Original Article Plasmid Profile and Enterobacterial Repetitive Intergenic Consensus-PCR Characterization of *Salmonella* Infantis Isolates Recovered From Poultry Sources

*Seyed Mostafa Peighambari¹ 💿, Azam Yazdani¹, Hanieh Taheri¹, Fereshteh Shahcheraghi² 💿

1. Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

2. Department of Bacteriology, Microbiology Centre, Pasteur Institute of Iran, Tehran, Iran.



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ABSTRACT

Background: Salmonella is known as one of the most important bacterial agents infecting both humans and animals. Salmonella Infantis has been reported as one of the 15 most prevalent serovars worldwide. Despite its clinical importance, there is little information on the molecular characteristics of *S*. Infantis in Iran.

Objectives: This study was conducted to characterize *S*. Infantis isolates collected from poultry sources in the last decade. The isolates were typified by plasmid profile and enterobacterial repetitive intergenic consensus (ERIC-PCR).

Methods: Forty *S.* Infantis isolates from poultry sources were subjected to plasmid profile and ERIC-PCR characterization. We used a commercial plasmid extraction kit to extract and purify plasmid DNA which then was separated by gel electrophoresis and viewed under a UV transilluminator. For ERIC-PCR, a commercial bacterial chromosomal DNA extraction kit was used. In this study, we chose ERIC2 primer for the ERIC-PCR test.

Results: The plasmid profile revealed that 35% of isolates did not contain any plasmids, but the rest (65%) carried a variable number of plasmids with different molecular weights. Six plasmid profiles were found among 40 *S*. Infantis isolates. Using ERIC2 primer, 7 profiles were found among 40 *S*. Infantis isolates in ERIC-PCR. Bands with molecular weights ranging from 400 to 3000 bp were observed.

Conclusion: This study provided some genetic data on *S*. Infantis isolates recovered from poultry sources, and these data can be used for a broader epidemiological study nationwide. These findings showed that although plasmid and ERIC profiles are valuable in epidemiological studies, they have some limitations, too.

Keywords: Epidemiological study, Enterobacterial repetitive intergenic consensus (ERIC)-PCR, Molecular typing, Plasmid profile, Salmonellosis

* Corresponding Author: Seyed Mostafa Peighambari Address: Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. Phone: +98 (21) 61117150 E-mail: mpeigham@ut.ac.ir

1. Introduction

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almonella species cause several diseases in most animal species worldwide (Gast & Porter, 2020). This bacterium is present in vertebrate gastrointestinal tracts, including mammals, birds, reptiles, and

fish, and can produce various diseases depending on the serotype, conditions, and host factors. *Salmonella* is excreted through the feces of infected humans or animals. The feces contaminate water, food, and the environment, and then the bacterium enters the gastrointestinal tract of other humans and animals. The prevalence of different *Salmonella* species in humans and animals varies from country to country (Crump et al., 2015; Mukherjee et al., 2019; Gast & Porter, 2020). Given the significance of *Salmonella* infection in humans and animals, including poultry, it is very important to identify the prevalence of *Salmonella* infections in industrial poultry flocks.

Non-typhoidal Salmonella species are among the major causes of morbidity and mortality worldwide (Jajere et al., 2019). According to WHO reports, about 94 million people worldwide are implicated with these infections annually, which results in about 155000 deaths (Crump et al., 2015; Kariuki et al., 2015; Jajere et al., 2019). It is estimated that out of the 94 million cases of non-typhoidal Salmonella infections, at least 80 million are of food origin (Jajere et al., 2019). Recent reports from different countries about the isolation of Salmonella from poultry or poultry-derived products have shown that Typhimurium, Enteritidis, Kentucky, Heidelberg, and Infantis serovars are among the most important non-typhoidal Salmonella epidemiologically (Crump et al., 2015; Jajere et al., 2019). These reports have also indicated that poultry and poultry-derived products are the major source of these infections and have been associated with most Salmonella infections that have occurred in human communities in recent years (Crump et al., 2015; Jajere et al., 2019).

