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

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Background: *Salmonella* are a genus of zoonotic bacteria of worldwide economic and health importance. Members of *Salmonella enterica* subspecies *enterica* are mainly associated with warm-blooded vertebrates and are usually transmitted by ingestion of food or water contaminated by infected feces.
Objectives: The aim of this study was to apply a PCR-RFLP method based on the *fliC* gene to identify the serotypes of *Salmonella* isolates from Karaj, Iran.
Materials and Methods: A total of 30 *Salmonella* isolates were serotyped by specific antisera. For the PCR-RFLP method based on the *fliC* gene, extracted DNA was used as the template for amplifying the *fliC* gene (1500 bp) using specific primers. PCR products were subjected to digestion using *HhaI* restriction endonuclease.
Results: This study determined 30 serotypes as *Salmonella durban* (56.6%), *Salmonella uno* (23.3%), *Salmonella enteritidis* (3.3%), *Salmonella tinda* (3.3%), *Salmonella mjimweme* (3.3%), *Salmonella Thompson* (3.3%), *Salmonella sIIO8* (3.3 %) and *Salmonella sIIO7* (3.3%). Observations indicated that *HhaI* is able to discriminate *Salmonella tinda* and *Salmonella thompson*, yet *Salmonella enteritidis*, *Salmonella durban* and *Salmonella mjimweme* had the same pattern with this enzyme. Also *Salmonella sIIO8*, *Salmonella sIIO7* and *Salmonella uno* showed the same pattern. Thus, regarding the size and the number of resulting fragments from this enzyme, four patterns were obtained for *HhaI*.
Conclusion: A large number of *Salmonella* serotypes need to be analyzed by the PCR-RFLP method and different enzymes must be used to give reliable results.

Keywords: Salmonella; Avian; *fliC* gene; PCR-RFLP; *HhaI*

1. Background

Non-typhoidal *Salmonella enterica* serovars are major causes of bacterial food-borne diseases worldwide (1). The current taxonomic scheme consists of the genus *Salmonella* containing two species, *Salmonella enterica* and *Salmonella bongori* (2). Within the species *Salmonella enterica*, there are seven groups or subspecies, which are further composed of *serovars*. There are 2,579 recognized *Salmonella serovars* (3).

Salmonella are one of the most important pathogens involved in human foodborne illness in the developed world (4). These bacteria cause disease in both humans and animals. The transmission of *Salmonella* to a susceptible host usually occurs through consumption of contaminated food. Human infections are usually associated with animal contact and the consumption of contaminated food products such as poultry, meat and other dairy products (5). Other *Salmonella serovars*, specifically *Salmonella enterica serovar typhimurium, Salmonella enterica serovar dublin*, *Salmonella enterica serovar enteritidis* usually do not cause disseminated, systemic disease in human yet clinically manifest as gastroenteritis and diarrhea (6). Poultry is commonly infected with a wide variety of *Salmonella serovars*. Chicken and related products are recognized as important reservoirs for *Salmonella* and are vehicles for salmonellosis. Some *Salmonella serovars* such as *Salmonella enteritidis*, *Salmonella infantis*, *Salmonella kentucky*, and *Salmonella heidelberg* appear to be more prevalent in poultry than in other food animals (7).

Many of the traditional methods used for typing bacteria such as morphological, physiological, and biochemical markers in conjunction with serology, are time-consuming, laborious, expensive and are often not able to discriminate between related outbreak strains (8-11). Also, methods of serotyping are expensive and require a high degree of training to interpret and understand (12). During the recent years, new molecular typing techniques have been developed, based on genomic differences between strains (10). Effective epidemiological surveillance and control of *Salmonella* and other zoonotic pathogens requires accurate subtyping of strains for identification of potential sources of infection (8, 13). The basic premise of these typing systems is that epidemiologically related isolates are derived from the clonal expansion of a single precursor and share characteristics that differ from those of epidemiologically unrelated isolates (13).

Therefore, in the present study we analyzed the diversity of the *fliC* gene of *Salmonella serovars* isolated from poultry based on the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. Tests using molecular tools have been useful in reducing the steps and time needed for the detection, identification and characterization of specific pathogens. The *fliC* gene, encoding the flagellin protein, has been used as a target gene in assays to test the genetic diversity in *Salmonella* (14). The *fliC* gene has a conserved terminal region and a variable central region, which determines antigenic specificity (7, 15).

2. Objectives

The aim of this study was to apply the PCR-RFLP method based on the *fliC* gene to identify the serotypes of the *Salmonella* isolates from karaj, Iran.

