British Microbiology Research Journal
4(4): 418-427, 2014

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Salmonella Enteritidis Flagella may Enhance Attachment and Invasion of Hen Ovarian Granulosa Cells and Induce Protective Immune Response in Egg-laying Hens

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Authors' contributions

This work was carried out in collaboration between all authors. Author AAA participated in the design of the study and conducted significant part of the laboratory work. Author MA conducted the statistical analysis and performed a part of the laboratory work. Author AMS designed the study, managed the entire work of the project and writing of the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 11th October 2013
Accepted 27th December 2013
Published 12th January 2014

ABSTRACT

Aims: To study the effect of flagellin on bacterial attachment and invasion of avian ovary cells in vitro by comparing the attachment and invasion of wild-type S. Enteritidis with non-motile mutants. To assess the immunogenic properties of extracted flagellin against *Salmonella* Enteritidis experimental infection in laying hens.
Methodology: Non-flagellated mutants for wild-type *S.* Enteritidis (phage type 8, 13A and 28) were produced by using a strain of *S.* Enteritidis, SA4502, which carried an fliC::Tn 10 to transfer fliC::Tn 10 insertion into the wild type strains using phage 22 (P22)-mediated transduction with selection for antibiotic resistance encoded within the mutant alleles. Granulosa cells were harvested from Single Comb White Leghorn hens between 18-45 weeks of age. Flagellin was purified from the studied bacterial cultures of *Salmonella* Enteritidis following reported methods. Laying hens were immunized with the flagellin with adjuvant
Results: Non-motile mutants of *S.* Enteritidis phage wild types were analyzed to confirm the elimination of H1 flagellin synthesis. Wild-type and fliC mutant strains were assessed for

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their ability to adhere to hen's ovarian granulosa cells. The adherence of the mutant strain was reduced nearly ten-fold compared with that of the wild-type phage 8. Similarly, light microscopic observation of fixed cover slips from wild-type phage types and its mutant strain revealed fewer numbers of the bacterial mutants adhered to the cultured granulosa cell monolayer. Light microscopy revealed similar findings for mutant phage types 28 and 13 A when compared to the wild-type control. There was five folds rise in the egg yolk antibody during the 2-3 weeks post-immunization. No rise was detected in the egg yolk samples from the control hens injected with the placebo mixture without flagellin.
Conclusion: It was concluded that Flagellin has an important role in the attachment and invasion of *Salmonella* Enteritidis to avian ovary cells and that it can be used as immunogenic components to induce a protective immune response in vaccinated hens against challenge infection with the wild type strains.

Keywords: Salmonella enteritidis; flagellin; mutants; attachment; immunization.

1. INTRODUCTION

Egg-laying hens are the major reservoir for *Salmonella enterica* serovar Enteritidis (S. Enteritidis). This *Salmonella* serotype has emerged during the last three decades to become a major cause of human gastroenteritis worldwide [1]. Several reports described *S*. Enteritidis to possess the unusual ability to infect the hen's ovary, thereby permitting the bacterium to contaminate the contents of intact eggs posing significant health risk to consumers [2-4]. The interplay of virulence factors in *S*. Enteritidis pathogenesis is still poorly understood, particularly with regard to the mechanisms and components of the bacterium which are involved with the transovarian transmission of the organism to the developing egg of infected hens. Preliminary investigations in our laboratory suggest that flagella expressed by *S*. Enteritidis are an important virulence factor for colonization of the hen's ovarian system [5]. The contributions of flagella to the virulence of *Salmonella* has been examined in mouse models [6-9] and to some extent in chickens [10,11] and for *S*. enterica serotype Typhimurium, flagella have been demonstrated to play a key role in pathogenesis. In addition to acting as organelles of motility and chemotaxis, which are not always required for virulence [12,13], flagella have been demonstrated to help bacteria survive within macrophages [14], up-regulate tumor necrosis factor alpha [8] and Mediate attachment to epithelial cells. However, there is a paucity of information on the role of flagella in the pathogenesis of *S*. Enteritidis in the avian species. [15] reported that flagella of *S*. Enteritidis are important for the adherence of the bacterium to chick gut explants. In this report, we have demonstrated that *S*. Enteritidis flagellin, the phase-1 protein filament of flagella, plays important role in the attachment of bacteria to chicken ovarian granulosa cells in vitro.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

