Infection, Genetics and Evolution 75 (2019) 104017

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Pathogenicity of fowl adenovirus serotype 4 (FAdV-4) in chickens

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ARTICLE INFO

Keywords:
Hepatitis-hydropericardium syndrome
Fowl adenovirus serotype 4
SPF chicken
Day-old
Experimental infection
Pathogenicity

ABSTRACT

Hepatitis-hydropericardium syndrome (HHS) is an acute infectious disease caused by fowl adenovirus serotype 4 (FAdV-4), which mainly infects broilers aged 3–5 weeks. In March 2018, a pathogenic disease, which was characterized by symptoms similar to HHS, broke out in 10-day-old broiler flocks in Shandong province. In this study, a strain of FAdV-4 (SDSG) was isolated from naturally infected broilers. To assess its pathogenicity, 10-day-old and 20-day-old specific pathogen-free (SPF) chickens were inoculated separately with the FAdV-4 virus fluid via oral and intramuscular injection routes.

The results show that typical hydropericardium and hepatitis were observed in experimental chickens. The titer levels of the virus antibody and the levels of inflammatory cytokines were upregulated, which may be caused by the infection and innate immune response. The detection of viral load showed that the presence of virus was detected in multiple organs, in which the liver contained the highest concentration of viral DNA, and the virus content in the intramuscular injection group was higher than that of the oral injection group.

In summary, these findings increase our understanding of the pathogenicity of FAdV-4 (SDSG) in chickens. The established model will be valuable for anti-viral drug testing and vaccine evaluation, which can prevent and reduce the spread of HHS in the poultry industry.

1. Introduction

Fowl adenoviruses (FAdVs), belonging to the Aviadenovirus genus of the family Adenoviridae, are non-enveloped double stranded DNA-viruses, which can be grouped into 5 species (FAdV-A to FAdV-E) with 12 serotypes (FAdV-1 to 12) (Hess, 2000; McFerran and Smyth, 2000). FAdV-4 is classified as a species of FAdV-C together with FAdV-10 based on the Ninth Report of the International Committee on Taxonomy of Viruses (Harrach and Kaján, 2011). FAdVs can cause acute avian infectious diseases, such as hydropericardium hepatitis syndrome (HHS), inclusion body hepatitis (IBH), and gizzard erosion in broilers and layers (Asthana et al., 2013; Domanska-Blicharz et al., 2011; Mase et al., 2010). FAdV-4 has been isolated from chickens with HHS, with each case showing similar clinical signs, including accumulation of slightly yellow transparent fluid in the pericardial sac, characteristic intranuclear inclusion bodies in hepatocytes and a high mortality of 20–80% (Ganesh et al., 2001; Toro et al., 2000; Vera-Hernandez et al., 2016). HHS was first reported in Angara Goth, Pakistan in 1987 and subsequently broke out in North America, Mexico, Eastern Europe, India, South Asia, China, Japan, and South Korea, causing economic losses mainly to the broiler industry worldwide (Choi et al., 2012; Mase et al., 2010; Mittal et al., 2014; Schachner et al., 2018).

Since mid-2015, sporadic outbreaks of HHS have been increasingly observed in commercial chicken farms in China, which causes high mortality, resulting in tremendous economic losses to poultry farmers. Therefore, strengthening the study of the disease is of great significance for protecting the development of the poultry industry in China. To determine different infection routes of FAdV-4 and the susceptibility of smaller-age-chickens, this study investigated the pathogenicity of 10 and 20-day-old SPF chickens to provide a theoretical basis for preventing and controlling this disease.

Abbreviations: HHS, Hepatitis-hydropericardium syndrome; FAdV-4, fowl adenovirus serotype 4; SPF, specific pathogen-free; IBH, inclusion body hepatitis; CAM, chorioallantoic membrane; EID 50, median embryo infectious dose; dpi, day post-injection; HE, hematoxylin and eosin; qPCR, quantitative real time polymerase chain reaction; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; OD, optical density; T cells, thymus-dependent lymphocytes; NK cells, natural killer cells

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https://doi.org/10.1016/j.meegid.2019.104017
Received 5 May 2019; Received in revised form 19 August 2019; Accepted 22 August 2019
Available online 26 August 2019

