IDENTIFICATION OF *Salmonella* sps. FROM CONTAMINATED MEAT SAMPLES BY MULTIPLEX PCR-BASED ASSAY

Behzad Nikbakht\(^1\) and Ali Mohamadi Sani\(^2\)*

\(^1\)Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran
\(^2\)Young Researchers and Elite Club, Quchan Branch, Islamic Azad University, Quchan, Iran

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**ABSTRACT**

Poultry and cattle are the principal hosts of pathogenic bacterium *Salmonella* species. Therefore, development and use of a precise and sensitive method for identifying this pathogen is one of the main concerns for researchers. In this study, total 1100 cattle and poultry meat samples were evaluated to examine the presence of *Salmonella* by using three methods viz pre-enrichment culture, medium culture and polymerase chain reaction. Among the collected 850 cattle and 250 poultry samples 4.35% and 5.6% of the cattle and poultry samples were contaminated by *Salmonella* respectively. Further, among the 51 *Salmonella* contaminated samples of cattle and poultry, 25% were infected by *S. enteritidis*, 35% by *S. typhimurium* and 17% by *S. infantis*. Among the various tested methods, combination of a conventional PCR technology and standard culture medium could be effective in providing a more and accurate profile of the prevalence of this pathogen in poultry and cattle meat.
1 Introduction

Salmonella, one of the major human pathogens, is widespread in the international food trade (Rhen, 2007). Generally, foodstuffs such as eggs, meat and dairy products are well known for spreading the infection of this bacterium among human being (Altekruse et al., 1997; Malorny et al., 2013). According to Hoffmann et al. (2012) annually more than one million people from United States were suffer from salmonellosis, among these 19,336 cases were reached up to hospitalization and 378 losses their life because of this disease. The European Union has confirmed that in 2011, total 95,584 European were suffered from salmonellosis and it was 4.5% less as compared to 2010 (EFSA, 2013).

Further, it was reported that annually 3.3 billion dollars losses occurred due to this disease (Hoffmann et al., 2012). Therefore, identification of this pathogenic microorganism in poultry and other meat products can play a vital role in preventing the infection and control the incidence of salmonellosis at the production or consumption level. Conventional and standard methods currently used for the detection of different Serotype of Salmonella include the application of non-selective and selective enrichment culture, and growth on selective media (FDA, 2007). Although, this practice is according to the standard method ISO6579 and the US Food and Drug Administration but it may take up to five days and in case of numerous samples, the identification approach will be exhausting and very laborious (ISO, 2002; FDA, 2007). As a result, optimization of a rapid, accurate and economical procedure can be a great help to the industry, laboratories and public (Temelli et al., 2012). Now in these days various rapid methods such as the use of polyclonal and monoclonal antibodies against the flagellar somatic antigens as well as identification by DNA hybridization with polynucleotide and oligonucleotide probes have been suggested for the detection of Salmonella, (Eriksson et al., 2007). Because of differences in sensitivity, specificity and analysis time, these methods did not get much popularity (Hoorfar et al., 2000).

According to recent literatures, PCR-based methods are much faster, accurate and inexpensive and these can employed widely in the identification of food pathogens, especially Salmonella (Löfström et al., 2004, Löfström et al., 2009, Krämer et al., 2011; Löfström et al., 2012; Jakociune et al., 2014). Among various identified Salmonella gene, INV gene contains a conserved sequence for this genus, which has been reported as the main indicator and global standard for the identification of Salmonella (Rhan et al., 1992; Malorny et al., 2003a). This gene produces the membrane protein responsible for host epithelial cells invasion (Darwin & Miller, 1999). Although PCR has been introduced as a rapid method to detect Salmonella in food but culture based methods are still used as principal and standard method to identify this pathogen. In present study, two methods viz PCR and medium culture methods were used to access the presence of Salmonella in 1100 collected cattle and poultry meat samples.

2 Material and Methods

2.1 Sample collection

Among total 1100 meat samples, 850 cattle meat samples were randomly collected from the selected slaughter houses from the city of Zahedan while the rest 250 samples of poultry meat were collected from the distribution centers of the same city. Collected samples were refrigerated and transported to the laboratory at 4°C. All the samples were collected confidentially without informing anything about the uses of these samples to the store owner. The pure culture of Salmonella spp. were established on Luria-Bertani agar medium and used as positive control.

