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Pulse-Field Gel Electrophoresis (PFGE) of *Salmonella* Serovar Infantis Isolates From Poultry

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Abstract:

BACKGROUND: *Salmonella* is one of the most important zoonotic agents known to infect humans and a wide range of animals, including poultry. *Salmonella* Infantis has been one of the 15 most frequently isolated serovars throughout the world. Despite its clinical importance, little is known about the molecular characteristics of *S.* Infantis strains from Iran.

OBJECTIVES: The purpose of this study was mainly to type a number of *S.* Infantis isolates obtained from Iranian poultry f ocks in the last decade by pulse-f eld gel electrophoresis (PFGE).

METHODS: Forty f ve *Salmonella* Infantis isolates, mostly from poultry origin, were subjected to PFGE according to protocol of the CDC PulseNet.

RESULTS: PFGE revealed 27 pulsotypes and eight clusters among 45 isolates based on the number of observed bands among the pulsotypes. The distribution of 45 isolates among the 27 pulsotypes was variable and included from one to nine isolates. One pulsotype included nine (20%) isolates. The genotypic similarity among 45 isolates was more than 90%.

CONCLUSIONS: This study showed the value of PFGE in determining the genotypic similarity among *S.* Infantis isolates. The high genotypic similarity shown by PFGE among the *S.* Infantis isolates of this study suggested that the majority of *S.* Infantis isolates studied may have descended from a common ancestor that has differed little and is responsible for the contamination of poultry f ocks and possibly humans as well.

Keywords:

Genotypic similarity, Poultry, Pulse-f eld gel electrophoresis (PFGE), Pulsotype, *Salmonella* Infantis

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Introduction

Salmonella is an important zoonotic agent known to infect humans and a wide range of animals, including poultry (Velge et al., 2005). More than 2600 serovars of *Salmonella enterica* have been recognized from all over the world and almost all are able to cause illness in humans and animals (Guibourdenche et al., 2010). *Salmonella enteritidis* and *Salmonella typhimurium* are considered as the most important *Salmonella* infecting poultry and its product worldwide. *Salmonella* control plan for the reduction of *Salmonella typhimurium* and *Salmonella enteritidis* in broilers was initiated in 2001 and resulted in a decrease of these serovars. In the last few years, the number of non-typhoidal Salmonellosis has increased in Iran and other parts of the world (Woo, 2005; Marimón et al., 2006; Zahraei Salehi et al., 2011; Sandt et al., 2013). Non-typhoidal Salmonellosis is one of the leading causes of hospitalization and death from foodborne illnesses. The Center for Disease Control and Prevention (CDC) has estimated that 9.4 million foodborne illnesses, 55,961 hospitalizations and 1351 deaths occur in the United States each year (Scallan et al., 2011). *Salmonella* Infantis is one of the 15 most frequently isolated serovars throughout the world (Hendriksen et al., 2011). *Salmonella* Infantis is a common serotype in livestock production and it is consistently isolated from broiler chickens (Hauser et al., 2012; Sasaki et al., 2012). The risk groups of infection with ser. Enteritidis are infants (under 3 months of age), the elderly, and the immunocompromised (CDC, 1990. Update). The number of infections and diseases caused by the serotype *Salmonella* Infantis started to increase in the last decades (Ungvári et al., 2007). So far, *Salmonella* Infantis is the most widespread serovar among animals and the third most common cause of human salmonellosis. *Salmonella* Infantis has been isolated from veterinary and human hospitals, foods such as vegetables and meat and production animals such as broiler chickens (Dunowska et al., 2007, Nógrády et al., 2008; Shahada et al., 2006). Besides the occurrence in animals, *S.* Infantis has been associated with cases of human salmonellosis and is implicated in nosocomial infections in veterinary hospitals (Fonseca et al., 2006; Dunowska et al., 2007) or food poisoning (Kohl and Farley, 2000; Najjar et al., 2012) in several countries. According to the global distribution of reported serotypes, *S.* Infantis was one of the highest ranked salmonellae (Galanis et al., 2006). Identi f cation 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 (Sasaki et al., 2012). Despite its clinical importance, little is known about the molecular characteristics of *S.* Infantis strains from Iran. Serotyping was the standard procedure for the classi f cation of *Salmonella* isolates in outbreak investigations prior to the development of molecular genotyping methods. However, serotyping has limited utility for epidemiologic analysis of *Salmonella* transmission, because it has poor discriminative ability for closely related isolates (Woo, 2005).

