Original Article

Assessment of *stn*, *sipB* and *sopB* Virulence Genes in Various *Salmonella* Serovars

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ABSTRACT

Salmonella is a zoonotic bacterium that is considered to be one of the most common causes of foodborne infections worldwide. Bearing in mind the genes involved in its virulence, identifying these genes can enable experts to better understand bacterial pathogenicity, which could subsequently help develop more efficient means to control and prevent infections. This study aimed to analyze *stn*, *sipB*, and *sopB* genes in various *Salmonella* serovars. To carry out this study, 103 *Salmonella* serovars were extracted from livestock, poultry, and humans from existing samples at the Department of Microbiology of the Razi Serum and Vaccine Research Institute in Karaj, Iran. These samples were cultured in selection and differential media, and their serovars were identified using specific antibodies based on Kaufman-White Tables. Utilizing PCR and specific primers, *stn*, *sopB*, and *sipB* genes were detected among these serovars. In this investigation, the most common human serovars were *Salmonella paratyphi A*, *Salmonella paratyphi B*, and *Salmonella enteritidis*; the most common serovars among livestock consisted of *Salmonella dublin* and *Salmonella typhimurium* and the most common *Salmonella* serovars among poultry consisted of *Salmonella infantis* and *Salmonella enteritidis*. The results of PCR on *stn*, *sipB*, and *sopB* genes demonstrated segments with 617bp, 875 bp, and 220 bp on agar gel, respectively. Based on the obtained findings, *stn*, *sipB*, and *sopB* genes were detected in 96.11%, 99.02%, and 98.05% of *Salmonella* serovars, respectively. Considering the fact that the aforementioned genes play significant roles in bacterial virulence, they can be used to develop diagnostic ELISA kits and recombinant vaccines.

Keywords: *Salmonella*, *SipB* gene, *SopB* gene, *Stn* gene, Zoonosis

1. Introduction

Salmonella is a common cause of foodborne infections and is considered a major health problem worldwide (1). Based on reports published by the CDC in the United States, 40,000 cases of salmonellosis occur annually. Moreover, nontyphi salmonellosis has been reported to be the second most frequent type of foodborne infection in Europe. *Salmonella* infections are more common in countries with low sanitation standards with regards to the preparation and transportation of food and sewage disposal. Foodborne salmonellosis commonly occurs due to the consumption of meat products, fruits, and vegetables (2). Although more than 250 *Salmonella* serovars have been identified, infections in humans are caused by a limited number of serovars. *Salmonella typhimurium* and *Salmonella enteritidis* are two of the most frequently encountered serovars. Various serovars have shown a high capability to colonize in various animal hosts. Many of these serovars have a wide variety of animal hosts, whereas some, especially those causing illnesses among humans, have a limited number of hosts. The factors involved in bacterial virulence and colonization in hosts are complicated, and some of these factors include bacterial genes, gene expression, the level of the host's immunity, bacterial response to immune systems, the host's environment, and interaction with intestinal microflora (3, 4).

Salmonella's virulence largely depends on its ability to invade its host and survive within the host's cells (1). Several genes are involved in creating a disease in the host, most of which are located in specific parts called pathogenicity islands in the bacterial chromosome (5). These pathogenicity islands are the result of bacterial evolution, and there are five major islands that play a role in virulence (6). The *sopB* gene is situated on pathogenicity islands 5, also known as SPI5 (7). This gene encodes a 62 kDa protein, which induces the rearrangement of the actin cytoskeleton and membrane ruffling (7, 8). Additionally, it aids in the internalization of bacteria (8). The *Salmonella* pathogenicity island 1 (SPI1) encodes a set of invasion effectors, among which the Sips (*Salmonella* invasion proteins A-D) can be found (9). In the process of bacterial invasion, SipB protein plays a crucial role (10). Its remarkable resemblance to IpaB highly indicates potential to trigger apoptosis (10). As a result, this protein is considered a strong contender for inducing the process of programmed cell death (10). The *stn* gene of *Salmonella* plays a significant role in the organism's virulence (11). As with other enterotoxins that cause an increase in cAMP levels, *stn* stimulates a similar response (12). This response leads to an increase in the synthesis and release of prostaglandins, which may also contribute to the organism's enterotoxic response (12). In addition to the SPI genes, virulence plasmids, adhesins, flagella, and proteins linked to biofilm have important roles in pathogenicity and bacterial survival in host cells (13).

