Virulent and attenuated strains of duck hepatitis A virus elicit discordant innate immune responses in vivo

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Previous studies of duck hepatitis A virus infection have focused only on the pathogenicity and host response of one strain. Here, we show that the virulent SH strain and the attenuated FC64 strain induced varied pathogenicity, apoptosis and immune responses in the livers of 1-day-old ducklings. SH infection caused apoptosis and visible lesions in the liver; serum aspartate aminotransferase, alanine transaminase, alkaline phosphatase, γ-glutamyltransferase and total bilirubin activities were markedly upregulated; and ducklings died at 36 h post-infection (p.i.). However, FC64 infection did not induce significant symptoms or impair liver function, and all of the infected ducklings remained healthy. In addition, both virus strains replicated well in the liver, spleen and intestine, whilst the SH strain replicated more efficiently than FC64. IFN-γ, IL-2, inducible nitric oxide synthase and nitric oxide were strongly induced by SH infection, and may be associated with the pathogenicity of the SH strain. IFN-α, IFN-β, IFN-stimulated transmembrane protein 1, IFN-stimulated gene 12, 2′,5′-oligoadenylate synthetase-like and IL-6 were moderately induced by SH infection at 24 h p.i., and dramatically induced by FC64 infection at 36 h p.i. The intensive induction of cytokines by FC64 may be involved in restriction of virus replication and stimulation of adaptive immune responses. Ducklings inoculated with FC64 produced high levels of antiviral antibodies within 45 days p.i. The low virulence and strong immune response of FC64 rendered this strain a good vaccine candidate, as confirmed by a protective assay in this study.

INTRODUCTION

Duck hepatitis A virus (DHAV; family Picornaviridae, genus Avihepatovirus) (Ding & Zhang, 2007; Kim et al., 2006; Tseng et al., 2007; Varghese et al., 2010–2011) was first identified in the United States in 1949 (Levine & Fabricant, 1950). DHAV strains are genetically divided into three different types: the original type DHAV-1 (Asplin, 1958; Levine & Fabricant, 1950), DHAV-2 isolated in Taiwan (Tseng & Tsai, 2007) and DHAV-3 isolated in South Korea and China (Fu et al., 2008; Kim et al., 2007). In China, duck viral hepatitis is mainly caused by DHAV-1 and DHAV-3, as DHAV-2 has not yet been isolated. The DHAV genome contains a single, long ORF encoding a polyprotein, which is cleaved into a leader protein, three structural proteins (VP0, VP1 and VP3) and eight non-structural proteins (2A1, 2A2, 2B, 2C, 3A, 3B, 3C and 3D). DHAV causes an acute, highly lethal infection in young ducklings usually < 4 weeks of age. Different DHAV strains cause diverse symptoms and pathology. Attenuated virus strains usually cause transient infection, whereas virulent strains often lead to severe contagious diseases resulting in mortality rates of up to 95% in 1-week-old ducklings (Li et al., 2013; Xu, 1990). Gross lesions tend to be restricted to the liver, which becomes swollen and presents multiple punctuate or coalescing haemorrhage sites (Bidin & Bidin, 2008; Woolcock & Crighton, 1979).

An early innate immune response is necessary to limit the initial infection and is a first-line defence response to virus infection (Everett & McFadden, 1999). Virus-associated molecules can be recognized by host pattern recognition receptors, which then initiate antiviral responses, including induction of type I IFNs (IFN-α/β), IFN-stimulated genes
ISGs) and ILs. ISGs act to inhibit viral infection at the early infection stage, where ILs activate adaptive immune responses and therefore eliminate virus infection at the later infection stage (Janeway & Medzhitov, 2002; Kawai & Akira, 2006; Theofilopoulos et al., 2005). Thus, the innate immune response plays an important role in defence against viral infection (Galiana-Arnoux & Imler, 2006).

Apoptosis is a crucial factor for disease onset and development as well as tissue homeostasis (Grossmann, 2002). Previous studies reported that DHAV infection causes hepatic necrosis and apoptosis, resulting in liver injury, which plays an important role in disease pathogenesis (Ding, 1996; Sheng et al., 2014).

