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Original Article

Detection of *invA*, *sivH,* and *agfA* Virulence Genes in *Salmonella* spp. Isolated from Broiler Breeder Farms in Alborz Province, Iran

Mashayekh, Z[1], Moradi Bidhendi, S[2 *], Khaki, P[2]

1. Department of Microbiology, North Tehran Branch, Islamic Azad University, Tehran, Iran
2. Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

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Corresponding Author: s.moradibidhendi@yahoo.com

Abstract

Salmonellosis, among poultry infectious diseases, not only imposes economic losses in the field of poultry breeding but also is considered a zoonotic disease. This study aimed to investigate the presence of *invA*, *sivH*, and *agfA* virulence genes in *Salmonella* species. The present study was conducted on 30 *Salmonella* strains. Samples were cultured on selective and differential media, and afterward, the isolates were serotyped using specific antisera based on the Kauffman-White table. Subsequently, the samples were analyzed to detect *invA*, *sivH*, and *agfA* genes by polymerase chain reaction technique. The results indicated that 30 (100%) isolates had *invA* and *agfA* virulence genes and 28 (93.33%) isolates had a *sivH* virulence gene. The highest frequency of serotypes was related to *Salmonella infantis*. Among the studied serotypes, *Salmonella uno* and *Salmonella O35* lacked the *sivH* virulence gene, unlike other serotypes. The findings of this study could pave the way for *Salmonella* monitoring and be used as a pattern to detect *Salmonella* bacteria-bearing genes encoding invasion and fimbria.

Keywords: *Salmonella*, *agfA* gene, *invA* gene, *sivH* gene, Serotyping

1. Introduction

Salmonella is one of the most common foodborne pathogens that infects humans and animals worldwide (1). Infected meat products are the main source of *Salmonella*, which imposes heavy economic losses on poultry, especially young birds, and is important because of its transmissibility from poultry to humans. Salmonellosis is mostly transmitted through the gastrointestinal tract. Therefore, contaminated water and food are a significant source of salmonellosis development (2).

According to the World Health Organization, up to 16-33 million cases of infection and 500-600 thousand deaths occur annually due to *Salmonella*, which is a major health problem in developing countries, including Iran (3). Epidemiological studies provide valuable information to establish criteria for controlling and preventing these pathogens. Kauffman-White serotyping is a suitable and valid method for the detection and epidemiological examination of *Salmonella* spp. However, there is a need to employ other methods, such as molecular techniques, for phylogenetic purposes to investigate the genetic association between different *Salmonella* serotypes (4).

Considering the importance of *Salmonella* in the incidence of Salmonellosis and its presence in various sources as a serious risk to humans, it is hoped that research findings in this area will be useful in the near future in preventing infectivity in poultry, and ultimately,

in humans by inhibiting virulence genes in *Salmonella* (5, 6). This bacterium is among the main agents transmitted from animals to humans, which is one of the most important and well-known causes of foodborne diseases due to the diversity of genetic and animal reservoirs (7). Invasion protein A (*invA*) is considered an invasion gene in *Salmonella* and plays the first role in attacking intestinal epithelial cells (8). The *sivH* gene is present as another invasion gene in *Salmonella* strains. The presence of this gene encodes an outer membrane protein that is associated with bacterial colonization in the host intestine (9). Aggressive factor A (*agfA*) gene is also involved as a gene contributing to the production of fimbriae in the bacterial colonization in the intestine and systemic infection in the host (10).

It should be noted that the presence of *Salmonella* in poultry farming has currently become one of the biggest bottlenecks and concerns of the healthcare community in the world, including Iran. Part of this problem can be attributed to the emergence of new strains due to subtle genetic changes in previous strains; in this respect, the rapid detection and identification of *Salmonella* spp. in different sources and the development of effective treatment are of particular importance (11, 12). The current study aimed to investigate the presence of *agfA*, *sivH*, and *invA* virulence genes in *Salmonella* spp. isolated from broiler breeder farms in Alborz Province, Iran. Continuous monitoring of these genes can help manage the spread of the disease. To the best of our knowledge, no study had been dedicated investigating the genotyping of *Salmonella* strains isolated from poultry in Alborz Province; Therefore, the study of the presence of *agfA*, *sivH*, and *invA* virulence genes in *Salmonella* strains isolated from broiler breeder farms in Alborz Province is a new topic and has no history. The findings of this study could pave the way for *Salmonella* monitoring and be used as a pattern to routinely detect *Salmonella* bacteria bearing genes encoding invasion and fimbriae in order to find a solution for the treatment of salmonellosis and make the necessary and appropriate arrangements in veterinary, health, and medical centers to improve performance and compare changes in treatment and resistance patterns in poultry.

