Research Article

# A Rapid Identification of *Salmonella enterica* in Chicken Skin Using an *invA* Molecular Marker

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## Abstract

*Salmonella* is a gram-negative bacillus that lives in the intestinal tract of human and animals and causes diarrhea. *Salmonella* could be found in undercooked products of poultry with no impact on the taste, smell, or appearance. Since poultry eggs and meat might be sources of *Salmonella* and pose a hazard to public health, it is important to accurately detect *Salmonella* infection. In this regard, the present study aimed to develop a rapid and sensitive method for the diagnosis of *Salmonella* spp. in samples from the poultry industry. To do so, the sensitivity of *S. enterica* serotype Enteritidis detection was assessed with ten-fold serial dilutions in peptone water to give suspensions containing $10^0$ to $10^5$ CFU/mL. For artificial inoculation, skin samples were sequentially inoculated with the serial dilutions, while a control sample was included to ensure that the skin was not naturally contaminated with *Salmonella*. 53 commercial chicken skin samples were obtained from different local shops. Then, DNA was extracted from all samples, and the quality of extracted DNAs was checked by spectrophotometry and confirmed by agarose gel electrophoresis. For PCR, a pair of oligonucleotide primers, INVA, was designed to amplify the *invA* gene. Results revealed a band of 796 bp in samples artificially contaminated with *S.* Enteritidis. Likewise, the 796 bp band was detected in 38 samples (71%) with deferent intensities, which presented different amounts of contamination. Accordingly, the present study provided a valuable method for the detection and control of *Salmonella* infection in the poultry industry, since results would be available in less time than with the conventional cultural method.

***Keywords*: *Salmonella enterica*, *invA* gene, PCR assay, public health**

## Introduction

*Salmonella* is a rod-shaped gram-negative bacillus that causes diarrhea in humans. They pass through the feces of humans or animals to others (Su and Chiu, 2007). Undercooked products of poultry including meat and eggs are food sources posing the greatest hazard to public health (Braun and Methner, 2011). The appearance of food, as well as the taste and smell, does not change by *Salmonella* Enteritidis. The bacteria live in the intestinal tracts of infected animals and humans (Callaway et al., 2008).

One of the most common etiologic agents of bacterial Enteritis in children is nontyphoid *Salmonella*. So far, several *Salmonella* serovars have been identified, in which *inv* gene exists that is capable of the bacteria attacking the cells (Galán and Curtiss 3rd, 1991). In addition, five *Salmonella* serovars have been identified that contain virulence plasmid carrying *spv* gene, such as Typhimurium, Choleraesuis, Dublin, Enteritidis, and Gallinarum-Pullorum (Gulig et al., 1992; Gulig et al., 1993). Most of the identified *Salmonella* spp. are etiologic agents of enteritis in humans except, Gallinarum-Pullorum, which is specific to fowl. For instances, Typhimurium is commonly seen in patients with diarrhea and Choleraesuis, and Dublin and Enteritidis are often found in bacteremia patients (Guiney et al., 1995). At present, bacterial culture methods used stool samples to detect *Salmonella* Enteritis. However, this method is very time-consuming and shows difficulties to perform. To overcome this problem and improve the diagnosis of *Salmonella* serovars in feces, selective culture media and enzyme-linked immunosorbent assay

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have been developed (Araj and Chugh, 1987; Aspinall et al., 1992) However, due to low sensitivity and low specificity of these methods, their application is very limited (Chiu and Ou, 1996). On the other hand, PCR with preincubation in an enrichment broth was introduced as a quick and sensitive method for analysis of human samples (Chiu and Ou, 1996; Lin and Tsen, 1999; Luk et al., 1997; Widjojoatmodjo et al., 1992), animal (Cohen et al., 1996; Stone et al., 1994; Stone et al., 1995), and stool and food (Aabo et al., 1995; Bennett et al., 1998; Chen et al., 1997; Gouws et al., 1998; Kimura et al., 1999; Rijpens et al., 1999) samples. PCR is a powerful tool in the field of pathogen detection that has been used in many PCR-based devices for 30 years (Khnouf et al., 2020).