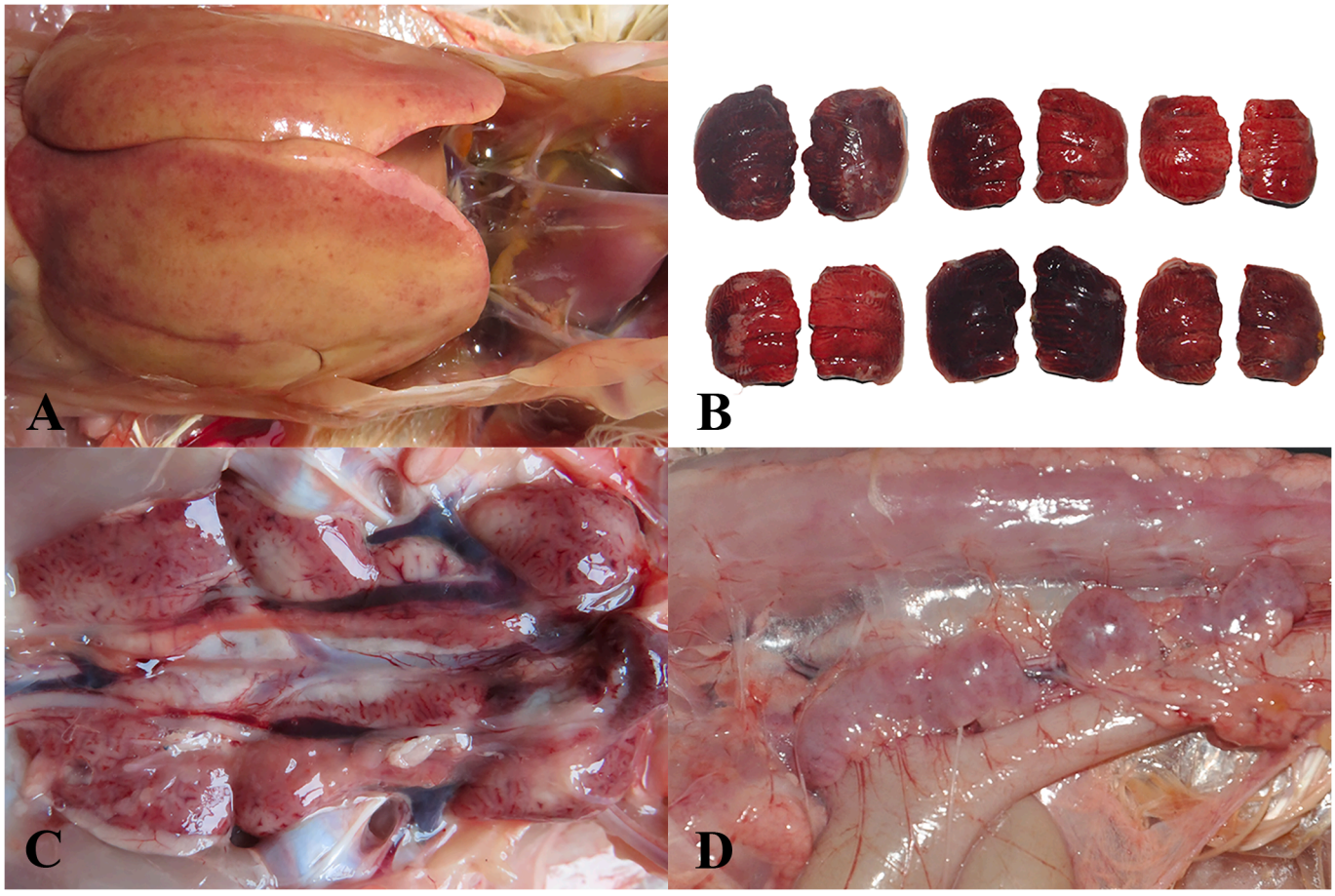

Fig. 1. Clinical investigation results. (A) Severe hydropericardium and enlarged livers with blood spots; (B, C) edema with congestion in lungs and kidney; (D) swelling and bleeding in thymus.

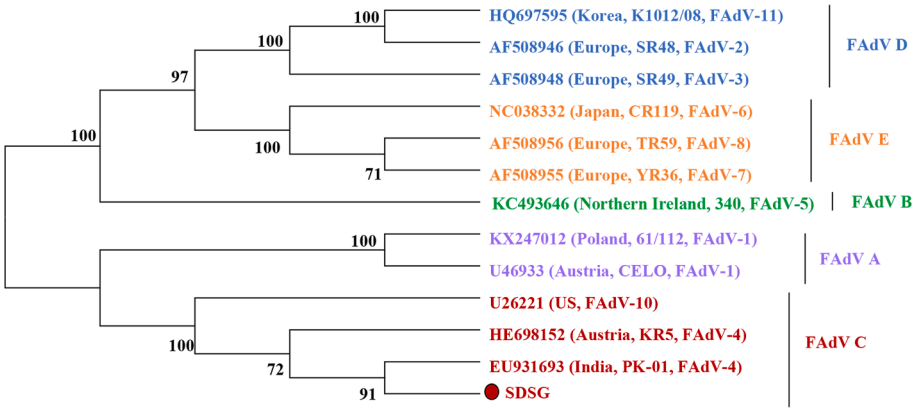

Fig. 2. Phylogenetic tree of hexon gene sequences of the isolate SDSG (marked by a red dot) and other avian strains. The phylogenetic tree was built using neighbor-joining analysis and MEGA 7.0, and the bootstrap confidence values were determined using 1000-bp replicates.

Table 1
Grouping and death status.

Grouping	Experiment	Quantity	Dead number	Mortality
10-day-old group	Oral injection	30	7	23.333%
	Intramuscular injection	30	15	50%
	Control	30	0	0
20-day-old group	Oral injection	30	5	16.667%
	Intramuscular injection	30	5	16.667%
	Control	30	0	0

2. Materials and methods

2.1. Ethics statement

All the animal infection experiments were carried out in accordance with international, national, and institutional guidelines. The animal procedure was approved by the Committee on the Ethics of Animals of Shandong (permit number: 2017360331). The chickens used in the experiment at Shandong Agricultural University were euthanized using rapid cervical dislocation.

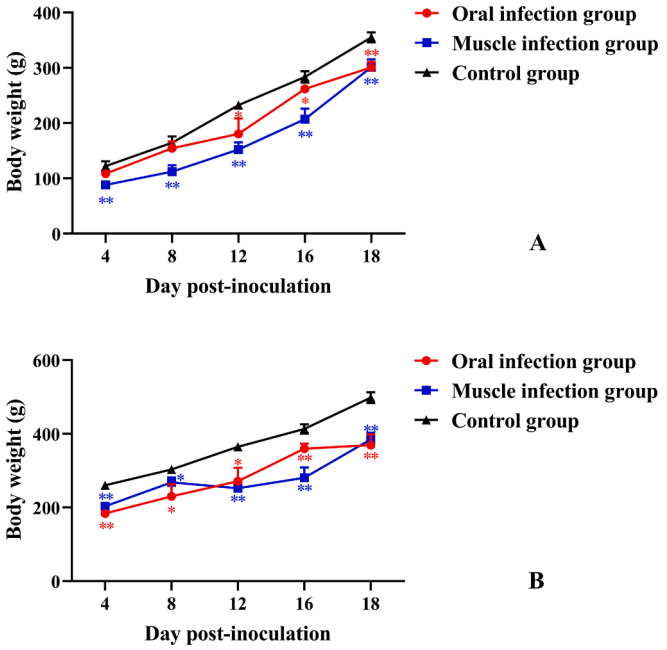

Fig. 3. Weight changes after injection. (A) 10-day-old group; (B) 20-day-old group.

2.2. Animals

All SPF White Leghorn line chickens were purchased from Poultry Science and Technology Co., Ltd., Jinan Spirax Ferrer. The chickens were maintained in specific pathogen-free (SPF) chicken isolators with ad libitum feeding. Serum and swab samples of experimental chickens were collected before inoculation, using serum neutralization and polymerase chain reaction (PCR) tests to confirm that all chickens used in this study were serologically negative for FAdV-4.

2.3. Samples collection

In March 2018, a pathogenic disease, which was characterized by symptoms similar to HHS, broke out in broiler flocks in several farms (located in Shenxian, Gaotang and Juxian) in Shandong province. The age of the diseased broilers was primarily around 10 days, and it is known that broilers are not vaccinated with FAdV. We collected 87 liver samples from the sick and dead chickens for FAdV detection. The collected samples were kept at −4 °C until being detected.

2.4. DNA sequencing

DNA was extracted by phenol-chloroform method from liver samples. To detect the virus, PCR was performed using the primer pair forward: 5′- GCCACTACCAACTTCTACTTCG −3′; reverse: 5′- AGAGG AACTTCTTGTAGCTGAGG −3′. The entire hexon open reading frame of isolate was amplified, sequenced, and analyzed to identify the serotypes according to previous study (Tang et al., 2009). PCR was carried out in a total volume of 20 μL containing 1 μL (10 pmol) of each primer, 10 μL of Taq HS Perfect Mix (TaKaRa, Beijing, China), 2 μL of DNA and 6 μL of nuclease-free water. Reactions were performed according to the following protocol: 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min 30 s, and a final elongation step of 3 min at 72 °C. All PCR products were cloned into pMD18-T vector and transformed into DH5α E. coli-competent cells, and the positive clone was sequenced (BGI Company Ltd., Beijing, China).

