Research Note: Correlation analysis of interleukin-6, interleukin-8, and C-C motif chemokine ligand 2 gene expression in chicken spleen and cecal tissues after *Eimeria tenella* infection in vivo

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ABSTRACT IL-6, IL-8, and C-C motif chemokine ligand 2 (**CCLi2**) are important factors in inflammatory and immune responses. To investigate their relationships in the spleen and cecum and between coccidiosis-infected and uninfected states, we performed quantitative real-time PCR to compare the relative expression difference of *IL-6*, *IL-8*, and *CCLi2* in the same tissues between the infection and control groups. In addition, the correlations of the relative expression levels of these 3 genes were determined in the same and different tissues within the same group. The results showed that the expression levels of *IL-6*, *IL-8*, and *CCLi2* in the spleen and cecum of the infected group were all higher than those of the uninfected group ($P < 0.05$). The correlation coefficients among the *IL-6*, *IL-8*, and *CCLi2* expression levels in the spleen or cecum were all positive in both the infection and control groups. In the spleen tissues, *CCLi2* expression was strongly correlated with *IL-6* and *IL-8* in the uninfected group ($P < 0.01$), and the correlation coefficients reached 0.853 ($R^2 = 0.728$) and 0.996 ($R^2 = 0.992$), respectively. The expression of *CCLi2* was also strongly correlated with *IL-8* (R reached 0.890, $R^2 = 0.792$) in the infected group. In the cecal tissues, the expression levels of the 3 genes were all extremely significantly correlated in the uninfected group ($P < 0.01$), and the correlation coefficients ranged from 0.498 to 0.765, indicating moderate correlations. The expression of *IL-6* was extremely significantly positively correlated with *IL-8* and *CCLi2* in the infected group ($P < 0.01$), with moderate correlations (R ranged from 0.469–0.639). In addition, the expression levels of the 3 genes were not significantly correlated ($P > 0.05$) between the spleen and cecum tissues in either the infection group or the control group. These results indicate that *IL-6*, *IL-8*, and *CCLi2* were correlated and play an important role in coccidiosis infection of Jinghai yellow chicken. Our data also provide a basis for further exploring the role of these 3 genes in genetic breeding for coccidiosis resistance.

Key words: *Eimeria tenella*, cecum, spleen, gene expression, correlation analysis

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INTRODUCTION

Chickens are one of the most important sources of meat. However, the poultry industry is constantly threatened by parasites, especially avian coccidiosis. It is estimated that the annual global economic loss caused by coccidiosis exceeds $3 billion (Sharman et al., 2010; Blake and Tomley, 2014), at least 70% of which is due to a decrease in production performance caused by subclinical symptoms of coccidiosis, including individual growth retardation and a reduction in the feed conversion ratio, and 24% of which is due to increased expenses for drug prevention and treatment (Li et al., 2005). In addition, the weak effects of anticoccidials against different strains of pathogens and the high cost of vaccine production are still concerns in the global poultry industry (Wang et al., 2019). Therefore, research on selecting birds with natural resistance to chicken coccidia and other pathogens is important.

Using the RNA-seq technique to screen the differentially expressed genes between *Eimeria tenella*–infected and uninfected cecal tissues of Jinghai yellow chickens on the seventh day, Lin (2015) revealed that the differentially expressed genes significantly enriched

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in cytokine-cytokine receptor pathway interactions included *IL-6*, *IL-8*, *IL-12b*, *IL-15*, *IL-17*, and *TGFB2*. Among their gene products, IL-6 is a multifunctional cytokine that plays a vital role in many acute-phase reactions, autoimmune diseases, and hematopoietic mechanisms, particularly inflammatory bowel disease (Nishimichi et al., 2006; Neurath and Finotto, 2011; Schett et al., 2013). Rose-John et al. (2017) discovered that the host defense against bacterial and fungal pathogen infection was primarily mediated by classical IL-6 signaling pathways. IL-8 is an important chemokine in birds. By binding to the receptor CXCR1 on immune cells, IL-8 attracts immune cells to the affected area and then participates in the immune response, achieving immune cell resistance and promoting wound healing; it is significantly associated with acute or chronic inflammatory diseases and autoimmune diseases (Grimm et al., 1996; Coussens and Werb, 2002; Savage et al., 2004). Studies have demonstrated that parasite replication was significantly inhibited in chickens given the pcDNA3-1E vaccine along with IL-8 plasmids (Lillehoj and Lillehoj, 2000; Min et al., 2001) and that IL-8 can be used as an adjuvant for the DNA vaccine of *E. tenella*. Chemokine C-C motif 2 (CCLi2) is a member of the chemokine CC subfamily. As a promoter of the inflammatory response, CCLi2 plays an important role in promoting the migration of monocytes by binding to the CCR2 receptor (Mahad et al., 2005; Qian et al., 2011; O'Connor et al., 2015). These findings suggest that cytokines (IL-6) and chemokines (IL-8 and CCLi2) play a key role in pathogen infection in chickens, including that of *E. tenella*. Swaggerty et al. (2015) reported that selection for the proinflammatory mediators *IL-6*, *IL-8* (*CXCLi2*), and *CCLi2* produces chickens more resistant to *E. tenella*. However, the relationships among these 3 genes in the coccidial-infected state and the uninfected state and between different tissues are unclear in current studies. Thus, determination of the expression and regulation of the chicken *IL-6*, *IL-8*, and *CCLi2* genes and the relationships among the expression levels of these 3 genes and coccidiosis should be performed.

