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S. enteritidis Genome Loads in the Tissues of Reproductive Organs in Laying Duck

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Abstract: The objective of this study was to determine the replication kinetics of *Salmonella enteritidis* genome loads (*S. enteritidis*) in reproductive organs of laying duck after oral challenge. We conducted serovar-specific real-time PCR for *S. enteritidis* to detect the genomic DNA of *S. enteritidis* in laying duck at different time points. To validate these results, the Indirect Fluorescent Antibody (IFA) technique was employed too. The results showed that *S. enteritidis* was consistently detected in all the samples. However, the number of bacteria started decreasing by 3-4 days and by 6 days, the concentration of *S. enteritidis* DNA was below the detection limits of the PCR assay except the spleen and vagina. In conclusion, this study will help in understanding the pathogenesis of *S. enteritidis* infection *in vivo*.

Key words: *Salmonella enteritidis*, reproductive organs, duck, PCR, China

INTRODUCTION

The international significance of contaminated eggs in the transmission of Salmonella enterica serovar enteritidis (*S. enteritidis*) infection to humans has been a focus for discussion, research and regulatory activity during the past two decades. Understanding the mechanism that leads to *S. enteritidis* colonization of eggs is essential to reduce the public health risk associated with consumption of infected eggs. However, the pathogenesis of egg contaminations still not completely understood. The current concept of vertical transmission of *S. enteritidis* in chickens considers contamination of the shell surface as the egg passes through the vagina and cloaca, contamination of the yolk in the ovary or contamination during passage through a colonized oviduct (Gast *et al*., 2004).

Contamination of eggs by Salmonella organisms could occur either on the surface of the egg shell or in the contents of eggs. Previous studies have shown the presence of Salmonella organisms in yolk and albumin of eggs laid by birds that were experimentally inoculated with these organisms (Takata *et al*., 2003). The deposition of *S. enteritidis* within eggs seems to result from the colonization of reproductive organs particularly the ovary and upper oviduct in systemically infected hens. The site of *S. enteritidis* deposition in eggs (Albumen or yolk) may be determined by the region of the hen's reproductive tract that is colonized (Gast *et al*., 2004). Although, the process of internal egg contamination has not been well explained, it is believed that internal contamination occurs in reproductive organs during egg formation. In China, the consumption of poultry products is high and the number of *S. enteritidis* cases in humans has increased considerably in recent years (Deng *et al*., 2008a). Up to day, it has not been previously described in the replication kinetics of *S. enteritidis* genome loads in reproductive organs of laying duck; we 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, samples, quantitative real-time PCR assay and IFA assay for detection of *S. enteritidis*: About 5 months old duck (3.3-3.8 kg) free from *S. enteritidis* infection were used in the study. The ducks were maintained in isolation units in a biosecure animal building. In brief, *S. enteritidis* cells were grown overnight in a Luria-Bertani broth (at 37°C). The cells were cultured overnight and then the presumptive live number of *S. enteritidis* cells was determined by the spread plate method. Thereafter, a group of 60 ducks were orally infected with a high-virulence *S. enteritidis* strain. (Animal experiments were reviewed by an Institutional Animal Care and Use Committee (IACUC) for humane use of animals for experimental purpose). Each duck was orally

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infected with a *S. enteritidis* strain at 4.0×10^4 cfu hen^{-1}. Another group of 60 duck was treated with an equal volume of sterile water and used as a control group. The ovary (Stroma, the largest, second and third largest preovulatory follicle), oviduct (Tubular region of the infundibulum and middle parts of magnum, isthmus, uterus and vagina), blood and spleen were analyzed by a FQ-PCR assay after 8, 12, 16, 20, 24, 28, 32, 36, 40, 48 and 60 h and 3, 4, 6 and 12 day postinoculation.

At each time point, 4 ducks were randomly selected from the infection and control groups and their tissue samples were collected and processed for further analyses. DNA extraction from the tissue samples was performed as described previously (Deng *et al*., 2008b).

Briefly, 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 (Deng *et al*., 2008b).

To validate the results, we simultaneously performed a quantitative bacteriological test (IFA) to determine the bacterial burden in the corresponding tissues and compare these data with the PCR data as described previously (Yan *et al*., 2008).

Statistical analysis: 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. A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The distribution of *S. enteritidis* within the reproductive organs after oral challenge was determined by means of FQ-PCR at different time points. The results showed that the spleen tested positive for *S. enteritidis* at 12 h PI firstly. The blood, vagina and uterus tested positive for *S. enteritidis* at 20 h PI. Thereafter, *S. enteritidis* was consistently detected in all the samples at 24 h PI; the last organ to show a positive result was the follicle at 32 h PI. The genome copy numbers of *S. enteritidis* in each tissue reached a peak at 36-60 h PI. The spleen, magnum, isthmus, uterus and vagina contained high concentrations of *S. enteritidis* whereas the stroma and follicle exhibited low concentrations. The numbers of bacteria decreased at 3-4 days PI. By 12 days, none of the samples were positive at day 12 except for the vagina and spleen. The reproductive organs of the duck in the control group did not show any positive results at any time point (Table 1).

