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Prevalence of Virulence Genes in Non-typhoidal *Salmonella* Isolates from Layers in Pantnagar, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study determined the prevalence of virulence genes in 23 non-typhoidal *Salmonella* (NTS) isolates obtained from layer breeds of an organized poultry farm in Pantnagar, India. Eight virulence genes *viz. sipA, sopE1, fli*C, *mgtC, spvC*, *gipA, sopB,* and *stn* were detected using the PCR technique. The most prevalent gene was *sipA* (95.65%), followed by *sopB* (73.91%), *sopE1* (60.87%), *stn* (56.52%), *fliC* (47.83%), *mgtC* (30.43%), *spvC* (13.04%) and *gipA* (13.04%). Among all the isolates, one isolate didn't show the presence of any virulence genes and none of the isolates harbored all the virulence genes screened. This study shows the high prevalence of virulence genes in NTS strains isolated from layers. Thus, continuous monitoring and surveillance of non-typhoidal *Salmonella* for virulence and its potential risks to humans and animals must be carried out.

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1. INTRODUCTION

Serotypes other than the typhoidal (Typhi) and paratyphoidal (Paratyphi A, B and C, and Sendai) serotypes of *Salmonella enterica* subspecies *enterica* are known as non-typhoidal *Salmonella* (NTS) [1]. NTS is a major public health hazard worldwide. Zoonotic *Salmonella* infections yearly result in 93.8 million cases of gastroenteritis and 155,000 fatalities [2]. People of all ages can get a variety of ailments from NTS, such as self-limiting gastroenteritis, which is characterized by vomiting, diarrhea, and abdominal pain. On the other hand, complicated extraintestinal illness, bacteremia, meningitis, and severe invasive disease can all occur in children, the elderly, and people with compromised immune systems [3,4]. Salmonellosis in humans is primarily transmitted by eating infected poultry products, such as meat and eggs [5]. Furthermore, several potential sites of disease transmission have been identified, such as contaminated poultry environments and faeces [6].

Pathogenicity of *Salmonella* has been associated with several virulence genes that involve a combination of chromosomal and plasmid factors [7]. These virulence genes encode products that facilitate NTS contact with the host cells [8] which in turn causes the characteristic symptoms of salmonellosis [9]. These genes induce colonization, immune evasion, inflammatory induction, host cell invasion, and host adaptability [10]. Large gene clusters called *Salmonella* pathogenicity islands (SPIs) are found within the chromosomes of *Salmonella,* which cause the bacteria to invade host cells [11]. About 21 SPIs have been found for *Salmonella*, and for *Salmonella* Typhimurium, about 12 SPIs with various virulence factors are known [12]. Acquisition of novel genes through horizontal gene transfer has contributed to the evolution of pathogenicity in *Salmonella* and thus, increases its ability to infect numerous hosts with greater severity [13].

To better understand *Salmonella*'s ability to cause disease, several researchers indicate that the identification of virulence genes in *Salmonella* typing is important [14,15]. Various genes have been identified as important virulence factors in *Salmonella* coding invasion (*sipA* and *sopE1*), motility (*fliC*), survival (*mgtC* and *spvC*), growth (*spvC*), enterotoxin (*stn*), and pathogenic processes (*sopB* and *gipA*). However, the type and rate of acquisition of these genes vary depending on various criteria such as serotype, source, and geographical location of the isolates. Hence, frequent monitoring is necessary to better understand the progression of *Salmonella* pathogenicity [16]. Therefore, this study was undertaken to detect different virulence genes in non-typhoidal *Salmonella* isolates obtained from different layer breeds of an organized poultry farm in Pantnagar, India.

