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Research Article

Isolation and Characterization of a Novel Duck Hepatitis A Virus Genotype 3 in Hebei Province, China

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2. Keywords

Duck hepatitis A virus; Isolation; Genotype; Vaccine; Phylogenetic analysis

1. Abstract

The HBGT/China/2014 strain of duck hepatitis A virus (DHAV) was isolated from infected ducklings with clinical symptoms in Hebei province, China. Pairwise comparisons and phylogenetic analyses demonstrated that the HBGT/China/2014 strain belongs to duck hepatitis A virus genotype 3, and it was most closely related to China isolate EY (98.5%) from geese, and was adjacent to Korean isolates in complete genes. Genome sequence analysis showed that DHAV-3 could be divided into four subtypes (I, II, III, and IV) and HBGT/China/2014 was belonged to DHAV-3-IV. Otherwise, the RGD sequence of VP1 of picornaviruses was mutated to QSD, suggesting that this mutation could change the relationship between viral particles and cell receptors, thus affecting the ability of the virus to replicate. Furthermore, animal experiments showed that the HBGT/China/2014 strain was high pathogenic to 3-day-old ducklings and caused typical clinical signs, including nervousness and opisthotonos, and died 4 days post-inoculation. Gross lesions of the dead ducklings that were mainly found in the liver displayed swelling and significant bleeding. In addition, histological examinations of the dead ducklings revealed significant numbers of diffuse confluent vacuoles in the liver and disordered liver cells that were lightly stained with concentrated or of a lack of nuclei, necrosis, and heterophilic granulocyte infiltration These findings provide evidence that the duck hepatitis virus was still in mutating, and it urgently needs to develop a multivalent vaccine to better control the infection of the disease.

3. Introduction

Duck hepatitis is a highly contagious disease caused by the duck hepatitis virus (DHV), which belongs to the family Picornaviridae, genus Enterovirus, and is particularly lethal to ducklings [1]. To date, three distinct DHV serotypes (1–3) of DHV with no cross-protective immunity have been described [2]. DHV-2 and DHV-3 have been reported in England and the US, while DHV-1 mainly circulates in China [3]. DHV-1 was recently renamed duck hepatitis A virus (DHAV) and is classified into three genotypes, serotype 1 (DHAV-1), the new Taiwan (DHAV-2) and the new South Korea (DHAV-3) [4]. These strains show no cross-protection or cross-neutralization among them [4,5]. At present, both DHAV-1 and DHAV-3 are circulating in China. Although a vaccine against DHV has been developed, it is primarily effective against DHAV-1, but insufficient to control outbreaks of DHAV [6,7]. Molecular testing has confirmed many new DHV strains as genotype 3, which have high similarity to the Korean new serotype of DHV.

The goal of this study was to isolate a virulent DHAV-3 strain from ducklings by virus titer assay for use in an animal experiment to elucidate the genetic background of the HBGT/China/2014 strain by sequencing the complete genome to provide a basis for the prevention and treatment of DHV.

4. Material and Methods

4.1. Bacterial Isolation

Liver samples collected from ducklings with fatal infections were cultured on tryptic soy agar (TSA) and incubated aseptically at 37°C for 48 h.

4.2. Viral Isolation

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Viruses were isolated from 6-day-old dead ducklings at a duck farm (3000) in Hebei province, China, in 2014. The livers of some sick ducklings demonstrated multiple sites of bleeding and hemorrhage. The liver samples were homogenated, frozen and thawed three times, and then centrifuged at 3000 rpm for 30 min. Antibiotics (2000 IU/ml of penicillin-streptomycin) were added to the supernatant, which was then incubated at 37°C for 30 min. Then, the supernatant was used to inoculate 11-day-old specific pathogen-free (SPF) duck embryos (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China) through the allantoic route with 0.2 ml per egg, which were then incubated for 5 days at 37°C. After a second passage was performed, the allantoic liquid of dead embryos was collected and stored at -80°C until use.

4.3. Hemagglutination Testing

Chicken, duck, and goose red blood cells were prepared by conventional methods and subjected to hemagglutination testing using a protocol prepared by the World Organization for Animal Health (http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/) [8].

4.4. Virus RNA Extraction and Real-Time (RT)-PCR

Total RNA was extracted from allantoic liquid and the liver homogenates using TRIzol reagent (Invitrogen Corporation, Calsbad, CA, USA), according to the manufacturer's instructions, and then treated with DNase to remove genomic DNA and reverse-transcribed using six random primers and Moloney murine leukemia virus reverse transcriptase (Takara Bio, Inc., Otsu, Shiga, Japan) following the manufacturer's instructions.

