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Original article

Molecular detection of *inv A* and *spv* virulence genes in Salmonella *typhimurium* isolated from human and animals in Iran

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ABSTRACT

In the present study, multiplex Polymerase Chain Reaction (m-PCR), simple PCR assay was used to confirm *Salmonella typhimurium* and detect Salmonella plasmid virulence (*spvA, spvB, spvC*) genes in human, bovine and poultry isolates. Fifty-five *S. typhimurium* isolates from bovine (n=15), poultry (n=20) and human (n=20) sources were isolated and analyzed with biochemical and serological testes. Firstly, (M-PCR) assay with four sets of primers was selected: ST139 - ST141 (284bp), specific for *invA* gene of Salmonella spp. the RFbj (663bp), Flic(183bp), Fljb(526 bp) specific for the *rfbj, fliC*, and *fljB* genes of *S. typhimurium*. In the second stage, simple PCR method with one set primer for SpvA (604bp) and another set primer for SpvB (1063bp) were applied to detect *spvA* and *spvB* genes. Also an M-PCR assay with two set primers InvA (244bp) and SpvC (571bp) was carried out to simultaneously detect and identify *invA* and *spvC* genes in S. *typhimurium.* Analysis of the samples shows that while the presence of *spvA*, *spvB*, and *spv*C genes in *S. typhimurium* from bovine source were 100% (15/15), these same genes were present in 65% (13/20), 100% (20/20) and 65%(13/20) of the poultry sources respectively.In addition, twenty (n=20) human isolates of *S. typhimurium* were obtained from Baghuyatallah University of Medical Science, Tehran, Iran. The study also shows that *spvA, spvB* and *spvC* genes were present in 85%, 100% and 85% of human source respectively. The

study represents the first report in Iran about the genotypic diversity of *spvA, spvB* and *spvC* genes of *S.typhimurium*.

1. Introduction

Salmonollosis is one of the most common infectious diseases in both humans and animals (Salehi Zahraei et al., 2006; cortez et al., 2006; Majtan et al. 2005). *Salmonella entrica* serovar Typhimurium is the most frequently isolated serovar worldwide (Salehi Zahraei et al., 2006; Gay et al., 1995). Therefore, it is necessary and important to discriminate *Salmonella* serovars from each other in order to ensure that each pathogen and epidemiology is correctly recognized (Lim et al. 2003; Pan et al. 2002). On the other hand, *Salmonella* control requires rapid and reliable methods (Soumet et al., 1998; Malkawi et al., 2004; Lim et al., 2003).Traditional *Salmonella* detection methods are based on cultures using selective media after overnight enrichment broth and characterization of suspicious colonies by chemical and serological tests. These methods are laborious and time-consuming (Aabo et al. 1993; Pan et al., 2005; Jamshidi et al., 2008; Soumet et al., 1998). The object of this study was to develop a PCR–based assay capable of simultaneously detecting *Salmonella* serovar and rapidly identifying *Salmonella typhimurium*(*S. typhimurium*).To this end, we selected M-PCR targeting four genes sequences namely *invA*, *rfbj*, *fliC*, and *fljB* specific for detecting genus *Salmonella* and serovar Typhimurium strains (Rahn et al.1992; Lim et al. 2003; Salehi Zahrai et al. 2008). *Salmonella entrica* serovar Typhimurium (88%) often harbor a serovar – specific virulence plasmid (90 kb) containing the spv operon (Chu et al., 2006; Gotoh et al., 2003; Hong *et al.*, 2008). Only a 7.8 kb region of *spv* is necessary to confer the virulence phenotype. The spv region harbors five genes *spvR, spvA, spvB, spvC, spvD* (Rotger et al., 1999; Ahmer et al., 1998; Boyed et al., 1998). Studies show that a major function of the spv operon is to potentiate the systemic spread of the pathogen (Gebreys et al., 1999; Salehi Zahraei et al., 2008; Yu et al. 2005). There are also studies describing the genetic contents of Spv, its role in the virulence, its association with antimicrobial resistance, and role in the multiplication of intracellular *Salmonella* (Chu et al. 1999; Gotoh et al., 2003). Brain et al. (1998) demonstration that virulence plasmid of *S. typhimurium* is self-transmissible, provides an example of horizontal gene transfer and hazards public health. This therefore necessitates investigating the plasmid profile for the presence of virulence genes (*spvA*, *spvB*, *spvC*) in *Salmonella* isolates. The presence of three of the most important genes of *spvA*, *spvB, spvC* of the operon was investigated in the study. In the second and most important stage of the study, simple and multiplex PCR (m-PCR) assays were utilized to detect the presence of the sequence *spvA*, *spvB, spvC* genes. We selected simple PCR with SpvA and SpvB primers that target *spvA ,spvB* genes sequences in *Salmonella* serovars (Dell Cerro et al. 2002). M-PCR with two pairs of oligonuclotide primers were performed according to the sequences of the chromosomal *invA* and plasmid *spvC* genes (Ziemer et al. 2003). The present study has two aims. Firstly, it aims at determining whether invA (invasion gene of the genus Salmonella) is specific for identification of Salmonella genus. Secondly, the study intends to assess S. *typhimurium* samples obtained and confirmed by multiplex PCR. 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. *typhimurium* isolates from poultry, bovine and human sources. This is the first report of the prevalence of these genes in Iran.