S. Enteritidis isolates used in our studies have been serotyped and phage typed at the National Veterinary Services Laboratories (NVSL) Ames. Iowa. USA. All isolates are known to be pathogenic to humans or chickens being isolated from human cases of gastroenteritis or chicken organs or egg yolk. Organisms were stored at -70ºC in tryptic soy broth containing 15 percent (v/v) glycerol. Before use the isolates were maintained on tryptic soy

agar slants at 4ºC till needed. Before testing, the bacterial strains were grown overnight in a nutrient broth at 37ºC. For mutation studies, in addition to phage type 8, phage types 28 and 13 A were constructed into fliC mutants.

2.2 Construction of Mutants

A mutant strain of *S*. Enteritidis, SA4502, which carried a fliC::Tn 10 insertions was obtained from Dr. Kenneth E. Sanderson, Director, *Salmonella* Genetic Stock Centre, University of Calgary, Canada. The fliC::Tn 10 insertion from this strain was transferred into the wild-type S. Enteritidis (phage type 8, 13A and 28) with phage 22 (P22)-mediated transduction with selection for antibiotic resistance encoded within the mutant alleles. Elimination of the synthesis of the H1 flagellin was verified by SDS-PAGE as described below. Mutants were also screened for motility in comparison to the wild type by light microscopy.

2.3 Isolation of Flagellin

Bacterial cultures of *Salmonella* Enteritidis were cultivated overnight in brain heart infusion broth (BHI) in a shaking incubator with 200 rpm at 37ºC under aerobic conditions. The bacterial culture was then heat shocked by incubating at 45ºC in a circulating water bath for one hour in 500 ml of phosphate buffered saline (PBS). The bacterial cell suspension was centrifuged at 10,000g for 30 min at 4ø C. The supernatant, which contained flagellin, was precipitated overnight with sulfate (53.2g/L) at 4ºC and then centrifuged at 23,000g for 25 min. Dialysis was carried out overnight at 4ºC against 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5). The protein content was routinely determined by the modified method of Lowry. Bovine serum albumin was used as a protein standard. The flagellin was lyophilized and stored at -20ºC [16].

2.4 SDS-polyacrylamide Gel Electrophoresis

SDS-PAGE (10 percent polyacrylamide separating gel and 4.5 percent stacking gel) was carried out by the methods of [17] to test for the purity of isolated flagellin. All samples were solubilized in 62.5 mM Tris hydrochloride buffer containing 5 percent beta-mercaptoethanol, 3 percent SDS, 10 percent glycerol and 0.01 percent bromophenol blue and heated for 5 minutes in a boiling water bath before loading onto the gel. The protein samples were electrophoresed at 50 mA along with molecular weight standards. The protein bands were stained with 0.025 percent Coomassie brilliant blue. SDS-PAGE analysis routinely revealed a single 56 kDa protein band.

2.5 Isolation of Hen Ovarian Granulosa Cells

Granulosa cells were harvested from Single Comb White Leghorn hens between 18-45 weeks of age. The hens were exposed to CO2 and then killed by cervical dislocation. Only the largest follicles (F1) were aseptically collected. The granulosa cell layer was separated from the theca layer as previously described by Gilbert et al. [18] and the cells were dissociated in Medium 199 with Hank's salts (Gibco, Grand Island, NY) containingNaHCO3 (350mg/L), HEPES (20mM) pH7.4, collagenase (500U/ml,) and trypsine inhibitor (.2mg/ml). Dispersed cells were washed three times in Medium 199 then re-suspended in Medium 199 containing one percent (wt/vol) BSA and NaHCO3 (350 mg/L), HEPES (20mM; pH 7.4). Cell viability was determined by the trypan blue exclusion method.

