

Full Length Research Paper

Molecular detection of *invA* and *spv* virulence genes in *Salmonella enteritidis* isolated from human and animals in Iran

Kumarss Amini¹, Taghi Zahraei Salehi^{1*}, Gholamreza Nikbakht², Reza Ranjbar³, Javid Amini⁴ and Shahrnaz Banou Ashrafganjooei⁵

¹Department of Microbiology, Faculty of Specialized Veterinary Science, Islamic Azad University, Science and Research Branch, Tehran, Iran.

²Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

³Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.

⁴Department of Microbiology, Islamic Azad University of Kerman, Iran.

⁵Department of Microbiology, Kerman University of Medical Science, Kerman, Iran.

Accepted 23 January, 2018

It is important to study the genotypic diversity of *Salmonella* plasmid genes which are responsible for its virulence. In the present study multiplex polymerase chain reaction (multiplex PCR) assay was carried out for detection of *Salmonella enteritidis* and presence of *invA* and *spv* genes. In the first stage of the study, 1001 poultry samples were collected from a slaughterhouse in Kerman province (southern Iran). Biochemical and serological tests were then performed for identification of *Salmonella* serovars and 6.79% (68/1001) were positive for *Salmonella*. Multiplex PCR with three set primers was then applied to confirm serovar enteritidis 51.4% (35/68). Simple-PCR was then applied to detect *spvA* (*Salmonella* plasmid virulence), and *spvB* genes. Finally, multiplex PCR assay was carried out to simultaneously detect and identify *invA* and *spvC* genes. The presence of *spvA*, *spvB* and *spvC* in *S. enteritidis* was 88.6% for each gene. In the second stage of the study, thirty-three bovine (n = 13) and human (n = 20) *S. enteritidis* strains were isolated from the culture collection in the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Iran. The analyses of the samples revealed that *spvA*, *spvB*, and *spvC* genes were present in 90% of *S. enteritidis* from human sources as compared to 100% in bovine sources. The study represents the first report in Iran about the genotypic diversity of *spvA*, *spvB* and *spvC* genes of *S. enteritidis*.

Key words: *Salmonella enteritidis*, multiplex PCR, virulence genes.

INTRODUCTION

Salmonellosis is associated with medium to severe morbidity and even mortality in farm animals representing a major economic productivity loss in the food and animal industries (Malkawi et al., 2004). *Salmonella enterica* subspecies *enterica* serovar enteritidis is a major cause of food-borne illness in animals and human disease worldwide (Agron et al., 2001). An important pathogen, *Salmonella* shows different disease syndromes and host specificities according to their antigenic profiles (Lim et al., 2003; Ranjbar et al., 2010). Poultry products have been recognized as a major source of human illness caused by this pathogen (Amavisit et al., 2001). During

the last decades, the isolation of *Salmonella* worldwide has been on the increase (Madadgar et al., 2008). Several gastroenteritis outbreaks have been reported in Iran due to the consumption of contaminated food products (Fardsanei et al., 2009). In fact, the incidence of food-borne cases of infection caused by *Salmonella enteritidis* has increased dramatically in the country during the past years. In a study of 480 broiler chicken farms around Tehran, 67% were reported to be contaminated with *S. enteritidis* (Bozorgmehri et al., 1992). The contamination rate in 171 commercial poultry farms around the country was reported to be 45% (Akbarian et al., 2007). Moreover, in a study of 146 clinical human fecal samples, 31 cases (21.23%) were confirmed as *Salmonella* spp, out of which 11 strains (35.48%) were *S. enteritidis* (Fardsanei et al., 2009).

Due to the increased prevalence of *Salmonella* serovar

*Corresponding author. E-mail: tsalehi@ut.ac.ir. Tel: 982166427517. Fax: 982166427517.

enteritidis, and its complex life cycle, many researchers emphasize the necessity and importance of finding a more rapid and effective detection method as a basis of control (Agron et al., 2001; Lim et al., 2003). Presently, *Salmonella* is detected by standard bacteriological, biochemical and serological tests. These tests are generally time-consuming, tedious and costly as they require hundreds of antisera as well as well-trained technicians (Echeita et al., 2002; Nori et al., 2010). Several rapid and sensitive methods have been developed for identification of *Salmonella* serotypes from clinical samples (Zahraei et al., 2007). These methods, however, still lack the necessary sensitivity and specificity (Widjoatmodjo et al., 1992; Aabo et al., 1993).

In vitro amplification of DNA by the polymerase chain reaction (PCR) method is a powerful tool in microbiological diagnostics (Malorny et al., 2003). Multiplex PCR provides us with a specific method and superior ability to detect *S. enterica* and the serovar *S. enteritidis* and/ or *Salmonella typhimurium* in the presence of other bacteria simultaneously (Yan et al., 2010; Malkawi et al. 2004). In this method several genes are used to detect *Salmonella* genus or serovars including: Virulent chromosomal genes such as *invA* (Malorny et al., 2003; Zahraei et al., 2006), *iroB* (Soumet et al., 1998), *invE* (Feder et al., 2001) and *slyA* (Del Cerro et al., 2003); fimbriae genes such as *fimY* (Yeh et al., 2002) and *sefA* (Pan et al., 2002); unique sequence such as Sdf I (Agron et al., 2001) and ST (Malkawi et al., 2004) and finally plasmid genes such as *spv* (Soumet et al., 1998). The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application (Jamshidi et al., 2008). This gene is recognized as an international standard for detection of *Salmonella* genus (Malorny et al., 2003). The *sefA* on the other hand, is a good candidate for specific detection of *S. enteritidis* (Pan et al., 2002; Woodward et al., 1996).

Strains of *S. enterica* serovar enteritidis often carry serovar-associated plasmids which encode a virulence operon consisting of five genes *spvR*, *spvA*, *spvB*, *spvC* and *spvD* (Asten et al., 2005; Aabo et al., 1999). The *spv* genes play a role in the virulence of the host strain (Chu et al., 1999; Saule et al., 1997). It is possible that virulence plasmid is sequentially or independently formed by recombination and hybridization (Hong et al., 2008; Del Cerro et al., 2003). The integration of resistance genes and additional replicons into a *Salmonella* virulence plasmid constitutes a new and interesting example of plasmid evolution posing a serious threat to public health. These genes can be horizontally transferred and mobilized by an F or F-like conjugative plasmid between the *Salmonella* strains and species (Hong et al., 2008; Chu et al., 2006). One main function of the *spv* operon is to potentiate the systemic spread of the pathogen (Heithoff et al., 2008). This potential is associated with multidrug-resistance with *spv* operon

which has been demonstrated in *Salmonella* strains (Chu et al., 2006). Some studies have provided evidence that the virulence plasmid plays a significant role in human disease (Guiney et al., 1994; Chu et al., 1996). Detection of these *spv* genes allows us to decide whether the pathogenesis of the isolates from positive clinical samples is attributable to chromosome or plasmid born virulence factor (Trafny et al., 2006).