The spleen, uterus and vagina exhibited a positive *S. enteritidis* signal by IFA at 28 h PI. Thereafter, a positive signal was detected in all the samples at 32-60 h (Fig. 1) a stronger positive signal was observed in the spleen, vagina, uterus and isthmus compared to the other organs. The positive *S. enteritidis* signal clearly decreased at 60 h PI and no positive results were detected in ovary. However, it was possible to detect a positive signal in the vagina and spleen at 6 days PI. Apparently, the results were similar to the results of FQ-PCR.

The *S. enteritidis* can be isolated from various organs including ovary and oviduct of the infected birds (Keller *et al*., 1997). It is possible that the *S. enteritidis*

Table 1: Kinetics of *S. enteritidis* genome loads in the reproductive tissues of ducks after oral infection with a high-virulence strain determined by quantitative real-time PCR

	Hours											Days			
Time	8	12	16	20	24	28	32	36	40	48	60	3	4	6	12
Stroma	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	2.0^{D}	2.2^{F}	2.3^{I}	3.4^{M}	3.2^{R}	3.4^{V}	2.8^{N}	0.0^{A}	0.0^{A}	0.0^{A}
Follicle[a]	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	2.0^{f}	2.2^{i}	3.7^{m}	3.9^{S}	3.5^{v}	2.4^{B}	0.0^{A}	0.0^{A}	0.0^{A}
Follicle[b]	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	1.9^{f}	2.2^{i}	3.6^{M}	3.4^{r}	3.7^{v}	2.5^{B}	0.0^{A}	0.0^{A}	0.0^{A}
Infundibulum	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	2.3^{d}	2.6^{G}	3.2^{J}	4.3^{O}	4.4^{T}	4.5^{W}	2.5^{B}	0.0^{A}	0.0^{A}	0.0^{A}
Magnum	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	2.4^{d}	2.6^{G}	3.7^{K}	4.6^{o}	4.2^{T}	4.7^{W}	3.6^{D}	2.3^{G}	0.0^{A}	0.0^{A}
Isthmus	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	2.1^{D}	2.5^{d}	3.1^{g}	3.3^{j}	4.7^{o}	4.5^{t}	5.8^{S}	4.2^{E}	2.4^{G}	0.0^{A}	0.0^{A}
Uterus	0.0^{A}	0.0^{A}	0.0^{A}	2.3^{B}	2.7^{e}	3.1^{E}	3.9^{H}	4.7^{L}	5.7^{Q}	5.4^{U}	5.9^{S}	4.5^{E}	3.6^{H}	2.2^{J}	0.0^{A}
Vagina	0.0^{A}	0.0^{A}	0.0^{A}	2.4^{B}	2.9^{e}	3.4^{e}	4.3^{h}	4.9^{l}	5.6^{q}	5.2^{U}	6.4^{Y}	5.2^{F}	4.4^{I}	3.3^{K}	2.5^{L}
Follicle[c]	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}	1.8^{f}	2.1^{i}	3.6^{M}	3.4^{r}	3.6^{W}	2.3^{B}	0.0^{A}	0.0^{A}	0.0^{A}
Blood	0.0^{A}	0.0^{A}	0.0^{A}	2.2^{B}	2.8^{e}	3.1^{E}	3.8^{H}	4.1^{M}	4.6^{o}	5.0^{D}	5.2^{J}	3.1^{A}	2.4^{G}	0.0^{D}	0.0^{A}
Spleen	0.0^{A}	2.1^{B}	2.4^{B}	2.8^{C}	3.8^{D}	4.0^{f}	4.2^{h}	5.1^{D}	5.6^{q}	6.1^{L}	7.1^{M}	5.1^{N}	4.2^{i}	3.6^{K}	3.3^{W}

The unit: log genome copies g^{-1} for each sample; Each time point represents the mean concentration of genomic DNA and is expressed as $\log_{10}$ of the bacterial genome copy number per gram of tissue tested obtained from 4 duck. Each sample was analyzed 3 times by the fluorescent quencher PCR. In this study, we get the mean from 12 tests for each sample and the 12 results were not different for each sample ($p>0.05$); Values with different superscripts within a column followed by different letters were significantly different between 11 groups ($p<0.01$), Values with different superscripts within a column followed by lower case and capital of the same letter were different between nine groups ($p<0.05$). Values within a column followed by same letters were not different between 11 groups ($p>0.05$); a = the largest preovulatory follicle, b = the second largest preovulatory follicle, c = the third largest preovulatory follicle

