Cytokine gene expression in the livers of ducklings infected with duck hepatitis virus-1 JX strain

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ABSTRACT Duck hepatitis virus type 1 (DHV-1) causes a highly contagious disease in ducklings and is often associated with liver necrosis, hemorrhages, and high mortality. In the current study, the expression levels of gene transcripts encoding proinflammatory cytokines and the virus were measured by quantitative reverse-transcription PCR in duck livers after infection with a DHV-1 JX isolate obtained from natural cases in Hubei Province, China. In addition, sera IL-1β, IL-6, and alanine aminotransferase levels were quantified. Liver histopathology was examined following DHV-1 infection. The ducklings died within 1 to 2 d postinfection (d.p.i.) because of typical liver degeneration, hemorrhage, necrosis, and bile-duct epithelial cell proliferation. Transcripts of the cytokines *IFN-α*, *IL-6*, *TNF-α*, and *IL-10* decreased by 0.5 d.p.i. and then gradually increased at 1 d.p.i. Similarly, DHV-1 JX *3D* gene levels in the liver sharply increased at 1 d.p.i. and then maintained a high level. In contrast, liver *TNF-α* and *IL-1β* transcripts showed no increased expression of the cytokine gene postinfection and significantly decreased compared with the expression at 0.25 d.p.i., only the expression of *IFN-α* transcripts increased 128-fold by 1 d.p.i. Changes in the serum IL-6 level remained relatively stable postinfection and not significantly different compared with that of the control ($P > 0.05$), whereas serum levels of IL-1β significantly decreased at 0.5 d.p.i. and increased from 1 d.p.i. onwards ($P < 0.05$). Serum alanine aminotransferase levels significantly increased 2 d.p.i. compared with that of the control group ($P < 0.01$), which seemed to keep with the number of dead ducks. The cytokines exhibited a biphasic pattern following DHV-1 JX infection. Taken together, the data indicated that duckling liver inflammatory responses were produced following experimental DHV-1 JX infection involving multiple cytokines.

Key words: duck hepatitis virus type 1 JX, cytokine, alanine aminotransferase, quantitative RT-PCR, liver injury

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INTRODUCTION

Duck hepatitis is a highly fatal, rapidly spreading viral infection of young ducklings characterized primarily by hepatitis. It is mainly caused by duck hepatitis virus type 1 (**DHV-1**), which was observed in 1945 in Peking ducklings in Long Island, New York (Levine and Hofstad, 1945). Outbreaks were subsequently reported in England, Germany, and Canada, among other countries. In 1963, the disease emerged in China, although its pathogen was not identified until 1984 (Guo and Pan, 1984). The DHV-1 strain, first isolated from chick embryos (Levine and Fabricant, 1950), has been shown to contain RNA and was classified as a picornavirus (McNulty, 2001; Woolcock, 2003; Monroe et al., 2005). It is the most widely distributed virus and can cause mortality rates above 90% in 1-wk-old ducklings (Hu et al., 2000; Jin et al., 2008).

Duck hepatitis is characterized by hemorrhage, degeneration, and necrosis of the liver. In the last few years, it has been suggested that, in most liver diseases, hepatocellular injury is not the damaging agent itself but the activated inflammatory cells that have attacked the stressed hepatocytes (Ramadori and Armbrust, 2001; Jia and Sheng, 2006). Similar to mammals, cytokines have a pivotal role in steering immune responses in poultry. It has been reported that the amount of the transcripts for cytokines was examined in the early days of infection of *Salmonella* Typhimurium (Sijben et al., 2003; Degen et al., 2005), *Eimeria maxima* (Hong et al., 2006), and Marek's disease virus (Kaiser et al., 2003; Parvizi et al., 2009) in chickens. Through current developments in molecular biology, many immune-related genes of ducks have been cloned and expressed

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in vitro (Wu et al., 2007; Chen et al., 2008; Wu et al., 2008). Yang et al. (2008a) reported that high levels of persistent *IFN-α* mRNA expression in the liver could be induced using the attenuated duck plague virus. Three proinflammatory cytokines are TNF, IL-1, and IL-6. With IL-10 being mainly an anti-inflammatory cytokine that forms part of a complex defensive network that protects the host against inflammatory agents, microbial invasion, and injury (Laskin and Pendino, 1995). An inflammatory response is mediated by the action of TNF-α and IL-1β, and these cytokines can induce an acute-phase response or modulate the effects of IL-6 (Simpson et al., 1997). However, overproduction or aberrant regulation of these cytokines may harm the host by inducing tissue injury or alteration of the immune system (Laskin and Pendino, 1995).

The DHV-1 invades and develops within hepatocytes, Kuffers' cells, or epithelial cells in sinuses (Cheng et al., 2007), eliciting antibody and cell-mediated immune responses (Liu, 2009). Previous studies have demonstrated that several proinflammatory cytokines, such as TNF-α and IL-2, are produced in response to experimental DHV-1 infections (Hu et al., 2006). However, little is known about the cytokines produced by duckling livers, and no information is currently available on DHV-1 infections. In 2008, a strain of DHV-1 was isolated from a natural case in Hubei Province, China, and named DHV-1 JX (Jin et al., 2008). In this work, we aimed to determine the expression levels of liver gene transcripts encoding proinflammatory TNF-α and IL-6, anti-inflammatory IL-10, and antivirus IFN-α, as well as serum levels of IL-1β and IL-6 during the course of DHV-1 JX infection. The relationships between virus-induced injury and immune response in the host were also discussed.

MATERIALS AND METHODS

Virus

The DHV-1 JX (accession no. EF585200) was isolated from an infected duck. The DHV-1 JX isolates were propagated in vitro, as described previously by Jin et al. (2008). The virus containing allantoic fluid was harvested from embryonated duck eggs 36 to 48 h postinoculation and centrifuged at 2,000 × *g* for 10 min. The suspension was stored at −80°C until it was used, and the egg median lethal dose was set to 10^{-5}/0.2 mL by the Reed Muench method (Reed et al., 1938).

