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Plasmid Profile and ERIC–PCR Characterization of *Salmonella* Infantis Isolates Recovered from Poultry Sources

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Running Title: Characterization of *Salmonella* Infantis isolates

Abstract

Backgrounds: *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 all over the world. Despite, its clinical importance, there is a paucity of information on the molecular characteristics of *Salmonella* Infantis in Iran.

Objectives: This study was conducted to characterize *Salmonella* Infantis isolates collected from poultry sources in last decade by plasmid profile and enterobacterial repetitive intergenic consensus (ERIC–PCR).

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

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

Conclusions: This study provided some genetic data on *Salmonella* Infantis isolates recovered from poultry sources and these data can be used for a broader epidemiological study nationwide. These findings showed that both plasmid- and ERIC profiles are valuable in epidemiological studies but also have some limitations.

Keywords: Epidemiological study, ERIC-PCR, Molecular typing, Plasmid profile, Salmonellosis

Introduction

Diseases caused by *Salmonella* species are found all over the world and in most animal species (Gast and Porter, 2020). This bacterium is present in vertebrate gastrointestinal tracts including mammals, birds, reptiles, and fish and can cause various diseases depending on the serotype, conditions, and host factors. *Salmonella* are excreted through the feces of humans or animals and cause contamination of water, food and the environment leading to the gastrointestinal tract of 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 and Porter, 2020). Given the importance of *Salmonella* infection in humans and animals including poultry, it is very important to identify the frequency of *Salmonella* infections in industrial poultry flocks.

Non-typhoidal *Salmonella* species are among the major causes of infection and mortality worldwide (Stanaway et al., 2019). According to WHO reports, about 94 million people worldwide are implicated with these infections annually, which results in about 155,000 deaths worldwide (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 products have shown that serovars Typhimurium, Enteritidis, Kentucky, Heidelberg, and Infantis, epidemiologically, are among the most important non-typhoidal

Salmonella (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 throughout the world 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 in the broiler flocks of Japan, Iceland, France, the Netherlands, the US, Australia, Turkey, Saudi Arabia, Algeria and Iran has also been reported (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, 2019b). *Salmonella* Infantis has been among the dominant serovars for the past ten years, accounting for a high proportion of human infections in the last decade (Ferrari et al., 2019; Jajere et al., 2019). Despite widespread control measures against *Salmonella* in Europe, *S.* Infantis ranked third in human *Salmonella* infection in 2002 (Anonymous, 2018). *Salmonella* Infantis is one of 15 serovars frequently isolated throughout the world (Galanis et al. 2006; Anonymous, 2018; Jajere et al., 2019; Stanaway et al., 2019).

Despite the clinical significance of *Salmonella* Infantis, the molecular characteristics of its strains are not well known in Iran. Nowadays, researchers are able to differentiate *Salmonella*

isolates within a specific serovar using DNA-based techniques. Techniques such as phage-typing, class 1 integron typing, plasmid profile determination, ribotyping, random amplified polymorphic DNA-PCR (RAPD-PCR), restriction fragment length polymorphism (RFLP), insertion sequence (IS) IS200 *fingerprinting,* enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), and pulsed-field gel electrophoresis (PFGE) and some other techniques have often been used by many researchers (Lukinmaa et al., 2004; Morshed and Peighambari, 2010; Fendri, et al. 2013; Golab et al., 2014). In our previous investigation (Peighambari et al., 2015), we recovered many *Salmonella* 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 purpose of the present investigation was to characterize 40 *Salmonella* Infantis isolates from our laboratory collection using plasmid profiling and ERIC-PCR methods. These data were analyzed to obtain a deeper understanding of the epidemiology of *S.* Infantis strains isolated from different poultry sources.

Materials and Methods

Samples

Forty *Salmonella* Infantis isolates recovered from different poultry sources, during the years 2010–2015, were used in this study. In our previous studies, these bacterial isolates had been identified as *Salmonella* Infantis by culture and PCR (Morshed and Peighambari, 2010; Peighambari et al., 2013, 2015). Detailed description of isolates has been provided in Table 1. Each frozen culture, kept in -80 °C, were defrosted and cultured on MacConkey agar plates for 24 hours. Then, a single colony of grown culture was streaked on LB agar medium, incubated for

24 hours and used for further works. Polymerase chain reaction (PCR) was performed to re-confirm all 40 isolates as serovar *Salmonella* 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 volts and then approximately two hours at 70 volts, 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 10,000 bp and an *E. coli* strain, AC11, containing three plasmids of 68 kb, 2.7 kb, and 1.7 kb (Peighambari et al., 1994) 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. ERIC2 primer (5′-AAG TAA GTG ACT GGG GTG AGC G-3 ′) was chosen for ERIC-PCR test in this study (Ungvári et al., 2007). For each isolate, amplification reaction for ERIC2 primer were carried out in a 20 µl reaction volume containing 2 µl 10 x PCR buffer, one µl of 10 mM dNTPs, one µl of 20 pmol of primer, one µl of *Taq* DNA polymerase (0.5 U), one µl of 50 mM $MgCl_2$, and 13 µl of deionized H_2O. One µl of extracted DNA template was added to the mixture. In all PCR reaction sets, negative controls (deionized H_2O instead of template DNA) were included. Amplification was programed 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 Gene Ruler 100 bp DNA Ladder Plus (Fermentas) was used as 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 on the same thermocycler. The primers and other materials used in PCR reaction were provided from Sinaclon.

