A comparative study on the colonization of *Salmonella enteritidis hilA* mutant and its parent strains in laying hens

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Abstract:

BACKGROUND: Several regulatory proteins are involved in *Salmonella* invasion. The key regulator of SPI-1 (*Salmonella* pathogenicity island 1) is *hilA*, a transcriptional activator encoded on SPI-1 that regulates the expression of the SPI-1 secretion system. **OBJECTIVES:** Importance of *hilA* mutation on *S. enteritidis* colonization and shedding in layer hens was evaluated in a long-term experiment. **METHODS:** Two groups of layer hens were orally inoculated with 10^{10} CFU of *hilA* and parent strains of *S. enteritidis*, consequently. At days 2, 7, 14, 21 and 35 post-inoculation samples were taken from cloaca and different parts of digestive and reproduction systems of euthanized birds. **RESULTS:** In the birds infected with parent strain, the higher numbers of colonizing bacteria in the liver, spleen, caecum, small intestine and cloaca-vagina were observed. Fecal shedding in this group was also higher than the *hilA* group. However, no significant differences were observed for the colonization of bacteria in magnum, isthmus and infundibulum of both groups. Using PCR method, *hilA* gene was only detected in tissues of parent group hens. **CONCLUSIONS:** This study has shown that the *hilA* mutant is able to colonize in internal organs; an implication of this is the possibility that genes other than *hilA*, or at least other mechanisms, might be involved in the invasion of *S. enteritidis* to the internal organs of birds.

Introduction

In 2006 there were 160,645 reported human cases of Salmonellosis in the 25 Member States of the European Union (equivalent to an incidence of 35.4 cases per 100,000 people) (Lahuerta et al., 2010). At that time salmonellosis was the second most commonly reported gastrointestinal zoonotic infection across the EU. Outbreaks of salmonellosis are mainly related to the consumption of contaminated eggs or egg-products and, less frequently, of poultry meat (Huneau-Salaün et al., 2009). The overall European Union prevalence of *Salmonella* in table eggs was 0.8% in 2006 and more than 90% of all egg isolates was *S. enteritidis*, and *S. enteritidis* is the most common serotype [52.3%] in the laying flock environment (Lahuerta et al., 2010). The persistence of this organism in poultry house environments poses a continuing threat of infection for laying hens (Lapuz et al., 2008). Additionally, there is suggestion that *S. enteritidis* has some intrinsic characteristics that allow a specific interaction with either the reproductive organs of laying hens or the egg components (Gantois et al., 2009).

Following oral ingestion, *Salmonella* colonizes the chicken gut, especially the caeca, where it

penetrates the mucosal epithelium (Bohez et al., 2008). *Salmonella* actively stimulates its own uptake into epithelial cells by inducing cytoskeleton rearrangements and membrane ruffling. These morphological changes are triggered by proteins of *Salmonella* secreted 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] (Aiastui et al., 2010). Several regulatory proteins that are involved in *Salmonella* invasion have been characterized (Lucas and Lee, 2000). 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 (Saini et al., 2010).

HilA knock-out mutants are unable to enter epithelial cells in vitro. Despite the numerous studies on regulation of *hilA* at the molecular level, studies of the effects of *hilA* expression on in vivo virulence are scarce (Bohez et al., 2006). The study of Lichtensteiger and Vimr (2003) showed that after early infection in pigs, a *hilA* mutant of the host-adapted S. choleraesuis failed to colonize in the intestine and spleen early after oral infection in a signature-tagged mutagenesis [STM] experiment (Lichtensteiger and Vimr, 2003). Furthermore, in mice and calves it was shown that colonization of Payers patches and spleen was reduced early after oral infection using the signature-tagged mutagenesis approach. SPI-1 mutants of S. typhimurium LT2, however, were still able to colonize tissues early after infection of young chicks (Morgan et al., 2004).

Bohez et al. (2006) reported that *hilA* is the key regulator of the *Salmonella enteritidis* pathogenicity Island I. Authors explained that very low numbers of *hilA* mutant strain of *Salmonella enteritidis* are able to colonize in the internal organs, and its shedding is significantly decreased relative to the parent strain when chicks are inoculated with *hilA* mutant of *S. enteritidis* and its parent at day of hatch (Bohez et al., 2006).

It is not known whether SPI-1 mutants are able to colonize layer hen's gastrointestinal and reproductive organs in the long-term after oral infection. So, the present study was designed to evaluate the importance of *hilA* mutation on *S. enteritidis* colonization and shedding in layer hens in a long-term experiment. Therefore, *S. enteritidis hilA* mutant and its parent strains were orally administered to layer hens. Fecal, egg shedding and organ colonization were monitored for 35-days post infection.