Due to the importance of *Salmonella* as one of the most important causative agents of food-borne diseases, a variety of phe-

notypic and genotypic methods have been used to trace the outbreak to the contaminated source and to elucidate the epidemiology of infection (Lukinmaa et al., 2004). Using DNA-based techniques, investigators are now able to better discriminate *Salmonella* isolates below the level of serotypes. Techniques such as plasmid profile, ribotyping, IS200 fingerprinting, PCR ribotyping, ribosomal DNA intergenic spacer amplification and heteroduplex analysis, amplified fragment length polymorphism, automated 5' nuclease PCR assay, random amplified polymorphic DNA (RAPD) analysis, enterobacterial repetitive intergenic consensus (ERIC – PCR) and pulsed-field gel electrophoresis (PFGE) have been frequently used by many researchers (Lukinmaa et al., 2004).

The aim of this study was to type 45 *S.* Infantis isolates obtained from poultry flocks in Iran by pulse-field gel electrophoresis (PFGE).

Materials and Methods

Bacterial isolates: Since 2005, specimens for *Salmonella* isolation were collected and cultured in our laboratory as previously described (Morshed and Peighambari, 2010; Abarian et al., 2012). All confirmed *Salmonella* isolates were stored in tryptic soy broth (TSB) with 25% glycerol at -70 °C for future use. In a subsequent study (Peighambari et al., 2015), 100 group C *Salmonella* isolates were selected from our laboratory collection and 79 isoaltes were identified as *Salmonella* Infantis by PCR as described by Kardos et al. (2007). In this study, 45 *Salmonella* Infantis isolates from our laboratory collection including isolates from broiler, broiler breeder and commercial layer farms and two human isolates were investigated (Table 1). All 45 selected isolates for this study were re-confirmed by PCR as *Salmonella* Infantis (Kardos et al., 2007).

Pulsed-Field Gel Electrophoresis (PFGE): Forty five *Salmonella* Infantis isolates were subjected to PFGE according to the standardized *Salmonella* protocol of the CDC PulseNet (PNL05, last upadated April 2013) but with some modifications. 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), pipetted into disposable molds and then stored at 4 °C for 20 to 30 min. 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, UK) with proteinase K (Fermentas, 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. Chromosomal DNA was digested with 30 U of XbaI (Fermentas, Lithuania) for 3 h in a water bath at 37 °C. 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 5 to 30 s. *Salmonella enterica* serotype Braenderup, strain H9812 was used as a size marker. The gels were visualized on a UV transilluminator, 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. DNA fragments patterns were analyzed with Gel Compare II software (Applied Maths, Kor-

Table 1. List of *Salmonella* Infantis isolates used in this study and the relevant data. [1]BCWRW = Broiler carcasses wash and rinse water; DOC = Day-old chicks. [2]Based on similarty more than 90%.

