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Contribution of flagella and motility to gut colonisation and pathogenicity of *Salmonella* Enteritidis in the chicken

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ABSTRACT

Salmonella Enteritidis causes fowl paratyphoid in poultry and is frequently associated to outbreaks of food-borne diseases in humans. The role of flagella and flagella-mediated motility into host-pathogen interplay is not fully understood and requires further investigation. In this study, one-day-old chickens were challenged orally with a wild-type strain *Salmonella* Enteritidis, a non-motile but fully flagellated (SE Δ*motB*) or non-flagellated (SE Δ*fliC*) strain to evaluate their ability to colonise the intestine and spread systemically and also of eliciting gross and histopathological changes. SE Δ*motB* and SE Δ*fliC* were recovered in significantly lower numbers from caecal contents in comparison with *Salmonella* Enteritidis at early stages of infection (3 and 5 dpi). The SE Δ*motB* strain, which synthesises paralysed flagella, showed poorer intestinal colonisation ability than the non-flagellated SE Δ*fliC*. Histopathological analyses demonstrated that the flagellated strains induced more *intense lymphoid reactivity* in liver, ileum and caeca. Thus, in the present study the flagellar structure and motility seemed to play a role in the early stages of the intestinal colonisation by *Salmonella* Enteritidis in the chicken.

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Introduction

Salmonella enterica subsp. *enterica* serovar Enteritidis (SE) is a broad-host range micro-organism which poses a threat to both public and animal health.[1] It causes fowl paratyphoid, which is often associated with extensive gut colonisation and bacterial shedding in the faeces.[2,3] SE is one of the commonest serovars related to worldwide food-borne outbreaks.[4]

Following oral infection flagellated strains of *Salmonella* spp. colonise the intestines and flagellin, the main flagellar protein, is recognised through Toll-like receptor (TLR)-5 leading to activation of a pro-inflammatory response and release of the cytokines necessary to initiate the innate and adaptive immune responses.[5] The intense local inflammation triggered during disease helps restrict the bacteria to the intestine and helps to control the systemic infection.[6]

Despite activating the innate immunity, possession of flagella is an important virulence trait which mediates bacterial attachment and invasion.[7,8] In addition, flagella-mediated motility has also been considered as a virulence determinant for gut-associated *Salmonella*. Thus, a non-motile SE strain showed reduced ability to attach to cells in comparison to the parental strain.[9]

The correlation between flagella and flagella-mediated motility and whether or not they contribute independently to Salmonella pathogenesis is unknown. To investigate this, non-motile but fully flagellated (SE Δ*motB*) and non-motile and non-flagellated (SE Δ*fliC*) mutant strains were constructed and the roles of flagella and flagella-mediated motility on intestinal colonisation and systemic invasion of chickens were assessed.

Materials and methods

Bacteria

This study used the spontaneous nalidixic acid resistant strain P125109 (SE). The parent strain was isolated from a case of food-poisoning in humans and is virulent for young chickens and capable of contaminating eggs when inoculated in laying hens.[10] All bacteria were cultured in lysogeny broth (LB – Becton Dickinson, Sparks, Maryland, USA) at 37 °C for 24 h at 150 revolutions per min (rpm).[11]

Mutant construction

Two mutant strains, SE Δ*fliC* and SE Δ*motB*, were constructed using the Lambda-red method[12] and transduction with the phage P22 was used to transfer the mutation to a clean genetic background. Putative mutants were selected in Lysogeny agar (LA – Difco™, Detroit, Michigan, US) containing 20 μg/mL chloramphenicol and confirmed by polymerase chain reaction (PCR). After selection, the chloramphenicol-resistance gene was eliminated by using a helper plasmid expressing the FLP recombinase (pCP20), which acts on the directly repeated FRT (FLP recognition target) sites flanking the resistance gene. Specific primers were designed through PrimerBlast tool[13] and are available in Table 1.

