Molecular characterization of virulence genes in broiler chicken originated *Salmonella* Enteritidis and *Salmonella* Typhimurium

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ABSTRACT

Salmonella Enteritidis and *Salmonella* Typhimurium are the most common serovars observed in human salmonellosis while contaminated poultry products are the major source of *Salmonella* transmission to humans. Therefore, high pathogenicity of poultry originated *S*. Enteritidis and *S*. Typhimurium strains poses a serious risk to human health. In this study, we investigated the virulence genes of *S*. Enteritidis and *S*. Typhimurium strains isolated from litter and environmental samples of broiler chicken flocks. *SipA, sipD, sopB, sopD, sopE, sopE2, sitC, sifA, ssaR, spvC,* and *pefA* genes were investigated in a total of 137 strains consisting of 105 *S*. Enteritidis and 32 *S*. Typhimurium. Nine strains (6.6%) had all genes. No negative strain was detected for all genes. *SopE* was found in all strains (100.0%). *SitC* (89.1%), *ssaR* (83.9%), *sipA* (70.1%), *sipD* (73.0%), *sopE2* (68.6%), *spvC* (68.6%), and *pefA* (73.0%) were also highly prevalent. Noticeable differences were observed between serovars in terms of *sopE2, spvC* and *pefA* prevalence: 77.1%, 80% and 82.9%, respectively, of *S*. Enteritidis strains were *sopE2, spvC* and *pefA* positive while 40.6%, 31.3% and 40.6% of *S*. Typhimurium strains were positive. This finding indicates that *S*. Enteritidis is more frequent than *S*. Typhimurium in poultry populations due to its higher virulence. Based on virulence gene distribution, the strains were divided into 44 different virulence genotypes, with the major genotype 4 (15.3%) carrying 8 of the 11 genes. The majority of strains (75.9%) were positive for at least 6 genes. *S*. Enteritidis and *S*. Typhimurium strains were highly virulent and pose a threat as zoonotic infection agents.

Introduction

Salmonella is a worldwide critical foodborne pathogen, responsible for more than 70% of foodborne outbreaks. Salmonellosis caused by non-typhoidal *Salmonella* is the second most common zoonotic infection worldwide, with 93.8 million cases and approximately 155.000 human deaths annually (1, 19). The genus consists approximately 2,600 serovars. According to the European Food Safety Authority (EFSA) 2019 zoonoses report, the most frequent serovars in human salmonellosis cases are *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) (50.3%) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (20.1%). The most frequencies of *S*. Enteritidis and *S*. Typhimurium in broilers are 67.8% and 34.8%, respectively, and 26.7% and 13.5%, respectively, in layers (3). Contaminated poultry products (meat and eggs) are considered the major source of *Salmonella* transmission to humans. Therefore, the characterization of pathogenicity-associated genes in poultry originated *S*. Enteritidis and *S*. Typhimurium is critical for controlling human salmonellosis outbreaks (6, 22).

Salmonella pathogenicity depends on the presence of virulence genes that encode various proteins responsible for adhesion, recognition, invasion, internalization, iron acquisition, neutralization, proliferation, and survival. They are located on plasmids or in *Salmonella* pathogenicity islands (SPIs). Among the 24 SPIs, SPI-1 and SPI-2 play major roles in *Salmonella* pathogenesis. SPI-1 and SPI-2 are DNA regions of approximately 40 kb located on the bacterial chromosome (12, 17). SPI-1

contains several virulence genes, including *sipA, sipD, sopB, sopD, sopE* and *sitC*, which encode effector proteins. *SipA, SopB, sopD* and *sopE*, are involved in adhesion to and invasion of host cells whereas *sipD* is responsible for translocation and *sitC* is involved in proliferation and iron acquisition (1, 14). SPI-2 contains virulence genes *sifA* and *ssaR,* associated with internalization, replication, and survival within host cells (1). Some *Salmonella* virulence genes are associated with plasmids, such as *spvC* and *pefA*. *SpvC* is carried on the 94.7 kb serovar-specific plasmid that can be used for serovar identification. It is responsible for survival and proliferation within macrophages. *PefA* encodes the fimbriae that mediate adhesion to host cells (2, 17).

This study analyzed the virulence genes of broiler chicken originated *S.* Enteritidis and *S.* Typhimurium strains in order to improve the detection of correlations between virulence genotypes, serovars, and pathogenicity.