This is a useful and more rapid method because it increases the number of viable *Salmonella* in the sample and, therefore, increases the sensitivity of the assay (Chiu and Ou, 1996; Gouws et al., 1998; Kimura et al., 1999; Soumet et al., 1999). In the present study, we employed a RCR method for identification of *Salmonella* spp. in skin samples from the poultry industry in Iran.

## Materials and Methods

### Bacterial strain, growth conditions, and preparation of inoculums

*Salmonella enterica* serovars Enteritidis 64K (ATCC 1980) was grown on buffered peptone water (BPW) 0.1% (Merck) at 37°C for 24h. An aliquot of 1 mL of bacterial suspension was exposed to spectrophotometer analysis for detection of the optical density (OD), followed by conversion of the OD to CFU/mL by the MacFarland scale ($2\times10^9$ CFU/mL). Ten-fold serial dilutions were prepared in peptone water to give suspensions containing $10^0$ to $10^5$ CFU/mL.

### Artificial inoculation of chicken skin sample

First, the chicken skin sample was autoclaved and then six sterile 100 mL glass bottles were labeled for each dilution, and 5g of skin chicken were weighed and placed in each glass bottle with 45 mL sterile BPW. A control sample was also included to ensure that the skin was not naturally contaminated with *Salmonella*. 1 mL of each bacterial dilution ($10^0$ to $10^5$ CFU/mL) was mixed and incubated at 37°C in a shaker incubator with 100 rpm for 24 h to the corresponding to the storage bottle. After 24 h, DNA extraction was done.

### Investigation of commercial chicken skin samples in the market

To check the contamination of chicken skin samples available in the market with *Salmonella* bacteria, the samples were obtained, all on the same date, from local shops in Mashhad, Khorassan province, Iran. They were placed into a cool bag (at 4°C) and transported to the laboratory. Then, DNA extraction was done.

### DNA extraction from chicken skin samples

The skin samples were ground to a fine powder with mortar and pestle under liquid nitrogen and genomic DNA was extracted according to instructions from AccuPrep Genomic DNA Extraction Kit (BIONEER, Korea). Briefly, tissues lysis buffer and proteinase K were added to samples and incubated at 60 °C until samples were completely lysed. After the addition of binding buffer and another incubation at 60 °C, isopropanol was used and then, lysates were centrifuged at 8,000 rpm. Finally, washing buffer was used to completely remove ethanol by centrifugation at 12,000 rpm. Quality of the extracted DNA was checked by ratio measurement of the spectrophotometric absorbances at 260 nm ($A_{260}$) to that of 280 nm ($A_{280}$) and confirmed by the performance of agarose gelelectrophoresis.

### PCR amplification conditions

Amplification was performed using a PCR Express thermal cycler (Bioer XP). Amplification reactions were performed in volumes of 20 µl, containing 5 µl of template DNA, 2 µl of 10× reaction buffer, 0.5 µl of 25µm MgCl2, 0.3 µl of 10 µm dNTPs and 10 pmol (0.9 µl) of each primer targeting the *invA* gene (GenBank accession no. KX788214), INVAF: CGGTGGTTTTAAGCGTACTCTT (Tm: 55.7) and INVAR: CGAATATGCTCCACAAGGTTA (Tm: 52.7), (Fratamico and Strobaugh, 1998) and 0.5 U of Taq DNA polymerase (Cinnagen). The reaction mixture was subjected to the following thermal cycling conditions: heat denaturation at 94°C for 2 min, and then 35 cycles with heat denaturation at 94°C for 40s, primer annealingat 58°C for 1 min, and DNA extension at 72°C for 1 min. After the last cycle, samples were maintained at

72°C for 7 min to complete synthesis of all strands. The PCR products (796 bp) were electrophoresed on agarose gel, 2 µl of PCR product and 1 µl of 100 bp molecular marker (Cinnagen), and visualized and photographed under the UV light.

## Results

In present study, sensitivity of *S.* Enteritidis detection was assessed with ten-fold serial dilutions. A band of 796-bp was detected after PCR assay in the samples of chicken skin artificially contaminated with *S.* Enteritidis (Figure 1). The commercial samples, 53 in total, of chicken skin, were prepared from different local shops (provided by fourteen different slaughterhouses) (Table. 1).