Flagella and flagella-mediated motility detection

SE, SE Δ*motB* and SE Δ*fliC* swimming motility was detected by propagation on semi-solid agar (SSA), after inoculation onto the surface of semi-solid plates consisting of 0.9% heart infusion broth (Oxoid, Basingstoke, Hampshire, UK) and 0.25% LA (Difco, Detroit, Michigan, US), after 24 h incubation at 28 °C assessed by bacterial spread through the soft agar. Flagella expression was additionally confirmed through serum-agglutination using specific anti-H:g,m antibodies (Remel, Dartford, Kent, UK).

Chickens

One hundred and seventy one one-day-old male chickens from a commercial line of egg layer were used in the two experiments. Birds were housed in acclimatised rooms and received water and feed *ad libitum*. On arrival, samples of faeces in the transport cardboard boxes were collected and processed to exclude infection with *Salmonella* spp.[11] In each experiment birds in infected groups received 1×10^9 colony forming units (CFU) of SE, SE Δ*motB* or SE Δ*fliC*, respectively, into the crop using oral gavage needles. Experiments were approved by the institutional ethical committee (Process 1.353/15; approved on 03 March 2015).

Experiment 1 – mortality, clinical signs and faecal shedding

Forty-five chickens were distributed randomly into three groups of 15 animals and then infected. Birds of infected groups received 1×10^9 CFU of SE, SE Δ*motB* or SE Δ*fliC*, respectively, into the crop as above. Birds were observed for four weeks. Mortality and other clinical signs were recorded daily and bacterial shedding in faeces was monitored by cloacal swabs twice a week.[11]

Experiment 2 – local and systemic infection and pathological changes

One hundred and five chickens were distributed randomly into three groups of 35 animals and then infected. Birds in infected groups were infected orally inoculated with 1×10^9 CFU of SE, SE Δ*motB* or SE Δ*fliC*, respectively. A fourth group of 21 chicks was kept as the uninfected control for histopathology. Birds of uninfected control group were also mock-infected with 0.2 mL of sterile lysogeny broth (LB – Becton Dickinson, Sparks, Maryland, USA). At 2, 3, 5, 7, 14, 21 and 28 days post-infection (dpi), five birds from each infected group were euthanased by cervical dislocation and samples of spleen, liver and caecal content collected for bacterial enumeration.[11] Gross pathologies were also recorded.

At the time points above samples of liver, caecum and ileum were collected from the same infected chicks and also from three non-infected animals for histopathology.[15] Samples were formalin-fixed and paraffin-embedded. Tissues were sectioned at 4-μm thickness, stained with haematoxylin and eosin and observed by light microscopy. Lesions were classified as mild, moderate and severe as described previously.[16]

Table 1 – Primer sequences used to construct the SE Δ*fliC* and SE Δ*motB* mutant strains.

Primer	Sequence	Reference
C1	5′-ttatacgcaaggcgacaagg-3′	12
C2	5′-gatcttccgtcacaggtagg-3′	12
motB F	5′-tgccgtggaatttggtcgta-3′	This study
motB R	5′-atccagagttgccgacagtg-3′	This study
motB75F	5′-atgaaaaatcaggctcatcccattgtcgtcgtaaaacgccgcaggcacaaaccgcacggcggcggggcgcgtgtaggctggagctgcttc-3′	This study
motB75 R	5′-tcacctcggttccgcttttggcgatgtgggtacgcttgccggcggggctgccgcaggctgttgtaatacacttaccatatgaatatcctccttag-3′	This study
fliC_ctr F	5′-gttatcggcaatctggaagc-3′	14
fliC_ctr R	5′-ggtgacaaaggcaggttcag-3′	14
fliC50 F	5′-gatacaagggttacggtgagaaaccgtgggcaacagcccaataagtgtaggctggagctgcttc-3′	14
fliC50 R	5′-ctttcgctgccttgattgtgtaccacgtgtcggtgaatcaatcgccggacatatgaatatcctccttag-3′	14

Long primers were used for amplifying antibiotic cassettes. Shorter primers were used for verifying cassette insertion.

