International Journal of Molecular and Clinical Microbiology 1 (2013) 255-260

International Journal of
Molecular and Clinical Microbiology

PCR–RFLP of isolated *Salmonella* from poultry with Sau3AI and HhaI restriction endonucleases in Arak

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ARTICLE INFO

Article history:
Received: 12 March 2013
Accepted: 30 April
Available online: 1 June 2013

Keywords:
Salmonella, Avian, fliC gene, PCR-RFLP, HhaI, Sau3AI, Arak.

ABSTRACT

Salmonella enterica is considered as one of the major pathogens in public health, worldwide. Diseases caused by *Salmonella enterica* serovars are especially prevalent in developing countries. It is one of the emerging pathogen in food-borne diseases, which is often found in contaminated chicken eggs. The aim of this study was to apply a PCR–RFLP method using Sau3AI and HhaI restriction endonucleases on the fliC gene to identify the serotypes of *Salmonella* isolates in avian living in Arak area in central Iran. Serotypes of 75 isolates included *Salmonella enteritidis* (45.33%), *Salmonella infantis* (44%), *Salmonella typhimurium* (5.33%), *Salmonella bardo* (2.67%) and *Salmonella bacongo* (2.67%). All the isolates showed fliC gene (1500bp) by using specific primers. The results of PCR-RFLP with restriction enzymes HhaI and Sau3AI for gene fliC showed 4 and 5 patterns. It was observed that Sau3AI is able to discriminate all serotypes of *Salmonella* but HhaI could not distinguish *Salmonella infantis* and *Salmonella typhimurium*. The prevalent *Salmonella* isolated in this study belonged to serotype enteritidis and infantis. The combination of the data obtained with both restriction enzymes could differentiate the serovars completely. These results showed that fliC gene is a suitable target gene for discriminating among these *Salmonella* serotypes by PCR-RFLP.

1. Introduction

Nontyphoidal *salmonella* (NTS) are of the major cause of food-borne illness and commonly cause self-limiting gastroenteritis, worldwide (Hendriksen et al., 2011). The ubiquitous *Salmonella enteritidis*, which affects both humans and animals, is one of the most commonly reported NTS serovars. In developing countries, NTS frequently cause severe illness, invasive infections and even death, especially among young children with underlying diseases or HIV infected immunocompromised adults (Graham 2010; Hohmann 2001). *Salmonella* causes a serious health problem in developing countries through a wide range of human diseases (Zaki et al., 2009). The most common sources of Salmonella include beef, poultry, eggs, dairy products and vegetables. Fruits and shellfish have also been implicated as sources of *Salmonella* (Uzzau et al., 2000).

The identification of *Salmonella* isolates into specific serovars is essential for epidemiologic studies and tracing the source of outbreaks (Hong et al., 2003). However, production and quality control of hundreds of antisera required for serotyping are laborious and time-consuming. To circumvent the problems, many genotyping techniques, including pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Multiplex PCR have been applied as alternative methods for *Salmonella* subtyping (Chansiripornchai et al., 2000; Gallegos-Robles et al., 2008; Luk et al., 1993). PCR is a rapid and

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specific method for detection of bacterial pathogens and biochemical methods have been complemented by DNA-based molecular techniques. The RFLP system is inexpensive and easy to perform but requires a unique set of restriction sites to be present in the amplicon of interest (Kwon et al., 2000). The method is rapid, simple, and reproducible and can potentially be applied for identification of isolates obtained from other production systems too (Gallegos-Robles et al., 2008). In *Salmonella*, flagella are a 55 kDa monomeric protein encoded by *fliC* and *fljB* genes that assemble to form the filament structure of the flagellar apparatus necessary for bacterial motility (Zeng et al., 2003).

The amino and carboxyl termini of flagellin are quite conserved, not only between *fliC* and *fljB*, but also in flagellins from different serotypes. In contrast, the central portion of the protein is hyper variable and contains most of the antigenic residues (Ciacci-Woolwine et al., 1998; McQuiston et al., 2004). The *fliC* gene has a conserved terminal region and a variable central region, which determines the antigenic specificity. For this, the fliC gene has been used as a target gene in assay to test the genetic diversity among *Salmonella* (Dauga et al., 1998). The aim of this study was to apply PCR-RFLP method based on the *fliC* gene to identify *salmonella* serotypes that isolated from poultry in Arak by using restriction endnucleases HhaI and Sau3AI.

2. Matherials and Methods

2.1. Salmonella isolates

A total of 75 *salmonella* isolates derived from poultry specimens sourced from various farms located in Arak areas in Central Iran, were obtained from Razi Type Culture Collection (RTCC), Karaj, Iran (Ezatpanah et al., 2013). *Salmonella enteritidis* (ATCC 13076), *Salmonella typhimurium* (ATCC 14028) and *Citrobacter freundii* (ATCC43864) were used as positive and negative controls in PCR-RFLP.