2. Materials and methods

2.1. Bacterial strain

Thirty five (n=35) isolate of *S. typhimurium* lyophilized form poultry (n=20) and bovine (n=15) sources were obtained from the culture collection in the department of microbiology, Faculty of Veterinary medicine, University of Tehran, Iran. In addition, 20 isolates of *S. typhimurium* from human (n=20) source were obtained from the Research Center of Molecular Biology, Bghuyatallah University of Medical Science, Tehran, Iran.

2.2. DNA plasmid preparation

Three colonies of each isolate on agar plate were picked and suspended in 200µl of distilled H2O. After vortexing, the suspension was boiled for 10 minutes, and 50µl of the supernant was collected after spinning for 10 minutes at 14.000 rpm in a microcentrifuge (Madadgar et al., 2008).

2.3. Oligonucleotid primers

In the first panel of M-PCR assay for identification of *S. typhimurium* four set primer were selected: ST139-ST141 (284bp) specific for Salmonella spp. (Salehi Zahraei et al., 2008) and the Rfbj (663bp), FliC (183 bp) and FljB (526 bp), specific for the *rfbj*, *fliC* and *fljB* genes of *S. typhimurium* or other *Salmonella* serovars with similar antigen properties (Lim et al. 2003). The primers sequences and their corresponding genes are shown in table 1.

Table 1
Nucleotide sequence and primers used for identification of *S. typhimurium* by m- PCR.

Primer	Gene	Sequence (5'-3')	Length bp	Reference
Rfbj-s	*rfbJ*	5'-CCAGCACCAGTTCCAACTTGATAC		Refrence
Rfbj-as		5'-GGCTTCCGGCTTTATTGGTAAGCA	663	No. 30
Flic-s	*fliC*	5'ATAGCCATCTTTACCAGTTCCCCC		Refrence
Flic-as		5'-GCTGCAACTGTTACAGGATATGCC	183	No. 30
Flijb-s	*fljB*	5'-ACGAATGGTACGGCTTCTGTAACC		Refrence No. 30
Flijb-as		5'-TACCGTCGATAGTAACGACTTCGG	526	
ST 139-s	*invA*	5'-GTGAAATTATCGCCACGTTCGGGCA		Refrence
ST141-as		5'-TCATCGCACCGTCAAAGGAACC	284	No. 30

In the second panel of m-PCR assay two set primers were selected: InvA (244 bp) for *invA* gene which is specific to *Salmonella* serovars and used to reconfirm *Salmonella* genus (Malorny et al. 2003) and SpvC (571bp) for *spvC* gene in *S. typhimurium* (Saroj et al. 2007). Moreover, Simple PCR with a pair of primer SpvA (604bp) for *spvA* gene (Gebreyes et al. 2008) and a pair of primer SpvB (1063bp) for *spvB* gene in *Salmonella* serovars were selected (Dell Cerro et al. 2002). The primers sequences and their corresponding genes are shown in table 2.

