Isolation and pathogenic characterization of duck adenovirus 3 mutant circulating in China

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ABSTRACT Duck adenoviruses (DAdVs) include serotype 1 (DAdV-1) in the genus Atadenovirus and serotypes 2-4 (DAdV-2, 3, and 4) in the genus Aviadenovirus. DAdV-3 was initially isolated from Chinese Muscovy ducks in 2014, whereby the infected ducks exhibited yellowing and hemorrhaging in the liver, along with slight pericardial effusion, swelling, and hemorrhaging in the kidneys. In recent years, duck adenovirus infections have appeared in Muscovy duck farms in Fujian, Zhejiang, Anhui, Guangdong, and other places in China. They have an incidence rate of 40 to 55% and a mortality rate of 35 to 43%, resulting in great losses to the duck breeding industry. In this study, 7 DAdV-3 strains, designated as TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, and GDHS were isolated from Muscovy ducks in different provinces of China during 2016–2019, and their complete genomics were sequenced. Their genomes all exhibited significant deletions in ORF67, which also had G to A transitions at the 41st and 977th nt positions, resulting in a stop codon. The pathogenicity of TZ193, a novel isolate of DAdV-3, was investigated in Muscovy ducks. TZ193 caused characteristic lesions of swelling as well as hemorrhagic liver and kidney in the infected ducklings. Moreover, the mortality rate of TZ193 in 5-day-old domestic ducks was 100%. Our data provide concrete evidence for the identification of the DAdV-3 novel variant mutant in China, which effects increased mortality in ducks. This highlights the necessity for monitoring the molecular epidemiology of novel DAdV-3 mutants and the development of new vaccines in the future.

Key words: duck adenovirus 3, isolation, pathogenicity, mutant, duck

INTRODUCTION

Duck adenoviruses (DAdVs) include serotype 1 (DAdV-1) in the genus Atadenovirus and serotypes 2-4 (DAdV-2, 3, and 4) in the genus Aviadenovirus. DAdV-3 was initially isolated from Chinese Muscovy ducks in 2014, whereby the infected ducks exhibited yellowing and hemorrhaging in the liver, along with slight pericardial effusion, swelling, and hemorrhaging in the kidneys. In recent years, duck adenovirus infections have appeared in Muscovy duck farms in Fujian, Zhejiang, Anhui, Guangdong, and other places in China. They have an incidence rate of 40 to 55% and a mortality rate of 35 to 43%, resulting in great losses to the duck breeding industry. In this study, 7 DAdV-3 strains, designated as TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, and GDHS were isolated from Muscovy ducks in different provinces of China during 2016–2019, and their complete genomics were sequenced. Their genomes all exhibited significant deletions in ORF67, which also had G to A transitions at the 41st and 977th nt positions, resulting in a stop codon. The pathogenicity of TZ193, a novel isolate of DAdV-3, was investigated in Muscovy ducks. TZ193 caused characteristic lesions of swelling as well as hemorrhagic liver and kidney in the infected ducklings. Moreover, the mortality rate of TZ193 in 5-day-old domestic ducks was 100%. Our data provide concrete evidence for the identification of the DAdV-3 novel variant mutant in China, which effects increased mortality in ducks. This highlights the necessity for monitoring the specific molecular epidemiology of novel DAdV-3 mutants and the development of new vaccines in the future.
data suggested that CH-GD-12-2014, along with the FJGT01, AHAQ13, ZJJH07, and GDMM10 strains, should be classified under DAdV-3.

In recent years, emerging DAdV-3 outbreaks—characterized by swelling as well as hemorrhagic liver and kidney have posed a great threat to Muscovy ducks in China. The morbidity of DAdV-3 ranges from 40 to 55 %, with a mortality rate of 35 to 43% (Wan et al., 2018; Shen et al., 2019). In this study, 7 DAdV-3 strains were isolated from Muscovy ducks in different provinces of China during 2016–2019, and their complete genomes were sequenced and identified as duck adenovirus 3 (DAdV-3). The sequenced genomes all exhibited significant deletions in the ORF67 gene that differed from all the other 5 DAdV-3 strains previously published. The molecular mechanisms underlying the infection and pathogenesis of these DAdV-3 strains remain unclear, hence prompting the investigation of the pathogenicity of TZ193, a novel isolate of DAdV-3 in Muscovy ducks.