2.2. Salmonella DNA Preparation

Salmonella DNA was extracted by a simple method as described by Ghaderi et al., 2013. Briefly a loopful of overnight culture on nutrient agar plates was placed in 0.5 ml of TE buffer and vortexed. The bacterial suspension was heat-inactivated at 90°C for 10 min and centrifuged at 11000 rpm for 4 min. A small portion (1/64 v/v) of a 10 mg/ml proteinase K solution (Roche, Germany) was added to the supernatant and this mixture was directly used for PCR-RFLP.

2.3. PCR primers

The primers used to amplify phase-1flagellin gene were AAGTCATTAATACMAACAGCC (Fsa-F) and TTAACGCAGTAAAGAGAGGAC (Fsa-R) (Dauga et al., 1998).

2.4. PCR amplification of the fliC gene

PCR was conducted in a volume of 25 µl containing 2 µl of genomic DNA from the *salmonella* isolates, 12.5 µl mastermix, 2 µl of primers specific for fliC gene and 8.5 µl of deionized distilled water (D.D.W). Amplification was performed in a thermal cycler (Eppendorf) programmed as follows: initial denaturation 94°C for 5 min, 35 cycles 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 electrophoresed on 0.8% agarose gel. Following electrophoresis, the gel was stained in ethidium bromide and photographed under ultraviolet (UV) light. A 100 bp DNA ladder (Fermentase) was used as a marker for determining the molecular weight of PCR products (Nair et al., 2002).

2.4. RFLP analysis

Digestion solution was prepared with 20µl of the PCR product, 3µl of either HhaI or Sau3AI buffer 10x, 1.5µl of either *HhaI* or *Sau3AI* enzyme, and 5.5µl of D.D.W. After incubation at 37ºC for 16h, RFLP were determined by electrophoresis of the digested DNA in 2.5% agarose gel for 4h at 50V. Size of the products was analyzed in comparison to the 100 bp plus DNA Ladder (Fermentase).

3. Result

3.1. Salmonella isolates

A total of 75 *Salmonella*, identified by biochemical tests and serotyped with specific antisera, included *Salmonella enteritidis* (45.33%),

Salmonella infantis (44%), *Salmonella typhimurium* (5.33%), *Salmonella bacongo* (2.67%), and *Salmonella bardo* (2.67%).

3.2 PCR amplification of the fliC gene

An approximately 1.5 kbp fragment was amplified from 75 isolates of *Salmonella*. As expected, all serotypes of *Salmonella* produced the respective band on the agarose gel (Figure 1).

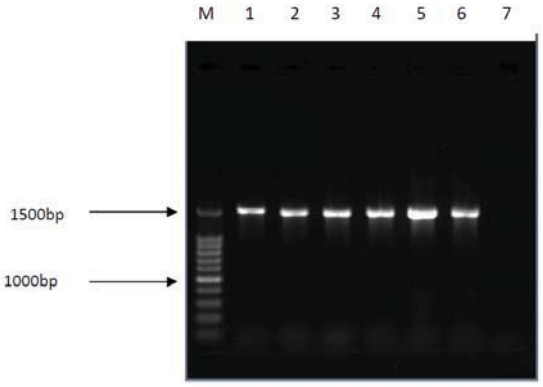

Figure 1. Electrophoresis of *fliC* gene amplicons from *Salmonella* isolates. M: Molecular marker 100bp DNA ladder (Fermentas); lane 1 (*Salmonella enteritidis* ATCC13076), lane 2(*Salmonella enteritidis*), lane 3 (*Salmonella infantis*), lane 4 (*Salmonella typhimurium*), lane 5 (*Salmonella bacongo*), lane 6 (*Salmonella bardo*), lane 7 Negative control *Citrobacter freundii* (ATCC43864).

3.3 RFLP analysis of fliC gene by HhaI

The results of PCR-RFLP with restriction enzyme *HhaI* for gene *fliC* showed 4 patterns between 75 isolated *Salmonella*. It was observed that *HhaI* is able to discriminate isolates including *Salmonella enteritidis* (bands: 110-810 bp), *Salmonella infantis* (bands: 110-790 bp), *Salmonella typhimurium* (bands: 110-790 bp), *Salmonella bacongo* (bands: 110-750 bp) and *Salmonella bardo* (bands: 110-320 bp). However, *Salmonella infantis* and *Salmonella typhimurium* produced the same band patterns (110-790 bp), as shown in Figure 2 and Table 1.