2. MATERIALS AND METHODS

2.1 *Salmonella* isolates

The study was conducted on 23 isolates of *Salmonella* previously isolated [17] from different layer breeds of an organized poultry farm in Pantnagar town of Uttarakhand State in India. The isolates consisted of *Salmonella* Typhimurium (n=21) and untypable strains (n=2) obtained from different samples *viz.* water (n=7), poultry faeces (n=6), caecal content (n=3), litter (n=4), feed (n=2) and egg surface (n=1) of layer breeds *viz.* Rhode Island Red (n=16), Uttara fowl (n=6), and Kadaknath (n=1). All the isolates were revived in nutrient broth (HiMedia, India) from the respective 20% glycerol stock kept at −80°C. After 24 hours of incubation at 37°C, cultures were streaked on XLT-4 agar (BD Difco, USA) and confirmed again for *Salmonella*.

2.2 Detection of Virulence Genes

All *Salmonella* isolates (n=23) were screened for eight virulence genes encoding various virulence mechanisms using PCR. Virulence genes screened were *sipA* [18], *sopE1, mgtC, spvC*, *gipA* [14], *fliC* [19], *sopB* [20] and *stn* [21]. Primer sequences, functional attributes, and amplicon size were used as described in Table 1. DNA was extracted using the thermal lysis method [22] with some modifications. Briefly, a loopful of culture from XLT-4 agar was mixed into 0·1 ml nuclease-free water (HiMedia, India) in sterile 1.5 ml Eppendorf tubes to obtain a turbid suspension of bacteria. The bacterial suspension was kept in a boiling water bath for 10 min, immediately cooled at −20°C for 15 min, and centrifuged at 12000g for 5 min. The supernatant containing genomic DNA was collected in a new tube and used as a DNA template for PCR reactions. The reaction mixture of 25 µl was set up for each gene containing 2.5 µl of 10X Taq buffer (Tris HCl with 15 mM $MgCl_2$) (GeNei, India), 1 µl of

Table 1. Details of the primers used for the detection of virulence genes

Gene	Functional attribute	Primer sequence (5' to 3')	Amplicon size (bp)	Reference
sipA	Invasion (actin-cytoskeletal dismantle)	F: TTCGACTAACAGCAGCA R: CCGTCGTACCGGCTTTATTA	449	18
sopE1	Invasion (encodes an effector protein)	F: CGGGCAGTGTTGACAAATAAAG R: TGTTGGAATTGCTGTGGAGTC	455	14
fliC	Motility (formation of flagella)	F: CGGTGTTGCCCAGGTTGGTAAT R: ACTGGTAAAGATGGCT	620	19
mgtC	Intramacrophage survival	F: TGACTATCAATGCTCCAGTGAAT R: ATTTACTGGCCGCTATGCTGTTG	677	14
spvC	Rapid growth and survival	F: ACTCCTTGCACAACCAAATGCGGA R: TGTCTTCTGCATTTCGCCACC	572	14
stn	Enterotoxin production	F: TTGTGTCGCTATCACTGGCAACC R: ATTCGTAACCCGCTCTCGTCC	617	21
sopB	Encodes an effector protein for pathogenesis	F: CGGACCGGCCAGCAACAAAACAAG R: TAGTGATGCCCGTTATGCGTGAGTGTATT	220	20
gipA	Peyer's patch-specific pathogenesis	F: ACGACTGAGCAGGCTGAG R: TTGGAAATGGTGACGGTAGAC	422	14

Table 2. PCR cycling conditions employed for different virulence genes

PCR steps	PCR conditions	Virulence genes							
		sipA	***sopE1***	***fliC***	***mgtC***	***spvC***	***stn***	***sopB***	***gipA***
Early Denaturation	Temperature (℃)	95	95	94	95	95	95	95	95
	Time (min)	5	1	5	1	1	3	3	1
Denaturation	Temperature (℃)	95	95	94	95	95	95	95	95
	Time (min)	0.5	0.5	0.5	0.5	0.5	1	1	0.5
Annealing	Temperature (℃)	55	58	55	58	58	57	57	58
	Time (min)	0.5	0.5	0.5	0.5	0.5	1	1	0.5
Extension	Temperature (℃)	72	72	72	72	72	72	72	72
	Time (min)	2	0.5	0.5	0.5	0.5	2	2	0.5
Final Extension	Temperature (℃)	72	72	72	72	72	72	72	72
	Time (min)	5	4	7	4	4	10	10	4
Number of cycles		35	35	30	35	30	30	30	35

each dNTP (2.5 mM) (GeNei, India), 1 µl of each primer (10 pmol) (IDT, India), 1U Taq DNA polymerase (GeNei, India) and 2 µl of DNA template (50-100 ng per µl). Nuclease-free water was added to make the final volume. Cycling conditions for PCR including annealing temperature for different genes were used as described in Table 2. Amplified PCR products were electrophoresed on 1.5% agarose gel visualized over a gel documentation system.