Materials and Methods

***Salmonella* strains and conventional confirmation:** *Salmonella* strains were derived from the culture collection at Ankara University Faculty of Veterinary Medicine, Microbiology Department. The strains originated from litter (n=102) and environmental (n=35) samples of different broiler chicken flocks in Türkiye, 2012-2021. A total of 137 strains were used, including 105 *S.* Enteritidis and 32 *S.* Typhimurium. *Salmonella* serovar confirmation was performed by conventional serotyping using commercial *Salmonella* antisera (SSI, Denmark) and Kauffmann-White-Le Minor scheme (5). The strains were stored in 20% glycerol at -80°C until molecular characterization.

Molecular characterization of virulence genes: The genomic DNA was extracted using by boiling the bacterial suspensions for 10 min at 100°C. DNA purifications and concentrations were measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Eleven different virulence genes, *sipA, sipD, sopB, sopD, sopE, sopE2, sitC, sifA, ssaR, spvC,* and *pefA*, were amplified by polymerase chain reaction (PCR) analyses (Table 1).

The PCR analyses were performed using the primers recommended by previous studies (Table 2). The reactions were conducted in a total mixture volume of 25 µl, comprising 0.2 µl Taq polymerase (2U/µl) (Thermo Scientific, USA), 0.5 µl 10 mM dNTPs, 1 µl of each 10 mM primer, 2.5 µl of 10xbuffer, 3 µl MgCl2, 14.8 µl nuclease free water, and 2 µl template DNA. The amplifications were conducted as follows: initial denaturation for 3 min at 95°C; 30 cycles of 60 s at 94°C; 20 s at 60°C; 1 min at 72°C; and final extension for 7 min at 72°C. The amplicons were analyzed by electrophoresis on 1.5% agarose gel (Thermo Scientific, USA) and visualized in G: Box Chemi gel imaging system (SynGene, India). The virulence genotypes were defined by the combinations of virulence genes for each strain.

Table 1. Gene locations, virulence genes, and virulence functions.

Gene locations	Virulence genes		Virulence functions
SPI-1	*sipA*	*Salmonella* inner protein A	Host adhesion/invasion
	sipD	*Salmonella* inner protein D	Host adhesion/invasion
	sopB	*Salmonella* outer protein B	Host adhesion/invasion
	sopD	*Salmonella* outer protein D	Host adhesion/invasion
	sopE	*Salmonella* outer protein E	Host adhesion/invasion
	sopE2	*Salmonella* outer protein E2	Host adhesion/invasion
	sitC	*Salmonella* iron transport C	Iron acquisition
SPI-2	*sifA*	*Salmonella*-induced filament A	Filamentous structure formation
	ssaR	Secretion system apparatus R	Replication and survival
Plasmid	*spvC*	*Salmonella* virulence plasmid C	Replication and survival
	pefA	Plasmid-encoded fimbriae A	Host adhesion/invasion

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Table 2. Virulence genes, primer sequences, amplicon sizes, and references.

Virulence genes	Primer sequences (5′-3′)	Amplicons sizes (bp)	References
sipA	F-ATGGTTACAAGTGTAAGGACTCAG R-ACGCTGCATGTGCAAGCCATC	2055	(12)
sipD	F-ATGCTTAATATTCAAAATTATTCCG R-TCCTTGCAGGAAGCTTTTG	1029	(12)
sopB	F-GCTCTAGACCTCAAGACTCAAGATG R-GCGGCCGCTACGCAGGAGTAAATCGGTG	1987	(11)
sopD	F-GAGCTCACGACCATTTGCGGCG R-GAGCTCCGAGACACGCTTCTTCG	1291	(11)
sopE	F-ATTGTTGTGGCGTTGGCATCGT R-AATGCGAGTAAAGATCCGGCCT	376	(22)
sopE2	F-TACTACCATCAGGAGG R-GAATGTTTTATGTGACGCAG	995	(11)
sitC	F-CAGTATATGCTCAACGCGATGTGGGTCTCC R-CGGGGCGAAAATAAAGGCTGTGATGAAC	768	(14)
sifA	F-ATGCCGATTACTATAGGCAATGG R-TTATAAAAAACAACATAAACAGCCG	1011	(7)
ssaR	F-GTTCGGATTCATTGCTTCGG R-TCTCCAGTGACTAACCCTAACCAA	1628	(7)
spvC	F-ACTCCTTGCACAACCAAATGCGGA R-TGTCTCTGCATTTCGCCACCATCA	571	(2)
pefA	F-GCGCCGCTCAGCCGAACCAG R-GCAGCAGAAGCCCAGGAAACAGTG	157	(14)

