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Comparative pathogenicity of different subtypes of duck hepatitis A virus in Pekin ducklings

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ABSTRACT

Duck hepatitis A virus (DHAV) is a major pathogen of viral hepatitis in ducks, which is a fatal and contagious disease of young ducklings. Despite the identification of numerous DHAV strains (e.g. DHAV-3, DHAV-2, DHAV-1 and DHAV-1a), the pathogenic differences among the different subtypes have not been evaluated. The objective of this study was to compare the pathogenic properties of three epidemic strains DHAV-3, DHAV-1, and DHAV-1a in mainland China, in a Pekin duckling infection model. We evaluated the pathogenicity of these different subtypes by investigating clinical signs, macroscopic and microscopic lesions, immunohistochemical examination, and viral RNA detection after experimental inoculation of Pekin ducklings with the three different DHAV strains. There was no significant difference in pathogenicity between DHAV-3 and DHAV-1. Pathogenicity of DHAV-1a differed significantly from that of classical duck hepatitis A (DHAV-3 or DHAV-1), in that there were no clinical signs of opisthotonos. More importantly, pancreatic bleeding or yellowing, and spleen swelling and bleeding were the predominant lesions in the DHAV-1a group, while liver and spleen lesions were the main signs in classical hepatitis (DHAV-1/3). Our findings indicate that there are differences in the pathogenicity of different subtypes of DHAV in ducklings, which may be useful for understanding the biological characteristics of the different subtypes of DHAV in ducks.

1. Introduction

Duck viral hepatitis is a fatal and contagious disease of young ducklings, especially those aged < 3 weeks. The disease is caused by duck hepatitis A virus (DHAV), which is the member of a novel genus *Avihepatovirus* in the family *Picornaviridae* (Chen et al., 2013). According to phylogenetic analyses and neutralization tests, DHAV is classified into DHAV-1, DHAV-2, and DHAV-3 serotypes. DHAV-1 is the classical serotype 1, which is the most widespread and virulent serotype that has caused enormous economic losses to the global poultry industry (Kim et al., 2006; Ding and Zhang, 2007; Tseng et al., 2007; Yugo et al., 2016). DHAV-2 has only been isolated in Taiwan (Tseng and Tsai, 2007). DHAV-3 was first isolated in South Korea (Kim et al., 2007), and it has recently become epidemic in mainland China (Zhang et al., 2017; Wen et al., 2014).

In general, ducklings infected with DHAV are characteristic by acute infection, causing high mortality and later neurological signs, as well as swelling and hemorrhages in the liver (Yugo et al., 2016). Also, a disease characterized by yellowing and bleeding of the pancreas, with a morbidity rate of 10%–30% and mortality rate of 25%–40%, has been occurring in 10–30-day-old Muscovy ducks or mule ducks (a cross between *Cairina moschata* and Pekin ducklings) in Fujian, Zhejiang, Shanghai, Guangdong and other parts of China, since September 2011 (Fu et al., 2012). Virus isolation and identification, genome sequencing, animal experimentation, serum cross-neutralization tests and antigenicity tests have shown that the pathogen causing these diseases is DHAV-1, which belongs to a new subtype of DHAV-1 (DHAV-1a) (Fu et al., 2012, 2014; Fu et al., 2015). Similar cases occurred in Muscovy ducks in the Loire River area of France in 2005 (Guérin et al., 2005); DHAV-1a was isolated from the ducks with encephalitis and yellowish

Abbreviations: DVH, Duck virus hepatitis; DHAV, Duck hepatitis A virus; DPI, days post infection; hpi, hours post infection; ELD_{50}, 50% embryo lethal dose; EDTA, ethylenediaminetetraacetic acid; RT-PCR, reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction

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pancreas, but there were no obvious pathological lesions in the liver. These studies have completely revised our understanding of duck hepatitis, and suggest that DHAV has emerged as a new pathogenic type.

In China, DHAV infection remains a major threat to the duck industry, despite a continuing official duck vaccination program. A recent epidemiological study has identified DHAV-3, DHAV-1, and DHAV-1a as the major cause of DHAV infection in China (Wen et al., 2014; Fu et al., 2018). More importantly, a new subtype of DHAV-1 (DHAV-1a) characterized by yellowing and bleeding of the pancreas was found in DHAV, which was significantly different from the previously reported DHAV characterized by swelling and hemorrhages of the liver in infected ducklings (Yugo et al., 2016). There is no doubt that this newly emerged pathogenic type will cause difficulty for diagnosis and prevention of the disease. There have been no comparative studies of the pathogenicity of the different DHAVs that are epidemic in ducklings in China. Therefore, in this study, we compared the pathogenicity of different DHAVs in infected ducklings and tissue tropism of the virus. We systematically investigated the clinical signs, gross lesions and histopathological changes, and analyzed virus shedding in different tissues of ducklings infected with DHAV-3, DHAV-1, and DHAV-1a.