Considering the zoonotic nature of infection, the danger posed to human health and financial burden placed on health systems around the world to combat infections, prompt diagnosis and treatment is of the utmost importance. Furthermore, diagnostic methods based on bacterial culture and extraction are time-consuming; therefore, developing methods to detect *Salmonella* in a rapid manner is a crucial part of disease prevention, particularly during epidemics. Nowadays, molecular methods are considered the most rapid and sensitive means of diagnosis, and PCR is one of the most common among them. Bearing in mind the significance of certain genes in bacterial virulence, identifying them could be considered an important step towards understanding the pathogenic process, and, subsequently, developing better means to control and prevent disease. In this particular study, the abundance of three genes, specifically *stn*, *sipB*, and *sopB* in 103 *Salmonella* serovars isolated from livestock, poultry and humans was analysed using PCR and Multiplex PCR.

2. Materials and Methods

2.1. Culture and collection of serovars

In this study, 103 *Salmonella* serovars were analysed at the Microbiology Department of the Razi Serum and Vaccine Institute in Karaj. They consisted of 30 serovars from livestock, 30 from poultry, and 43 from humans (lyophilized or freeze-dried at -70°C). To culture the lyophilized samples, 10% horse serum was initially added to the TSB culture medium, and 2 ml of the mixture were added to the vials containing bacteria. The obtained suspension was cultured on blood, nutrient, and MacConkey agars. The plates were then placed inside an incubator at a temperature of 37°C for 24 h. The samples that had been freeze-dried at -70°C were cultured in the mentioned media and incubated at 37°C. Biochemical tests, including IMViC, TSI, catalase, oxidase, urease, and lysin were used to confirm *Salmonella*.

2.2. Serotyping of the samples

Salmonella serovars were identified based on H, O, and Vi antigens according to Kaufman-White guidelines. In this process, rapid slide agglutination with polyvalent antisera was used to confirm bacterial serology. Then, bacterial serogroups and serovars were determined using O and H antisera (phases 1 and 2), respectively.

2.3. DNA extraction

After performing confirmatory tests, bacterial precipitate was obtained by culturing bacteria overnight in nutrient agar, and was later used to extract DNA via boiling (14). Furthermore, after DNA was extracted, it was quantitatively assessed using spectrophotometry and qualitatively analysed by electrophoresis on gel agar.

2.4. Detection of virulence genes in serovars

Genotyping and identifying virulence genes *stn*, *sipB*, and *sopB* in the serovars under investigation was performed using PCR. In this process, 6 microliters of the master mix, one microliter of each of the forward and reverse primers (Table 1) with a concentration of 10 pmol and 2 microliters of template DNA with a concentration of 100 ng were combined, and double-distilled water was added to this combination to obtain a volume of 12 microliters. The PCR thermal cycles consisted of denaturation at 94 degrees, annealing temperatures according to the Tm of each primer, and expansion at 72 degrees centigrade. The end product of PCR was placed on agar gel, and the existence of specific bands in the serovars was assessed after electrophoresis.