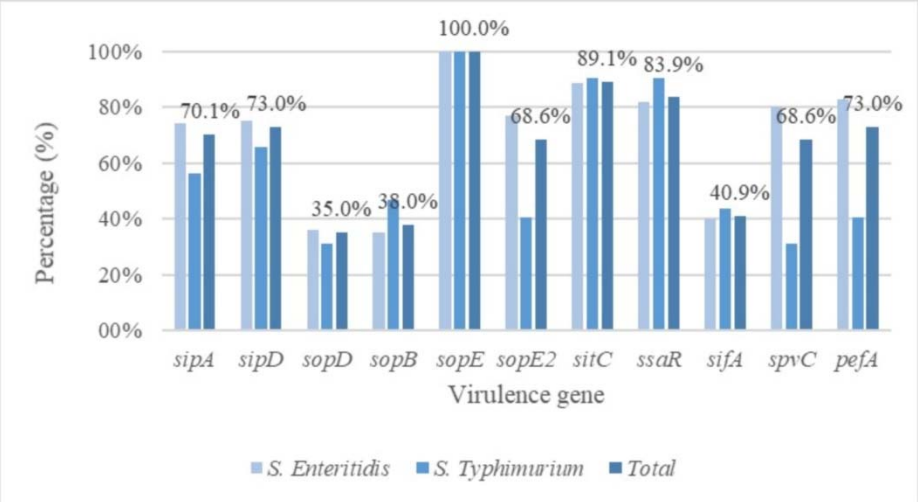

Figure 1. Percentages of the virulence genes in *S.* Enteritidis and *S.* Typhimurium strains.

Results

Figure 1 presents the PCR findings for the 11 virulence genes. All virulence genes were found in nine strains (6.6%), specifically eight *S.* Enteritidis strains and one *S.* Typhimurium strain. No strain was negative for all genes. *SopE* was found in all strains (100.0%), followed by *sitC* (89.1%) and *ssaR* (83.9%) while *pefA* (73.0%), *sipD* (73.0%), *sipA* (70.1%), *sopE2* (68.6%), and *spvC* (68.6%) all had prevalence levels above 50%. The lowest prevalences were for *sopD* (35.0%), *sopB* (38.0%), and *sifA* (40.9%).

The virulence genotypes were investigated in all *Salmonella* strains. All strains were divided into 44 virulence genotypes (genotypes 1-44) (Table 3). Thirty-one different genotypes were detected in *S.* Enteritidis strains while 21 different genotypes were detected in *S.* Typhimurium strains. The dominant genotype (15.3%, 21/137) was genotype 4, namely *sipA, sipD, sopE, sopE2, sitC, ssaR, spvC* and *pefA* (+), and *sopD, sopB* and *sifA* (-). The next two most highly prevalent genotypes were genotype 21 (9.5%, 13/137) and genotype 1 (8.8%, 12/137).

Table 3. Combinations, numbers, and percentages of virulence genotypes.