3. RESULTS AND DISCUSSION

Salmonella organism possesses certain genetic determinants to invade, proliferate, and survive in a host cell which are responsible for its virulence. All 23 isolates were screened for the presence of eight virulence genes of which *sipA* (22/23; 95.65%) was the most prevalent gene found followed by *sopB* (17/23; 73.91%), *sopE1* (14/23; 60.87%), *stn* (13/23; 56.52%),

fliC (11/23; 47.83%), *mgtC* (7/23; 30.43%), *spvC* (3/23; 13.04%) and *gipA* (3/23; 13.04%) (Fig. 1). One isolate (S-1) was found to be avirulent, i.e., devoid of any virulence genes screened. None of the isolates harbored all the eight virulence genes elucidated during this study. A maximum of 7 virulence genes were recorded in an isolate. The distribution of virulence genes in the *Salmonella* isolates examined is shown in Table 3.

Table 3. Distribution of virulence genes in tested *Salmonella* isolates (n=23)

Isolate Id.	Layer Breed	Sample type	Virulence genes								Total positive	Serotype
			sipA	***sopE1***	***fliC***	***mgtC***	***spvC***	***gipA***	***sopB***	***stn***		
S-1	Rhode Island Red	Egg surface	-	-	-	-	-	-	-	-	0	Typhimurium
S-2	Uttara fowl	Feed	+	-	-	-	-	-	+	+	3	Typhimurium
S-3	Uttara fowl	Feed	+	-	-	-	-	-	+	+	3	Typhimurium
S-4	Uttara fowl	Faeces	+	-	-	+	-	-	+	+	4	Typhimurium
S-5	Uttara fowl	Faeces	+	+	-	+	-	-	+	+	5	Untypable
S-6	Kadaknath	Caecal content	+	-	+	-	+	+	+	+	6	Untypable
S-7	Uttara fowl	Caecal content	+	-	+	+	+	+	+	+	7	Typhimurium
S-8	Uttara fowl	Caeca content	+	-	-	-	+	+	+	-	4	Typhimurium
S-9	Rhode Island Red	Water	+	+	+	-	-	-	+	+	5	Typhimurium
S-10	Rhode Island Red	Water	+	+	-	-	-	-	-	-	2	Typhimurium
S-11	Rhode Island Red	Water	+	+	+	-	-	-	+	+	5	Typhimurium
S-12	Rhode Island Red	Water	+	+	-	-	-	-	-	-	2	Typhimurium
S-13	Rhode Island Red	Water	+	+	-	-	-	-	+	-	3	Typhimurium
S-14	Rhode Island Red	Water	+	+	+	-	-	-	+	+	5	Typhimurium
S-15	Rhode Island Red	Water	+	+	+	-	-	-	+	+	5	Typhimurium
S-16	Rhode Island Red	Litter	+	+	+	-	-	-	+	-	4	Typhimurium
S-17	Rhode Island Red	Litter	+	-	-	-	-	-	-	-	1	Typhimurium
S-18	Rhode Island Red	Litter	+	+	-	+	-	-	-	-	3	Typhimurium
S-19	Rhode Island Red	Litter	+	+	+	+	-	-	+	-	5	Typhimurium
S-20	Rhode Island Red	Faeces	+	+	+	-	-	-	+	+	5	Typhimurium
S-21	Rhode Island Red	Faeces	+	+	-	+	-	-	-	-	3	Typhimurium
S-22	Rhode Island Red	Faeces	+	+	+	+	-	-	+	+	6	Typhimurium
S-23	Rhode Island Red	Faeces	+	-	+	-	-	-	+	+	4	Typhimurium