Experimental Birds and Design

In total, ninety-six 4-d-old ducklings were purchased from Dadi Breeding Duck Farm (Wuhan, China). The ducklings were determined to be free of DHV-1 using reverse-transcription (RT) PCR (Yang et al., 2008b; Anchun et al., 2009). The ducklings were divided into 2 groups. Sixty ducklings were intramuscularly inoculated with 0.2 mL of allantoic liquid containing DHV-1 JX isolates. The rest of the ducklings were kept in a separate room as uninfected controls. In a pilot experiment, inoculation with this dose of the virus resulted in the death of half of the infected birds by classic hepatitis 2 d postinfection (**d.p.i.**). Many reports have pointed out that infected ducks die within 1 to 3 d.p.i. (Hu et al., 2000; Cheng et al., 2007; Jin et al., 2008). Sampling was performed 0.25, 0.5, 1, 2, 3, and 5 d.p.i. At each time point, equal numbers of birds (n = 6) from each group were anesthetized with sodium pentobarbital and blood sera were obtained by jugular-vein puncture. Livers were collected from the ducks, some of which were stored at −80°C for RNA extraction while others were fixed with 10% formaldehyde in PBS for histopathological examinations. Clinical symptoms and results of the autopsy were recorded. All surviving ducklings were anesthetized until the experiment was completed.

ELISA for Cytokines IL-1β and IL-6

Levels of IL-1β and IL-6 in sera were measured using the ELISA kit (Shanghai Hengyuan Biotech Co. Ltd., Shanghai, China) according to the manufacturer's specifications. Serum samples containing high cytokine levels were repeatedly measured after dilution to ensure that the assay results remained within the standard curve.

Serum Enzyme Activity Determination

Serum alanine aminotransferase (**ALT**) was assessed by a commercial ALT assay kit (Nanjing Jiancheng Bioengineering Institute, Nangjing, China). To check the accuracy of results, reduplications for each sample were performed.

Histology

Liver samples were taken from all birds and fixed with 10% formaldehyde in PBS for 24 h. They were then washed with tap water, dehydrated in alcohol, and embedded in paraffin. Following paraffin removal, 5-μm liver sections were mounted in glass slides covered with saline and then subjected to hematoxylin and eosin staining for histological examination under a light microscope.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the livers using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The quality and quantity of RNA were estimated by ND-1000 spectrophotometry (NanoDrop Technologies Inc., Wilmington, DE). The RNA was reverse-transcribed using oligo(dT)18 primers (SuperScript First-Strand synthesis System, Invitrogen) following the manufacturer's instructions.

Table 1. Sequence of the oligonucleotide primers used in quantitative reverse-transcription PCR

RNA target	Primer sequence		Accession no.
	Forward (5′–3′)	Reverse (5′–3′)	
β-actin	TACGCCAACACGGTGCTG	GATTCATCATACTCCTGCTTG	EF667345.1
IFN-α	TCCACCTCCTCCAACACCTC	TGGGAAGCAGCGCTCGAG	AY879230.1
IL-6	CGTGTGCGAGAACAGCATG	GTCTCGGAGGATGAGGTG	AB191038
IL-10	GCTGTCACCGCTTCTTCACCT	GGCTCACTTCCTCCTCCTCATC	EF554720.1
TNF-α	CATTTGGAAGCAGCGTTTGG	GGTTGTGGGACAGGGTAGGG	NM-204267
DHV-1	ACAATGACCCAGCCTTAG	CCACTGTATCTTCCCTTC	EF093502

Quantitative RT-PCR

Quantitative (**q**) RT-PCR oligonucleotide primers for the duck cytokines, the β-actin control, and the DHV 3D gene (***3D***) are listed in Table 1. (Abdul-Careem et al., 2007; Li et al., 2007; Luo et al., 2008; Yang et al., 2008b). The primers were synthesized by Invitrogen-Shanghai China, Ltd. Serial dilutions of the standards were used in each real-time PCR assay. All of the real-time PCR reactions were carried out in 96-well plates at a final volume of 25 μL of SYBR green real-time PCR Master Mix Plus (Toyobo Co. Ltd., Osaka, Japan) using a real-time PCR detection system (Slan, Hongshi Medical Technology Co. Ltd., Shanghai, China). Each reaction system consisted of 0.25 μ*M* of each gene-specific primer and 2 to 5 μL of a 1:10 dilution of cDNA and PCR-grade water. Each analysis was performed in triplicate.

The optimum thermal cycling parameters varied according to the gene under study and consisted of segment 1, denaturation at 95°C for 2 min, 40 cycles of amplification at 95°C for 15 s, 58°C for 5 s (59°C for 5 s for *INF-α* and 60°C for 5 s for *IL-10*), 72°C for 15 s (72°C for 45 s for *3D* and *IL-10*), melting curve analysis at 95°C for 1 s (95°C for 5 s for *3D*); segment 2, 65°C for 15 s (65°C for 1 s for *3D*), and 95°C for 1 s, except for *3D* that needed segment 3, 97°C for 1 s; and cooling at 10°C for 30 s. Fluorescence acquisition was done at 75°C for 5 s for β-actin and *IL-10*, 80°C for 3 s for *IL-6* and *IFN-α*, 72°C for 5 s for *TNF-α*, and 72°C for 10 s for *3D*, depending on the melting temperature of the PCR product of the target or the reference genes.

