



RESEARCH ARTICLE

Comparative Pathogenicity of Duck Hepatitis A Virus Type 1 and 3 Infections in South Korea

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ABSTRACT

Despite the impact of duck hepatitis virus type 1 (DHAV) as economically important and devastating pathogen in duck industry, there is a scarcity of data on the pathogenesis of DHAV. In this study, 1-day old ducklings were orally inoculated with DHAV-3 isolate (DHAV-3/duck-wt/KOR/Y02-1/2012) and DRL-62 strain as control virus in addition to mock-inoculated group. These strains induced the same clinical signs and gross lesions in virus-inoculated ducklings. The clinical signs appeared at HPI 24 and the mortalities were recorded by HPI 36. Histopathologically, both strains induced the same characteristic liver lesions: hepatocyte necrosis, lymphoid cell infiltration in the portal triad and bile duct hyperplasia. Different microscopic lesions were also observed in extra-hepatic organs and tissues. By RT-PCR and nested PCR, viral RNA was detected in liver, spleen, brain, kidney, lung, heart, pancreas, bursa of Fabricius and thymus. An immunofluorescence assay confirmed virus antigen localization in these organs and tissues. Serum biochemical analysis revealed a significant hypoalbuminemia which reached the lowest level by HPI 24. These results indicated that DHAV-1 or 3 have a strong liver tropism and a pantropic effect with wide damage to various organs and tissues. The lesions of spleen, bursa of Fabricius and thymus indicated that the immune function might be affected by the DHAV infection.

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INTRODUCTION

Duck viral hepatitis (DVH) is an acute, contagious, rapidly spreading viral infection of young ducklings that is characterized primarily by ecchymotic hemorrhage and liver necrosis (Chen *et al.*, 2013; Xue *et al.*, 2019). DVH is mainly caused by duck hepatitis A virus (DHAV), which is the only member of a novel genus Avihepatovirus in the family Picornaviridae (Li *et al.*, 2013, Wang *et al.*, 2018; Xue *et al.*, 2019). It is caused by three types of RNA viruses, namely, duck hepatitis virus (DHV) types 1, DHV-2 and DHV-3 (Woolcock and Fabricant, 1991; McNulty, 2001; Ding and Zhang, 2007). There is no cross-neutralization between DHAV-1 and DHAV-2 (Tseng and Tsai, 2007; Zhang *et al.*, 2014) and limited cross-neutralization between DHAV-1 and DHAV-3 (Kim *et al.*, 2006, 2007a).

DHAV usually affects ducklings under 6 weeks of age. It causes 100% morbidity and although mortality

varies. Clinical manifestations in young ducklings include lethargy, squat down with their eyes partially closed, fall on their sides, kick spasmodically, and die with their heads drawn back (opisthotonos). Gross pathological changes appear chiefly in the liver, which is enlarged and displays multiple distinct petechial to ecchymotic haemorrhages, hepatic necrosis and apoptosis (Woolcock, 2003; Sheng *et al.*, 2014)). However, the clinical signs, gross lesions and histopathological lesions of the duck hepatitis caused by DHAV-1 and DHAV-3 could not be differentiated, making the molecular methods necessary to detect and differentiate between DHAV infections (Kim *et al.*, 2007b, 2008; Yang *et al.*, 2008; Anchun *et al.*, 2009; Huang *et al.*, 2012).

Since the DHAV-1 was reported in 1985, South Korea, it was believed to be the only serotype present because of vaccination with attenuated DHAV-1 strain did provide efficient protection until 2000 (Kim *et al.*, 2008). However, the incidence of duck hepatitis has recently increased both in vaccinated and non-vaccinated flock, and a DHAV-3 was isolated from ducklings showing

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typical clinical signs of DHAV (Kim *et al.*, 2008). Subsequently, Korean DHAV-3 strains are known to be completely different from the DHAV-1 based on the molecular identification and serological tests (Kim *et al.*, 2007a). Although DHAV-3 has represented a serious threat and has had a significant economic impact on the duck industry in South Korea (Kim *et al.*, 2008), the pathogenicity of DHAV-3 has to date remained largely unclear. Hence, this prompted us to investigate the pathogenicity of DHAV-3 which can provide a valuable basis for prevention and control of DHAV infection. In this study, the hepatic and extra-hepatic pathogenicity of DHAV-3 strain was evaluated in comparison with that of DHAV-1 strain. For this experiment, major organs and tissues were sequentially sampled from experimental animals to analyze the morphological changes, antigen distribution using an immunofluorescence assay, and viral RNA presence by RT-PCR and nested PCR. To obtain a better understanding of the potential consequences of liver function failure, sequential serum albumin levels were compared between DHAV-3 and DHAV-1 infected ducklings.

