

# Prevalence of Virulence Genes in Non-Typhoidal

## *Salmonella* Isolated from Layers

Comment [a1]: In Uttarakhand, India.

### ABSTRACT

**Aims:** This study determined the prevalence of virulence genes in 23 non-typhoidal *Salmonella* (NTS) isolates obtained from layer breeds of an organized poultry farm.

**Study design:** The study was conducted on *Salmonella* isolates previously isolated from different layer breeds.

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**Place and Duration of Study:** Department of Veterinary Public Health and Epidemiology, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, between July 2018–June 2019.

**Methodology:** Eight virulence genes viz. *sipA*, *sopE1*, *fliC*, *mgtC*, *spvC*, *gipA*, *sopB*, and *stn* were detected using the PCR technique.

**Results:** 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.

**Conclusion:** This study shows the 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.

**Keywords:** Prevalence; virulence genes; non-typhoidal *Salmonella*; layer breeds; PCR.

### 1. INTRODUCTION

Serotypes other than the typhoidal (Typhi) and paratyphoidal (Paratyphi A, B and C, and Sendai) serotype of *S. enterica* within this *S. enterica* subspecies are known as non-typhoidal *Salmonella* (NTS). Globally, non-typhoidal *Salmonella* (NTS) is a significant public health hazard.

Comment [a3]: Please add this sentence to make the introduction meaningful to a wider range of readers.

Every year, *Salmonella* infections cause 155,000 fatalities and 93.8 million instances of gastroenteritis

Comment [a4]: Insert the reference Sedrakyan, A.; Ktsoyan, Z.; Arakelova, K.; Gevorgyan, Z.; Zakharyan, M.; Hakobyan, S.; Hovhannisyanyan, A.; Arakelyan, A.; Aminov, R. Molecular Epidemiology and Virulence of Non-Typhoidal *Salmonella* in Armenia. Int. J. Mol. Sci. 2022, 23, 9330. <https://doi.org/10.3390/ijms23169330>

[1]. NTS causes a wide range of illnesses among people of all ages including self-limiting

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gastroenteritis characterized by diarrhoea, abdominal pain, and vomiting. However, children, the elderly, and individuals with impaired immune systems can experience severe invasive disease along with complex extraintestinal illness, bacteraemia, and meningitis [2,3]. Poultry has been identified as the primary source of human salmonellosis transmitted through ingesting contaminated poultry meat and eggs [4]. In addition, several possible transmission sources for this disease have been found, including contaminated poultry surroundings and faeces [5].

Pathogenicity of *Salmonella* has been associated with several virulence genes that involve a combination of chromosomal and plasmid factors [6]. These virulence genes encode products that facilitate NTS contact with the host cells [7] which in turn causes the characteristic symptoms of salmonellosis [8]. These genes cause colonization, immune evasion, inflammatory induction, host cell invasion, and host adaptability [9]. Large gene clusters called *Salmonella* pathogenicity islands (SPIs) are found within the chromosomes of *Salmonella*, which cause the bacteria to invade host cells [10]. For *Salmonella*, about 21 SPIs have been found, and for *Salmonella* Typhimurium, about 12 SPIs with various virulence factors are known [11]. Through the process of horizontal gene transfer, *Salmonella* has evolved to become more pathogenic, which makes it more capable of causing severe infections in a wider range of hosts [12].

To better understand *Salmonella*'s ability to cause disease, several researchers indicate that the identification of virulence genes in *Salmonella* typing is important [13,14]. Various genes have been identified as important virulence factors in *Salmonella* coding invasion (*sipA* and *sopE1*), motility (*fliC*), survival (*mgcC* 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 [15]. 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.

## **2. MATERIAL AND METHODS**

### ***Salmonella* isolates**

The study was conducted on 23 isolates of *Salmonella* previously isolated [16] from different layer breeds of an organized poultry farm in Pantnagar town of Uttarakhand State. 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*.

### 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* [17], *sopE1*, *mgtC*, *spvC*, *gipA* [13], *fliC* [18], *sopB* [19] and *stn* [20]. Primer sequences, functional attributes, and amplicon size were used as described earlier in respective references. DNA was extracted using the thermal lysis method [21] 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<sub>2</sub>) (GeNei, India), 1 µl of 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 earlier in respective references. 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 1.

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Comment [a7]: If you provided the target gene, sequence, amplicon size (bp), thermal profile, and reference in a tabulated form, it would be useful for beginners in this technology to learn from online literature.

Comment [a8]: Please give the reference.

