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Detection and Molecular Identification of Plasmid Virulence Genes in *Salmonella enterica* serovar *typhimurium* Isolated from Human and Animals by Multiplex PCR Method

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ABSTRACT

Background: *Salmonella enterica* is a zoonotic species that can acquire its resistance in livestock. In humans, *Salmonella typhimurium* is a major etiological agent of food-borne salmonellosis. The identification of *Salmonella* spp. by traditional cultural techniques requires 4 to 5 days. The polymerase chain reaction (PCR) offers a simple tool for the rapid detection of *Salmonella*.

Methods: Fifty-five *S. typhimurium* isolates from bovine, poultry and human sources were isolated and analyzed with biochemical and serological tests. Firstly, multiplex PCR assay with four sets of primers was selected for *invA*, *rfbj*, *fliC* and *fljB* genes. In the second stage, a simple PCR method with one set primer was applied to detect *spvA* and *spvB* genes. Also, multiplex PCR assay with two set primers was carried out to simultaneously detect and identify *invA* and *spvC* genes in *S. typhimurium*.

Results: Analysis of the samples showed that while the presence of *spvA*, *spvB* and *spvC* genes in *S. typhimurium* from the bovine source was 100% (15/15), these same genes were present in 65% (13/20) of the poultry sources. The study also showed that *spvA*, *spvB* and *spvC* genes were present in 85% of human source.

***Conclusion*:** This study showed that M- PCR of *invA*, *rfbJ*, *fljB*, and *fliC* genes were fast, simple, less expensive, accurate and specific in identification *S. typhimurium*. The advantage of multiplex PCR was that it could simultaneously identify the *Salmonella* strains which had a virulence plasmid thus facilitating the search for specific etiologic *Salmonella* serovars. The higher prevalence of *spv* genes among bovine sources can be injurious for public health.

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Introduction

Salmonellosis is one of the most common infectious diseases in both humans and animals (1- 2). *Salmonella entrica* serovar *typhimurium* is the most frequently isolated serovar worldwide (1, 3). 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 (4-5). On the other hand, *Salmonella* control requires rapid and reliable methods (4, 6-8). 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 (5-6, 9-10). The *S. typhimurium* often harbor a serovar – specific virulence plasmid (90 kb) containing the *spv* operon (11-13). 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* (14-16). Studies showed that a major function of the *spv* operon is to potentiate the systemic spread of the pathogen (17). There are also studies describing the genetic contents of *spv*, its role in the virulence and multiplication of intracellular Salmonella (14, 18). Brain et al. demonstrated that virulence plasmid of *S. typhimurium* which is self-transmissible, provides an example of horizontal gene transfer (19). This therefore, necessitates investigating the plasmid profile for the presence of virulence genes (*spvA*, *spvB*, *spvC*) in *Salmonella* isolates. Simple and multiplex PCR (M-PCR) assays were utilized to detect the presence of the sequence *spvA*, *spvB*, *spvC* genes in the study. We selected simple PCR with SpvA and SpvB primers that target *spvA*, *spvB* gene sequences in *Salmonella* serovar (20). M-PCR with two pairs of oligonucleotide primers were performed according to the sequences of the chromosomal *invA* and plasmid *spvC* genes (21). We selected M-PCR targeting four genes sequences namely *invA*, *rfbj*, *fliC* and *fljB* specific for detecting genus *Salmonella* and serovar *typhimurium* strain (4, 22-23). The present study had three aims as follows: detection the *Salmonella* genus with *invA* gene, detection of *S. typhimurium* with *rfbj*, *fliC* and *fljB* genes by multiplex PCR and determining of the distribution of *spvA*, *spvB* and *spvC* genes in *S. typhimurium* isolates from poultry, bovine and human sources.

Materials and Methods

Bacterial strain

Thirty five (n=35) isolates 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) sources were obtained from the Research Center of Molecular Biology, Baqiyatallah University of Medical Sciences, Tehran, Iran.

DNA plasmid preparation

Three colonies of each isolate on an agar plate were picked and suspended in 200 µl of distilled water. After vortexing, the suspension was boiled for 10 minutes, and 50 µl of the supernatant was collected after spinning for 10 minutes at 14.000 rpm in a micro-centrifuge (24).