Statistical analysis

Data on mortality and faecal shedding were compared by chi-square test.[17] Statistical differences amongst viable bacteria numbers recovered from caecal contents, livers and spleens were determined using Tukey's test.[6] Statistical tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

Results

Mutagenesis – flagella and flagella-mediated motility assessment

Deletion of *fliC* and *motB* genes from the SE chromosome was first confirmed by PCR. The mutant strains showed impairment in their ability to spread throughout the SSA after 24 h incubation and only a small halo, nearly 6-mm diameter, was noticeable in the centre of the agar. By contrast, SE was able to cover the whole semi-solid surface after 24 h of incubation. The serum agglutination test targeting the flagellar antigens (H: g,m) was positive for SE and SE Δ*motB* strains and negative for SE Δ*fliC*.

Experiment 1 – mortality and faecal shedding

Clinical manifestations began at 4 dpi in all infected chickens in which somnolence, closed eyes and persistent diarrhoea (up to 13 dpi) containing smears of blood were observed. SE and SE Δ*fliC* infections produced 13% mortality ($n = 2/15$ infected) whereas no mortality occurred amongst SE Δ*motB*-infected chickens. Despite this, no statistical significance was found between the mortality rates ($p > 0.05$). Additionally, the number of positive cloacal swabs from which the inoculated strain was recovered was very similar amongst the animals infected with SE (92.5%), SE Δ*motB* (87.5%) and SE Δ*fliC* (93.3%), and it was not statistically significant ($p > 0.05$).

Experiment 2

Caecal colonisation and systemic invasion

The results of bacterial enumeration in livers, spleens and caecal contents are shown in Fig. 1. There was no statistically significant difference between the bacterial numbers in caecal content ($p = 0.7225$), liver ($p = 0.5618$) and spleen ($p = 0.5294$) at 2 dpi. SE colonised the caecal contents in higher numbers early (3, 5 and 7 dpi) in infection ($p < 0.05$). However, from 14 dpi onward the bacterial counts of all strains in caecal contents decreased to similar numbers ($p = 0.6257$). Bacterial recovery from livers and spleens was very similar for all three strains. SE reached the spleens in higher numbers at 3 dpi ($p < 0.05$) but at 5 dpi onward all strains showed a similar behaviour ($p = 0.1880$). The bacterial numbers in the livers were low (10^3 CFU/g) throughout the experiment for all three strains and no statistical significance was found ($p = 0.3513$).

Pathological changes

No gross pathology was observed in any infected animal at 2 dpi. From 3 dpi, mild hepatosplenomegaly and mild haemorrhagic enteritis were observed in SE-infected chickens, whereas no noticeable changes occurred in the intestines of SE Δ*fliC*- and SE Δ*motB*-infected chickens. The greatest changes, however, were noticed at 7 dpi when congestive hepatosplenomegaly and thickened intestinal mucosa were noticeable in all necropsied animals. From 14 to 28 dpi gross pathologies became mild but present in all infected animals.

The most severe histopathological changes were observed in the liver, ileum and caeca of SE-infected chickens. SE induced hepatocyte degeneration and lymphoid reactivity from 2 dpi, but at 7 dpi, the former became severe and diffused and the latter moderate and mostly surrounding the portal triads and perivascular areas. During this same span of time (2–7 dpi) SE Δ*motB* induced milder hepatocyte degeneration and moderate lymphoid reactivity surrounding the portal triads and perivascular areas whereas SE Δ*fliC* provoked mild foci of necrosis with mild adjacent infiltration of mononuclear cells in the hepatic parenchyma. At 14, 21 and 28 dpi mild hepatocyte degeneration with lymphoid reactivity at parenchyma was seen in livers in all infected animals.

