



Characteristics of systemic infection and host responses in chickens experimentally infected with *Salmonella enterica* serovar Gallinarum biovar Gallinarum

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ABSTRACT. *Salmonella enterica* serovar Gallinarum biovar Gallinarum (*S. Gallinarum*) is a host-specific pathogen causing systemic infection in poultry, which leads to significant economic losses due to high mortality. However, little is known about the dynamic process of systemic infection and pathogenic characteristics of *S. Gallinarum* in chickens. In the present study, we developed an oral infection model that reproduces the pathology of *S. Gallinarum* and clarified the host immune response of the infected chickens. Chickens at 20 days of age orally inoculated at a dose of 10^8 colony forming unit (CFU) showed typical clinical signs of fowl typhoid and died between 6 and 10 days post infection. The inoculated *S. Gallinarum* rapidly disseminated to multiple organs and the bacterial counts increased in the liver and spleen at 3 days post infection. Pathological changes associated with inflammation in the liver and spleen became apparent at 4 days post infection, and increased expression of interferon (IFN)- γ and interleukin (IL)-12 in the liver and spleen did not observed until 3 days post infection. These results indicate that *S. Gallinarum* rapidly spread to entire body through intestine, and the low-level of inflammatory responses in the liver during the early stage of infection may contribute to rapid, systemic dissemination of the bacteria. Our infection model and findings will contribute to the better understanding of the pathogenic mechanism of *S. Gallinarum*, and provide new insights into the prevention and control of fowl typhoid.

KEY WORDS: chicken, fowl typhoid, *Salmonella* Gallinarum, systemic infection

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Salmonella enterica serovar Gallinarum biovar Gallinarum (*S. Gallinarum*) is an important Gram-negative rod that causes a systemic infection called fowl typhoid in poultry and other birds, which leads to substantial economic losses due to high mortality [11, 15, 19, 28]. Although fowl typhoid has been eradicated or markedly controlled amongst commercial poultry in developed countries, it is still common in many developing countries, mainly including some countries in Central and South America, Africa and South-East Asia [3, 29]. To promote effective breeding of poultry flocks and their global trade, more effective control of fowl typhoid worldwide is one of the challenges to be solved. Vaccination is one of the control strategies against fowl typhoid. Some attenuated strains of *S. Gallinarum* have been used as a live vaccine for prevention of the disease [12, 34]. However, the protective effect of the vaccines is not yet completely satisfactory, and the retaining some virulence is still an important problem [10, 23]. Better understanding of the pathogenesis and infective mechanism of *S. Gallinarum* is necessary to develop more effective and safe vaccine.

S. Gallinarum is a host-specific bacterium that produces a severe, septicemic, often fatal, systemic infectious disease in both chicks and adults of poultry [33]. Chickens are the natural hosts for *S. Gallinarum*. Unlike *S. Typhimurium* which is a broad host range serovar and produces mainly gastrointestinal infection in many kinds of animals and birds, *S. Gallinarum* does not cause

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severe systemic disease in mice or other laboratory mammals and has only rarely been reported to cause enteritis in humans [2, 15, 25]. Although the mechanism of *S. Typhimurium* causing enteritis in mammals and typhoid-like diseases in mice have been extensively studied, little is known about the host-specific mechanism of *S. Gallinarum* and the process of the lethal systemic infection in chicken. To shed some light on the pathogenic mechanism and process of systemic infection in chickens, in this study, we investigated the dynamic process and pathogenic characteristics of *S. Gallinarum* systemic infection *in vivo* that mimics the natural infection in chickens. In this study, chickens were orally infected with different doses of *S. Gallinarum*, and the mortality, clinical signs, bacterial counts in organs, histopathological changes, and host immune responses were evaluated. Our results demonstrate that oral inoculation of chickens with *S. Gallinarum* causes rapid spread of the bacteria to the whole body through the intestine, and the low-level of cytokine production and inflammatory response in the early stage of infection may contribute to the rapid, systemic dissemination of the orally infected *S. Gallinarum* and led to the host death.

MATERIALS AND METHODS

Bacterial strains

S. Gallinarum 287/91, a spontaneous nalidixic acid-resistant strain [1], and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) SL1344 were grown in heart infusion broth (HIB) (Eiken Chemical, Tokyo, Japan) at 37°C with shaking (at 150 rpm). For experimental infection in chickens, the strains were cultured at 37°C in HIB to logarithmic phase, and then collected by centrifugation and washed twice with sterile 0.01 M phosphate-buffered saline (PBS). The washed bacteria were suspended with PBS, adjusted spectrophotometrically at 600 nm to reach 1×10^9 CFU/ml, and then were 10-fold serially diluted to 1×10^5 colony forming unit (CFU)/ml.