3.4 RFLP analysis of fliC gene by Sau3AI

The results of PCR-RFLP with *Sau3AI* on *fliC* showed 5 patterns among the isolates. It was observed that *Sau3AI* is able to discriminate isolates including *Salmonella enteritidis* (bands: 110-850 bp), *Salmonella infantis* (bands: 190-880 bp), *Salmonella typhimurium* (bands: 330-1100 bp), *Salmonella bacongo* (bands: 110-900 bp) and *Salmonella bardo* (bands: 530-1100 bp). This restriction enzyme was able to differentiate all serotypes as each produced a different RFLP pattern (Figure 3).

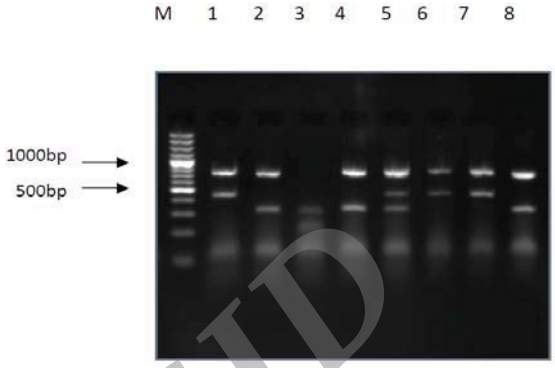

Figure 2. PCR-RFLP profiles of *fliC gene* with *HhaI* restriction endonuclease. M: Molecular Weight Marker DNA Ladder (plus); Lane 1, *Salmonella enteritidis*, Lane 2, *Salmonella infantis*, Lane 3, *Salmonella bardo*, Lane 4, *Salmonella typhimurium*, Lane 5, *Salmonella bacongo*, Lane 6, *Salmonella enteritidis*, Lane 7, *Salmonella enteritidis (*ATCC 13076*),* Lane 8, *Salmonella typhimurium (*ATCC 14028).

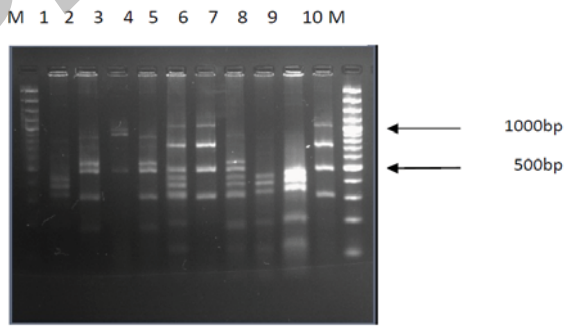

Figure 3. PCR-RFLP profiles of *fliC gene* with *Sau3AI* restriction endonuclease. M: Molecular Weight Marker DNA Ladder (plus); Lane 1, *Salmonella enteritidis*, Lane 2, *Salmonella infantis*, Lane 3, *Salmonella bardo,* Lane 4, *Salmonella infantis*, Lane 5, *Salmonella* sp., Lane 6, *Salmonella typhimurium*, Lane 7, *Salmonella bacongo*, Lane 8, *Salmonella enteritidis*, Lane 9 *Salmonella enteritidis (*ATCC 13076*),* Lane 10, *Salmonella typhimurium (*ATCC 14028).

4. Discussion

Salmonellosis is one of the major public health problems. Strains of *Salmonella* are considered as important pathogens and a leading cause of bacterial food-borne illness. Since genomic variations play an important role in bacterial identification, it is

Table 1. PCR-RFLP profiles of *Salmonella fliC* gene digested with *Sau3AI* and *HhaI* restriction enzymes

Salmonella enterica serotypes	***fliC* PCR- RFLP bands (*HhaI*)**	***fliC* PCR- RFLP bands (*Sau3AI*)**
Salmonella enteritidis	110-480-810	110-200-340-390-450-850
Salmonella infantis	110-350-790	190-320-490-550-880
Salmonella typhimurium	110-350-790	330-520-790-1100
Salmonella bacongo	110-350-450-750	110-190-340-400-450-530-590-900
Salmonella bardo	110-230-320	530-1050-1100

necessary to use molecular techniques capable of detecting genetic variation to identify different species of bacteria (Akbarmehr et al., 2010). Antigenic polymorphism of flagella seems to have been generated by the accumulation of ordinary genetic events in flagellin genes, such as point mutations, deletion and insertions (Dilmaghani et al., 2010).