PCR assay showed the 796 bp band in 38 samples (71%) indicating of contamination these samples with *S. enterica*. Gel electrophoresis analysis of PCR products performed on genomic DNA from commercial chicken skins, using primers aiming a DNA fragment of *Salmonella* spp. The codes on top of each lane correspond to the samples from local shops. Visualized bands indicate contamination of the samples with bacteria, and different rate shows different intensities (Figure 2).

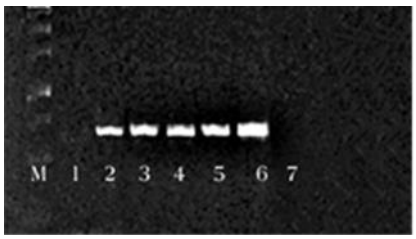


**Figure 1.** Sensitivity of *S.* Enteritidis detection; Lanes 1-6: PCR results from a serial of 10-fold dilutions prepared on buffered peptone water containing $10^0$ to $10^5$ CFU of *S.* Enteritidis. Lane 7: negative control. M: marker

**Table 1-** Molecular detection of *Salmonella* spp. by PCR on different samples. C: The name of samples is on the basis of slaughterhouses 's.

| Samples | PCR assay |
|---|---|
| | |
| $(Ka_1)c$ | + |
| $(Ka_2)$ | + |
| $(Ka_3)$ | - |
| $(Ka_4)$ | + |
| $(Ka_5)$ | + |
| $(Ka_6)$ | + |
| $(M_1)$ | + |
| $(M_2)$ | - |
| $(M_3)$ | + |
| $(M_4)$ | - |
| $(SP_1)$ | + |
| $(SP_2)$ | - |
| $(SP_3)$ | - |
| $(SP_4)$ | + |
| $(SP_5)$ | + |
| $(SP_6)$ | + |
| $(F_1)$ | - |
| $(F_2)$ | + |
| $(B_1)$ | + |
| $(B_2)$ | + |
| $(B_3)$ | + |
| $(B_4)$ | + |
| $(B_5)$ | + |
| $(D_1)$ | - |
| $(D_2)$ | + |
| $(D_3)$ | + |
| $(D_4)$ | - |
| $(D_5)$ | + |
| $(D_6)$ | + |
| $(TK_1)$ | + |
| $(TK_2)$ | + |
| $(TK_3)$ | - |
| $(TK_4)$ | + |
| $(TK_5)$ | + |
| (H) | + |
| $(A_1)$ | - |
| $(A_2)$ | + |
| $(L_1)$ | + |
| $(L_2)$ | + |
| $(L_3)$ | + |
| $(L_4)$ | + |
| $(Bsh_1)$ | + |
| $(Bsh_2)$ | - |
| $(TU_1)$ | + |
| $(TU_2)$ | - |
| $(TU_3)$ | + |
| $(TU_4)$ | - |
| $(TU_5)$ | + |
| $(TU_6)$ | - |
| (Ra) | + |
| $(N_1)$ | + |
| $(N_2)$ | - |
| $(N_3)$ | + |