Material and methods

Bacterial strain: *S. enteritidis* phage type 4, strain NIDO 76Sa88 Nalr [parent strain] and its *hilA* mutant which is used in this experiment, were obtained from Ghent University, Belgium. The nalidixic acid resistant strain is well-characterized (Van Immerseel et al., 2002).

Hens: Fifty 26-week-old broiler breeder hens were selected from an Arian Grand Parent farm that is under strict control for *Salmonella* and other infectious diseases. They were free of any apparent disease throughout the growing and laying periods. Before the start of the experiment cloacal swabs were taken from all hens and checked for *Salmonella* infection to confirm that the animals were *Salmonella*-free.

Hens were randomly divided in two groups of 25 birds. Each group of birds was inoculated by oral route in the crop, using a plastic tube with 10^{10} colony forming units [CFU] of *S. enteritidis* 76Sa88 Nalr parent strain [St] and *S. enteritidis hilA* mutant [hA] in a volume of 1 mL of PBS as reported previously (Bohez et al., 2006).

At days 2, 7, 14, 21 and 35 post-inoculation, cloacal swabs were taken from survived hens and examined for *S. enteritidis*. Additionally, egg production was reported per group, and 10 eggs were pooled and cultured. On the same days two hens per group were euthanized and post-mortem examinations were carried out. For bacterial analysis, samples were taken from different parts of digestive [caecal, small intestine, liver and spleen] and reproduction [infundibulum-ovules, magnum, isthmus and cloacal-vagina] systems separately.

Bacteriological analysis: Swabs from the cloacae were placed in 5 mL selenite cystein broth and after 24h incubation at 37°C, were cultured on *Salmonella* - Shigella [SS] agar plates. Suspected colonies were cultured in Triple sugar iron agar [TSI] and urea broth tubes. Samples of internal organs were homogenized, and 10-fold dilutions were made in PBS. From each dilution, 100 µL was cultured on SS agar plates with 20µg/mL nalidixic acid. After

overnight incubation [37 °C], the number of CFU/g tissue was determined by counting the bacterial colonies (Bohez et al., 2006). For samples which were negative after titration, pre-enrichment and enrichment were performed in selenite cystein broth. Samples that were negative after titration but positive after *Salmonella* enrichment were presumed to contain 10^1 CFU/g organs. Samples that were negative after enrichment were presumed to have 0 CFU/g. The mean CFU/g tissue was calculated for each group.

On the experimental daily basis, every 10 eggs were pooled together into sterile honey jars and the contents were mixed and homogenized by shaking the jars. These were incubated at 37°C for 48 hours and then plated onto the antibiotic containing SS agars.

PCR: DNA was extracted from tissue samples using DNA Purification kit [Fermentas, Made in EU] and was used as a template to detect ST, spv, *SefA* and *hilA* genes by mono and multiplex PCR [Table 1].

For multiplex PCR, three sets of primers [Table 1] were selected from different genomic sequences amplifying a 429 bp fragment specific for the genus *Salmonella* within a randomly cloned sequence [ST gene], a 250 bp fragment within a spv gene, and a 310 bp fragment within the *sefA* gene specific for *Salmonella enteritidis* (Pan and Liu, 2002).

The polymerase chain reaction was developed for detection of *hilA* gene [401 bp] in parent strain of *S. enteritidis* that was inoculated orally in standard group hens [Table 1]. The *hilA* primers were designed according to the *hilA* gene sequence found at Genome Net [www.genome.ad.jp], accession number U25352.

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 specific forward and reverse primers.

Amplification was carried out using a Techne TC-512 thermocycler [Techne Industrial, UK], as follows: 35 cycles of 30 s for denaturation at 94 °C, 90 s for annealing at 56 °C, and 30 s for primer extension at 72 °C, 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 gels were stained with ethidium bromide [2 µg mL-1] to visualize fluorescent bands while using UV in the gel document system [BIORAD Laboratories, UK].

Results

Egg production and egg culture: Following oral inoculation productivity decreased to a low level in both groups of hens, but was more pronounced in the standard group [Table 2]. Egg pool cultures were positive at 7 and 14 dpi in the standard group whereas it was positive at 2 and 14 dpi for *hilA* group hens.