Although DHAV has been studied extensively, previous studies of DHAV have focused only on the pathogenicity and host response of one strain; relatively few reports have compared the host response and pathology induced by different virus strains to variation in virulence. Here, we hypothesized that the host immune responses and pathogenicity of different virulent DHAV strains are diverse. We compared the pathology, apoptosis, virus replication, cytokine induction and nitric oxide (NO) production in 1-day-old ducklings infected with the virulent SH strain (GenBank accession number HQ265433) (Song et al., 2011b) or attenuated FC64 strain (GenBank accession number HQ232302) (Song et al., 2011a). The liver, spleen and intestine were found to be the main target organs of both DHAV strains. Infection with the SH strain was lethal to the ducklings at 36 h post-infection (p.i.), whilst the FC64 strain was non-pathogenic. The induction of type I IFNs, ISGs and IL-6 by FC64 infection was several hundred-fold greater than that of SH infection. Inducible nitric oxide synthase (iNOS) and NO production were strongly upregulated by SH infection, which may contribute to the pathogenicity of SH. The low virulence and strong immune response rendered FC64-infected ducklings capable of being protected against attack by virulent SH. The aim of this study was to provide a basis to elucidate the pathogenesis of DHAV and facilitate vaccine development.

RESULTS

Clinical signs and histopathological analysis of DHAV-infected ducklings

All 1-day-old ducklings infected with SH showed neurological symptoms as early as 24 h p.i. and died by 36 h p.i. Post-mortem examination revealed typical visible lesions in the 'piebald liver', as described previously (Song et al., 2013), whilst none of the FC64-infected or mock-infected ducklings displayed clinical signs or died.

Histological analysis of the liver specimens revealed significant differences between virulent SH- and attenuated FC64-infected ducklings. All SH-infected ducklings exhibited asystematic cell swelling and vesicular degeneration (hydropic degeneration) in the liver at 16 h p.i. (Fig. 1a). More severe blistering, degeneration, local steatosis and a small amount of local heterophilic neutrophil infiltration in the small blood vessels were observed at 24 h p.i. (Fig. 1b). Moreover, hepatocyte morphology was characterized by fuzzy cell boundaries, necrosis, collapse, perivascular lymphocyte infiltration, wide spread of large numbers of granulocytes (marked by arrow) and many necrotic focal points in liver specimens at 36 h p.i. (Fig. 1c).

However, fewer pathological changes were observed in liver specimens from FC64-infected ducklings compared with SH-infected ducklings. The livers of FC64-infected ducklings appeared only slightly inflamed, with vesicular degeneration and a small number of inflammatory lesions (Fig. 1d–f). These results confirmed that the livers of SH-infected ducklings had more lesions than those infected with FC64.

Biochemical detection of serum aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT) and total bilirubin (T-Bil)

Levels of the serum biochemical markers AST, ALP, ALT and T-Bil are associated with liver function (Hu & Cheng, 1996). As liver lesions were observed in SH-infected ducklings, each of these biochemical markers of liver function were measured in ducklings infected with SH or FC64. Mock-infected liver specimens were included as negative controls. As shown in Fig. 1(g), there were higher serum ALT, AST, ALP, GGT and T-Bil levels in liver specimens from SH-infected ducklings than those from FC64- or mock-infected ducklings, indicating that liver function was severely impaired by SH infection. There were no significant differences in AST, ALT, ALP, GGT and T-Bil levels between FC64- and mock-infected ducklings, demonstrating that the liver function of FC64-infected ducklings was not impaired.

Apoptosis in SH- or FC64-infected duckling livers

Apoptosis in liver specimens from SH- and FC64-infected ducklings was examined using the terminal deoxynucleotidyltransferase dUTP nick end labelling (TUNEL) assay. In SH-infected ducklings, many TUNEL-positive cells with brown-stained nuclei were observed in the sliced liver specimens at 24 and 36 h p.i., which appeared as small rounded or granular patterns (Fig. 2a, b). However, few TUNEL-positive cells were found in sliced liver specimens of FC64-infected ducklings (Fig. 2c, d). These results demonstrated that SH infection, but not FC64 infection, induced apoptosis in the duckling liver.