Oligonucleotide primers

In the first panel of M-PCR assay for identification of *S. typhimurium*, four sets of primers were selected for detection of: *ST139-ST141* (284bp) (24) *rfbJ* (663bp), *fliC* (183 bp) and *fljB* (526 bp) genes (4). The primers sequences and their corresponding genes are shown in Table 1. In the second panel of M-PCR assay two sets of primers were selected for detection of *invA* (244 bp) (25) and *spvC* (571bp) genes (12). Moreover, simple PCR was selected with a pair of primer for *spvA* gene (604bp) (17) and a pair of primer for *spvB* gene (1063bp) were

selected (20). The primer sequences and their corresponding genes are shown in Table 2.

DNA amplification

M-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 □M dNTPs, 1 U Taq polymerase (CinaGen, Iran) and 1 µm of each primer (CinaGen, Iran). On the other hand, the M-PCR program for *invA+spvC* gene conditions was 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 30s at 94°C, 30s at 60 °C, 1 min 72 °C and final extension 5 min at 72 °C (24). The positive control *S. typhimurium* ATCC 14028 isolate and negative controls *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 70603 *Pseudomonas aeruginosa* ATCC 27853 were used.

Results

Confirmation 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* (Figure1). M-PCR assay confirmed the isolates were *S. typhimurium*.

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

M-PCR to detect both *invA* and *spvC* genes in the samples are shown in Figure 2 and Table 3. Simple PCR to detect virulence gene *spvA* and *spvB* with one pair of primers are shown in Table 3.

Discussion

Our study confirmed the presence of *invA*, *fliC*, *rfbj* and *fljB* genes in all *S. typhimurium*. Lim et al. used three primer sets specific to *rfbJ*, *fliC* and *fljB* genes and observed simultaneous presences of all genes were specific and necessary to detect *S. typhimurium* (4). Soumet et al. observed that the *fliC* gene alone was sufficient in the detection of *S. typhimurium* (6). Other researchers have also reported that *invA* and *fliC* genes were specific for the detection of *Salmonella* genus and *S. typhimurium* respectively (1, 7, 26). Hughes et al. confirmed the presence of the *invA* gene in all *Salmonella* isolates from wild birds in England (27). Rhan et al. did not detect the *invA* gene in *S. seftenburg* and *S. litchfield* (11). In addition to *invA* and *fliC*, it is therefore necessary to use more specific genes for the detection of *S. typhimurium*. Invasion gene operon, *invA* was detected in all *Salmonella* isolates in our study.

Amplification of the *invA* is now recognized as an international standard procedure for the detection of the *Salmonella* genus (28). This increases the value of the present research because of the virulence properties and clinical importance of the *invA* gene. According to the results of this study PCR method based on *invA* gene is useful for rapid identification of *Salmonella*.

The *spv* operon, which contains five genes (R, A, B, C, D) is present in the virulence plasmid (11). There are some reports about the distribution of these genes worldwide. The distribution of the virulence plasmid (90kb) in *S. typhimurium* was reported by other researchers as 88% and 91.5% (15, 29). Derakhshandeh et al. showed that among 60 different *Salmonella* isolates, the prevalence of *spvB* and *spvC* genes were (46.3 %) and (73.3), respectively (30).

Zahraei et al. revealed that *spvB* and *spvC* genes were present in 90 % of *S. enteritidis* (31). Moussa et al. reported the prevalence of the *spvC* gene 30% in 20 *S. typhimurium* (32).