MATERIALS AND METHODS

Virus inoculums: The DHAV-3 strain (DHAV-3/duck-wt/KOR/Y02-1/2012) was originally isolated and identified from liver sample collected from dead ducklings with duck hepatitis in South Korea during 2012 (GenBank accession number: KM216052) (Soliman *et al.*, 2015) and DHAV-1 strain DRL-62 was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The virus stock with titer of 2×10^4 ELD₅₀/ml for DHAV-3/duck-wt/KOR/Y02-1/2012 strain and 2×10^3 ELD₅₀/ml for DRL-62 strain were used as the inocula in this study.

Animal and experiment design: A total of 120 one-day old commercial Peking ducklings were used to evaluate the pathogenicity of DHAV-3 strain in comparison with that of DHAV-1 strain. The ducklings were divided into three groups: first group (45 ducklings) were orally inoculated with 1 ml of the infectious allantoic fluid with virus titer 2×10^4 ELD₅₀/ml of the DHAV-3/duck-wt/KOR/Y02-1/2012 isolate; second group (45 ducklings) were orally inoculated with 1 ml of DRL-62 strain with virus titer of 2×10^3 ELD₅₀/ml; third group (30 ducklings) were orally inoculated with sterile PBS and served as mock-infected control. The inoculated ducklings were euthanized at HPI 1, 3, 6, 12 and 24 and at DPI 3, 5, 7 and 14. All animal procedures were conducted in accordance with the university guidelines of the local ethical committee at Chonnam National University (CNU IACUC-YB-2013-49).

Histological examination: The formalin-fixed samples were routinely processed for H&E staining. Histopathological lesion score of livers was performed according to hepatocyte necrosis, lymphoid cell infiltration and bile duct hyperplasia in the portal triad. The hepatocyte necrosis was measured as follows: 0 = normal, 1 = mild (1-25% necrosis), 2 = moderate (26-50% necrosis), 3 = marked (51-75% necrosis) and 4 = severe (76-100%

necrosis). On the other hand, the lymphoid cell infiltration and bile duct hyperplasia in the portal triad were measured as follows: 0 = normal, 1 = involvement of <1/3 of portal triads, 2 = involvement of 1/3-2/3 of portal triads and 3 = involvement of >2/3 of portal triads.

Immunofluorescence assay (IFA): The immunofluorescence assay was performed for the detection of viral antigen in each tissue and organ sampled from experimental ducklings as described elsewhere (Kim *et al.*, 2011). Fluorescence was examined under the UV light illumination with a Leica microscope (Leica Microsystems, Wetzlar, Germany).

RT-PCR and nested PCR: RT-PCR assay was performed using a standard one-step RT-PCR and nested PCR as previously described (Cho *et al.*, 2001). The sequences of four primer pairs used were as follows; (1st set for DHAV-1, 755bp) F: TATGGAAATTTGCAG ATGGCA, R: ATTTGGTCAGATTCAATTTCCA; (2nd set for DHAV-1, 339bp) nF: ACACCAGCAATGGT AGCACA, nR: CTACTTCATCCCCAGACT GAT; (3rd set for DHAV-3, 737bp) F: GTATGGGAACCTGCAGA TGGC, R: AGCTCAAAGGCAAGTGTTTTAAGT; (4th set for DHAV-3, 352bp) nF: GAGACTGCAAATGTGC CAATACAAGG, nR: GTGGGGCGTAGAGGTGTGA CAGAAT). The amplified products were analyzed by 1.2% agarose gel electrophoresis added with Red Safe (nucleic acid stain) and were visualized using UV illuminator.

Serum biochemical analysis: Serum biochemical examination was used to assess serum albumin using a commercial BCP albumin assay kit (Abnova, Walnut, CA, USA) and compared with bovine serum albumin standard at 620 nm. After separating the serum by centrifuge, 20 μ l of the serum samples were mixed well with 200 μ l of the working reagent. Afterwards, the mixture was incubated at room temperature for 5 min and the serum level was detected at optical density (OD) value 620 nm.

Statistical analysis: Statistical differences in the hepatic histopathological lesions were analyzed by Tukey's multicomparison post-test using GraphPad Software, Inc. Version 3.0 (La Jolla, USA). Data are summarized as mean \pm standard deviation (SD). A $P < 0.05$ was considered significant.

RESULTS

Clinical signs and gross pathology: The clinical signs and gross findings of ducklings inoculated with DHAV-3 were similar to those seen in ducklings inoculated with DHAV-1. The clinical signs including the anorexia, lethargy, ataxia and opisthotonos were observed at HPI 24. The first dead case was recorded at HPI 36.

The most striking gross findings in duckling inoculated DHAV-3 or 1 at necropsy were hepatomegaly with multiple petechial to ecchymotic hemorrhages (Fig. 1). Congestion and swelling of the spleen and kidney were also observed. No changes were detected in any organs and tissues from mock-inoculated ducklings.

