The pathogenicity of duck hepatitis A virus types 1 and 3 on ducklings

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ABSTRACT Duck hepatitis A virus (**DHAV**) is one of the pathogens that cause fatal duck viral hepatitis (**DVH**) in ducklings, which is an acute and contagious disease with a high mortality rate. Despite a continuing official duck vaccination program, DHAV infection remains a major threat to the duck industry. Considerable changes were observed in the epidemiology of DHAV-1/-3 in China over time. Therefore, comparing the pathogenicity of different DHAV serotypes can provide a theoretical basis for the diagnosis and prevention of DVH. In this study, we systematically investigated the effects of infection with DHAV-1/-3 field strains on clinical signs, gross lesions, histopathological changes, viral RNA detection, enzymatic systems, and metabolite concentrations. The results demonstrated that the major macroscopic and microscopic lesions in ducks infected with DHAV-1/-3 in the liver, brain, spleen, pancreas, and kidneys exhibited no significant differences. After 24 h of infection, DHAV quickly appeared in blood and major organs. Significant changes in clinical chemical markers together with histopathological lesions and viral RNA detection indicated that the liver is the major target organ for both viruses, resulting in impaired of liver integrity and function. In addition, we found that both viruses were able to invade both central and peripheral immune organs. Also lipase plasma activity was substantially affected by DHAV-1/-3, indicating that the integrity and function of the pancreas was compromised. However, there was no significant difference in pathogenicity between DHAV-1 and -3. The results of this study provide new insights into the pathogenesis of DHAV-1/3, two viruses that cause serious depression, metabolic disorders, and immunosuppression.

Key words: duck hepatitis A virus, pathogenicity, metabolic derangements

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INTRODUCTION

Duck virus hepatitis (**DVH**) is characterized by liver enlargement, necrosis, and hemorrhage and is a lethal, acute, and contagious disease with a high mortality rate of 1 to 21-day-old ducklings. The disease is caused by the duck hepatitis A virus (**DHAV**), which is the only member of the novel genus *Avihepatovirus* in the family *Picornaviridae* (Chen et al., 2013). Currently, with phylogenetic analyses and neutralization tests, DHAV has been classified into 3 serotypes: DHAV-1, DHAV-2, and DHAV-3 (Kim et al., 2006; Ding and Zhang, 2007; Tseng et al., 2007; Wang et al., 2008). In the literature, DHAV-1 is described as the classical serotype and exhibits the most significant impact on the local poultry industry (Wang et al., 2008; Swayne et al., 2013). In contrast, DHAV-2 has only been found in Taiwan (Tseng and Tsai, 2007), and DHAV-3 was first recorded in South Korea (Kim et al., 2007; Cha et al., 2013), and has recently occurred in mainland China (Liu et al., 2011) and Vietnam (Roan et al., 2015).

To date, the clinical and pathologic lesions are characteristic of DHAV-1 infections and DHAV-3 infections are increasingly recognized as being more common in China than previously though. So far, DHAV infection remains a major threat to the duck industry, despite a continuing official duck vaccination program (Wen et al. 2017; Liu et al., 2019). Therefore, comparing the pathogenicity of different DHAV serotypes can provide a theoretical basis for the diagnosis and prevention of DVH. In this study, we systematically investigated the effects of infection with DHAV-1/-3 field strains on clinical signs, gross lesions, histopathological changes, viral RNA detection, enzymatic systems, and metabolite concentrations.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Medical Ethics Committee of Affiliated Hospital of Qingdao University. All ducklings were carried out in accordance with the

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approved guidelines. The ducklings were euthanized after anesthesia with intraperitoneal injection of sodium pentobarbital (150 mg/kg) used in animal experiment.

