Original Article

Identification of Avian *Salmonella* Isolates by PCR-RFLP Analysis of a fliC Gene Fragment

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**ABSTRACT**

The genus of *Salmonella* is very polymorphic and comprised of a number of genetically closely related serotypes. It is one of the emerging pathogens in food-borne disease which is often found in contaminated chicken eggs. *Salmonella enterica* is considered one of the major pathogens in public health worldwide. A total of 31 *Salmonella* isolates identified by specific antisera, which included *Salmonella enteritidis* (51.6%), *Salmonella typhimurium* (25.8%), *Salmonella infantis* (19.4%) and *Salmonella colindale* (3.2%). DNA was extracted using phenol- chloroform- isoamyl alcohol method. All the isolates showed fliC gene (1500bp) by using specific primers. PCR products were subjected to digestion using *HhaI* restriction endonuclease. PCR- RFLP results showed 3 patterns between all isolates. Our research gained in this study demonstrated that using *HhaI* restriction endonuclease could differentiate *Salmonella enteritidis* and *Salmonella colindale* but there is similarity between pattern of *Salmonella typhimurium* and *Salmonella infantis*.

**Keywords:** *Salmonella*, Avian, fliC gene, PCR-RFLP, *HhaI* restriction endonuclease

**INTRODUCTION**

Food-borne diseases caused by nontyphoid *Salmonella* represent an important public health problem worldwide. Most *Salmonella* infections in humans result from the ingestion of contaminated foods of animal origin (Kimura *et al* 2004, Braden 2006). *Salmonella* is one of the emerging pathogen in food borne disease (Bhunia *et al* 2008). Animals and their products, particularly chicken, meat and eggs, are considered to be major sources of human infections caused by this pathogen (Mahe *et al* 2008). Due to its endemic nature, high morbidity and association with a wide range of foods, this zoonotic disease is of high public health concern (Kottwitz *et al* 2008, Aarestrup *et al* 2007). Both the presence as well the dissemination of *Salmonella* spp. in foods represent an important issue to the poultry industry, since they could determine a decrease in the consumption of poultry meat, posing a threat to the national and international poultry trading (Ikuno *et al* 2004). The investigation of phenotypic and molecular profiles can provide the epidemiological characterization of *Salmonella* strains, and can help elucidate their cycle of transmission (Michael *et al* 2006). However, to achieve a higher confidence level on the results, epidemiological tracing should be based
on the association of independent genotypic and phenotypic characteristics (Harbottle et al 2006). Biochemical properties, serotyping and phage typing are routinely used in reference laboratories for the identification and characterization of Salmonella isolates, but their overall low discriminative power mean that these methods are of limited use as discriminative tools in epidemiological studies (Rementeria et al 2009). A number of genotyping and genetic methods represent the major techniques for the characterization of bacteria. Different methods such sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pulsed field gel electrophoresis (PFGE), plasmid profiling, DNA amplification finger printing (DAF), random amplified polymorphic DNA analysis (RADP-PCR) and restriction fragment length polymorphism (RFLP) analysis are the molecular techniques used for the characterization of bacterial macromolecules (Durrani et al 2008, Foley et al 2009). The RFLP system is inexpensive and easy to perform but requires that a unique set of restriction sites be present in the amplicon of interest. The method is rapid, simple, and reproducible and can potentially be applied for identification of isolates obtained from other production systems. More extensive studies need to be performed examining a larger number of farms and samples to determine the prevalence of Salmonella in agricultural production systems (Gallegos-Robles et al 2008). The fliC gene has a conserved terminal region and a variable central region, which determines the antigenic specificity. For this, the fliC gene, encoding for the flagellin protein, has been used as a target gene in assays to test the genetic diversity in Salmonella (Dauga et al 1998). The aim of this study was identification of Salmonella isolated from avian by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) by using restriction endonuclease HhaI.