Table 1. Primer sequences used in this research

Gene	Primer Sequences	Fragment Length	Reference
Stn	F: TTGTGTCGCTATCACTGGCAACC R: ATTCGTAACCCGCTCTCGTCC	617 bp	**(18)**
sopB	F: CGGACCGGCCAGCAACAAAACAAGAAGAAG R: TAGTGATGCCCGTTATGCGTGAGTGTATT	220 bp	**(25)**
sipB	F: GGACGCCGCCCGGGAAAAACTCTC R: ACACTCCCGTCGCCGCCTTCACAA	875 bp	**(29)**

2.5. Multiplex PCR

To carry out Multiplex PCR, 25 microliters of master mix, 1 microliter of each primer, and 3 microliters of template DNA were initially added to double-distilled water to reach a combined volume of 50 microliters. The Multiplex PCR protocol consisted of the following steps: (i) a host start step of 5 min at 94°C; (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C; and (iii) a final extension step of 10 min at 72°C.). The final product of Multiplex PCR was placed on agar gel, and bands were assessed after electrophoresis.

3. Results

3.1. Biochemical results in *Salmonella* serovars

In this study, 103 serovars were analysed, consisting

of 43 human serovars, 30 serovars from livestock, and 30 from poultry. The VP, indole, urease and lactase tests were negative in all samples, whereas the MR test was positive in all of them. Around 97.8% of serovars were lysin positive. Moreover, 89.32% of serovars changed the colour to blue in the citrate test, and 97.08% of serovars produced H2S in the TSI test.

3.2. Serotyping

Serotyping was conducted using slide agglutination with O and H antigens (phases 1 and 2); the serovars assessed are shown in Table 2.

Table 2. Serovars studied in this research

Number	Poultry serovars	No.	Number	Livestock Serovars	No.	Number	Human Serovars	No.
7	*Salmonella infantis*	1	4	*Salmonella typhimurium*	1	8	*Salmonella paratyphi A*	1
4	*Salmonella enteritidis*	2	4	*Salmonella dublin*	2	8	*Salmonella enteritidis*	2
2	*Salmonella typhimurium*	3	3	*Salmonella enteritidis*	3	7	*Salmonella paratyphi B*	3
2	*Salmonella muenchen*	4	2	*Salmonella abortusovis*	4	4	*Salmonella typhi*	4
2	*Salmonella rostock*	5	2	*Salmonella tsevie*	5	4	*Salmonella typhimurium*	5
1	*Salmonella arizonae*	6	1	*Salmonella sandow*	6	3	*Salmonella infantis*	6
1	*Salmonella blegdam*	7	1	*Salmonella anatum*	7	2	*Salmonella Jaffna*	7
1	*Salmonella havana*	8	1	*Salmonella duesseldorf*	8	2	*Salmonella nigeria*	8
1	*Salmonella mbandaka*	9	1	*Salmonella derby*	9	2	*Salmonella arizonae*	9
1	*Salmonella moscow*	10	1	*Salmonella eastbourne*	10	2	*Salmonella blegdam*	10
1	*Salmonella tinda*	11	1	*Salmonella cubana*	11	1	*Salmonella agona*	11
1	*Salmonella hessarak*	12	1	*Salmonella augustenborg*	12	43		Total
1	*Salmonella montevideo*	13	1	*Salmonella infantis*	13			
1	*Salmonella colindale*	14	1	*Salmonella aberdeen*	14			
1	*Salmonella kuilsrivier*	15	1	*Salmonella paratyphi B*	15			
1	*Salmonella mjimwema*	16	1	*Salmonella ndolo*	16			
1	*Salmonella adeliade*	17	1	*Salmonella daytona*	17			
30		Total	1	*Salmonella adelaide*	18			
			1	*Salmonella bovismorbificans*	19			
			1	*Salmonella newport*	20			
			30		Total			

3.3. PCR results regarding virulence genes

Based on PCR on the *stn* gene, the 617 bp band was observed in all 43(100 %) human serovars, 27(90%) out of 30 serovars from livestock and 29(96.6%) out of 30 serovars from poultry. In total, 96.11% of samples had this gene. The *sopB* gene, consisting of 220 bp, was detected in 43(100%) human serovars, 30(100%) serovars from poultry and 28(93.3%) out of 30 serovars from livestock. In total, 98.05% of the serovars possessed this gene. Regarding the *sipB* gene, which consisted of 875 bp, 43(100%) human serovars, 30(100%) serovars from poultry and

29(96.6%) out of 30 serovars from livestock possessed this gene. Overall, 99.02% of the serovars in the study had this particular gene (Figure 1).

