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Populations of *Salmonella enteritidis* in the Tissues of Reproductive Organs in Laying Hens After Oral Challenge: A Real-Time PCR Study

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Abstract: The objective of this study was to determine the populations of *Salmonella enteritidis* (*S. enteritidis)* in reproductive organs of laying hens after oral challenge. Researchers conducted serovar-specific Real-time PCR for *S. enteritidis* to detect the genomic DNA of *S. enteritidis* from laying hens 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. 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 days and by 6 days, the concentration of *S. enteritidis* DNA was below the detection limits of the PCR assay except the vagina. In conclusion, the results provided insights into the *S. enteritidis* populations in the reproductive organs. This study will help in understanding the pathogenesis of *S. enteritidis* infection *in vivo*.

Key words: *Salmonella enteritidis*, reproductive organs, population, vagina, concentration, China

INTRODUCTION

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 that were experimentally inoculated with those organisms (Gast and Holt, 2000; Takata *et al*., 2003).

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. 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). Further, this disease has had significant economic impacts on the poultry industry especially the egg industry. Up to day, it has not been previously described in the populations of *S. enteritidis* in reproductive organs of laying hens; it is believed 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: About 5 months old hens (2.1-2.3 kg) free from *S. enteritidis* infection were used in the study. Prior to challenge with *S. enteritidis* all hens were found to be negative for *S. enteritidis*-specific antibodies and *S. enteritidis*-specific antigens by an enzyme-linked immunosorbent assay and PCR, respectively (Gast and Beard, 1990; Deng *et al*., 2008b). The hens 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 method. Thereafter, a group of 60 hens were orally infected with a high virulence *S. enteritidis* strain (phage type 4; No: 50338).

Animal experiments were reviewed by an Institutional Animal Care and Use Committee (IACUC) for humane use of animal for experimental purposes. Each hen was orally infected with a *S. enteritidis* strain (No: 50338) at 4.0×10^4 cells per hen. Another group of 60 hen was 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 analyzed 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.

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At each time point, four hens 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, 0.2 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. Finally with a conventional phenol/chloroform/isoamyl alcohol method (Deng *et al*., 2008b) 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, researchers have established a serovar specific Real-time PCR assay (Genbank Accession No. AF 370707.1), the limit of detection was 7 copies μL^{-1} (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 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, it 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 reproductive organs of hens 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 g 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

Clinical signs and gross lesions at necropsy: *S. enteritidis*-inoculated hens appeared to be clinically normal and there were no signs of depression or diarrhea moreover, the feeding and drinking behaviors were normal at 8 h until 16 h and at 6 days until 12 days PI.

However at 28 h and until 3 days PI, there were clinical signs of *S. enteritidis* infection. At necropsy, gross lesions were observed in all of the hens during this period, e.g., in testinal hyperemia.

Distribution of *S. enteritidis* in the reproductive organs (PCR assay): The distribution of *S. enteritidis* within the reproductive organs after oral challenge was determined by means of FQ-PCR over a 12 days 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 36-60 h PI. The magnum, isthmus, uterus and vagina contained high concentrations of *S. enteritidis* whereas, the stroma and follicle exhibited low concentrations. The numbers of bacteria decreased at 3-4 days. By 12 days, all the sample did not show positive results except the vagina. The reproductive organs of the hens in the control group did not show any positive results at any time point. The details are shown in Table 1.

Distribution of *S. enteritidis* in the reproductive organs (IFA technique): 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

Table 1: Kinetics of *S. enteritidis* DNA loads in the tissues of reproductive of hens after orally infected with a high-virulence strain determined by quantitative Real-time PCR

Time	8 h	12 h	16 h	20 h	24 h	28 h	32 h	36 h	40 h	48 h	60 h	3 days	4 days	6 days	12 days
Stroma	0.0	0.0	0.0	0.0	0.0	1.9	2.0	2.4	3.2	3.1	3.3	2.6	0.0	0.0	0.0
Follicle[a]	0.0	0.0	0.0	0.0	0.0	0.0	1.8	2.0	3.6	3.5	3.6	2.1	0.0	0.0	0.0
Follicle[b]	0.0	0.0	0.0	0.0	0.0	0.0	1.9	2.1	3.4	3.3	3.5	2.3	0.0	0.0	0.0
Infundibulum	0.0	0.0	0.0	0.0	0.0	2.0	2.6	3.1	4.0	4.2	4.3	2.2	0.0	0.0	0.0
Magnum	0.0	0.0	0.0	0.0	0.0	2.3	2.8	3.9	4.4	4.3	4.5	3.5	2.1	0.0	0.0
Isthmus	0.0	0.0	0.0	0.0	2.0	2.6	3.0	3.4	4.8	4.6	5.2	4.1	2.3	0.0	0.0
Uterus	0.0	0.0	0.0	2.1	2.5	3.0	3.7	4.5	5.4	5.2	5.8	4.3	3.5	2.0	0.0
Vagina	0.0	0.0	0.0	2.3	2.8	3.3	4.1	4.8	5.6	5.3	6.1	5.0	4.3	3.1	2.2

The unit: 1 g 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 hens; each sample was analyzed 3 times by the fluorescent quencher PCR in this study, 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 3rd largest preovulatory follicle