Chickens and experimental infection

Conventional female Boris Brown chickens, originated from Rhode Island Red chicken, which is well known to be susceptible to salmonellosis [30], were obtained from commercial farm. They were housed and provided water and food *ad libitum*. In order to ensure whether the chickens were free from *Salmonella*, fecal swabs were taken from the transport box for the bacteriological detection of *Salmonella* before experimental infection. For oral infection, each chicken was inoculated by oral gavage either with 1×10^5 to 10^9 CFU of *S. Gallinarum* or *S. Typhimurium* in a volume of 1.0 ml at 20 days old. After inoculation, chickens were reared for 14 days and observed twice a day for monitoring their clinical signs and mortality. To analyze the bacterial counts and host responses in tissues of the chickens post infection, chickens were inoculated by oral gavage with 10^8 CFU of *S. Gallinarum* or *S. Typhimurium* as described above and were euthanized on 1, 2, 3, 4, 5 or 6 days post infection. Five chickens in each group were euthanized at each time point. The cecal contents, cecal tonsil, liver and spleen were collected aseptically for determination of viable *Salmonella*, pathological examination and quantitative real-time RT-PCR analysis of cytokines. Animal experimentation protocol was approved by the President of Kitasato University through the judgment by Institutional Animal Care and Use Committee of Kitasato University (Approval no. 19-140 and 20-055).

Isolation and enumeration of *Salmonella*

For detection of *Salmonella* in the infected chickens, the samples of cecal tonsil were washed three times in sterile PBS to remove as much of the intestinal contents and tissue surface bacteria as possible. These samples were then incubated for 30 min at 37°C with gentamicin (200 µg/ml) to kill any remaining tissue surface bacteria. The samples were washed with sterile PBS to remove residual gentamicin, homogenized, then treated with 0.5% Triton-X 100 to lyse epithelial cells and release intracellular bacteria. The collected cecal contents, liver and spleen were homogenized in 9 volume of HIB, serially diluted 10-fold with HIB and spread on desoxycholate-hydrogensulfide-lactose agar plates (DHL) containing 25 µg/ml nalidixic acid. After 24 hr of incubation at 37°C, colonies on the plates were counted as colony forming unit (CFU).

Clinical evaluation and histopathological examination

The clinical changes in chickens infected with *S. Gallinarum* or *S. Typhimurium* were observed and evaluated for onset of systemic infection. Clinical signs, redness, and discoloration of the comb and feathers were observed and recorded. Monitoring of chickens for morbidity and mortality was carried out up to 14 dpi. Five chickens in each group were euthanized at 1 to 6 days after infection and investigated for the extent of inflammation, by observing redness, swelling, congestion, bleeding, and discoloration of the tissues.

To estimate the inflammation levels and histological changes, the tissues, cecal tonsil, liver and spleen of each group were collected and fixed in 10% neutral buffered formalin for 24 hr, before being processed with an automatic tissue processor, and was embedded in paraffin wax. Sections were cut at three levels to a thickness of 4 µm and stained by the haematoxylin-eosin (HE) staining. Histological changes such as infiltration of inflammatory cells and tissue damages were recorded for each section.

Quantitative real-time RT-PCR analysis

Each tissue sample was immersed separately in 0.5 ml of RNA_{later} (Invitrogen, Carlsbad, CA, USA) and stored at -80°C until use. Total RNA was extracted from 5 mm × 5 mm of the tissue using RNA iso Plus (TaKaRa, Kusatsu, Japan) according to the manufacturer's instructions. The quantity and quality of RNA were determined by spectral analysis (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). The RNA samples with purity of 2 for A260/A280 ratio and above 2 for