The present study has three aims. Firstly, it aims at determining whether *invA* (invasion gene of the genus *Salmonella*) is specific for identification of *Salmonella* genus. It also intends to determine if genes *sefA* (fimbrial antigen of *S. enteritidis*) and *spv* (S1-S4 primers) are specific for detection of *S. enteritidis* serovars. Secondly, the study tends to assess the occurrence of *Salmonella* spp. and *S. enteritidis* in a chicken slaughterhouse in Kerman, Iran by multiplex PCR. This assay will then be compared with conventional culture and biochemical methods. The third and more important aim of the present study is detection and determining of the distribution of *spvA*, *spvB* and *spvC* genes in *S. enteritidis* isolates from poultry, bovine and human sources. This is the first report of the prevalence of these genes in Iran.

MATERIALS AND METHODS

Samples and bacterial strains

A total of 1001 poultry samples including feces, liver, spleen, caecal content, and bile (feces dominant) were collected from two poultry slaughterhouses located in Kerman, Iran. Two days a week for a period of nine months starting from January (2009) to September (2009), 15 samples were randomly obtained from 15 animals.

In addition, 33 isolates of *S. enteritidis* lyophilized from human (n = 20) and bovine (n = 13) sources were obtained from the culture collection in the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Iran. The positive control *S. enteritidis* isolate and negative control *Escherichia coli* ATCC 35218 or *Klebsiella pneumoniae* were also obtained from the culture collection in the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Iran.

Microbiological methods

The samples, collected twice a week (15 samples in the beginning and 15 samples in the middle of the week), were immediately placed into sterile polyethylene bags. To determine the presence of *Salmonella* spp. in the samples, conventional culture method was used. The feces, caecal content, and bile samples were directly inoculated into Rappaport-Vasisiliadis (RV) broth (Merck, Germany) and Selenite-Cystein broth (Merck, Germany) media. The ratio of the sample volume to that of the medium were 0.01:0.1 for RV broth and Selenite-Cystein broth respectively. Other samples (liver and spleen) were analyzed for *Salmonella* according to ISO 6579 (25 g of each sample), and then were placed in sterile stomacher bags. 225 mL of buffered peptone water (BPW, Merck) was added in each sample. Each sample was then homogenized (Germany), Rambach agar (Hi-Media, India), and Hi-Chrome 37°C. Then 0.1 and 1 mL, respectively of the pre-enriched broth were simultaneously transferred into 10 mL of RV and 10 mL of

Table 1. Nucleotide sequence and primers used for identification of *S. enteritidis* by multiplex PCR (Pan et al., 2002).

Primer	Target gene	Sequence	Amplified fragment size (bp)
ST11 ST14	Random ^a Sequence	5'-GCCAACCATTGCTAAATTGGCGCA 5'-GGTAGAAATTCACGCGGGTACTGG	429
SEFA2 SEFA4	<i>sefA</i> ^b	5'-GCCGTACACGAGCTTATAGA 5'-ACCTACAGGGGCACAATAAC	310
S1 S4	<i>spv</i> ^c	5'-GCCGTACACGAGCTTATAGA 5'-ACCTACAGGGGCACAATAAC	250

a. Randomly cloned sequence specific for the genus *Salmonella*, b. *S. enteritidis* fimbrial antigen gene (specific for the *S. enteritidis*) and c. *Salmonella* plasmid virulent gene.

Table 2. Nucleotide sequence used as primers in the multiplex PCR *invA+spvC* genes and simple-PCR *spvA*, *spvB* genes in *S. enteritidis*.

Name of primer	Gene	Sequence	Length (bp)	Reference
Multiplex <i>invA</i> and <i>spvC</i>	<i>invA+ spvC</i>	f 5'-ACAGTGCTCGTTTACGACCTGAAT-3' r 5'-AGACGACTGGTACTGATCTAT-3'	244	Chiu et al. (1996)
		f 5'-GTCCTTGCTCGTTTACGACCTGAAT-3' r 5'-TCTCTTCTGCATTTTGTCA-3'	571	Chiu et al. (1996)
Simple- <i>spvA</i> -f/B	<i>spvA</i>	f 5'-GTCAGACCCGTAACAGT-3' r 5'-GCACGCAGAGTACCCGCA-3'	604	Del Cerro et al. (2003)
Simple- <i>spvB</i> -f/B	<i>spvB</i>	f 5'-ACGCCTCAGCGATCCGCA-3' r 5'-GTACAACATCTCCGAGTA-3'	1063	Del Cerro et al. (2003)

Selenite-Cystein broth respectively for overnight enrichment at *Salmonella* agar (Hi-Media, India). Suspicious colonies were identified with biochemical tests (IMVIC, TSI, SIM, Urease, Phenylalanine deaminase, and Ornithine decarboxylase). The isolates identified as *Salmonella* were serotyped by slide agglutination tests. Serogroup D₁ was serotyped with specific O and H antisera (*S. enteritidis*) and the other serogroups were serotyped with only O antisera (Difco Detroit, MI, USA).

Bacterial growth

Lyophilized or recently isolated strains, after one-night at 37°C incubation in 2 mL brain-heart infusion broth (BHI, Difco, Detroit, MI, USA), were transferred to Luria-Bertani (LB) agar (Difco, France) for one- night at 37°C to isolate single colony.

DNA preparation

Three colonies of each isolate on agar plate were picked and suspended in 200 µl of distilled H₂O. After vortexing, the suspension was boiled for 10 min, and 50 µl of the supernate was 37°C. The enrichment samples for primary diagnosis were then applied on to Xylose-Lysine-Sodium-Deoxycholate agar (Merck, using a stomacher for 2 min, followed by incubation for 24 h at collected after spinning for 10 min at 14,000 rpm in a microcentrifuge (Madadgar et al., 2008).