3. Materials and Methods

3.1. Salmonella Strains

Thirty isolates of *Salmonella* were obtained from Razi Type Culture Collection (RTCC), at the Microbiology Department of Razi Vaccine & Serum Research Institute, Karaj, Iran. *Salmonella enterica* serotype *enteritidis* (ATCC 13076), *Salmonella enterica*, serotype *typhimurium* (ATCC 14028) and *Citrobacter freundii* (ATCC43864) were used as positive and negative controls in the PCR-RFLP.

3.2. Bacterial DNA Preparation

DNA was extracted from centrifuged bacterial colonies with some modification. Briefly, for each isolate, a loopful of an overnight pure culture of bacteria was inoculated in 1 mL of deionized distilled water (DDW) and then boiled at 95°C for 10 minutes in order to release bacterial DNA from the bacterial cells. The bacterial DNA suspension was centrifuged for 5 minutes and 5 μL of the supernatant was used as the template for further PCR experiments (7).

3.3. PCR Primers

For amplification of the phase-1 flagellin gene, the primers used were CAAGTCATTAATACMAACAGCC (Fsa-F) and TTAACGCAGTAAAGAGAGGAC (Fsa-R) (16).

3.4. PCR Amplification of the fliC Gene

PCR was conducted in a 25 μL volume containing 2 μL of genomic DNA from the *Salmonella serovar* isolates, 12.5 μL master mix, 2 μL of primers special for the *fliC* gene and 8.5 μL of DDW. Amplification was performed in a thermal cycler (Biosystem) programed as follows: initial denaturation 94°C for 5 minutes, 35 cycles consisting of 1 minute at 94°C, 1 minute at 55°C, 35 sseconds at 72°C and a final extension step of 1 minute at 72°C. Amplified products were resolved in 0.8% agarose gel. Following electrophoresis, the gel was stained with 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 (17).

3.5. PCR-RFLP Analysis

Digestion solution was prepared with 20 μL of the PCR product, 3 μL of *HhaI* buffer 10 X, 1.5 μL of *HhaI* enzyme and 5.5 μL of DDW. After incubation at 37 °C for 16 hours, RFLP were determined by electrophoresis of the digested DNA in 2.5% agarose gel for 4 hours at 50 V. Size of the products was analyzed in comparison to the DNA Ladder.

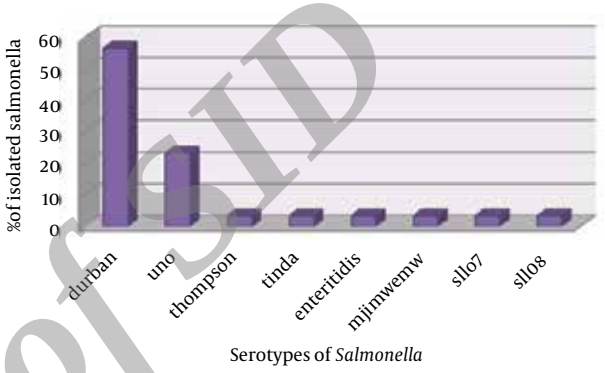

Figure 1. *Salmonella* Serotypes Isolated From Avian Samples in Karaj

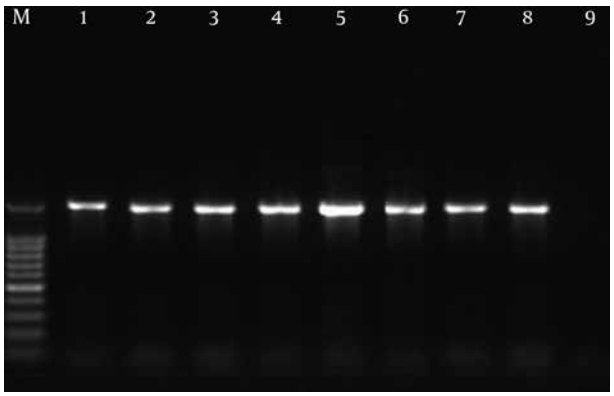

Figure 2. Electrophoresis of fliC Gene Amplicons From Salmonella SamplesM: Molecular marker 100 bp DNA ladder (Fermentas); lane 1, Salmonella enteritidis ATCC13076; lane 2, *Salmonella enteritidis* ; lane 3, *Salmonella uno*; lane 4, *Salmonella durban*; lane 5, *Salmonella tinda*; lane 6, *Salmonella mjimweme*; lane 7, *Salmonella thompson*; lane 8, *Salmonella sII08*; lane 9, negative control, *Citrobacter Freundii* (ATCC43864).

