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Populations of *Salmonella enteritidis* in Orally Infected White Chinese Goose

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Abstract: The objective of this study was to understand populations of *Salmonella enteritidis* (*S. enteritidis*) in the internal organs of geese after oral challenge. Researchers conducted serovar-specific Fluorescent Quantitative Real-Time PCR (FQ-PCR) for *S. enteritidis* to detect the genomic DNA of *S. enteritidis* in the blood and the internal organs including heart, liver, kidney, duodenum, jejunum, ileum, cecum, the bursa of fabricius, thymus, spleen and harderian gland from geese after oral challenge at different time points. To validate these results, the Indirect Fluorescent Antibody (IFA) technique was employed. The results showed that *S. enteritidis* was consistently detected in all the samples. Ileum and jejunum were positive at 8 h PI and the last organ to show a positive result was the thymus at 36 h PI. The copy numbers of *S. enteritidis* DNA in each tissue reached a peak at 24 h and 2nd day PI with the blood, jejunum, ileum, liver and spleen containing higher concentrations than other tissues. However, the number of bacteria started decreasing by 2-3 days and by 6 days, the concentration of *S. enteritidis* DNA was below the detection limits of the FQ-PCR assay except the spleen. The IFA results were similar to those of the FQ-PCR. This study will help in understanding the pathogenesis of *S. enteritidis* infection *in vivo*.

Key words: *Salmonella enteritidis*, internal organs, distribution, goose, detection, tissues, China

INTRODUCTION

The Chinese goose is a breed of domesticated goose descended from the wild Swan goose. Chinese geese differ from the wild birds in much larger size (up to 5-10 kg in males, 4-9 kg in females) and in having an often strongly developed basal knob on the upper side of the bill. The knob at the top of the beak is more prominent on males than females. By 6-8 weeks of age, the knob is already pronounced enough that it can be used for mating. Chinese geese are a close cousin of the African goose, a heavier breed also descended from the Swan goose. In Gui Zhou, China, the consumption of goose products is high and the number of *S. enteritidis* cases in humans has increased considerably in recent years. Further, this disease has had significant economic impacts on the poultry industry especially the egg industry (Gillespie *et al*., 2005). Due to the increased prevalence of *S. enteritidis* and its complex pathogenesis, it is important to understand the correlation between the levels of this bacterium in internal organs and the progression of the infection (Deng *et al*., 2008a), this has not been previously described in geese. Generally, little is known about the pathogenesis of *S. enteritidis* in geese after *S. enteritidis* infected (Takata *et al*., 2003). Up to day, the mechanisms by which *S. enteritidis* and other serotypes persist within the host and the reasons for the absence of immune clearance are not known. In a susceptible host, *S. enteritidis* replicates primarily in the mucosa of the digestive tract after oral challenge and then spreads to the spleen, liver and various other organs and tissues after oral challenge (Dibb-Fuller *et al*., 1999). The objectives of the present study were to determine the pathogenesis of a high-virulence strain of *S. enteritidis* (No. 50338; phage type 4) in geese by a time-course study using a quantitative TaqMan assay (Deng *et al*., 2008b); researchers believe that this analysis will help provide valuable insights into the etiology of *S. enteritidis* infections.

MATERIALS AND METHODS

Bacterial strains: A high-virulence strain of *S. enteritidis* (phage type 4; No.: 50338) was purchased from the National Center for Medical Culture Collection.

Experimental animals and samples: Three months old white Chinese geese free from *S. enteritidis* infection were used in the study. Prior to challenge with *S. enteritidis*, all geese were found to be negative for *S. enteritidis*-specific antibodies and *S. enteritidis* specific antigens by an enzyme-linked immunosorbent assay and PCR, respectively (Deng *et al*., 2008a; Gast and Beard, 1990). The geese were maintained in isolation units in a biosecure animal building. In brief, *S. enteritidis* cells were grown overnight in a Luria-Bertani broth. The cells were cultured overnight and then the presumptive live number of *S. enteritidis* cells was determined by the spread plate