The presence of potential pathogens, such as avian influenza virus (AIV) (Standard), Newcastle disease virus (NDV) [9], duck reovirus (DRV) [10], duck circovirus (DuCV) [11], DHAV [12], duck flavivirus (DFV) [13], duck hepatitis B virus (DHBV) [14], duck parvovirus (DPV) [15], and duck enteritis virus (DEV) [16] were detected using specific primers.

4.5. Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequence of the whole genome of the HBGT/China/2014 strain isolated in this study was RT-PCR amplified and sequenced using the primers listed in Table 1 and deposited into the Gen Bank database. Then, this sequence was aligned with reference sequences to generate genotype classifications. Phylogenetic trees based on the complete genomes of the isolate together with other DHAVs from the Gen Bank database were constructed using Meq software, version 7.0.14.

Table 1: Primers were designed and used for amplification and sequencing.

	Sequence (5'—— 3')
A	TTTGAAAGCGGCTGTGGTGTAGAYCATTTTCTGGCACTT
	GGATAGATTTGCAGCTCCTGG
B	ACACATGGGTCAGGAACCTC
	TTTTAAGTCTCAAACAGAAGCG
C	GACAATCTCACTTCTGAATATGC
	GCATCTTTCCCAGGAAATGAT
D	GAGAAGTGGAATGGCAATTGG
	GCGGTCAAAATCAAAATCAAGTT
E	GTTGCTACTGCAATGAGGGATGGC
	AATTTGAGCACTCAGAGACCCGTC
F	TTATTTTATCAGGCTGTGCTGTTGG
	GCGGCCGCGCGCGCCAGCTGTTTTTTTTTTTTTTTTTTTTTTTTT

4.6. Animal Experiments

A total of 20 three-day-old SPF ducklings were separated into two groups of 10 ducklings each. The ducklings in the experimental group were intramuscularly inoculated with 1000 median embryo lethal dose (ELD_{50})/0.2 ml virus, while those in the control group were inoculated with sterile phosphate-buffered saline (PBS). Then, the ducklings were observed for 10 days and the mortality, clinical symptoms, and presence of gross and histological lesions were recorded. The livers of the dead and surviving ducklings were collected and some sections were stored at -80°C for RNA extraction and others were fixed with 10% formaldehyde in PBS for histopathological examination. All experimental and animal management procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science.

5. Results

5.1. Bacterial Isolation

The bacterial cultures on TSA remained negative and no bacteria were observed in the liver sections, as demonstrated by Wright's

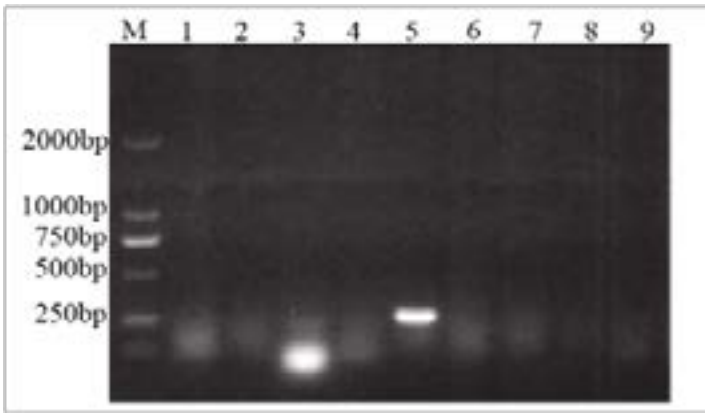

Figure 1: Virus detection from HBGT/China/2014 by RT-PCR or PCR: M: DNA Marker (DL2000, TaKaRa); 1: Avian Influenza Virus (AIV); 2: Newcastle disease virus (NDV); 3: duck reovirus (DRV); 4: duck circovirus (DuCV); 5:DHAV; 6: duck flavivirus (DFV); 7: duck hepatitis B virus (DHBV); 8: duck parvovirus (DPV); and 9: duck enteritis virus (DEV)