MATERIALS AND METHODS

Sample Collection and Virus Isolation

Liver samples were collected from diseased Muscovy ducks in the Jiangsu, Fujian, Guangxi, Guangdong, and Anhui provinces of China. Total DNA and RNA of supernatants were extracted using the OMEGA E.Z.N. A Viral DNA/RNA Kit. Other potential pathogens such as duck enteritis virus (DEV), duck adenovirus serotype 4 (DAdV-4), avian influenza virus (AIV), novel duck parvovirus (NDPV), duck Tembusu virus (DTMUV), duck virus hepatitis A type 1 (DHAV-1), Newcastle disease virus (NDV), duck adenovirus serotype 3 (DAdV-3), fowl adenovirus serotype 4 (FAV-4), and duck circovirus (DuCV) were detected via PCR or reverse transcription PCR (RT-PCR) assays using the primers listed in Table 1. In order to purify the isolate, the viruses were initially amplified in LMH cells, followed by the selection of 20 plaques for genomic DNA identification. Subsequently, the clones were blindly picked, passed into LMH cells, and purified twice. The purified clones were amplified in SPF duckling embryo eggs purchased from the Harbin Research Institute, CAAS.

Electron Microscopy

For transmission electron microscopy (TEM) examination, the cell culture fluid of the TZ193 virus infected LMH cells was harvested, centrifuged at 8,000 rpm at 4°C for 30 min to remove impurities, and the supernatant was overlaid onto a 60% sucrose solution, and then ultracentrifuged at 31,000 rpm at 4°C for 3 h to obtain a virus sucrose solution. The virus sucrose solution was diluted in PBS (phosphate buffer saline), centrifuged at 31,000 rpm for 2 h at 4°C, and the virus pellet was harvested. The virus pellet was resuspended in PBS and evenly covered with copper mesh. After staining with 2% phosphotungstic acid (Ted Pella Inc., Redding, CA) for 2 min, bake at 65°C for 15 min, and assessed via TEM.

Indirect Immunofluorescence Assay

To perform the immunofluorescence assays, LMH cells cultured in 96-well-plate were mock-infected or infected with the TZ193 virus at a multiplicity of 1 PFU per cell. At 48 hpi, the cells were fixed with 3% paraformaldehyde at room temperature for 15 min and then incubated with 1% TritonX-100 in PBS at room temperature. The cells were subsequently incubated for 1 h with the primary antibody—a monoclonal antibody (mAb) against DAdV-3 Fiber-2 proteins—that was prepared in the lab itself, followed by incubation with 1:2,000 diluted goat anti-mouse IgG (H + L) secondary antibody, Alexa Fluor 488. Finally, cells were incubated with DAPI. Preparations were examined using a Bio-Rad Radiance 2000 confocal laser-scanning microscope and the resulting images were processed using Adobe Photoshop software.

Determination of Nucleotide Sequences and Genome Organization

For whole-genome sequencing, 14 pairs of primer pairs (Table 2) were designed for PCR amplification of genomic fragments covering the entire genomes of the viruses. All fragments were sequenced by GENEWIZ Biotechnology Co., Ltd, Suzhou, Jiangsu province, China. The whole sequences of the viral genomes were edited and assembled using SnapGene v4.1.9. Sequences of the reference adenovirus strains were retrieved from GenBank. Furthermore, the nucleotide or amino acid sequences were aligned using the SnapGene v4.1.9.

| Table 1. List of detection primers designed in this study. |
|-----------------------------|-----------------------------|
| Name | Sequence | Size |
| DEV-F | ATGTCCTATTCTTGGAGGTTA | 354 bp |
| DEV-3544R | TATATCTGCTGATGCTGCT | |
| DAdV-J-F | GGGCARTGATGAGCATG | 1,200 bp |
| DAdV-J-1200R | CCTATGGTCGATGCTGCTG | |
| NDPV-F | CCTATGGTCGATGCTGCTG | 661 bp |
| NDPV-661R | TAGGGCGCCCCAAGGAGGCT | |
| AIV-F | CTGGAGGCGAACAGGAGGCT | 1,413 bp |
| AIV-1148R | GAGGGCAGGCGAACAGGAGGCT | |
| DTMUV-F | GAGGTAGCGGCGAACAGGAGGCT | 567 bp |
| DTMUV-567F | AAGGTAGGCGGCGAACAGGAGGCT | |
| DHAV-I-F | GGTTGAGGACGAATGCTG | 360 bp |
| DHAV-I-360R | ATAGGATGTCGACGCTG | |
| NDV-F | CGACAAAGGCGAACAGGAGGCT | 493 bp |
| NDV-1958R | TGGGTCTCACGACAGGAGGCT | |
| DAdV-1874F | ATGCGCCGCTGCTGCTGCTG | 2,814 bp |
| DAdV-1874R | TATACGCGGCTGCTGCTGCTG | |
| FAdV-4-F | CGACGGCTAACGATGCTGCTG | 1,048 bp |
| FAdV-4-1048R | CCGGGGCTCCGCTGCTGCTG | |
| DuCV-F | CCAATGATGACCGCGAACAGGCTG | 980 bp |
| DuCV-980R | AGCAAGGCTGACATGCTGCTG | |
| pfl-TZI98F | CGTTCCGCGGCCGCTGCTGCTG | 92 bp |
| pfl-TZI98R | CCGGTCGCGGCCGCTGCTGCTG | |
| FAM-pfl2-Probe | CACAGTACGACATCGACATCGACATCGACATCGAC | 567 bp |
| DTMUV-F | GAGGTAGCGGCGAACAGGAGGCT | |
| FAdV-4-F | CGACGGCTAACGATGCTGCTG | |
Phylogenetic trees were generated using the maximum likelihood method implemented in MEGA v7.0.

