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Molecular detection of *invA, ssaP, sseC and pipB* genes in *Salmonella typhimurium* isolated from human and poultry in Iran

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Infections due to *Salmonella* serovars are a great risk to public health. It is known that genes involved in pathogenesis of *Salmonella* serovars are clustered within *Salmonella* pathogenicity Islands. We have characterized four genes (*invA*, *ssaP*, *sseC*, and *pipB*) coded by 3 pathogenicity Islands among 54 *Salmonella typhimurium* isolated from poultry samples in Iran. A set of tetraplex polymerase chain reaction (PCR) was developed. Results show that *invA*, *ssaP*, *sseC*, and *pipB* genes were 100% prevalent in this study. This assay is a reproducible and sensitive assay for the detection of *Salmonella* by screening several virulent genes present in SPIs. Because of rapidity, high specificity and sensitivity of M-PCR method, by standardization of this method, it could be considered as an alternative to conventional culture and serotyping methods to confirm the presence of *Salmonella* in human and poultry samples. This is the first study on the distribution of *ssaP*, *sseC* and *pipB* genes in isolates from human and poultry sources in Iran.

Key words: *Salmonella typhimurium*, multiplex PCR, *invA*, *ssaP*, *sseC*, *pipB*, gene.

INTRODUCTION

Salmonella is a facultative intracellular pathogen that causes a variety of infectious diseases. The most common of such diseases is gastroentritidis, with bacterial multiplication in intestinal submucosa and diarrhea, caused by the inflammatory response and perhaps also by toxins. In specific hosts, adapted *Salmonella* produces systemic diseases such as typhoid and paratyphoid fevers in humans (Rotger et al., 1999). The genus *Salmonella* is composed of two distinct species, *Salmonella bongori* and *Salmonella enterica* (Faucher et al., 2009). *Salmonella enterica* subspecies enterica serovar Typhimurium is the main cause of human food-born illness that is mainly associated with the consumption of contaminated poultry meat and eggs (Desin et al., 2009). *Salmonella* has a complex life cycle in infected humans and birds, and a large number of genes have been implicated in *salmonella* virulence. Several of these virulence determinants are clustered within pathogenicity Islands, that is, large segments of horizontally acquired sequences present in pathogenic species but absent from closely related nonpathogenic species. SPIs are insertions of large regions of DNA containing virulence genes located on the bacterial chromosome. 15 SPIs have been identified in *Salmonella enterica* serovars. SPI-1 and SPI-2, which are present in all *S.enterica* Serovars, represent 2 major pathogenesis

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determinants that encode type III secretion systems (Daigle, 2008) Whereas SPI-1 governs the ability to invade epithelial cells and is required for *Salmonella* induced macrophage apoptosis, SPI-2 harbors genes required for intramacrophage survival and systemic infection (Blanc-Potard et al., 1999). SPI-5 genes encode proteins that are associated with enteropathogenes which is most likely involved in fluid secretion and neutrophile recruitment (Rotger and Casadesus, 1999). *invA* gene allows *S. typhimurium* to enter cultured epithelial cells. Virulent strains of *Salmonella* carrying defined mutations in inv genes (*invA, invB, invC* and *invD*) were shown to contain 50% higher lethal doses than their parent strains when administered orally to mice (Ginocchio et al., 1992). Studies also showed that *invA* gene are present and functional in most -if not all- *Salmonella* serovars (Ginocchio et al., 1992). *sseC* has a crucial role in the intracellular survival of *Salmonella*. Strains carrying mutation within SPI-2 genes (*ssaP* and *sseC*) are attenuated for virulence, which confirms the importance of these genes at different stages of infection and for survival of bacteria inside the host cells (Bhowmick et al., 2010). Finally, *PipB* gene contributes to enteric but not to systemic salmonellosis (Wood et al., 1998). Traditional detection techniques of *Salmonella* are based on cultures using selective media and the biochemical characterization of the colonies obtained. These conventional methods are time consuming and do not enjoy high sensitivity (Rahn et al., 1992). A lot of attention is being paid to on rapid detection of *Salmonella* using molecular techniques like polymerase chain reaction (PCR) as it is highly sensitive, specific and reproducible. The use of multiplex PCR as a tool for pathogen detection in clinical and environmental samples is well documented (Amini et al., 2010). However, there are very few reports highlighting the use of multiplex-PCR for detection of virulence genes of *Salmonella* serovars isolates in Iran. Therefore this study was aimed to develop a sensitive and rapid multiplex PCR technique for detection of *Salmonella* pathogenicity islands coding virulence genes (*invA*, *ssaP*, *sseC*, *pipB)* in human and poultry associated *Salmonella* serotypes in Iran.