Table 1. Primers used for identification of *S. typhimurium* by M- PCR**.**

Gene	Primer	Sequence (5'-3')	Length bp	Reference
rfbJ	Rfbj-s Rfbj-as	5'-CCAGCACCAGTTCCAACTTGATAC 5'-GGCTTCCGGCTTTATTGGTAAGCA	663	4
fliC	Flic-s Flic-as	5'ATAGCCATCTTTACCAGTTCCCCC 5'-GCTGCAACTGTTACAGGATATGCC	183	4
fljB	Flijb-s Flijb-as	5'-ACGAATGGTACGGCTTCTGTAACC 5'-TACCGTCGATAGTAACGACTTCGG	526	4
invA	ST 139-s ST141-as	5'-GTGAAATTATCGCCACGTTCGGGCAA 5'TCATCGCACCGTCAAAGGAACC	284	24

Table 2. Primers 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 AGACGACTGGTACTGATCGATAAT + ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	244 571	21
Simple *spvA-f/B*	*spvA*	GTCAGACCCGTAAACAGT GCACGCAGAGTACCCGCA	604	20
Simple *spvB-f/B*	*spvB*	ACGCCTCAGCGATCCGCA GTACAACATCTCCGAGTA	1063	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%(15/15)	0

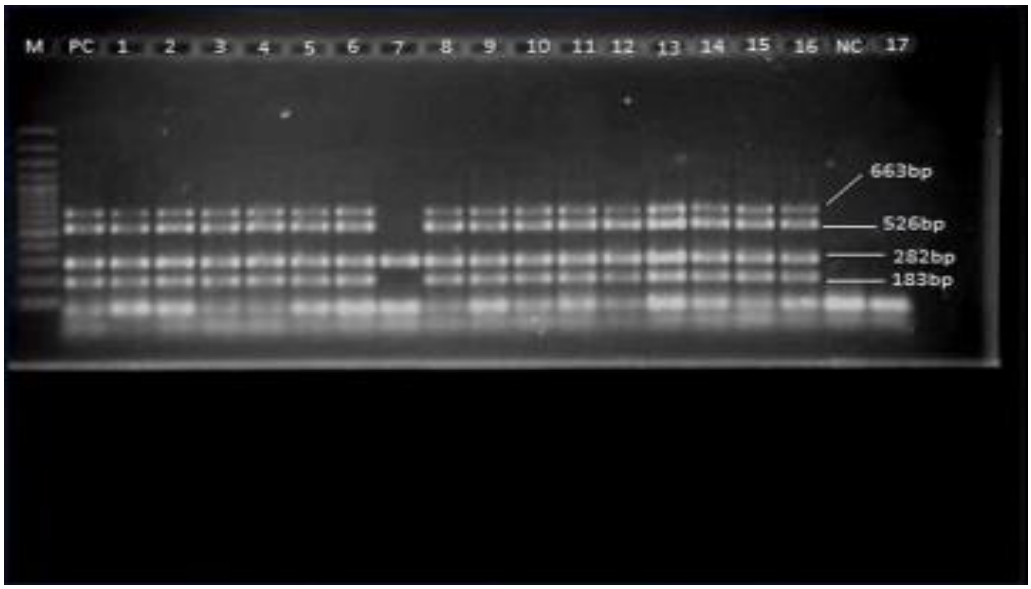

Figure 1. Multiplex PCR with four pairs of primers for *S. typhimurium* isolates: M marker (100bp); PC positive control; NC: negative control (*E. coli*); lane 17 free PCR Control (without DNA); lane 7 *Salmonella* spp. and another lane for positive *S. typhimurium*.