3.4. Results of Multiplex PCR

To obtain more rapid results, multiplex PCR was performed. The results of Multiplex PCR on the products of electrophoresis are shown in Figure 2. As observed in the figure, sharp bands were observed for each of the three genes using this method.

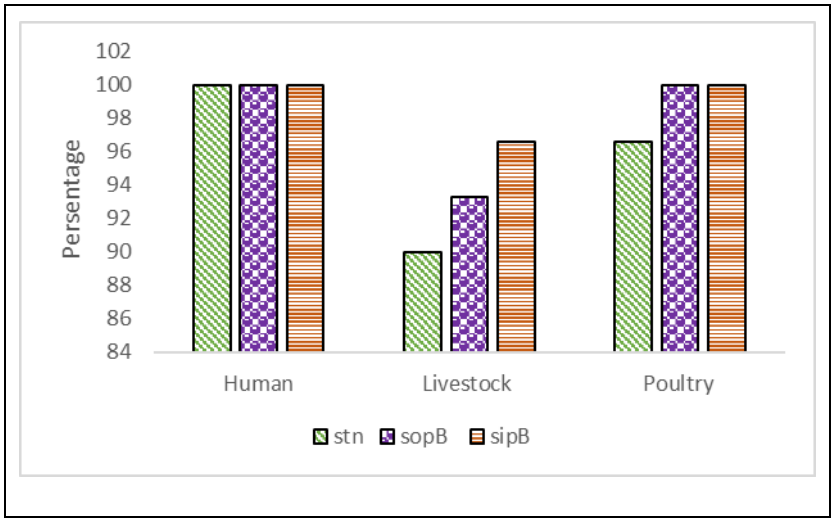

Figure 1. Assessment of the presence of virulence genes *stn, sopB,* and *sipB* in the *Salmonella* serovars under investigation based on sources

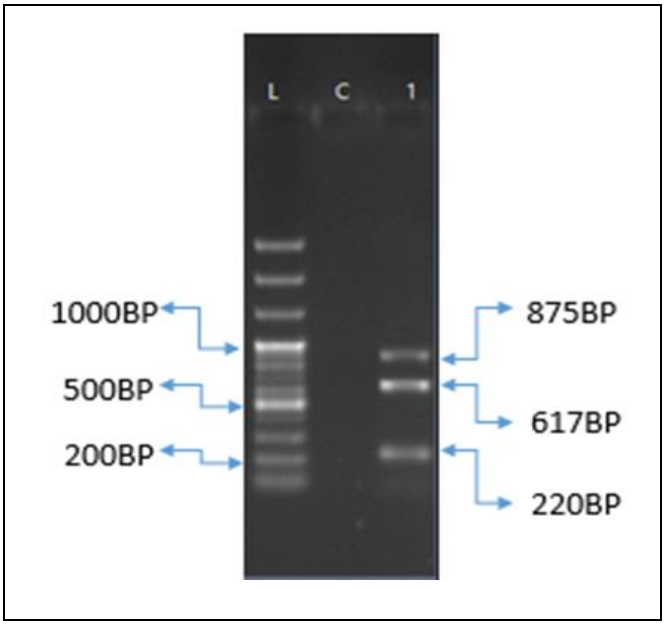

Figure 2. Results obtained by electrophoresis of Multiplex PCR products for the virulence genes *stn*, *sopB* and *sipB*