Histopathological changes and antigen-distribution in the liver: In DHAV-3 or DHAV-1 inoculated ducklings, histopathological evaluation of the liver revealed mild to severe hepatocyte necrosis, lymphoid cell infiltration and bile duct hyperplasia in the portal triad (Fig. 2). Both DHAV-1 and -3 tended to have similar histopathological changes in the liver (Table 1). Hepatocyte necrosis appeared from HPI 3 as mild lesions, surged sharply at HPI 12 which lasted to HPI 36 ($P<0.001$) (Fig. 2B), and then gradually decreased (Table 1). In contrast, bile duct hyperplasia and lymphoid cell infiltration in and around the portal triad were not detected in the initial stage (HPI 1-36). As time elapsed, the bile duct hyperplasia and lymphoid cell infiltration in the portal triad became prominent and severe ($P<0.001$) (Fig. 2C and D) while there was mild or no hepatocyte necrosis by DPI 7 and 14 (Table 1). The mock-inoculated ducklings did not exhibit any changes (Fig. 2A).

To assess the antigen localization of DHAV-1 or -3 in the liver, IFA was carried out. Antigen-positive cells were detected in the necrotic hepatocytes in the liver from DHAV-3 or DHAV-1 inoculated ducklings (Fig. 3B and D). However, no positive cells were observed in the mock-inoculated ducklings (Fig. 3A and C).

Extra-hepatic histological changes and antigen distribution: In order to determine whether DHAV have extra-hepatic tropisms and/or virulence, histological changes and antigen distribution were evaluated with extra-hepatic organs and tissues sampled from virus-inoculated ducklings. Both DHAV-1 and DHAV-3 showed very similar extra-hepatic lesions and antigen-distribution.

Compared to mock-infected ducklings (Fig. 4A), DHAV-3 or 1 inoculated ducklings showed lymphoid cell necrosis in the white pulp (Fig. 4B) with activated macrophages (starry-sky appearance) in the spleen. DHAV-positive lymphoid cells were also detected in the spleen (Fig. 4C). No lesions were observed in the brain of mock-inoculated ducklings (Fig. 4D). In contrast, the infected brain tissues displayed congestion of the blood vessels and perivascular-lymphoid cell infiltration (Fig. 4E). Antigen-positive cells were also detected in the brain from virus-inoculated ducklings (Fig. 4F). The kidney sampled from the mock-inoculated ducklings showed normal. (Fig. 4G). Histological sections of the kidney revealed degeneration and necrosis of renal tubular epithelial cells with congestion of the blood vessels and nephrons (Fig. 4H). DHAV antigens were detected in the

degenerative and/or necrotic renal tubules (Fig. 4I). No changes were observed in the lung from mock-inoculated ducklings (Fig. 4J). In virus-inoculated ducklings, lymphoid cell infiltration and severe multiple hemorrhages (Fig. 4K) with deposition of proteinaceous material in the lumen of parabronchus (lung edema) were observed in the lung (Fig. 4K). DHAV antigens were detected in the infiltrated lymphoid cells in the lung (Fig. 4L). Heart from the mock-inoculated ducklings had no histopathological changes (Fig. 5A). In virus-inoculated ducklings, moderate congestion of the blood vessels was seen in the heart (Fig. 5B). Antigen-positive cell was detected in the heart (Fig. 5C). No lesions were observed in mock pancreas (Fig. 5D), whereas virus-inoculated ducklings showed degeneration and necrosis of acinar epithelial cells with moderate congestion (Fig. 5E). DHAV-antigen was also detected in the acinar epithelial cells (Fig. 5F). Compared to mock-inoculated ducklings (Fig. 5G), the bursa of Fabricius sampled from virus-inoculated ducklings showed lymphoid cell depletion in the medulla of bursal follicle (Fig. 5H). DHAV-positive lymphoid cells were detected in the medullary B cells in the bursa of Fabricius (Fig. 5I). In contrast to mock-inoculated ducklings (Fig. 5J), thymus sampled from virus inoculated ducklings showed moderate lymphoid cell depletion and congestion (Fig. 5K). DHAV-positive lymphoid cells were also detected in the T cells of the thymus (Fig. 5L). No specific lesions were observed in the esophagus, trachea, proventriculus, gizzard and intestine either from virus-inoculated or mock-inoculated birds.

Viral RNA detection by RT-PCR and nested PCR: RT-PCR and nested PCR assays were performed to detect the viral RNA in the hepatic and extra-hepatic organs or tissues. Viral RNA was detected from the immediately early phase in the organs and tissues sampled from DHAV-3 or DHAV-1 inoculated ducklings. Liver, spleen, lung, bursa of Fabricius, and thymus contained viral RNA as early as HPI 3, which lasted 14 days (Table 2). In other organs and tissues, viral RNA was detected from HPI 6 or 12 and persisted to DPI 7 or 14 (Table 2).

Serum albumin analysis: Serum albumin levels were determined whether hepatocyte necrosis due to DHAV infection influences serum albumin levels. Compared to mock-inoculated group, serum albumin levels in DHAV-3 or DHAV-1 inoculated ducklings began to decrease at HPI 3 and then reached the lowest by HPI 24. By DPI 3, the levels of serum albumin gradually increased until DPI 14 (Fig. 6).