DNA primers

In the first panel of multiplex PCR assay for identification of *S. enteritidis* three set OF primers were selected: ST11-ST14 (429 bp), SEFA2-SEFA4 (310 bp), and S1-S4 (250 bp). In the second panel of multiplex PCR assay, two set primers were selected: for *invA* gene (244 bp), which is specific to *Salmonella* genus, and for *spvC* gene (571 bp) in *S. enteritidis* (Chiu et al., 1996). Moreover, simple-PCR with a pair of primer for *spvA* gene (604 bp) and a pair of primer for *spvB* gene (1063 bp) in *S. enteritidis* were selected (Del Cerro et al., 2003). The primers sequences and their corresponding genes are shown in Tables 1 and 2.

DNA amplification

Multiplex PCR was performed in a reaction of 25 µl containing reaction buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH = 8.3) (CinaGen, Iran), 2 µl of DNA sample, 200 µM dNTPs, 1 U *Taq* polymerase (CinaGen, Iran) and 1 µM of each primer (CinaGen, Iran). The multiplex PCR amplification program for *S. enteritidis* confirmation was similar to the protocol by Pan et al. (2002). On the other hand, the multiplex PCR program for *invA+spvC* genes conditions were 1 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, 2 min at 72°C and final extension 10 min at 72°C. The PCR program for *spvA* and *spvB* conditions were 5 min at 94°C followed by 30 s at 94°C, 30 s at 60°C, 1 min 72°C and final extension 5 min at 72°C.

Table 3. The results of serotyping evaluation and identification of *S. enteritidis* by multiplex PCR in poultry samples

Total (serogroup)	<i>Salmonella</i> spp. with one bond: ST11-ST14 (429 bp)	<i>Salmonella</i> spp. with two bonds: ST11-ST14 (429 bp), S1-S4 (250 bp)	<i>Salmonella enteritidis</i> with two bonds: ST11-ST14 (429 bp), SEFA2-SEFA4 (310 bp)	<i>Salmonella enteritidis</i> with three bonds: ST11-ST14 (429bp), SEFA2-SEFA4 (310 bp), S1-S4 (250 bp)	Serogroup
35/68 (51.4%)	-	-	4/68 (5.9%)	31/68 (45.6%)	D ₁ (<i>S. enteritidis</i>)
19/68 (27.9%)	18/68 (26.4%)	1/68 (0.68%)	-	-	C ₁ and C ₄ (<i>Salmonella</i> spp.)
7/68 (10.2%)	7/68 (10.2%)	-	-	-	A ₁ (<i>Salmonella</i> spp.)
7/68 (10.2%)	7/68 (10.2%)	-	-	-	E (<i>Salmonella</i> spp.)
68 (100%)	31/68 (45.6%)	4/68 (5.9%)	1/68 (0.68%)	32/68 (47%)	Total (Multiplex PCR)

The PCR product was electrophoresed in 1.2% agarosis gel (Fermentas) and afterward stained with ethidium bromide and visualized by UV light illumination (Bio- rad, Molecular Imager, Gel Doc™, XR Imaging system, USA).

RESULTS

Panel 1

Detection of *S. enteritidis* by culture and serotyping

Sixty-eight out of the 1001 poultry samples (6.79%) were culture positive for *Salmonella* spp. Serotyping evaluation showed that thirty-five out of sixty-eight samples (51.4%) were serogroup D₁ (*S. enteritidis*) with O and H antisera. Moreover, nineteen out of sixty-eight samples (27.9%) were serogroups C₁ and C₄. Seven out of sixty eight samples (10.2%) were serogroup E. Also, seven out of sixty eight samples (10.2%) were serogroup A₁. Finally, no serogroup B was observed in the study (Table 3)

Identification of *S. enteritidis* by multiplex PCR

Multiplex PCR assay was applied to confirm

S. enteritidis in sixty-eight poultry samples which were confirmed to be *Salmonella* positive through culture and serotyping method. Results showed that thirty-one out of sixty-eight samples (45.6%) were positive for *S. enteritidis* with three bands (ST11-ST14, SEFA2-SEFA4, and S1-S4) amplifying the expected 429 310 and 250 bp fragments respectively. Four out of sixty-eight samples (5.9%) were positive *S. enteritidis* with two bands (ST11-ST14 and SEFA2-SEFA4). One sample (0.68%) was *Salmonella* spp. with two bands (ST11-ST14 and S1-S4). Finally, thirty-two out of sixty-eight samples (47%) were *Salmonella* spp. with only one band ST11-ST14 (Table 3 and Figure 1).

Panel 2

Detection of *spvA*, *spvB* and *invA+spvC* genes in *S. enteritidis*

Simple-PCR to detect virulence gene *spvA* and *spvB* with one pair primer and multiplex PCR to detect both *invA* and *spvC* genes in the samples yielded the following results:

Poultry isolated *S. enteritidis*

The study showed that *spvA*, and *spvB*, genes

were present in 88.6% (31/35) of the samples respectively (Figure 2). In 88.6% of the samples (31/35) *spvC* and *invA* were present. In the same samples (without *spvC*) *invA* genes were present in 11.4% of the samples (4/35), (Table 4 and Figure 3).

Human isolated *S. enteritidis*

The study showed that *spvA*, and *spvB*, genes were present in 90% of the samples (18/20). Similarly, *spvC* and *invA* were present in 90% of the samples (18/20). In the same samples (without *spvC*) *invA* genes were present in 10% (2/20), (Table 4).

Bovine isolated *S. enteritidis*

As Table 2 shows, positive band appears for *spvA*, *spvB* and *invA + spvC* genes in 100% (13/13) of the all isolates (Figure 3).