Results

Plasmid Profile

In Table 2, the plasmid profiles of 40 *Salmonella* Infantis isolates from different poultry sources is shown. In 14 (35%) out of 40 isolates, no plasmid was detected. Among the remaining 26 (65%) isolates in which plasmids were found, 23 isolates carried 68 kb plasmids. Four (10%) isolates had a plasmid weighing more than 68 kb. Three isolates contained two plasmids and one isolate had three plasmids. The MW 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%) has 68 kb plasmid, profile C (10%) has plasmids weighing more than 68 kb, profile D (2.5%) has three plasmids weighing 2.6, 7 and 68 kb, profile E (5%) has plasmids weighing 5 and 68 kb; and profile F (5%) had plasmids weighing 3 and 68 kb, respectively. No plasmid profile could be attributed to any specific poultry source.

ERIC-PCR

Using the ERIC2 primer, seven different ERIC profiles were identified among the 40 *Salmonella* Infantis isolates tested in ERIC-PCR (Table 3). Bands with a MW ranging from 400 to 3000 bp were observed. Twenty-nine (72.5%) isolates had a band of 1300 bp. The most

frequent profile was ERIC profile A, which showed a band of 1300 bp only and found in 12 isolates recovered from different poultry sources. No ERIC profile could be attributed to any specific poultry source.

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Discussion

Recent studies on *Salmonella* infection status of poultry flocks in Iran have led to the recovering 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
180 al., 2015; Alzwghaibi et al., 2018; Peighambari et al., 2018; Ghoddusi et al., 2019; Peighambari et al., 2019a, 2019b).

In 2015, Peighambari et al., by using species-specific PCR, found that 70 of 100 *Salmonella* group C isolates belonged to *Salmonella* Infantis serovar. Emadi et al. (2009) recovered 1125 *Salmonella* isolates from backyard poultry in North of Iran and revealed that
185 7.4% of the isolates belonged to *Salmonella* 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 *Salmonella* Infantis.

In Iran, there has been no comprehensive research on the molecular and genotypic characteristics of *Salmonella* Infantis strains from various sources and most studies have been
190 done on other *Salmonella* serovars such as *Salmonella* Enteritidis. Of course, several studies have reported the frequency of *Salmonella* 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; Peighambari et al., 2019a, 2019b). For this reason, this study was designed to identify

some of the genotypic characteristics of *Salmonella* Infantis strains from different poultry sources that can provide valuable data for further epidemiological studies. These findings and further complementary genotypic studies will be very useful in comparative studies with isolates from human and other sources. The obtained data on correlation of *Salmonella* Infantis recovered from different sources, ultimately, will help health authorities to design appropriate prevention and control policies.

Numerous techniques have been used for genotypic studies of *Salmonella* spp. as mentioned above. 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 that are 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 *S.* Typhimurium isolates, plasmid profiling has been frequently used for *S.* Typhimurium. However, after the year 2000, this subtyping method was also commonly used and found to be valuable for *S.* Enteritidis isolates. Nógrády et al. (2008) examined 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 *Salmonella* Infantis isolates. In the present study, the plasmid content of 40 *Salmonella* Infantis isolates was investigated. It was found that 14 isolates did not contain any plasmids but the other 26 isolates contained at least one plasmid that 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 that are 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 *Salmonella* Infantis isolates (Ungvári et al., 2007; Almeida et al., 2013). Almeida et al. (2013) studied 34 *Salmonella* Infantis isolates using ERIC-PCR and observed 93.7% genetic similarity among the 34 isolates that were distributed into eight ERIC profiles (Almeida et al., 2013). Ungvári et al. (2007) genotyped 31 *Salmonella* Infantis isolates collected from 21 different farms using ERIC-PCR (ERIC2 primer) and concluded that all *Salmonella* Infantis studied isolates were genetically close to each other and ERIC-PCR was not able to accurately differentiate the isolates from each other (Ungvári et al., 2007). Johnson et al. (2001) examined the genotyping of 70 *Salmonella* Infantis isolates isolated from different locations by using Rep-PCR and the ERIC2 primer and observed 96% genetic similarity among the isolates. In Iran, Ranjbar et al. (2014) studied 40 *Salmonella* Infantis isolates from human sources by ERIC-PCR and observed eight different profiles among the isolates. In the present study, seven ERIC

profiles were found among the 40 *Salmonella* 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 increasing importance of paratyphoidal *Salmonella*e, especially *Salmonella* Infantis in human 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 human. This study provided some genetic data on *Salmonella* Infantis isolates recovered from different poultry sources. These data can be used for a broader epidemiological study nationwide. Both plasmid profile determination and the ERIC-PCR are valuable in epidemiological studies but also have some limitations. 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 of the infection and the foci of infection that can be used to improve infection control strategies.