Salmonella Infantis is one of the most prevalent Salmonella serovars isolated from pigs, poultry, beef, and seafood worldwide (Ferrari et al., 2019). Since 1970, this serovar has been spreading worldwide in countries including Argentina, Australia, Brazil, the Netherlands, Finland, Canada, Japan, and Russia (Ferrari et al., 2019). Salmonella Infantis is commonly found in hospitals, especially in the pediatric ward, but is associated with septicemia and death if adults are involved (Jajere et al., 2019). The major source of this bacterium is animals, especially industrial poultry populations (Ferrari et al., 2019). The presence of this bacterium has also been reported in the broiler flocks of Japan, Iceland, France, the Netherlands, the US, Australia, Turkey, Saudi Arabia, Algeria, and Iran (Galanis et al., 2006; Peighambari et al., 2013; Ghoddusi et al., 2015; Peighambari et al., 2015; Alzwghaibi et al., 2018; Anonymous, 2018; Peighambari et al., 2018; Jajere et al., 2019; Peighambari et al., 2019a, Peighambari et al., 2019b). S. Infantis has been among the dominant serovars for the past 10 years, accounting for a high proportion of human infections in the last decade (Ferrari et al., 2019; Jaiere et al., 2019). Despite widespread control measures against Salmonella in Europe, S. Infantis ranked third in human Salmonella infection in 2002 (Anonymous, 2018). S. Infantis is one of 15 serovars frequently isolated worldwide (Galanis et al., 2006; Anonymous, 2018; Jajere et al., 2019).

Despite the clinical significance of S. Infantis, the molecular characteristics of its strains are not well known in Iran. Nowadays, researchers can differentiate Salmonella isolates within a specific serovar using DNA-based techniques. Many researchers have often used techniques such as phage-typing, class 1 integron typing, plasmid profile determination, ribotyping, random amplified polymorphic DNA-PCR, restriction fragment length polymorphism, insertion sequence IS200 fingerprinting, enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), and pulsed-field gel electrophoresis and some other techniques (Lukinmaa et al., 2004; Morshed & Peighambari, 2010; Fendri et al. 2013; Golab et al., 2014). In our previous investigation (Peighambari et al., 2015), we recovered many S. Infantis isolates from poultry sources in different parts of Iran between 2010 and 2015 and confirmed the identity of the isolates by culture and PCR. The present investigation aimed to characterize 40 S. Infantis isolates from our laboratory collection using plasmid profiling and ERIC-PCR methods. These data were analyzed to understand the epidemiology of S. Infantis strains isolated from different poultry sources.

2. Materials and Methods

Study samples

Forty S. Infantis isolates recovered from different poultry sources during 2010-2015 were used in this study. In our previous studies, these bacterial isolates had been identified as S. Infantis using culture and PCR (Morshed and Peighambari, 2010; Peighambari et al., 2013; Peighambari et al., 2015). A detailed description of isolates has been provided in Table 1. Each frozen culture, kept at -80°C, was defrosted and cultured on MacConkey agar plates for 24 h. Then, a single colony of grown culture was streaked on LB agar medium, incubated for 24 h, and used for further analyses. Polymerase chain reaction (PCR) was performed to re-confirm all 40 isolates as serovar *S*. Infantis (Peighambari et al., 2015).

Plasmid profile

A commercial plasmid extraction kit (Bioneer, South Korea) was used to extract and purify plasmid DNA from the bacterial isolates. Plasmids were separated by gel electrophoresis (Paya Pajouhesh, Iran) in 1% agarose gel. The gels were run for 10 minutes at 100 V and then approximately 2 hours at 70 V, stained with DNA safe stain (Sinaclon, Iran), exposed to ultraviolet light, and photographed. One kb DNA ladder (Fermentas, Lithuania) containing 13 bands ranging from 250 to 10000 bp and an *E. coli* strain, AC11, containing three plasmids of 68 kb, 2.7 kb, and 1.7 kb (Peighambari et al., 1995) were used as molecular-weight (MW) markers in each gel running. The MW of plasmids was determined by SEQAID II software (Kansas State University, ver. 3.5, USA).