Virus Isolation and Propagation

The DHAV-1 and DHAV-3 isolates used in the present study were isolated from naturally infected ducks who were characterized by liver hemorrhage and neurological symptoms in Shandong, China in 2016. The virus was purified and propagated three times in the allantoic cavities of 10-day-old specific-pathogen-free (**SPF**) embryonated duck egg. Polymerase chain reaction (**PCR**) and reverse transcription-PCR (**RT-PCR**) were performed to distinguish the genotypes of DHAV and confirm the absence of contamination other viruses. The DHAV-1 and DHAV-3 titers were determined to be $10^{7.25}$ and $10^{8.75}$ duck embryos lethal median dose (ELD_{50})/0.2 mL, respectively. The viruses were stored at −80°C for further use.

Animal Experiments

The SPF duck embryos purchased by the Harbin Veterinary Research Institute (Harbin, China) were incubated at 37°C for 28 D and then moved to an isolator. At 5 D old, 79 SPF ducklings were randomly divided into 3 groups (control (n = 19), DHAV-1 (n = 30), and DHAV-3 (n = 30)). The ducklings of groups DHAV-1 and DHAV-3 were infected intranasally with 0.2 mL of embryo culture supernatant containing 10^5 ELD_{50} of DHAV. The control group was inoculated in the same manner with 0.2 mL of sterile phosphate buffered saline (pH 7.4). A total of 10 birds from the 3 groups were monitored twice daily and scored for clinical signs for 6 D. Clinical signs were given daily clinical scores: 0 (normal), 1 (mild depression), 2 (severe depression), 3 (paralysis/prostration), and 4 (death). Dead ducklings in the DHAV-1 and DHAV-3 groups were not considered. At 1, 2, 3, 4, 5, and 6 D post infection (**dpi**), 3 serum samples from groups DHAV-1 and DHAV-3 were used to determine the viremia by RT-PCR. At 1, 3, and 5 dpi, 3 ducklings from each group with DHAV or mock infection were euthanized and necropsied. The livers, brains, spleen, kidneys, and pancreas were collected and divided into 2 parts: one for histopathological examination and the other for viral titration. The other organs (heart, lung, and bursa of Fabricius) were collected only for viral titration.

Quantification of Viral Titration Using Duck Embryos

The 0.5 g tissues (livers, brain, heart, lung, spleen, kidney, pancreas, and bursa of Fabricius) from ducks were homogenized at 1:10 dilution in PBS and centrifuged (12,000 × g for 15 min). For quantification of viral titration, ELD_{50} was determined using the method of Reed and Muench (1938). Ten 10-fold serial dilutions of supernatants were performed ranging from 10^{-1} to 10^{-10}, and then the supernatants were inoculated into 10-day-old SPF duck embryos via allantois sac route. Eggs inoculated with PBS were used as negative controls. The inoculated eggs were incubated at 37°C for 144 h and dead eggs were discarded within 24 h of incubation.

Detection of DHAV in Serum by RT-PCR

Viral RNA of the serums were extracted for the detection of DHAV using the EasyPure viral DNA/RNA Kit (TransGen Biotech Co., Ltd., Beijing, China), in accordance with the manufacturer's protocol. The primers for DHAV were designed using Primer 6.0 software (http://bioinfo.ut.ee/primer3–0.4.0/) (Forword: 5′- CAGTTTACCGCCCCACTCTAT-3′, Reserved: 5′-TGGCTTCCACCTCCTCTTCAT-3′). RT-PCR amplifications were conducted with the EasyScript One-step RT-PCR SuperMix (TransGen Biotech), and the following cycle conditions were used for all three reactions: 45°C for 30 min, followed by 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s. A final extension step was conducted at 72°C for 10 min. The PCR products were separated on a 1.2% agarose gel.

Biochemical Assays

At 1, 3, and 5 dpi, prior to euthanasia, blood was collected from the jugular vein of the ducklings. Then, the serum was separated, and the values of the following clinical chemistry analytes were investigated by a fully selective clinical chemistry analyzer (Roche Diagnostics, Shanghai, China), and included: total protein (**TP**), albumin (**ALB**), alanine aminotransferase (**ALT**), aspartate aminotransferase (**AST**), lipase, and bile acids. All assays were performed according to the manufacturer's instructions.