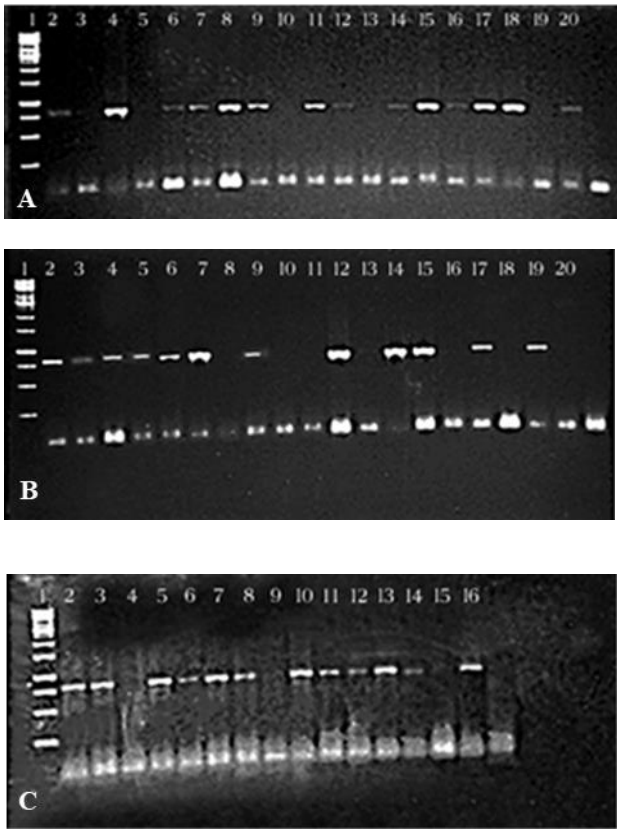


**Figure 2**. **A**: Lane 1 represents 100bp molecular marker. Lanes 2-20 represent PCR products from samples M1, M2, M3, M4, H1, D1, D2, D3, D4, D5, D6, A1, A2, L1, L2, L3, L4, F1, F2 respectively. **B**: Lane 1 represents 100bp molecular marker. Lanes 2-20 represent PCR products from samples B1, B2, B3, B4, B5, Bsh1, Bsh2, Sp1, Sp2, Sp3, Sp4, Sp5, Sp6, TU1, TU2, TU3, TU4, TU5, TU6 respectively. **C**: Lane 1 represents 100bp molecular marker. Lanes 2-16 represent PCR products from samples TK1, TK2, TK3, TK4, TK5, Ka1, Ka2, Ka3, Ka4, Ka5, Ka6, Ra1, N1, N2, N3 respectively. The last column in each picture corresponds to the negative control.

## Discussion

As indicated in previous reports, the application of boling method for extraction of bacterial genomic DNA from the contaminated chicken skins was inefficient compared to the commercial kits (Silva et al., 2011). It seems that the loss of DNA during the different isolation procedures plays a decisive role in the detection limit of *Salmonella* (Klerks et al., 2006). Effective methods for *Salmonella* detection in foods should be as specific and sensitive as conventional culture methods, and also analyze different food matrices. PCR is a reliable method for the detection of food-born pathogens and has international regulatory standards (Germini et al., 2009). The absence of the *Salmonella invA* sequence in other invasive bacteria such as *Yersinia* spp, which also can invade chicken, demonstrates the particular specificity and utility of this primer pair (INVA) for the detection of *Salmonella* spp. (Gallegos-Robles et al., 2009). A culture-based method is used to detect *Salmonella* in food, a process that takes several days and involves non-selective and selective enrichment, differential selective plating, and biochemical confirmation (Whyte et al., 2002). Quite unlike, PCR is feasible and makes it possible to identify damaged or dead cells (de Freitas et al., 2010; Silva et al., 2011). The *invA* gene, presented in most *Salmonella* species and essential for invading cells, is located in pathogenicity island I. This gene encodes type-III proteins of the secretion system. One of the methods for detecting *Salmonella* spp. in human and animal food matrices, as well as in water and faecal samples, is the use of sequences complementary to the *invA* gene as a primer (Germini et al., 2009; Maciorowski et al., 2005; Malorny et al., 2003). Previous microbiological studies conducted in cantaloupe rinse samples suggested that PCR assay is more sensitive for the detection of *Salmonella* spp. than the cultural method (Gallegos-Robles et al., 2009). Moreover, another study showed that PCR assay has an efficiency of 95%, which is much higher than the 60% by culture alone for identification of *Salmonella* serovars in feces (Chiu and Ou, 1996). A similar study was conducted on the poultry market, and 440 chicken meat samples were examined for virulent antibiotic-resistant *Salmonella*. spp strain by PCR and classical bacteriology methods (Nazari Moghadam et al., 2023).

In conclusion, although chicken skin is usually not consumed raw, there is risk of *S. enterica* infection if the skin is improperly cooked, so it is better to separate chicken skin before consumption. Just as Gallegos- Robles *et al*., (2009) discussed, the current study indicates that PCR assay is a reliable screening test, and outcomes are available more quickly than with the cultural method.

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