To confirm that apoptosis was caused by SH infection, caspase-3 cleavage was analysed by Western blot analysis of duckling liver specimens infected with SH or FC64. As shown in Fig. 2(e), the 17- and 19 kDa caspase-3 cleavage forms were detected in the SH-infected duckling liver at

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36 h p.i., but not in the FC64-infected duckling liver. These results demonstrated that SH infection, but not FC64 infection, induced apoptosis in the duckling liver, which was in agreement with the TUNEL assay results. Apoptosis induced by virulent SH infection may play a critical role in pathogenesis.

Fig. 1. Histopathological analysis of duckling livers and liver function serum marker levels in ducklings infected with strain SH or FC64. (a) Vesicular degeneration in duckling liver infected with SH at 16 h p.i. is marked with an arrow. (b) Widespread and scattered steatosis in various eosinophilic granulocytes in duckling liver infected with SH at 24 h p.i. is indicated with arrows. (c) Necrosis, collapse, perivascular lymphocyte infiltration and many necrotic focal points in duckling liver infected with SH at 36 h p.i. are indicated. (d–f) Slight hepatitis lesions in duckling livers infected with FC64 at 16, 24 and 36 h p.i. respectively, exhibited vesicular degeneration and inflammatory lesions (arrow). Bar, 200 µm. (g) Sera of infected ducklings were collected and subjected to chemical analysis, and the results were analysed using SPSS v. 13.0 software. Values are presented as mean ± SD (n=4). *P<0.05 indicates significant difference using Student’s t-test.
DHAV replicates most efficiently in the liver; therefore, SH and FC64 invasive efficiencies in the liver were compared using a time-course assay. Total RNA was extracted from liver tissue at 16, 24 and 36 h p.i., and levels were determined by qRT-PCR. As shown in Fig. 3(c), there were significantly higher levels of SH RNA copies than FC64 at all three time points, especially at 24 and 36 h p.i. These results further confirmed that SH replicated more efficiently than FC64 at all time points in duckling liver.

### Innate immune response in DHAV-infected duckling liver

To determine the mechanisms of replication efficiency disparities between SH and FC64, the levels of cytokines and ISGs in the infected duckling livers were examined. The RNA levels of five cytokines (IFN-α, IFN-β, IFN-γ, IL-6 and IL-2) and three ISGs [IFN-stimulated transmembrane protein 1 (IFITM1), IFN-stimulated gene 12 (ISG12) and 2′,5′-oligoadenylate synthetase-like (OASL)] were examined by qRT-PCR. As shown in Fig. 4(a), all of the cytokines and ISGs were induced in a time-dependent manner following infection by either virus strain. In SH-infected ducklings, cytokine production was increased until 24 h p.i. (increases of 26-fold for IFN-α, 23-fold for IFN-β, 19-fold for IFN-γ, 170-fold for IL-6 and 130-fold for IL-2), when peak values were reached. After this peak, the concentration of each cytokine decreased by 36 h p.i. However, production of ISGs increased until 36 h p.i. (increases of 13-fold for IFITM1, 22-fold for ISG12 and 48-fold for OASL), when peak values were reached. However, in FC64-infected ducklings, dramatically increased levels of cytokines and ISGs (increases of 1150-fold for IFN-α, 165-fold for IFN-β, 14.5-fold for IFN-γ, 15414-fold for IL-6 and 4.8-fold for IL-2) and ISGs (increases of 329-fold for IFITM1, 7963-fold for ISG12 and 7250-fold for OASL) were observed until 36 h p.i., when peak values were reached. These results indicated that SH infection induced an earlier innate immune response than FC64 and that FC64 induced a significantly stronger innate immunity response at a later infection stage than SH. Notably, concentrations of ISGs (IFITM1, ISG12 and OASL) peaked at 36 h p.i. by both SH and FC64 infection. The differences in innate immune responses were correlated with replication efficiency and virulence of these two virus strains, and contributed to the variation in pathogenesis.