Statistical Analysis

All data are expressed as the mean ± standard deviation. Statistical comparisons were analyzed by ANOVA using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). The GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for chart and $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Symptoms, Gross Lesions, and Details of the Deaths of Ducklings With DHAV-1 or DHAV-3 Infection

Both DHAV serotypes caused very similar clinical symptoms, such as depression, lethargy, and anorexia

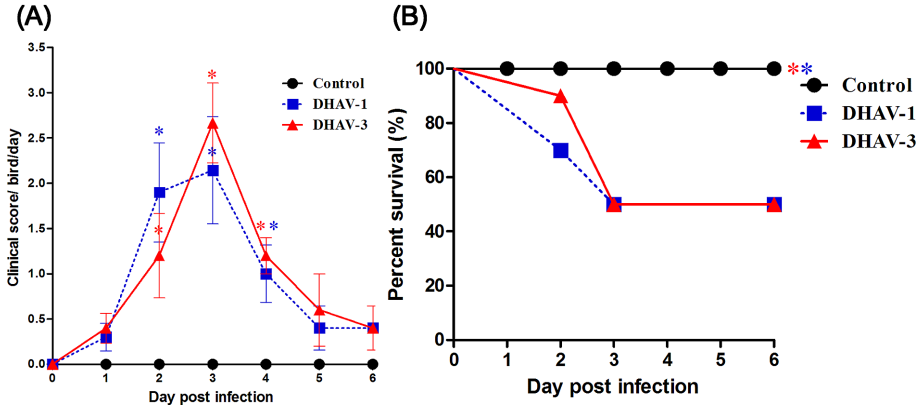

Figure 1. The clinical indices of ducklings after infection with DHAV-1 and DHAV-3. **(A)** Clinical scoring: 0, for normal; 1, mild depression; 2, severe depression; 3, paralysis/prostration; and 4, death. The mean scores per group per day are shown. **(B)** The percentage of ducklings that survived in the infected groups was significantly lower than in the control group. The error bars indicate standard deviations. Asterisks indicate statistical significant difference ($P < 0.05$).

at 1 dpi. High clinical scores were reached at 3 dpi; however, the symptoms improved at 4 to 6 dpi. At 2 to 4 dpi, the DHAV-1 and DHAV-3 groups exhibited significant clinical symptoms ($P < 0.05$, Figure 1A). Mortality rates in the DHAV-1 and DHAV-3 groups were 50%. The ducklings began to die at 2 dpi and peaked at 3 dpi, and no ducklings died after 4 dpi (Figure 1B). Most of the dead ducks showed an angular arch posture (Figure 2A). Anatomical analysis revealed that infected ducklings had similar and typical visible lesions in the liver, brain, spleen, and pancreas throughout in the DHAV-3 and DHAV-1 groups. The most livers were typically enlarged with a color ranging from yellow to brown, and petechial and ecchymosis hemorrhages were visible (Figure 2B). Some ducklings exhibited severe meningeal hyperemia and hemorrhage (Figure 2C). The spleen showed serious splenectasis and was reddish brown with congestion and gray-white with necrosis (Figure 2D). The pancreas of individual ducks was covered with gray-white focal necrosis (Figure 2E).

Histopathological Analysis

Significant pathological changes were detected in the liver of dead ducklings from the DHAV-1 and DHAV-3 groups. There was an increase in cytoplasmic vacuoles and necrosis in hepatocytes accompanied by severe hemorrhage (Figure 3B). Some histological section of the liver revealed proliferation of basophilic bile ductular cells around the portal areas (Figure 3C). Severe infiltration of lymphocytes under the meninges (Figure 3E), necrosis of brain nerve cells, and local microglial proliferation (Figure 3F) could be observed in the DHAV-1 and DHAV-3 groups. The DHAV-1 and DHAV-3 groups also exhibited severe necrosis of lymphocytes, hyperemia, and structural disorders (Figure 3H-I) as well as significant necrotizing pancreatitis with a large area of necrosis and degeneration of pancreatic epithelial cells in the pancreas (Figure 3K–L). Changes in the kidneys were variable. Some ducklings showed severe hyperemia, and massive degeneration of renal epithelial cells (Figure 3N), while in other ducklings, the tubular epithelium was intact but separated from the basement membrane by edema fluid (Figure 3O). No histological microscopic lesions were seen in the control group (Figure 3A, D, G, J, M).