Data Analysis

The efficiency of real-time PCR and relative quantification of cytokine and viral genes were calculated based on $2^{-\Delta\Delta Ct}$ methods described previously (Livak and Schmittgen, 2001). The mRNA expressions of cytokine and viral *3D* gene were quantified relative to β-actin, employed as the reference gene in the same sample preparation. The relative dynamical expressions of cytokines in DHV-infected ducklings were compared with that of infected ducklings 0.25 d.p.i. The relative dynamic expression of the virus in DHV-infected ducklings was compared with that of infected ducklings 0.5 d.p.i. Serum cytokine levels and ALT in the test ducklings were compared with those of the control group on the same day. Mean ± SD values for each group were calculated (n = 6), and SPSS software *t*-test was used to analyze the differences between 2 samples, where $P < 0.01$ or $P < 0.05$ were considered significant (Zhou et al., 2007).

RESULTS

Liver Injury Caused by DHV-1 JX Infection

Infected ducklings appeared depressed and showed little desire for food 0.5 d.p.i., and then they began to die 1 d.p.i. The death peak arrived at 30 h or so, and the mortality rate declined dramatically by 2 d.p.i. Seventeen ducklings infected with DHV-1 JX died within 1 to 2 d.p.i. Anatomical analysis showed marked hepatic fatty degeneration and point-like hemorrhage (Figure 1A). Histopathology showed circular lipid droplets in the normal liver cells of 6-d-old ducklings (Figure 1B). Early histological examinations of dead ducklings 1 d.p.i. revealed liver degeneration, extensive irregularities in cytoplasmic vacuoles, hemorrhage (Figure 1C), and mild bile-duct epithelium proliferation (Figure 1D). Liver injury gradually increased, and liver degeneration, necrosis, and hemorrhage were observed 2 d.p.i. (Figure 1E). Liver bile-duct epithelium hyperplasia was observed 3 d.p.i. (Figure 1F). Swollen liver cells were reduced and bile-duct epithelium proliferation was maintained 5 d.p.i. Serum ALT levels increased slightly 0.5 d.p.i. and were 5 to 7 times higher than those of the control samples after 2 d ($P < 0.01$). They then began to decrease 3 d.p.i. (Figure 2). Increased levels of ALT seemed to correlate with the severity of pathological liver damage.

Serum Cytokine Response During DHV-1 JX Infection

Serum *IL-1β* expression demonstrated a tendency to decrease at 0.25 d.p.i. and was minimal by 0.5 d.p.i. However, a rapid increase, significantly higher than that of the control samples, was observed at 1 d.p.i. ($P < 0.01$). Serum expression rapidly decreased 2 d.p.i. (Figure 3A). Figure 3B shows that changes in serum *IL-6* level were not observed in ducklings infected with DHV-1 JX within the first 5 d after infection. Only a