Figure 1 describes the percentages of *S. enteritidis* isolation from cloacal swabs of the two groups of hens. The percentage of isolation was higher in standard compared to *hilA* group hens throughout the experimental period. *Salmonella* isolation from cloacal swabs was 64% positive at 2 dpi and decreased to 25% at 35 dpi in standard group hens, while in the *hilA* group birds, the isolation rate was 24% positive at 2 dpi and decreased to 0 % at 14 dpi, which remained negative to about 35 dpi.

Internal organs' culture: Table 3 indicates the recovery and colony counts of *S. enteritidis* from the different parts of the digestive system. *S. enteritidis* was isolated from different parts of the gastro-intestinal system throughout the sampling times, but the majority of detection was from the hens of the standard group compared to the *hilA* group hens. In the standard group, the highest recovery rate of *S. enteritidis* was performed from caecum and small intestine until 14 dpi, whereas in the liver-spleen it was done until 7 days post infection. In the *hilA* infected hens, the highest *S. enteritidis* recovery was observed from the caecum and small intestine tissues at 2 dpi, while bacterial isolation from the liver-spleen was not observed permanently [Table 3].

The total recovery of *S. enteritidis* from different parts of the reproduction system was lower than from the digestive system and the majority of isolates of this system were from the standard group hens, compared with *hilA* group hens [Table 4]. In the reproduction system, generally the highest recovery of *S. enteritidis* was performed from the cloaca-vagina and thereafter in infundibulum-ovules tissues.

PCR detection of *S. enteritidis*: Figures 2 and 3 show some results of PCR that were carried out on

Table 1. Primers used for identification of *Salmonella enteritidis* by polymerase chain and multiplex polymerase chain reaction (Pan and Liu, 2002).

Amplification product [bp]	Sequence	Primer	
429 bp	5' -GCCAACCATTGCTAAATTGGCGCA- 3' 5' -GGTAGAAATTCCCAGCGGGTACTGG- 3'	*ST*11 *ST*14	*ST*
250 bp	5' -GCCGTACACGAGCTTATAGA-3' 5' -ACCTACAGGGGCACAATAAC- 3'	S1 S4	Spv
310 bp	5' -GCAGCGGTTACTATTGCAGC- 3' 5' -TGTACAGGGACATTTAGCG- 3'	*SEFA*2 *SEFA*4	*SefA*
401 bp	5' -ACGGCTCCCTGCTACGCTCA- 3' 5'-GCTCAGGCCAAAGGGCGCAT- 3'	*hilA*-F *hilA*-R	*hilA*

Table 2. Percentages of egg production and egg pooled culture after infection of hens by standard [St] or *hilA* [hA] strains of *Salmonella enteritidis*. (*) Daily percentage of egg production/ number of hens.

Days post infection/ groups	Percentages %		Egg pooled culture	
	St	hA	St	hA
2	42/25*	44/25	-	+
7	56/23	62/23	+	-
14	55/20	57/21	+	+
21	50/18	47/19	-	-
35	62/16	65/17	-	-

Table 3. Recovery and counts [log10 CFU/g] of *Salmonella enteritidis* from different parts of digestive system of standard [St] and *hilA* [hA] group hens. dpi = days post infection.

Digestive system/ groups	2 dpi		7 dpi		14 dpi		21dpi		35 dpi	
	St	hA	St	hA	St	hA	St	hA	St	hA
Small intestine	2	2.3	2	0	2.7	0	1	1	1	0
Caecum	4.2	2.3	3.5	1	4	1	1	1	1	0
Liver & spleen	2.6	1	2.3	1	0	1	1	1	1	0

Table 4. Recovery and counts [log10 CFU/g] of *Salmonella enteritidis* from different parts of reproduction system and the cloacal swaps in standard [st] and *hilA* [hA] group hens.

Reproduction system	2 dpi		7 dpi		14 dpi		21dpi		35 dpi	
	St	hA	St	hA	St	hA	St	hA	St	hA
Infundibulum-ovules	0	1	1	1	1	1	1	0	0	0
Magnum	0	1	0	0	0	0	1	1	0	0
Isthmus	0	1	1	0	0	0	0	1	0	0
Cloaca-vagina	2	0	1	1	0	0	1	0	1	0

DNAs extracted from different tissues. In most tissues of the two groups of hens, *S. enteritidis* was detected at 2 and 7 dpi while PCR results were negative at 14 and 35 dpi. Three genes for *S. enteritidis* identification were detected in the tissues related to both *hilA* and standard group hens (Figure 2A, B), while as was expected *hilA* gene was detected only in tissues of standard group but not in *hilA* group hens (Figure 3A, B).

