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Originall 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 pathogen 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- isoamylalchol 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

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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 endnuclease *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 ºC 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) programed 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).

Table1. The number of isolates and PCR-RFLP profiles of isolated *Salmonella.*

Salmonella serotype	No. of isolate	No. of bands	*fliC* RFLP *HhaI*
Salmonella enteritidis	16	3	110-480-810
Salmonella infantis	6	3	110-350-790
Salmonella typhimurium	8	3	110-350-790
Salmonella colindale	1	4	110-350-650-700

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).

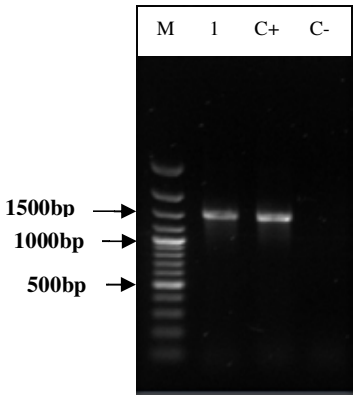

Figure 1. Agarose gel electrophoresis of PCR products obtained from amplification of the *fliC* gene. Lane M: 100bp-plus DNA Ladder, 1: *Salmonella enteritidis* isolates, C+: Positive control (*Salmonella enteritidis* ATCC13076), C- : Negative control (*Citrobacter freundii* ATCC43864). %0.8 gel agarose.

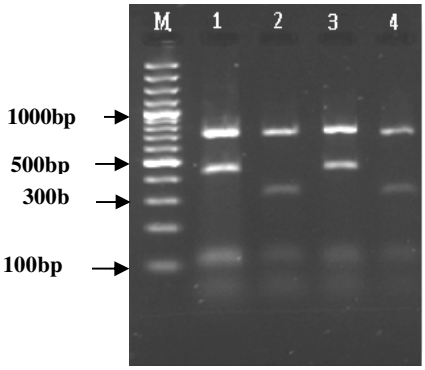

Figure 2. PCR-RFLP profiles of *fliC gene* with *HhaI* restriction endonuclease. Lane M: 100bp-plus DNA Ladder, 1: *Salmonella enteritidis* 2: *Salmonella infantis* 3: Positive control (*Salmonella enteritidis* ATCC13076), 4:*Salmonella typhimurium*, 2.5% gel agarose.

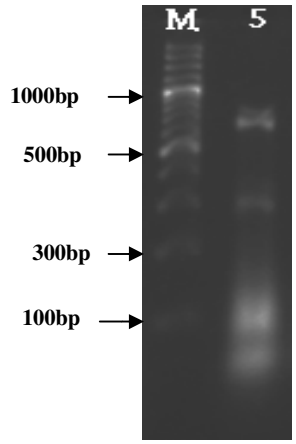

Figure 3. PCR-RFLP profiles of *fliC* gene with *HhaI* restriction endonuclease.

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. Ninty 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 belongs 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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