MATERIALS AND METHODS

Sample collection and bacterial strains

A total of 100 poultry samples were collected from a poultry slaughterhouse located in Tehran. Samples were transported in individual bags in ice and stored in an icebox prior to bacteriological analysis. In addition, 54 isolates of *Salmonella typhimurium* Lyophilized from human and poultry sources were obtained from the culture collection in the Department of Microbiology, Faculty of Veterinary Medicine, university of Tehran and Department of Microbiology, Faculty of Specialized Veterinary Science, Islamic Azad university, Science and Research Branch, Iran.

Microbiology methods

To determine the presence of *Salmonella* serovars in the samples, conventional culture method was used. The intestine content, cecal content, bile, liver and spleen samples were directly inoculated into Rappaport-vasisiliadis (RV) broth (Merck, Germany) media and incubated at 37°c for 24 h. Each sample was then inoculated on to *Salmonella* – *Shigella* agar (SS) plates. The plates were incubated at 37°C for 24 – 48 h. Suspicious colonies morphologically similar to *Salmonella* were identified with biochemical tests (IMVIC, SIM, TSI, and Urease). The *Salmonella* isolates were first cultured into TSI slant medium and growth overnight at 37°C , then were tested by using antisera 0 (B, D, E, C) and H based on slide and tube agglutination tests to determine O and H antigens, respectively .

Multiplex PCR

DNA extraction

Prior to DNA extraction, *Salmonella* isolates from human and poultry were cultured into Luria Bertani (L.B) agar plates and incubated at 37°C for 24 h. For DNA extraction, a loopful of each sample from L.B agar was suspended in 200 µl sterile distilled water. In order to have uniform turbidity, the samples were vortexed, then were boiled for 10 min and centrifuged at 1400 rpm for 10 min, supernatants were collected and saved for the multiplex PCR analysis.

DNA primers

In the panel of multiplex PCR assay for identification of *Salmonella* serovars four sets of primers were selected for *invA*, *ssaP*, *sse C*, and *PiPB* genes in *Salmonella* spp. (Bhowmick et al., 2011). The primer sequences and their corresponding genes are shown in Table 2.

DNA amplification

The cycling condition of each primer was standardized in a singleplex PCR followed by the multiplex PCR format using a gradient thermocycler (MJ Research, USA). The cycling condition is mentioned in Table 1. A set of tetraplex PCR was developed. 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) using a gradient thermocycler (Bio-rad USA). The PCR products were resolved on a 1.5% agarose gel, stained with ethidium bromide (5 ng ml^{-1}) and visualized by UV light illumination (Bio-rad Molecular Imager, Gel Doc TM, XR Imaging system, USA).

RESULTS

Panel 1

Detection of *Salmonella* by culture and serotyping: Three out of the 100 poultry samples (3%) were culture positive for *Salmonella*. Serotyping evaluation showed that 100% samples were serogroup C_1 (*S. infantis*) with O and H antisera.

Panel 2

Detection of *invA*, *ssaP*, *sseC*, and *pipB* genes in

Table 1. PCR conditions of one set of optimized multiplex PCR.