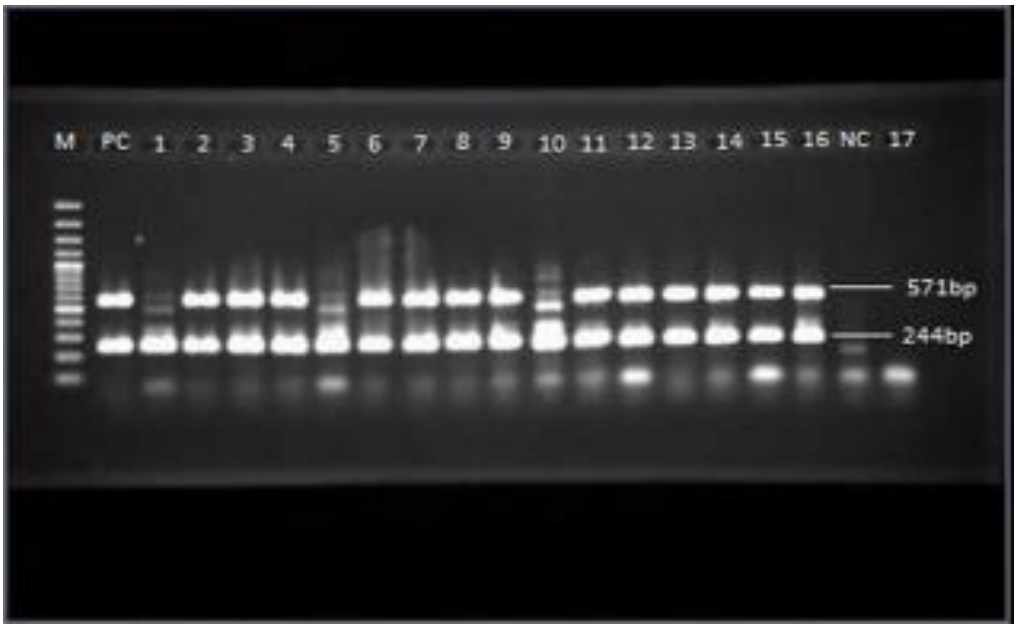

Figure 2. Multiplex PCR *invA, spvC* genes in *S. typhimurium* lane M: l00bp marker; lane PC: positive control; lane NC: negative control (*E. coli*); lane 17: free PCR control; lanes 1, 5, 10: *S. typhimurium* (*invA*+ *spvC*-); other lanes: *S. typhimurium* (*invA*+ *spvC*+).

Namimatsu et al. 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 (33). Many studies have focused on the simultaneous presence of *invA* and *spvC* genes. Ziemer et al. analyzed *Salmonella* spp. from human source and reported this as 17.3% (9/52) (23). Saroj et al. reported the simultaneous presence of *invA* and *spvC* genes in food-born isolates of *S. typhimurium* as 85% (22/28) (12). The simultaneous presence of *invA* and *spvC* genes in *S. typhimurium* isolates separated from food and environmental samples were reported as 22% (10/46) (34). In another study, Gebreyes et al. reported the presence of the *spvA* gene only in serogroup B (*S. typhimurium*) (17). Nikbakht et al. studied the *spvR* gene (890 bp) in different *Salmonella* serovars in Iran confirming its presence in *S. typhimurium* and *S. enteritidis* (35). In another study by Del cerro et al. from a total of 56 *Salmonella* isolates of animal source, 21 isolates contained *spvA, spvB, spvC* genes (20). Studies suggest a direct relationship between the presence of virulent plasmid genes with multiple-drug resistance factors, systemic disease, source of *Salmonella* (human or animals) and bacterial virulence (12, 14, 16-17, 20, 26, 30- 31, 36-37). There are some discrepancies about the distribution of virulence plasmid of various *Salmonella* serovars between samples from human and animal origins. In some studies results, showed a higher distribution for the virulence plasmid from animal-origin isolates than that of human-origin (20). Drastic genetic variations in *Salmonella* could be derived from transfer of this organism between human-origin and animal-origin strains. 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 *S. typhimurium* and *S. enteritidis*) which carry virulence plasmid can cause systemic disease, while plasmidless strains can cause local or asymptomatic disease (38). 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. 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 the type and race of the host, selective pressures, consuming too many antibiotics and the regional situations. Our study has some limitations. Firstly, our sample size was small. Secondly, other genes in *spv* operon were not determined. Further studies are recommended to clarify if the presence of *spv* genes in other serovars of *Salmonella*.

Conclusion

Our results showed that all of the isolates were confirmed by M-PCR as *S. typhimurium* and highlight the usefulness of the M-PCR of specific primers *invA*, *fliC*, *rfbJ* and *fljB* for concurrent and rapid detection of *Salmonella* spp and *S. typhimurium*. The prevalence among animals and humans of *S. typhimurium* harboring the virulence plasmid in this study is a significant concern for public health. The epidemiological survey, identification of *S. typhimurium* and screening of *spv* gene through PCR-based procedures can have a major benefit in public health specifically for rapid diagnosis, etiology, epidemiological investigations, ideal vaccine, development of treatment and prophylactic strategies for salmonellosis in Iran.

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Conflict of interest

Authors declare no conflict of interest in this study.

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