DISCUSSION

The endemic prevalence of *S. enteritidis*

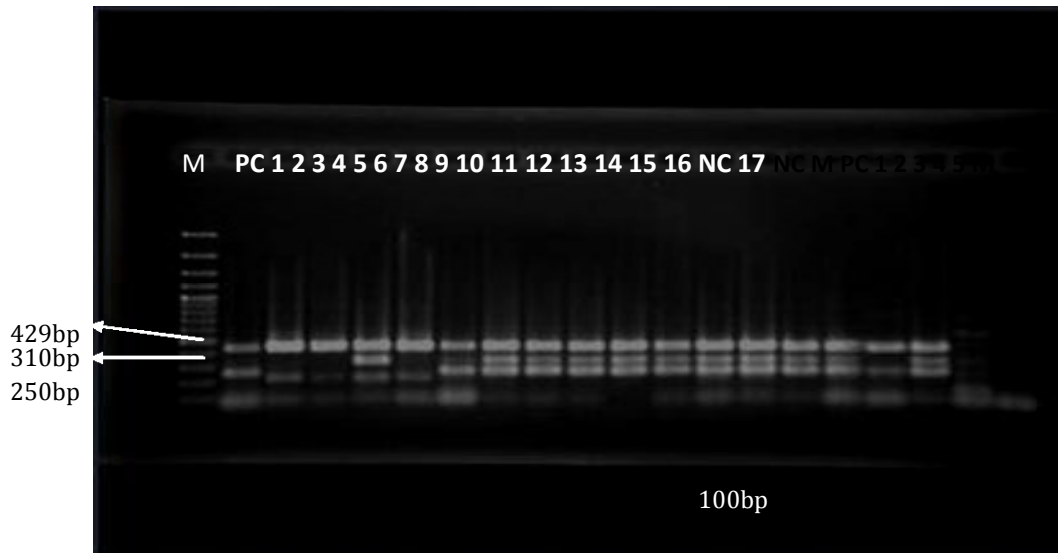


Figure 1. Multiplex PCR with three pairs of primers for detected *S. enteritidis* isolated (poultry source); M: marker 100 bp; PC: positive control; NC: negative control (*E. coli*); lane 17: Product without the DNA template; lane 1, 2, 4, 15: *Salmonella* spp. and other lane for positive *S. enteritidis*.



Figure 2. Simple- PCR with one pair of primer for *spvA* gene (604 bp) in *S. enteritidis* (poultry source) ; M: 100 bp marker; lane PC: positive control; lane NC: negative control (PCR product without the DNA); lanes 5, 6, 8, 10: negative *spvA* gene; other lanes: positive *spvA* gene.

Table 4. Distribution of *spvA*, *spvB*, *invA* + *spvC* genes in *S. enteritidis*.

<i>invA</i> + <i>spvC</i>		<i>spvB</i> (+) (%)	<i>spvA</i> (+) (%)	Total	Source	Serogroup	Serotype
<i>spvC</i> (+) <i>invA</i> (+) (%)	<i>spvC</i> (-) <i>invA</i> (+) (%)						
88.6 (31/35)	11.4 (4/35)	88.6 (31/35)	88.6 (31/35)	35	Poultry	D ₁	<i>S. enteritidis</i>
90 (18/20)	10 (2/20)	90 (18/20)	90 (18/20)	20	Human	D ₁	<i>S. enteritidis</i>
100 (13/13)	0 (0/13)	100 (13/13)	100 (13/13)	13	Bovine	D ₁	<i>S. enteritidis</i>

(+) – positive; (-) – negative.

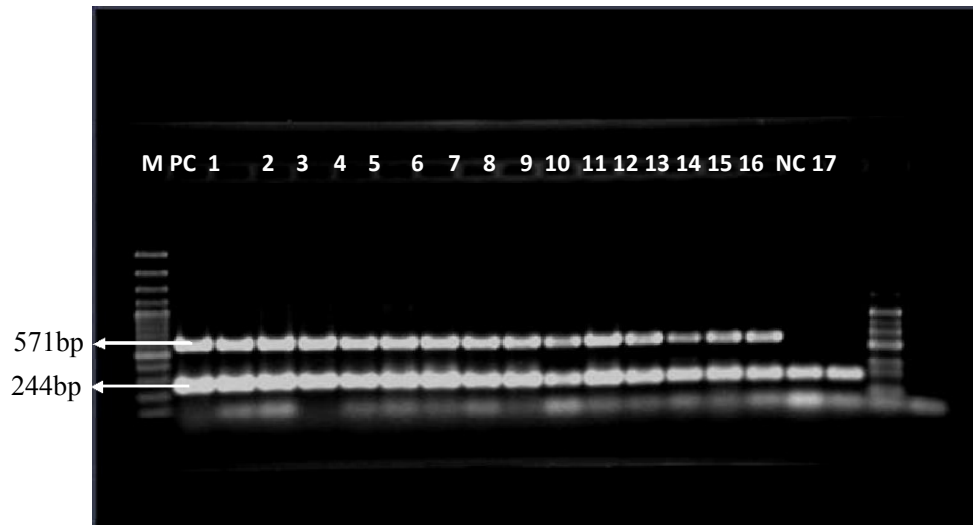


Figure 3. Multiplex PCR with two pairs of primers for *invA* (244bp), *spvC* (571 bp) virulence genes in *S. enteritidis* (bovine, and poultry source); lane M: 100 bp marker; lane PC: positive control; lane NC: negative control (*Klebsiella pneumoniae*); lane 17: PCR product without the DNA template; lanes 15, 16: *S. enteritidis* (positive *invA* and negative *spvC* genes); other lanes: *S. enteritidis* (positive *invA* and *spvC* genes).

in intensive livestock production presents explicit public health risks in addition to food industry losses. Multiplex PCR provides a rapid means to monitor specific microorganisms in a variety of samples. This assay is an epidemiologically useful tool to distinguish serovar enteritidis. In previously reported studies where S1-S4 primers were chosen to detect the *spv* gene of a virulent plasmid of *S. enteritidis* through multiplex PCR assay; this gene was not confirmed in all isolates (Woodward et al., 1999). This is also confirmed by the results obtained in the present study (Figure 1).

Wood et al. (1994) found that this gene is only present in 30% of *S. enteritidis* strains isolated from poultry. The present study yielded different and unexpected results. The *spv* genes were not detected in 5.9% of isolates of the *S. enteritidis* as compared with 7.4% of the isolates reported by Pan et al. (2002) and 70% of the isolates reported by Wood et al. (1994). The study suggests that *spv* gene for *S. enteritidis* is on the increase in recent years.