Phylogenetic analysis based on the nucleotide sequences of hexon genes of the positive samples and other FAdV was constructed using MEGA7 software with neighbor-joining method.

2.5. Virus isolation

Samples that were positive by PCR detection were used for virus isolation. In brief, the homogenates were centrifuged at 8000 ×*g* for 10 min, and the supernatants were filtered through 0.22-μm filters. The isolates were passaged 5 times in SPF chicken embryos via the chorioallantoic membrane (CAM) route. The isolated strain was designated as SDSG and the median embryo infectious dose (EID 50) was determined to be $10^{-7.56}$/0.2 mL by the Reed and Muench method (Reed and Muench, 1938). Allantoic fluids were harvested as the challenge virus in the present study and stored at −80 °C.

2.6. Experimental design

Animal regression experiment was performed with SDSG strain virus. Ninety 10-day-old SPF chickens were randomly divided into 3 groups: oral injection group (30), muscle injection group (30), and control group (30). The chickens in the experimental group were inoculated with 1 mL of FAdV-4 (SDSG strain; EID 50, 10–7.56/0.2 mL) either orally or by intramuscular route. The control group was inoculated with equal doses of physiological saline at the same injection site. The 3 groups were separately reared in different SPF chicken isolators. Water and food were autoclaved before feeding and automatically refilled. The temperature of each house was maintained using radiators at 20–24 °C. The chicken feces was manually cleaned every day. The same inoculation dose and test method were adopted in 20-day-old chickens. The chickens were observed daily for clinical signs.

2.7. Body weight and histopathology

Five chicks from the infected and control groups were respectively and randomly drawn for weight detection and necropsy in each of 4th, 8th, 12th, 16th, and 20th days post-injection (dpi) (chicks were euthanized). Chicks were examined for the presence of gross lesions of the heart, liver, spleen, lung, kidney, thymus, pancreas, and bursa of Fabricius. Each organ was sectioned into 3 portions: one was placed into 10% formalin, routinely processed, and embedded in paraffin-wax, sections were cut approximately 4-μm-thick and stained with hematoxylin and eosin (HE), and all stained sections were examined by light microscopy; the second was stored at −80 °C for virology; and the third was stored at −80 °C for quantitative real time PCR.

2.8. Viral DNA extraction

The viral DNA of tissue samples was extracted from the previously stored organs using the Universal Genomic DNA Kit (Beijing ComWin Biotech Co., Ltd.) according to the manufacturer's instructions. The concentration of high-quality DNA samples (i.e., A260/280 was between 1.8 and 2.2, A26/230 ≧2.0) was normalized to 50 ng/ul, and used in subsequent experiments.

2.9. QPCR analysis

To determine the viral load in tissues, real-time PCR was performed using the method previously established in our laboratory for the detection of FAdV-4. The primers used in this study are as follows: forward: 5′ -CGTCAACTTCAAGTACTC-3′ and reverse: 5′ -AGAGGATGCT CATGTTAC-3′. The relative TaqMan probe was a 24 bp oligonucleotide,5′ FAM-CCTACTCAGATGGAGGCTTCTACC-3′ TAMRA, which was labeled with FAM at the 5 end and TAMRA at the 3 end (Yu et al., 2018). QRT-PCR analysis was performed with Premix Ex Taq™ (Probe qPCR) (TaKaRa, China) following the manufacturer's instructions, which contained 10 μL of Premix Ex Taq, 0.4 μL of forward primer, 0.4 μL of reverse primer, 0.2 μL of ROX Reference Dye II, 6.2 μL of ddH2O, 0.8 μL of probe, and 2.0 μL of DNA template. Amplification and detection were performed with an Applied Biosystems® 7500 FAST