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

To extract bacterial chromosomal DNA, a commercial bacterial DNA extraction kit (MBST, Iran) was used. Enterobacterial Repetitive Intergenic Consensus ERIC2 primer (5'-AAG TAA GTG ACT GGG GTG AGC G-3') was chosen for the ERIC-PCR test in this study (Ungvári et al., 2007). For each isolate, amplification reactions for ERIC2 primer were carried out in a 20-µL reaction volume containing 2 µL of 10 x PCR buffer, 1 µL of 10 mM dNTPs, 1 µL of 20 pmol of primer, 1 µL of Taq DNA polymerase (0.5 U), 1 µL of 50 mM MgCl₂, and 13 µL of deionized H₂O. One microliter of extracted DNA template was added to the mixture. In all PCR reaction sets, negative controls (deionized H₂O instead of template DNA) were included. Amplification was programmed in a thermocycler (SensoQuest, Germany) as follows: 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 65°C for 8 min, and a final extension at 65°C for 15 min. The amplification products were detected by gel electrophoresis in 1% agarose gel, stained with DNA safe stain (Sinaclon), exposed to ultraviolet light, and photographed. The commercial GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as the MW marker in each gel running. The MW of DNA bands was determined by SEQAID II software. Reproducibility of the ERIC-PCR patterns was confirmed using duplicate runs by two operators on separate occasions but the same thermocycler. Sinaclon provided the primers and other materials used in the PCR reaction.

3. Results

Plasmid profile

In Table 2, the plasmid profiles of 40 S. Infantis isolates from different poultry sources are shown. In 14 isolates (35%), no plasmid was detected. Among the remaining 26 isolates (65%) in which plasmids were found, 23 isolates carried 68 kb plasmids. Four isolates (10%) had 1 plasmid weighing more than 68 kb. Three isolates contained 2 plasmids, and 1 isolate had 3 plasmids. The molecular weight of the plasmids varied from 2 kb to more than 68 kb. Six plasmid profiles were named from A to F (Table 2). Profile A (14%) had no plasmid, profile B (42.5%) had one 68 kb plasmid, profile C (10%) had plasmids weighing more than 68 kb, profile D (2.5%) had 3 plasmids weighing 2.6, 7, and 68 kb, profile E (5%) had plasmids weighing 5, and 68 kb; and profile F (5%) had plasmids weighing 3 and 68 kb. No plasmid profile could be attributed to any specific poultry source.

ERIC-PCR

Using the ERIC2 primer, 7 different ERIC profiles were identified among the 40 *S*. Infantis isolates tested in ERIC-PCR (Table 3). Bands with molecular weights ranging from 400 to 3000 bp were observed. Twentynine isolates (72.5%) had a band of 1300 bp. The most frequent profile was ERIC profile A, showing a single band of 1300 bp in 12 isolates recovered from different poultry sources. No ERIC profile could be attributed to any specific poultry source.

4. Discussion

Recent studies on the *Salmonella* infection status of poultry flocks in Iran have recovered many *Salmonella* isolates belonging to different serovars, mainly *S. Enteritidis* and *S.* Infantis (Peighambari et al., 2013; Rahmani et al., 2013; Ghoddusi et al., 2015; Peighambari et al., 2015; Alzwghaibi et al., 2018; Peighambari et al., 2019; Peighambari et al., 2019a; Peighambari et al., 2019b).

Using species-specific PCR, Peighambari et al. (2015) found that 70 of 100 *Salmonella* group C isolates belonged to *S*. Infantis serovar. Emadi et al.