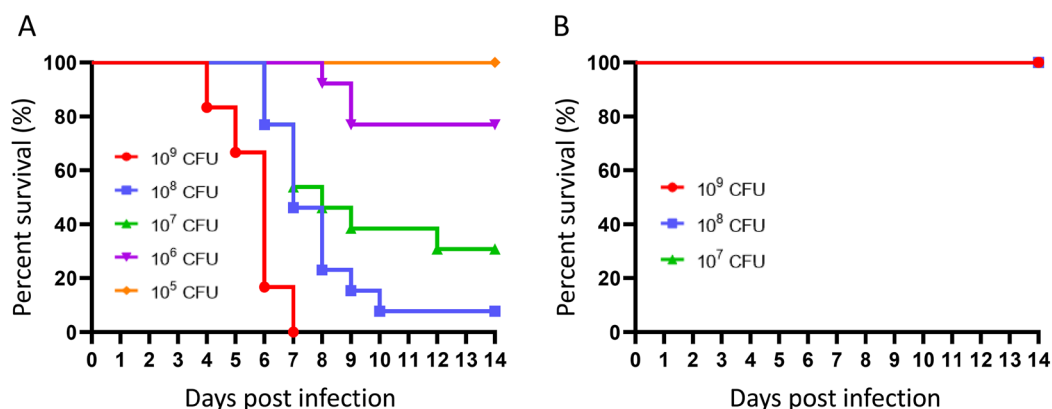


Fig. 1. Survival of chickens infected with *Salmonella Gallinarum* and *Salmonella Typhimurium*. Chickens were orally inoculated with 10⁵ to 10⁹ colony forming unit (CFU) of *S. Gallinarum* (A) or 10⁷ to 10⁹ CFU of *S. Typhimurium* (B). The clinical symptoms and survival of chickens were monitored and recorded for 14 days post infection.

A260/A230 ratio were used. After being treated with DNase, RNA was transcribed to complementary DNA (cDNA) using the ReverTra Ace[®] qPCR RT Master Mix (Toyobo, Osaka, Japan) following the manufacturer's instructions. Expression of mRNA for interleukin (IL)-12 and interferon (IFN)- γ in the tissues was measured using quantitative real-time RT-PCR. Primer sequences of chicken IL-12, IFN- γ and GAPDH are as follows: IL-12 forward, 5'-AAGTAGACTCCAATGGGCAAATG-3', IL-12 reverse, 5'-ACGTCTTGCTTGGCTCTTTATAGC-3'; IFN- γ forward, 5'-ATGTAGCTGACGGTGGACCT-3', IFN- γ reverse, 5'-CCAAGTACATCGAAACAATCTGGC-3'; GAPDH forward, 5'-GGCACTGTCAAGGCTGAGAA-3', GAPDH reverse, 5'-TGCATCTGCCCATTTGATGT-3'. Twenty μ l reaction mixture, which contained 2.0 μ l cDNA, 10 μ l THUNDERBIRD[®] SYBR[®] qPCR Mix, 0.6 μ l of each primer (at 10 μ M), 0.4 μ l 50 \times ROX reference dye and 6.4 μ l Nuclease-free water, were prepared using the THUNDERBIRD[®] SYBR[®] qPCR Mix (Toyobo). Duplicate reactions were set up for each sample. Quantitative real-time RT-PCR was performed on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following reaction profile: one cycle at 95 $^{\circ}$ C for 20 sec, and 40 cycles at 95 $^{\circ}$ C for 3 sec and 60 $^{\circ}$ C for 30 sec. To check the specificities of amplified products, the melt-curve mode was used (one cycle at 95 $^{\circ}$ C for 15 sec, 60 $^{\circ}$ C for 1 min, and 95 $^{\circ}$ C for 15 sec) after amplification. The expression of the target genes was determined using the cycle threshold value relative to that of the housekeeping gene GAPDH. The results were expressed as fold-changes in corrected target gene expression in the infected chickens relative to the uninfected controls.

Statistical analysis

The bacterial counts were converted logarithmically and the differences between means obtained for each day were analyzed using one-way ANOVA analysis followed by Tukey's multiple comparison test. For analysis of cytokine expressions, statistical comparison was made by Student's *t* test compared with uninfected control group. Both analyses were performed using GraphPad Prism 8.43 (GraphPad Software, San Diego, CA, USA), and the *P* values of <0.05 were considered statistically significant.

