

Molecular Typing of *Salmonella* Isolates in Poultry by Pulsed-Field Gel Electrophoresis in Iran

Narges Golab¹; Pejvak Khaki^{2,*}; Fatemeh Noorbakhsh¹

¹Department of Microbiology, Biology Faculty, Varamin Branch, Islamic Azad University, Varamin, IR Iran

²Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, IR Iran

*Corresponding author: Pejvak Khaki, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, IR Iran. Tel: +98-9123894986, Fax: +98-2634552194, E-mail: 2p.khaki@rvisri.ac.ir

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Background: *Salmonella* is one of the most widespread zoonotic enter pathogenic microorganisms found in the global food chain. Poultry and Poultry products have been identified as one of the important foodborne sources of *Salmonella*. Pulsed-Field Gel Electrophoresis (PFGE) is a gold standard typing method for identification of *Salmonella* isolates during outbreaks and epidemiological investigations.

Objectives: The aim of this study was to carry out molecular typing of *Salmonella enterica* spp. by PFGE technique.

Materials and Methods: All 47 *Salmonella* isolates were serotyped and then subjected to PFGE. Total isolates were analyzed by means of the molecular technique *Xba*I PFGE.

Results: In the current work, PFGE and serotyping were used to subtype 47 *Salmonella* isolates belonging to 22 different serotypes and derived from poultry. Thirty-nine PFGE patterns out of 47 isolates were obtained. The Discrimination Index (DI) by serotyping (0.93) was lower than PFGE (DI = 0.99).

Conclusions: In conclusion, molecular methods such as PFGE can be used for epidemiological characterization of *Salmonella* serotypes.

Keywords: *Salmonella enterica*; Poultry; Molecular typing; Iran

1. Background

Salmonellosis is one of the most frequently occurring foodborne diseases worldwide (1, 2). The commonest serotypes causing this disease are non-typhoidal *Salmonella enterica* subsp. *enterica*. It is the only group with clinical importance and is represented by 1,478 serotypes (3). Contaminated poultry meat and eggs are important vehicles of *Salmonella* infections (4). In the last few years in Iran (5) and other Asian (6, 7) or European countries (8) as well as in the United States of America (9, 10), the number of non-typhoidal *Salmonella* isolates has increased and is one of leading causes of hospitalization and death from foodborne illnesses. The industrial production of poultry is very diverse. It has been reported that in addition to mishandling of poultry products and raw poultry carcasses, undercooked poultry meat is also one of the most frequent causes of human infection by *Salmonella* species (1). Identification of different strains is essential for the successful epidemiological investigation of *Salmonella enterica* outbreaks. Therefore *Salmonella* control has become an important objective for the poultry industry from both public health and economic perspectives (11). Conventional typing methods based on phenotypic characteristics such as, biotyping, serotyping and phage typing, have been widely used (12, 13), but are often not able to discriminate between related outbreak strains. Recently molecular

typing methods such as Random Amplified Polymorphic DNA-PCR (RAPD-PCR), Repetitive -PCR (Rep-PCR), Enter bacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Pulsed Field Gel Electrophoresis (PFGE), and Amplified Fragment Length Polymorphism (AFLP) have been developed for characterization of *Salmonella* serotypes (5, 14, 15). The PFGE is currently used by the CDC PulseNet (<http://www.cdc.gov/pulsenet>), surveillance program and is generally accepted as the "gold standard" for molecular typing of *Salmonella* (16-19). Although some molecular typing methods have been used for epidemiological studies of *Salmonella* serotypes in Iran (20-22), only a few recently published reports involve the use of PFGE for characterization of *Salmonella* serotypes in poultry in Iran (5, 23).

2. Objectives

The aim of this study was to evaluate the usefulness of PFGE in molecular typing of *Salmonella enterica* spp.

3. Materials and Methods

3.1. Bacterial Isolates

A total of 47 *Salmonella enterica* isolates were obtained from the Razi Type Culture Collection (RTCC), Razi Vac-

cine and Serum Research Institute, Karaj, Iran (Table 1). All isolates were recovered from poultry. The bacteria were inoculated directly onto the tryptic soy agar (TSA) (Merck, Germany) and incubated at 37°C for 24 hours.

Table 1. The *Salmonella* Isolates Used in This Study From Poultry

Serotype	No
<i>Salmonella Gallinarum</i>	11
<i>Salmonella Pullorum</i>	4
<i>Salmonella Typhimurium</i>	4
<i>Salmonella Enteritidis</i>	3
<i>Salmonella Infantis</i>	3
<i>Salmonella Abortusovis</i>	2
<i>Salmonella Derby</i>	2
<i>Salmonella Kuilsriver</i>	2
<i>Salmonella Rostock</i>	2
<i>Salmonella Thompson</i>	2
<i>Salmonella Bardo</i>	1
<i>Salmonella Calvinia</i>	1
<i>Salmonella Colindale</i>	1
<i>Salmonella Durban</i>	1
<i>Salmonella Mijiwema</i>	1
<i>Salmonella Neinstedton</i>	1
<i>Salmonella Newport</i>	1
<i>Salmonella Oysterben</i>	1
<i>Salmonella Strenbos</i>	1
<i>S. tinda</i>	1
<i>S. uno</i>	1
<i>S. virchow</i>	1
Total	47

3.2. Serotyping

All *Salmonella* isolates were serogrouped and serotyped according to the Kauffmann-White scheme (24).