Combinations of virulence genes												Number of strains			
Genotypes	***sipA***	***sipD***	***sopB***	***sopD***	***sopE***	***sopE2***	***sitC***	***sifA***	***ssaR***	***spvC***	***pefA***	***S.* Enteritidis**	***S.* Typhimurium**	**Patterns (%)**	
1	+	+	+	+	+	+	+	+	-	+	+	12	0	12	8.8%
2	+	+	+	+	+	+	+	+	+	+	+	8	1	9	6.6%
3	+	+	+	+	+	+	+	-	-	+	+	2	0	2	1.5%
4	+	+	-	-	+	+	+	-	+	+	+	20	1	21	15.3%
5	-	+	-	-	+	+	+	-	+	+	+	4	0	4	2.9%
6	-	-	-	-	+	+	+	-	-	+	+	1	0	1	0.7%
7	+	-	-	-	+	+	-	-	-	+	+	2	0	2	1.5%
8	-	+	-	-	+	+	+	+	+	+	+	1	0	1	0.7%
9	+	+	-	+	+	+	+	+	+	+	+	4	1	5	3.6%
10	+	+	+	-	+	+	+	+	+	+	+	3	3	6	4.4%
11	-	-	-	-	+	+	+	+	+	+	+	3	0	3	2.2%
12	-	-	-	-	+	+	-	-	+	+	+	1	0	1	0.7%
13	-	+	+	+	+	+	+	+	+	+	+	2	0	2	1.5%
14	+	+	+	+	+	+	+	+	+	-	+	3	2	5	3.6%
15	+	+	+	+	+	+	+	-	+	-	+	3	0	3	2.2%
16	+	-	+	+	+	+	+	-	+	-	+	1	0	1	0.7%
17	-	+	-	-	+	+	+	+	+	-	+	1	0	1	0.7%
18	-	+	-	-	+	-	+	-	+	+	-	3	1	4	2.9%
19	-	+	-	-	+	-	+	-	+	-	-	2	2	4	2.9%
20	-	-	-	-	+	-	-	-	+	+	+	1	0	1	0.7%
21	-	-	-	-	+	-	+	-	+	-	-	7	6	13	9.5%
22	+	-	-	-	+	-	+	-	+	+	+	6	0	6	4.4%
23	+	-	-	-	+	-	+	-	+	+	-	1	0	1	0.7%
24	-	+	-	-	+	-	+	-	+	-	+	1	0	1	0.7%
25	+	-	-	+	+	-	+	-	+	+	+	1	0	1	0.7%
26	+	+	-	-	+	-	+	+	+	+	+	1	0	1	0.7%
27	+	+	-	-	+	-	-	-	+	+	-	1	0	1	0.7%
28	+	+	-	-	+	+	-	-	+	+	-	3	0	3	2.2%
29	+	+	-	-	+	+	-	-	+	+	+	4	0	4	2.9%
30	+	+	+	-	+	+	+	+	+	-	-	1	0	1	0.7%
31	+	-	+	+	+	+	+	+	-	-	+	2	1	3	2.2%
32	+	+	-	+	+	-	+	+	+	-	-	0	1	1	0.7%
33	-	+	-	+	+	+	+	-	+	-	+	0	1	1	0.7%
34	+	-	-	-	+	+	+	-	-	-	-	0	1	1	0.7%
35	+	-	-	-	+	-	+	+	+	+	+	0	1	1	0.7%
36	+	+	-	-	+	-	+	-	+	-	-	0	1	1	0.7%
37	+	+	-	-	+	-	+	+	+	+	-	0	1	1	0.7%
38	+	+	+	-	+	-	+	+	+	-	-	0	1	1	0.7%
39	+	+	+	-	+	-	-	-	+	-	+	0	1	1	0.7%
40	-	-	+	-	+	-	+	+	+	-	-	0	1	1	0.7%
41	-	+	+	+	+	-	+	-	+	-	-	0	1	1	0.7%
42	-	+	+	-	+	-	-	-	-	-	-	0	1	1	0.7%
43	-	+	+	-	+	+	+	-	+	-	+	0	1	1	0.7%
44	+	-	+	+	+	+	+	+	+	+	-	0	1	1	0.7%
Total number of strains												105	32	137	

Discussion and Conclusion

Salmonellosis is one of the most common foodborne infections worldwide, transmitted to humans through infected animals and consumption of contaminated food, most commonly due to contaminated poultry products (18). Therefore, monitoring of poultry originated *Salmonella* pathogenicity is recommended to control human salmonellosis (6).

In this study, we analyzed the virulence genes of *S.* Enteritidis and *S.* Typhimurium strains isolated from litter and environmental samples of broiler chicken flocks. Eleven different virulence genes (*sipA, sipD, sopB, sopD, sopE, sopE2, sitC, sifA, ssaR, spvC,* and *pefA*) were investigated in 137 strains. The detected high prevalences for most genes were compatible with previous studies (1, 8). These genes are also commonly detected in clinical isolates. Some previous studies have reported poultry isolates that have similar virulence genes as human isolates and are human pathogens (4, 10, 14). *S.* Enteritidis and *S.* Typhimurium are the most common serovars observed in salmonellosis cases of humans, as reported by both the EFSA zoonoses report and recent clinical studies (3, 16, 20). This suggests that the high pathogenicity of poultry originated *S.* Enteritidis and *S.* Typhimurium strains poses a serious risk to human health.

Regarding specific bacterial genes, *sipA* and *sipD*, which are responsible for the invasion of host cells and significantly contribute to salmonellosis, were found in approximately 70% of all strains in our study. Shah et al. (12) reported expression levels of *sipA* and *sipD* that were respectively 16 and 11-fold higher in more invasive strains than less invasive strains. Thus, the high prevalences we found are worrisome in terms of pathogenicity.

The prevalence of *sopB*, which is responsible for adhesion to and internalization into host cells, had 38.0% prevalence in all strains. This contrasts with previous studies. Skyberg et al. (14) and Zou et al. (22) respectively reported 100.0% and 79.0% *sopB* prevalences in poultry originated *Salmonella* strains. Mezal et al. (10) reported 100.0% *sopB* prevalence in *S.* Enteritidis strains while Krawiec et al. (9) and Farahani et al. (4) respectively reported 94.45% and 99.6% *sopB* prevalence in *S.* Enteritidis strains. The low *sopB* prevalence detected in our study could be due to the presence of endemic strains.