RESULTS

Systemic infection and mortality of chickens infected with *S. Gallinarum*

We firstly investigated the mortality and clinical symptoms in chickens inoculated with different doses of *S. Gallinarum* or *S. Typhimurium*. Chickens inoculated with 10⁹ CFU of *S. Gallinarum* showed a mortality rate of 100% and died on days 4 to 7 post infection (Fig. 1A). The chickens died on day 4 post infection had no obvious clinical symptoms. The group infected with 10⁸ CFU *S. Gallinarum* showed mortality rate of 90% and died between 6 and 10 days post infection. The groups infected with 10⁷ and 10⁶ CFU of *S. Gallinarum* showed mortality rates as 70% and 40%, respectively, and most of the chickens died between 6 and 12 days post infection (Fig. 1A). The chickens that died after 4 days of infection showed significant clinical symptoms of fowl typhoid, such as feather disturbance and depression. In contrast, in the chickens that infected with *S. Typhimurium*, there were no death and no significant clinical changes even at the dose of 10⁹ CFU of *S. Typhimurium* (Fig. 1B).

Enumeration of *Salmonella* in the infected chickens

To investigate the spreading and proliferation of *S. Gallinarum* in the infected chickens, we detected the bacterial burdens in the cecal contents, cecal tonsils, liver and spleen of chickens on 1 to 6 days after oral infection with 10⁸ CFU of *S. Gallinarum* or *S. Typhimurium*. The results showed that the numbers of bacteria in the cecal contents and cecal tonsils of chickens infected with *S. Gallinarum* were lower compared with that infected with *S. Typhimurium*. In contrast, the bacterial counts of *S. Gallinarum* in the liver and spleen were higher than those of *S. Typhimurium* and continued to increase up to 6.0 log₁₀ CFU/g significantly (*P*<0.05) from day 2 to 6 post infection, indicating that orally infected *S. Gallinarum* rapidly spread to the systemic sites through the