4. Results

4.1. Salmonella Strains

A total of 30 *Salmonella* isolates were identified by biochemical tests. All the biochemically confirmed isolates

were serotyped using slide agglutination with standard antisera (Mast, Bootle, England) for somatic and flagellar antigen identification according to the Kauffman-White classification scheme, which resulted the identification of *Salmonella durban* (56.6%), *Salmonella uno* (23.3%), *Salmonella enteritidis* (3.3%), *Salmonella tinda* (3.3%), *Salmonella mjimweme* (3/3%), *Salmonella Thompson* (3/3%), *Salmonella sIIO8* (3/3 %) and *Salmonella sIIO7* (3/3%). Figure 1 show the prevalence of *Salmonella* serotypes that were isolated from avian samples in karaj, Iran (Figure 1).

4.2. PCR Amplification of the fliC Gene

PCR was carried out using a specific primer. An approximately 1.5 kbp fragment was amplified from 30 isolates of *Salmonella*. As expected, all serotypes of *Salmonella* produced 1.5 kbp bands of the respective size on the agarose gel (Figure 2).

4.3. PCR-RFLP Analysis

PCR-RFLP was carried out on the 30 isolates , belonging to 8 different *Salmonella* serotypes. PCR-RFLP analyses with *HhaI* on *Salmonella* isolates of eight different known serotypes yielded four distinct restriction profiles for the *fliC* gene. In Figure 3 , different electrophoretic analysis of isolated serovars were shown. In this Figure *Salmonella durban*, *Salmonella enteritidis* and *Salmonella mjimweme* had the same pattern and *Salmonella uno*, *Salmonella sIIO8* and *Salmonella sIIO7* showed the same pattern. Figure 4A and 4B indicates the pattern of *Salmonella thompson* and *Salmonella tinda*, which showed different patterns.

Nineteen out of 30 isolated *Salmonella serovars*, belonged to serogroup D1 followed by 7 *Salmonella serovars* that belonged to the serogroup C2 while 1 *Salmonella serovar* belonged to serogroup C3 and 2 *Salmonella serovars* belonged to the serogroup C1 and 1 serovar belonged to serogroup B (Table 1). These data showed that serogroup D1 produced three *HhaI* restriction bands and serogroups C1, C2 and C3 produced three different *HhaI* restriction bands from serogroup D1. *Salmonella Thompson* (serogroup C1) and *Salmonella tinda* (serogroup B) showed different *HhaI* restriction bands.

5. Discussion

Salmonella spp. has been isolated from a variety of food such as milk, beef, pork and chicken. The ingestion of contaminated food can cause salmonellosis (18). It is believed that the presence of *Salmonella* spp. in chicken makes it unsafe for human consumption (19). It is necessary to use molecular techniques capable of detecting genetic variation in different species of bacteria. According to Table 1 *Salmonella* serogroup D1 with 70% frequency and *Salmonella* serogroup C1 with 20% frequency are the most dominant *Salmonella* serogroups in poultry farms of Karaj followed by serogroup C2, C3 and B. In a study conducted in Azarbayjan, Iran, *Salmonella* serogroup D1 with 62.06% frequency

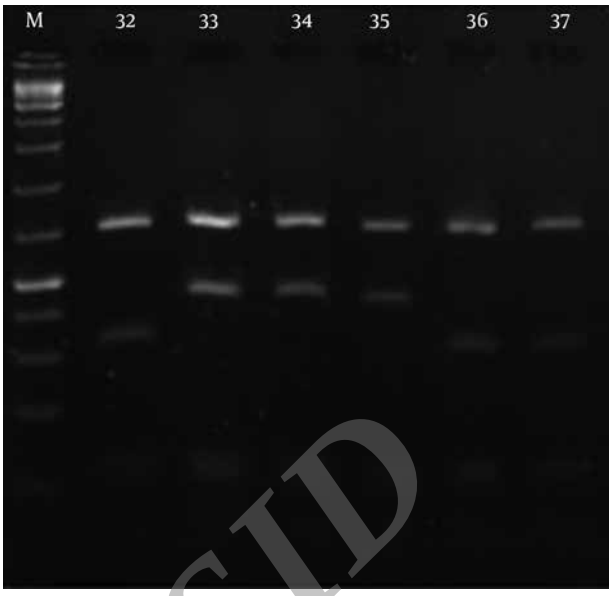

Figure 3. Eletrophoretic Analysis of the fliC Gene After Enzymatic Treatment With HhaI Restriction Enzyme. M: molecular weight marker DNA ladder (plus); lane 32: *Salmonella sIIO7*; lane 33: *Salmonella Durban;* lane 34: *Salmonella enteritidis*; lane 35: *Salmonella mjimweme;* lane 36: *Salmonella uno*; lane 37: *Salmonella sIIO8*.