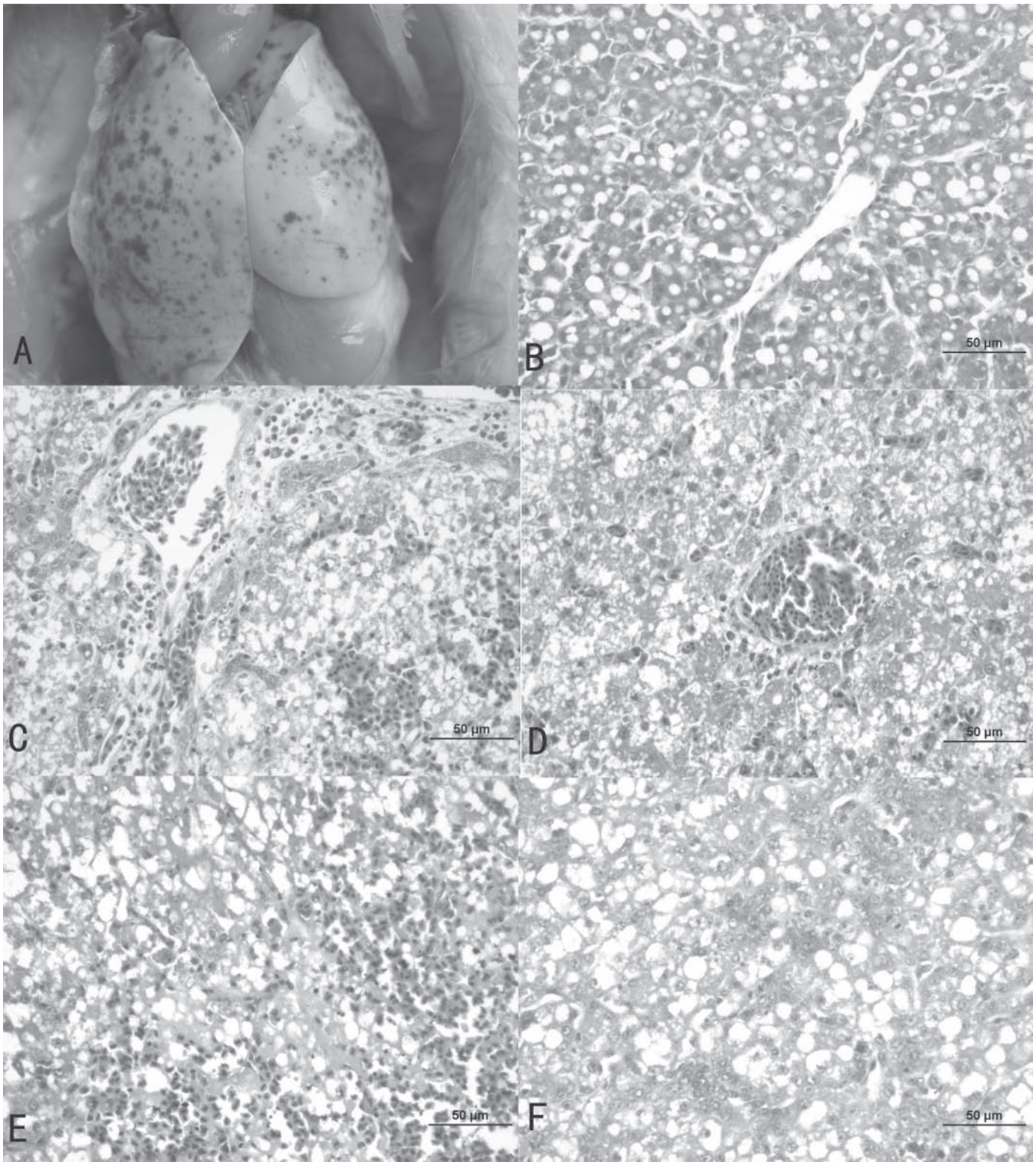

Figure 1. Liver injury in ducklings infected with duck hepatitis virus type-1 JX isolate. Gross lesions of the dead duckling included A) yellow liver swelling and hemorrhage. Histopathological changes included B) circular lipid droplets existing in the hepatocytes of 6-d-old normal ducklings; C) extensive vacuoles within the swollen hepatocytes, accumulation of large numbers of lymphocytes within the portal tracts, and diffuse accumulation of red cells in the liver 1 d postinoculation; D) bile duct proliferation around portal areas 2 d postinoculation; E) accumulation of large numbers of red cells 2 d postinoculation; and F) bile-duct epithelial cell proliferation in liver lobules 3 d postinoculation. Images shown with hematoxylin and eosin staining, bar = 50 μm.

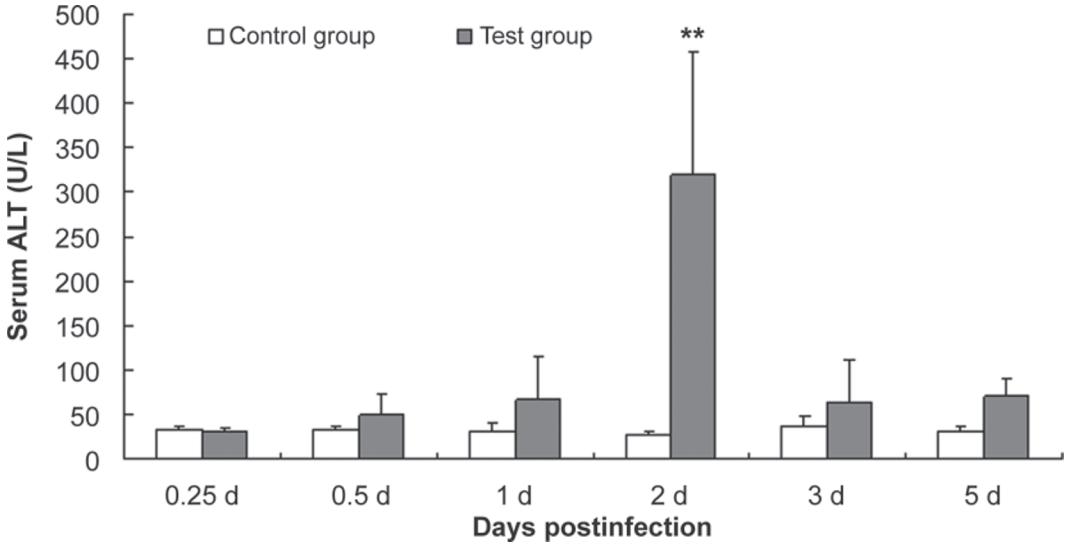

Figure 2. Serum alanine aminotransferase (ALT) changes at different time points from ducklings infected with duck hepatitis virus type-1 JX isolate. $^{**}P < 0.01$ compared with the control group.

slight increase was found at1 d.p.i. in comparison with the control group, but this was insignificant.

Expression of 3D Gene in Livers of DHV-1 JX-Infected Ducklings

Using real-time PCR for the detection of the DHV-1JX *3D* gene, we determined that all experimental ducklings had remained DHV-1 antibody- and DHV-1-free. Liver RNA from the infected ducklings was subsequently extracted and assessed for the expression of the DHV *3D* gene using real-time qRT-PCR (Figure 4). The DHV *3D* gene transcripts were detected 0.25 d.p.i. An approximate 32- to 256-fold increase in virus content was detected from 0.25 to 0.5 d.p.i. ($P < 0.05$).

Expression of IFN-α, IL-6, TNF-α, and IL-10 Cytokine Genes in the Liver

To evaluate the influence of DHV-1 JX in the activation of inflammation-related cells in the liver, cytokine transcript levels of genes encoding *IFN-α*, *IL-6*, *TNF-α*, and *IL-10* were quantified after infection, as shown in Figure 5. Transcript levels of cytokines *IFN-α*, *TNF-α*, and *IL-10* significantly decreased following inoculation compared with those of noninfected animals. The decrease ranged from 4- to 64-fold at 0.5 d.p.i., after which the levels gradually increased but at a rate lower than that of the control samples. Only *IFN-α* gene expression was significantly upregulated in the livers of DHV-1 JX-infected ducklings compared with that of the control group ($P < 0.01$), reaching a maximum at 1 d.p.i. In contrast, *IFN-α* transcript levels began to decrease by 2 d.p.i., and this downregulation was statistically significant ($P < 0.05$).

DISCUSSION

In this study, liver indices were investigated in ducklings infected with DHV-1 JX, a virulent strain of DHV-1. The results showed that serum ALT levels dramatically increased 1 to 2 d.p.i. This indicates time of death, which occurred in infected birds but not in control birds. The ducklings that died 1 to 2 d.p.i. showed classic hepatitis signs in agreement with those described in the literature (Levine and Fabricant, 1950; Hu et al., 2000).

