Salmonella enterica serovar Enteritidis live vaccine strain in the reproductive organs of laying goose after subcutaneous vaccination

He, G. Z.[1*]; Deng, S. X.[2] and Tian, W. Y.[1]

[1]Department of Microbiology, Guiyang College of Traditional Chinese Medicine, Guiyang 550002, Guizhou Province, China; [2]Agricultural Office of Dalingshan Town, Dongguan 523830, Guandong Province, China

*Correspondence: G. Z. He, Department of Microbiology, Guiyang College of Traditional Chinese Medicine, Guiyang 550002, Guizhou Province, China. E-mail: heguangzhi7711@163.com

Summary

Serovar-specific real-time PCR for *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) was conducted to detect the genomic DNA of *S.* Enteritidis from laying goose after subcutaneous vaccination at different time points. Indirect fluorescent antibody (IFA) technique and immunohistochemical localization were employed to validate the results. The results showed that *S.* Enteritidis was consistently detected in all the samples. Vagina and uterus were positive at 20 h PI, and the last organ to show a positive result was the largest and third largest preovulatory follicle, at 32 h PI. The copy numbers of *S.* Enteritidis DNA in each tissue reached a peak at 36-60 h PI, with the vagina and uterus containing higher concentrations than other tissues. However, the number of bacteria started decreasing by 3-4 d, and by 6 d, the concentration of *S.* Enteritidis DNA was below the detection limits of the PCR assay, except the vagina. The real-time PCR analysis of a variety of tissues is significant for further investigation of the mechanism of vaccine protection and the optimization of vaccination regimes.

Key words: *Salmonella* Enteritidis, Reproductive organs, Population, Goose

Introduction

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. Contamination of eggs by *Salmonella* organisms could occur either on the surface of the eggshell or in the contents of eggs. Previous studies have shown the presence of *Salmonella* organisms in yolk and albumin of eggs laid by birds experimentally inoculated with those organisms (Gast and Holt, 2000; Takata *et al*., 2003; Deng *et al*., 2008a; Atyabi *et al*., 2012). Few data are present in the literature about the quantitative analysis of live *S.* Enteritidis vaccine strain in poultry at different times following vaccination. The mechanism for the rapid induction of immunity of attenuated *S.* Enteritidis vaccine strain remain unclear. The quantification of live vaccine number *in vivo* may provide a complement for understanding this mechanism.

A better understanding of the kinetics of *S.* Enteritidis live vaccine strain replication, the quantification of live vaccine load will be required as an aim towards more effective vaccines. With this in mind, DNA loads were examined as a measure of the kinetics of attenuated *S.* Enteritidis vaccine strain replication in experimentally vaccinated goose. Various tissues were examined in order to investigate the different stages in the course of vaccine infection and replication. Although the process of contamination of internal egg components has not been well explained, it is believed that internal contamination occurs in reproductive organs during egg formation.

Materials and Methods

Vaccine

A commercially available *S.* Enteritidis live vaccine strain (Avipro *Salmonella* vac E) was purchased from the National Center for Medical Culture Collection of China.

Experimental animals and samples

Five-month-old geese (3.3-3.5 kg, Chinese white goose) 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 immuno-sorbent assay and PCR, respectively (Gast and Beard, 1990; Zheng *et al.*, 2001; Deng *et al.*, 2008b). The geese were maintained in isolation units in a biosecure animal building. A group of 68 geese was vaccinated with one commercial dose of *S.* Enteritidis live vaccine (0.5 ml) subcutaneously. Another group of 60 geese were treated with an equal volume of water and used as a control group. The ovary (stroma, the largest and third largest preovulatory follicle) and oviduct (tubular region of the infundibulum and middle parts of magnum, isthmus, uterus, and vagina) were analysed by a fluorescent quencher PCR assay at postinoculation times of 8, 12, 16, 20, 24, 28, 32, 36, 40, 48 and 60 h; and 3, 4, 6 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 extraction from the tissue samples was performed as described previously (Deng *et al.*, 2008b).

Quantitative real-time PCR assay for detection of *S.* Enteritidis DNA

In our previous study, we have established a serovar specific real-time PCR assay, the limit of detection was 7 copies/µL (Deng *et al.*, 2008b). 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 iQTM Real-time PCR Detection System (version 3.1, Bio-Rad, USA) and was performed as described previously (Deng *et al.*, 2008b).