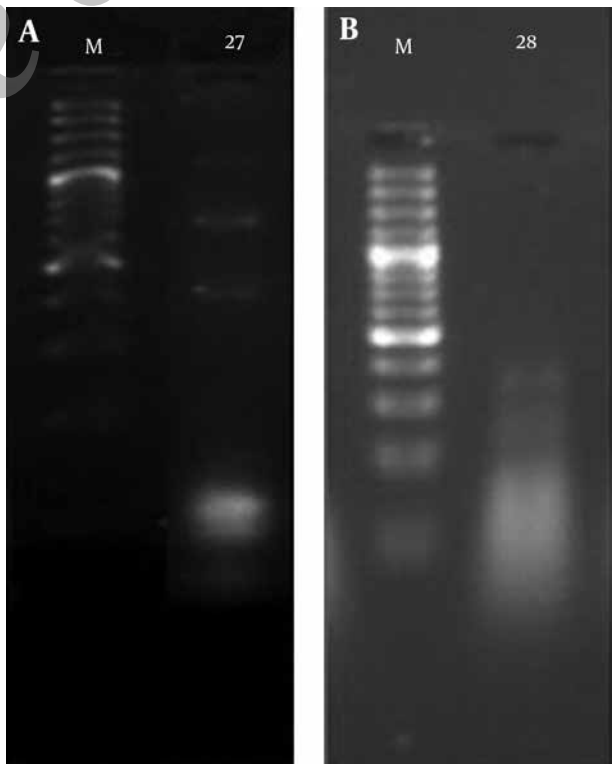

Figure 4. Eletrophoretic Analysis of the fliC Gene After Enzymatic Treatment With HhaI Restriction Enzyme. Ladder: marker DNA ladder (plus); lane 27: *Salmonella thompson*. M: marker DNA Ladder (plus); lane 28: *Salmonella tinda*.

Table 1. Number of *PCR-RFLP* Bands in Isolated *Salmonella* Serogroups

Strain No.	*Salmonella* serogroup	*Salmonella* serovar	Number of *PCR-RFLP* bands (*fliC HhaI*)	Strain No.	*Salmonella* serogroup	*Salmonella* serovar	Number of *PCR-RFLP* bands (*fliC HhaI*)
1	B	*Tinda*	**2**	16	D1	*Durban*	3
2	C1	*SIIO7*	3	17	D1	*Durban*	3
3	C1	*Thompson*	4	18	D1	*Durban*	3
4	C2	*Uno*	3	19	D1	*Durban*	3
5	C2	*Uno*	3	20	D1	*Durban*	3
6	C2	*Uno*	3	21	D1	*Durban*	3
7	C2	*Uno*	3	22	D1	*Durban*	3
8	C2	*Uno*	3	23	D1	*Durban*	3
9	C2	*Uno*	3	24	D1	*Durban*	3
10	C2	*Uno*	3	25	D1	*Durban*	3
11	C3	*SIIO8*	3	26	D1	*Durban*	3
12	D1	*Enteritidis*	**3**	27	D1	*Durban*	3
13	D1	*Durban*	3	28	D1	*Durban*	3
14	D1	*Durban*	3	29	D1	*Durban*	3
15	D1	*Durban*	3	*30*	D1	*Mjimwema*	3

and *Salmonella* serogroup B with 27.58% were the most dominant *Salmonella* serogroups in poultry farms. Another study in Shiraz, Iran, showed that *Salmonella* sergroup D1 with 70% frequency was dominant (20). These results were the same as our results and reveal that *Salmonella* serogroup D1 is the most common serogroup in poultry farms of most geographical areas of Iran. Genomic variations play an important role in bacterial identification. It is necessary to use molecular techniques capable of detecting genetic variation in different species of bacteria. Thus, a PCR based technique, PCR-RFLP was applied for identification of *Salmonella* isolated from poultry farms of Karaj, Iran. In our study, regarding the size and the number of resulted fragments from the *HhaI* restriction enzyme, four patterns were obtained. The results showed that *HhaI* is able to discriminate *Salmonella tinda* and *Salmonella thompson* with different patterns while *Salmonella enteritidis*, *Salmonella Durban* and *Salmonella mjimweme* had the same pattern with this enzyme. Also *Salmonella uno*, *Salmonella sIIO8* and *Salmonella sIIO7*, showed the same pattern. In another study, PCR-RFLP method based on the *fliC* gene was used to determine 55 serotypes amongst isolates from cantaloupe and chile pepper production systems in Mexico with the restriction enzyme *Sau3AI*. *Salmonella typhimurium* was the only serotype found to be associated with the cantaloupe production system, whereas both *Salmonella typhimurium* and *enteritidis* serotypes were found to be associated with the chile pepper production system. Results showed that 91% (20 of 22) and 9% (2 of 22) of the isolates from both agricultural systems matched with the *Salmonella typhimurium* and *Salmonella enteritidis* reference strain restriction profiles. PCR-RFLP analyses with *Sau3AI* on *Salmonella* strains of seven different known serotypes yielded seven distinct restriction profiles for the *fliC* gene, demonstrating that *fliC* is a suitable target gene for discrimination of *Salmonella* serotypes by PCR-RFLP. This study demonstrated the utility of the PCR-RFLP technique for determining the serotypes of *Salmonella* isolates obtained from cantaloupe and chile pepper production systems by comparison to restriction profiles of known reference strains. Researchers showed that this method is rapid, simple and reproducible and can potentially be applied for identification of isolates (21). Their results are in contrast to our results because in their study two serotypes were isolated yet in our study weight different serotypes with four restriction profiles were observed. This shows that this method with the mentioned enzyme does not have the potential be applied for identification of our isolates.

Nair et al. using PCR-RFLP on the *groEL* gene amplicon of *Salmonella* digested with HaeIII, found a low discriminatory capacity, as only three different profiles were obtained, and different serotypes of *Salmonella* shared the same restriction profile (17). In our study we had eight different serotypes with four restriction profiles, which showed that our study is in agreement with their study. Dauga et al. and Hong et al. achieved good discriminatory ability with the *fliC* and fljB genes, particularly with the use of double digestion with endonucleases, *HhaI* and HphI (16, 23). In this study, use of a single digestion with endonuclease *HhaI* could not differentiate all serotypes of isolated *Salmonella*.

Akbarmehr et al. isolated 58 *Salmonella* strains from poultry. They evaluated hsp *groEL* gene diversity by PCR-RFLP analysis with HaeIII restriction enzyme. They showed that there were differences in the Hae III restriction sites within the *groEL* gene of strains belonging to serovars *Salmonella typhimurium* and *Salmonella enteritidis* but clear discrimination between the serovars of

different *Salmonella* serogroups was not observed (20). Their results showed that use of *groEL* gene like *fliC* gene did not result good discriminatory ability, such as that achieved by our study. Shah et al. developed a PCR-RFLP method with HinfI restriction enzyme and identified six serotypes of *Salmonella choleraesuis*. Their method proved to be specific and rapid for identifying bacteria based on stable genetic characteristics. This study demonstrates the potential of their method for subspecies discrimination of other bacteria as well (24). Their study showed that HinfI restriction enzyme is better than *HhaI* restriction enzyme that we used in this study, for identification of *Salmonella choleraesuis*.

Tiba et al. used the *fliC* PCR-RFLP with *HhaI* and RsaI restriction enzymes in *E. coli*. They showed that the *fliC* gene could be amplified in all non-typable *E. coli* strains, and considerable polymorphism of the *HhaI* and RsaI restriction products of the amplified *fliC* gene could be detected. The diversity of amplification products was examined with the use of *HhaI* and RsaI, which demonstrated this method as a feasible and rapid method for identification and subtyping of H-antigens in *E. coli* (25). Their study showed that this method with the two mentioned enzymes is more applicable to differentiate *E. coli* not *Salmonella*.

The data from these studies and our study showed that although serotyping is the "gold standard" in typing of *Salmonella*, but serotyping itself is expensive and labor intensive. For epidemiological investigation and genotyping of *Salmonella* isolates, we can use genotyping methods such as RFLP. Many researches have performed this method with different genes and restriction endonuclease enzymes with different results. In this study, we found a low discriminatory capacity with the *fliC* gene, because only four different profiles were obtained from eight different serotypes. On the other hand, other researchers have found both good and bad discriminatory ability with the *fliC* gene. Thus, a large number of *Salmonella* serotypes need to be analyzed by the PCR-RFLP method and different enzymes must be used to give reliable results. Also typing of isolates by other methods such as pulsed-field gel electrophoresis might be useful.

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