Table 1: Sequential histopathological changes in the livers from ducklings infected with DHAV-3 (DHAV-3/duck-wt/KOR/Y02-1/2012) or 1 (DRL-62) strains

Inoculum	Criteria	Mean \pm SD										
		Mock	HPI 1	HPI 3	HPI 6	HPI 12	HPI 24	HPI 36	DPI 3	DPI 5	DPI 7	DPI 14
DHAV-3	HN ^a	0.00 \pm 0.00	0.00 \pm 0.00	0.60 \pm 0.54	1.00 \pm 0.81	2.80 \pm 0.44 ^f	3.20 \pm 0.44 ^f	4.00 \pm 0.00 ^f	2.60 \pm 0.54 ^f	1.75 \pm 0.54 ^e	1.20 \pm 1.09	0.60 \pm 0.54
	LCI ^b	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	1.60 \pm 0.54	2.50 \pm 1.00 ^f	3.00 \pm 0.70 ^f	3.20 \pm 0.44 ^f				
	BDH ^c	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	1.80 \pm 0.44 ^d	2.25 \pm 0.50 ^f	2.40 \pm 0.54 ^f	2.80 \pm 0.44 ^f				
DHAV-1	HN	0.00 \pm 0.00	0.00 \pm 0.00	0.40 \pm 0.54	1.00 \pm 0.81	2.20 \pm 0.44 ^f	3.00 \pm 0.00 ^f	4.00 \pm 0.00 ^f	2.20 \pm 0.44 ^f	1.60 \pm 0.54 ^d	1.00 \pm 0.00	0.60 \pm 0.54
	LCI	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	1.80 \pm 0.44 ^d	2.40 \pm 0.89 ^f	3.25 \pm 0.95 ^f	3.40 \pm 0.54 ^f				
	BDH	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	1.80 \pm 0.44 ^e	2.50 \pm 0.54 ^f	2.75 \pm 0.50 ^f	3.00 \pm 0.00 ^f				

^aHN: hepatocyte necrosis. ^bLCI: lymphoid cell infiltration. ^cBDH: bile duct hyperplasia. ^dSignificantly different value compared to mock-treated group ($P<0.05$); ^eVery significantly different value compared to mock-treated group ($P<0.01$); ^fHighly significantly different value compared to mock-treated group ($P<0.001$).

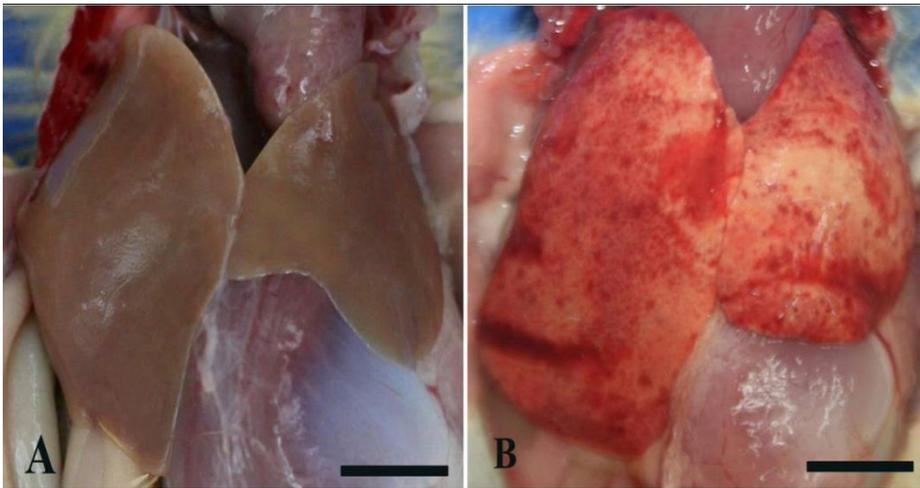


Fig. 1: (A) Liver from mock-inoculated ducklings. (B) Liver from DHAV-3/duck-wt/KOR/Y02-1/2012 strain-inoculated ducklings. Liver shows hepatomegaly and mottled with multiple petechial to ecchymotic hemorrhages. Bars A -B =4 cm.