Table 2
Nucleotide sequence used as primer in the m-PCR *invA*+*spvC* genes and Simple PCR *spvA*, *spvB* genes in *S. typhimurium.*

Name of primer	Gene	Sequence (5'-3')	Length Bp	Reference
Multiplex *InvA* and *SpvC*	*invA* + *spvC*	ACAGTGCTCGTTTACGACCTGAAT/AGACGACTGGTACTGATCTAT ACTCCTTGCTCGTTTACGACCTGAAT/TCTCTTCTGCATTTCGTCA	244 571	Ziemer et al. (2003) Ziemer et al. (2003)
Simple *SpvA-f/B*	*spvA*	GTCAGACCCGTAAACAGT/GCACGCAGAGTACCCGCA	604	Del cerro et al. (2003)
Simple *SpvB-f/B*	*spvB*	ACGCCTCAGCGATCCGCA/GTACAACATCTCCGAGTA	1063	Del cerro et al. (2003)

Primers are from Del cerro et al. (2003) and Ziemer et al. (2003).

2.4. DNA amplification

M-PCR was performed in a reaction of 25 µl containing reaction buffer (50mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl PH=8.3) (CinaGen, Iran) , 2µl of DNA sample, 200µM dNTPs, 1 U *Taq* polymerase (CinaGen, Iran) and 1µm of each primer (CinaGen, Iran). The m-PCR amplification program for *S. typhimurium* confirmation was similar to the protocol by Madadgar *et al.* (2008). On the other hand, the m-PCR program for *invA+spvC* gene conditions were 1 min at 94°c followed by 30 cycles of 30s at 94°C , 30s at 56°C ,2min at 72°C and final extension 10 min at 72°C. The PCR program for *spvA* and *spvB* conditions were 5min at 94°C followed by 30s at 94°C, 30s at 60°C, 1 min 72°C and final extension 5 min at 72°C. The positive control *S. typhimurium* ATCC 14028 isolate and negative control *E.*coli ATCC 25922, *klebciella pneumonia* ATCC 70603 *pseudomonas aeroginosa* ATCC 27853 were obtained from the culture collection in the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran. The PCR product was electrophoresesed in 1.2% agaros (Fermentase) and after staining with ethidium bromide and visualized by UV light illumination (Bio Rad-USA).

3. Results

3.1. Confirm of *S. typhimurium* by m-PCR

M-PCR assay was applied to all isolates obtained from poultry, human and bovine sources for confirming *S. typhimurium.* A total of 55 (n=55) isolates including 20 poultry (n=20), 20 human (n=20) and 15 bovine (n=15) isolates were designated for the study (Figure1). M-PCR assay confirmed the isolates being *S. typhimurium*.

3.2. Identification of *spvA, spvB* and *invA+spvC* genes in *S. typhimurium*

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

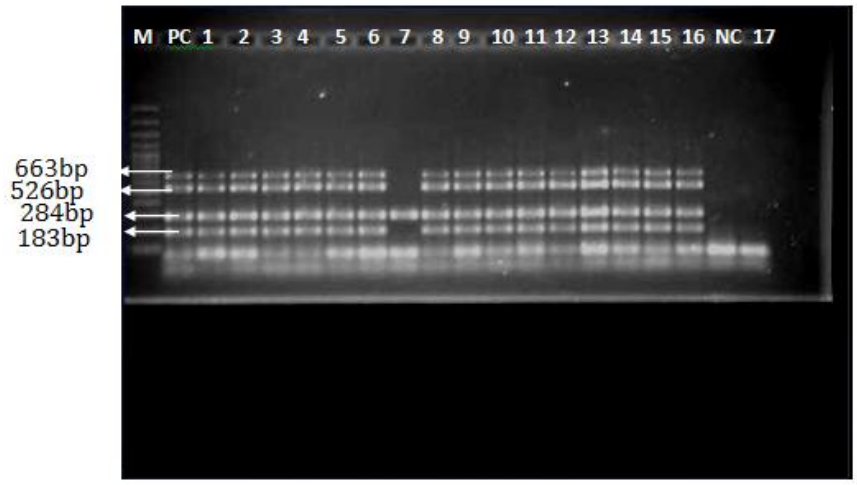

Fig. 1. Multiplex PCR with four pairs of primers for *S.typhimurium* isolated (Human, Bovine and Poultry source); M: marker (100bp); PC: positive control; NC: negative control (*E.coli*); lane 17 free PCR Control (without DNA) ; lane 7 is Salmonella SPP. and other lane for positive S. typhimurium.