In the gut SE elicited moderate multifocal lymphocyte infiltration in the ileal lamina propria mucosa from 2 to 7 dpi. At 5dpi SE Δ*motB* elicited mild multifocal lymphocyte infiltration in the ileal lamina propria (Fig. 2). Meanwhile in SE Δ*fliC*-infected chickens this alteration was observed later, at 7 dpi. From 14 dpi onwards, lymphocyte infiltration in the ileal lamina propria became moderate in birds infected with both mutant strains. By contrast, SE caused diffuse and moderate lymphocyte infiltration in caecal lamina propria during all

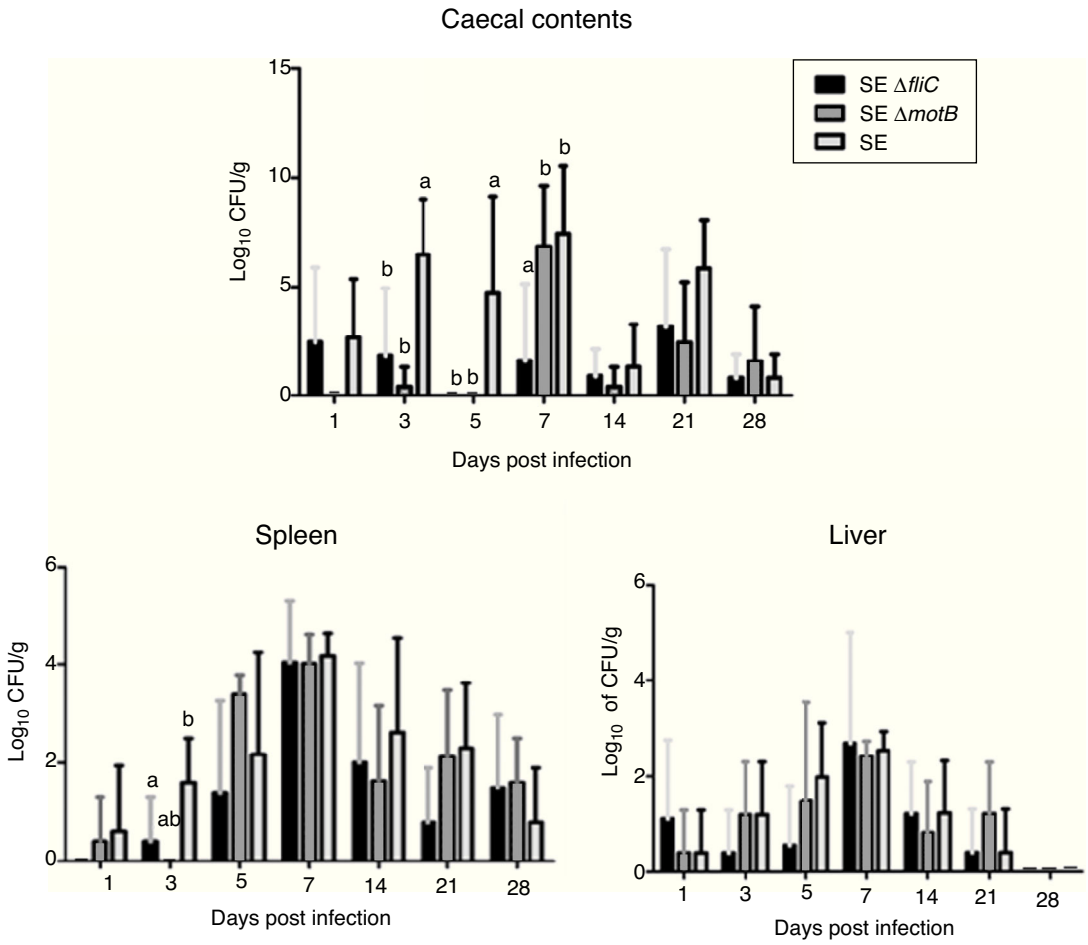

Fig. 1 – Bacterial counts (log_{10} CFU/g) in livers, spleens and caecal contents collected from one-day-old chicks infected with SE, SE Δ*fliC* and SE Δ*motB*. Different letters on the plots mean there was statistical significance by Tukey's test ($p < 0.05$) between distinct treatments by day.

experiments. The SE Δ*fliC* and SE Δ*motB* strains caused the same lesions, but at 3 and 5 dpi mild lymphocyte infiltration in the caecal lamina propria was observed. Mononuclear cell infiltration in lamina propria in addition to villus fusion and submucosal oedema became mild and similar in all infected birds after 21 dpi.