Discussion

Following *Salmonella* inoculation, egg production decreased to a low level that was more pronounced in standard group birds compared to *hilA* group birds. Whether this was a result of *S. enteritidis* infection or from handling during the inoculation of bacteria and sampling is unclear. The eggs' contents culture results were not consistent at different days, but it seems during the period when *Salmonella* was isolated from the reproduction system, *Salmonella* in egg cultures was positive.

Natural infection of poultry by *Salmonella* occurs via oral route, and *Salmonella* colonizes in the intestinal tract with the ceca being the primary site of colonization (Impey and Mead, 1989). At this site, individual and groups of *Salmonella* bacteria lying

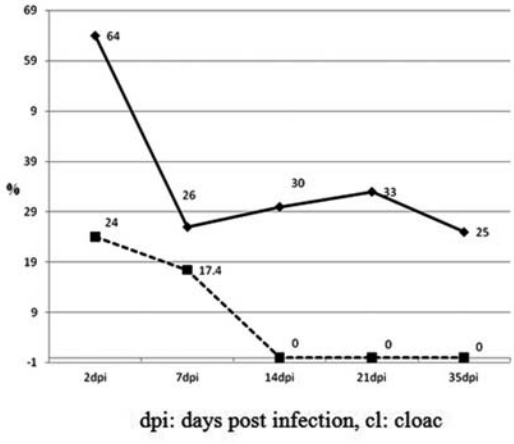

Figure 1. The percentage of S.Enteritidis isolation from cloacal swabs of standard [St] and hilA [hA] group hens.

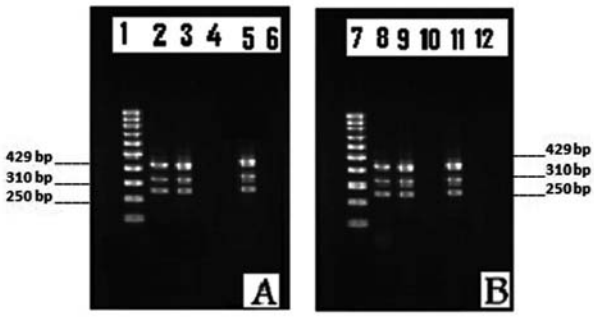

Figure 2. Multiplex polymerase chain reaction for detection of S.Enteritidis in standard (A) and hilA (B) groups hens: 1,7= Gene Ruler; 2,8= Control (+); 3,9= Liver (2dpi); 4,10= Liver (14dpi); 5,11= Caecum (7dpi); 6,12= Caecum (35dpi).

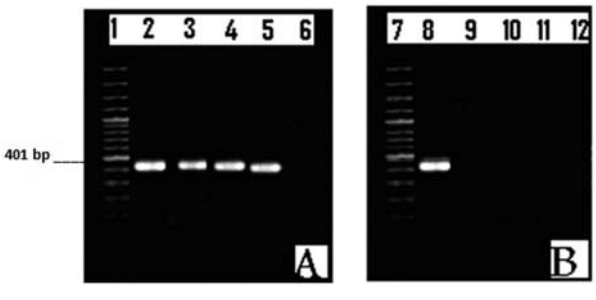

Figure 3. Polymerase chain reaction for detection hilA Gene of S.Enteritidis in standard (A) and hilA (B) groups hens: 1,7= Gene Ruler; 2,8= Control (+); 3,9= Liver (2dpi); 6,12= Liver (35 dpi).

free within the cytoplasm and in membrane-bound vesicles of caecal epithelial cells, as well as in the extracellular space of the lamina propria, have been detected by electron microscopy (Desmidt et al., 1996).

Bohez et al. (2006) reported that *hilA* is required for caecal colonization and long-term shedding of *S. enteritidis* in broiler chickens, as very few *hilA* mutant bacteria would be able to colonize in the internal organs(Bohez et al., 2006). The current study indicated that recovery and the counts of *Salmonella enteritidis* from different parts of gastrointestinal organs in standard group hens were much higher than *hilA* group hens; recovery rates of *S. enteritidis* from the caecum of standard group birds was clearly higher than the *hilA* group birds during the first two-week post infection. Since the *hilA* mutant of *Salmonella* could still be able to colonize in internal organs of birds, in agreement with the report of Bohez et al. (2006), the ability of the *hilA* mutant strain for colonization might be due to the existence of additional mechanisms like sipC and invF proteins for *Salmonella* pathogenicity and internal organ invasion (Murray and Lee, 2000).