Pathogenicity of TZ193 Strain in Muscovy Ducks

All animal experiments were performed in compliance with the regulations and guidelines of the Experimental Animal of Shanghai Science and Technology Committee of Shanghai Veterinary Research Institute (Permit Number: SHVRI-SZ-20200326-01). Five-day-old Muscovy ducks (n = 26) were randomly divided into 2 groups (13 ducklings per group). The ducklings in the infection group were inoculated with 500 \( \mu L \) of TZ193 virus containing \( 10^6 \) TCID50/mL via the intramuscular route. Another group was inoculated intramuscularly with 500 \( \mu L \) PBS as a control. All ducks were monitored for 14 d. Upon attaining 3 dpi, the heart, liver, spleen, lung, kidney, bursa of Fabricius, small intestine, cecum, thymus, trachea, and brain samples of 3 ducks in both groups were collected separately. All animals were housed in a specific pathogen-free facility with free access to water and food.

Histopathology Assays

All tissue samples were fixed in 10% neutral formalin buffer (pH 7.2) for histological examination. The tissues were embedded in paraffin wax, dissected into 4-\( \mu m \) sections, stained with H & E, and examined using light microscopy.

Immunohistochemistry Assays

The liver slides with typical lesions was carried out the immunohistochemistry (IHC) assays. Briefly, the IHC analysis was performed with DaDv-3 Fiber-2 mAb and then incubation with 1:2,000 diluted HRP-goat anti-mouse IgG (H + L) secondary antibody.

The qPCR Assay

All tissue samples were stored at \(-80^\circ C\) for DNA extraction in order to detect viral distribution in various organs by qPCR assay. The qPCR assay was designed by our lab based on the fiber-2 gene of DaDv-3, involving the following primers: pf2-TZ193F, pf2-TZ193R, FAM-pf2-Probe (sequences presented in Table 1), and the standard plasmid pet32a-fiber2 for qPCR was stored in our lab.

RESULTS

Identification and Characterization of DaDv-3 Variant Isolates

During 2016–2019, liver samples of diseased Muscovy ducks demonstrating pathological changes in the liver, including multifocal areas of necrosis and hemorrhage, were collected from several provinces in China (Figure 1A). The samples tested negative for DEV, (DaDv-4, AIV, NDPV), DTMUV, DHAV-1, NDV, FAdV-4, and DuCV, whereas they tested positive only for the DaDv-3 virus, with the primer pairs for gene

### Table 2. Primers for whole genome sequencing.

| Number | Name          | Sequence                  | Size   |
|--------|---------------|---------------------------|--------|
| 1      | pD3-1F        | CATCATCATATATATATACCATC   | 1-3272 |
| 2      | pD3-3272R     | ACTTTATGTCGCAATAAAAAT     |        |
| 3      | pD3-6044F     | CTCATTGCAATATATATATACCATC | 11712-14708 |
| 4      | pD3-6044R     | ACTTTATGTCGCAATAAAAAT     |        |
| 5      | pD3-1105F     | GCCCTGATATATATATATACCATC | 11712-14708 |
| 6      | pD3-1105R     | GCCCTGATATATATATATACCATC | 11712-14708 |
| 7      | pD3-20358F    | GTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 8      | pD3-20358R    | GTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 9      | pD3-27100F    | GGTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 10     | pD3-27100R    | GGTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 11     | pD3-30336F    | GGTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 12     | pD3-30336R    | GGTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 13     | pD3-36729F    | GGTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
| 14     | pD3-36729R    | GGTGTCGTCGACCTTTCTAGCG    | 23857-27350 |
hexon, DAdV3-F and DAdV3-2814R (Figure 1B). The alignment result of the PCR product sequence showed that the amplified hexon gene was highly homologous (100%) to the strains from DAdV-3.

LMH cells were prepared as stated, and the DAdV-3 positive liver samples were inoculated onto LMH cells, and 7 virus isolates, designated as TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, and GDHS, were isolated successfully. Compared to normal LMH cells, infected cells attained a spherical shape and grape-like clusters (Figure 1D), which is a classical characteristic of the cytopathic effect in adenovirus-infected cells (Hematian et al., 2016; Shen et al., 2019). Electron microscopy revealed the presence of numerous icosahedral, non-enveloped viral particles measuring approximately 80 nm in diameter. These virions were accumulated and arranged in crystal lattice formations in the nuclei of the LMH cells (Figure 1E), which is consistent with adenoviral infection observed under normal conditions. Moreover, the TZ193 strain was successfully amplified in SPF duckling embryo eggs, resulting in spontaneous embryo death at 8 to 13 dpi (Figure 1C). