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Cytokine activation during embryonic development and in hen ovary and vagina during reproductive age and *Salmonella* infection

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ABSTRACT

Salmonellosis is one of the most important zoonotic diseases and is usually associated with consumption of *Salmonella* Enteritidis (SE) contaminated poultry meat or eggs. Contamination with SE is usually the result of infection of the digestive tract, or reproductive organs, especially the ovary and vagina. Thus, knowledge of endogenous innate immune mechanisms operating in the ovary and vagina of hen is an emerging aspect of reproductive physiology. Cytokines are key factors for triggering the immune response and inflammation in chicken to *Salmonella* infection. The aim of this study was to investigate the expression profile of 11 proinflammatory cytokines in the chicken embryos during embryonic development, as well as in the hen ovary and vagina *in vivo*, to investigate whether sexual maturation affects their ovarian and vaginal mRNA abundance and to determine whether cytokine expression was constitutive or induced in the ovary and vagina as a response to SE infection. RT-PCR analysis revealed that several cytokines were expressed in the chicken embryos, and in the ovary and vagina of healthy birds. Expression of various cytokines during sexual maturation appeared to be developmentally regulated. In addition, a significant up-regulation of several cytokines in the ovary and vagina of sexually mature SE infected birds compared to healthy birds of the same age was observed. These results suggest a cytokine-mediated immune response mechanism against *Salmonella* infection in the hen reproductive organs.

1. Introduction

Salmonellosis is a major cause of food-poisoning worldwide with outbreaks usually associated with *Salmonella* Enteritidis (SE) and connected to the consumption of contaminated poultry meat or eggs (Guard, 2001). Infection of the reproductive organs and especially the chicken ovary and vagina is often the major cause of production of contaminated eggs with SE in laying hens (Miyamoto et al., 1998; van Immerseel, 2010). Systemic SE infection in laying hens can lead to colonization of the ovary or vagina (Keller et al., 1995; De Buck et al., 2004), which can result to ovarian disorders, functional disorders for egg formation and concomitant production of contaminated eggs (Gantois et al., 2008; Feberwee et al., 2009; Neubauer et al., 2009; Ozaki and Murase, 2009). Thus, knowledge of the innate immune mechanisms operating in the ovary and vagina of birds, functioning naturally to protect them from colonization, infection and transmission of microbial pathogens, as well as to maintain their normal functions, is an emerging aspect of reproductive physiology.

Innate immune system plays an essential role in host defense against infection. During recent years, Toll-like receptors (TLRs) have been identified as one of the key components of innate immune recognition in vertebrate species (Akira et al., 2001; Brownlie and Allan, 2010) and have been reported to be expressed in the reproductive organs of various vertebrate female species, including chickens (Fazeli et al., 2005; Andersen et al., 2006; Shimada et al., 2006; Soboll et al., 2006; Herath et al., 2007; Ozoe et al., 2009; Michailidis et al., 2010, 2011). TLRs recognize a range of microbial molecular patterns and generate intra-cellular signals through nuclear factor-kB (NF-kB) dependent pathways, to induce chemokine and cytokine expression, which activate a range of host responses (Zarember and Godowski, 2002). Interaction of TLRs and their ligands induces cellular responses such as synthesis of antimicrobial peptides and proinflammatory cytokines (Kogut et al., 2006; Berndt et al., 2007; Cheeseman et al., 2008; Abdel Mageed et al., 2011). Cytokines are key factors for triggering the immune response and inflammation. They are a group of mediators regulating cellular function that are secreted by specific cells to affect the behaviour of other cells, playing a role in the regulation of immune and inflammatory processes (Giansanti et al., 2006). Proinflammatory cytokines, such as interleukins, play a key role in initiating innate and adaptive immune responses and assist in generating a local inflammatory response (Staeheli et al., 2001; Ferro et al., 2004; Hughes et al., 2007).

Previous studies have reported expression and stimulation of TLRs in chicken ovary and vagina, suggesting the presence of immune system

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mediated by TLRs in the hen reproductive organs (Subedi et al., 2007; Ozoe et al., 2009; Michailidis et al., 2010, 2011). In chickens, a common downstream result of TLR stimulation is the induction of pro-inflammatory cytokines (St. Paul et al., 2012). However, whether stimulation of TLRs induced proinflammatory cytokines that may be responsible to initiate innate and adaptive immune response in the chicken reproductive organs has not been studied extensively.

As cytokines are an integral part of the immune response in avian species to *Salmonella* infection (Swaggerty et al., 2006) and activation of the innate immune system is characterized by production of proinflammatory cytokines, such as interleukins, the aim of this study was to investigate the expression profile of cytokines in the hen ovary and vagina *in vivo*, as well as in the chicken embryos *de novo* during early embryogenesis, to investigate whether sexual maturation affects their ovarian and vaginal mRNA abundance and to determine whether cytokine expression was constitutive or induced in the hen ovary and vagina *in vivo*, as a response to *Salmonella enteritidis* infection.