2. Materials and Methods

2. 1. Bacterial Strains Studied

In this study, 30 lyophilized cloacal samples were prepared from the microbial bank of the Microbiology Department of Razi Vaccine and Serum Research Institute-Karaj-Iran. Two standard strains of *Salmonella serovar enteritidis* RTCC 1621 (ATCC 13076) and *Salmonella typhimurium* RTCC 1735 (ATCC 14028) were also used as positive controls. Biochemical tests were performed to confirm the detection of *Salmonella*.

2.2. Serotyping the Strains

Salmonella-specific antisera (MAST Company-UK) were used for serotyping of existing samples. First, a sample was taken from the nutrient agar medium and a concentrated bacterial suspension in normal saline was prepared on a slide. Afterward, a drop of monovalent O serum was added to the complex and the formation of agglutination in less than a few minutes was investigated. At the next step, the sample was exposed to H antisera (phases 1 and 2) and agglutination was re-examined. Bacterial serotype was determined based on the Kauffman-White table.

2.3. Detection of Virulence Genes in the Isolates

To genotype the strains based on three virulence genes of *sivH*, *invA*, and *agfA*, bacterial DNA was first extracted using overnight bacterial culture in triple sugar iron medium and boiling method. The quantity and quality of the extracted DNA were evaluated using NanoDrop and agarose gel electrophoresis, respectively. Polymerase chain reaction (PCR) was performed with Taq DNA l 2.0X Master Mix red. The sequence of primers used is also listed in table 1. The thermal cycle was defined for a thermocycler as 94°C for denaturation, annealing temperature based on Tm per primer, and 73°C for the extension. The products of PCR were electrophoresed on agarose gel with 1X TBE buffer and 80-V voltage and 30-mA current for 75 min, and the bands formed on the gel were examined using a gel documentation device.

Table 1. Oligonucleotide sequence of used primers

Gene	Virulence Factor	Tm (ºC)	5' → 3'(Primer Sequence)	Amplicon length	Ref.
sivH	Invasive	53	F:CAGAATGCGAATCCTTCGCAC R:GTATGCGAACAAGCGTAACAC	763 bp	(13)
invA	Invasive	63	F:GTGAAATTATCGCCACGTTCGGGCAA R:TCATCGCACCGTCAAAGGAACC	284 bp	(13)
agfA	Aggressive fimbriae	59	F:TCCGGCCCGGACTCAACG R:CAGCGCGGCGTTATACCG	261 bp	(14)

2.4. Determination of Gene Sensitivity

In the susceptibility test, DNA purification was performed for the *invA* gene from *Salmonella serovar havana*, for the *agfA* gene from *Salmonella serovar mbandaka*, and for the *sivH* gene from *Salmonella serovar arizonae C1*, and the purified DNA was diluted from 100 ng to 0.1 pg. Subsequently, PCR was performed and band formation was evaluated for each concentration.

2.5. Determination of Primer Specificity

To determine the specificity of the primers, PCR was performed on the DNA extracted from *Salmonella serovar enteritidis* and three samples of *Shigella*, *Escherichia coli*, and *Citrobacter*, along with the specific primer of *invA*, *agfA*, and *sivH* genes.

3. Results

3.1. Identification of Isolates

All biochemical tests confirmed the strains prepared as *Salmonella*. The results of these tests are summarized in table 2. These results matched the identification key for *Salmonella*. As it was revealed, these strains were H_2S, Lysine, citrate, methyl red (MR) positive, Urea and Voges–Proskauer (VP) negative.

3.2. Serotyping the Strains

The obtained serotypes are tabulated in table 3. According to the results, Infantis, Enteritidis, Infentis, and Typhimurium serotypes had 6, 4, 2, and 2 isolates, respectively, and Rostock serotype had 2 strains. The other serotypes listed in table 3 each had 1 sample (3.33%).