Isolate #	Isolate Source	Plasmid Profile	ERIC Profile	Isolate #	Isolate Source	Plasmid Profile	ERIC Profile
1	DOC	В	С	21	Abattoir	А	D
2	DOC	В	D	22	Abattoir	А	E
3	Broiler liver	В	С	23	Abattoir	А	D
4	Broiler flock	А	G	24	Abattoir	В	D
5	Broiler flock	E	С	25	Abattoir	В	А
6	Broiler flock	В	А	26	Abattoir	А	А
7	Broiler flock	А	А	27	Abattoir	В	А
8	Broiler flock	В	А	28	Abattoir	D	С
9	Broiler flock	А	А	29	Abattoir	E	С
10	Broiler flock	С	А	30	Abattoir	В	В
11	Broiler flock	А	А	31	Abattoir	В	С
12	Broiler flock	А	А	32	Abattoir	F	В
13	Broiler flock	В	А	33	Abattoir	С	В
14	Broiler flock	В	С	34	Abattoir	С	F
15	Broiler flock	В	А	35	Abattoir	В	В
16	Broiler flock	А	С	36	Abattoir	В	В
17	Broiler flock	F	D	37	Abattoir	А	В
18	Broiler flock	А	С	38	Abattoir	А	В
19	Abattoir	В	E	39	Abattoir	В	В
20	Abattoir	С	D	40	Abattoir	А	В

Table 1. Description of S. Infantis isolates used

DOC: day-old chicks; ERIC: enterobacterial repetitive intergenic consensus.

(2009) recovered 1125 Salmonella isolates from backyard poultry in the north of Iran and reported that 7.4% of the isolates belonged to S. Infantis. From 2007 to 2001, Rahmani et al. (2013) collected 36 Salmonella isolates from broilers in three regions in the north of Iran, of which 25% were Salmonella Enteritidis, and 75% were S. Infantis.

In Iran, no comprehensive research has been conducted on the molecular and genotypic characteristics of *Salmonella* Infantis strains from various sources, and most studies have been done on other *Salmonella* serovars, such as *S*. Enteritidis. Of course, several studies have reported the frequency of *S*. Infantis and the related drug resistance patterns among Iranian poultry flocks (Peighambari et al., 2013; Rahmani et al. 2013; Ghoddusi et al., 2015; Peighambari et al., 2015; Alzwghaibi et al., 2018; Peighambari et al., 2018; Ghoddusi et al., 2019). For this reason, this study was designed to identify some of the genotypic characteristics of *S*. Infantis strains from different poultry sources that can provide valuable data for further epidemiological studies. These findings and further complementary genotypic studies will be useful in comparative studies with isolates from humans and other sources. The obtained data on the correlation of *S*. Infantis recovered from different sources, ultimately, will help health authorities to design appropriate prevention and control policies.

No. (%)	- Malacular Misiaht of Danda (Irku)	Plasmid Profile	
Isolates	 Molecular Weight of Bands (kbp) 		
14 (35)	-	А	
17 (42.5)	68	В	
4 (10)	>68	C	
1 (2.5)	2.8, 7, 68	D	
2 (5)	5, 68	E	
2 (5)	3, 68	F	

Table 2. Distribution of 6 plasmid profiles among S. Infantis isolates

As mentioned above, numerous techniques have been used for genotypic studies of Salmonella spp. Plasmid profile determination is usually used as one of the methods for Salmonella subtyping (Morshed and Peighambari, 2010; Peighambari et al., 2013). Plasmid profiles may be of value as epidemiologic markers and may also suggest the presence of virulence plasmids. However, because some isolates may lack plasmids or there may be no correlation between plasmid content and the virulence factors, plasmid profiling may not always be considered valuable for Salmonella subtyping. Some Salmonella strains may carry virulence plasmids that play an important role in the invasion and survival of Salmonella within the host. Virulence plasmids encode genes involved in Salmonella's ability to cause disease. Some high molecular weight plasmids are responsible for resistance to antimicrobial agents (Peighambari et al., 2013). Isolates of S. Infantis expressing antimicrobial resistance have been