Discussion

Flagella and flagella-mediated motility are considered important factors for salmonellosis.[18] Their contribution to S. Enteritidis (SE) pathogenicity in poultry has been evaluated in separate studies,[8,19,20] but the role of flagella as opposed to motility still requires further investigation. To shed light on this subject, the present study compared the infection biology of the motile and fully flagellated SE strain P125109 and its derivative mutant strains, one non-motile and non-flagellated (SE Δ*fliC*) and other non-motile but flagellated (SE Δ*motB*) using one-day-old male chickens as the model.

Over the 4-week experiment 1 bacterial recovery from faeces was similar for all strains independent on the phenotype. In agreement with this result, a previous report showed that the infection of chicks by a wild-type SE and a non-motile flagellated resulted in a similar degree of faecal excretion.[20] These findings, combined with the absence of significant mortality in the present study, show that neither the absence of flagella nor its related motility alter the faecal excretion ability of SE in chickens.

Although the mutations introduced into the SE chromosome did not impair bacterial shedding by faeces, the ability to colonise the caeca early was altered since the counts of SE Δ*fliC* and SE Δ*motB* in caecal contents at 3 and 5 dpi were significantly lower. Previous studies using chicken infection, chicken gut explants or cultured epithelial cells infected with non-motile strains of *Salmonella*, also reported the reduced ability of these mutant strains to colonise/adhere to the cells when comparing to the wild type flagellated strains, in the early stages of colonisation.[7,19,21] Taken together these results suggest that flagella and flagella-mediated motility would play important roles early (up to 5 dpi), but not later, during SE infection in chickens. This conclusion is also supported by the fact that at 7 dpi both mutant strains started to cause intestinal histopathological changes similar to those induced by the wild-type strain.