3.3. Identification of Virulence Genes in Strains

All 30 studied strains, in addition to both standard positive control strains, had *invA* and *agfA* genes. All strains, except strains 24 and 28 (*Salmonella O35* and *Salmonella uno*), had the *sivH* gene (Figure 1).

3.4. Determination of Gene Sensitivity

As shown in figure 2, the sensitivity of PCR was up to a dilution of 0.1 pg, and all dilutions prepared from DNA were able to form a band for all three studied genes.

3.5. Determination of Primer Specificity

The PCR results revealed that *invA*, *agfA*, and *sivH* genes were present in *Salmonella enteritidis*, however, not in other tested bacteria (Figure 3).

Table 2. Results of biochemical tests for *Salmonella* strains

Sample	TSI	Lysine	Urea	Citrate	MR	VP	P
Salmonella havana	H2S+Alk/AG+	+	-	+	+	-	-
Salmonella mbandaka	H2S+Alk/AG+	+	-	+	+	-	-
Salmonella enteritidis	H2S+Alk/AG+	+	-	+	+	-	-

TSI: Triple sugar iron

Table 3. Serotyping results of strains

Number	Species name	Number	Species name
1	*Salmonella blegdam*	16	*Salmonella thompson*
2	*Salmonella colindale*	17	*Salmonella typhimurium*
3	*Salmonella enteritidis*	18	*Salmonella infantis*
4	*Salmonella infantis*	19	*Salmonella infantis*
5	*Salmonella arizonae C1*	20	*Salmonella entritidis*
6	*Salmonella group E1*	21	*Salmonella hesarak*
7	*Salmonell havana*	22	*Salmonella typhimurium*
8	*Salmonella kuilsriver*	23	*Salmonella infantis*
9	*Salmonella mbandaka*	24	*Salmonella O35*
10	*Salmonella enteritidis*	25	*Salmonella enteritidis*
11	*Salmonella meunchen*	26	*Salmonella infantis*
12	*Salmonella montevideo*	27	*Salmonella infantis*
13	*Salmonella mosco*	28	*Salmonella uno*
14	*Salmonella rostock*	29	*Salmonella tinda*
15	*Salmonella rostock*	30	*Salmonella mjimwema*

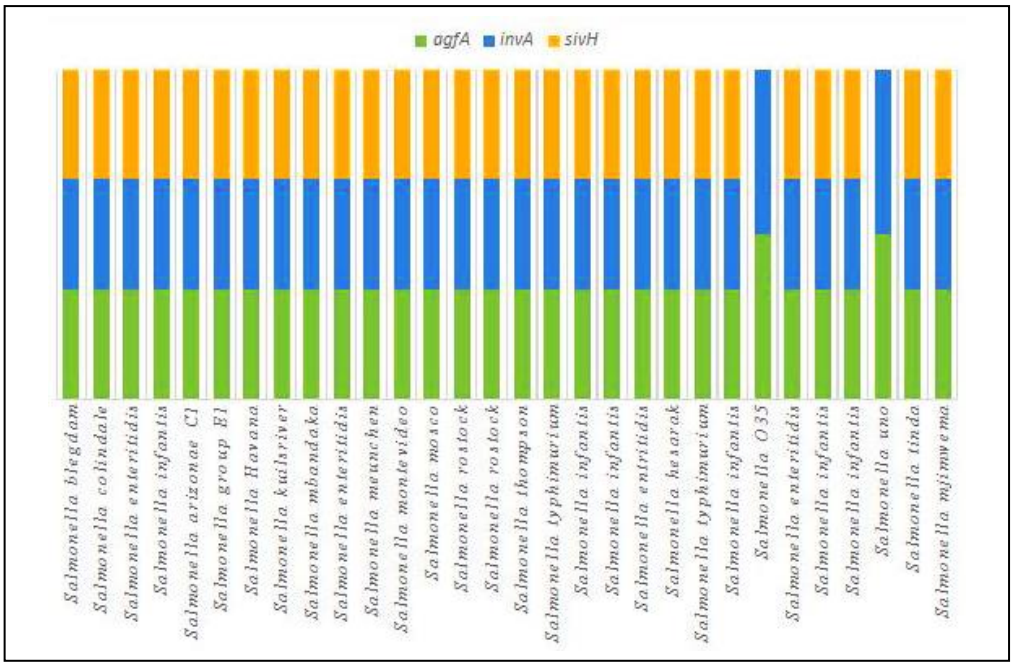

Figure 1. Frequency of *agfA*, *invA*, and *sivH* genes in *Salmonella* serotypes