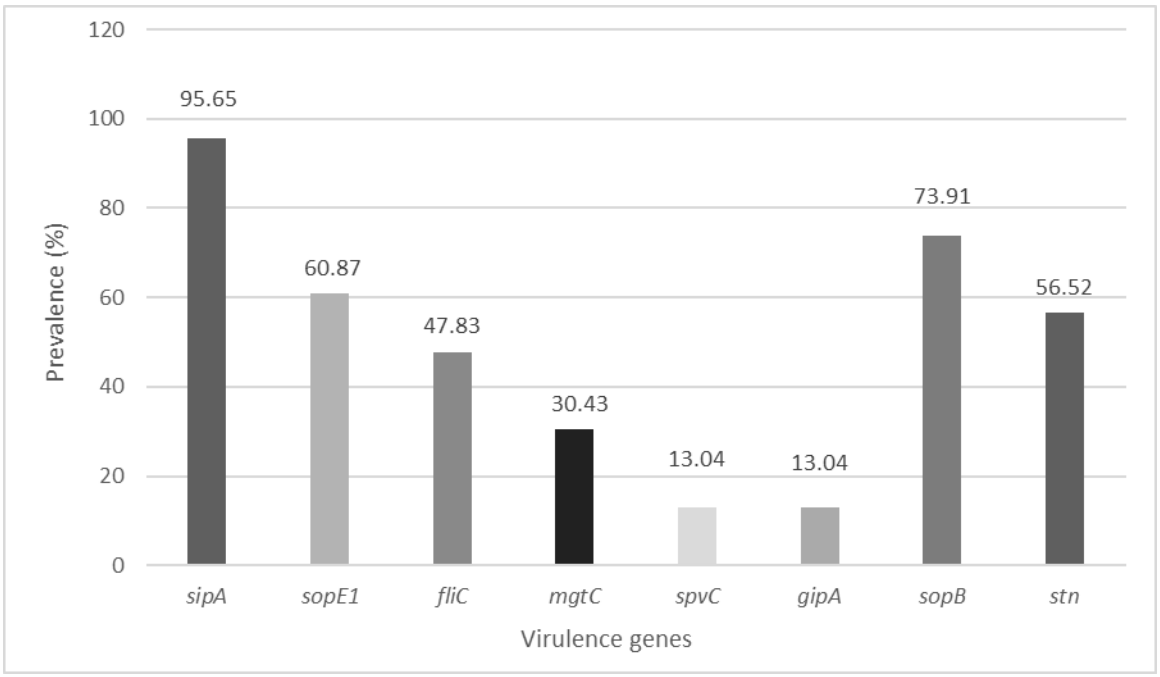

Fig. 1. Prevalence of virulence genes in the *Salmonella* isolates (n=23)

The *sipA* gene encoding for *Salmonella* invasion protein A induces apoptosis in macrophages and plays a vital role in *Salmonella* pathogenesis [23]. The *sipA* gene was detected in the majority of *S.* Typhimurium (95.24%; 20/21) and two untypable isolates. Sharma et al., [24] reported this gene in all the Typhimurium isolates. Shekhar and Singh [25] found the presence in 85.71% of *Salmonella* isolates. Thus, the presence of this gene in the *Salmonella* isolates confirms majority possesses the ability to invade and colonize the host intestine. The SopB protein is an inositide phosphate phosphatase, one of the effector proteins responsible for the induction of macropinocytosis [26]. The gene *sopB* was identified in 71.43% (15/21) of the *S*. Typhimurium and two untypable isolates. Different studies reported this gene to be commonly expressed by Typhimurium isolates. A presence of 44.44%, 60%, 96.7%, and 100% have been observed to harbor this gene by Ammar et al., [27], Sharma et al., [24], Ahmed et al., [28] and Borges et al., [29], respectively. Thus, the data obtained indicates the extensive distribution of *sopB* gene among *Salmonella* isolates that leads to diarrhoea.