### Induction of iNOS and NO by SH infection

Accumulated evidence suggests that NO and oxygen radicals are key molecules in the pathogenesis of various infectious diseases. NO biosynthesis, particularly through expression of iNOS, occurs in a variety of virus infections.
Fig. 3. Viral 3D gene expression. (a) Log of virus RNA copies in 1 LD50 SH and 1 ELD50 FC64. RNA was extracted from 1 LD50 SH or 1 ELD50 FC64 and subjected to qRT-PCR. (b) Log of virus RNA copies in the heart, spleen, lung, kidney, brain, intestine and muscle of SH- or FC64-infected ducklings. Total RNA was extracted from different tissues at 36 h p.i. and subjected to qRT-PCR using specific primers. The error bars show the mean ± SD from four liver specimens (n=4). (c) The log of virus RNA copies in livers of SH- and FC64-infected ducks. Total RNA was extracted from livers at 16, 24 and 36 h p.i., and subjected to qRT-PCR using specific primers. Values represent mean ± SD (n=4). Virus titres were determined by comparing input RNA : C1 ratios. **P<0.01 and *P<0.05 indicate significant difference in gene expression using Student’s t-test.

Fig. 4. Upregulation of IFN genes, ISGs, pro-inflammatory cytokines and iNOS mRNA in duckling livers and NO production in duckling serum infected with SH or FC64. (a) Total RNA was extracted from livers at 16, 24 and 36 h p.i. and subjected to qRT-PCR using specific primers. Gene fold changes were quantified and normalized with those in mock-infected ducklings. Values represent mean ± SD (n=4). *P<0.05 and **P<0.01 indicate cytokines with significant increases, as indicated by Student’s t-test. (b) Total nitrate and nitrite concentrations produced by NO in serum samples were detected at 16 and 24 h p.i., and normalized to the total protein content in the filtered sera. Values are presented as the mean ± SD (n=4). Different lower-case italic letters indicate significant differences of the means (P<0.05).
iNOS is an indicator of NO in the tissues of infected animals (Burggraaf et al., 2011). To examine whether SH and FC induced iNOS and NO production, iNOS expression in DHAV-infected duckling liver was determined by qRT-PCR. As shown in Fig. 4(a), iNOS expression levels gradually increased in a time-dependent manner by SH infection, with increases of sixfold at 16 h p.i., 18-fold at 24 h p.i. and 23-fold at 36 h p.i. However, in FC64-infected ducklings, iNOS induction was at a relatively low level, with increases of fourfold at 16 h p.i., sixfold at 24 h p.i. and ninefold at 36 h p.i. These results demonstrated that SH infection induced higher levels of iNOS than FC64 infection.

These data were further verified by measuring serum NO levels in SH- and FC64-infected ducklings. In SH-infected ducklings, serum NO concentrations increased in a time-dependent manner, with peak values at 24 h p.i. (Fig. 4b). In FC64-infected ducklings, NO levels were maintained at low levels similar to those in mock-infected ducklings. SH infection induced significantly greater NO production than FC64 infection. The high level of NO production induced by SH infection may correlate with apoptosis and pathogenesis.

**FC64-immunized ducklings resist virulent SH virus infection**

As FC64 has low virulence and induced a very intensive innate immunity response, it is a potential vaccine candidate. To assess the protective effect of the FC64 strain against the virulent SH strain, 1-day-old ducklings were immunized with diluted FC64 and then challenged with virulent SH virus 5 days p.i.. As shown in Table 1, all ducklings challenged directly with SH died quickly, with typical clinical features such as appetite loss, wasting, ataxia, wryneck and opisthotonus. When the ducklings were immunized with FC64 at $10^{4.5}$ or $10^{5.5}$ ELD$_{50}$ (0.2 ml)$^{-1}$, none died after SH challenge, demonstrating the protective effect of FC64 against SH infection. When the inoculation of FC64 copy numbers was reduced to $10^{2.5}$, $10^{3.5}$ or $10^{4.5}$ ELD$_{50}$ (0.2 ml)$^{-1}$, fewer ducklings survived after challenge with SH, showing that the protective effect of FC64 against SH was dose-dependent.