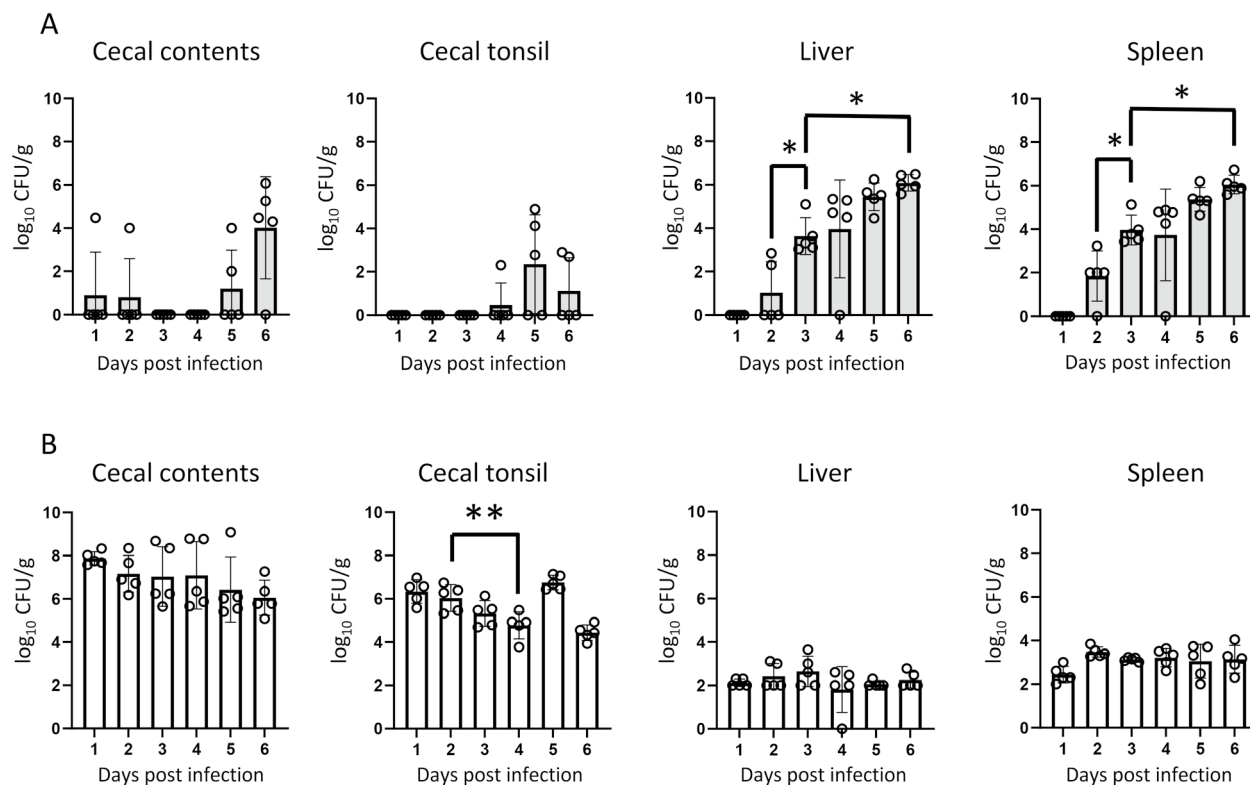


Fig. 2. Viable bacterial counts in the organs of chickens infected with *Salmonella Gallinarum* or *S. Typhimurium*. Chickens were orally inoculated with 10^8 colony forming unit (CFU) of *S. Gallinarum* (A) or *S. Typhimurium* (B). The numbers of the bacteria in the cecal contents, cecal tonsil, liver and spleen were determined on days 1 to 6 post infection. The data are means \pm standard deviations based on five chickens per group at each time point. Statistical comparison was made using one-way ANOVA followed by Tukey's multiple comparison test. The significant difference was shown as * $P < 0.05$, ** $P < 0.01$.

intestine (Fig. 2A). In contrast, *S. Typhimurium* showed higher bacterial numbers in the cecal contents and cecal tonsils but lower in the liver and spleen, and did not increase significantly during the observation (Fig. 2B).

Pathological findings and histological changes in the infected chickens

In the natural infection of *S. Gallinarum*, hypertrophy, white lesions, and small necrotic foci are observed in the liver, which are the most characteristic pathological lesions of fowl typhoid [6, 7]. These lesions were also observed in our infection model. In particular, these lesions become detectable at 4 days after infection in the chickens infected with 10^8 and 10^7 CFU of *S. Gallinarum* (Fig. 3A). The lesions were characterized by marked infiltration of heterophils and lymphocytes with degeneration and necrosis of hepatocytes (Fig. 3B). The angiectasis and vacuolar degeneration of hepatocytes were observed at 5 days after infection (Fig. 3C). In contrast, no significant pathological change was observed in the liver of *S. Typhimurium*-infected chickens (data not shown).

For histological changes in the cecal tonsils, the infiltration of heterophils in the lamina propria was found on day 1 and 3 post infection (Fig. 3D and 3E). Dehydration of epithelial cells and a starry sky appearance in lymphoid follicles were observed on days 3 and 5 after infection (Fig. 3E). In the spleen, no tissue damage and inflammation were observed even at 6 days after infection, but extramedullary hematopoiesis of the red pulp was observed 5 days after infection (Fig. 3F).

Expression of immune genes in the organs of *S. Gallinarum*-infected chickens

To further analyze the immune responses in the systemic level of the chickens infected with *S. Gallinarum*, we determined the expression of selected cytokine genes of IFN- γ and IL-12, as markers of immune responses for elimination of intracellular bacteria, in cecal tonsils, liver and spleen on days 1 to 6 after infection (Fig. 4A and 4B). *S. Gallinarum* infection did not increase the expression of IL-12 and IFN- γ in the cecal tonsils of the infected chickens with no significant difference compared to the uninfected controls. The expression of IFN- γ and IL-12 in the liver and spleen showed no differences in the first 3 days and started to increase at day 4 post infection, which were significantly higher than those of uninfected chickens between days 4 and 6 after infection ($P < 0.05$). Upregulation of IFN- γ , but not IL-12, in the spleen of infected chickens was also observed between days 4 and 6 post infection. The delayed expression of cytokines is consistent with the onset of the inflammatory response in the tissues which became apparent at 4 days after infection (Fig. 3).