3.3. Poultry isolates *S. typhimurium*

The *spvA* and *spvB* genes were present in 65% (13/20) respectivly of the isolates. In 65% (13/20) of the isolates *spvC* and *invA* genes were present respectively (Figure 2). In the same isolates (those with no *spvC*) *invA* genes were present in 35% of the isolates (7/20), (Table 3).

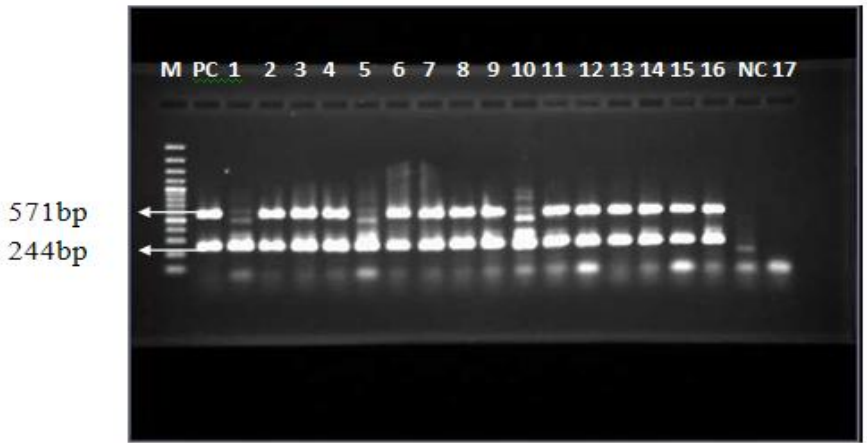

Fig. 2. Multiplex PCR with two pairs of primers for *invA*, *spvC* virulence genes in *S. typhimurium* (Human, Bovine and Poultry source): lane M: l00bp marker; lane PC: positive control; lane NC: negative control (*E.coli*); lane 17: free PCR control; lanes 1, 5, 10: *S. typhimurium* ($invA_+ spvC_-$); other lanes: *S.typhimurium* ($invA_+ spvC_+$).

3.4. Human isolates *S. typhimurium*

The study showed that *spvA*, and *spvB*, genes were present in %85 (17/20) and 85% (17/20) of the samples respectively (Figure 3). In 85% (17/20) of the samples *spvC* and *invA* were present. In the same samples (those with no *spvC*) *invA* genes were present 15% (3/20).

Table 3
Distribution of *spvA, spvB*, *invA* + *spvC* genes in *S. typhimurium.*

Serotype	Serogroup	Source	Total	Present spvA (641bp)	Present spvB (1063bp)	M-PCR invA(244bp)+spvC(571bp)	
						***spvC*(+), *invA*(+)**	***spvC*(-), *invA*(+)**
S.typhimurium	B	Poultry	20	65%(13/20)	65%(13/20)	65%(13/20)	25% (7/20)
S.typhimurium	B	Human	20	85%(17/20)	85%(17/20)	85%(17/20)	15%(3/20)
S.typhimurium	B	Bovine	15	100%(15/15)	100%(15/15)	100%(15/5)	0

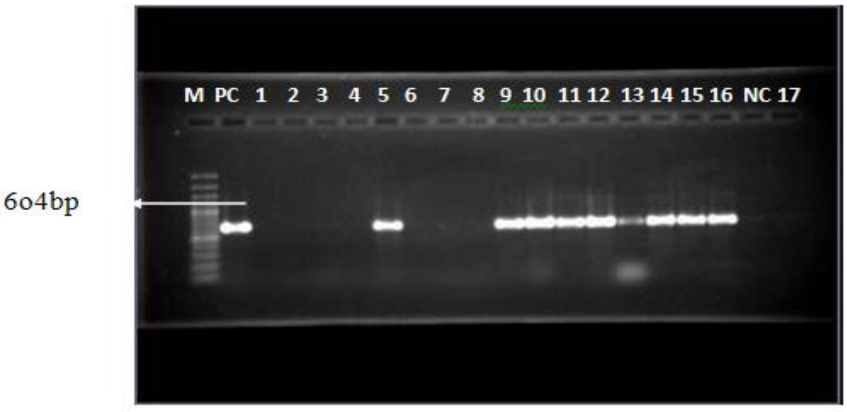

Fig. 3. Simple PCR with one pairs of primer for *spvA* gene in *S.typhimurium* (Human, Bovine and Poultry source): M: 100bp marker; lane PC: positive control; lane NC: negative control (*kelebsiella pneumonia*); lane 17: free PCR control; lanes 1, 2, 3, 4, 6, 7 and 8: negative *spvA* gene; other lanes: positive *spvA* gene.