No.	Lab no.	Dendogram no.	Source1	Farm/House	Province/ Isolation date	Pulsotype	Cluster2
1	77	32	Broiler feces	F5/H4	Tehran/ 11.2005	1	-
2	89	34	Broiler feces	F6	Tehran/12.2005	2	-
3	72	11	Broiler feces	F5/H4	Tehran/11.2005	3	I
4	38	22	BCWRW	Abattoir	Tehran/09.2005	3	I
5	25	4	BCWRW	Abattoir	Tehran/09.2005	3	I
6	140	26	BCWRW	Abattoir	Tehran/09.2005	4	I
7	107	15	DOC	F8	Tehran/03.2006	5	I
8	108	16	DOC	F8	Tehran/03.2006	6	I
9	7	1	BCWRW	Abattoir	Tehran/09.2005	7	I
10	8	2	BCWRW	Abattoir	Tehran/09.2005	7	I
11	50	3	BCWRW	Abattoir	Tehran/09.2005	7	I
12	148	43	BCWRW	Abattoir	Tehran/09.2005	7	I
13	151	44	BCWRW	Abattoir	Tehran/09.2005	7	I
14	152	46	BCWRW	Abattoir	Tehran/09.2005	7	I
15	155	47	BCWRW	Abattoir	Tehran/09.2005	7	I
16	157	48	BCWRW	Abattoir	Tehran/09.2005	7	I
17	159	49	BCWRW	Abattoir	Tehran/09.2005	7	I
18	84	19	Broiler feces	F5/H5	Tehran/11.2005	8	I
19	83	24	Broiler feces	F5/H5	Tehran/11.2005	9	I
20	65	9	Broiler Liver	F5/H3	Tehran/11.2005	9	I
21	70	42	Broiler feces	F5/H4	Tehran/11.2005	10	I
22	75	21	Broiler feces	F5/H4	Tehran/11.2005	11	I
23	36	6	BCWRW	Abattoir	Tehran/09.2005	11	I
24	88	12	Broiler feces	F5/H5	Tehran/12.2005	12	I
25	184	37	BCWRW	Abattoir	Tehran/09.2005	13	II
26	185	38	BCWRW	Abattoir	Tehran/09.2005	13	II
27	87	33	Broiler feces	F5/H5	Tehran/12.2005	14	II
28	31	29	BCWRW	Abattoir	Tehran/09.2005	15	III
29	48	30	BCWRW	Abattoir	Tehran/09.2005	15	III
30	73	31	Broiler feces	F5/H4	Tehran/11.2005	16	IV
31	163	36	BCWRW	Abattoir	Tehran/09.2005	17	IV
32	80	23	Broiler feces	F5/H4	Tehran/11.2005	18	-
33	290	50	BCWRW	Abattoir	Ghazvin/10.2008	19	-
34	69	10	Broiler feces	F5/H4	Tehran/11.2005	20	V
35	86	25	Broiler feces	F5/H5	Tehran/11.2005	21	V
36	52	7	BCWRW	Abattoir	Tehran/09.2005	22	VI
37	53	8	BCWRW	Abattoir	Tehran/09.2005	22	VI
38	39	17	BCWRW	Abattoir	Tehran/09.2005	23	VI
39	85	20	Broiler feces	F5/H5	Tehran/11.2005	23	VI
40	5	45	Broiler Liver	F3	Tehran/07.2005	24	VI
41	143	27	BCWRW	Abattoir	Tehran/09.2005	25	VII
42	142	28	BCWRW	Abattoir	Tehran/09.2005	25	VII
43	35	5	BCWRW	Abattoir	Tehran/09.2005	26	-
44	339	39	Human feces	Hospital	Tehran/07.2006	27	VIII
45	340	40	Human feces	Hospital	Tehran/07.2006	27	VIII

trijk, Belgium). Isolates that exhibited similarity cut-off 80% were considered as a pulsotype (Tenover et al., 1995). Reproducibility power was confrmed by comparing the fngerprint patterns obtained from duplicate runs of the same isolates.

Results

We analyzed the 45 samples of *Salmonella* Infantis by PFGE and then by Video Gel-Doc System. The data was analyzed with Bio-Rad software and a dendogram was drawn. PFGE revealed 27 pulsotypes and eight clusters among 45 isolates based on the number of observed bands among the pulsotypes and has been demonstrated in Table 1 and Fig. 1. The distribution of 45 isolates among the 27 pulsotypes was variable. Seventeen (37.8%) isolates each belonged to a single pulsotype, 16 (35.5%) isolates belonged to eight pulsotypes each including two isolates, one pulsotype contained three (6.7%) isolates and the remaining nine isolates (20%) were placed in one pulsotype (Fig. 2). The genotypic similarity among 27 pulsotypes was more than 90%. Most of the pulsotypes that included more than one *Salmonella* Infantis isolate had been recovered from broiler carcasses wash and rinse water in poultry abattoirs (Table 1). There was only one pulsotype with two isolates from the same farm. Majority of isolates, even in some cases from the same farm, were distributed in different pulsotypes.

Discussion

This study examined the molecular epidemiology of 45 *Salmonella* Infantis isolates obtained since the year 2005 from poultry sources in different regions of the country using PFGE.

Salmonella Infantis is the third most common serovar isolated from humans in Europe since 2006 with an increased rate of infection from 1% in 2006 to 2.2% in 2010 (Rašeta et al., 2014). The application of pulsed-feld gel electrophoresis (PFGE) has been proven to be useful for establishing genetic relatedness of different bacterial strains including *Salmonella enterica* strains (Fonseca et al., 2006; Foley et al., 2009; Gal-Mor et al., 2010; Almedia et al., 2013). PFGE is a powerful and reliable method, and is able to analyze the entire microbial genome and is very effcient due to its repeatability, reproducibility and ability to discriminate between different bacterial strains. However, PFGE is expenseive and time consuming because it takes more than 5 days to get the end results. Currently, PFGE is commonly used in epidemiological studies to trace back outbreaks associated with a particular pathogen. PFGE is the current gold standard subtyping method for foodborne bacterial pathogens used by PulseNet, the national molecular subtyping network for foodborne disease surveillance in the United States (Swaminathan et al., 2001).