MATERIALS AND METHODS

Bacterial strain. Thirty-one isolates of Salmonella were obtained from Razi Type Culture Collection (RTCC), Karaj, Iran. Biochemical tests and serotyping of all isolates were performed at the Microbiology Department of Razi Vaccine & Serum Research Institute. Salmonella enteritidis (ATCC 13076) and Citrobacter freundii (ATCC 43864) were used as positive and negative control in PCR-RFLP.

DNA extraction. Each Salmonella strains in this study was streaked onto trypticase soy agar (Merck, Germany) plates and incubated at 37 °C for 24 h. Colonies from each agar plate were removed with a loop and then suspended in 1 ml of lysis buffer. DNA extraction of all Salmonella isolates were performed by phenol-chloroform-isoamylalcohol (25: 24: 1) method (Sambrook et al 2001). DNA concentrations were estimated by the nanodrop (Biotek, USA). The purity of DNA checked at 260nm/280nm>1.8. The DNA template (100ng) used immediately for PCR amplification.

PCR – RFLP. PCR was conducted in a volume of 25 µl containing 2 µl of genomic DNA from the Salmonella serovar isolates, 12.5 µl mastermix, 2 µl of primers specific for fliC gene, 1 µl FSa-F (5CAAGTCATTAATACAAACAGCC-3), 1 µl FSa-R (5-TTAACGCAGTAAAGAGAGGAC-3) (Zahraei Salehi et al 2007) and 8.5 µl of dionized distilled water. Amplification was performed in a thermal cycler (Eppendorf) programead as follows: initial denaturation 94 °C for 5 min, 35 cycles with consisting of 1 min at 94 °C, 1 min at 55°C, 35 s at 72 °C and a final extension step of 1 min at 72 °C. Amplified products were resolved in 0.8% agarose gel. Following electrophoresis the gel was stained in ethidium bromide(1 µg/ml) and photographed under ultraviolet (UV) light. A 100 bp-plus DNA ladder (Fermentase) was used as a marker for determining the molecular weight of PCR products. Then, the fliC PCR products were directly digested by the restriction endonuclease HhaI (Fermentase). Each digestion was performed in a reaction volume of 30 µl consisting of 20 µl of PCR product, 3µL of HhaI buffer 10x, 1.5 µl HhaI enzyme and 5.5µl of dionized distilled water. After incubation at 37 °C for 16 h RFLP were
determined by electrophoresis of the digested DNA in 2.5% agarose gel for 4h at 60V. The 100 bp- plus DNA ladder (Fermentase) was used as the molecular weight marker in the PCR-RFLP analysis.

RESULTS

A total of 31 Salmonella isolates identified by specific antiserum, which included Salmonella enteritidis (51.6%), Salmonella typhimurium (25.8%), Salmonella infantis (19.4%) and Salmonella colindale (3.2%). In all 31 Salmonella isolates, a 1500 bp fliC fragment was amplified from all isolates (Figure 1). The results of PCR-RFLP with restriction enzyme HhaI for gene fliC showed 3 pattern between 31 isolated Salmonella. It was observed that HhaI is able to discriminate isolated including Salmonella enteritidis bands(110-810bp), Salmonella colindale bands between (110-700bp), and none of these represented strains have the same patterns, but Salmonella infantis and Salmonella typhimurium bands(110-790bp) were the same with their patterns (Table 1) and these two independent serotypes have the same profile (Figures 2, 3).

Table 1. The number of isolates and PCR-RFLP profiles of isolated Salmonella.

<table>
<thead>
<tr>
<th>Salmonella serotype</th>
<th>No. of isolate</th>
<th>No. of bands</th>
<th>fliC RFLP HhaI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella enteritidis</td>
<td>16</td>
<td>3</td>
<td>110-480-810</td>
</tr>
<tr>
<td>Salmonella infantis</td>
<td>6</td>
<td>3</td>
<td>110-350-790</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>8</td>
<td>3</td>
<td>110-350-790</td>
</tr>
<tr>
<td>Salmonella colindale</td>
<td>1</td>
<td>4</td>
<td>110-350-650-700</td>
</tr>
</tbody>
</table>

DISCUSSION

Salmonellosis is one of the most common food-borne bacterial diseases in the world (Fitzgerald et al 2003). Members of the genus Salmonella colonizes vertebrate hosts, with outcomes ranging from subclinical to systemic infection with high mortality. Animal infection has direct economic consequences, but asymptomatic carriage, leading to direct or indirect transmission to humans, maybe even more important (Songer et al 2005).