2. Material and methods

2.1. Collection of tissues and embryos

The poultry (Rhode Island Red) used in this study were supplied by a commercial supplier. Birds were housed in cages under a light regimen of 14 h light:10 h dark. Feed and water were given *ad libitum*. Management of experimental animals was in concordance with the accepted institutional Welfare Guidelines. Before the experiment was initiated, fecal samples were cultured and were confirmed, using *Salmonella*/*Shigella* (SS) agar (Fluka), to be negative for *Salmonella* organisms. Experimental groups consisted of immature (12-week-old, before sexual maturity), mature (26-, 28- and 52-week-old, sexual maturity) and aged (104-week-old, decline in reproductive function) female chickens ($n = 6$ at each age). Birds were sacrificed by cervical dislocation. Total ovary and vagina were removed from each bird, snap frozen in liquid nitrogen and stored at −80 °C until analysed. Large yolky preovulatory follicles from the ovary of sexually mature chickens were removed before storing in liquid nitrogen.

Freshly-laid fertile chicken eggs (Rhode Island Red) were obtained from a commercial supplier and incubated at 38 °C and 65–75% relative humidity in a forced air incubator for three to ten days. Embryos were removed from eggs at day 3 until day 10 of incubation ($n = 4$ at each day). Because after "day 10" the chicken embryo becomes too large for whole embryo expression to be but an average of differential expression in many tissues (Meade et al., 2009), the focus of this study was on differential gene expression during early embryogenesis (up to and including day 10 of embryonic development). The allantoic fluid from the eggs was removed, and the embryos were sacrificed, snap frozen in liquid nitrogen and stored at −80 °C until analysed.

2.2. Experimental infections

Two groups of 28- and 104-week-old female birds ($n = 6$ per group) were orally gavaged with 0.1 ml of inoculum containing approximately 5×10^6 organisms of *Salmonella enteritidis* (SE). Age-matched, non-infected control birds were housed under similar environmental conditions, and 0.1 ml of phosphate buffered saline (PBS) was introduced by gavage. The *in vivo* bacterial challenge of experimental animals was in concordance with the institutional accepted welfare guidelines. Chicks were sacrificed on the fourth day after infection and the ovary and vagina of each bird were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until analysed.

Presence of *Salmonella* in the ovary and vagina of infected birds was confirmed using *Salmonella*/*Shigella* (SS) agar (Fluka). The ovary and vagina of each infected bird were tested for *Salmonella*, by plating different dilutions of homogenised ovarian samples, resuspended in 1× PBS, on SS selective agar plates. Using this medium, growth of the *Salmonella enteritidis* species is uninhibited and appears as a colourless colony with a black centre.

2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated from chicken embryos and ovarian and vaginal tissues and stored at −80 °C. Initially, the embryos and tissues were ground to a fine powder, and the RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion), according to manufacturer's instructions. Preliminary quantity and purity of extracted RNA were measured at 260 and 280 nm using the BioPhotometer (Eppendorf) and RNA integrity was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. To reduce degradation, RNase inhibitor (Invitrogen) was added to each sample (1 unit per μg of RNA) before storage at −80 °C. All samples were pre-treated, before reverse transcription (RT) with DNAse (Fermentas) at a concentration of 1 unit per μg of RNA. One μg of total RNA was reverse transcribed to cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen), according to the manufacturer instructions. PCR amplification of cytokine genes was performed using the primers and conditions as previously described (Michailidis et al., 2014). Amplification of the chicken β-actin gene was performed as a control to assess the quality and quantity of the synthesized cDNAs. PCR amplification was performed using 1 μl of cDNA as template, 200 nM primers each, 1 mM dNTPs and 1 unit Taq DNA Polymerase Recombinant (Invitrogen) in 25 μl total volume reaction. The cDNA template negative controls were included to monitor genomic DNA contamination. RT-PCR products were resolved by electrophoresis using 1.5% TBE agarose gels, visualised with ethidium bromide and imaged under UV illumination.

2.4. Real-time PCR analysis

Quantitative expression analysis of cytokines was performed with real-time PCR, using a LightCycler real-time PCR machine (Roche Molecular Biochemicals). Prior to quantitative expression analysis experiments and following oral infection with SE, the experimental birds were examined for SE colonization. Fecal, ovarian and vaginal samples of each experimentally SE infected and control (PBS) birds were cultured. *Salmonella* organisms were detected only in the fecal, ovarian and vaginal samples of SE infected birds, indicating the successful colonization of SE in the reproductive organs of the inoculated birds.

Real-time PCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems) and 0.2 pmol of each primer in a final volume of 20 μl using as template 1/10 of the initial cDNA synthesis reaction. Gene expression levels were quantified using β-actin as an internal standard for cDNA normalization. The cycling parameters were, incubation at 95 °C for 10 min, followed by 45 cycles of incubation at 95 °C for 10 s, 56 °C for 8 s, 72 °C for 8 s and read at 60 °C. For identification of the PCR products, a melting curve was performed from 65 to 95 °C with read every 0.2 °C and 5 s hold between reads. All the reactions were performed 6-times using ovary or vagina total tissue RNA based cDNA isolates from different birds. Mean threshold cycle (Ct) values were determined and relative quantification of the transcript levels was performed using the comparative Ct method (Livak and Schmittgen, 2001). Real-time PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) to calculate relative level of each mRNA in each sample and expressed as a ratio relative to β-actin housekeeping gene. To examine the differences among various developmental stages, fold changes in the gene transcription were as relative values to the 12-week-old ovarian mRNA. Infection experiments were performed, as described previously. Expression levels of 28- and 104-week-old birds were indicated as relative values following treatment with PBS, compared to SE infection; as also difference in expression levels following SE infection among laying and non-laying birds.