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method. Thereafter, a group of 52 geese were orally infected with a high-virulence *S. enteritidis* strain (No. 50338; phage type 4). Animal experiments were reviewed by an Institutional Animal Care and Use Committee (IACUC) for humane use of animal for experimental purposes. Each goose was orally infected with a *S. enteritidis* strain (No. 50338), at 4.0×10^5 cells goose^{-1}. Another group of 56 goose was treated with an equal volume of water and used as a control group. The blood, heart, liver, kidney, duodenum, jejunum, ileum, cecum, the bursa of fabricius, thymus, spleen and harderian gland were analyzed by a FQ-PCR assay at postinoculation times of 30 min; 4, 8, 10, 12, 24, 36 and 60 h and 2, 3, 6, 9 and 12 days. At each time point, 4 geese were randomly selected from the infection and control groups and their tissue samples were collected and processed for further analyses. DNA from the tissue samples was performed as described previously (Deng *et al*., 2008b). Briefly, 0.5 g of the tissue sample was ground up using a tissue grinder in the 1.5 mL Eppendorf tube. The pellet was resuspended in 500 μL TE buffer (pH 8.0) with 10 μL Proteinase K (30 g L^{-1}) and incubated at 37°C for 2 h. A 0.5 mL blood sample was suspended in 500 μL PBS directly. Finally with a conventional phenol/ chloroform/isoamyl alcohol method to extract the genomic DNA of *S. enteritidis* from tissue used 5 μL aliquot of DNA template for FQ-PCR detection.

Quantitative real-time PCR assay for detection of *S. enteritidis* DNA: In the previous study, there have established a serovar specific real-time PCR assay (GenBank Accession No. AF370707.1), the limit of detection was 70 copies g^{-1} (Deng *et al*., 2008a). Brifely, a real-time PCR assay was carried out using a real-time PCR core kit (R-PCR Version 2.1, Takara, Japan) with an Icycler iQ™ real-time PCR detection system (Version 3.1, Bio-rad, USA) and was performed as described previously. PCR amplification was performed in a 25 μL reaction mixture containing 0.6 μL of each primer (10 μmol L^{-1}), 0.75 μL deoxyribonucleotide triphosphates (dNTPs) (10 mmol L^{-1}), 1.25 U Ex Taq DNA Polymerase (Takara Ex Taq Hot Start Version, Takara, Japan), 5 μL of 5×PCR buffer (free Mg^{2+}), 0.8 μL TaqMan probe (5 μmol L^{-1}), 0.5 μL Mg^{2+} (250 mmol L^{-1}) and 5 μL templates. The reaction mixture was subsequently made up to a volume of 25 μL with deionized water. Each PCR run consisted of a 5 min hot start at 95°C which activated the conjugated polymerase followed by 40 cycles consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C and a fluorescent read step.

Differences between the FQ-PCR and IFA assay results: To validate the results, researchers simultaneously performed a quantitative bacteriological test to determine the bacterial burden in the corresponding tissues and compare these data with the PCR data. In the previous study, there was also established a specific method of IFA staining for *S. enteritidis* (Yan *et al*., 2008). At present, researchers relied on the IFA assay to study the distribution pattern and quantity of *S. enteritidis* in the internal organs of geese after oral challenge.

Statistical analysis: The PCR assay and data acquisition and analysis were performed using the iCycler iQ Optical system software (Version 3.1; Bio-Rad, USA). The number of target copies in the reaction was deduced from the threshold cycle values. The threshold cycle value corresponds to the fractional cycle number at which the fluorescence emission exceeds the standard deviation of the mean baseline emission by 15-fold. Plasmid DNA containing the target amplicon was diluted to contain 7.0×10^2-7.0×10^8 copies of the target DNA per test tube and used as the plasmid standard series. All samples were analyzed 3 times by the real-time PCR assay and concentrations of the target DNA detected were expressed as the mean $\log_{10}$ of the bacterial genome copy number per gram of tissue tested. The real-time PCR data were analyzed using Version 11 of the SPSS software. The comparison of means was performed using Duncan's multiple-range test. The $p<0.05$ was considered statistically significant.

RESULTS

The distribution of *S. enteritidis* within the internal organs after oral challenge was determined by means of FQ-PCR over a 12 days period at intervals. The results showed that the jejunum and ileum tested positive for *S. enteritidis* at 8 h PI. Thereafter, *S. enteritidis* was consistently detected in all the samples at 12 h PI; the last organ to show a positive result was the thymus at 36 h PI. The copy numbers of *S. enteritidis* in each tissue reached a peak at 24 h and 2nd day PI.