Figure 2. Susceptibility test for different dilutions of purified DNA of *Salmonella serovar mbandaka*; left to right for *invA*, *agfA*, and *sivH* genes (100-bp L-DNA marker; other bands from left to right: 100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng, 1 pg, and 0.1pg)

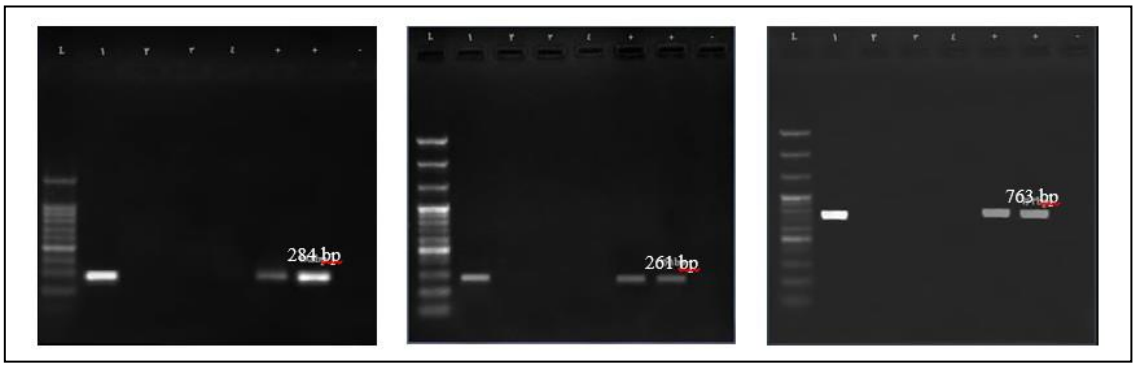

Figure 3. Results of 1% agarose gel electrophoresis for PCR products with the primer of *invA*, *agfA*, and *sivH* genes (100-bp L-DNA marker, left to right: *Salmonella enteritidis*, Citrobacter, *Escherichia coli*, *Shigella*, positive control, and negative control)

4. Discussion

Isolation, identification, control, and prevention of *Salmonella* strains in animal source foods is of great public health importance. Among diagnostic methods, the PCR technique is a suitable tool in identifying food contaminated with *Salmonella* strains due to its sensitivity and high rapidity. In the present study, based on the needs of the Iran Veterinary Organization to the study of salmonellosis, three virulence genes of *agfA*, *sivH*, and *invA* using PCR technique showed that all 30 isolates (100%) had the *agfA* and *invA* virulence genes and 28 isolates (93.33%) had the *sivH* virulence gene. There are different reports of poultry contamination in various parts of the world and Iran. The results of some of these studies have reported lower virulence capability for different genes and serotypes than those in the present study, while the findings of other pieces of research have indicated more in this regard. In a study conducted by Borges et al. in southern Brazil, the results of PCR-based experiments showed that the *sefA*, *sivH*, *hilA*, *invA*, and *avrA* genes were present in 100%, the *lpf* and *sopE* genes in 99%, the *agfA* gene in 96%, and the *spvc* gene in 92% (13) of the isolates. In a study carried out by Crăciunaş et al. (2012), the amplification of *hilA*, *agfA*, *spvC*, and *sef* genes was performed by PCR as a method to detect *Salmonella* strains. The results demonstrated that all *Salmonella* strains were positive for the presence of the *hilA* gene, and the use of the *sef* and *spvc* genes or the *spvc* and *agfA* genes was introduced as a valuable diagnostic tool for *Salmonella enteritidis* strains (14). In a study performed by Rocha-e-Silva et al. to generate a genotypic profile for *Salmonella* Gallinarum, 15 strains were obtained from previously isolated and confirmed commercial poultry reared in Brazil. The *agfA*, *hilA*, *invA*, and *sivH* genes were observed in all isolates (15). In another study conducted by Webber et al. (2019), 126 strains of *Salmonella* Heidelberg isolated from chicken carcasses were examined, and the presence of 24 virulence genes in these isolates was investigated. According to PCR results, the results of the mentioned study showed that *invA* and *sivH* genes were present in all isolates (16). In general, the comparison of the findings in the present study with those in the mentioned studies revealed that the frequency rate of *invA*, *agfA*, and *sivH* genes indicated the high frequency of these genes in *Salmonella* spp.