In our study, quantitative real-time PCR (qRT-PCR) was applied to detect the relative mRNA expression levels of the proinflammatory cytokine *IL-6* and the chemokines *IL-8* and *CCLi2* in the spleen and cecal tissues of Jinghai yellow chickens (*Gallus gallus*). The differences in relative expression levels of the 3 genes were compared between the infection and control groups, and correlations of the relative expression levels were analyzed. The objective of this study was to provide a basis for subsequent research on resistance to coccidia in chickens.

MATERIALS AND METHODS

Animals

A total of 80 (40♂, 40♀) 1-day-old healthy and physiologically similar Jinghai yellow chickens were selected from the Jinghai Yellow Chicken Resource Farm, Haimen, Jiangsu Province, China. All the chicks were randomly divided into the infected and uninfected groups and were kept in single cages free of coccidiosis by flame disinfection with a gasoline torch and fed antibiotic-free feed and water for up to 30 D (Wang et al., 2019). Each chicken in the infected group was orally infected with 2.5×10^4 *E. tenella* sporulated oocysts. The uninfected group received the same amount of normal saline. All protocols for animal sample collection were approved by the Animal Welfare Committee of Yangzhou University (permit number: SYXK (Su) IACUC 2012-0029), and all efforts were made to minimize the suffering of the chickens.

Tissue Collection

The animals were slaughtered on the seventh day after infection, and the spleen and cecal tissues of all chickens in the infected and uninfected groups were collected in a cryotube, immediately stored in liquid nitrogen, and then transferred to a freezer for storage at −70°C.

RNA Isolation and Quality Assessment

Total RNA from chicken tissues was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A NanoPhotometer spectrophotometer (Implen, Inc., Westlake Village, CA) was used to assess the optical density value (A260/A280) of the total RNA. RNA integrity was assessed using an RNA Nano 6000 Assay Kit with a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). RNA degradation was monitored with 1% agarose gels. Qualified RNA samples were diluted to 100 ng/μL and stored at −70°C.

Primer Design and qRT-PCR Performance

Based on the published chicken *IL-6*, *IL-8*, and *CCLi2* gene sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), qRT-PCR primers for the 3 target genes were designed using the Primer Premier 5 software (PREMIER Biosoft, Palo Alto, CA). The reference genes *GAPDH* and *β-actin* were used as previously described (Fang, 2012). All primers were synthesized by Sangon Biotech Co. (Shanghai, China), and the primer information is presented in Table 1.

cDNA Synthesis

The extracted total RNA was reverse transcribed with PrimeScript RT Master Mix (Takara Bio Inc, Otsu, Japan) according to the manufacturer's instructions, and reverse transcription was carried out in a final volume of 40 μL assembled on ice containing 8 μL of 5 × PrimeScript RT Master Mix, 2000 ng of RNA template, and RNase-free ddH_2O added to 40 μL. The reaction conditions were 37°C for 15 min and 85°C for 5 s. The samples were stored at −20°C.

Table 1. Quantitative real-time PCR primers used in this study.

Gene amplified	Primer name	Primer sequence (5'→3')	Amplification size (bp)	GenBank accession no.
IL-6	F	CAAGGTGACGGAGGAGGAC	254	AJ309540
	R	TGGCGAGGAGGGATTTCT		
IL-8	F	GGCTTGCTAGGGGAAATGA	136	AJ009800
	R	AGCTGACTCTGACTAGGAAACTGT		
CCLi2	F	GGCAGACTACTACGAGACCAACAG	70	L34553
	R	ACGGCCCTTCCTGGTGAT		
GAPDH	F	GGTGGTGCTAAGCGTGTTAT	264	K01458
	R	ACCTCTGTCATCTCTCCACA		
β-actin	F	ACACGGTATTGTCACCAACT	263	L08165
	R	TAACACCATCACCAGAGTCC		