The blood, spleen, liver, jejunum and ileum contained high concentrations of *S. enteritidis* whereas the kidney, Harderian gland, bursa of Fabricius and Thymus exhibited low concentrations. The numbers of bacteria decreased at 2nd and 3rd day. By 6 days, all the sample did not show positive results except the spleen. The internal organs of the geese in the control group did not show any positive results at any time point. The details are shown in Table 1.

The jejunum and ileum exhibited a positive *S. enteritidis* signal by IFA at 10 h PI. Thereafter, a positive signal was detected in all the samples at 36 h to 2 days; a stronger positive signal was observed in the spleen, jejunum, ileum and liver compared to the other organs. The positive *S. enteritidis* signal clearly decreased at 60 h PI and no positive results were detected in thymus. However, it was possible to detect a positive

Table 1: Kinetics of *S. enteritidis* DNA loads in geese orally infected with a high-virulence strain determined by quantitative real-time PCR

Time	30 min	4 h	8 h	10 h	12 h	24 h	36 h	2 days	60 h	3 days	6 days	9 days	12 days
Heart	0.0	0.0	0.0	0.0	2.8	3.3	4.3	5.3	4.4	2.7	0.0	0.0	0.0
Liver	0.0	0.0	0.0	0.0	2.6	4.1	4.8	6.5	4.1	3.3	0.0	0.0	0.0
Kidney	0.0	0.0	0.0	0.0	2.0	2.9	3.6	4.6	3.4	0.0	0.0	0.0	0.0
Duodenum	0.0	0.0	0.0	0.0	2.4	3.2	4.7	5.7	4.5	3.0	0.0	0.0	0.0
Jejunum	0.0	0.0	2.1	2.5	3.7	4.5	6.4	8.1	5.9	4.7	0.0	0.0	0.0
Ileum	0.0	0.0	2.3	2.7	3.6	4.5	6.3	7.9	6.3	4.2	0.0	0.0	0.0
Cecum	0.0	0.0	0.0	0.0	2.4	3.6	4.7	5.7	4.2	2.3	0.0	0.0	0.0
BF	0.0	0.0	0.0	0.0	0.0	1.9	2.5	2.9	1.8	0.0	0.0	0.0	0.0
Thymus	0.0	0.0	0.0	0.0	0.0	0.0	2.3	2.6	0.0	0.0	0.0	0.0	0.0
Spleen	0.0	0.0	0.0	2.1	2.6	3.9	5.3	7.2	6.4	3.4	2.9	2.3	1.9
HD	0.0	0.0	0.0	0.0	0.0	1.9	2.6	4.9	1.9	0.0	0.0	0.0	0.0
Blood	0.0	0.0	0.0	0.0	1.9	3.1	5.2	6.9	4.8	3.6	0.0	0.0	0.0

The unit: $\log_{10}$ copies mL^{-1} for blood and l g copies g^{-1} for others each time point represents the mean concentration of genomic DNA and is expressed as $\log_{10}$ of the bacterial genome copy numbe/g/mL of tissue tested obtained from 4 geese. Each sample was analyzed 3 times by the FQ-PCR assay. In this study, researchers get the mean from 12 tests for each sample and the 12 results were not different for each sample ($p>0.05$); min = minute, h = hour, d = day, BF = Bursa of Fabricius, HD = Harderian gland