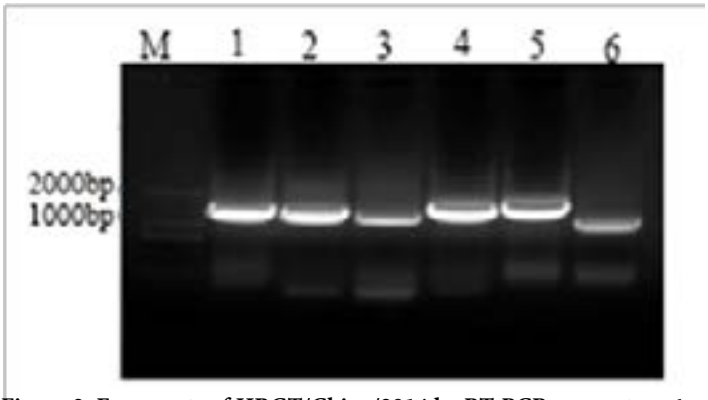

Figure 2: Fragments of HBGT/China/2014 by RT-PCR: M: DNA Marker (DL2000, TaKaRa); 1-6: fragments A–F, respectively

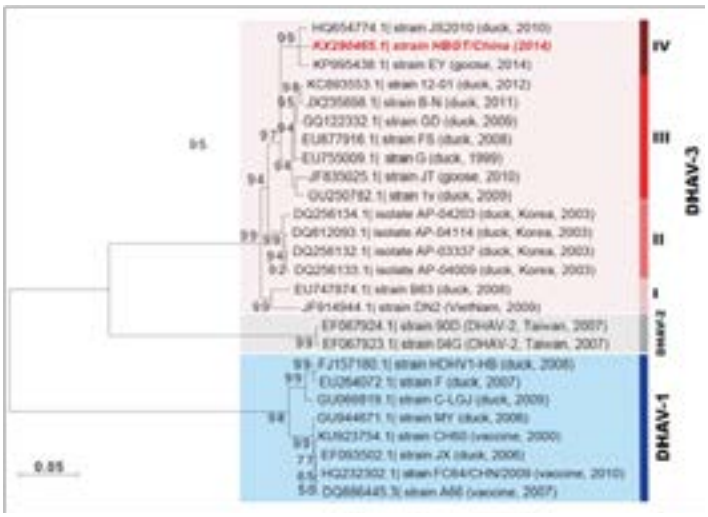

Figure 3: Phylogenetic relationships between HBGT/China/2014 and other strains of duck hepatitis virus

staining.

5.2. Virus Isolation

The isolated virus was shown to induce significant symptoms in embryonated SPF duck eggs, which included congestion, swelling, and hemorrhage. Death of the embryos occurred between 60 and 96 h post-inoculation. The virus was identified by PCR or RT-PCR, and only the DHAV assay was positive, which revealed a genome of 314 bp, while AIV, NDV, DRV, DuCV, DFV, DHBV, DPV, and DEV were negative (Figure 1). Furthermore, the HA assay demonstrated that allantoic liquid has no hemagglutination to chicken, duck, and goose erythrocytes. The isolate was designated as HBGT/China/2014 and uploaded to the GeneBank database under the accession number KX290465. The ELD_{50} of the virus was $10^{4.6}$/0.2 ml.

5.3. Sequence and Phylogenetic Analyses

Six HBGT/China/2014 strain fragments (Figure 2) obtained by RT-PCR were sequenced and aligned. The nearly complete genome sequence of HBGT/China/2014 strain contained 7802 nucleotides, which encoded for 2516 amino acids. Phylogenetic analysis of the nucleotide sequence of the complete genome revealed that the isolate had 98.5% genetic similarity to goose EY strain (2014) and 94.8%–94.9% similarity to Korean strains. The HBGT/China/2014 strain was identified as genotype 3, which was adjacent to DHAV-2 (78%), but differed from DHAV-1 (72.6%–72.8% similarity) (Figure 3 and 4).

The sequences of the VP1 gene of HBGT/China/2014 strain and reference strains were aligned with MegAlign software, which showed that the conserved sequence RGD in the hypervariable region of VP1 of Picornaviridae had been mutated to QSD (Fig. 5).

5.4. Animal Experiments

Three-day-old ducklings inoculated with allantoic liquid containing the HBGT strain demonstrated typical clinical signs, including nervousness and opisthotonos (Figure 6a), and died 4 days post-inoculation. Gross lesions of the dead ducklings that were mainly found in the liver displayed swelling and significant bleeding (Figure 6b). DHAV was detected in the livers of the dead ducklings by RT-PCR. In addition, histological examinations of the dead ducklings revealed significant numbers of diffuse confluent vacuoles in the liver and disordered liver cells (Figure 7a) that were lightly stained with concentrated or of a lack of nuclei, necrosis, and heterophilic granulocyte infiltration (Figure 7b), while the control livers were complete with clearly organizational structures and no obvious lesions (Figure 7c).