Previous studies suggest that selection of S1-S4 primers for the multiplex PCR as marker for the presence of serovar enteritidis is not a suitable candidate as they were not detected in some *S. enteritidis* serovars (Pan et al., 2002; Wood et al., 1994). In order for the presence of strains of *S. enteritidis* to be confirmed, a new pair of primers is needed. The set of primer suggested in a number of studies is *S. enteritidis* fimbrial antigen (SEFA) (Pan et al., 2002). Agron et al. (2001) found *sef* gene in other *Salmonella* serovars non-enteritidis. Consequently, they suggested that *sef* gene is not specific for detection of *Salmonella* serovar enteritidis and proposed a novel *S. enterica* serovar enteritidis locus that serves as a marker

for DNA-based identification of *S. enteritidis*. While these researchers suggest that *Salmonella* difference fragment (Sdf I - 333 bp) primer is a highly specific marker for *Salmonella* serovar enteritidis and yield's clear results in laboratory testing, this fragment (Sdf I) cannot detect clearly infectious *S. enteritidis* isolates (Agron et al., 2001). On the other hand, results obtained from other researchers suggest that *sef* gene is a robust marker for detection of *Salmonella* serovar enteritidis (Soumet et al., 1999; Pan et al., 2002; Malkawi et al., 2004). This gene was detected in all of the isolated *S. enteritidis* in the present study (Figure 1).

Invasion gene operon, *invA* was detected in all *Salmonella* spp. isolates in our study. This gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for invasion of deeper tissue (khan et al., 1999). There are studies reporting the detection of this gene in all *Salmonella* spp. isolates (Zahreai et al., 2006; Nashwa et al., 2009; Trafny et al., 2006; Jamshidi et al., 2008). Oliveira et al. (2003) reported that PCR assay using the *invA* primers specific for *Salmonella* spp. considerably decreases the number of false-negative results which commonly occur in diagnostic laboratories. Amplification of *invA* is now recognized as an international standard procedure for detection of *Salmonella* genus (Malorny et al., 2003). This increases the value of the present research because of virulence properties and clinical importance of *invA* gene. According to the results of this study, PCR method based on *invA* gene is useful for rapid identification of *Salmonella* serovares.

In a study of poultry samples from among a total of 288

samples, 52 samples (18%) were positive for *Salmonella* spp. by culture method (Cortez et al., 2006). From these, 5.6 and 2.4% were positive for *S. enteritidis* and *S. typhimurium*, respectively by multiplex PCR assay. Another study shows that from among a total of 93 samples collected from poultry carcass, 19/93 (20%) were detected as *S. enteritidis* (Malkawi et al., 2004). Yet in another study in U.S.A, prevalence of *Salmonella* in poultry was reported as 25 to 29% (Harrison et al., 2001). Agron et al. (2001) using three pair primers Sdf I, Sdf II, and Sdf III by S.S.H (suppression subtractive hybridization), which is a PCR-based technique, identified 81 *S. enterica* isolates with various serovars. They detected and amplified Sdf II and Sdf III in a few *S. enterica* serovars. They could detect and amplify Sdf I in only one pathogenic *Salmonella* serovar *enteritidis* (Agron et al., 2001). In a 1996 report from Italy, the prevalence of food-borne *Salmonella* was reported 81% from which, 50 samples were *S. enteritidis* and only 3 samples were *S. typhimurium* (Scuderi et al., 1996).

According to the results of the present study, 6.8% of the samples were *Salmonella* serovars. This may suggest a drop in the incidence of *Salmonella* as compared with the previous years. There is also the possibility of cross-contamination of products, differences in sample origin, detection methods, sampling procedure, and level of processing in the previous studies (Brayan and Doyle., 1995).

From among 68 poultry source *Salmonella* spp. isolates, no *S. typhimurium* serovar was detected after serotyping and multiplex PCR. This is similar to a study by Soltan et al. (2010) who did not detect *S. typhimurium* in 22 *Salmonella* spp. isolates taken out of 1950 fecal samples from diarrheic children in Tehran, Iran. This does not suggest that *S. typhimurium* is an insignificant pathogen. The pathogen is in fact detected in a number of epidemics worldwide. In Iran, Yousefi Mashoof et al. (2005) detected *S. typhimurium* in 20% of 100 *Salmonella* spp. isolates from human source. The detected *S. typhimurium* from poultry source by Jafari et al. (2005) was 8.5% in Ahwaz, Iran. In their studies in Iran, Zahraei et al. (2006) detected this pathogen in 66% of a total of 33 *Salmonella* spp. detected from 400 bovine fecal samples. Soltan et al. (2008) detected 9.1% *S. typhimurium* in 195 raw beef samples in Tehran. Namimatso et al. (2005) in a report about the prevalence of *S. typhimurium* from porcine source in Japan detected this pathogen in 35.8% of a total of 106 porcine isolates. Hughs et al. (2007) detected this pathogen in 90.6% of 32 *S. enterica* isolates from wild birds in northern England.

Operon *spvR*, *spvA*, *spvB*, *spvC*, *spvD* (7.8 kb) in virulence plasmid (*S. enteritidis*, 60 kb) which are present in a few serovars of subspecies of *S. enterica* are responsible for the systemic infection and multidrug-resistance in both human and animals (Boyed et al., 1998; Rotger et al., 1999; Chiu et al., 2006; Gebreyes et al., 2009). They are also responsible for the induction of

intracellular bacterial proliferation and apoptosis of infected macrophages (Kurita et al., 2003; Heithoff et al., 2008). The carriage of *spv* gene may increase the propensity of *Salmonella* strains to be of major clinical relevance (Gebreyes et al., 2009). Heithof et al. (2008) showed that *Salmonella* serovar *typhimurium* isolates driven from human gastroenteritis patients often lose the *spv* gene and, accordingly, lack the capacity to cause systemic disease in mice. In the present study, presence of *spvA*, *spvB*, and *spvC* genes in *S. enteritidis* from human source was 90%. *SpvA*, *spvB*, and *spvC* genes were present in 100% of the bovine source isolates. In the case of *S. enteritidis* in poultry source, presence of *spvA*, *spvB*, and *spvC* was 88.6%. Regarding the presence of virulent plasmid genes in *S. enteritidis*, another study has reported lack of *spvC* gene in 8/110 (7.2%) of the samples from human, pig, and poultry sources (Castilla et al., 2006). Chiu et al. (1996) analyzing 38 isolates from *Salmonella* serovars with two primers *invA* and *spvC* reported the presence of *invA* in all strains 100% (38/38). The same study reports that in only 21 strains 21/38 (55.26%) there were *spvC* together with *invA*. A study reported in 2000 has shown that from among a total of 17 isolates of *S. enteritidis* and *S. typhimurium*, all samples possess *invA* and *spvC* together (Jenikova et al., 2000). Ling et al. (2009) analyzed 152 isolates of *S. enteritidis* from human feces in which only 4 isolates (8%) lacked *spvC* and possessed *invA*. Del Cerro et al. (2003) reported that of 56 *S. enteritidis* samples from human feces only 2 isolate lacked *spvA*, *spvB*, and *spvC*. This last study is also in line with the present study. Finally, the only report in Iran about detection of *spvR* gene has confirmed that the gene was present in 100% (16/16) of the *Salmonella* serovars samples (Nikbakht et al., 2004). The present study shows a remarkably high distribution for *spvA*, *spvB*, and *spvC* in poultry, human, and bovine sources (88 to 100%).