Differences between the FQ-PCR and IFA assay results

To validate the results, we simultaneously performed a quantitative bacteriological test to determine the bacterial burden in the corresponding tissues and compared these data with our PCR data. In our previous study, we also established a specific method of IFA staining for *S.* Enteritidis (Yan *et al.*, 2008). At present, we relied on the IFA assay to study the distribution pattern and quantity of *S.* Enteritidis in the reproductive organs of goose after oral challenge.

Immunohistochemical localization of *S.* Enteritidis antigen

Small pieces of tissues were collected and fixed in 10% neutral buffered formalin, processed for paraffin embedding, and sectioned at a thickness of 5 µm. The sections were stained for *S.* Enteritidis antigen by using the avidin-biotin-peroxidase complex method as previously described (Islam *et al.*, 1993).

Statistical analysis

The real-time PCR data were analysed using version 11 of the SPSS software. The comparison of means was performed using Duncan's multiple-range test. A P-value <0.05 was considered statistically significant.

Results

The distribution of *S.* Enteritidis within the reproductive organs after oral challenge was determined by means of FQ-PCR over a 12 d period at intervals. The results showed that the 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 copy numbers of *S.* Enteritidis in each tissue reached a peak at 36 h-60 h PI. The magnum, isthmus, uterus and vagina contained high concentrations of *S.* Enteritidis, whereas the stroma and follicle exhibited low concentrations. The numbers

Table 1: Kinetics of *S.* Enteritidis DNA loads in the tissues of reproductive of goose after subcutaneous vaccination

Tissue	Time														
	8 h	12 h	16 h	20 h	24 h	28 h	32 h	36 h	40 h	48 h	60 h	3 d	4 d	6 d	12 d
Stroma	0.0	0.0	0.0	0.0	0.0	2.1	2.2	2.5	3.3	3.2	3.5	2.7	0.0	0.0	0.0
Follicle a	0.0	0.0	0.0	0.0	0.0	0.0	1.9	2.1	3.7	3.7	3.8	2.3	0.0	0.0	0.0
Follicle b	0.0	0.0	0.0	0.0	0.0	0.0	2.1	2.2	3.5	3.4	3.6	2.4	0.0	0.0	0.0
Infundibulum	0.0	0.0	0.0	0.0	0.0	2.2	2.7	3.4	4.2	4.4	4.5	2.3	0.0	0.0	0.0
Magnum	0.0	0.0	0.0	0.0	0.0	2.4	2.9	4.1	4.5	4.5	4.7	3.7	2.3	0.0	0.0
Isthmus	0.0	0.0	0.0	0.0	2.2	2.8	3.2	3.6	4.9	4.8	5.4	4.3	2.5	0.0	0.0
Uterus	0.0	0.0	0.0	2.5	2.6	3.1	3.9	4.7	5.6	5.4	5.9	4.5	3.7	2.3	0.0
Vagina	0.0	0.0	0.0	2.7	2.9	3.5	4.3	5.0	5.8	5.6	6.3	5.3	4.5	3.4	2.4

Note: The unit: log 10 copies/g 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 geese. Each sample was analysed 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). h: hour, d: day, a: the largest preovulatory follicle, and b: the third largest preovulatory follicle

of bacteria decreased at 3-4 d. By 12 d, none of the samples showed positive results, except the vagina. The reproductive organs of the goose in the control group did not show any positive results at any time point. All these data have been presented in Table 1.

A side-by-side IFA technique was performed to determine the bacterial burdens in all corresponding tissues and these data were compared with the PCR data. The 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 h to 60 h; a stronger positive signal was observed in the 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 at 6 d 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).

A immunohistochemical localization assay was performed simultaneously to determine the bacterial burden in all the corresponding tissues. The *S.* Enteritidis immunoreaction product was frequently found in the fibroblast-like and macrophage-like cells in the stroma and surface layer of follicles (Fig. 2). The *S.* Enteritidis immunoreaction products were identified on the mucosal surface, in the mucosal epithelium, and in the stromal tissues in all segments of the oviduct. Many of the

bacteria were present in the cytoplasm of mucosal epithelial cells and stromal cells in those tissues.

Discussion

The *S.* Enteritidis reaction products were localized in the stromal connective tissue and follicular wall in the ovaries, as well as mucosal epithelium and stroma in the oviduct. The presence of *S.* Enteritidis in the ovarian stroma and follicular wall suggest that the *S.* Enteritidis in the peritoneal cavity invaded the tissues through superficial epithelium (Takata *et al*., 2003; Bagherzadeh Kasmani *et al*., 2012).