3.3. Sample Preparation

Pulsed-Field Gel Electrophoresis was done as described with some modifications (7, 14). Briefly, the cell suspension buffer (100 mM Tris, 100 mM EDTA, and pH 8.0) was adjusted to a turbidity reading of 1 to 1.3. This suspension was mixed in equal parts with molten 2% low-melting-point agarose (Sigma, USA) and pipetted into disposable molds then stored at 4°C for 20-30 minutes. These agarose plugs were incubated overnight at 56°C in 1 ml of lysis buffer (0.5 M EDTA, 0.5 M Tris, 1% N-laurylsarcosine) [Sigma, U K] with proteinase K (Fermentase, Spain) at a final concentration of 250 µg/mL. A total of six washes (twice with sterile ultrapure water and four times with 0.01 M Tris-EDTA buffer, pH 8.0) were used to remove excess reagents and cell debris from the lysed plugs.

3.4. DNA Restriction

Chromosomal DNA was digested with 30 U of *Xba*I (Fermentase, Lithuania) for 3 hours in a water bath at 37°C.

3.5. PFGE Electrophoresis

Electrophoresis was carried out with 0.5x TBE buffer at 6 V/cm and 14°C by CHEF DRIII system (Bio-Rad, USA). The running time was 20 h and the pulse ramp time was 2.16-63.8 s. A lambda ladder PFG marker (48.5 to 1,018.5kb) was used as a molecular size marker (New England Biolabs, USA). The gels were visualized on a UV Tran illuminator, and photographs were captured by a digital imaging system (Video gel doc system, Bio-Rad) and conversion of gel images to the TIFF file format. Reproducibility power was confirmed by comparing the fingerprint patterns obtained from duplicate runs of same isolates.

3.6. Gel Analysis

Gel images were analyzed using Gel Compare II software. A dendrogram based on the Dice coefficient was generated using the unweight pair group with arithmetic mean (UPGMA) algorithm at 1% position. PFGE patterns were analyzed with Tenover's categorization (25). Isolates were designated genetically on the basis of DNA fragment patterns in 4 categories of genetic and epidemiologic relatedness, Indistinguishable, Closely related, possibly related and unrelated. Isolates were considered identical when their PFGE patterns contained the same number and size of fragments. Discrimination power was calculated by determining the Simpson discrimination index (DI) according to Hunter et al. (26).

4. Results

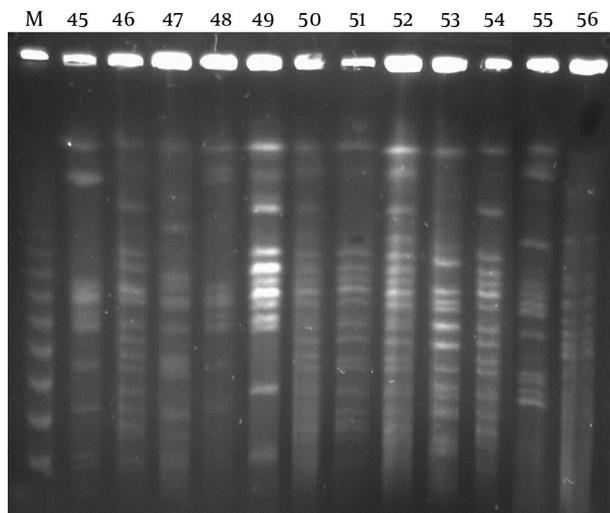
Twenty-two serotypes out of 47 isolates were observed. *Salmonella gallinarum* (n = 11), *Salmonella pullorum* (n = 4) and *Salmonella typhimurium* (n = 4) were the main serotypes (Table 1). The serotyping discrimination index was DI = 0.93.

Thirty nine PFGE patterns out of 47 *Salmonella* isolates generated from the *Xba*I enzyme were identified (Ps1 to 39). The Pulsotypes consisted of 7 to 17 fragments with sizes ranging from 33.4 -135.0 kb (Figure 1). The majority of PFGE profiles appeared to be unique to the individual isolates (Ps8 to 39). Seven clusters (Ps1-Ps7) out of 39 pulsotypes had more than one isolate. The largest cluster consisted of three isolates with different serotypes (Ps1). Six clusters had two isolates with the same serotypes (Ps2-Ps7). The similarity range had been between 46-100%. Six clusters showed 90% > similarity (Ps12, Ps13, Ps15 to 18).