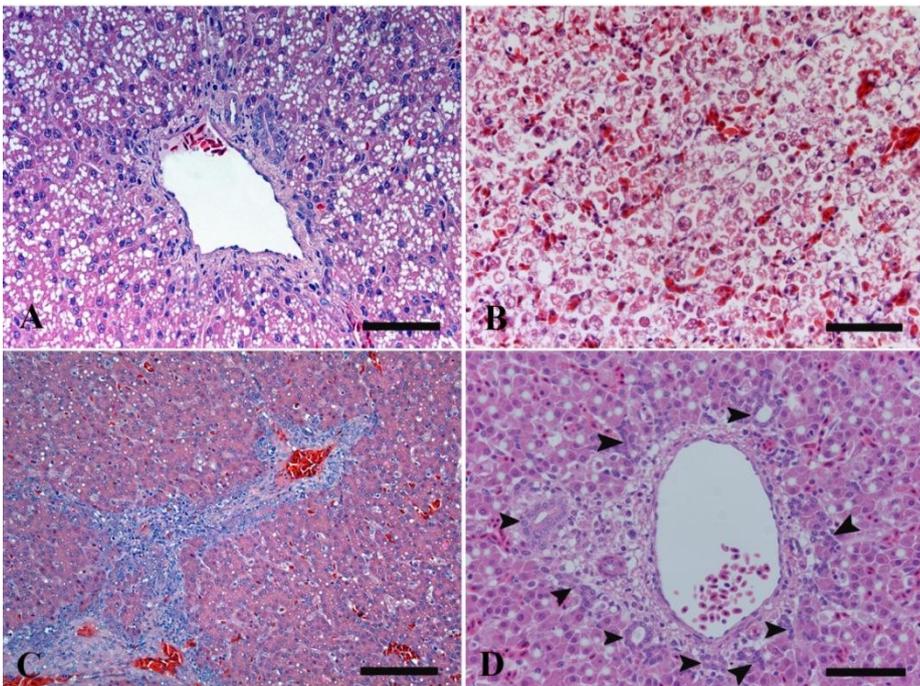


Fig. 2: Histopathological changes in the liver of DHAV infected ducklings. (A) Mock-inoculated duckling's liver. (B) Liver sampled from virus-inoculated ducklings showed severe hepatocyte necrosis associated with hemorrhage at hour post-inoculation (HPI) 36. (C) Lymphoid cell infiltration in the portal triad observed at day post-inoculation (DPI) 7. (D) Bile duct hyperplasia (arrows head) seen at DPI 7. Hematoxylin and eosin stained. Bars A, B and D =400 μ m. Bar C =200 μ m.

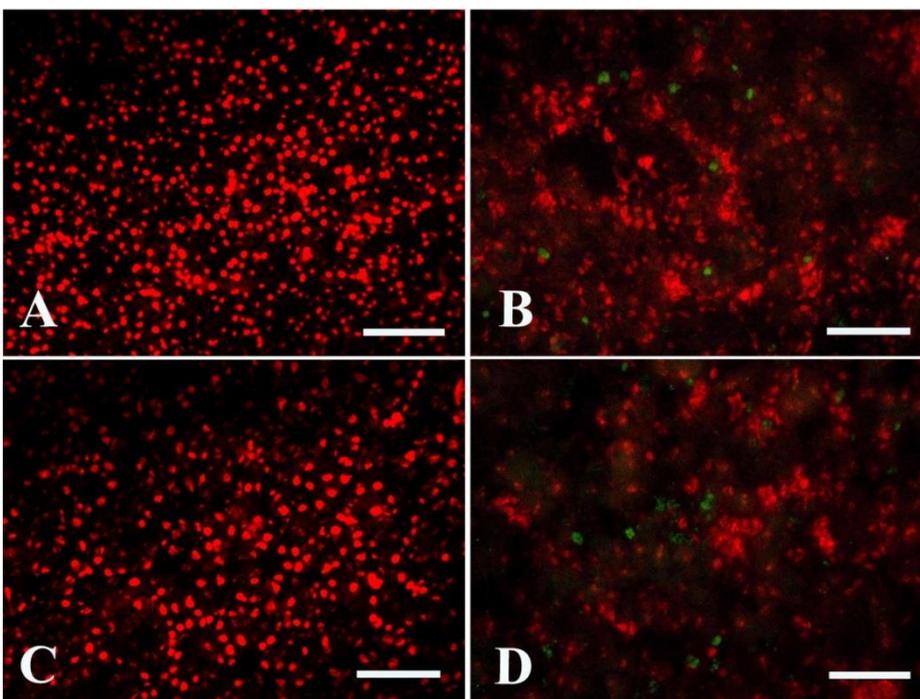


Fig. 3: Distribution of DHAV antigen-positive cells of DHAV-3/duck-wt/KOR/Y02-1/2012 and DRL-62 strains in the liver. DHAV-positive cells were detected in the necrotic hepatocytes in the liver sampled from ducklings inoculated with DRL-62 strain (B) or DHAV-3/duck-wt/KOR/Y02-1/2012 strain (D) at 36-hour post-inoculation (HPI). Mock-inoculated ducklings contained no antigen-positive cells in the liver (A) and (C). Bars A and D =400 μ m.