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with the recommendations of the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines). The animal experiments were approved by the Committee of Experiment Operational Guidelines and Animal Welfare of Fujian Academy of Agricultural Sciences, China (No.160521FAAS). Animals were raised in compliance with the animal welfare regulations and maintained according to standard protocols. The ducks were anesthetized by intravenous injection of sodium pentobarbital (40 mg/kg) before being humanely euthanized.

2.2. DHAV field viruses

All DHAV isolates were obtained from naturally infected ducks in China. The serotype 1 DHAV strain FZ86 (JX390982) was isolated by our laboratory in Fujian, China in 1986, and was characterized by causing liver hemorrhage in infected ducklings. The serotype 1a DHAV strain ZJ1206 (KF924552) was isolated by our laboratory in Fujian, China in 2012, and was characterized by causing pancreatitis in infected ducklings (Fu et al., 2012). The serotype 3 DHAV strain G (EU755009) was isolated from infected ducks with typical hepatitis clinical signs in Fujian, China in 1999 (Shi et al., 2009). All the viruses were propagated in healthy Muscovy duck embryos with no pathogenic infection.

2.3. Animals

Pekin ducklings aged 7 days were screened and selected based on antibody negative response to both DHAV-1 and DHAV-3 using an indirect ELISA (Shen et al., 2015). The ducks were also tested for preexisting virus infection using polymerase chain reaction (PCR) as previously described (Liu et al., 2018). The selected ducks were further subdivided into four groups of 14. The DHAV-3 group was inoculated with strain G; the DHAV-1 group was inoculated with strain FZ86; and the DHAV-1a group was inoculated with strain ZJ1206. Ducks of groups DHAV-3, DHAV-1 and DHAV-1a were inoculated intramuscularly with 0.5 ml embryo culture supernatant containing 10^5 ELD_{50} of isolates G, FZ86 and ZJ1206, respectively; the control group was inoculated intramuscularly with the same volume of 0.5 mM phosphate-buffered saline (PBS; pH 7.4). The ducks were housed individually and fed twice daily; water was freely available at all times.

2.4. Clinical observation, autopsy, and sample collection

The clinical signs (e.g. feed intake, appearance of ducks, and the clinical signs of ducklings before and after death) of each animal were monitored daily for 15 days or until death from DHAV infection. The dead (or dying) ducks were dissected and the histological lesions were recorded. The tissues (heart, liver, spleen, lung, kidney, brain, pancreas, duodenum, thymus, and bursa) of dead ducks at 48 h post-infection (hpi) were collected and divided into two parts: one for quantification of viral RNA, and the other for histopathological and immunohistochemical examination. In addition, five cloacal swab samples were collected randomly from each group at 1, 3, 5, 7, 9, 14 days post-infection (dpi), and the samples were also used for quantification of viral RNA with TaqMan reverse transcription-PCR (RT-PCR). All the samples were made in triplicate.

2.5. Histopathological and immunohistochemical examination

The tissues were fixed in a buffered formalin solution, then processed, embedded, and sectioned according to the conventional methods for light microscopic examination. From each block, one section was stained with hematoxylin and eosin using standard procedures; the other section was used for immunohistochemistry to detect DHAV antigen. Paraffin-embedded tissues were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval, slides were boiled in Tris/EDTA pH 9.0 for 20 min. HCl (0.01 M) was used to block endogenous alkaline phosphatase for 15 min at room temperature. The slides were incubated in 5% bovine serum albumin blocking solution followed by overnight incubation with rabbit anti-DHAV polyclonal antibody (1:100 dilution). The slides were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution, Life Technologies, Carlsbad, CA, USA) for 30 min at 37 °C. Finally, the sections were stained with hematoxylin, and then analyzed under a microscope. The presence of viral antigen was assessed semi-quantitatively (−: negative; +: weakly positive; ++: positive; +++: strongly positive).