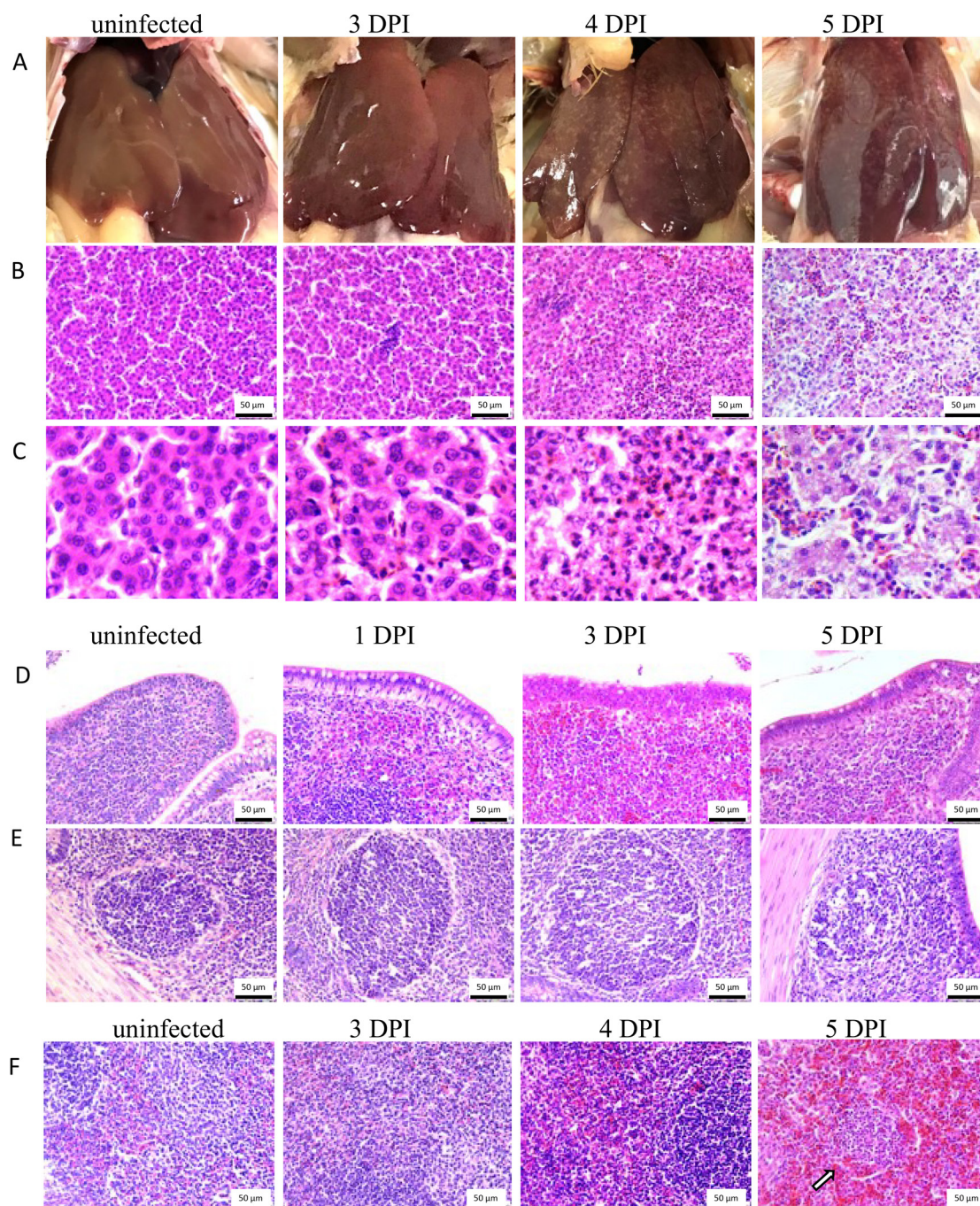


Fig. 3. Pathological finding and histological lesions in *Salmonella Gallinarum*-infected chickens. Chickens were inoculated orally with 10^8 colony forming unit (CFU) of *S. Gallinarum* and the cecal tonsil, liver and spleen were collected at 1 to 6 days post infection (DPI). The organs of uninfected chickens were used as the controls. Paraffin sections of the organs were prepared and stained with hematoxylin and eosin. Gross pathological changes (A) and microscopic lesions (B, magnification $\times 400$) in the livers, (C) is an enlarged photo from (B); histopathological changes and microscopic lesions in the cecal tonsils (D and E, magnification $\times 400$); histopathological changes and microscopic lesions in the spleens (F, magnification $\times 400$), and the arrow shows extramedullary hematopoiesis of the red pulp in the spleen.