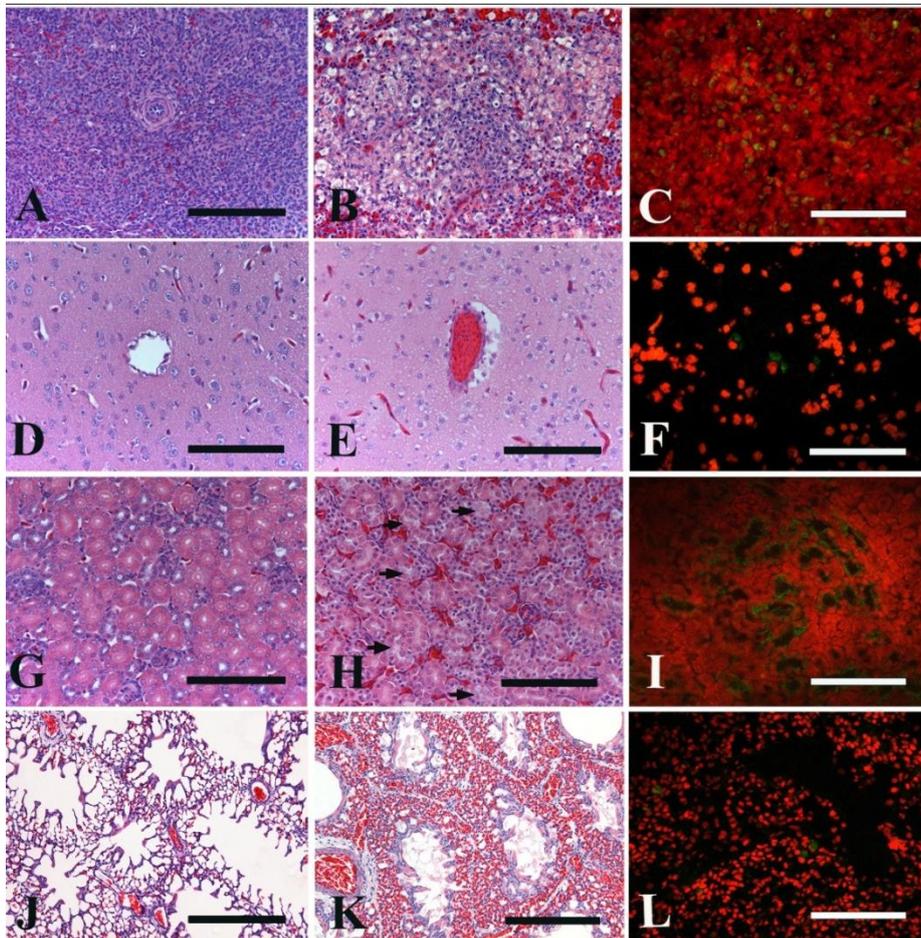


Fig. 4: Histopathological findings and antigen distribution in the extra-hepatic organs and tissues. (A) Spleen from mock-inoculated ducklings. (B) Spleen sampled from virus-inoculated ducklings showed lymphoid cell necrosis in the white pulp and moderate congestion. (C) DHAV-positive lymphoid cells were scattered in the spleen. (D) Brain from mock-inoculated ducklings. (E) Congestion of the blood vessel and perivascular-lymphoid cell infiltration in the brain of virus-inoculated ducklings. (F) Antigen-positive cells were detected in the brain. (G) Kidney from mock-inoculated ducklings. (H) Kidney sampled from virus-inoculated ducklings showed degeneration and necrosis of renal tubular epithelial cells (arrows) with congestion of the blood vessels and nephrons. (I) DHAV antigens were detected in the necrotic renal tubules. (J) Lung from mock-inoculated ducklings. (K) Lung tissue sampled from virus-inoculated ducklings showed severe multiple hemorrhage and mild diffuse lung edema. (L) DHAV antigens were detected in the infiltrated lymphoid cells in the lung. Bars A - I =400 μm . Bar J - L =200 μm .