In a study, PCR-RFLP method based on the *fliC* gene was used to determine 55 serotypes of *Salmonella* in Mexico with the restriction enzyme *Sau3AI*. The results showed that 91% and 9% of the isolates matched with the *Salmonella typhimurium* and *Salmonella enteritidis* reference strain restriction profiles. PCR-RFLP analyses with *Sau3AI* demonstrated that *fliC* is a suitable target gene for discriminating among *Salmonella* serotypes by PCR-RFLP. Researchers showed that this method is rapid, simple, and reproducible and can potentially be applied for identification of isolates (Gallegos-Robles et al., 2008). In our study, 75 isolates from poultry that belongs to 5 serotypes were investigated by PCR-RFLP. The *fliC* gene was able to differentiate *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella bacongo*, *Salmonella bardo* and *Salmonella infantis*. These results showed that this method can potentially be applied for identification of these serotypes.

Akbarmehr et al., (2010) isolated 58 *Salmonella* starins from poultry. They evaluated hsp *groEL* gene diversity by PCR-RFLP analysis using *HaeIII* restriction enzyme. They showed that there were differences in the *HaeIII* restriction sites within the groEL gene of strains belonging to serovars *Salmonella typhimurium* and *Salmonella enteritidis;* however, clear discrimination between the serovars of other *Salmonella* serogroups was not observed. Their results showed that *groEL* lacked the ability to discriminate between *Salmonella* serovars, while the results obtained by our study shows that PCR-RFLP on *fliC* gene using *Sau3AI* restriction enzyme is able to differentiate *Salmonella* serovars.

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 5 different serotypes of *Salmonella*, and PCR-RFLP method on *fliC* gene showed 4 and 5 different patterns using *HhaI* and *Sau3AI*, respectively.

Hong et al., (2003) studied on 52 serotypes of *Salmonella* spp. The phase 1 (*fliC*) and phase 2 (*fljB*) *Salmonella* flagella genes were analyzed by RFLP-PCR to aid in identification of different *Salmonella* serotypes with restriction endonucleases *Sau3AI* and *HhaI*. Ninety percent of the *Salmonella* serotypes were identified by the restriction enzyme RFLP analysis of *fliC* and *fljB* genes. They concluded that PCR-RFLP could be a fast, accurate, and economical alternative approach for serotyping of *Salmonella* spp. Our serotyping results showed that PCR-RFLP technique was able to differentiate all serovars of *Salmonella* in this study.

Dauga et al., (1998) and Hong et al., (2003) found good discriminatory ability with the fliC and *fljB* genes, particularly using double digestion with either *HhaI/ HphI* or *Sau3AI*/*HhaI* endonucleases. Similarly in our study, double digestion with endonucleases *HhaI* and *Sau3AI* was used. These 2 enzymes were able to differentiate all serotypes of isolated *Salmonella*.

Forty-seven *Salmonella* isolates of 20 different serovars, derived from chicken samples in Thailand, were studied by PCR-RFLP assay using *MboI* and *HhaI* restriction endonucleases on *fliC*/*fljB* genes by Jong et al., (2010). The *fliC* showed 11 and 9 patterns, while the *fljB* showed 6 and 7 patterns. They demonstrated that PCR-RFLP was not able to

differentiate *Salmonella hadar*, *Salmonella virchow*, *Salmonella emek* and *Salmonella albany*. Therefore, they concluded that PCR-RFLP test could not replace by 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. According to our research this method can be considered as an alternative typing along with serotyping.

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 the results obtained by Matsui et al., (2001) and Sumithra et al., (2013), PCR-RFLP with more than one endonuclease and genes give good typeability and increase the differentiating power.

Serotyping of our isolates showed 5 different serotypes of *Salmonella*. Based on our results, PCR-RFLP using *Sau3AI* was able to differentiate all 5 serotypes but using *HhaI* was unable to differentiate between *Salmonella infantis* and *Salmonella typhimurium*. Our data and other research in this field emphasized that PCR-RFLP by using *Sau3AI* could differentiate *Salmonella enteritidis* and *Salmonella typhimurium*. Serotyping is laborious, expensive and time-consuming but it must be done with molecular techniques. Also more studies need to be performed examining a large number of Salmonella serotypes.

Acknowledgement

The authors thank all members of Microbiology department of Razi Vaccine & Serum Research Institute, Karaj, Iran for assistance in this study.

Grant support

This work was fully financed with the state funds from Razi Vaccine and Serum Research Institute, under grant no. 12-18- 18-8901-89003.

Conflict of interest

The authors declare that they have no conflict of interest.

Financial Disclosure

Authors have no relevant financial interests related to the materials in the manuscript.

Authors' Contribution

Soheila Moradi Bidhendi developed the original idea and the protocol, abstracted and analyzed the data, revised the manuscript, and was the sponsor. Pejvak Khaki was the advisor and helped to design the protocol. Elham Ezatpanah performed the experiments.

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