3.5. Bovine isolates *S. typhimurium*

As table 2 shows, positive band appears for *spvA, spvB* and *invA + spvC* genes in 100% (15/15) of the all isolates (Figure 4).

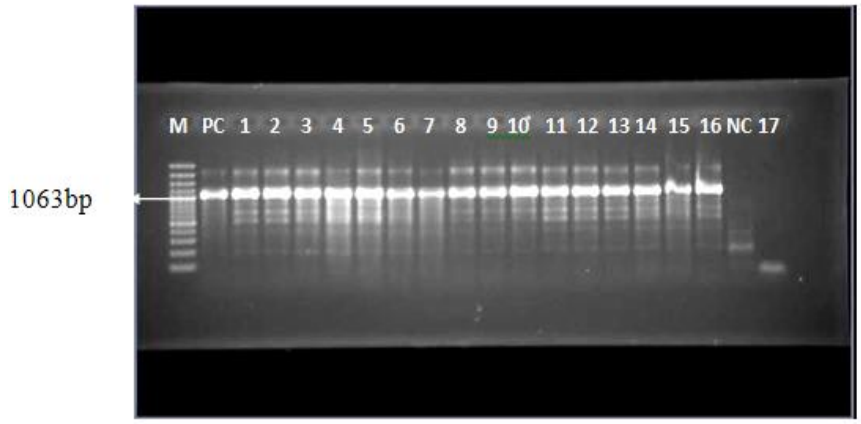

Fig. 4. Simple PCR with one pairs of one primer for *spvB* gene (spvB-1063bp) in *S. typhimurium* (Human, Bovine and Poultry source): lane M: 100bp marker; lane PC: positive control; lane NC: negative control (pseudomonas aeroginosa); lane 17: free PCR control; other lanes: positive *spvB* gene.

4. Discussion

Our study confirms the presence of *invA, fliC, rfbj* and *flgB* genes in all Typhimurium serovars. Lim et al. (2003) using 3 set primers specific to *rfbJ, fliC*, and *fljB* genes observed that the simultaneous presence of all 3 genes were specific and necessary to detect *S. typhimurium*. This was despite an earlier report by Soumet *et al.* (1999) who observed that *fli*C gene alone was sufficient in detection of Typhimurium serovar. Other researchers have also reported that *invA* and *fli*C genes are specific for the detection of *Salmonella* genus and serovar Typhimurium respectively (Malorny et al., 2001; Malorny et al., 2003; Malkawi et al., 2008; Oliviera et al., 2003; Salehi Zahraei et al., 2006; Khan et al., 1999; Holger et al., 2001; Jamshidi et al. 2008). Houges et al., (2008) were also able to confirm the presence of *invA* gene in all *Salmonella* isolates from wild birds in England. Rhan et al. (1992) on the other hand, did not detect *invA* gene in Sefetnburg and Litchfield serovars concluding that this gene is not specific to *Salmonella* genus. This was also the case with some more recent studies which did not find *invA* and *fliC* genes alone being specific for the detection of *Salmonella* genus and serovar Typhimurium (Bolton et al. 1999; Rahn et al., 1992). A reasonable consequence for the conflicting findings is that using *invA* and *fliC* genes alone are not sufficient for confirming *Salmonella* and Typhimurium serovar. In addition to these two genes, it is therefore necessary to use more specific genes for the detection of Typhimurium serovar. 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 tissues (khan et al. 1999). Amplification of *invA* is now recognized as an internationally 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.