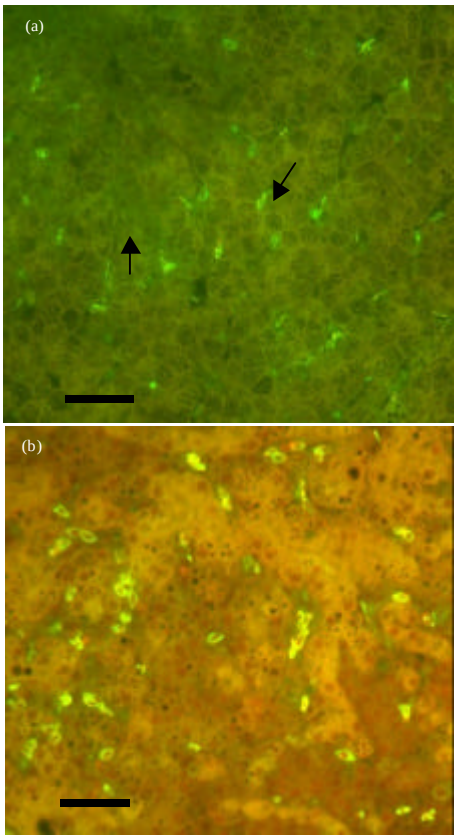

Fig. 1: Used indirect immunofluorescent antidody staining assay to determine the bacterial burden. Bar = 50 μm; A: uterus from 32 h PI, presented positive signal; B: vagina from 32 h PI, presented positive signal

invades the tissues of these organs; electron microscopic studies of chicks have shown passage of salmonella through ileocecal mucosa. The attachment and invasion of cultured chicken ovarian granulosa cells by *S. enteritidis* and colonization of *S. enteritidis* in the ovarian and oviductal cells of birds inoculated with *S. enteritidis* orally (Keller *et al*., 1995) also have been reported. Up to day, it has not been previously described in the replication kinetics of *S. enteritidis* genome loads in reproductive organs of laying duck.

Two possible routes of egg contamination with Salmonella are known. The horizontal transmission route implies Salmonella penetration through the eggshell after the eggs are covered by the shell (De Reu *et al*., 2006). In contrast, the vertical transmission route refers to direct contamination of the egg content before oviposition as a result of Salmonella infection of the reproductive organs. It is believed that the most important route of egg contamination is via infected reproductive tissues, both the oviduct and the ovary (Gantois *et al*., 2008).

In this studies, the spleens were positive at 12 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. Pathogens presented subepithelially or even luminally by dendritic cells or CD18-expressing phagocytes can be transferred directly to the liver and spleen (Vasquez-Torres *et al*., 2000).

The oviduct consists of the infundibulum, magnum, isthmus, uterus and vagina. Because the cloaca is the common opening to the digestive and reproductive tracts, microorganisms in the digestive tract can reach the cloaca and then may migrate into the vagina. Sperm inseminated in the uterus are transported to the infundibulum by actions of the oviduct. Thus, it could be possible that Salmonella which invaded the lower part of the oviduct are transported to the infundibulum followed by movement through the peritoneal cavity to the ovary and other organs. It is also assumed that Salmonella organisms invade circulating blood and are transported to the ovarian follicles (Takata *et al*., 2003).

The *S. enteritidis* populations in the isthmus, uterus and vagina were higher (by 10-100 times) than those in other regions of the reproductive organs. 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*., 2004). The immune mechanisms involved in the defense against Salmonella infection are not well understood in chickens. Significance of phagocytosis by heterophils andresponse of T-cell subsets and B cells in defending against *S. enteritidis* have been suggested. The presence of immunocompetent cells including antigen-presenting cells and T and B cells has been shown in the ovary and oviduct (Zheng *et al*., 2001; Deng *et al*., 2008c). The current study showed that there were no signs of depression or diarrhea moreover, the feeding and drinking behaviors were normal at 6 days until 12 days PI. However, at 28 h and until 3 days PI there were clinical signs of *S. enteritidis* infection. The PCR results showed that the numbers of bacteria decreased at 3-4 days. By 12 days, all the sample did not show positive results, except the vagina. Thus far, enhancement of immunity in the reproductive organs may be needed to protect them from infection. Also, the present study indicate that different regions of the reproductive organ differ in their susceptibility to *S. enteritidis* colonization and invasion. In the previous study, it showed that the isthmus has higher numbers of intracellular *S. enteritidis* after an intravenous infection and after inoculation into ligated oviductal loops and that

the tubular epithelial cells of the isthmus are more readily invaded *in vitro* (He *et al*., 2010). These observations are in accordance with observations of others after experimental infections where the isthmus is more frequently or heavily contaminated. Analysis of eggs laid by infected hen has shown that the shell containing the egg shell membranes and produced by the isthmus is often the most heavily infected site of surface decontaminated eggs (He *et al*., 2011a, b). Thus, from the results of bacterial culturing of oviduct segments and eggs after experimental infections and from the results of this study, *S. enteritidis* is suggested to have adapted best to the isthmus segment of the chicken oviduct.

CONCLUSION

Obviously, the results of the study is similar to the previres as described. The mechanism of colonization by *S. enteritidis* in the reproductive organs is not clear and requires further studies. We believe that the study will help provide valuable insights into the etiology of *S. enteritidis* infections.

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