2.6. Quantification of viral RNA using TaqMan RT-PCR

Each sample from the heart, liver, spleen, lung, kidney, brain, pancreas, duodenum, thymus, and bursa was individually weighed prior to processing. The tissues from ducks were homogenized or vortexed at a 1:10 dilution in phosphate buffered saline (pH 7.2) and centrifuged (12,000 × g for 5 min). The supernatants of centrifuged samples were collected and stored at −80 °C. The total RNA was extracted using the EasyPure Viral DNA/RNA kit (Trans Gen Biotech Company, Beijing, China). A one-step TaqMan RT-PCR assay with a primer pair specific to the 3D gene of DHAV to quantify the RNA of DHAV in the samples was performed as described previously (Yang et al., 2008).

2.7. Statistical analysis

The data were analyzed using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The mean and standard deviation (SD) were used as descriptive statistics. Student's *t*-test was used for normally distributed variables. One-way analysis of variance was used to detect significant changes and differences between diseased and normal animals, and $p < 0.05$ was considered statistically significant.

Table 1
Comparison of clinical signs and gross lesions of ducklings infected with different serotypes of duck hepatitis A virus strains in Mainland China.

Strains		DHAV-3	DHAV-1	DHAV-1a
Morbidity/mortality (%)		100 (35.7)	100 (35.7)	100 (28.6)
Clinical signs	Lethargy and anorexia[a]	+ + +	+ + +	+ + +
	Opisthotonos	+ + +	+ + +	–
Gross lesions	Liver	+ + +	+ + +	+
	Spleen	+ + +	+ + +	+ + +
	Kidney	+ + +	+ + +	+
	Brain	+ + +	+ + +	+
	Pancreas	+	+	+ + +

–: No duck showed the clinical signs or lesions;
+: 5 ~ 35% of dead ducks showed the clinical signs or lesions;
+ +: 35 ~ 65% of dead ducks showed the clinical signs or lesions;
+ + +: More than 65% of dead ducks showed the clinical signs or lesions;
[a] All the diseased duck showed the clinical signs.

3. Results

3.1. The DHAV-1a caused lower mortality rates in Pekin ducklings than DHAV-1 or DHAV-3, but with no opisthotonos clinical signs

The major differences in clinical features and gross lesions caused by the three different serotypes of DHAV in mainland China are summarized in Table 1. Most diseased ducks exhibited clinical signs of lethargy and anorexia, as well as opisthotonos; however, the clinical signs of opisthotonos were not present in the DHAV-1a group. The major diseased organs in the DHAV-3 and DHAV-1 groups were liver, spleen, kidney and brain; however, the major diseased organs in the DHAV-1a group were the pancreas and spleen (Table 1). Mortality rates in the DHAV-3, DHAV-1 and DHAV-1a groups were 35.7%, 35.7% and 28.6%, respectively. The ducklings began to die at 24 hpi and peaked at 48 hpi, and no ducklings died after 72 hpi (Fig. 1). The mean time for a duck to begin exhibiting clinical signs was 48 hpi, and the mean time to death after showing clinical signs was 6–12 h (Fig. 2). The death time of infected ducks is concentrated at 2–3 dpi (Fig. 1).

3.2. DHAV-1a caused pancreatic bleeding or yellowing, and spleen swelling and bleeding of infected ducklings

Gross lesions in Pekin ducklings inoculated with the different serotypes of DHAV strains at 48 hpi are shown in Fig. 3A–T. The liver was typically enlarged with petechial and ecchymosis hemorrhages throughout in the DHAV-3 and DHAV-1 groups (Fig. 3A, B). The liver in the DHAV-1a group had slight swelling and congestion only (Fig. 3C). The pancreas showed visible bleeding and yellowing in the DHAV-1a group (Fig. 3G), but the DHAV-3 and DHAV-1 groups were similar to the control group (Fig. 3E, F, H). In the kidneys, the DHAV-3 and DHAV-1 groups both showed obvious swelling and bleeding (Fig. 3I, J). Nevertheless, only slight hydroncus was seen in the DHAV-1a group (Fig. 3K). The main lesions in the brain in the DHAV-3 and DHAV-1 groups were meningeal hyperemia and hemorrhage (Fig. 3M, N). The brain in the DHAV-1a group was similar to that in the control group (Fig. 3O, P). In addition, there was serious splenectasis and the spleen had a reddish brown color with congestion in all inoculated groups (Fig. 3Q–S); there were no obvious gross lesions in the other tissues of dead ducklings; no lesions were found in any of the control tissues (Fig. 3D, H, L, P, T).