We found virus replication in the livers of the ducklings similar to the results obtained by Cheng et al. (2007). The viral *3D* gene is a highly conserved domain of serotype I DHV that encodes RNA-dependent RNA polymerase (Kim et al., 2006; Ding and Zhang, 2007; Tseng et al., 2007). Our findings showed that the expression of the *3D* gene was significantly higher in duckling livers, especially on 1, 2, 3, and 5 d.p.i. compared with the expression 0.25 d.p.i. The expression of the *3D* gene in the livers may indicate viral replication (Anchun et al., 2009). Despite the high virus content in the liver at the specific time, the infected ducklings survived 2 d.p.i. Our data showed that liver injury was not only the damaging agent itself, but also its stress effect, similar to the results obtained by Jia and Sheng (2006) and Ramadori and Armbrust (2001).

The expression of cytokines has been demonstrated in peripheral blood or spleen mononuclear cells in ducklings in vitro (Wu et al., 2007; Chen et al., 2008; Wu et al., 2008). Our work is the first report on the cytokine repertoire in duckling livers in the context of DHV infection. In the present study, we examined several cytokines, namely IL-1β, IL-6, IFN-α, TNF-α, and IL-10. The IL-1β and IL-6, which are classified as proinflammatory cytokines, are involved in the activation of B and T cells and the development of macrophages

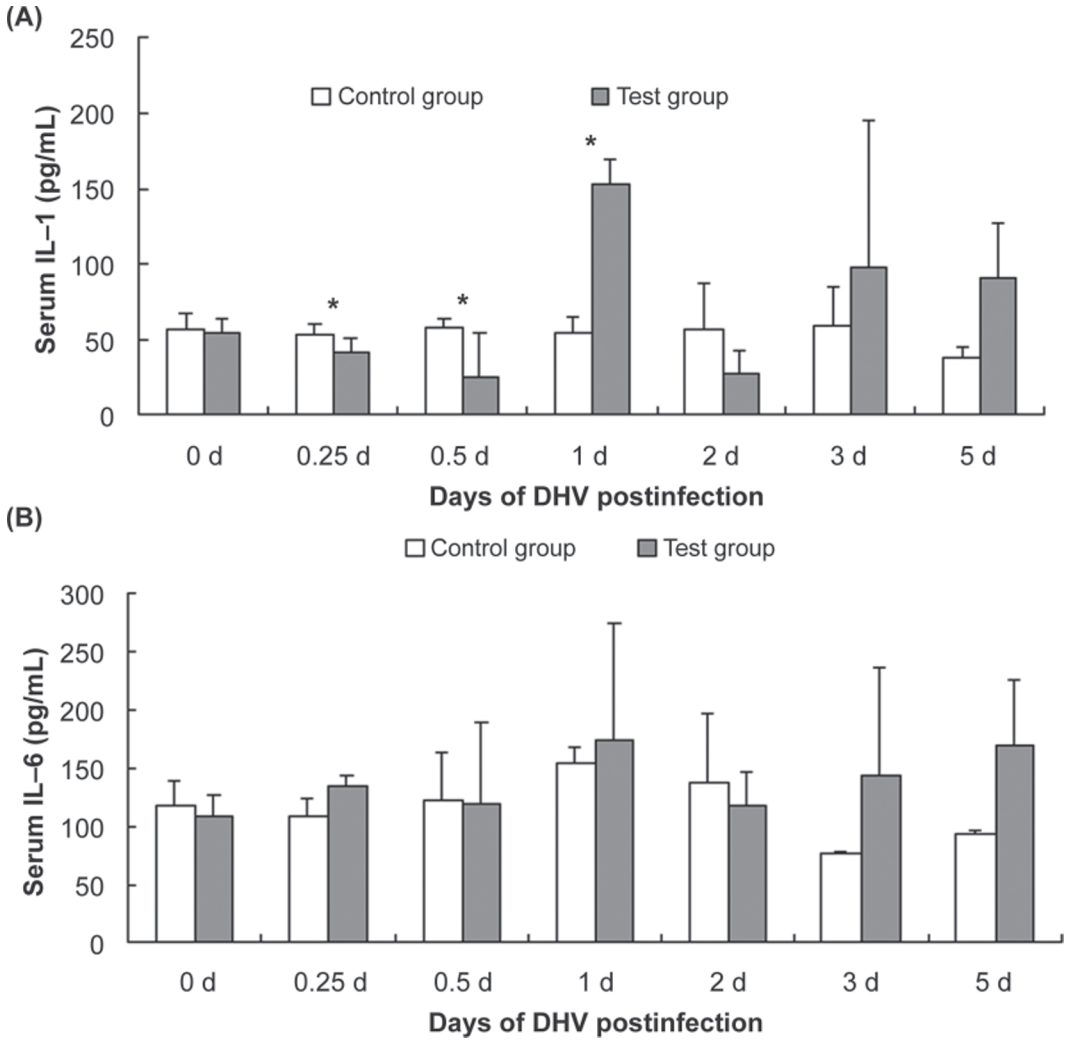

Figure 3. Serum cytokines were determined by ELISA at different time points from ducklings infected with duck hepatitis virus type-1 JX isolate (DHV). A) and B) show serum IL-1 and IL-6 concentrations, respectively. $^{*}P < 0.05$ compared with the control group.