PFGE has been successfully used worldwide to type *S.* Infantis strains isolated from different sources, elucidating outbreaks and their epidemiology (Lindqvist and Pelkonen, 2007; Nógrády et al., 2007, 2008, 2012; Ungvari et al., 2007; Abbasoglu and Akcelık, 2011; Hauser et al., 2012; Rašeta et al., 2014). Abbasoglu and Akcelık (2011) showed three distinct PFGE patterns among 20 *S.* Infantis isolates after digestion of each isolate's chromosomal DNA with XbaI. Rašeta et al. (2014) used PFGE to determine genetic similarity between fve *S.* Infantis isolates from diseased humans and 22 iso-

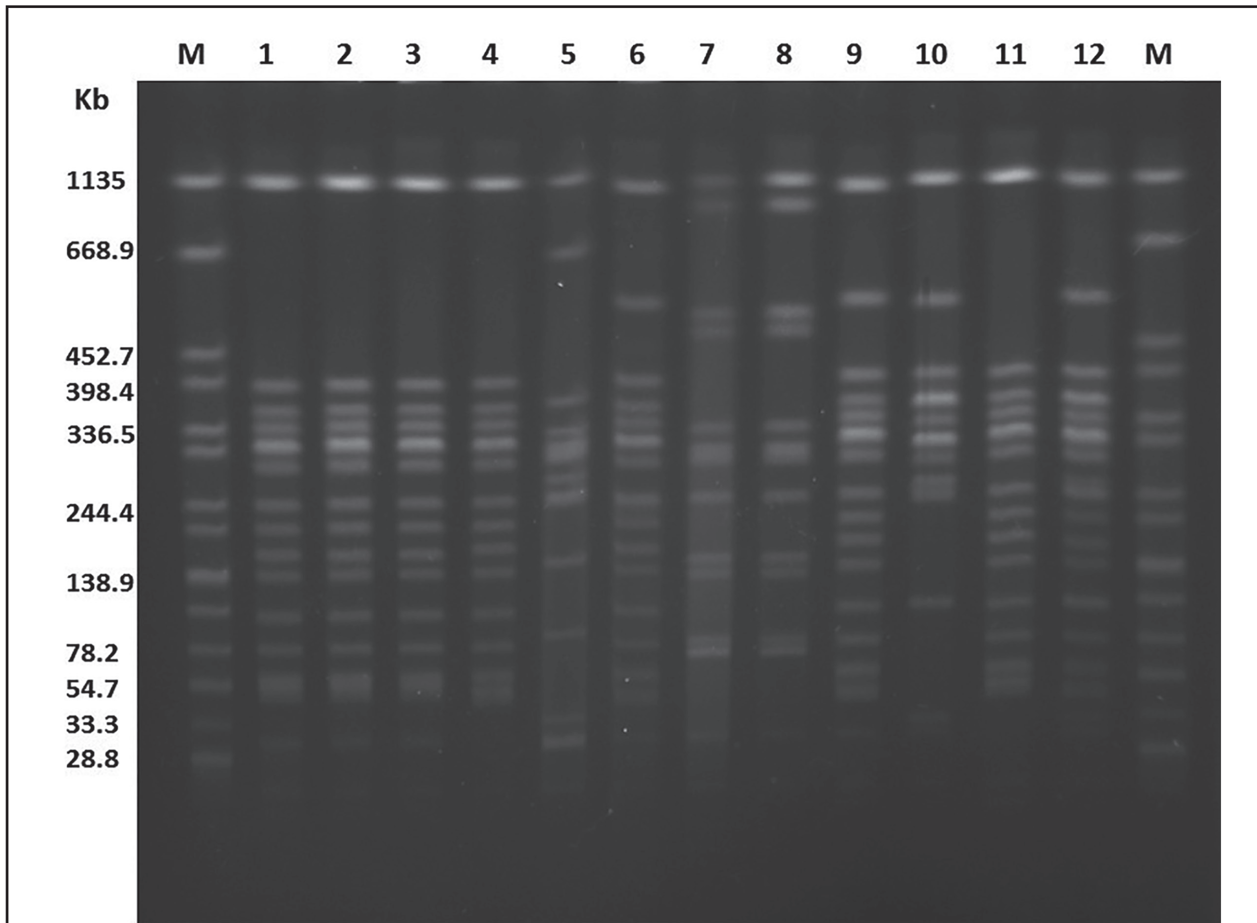

Figure 1. PFGE of 12 S. Infantis isolates' chromosomal DNA digested with XbaI enzyme. M in both sides represent marker, S. Braenderup strain H9812.