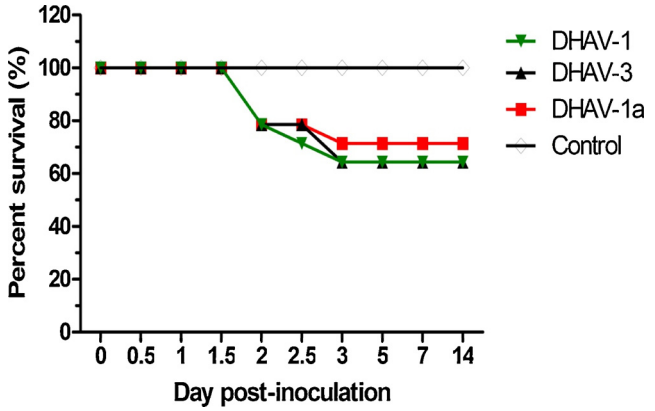

Fig. 1. Survival rates of 7-day-old Pekin ducklings inoculated with different serotype of duck hepatitis A virus strains.

3.3. Histological lesions of ducklings infected with DHAV-1a were significantly different than those infected with DHAV-1 or DHAV-3

Histological lesions of Pekin ducklings inoculated with the different subtypes of DHAV at 48 hpi are shown in Fig. 4. Significant pathological changes were detected in the liver of dead ducklings in the DHAV-3 and DHAV-1 groups. There was an increase in cytoplasmic vacuoles in hepatocytes and a large number of inflammatory cells infiltrating the blood vessels (Fig. 4A, B). Mild degeneration of hepatocytes and an increase in basophilic cells were observed in the DHAV-1a group (Fig. 4C). In the pancreas, in the DHAV-3 and DHAV-1 groups, the nuclei disappeared, and a small number of pancreatic epithelial cells were denatured (Fig. 4E, F). There was significant necrotizing pancreatitis with a large area of necrosis and degeneration of pancreatic epithelial cells in the DHAV-1a group (Fig. 4G). Severe infiltration of renal inflammatory cells and swelling and degeneration of renal epithelial cells was observed in the DHAV-3 and DHAV-1 groups (Fig. 4I, J). The tubular epithelial cell structure was loose, with granular-like vesicular degeneration in the DHAV-1a group (Fig. 4K). Necrosis of brain nerve cells and local microglia proliferation were seen in the DHAV-3 and DHAV-1 groups (Fig. 4M, N); only slight degeneration and swelling of nerve cells were observed in the DHAV-1a group (Fig. 4O). The spleen showed severe necrosis, hyperemia, and structural disorder in all groups (Fig. 3Q–S). No histological microscopic lesions were observed in the control group (Fig. 3D, H, L, P, T).

3.4. Viral antigen contents in liver and pancreas of ducklings infected with DHAV-1a were significantly different than in those infected with DHAV-1 or DHAV-3

DHAV antigens in different tissues of ducks at 48 hpi were examined using immunohistochemistry. The IHC staining images of the liver and pancreas are shown in Fig. 4. Yellowish brown reactants were observed in DHAV-positive tissues, and positive staining was present in the cytoplasm. No immunoreactivity for DHAV antigen was detected in the tissue sections from the control ducklings. DHAV was positive in all tissues from inoculated ducks (Table 2). In the DHAV-1 and DHAV-3 groups, strongly positive staining was present in the liver, spleen, kidneys and brain; heart, lungs, duodenum, thymus and bursa had positive staining; and the pancreas had weakly positive staining. There was no significant difference between the DHAV-1 and DHAV-3 groups. In the DHAV-1a group, the positive staining in the pancreas, spleen and heart was stronger than in other tissues. The lungs, duodenum, thymus and bursa had positive staining, while the liver, kidneys and brain had weakly positive staining. In the DHAV-1 and DHAV-3 groups compared to the DHAV-1a group, there were significant differences in DHAV antigen content of the liver, pancreas, kidneys and brain.

3.5. Viral loads in liver and pancreas of ducklings infected with DHAV-1a were significantly different than in those infected with DHAV-1 or DHAV-3

The viral RNA loads in different organs of dead (or dying) ducklings at 48 hpi were detected and analyzed using TaqMan RT-PCR. Viral RNA

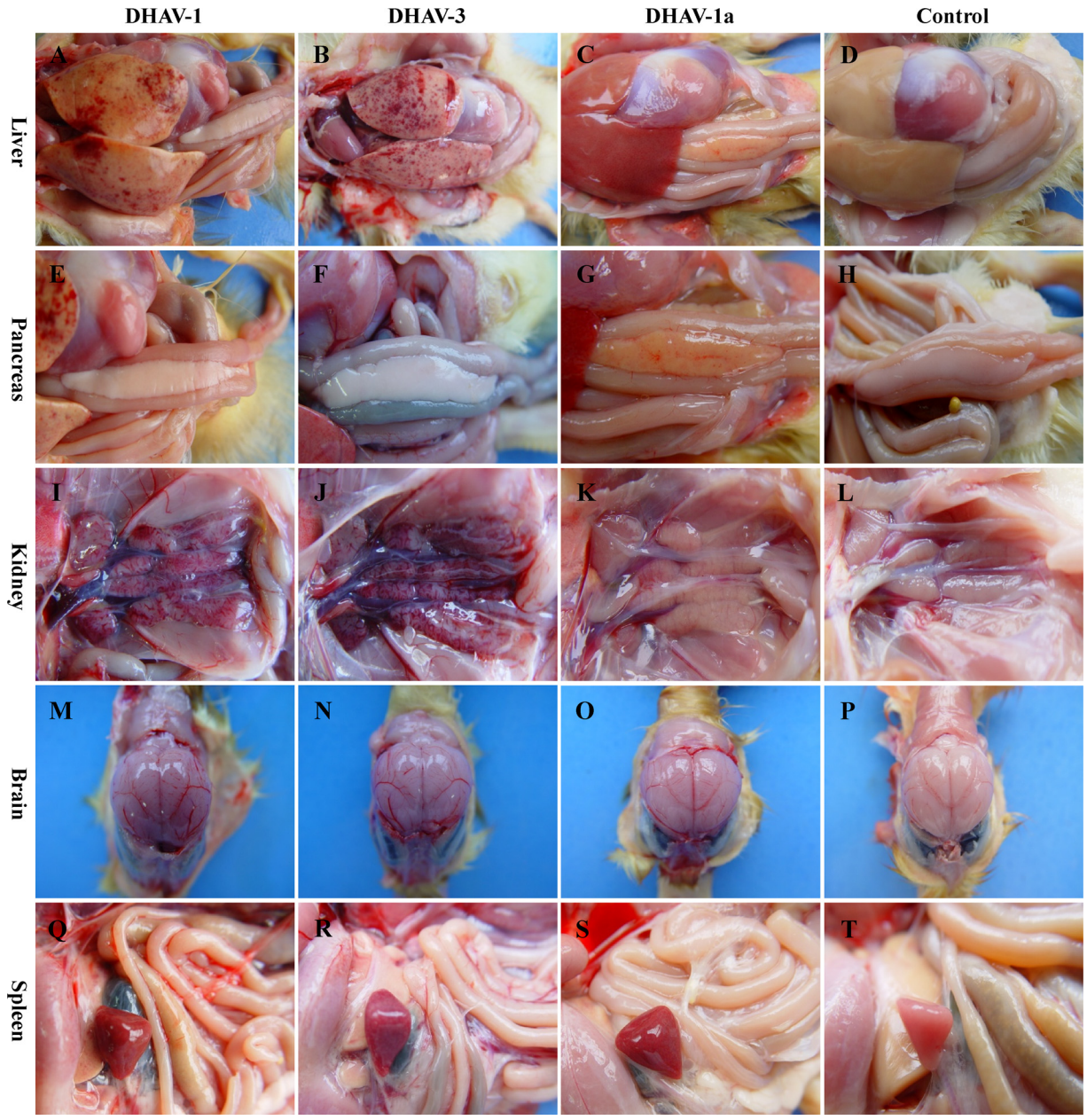

Fig. 2. Gross lesions of Pekin ducklings inoculated with different serotypes of duck hepatitis A virus strains at 48 h post-infection.

was detected in all 10 tissues, and the viral RNA loads varied among the different virus strains and from tissue to tissue (Fig. 5A). In the DHAV-1 and DHAV-3 groups, the most obvious difference in viral RNA loads between various tissues was the liver, spleen, kidney, and brain, which were significantly higher than in other tissues. There was no significant difference in viral load between different tissues in the DHAV-1 and DHAV-3 groups. In the DHAV-1a group, the viral load in the pancreas, spleen and heart was higher than in other tissues. A more detailed comparison found that, compared with the DHAV-1 and DHAV-3 groups, the viral load of the DHAV-1a group in the pancreas and heart was relatively high, while the viral load in the liver, kidneys and brain was relatively low. Viral RNA in the cloacal swab from ducks inoculated with DHAV was detected from 1 to 14 dpi in all three groups (Fig. 5B). In the DHAV-1 and DHAV-3 groups, viral RNA in the cloacal swabs decreased gradually as the time after inoculation increased. In the DHAV-1a group, the viral RNA was first detected at 1 dpi, reaching a peak at 3 dpi and gradually decreasing after 5 dpi. Compared with the DHAV-1a group, the viral load of the DHAV-1 and DHAV-3 groups in cloacal swabs was relatively high at 1 dpi, but relatively lowly at 7 dpi. However, viral RNA was still detected in all groups at 14 dpi.

4. Discussion

The clinical signs, gross lesions, histopathological lesions and tissue viral load of the original DHAV-3 and DHAV-1 strains have been well documented, and the clinicopathological features of these two strains are characterized by swelling or hemorrhage of the liver and spleen (Zhang et al., 2012, 2018). However, it has also been reported in South Korea that low-pathogenicity DHAV-3 was isolated from dead ducklings characterized by swollen spleen and did not display typical clinical signs of DHAV-1 infection (Cha et al., 2013). DHAV-3 has only become epidemic in China in recent years, and DHAV-1a infection, which is characterized by yellowing and bleeding of the pancreas, was first reported by our team in South China from 2011 (Fu et al., 2012, 2014). A direct comparison of the pathogenicity between these different DHAV subtypes has never been conducted. Therefore, in this study, we

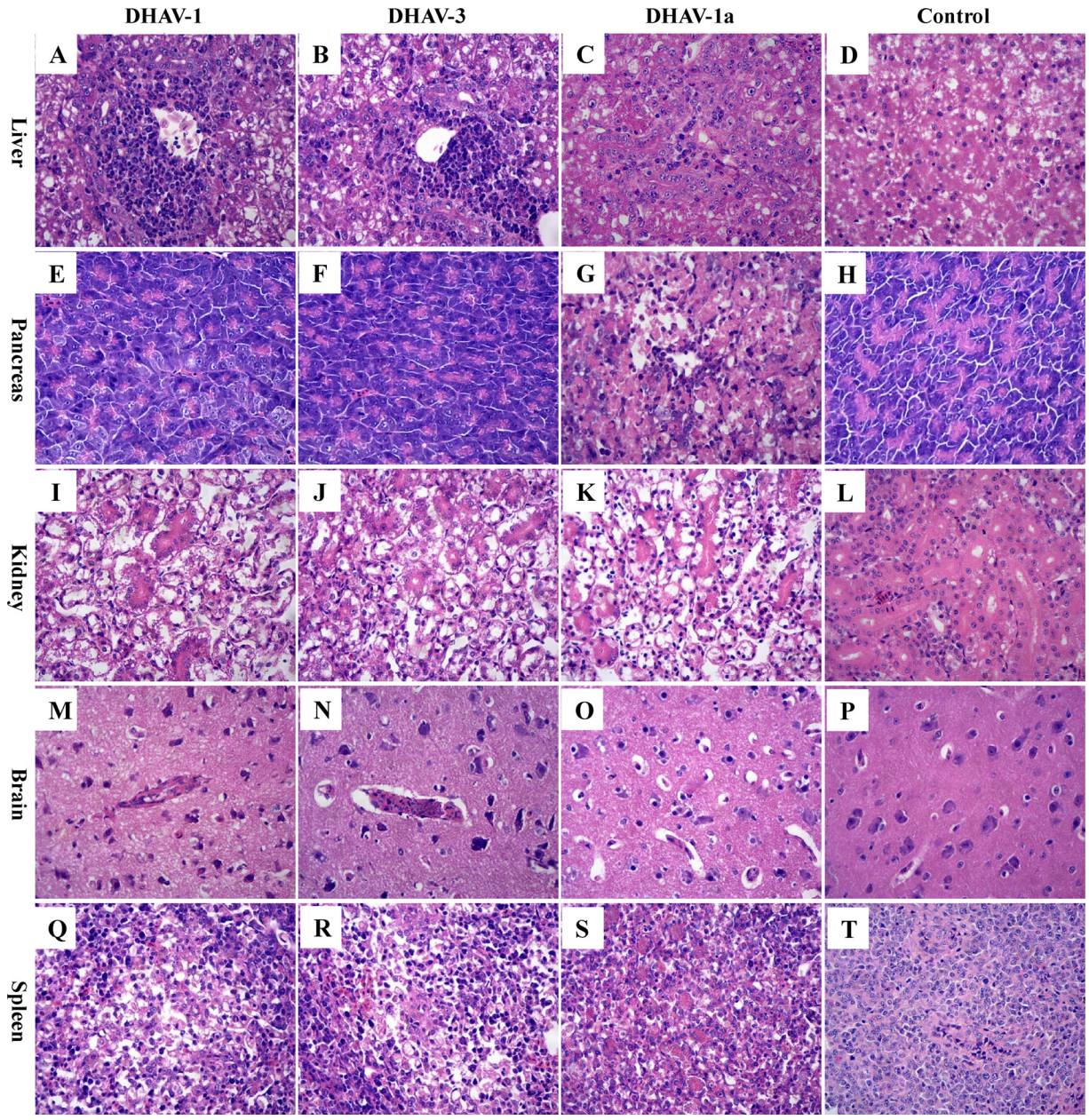

Fig. 3. Histological lesions of Pekin ducklings inoculated with different serotypes of duck hepatitis A virus strains at 48 h post-infection.

compared the pathology of infection with different subtypes of DHAV in Pekin ducklings, which may be useful to understand the DHAV strains further.

The conventional commercial Pekin duckling model used in this study, although probably less sensitive than the specific pathogen free duck model, is considered to be an excellent system to study and compare clinical signs and pathology caused by DHAV (Cha et al., 2013; Ou et al., 2017). Using this model, we found differences in the clinical and pathological outcomes of infection by the different subtypes of DHAV. Although the majority of infected ducks in this study exhibited clinical signs with lethargy, anorexia and opisthotonos, the latter was not