There are some discrepancies about distribution of virulence plasmid of various *Salmonella* spp. serovars between samples from human and animal origins. In some studies results show a higher distribution for the virulence plasmid from animal-origin isolates than that of human-origin (Del Cerro et al., 2003). The findings of the present study show a higher distribution of *spvA*, *spvB*, and *spvC* genes in bovine sources and lower distribution in poultry sources compared with human-origin sources. Drastic genetic variations in *Salmonella* could be derived from transfer of this organism between human-origin and animal-origin strains (Chiu et al., 2006). Whether this can transfer virulence plasmid from animal-origin strains to human-origin strains or vice versa remains to be investigated. Strains of *Salmonella* bacterium (Particularly *typhimurium* and *enteritidis* serovars) which carry virulence plasmid can cause systemic disease, while plasmidless strains can cause local or asymptomatic disease (Heitoff et al., 2008).

The findings of this study indicates that currently

prevalence of *S. enteritidis* in Iran is more than that of *S. typhimurium* while the trend was opposite in the earlier studies (Zahraei et al., 2008; Yoosefi Mashoof et al., 2005). This study also shows that poultry carcasses have potential for salmonellosis. Food hygiene necessitates more control on *Salmonella* as this contributes to safe food production and can help improve public health. This is why a more reliable and rapid method for the detection of *Salmonella* is needed in epidemiological studies and monitoring of *S. enteritidis* in Iran. This study confirms that compared with bacteriological culture method, multiplex PCR is remarkably faster saving precious time to control *S. enteritidis* which is the predominant serotype in Iran. Multiplex PCR provides us with reliable, rapid, specific, and reproducible results about the status of the sample and detection of certain microorganisms in large-scale epidemiological studies involving several laboratories (Ebner et al., 2001; Chiu et al., 2006).

Multiplex PCR assay carried out in this study suggest that set primers ST11-ST14 and *invA* are specific for detection of *Salmonella* spp. Also set primers SEFA2-SEFA4 are specific for detection of *S. enteritidis* serovar. However, the study shows that presence of *spv* gene (with set primers S1-S4) is not specific for detection of *S. enteritidis* as it is seen in other *Salmonella* non-enteritidis serovars. This is contrary to an earlier report by Pan et al. (2002) who considered presence of set primers S1-S4 specific for detection of *S. enteritidis*.

In conclusion, epidemiological survey, identification of *S. enteritidis*, and screening of *spv* gene through PCR-based procedures can have major benefit in public health specifically for rapid diagnosis, etiology, epidemiological investigations, ideal vaccine, development of treatment, and prophylactic strategies for salmonellosis in Iran. This is the first study on the distribution of genotypes of *spvA*, *spvB*, *invA+spvC* genes in isolates from poultry, human and bovine sources in the country.

ACKNOWLEDGEMENTS

This work was supported by a grant from Faculty of Specialized Veterinary Science, Islamic Azad University, Science and Research Branch, Tehran, Iran. The authors wish to thank Professor I. Sohrabi Haqdoost, Mr. M. Abedi, Mr. A. Mokhtary, and Mr. A. H. Jangjou. Special thanks are given to Mr. Mohammad Reza Masrouf for his considerable support during the preparation of the final draft of the paper.