reported by various researchers (Abbasoglu and Akcelık, 2011; Rahmani et al., 2013; Rašeta et al., 2014; Shah et al., 2017). The size of Salmonella Enterica plasmids may vary from 2 to 200 kb. Due to plasmid content diversity among Salmonella Typhimurium isolates, plasmid profiling has been frequently used for Salmonella Typhimurium. However, after 2000, this subtyping method was also commonly used and found to be valuable for Salmonella Enteritidis isolates. Nógrády et al. (2008) examined the plasmid content of 145 S. Infantis isolates and, in all but one isolate observed a large plasmid of >168 kb in size. In another study, Abbasoglu and Akcelık (2011) showed the presence of a mega-plasmid with a molecular weight of 206 kb in 20 S. Infantis isolates. In the present study, the plasmid content of 40 S. Infantis isolates was investigated. It was found that 14 isolates did not contain any plasmids but the other 26 isolates

No. (%)		ERIC Profile	
Isolates	— Molecular Weight of Bands (bp)		
12 (30)	1300	А	
9 (22.5)	700, 1000, 1300, 2300, >3000	В	
9 (22.5)	-	С	
6 (15)	400, 700, 1300	D	
2 (5)	400, 700	Е	
1 (2.5)	400, 700, 1000, 1300	F	
1 (2.5)	400, 700, 1300, 1600, 2300	G	

DOC: day-old chicks; ERIC: enterobacterial repetitive intergenic consensus.

contained at least one plasmid, which is consistent with previous investigations.

The discovery of repetitive sequences such as the ERIC sequence in prokaryotic genomes has expanded the molecular biology tools used to evaluate the clonal diversity of many bacterial species, such as *Salmonella* (Versalovic et al., 1991; Fendri et al., 2013). The ERIC sequences are 126 bp conserved motifs that, when homologous primers with these sequences are used to amplify them by PCR, show a pattern of amplified bands specific to each isolate (Versalovic et al., 1991). ERIC-PCR is a simple, fast, and inexpensive method that can be used in all laboratories equipped with molecular biology tools.

Many researchers have used ERIC-PCR for subtyping S. Infantis isolates (Ungvári et al., 2007; Almeida et al., 2013). Almeida et al. (2013) studied 34 S. Infantis isolates using ERIC-PCR and observed 93.7% genetic similarity among the 34 isolates distributed into 8 ERIC profiles (Almeida et al., 2013). Ungvári et al. (2007) genotyped 31 S. Infantis isolates collected from 21 different farms using ERIC-PCR (ERIC2 primer) and concluded that all S. Infantis studied isolates were genetically close to each other and ERIC-PCR could not accurately differentiate the isolates from each other (Ungvári et al., 2007). Johnson et al. (2001) examined the genotyping of 70 S. Infantis isolates sequestered from different locations using the repetitive sequencebased PCR and the ERIC2 primer and observed 96% genetic similarity among the isolates. In Iran, Ranjbar et al. (2014) studied 40 S. Infantis isolates from human sources by ERIC-PCR and observed 8 different profiles among the isolates. In the present study, 7 ERIC profiles were found among the 40 S. Infantis isolates using ERIC-PCR and ERIC2 primer, which is consistent with the findings of previous investigators, especially with those of Ranjbar et al. (2014).

Given the growing significance of *Salmonella* paratyphoid, especially *S*. Infantis, in humans and poultry in recent years throughout the world, including Iran, there is a need for extensive and advanced research to reduce and control infection in poultry and, subsequently, in humans. This study provided genetic data on *S*. Infantis isolates recovered from different poultry sources. These data can be used for a broader epidemiological study nationwide. Although plasmid profile determination and the ERIC-PCR are valuable in epidemiological studies, they have some limitations, too. For ERIC-PCR, using more repeat primers may better differentiate the bacterial isolates. One of the important applications of bacterial subtyping techniques in epidemiological studies is to find the origin and the foci of infection that can be used to promote infection control strategies.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Methodology, Data collection, and Data analysis: Seyed Mostafa Peighambari, Fereshteh Shahcheraghi, and Hanieh Taheri; Conceptualization and Writin– original draft: Hanieh Taheri and Azam Yazdani; Writing–review & editing: Seyed Mostafa Peighambari. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declared no conflict of interest.

