2/30	0/30	3/90
Other (s) <i>Salmonella</i> *	0/30	5/30	4/30	9/90
<i>Salmonella</i> 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.

Acknowledgments

This research was supported by CNPq/MAPA (Process #578315/2008-4) and Araucária Foundation/CNPq Pronex (Protocol #09.277). FGP holds a postdoctoral CNPq scholarship (Process 151809/2008-9) and MS is a CNPq Research Fellow. We thank Dr Tereza Cristina Rocha Moreira de Oliveira from Londrina State University for the kind supply of the bacterial strains used in this work.

References

- Aabo S, Rasmussen OF, Rossen L, Sørensen PD, Olsen JE (1993) *Salmonella* identification by the polymerase chain reaction. *Mol Cell Probes* 7:171-178.
- Araj GF, Chugh TD (1987) Detection of *Salmonella* spp. in clinical specimens by capture enzyme-linked immunosorbent assay. *J Clin Microbiol* 25:2150-2153.
- Aspinall ST, Hindle MA, Hutchinson DN (1992) Improved isolation of salmonellae from feces using a semisolid Rappaport-Vassiliadis medium. *Eur J Clin Microbiol Infect Dis* 11:936-939.
- Cardona-Castroa N, Sánchez-Jimenez M, Lavaletta L, Munhoz N, Morenoc J (2009) Development and evaluation of a multiplex polymerase chain reaction assay to identify *Salmonella* serogroups and serotypes. *Diag Microb Infect Dis* 65:327-330.
- Doyle MP, Erickson MC (2006) Reducing the carriage of food-borne pathogens in livestock and poultry. *Poultry Sci* 85:960-973.
- Frankel G, Riley L, Giron A, Valmassoi J, Friedmann AN, Strockbine NA, Falkow S, Schoolnik GK (1990) Detection of *Shigella* in feces using DNA amplification. *J Infect Dis* 16:52-1256.
- Fratamico PM (2003) Comparison of culture, polymerase chain reaction (PCR), TaqMan *Salmonella*, and Transia Card *Salmonella* assays for detection of *Salmonella* spp. in naturally-contaminated ground chicken, ground turkey, and ground beef. *Mol Cell Probes* 17:215-221.
- Fratamico PM, Strobaugh TP (1998) Simultaneous detection of *Salmonella* spp. and *Escherichia coli* O157:H7 by multiplex PCR. *J Food Ind Microbiol Biotech* 21:92-98.
- Germini A, Masola A, Carnevali P, Marchelli R (2009) Simultaneous detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Cont* 20:733-738.
- Hald T, Vose D, Wegener HC, Koupeev T (2004) A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. *Risk Anal* 24:255-269.
- Kottwitz LBM, Oliveira TCRM, Alcocer I, Farah SMSS, Abrahão WSM, Rodrigues DP (2010) Avaliação epidemiológica de surtos de salmonelose ocorridos no período de 1999 a 2008 no estado do Paraná, Brasil. *Acta Scientia* 32:9-15.
- Kumar R, Surendran PK, Thampuran N (2009) Detection and characterization of virulence factors in lactose positive and lactose negative *Salmonella* serovars isolated from seafood. *Food Cont* 20:376-380.
- Malorny B, Huehn S, Dieckmann R, Kramer N, Helmuth R (2009) Polymerase chain reaction for the rapid detection and serovar identification of *Salmonella* in food and feeding stuff. *Food Anal Meth* 2:81-95.
- Lim YH, Hirose K, Izumiya H, Arakawa E, Takahashi H, Terajima J, Itoh KI, Tamura K, Kim SI, Watanabe H (2003) Multiplex polymerase chain reaction assay for selective detection of *Salmonella enterica* serovar Typhimurium. *Jpn J Infect Dis* 56:151-155.
- Popoff MY, Bockemuhl J, Gheesling LL (2003) Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Res Microbiol* 154:173-174.
- Rahn K, De Grandis SA, Clarke RC, Curtiss R, Gyles CL (1992) Amplification of an *invA* gene sequence of *Salmonella* Typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes* 6:271-279.
- Rasschaert G, Houf K, Godard C, Wildemaue C, Pastuszczyk-Frak M, De Zutter L (2008) Contamination of carcasses with *Salmonella* during poultry slaughter. *J Food Prot* 71:146-152.
- Rozen S, Skaletsky JH (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, pp 365-386.
- Silva DSP, Canato T, Magnani M, Alves J, Hirooka EY, Oliveira TCRM (2011) Multiplex PCR for the simultaneous detection of *Salmonella* spp. and *Salmonella* Enteritidis in food. *Int J Food Sci Tech* 46:1502-1507.
- Smith HW (1965) The development of the flora of the alimentary tract in young animals. *J Pathol Bacteriol* 90:495-513.
- Soumet C, Ermel G, Rose V, Rose N, Drouin P, Salvat G, Collin P (1999) Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium and *Salmonella* Enteritidis strains from environmental swabs poultry houses. *Lett App Microbiol* 29:1-6.
- Sterzo EV, Paiva JB, Penha-Filho RAC, Berchieri-Junior A (2005) Time required to protect the intestinal tract of chicks against *Salmonella enterica* serovar Enteritidis using competitive exclusion. *Braz J Poultry Sci* 7:119-122.
- Stone GG, Oberst OD, Hays MP, McVey S, Chengappa MM (1994) Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. *J Clin Microbiol* 32:1742-1749.
- Wang SJ, Yeh DB (2002) Designing of polymerase chain reaction primers for the detection of *Salmonella* Enteritidis in foods and faecal samples. *Lett App Microbiol* 32:422-427.