DISCUSSION

S. Gallinarum is an intrinsically flagellate *Salmonella* that causes serious systemic infection, affecting domestic fowl of all ages and leading to high mortality [5, 9, 28]. In order to reveal the pathogenic mechanism and process of systemic infection in chickens, we investigated the dynamic process and pathogenic characteristics of *S. Gallinarum* systemic infection *in vivo* that mimics the natural infection in chickens. In the oral infection model, *S. Gallinarum* was rapidly disseminated to the systemic sites, with a

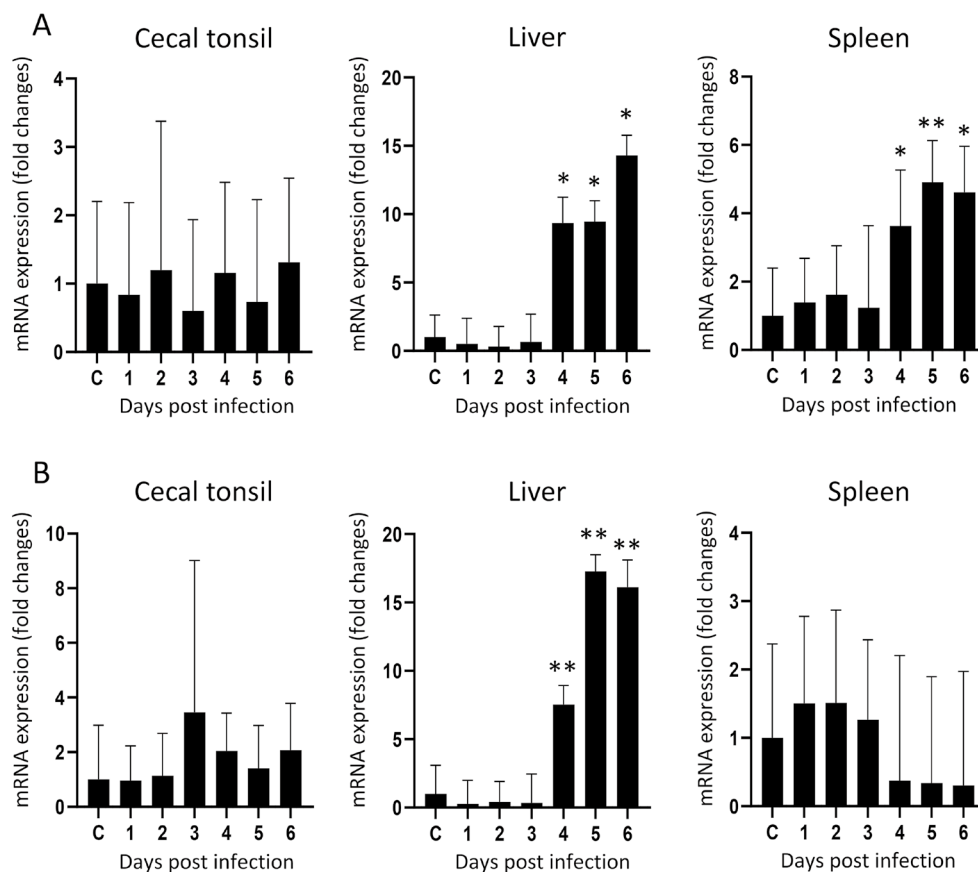


Fig. 4. Expression of cytokines in chickens infected with *Salmonella Gallinarum*. Chickens were inoculated orally with 10^8 colony forming unit (CFU) of *S. Gallinarum*. The cecal tonsil, liver and spleen of the chickens were collected and the expression of interferon (IFN)- γ (A) and interleukin (IL)-12 (B) were determined by quantitative RT-PCR on days 1 to 6 post infection. Data were expressed as means \pm standard deviations of fold-changes in gene expression in the cecal tonsil, liver and spleen from infected groups relative to those from uninfected control group (five chickens per group at each time point). Statistical analysis was performed using the Student's *t* test to compare infected chickens with uninfected controls. The significant difference was shown as * $P < 0.05$, ** $P < 0.01$. C: uninfected controls.

marked increase in the number of bacteria in the liver and spleen, which led to high mortality and severe pathological changes in the liver of infected chickens. The expression of IFN- γ and IL-12 in the liver and spleen was initially unchanged, but increased after 4 days post infection when inflammatory response in the tissues began to become apparent. These results suggest that *S. Gallinarum* spread rapidly from the intestinal tract to the systemic sites after oral infection, and that the low-level of cytokine production and inflammatory responses in the early stage of infection may lead to systemic infection and death of the infected chickens.