To investigate the underlying mechanisms of the protective effect, livers of four ducklings were collected to examine the expression levels of innate immune response-related genes 5 days after FC64 inoculation. Results showed that levels of all innate immune response-related genes were upregulated by FC64 infection in a dose-dependent manner (Fig. 5). Compared with the peak induction level at 36 h p.i. (Fig. 4a), the induction of these genes was notably reduced to a low level at 5 days p.i. (Fig. 5), showing that the innate immune response is an early defence response to infection.

To examine the antibody levels of FC64-vaccinated ducklings, sera of ducklings immunized with $10^{3.5}$ ELD$_{50}$ (0.2 ml)$^{-1}$ FC64 were collected 5, 10, 15, 30 and 45 days...
p.i. and subjected to a neutralization antibody assay. The results in Table 2 show that the neutralization titre of antibodies in serum was 23.8 at 5 days post-immunization and gradually increased to 24.7 at 10 days post-immunization, 25.1 at 15 days post-immunization and peaked at 26.5 at 30 days post-immunization. At 45 days post-immunization, the neutralization titres of FC64 antibodies slightly decreased to 25.6, which was higher than the protective titre of 23.8 at 5 days. The results showed that the immune duration was maintained for 45 days by a single immunization of FC64, which effectively protected the ducks against DHAV infection during the susceptible period. Altogether, these results demonstrated that FC64 provided effective protection against SH infection in ducklings by inducing the adaptive immune response.

DISCUSSION

Different DHAV strains cause disease ranging from mild to severe in infected ducklings. The severe form is life-threatening and causes significant economic losses to the duck-breeding industry. However, most previous studies of DHAV infection have focused only on the pathogenicity and host response of one virus strain (Song et al., 2013). In the current study, we compared differences in pathogenicity and innate immune responses induced by the virulent SH strain and attenuated FC64 strain in ducklings. As the attenuated FC64 strain did not cause fatalities, an inoculation of 10^3.5 ELD50 was used in this study. Although fewer SH virus copies were used for inoculation of the ducklings than FC64 virus copies (determined by genomic RNA level), greater numbers of SH virus RNA copies were detected in different organs at 36 h p.i., suggesting that the SH strain replicated more efficiently than the FC64 strain in ducklings. Our results showed that the liver, spleen and intestine are the primary target organs of these two DHAV strains. Increased liver lesions, liver dysfunction, apoptosis, serum NO levels and death were observed by SH infection, but not by FC64 infection. SH induced a moderate innate immune response at 24 h p.i., whilst FC64 induced an intensive innate immunity response at 36 h p.i. Moreover, FC64 infection stimulated production of high anti-DHAV antibody levels in infected ducklings. The strong immunostimulatory effect and low virulence of FC64 indicates that it is a good candidate for vaccine development.

The liver is the primary target organ of DHAV infection, resulting in liver dysfunction. Microscopic analysis revealed condensed nuclei and focal necrosis of hepatocytes together with the presence of acidophilic bodies and increased heterophilic infiltration after infection with SH, in accordance with the findings of a previous study (Hu et al., 2002). Less pathological change was observed in FC64-infected duckling livers. The impaired liver function by SH infection was also reflected by enhanced expression of the serum biochemical markers ALT, AST, ALP, GGT and T-Bil. The TUNEL assay results and caspase-3 cleavage showed that SH infection induced apoptosis in the duckling liver, which was the most prominent site of apoptosis and sustained the most severe DHAV-induced pathological change. No apoptosis was observed in FC64-infected duckling livers. The induction of apoptosis by SH infection may be correlated with the observed liver lesions and subsequent liver dysfunction, and may be attributed to virus virulence (Ito et al., 2002).

The innate immune response is an early host defence response to pathogen infection and can constrain the initial
infected cells and provoke uninfected cells to mount an antiviral proteins (ISGs), including IFITM1, ISG12 and OASL. IFN and ISGs may inhibit virus replication in the infected cells and provoke uninfected cells to mount an antiviral defence (Darnell et al., 1994; Takaoka & Taniguchi, 2003). In FC64-infected ducklings, IFITM1 expression was increased up to 329-fold, ISG12 up to 7963-fold and OASL up to 7250-fold. The robust induction of ISGs by FC64 infection is key to restricting FC64 to low replication levels, thereby aiding the host duckling to clear the virus and remain healthy. Meanwhile, the relatively low induction level of IFN and ISGs by SH infection may contribute to the robust replication and pathogenicity observed with this strain.