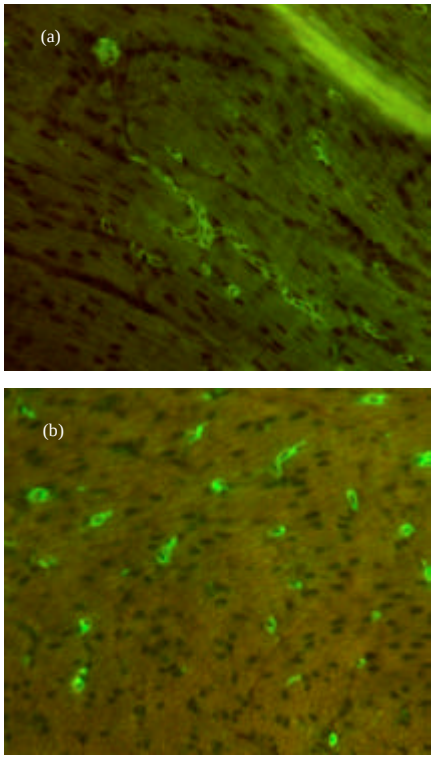

Fig. 1: Used indirect immunofluorescents antidody staining assay to determine the bacterial burden; Bar = 50 μm; a) uterus from 28 h PI, presented positive signal and b) vagina from 28 h PI, presented positive signal

at 32-60 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 days 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). 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; Takata *et al*., 2003). Therefore, what it is described above may be the reason for why *S. enteritidis* cells was consistently detected in all the samples in this study.

Over the 12 days period, 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*., 2004a, b). The immune mechanisms involved in the defense against Salmonella infection are less 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 (Andreasen *et al*., 2001).

The presence of immunocompetent cells including antigen-presenting cells and T and B cells has been shown in the ovary (Barua *et al*., 2001) and oviduct (Zheng *et al*., 2001; Takata *et al*., 2003).

The present study indicate that different regions of the reproductive organ differ in their susceptibility to *S. enteritidis* colonization and invasion. *S. enteritidis* cells were still present up to 12 days 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. FQ-PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility and accuracy (De Medici *et al*., 2003). However, variation results may be due to either the PCR inhibitors or a large amount of DNA from background organism DNA. In preliminary experiments, it was used phenol/chloroform/isoamyl alcohol method to extract DNA of tissue from several control group samples and added 7.0×10^5 copies of the standard DNA for each. Finally, fluorimetic cycler measurements were performed as described previous.

CONCLUSION

The results showed that all the tests can obtain the expected datas and the variability was statistically low, at <2.5%. So, this methodology is very accuracy for studying on the distribution of *S. enteritidis* in the reproductive organs. In conclusion, this study will help to further understanding of the mechanisms of action of *S. enteritidis*.

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REFERENCES

Andreasen, C.B., J.K. Akunda and T.T. Kramer, 2001. Comparison of heterophil phagocytosis for heterophil-adapted *Salmonella enteritidis* (HASE) and wild-type *Salmonella enteritidis* (SE). Avian Dis., 45: 432-436.

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

De Buck, J., F. van Immerseel, F. Haesebrouck and R. Ducatelle, 2004a. Effect of type 1 fimbriae of *Salmonella enterica* serotype Enteritidis on bacteraemia and reproductive tract infection in laying hens. Avian Path., 33: 314-320.

De Buck, J., F. Pasmans, F. van Immerseel, F. Haesebrouck and R. Ducatelle, 2004b. Tubular glands of the isthmus are the predominant colonization site of *Salmonella enteritidis* in the upper oviduct of laying hens. Poult. Sci., 83: 352-358.

De Medici, D., L. Croci, E. Delibato, S. di Pasquale, E. Filetici and L. Toti, 2003. Evaluation of DNA extraction methods for use in combination with SYBR green I real-time PCR to detect *Salmonella enterica* serotype enteritidis in poultry. Applied Environ. Microbiol., 69: 3456-3461.

Deng, S.X., A.C. Cheng, M.S. Wang, P. Cao and B. Yan *et al*., 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 polymerase chain reaction. World J. Gastroenterol., 14: 782-789.

Deng, S.X., A.C. Cheng, M.S. Wang and P. Cao, 2008b. Serovar-specific real-time quantitative detection of *Salmonella enteritidis* in the gastrointestinal tract of ducks after oral challenge. Avian Dis., 52: 88-93.

Gast, R.K. and C.W. Beard, 1990. Serological detection of experimental *Salmonella enteritidis* infections in laying hens. Avian Dis., 34: 721-728.

Gast, R.K. and P.S. Holt, 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.

Takata, T., J. Liang, H, Nakano and Y. Yoshimura, 2003. Invasion of *Salmonella enteritidis* in the tissues of reproductive organs in laying Japanese quail: An immunocytochemical study. Poult. Sci., 82: 1170-1173.

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

Yan, B., A.C. Cheng, M.S. Wang, S.X. Deng and Z.H. Zhang *et al*., 2008. Application of an indirect immunofluorescent staining method for detection *Salmonella enteritidis* in paraffin slices and antigen location in infected duck tissues. World J. Gastroenterol., 14: 776-781.

Zheng, W.M., M. Nishibori, N. Isobe and Y. Yoshimura, 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.