A previous study has reported that orally infected *S. Typhimurium* reaches the intestinal tract, adheres to and passes through the intestinal epithelium, and then reaches the lamina propria, where it replicates or penetrates into deeper tissues [32]. In the present study, the bacterial counts in the cecal contents and cecal tonsils of *S. Gallinarum* infected chickens were lower than those of the chickens infected with *S. Typhimurium* (Fig. 2). In contrast, bacterial counts in the liver and spleen of the chickens infected with *S. Gallinarum* increased markedly from 2 to 6 days after infection, which was not observed in *S. Typhimurium*-infected chickens (Fig. 2). Previous studies have reported that the flagella of *Salmonella* can activate Toll-like receptor (TLR)-5 in intestinal epithelial cells, and the resulting host immune responses are important for defense against *Salmonella* infection. [26, 31]. In this study, *S. Gallinarum*, a non-flagellated *Salmonella*, is conducive to systemic spread of the bacteria in the early stage of infection because the lack of flagella does not activate TLR-5 in the intestinal epithelial cells [3, 35, 36]. Although infiltration of heterophils in the lamina propria of cecal tonsil were observed on 1 day post infection, this immune response did not reduce the spread of *S. Gallinarum* to systemic organs. These results suggest that *S. Gallinarum* can invade and rapidly reach systemic organs from the intestine, and the first 3 days post infection are the critical period for the proliferation of *S. Gallinarum* in the infected chickens. In addition, our results showed that in the liver of *S. Gallinarum*-infected chickens, inflammation, tissue damages and lesions such as white foci developed following the increase of the number of the bacteria, suggesting that the liver is the pivotal organ with the most significant changes in bacterial invasion and host immune responses (Fig. 3A–C). Hematopoiesis in the spleen was also observed at 5 days post infection (Fig. 3F), which might be a response to hemolytic anemia caused by *S. Gallinarum* [6]. Previous reports have shown that histopathological changes such as necrosis occurred in the liver and spleen in *S. Gallinarum*-infected

chickens [6, 33]. These results indicate that pathological changes and viable counts of *S. Gallinarum* in the liver and spleen can be parameters for assessing the extent of systemic infection [15, 33]. Liver is considered to be a functional organ for innate immune defense, with kupffer cells which are liver-specific macrophages, and pit cells which are liver-specific natural killer cells [20, 39]. Da Silva *et al.* has reported that acetylcholinesterase activity in the liver from *S. Gallinarum*-infected laying hens decreased compared with uninfected laying hens, suggesting that reduced acetylcholinesterase activity in the liver decreases the induction of pro-inflammatory cytokines and inhibits the immune responses in *S. Gallinarum*-infected hens [7, 8]. Together with our results, it is indicated that the induction of pro-inflammatory cytokines and acetylcholinesterase activity in liver may be important for the defense against *S. Gallinarum* infection in chickens.

Salmonella, especially for serovars having broad host range such as *S. Typhimurium*, invades the chicken intestine and induces an inflammatory process, resulting in the expression of pro-inflammatory cytokines and chemokines [24, 27, 37, 38]. The outcome of this innate immune activation is an initial major infiltration of heterophils to the intestine, thereby limiting bacterial invasion [4, 21, 22]. In the present study, intriguingly, the expression of IFN- γ and IL-12 in the cecal tonsil, liver and spleen in the chickens infected with a highly host-restricted serovar *S. Gallinarum* did not increase until 4 days post infection, and the inflammatory response in the tissues began to become apparent after 4 days post infection. Previous reports have shown that IFN- γ and IL-12 as the important factors to eliminate *Salmonella* in the cecal tonsil, liver and spleen, and lack of these cytokines resulted in high susceptibility to *Salmonella* and *Mycobacterium* [16, 18]. Huang *et al.* reported that *S. Gallinarum*-infected chicken macrophage cell line, HD11, exhibited lower induction of pro-inflammatory cytokine IL-1 β , CXCL1 and CXCL2 compared to *S. Typhimurium*- and *S. Dublin*-infected cells [13, 14]. Recently, Kaiser *et al.* described that *S. Gallinarum* infection induces low inflammatory response *in vitro* compared with *S. Typhimurium* and *S. Enteritidis*, which seldom cause clinical disease in poultry [17]. The present study demonstrated that the low-level of cytokine production and inflammatory responses in the organs during the early stage of infection may be permissive for systemic spread of *S. Gallinarum*, which could cause typhoid-like disease with considerable mortality in the infected chickens. Further studies on the interactions between *S. Gallinarum* and host immune responses in the cecal tonsil and liver of infected chickens will be contribute to the better understanding of the host-specific mechanism in the pathogenesis of fowl typhoid, and provide new insights into the prevention and control of this disease.

CONFLICT OF INTEREST. The authors declare no competing interests.

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