Amongst the cytokines induced by DHAV infection, only IL-2 and IFN-γ were strongly upregulated by SH infection, and IL-2 may be the inducer of IFN-γ (Ye et al., 1995). Previous reports have shown that IFN-γ production upregulated iNOS mRNA expression (Chan & Riches, 2001; Lorsbach et al., 1993; Melillo et al., 1994). The high levels of iNOS and NO in SH-infected ducklings may be correlated with the induction level of IFN-γ. NO is produced primarily as an effector molecule as part of the host defence response against viral infection (Bogdan et al., 2000; Karupiah et al., 1993; Kreil & Eibl, 1996) and also plays a role in pathogenesis (Suzuki et al., 2014). An abundance of NO reacts with superoxide anion to form peroxynitrite, which has a wider range of chemical targets. Peroxynitrite is able to change the catalytic activity of enzymes, alter cytoskeleton organization, impair cell signal transduction, and induce apoptosis and necrosis. Peroxynitrite can also break DNA strands and inhibit DNA ligase activity, which increases the frequency of DNA strand breaks and activates DNA repair mechanisms, including poly(ADP) ribose polymerase. Therefore, in SH-infected ducklings, upregulation of IL-2 and IFN-γ expression may induce expression of iNOS and NO production, contributing to apoptosis and pathologies.

In conclusion, our findings demonstrated that the innate immune response contributes to disease severity and viral replication efficiency in ducklings infected with different DHAV strains. However, further studies are required to

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**Table 2. Antibody levels of ducklings immunized with FC64**

Sera from FC64-immunized ducklings were collected at 5, 10, 15, 30 and 45 days p.i., and subjected to neutralization assay using chicken embryos. Sera from ducklings vaccinated with AV2111 and from normal ducklings were used as positive and negative controls, respectively. Chick embryos inoculated with FC64 were also included as controls.

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Amongst all the cytokines induced by DHAV infection, IL-6 functions to stimulate the immune response and induces production of major acute-phase proteins (Andus et al., 1987; Gauldie et al., 1987), as well as the production of neutrophils in the bone marrow. IL-6 also supports the growth of B cells and is antagonistic to regulatory T cells, as well as playing an important role against pathogen infection (Wong et al., 1988). In this study, SH infection induced a 170-fold increase in IL-6 expression at 24 h p.i. and maintained this level until 36 h p.i. However, FC64 infection induced a 44-fold increase in IL-6 expression, which dramatically increased to 15 414-fold at 36 h p.i. The intensive induction of IL-6 by FC64 infection may help the host animal to clear the viral infection and activate the adaptive immune response. Indeed, antiviral antibodies were produced in FC64-infected ducklings by 5 days p.i.; the antibodies were persistently elevated and peaked by 30 days p.i., effectively protecting them from SH attack.

The induction of IFN induced expression of a set of antiviral proteins (ISGs), including IFITM1, ISG12 and OASL. IFN and ISGs may inhibit virus replication in the infected cells and provoke uninfected cells to mount an adaptive immune response. Indeed, antiviral antibodies were produced in FC64-infected ducklings by 5 days p.i.; the antibodies were persistently elevated and peaked by 30 days p.i., effectively protecting them from SH attack.

As previously reported, IFN-β is induced by SH infection, which may help the host to limit FC64 replication and further induce the adaptive immune response.
identify viral factors that contribute to the different host innate immune responses. Considering that the attenuated FC64 strain replicates at low levels in vivo, induces slight liver damage, provokes a strong innate immune response, induces high levels of antibodies and provides ducklings with effective protection against virulent DHAV strain infection, it is a good DHAV vaccine candidate. Hence, further work is in progress to develop DHAV as a mature vaccine.