Viral Titration in Different Tissues of the Ducklings

Live virus titration demonstrated that both DHAV serotypes could be detected in various organs at 1 dpi. However, there were no apparent differences between DHAV-1 and DHAV-3 or between different organs (Figure 4A). At 3 dpi, DHAV was detected in all of the organs and the DHAV titers were significantly higher in the liver than in other organs ($P < 0.01$); however, there were no significantly difference between DHAV-1 and DHAV-3 within the same organ (Figure 4B). None of the tissues from ducklings in the control group contained any live viruses.

Detection of Viremia

Viral RNA in serum was detected by RT-PCR and the results are summarized in Table 1. In the control group, no viral RNA was detected. At 1 dpi, viremia was detected in one (1/3) of each DHAV group. At 2 to 3 dpi, 2 (2/3) serum samples were positive for viral RNA in the DHAV-1 group. At 4 dpi, all (3/3) of the serum samples possessed viral RNA. However, afterward, the detection rate decreased; at 5 dpi, only 1 sample (1/3) was positive and at 6 dpi, no viral RNA was detected. For the DHAV-3 group, at 2 to 4 dpi, all serum samples (3/3) were positive and at 5 to 6 dpi, all serum samples were negative for viral RNA.

Clinical Chemistry

The clinical chemistry results are presented in Figure 5. Total plasma protein values were significantly decreased in groups DHAV-1 and DHAV-3 at 1 to 3

