



Comparison Study on Colonization of *hilA* Mutant and Parent Strains of *Salmonella enteritidis* in Vertically Infected Broiler Chickens

Mohammad Sadegh Madadi^{1*}, Sara Mirzaei², Mohammad Hassanzadeh³

¹Department of Clinical Sciences, Faculty of Veterinary medicine, University of Tabriz, Tabriz, IR Iran

²Department of Animal, Poultry and Aquatic Sciences, Institute for Agricultural Research, Iranian Organization for Science and Technology, Tehran, IR Iran

³Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Tehran, IR Iran

ARTICLE INFO	ABSTRACT
Article type: Original Article	Background: Salmonella actively stimulates its own uptake into the epithelial cells by inducing cytoskeleton rearrangements and membrane ruffling triggered by some proteins secreted by Salmonella into the cytosol of the epithelial cells via a type III secretion system (TTSS) encoded by genes of the Salmonella pathogenicity island 1 (SPI-1). <i>hilA</i> is a transcriptional activator encoded on Salmonella Pathogenicity Island 1 (SPI-1) genes.
Article history: Received: 10 Sep 2014 Revised: 22 Sep 2014 Accepted: 17 Oct 2014	Methods: To assess the importance of <i>hilA</i> in a simulation modeling of vertical infection and shedding of <i>S. enteritidis</i> in broiler chickens a long-term experiment was designed. Two groups of 200 fertile eggs were inoculated with 20 colony forming units (CFU) of <i>hilA</i> mutant of <i>S. enteritidis</i> or its parent strain just prior to incubation. Thirty five birds of each group were housed in separate rooms. On days 2, 4, 7, 14, 21, 28 and 35 of age, cloacal swabs from live birds as well as samples from internal organs (intestinal tract, liver and spleen) were evaluated by bacteriological or molecular methods.
Keywords: <i>Salmonella enteritidis</i> <i>hilA</i> mutant Vertical Broilers PCR	Results: In most of sampling days colonization and invasion of parent strain <i>S. enteritidis</i> in intestine (especially caeca) and internal organs of chickens were higher with compared to its <i>hilA</i> mutant but this mutant strain could still colonize in intestinal tract and even invade liver or spleen. Conclusion: Colonization of <i>hilA</i> mutant of <i>S. enteritidis</i> indicated that <i>hilA</i> gene is only one part of the modulators in Salmonella invasion mechanism. The ability of <i>hilA</i> mutant to multiply and persist in host internal organs including caeca may promise further research for potential of <i>hilA</i> mutant to prevent the initial colonization of the intestinal tract by a virulent <i>S. enteritidis</i> strain

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Introduction

Salmonella serotype enteritidis is one of the most common serotypes of Salmonella bacteria reported worldwide. Many serotypes of Salmonella are zoonotic agents but the commonest serotypes causing disease in humans are *S. enteritidis* and *S. typhimurium*. Since 1987, *S. enteritidis* has been the main cause of Salmonella poisoning in humans from poultry products. During the 2012 there were 91,034 confirmed human cases of salmonellosis in the European Union. A statistically significant decreasing trend in the European Union was observed over the period 2008-2012. It is assumed that the observed reduction in salmonellosis cases is mainly a result of the successful Salmonella control programmes in poultry populations. (1). The persistence of Salmonella in poultry house environments poses a continuing threat of infection for birds and subsequently to humans. One important route for introducing Salmonella into poultry houses is free flying wild birds like house sparrows (1). In a recent study, 44.4% of Salmonella isolates from house sparrows which were captured around broiler chickens farms were belonged to *S. enteritidis* (2). An important step in Salmonella pathogenesis is bacterial entry in the epithelial cells of the intestinal tract, especially the caeca. Salmonella actively stimulates its own uptake into the epithelial cells by inducing cytoskeleton rearrangements and membrane ruffling. These morphological changes are triggered by some proteins secreted by Salmonella into the cytosol of the epithelial cells via a type III secretion system (TTSS) encoded by genes of the Salmonella pathogenicity island 1 (SPI-1). Several regulatory proteins that are involved in Salmonella invasion have been characterized. The key regulator of SPI-1 is *hilA*, a transcriptional activator encoded on SPI-1 that regulates the expression of the SPI-1 secretion system as well as many of its secreted effectors (3). It has been shown that *hilA* knock-out mutants are unable to enter epithelial cells in vitro. In addition in a study *hilA* mutant of *S. enteritidis* had no virulence in

one- day- old chicks following oral infection and at the age of 4 weeks, the *hilA* mutant strain was largely cleared from the chicken gut (3). Since few studies have been done to assess the effects of *hilA* expression on in vivo virulence, the goal of the present study was to compare the long- term colonization and shedding of *hilA* mutant and parent strain of *S. enteritidis* in broiler chickens by simulating vertical transmission of the organism.

Material and methods

Salmonella strains

S. enteritidis phage type four, strain NIDO 76Sa88 NaI^r, a well-characterized strain isolated from poultry and its isogenic *hilA* mutant derivative, had been obtained from Ghent University, Belgium. The virulence of this strain had been tested before (4).

Experimental procedure

Four hundred fertile eggs were obtained from specific parent farms that were under strict control for Salmonella and other infectious diseases (Arian GGP farms, north of Iran). Broiler parents were free of any apparent disease throughout the growing and laying periods. Before the start of the study, parent flock was tested by taking cloacal swabs and egg culture to confirm that birds were Salmonella-free. Eggs randomly divided in two groups of 200 eggs. The eggs were inoculated with 20 colony forming units (CFU) Salmonella in a volume of 0.1 ml of PBS a few hours before incubation by injection into the albumin with either parent strain or *hilA* mutant of *S. enteritidis*. Soon after injection, two groups of eggs were transferred to separate incubators with the same conditions. After incubation period, 35 apparently normal newly hatched chicks of each group were housed in separate rooms with similar conditions. The chicks had ad libitum access to commercial feed and water throughout the study. Lighting in each room was scheduled to provide 23 h light/1 h dark photoperiod. The room temperature was

adjusted according to Arian broiler rearing recommendations. Shedding of *Salmonella* was evaluated throughout the study by taking cloacal swabs on days 2, 4, 7, 14, 21, 28 and 35 of life. For bacteriological examination of internal organs, two broilers per group were euthanized at the mentioned days and samples of liver and spleen mixture, small intestine and caeca were separately tested for the recovery of *S. enteritidis* (3).

Bacteriological analysis

Cloacal swabs from live birds were placed in test tubes containing 5 ml selenite cystine broth and after 24 h of incubation at 37 °C, were streaked on *Salmonella-Shigella* (SS) agar plates. Homogenized samples of internal organs were serially diluted in PBS and each dilution was cultured on SS agar plates. After overnight incubation at 37 °C, the number of CFU per gram of tissue was determined by counting the bacterial colonies. Enrichment in selenite cystine broth was carried out for negative tissue samples and those that were negative at first but showed positive results after enrichment, were presumed to contain 10¹ CFU per gram of tissue. The mean CFU per gram of tissue was calculated for each group (3).

Polymerase chain reaction

To determine the presence of *Salmonella* genomic DNA in tissues, samples of internal organs from 2 chicks per group were tested. DNA extraction was done by using of genomic DNA purification kit (Fermentas, EU). Primers which were used in this study are shown in Table 1. Three sets of primers specific for randomly cloned sequence (429 bp), *spv* (250 bp) and *sefA* (310 bp) genes were used together in multiplex PCR and one set of primer which was designed based on complete sequence data of *hilA* gene (exist in the GenomeNet with accession number U25352), was used in another PCR reaction for detection of *hilA* gene (410 bp). Both reactions were performed in a final volume of 25 µl containing template DNA, PCR buffer (20

mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 0.25 mM of dNTPs, and 1 U of *Taq* DNA polymerase, 20 pmole of a specific forward and reverse primers. Amplification was carried out by using of TC- 512 thermocycler (Techne, UK), as follows: Initial denaturation at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 90 s, primer extension at 72 °C for 30 s, followed by a terminal extension at 72 °C for 10 min. The amplification products were electrophoresed on 1.2% agarose gels and 100-bp ladder was used as a molecular weight marker. The gel was stained with ethidium bromide (2 µg mL⁻¹) to visualize fluorescent bands while using UV light in the gel document system (BIORAD, UK) (5, 6).

Results

Bacteriological analysis

The results of recovery and colony counts of *S. enteritidis* from cloacal swabs and different parts of intestinal tract are shown in Table 2. At two days of age *Salmonella* isolation rates for cloacal samples of chickens infected with parent strain of *S. enteritidis* were 100%. This rate decreased to 50% at 35 days of age, while the isolation rate for chickens infected with *hilA* mutant strain were 34% and 0% in the mentioned sampling days respectively. *S. enteritidis* could be retrieved more from intestinal tract especially caeca than from other sampled tissue. The recovery rate of *S. enteritidis* decreased over time post infection. Isolation rate by colony counting of *S. enteritidis* from small intestine and caeca was higher in parent strain compared to *hilA* mutant group in most of the sampling days. However recovery of *S. enteritidis* from liver- spleen mixed samples of *hilA* mutant group was greater than parent strain group at days 4, 7 and 14 indicating higher colonization of *hilA* mutant with the compare to parent strain at those days.