2.6 Bacterial Attachment Assays

The granulosa cells were grown on 12 mm glass cover slips at density of approximately .7-2 x105 live cells/ml in 24 –well Nunclon plates. In vitro attachment assays and invasions assays were carried our essentially as described by Thiagarajan et al. [19,20]. The cells were incubated overnight a 37ºC with 5 percent CO2. The following day, the cells were washed and 1 ml of fresh Medium 199 was added to the wells. The plate wells were inoculated with approximately 2 x107 CFU of S. Enteritidis phage type 8, 13A and 28 wild-type and/or mutant strain in triplicate and then incubated at 37ºC for 3 hours with 5 percent CO2 after which the cells were washed at least five times with plain Medium 199. For light microscopic examination, the cover slips were fixed in ethanol, stained with 10 percent Giemsa and then mounted onto glass slides. Variations to the procedure included running the assay at 42ºC and adding purified flagellin (5-50 mg) to plate wells at the same time as bacterial inoculation.

2.7 Quantification of Bacterial Adherence for Mutation Studies

The number of S. Enteritidis organisms adhering to granulosa cell monolayers was quantified as described previously [21]. Non-adherent organisms were removed with five rinses of Medium 199. The cells were solubilized with 0.5 percent sodium desoxycholate (Sigma Chemical Co., St. Louis, Mo.). The suspensions were diluted and viable bacteria were determined by counting the CFU on MH agar plates. Results are expressed as the average number of bacteria adhering to granulosa cells from three determinations.

2.8 Preliminary Immunization Study

Twenty-five Single Comb White Leghorn hens approximately 25 weeks of age were obtained from a local farm and ascertained to be free from *S*. Enteritidis infection by serological methods and fecal culture. The birds were housed in individual wire mesh cages in a windowless, air conditioned room with 14h light/10h darkness cycle. They had unlimited access to tap water and a *Salmonella*-free feed (NRC) containing no antibiotics. Birds were randomly assigned into two groups; group 1 was comprised of 15 birds. Each bird received 0.1 ml of adjuvanted immunogen containing .5 mg of purified flagellin subcutaneously. Group 2 was comprised of 10 birds and each bird received a similar amount of adjuvanted BSA as placebo control. Adjuvanted immunogen was prepared by mixing 1.5 ml sesquiloleate (Sigma Chemical Co., St. Louis, MO.) and 0.5 ml of Tween 80 (Sigma Chemical Co., St. Louis, MO.) with 150 mg of flagellin in 5 ml of PBS. The adjuvanted BSA placebo was prepared similarly. Inoculum was given subcutaneously in the neck fold at 25 weeks of age and repeated two weeks later. Three weeks after this booster immunization all birds were orally inoculated with 1 X 10^8 CFU of wild type *S*. Enteretidis phage type 8 in .5 ml of solution as previously reported by Lindell et al. [22].

Eggs were collected daily for 28 days and each bird's weekly production was pooled for bacteriological analysis according to Lindell et al. [22]. In addition 0.1 ml of egg yolk from each bird was added to 0.9 ml of 0.85 percent saline and stored frozen at -20ºC for ELISA analysis as described below. Feces were also collected weekly and cultured in a similar manner. To assess organ carriage, birds' form each group were euthanatized at weekly intervals and liver, spleen, ovary and ceca were collected for bacteriological analysis [22,19].

2.9 Flagellin-specific ELISA

An ELISA specific for flagellin [23] was used to demonstrate the antibody response in egg yolk. Ninety-six well plates were coated with 2 mg of purified flagellin in carbonate buffer (pH 9.6) and allowed to incubate overnight at 4ºC. Serial dilutions of egg yolk extract in Tween 20 PBS were added in triplicates to the labeled wells and plates were incubated at 37ºC for one hour before washing with Tween 20 PBS to remove non-specifically bound reagents. Specifically-bound antibody was detected with alkaline phosphatase-conjugated rabbit anti-chicken I gY (1:10,000). Visualization was performed with p- Nitrophenyl phosphate, reading optical density at 405 nm after 30 min of incubation at 37ºC in a ThermoMax kinetic micro plate reader.