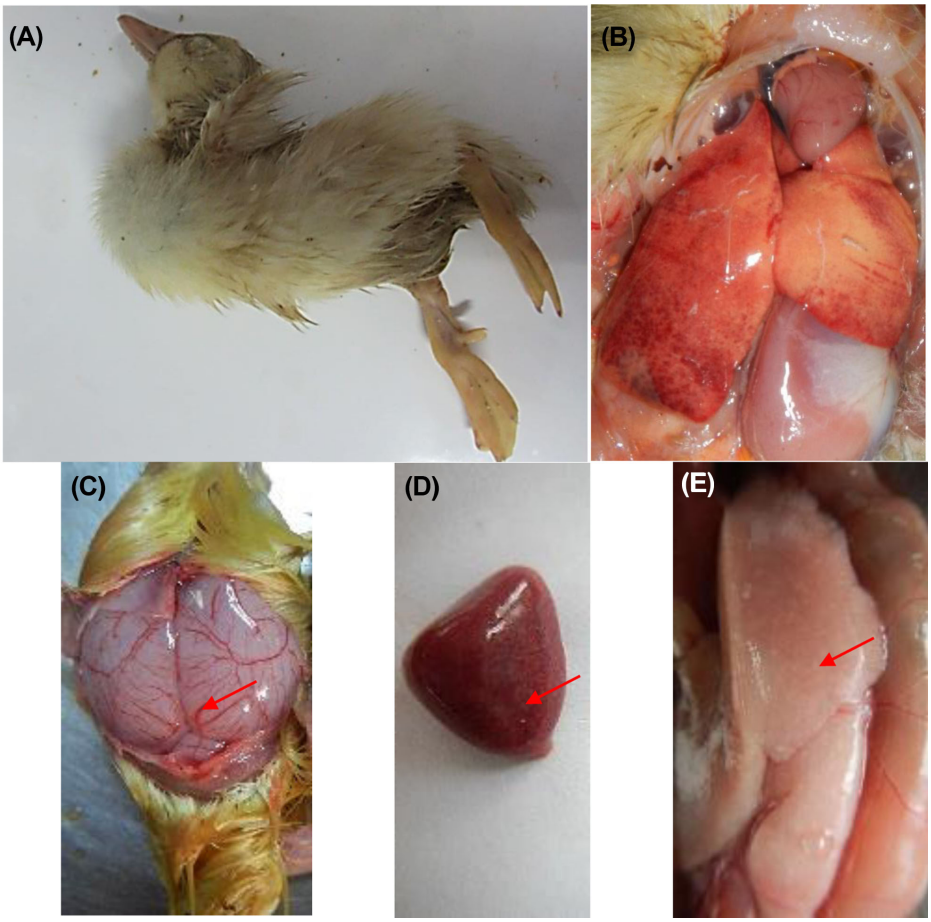

Figure 2. Gross lesions of ducklings infected with DHAV-1 and DHAV-3 at 48 h post-infection. **(A)** The dead ducklings neck back and opisthotonos. **(B)** The liver of dead ducklings is typically enlarged with petechial and ecchymosis hemorrhages throughout. **(C)** Severe meningeal hyperemia and hemorrhage was in some dead ducklings (red arrow). **(D)** The spleen presented reddish brown with congestion and gray-white with necrosis (red arrow). **(E)** The pancreas were covered with gray-white focal necrosis (red arrow).

dpi compared to the control group (Figure 5A). Albumin values decreased in groups DHAV-1 and DHAV-3 at 1 dpi, however, these values did not exhibit statistically significant differences compared to the control group. At 3 to 5 dpi, these values were significantly lower in groups DHAV-1 and DHAV-3 than the values from control ducklings (Figure 5B). The contents of the two liver enzymes, ALT and AST, in the serum were significantly higher in the DHAV-1 and DHAV-3 groups at 3 to 5 dpi and 1 to 3 dpi, respectively, compared with those of the control group (Figure 5C–D). Overall, the infected groups exhibited a tendency towards higher enzyme activities. Significantly high bile acids and uric acid concentration was recorded in the DHAV-1 and DHAV-3 groups at 1 to 3 dpi in comparison to control ducklings (Figure 5E–F). Interestingly, the 6 chemical indicators exhibited no significant differences between the DHAV-1 and DHAV-3 groups.

DISCUSSION

DHAV infection is still common in duck industry in China (Chen et al., 2013; Chen et al., 2014; Gan et al., 2014). It has been reported that the outbreaks of DHAV-1-associated DVH occurred in China from 2010 to 2012, after which DHAV-3-associated DVH predominated from 2013 to 2015 (Wen et al., 2017). Furthermore, mixed infections caused by DHAV-1/-3 have become common in domestic ducks (Lin et al., 2016; Zhong et al., 2016; Liu et al., 2019). Therefore, the aim of the present study was to characterize and compare clinical signs, gross lesions, histopathological lesions, tissue viral load, enzyme systems, and metabolite concentrations of SPF ducklings that were infected with the DHAV-1 and DHAV-3 field strains to experimentally induce DVH. We found that the major macroscopic and microscopic lesions in the liver, brain, spleen, pancreas, and kidneys of ducks infected with DHAV-1/-3 exhibited no significant differences. These results are consistent with previous reports (Zhang et al., 2012).

In order to further compare the pathogenicity of DHAV-1 and DHAV-3, we also analyzed the distribution and changes of the virus *in vivo*. In clinically, transmission via the respiratory tract is one of the main routes for the spread of DHAV, therefore, we simulated this transmission mode to infect ducklings. Interestingly, both DHAV serotypes spread and replicated

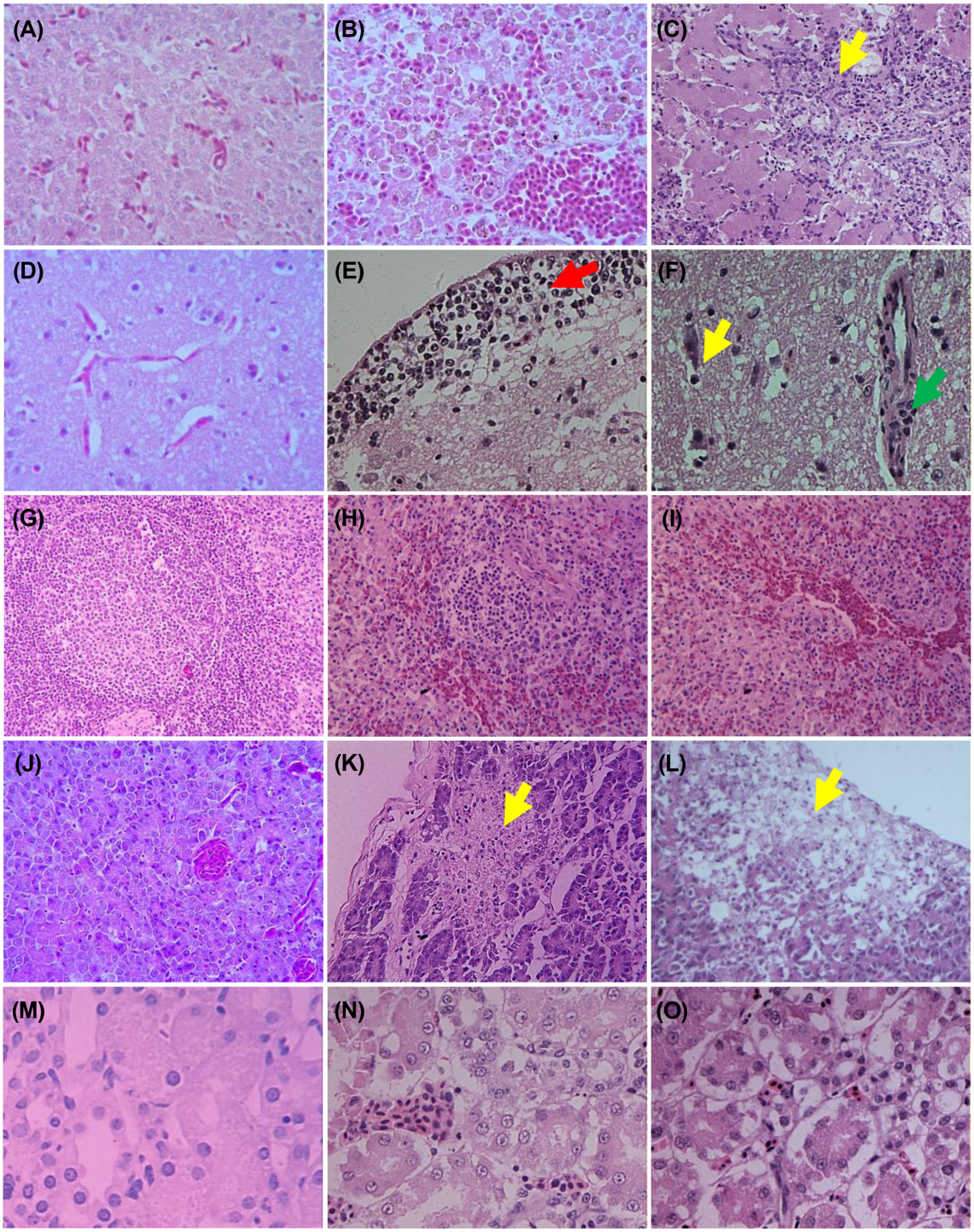

Figure 3. Pathological changes of the DHAV-1and DHAV-3 infected ducklings at 48 h post-infection. **A**, **D**, **G**, **J**, **M** represented the liver (hematoxylin and eosin (H&E) staining, 200 ×), brain (H&E, 200 ×), spleen (H&E, 100 ×), pancreas (H&E, 200 ×), and kidney (H&E, 400 ×) of ducklings from the control group, respectively. The liver showed diffuse degeneration, hemorrhage, **(B)** and necrosis of hepatocytes with proliferation of basophilic bile ductular cells around the portal areas (yellow arrow) **(C)**. Severe infiltration of lymphocytes in under the meninges (red arrow) **(E)**, necrosis of brain nerve cells (yellow arrow) and local microglia proliferation (green arrow) **(F)** were observed. Necrosis of lymphocytes, hyperemia, and structural disorder were observed in spleen **(H-I)**. Diffuse degeneration and necrosis of pancreatic epithelial cells were seen in the pancreas **(K-L)**. The hyperemia and tubules degeneration were observed in kidneys **(N-O)**.