present in the DHAV-1a group. This was the most marked difference in clinical signs between DHAV-1/DHAV-3 and DHAV-1a. To reveal further their pathogenicity differences, we dissected dead (or dying) ducks and found that the major macroscopic lesions in ducks infected with DHAV-3/DHAV-1 were in the liver, spleen, kidneys and brain, which was consistent with previous reports (Zhang et al., 2012; Yugo et al., 2016). However, the major lesions in ducks infected with DHAV-1a were seen in the spleen and pancreas only, which was consistent with our previous clinical reports and animal experiments (Fu et al., 2012, 2014). These results indicate that the new DHAV-1a subtype differed significantly from DHAV-1/DHAV-3 in pathogenicity and tissue tropism.

To verify the results for gross lesions, we took pathological sections of the major parenchymal organs. Only ducklings infected with DHAV-1/DHAV-3 showed typical microscopic lesions of hepatitis, mainly in the liver, spleen, kidneys and brain, and other tissue lesions were minor. These results were consistent with previous reports (Zhang et al., 2012; Yugo et al., 2016). However, the microscopic lesions of the DHAV-1a-infected group were obviously different from those in the aforementioned two groups; the microscopic lesions were mainly in the pancreas and spleen, and the lesions in other tissues were not obvious. The immunohistochemical examination showed that there was no significant difference between the DHAV-3 and DHAV-1 groups, but the IHC positive staining of the DHAV-1a group was significantly different in liver, pancreas, kidney and brain, compared to the DHAV-3 and

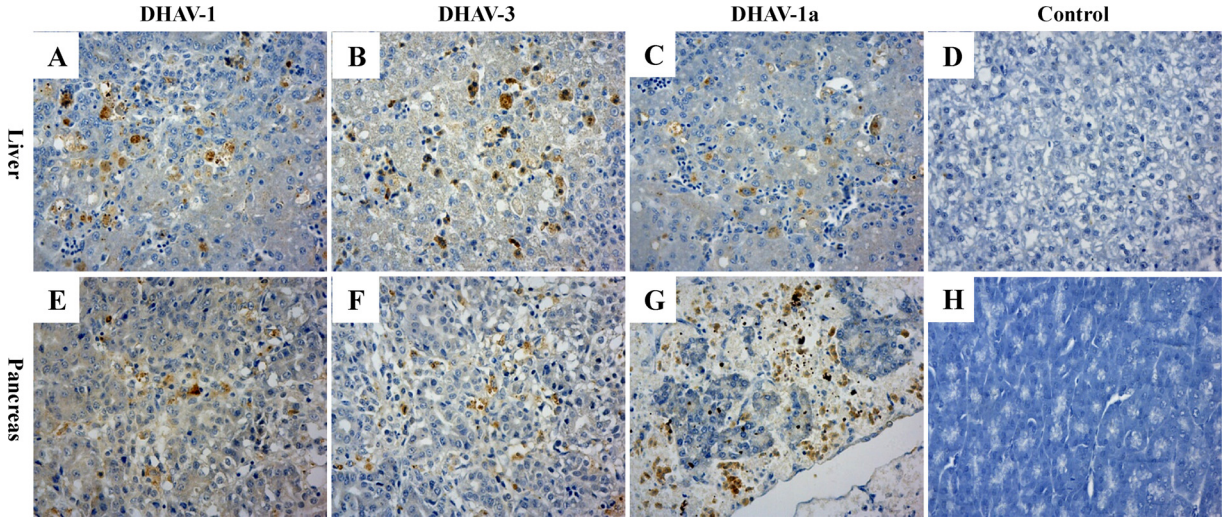

Fig. 4. Immunohistochemical examination of inoculated Pekin ducklings for the duck hepatitis A virus (DHAV) antigen.