3. RESULTS AND DISCUSSION

3.1 Characterization of FliC Mutants

Non-motile mutants of *S*. Enteritidis phage type 8 were analyzed to confirm the elimination of H1 flagellin synthesis. Equal amounts of wild-type and mutant phage type 8 were exposed to the same flagellin isolation procedure and the purified product was analyzed by SDS-PAGE. The wild type strain demonstrated the characteristic single 56-kDa protein band (Fig. 1).

This protein band was absent for the mutant strain. *S*. Enteritidis phage type 8 flagellin control that has been analyzed by amino acid composition and partial N-terminal protein sequence analysis and determined to be 56-kDa. The in vitro growth rates of all mutant stains in nutrient broth incubated at 37ºC were similar to the wild-type strain. The fliC mutants exhibited no motility when viewed directly by light microscope.

3.2 Adherence of Mutant Strains of S. Enteritidis to In vitro Hen's Ovarian Granulosa Cells

Wild-type and fliC mutant strains were assessed for their ability to adhere to hen's ovarian granulosa cells. The adherence of the mutant strain was reduced nearly ten-fold compared with that of the wild-type strain Light microscopic observation of fixed cover slips from wild-type phage type 8 and its mutant strain revealed fewer numbers of the bacterial mutants adhered to the cultured granulosa cell monolayer (Figs. 2 and 3). Light microscopy revealed similar findings for mutant phage types 28 and 13 A when compared to the wild-type control.

3.3 Effect of Exogenous Purified Flagellin on Bacterial Attachment Assays

Addition of 25 mg of purified flagellin (determined to be optimum amount), concomitantly with S. Enteritidis phage type 8 to in vitro cell cultures of hen's ovarian granulosa cells resulted in enhanced bacterial attachment as observed by light microscopy (Fig. 3). The patterns of bacterial attachment to granulosa cell monolayer changed from a diffuse pattern of attachment to an aggregative pattern with increased numbers of bacteria adhering in clumps [19]. By counting individual granulosa cells in five random fields with 50 or more attached bacteria, it was revealed that addition of flagellin to cell cultures resulted in approximately 50 percent increase when compared to the level of attachment of the wild type strain. Enhanced bacterial attachment was also observed using Hep-2 and CaCo-2 cells instead of granulosa cells (data not shown).

3.4 Egg Yolk Antibody Response to Immunization of Hens with Adjuvanted-flagellin

Fig. 4 shows the flagellin- specific egg yolk antibody measured by ELISA, from eggs laid by the hens immunized with adjuvanted flagellin. There was five folds increase in the egg yolk antibody during the 2-3 weeks post-immunization. No rise was detected in the egg yolk samples from the control hens injected with the placebo mixture without flagellin. Eggs from all birds in each group were collected on a weekly basis. Yolk was pooled from 4-5 eggs from each bird and sampled separately for determination of flagellin specific antibody reactivity using indirect ELISA as described.

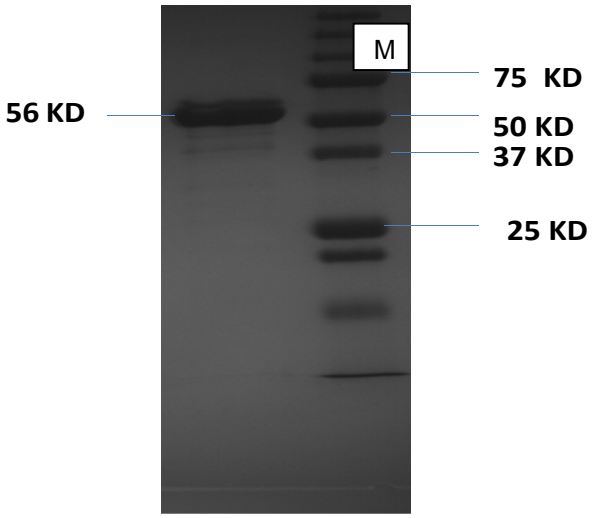

Fig. 1. SDS-PAGE (10%W/V) of purified flagellin preparation from *Salmonella* Enteritidis phage type 8, at 200 V for 40 minutes. Lane M: Molecular weight latter 250 KD. Lane 1: purified flagellin