It is generally believed that colonization of the reproductive organs is a consequence of systemic spread of *Salmonella* from the intestine (Vazquez-Torres et al., 1999). In the present study, *S. enteritidis* recovery from infundibulum-ovules and cloac-vagina were higher in comparison with the magnum and isthmus. In the majority of studies, a higher frequency of ovary colonization was reported, compared with the frequency of recovery from other sections of the oviduct (Gast et al., 2007). It is strongly believed that *S. enteritidis* must interact with the cellular components of the pre-ovulatory follicles, hence the extensive permeability of the vascular endothelia observed in the ovary may contribute to the higher colonization rate at this site (Griffin, 1984).

In the reproduction system, only the recovery rates of *S. enteritidis* from cloac-vagina of standard group hens were clearly higher than the *hilA* group hens, which persisted up to the 35th day post infection. This coincides with the higher contamination of the caecum in standard group hens that was accompanied with further shedding of *S. enteritidis* in faeces of these hens as well. Therefore, better recovery of *Salmonella* was observed from their

cloacal swab samples.

In PCR test, *S. enteritidis* was identified by detection of three related genes in tissues of both standard and *hilA* group hens. However, the lack of *hilA* gene in the tissues of *hilA* group hens was indicative of not cross-contamination of *hilA* group hens with standard strain of *Salmonella* in this study.

The results of this study indicated that, *hilA* mutant of *S. enteritidis* has reduced ability of contamination in the digestive and reproduction system organs preferably in the caecum of the laying hens. Since *hilA* mutant of *Salmonella* could still be able to contribute in colonization of the hens' internal organs, it is suggested that other genes and mechanisms besides *hilA* might be involved in the invasion of *S. enteritidis*. Further studies must be carried out to reveal the effects of other factors which might be involved in the process of *Salmonella* virulence mechanisms.

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مطالعه مقایسه ای کلونیزاسیون سویه های استاندارد و موتانت *hilA* سالمونلا انتریتیدیس در مرغ های تخم‌گذار

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چکیده

زمینه مطالعه: مقایسه بیماریزایی بین سالمونلا انتریتیدیس و سویه باکتری موتانت یافته ژن *hilA* آن، در مرغ های تخم گذار. **هدف:** بررسی اهمیت ژن *hilA* باکتری سالمونلا انتریتیدیس، بر میزان کلونیزاسیون در امعاء و احشای داخلی مرغ های تخم گذار و میزان دفع آن از طریق مدفوع و تخم مرغ های تولیدی. **روش کار:** در دو گروه از مرغ های تخم گذار، تعداد 10^{10} cfu باکتری از دوسویه سالمونلا انتریتیدیس موتانت یافته در ژن *hilA* و باکتری سالمونلا اصلی یا استاندارد آن، بصورت داخل دهانی تلقیح و سپس میزان جایگزینی این دوسویه باکتری در ارگان های مختلف آنها با هم مقایسه شدند. **نتایج:** میزان کلونیزاسیون باکتری در بافت های کبد، طحال، سکوم، روده کوچک، واژن و کلواک آن گروه از پرنده هایی که با سویه سالمونلا موتانت یافته آلوده شدند کمتر از پرندگانی بود که با سوش اصلی آن باکتری تلقیح شده بودند. میزان سالمونلای جدا شده از سواب های کلواکی مرغ های گروه *hilA* کمتر از مرغ هایی بود که با باکتری سالمونلای استاندارد آلوده شده بودند. اما تفاوت قابل ملاحظه‌ای در میزان جداسازی باکتری از نواحی رحمی (مگنوم و ایستموس) دوگروه از مرغ های مورد مطالعه مشاهده نشد. ضمن اینکه با استفاده از آزمایش PCR، ژن *hilA* سالمونلا، فقط در بافت های آلوده پرنده هایی که سالمونلا استاندارد را دریافت کرده بودند، مورد شناسایی قرار گرفت و ژن *hilA* در بافت های گروه دیگر از مرغان مشاهده نگردید. **نتیجه گیری نهایی:** این مطالعه نشان داد که ژن *hilA* باکتری سالمونلا انتریتیدیس در جایگزینی باکتری در ارگان های داخلی مرغ های تخم‌گذار نقش بارزی دارد ولی این تاثیر انحصاری نبوده و احتمالاً مکانیسم ها و ژن های دیگری هم در تهاجم این باکتری نقش ایفا می کنند.

واژه های کلیدی: سالمونلا انتریتیدیس، مرغ های تخم گذار، ژن *hilA*.

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