5.5. Discussion and Conclusion

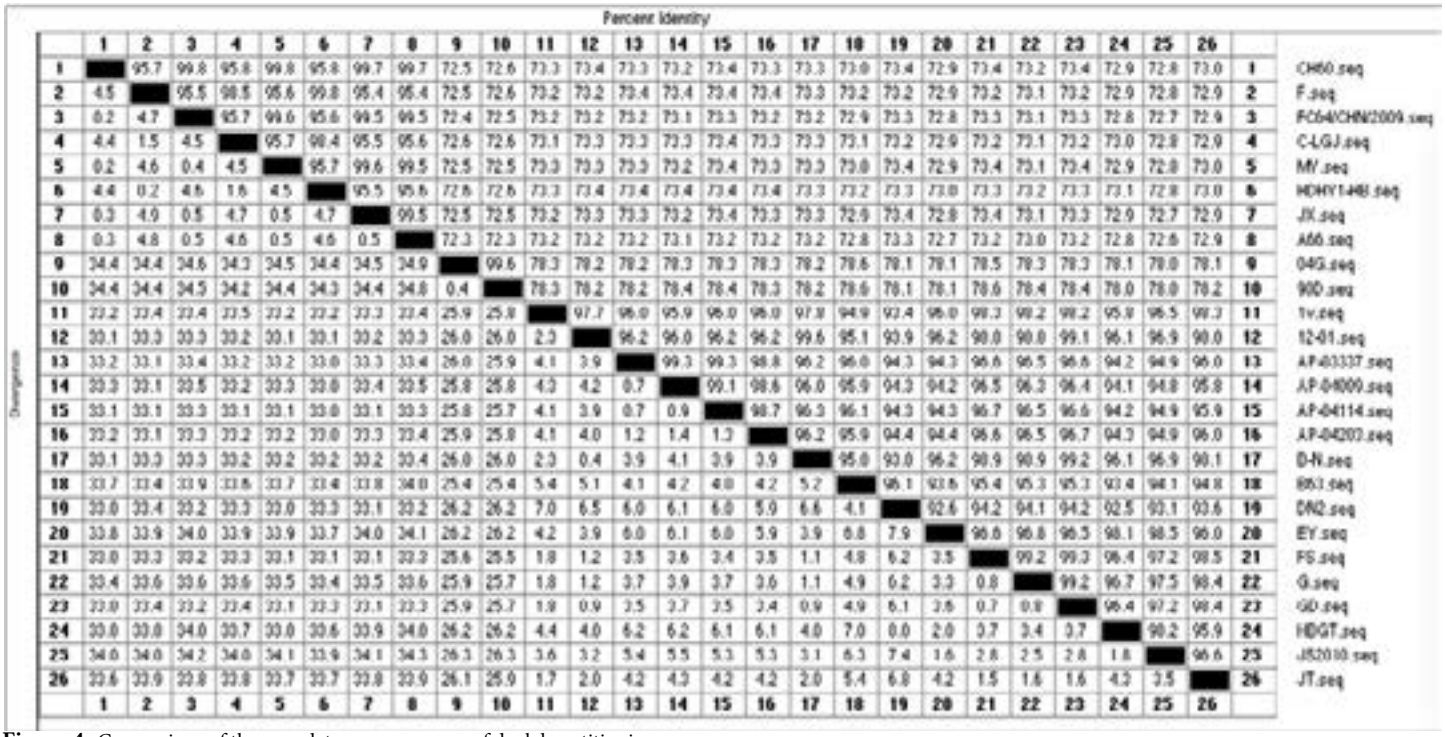

Figure 4: Comparison of the complete gene sequence of duck hepatitis virus

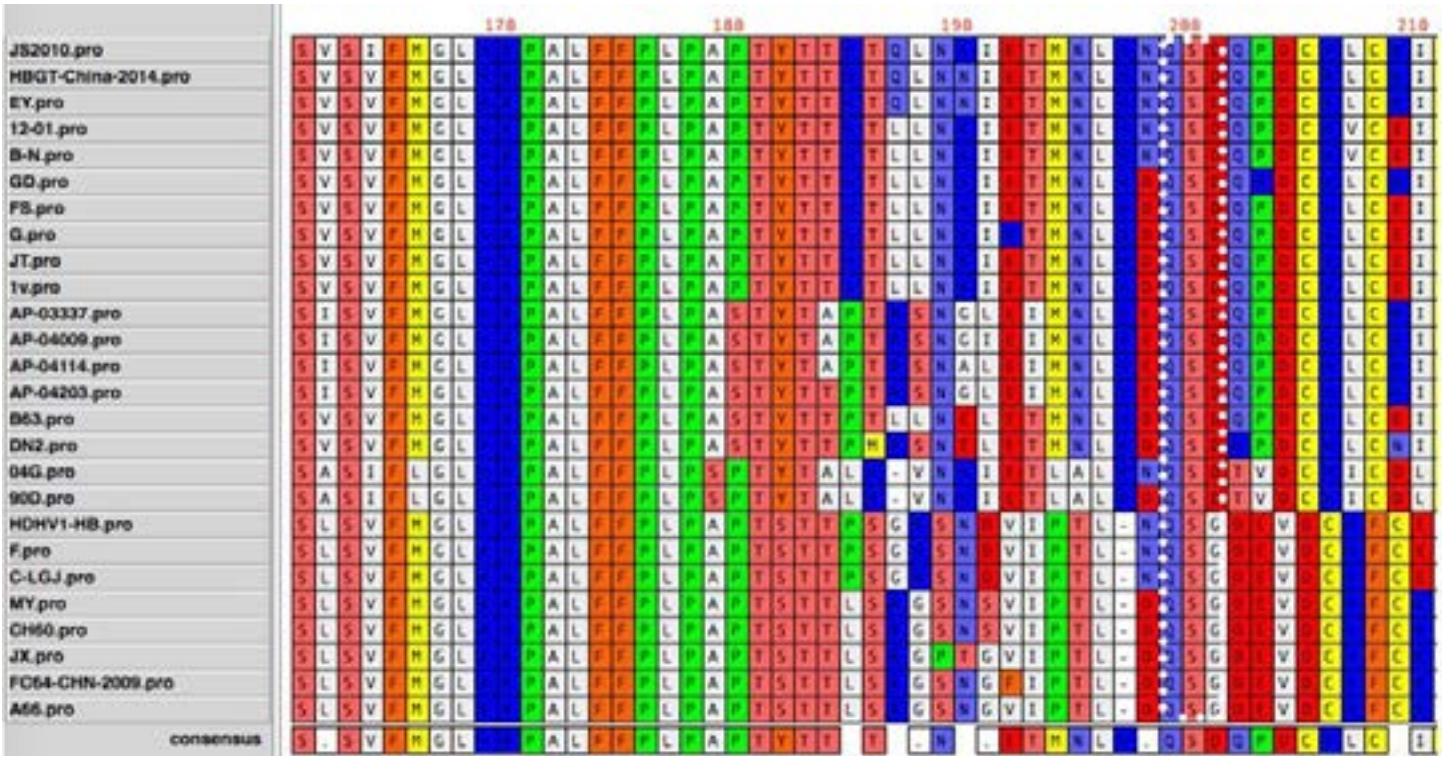

Figure 5: Amino acid sequence alignment of the DHAV VP1 protein.

In 2002, Su et al. isolated two strains of DHV in Beijing and Guangxi, which were not serologically related to DHAV-1 or DHAV-3, and were identified as new DHV strains. In 2007, new DHV strains were isolated in Taiwan [4, 17] and Korea [1], which was classified as picornaviruses. The clinical symptoms of the new DHV strains were consistent with those of DHAV-1, but demonstrated no cross-reactivity by cross-serum testing and homology analysis.