4. Discussion

Salmonella is a prevalent food-borne pathogen that continues to pose a significant public health concern worldwide (15). The infection can manifest in human subjects as typhoid fever, gastroenteritis, bacteremia, and other associated extraintestinal complications (15). Given the presence of *Salmonella* in a variety of sources

and its role as a zoonotic disease, timely diagnosis is crucial (16). Traditional serotyping techniques necessitate specialized knowledge and a range of antisera (17). Due to the time-consuming nature of traditional methods, such as culture and isolation, it has become necessary to use rapid techniques to identify the presence of *Salmonella* in suspected samples (16).This is essential for preventing the spread of the disease and potential epidemics (16). On the other hand, molecular techniques, such as PCR, afford a rapid, cost-effective, specific, and remarkably sensitive methodology, effectively overcoming their limitations (16). This study aimed to investigate the presence of three virulence genes, namely *sopB*, *stn*, and *sipB*, in multiple *Salmonella* serovars. In this study, the frequency of three genes (*stn*, *sopB*, and *sipB*) in 103 *Salmonella* serovars from humans, poultry, and livestock was assessed, and the results demonstrated that *stn*, *sipB*, and *sopB* could be detected in 96.11%, 99.02%, and 98.05% of *Salmonella* serovars, respectively.

The *stn* gene is frequently detected in various strains of *Salmonella* and codes the enterotoxin protein. Other researchers have shown that this particular gene is preserved in various *Salmonella* serotypes (11). In a study conducted by Naik et al. to detect the virulent *stn* gene in goats and chickens in 2015, 32 isolates were identified, among which 100% possessed this particular gene. The results obtained in various studies showed that the *stn* gene is frequently detected in *Salmonella* isolates regardless of their source (18). In this study, 29 isolates out of 30 from poultry and 27 isolates out of 30 from livestock possessed *stn*, which was consistent with the results of previous studies. Another study conducted by Chaudhary et al. investigated the virulence genes in *Salmonella* serovars from pigs in 2015. Their results showed that from a total of 270 samples, *Salmonella enteritides* and *typhimurium* were detected in 37 samples. Furthermore, all 37 samples possessed *stn*, which signifies the importance of this specific gene in causing gastroenteritis in humans and also highlights the fact that it could be used to detect salmonellosis (19). Our results showed that 27 out of 30 serovars from livestock possessed *stn* and were consistent with the results of the mentioned study. Muthu et al. carried out a research to detect the *stn* gene in isolates of three different strains of *Salmonella* in humans in 2014. Overall, although this gene was present in 79.5% of the strains, the 2 *typhimurium* strains in their study lacked *stn* (20). Our study revealed that this gene was present in 100% of serovars from humans, regardless of the type of serovar; therefore, our results do not contradict their findings. Another investigation was performed by Makwana et al. with the objective of serotyping bacteria and detecting genes involved in virulence. A total of 284 samples of meat were assessed, and 13 *Salmonella* isolates were obtained, all of which possessed the *stn* gene (21). Overall, it appears that *stn* is an important factor in bacterial virulence, regardless of the source and serovars, and that it can commonly be detected.

The *sopB* gene codes a protein with inositol phosphate phosphatase activity, which is transported via a SPI-1 dependent pathway inside host cells (8, 22). This specific protein causes the secretion of chloride ions and leads to diarrhea in the host (7). Osman et al. conducted a study to identify the integrons and gene cassettes responsible for drug resistance among multiple drug resistant serovars isolated from humans in Egypt in 2014. The *SopB* was detected in all the *Salmonella* isolates, which was similar to the findings of the present study (23). Elemfareji et al. carried out a study to identify virulence genes among *Salmonella typhi* and nontyphi strains in 2013, and the results demonstrated the presence of the *sopB* gene in all the *Salmonella typhi* and *enteritidis* strains, regardless of the source (24). In our study, the *sopB* gene was detected in 101 serovars, regardless of the source, which is consistent with their findings. Mezal et al. carried out an investigation to detect *Salmonella enteritidis* in poultry in 2014. In this study, 60 samples were assessed in terms of antibiotic resistance and virulence genes, and all isolated were found to possess *sopB* and *sipB* (25). In