Quantitative Real-Time PCR

Fluorescence quantitative analysis was performed using the SYBR Green I dye method (Bio-Rad Co., Hercules, CA) with a total volume of 20 μL, which contained 10 μL of SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.8 μL each of upstream and downstream primer, 0.4 μL of ROX Reference Dye II dye, 2 μL of cDNA template, and ddH_2O added to 20 μL.

qRT-PCR was carried out as follows: preliminary denaturation at 94°C for 30 s, followed by 40 cycles of denaturation for 5 s at 95°C and annealing for 34 s at 60°C. Data at multiple points were collected for dissolution curve analysis, and the procedure was as follows: 95°C for 15 s; 60°C for 1 min; and 95°C for 15 s and 60°C for 15 s. Each sample was analyzed in triplicate.

Statistical Analysis

We used the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Adnan et al., 2011) to analyze the qRT-PCR results. SPSS 25.0 was used for correlation analysis of the relative expression of the target genes in different tissues.

RESULTS

RNA Isolation Results

The extracted RNA was monitored with formaldehyde-denatured agarose gel electrophoresis, and the results showed that the 28S and 18S bands of the total RNA samples were bright, clear, and sharp. The image is shown in Figure 1. We used a UV spectrophotometer to assess the extracted RNA. The A260/A280 value of the RNA sample was found to be between 1.8 and 2.0, suggesting that the extracted RNA had a high purity and could be used for the next step of the experiment.

Differences in IL-6, IL-8, and CCLi2 Gene Expression in the Spleen and Cecum of the Infection and Control Groups

As shown in Figure 2A, the *IL-6*, *IL-8*, and *CCLi2* genes were all expressed in the spleen and cecal tissues of Jinghai yellow chickens in the infection and control groups. In the spleen tissues, the relative expression levels of the *IL-6*, *IL-8*, and *CCLi2* genes in the infected group were all significantly higher than those in the control group ($P < 0.05$), and the relative expression level of the *IL-6* gene in the infected group was extremely significantly different from that in the control group ($P < 0.01$). In the cecal tissues, the relative expression levels of the *IL-6*, *IL-8*, and *CCLi2* genes of the infected group were all extremely significantly different from those of the control group ($P < 0.01$).

As shown in Figure 2B, the relative expression levels of other genes were significant or extremely significant in both the control group and the infected group ($P < 0.05$ or $P < 0.01$), while the relative expression levels of *IL-6* in the spleen and cecum of the controls were not significant ($P > 0.05$). Moreover, the relative expression levels of each gene in the cecum were higher than those in the spleen.

Correlation Analysis of IL-6, IL-8, and CCLi2 Gene Expression in the Spleen and Cecal Tissues

As shown in Table 2, the expression levels of the *IL-6*, *IL-8*, and *CCLi2* genes in the spleen and cecal tissues were all positively correlated in both the infection group and the control group. In the spleen tissues of the control group, the relative expression levels of the *IL-8* and *IL-6* genes did not show a significant positive correlation ($P > 0.05$), but there was an extremely significant

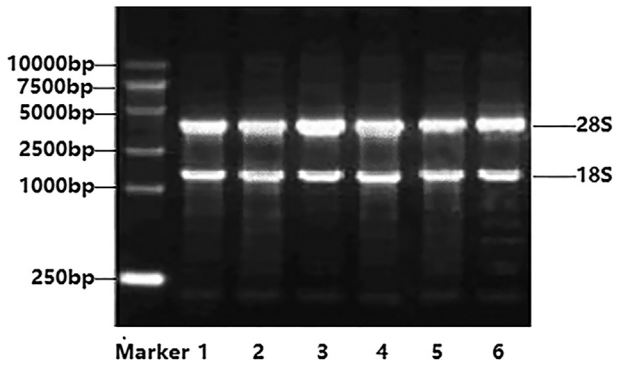

Figure 1. Agarose gel electrophoresis image of the total RNA. The marker used here was the DL15,000 DNA marker. Electrophoretic profiles showed the extracted RNA from 6 chickens selected randomly.