Table 1. Primer sequences used for the detection of *Salmonella enteritidis* wild type and *hilA* mutant strains

Primer	Target gene	Length	Sequence (5'- 3')	Amplification product (bp)
ST11	Random sequence*	24	GCCAACCATTGCTAAATTGGCGCA	429
ST14	Random sequence	25	GGTAGAAATTCCCAGCGGGTACTGG	429
S1	<i>Spv</i> **	20	GCCGTACACGAGCTTATAGA	250
S4	<i>Spv</i>	20	ACCTACAGGGGCACAATAAC	250
SEFA2	<i>SefA</i> ***	20	GCAGCGTTACTATTGCAGC	310
SEFA4	<i>SefA</i>	19	TGTACAGGGACATTTAGCG	310
<i>hilA-F</i>	<i>hilA</i>	20	ACGGCTCCCTGCTACGCTCA	410
<i>hilA-R</i>	<i>hilA</i>	20	GCTCAGGCCAAAGGGCGCAT	410

* Randomly cloned sequence specific for the genus *Salmonella*

** *Salmonella* plasmide virulent gene

*** *Salmonella enteritidis* fimbrial antigen gene

Table 2. Recovery and counts (log₁₀ CFU/g) of SE from cloacal swabs and different parts of digestive tract of parent [P] and *hilA* strain [hA] infected chicken groups

Days of age	2		4		7		14		21		28		35		
	P	hA	P	hA	P	hA	P	hA	P	hA	P	hA	P	hA	
Number of positive cloacal samples/ total chickens (Percent of positive samples)	34/34 100%	12/34 34%	32/32 100%	10/31 32%	28/28 100%	9/29 31%	22/25 88%	7/26 27%	21/23 91%	5/23 22%	15/20 75%	3/21 14%	8/16 50%	0/17 0%	
Counts of SE (log₁₀ CFU/g) of tissue samples	Liver-spleen mixture	5.8	5	1	4.3	1.4	4.3	1	2.6	1	1	1	0	0	0
	Small intestine	6.4	5.7	2.4	4.6	2.3	1	3.8	1	2.5	0	2.4	1	0	0
	Caeca	9.58	7.3	7.8	6.5	6.5	6.7	3.5	5	4.4	3.1	4.5	3.1	3.4	3.3

PCR detection of *Salmonella enteritidis*

PCR amplified 429, 250 and 310 base pair products from a random sequence (specific for the genus *Salmonella*), *spv* and *sefA* in all of extracted positive tissue samples, while as was expected 410 bp longed *hilA* gene was detected only in tissues of parent strain group (Figures 1 and 2). By testing tissue samples of liver- spleen mixture, *S. enteritidis* was detected from 2 to 14 days of age in *hilA* mutant group while it was detectable at days 2 and 7 of age in the same samples from parent strain group. However in samples from small intestine, PCR results for *hilA* mutant group were positive only at 2 and 4 days of age unlike samples from the other group which were showed to be positive until day 28 of age. *S. enteritidis* infection of ceecal samples could be detectable throughout the sampling period regardless of experimental group.

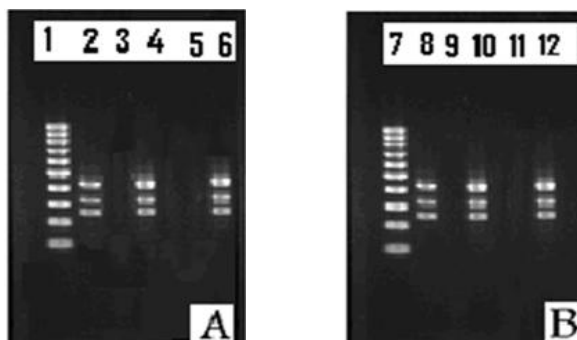


Figure 1. Multiplex polymerase chain reaction for the detection of *S. enteritidis* in standard (A) and *hilA* (B) chicken groups; 1 and 7 gene ruler, 2 and 8 control, 4 and 10 caecum (2 days of age), 3 and 9 small intestine (35 days of age), 5 and 11 liver (35 days of age)