Set of PCR	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
Tetraplex set:	95°C, 5 min	95°C, 1 min	50 °C, 1 min	72°C, 1 min	72°C, 5 min	35

Table 2. Primer sequences used to detect the virulence genes of *S. typhimurium.*

Multiplex PCR set	Gene	Pathogenicity island	Primer sequence (5'-3')	Product size (bp)
Tetraplex set	*invA*	SPI-1	F:GTTGTACCGTGGCATGTCTG R:GCCATGGTATGGATTTGTCC	930
	ssaP	SPI-2	F:ATGCGTATTACCAAAGTTGA R:TCATTCGCTATTCTTAACAT	375
	sseC	SPI-2	F:TATGGTAGGTGCAGGGGAAG R:CTCATTCGCCATAGCCATTT	121
	pipB	SPI-5	F:AATATCGGATGGGGGAAAAG R:AACCTGACTCACGCAGACCT	230

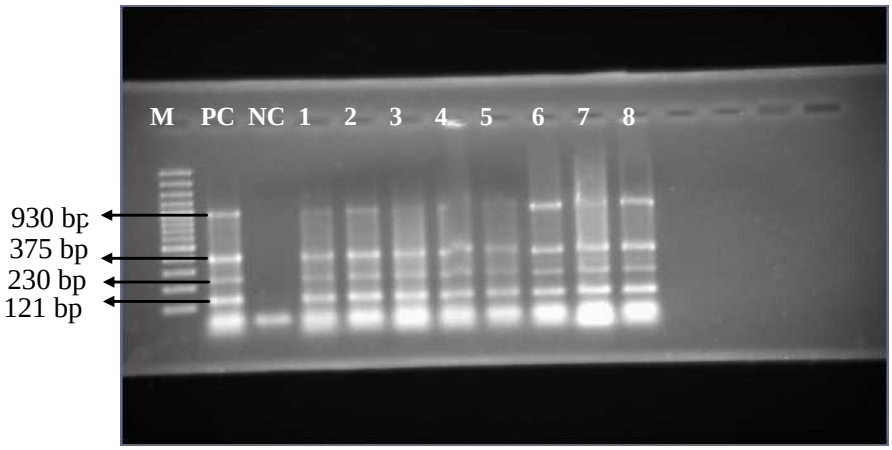

Figure 1. Multiplex PCR with four pairs of primers for invA, ssap, pipB and sseC virulence genes in *S. typhimurium* (Human source): lane M: 100 bp marker; lane PC: positive control; lane NC: negative control (Water); lane 1-8: positive four genes in *S. typhimurium.*

S. *typhimurium*: Multiplex PCR to detect *invA*, *ssaP*, *sseC*, and *pipB* genes in the samples yielded the following results.

Poultry isolated *S. typhimurium*

The study shows that *invA*, *ssaP*, *sseC*, and *PiPB* genes were present in 100% (22/22) of the samples (Figure 1).

Human isolated *S. typhimurium*

The study shows that *invA*, *sseC*, *sseP*, and *PiPB* genes were present in 100% (32/32) of the samples.