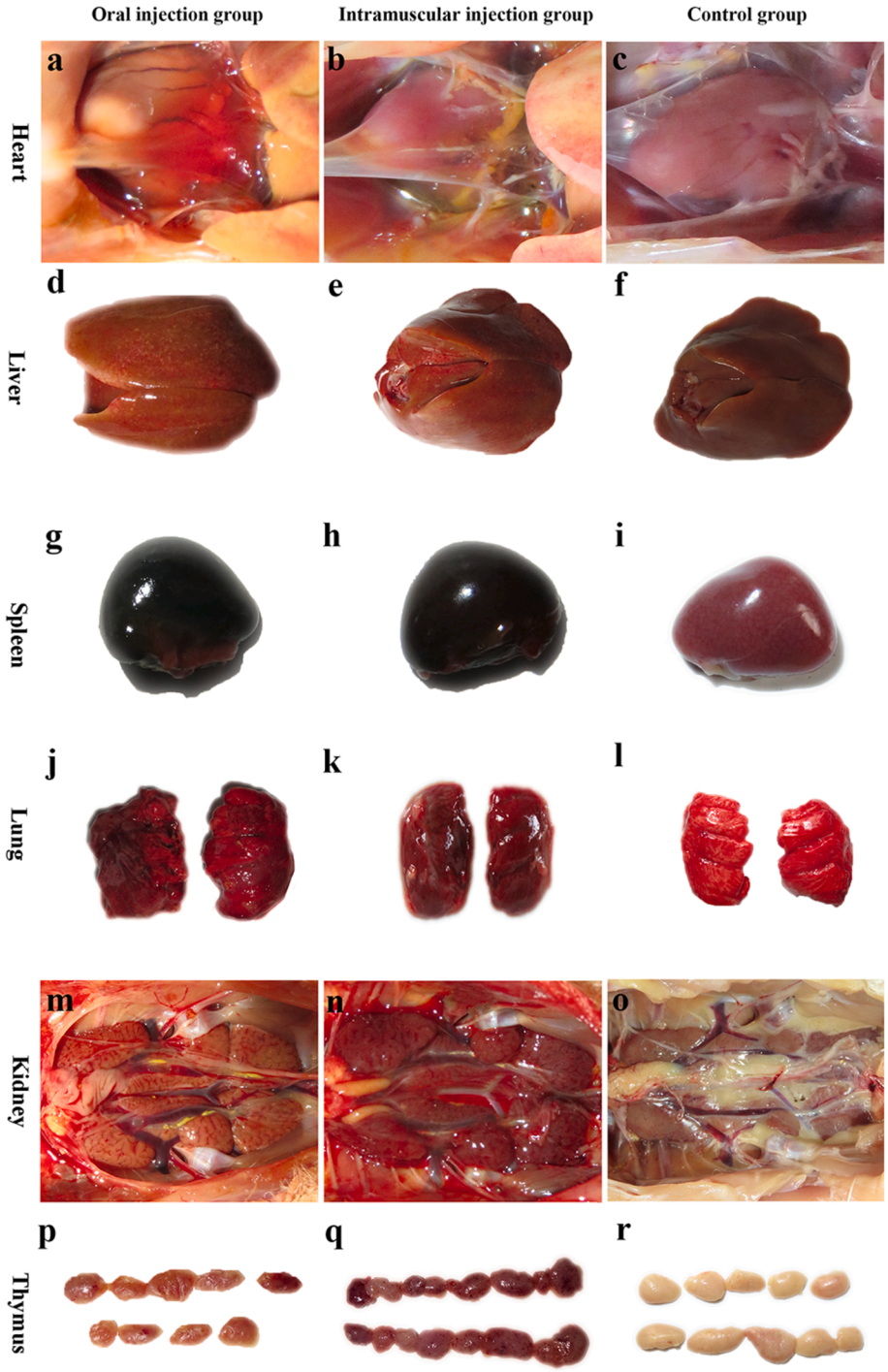

Fig. 4. Gross lesions of 10-day-old group chickens inoculated with FAdV-4.

Real-Time PCR System under the following conditions: 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 62 °C for 32 s. The fluorescent signals were measured during the annealing step. Each DNA sample was processed with 3 replicates to guarantee the reproducibility of the amplification.

2.10. Determination of biochemical indexes, cytokines, and antibodies

On the 4th, 8th, 12th, 16th, and 20th days after the injection, 5 chickens were selected randomly, bled, and euthanized. Serum samples were harvested for detecting and recording the changes of blood biochemical indexes, cytokines, and antibodies.

Biochemical indexes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), were detected through the testing organization ADICON CLINICAL LABORATORIES, INC (Jinan, China).

Cytokines were detected using the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit of chicken interleukin 6 (IL-6) and interferon-γ (IFN-γ) (Lengton, Shanghai, China) according to the manufacturer's directions.

The FAdV-4-specific antibodies were determined by the indirect ELISA which developed in previous work in our laboratory (Niu et al., 2017). In brief, 47 μg/mL of recombinant hexon protein in carbonate buffer solution was incubated in black Maxisorb 96-well plates (Greiner

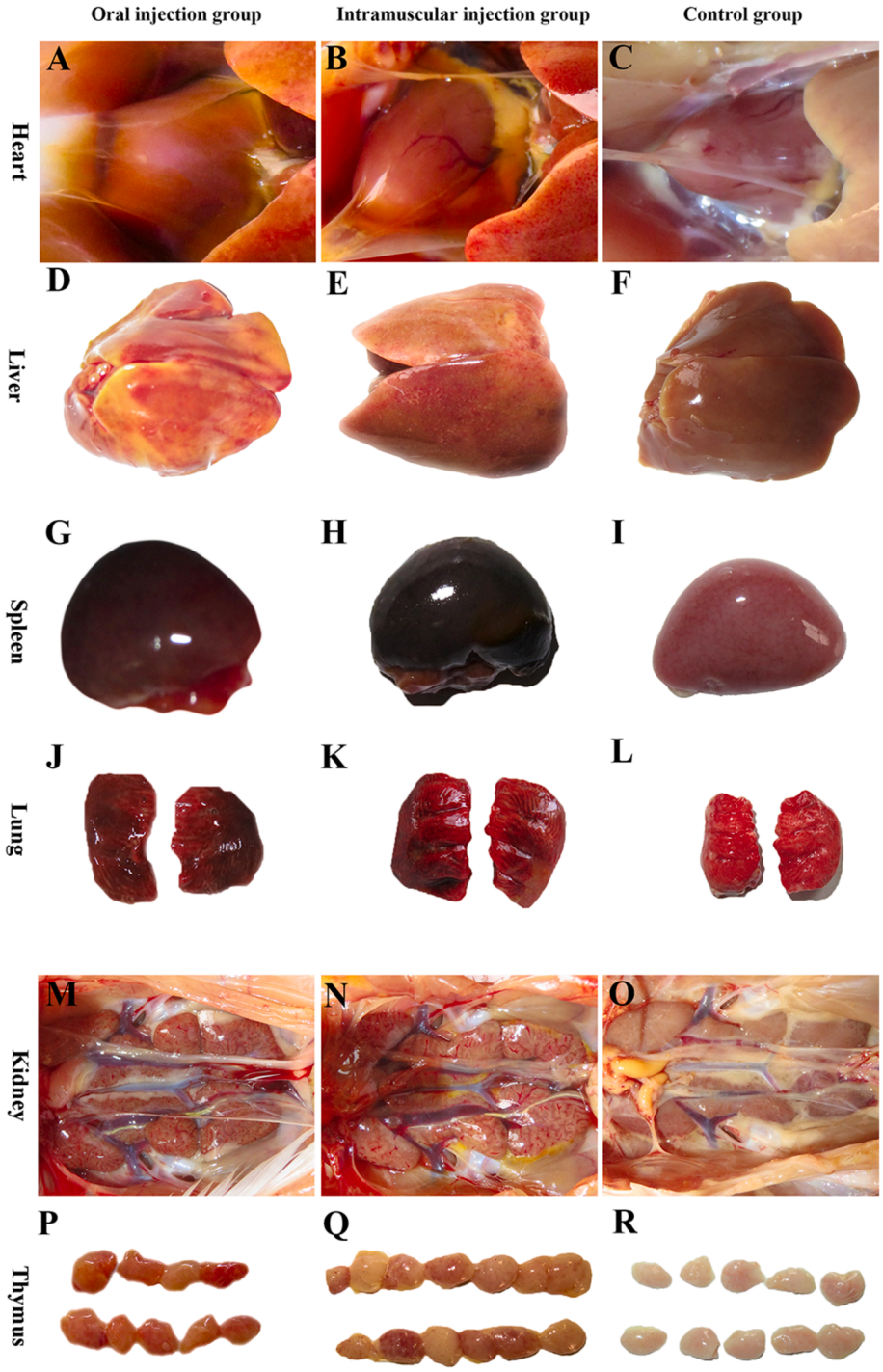

Fig. 5. Gross lesions of 20-day-old group chickens inoculated with FAdV-4.