**METHODS**

**Virus preparation.** Two DHAV strains were used in this study: SH (GenBank accession number HQ265433) and FC64 (GenBank accession number HQ232303). Both SH and FC64 strains belong to DHAV-1, and were propagated in embryonated duck eggs and embryonated chicken eggs, respectively (Song et al., 2011a, b). The SH strain was isolated from a duckling with liver ascites in Shanghai, China, whilst the attenuated FC64 strain was originally passaged for 64 generations in specific pathogen-free eggs, respectively (Song et al., 2011a, b). Both SH and FC64 strains belong to DHAV-1, and the FC64 strain was originally passaged for 64 generations in specific pathogen-free eggs, respectively (Song et al., 2011a, b). Both SH and FC64 strains belong to DHAV-1, and the FC64 strain was originally passaged for 64 generations in specific pathogen-free eggs, respectively (Song et al., 2011a, b). Both SH and FC64 strains belong to DHAV-1, and

**Duckling and virus inoculation.** One-day-old Peking ducklings (n=36) were purchased from Zhihang Hang Duck Farm, and housed in cages with free access to food and water during the study period. The temperature was maintained at 28–30 °C and the air was changed 8–10 times h⁻¹. All ducklings were obtained from flocks with no prior exposure to DHAV, as confirmed by negative antibody test results. The ducklings were randomly divided into three groups: two experimental groups received subcutaneous injections of 10⁵ LD₅₀ (SH strain) or 10⁷ ELD₅₀ (FC64 strain), respectively, to the neck, whilst the third group served as a control. All ducklings were maintained in accordance with the Institutional Animal Care and Use Committee guidelines of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science.

**Pathological identification and histological examination.** Clinical signs of the experimental and control duckling groups were recorded daily following DHAV infection. Infected ducklings were sacrificed and the livers were collected for histological analysis, which was performed as described previously (Cheng et al., 2001). After fixation in 4% formalin for 24 h, the liver samples were dehydrated with graded alcohol, embedded in paraffin and sliced into 4 μm sections, which were stained with haematoxylin and eosin and observed under a light microscope (Eclipse TS100; Nikon).

**Measurement of AST, ALT, ALP, GGT and T-Bil activity.** The serum concentrations of the enzymes ALT, AST, ALP, GGT and T-Bil were determined using a BS-200 chemical analyser (Mindray). All statistical analyses were performed using SPSS v. 13.0 software (SPSS). The results are expressed as mean ± SD (P<0.05).

**TUNEL assay.** The 4 μm sections were examined by TUNEL whilst mounted on poly-l-lysine-coated slides, subsequently de-waxed by immersion in fresh xylene for 5 min at room temperature and then rehydrated in a series of graded alcohol. In situ detection of fragmented DNA was performed using a Cell Death Detection kit (POD; Roche Diagnostics) according to the manufacturer's instructions. Apoptotic cells were observed under a light microscope.

**Western blot analysis.** Western blot analysis was performed to assess caspase-3 cleavage. Briefly, 1 g infected liver tissue was harvested at 16, 24 and 36 h p.i., ground, and then lyzed in lysis buffer (Beyotime). The samples were subjected to 10% SDS-PAGE and Western blot analysis. Briefly, proteins were transferred onto PVDF membranes (Bio-Rad) and the membranes were blocked with 5% BSA in Tris-buffered saline with Tween 20 (TBST) for 1 h. Membranes were then incubated with anti-caspase-3 antibody (Abcam) at a dilution of 1:1000 in blocking buffer for 1 h at room temperature. After washing three times with TBST, the membranes were incubated with anti-rabbit HRP-conjugated secondary antibodies (diluted to 1:1000 in blocking buffer) at room temperature for 1 h. After washing three times with TBST, signals were developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and exposed to X-ray film (FujiFilm).