The *sopE1* gene encodes an effector protein that facilitates *Salmonella* to invade the host cell by restructuring the cell's actin cytoskeleton and membranes and activates certain receptors that lead to the production of pro-inflammatory cytokines in the host cell [12]. This gene was found in 61.9% (13/21) of *S.* Typhimurium and one untypable isolate. This finding is in agreement with Borges et al., [29] and Sharma et al., [24] who reported 62.5% and 60% of Typhimurium isolates to exhibit the *sopE1* gene, respectively. Contrary to this, Osman et al., [30] found no recovered *Salmonella* isolates to carry this gene. The *stn* gene in *Salmonella* is one of the chromosomally encoded genes that codes for the production of enterotoxins [23]. This gene was found in 52.38% (11/21) of *S*. Typhimurium and two untypable isolates designating the enterotoxigenic property in the isolates. Sharma et al., [24] reported the presence of the gene in 60% of *S.* Typhimurium which is in agreement with the present study. The high presence of *stn* gene in Typhimurium isolates (80.95%) was found by Shekhar and Singh [25]. Many other reports have found the *stn* gene in all the tested Typhimurium isolates [21,31,32]. In contrast, no *stn* gene was recovered in any of the *Salmonella* isolates from chicken by Gharieb et al., [9].

The *fliC* gene encodes for the flagellin subunit protein that forms a major component of the bacterial flagellum and plays a significant role in the motility of *S.* Typhimurium organism [33]. This gene was observed in 47.62% (10/21) of

S. Typhimurium and one untypable isolate. Jamshidi et al., [34] reported its presence in all the examined Typhimurium isolates. On the contrary, Sharma et al., [24] could not detect its presence in any of the Typhimurium isolates tested. The MgtC virulence protein encoded by the mgtCB operon (located in SPI-3) aids in the survival of *Salmonella* inside macrophages [35]. The present study revealed the presence of the *mgtC* gene in 7 (30.43%) *Salmonella* isolates that consisted of 6 (28.57%) *S.* Typhimurium and one untypable isolate. On the contrary, Ahmed et al., [28], and Sharma et al., [24] reported the presence of the gene in all Typhimurium isolates examined.

The *spv* (*Salmonella* plasmid virulence) operon consists of 5 genes i.e., *spvRABCD* which helps in the systemic spread and replication of *Salmonella* organisms [36]. This study detected the *spvC* gene in 9.52% (2/21) *S*. Typhimurium and one untypable isolate. A similar finding was reported by Capuano et al., [37] who detected the gene in 10.2% of the isolates. On the contrary, Yang et al., [38] observed this gene in 65.5% of isolates whereas, Ammar et al., [27] revealed the absence of the gene in all Typhimurium isolates. The gene *gipA* is a Peyer's patch-specific virulence factor encoded by phage Gifsy-1, which is induced specifically in the small intestine. This gene plays a role in the initial colonization, invasion, survival, and dissemination of *S.* Typhimurium during its pathogenic course [39]. The absence of the *gipA* gene was detected in Typhimurium and untypable isolates by Osman et al., [30] unlike our study which showed *S*. Typhimurium (9.52%; 2/21) and one untypable isolate bearing the gene. In contradiction with the present finding, Huehn et al., [14], and Yang et al., [38] reported the presence of the *gipA* gene in 50%, 82.8%, and 100% of *S.* Typhimurium isolates, respectively. Hence, a wide difference in the presence of the *gipA* gene in *Salmonella* isolates has been observed.

4. CONCLUSION

The findings of this study reflect the high prevalence of virulence genes in the recovered *Salmonella* isolates from layers that majorly involve *S*. Typhimurium, pointing to a potential threat to humans and animals. Most isolates in this investigation included numerous virulence genes in various combinations, suggesting their potential role as a pathogen in the host species. Thereby, continuous monitoring and surveillance of non-typhoidal *Salmonella* for virulence factors is required to assess its pathogenicity. In addition, several other recognized genes for virulence in NTS, which are not targeted in the study, can be evaluated to enhance future research.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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