lates from broiler carcasses. Cluster analysis showed the presence of seven profiles and 92% genetic similarity among all isolates indicating *S.* Infantis as a hazard to human health. In Brazil, between 1984-2009, Almedia et al (2013) investigated the molecular epidemiology of *S.* Infantis isolates, 25 from human sources and 10 from food items, using ERIC-PCR, PFGE and MLST. Thirty-two *S.* Infantis isolates demonstrated a similarity 80.6% in PFGE while 34 isolates showed a high genetic similarity of 93.7% in ERIC-PCR. Due to high genetic similarity among the isolates, Almedia et al (2013) suggested that a prevalent subtype was the cause of human disease and food contamination during the 25 year period in São Paulo State, Brazil. These researchers expressed that both ERIC-PCR and PFGE were sufficienty adequate methods for long-term epidemiological surveys but concluded that PFGE was more efficient for *Salmonella* subtyping due to its higher discrimination power. In another study (Nógrády et al., 2012), a high genetic similarity of 92% was found between the 76 isolates of *S.* Infantis in the period of 2004 to 2009 from broiler meat and broiler feces. In Hungary, during 2006-2007, 164 isolates of *S.* Infantis were divided in two clusters, whereas the genomes showed similarity more than 88.7% (Nogrady et al., 2007; 2008)

In Iran, only a few recently published reports involve the use of PFGE for characterization of *Salmonella* serotypes isolated from poultry (Zahraei Salehi et al., 2011;