In this study, a new strain was isolated that was identified as duck hepatitis A virus genotype 3 and named HBGT/China/2014. Animal experiments confirmed that the clinical symptoms of ducklings infected with HBGT/China/2014 were similar to those

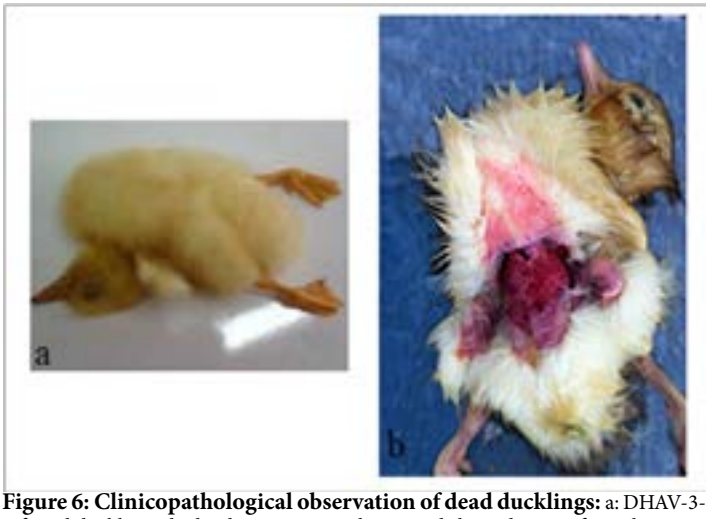

Figure 6: Clinicopathological observation of dead ducklings: a: DHAV-3-infected ducklings died 4 days post-inoculation and showed signs of opisthotonos; b: the liver of the dead duckling were swollen with diffuse bleeding, known as "tabby liver."

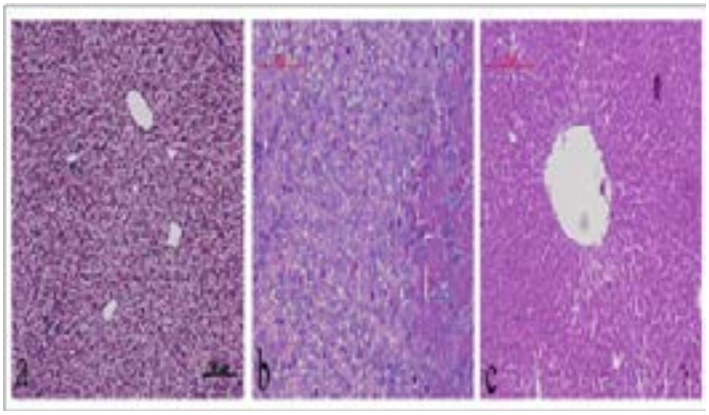

Figure 7: Histopathological observation of dead duckling livers: a: Confluent vacuoles and disordered liver cells; b: Necrosis and heterophilic granulocyte infiltration; c: No histopathological changes were observed in the livers of control ducklings.

of DHAV-1. Furthermore, HBGT/China/2014 was isolated from infected ducklings and the complete genome was sequenced. Phylogenetic trees constructed from the complete nucleotide sequences showed high sequence similarity with one another and demonstrated the existence of three DHV genotypes. In addition, obvious geographical characteristic appeared. Classic DHV-1 strains mainly in China belong to genotype 1, while isolates from Taiwan (04G and 90D) and Korea (AP04009, AP03337, AP04114, and AP04203) clustered in genotypes 2 and 3. Genome sequence analysis showed that DHAV-3 could be divided into four subtypes (I, II, III, and IV). China vaccine strain B63 and VietNam strain DN2 belong to DHAV-3-I, and Korean strains were DHAV-3-II, while most isolates were DHAV-3-III. HBGT/China/2014 was DHAV-3-IV.

The VP1 protein is the key immunogenic protein and contains B and T cell epitopes, which can induce the production of neutralizing antibodies. In addition, VP1 is used as a marker of biodiversity of viral genotypes and serotypes [18,19]. The RGD sequence is a cell surface ligand of the VP1 protein and is necessary for the virus to invade cells [20]. In this study, the RGD sequence of picornaviruses was mutated to QSD, suggesting that this mutation could change the relationship between viral particles and cell receptors, thus affecting the ability of the virus to replicate. HBGT/China/2014 strain from 6-day-old ducklings in Hebei province shared low homology with the Taiwan strains, but shared high homology with the EY (goose) (98.5%) and Korean strains, and was therefore grouped into DHAV-3-IV, suggesting that the HBGT/China/2014 isolate recently evolved and DHV continues to mutate, possibly due to immune pressure. Taken together, the data from the present study confirmed the circulation of DHAV genotype 3 in China with DHAV-1 and DHAV-3 as the predominant strains. While a vaccine was found to be effective against DHAV-1, it was insufficient to protect against DHAV-3 and may enhance the risk of infection in ducklings. The finding of this study was help to further elucidate the evolutionary mechanisms of DHAV via molecular testing in order to better control the disease.

6. Acknowledgements

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