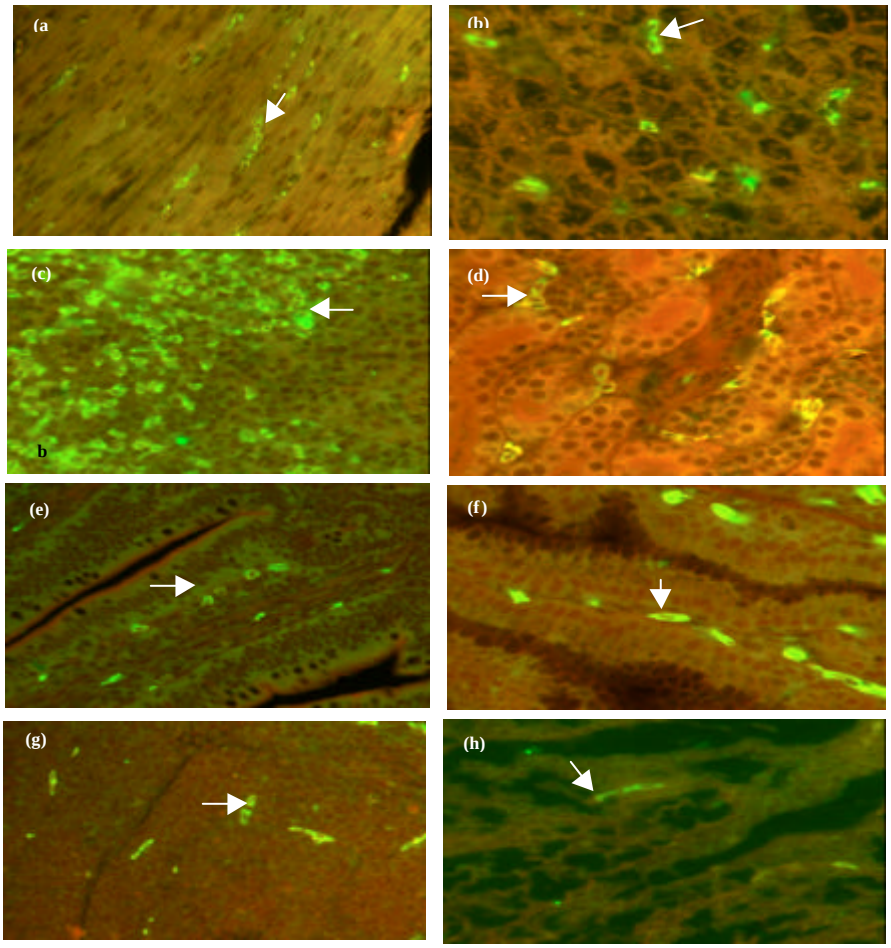

Fig. 1: An IFA staining assay was used to detect *S. enteritidis* in the infected-group samples at 36 h PI. Samples from infected-group showing positive result; images were acquired by using a 60×objective; a = Heart, b = Liver, c = Spleen, d = Kidney, e = Cecum, f = Harderian gland, g = Bursa of fabricius, h = Thymus

signal in the spleen at 6th day PI. Apparently, the results were similar to the results of FQ-PCR. Therefore, the FQ-PCR assay was considered to be a more sensitive and accurate method for this study (Fig. 1).

DISCUSSION

Many studies confirmed that the bursa of Fabricius, harderian gland and thymus have significant roles to play in the development of adaptive immunity (Cooper, 2002; Glick, 1995). It has also been determined that M cells are interspersed within the follicle-associated epithelium of each follicle which explains the movement of antigens from the lumen into the medulla of the follicle where immature B cells develop (Sayegh and Ratcliffe, 2000; Scott, 2004). Furthermore, it has been reported that the limited gene rearrangement of pre-bursal cells and the gene conversion events primarily produce antibody diversity in the bursal cells of poultry (Weill and Reynaud, 1987). In this study, the results showed that the *S. enteritidis* populations in the bursa of fabricius, harderian gland and thymus were much lower than that in the other organs sampled with copies of *S. enteritidis* being 10000-100000 times less than those in other regions. The possible reasons for this occurrence are described above.

Interestingly, *S. enteritidis* organisms have a higher tropism in the Harderian gland than in the thymus and bursa of fabricius with copies of *S. enteritidis* being 100 times more than those described pervious. It has been reported that the Harderian gland plays an important role in controlling mucosal immunity in chickens (Li *et al*., 2004). IgA-positive lymphocytes migrate from the Harderian gland to the caecal tonsils; the route of lymphocyte migration from the Harderian gland to the intestine is not clear (Akaki *et al*., 1997). Moreover, it has been reported that significant IgG and IgA antibody responses were induced in both the serum and intestinal mucosa (Akaki *et al*., 1997). Previous studies suggested that lymphocytes in the bursa of fabricius differentiate into Ig-producing cells in the Harderian gland and immunization with liposomes stimulates the Harderian gland lymphocytes to produce IgA and IgG thereafter, lymphocytes in the caecal tonsil that may have migrated from the Harderian gland induce an immune response in the lamina propria of the intestinal wall (Glick, 1978). Thus far, the colonization mechanism of *S. enteritidis* in the immune organs is not clear and further studies are required to understand the same.

Salmonella cells have to attach to or form a close association with the intestinal epithelium in order to colonize the gut and sub-sequently invade the underlying tissues. Previous studies suggested that *S. enteritidis* cells were removed through circulation in the lymphatic system, especially the Peyer's patches in the gut (Takata *et al*., 2003). In the studies, the spleens were positive at 10 h PI but not the blood. This indicates that the *S. enteritidis* cells may have been removed through circulation in the lymphatic system primarily and this finding is similar to the results obtained in previous researches (Deng *et al*., 2008b).