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مقاله پژوهشی

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مطالعه خصوصیات جدایههای سالمونلا اینفنتیس بدست آمده از منابع طیوری با استفاده از تعیین الگوی پلاسمیدی و ERIC-PCR

•سید مصطفی پیغمبری ای، اعظم یزدانی ، هانیه طاهری ، فرشته شاهچراغی ای

۱. گروه بیماریهای طیور، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران. ۲. گروه میکروبشناسی، مرکز میکروب شناسی، موسسه پاستور ایران، تهران، ایران.

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زمینه مطالعه: سالمونلا به عنوان یکی از مهمترین عوامل باکتریایی که هم انسان و هم حیوانات را آلوده می نماید، شناخته شده است. سالمونلااینفنتیس به عنوان یکی از ۱۵ سرووار شایع سالمونلا در سراسر جهان گزارش شده است. علیرغم اهمیت بالینی آن، اطلاعات محدودی مورد ویژگی های سالمونلا اینفنتیس در ایران موجود است.

هدف: هدف از این مطالعه بیشتر دستهبندی نمودن سالمونلااینفنتیس جدا شده از گله های مختلف در ایران در دهه گذشته بااستفاده از تکنیک های تعیین محتوی پلاسمیدی و ERIC-PCR بود.

روش کار: تعداد ۴۰ جدایه سالمونلا اینفنتیس، از منبع مختلف مرتبط با طیور در ایران، با استفاده از تکنیک.های تعیین محتوی پلاسمیدی و ERIC-PCR مورد مطالعه قرار گرفتند. یک کیت تجاری استخراج پلاسمید برای استخراج و خالص سازی پلاسمید از جدایه ها مورد استفاده قرار گرفت. سپس، پلاسمیدها با ژل الکتروفورز از هم جدا شدند و با اشعه ماوراء بنفش در یک دستگاه ترانس ایلومیناتور مشاهده شدند. برای انجام ERIC-PCR، کیت تجاری استخراج DNA کروموزومی مورد استفاده قرار گرفت.

نتایج: از پرایمر ERIC2 برای ازمایش ERIC-PCR استفاده شد. تعیین محتوی پلاسمیدی جدایه ها مشخص نمود که ۳۵٪ جدایه ها فاقد پلاسمید بودند و ۶۵٪ بقیه دارای تعداد متنوعی پلاسمید در اوزان ملکولی متفاوت بودند. با استفاده از آغاز گر ERIC2 تعداد هفت الگو در بین ۴۰ جدایه سالمونلا اینفنتیس مورد آزمایش در ERIC-PCR مشاهده شد. باندهای با دامنه وزنی بین ۴۰۰ تا ۳۰۰۰ جفت باز مشاهده شدند.

نتیجه گیری نهایی: این مطالعه برخی از داده های ژنتیکی در مورد جدایه های سالمونلا اینفنتیس با منشاء طیوری را ارائه داده است. تاریخ دریافتد ۵۰ مرداد ۲۱۰۱ تاریخ پذیرش: ۲۷ مهر ۲۱۰۱ در مطالعات اپیدمیولوژیک با ارزش هستند، اما برخی از محدودیت ها را نیز دارا می باشند. از محدودیت ها را نیز دارا می باشند. در مطالعات اپیدمیولوژیک، ۱۹۰۲ در مطالعات اپیدمیولوژیک، ERIC-PCR

سید مصطفی پیغمبری **نشانی:** تهران، دانشگاه تهران، دانشکده دامپزشکی، گروه بیماریهای طیور. **تلفن: ۱۱۱۷۱۵۹ (۲۱) ۹۸**+ **رایانامه:** mpeigham@ut.ac.ir

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