2.2 Culture method

Accurate detection of bacterium in food samples required a selection of a suitable method to increase the population of Salmonella in cattle and poultry meat. For this purpose, four stages of enrichment protocol were used for the collected samples (Malorny et al., 2003b). These steps of enrichment proposal are (EP1): homogenized meat samples were supplemented with 25g of the enrichment medium containing buffered peptone and final volume of 225ml was prepared by adding water. Prepared combination were incubated at 37°C for 24 hours; 10ml of the previous culture was transferred to 100ml of the enrichment media for Salmonella (RV) (Rappaport-Vassiliadis Salmonella Enrichment Broth) and incubated at 42°C for 24 hours (EP2); 10ml of the previous culture medium was transferred to100ml of the enrichment media for Salmonella (SC) (selenite cystine broth) and incubated at 42°C for 24 hours (EP3); finally streaking was carried out using a sterile loop from the two enrichment media on the media Brilliant-Green Phenol-Red Lactose Sucrose Agar (BplS) and Xylose lysine deoxycholate agar (XLD) (EP4). These plates were then incubated at 37°C for 24 hours. Five colonies from each plate were transferred to the Nutrient agar medium and cultured at 37°C for 24 hours.

2.3 Biochemical tests

In order to identify the species of Salmonella in the samples, the bio chemical test Triple Sugar Iron Agar (TSI), Urea agar, Simmon Citrate, MRVP Broth, Lysine Iron Agar and tryptone water test were used (ISO 6579, 2002). All of these tests are based on color change in the medium. Positive samples for the presence of Salmonella at this stage were evaluated to determine the serotype using molecular methods.

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Table 1 Features and sequences of the primers used in this study.

| Serotype       | Gene | Primer Sequence                      | Gene length (bp) |
|----------------|------|--------------------------------------|------------------|
| *Salmonella*   | *Inv A* | 5’ AAA CGT TGA AAA ACT GAG GA3’        | 199              |
|                |       | 5’ TCG TCA TTC CAT TAC CTA CC3’        |                  |
| *S. enteritidis* | SDF   | 5’ AAA TGT GTT TTA TCT GAT GCA AGA GG 3’ | 399              |
|                |       | 5’ GTT CTT TCT TCT GGT ACT TAC GAT GAC 3’ |                |
| *S. typhimurium* | STM   | 5’ ACA GCT TGG CCT ACG CGA G 3’        | 759              |
|                |       | 5’ AGC AAC CGT TCG GCC TGA C 3’        |                  |
| *S. infantis*  | S1    | 5’ TTG CTT CAG CAG ATG CTA AG 3’       | 413              |
|                |       | 5’ CCA CCT GCC CCA ACG CT 3’           |                  |

2.4 Polymerase chain reaction

DNA was extracted from positive samples of *Salmonella* by using standard protocol proposed by Rodriguez-Lazaro et al. (2014). Briefly, Bacteria were cultured on LB agar for 24h at 37°C and it was followed by the inoculation of a loopful of bacteria with 300 microliters of distilled water, and boiled it for 10 min, then centrifuged at 6000 rpm for 5 min. 1.5 microliter of the supernatant were used for amplification by PCR. In order to detect *Salmonella* spp., *S. Enteritidis*, *S. Typhimurium* and *S. infantis*, specific primers were synthesized (Table1).

Reaction with these primers were carried out in a 50 microliter amplification mixture consisting of 5 µl of 10X PCR buffer (500mM Kcl, 200mM Tris HCl), 2.5 µl of dNTPs (10mM), 3 µl of MgCl2, 2 µl of each primer, 4 U of Taq DNA polymerase (Fermentase) and 3 µl of DNA extraction for each isolate. The mixture was subjected to 35 PCR cycles in a T-Personal Thermal Cycler (Germany). The variables for the amplification cycles were as follows: denaturation for 45s at 95°C, annealing of primers for 45s at 55°C, and primer extension for 1 min at 72 °C. Prior to the first cycle, the PCR mixture was incubated for 5 min at 94°C. After the last cycle, the mixture was incubated for 5 min at 72°C. The amplified products were detected by 1% agarose gel electrophoresis pre-stained with GelRed, at 100 V for 45 min. A positive response was defined by the presence of a visible band at the expected size.

3 Results

The enrichment medium was spiked into samples and the enrichment PCR method used to detect *Salmonella* after enrichment. Cultural and biochemical test results showed that out of 850 cattle samples, 37 samples (4.35%) and among 250 poultry samples, 14 samples (5.6 %) were infected by *Salmonella*. Electrophoresis of PCR products for the gene *invA* indicated a199-bp fragment on the agarose gel (Figure1). Based on the positive results obtained from the culture step, PCR was performed for 51 samples of cattle and poultry and the electrophoresis results of their products demonstrated that all 51 samples examined were positive for *invA* gene (100%).