DISCUSSION

The rapid detection of bacterial pathogens is critical since

people's life may depend on it. Thus there is a need for more sensitive and faster methods. Molecular methods are increasingly important in the detection and typing of *Salmonella*. Assays were published with the aim of replacing the traditional serotyping by direct detection of different genes (Kardos et al., 2007). For rapid identification of organisms multiplex PCR is a modified form of PCR in which several segments of target DNA are amplified simultaneously in a single PCR reaction to conserve template DNA and to save the time and cut expenses. Multiplex PCR has been used for epidemiological typing of *Salmonella* (Bhowmick et al., 2011). Not many studies are reported on using multiplex PCR for detection of virulence genes of *Salmonella* in Iran. In this study we standardized a set of tetraplex PCR using 54 human and poultry isolates. In a study of poultry samples from among a total of 10,132 samples from 561 slaughterhouses in the European Union 3.6% samples were positive for *Salmonella* serovars, and *S. infantis* and *S. enteritidis* were the two most frequently isolated serovars on carcasses in the European Union member states. This accounted for about one-third and one-sixth of the *Salmonella* isolates, respectively (Marta and Eirini, 2008). In another study, from among a total of 1125 samples collected from chickens, 27 cases (2.4%) of *Salmonella* were isolated and the subsequent analysis revealed that of these, 55.5% were identified as serovars of *S. enteritidis*, 22.2% as *S. typhimurium* and 7.4% as *S. infantis* (Emaddi-Chashni et al., 2009). Yet in another study in Spain, prevalence of *Salmonella* in chickens was reported as 17.9 sing serological and molecular methods it was shown that, 36 (60%), 6 (10%), and 3 (5%) of the detected isolates of *Salmonella* were positive for *S. enteritidis, S. infantis*, and *S. typhimurium*, respectively by (Capita et al., 2007). According to the result of the present study, 3% of the samples were salmonella. This may suggest a drop in the incidence of *Salmonella* as compared with the previous studies. 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 (Bryan and Doyle, 1995). From among 3 poultry sources *Salmonella* serovars isolates in this study, no *S. typhimurium* serovar was detected after serotyping. This is similar to the study of Amini et al. (2010) who did not detect *S. typhimurium* in 68 *Salmonella* serovars isolates taken out of 1001 poultry samples from a slaughterhouse in Kerman, Iran. This does not suggest that *S. typhimurium* is an insignificant pathogen. The pathogen is in fact detected in a number of epidemics worldwide. This study aimed at detecting the prevalence of four different virulence gens coded by three pathogenicity Islands, namely, SPI-1, SPI-2, and SPI-5 among 54 *S. typhimurium* isolated in Iran. In the present study, presence of *invA* gene in *S. typhimurium* from human and poultry sources was 100%. Several reports in Iran as well as other countries about detection of inv*A* gene have confirmed that the gene was present in 100% of the *Salmonella* serovars samples (Zahraei et al., 2006; Amini et al., 2010; Jamshidi et al., 2009; Trafny et al., 2006; Nashwa et al., 2009). *invA* gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for invasion of dipper tissues (Khan et al., 2000). Another study showed that *S. typhimurium* serovars carrying mutation in *invA* genes are unable to selectively invade and destroy M cells in the follicle-associated epithelium of murine peyer's patches (Nashwa et al., 2009). Two genes of SPI-2 (*sseC* and *ssaP*) were targeted in this study. SseC was present in all the samples studied. This is similar to the study of Bhowmick et al. (2011) who detect *sseC* gene in 96% of samples among *Salmonella* serovars in India. Expression of *sseC* was upregulated during the intracellular phase in *S. typhimurium*. Paulin et al. (2007) showed sseC to be expressed by *S. typhimurium* macrophages 4 h after infection. Bhowmick et al. (2011) showed that deletion of the sseC gene in mutants resulted in loss of ability to survive intracellular in Hela cells, and that expression of this gene plays a crucial role in survival of *Salmonella* within the host cells. *ssaP* gene was detected in all of the isolated *S. typhimurium* in the present study. This is consistent with the study of Bhowmick et al. (2011) who detect *ssaP* gene in 100% of the samples drawn from seafood associated *Salmonella* isolates in India. *PipB* gene was detected in all of the isolated *S. typhimurium* cases in the present study. This is similar to the study of Bhowmick et al. (2011) who deteceted *pipB* gene in 100% samples of *Salmonella* isolates in India. *pipB* gene is a chicken host - specific colonization factor of *S. typhimurium*. This gene is targeted to detergent – resistant microdomains of intracellular membranes, which leads to the speculation of a possible interaction between pipB and host cell signaling molecules (Lesley et al., 2011). With multiplex PCR, multiple gene products can be amplified in a single PCR reaction and in this study it was shown as a rapid method to identification of four virulence associated genes. Such early detection can have major benefit in public health especially for rapid diagnosis and finding ideal vaccine, epidemiological investigations and prophylactic strategies for Salmonellosis in Iran. In sum, the findings of the present study show a high (100%) distribution of *invA*, *ssaP*, *sseC*, and *pipB* genes in human and poultry sources (Table 3). This necessitates planning and implementation of control programs to prevent and control infection.

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Table 3. Distribution of virulence genes found among *S. typhimurium* by multiplex PCR.

Percentage of prevalence	Gene	*S. typhimurium* (n-=54)
100	*invA*	54
100	*ssaP*	54
100	*sseC*	54
100	*pipB*	54

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