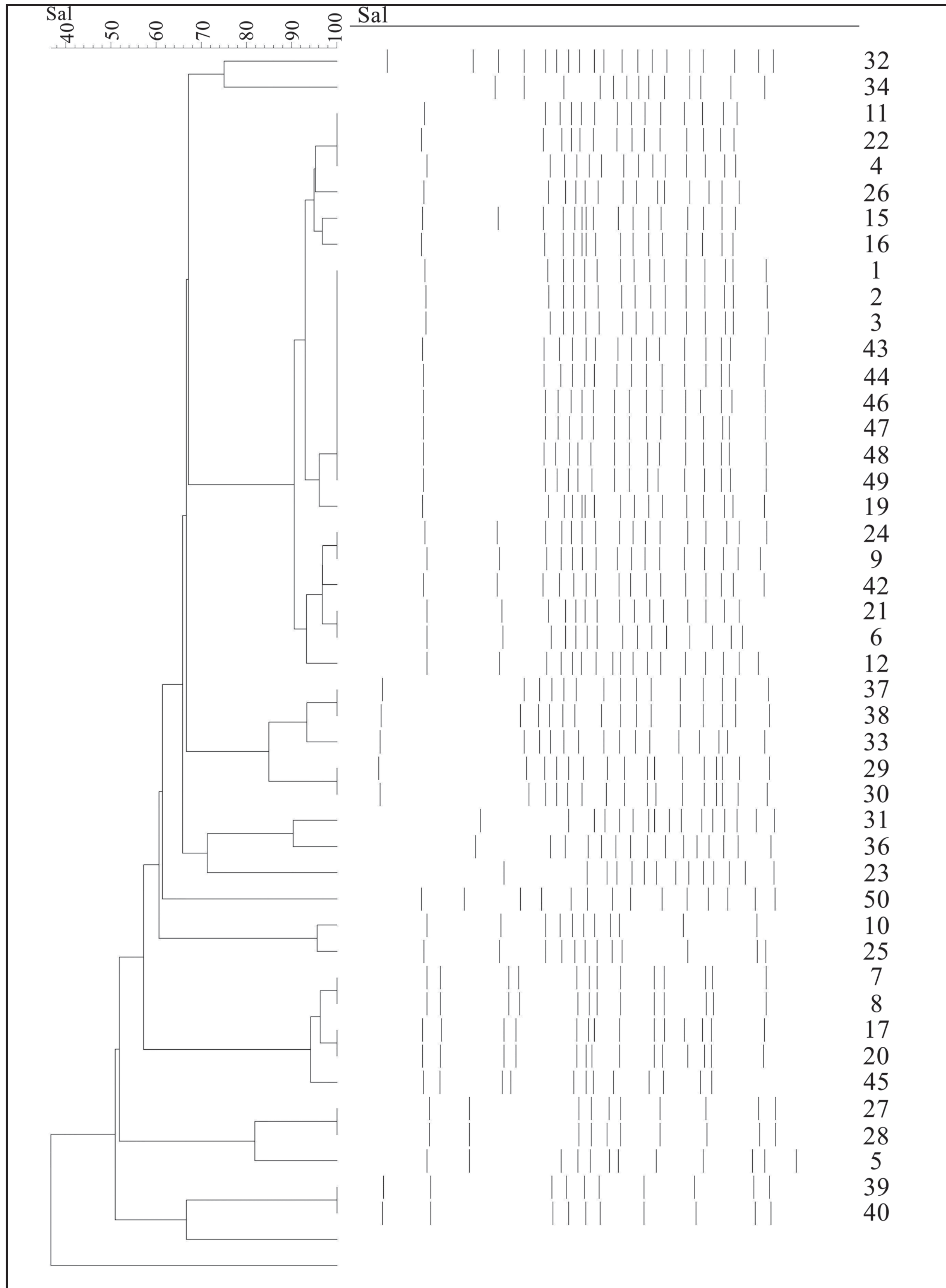

Figure 2. Dendrogram of PFGE types obtained by XbaI-PFGE of 45 S. Infantis isolates. The top bar on the left hand side indicates the percentage of similarity. The bands on the right hand side show the bands observed for each isolate with variable molecular sizes (kb). The numbers on the right hand side of the dendrogram show the isolates numbers.

Rahmani et al., 2013; Golab et al., 2014). Zahraei Salehi et al. (2011) studied *Salmonella enterica* spp. isolates from human and animal origin using PFGE and ERIC-PCR and reported the PFGE as the most effective molecular typing technique (Zahraei Salehi et al., 2011). Rahmani et al. (2013) expressed the value of PFGE typing to determine the epidemiologic distribution of 27 *S.* Infantis isolates from three northern provinces of Iran, and showed two distinct PFGE patterns among the 27 isolates and revealed highly similar PFGE patterns indicating clonal relatedness across different geographical locations.

Golab et al. (2014) used PFGE and serotyping to subtype 47 *Salmonella* isolates belonging to 22 different serotypes derived from poultry. Thirty-nine PFGE patterns among 47 isolates were demonstrated. They indicated that PFGE testing played a key role in distinguishing outbreak-related *Salmonella* isolates from unrelated sporadic isolates.

In the present study, PFGE revealed 27 profles and eight clustes with a genotypic similarity more than 90%. The increasing rate of *Salmonella* Infantis infection among poultry focks in Iran have been reported previously (Rahmani et al., 2013; Peighambari et al, 2015). Our results are in agreement with other workers who reported that PFGE is one of the most reliable techniques for discriminating different serotypes of *Salmonella* (Chen et al., 2011; Almedia et al., 2013; Fendri et al., 2013). In fact, using PFGE is a more preferable and logical approach in analysis of a bacterial genome because it covers almost the whole genome compared to other techniques that only study part of the genome. Our fndings showed that most of the pulsotypes which included more than one single isolate had been recovered from poultry abattoirs. This fnding indicates that during the processing of poulty carcasses, the contamination speads in the abattoir and, therefore, the origin of isolates might rationally be from a single clone. Most of the isolates even in some cases from the same farm belonged to different pulsotypes but still there was more than 90% genotypic homogenicity among the isolates. There were two human isolates of *S.* Infantis, both of which belonged to one pulsotype other than pulsotypes from poultry origin, but these two isolates also showed more than 90% genotypic similarity with poultry-originated isolates. This fnding reinforces previous investigations that considered *S.* Infantis as a hazard to human health (Almedia et al., 2013; Rašeta et al., 2014). Like us, researchers around the world have reported the high genetic similarty among *S.* Infantis isolates recoverd from different sources in their own countries (Fonseca et al., 2006; Gal-Mor et al., 2010; Almedia et al., 2013).

This study showed the value of PFGE in determining the genotypic similarity among *S.* Infantis isolates. The high discriminatory power of PFGE methodology was also demonstrated in this work. The higher efciency of PFGE in comparison to other typing methods such as plasmid profle, ERIC-PCR or RAPD-PCR in discriminating *Salmonella* isolates has also been reported by other researchers (Zahraei Salehi et al., 2011; Almedia et al., 2013; Rašeta et al., 2014). Therefore, PFGE is still considered the preffered method for typing *Salmonella* isolates.

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