All strains (100%) presented the *invA* gene which was related to the host recognition and internalization of the bacterium during the invasion of epithelial cells. This gene is associated with the structure of the Type Three Secretion System and is considered the main target gene for the detection of strains belonging to this genus by PCR (13, 16). It was found that 93.33% of these isolates of *Salmonella* were *sivH* gene positive. Although there are few studies on the frequency of this gene in the populations of *Salmonella* spp., our results were in line with those of a study carried out by Kingsley, Humphries (17).

Many of these effector proteins were shown to play an important role in *Salmonella* virulence. The *agfA* gene is one of the genes encoding the presence of fimbriae, which also have properties related to pathogenesis and auto-aggregation, are pro-inflammatory and increase invasion of eukaryotic cells (16). The *agfA* gene is also associated with bacterial adhesion to various inert surfaces, including those used in food production. For this reason, it is also considered an important gene for the production of biofilms and the maintenance of bacteria in the environment (18). The findings of other studies have reported a high detection frequency of the *agfA* gene in different serotypes of *Salmonella* spp. (13, 16), which was in agreement with those of our study.

The present study also analyzed *Salmonella* serotypes. Out of 32 available samples, including 2 standard strains and 30 poultry samples, serotyping results were as follows: 2 cases (66.6%) of *Salmonella rostock*, 2 cases (66.6%) of *Salmonella typhimurium*, 6 cases (20%) *Salmonella infantis*, 4 cases (13.33%) *S. enteritidis*, and 16 other serotypes, including *Salmonella blegdam, Salmonella colindale, Salmonella arizonae C1, Salmonella* group *E1, Salmonella Havana, Salmonella kuilsriver, Salmonella mbandaka, Salmonella meunchen, Salmonella montevideo, Salmonella mosco, Salmonella thompson, Salmonella hesarak, Salmonella O35, Salmonella uno, Salmonella tinda*, and *Salmonella mjimwema*, each of which had 1 sample (3.33%). Accordingly, the highest frequency of serotypes belonged to *S. infantis*. The PCR findings in this study revealed that 30 isolates (100%) had *agfA* and *invA* virulence genes, while the other isolates had the *sivH* virulence gene, except for *S. uno* (3.33%) and *S. O35* (3.33%) isolates that lacked the *sivH* virulence gene.

Some other studies have also been previously performed on *Salmonella* serotyping. A study was performed on 100 local egg samples in Urmia, Iran, for the presence of *Salmonella*, of which 6 *Salmonella* samples were isolated. Consistent with serotyping findings, all 6 isolates were identified as *S. enteritidis*, indicating a high frequency of this serotype. A high frequency of this serotype was also observed in the present study (19). Among poultry products, eggs have been considered the best product; regarding this, within 1985-1989, eggs were recognized as 82% of *S. enteritidis* infections in the United States. The incidence rate of *S. enteritidis* infections in the United States on broiler breeder farms and human cases has also been increasing since the early 1990s. Studies have documented that this serotype has been suggested as the second most common salmonellosis serotype in humans, accounting for 17% of salmonellosis cases reported in the United States in 2006 (20). The results of studies conducted in Iran have also confirmed these findings; accordingly, 480 (67.1%) out of the 715 broiler breeder flocks studied in 1993 were infected with *S. enteritidis*. On the other hand, the findings of a study performed in Shiraz, Iran, showed that the most common serotypes among 360 *Salmonella* strains isolated from broiler breeder farms around this city were *S. enteritidis* and *S. typhimurium* in descending order. After serotyping 658 isolated *Salmonella* samples, 612 (93%) cases of *S. enteritidis* and 46 (7%) cases of *S. typhimurium* were detected in broiler breeder farms of Chaharmahal and Bakhtiari Province, Iran (21).

In the present study, *S. enteritidis* and *S. typhimurium* serotypes also had a higher frequency than other serotypes. Due to the presence of *invA, sivH*, and *agfA* virulence genes in the majority of samples, it can be concluded that the identification and confirmation of these genes in the bacteria of the region can play a role in extensive epidemiological studies, vaccine production, virulence rate, prevention, and treatment. It is really important to detect these genes in the samples because of being the virulence marker.

Authors' Contribution

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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