Bio-One, Germany) overnight at 4 °C, and the plates were blocked by 5% non-fat dry milk (Solarbio, Beijing, China, w/v) for 2 h at 37 °C. Then, serum (1:10) was added at 200 μL/well and incubated at 37 °C for 1 h. Afterward, the plates were incubated with rabbit anti-chicken immunoglobulin G (IgG, 1: 800) (Solarbio, Beijing, China) conjugated to horseradish peroxidase (Sigma-Aldrich) at 100 μL/well. The plates were incubated at 37 °C for 1 h, and 3′, 3′, 5′, 5′-tetramethylbenzidine substrate solution (TransGen, Beijing, China) was added to each well (100 μL/well). Fifty microliters of 3 M H2SO4 (Wuxi JINGKE Chemical CO., LTD.) was added to stop the reaction, and the optical density (OD) values were observed at 450 nm using an automated ELISA plate reader (Thermo, Waltham, MA, USA).

2.11. Statistical analysis

All results are presented at means ± standard deviations and analyzed using the one-way analysis of variance (ANOVA) procedure of GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). *P* values of < 0.05 or < 0.01 were used to define statistical significance.

3. Results

3.1. Clinical investigation

The PCR results showed that 79 of the 87 samples were positive for

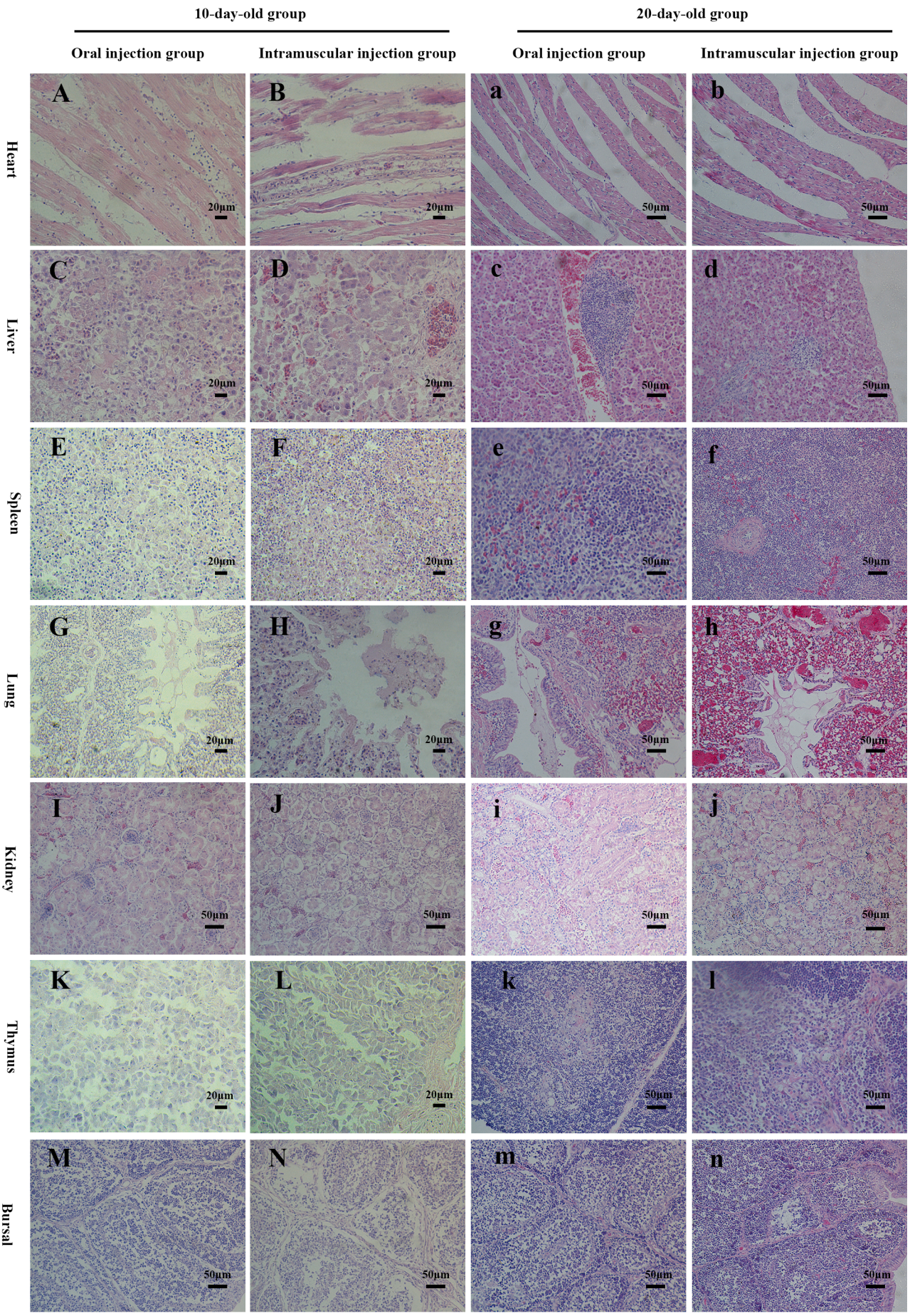

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Fig. 6. Histopathologic changes of FAdV-4-infected chickens. (A–H) (K) and (L) Magnification, ×400; other magnification, ×200.

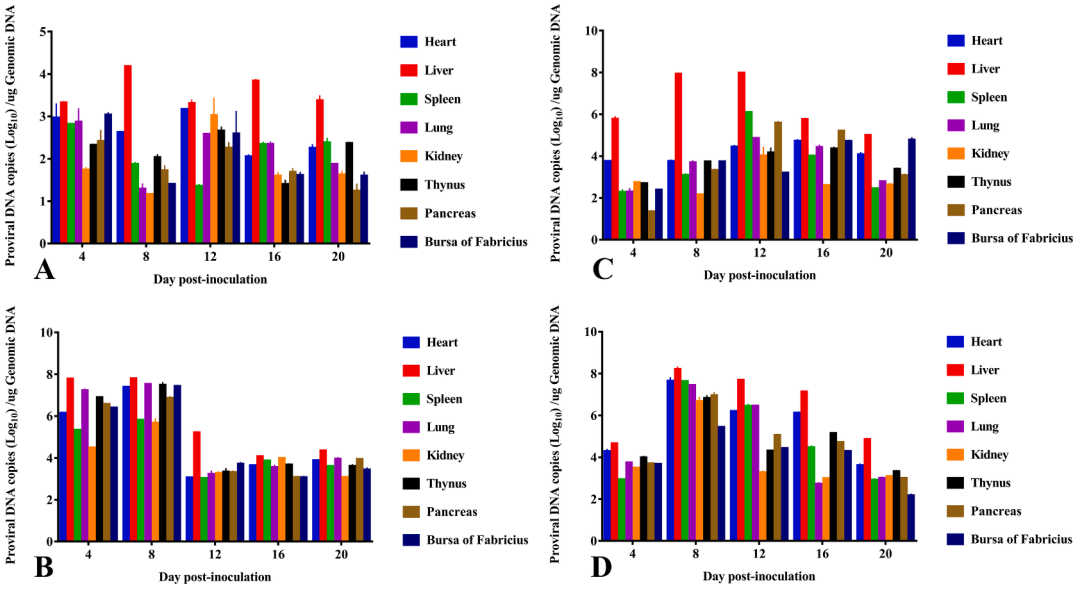

Fig. 7. The viral copies in tissue samples of the challenge group. (A) 10-day-old oral injection group; (B) 10-day-old intramuscular injection group; (C) 20-day-old oral injection group; (D) 20-day-old intramuscular injection group.