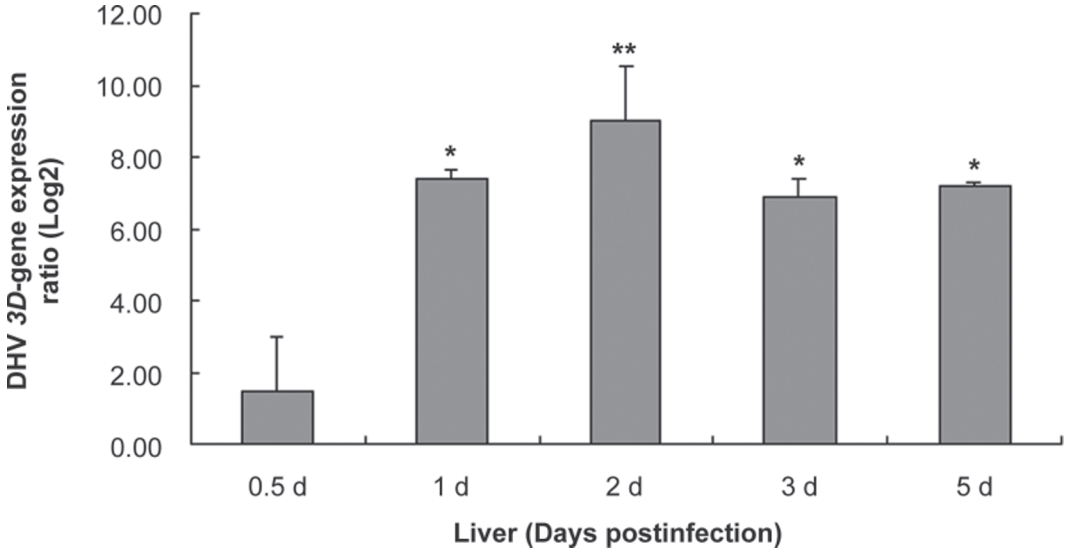

Figure 4. Relative quantification of liver RNA of genes encoding *3D* of duck hepatitis virus type-1 JX (DHV) isolate-infected ducklings as measured by quantitative reverse-transcription PCR. Expression of genes encoding *3D* was significantly increased compared with the levels found 0.5 d postinfection. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate genes with significant increases in RNA expression.

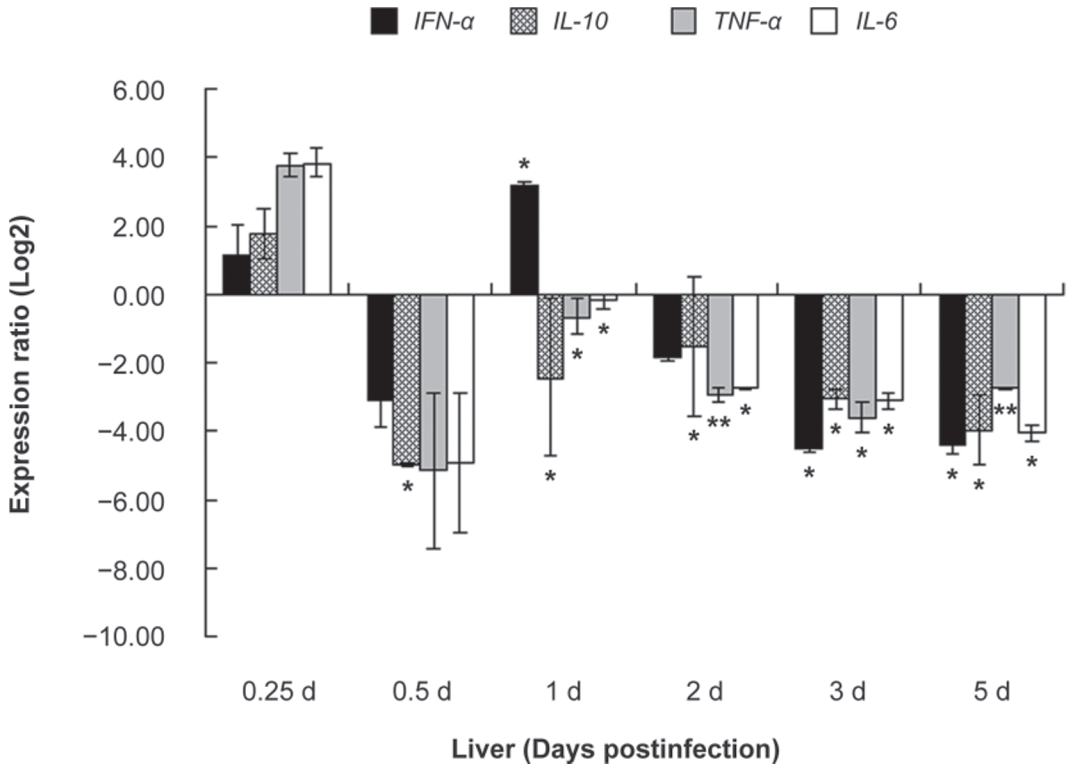

Figure 5. Relative quantification of liver RNA of genes encoding inflammatory mediators A) *TNF-α*, B) *IL-6*, C) *IL-10*, and D) *IFN-α* from duck hepatitis virus type-1 JX isolate-infected ducklings as measured by quantitative reverse-transcription PCR. Expression of genes encoding *TNF-α*, *IL-10*, *IL-6*, and *IFN-α* was significantly increased. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with those 0.25 d postinfection.

in chickens (Wigley and Kaiser, 2003; Giansanti et al., 2006). The IL-6 cytokine is produced in response to various infections in chickens (Zhou et al., 2007) and mammals (Jia and Sheng, 2006). The serum IL-6 levels of infected ducklings obviously increased, which could be both an indication of the induction of inflammation and an immune response against the virus. We noted that the serum IL-1β decreased by 0.5 d.p.i. and increased at 1 d.p.i. in the birds, which may take part in liver inflammation postinfection. The increase in *IL-1β* transcript levels was not observed in the livers but it might be very high in other immune or immune-related organs of infected ducklings.

In many species of animals, IFN is a key antiviral factor in vivo. Increased expression of *IFN-α*, rather than *IFN-γ*, has been observed in the livers of ducks infected with the duck plague virus (Yang et al., 2008a). An enhanced expression of *IFN-α* in the liver was noted during the first few days postinfection. The *IFN-α* expression has been demonstrated to increase during the first few days postinfection and several functions have been attributed to this cytokine, including the induction of nitric oxide in macrophages (Xing and Schat, 2000), leading to the inhibition of virus replication (Djeraba et al., 2002). In our study, a considerable increase in *IFN-α* expression was observed in the livers at 1 d.p.i. The increase in *IFN* expression was in agreement with previous studies on chicken diseases (Kaiser et al., 2003); therefore, *IFN-α* is speculated to play a crucial role in DHV-induced hepatitis.