**RNA extraction and qRT-PCR.** Total RNA from 0.1 g SH- or FC64-infected liver tissues was extracted using TRizol reagent (Invitrogen) according to the manufacturer's instructions and then treated with DNase to remove genomic DNA. Total RNA (3 μg) was used to perform reverse transcription using Moloney murine leukemia virus reverse transcriptase (TaKaRa) and oligo-dT primers. An equal volume of cDNA was then subjected to qRT-PCR using SYBR Green Real-Time RT-PCR Master Mix Plus (Promega) and appropriate primers. The primers for IFN-α (Cagle et al., 2011), IFN-β, IFN-γ, IFITM1, ISG12, OAS1 (Vanderven et al., 2012), IL-6, IL-2, iNOS and Arhp (acidic ribosomal phosphoprotein) are listed in Table 3. Arhp was used as a control (Sun et al., 2013). Reactions were performed in triplicate using an ABI PRISM 7500 Real-Time RT-PCR System (Applied Biosystems). Expression fold was determined using the 2⁻ΔΔCt method with Arhb as an endogenous reference gene to normalize the level of target gene expression.

Virus titers of inoculation and virus titers in heart, spleen, lung, kidney, brain, intestine and muscle were determined by detection of the expression levels of the DHAV 3D gene using a one-step real-time TaqMan RT-PCR assay (Yang et al., 2008). Primers for DHAV 3D and DHAV FP are listed in Table 1. Briefly, a standard plasmid encoding the DHAV 3D gene was constructed, serially diluted 10-fold and quantified using real-time TaqMan RT-PCR assay. A regression curve was constructed to plot the threshold cycle values versus the logarithm of the copies. Analysis was based on the data for generating the standard curve to obtain an equation and Cn values for each sample were determined. Reactions were performed in triplicate using an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems).

**NO assay.** NO level was measured in serum collected at 16 and 24 h p.i. from four ducklings from each group. Serum was passed through a 10 kDa ultrafilter (Millipore) prior to analysis. The total protein content in the filtered serum was measured using a DC Protein Assay (Bio-Rad). A NO Synthase Assay kit (Calbiochem-Novabiochem) was used to measure the NO content in 40 μl filtered serum according to the manufacturer's instructions (Cagle et al., 2011; Pantin-Jackwood et al., 2012). Values were normalized to the total protein content in the filtered serum.

**Protective test.** One-day-old ducklings were randomly divided into experimental and control groups (n=10 each). Ducklings in the experimental group were subcutaneously inoculated with attenuated FC64 virus in 10-fold serial dilutions. Five days later, all ducklings were challenged with 10⁵ LD₅₀ of virulent SH virus to observe the protective effects of the attenuated FC64 strain.

**Neutralization test.** After immunization with 10⁻⁵ ELD₅₀ (0.2 ml)⁻¹ FC64, sera of ducklings were collected at different time points to examine the antibody levels by neutralization test; 10⁻⁵ ELD₅₀ (0.2 ml)⁻¹ FC64 was used as the virus copy number sufficient to protect ducklings from SH attack (Table 2). Briefly, serum from four FC64-immunized ducklings was mixed and inactivated at 56 °C for 30 min, and diluted in 2⁻¹, 2⁻², 2⁻³, 2⁻⁴, 2⁻⁵, 2⁻⁶, 2⁻⁷ and 2⁻⁸
serial dilutions. The diluted sera were then mixed with FC64 strain [final concentration 100 ELD$_{50}$ (0.2 ml)$^{-1}$] in a 1 : 1 ratio at 37 °C for 1 h. Each mixture was inoculated into five 9-day-old chicken embryos through the urinary cavity. The number of dead chicken embryos was recorded and neutralization titres were calculated using the Reed–Muench method. Sera from four ducklings immunized with the AV2111 strain (purchased from China Institute of Veterinary Drugs Control) were included in the neutralization test as positive controls and sera from four normal ducklings were included in the neutralization test as negative controls. Chick embryos directly inoculated with 100 ELD$_{50}$ (0.2 ml)$^{-1}$ FC64 were also included as controls.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


### Table 3. Primers used in this study

<table>
<thead>
<tr>
<th>Targets</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
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</thead>
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<tr>
<td>IFN-β</td>
<td>CCTCAACGAGAATCCAGATT</td>
<td>GGATGCAGCTGAAAGAAGGAGG</td>
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<tr>
<td>IFN-γ</td>
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<tr>
<td>IL-6</td>
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<td>DHAV FP</td>
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