All the strains in our study were *sopE* positive (100.0%). Since all strains studied have *sopE* and the major cause of human salmonellosis is poultry originated *S.* Enteritidis and *S.* Typhimurium, this finding indicates a significant risk for human health. Moreover, *sopE2* was positive in 68.6% of all strains, although the serovars differed significantly: while 77.1% of *S.* Enteritidis strains were *sopE2* positive, only 40.6% of *S.* Typhimurium strains were.

The other genes located in SPIs are *sitC, ssaR,* and *sifA*. We found *sitC, ssaR,* and *sifA* in 89.1%, 83.9% and 40.9% of all strains, respectively. Mezal et al. (10) reported that *sitC* belongs to SPI-1 and is associated with iron acquisition, which is thought to be important in pathogenesis. The high *sitC* prevalence in our study is similar to that reported by Mezal et al. (10) (91.6%), Krewiec et al. (9) (94.45%), and Farahani et al. (4) (97.9%). The SPI-2-located *ssaR* facilitates proliferation, survival in phagocytic cells and the spread of systemic infection. Almeida et al. (1) and Sever and Akan (8) also detected high prevalence of ssaR. SPI-2-located *sifA* are associated with filamentous structure formation (8, 21). The high positivity detected in our study is compatible with Mezal et al. (10). Furthermore, *sifA* has previously been detected in 93.3% and 97.9% of all *S.* Enteritidis strains, and 100% of all *S.* Typhimurium strains (4, 9, 10). In short, the high *sifA* prevalence detected in our study is compatible with previous studies.

SpvC, which is associated with survival in macrophages, infects reticuloendothelial system organs, such as spleen, lymph nodes, and liver. The other plasmid virulence gene, *pefA*, plays an essential role in adhesion to host cells. We detected *spvC* and *pefA* positivity in 68.6% and 73.0% of all strains, which are quite high rates compared with previous findings (14, 15). This may reflect differences in strain origins, and the pathogenicity of epidemic strains and serovars. We found lower *spvC* and *pefA* prevalence compared with virulence genes on the chromosome, except for *sopD* (35.0%), *sopB* (38.0%) and *sifA* (40.9%). Both genes are carried and transferred by virulence plasmids, which can be found on serovars or be strain specific. Therefore, *spvC* and *pefA* are likely to be less prevalent than the virulence genes carried by the chromosome.

We also detected a difference in the prevalence of *spvC* and *pefA* between serovars. While 80.0% and 82.9% of *S.* Enteritidis strains were *spvC* and *pefA* positive, respectively, 31.3% and 40.6% of *S.* Typhimurium strains were *spvC* and *pefA* positive, respectively. Yue et al. (20) also reported higher positivity for *spvB, spvR,* and *pefA* in *S.* Enteritidis strains compared to *S.* Typhimurium strains. Similarly, Siddiky et al. (13) reported that 92.0% of *S.* Enteritidis strains had *spvC* whereas only 28.0% of *S.* Typhimurium did. These findings suggest that the reason *S.* Enteritidis (50.3%) is more commonly observed than *S.* Typhimurium (20.1%) in poultry populations is due to the higher adhesion and invasion ability of *S.* Enteritidis. In other words, the higher prevalence of *S.* Enteritidis strains in poultry samples increases the possibility of virulence gene acquisition by horizontal transfer. This explains the higher prevalences of virulence genes in *S.* Enteritidis than *S.* Typhimurium strains.

Based on the distribution of 11 virulence genes, *S.* Enteritidis and *S.* Typhimurium strains were genotyped into 44 different virulence genotypes (genotypes 1-44). Thirty-one different genotypes were detected in 105 *S.* Enteritidis strains while 21 different genotypes were detected in 32 *S.* Typhimurium strains. The *S.* Typhimurium strains had greater diversity of genotypes (68.8%) than *S.* Enteritidis strains (29.5%). The major genotype 4 (15.3%, 21/137) was positive for all other genes except *sopD, sopB,* and *sifA*. The majority of strains (75.9%) were positive for at least six genes in 31 different genotypes.

In this study, we analyzed the virulence genes of broiler chicken originated *S.* Enteritidis and *S.* Typhimurium strains. The findings contributed to understanding the pathogenicity and epidemiology of *Salmonella* strains in broilers. The high positivity detected for virulence genes provides clear evidence of the high pathogenicity of the serovars, which are common among both animals and humans. *S.* Enteritidis *and S.* Typhimurium strains were highly virulent and therefore pose a threat as a zoonotic infection. However, these findings need further support from molecular characterization and epidemiological analysis of virulence genes in human strains.

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Author Contributions

SSI carried out the experiments, writing, and editing. MA contributed to the interpretation of the results and editing. SSI took the lead of writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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