rapidly in vivo. After 24 h of infection, DHAV quickly appeared in blood and major organs (Table 1 and Figure 4A). After 3 dpi, the viral titers of DHAV-1 and DHAV-3 in liver were significantly higher than in other organs (Figure 4B), which indicates that the liver of ducklings is the major target organ for both viruses. In addition, the viral titers of DHAV-1 and DHAV-3 in spleen and bursa of Fabricius were higher than $10^{3.7}$ ELD_{50}/mL, which indicates that both viruses can invade both central and peripheral immune organs, which

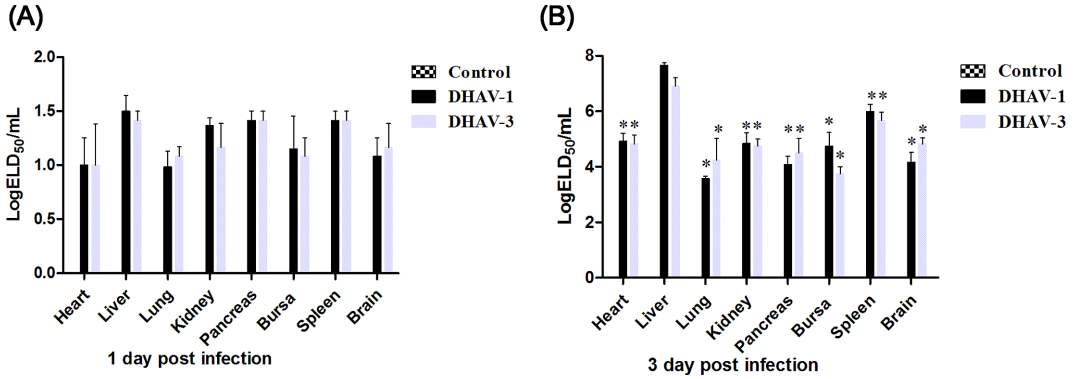

Figure 4. Viral titration in different tissues of the ducklings at 1 **(A)** and 3 **(B)** day post infection. Note: Data are expressed as the mean ± SD. Asterisks indicate statistically significant differences compared with the each group's liver ($P < 0.05$).

Table 1. Dynamic changes of viremia.

	Day post infection					
Group	1	2	3	4	5	6
Control	0/3	0/3	0/3	0/3	0/3	0/3
DHAV-1	1/3	2/3	2/3	3/3	1/3	0/3
DHAV-3	1/3	3/3	3/3	3/3	0/3	0/3

Data are no. of positive samples/no. of tested samples.

consequently caused the immunity dysfunction in infected ducklings. These results are consistent with previous reports (Zhang et al., 2018), and demonstrate that even if ducklings recover from DHAV infection, they are still vulnerable to other pathogens afterwards, due to an impaired immune system.

Our study went on to further elucidate the pathogenesis of DHAV-1/-3 and its influence on metabolism. Liver and pancreas are important target organs for DHAV (Liu et al., 2019), therefore, we selected 6 reliable indicators for liver and pancreas function. To our knowledge, TP and ALB (protein produced by the liver) are associated with the synthesis functions of the liver. Once hepatic cells are destroyed and the synthesis of liver is impaired, serum levels of these will decrease rapidly (Campbell, 2012). ALT is a cytoplasmic enzyme of the liver that is released into the blood stream during early cell degeneration (Lumeij, 2008). High bile acid concentration had a higher association with confirmed hepatic disease in birds when compared to AST, which is routinely used to assess hepatocellular injury (Matos et al., 2016). In the present study, we observed decreases in TB and ALB concentrations and an increase in the plasma enzyme activity, indicating a direct correlation with the presence of hepatic injury in the infected groups.