Moreover, recent studies have shown that the systemic spread of Salmonella can occur to some extent without removal through the lymphatic system and blood circulation (Vasquez-Torres *et al*., 2000). Pathogens presented subepithelially or even luminally by dendritic cells or CD18-expressing phagocytes can be transferred directly to the liver and spleen (Sierro *et al*., 2001).

Previous studies showed that there is an association between Salmonella infection and intracellular survival of the organism in macrophages which can be regarded as safe sites for bacterial multiplication (Dunlap *et al*., 1991). The mechanism underlying the survival of *S. enteritidis* within macrophages is unclear and it seems likely that the type 3 secretion system encoded by Salmonella pathogenicity island two may play a major role in this survival (Hensel *et al*., 1998).

One of the functions of the Salmonella pathogenicity island 2 is to inhibit NADPH oxidase-dependent killing of Salmonella (Vasquez-Torres *et al*., 2000). Furthermore, it has been reported that the *S. typhimurium* genome encodes many genes responsible for resistance against the stressful environment encountered within the macrophage. Such stress includes the production of superoxide anions (O_2^-) and other Reactive Oxygen Species (ROS) by NADPH oxidase and production of NO and other Reactive Nitrogen Species (RNS) by inducible Nitric Oxide (NO) synthase (iNOS) (Gilberthorpe *et al*., 2007). A number of important bacterial pathogens infect, replicate and persist within nucleated cells of the host and T cell-mediated immunity has proven to be a critical factor in the effective clearance of many such intracellular bacterial pathogens. Moreover, Salmonella can induce the suppression of cellular responses (Zhang *et al*., 2000). Simultaneously, it has been reported that chicken macrophages display differences in their responses to *S. enteritidis* and *S. typhimurium* and contribute to the differential pathogenesis of these Salmonella serovars (Bihl *et al*., 2003). Further, it has been reported that *S. enteritidis* infection induces less inflammation resulting in a more commensal environment in the host while an *S. typhimurium* infection can be cleared more rapidly by induction of inflammatory molecules (Okamura *et al*., 2005). *S. enteritidis* produced an increase in splenic CD3 and reduced B populations, it was difficult to associate this increase with *S. enteritidis* clearance due to lack of any significant changes in $CD4^+$ or $CD8^+$ cells (Mittrucker *et al*., 2002). The functions of the spleen in filtration, immune responsiveness and activation of

complement have been well documented. The spleen is made up of a lymphocyte-rich white pulp and macrophage-rich red pulp it is comprised of distinctive B cells and macrophages. Therefore, what researchers described above may be the reason for why a significant number of *S. enteritidis* cells can be tested in the spleen in this study.

Over the 12 days period, the *S. enteritidis* populations in the jejunum and ileum were higher (by 10-100 times) than those in other regions of the gut. It has been reported that the differences in the lymphoid tissue (especially Peyer's patches), villi, mucosal layer and the ganglioside GM3 and neutral glycolipids in various regions of the gut may be important factors influencing *S. enteritidis* invasion (Carter and Collins, 1974). Further, it has been reported that in chickens, *S. enteritidis* has an unusual tendency to alter the heterogeneity of the LPS O-chain and the fimbriae of *S. enteritidis* have high affinity for the vaginal epithelium (De Buck *et al*., 2004a, b). The present study indicate that different regions of the gut differ in their susceptibility to *S. enteritidis* colonization and invasion. Thus far, the mechanism of colonization by *S. enteritidis* in the gut is not clear and requires further studies.

In the previous study, a same type of investigation have been performed in duck, chicken, pigeon and mouse after oral challenge by a real-time PCR assay (Deng *et al*., 2008a; He *et al*., 2010, 2011a, b). The results of these researches were similar to the results obtained in the present study. *S. enteritidis* DNA was consistently detected in all the samples. The copy number of *S. enteritidis* DNA in each tissue reached a peak at 24-36 h PI with the ileum, jejunum and spleen containing high concentrations of *S. enteritidis* cells.

CONCLUSION

In this study will help in understanding the pathogenesis of *S. enteritidis* infection *in vivo* and may help in the development of a live Salmonella vaccine in future.

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