Rothwell et al. (2004) reported chicken *IL-10* mRNA expression occurred mainly in the bursa of fabricius and cecal tonsils as well as in lipopolysaccharide-stimulated monocyte-derived macrophages. Although it was statistically significant, *IL-10* expression was low throughout the course of our study, especially after 0.5 d.p.i. The IL-10 cytokine has been shown to have immunomodulatory functions in mammals (Murphy and Reiner, 2002). In the study, *IL-10* downregulation in the livers could indicate acute liver inflammation and cause rapid death in ducklings because IL-10 is one of the main anti-inflammation factors (Lalani et al., 1997). It has been reported to participate in the persistence of the hepatitis-C virus infections in mammals (Jia and Sheng, 2006).

Also, TNF-α is involved in activating neutrophils and natural killer cells in mammals (Okamura et al., 1995). Our results showed that transcript levels of cytokine TNF-α significantly decreased 0.5 d.p.i. This was followed by a slight increase, but the resulting levels were still lower than those of the controls. Interestingly, proinflammatory cytokine levels of ducks infected with duck plague virus in vivo were found to be significantly increased (Yang et al., 2008a). We observed that the expression of *TNF-α* sharply declined 12 h after infection with DHV-1 JX. This was followed by an increase in its expression, but this increase was no higher than that of the control samples. This may be related to several factors. First, the immune systems of the ducklings were not fully developed, making them vulnerable to

virus attacks. According to the literature, the immune level of a 7-d-old chicken could be close to that of an adult chicken (Abdul-Careem et al., 2007). Transcription levels of cytokines gradually increased 1 d.p.i. in our study, but no significant differences were found between different time points. Second, Kaiser et al. (2003) measured increasing cytokine expressions in total chicken splenocyte populations, whereas we measured cytokine expressions in the duckling liver. Finally, the target cells of DHV are mainly liver cells, which contain a large amount of lipids. The DHV may stimulate liver cells to induce peroxidation damage, resulting in rapid death in ducklings 1 to 2 d.p.i (Sijben et al., 2003).

The results showed a large number of virus replications in the livers of ducklings infected with DHV-1 JX and liver *INF-α* expression. The infected ducklings died within 2 d.p.i. If the ducklings can be made resistant to the virus for 2 d, gradual maturation of their immune systems may yield better survival rates. Further work is necessary to shed more light on the interaction of cytokines and DHV in liver damage or immune-related liver cells in ducklings.

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REFERENCES

Abdul-Careem, M. F., B. D. Hunter, P. Parvizi, H. R. Haghighi, N. Thanthrige-Don, and S. Sharif. 2007. Cytokine gene expression patterns associated with immunization against Marek's disease in chickens. Vaccine 25:424–432.

Chen, H. Y., B. A. Cui, P. A. Xia, X. S. Li, G. Z. Hu, M. F. Yang, H. Y. Zhang, X. B. Wang, S. F. Cao, L. X. Zhang, X. T. Kang, and K. Tu. 2008. Cloning, in vitro expression and bioactivity of duck interleukin-18. Vet. Immunol. Immunopathol. 123:205–214.

Anchun, C., W. Mingshu, X. Hongyi, Z. Dekang, L. Xinran, C. Haijuen, J. Renyong, and Y. Miao. 2009. Development and application of a reverse-transcriptase polymerase chain reaction to detect Chinese isolates of duck hepatitis virus type 1. J. Microbiol. Methods 76:1–5.

Cheng, H. J., A. C. Cheng, M. S. Wang, and X. Y. Chen. 2007. Development of an indirect immunoperoxidase staining technique for the detection of duck hepatitis virus type 1. Vet. Sci. China 37:369–373.

Degen, W. G., N. Daal, L. Rothwell, P. Kaiser, and V. E. Schijns. 2005. Th1/Th2 polarization by viral and helminth infection in birds. Vet. Microbiol. 105:163–167.

Ding, C., and D. B. Zhang. 2007. Molecular analysis of duck hepatitis virus type 1. Virology 361:9–17.

Djeraba, A., E. Musset, N. Bernardet, Y. Le Vern, and P. Quere. 2002. Similar pattern of iNOS expression, NO production, and cytokine response in genetic and vaccination-acquired resistance to Marek's disease. Vet. Immunol. Immunopathol. 85:63–75.

Giansanti, F., M. F. Giardi, and D. Botti. 2006. Avian cytokines—An overview. Curr. Pharm. Des. 12:3083–3099.

Guo, Y. P., and W. S. Pan. 1984. Preliminary identifications of the duck hepatitis virus serotypes isolated in Beijing, China. Chin. J. Vet. Med. 10:2–3.

Hong, Y. H., H. S. Lillehoj, E. P. Lillehoj, and S. H. Lee. 2006. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet. Immunol. Immunopathol. 114:259–272.

Hu, X. Y., G. F. Cheng, S. Q. Zhou, and D. H. Xiong. 2000. Studies on histopathological changes of inoculated ducklings with DHV-1. J. Huazhong Agric. Univ. 19:48–50.

Hu, X. Y., Y. H. Zheng, D. H. Wang, C. Q. Gu, J. L. Su, R. P. She, and G. F. Cheng. 2006. Relationship between the dynamic changes of NO, TNF, IL-2, and tissue hurt of ducklings infected with new type hepatitis virus. Chin. J. Vet. Sci. 26:420–423.

Jia, H. Y., and J. F. Sheng. 2006. Study on the changes of serum cytokines in patients with chronic hepatitis C before and after IFN treatment. Modern Medical Health 22:1919–1921.