Conflict of interest

There is no conflict of interest over this manuscript.

Acknowledgements

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Table 1. Description of *Salmonella* Infantis isolates characterized in the present study.

Table 2. Distribution of six plasmid profiles among *Salmonella* Infantis isolates of this study.

Table 3. Distribution of seven ERIC profiles among *Salmonella* Infantis isolates of this study.

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Table 1. Description of *Salmonella* Infantis isolates used in this study. DOC = Day-old chicks

Isolate #	Isolate source	Plasmid profile	ERIC profile	Isolate #	Isolate source	Plasmid profile	ERIC profile
1	DOC	B	C	21	Abattoir	A	D
2	DOC	B	D	22	Abattoir	A	E
3	Broiler liver	B	C	23	Abattoir	A	D
4	Broiler flock	A	G	24	Abattoir	B	D
5	Broiler flock	E	C	25	Abattoir	B	A
6	Broiler flock	B	A	26	Abattoir	A	A
7	Broiler flock	A	A	27	Abattoir	B	A
8	Broiler flock	B	A	28	Abattoir	D	C
9	Broiler flock	A	A	29	Abattoir	E	C
10	Broiler flock	C	A	30	Abattoir	B	B
11	Broiler flock	A	A	31	Abattoir	B	C
12	Broiler flock	A	A	32	Abattoir	F	B
13	Broiler flock	B	A	33	Abattoir	C	B
14	Broiler flock	B	C	34	Abattoir	C	F

15	Broiler flock	B	A	35	Abattoir	B	B
16	Broiler flock	A	C	36	Abattoir	B	B
17	Broiler flock	F	D	37	Abattoir	A	B
18	Broiler flock	A	C	38	Abattoir	A	B
19	Abattoir	B	E	39	Abattoir	B	B
20	Abattoir	C	D	40	Abattoir	A	B

Table 2. Distribution of six plasmid profiles among *Salmonella* Infantis isolates of this study.

Plasmid profile	Molecular weight of bands (kbp)	Number (%) of isolates
A	-	14 (35)
B	68	17 (42.5)

C	<68	4 (10)
D	2.8, 7, 68	1 (2.5)
E	5, 68	2 (5)
F	3, 68	2 (5)

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450 **Table 3.** Distribution of seven ERIC profiles among *Salmonella* Infantis isolates of this study.

ERIC profile	Molecular weight of bands (bp)	Number (%) of isolates
A	1300	12 (30)
B	700, 1000, 1300, 2300, < 3000	9 (22.5)
C	-	9 (22.5)
D	400, 700, 1300	6 (15)
E	400, 700	2 (5)
F	400, 700, 1000, 1300	1 (2.5)
G	400, 700, 1300, 1600, 2300	1 (2.5)

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

عنوان کوتاه: مطالعه خصوصیات جدایه‌های سالمونلا اینفنتیس

چکیده

زمینه مطالعه: سالمونلا به عنوان یکی از مهم ترین عوامل باکتریائی که هم انسان و هم حیوانات را آلوده می‌نماید، شناخته شده است. سالمونلا اینفنتیس به عنوان یکی از 15 سروار 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 دسته بندی نمودن سالمونلا اینفنتیس جدا شده از گله های مختلف در ایران در دهه گذشته با استفاده از تکنیک‌های تعیین محتوی پلاسمیدی و ERIC-PCR بود.

روش کار: تعداد 40 جدایه سالمونلا اینفنتیس، از منبع مختلف مرتبط با طیور در ایران، با استفاده از تکنیک‌های 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.

از پرایمر ERIC2 برای ازمایش ERIC-PCR استفاده شد.

نتایج: تعیین محتوی پلاسمیدی جدایه ها مشخص نمود که 35٪ جدایه ها فاقد پلاسمید بودند و 65٪ بقیه دارای تعداد

متنوعی پلاسمید در اوزان ملکولی متفاوت بودند. با استفاده از آغازگر ERIC2 تعداد هفت الگو در بین 40 جدایه سالمونلا اینفنتیس مورد آزمایش در ERIC-PCR مشاهده شد. باندهای با دامنه وزنی بین 400 تا 3000 جفت باز مشاهده شدند.

نتیجه گیری نهائی: این مطالعه برخی از داده های ژنتیکی در مورد جدایه های سالمونلا اینفنتیس با منشاء طیوری را ارائه داده است. این داده ها را می توان برای یک مطالعه اپیدمیولوژیک گسترده تر در سراسر کشور مورد استفاده قرار داد. این یافته ها نشان داد که هر دو روش تعیین پروفایل پلاسمیدی و ERIC در مطالعات اپیدمیولوژیک با ارزش هستند، اما برخی از محدودیت ها را نیز دارا می باشند.

کلمات کلیدی: پروفایل پلاسمیدی، تایپینگ ملکولی، سالمونلوز، مطالعه اپیدمیولوژیک، ERIC-PCR