Table 2
Summary of immunohistochemical detection of duck hepatitis A virus in Pekin ducklings at 48 h post-inoculation.

Challenge virus	Heart	Liver	Spleen	Lung	Kidney	Brain	Pancreas	Duodenum	Thymus	Bursa
DHAV-1	++	+++	+++	++	+++	+++	+	++	++	++
DHAV-3	++	+++	+++	++	+++	+++	+	++	++	++
DHAV-1a	+++	+	+++	++	+	+	+++	++	++	++
Control	–	–	–	–	–	–	–	–	–	–

−: negative; +: weakly positive; ++: positive; +++: strongly positive.

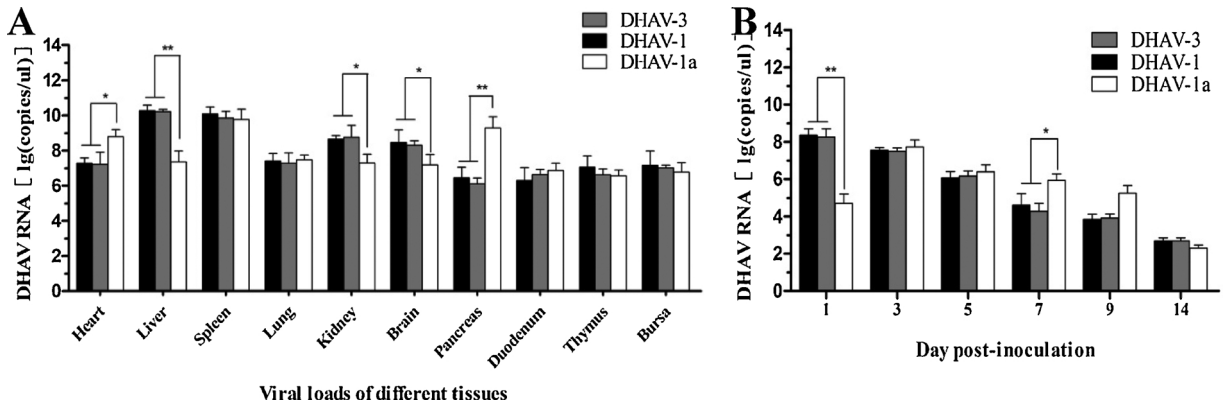

Fig. 5. Quantification of duck hepatitis A virus RNA in the cloacal swabs and different tissues, at each sampling point. RNA copies of three individuals in each group were detected and calculated as mean ± standard deviation. (A) Viral loads of different tissues were sampled from ducks at 48 h post-inoculation; (B) viral loads of cloacal swabs at different times post-inoculation.

DHAV-1 groups. These results were further verified by the TaqMan PCR, and we showed that, compared with the DHAV-3 and DHAV-1 groups, the viral load of the DHAV-1a group in the pancreas and spleen was high, while that in the liver, kidneys and brain was low.

In summary, our comparison of the major DHAV subtypes in China (DHAV-3, DHAV-1 and DHAV-1a) indicated that there was no significant difference in pathogenicity between DHAV-3 and DHAV-1. Nevertheless, this study also revealed that the pathogenicity of DHAV-1a was significantly different from that of the classical DHAV-3 and DHAV-1, as demonstrated by the absence of opisthotonos clinical signs. More importantly, according to the results of macroscopic, microscopic and immunohistochemical examination and viral RNA detection in different tissues in inoculated ducks, we found that the pancreatic and splenic lesions were predominant in the DHAV-1a group, while lesions in the liver and spleen were predominant in classical hepatitis (DHAV-1/3 groups).

Our study had some limitations, in that we did not include DHAV-3 or DHAV-1 isolated in recent years. A larger study, involving more isolates, would confirm our findings. Nevertheless, this is the most extensive comparative study of the pathogenicity of different subtypes of DHAV carried out to date. Therefore, our study may contribute to understanding the biological characteristics of different subtypes of DHAV in ducks.

Acknowledgments

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