This bacterium is a major cause of diarrheal disease in both industrialized and developing countries (Goldman et al 2009). Chicken and related products are recognized as important reservoirs for Salmonella and vehicles for salmonellosis (Foley et al 2011). Hong et al (2003) studied on 52 serotypes of Salmonella. They were
analyzed their serotypes by PCR-RFLP with restriction endonucleases Sau3AI and HhaI for fliC and fljB genes. Ninety percent of the Salmonella serotypes could be identified. They concluded that PCR-RFLP could be a fast, accurate, and economical alternative approach to serotyping of Salmonella spp. In our study, 31 isolates from poultry that belong to 4 serotypes were investigated by PCR-RFLP. The fliC gene was able to differentiate Salmonella enteritidis, Salmonella typhimurium and Salmonella colindale. These results showed that this method can potentially be applied for identification of these serotypes. Dilmaghani et al (2010) identified the polymorphism of fljB gene among avian in different regions by PCR-RFLP method. Two RFLP patterns obtained. Pattern A was observed in 33 (63.46%) and pattern B in 19 (36.54%) of isolates. Salmonella typhimurium recovered from 13 broilers and 8 sparrow showed both A and B patterns. Our study showed 4 different serotypes of Salmonella and PCR-RFLP method on fliC gene showed 3 different patterns using HhaI, respectively. Forty-seven Salmonella isolates of 20 different serovars, derived from chicken samples in Thailand, were studied using the fliC/fljB PCR-RFLP assay with two restriction endonucleases, Mbo I and HhaI by Jong et al (2010). They demonstrated that PCR-RFLP was not able to differentiate Salmonella hadar, virchow, emek and albany. According to their research PCR-RFLP cannot replace serotyping. They showed that this assay was reproducible and successfully applied to simply screen Salmonella serovars as an alternative subtyping test for rapid traceability of Salmonella contamination in chicken production. In our research Salmonella typhimurium and infantis had same pattern and was not able to distinguish. Sumithra et al (2013) used RFLP to analysis of typing, heterogeneity, typeability and polymorphism of the 16S rRNA, fliC and fimH genes in Salmonella typhimurium isolates from different origin. Their results demonstrated that PCR-RFLP of these genes had good typeability but low discriminatory power. Based on our study, using two enzymes and two genes is more suitable to typing samples in PCR-RFLP. Gallegos-Robles et al (2008) worked on 22 Salmonella isolated from cantaloupe and chile pepper production systems by PCR–RFLP based on the fliC gene. Their results showed that the pattern of Salmonella typhimurium and Salmonella enteritidis was as the same as reference strain restriction profiles. We also had the same result for the profile of Salmonella enteritidis and Salmonella typhimurium. Our research gained in this study demonstrate that using HhaI restriction endonuclease could differentiate Salmonella enteritidis and Salmonella colindale but there is similarity between pattern of Salmonella typhimurium and Salmonella infantis. Many researchers used this method by different genes and restriction endonuclease enzymes and obtained different results. Our results confirm studies were done by Hong et al (2003) and Dauga et al (1998). According to the results based on Matsui et al (2001) and Sumithra et al (2013) research showed that PCR-RFLP with more than one endonuclease and genes give good typeability and increase the differentiating power. Although serotyping is gold standard in typing of Salmonella, but it is expensive and time-consuming and it must be done beside molecular techniques such as PCR-RFLP to differentiate all serovars of Salmonella. Also more studies need to be performed examining a large number of Salmonella serotypes. Our results in comparison with the other researches state that a large number of Salmonella serotypes must be analyzed by PCR-RFLP method and different enzymes must be used to give reliable results. Also typing of isolates by other methods might be useful.

**Ethics**

I hereby declare all ethical standards have been respected in preparation of the submitted article.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

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