Various biochemical and m -PCR assays have been carried out to detect *Salmonella* genus and Typhimurium serovar around the world. Compared with bacteriological culture method, m-PCR is more cost effective and remarkably fast, saving precious time needed to control *S. typhimurium*. While it takes 5 to 6 days to confirm the presence of *S. typhimurium* through bacteriological culture method (Malorny et al., 2003; Salehi Zahraei et al., 2006), M-PCR assays are generally carried out within 5 to 6 hours. M-PCR also provides us with reliable, specific, reproducible results about the status of the sample and recognition of certain microorganisms in large-scale epidemiological studies involving several laboratories (Ebner et al., 2001; Malkavi et al., 2004; Soumet et al., 1998).

Spv operon plays a role in multiplex drug resistance, systemic disease, and bacterial virulence (Boyed et al., 1998). This operon, which contains 5 genes (R, A, B, C, D), is present in the virulence plasmid (Chu et al. 2006). It

exists in a few serovars of subspecies of *Salmonella* entrica. Distribution of this plasmid and the genes associated with spv operon in various serovars and strains of a particular serovar is dependent on the geographical conditions, selective pressures, and the host type - whether the host is a human or an animal (Kurita et al. 2003). There are some reports about the distribution of these genes worldwide. The distribution of the virulence plasmid (90kb) in *S. typhimurium* was reported by Majtan et al., (2005) Ahmer et al. (1998) ,Chiu et al. (2006) and Chiu et al. (1996) as 89.6%, 88%, 91.5% and 55% respectively. Namimatsu et al., (2005) compared the presence of the virulence plasmid in the *S. typhimurium* isolates from systematically infected, diarrheic and healthy pigs. They reported the virulence plasmid in 92%, 18.8%, and 17.6% of the systematically infected, diarrheic and healthy pig isolates respectively. Many studies have focused on the simultaneous presence of *invA* and *spvC* genes. While Chiu et al. (1996) have reported the simultaneous presence in children fecal sample as 55/2% (21/38), Ziemer et al. (2003) analyzing *Salmonella* spp. from human source have reported this as 31.2% (15/48). Saroj et al. (2007) reported the simultaneous presence of *invA* and *spvC* genes in food-born isolates of *S. typhimurium* serovares as 85% (22/28). They further reported the presence of negative *spvC* isolates as 6/28. The simultaneous presence of *invA* and *spvC* genes in *S. typhimurium* isolates separated from food and environmental samples was reported as 10/46 (22%) by Ling et al. (2009). Bolton *et al.* (1999) reported the presence of *invA* and *spvC* in all Typhimurium serovas as 88% and 98% respectively. In another study Gebreyes et al. (2008) have reported the presence of *spvA* gene only in serogroup B (Typhimurium). They have also reported the presence of this gene in acute clinical isolates. Nikbakht et al. (2004) studied *spvR* gene (890 bp) in different *Salmonella* serovars in Iran confirming its presence in *Salmonella* Typhimurium and Enteritidis. In yet another study by Del cerro et al., (2003), from a total of 56 *Salmonella* isolates of animal source, 21 isolates contained *spvA, spvB, spvC* genes. Finally, Jenikova et al. (2000) reported observing *invA* and *spvC* genes only in *Salmonella* and not in other genus.

Studies suggest a direct relationship between the presence of virulent plasmid genes with multiplex-drug resistance factors, systemic disease, the source of Salmonella (human or animals), and bacterial virulence (Namimatso et al., 2008; Boyed et al., 1998; Matsu et al., 2001; Gebreyes et al., 2008; Gotoh et al., 2003; Chiu et al. 2006; Rotger et al. 1999; Yu et al. 2005; Del cerro et al. 2002; Halawani et al. 2008; Heithoff et al., 2008). 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). Drastic genetic variations in Salmonella could derive 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 serovares) which carry virulence plasmid can cause systemic disease, while plasmidless strains can cause local or asymptomatic disease (Heitoff et al., 2008).

This study performed simple PCR and m-PCR genotype, plasmid profiles for the presence of virulence genes (*spvA, spvB, spvC*) in *S. typhimurium* isolates from poultry, human and bovine sources. Our findings were largely in line with those of other researchers. However, the distribution of *spvA* and *spvC* genes from the poultry source (65%) was lower than what the other researchers have reported. This lower distribution of the strains without virulence plasmid in Iran can be attributed to type and the race of the host, selective pressures, consuming too much antibiotics and the regional situations (Heithoff et al., 2008). Epidemiological survey, identification of *S. typhimurium* , and screening *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 sallmonelosis 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.

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