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 2004a, b). The immune mechanisms involved in the defense against *Salmonella* infection are less well understood in chickens, and significance of phagocytosis by heterophils and response of T-cell subsets and B cells in defending against *S.* Enteritidis have been suggested (Andreasen *et al*., 2011). The presence of immunocompetent cells, including antigen-presenting cells and T and B cells, has been shown in the ovary and oviduct (Barua *et al*., 2001; He *et al*., 2010). The present study indicates that different regions of the reproductive organs differ in their
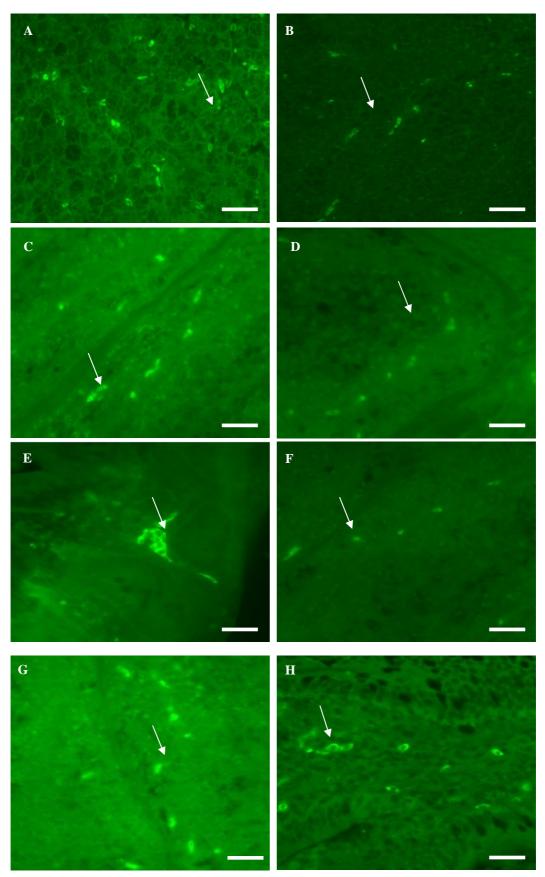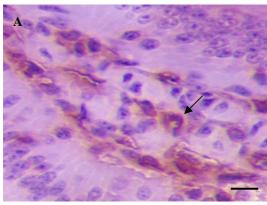
Fig. 1: Results of indirect immunofluorescents antibody staining assay to determine the bacterial burden. A-H: Uterus, vagina, stroma, the largest preovulatory follicle, the third largest preovulatory follicle, infundibulum, magnum, isthmus from 28 h PI, presented positive signal, (Bar: 50 μm)
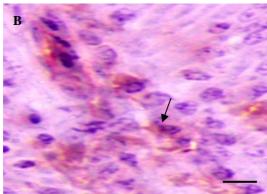
Fig. 2: Used immunohistochemical localization assay to detected infected-groud at 28 h postinoculation. Images were acquired by using 60 * objective. A: uterus, sheets of epithelial cells displaced from the villi surface were observed, and B: vagina, the *S.* Enteritidis antigen in the epithelial cells and lymphocytes, (Bar: 100 μm)

susceptibility to *S.* Enteritidis colonization and invasion. *S.* Enteritidis were still present up to 12 d for the vagina without causing apparent symptoms. Thus far, the mechanism of colonization by *S.* Enteritidis in the reproductive organs is not clear and requires further studies.

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 would be possible that *Salmonella* that 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 (Thiagarajan *et al*., 1996; He *et al*., 2011a, b).

The next step is to confirm that vaccine load in the vaccinated goose correlates well with specific immune responses induced by *Salmonella* Enteritidis vaccine. Once this relationship is understood, the quantitative PCR test could provide data to assist optimization of existing vaccination strategies in terms of delivery route and choice of the route of vaccine administration.

Serovar-specific real-time PCR was conducted for *S.* Enteritidis to detect the genomic DNA of *S.* Enteritidis from laying goose at different time points. To validate these results, the indirect fluorescent antibody (IFA) technique and immuno-histochemical localization were employed too. This study will help in better understanding of the mechanisms of action of *S.* Enteritidis.

References

Andreasen, CB; Akunda, JK and Kramer, TT (2011). Comparison of heterophil phagocytosis for heterophil-adapted *Salmonella enteritidis* (HASE) and wild-type *Salmonella enteritidis* (SE). Avian Dis., 45: 432-436.