REFERENCES

- Aabo S, Rasmussen F, Rossen, Sorensen LPD, Olsen, JE (1993). *Salmonella* Identification by the polymerase chain reaction. *Molecular and Cellular Probs.*, 7: 171-178.
- Aabo S, Brown D J, Olsen E J (2000). Virulence characterization of a strain of *Salmonella enterica* subspecies houten with chromosomal integrated *Salmonella* plasmid virulence (*spv*) genes. *Res. Microbiol.*, 151: 183-189.
- Agron GP, Walker RL, Kind H, Sawyer SJ, Hayes DC, Wollard J, Andersen GL (2001). Identification by subtractive hybridization of sequences specific for *Salmonella* serovar Enteritidis. *Appl. Environ. Microbiol.*, 67(11): 4984-4991.
- Amavisit P, Browing GF, Lightfoot D, Anderson CS (2001). Rapid PCR detection of *Salmonella* in horse faecal samples. *Vet. Microbiol.*, 79: 63-74.
- Asten AJ, Dijk JE (2005). Distribution of "Classic" virulence factors *Salmonella* serovars. *fems Immunol. Med. Microbiol.*, 44: 251- 259.
- Boyed EF, Hartl DL (1998). *Salmonella* virulence plasmid Modular Acquisition of the *spv* virulence Region by an F-Plasmid in *Salmonella enterica* subspecies I and Insertion into the chromosome of subspecies II, III a, IV and VII Isolate. *Gen. Soc. Am.*, 149: 1183-1190.
- Bozorgmehri (1992). An introduction of contamination rate of *Salmonella* Enteritidis in broiler chicken from of Tehran. *Int. J. Fac. Vet. Med. Univ. Tehran*, 47(1): 70-76.
- Brayan FL, Doyle MP (1995). Health risk and consequences of *Salmonella* and *Campylobacter* Jeujeni in raw poultry. *J. Food Protect.*, 58: 326-344.
- Castilla KS, Ferreira CSA, Moreno AM, Nunes IA, Ferreira AJP (2006). Distribution of virulence genes *sefC*, *pefA* and *spvC* in *Salmonella* Enteritidis phage type 4 strains isolated in Brazil. *Br. J. Microbiol.*, 37(2): 1-7.
- Chiu CH, Ou JT (1996). Rapid identification of *Salmonella* serovars in faeces by specific detection of virulence genes *invA*, and *spvC*, by an enrichment broth culture-Multiplex PCR combination assay. *J. Clin. Microbiol.*, 96: 2619-2622.
- Chiu CH, Su LH, Chu CH, Wang MH, Yeh CM, Weill FX, Chu C (2006). Detection of Multidrug-Resistant *Salmonella enterica* serovar Typhimurium phage types DT102, DT 104 and U302 by Multiplex PCR. *J. Clin. Microbiol.*, 10: 2354-2355.
- Chu C, Hong SF, Tsai C, Lin WS, Liu TP, Ou J (1999). Comparative physical and genetic maps of the virulence plasmids of *Salmonella enterica* serovar Typhimurium, Enteritidis, Choleraesuis and Dublin. *Infect. Immun.*, 99: 2611- 2614.
- Chu C, Chiu CH (2006). Evolutions of the Virulence Plasmid of non-typhoid *Salmonella* and its association with antimicrobial resistance. *Microb. Infect.*, 8: 1931-1935.
- Cortez AL, Carvalho AC, Ikuno AA, Burger KP, Idal-Marthins AM (2006). Identification of *Salmonella serovars* Isolate from chicken abattoirs by Multiplex PCR. *Res. Vet. Sci.*, 81: 340-344.
- Del Cerro A, Soto SM, Mendoza MC (2003). Virulence and antimicrobial-resistance gene Profiles determined by PCR-Based Procedures for *Salmonella* isolated from samples of animal origin. *Food Microbiol.*, 20: 431-436.
- Ebner PD, Mathew AG (2001). Three molecular methods to identify *Salmonella enterica* serotype Typhimurium DT104: PCR fingerprinting multiplex PCR and rapid PFGE. *FEMS Microbiol. Lett.*, 205: 25-29.
- Echeita MA, Herrera S, Garaizar J, Usera MA (2002). Multiplex PCR-based detection an identification of the most common *Salmonella* second-Phase flagellar antigen. *Res. Microbiol.*, 153: 107-113.
- Fardsanaei F, Soltan Dallal M, Eeshraqi SS, Zahraei T, Ranjbar R, Akbari A, Abdosamadi Z, Amin Harati F, Nikmanesh FB (2010). Study of *Salmonella* Enteritidis prevalence in patient referred to TUMS hospitals. *10TH Iranian congers of Microbiology*, 42: 37-38.
- Feder I, Nietfeld JC, Gallan J, Yeary T, Sargeant JM, Oberst R, Tamplin ML, Luchansky JB (2001). Comparison of cultivation and PCR hybridization for detection of *Salmonella* in porcine faecal and water sample. *J. Clin. Microbiol.*, 39: 2477-2484.
- Gebreyes WA, Thakur S, Dorr P, Tadesse DA, Post K, Wolf L (2009). Occurrence of *spvA* virulence Gene and clinical significance for Multidrug-Resistant *Salmonella* strains. *J. Clin. Microbiol.*, 10: 777-780.
- Guiney D, Fang F, Krause, Libby S (1994). Plasmid mediated virulence genes in non-typhoid *Salmonella* serovares. *FEEMS Microbiol. Lett.*, 124: 1-9.
- Harrison AW, Griffith CJ, Tennant D, Peters AC (2001). Incidence of *Campylobacter* and *Salmonella* isolated from retail chicken and associated packing in south wale's. *Lett. Appl. Microbiol.*,

33: 450-454.