From field cases and experimental studies (Zhang et al., 2018; Liu et al., 2019), necrosis of pancreatic acinar cells has been observed before, suggesting an impact on the exocrine function of the pancreas. Lipase is a digestive pancreatic enzyme that leaks from injured

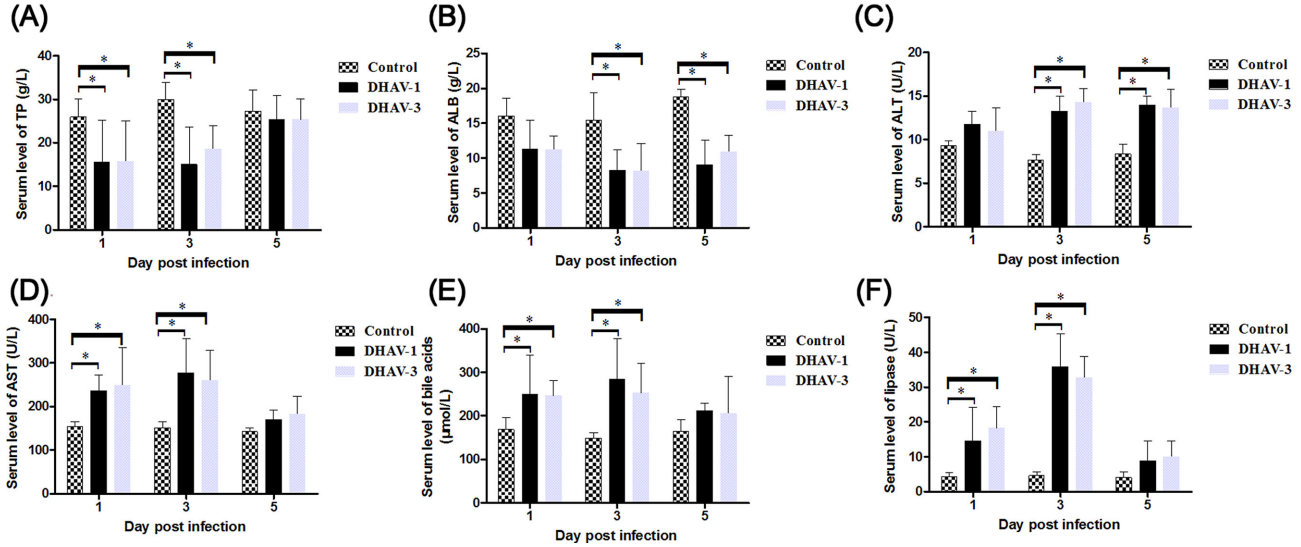

Figure 5. Graphical illustration of the means and standard deviations of the clinical chemistry analytes at 1, 3, and 5 day post infection. **(A)** total protein, **(B)** albumin, **(C)** alanine aminotransferase, **(D)** aspartate aminotransferase, **(E)** bile acids, and **(F)** lipase contents. Note: Data are expressed as the mean ± SD. Asterisks indicate statistically significant differences compared with the control group ($P < 0.05$).

pancreatic cells into the blood (Campbell, 2012). In our study, we monitored plasma lipase activity as an indicator of pancreatic involvement during the course of the disease. The plasma activity of lipase differed substantially from the control group at 1 and 3 dpi in the DHAV-1 and DHAV-3 groups, indicating that the integrity and function of the pancreas was compromised. In addition, the occurrence of pancreatitis can also affect liver function (Restrepo et al., 2016). Therefore, the digestive and metabolic functions of ducklings were severely impaired, which partly explains the short course and rapid death of DVH.

CONCLUSION

In this study, we compared the pathogenicity and assessment indicators of two different DHAV serotypes and demonstrated that there are no significant differences in pathogenicity between DHAV-3 and -1. Thus, a continued surveillance of DHAV is required to understand the epidemiology of the viruses associated with DVH. However, the limitation of our study was that we did not provide scientific evidence for the molecular epidemiology of DHAV in China. Nevertheless, this study systematically compared the pathogenicity of both DHAV-1 and DHAV-3 and the involvement of the liver and pancreas in metabolic disorders.

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