The discrimination index was high for PFGE (DI = 0.99) and the technique was able to distinguish between isolates and further subtype of the serotypes. There was a high genetic diversity among the *Salmonella typhimurium* as the 4 isolates were subtyped into 4 pulsotypes (Ps 10, 12, 36, 37). Each of them was different in pattern and unre-

lated clonal isolates. Nine pulsotypes out of 11 *Salmonella gallinarum* serotypes were also identified. Although they were the same serotype, they were not in the same pulsotype.

Figure 1. Representative PFGE-XbaI Profiles of *Salmonella* isolates From Poultry.



M: lambda ladder PFGE marker (NO340S, New England Biolabs, USA; band size: 48.5-1018.5 kb). 45-*S. colindale* 46-*S. strenbos* 47-*S. uno* 48-*S. durban* 49-*S. mijiwema* 50-*S. newport* 51-*S. nienstedton* 52-*S. oysterben* 53-*S. calvinia* 54-*S. tinda* 55-*S. bardo* 56-*S. gallinarum*.

5. Discussion

The poultry industry is a large and well-organised system for the efficient production of animal protein foods (12). Non-typhoidal *Salmonella* contamination in poultry is also a major problem in Asian countries as well as the other countries, in terms of both morbidity and economic costs (7, 27). Most of these infections have been attributed to the consumption of poultry meat and eggs (28, 29). Although *Salmonella* serotypes typhimurium and enteritidis are the most common causes of salmonellosis worldwide, other *Salmonella* serotypes associated with food poisoning cases are becoming important in recent years (29, 30). Both understanding of the risk factors and subsequent reduction of *Salmonella* transmission may decrease the risk of contamination throughout the food chain. Therefore, a reliable and powerful method for molecular typing of *S. enterica* in poultry is significantly necessary for the identification and characterization of certain serotypes circulating in population. In recent years, phenotypic typing methods have been found to lack discriminatory power due to the expanded diversity of isolates. For this reason, molecular typing methods have been developed for the differentiation of *Salmonella* serotypes (14, 31-33). Since not all the molecular techniques are equally effective, the reasons below can clearly justify why we have selected PFGE over other methods. For

instance, results provided by pulsed-field gel electrophoresis (PFGE) that analyze the entire microbial genome (used in this study). An additional advantage of PFGE is its effective ability to distinguish *Salmonella* serotypes. PFGE is the most useful as a confirmatory method, due to its repeatability, reproducibility and ability to discriminate between serotypes. However the process of getting to the end results in PFGE may take more than 5 days. This can be one of the disadvantages of this method compare to other methods.

In spite of increasing rates of the *Salmonella* infection between fowl in Iran, the PFGE was not practiced very much on epidemiological studies of *Salmonella* spp. in poultry in this country (5, 23). Therefore, our study was to do molecular typing of the *Salmonella* serotypes in poultry by PFGE technique. In the present study, 39 patterns out of 47 isolates were observed. *XbaI* enzyme produced fragment patterns consisting of 7-17 bands in the range of 33.4 -1135.0 kb. The maximum fragments for *S. typhimurium* belonging to Ps10 were 17 bands. The minimum belonging to *S. rostock* (Ps11) was 7 bands. The results showed that the PFGE was able to distinguish between isolates and further subtype of the serotypes and the majority of PFGE profiles appeared to be unique to the individual isolates except 7 clusters that had more than one isolates. One cluster with three isolates (Ps1) was shown to be largest pulsotype in our isolates, their similar patterns showed an indistinguishable profile which is probably caused by the lack of discriminatory power of PFGE which resulted in its incapability in identifying different serotypes.

Previous studies such as Zahraei Salehi et al. (2011), in Iran, which evaluated *Salmonella enterica* spp. from animal and human by PFGE as well as the other methods, proved that PFGE is probably the most effective molecular technique (5). Similarly, in recent work in Iran, Rahmani et al. in 2013 reported that the results obtained from serotyping and PFGE patterns are practical for determining the current distribution of MDR serotypes of *Salmonella* and epidemiological state of isolates circulating among poultry (23). These two results were the same as our results. Sandt et al. in 2013 isolated non-typhoidal *Salmonella enterica* starins from different sources such as poultry during 6 years in Pennsylvania. They compared clinical isolates of non-typhoidal *Salmonella* recovered from human with *Salmonella* isolates recovered from animals. They concluded that the PFGE can provide information that is helpful in identification of source infection and outbreak investigations (9). Previously in 2006 the same author had revealed that PFGE testing played a key role in distinguishing outbreak-related *Salmonella* isolates from unrelated sporadic isolates (34).

Our results has highlighted the capability of PFGE with high discrimination index (DI = 0.99) for subtyping to differentiate *Salmonella* isolates of the same serotypes among poultry. These results are in agreement with other workers who reported that PFGE is one of the most reli-

able techniques for discriminating different serotypes of *Salmonella* (10, 13, 35, 36).

Finally our findings revealed that PFGE is a type able and reproducible technique and had higher discriminatory power than serotyping method to characterization of *Salmonella* isolates in poultry. The results of this study also suggested that combined analysis by both phenotypic (serotyping) and genotyping (PFGE) methods are required for epidemiological investigations of *Salmonella* isolates (37).

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