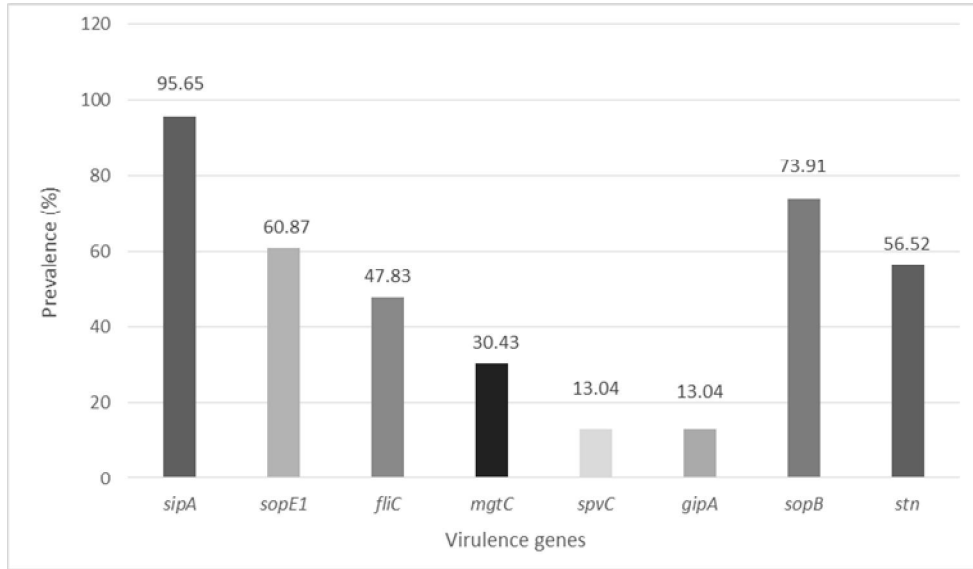


Fig. 1. Prevalence of virulence genes in the *Salmonella* isolates (n=23)

Table 1. Distribution of virulence genes in tested *Salmonella* isolates (n=23)

Isolate Id.	Layer Breed	Sample type	Virulence genes								Total positive	Serotype
			<i>sipA</i>	<i>sopE1</i>	<i>fliC</i>	<i>mgtC</i>	<i>spvC</i>	<i>gipA</i>	<i>sopB</i>	<i>stn</i>		
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	Water	+	+	-	-	-	-	-	-	2	Typhimurium
	Island Red											
S-11	Rhode	Water	+	+	+	-	-	-	+	+	5	Typhimurium
	Island Red											
S-12	Rhode	Water	+	+	-	-	-	-	-	-	2	Typhimurium
	Island Red											
S-13	Rhode	Water	+	+	-	-	-	-	+	-	3	Typhimurium
	Island Red											
S-14	Rhode	Water	+	+	+	-	-	-	+	+	5	Typhimurium
	Island Red											
S-15	Rhode	Water	+	+	+	-	-	-	+	+	5	Typhimurium
	Island Red											
S-16	Rhode	Litter	+	+	+	-	-	-	+	-	4	Typhimurium
	Island Red											
S-17	Rhode	Litter	+	-	-	-	-	-	-	-	1	Typhimurium
	Island Red											
S-18	Rhode	Litter	+	+	-	+	-	-	-	-	3	Typhimurium
	Island Red											
S-19	Rhode	Litter	+	+	+	+	-	-	+	-	5	Typhimurium
	Island Red											
S-20	Rhode	Faeces	+	+	+	-	-	-	+	+	5	Typhimurium
	Island Red											
S-21	Rhode	Faeces	+	+	-	+	-	-	-	-	3	Typhimurium
	Island Red											
S-22	Rhode	Faeces	+	+	+	+	-	-	+	+	6	Typhimurium
	Island Red											
S-23	Rhode	Faeces	+	-	+	-	-	-	+	+	4	Typhimurium
	Island Red											

The *sipA* gene encoding for *Salmonella* invasion protein A induces apoptosis in macrophages and plays a vital role in *Salmonella* pathogenesis [22]. The *sipA* gene was detected in the majority of S.

Typhimurium (95.24%; 20/21) and two untypable isolates. Sharma et al., [23] reported this gene in all the Typhimurium isolates. Shekhar and Singh [24] 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 inositol phosphate phosphatase, one of the effector proteins responsible for the induction of macropinocytosis [25]. 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., [26], Sharma et al., [23], Ahmed et al., [27] and Borges et al., [28], 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 [11]. 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., [28] and Sharma et al., [23] who reported 62.5% and 60% of Typhimurium isolates to exhibit the *sopE1* gene, respectively. Contrary to this, Osman et al., [29] 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 [22]. 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., [23] 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 [24]. Many other reports have found the *stn* gene in all the tested Typhimurium isolates [20,30,31]. In contrast, no *stn* gene was recovered in any of the *Salmonella* isolates from chicken by Gharieb et al., [8].

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 [32]. This gene was observed in 47.62% (10/21) of *S. Typhimurium* and one untypable isolate. Jamshidi et al., [33] reported its presence in all the examined Typhimurium isolates. On the contrary, Sharma et al., [23] 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 [34]. 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., [27], and Sharma et al., [23] 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 [35]. 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., [36] who detected the gene in 10.2% of the isolates. On the contrary, Yang et al., [37] observed this gene in 65.5% of isolates whereas, Ammar et al., [26] 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 [38]. The absence of the *gipA* gene was detected in *Typhimurium* and untypable isolates by Osman et al., [29] 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., [13], and Yang et al., [37] 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.

#### **ETHICAL APPROVAL**

This research study was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar. This

committee functions based on the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

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