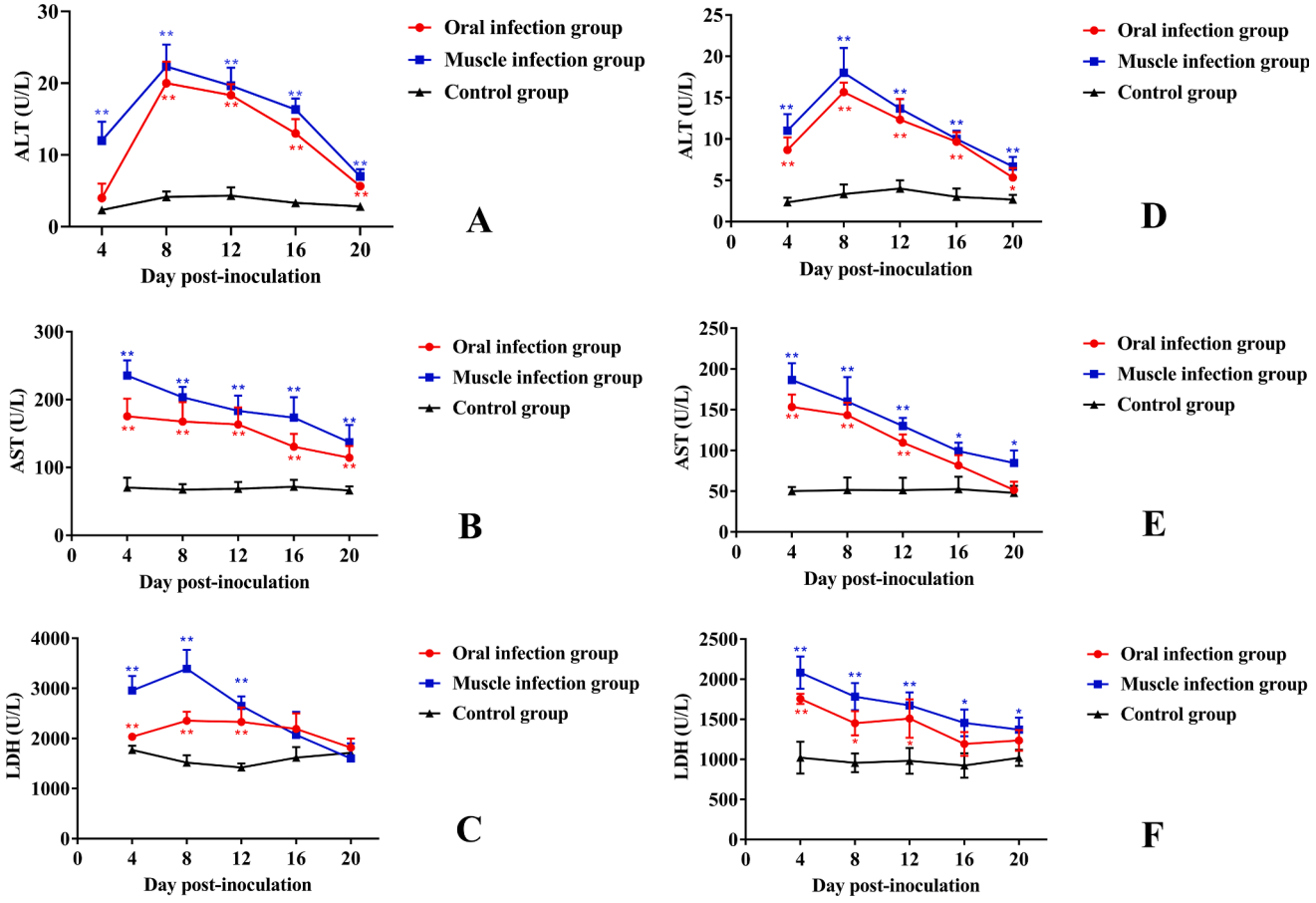

Fig. 8. Dynamics of ALT, AST, and LDH in serum of the chickens post artificially infected with FAdV-4. (A–C) 10-day-old group; (D–F) 20-day-old group.

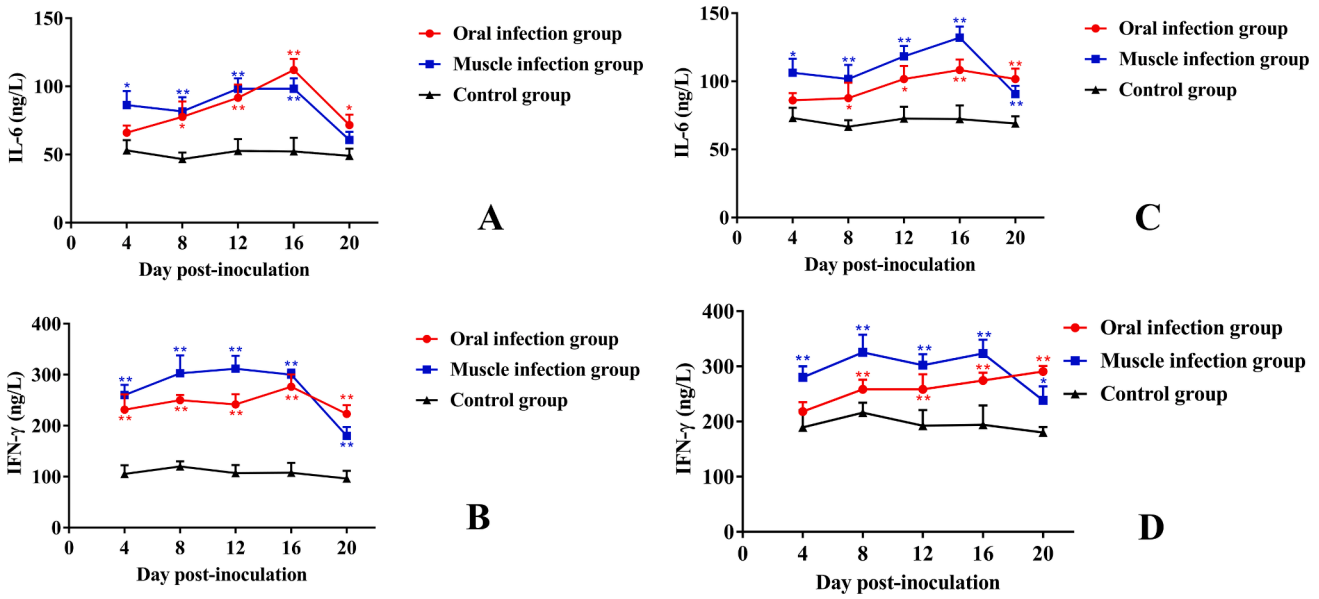

Fig. 9. Dynamics of IL-6 and IFN-γ in serum of the chickens post artificially infected with FAdV-4. (A–B) 10-day-old group; (C–D) 20-day-old group.