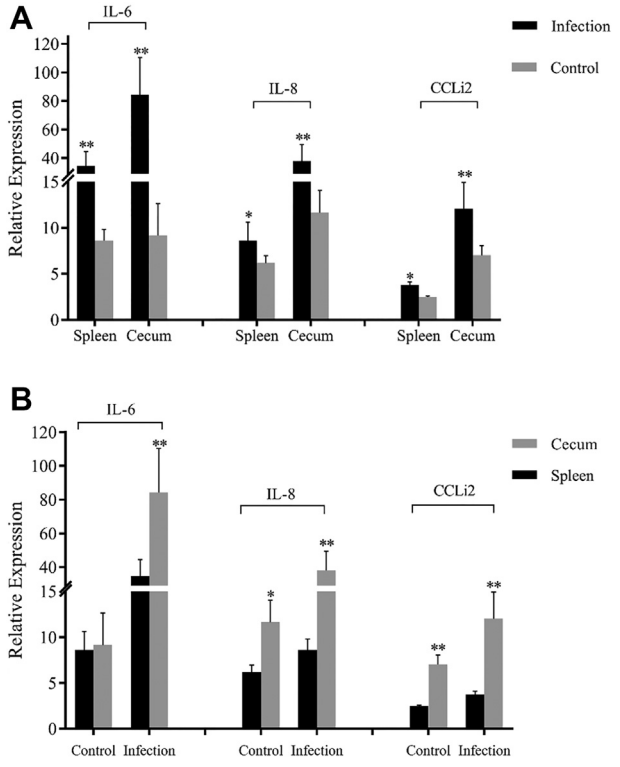

Figure 2. Comparison of the expression of the *IL-6*, *IL-8*, and *CCLi2* genes between the spleen and cecum of the Jinghai yellow chickens in the infected group and the control group. *Indicates a significant difference ($P < 0.05$) and **indicates an extremely significant difference ($P < 0.01$). (A) A comparison of the relative expression of genes in different groups in the same tissue. (B) A comparison of the relative expression of genes in different tissues in the same group. CCLi2, C-C motif chemokine ligand 2.

positive correlation ($P < 0.01$) between the relative expression of *IL-8* and *IL-6* and that of *CCLi2*; in the spleen tissues of the infected group, the correlation coefficient between the expression of *IL-6* and *IL-8* was positive ($P < 0.01$), and extremely significantly positive correlations were found between the expression of *IL-6* and *IL-8* and that of *CCLi2* ($P < 0.01$). In the cecal tissues of the control group, there were extremely significant positive correlations ($P < 0.01$) between the relative expression of *IL-8* and *CCLi2* and that of *IL-6*, and the relative expression levels of the *IL-8* and *CCLi2* genes were also significantly correlated ($P < 0.05$); in the cecal tissues of the infected group, there were extremely significant positive correlations ($P < 0.01$) between the relative expression of *IL-8* and *CCLi2* and that of *IL-6*, but the relative expression correlation between *IL-8* and *CCLi2* genes were not significantly correlated ($P > 0.05$).

Correlation Analysis of IL-6, IL-8, and CCLi2 Gene Expression in the Spleen and Cecal Tissues

The correlations of the relative expression of the 3 genes were determined in the spleen vs. the cecum of the infected group (Table 3). The results showed that there was no significant positive correlation ($P > 0.05$) between the relative expression of *IL-6*, *IL-8*, and *CCLi2* in the spleen and that of *IL-6* in the cecum, and the relative expression of *IL-8* and *CCLi2* in spleen and that of *IL-8* in cecum were not significantly negatively correlated ($P > 0.05$). The relative expression of *IL-6* in the spleen and that of *IL-8* in the cecum were not significantly negatively correlated ($P > 0.05$), and the relative expression levels of *IL-6*, *IL-8*, and *CCLi2* in the spleen were not significantly negatively correlated with *CCLi2* in the cecum ($P > 0.05$). The correlations among the relative expression levels of the 3 genes in the spleen and the same genes in the cecum of the control group were examined, and we found that the expression levels of the *IL-6*, *IL-8*, and *CCLi2* genes were not significantly correlated ($P > 0.05$) between the spleen and cecal tissues. Moreover, in the control group, only the *CCLi2* gene in the cecum had no significant positive correlation with *IL-8* and *CCLi2* in the spleen ($P > 0.05$), and there was no significant negative correlation between the other genes ($P > 0.05$).

Table 2. Correlation analysis of the expression of the 3 genes in the spleen and cecal tissues of the infected and control groups[1].

Genes	*IL-6*	*IL-8*	*CCLi2*
IL-6	1 (1)	0.475** (0.639**)	0.579** (0.469**)
IL-8	0.261 (0.667**)	1 (1)	0.890** (0.324)
CCLi2	0.853** (0.498**)	0.996** (0.765**)	1 (1)

*Significant correlation ($P < 0.05$), **extremely significant correlation ($P < 0.01$), and "no superscript designator" indicates no significant correlation ($P > 0.05$).

[1]The correlation coefficients above and below the diagonal are based on the infected group and the control group, respectively. The data in brackets are correlation coefficients of expression of the 3 genes in the cecum.