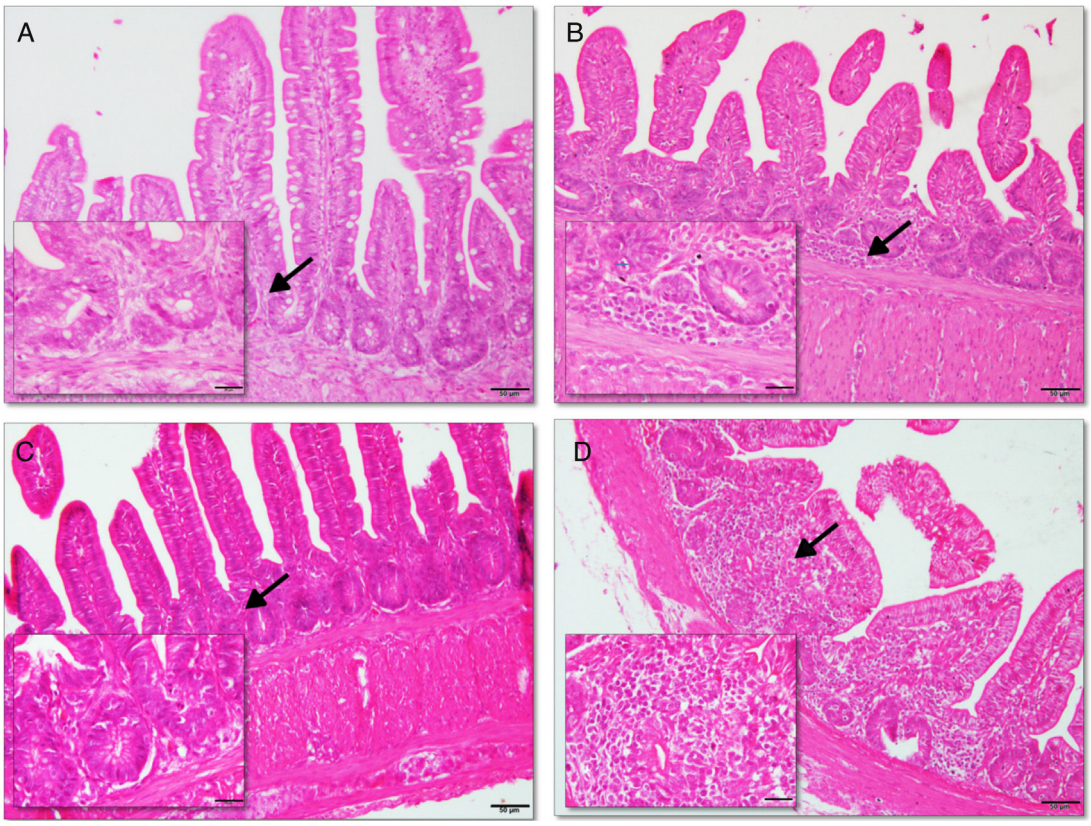

Fig. 2 – Transverse sections of ilea collected at 5 dpi from chicks infected at 1 day of life with SE, SE Δ*motB* or SE Δ*fliC*. (A) Healthy ileum collected from an uninfected bird showing no mononuclear infiltration (arrow); (B) presence of mild mononuclear inflammatory infiltrate in the mucosa (arrow) of a SE Δ*motB*-infected chick; (C) no detectable changes in ileal mucosa (arrow) of a SE Δ*fliC*-infected chick; (D) presence of severe mononuclear inflammatory infiltrate (arrow) in fused and shortened villi in the ileal mucosa of a SE-infected chick. Haematoxylin and eosin staining. Scale bars: 100 μM (small images) and 40 μM (large images).

It has been hypothesised that recognition of flagellated *Salmonella* strains through intestinal TLR5 leads to activation of pro-inflammatory response which in turn helps to restrict the bacteria to the intestine and to prevent systemic infection.[5,7] However, in the present study, at 3 dpi the wild type SE strain was recovered from spleen in higher counts than the non-flagellated SE Δ*fliC*. Very similar results were reported in rats infected with flagellated and non-flagellated-SE strains.[20] It seems that the absence of flagella in SE was in fact disadvantageous in establishing systemic infection. Further studies must be carried out in order to better characterise the immunological bases of the infection by these strains and to assess whether or not the lower systemic colonisation of SE Δ*fliC* is a consequence of its reduced invasion ability due to the absence of motility.[9]

Interestingly, SE Δ*fliC*, but not SE Δ*motB*, showed improved ability to colonise the caeca at 7 dpi. Similar results were previously reported for *Salmonella* Typhimurium (STM) in a murine ligated-loop invasion assay. It was postulated that the extracellular electrostatic repulsion produced around the paralysed flagella prevented the contact between bacteria and intestinal cells, thus affecting the gut colonisation.[22] This phenomenon, designated as steric hindrance, could also be the possible explanation for the longer lower level caecal recovery of SE Δ*motB* compared to SE Δ*fliC* observed in the present study.

The wild-type strain induced, at the early stages of infection, more severe hepatic lesions. According to Xiao et al.,[23] flagella expression is inhibited in the liver although a minimal amount of flagellin released by flagellated strains is sufficient to stimulate the immune system *via* TLR5 recognition and induce subsequent function abnormality and damage to the liver. SE Δ*fliC* does not produce flagellin which is thought to be the reason by which only mild hepatic lesions were produced by this strain early in infection. In this study SE Δ*motB* induced inflammatory infiltration in ileal and caeca mucosae. This result agrees with that shown by Xiao et al.[23] in which paralysed flagella were associated with a significant reduction in *in vitro* invasiveness although presumably still able to signal through TLR5.

Data generated in this study showed that the lack of either flagella or flagella-mediated motility impairs SE pathogenicity in young chickens, chiefly in the intestine and early during infection. The paralysed flagella also appeared to be more detrimental than the complete absence of flagella, a fact demonstrated previously in epithelial cells.[9] These results imply that motility in *Salmonella* contributes to the early stages of intestinal colonisation.

Conflicts of interest

There is no conflict of interest.

Acknowledgments

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