Atyabi, N; Zahraei Salehi, T; Ghazisaeedi, F and Ashrafi, I (2012). The molecular investigation of widespread *Salmonella* serovars, *S. typhimurium* and *S. enteritidis*, involved in salmonellosis of cattle and sheep in farms around Tehran, Iran. Iranian J. Vet. Res., 13: 126-133.

Bagherzadeh Kasmani, F; Karimi Torshizi, MA; Allameh, AA and Shariatmadari, F (2012). Aflatoxin detoxification potential of lactic acid bacteria isolated from Iranian poultry. Iranian J. Vet. Res., 13: 152-155.

Barua, A; Michiue, H and Yoshimrua, Y (2001). Changes in the localization of MHC class ‖ positive cells in hen ovarian follicles during the process of follicular growth, postovulatory regression and atresia. Reproduction. 121: 953-957.

De Buck, J; Pasmans, F and Van Immerseel, F (2004a). Tubular glands of the isthmus are the predominant colonization site of *Salmonella enteritidis* in the upper oviduct of laying hens. Poultry Sci., 83: 352-358.

De Buck, J; Van Immerseel, F and Haesebrouck, F (2004b). Effect of type 1 fimbriae of *Salmonella enterica* serotype Enteritidis on bacteraemia and reproductive tract infection in laying hens. Avian Pathol., 33: 314-320.

Deng, SX; Cheng, AC; Wang, MS and Cao, P (2008b). Serovar-specific real-time quantitative detection of *Salmonella enteritidis* in the gastrointestinal tract of ducks after oral challenge. Avian Dis., 52: 88-93.

Deng, SX; Cheng, AC; Wang, MS; Cao, P; Yan, B; Yin, NC; Cao, SY and Zhang, ZH (2008a). Quantitative studies of the regular distribution pattern for *Salmonella enteritidis* in the internal organs of mice after oral challenge by a specific real-time PCR. World J. Gastroenterol., 14: 782-789.

Gast, RK and Beard, CW (1990). Serological detection of experimental *Salmonella enteritidis* infections in laying hens. Avian Dis., 34: 721-728.

Gast, RK and Holt, PS (2000). Deposition of phage type 4 and 13a *Salmonella enteritidis* strain in the yolk and albumen of eggs laid by experimentally infected hens. Avian Dis., 44: 706-710.

He, GZ; Tian, WY; Qian, N; Cheng, AC and Deng, SX (2010). Quantitative studies of the distribution pattern for *Salmonella* Enteritidis in the internal organs of chicken after oral challenge by a real-time PCR. Vet. Res. Commun., 34: 669-676.

He, GZ; Tian, WY; Qian, N and Deng, SX (2011a). The pathogenesis of *Salmonella enteritidis* serovar enteritidis in sub-cutaneously infected pigeon: a quantitative time-course study using real-time PCR. J. Anim. Vet. Adv., 7: 909-913.

He, GZ; Tian, WY; Qian, N and Deng, SX (2011b). Populations of *Salmonella enteritidis* in the tissues of reproductive organs in laying hens after oral challenge: a real-time PCR study. J. Anim. Vet. Adv., 8: 945-948.

Islam, MR; Nessa, J and Halder, KM (1993). Detection of duck plague virus antigen in tissues by immunoperoxidase staining. Avian Pathol., 22: 389-393.

Takata, T; Liang, J; Nakano, H and Yoshimura, Y (2003). Invasion of *Salmonella* Enteritidis in the tissues of reproductive organs in laying Japanese quail: an immunocytochemical study. Poultry Sci., 82: 1170-1173.

Thiagarajan, D; Saeed, M; Turek, J and Asem, E (1996). *In vitro* attachment and invasion of chicken ovarian granulose cells by *Salmonella enteritidis* phage type 8. Infect. Immun., 64: 5015-5021.

Yan, B; Cheng, AC and Wang, MS (2008). Application of indirect immunofluorescent staining method for detection of *Salmonella enteritidis* in paraffin slices and antigen location in infected duck tissues. World J. Gastroenterol., 14: 776-781.

Zheng, WM; Nishibori, M; Isobe, N and Yoshimura, Y (2001). An *in situ* hybridization study of the effects of artificial insemination on the localization of cells expressing MHC class II mRNA in the chicken oviduct. Reproduction. 122: 581-586.