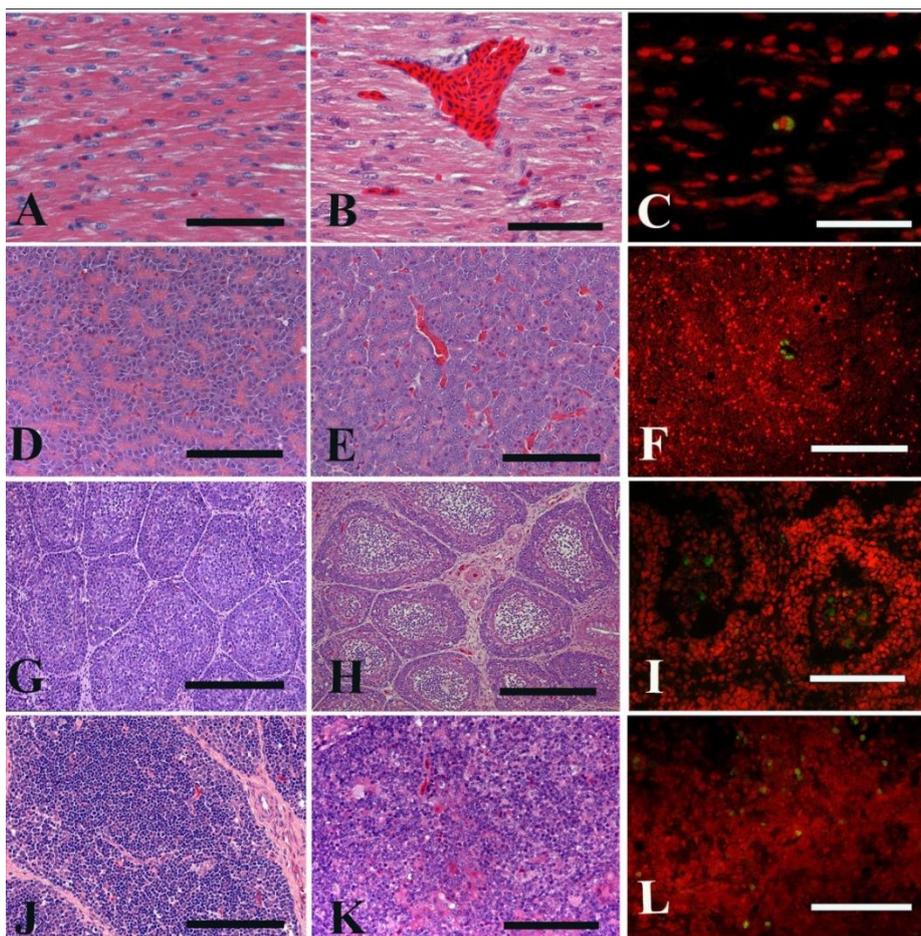
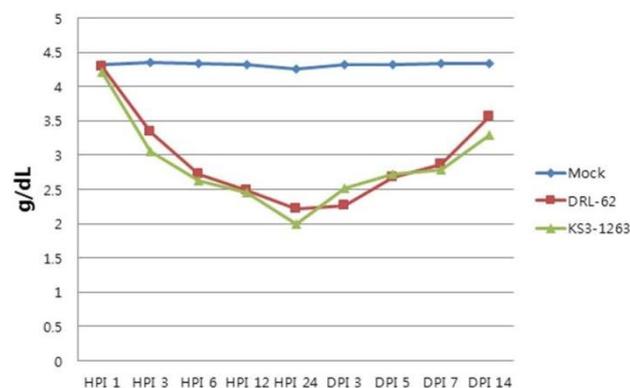


Fig. 5: Histopathological findings and antigen distribution in the extra-hepatic organs and tissues. (A) Heart from mock-inoculated ducklings. (B) Moderate congestion of the blood vessels in the heart sampled from virus-inoculated ducklings. (C) Antigen-positive cells were detected in the heart. (D) Pancreas from mock-inoculated ducklings. (E) Pancreas sampled from virus-inoculated ducklings revealed degeneration and necrosis of acinar epithelial cells with moderate congestion. (F) DHAV antigens were detected in the acinar epithelial cells of the pancreas. (G) Bursa of Fabricius from mock-inoculated ducklings. (H) Infected bursa of Fabricius showed lymphoid cell depletion in the medulla of bursal follicle. (I) DHAV antigens were detected in the medullary B cells in the bursa of Fabricius. (J) Thymus from mock-inoculated ducklings. (K) Moderate lymphoid cell depletion and congestion were observed in the thymus sampled from virus-inoculated ducklings. (L) DHAV antigens were detected in the T cells of the thymus. Representative samples from each organ were collected at day post-inoculation 7. Bars A -F and I - L =400 μm . Bar G and H =200 μm .

Table 2: Summary of viral RNA detection in different organs or tissues of ducklings inoculated with DHAV-3 (DHAV-3/duck-wt/KOR/Y02-1/2012) or I (DRL-62) strains

Organ or tissue	DHAV-3		DHAV-1		Mock	
	RT-PCR onset (duration)	Nested PCR onset (duration)	RT-PCR onset (duration)	Nested PCR onset (duration)	RT-PCR onset (duration)	Nested PCR onset (duration)
Liver	HPI 3 (DPI 14)	HPI 3 (DPI 14)	HPI 6 (DPI 14)	HPI 3 (DPI 14)	None	None
Spleen	HPI 3 (DPI 7)	HPI 3 (DPI 14)	HPI 6 (DPI 7)	HPI 3 (DPI 14)	None	None
Brain	HPI 12 (DPI 7)	HPI 12 (DPI 14)	HPI 24 (DPI 7)	HPI 12 (DPI 14)	None	None
Kidney	HPI 12 (DPI 7)	HPI 12 (DPI 14)	HPI 12 (DPI 7)	HPI 12 (DPI 14)	None	None
Lung	HPI 3 (DPI 7)	HPI 3 (DPI 14)	HPI 6 (DPI 7)	HPI 3 (DPI 14)	None	None
Heart	HPI 6 (DPI 5)	HPI 6 (DPI 7)	HPI 6 (DPI 5)	HPI 6 (DPI 7)	None	None
Pancreas	HPI 12 (DPI 7)	HPI 6 (DPI 7)	HPI 12 (DPI 7)	HPI 12 (DPI 7)	None	None
Bursa of Fabricious	HPI 3 (DPI 7)	HPI 3 (DPI 14)	HPI 6 (DPI 7)	HPI 3 (DPI 14)	None	None
Thymus	HPI 3 (DPI 7)	HPI 3 (DPI 14)	HPI 6 (DPI 7)	HPI 3 (DPI 14)	None	None

**Fig. 6:** Dynamics of the serum albumin levels in the ducklings inoculated with DRL-62 strain and DHAV-3/duck-wt/KOR/Y02-1/2012 strain in comparing with mock-inoculated ducklings.