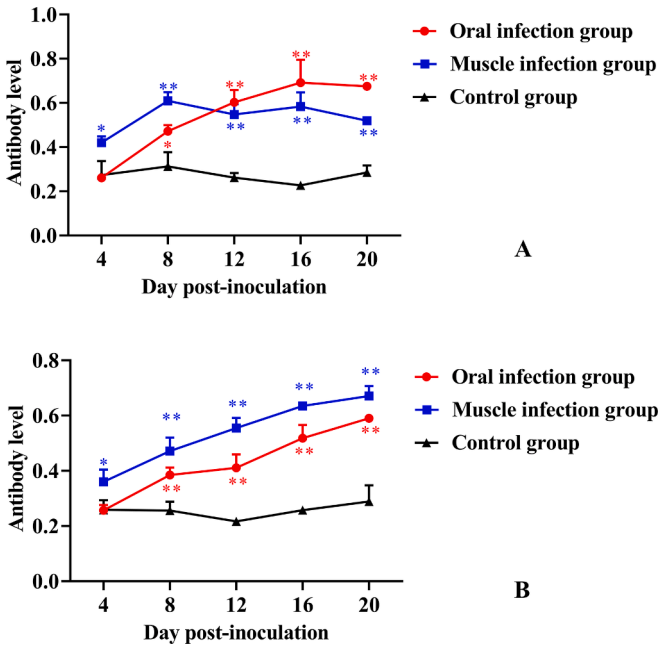

Fig. 10. Dynamics of antibodies against FAdV-4 in serum of the chickens post artificially infected with FAdV-4. (A) 10-day-old group; (B) 20-day-old group.

FAdV. Postmortem examination of the 79 chickens showed severe lesions, including hydropericardium in the heart, hemorrhaging and enlargement of livers, edema with congestion in lungs and kidneys, and swelling and bleeding in the thymus (Fig. 1(A)–(D)). The remaining 8 had no obvious lesions.

3.2. Sequence analysis of hexon gene

The isolate was designated as SDSG (GenBank accession: MK424834). Multiple alignments showed that the hexon genome of the isolate shared 60.7–99.7% of the nucleotides and 66.4–99.9% of the deducted amino acid sequences with those of representative strains from different lineages. As shown in Fig. 2, the phylogenetic tree for hexon genes was constructed based on the sequences obtained from the GenBank database, and it revealed that the isolate SDSG was clustered together with chicken FAdV-4 isolates (PK-01 and KR5) (Steer et al., 2009).

Notably, the SDSG strain shared 99.7% of the nucleotide and amino acid sequences with the India-isolated strain (EU931693, PK-01), which indicated that this strain might have originated from early India isolates.

3.3. Clinical symptoms

After infection with FAdV-4, the injection group of 10-day-old and 20-day-old SPF chickens showed signs of depression, lack of appetite, and stunted growth. The chickens of the control group did not demonstrate any clinical symptoms throughout the experiment. The mortality rate of the 10-day-old muscle injection group reached 50% (Table 1). Chickens in the injection group grew slowly, and the body weights were lower than those of the control group (Fig. 3).

3.4. Pathologic change

Dissecting the chickens showed that hearts have a severe pericardial effusion; livers are swollen with bleeding spots. In addition to the above typical clinical symptoms, the infected chickens also presented significant lesions characterized by swelling with hemorrhage in spleens, edema with varying degrees of congestion in lungs and kidneys, and swelling with different extents of bleeding in the thymus. The chickens of the control group had no obvious necrotic lesions (Figs. 4 and 5).

3.5. Histopathologic analysis

Histopathological analysis showed that chickens from both experimental groups had obvious histopathological changes (Fig. 6). The heart showed interstitial edema accompanied with lymphocytic infiltration. Also, the 10-day-old muscle injection group (Fig. 6(B)) exhibited myocardial fiber granular degeneration, necrosis, and hemorrhage. The livers exhibited hepatocyte steatosis, necrosis, and hemorrhage, and the 10-day-old muscle injection group (Fig. 6(D)) also exhibited hepatocellular nuclear concentration and dissolution necrosis. Lesions in the spleens of 10-day-old injection group (Fig. 6(E), (F)) were lymphocyte of white pulp decreased and necrosis, but in the 20-day-old injection group (Fig. 6(e), (f)) were hemorrhage in the white pulp and increase in the number of lymphocytes; In the lungs, there were exudates in the bronchi, as well as capillary congestion. Lesions in

the kidneys were characterized by renal tubular epithelial cell degeneration and renal interstitial hemorrhage. In the thymus, a large amount of necrosis of lymphocytes in the medulla was observed. Lesions in bursas were characterized by reduction and necrosis of lymphocytes; in particular, the muscle injection group was more serious than the oral injection group.

3.6. Real-time PCR analysis

After injection, the presence of virus was detected in the heart, liver, spleen, lung, kidney, thymus, pancreas and bursae of both the oral and intramuscular groups (Fig. 7). Notably, more FAdV-4 copies were found in the liver than in other tissues. Moreover, the overall number of virus copies in the tested tissues was higher in the intramuscular group than in the oral group.

3.7. Detection of biochemical indices

The results of serum biochemical indices is presented in Fig. 8. In the 10-day-old group, the AST, ALT, and LDH of experimental groups were higher than those of the control group. Briefly, ALT reached the peak at 8 dpi ($p < .01$), AST at 4 dpi ($p < .01$), and LDH at 8 dpi ($p < .01$). After reaching peak levels, the above biochemical indices declined with time. The results of serum biochemical indicators in the 20-day-old group were similar to those in the 10-day-old group, except the LDH reached the peak at 4 dpi ($p < .01$).

3.8. Detection of cytokines in serum

As Fig. 9 shows, in the 10-day-old group, the levels of IL-6 in the serum reached the peak at 16 dpi and then tended to decline. The level of the IFN-γ in the intramuscular group was higher than that in the oral group, which showed a slowly upward trend, but decreased rapidly after reaching the peak, and was finally lower than the oral group. In the 20-day-old group, IL-6 levels rose slowly and peaked at 16 dpi. The IFN-γ levels were significantly higher than in the control group ($P < .01$) at 8 and 16 dpi, but the IFN-γ level in the intramuscular injection group was downregulated rapidly after 16 dpi.