our study, 14 out of 15 samples of *Salmonella enteritidis* had *sopB* and *sipB*, which was consistent with the results obtained by this group of researchers. Rasha et al. conducted a study to investigate the virulence genes in nontyphi *Salmonella* strains in 2015. Their results showed the presence of *sopB* in 5 strains isolated from chickens and humans. *Salmonella typhimurium* also possessed the *stn* gene (26). In the present study, the results of PCR for *sopB* and *stn* genes in nontyphi isolates (in humans and poultry) showed that *sopB* was present in all serovars, and that only one of the serovars from poultry lacked *stn*, which was in contradiction with the results obtained by this particular group of researchers.

The *sipB* gene codes a protein responsible for the invasion of host cells. This specific protein is necessary for the transport of other proteins, and in addition to this role, it possesses effector activity in pathogenicity (10). Kim et al. performed a study to assess the molecular characteristics of drug resistant nontyphi *Salmonella* isolates from chickens in Korea in 2017 (27). Their results revealed the presence of *sopB* and *sipB* in all serovars isolated from poultry, which was consistent with our findings. Beshiru et al. conducted a study to investigate virulence genes in *Salmonella* serovars from shrimps in 2019, the results of which revealed the presence of *sipB* in 78.6% of isolates (28). In our study, 99.02% of all serovars possessed *sipB*, which was in contradiction to the findings of this group of researchers. Ed-Dra et al. carried-out research to assess antibiotic resistance and detect virulence genes in *Salmonella* strains. Their findings demonstrated that all isolates possessed *sipB* and were resistant to at least one antibiotic (29). In the present study, *sipB* was detected in 102 out of 103 serovars (regardless of the source); therefore, our findings were consistent with those reported by the mentioned group of investigators.

A study was conducted by Najafi et al. to identify *cmlA/tetR*, bla_{TEM}, bla_{PSE-1}, and *sipB* in *Salmonella* strains in Iran in 2017. In this particular investigation, 163 clinical samples from patients who had been referred to hospital were collected, among which 48 cases of *Salmonella* were detected. Out of these 48 samples, 25 had *Salmonella enteritidis*, while 14 had *Salmonella typhimurium*, and 9 had *Salmonella infantis*. The M-PCR results demonstrated that 16.6% of isolates had *sipB (30)*. In our study, all 43 isolates from humans possessed *sipB*, which was contradictory to the results obtained by this group of researchers.

In summary, the findings of this study revealed the presence of *stn*, *sopB*, and *sipB* in various *Salmonella* serovars. The frequency of these in different studies has been varied, which could have been due to different study regions, various sources or differences in terms of serovars. Having said that, one noteworthy fact is the high prevalence of these specific genes, and this could be taken into consideration when adopting policies to fight salmonellosis. Our study results revealed that *stn*, *sopB*, and *sipB* were present in all isolates from humans. Moreover, *sopB* and *sipB* were detected in all isolates from poultry, whereas *stn* was detected in 96.6% of these isolates. Among isolates from livestock, *stn* was present in 90%, while *sopB* and *sipB* were detected in 93.33% and 96.66%, respectively. Based on our findings, these genes can be detected in *Salmonella* isolates regardless of the source. All *Salmonella typhi* serovars (4 isolates), *typhimurium* serovars (10 isolates), *paratyphi A* serovars (8 isolates), *paratyphi B* serovars (8 isolates), and *infantis* serovars (10 isolates) in this investigation possessed *stn*, *sopB*, and *sipB*, which showed the presence of these genes in various isolates, regardless of serovars. Considering the significance of these genes in bacterial virulence, their presence in serovars isolated from different sources and the importance of detecting them in understanding pathogenicity and potentially preventing disease, they could be used for molecular techniques such as PCR.

Authors' Contribution

This article was the result of M. J. S.'s master's thesis, and S. M. B. was the supervisor and P. K. was the advisor.

Ethics

The authors declare all ethical considerations were respected in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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