- Heithoff DM, Shimp WR, Iau PW, Badie G, Enioutina EY, Daynes, Barbara RA, Byrne A, House J, Mahan MJ (2008). Human *Salmonella* Clinical Isolates Distinct from Those of Animal origin. *Appl. Environ. Microbiol.*, 10: 1757-1766.
- Hong SF, Chiu H, Chu C, Feng C, Ou TJ (2008). Complete nucleotide sequence of a virulence plasmid of *Salmonella enterica* serovar Dublin and its phylogenetic relationship to the virulence plasmids of serovars Choleraesuis, Enteritidis, Typhimurium. *FEMS Microbiol.*, 282: 39-43.
- Hughes AL, Shopland S, Wigley Paul, Bardon H, Leatherbarrow LA, William N, Bennett M, Pinna E, Lawson B (2008). Characterisation of *Salmonella enterica* serovar Typhimurium isolates from wild bird in northern England from 2005-2006. *BMC Vet. Res.*, 4: 4.
- Jafari A, Ghorbanpour M, Jaidrei A (2005). A survey of the *Salmonella* Infection in backyard chickens in Iran. *Int. J. Ahvaz Vet. Microbiol.*, (35): 145.
- Jamshidi A, Bassami MR, Afshari-Nic S (2008). Identification of *Salmonella* serovars Typhimurium by a multiplex PCR-Based assay from Poultry carcasses in Mashhad-Iran. *Int. J. Vet. Res.*, 3(1): 43-48.
- Jenikova G, Pazlarova J, Demnerova K (2000). Detection of *Salmonella* in food sample by the combination of immunomagnetic separation and PCR assay. *Int. Microbiol.*, 3: 225-229.
- Karimi A, Ranjbar R, Ahmadi Z, Safiri Z (2007). Rapid Detection of Different Serovars of *Salmonella enterica* by Multiplex PCR. *Iranian J. Publ. Health*, 36(2): 38-42.
- Khan AA, Nawaz MS, Khan S, Sernigelia CE (1999). Detection of Multidrug Resistant *Salmonella* Typhimurium DT104 by Multiplex polymerase chain reaction. *FEEMS Microbiol. Lett.*, 182: 355-360.
- Kurita AI, Gotoh H, Eguchi M, Okada N, Matsuura S, Matsui H, Danbara H, Kikuchi Y (2003). Intracellular expression of the *Salmonella* plasmid virulence protein, SPVB, causes apoptotic cell death in eukaryotic Cells. *Microb. Pathol.*, 35: 43-48.
- Lim YH, Hirose K, Izumiya H, Arakawa, Shi HT, Terajima J, Itoh K, Tamura K, Kim S, Watanabe H (2003). Multiplex Polymerase chain Reaction Assay for selective of *Salmonella enterica* serovar Typhimurium. *Jpn. J. Infect. Dis.*, 56: 151-155.
- Ling JML (2009). Rapid detection of food-borne pathogens in clinical specimens, food and environmental samples Hong Kong Med. J., 15(1): 26-29.
- Madadgar O, Zahraei Salehi T, Tadjbakhsh H, Mahzounieh M, Feizabadi M (2008). Genomic and Phenotypic evaluation of *Salmonella* Typhimurium and *Salmonella* Enteritidis in Iran. *Com. Clin. Pathol.*, 17: 2298-235.
- Malkawi HI, Gharaibeh R (2004). Rapid and simultaneous Identification of two *Salmonella enterica* serotypes Enteritidis and Typhimurium from chicken and meat product by multiplex PCR. *Biotechnol.*, 3(1): 44-48.
- Namimatsu T, Asai T, Osumi T, Imai Y, Soto S (2006). Prevalence of the virulence plasmid in *Salmonella* Typhimurium isolates from pigs. *J. Vet. Med. Sci.*, 68(2): 187-188.
- Nashwa HM, Mahmoud AH, Sami, Adawy S (2009). Application of Multiplex Polymerase Chain Reaction (M-PCR) for Identification and Characterization *Salmonella enteritidis* and *Salmonella typhimurium*, 5(12): 2343-2348.
- Nikbakht G, Tadjbakhsh H (2004). Study of plasmid virulence genes (*spvR*) in *Salmonella enterica* serovars isolated in Iran. *J. Fac. Vet. Med. Univ. Tehran*, 59 (2): 137-140.
- Nori EEM, Thong KL (2010). Differentiation of *Salmonella enterica* based on PCR detection of selected somatic and flagellar antigen. *Afr. J. Microbiol. Res.*, 4(9): 871-879.
- Oliveira SD, Rodenbusch CR, Ce MC, Rocha SLS, Canal CWI (2003). Evaluation of selective and non-selective enrichment PCR. *Lett. Appl. Microbiol.*, 36(4): 217-221.
- Pan TM, Iiu YJ (2002). Identification of *Salmonella* Enteritidis isolate by polymerase chain reaction and multiplex polymerase chain reaction. *J. Microbiol. Immunol. Infect.*, 35: 147-151.
- Ranjbar R, Giammanco GM, Aleo A, Plano MR, Naghoni A, Owlia P, Mammia C. (2010). Characterization of the first extended – spectrum beta lactamase – producing non-typhoidal *Salmonella* serovar isolated in Tehran, Iran. *Foodborne Pathog. Dis.*, 7(1): 91-95.
- Rotger R, Casadesus J (1999). The virulence plasmids of *Salmonella*. *Internal Microbiol.*, 2: 177-184.
- Saul V R, Schaeffer F, Kowarz L, Norel F (1997). Relationships between H-NS, SPVR and growth phase in the control of *spvR*, the regulatory gene of the *Salmonella* plasmid virulence operon. *Mol. Gen. Genet.*, 25b: 333-347.
- Scuderi G, Fantasia M, Filetici E, Anastasio MP (1996). Food-borne outbreaks caused by *Salmonella* in Italy, 1991-1996. *Epidemiol. Infectol.*, 116: 257-265.
- Shakerian A, Sarofzade A, Rahimi E, Kargar Y (2010). *Salmonella* serotype as a potential pathogen in poultry and cow carcasses slaughtered in Isfahan-Iran. 10TH Iranian congress of Microbiology. 42: 333.
- Soltan DM, Fardsanaei F, Eesraqi SS, Zahraei T, Ranjbar R, Akbari A, Abdosamadi Z, Amin harati F, Nikmanesh B (2009). The antimicrobial resistance pattern among *Salmonella* Enteritidis isolate from 1950 diarrhoeic children in Iran. *J. Med. Univ. Tehran*, 67(12): 35-48.
- Soltan Dallal M, Vahedi S, Zeraati H, Kalantar E (2008). Incidence of *Salmonella* serovars and its antimicrobial pattern in barbecued meat and ground beef burgers in Tehran. *Iranian J. Microbiol.*, 1: 37-41.
- Soumet C, Ermel G, Rose N, Rose V, Drouin P, Salvat G, Colin P (1999). Evaluation of a Multiplex PCR assay for simultaneous identification of *Salmonella* serovar *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swab of poultry houses. *Lett. Appl. Microbiol.*, 25: 113-117.
- Trafny EA, Kozłowska k, Szpakowska (2006). A novel Multiplex PCR assay for the detection of *Salmonella enterica* serovar Enteritidis in human faeces. *Lett. Appl. Microbiol.*, 43: 673-679.
- Widjojoatmodjo, N M, Fluit A C, Torensma R, Verdonk G P and Verhoef J (1992). The magnetic immune polymerase chain reaction assay for direct detection of *Salmonella* in faecal sample. *J. Clin. Microbiol.*, 30: 3195-3199.
- Wood MW, Mahon J, Lax AJ (1994). Development of a probe and PCR primers specific to virulence plasmid of *Salmonella* Enteritidis. *Mol. Cell Probes*, 8: 473-479.
- Woodward MJ, Kirwan SE (1996). Detection of *Salmonella* Enteritidis in eggs by the polymerase chain reaction. *Vet. Record*, 138: 411-413.
- Yan, Yusof MY, Sekaran SD (2010). Multiplex polymerase chain reaction (M-PCR) assay for the detection of *Enterobacteriaceae* in clinical samples. *Afr. J. Microbiol. Res.*, 4(11): 1186-1186-1191.
- Yeh KS, Chen TH, Liao CW, Chang CVS, Lo HC (2002). PCR amplification of the *Salmonella* Typhimurium *fimY* gene sequence to detect the *Salmonella* species. *Int. J. Food Microbiol.*, 78: 227-234.
- Yousefi M, Goodarzi A, Azadi S (2006). Identification and non-typhoid *Salmonella* typing by SDS-PAGE method. *Int. J. Fac. Med. Uni. Iran-Hamadan*, 12(3): 48.
- Zahraei T, Mahzoonae MR, Ashrafi A (2006). Amplification of *invA* gene of *Salmonella* by polymerase chain reaction (PCR) as a specific method for detection of *Salmonella*. *J. Fac. Vet. Med. Univ. Tehran.*, 61(2): 195-199.
- Zahraei T (1999). *Salmonella*. Tehran University press, pp. 146-150.