3.9. Detection of antibodies in serum

FAdV-4 antibody levels in the serum were detected throughout the experiment. As Fig. 10 shows, the positive serum could be detected earlier in the intramuscular injection group at 4 dpi, compared with the oral group. The antibody level in the experimental group continued to increase throughout the experiment; however, the antibody level in the 10-day-old intramuscular injection group showed a downward trend at 8 dpi and 16 dpi.

4. Discussion

HHS caused by FAdV-4 is a highly pathogenic communicable disease characterized by pericardial effusion and IBH (Mase et al., 2010; Schachner et al., 2018). HHS mainly infects broilers aged 3–5 weeks with a high mortality of 20–80%. Currently, this disease can also occur in hybrid chickens, breeders, and layers (Ye et al., 2016), resulting in considerable economic losses in China.

At present, a few studies have been reported on the pathogenicity of FAdV-4 in chickens such as one on oral infection of 3-week-old SPF chickens (Zhao et al., 2016), and another on both oral and intramuscular infection of 40-day-old chickens (Li et al., 2016). However, these studies were all conducted on chickens older than 3 weeks of age. There is no article about the pathogenicity of smaller-day-old SPF chickens via different injection routes. Some studies included a small portion of data on the pathogenicity of FAdV-4 to chickens (Li et al., 2018a,b; Ren et al., 2019), but they did not systematically and comprehensively test cytokines, biochemical indices, and antibody levels in infected chickens. Existing articles lack this part of the data. The pathogenesis of the virus in chickens is not comprehensively understood, and it is necessary to better understand the pathogenic mechanisms of FAdV-4 to effectively control a viral epidemic.

Because the pathogenicity of FAdV-4 isolated from small-day-old chickens and the pathogenic difference of this strain to small-day-old and large-day-old chickens remains unclear, this study established animal regression experiments. Ten-day-old and 20-day-old SPF chickens were injected separately with the same doses of the FAdV-4 serum via oral and intramuscular injection routes.

From the gross lesions, body weight, mortality percent, and viral load we observed that the pathogenicity of FAdV-4 in SPF chickens is related to the age and the route of injection. Ten-day-old chickens are more susceptible than 20-day-old chickens, and the muscular injection group was more serious than the oral injection group.

Most chickens in the 2 experimental groups showed obvious clinical symptoms, including hydropericardium and hepatitis manifestation, bleeding,and swelling of the spleen, lungs, and thymus. The inoculated chickens in the 10-day-old and 20-day-old groups started to die on the 4th and 7th days after injection, respectively. Mortality rates among the 10-day-old chickens inoculated intramuscularly were 50%. Histopathological analysis also indicated obvious histopathological changes, including the interstitial edema around cardiocytes, visible inflammatory cell infiltration in the liver and spleen, and lymphocyte necrosis in the thymus and bursae. The results demonstrate that FAdV-4 can invade multiple organs and lead to the pathogenesis of chickens. The clinical manifestations and pathological changes after infection were highly consistent with those of other scholars, such as Abdul-Aziz (Abdul-Aziz and Hasan, 1995).

Real-time PCR results show that the virus is widely distributed in the body, and virus DNA can be detected in various organs, such as the heart, liver, spleen, lung, kidney, thymus, and bursae, which is consistent with the necropsy and histopathological changes of the infected chickens. The quantity of virus in the liver was the highest, so the liver is the primary choice for clinical detection of adenovirus. Notably, the quantity of virus in the intramuscular injection group was higher than that in the oral injection group, indicating that the pathogenicity of the body was stronger by intramuscular injection of the virus. In the natural infection model, there is no intramuscular injection route. However, the established 10-day-old muscle infection model is more infectious and pathogenic to the body, so this model can be valuable for anti-viral drug testing and vaccine evaluation.

AST mainly exists in cardiomyocytes, but also in mitochondria of hepatocytes (Fan et al., 2014). If hepatic cells or myocardial cells are destroyed, the enzymes will leak into the blood and levels of it will increase rapidly. As the cells are repaired, its content gradually decreases. ALT is mainly distributed in the body's hepatic cytoplasm. The activity of this enzyme is approximately 100 times higher in the liver than in serum. Therefore, 1% hepatocyte necrosis results in a 1-fold increase in serum ALT (Kew, 2000; Rosenthal, 1997). LDH exists in the cytoplasm of all histiocytes in the body, and its content is highest in the kidney. Therefore, LDH levels can reflect tissue damage, especially kidney damage. The detection results of this study showed that almost all blood biochemical indices of experimental groups were higher than those of the control group and then decreased with time. As the cells self-repair, the levels of enzymes in the plasma are reduced, which may be due to the damage of the heart, liver, and kidney after injection. These were highly consistent with Asrani's report (Asrani et al., 1997). Furthermore, this also supports the changes of histopathological damage.

IL-6 was produced by a variety of lymphocytes and non-lymphocytes, which can promote the proliferation of B cells, enhance their ability to produce antibodies, and promote humoral immunity. The level of secretion can reflect the body's humoral immune function (Thacker et al., 2009). IFN-γ is produced by thymus-dependent

lymphocytes (T cells) and natural killer cells (NK cells). It is a pleiotropic factor for immune regulation that can participate in the body's immune regulation and improve antigen presentation ability. In this study, the levels of IL-6 in the 2 injection groups first rose and then decreased. This shows that the body is in a normal state of immunity at the initial stage after injecting the virus, but at the late stage, immunosuppression appears (Schonewille et al., 2008). The IFN-γ in the intramuscular group was significantly higher than that in the control group and was maintained in the first 16 dpi, which reflected that many of these cytokines with high concentrations were produced by T cells and NK cells under the stimulation of FAdV-4. However, with the constant invasion and colonization of the SDSG strain, the immune organs of the infected chickens were severely damaged, resulting in a decrease in IFN-γ levels. After the injection, the antibody levels also showed an increasing trend, indicating that humoral immunity plays an important role in host antiviral properties.

In conclusion, this experiment determined the accurate value of viral load, cytokines, biochemical indicators and antibodies of FAdV-4 (SDSG)-induced chickens at 10 and 20 days old. Filling in the gaps in this aspect of data may provide references for future research.

Funding

This work was supported by Tai'shan Industry Leader (LJNY201610),; the National Key Research and Development Program of China (2018YFD0501506),; the China Agriculture Research System (CARS-42-19), and; Funds of Shandong "Double Top" Program.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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