Jin, X., W. Zhang, W. P. Zhang, C. Q. Gu, G. F. Cheng, and X. Y. Hu. 2008. Identification and molecular analysis of the highly pathogenic duck hepatitis virus type I in Hubei province of China. Res. Vet. Sci. 85:595–598.

Kaiser, P., G. Underwood, and F. Davison. 2003. Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. J. Virol. 77:762–768.

Kim, M. C., Y. K. Kwon, S. J. Joh, A. M. Lindberg, J. H. Kwon, J. H. Kim, and S. J. Kim. 2006. Molecular analysis of duck hepatitis virus type 1 reveals a novel lineage close to the genus *Parechovirus* in the family Picornaviridae. J. Gen. Virol. 87:3307–3316.

Lalani, I., K. Bhol, and A. R. Ahmed. 1997. Interleukin-10: Biology, role in inflammation, and autoimmunity. Ann. Allergy Asthma Immunol. 79:469–483.

Laskin, D. L., and K. J. Pendino. 1995. Macrophages and inflammatory mediators in tissue injury. Annu. Rev. Pharmacol. Toxicol. 35:655–677.

Levine, P. P., and J. Fabricant. 1950. A hitherto-undescribed virus disease of ducks in North America. Cornell Vet. 40:71–86.

Levine, P. P., and M. S. Hofstad. 1945. Pages 55–56 in Duck disease investigation. Annual Report of the New York State Veterinary College, Ithaca, NY.

Liu, X. L. 2009. Dynamics of antibody against duck hepatitis virus-1 in breed ducks and ducklings. Pages 20–25 in MSc Thesis. Huazhong Agricultural University, Wuhan, China.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Luo, Y., G. Zhang, X. Xu, J. Chen, and M. Liao. 2008. Molecular characterization and SYBR green I-based quantitative PCR for duck hepatitis virus type 1. Agric. Sci. China 7:1140–1146.

McNulty, M. S. 2001. Picornaviridae. Pages 305–318 in Poultry Diseases. F. Jordan, M. Pattison, D. Alexander, and T. Faragher, ed. 5th ed. W.B. Saunders, London, UK.

Monroe, S. S., M. J. Carter, J. Herrmann, D. K. Mitchel, and A. Sanchez-Fauquier. 2005. Family Astroviridae. Pages 859–864 in Virus Taxonomy. 8th Report of the Int. Committee on Taxonomy of Viruses. Elsevier/Acad. Press, London, UK.

Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. Nat. Rev. Immunol. 2:933–944.

Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, and T. Tanimoto. 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 378:88–91.

Parvizi, P., L. R. Read, M. F. Abdul-Careem, A. J. Sarson, C. Lusty, M. Lambourne, N. Thanthrige-Don, S. C. Burgess, and S. Sharif. 2009. Cytokine gene expression in splenic $CD4^+$ and $CD8^+$ T-cell subsets of genetically resistant and susceptible chickens infected with Marek's disease virus. Vet. Immunol. Immunopathol. 132:209–217.

Ramadori, G., and T. Armbrust. 2001. Cytokines in the liver. Eur. J. Gastroenterol. Hepatol. 13:777–784.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hygiene 27:493–497.

Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173:2675–2682.

Sijben, J. W. C., K. C. Klasing, J. W. Schrama, H. K. Parmentier, J. J. van der Poel, H. F. J. Savelkoul, and P. Kaiser. 2003. Early in vivo cytokine genes expression in chickens after challenge with *Salmonella* Typhimurium lipopolysaccharide and modulation by dietary n-3 polyunsaturated fatty acids. Dev. Comp. Immunol. 27:611–619.

Simpson, K. J., N. W. Lukacs, L. Colleti, R. M. Strieter, and S. L. Kunkel. 1997. Cytokines and the liver. J. Hepatol. 27:1120–1132.

Tseng, C. H., N. J. Knowles, and H. J. Tsai. 2007. Molecular analysis of duck hepatitis virus type 1 indicates that it should be assigned to a new genus. Virus Res. 123:190–203.

Wigley, P., and P. Kaiser. 2003. Avian cytokines in health and disease. Braz. J. Poult. Sci. 5:1–14.

Woolcock, P. R. 2003.Duck hepatitis. Pages 343–354 in Disease of Poultry. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne ed. 11th ed. Iowa State Univ. Press, Ames.

Wu, Y. F., H. J. Liu, S. H. Chiou, and L. H. Lee. 2007. Sequence and phylogenetic analysis of interleukin (IL)-1b-encoding genes of five avian species and structural and functional homology among these IL-1b proteins. Vet. Immunol. Immunopathol. 116:37–46.

Wu, Y. F., J. H. Shien, H. H. Yin, S. H. Chiow, and L. H. Lee. 2008. Structural and functional homology among chicken, duck, goose, turkey, and pigeon interleukin-8 proteins. Vet. Immunol. Immunopathol. 125:205–215.

Xing, Z., and K. A. Schat. 2000. Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. Immunology 100:70–76.

Yang, F. L., X. L. Xie, C. Tang, B. Jin, and H. Yue. 2008a. Dynamics of *IFN-α* mRNA expression in liver of ducks infected with duck plague virus of different virulence. Chin. J. Prev. Vet. Med. 8:647–650.

Yang, M., A. Cheng, M. Wang, and H. Xing. 2008b. Development and application of a one-step real-time Taqman RT-PCR assay for detection of duck hepatitis virus type 1. J. Virol. Methods 153:55–60.

Zhou, Q., C. Q. Gu, X. Y. Hu, D. H. Wang, S. Q. Zhou, and G. F. Cheng. 2007. Role of interleukin-6 in the pathogenesis of an avian model of an avian model of *Staphylococcus aureus* arthritis. Poult. Sci. 86:1245–1250.