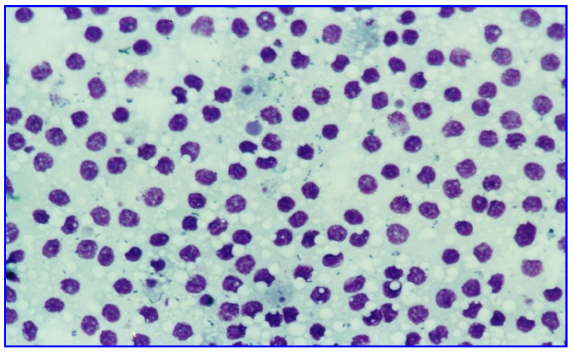

Fig. 2. Light microscopy of fixed Giemsa-stained avian ovary granulosa cells monolayer after infection with the *Salmonella* Enteritidis fliC mutants revealing the reduced attachment of the mutant to hen's ovary granulose Cells in tissue culture. (500X).

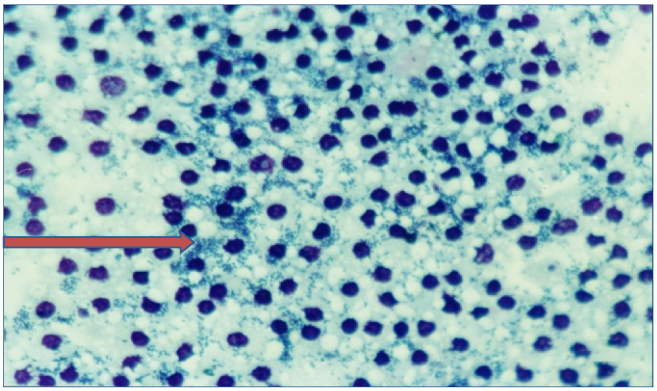

Fig. 3. Light microscopy of fixed and Giemsa-stained avian ovary granulose Cells showing the enhanced attachment of wild-types *Salmonella* Enteritidis with addition of 25 mg of purified flagellin. Arrow points to the aggregating bacterial cells.(500X).