![Figure 1](image-url)
Electrophoresis of PCR products for the genes SDF and STM in the form of multiplex PCR showed 399 and 759bp fragments on the agarose gel (Figure 2). Electrophoresis of PCR products for gene invA and S1 is shown in Figure 3.

Among 51 Salmonella positive samples, 13 samples (25%) were infected by S. Enteritidis, 18 samples (35%) by S. Typhimurium and 9 samples (17%) by S. infantis. Molecular analysis results of the cultured cattle samples indicated that among the various serotypes under this study, S. typhimurium composes the largest contaminating population of red meat (1.17 percent). Additionally, strains of S. Enteritidis and S. infantis were found in 0.58% and 0.11% of cattle samples, respectively. Molecular analysis of the cultured poultry samples was an indication of equal populations of these serotypes (3.2%).

Percentages of contaminated meat samples are provided in Table 2. Results of Table 2 showed that 62.1% of Salmonella subspecies detected in cattle appertain to the subspecies not investigated in this study and only 37.9% of the Salmonella identified in cattle are related to the subspecies of S. Enteritidis, S. Typhimurium and S. infantis. Inspection of positive samples in chicken shows that 78.58% of the Salmonella detected in poultry belongs to the subspecies of S. enteritidis, S. typhimurium and S. infantis. Therefore, it appears that these three subspecies of S. enteritidis, S. typhimurium and S. infantis are more common in poultry than cattle and they can be identified as the primary subtypes of Salmonella entangling chicken meat.

4 Discussion

Results of present study are in agreement with the findings of Jamshidi et al. (2008) who has reported 3 to 66% Salmonella contamination in cattle and poultry meat sample. Although the results of this research correspond with these studies, the identification methodology, sample size and selection of target...
Identification of *Salmonella* sps. from contaminated meat samples by multiplex PCR-based assay.

Table 2 Status of Contamination level in collected meat samples and available serotypes.

| No of Studied samples | Response | S. infantis | S. Typhimurium | S. enteritidis | S. enteritidis & S. infantis | S. infantis & S. Typhimurium | S. enteritidis & S. Typhimurium | S. enteritidis & S. Typhimurium & S. infantis | Other serotypes |
|-----------------------|----------|-------------|----------------|---------------|-----------------------------|-------------------------------|-------------------------------|------------------------------------------------|---------------|
|                       | Positive sample | 37          | 1              | 10            | 5                          | 0                            | 1                            | 1                                            | 0              |
|                       | Percentage     | 4.35        | 0.11           | 1.17          | 0.58                        | 0                            | 0.11                          | 0.11                                         | 0              |
|                       | Contamination percent in positive Sample | -           | 2.7            | 27            | 13.51                       | 0                            | 2.7                           | 2.7                                          | 0              |
|                       | Percentage     | 5.6         | 3.2            | 3.2           | 3.2                        | 2                            | 2.8                           | 2.8                                          | 16             |
|                       | Contamination percent in positive Sample | -           | 57.14          | 57.14         | 57.14                       | 35.7                         | 50                            | 35.7                                         | 28.57          |
|                       |               |             |                |               |                             |                              |                               |                                               |                |

One of the limitations in most of the studies is the number of samples being evaluated. Therefore, in this research revealed that results of small sample size cannot be generalized to the large population and these show very high percentage of contamination which may not be applicable for large population. Analysis of 850 cattle and 250 poultry samples in this study offered acceptable results on contamination status of meat by *Salmonella* in Zahedan, Iran. In some studies *femA* gene was used to identify salmonella (Cohen et al., 1996) but in majority of studies *invA* gene was used to detect *Salmonella*. Some strains of *Salmonella* such as *S.litchfield* and *S. senftenberg* cannot be identified using *invA* gene (Gala’n et al., 1992). So the application of PCR for the direct detection of *Salmonella* in food does not provide researchers with correct estimations. Therefore, understanding *Salmonella* subspecies present in each area, according to the studies of other researchers can be applied in the selection of appropriate target genes.

**Conclusion**

The results of this study demonstrate that every method of *Salmonella* detection in food has a number of limitations. However, culture method is suggested as the golden method for *Salmonella* detection in food, but to identify subtypes of *Salmonella*, the precise and sensitive PCR technique is required. In conclusion, the combined use of these methods can lead to the accurate identification of Salmonella.

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**Conflict of interest**

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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