DISCUSSION

Recently, DHAV-3 infection, a new genotype of DVH with high mortality, has spread in Southeast Asia, which caused great economic losses in the local duck industry (Huang *et al.*, 2012; Kim *et al.*, 2007a). Although DHV has been known for more than fifty years, the information about the DHV pathogenesis remains unclear. In the present study, one-day old ducklings experimentally infected with DHAV-3 strain DHAV-3/duck-wt/KOR/Y02-1/2012 (isolate) showed similar clinical signs and gross pathological lesions to that of DRL-62 strain (Kim *et al.*, 2008). The typical clinical signs appeared 24 hours post-inoculation, whereas death occurred after 36 hours post-infection. In other studies, the signs in DHAV-3 strain SWUN 3504-infected ducklings were observed 24 hours post-infection and ducklings began to die at HPI 32 (Huang *et al.*, 2012). However, comparing to D11-Jw-018 strain (low pathogenic DHAV-3), the clinical signs appeared at DPI 3-4 and death occurred within 4 days post-inoculation (Cha *et al.*, 2013).

In the early phase of infection, the hepatocyte necrosis was severe which decreased in severity and became mild at the late phase. In contrast, the lymphoid cell infiltrations in the portal triad and bile duct hyperplasia became more prominent in the late phase when the severity of hepatocyte necrosis was eroded.

Although the prominent and the prevailing hepatocyte necrosis in the early stage of infection in virus-inoculated ducklings, there was few DHAV antigen-positive cells detected by IFA in the liver. The mechanism of DHAV

causes massive hepatocyte necrosis remains largely unknown. The one possibility is that the hepatocyte necrosis in the patients with hepatitis C virus infection is due to viral replication as well as super-antigen of HCV (Sautto *et al.*, 2012). It has been postulated that the anti-HCV IgG complexes with the virus and HCV lipoproteins (VLDL) complexes may act as another B-cell super-antigens (Thomssen *et al.*, 1993). These super-antigens can activate cytokine production from macrophages with free radicals and other inflammatory mediators release which can aggravate the lesion (Samarkos and Vaiopoulos, 2005).

In contrast, at the late stage of infection, the immune defense response became more developed with successful regeneration of the hepatic cells which proceeded not only from the surviving parenchymal cells but also from bile duct epithelium (Malato *et al.*, 2011; Kang *et al.*, 2012).

Although DHAV can cause extra-hepatic lesions, the antigen distributions and the levels of viral RNA copy numbers in the extra-hepatic organs and tissues caused by either DHAV-1 or -3 are largely unknown. In the present study, both DHAV-1 and 3 induced almost similar extra-hepatic lesions. In these lesions, DHAV-antigens and -RNA were detected in the neuronal cells. These data imply that how DHAV induce nervous signs (ataxia and opisthotonos). Besides, DHAV antigen positive cells and RNA were also detected, particularly in the spleen. These results are in consistent with that the immune function might be affected by the DHAV infection (Huang *et al.*, 2012).

There were significant changes in the serum albumin level in the virus-inoculated ducklings comparing with mock-inoculated group. In the early phase, the marked decline in the serum albumin level was attributed primarily to a deranged liver function caused by a massive hepatocyte necrosis associated with duck hepatitis virus infection. In the human hepatitis cases, massive hepatocyte necrosis cause the decrease of albumin production which in turn can induce multiple edemas including the ascites, hydrothorax, hydropericardium and brain edema (Nagao and Sata, 2010). Like human cases, this hypoalbuminemia associated with DHAV infection can elucidate the occurrence of edema in the lung. In contrast, the albumin level was gradually increased during the late phase which might be due to successful regeneration of the parenchymal cells of the liver.

Conclusions: The DHAV-3 and DHAV-1 both induce similar hepatic and extra-hepatic lesion changes. These

lesion changes were elicited by virus replication. In particular, massive hepatocyte necrosis is not only by virus replication but also other virus-associated mechanism such as viral or host endogenous superantigens. In addition, DHAV-associated lymphoid cell necrosis in the spleen, bursa of Fabricius and thymus may indicate the host immune suppression. The present results may contribute to increased understanding of infection, pathology, disease and testing of prospective vaccines against DHAV-3.

Authors contribution: All authors designed and performed this study. All authors edited and approved final version of the manuscript.

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