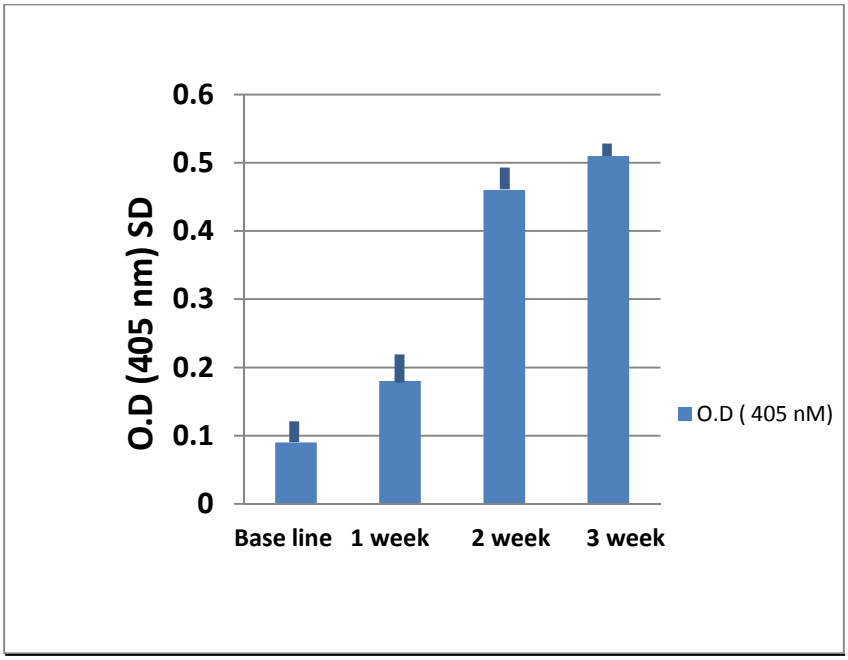

Fig. 4. Egg yolk Enzume-Linked Immunosorbant Assay (ELISA) measurement of S Enteritidis flagellin-specific antibody in yolks from eggs laid by hens immunized with flagellin.Red areas represent the standard deviation. Small bars= standard deviation.

Flagella are highly complex bacterial organelles, which are usually well conserved among diverse bacterial species. Their preservation suggests a key role in the survival of many organisms. Flagella have a role in chemotaxis and motility and also facilitate the acquisition of essential nutrients; thus it seems likely that these organelles have a role in the virulence of pathogenic organisms [24,25]. A role for flagella in the bacterial adherence to epithelial cells has been reported for *Salmonella* species as well as other mucosal pathogens such as

P. aeruginosa and pathogenic E. coli [24,26]. Our attachment assay data suggest that flagella are important for *S*. Enteretidis adherence avian ovary cells as fliC mutants were less able to adhere. This may be due to the loss of motility, to the loss of an adhesion properties or an effect mediated by the morphology of these organelles (i.e. increasing surface area for initial contact). It has been reported that it's unlikely that flagella possess adhesion properties [15,27]. However it was reported that *P. aeruginosa* purified flagellin binds to glycolipids, particularly to the common membrane constituent GM1. The flagella may act as a tether to epithelium, especially if there is epithelial damage and GM1 is exposed [13,24]. It is conceivable that flagellin may stimulate the host cell in some manner, for instance, up regulation or exposure of surface receptors or release of secretary proteins and chemokines since flagella do not need to be intact to result in enhanced bacterial adherence to cultured granulosa cells. The ability of salmonellae to induce tumor necrosis factor alpha (TNFa) is well documented and flagellin is suspected as the TNFa inducing polypeptide [8,28]. Flagellin from *P. aeruginosa* can induce interleukin-8 production in respiratory epithelial cells [13,29]. It is interesting that host cells appear to respond to flagellin from two distantly related organisms, salmonellae and pseudomonads in a similar way. Hyper-flagellation of *S*. Enteritidis has been observed when SE6-E21 is grown in complement at 42ºC versus 37ºC and ten-fold more protein, including flagellin, could be recovered from SE6-E5 when grown under the same conditions [30]. We have observed similar results in our laboratory. We are able to isolate more flagellin when bacteria are heat shocked without complement at 45 C versus 21ºC or 37ºC. At 55ºC, less flagellin was recovered than at any of the temperatures and this was probably due to the death of the organism. Since we are concerned with the elimination of transovarian transmission of *S*. Enteritidis to the developing egg, we tested egg-yolk from flagellin immunized hens for antibody reactivity. Flagellin- vaccinated hens had high egg yolk flagellin-specific antibody. Preliminary investigations, including experimental challenge to see if flagellin has an immunoprotective response are currently being pursued by our laboratory. Although elimination of H1 flagellin synthesis did not completely inhibit bacterial attachment, other surface structures may be involved in bacterial attachment. Thiagarajan [20] has demonstrated a reduction in adherence of *S*. Enteretidis to hen's ovarian granulosa cells in vitro by pre-incubation of the bacteria with purified SEF14 fimbriae.

4. CONCLUSION

It was concluded that flagellin of Salmonella Enteritidis play an important role in the attachment of different phage types of this serotype to avian ovary granulosa cells and that it is possible that flagella and other surface structures may provide a useful target for immune intervention against Salmonella infection of egg laying hens.

COMPETING INTERESTS

Authors have declared that no competing interest exist regarding their work in this project and manuscript.

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