DISCUSSION

Chickens have no lymph nodes, only primitive lymphoid tissues, and the cecal tonsils and spleens play a dominant role in the immune response. Moreover, T lymphocytes in the spleens of chickens show proliferation and immune functions from 4 D of age and have perfected these functions at 30 D of age (Luan et al., 2008). The main target of *E. tenella* is in the cecum, and the spleen is an important immune organ in chickens (Jarosinski et al., 2005; Guo et al., 2013). Therefore, we selected chicks aged 30 D and detected the relative expression of 3 genes in the spleen and cecum on the seventh day after oral feeding of *E. tenella* to explore the correlations between these 3 genes and coccidiosis infection in chickens.

Many studies have shown that the relative expression levels of *IL-6*, *IL-8*, and *CCLi2* are higher in infectious conditions than in noninfectious conditions after challenge with different pathogens (Sadeyen et al., 2004; Kim et al., 2008; Fernando et al., 2015). *E. tenella* can strongly induce an immune response and increase *IL-8* expression in the cecum (Cornelissen et al., 2009). The

Table 3. Correlation analysis of the expression of the 3 genes in the spleen and cecum of the infected and control groups.

	Spleen in the infection group			Spleen in the control group		
Cecum	*IL-6*	*IL-8*	*CCLi2*	*IL-6*	*IL-8*	*CCLi2*
IL-6	+0.091	+0.062	+0.297	−0.284	−0.146	−0.113
IL-8	−0.014	+0.169	+0.203	−0.380	−0.101	−0.067
CCLi2	−0.162	−0.110	−0.023	−0.442	+0.194	+0.207

+ indicates a positive correlation; – indicates a negative correlation. There are no significant correlations ($P < 0.05$).

transcripts of *IL-6* were increased up to 2020-fold after primary *E. tenella* infection. These results are consistent with our current data; meanwhile, we also observed that in the cecal tissue, the expression of *IL-6* was the highest in the infected group, higher than that of *IL-8*, and *CCLi2* showed the lowest expression. The same pattern was observed in the spleen.

The body's defense tends to function as a whole, and thus, the interaction between immune factors is very important in pathogen infection. Baron et al. (2015) suggested that synergistic expression of *IL-8* and *IL-6* contributes to disease recovery and resistance to pathogens. Kogut et al. (2003) confirmed that the mRNA expression of *IL-6* and *IL-8* was significantly upregulated in chickens after *Salmonella* infection, and *IL-6* expression was significantly positively correlated with *IL-8*. Kim et al. (2008) clearly state that coexpression of *IL-6* and *IL-8* was associated with enhanced resistance to *Eimeria maxima* infection in chickens. CCLi2 could promote the secretion of the anti-inflammatory cytokines IL-4 and IL-6 (Luther and Cyster, 2001). In this study, the expression levels of *IL-6*, *IL-8*, and *CCLi2* in the spleen or cecum were all positively related, either in the infection or the control group. Swaggerty et al. (2015) developed a novel selection method based on the identification and selection of chickens with an inherently high and low phenotype of proinflammatory mediators, such as *IL-6*, *IL-8*, and *CCLi-2*. The results showed that the positive synergistic effect of the 3 genes increased the resistance to pathology associated with coccidial infections compared with the low-line birds. Swaggerty et al. (2004) also found that the expression of *IL-6* and *IL-8* in chickens with resistance to *Salmonella* was higher than that in susceptible chickens and that most of the resistant chickens had a strong positive correlation between *IL-6* and *IL-8* gene expression. Similarly, in the spleen tissue of this study, the expression of *CCLi2* was strongly correlated with *IL-6* and *IL-8* in the uninfected group and also with *IL-8* in the infected group. Another study by Laurent et al. (2001) found that the mRNA expression of the proinflammatory cytokines *IL-8* and *CCLi2* was upregulated in the cecum of chickens starting at 3 to 4 D after infection with *E. tenella*. In our study, the expression levels of the 3 genes in the cecal tissues were moderately correlated in the uninfected group. The expression of *IL-6* was positively moderately correlated with *IL-8* and *CCLi2* in the infected group. In addition, the expression levels of the 3 genes were not correlated between the spleen and cecal tissues, either in the infection or the control group. We speculate that this may be because the regulatory mechanisms of the expression of the 3 genes are different in different organs. In conclusion, the expression levels of *IL-6*, *IL-8*, and *CCLi2* are all correlated with each other in the spleen and cecum. The findings of this study can enhance our understanding of the expression relationships of immune-related genes and provide a basis for genetic breeding strategies of coccidiosis resistance.

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