Discussion

The present study's plan was a model of vertical infection of chickens which occur naturally as one of the transmission routes of paratyphoid infection. As one of paratyphoid *Salmonella* serotypes, *S. enteritidis* is highly invasive and can be systemically disseminated to numerous internal tissues including liver and spleen, so samples from these organs can be assessed alongside of digestive tract for studies on *Salmonella* pathogenicity and invasion (1). Our results in agreement with previous reports indicated that although colonization and invasion of parent strain *S. enteritidis* in internal tissues was higher with compared to its *hilA* mutant but this mutant strain could still colonize in intestinal tract and even invade liver or spleen. Bohez et al. (3) reported that *hilA* is the key regulator of *S. enteritidis* pathogenicity island I (SPI-1) gene. After inoculation of chickens at day of hatch with *hilA* mutant of *S. enteritidis* and its parent, very low numbers of *hilA* mutant strain of *S. enteritidis* were able to colonize in the internal organs shortly after infection, but they were not eliminated from internal organs until 4 weeks post infection.

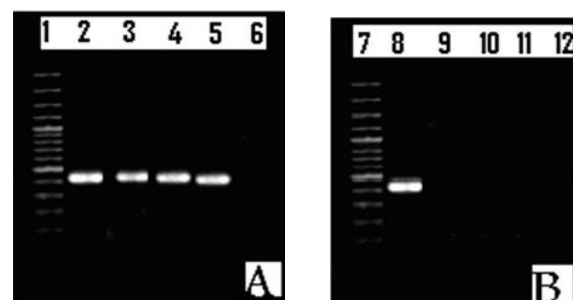


Figure 2. Polymerase chain reaction for detection of *hilA* gene of *S. enteritidis* in standard (A) and *hilA* (B) chicken groups; 1 and 7 gene ruler, 2 and 8 control, 3 and 9 liver (2 days of age), 4 and 10 liver (7 days of age), 5 and 11 caecum (2 days of age), 6 and 12 liver (35 days of age)

Their results also showed that shedding rate of *hilA* mutant is lesser than the parent strain. Madadi et al., (7) reported that following oral inoculation of *hilA* mutant and parent strains of *S. enteritidis* to layer hens, higher number of colonizing bacteria in the internal organs and higher rate of fecal shedding was observed in birds infected with parent strain compared with the *hilA* mutant. However no significant difference was reported for the colonization of bacteria in oviduct between the two strains and both parent and *hilA* mutant strains were isolated from eggs. The study of Lichtensteiger and Vimr, (8) showed that after early infection in pigs, a *hilA* mutant of the host-adapted *S. Cholerasuis* failed to colonize in the intestine and spleen early after oral infection in a signature-tagged mutagenesis (STM) experiment. Also in mice and calves it was shown that colonization of Payer's patches and spleen was reduced early after oral infection using the STM approach. SPI-1 mutants of *S. Typhimurium* LT2, however, were still able to colonize tissues early after infection of young chicks (9). Our results in agreement with previous reports indicated that *hilA* mutant of *S. enteritidis* were still able to colonize bird internal tissues in addition to digestive tract. Colonization ability of *hilA* mutant of *S. enteritidis* indicated that *hilA* gene is only one part of the modulators in Salmonella invasion mechanism. However our results unlike the previous studies showed that colonization of liver and spleen by *hilA* mutant was even higher than its parent strain although this colonization wasn't related to clinical diseases. By implementing PCR, *S. enteritidis* was identified through detection of three related genes in tissues of both chicken groups. Similar to bacterial culture, this test also showed that *hilA* mutant of *S. enteritidis* could able to colonize internal tissue especially caeca. The lack of *hilA* gene's related band in the PCR result of tissues from *hilA* strain infected chicken group was indicative of not cross- contamination of mentioned group with parent strain of *S. enteritidis*. In a study by Bohez et al. (10), *S. enteritidis hilA* mutant was evaluated for its potential to induce protection against challenge by

homologous virulent strain in young chickens. The results showed that mutant strain could colonize the gut and although significant degree of resistance against caecal and internal organ colonization by the challenge strain was found but due to limited persistency, the mutant strain was not useful to induce broiler protection.

Conclusion

Nevertheless the observed persistency of *hilA* mutant in caeca in the present study may promise further studies to reveal the potential of *hilA* mutant strain to prevent colonization of the intestinal tract by a virulent *S. enteritidis* strain.

Acknowledgement

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Conflict of interest

None declared conflicts of interest.

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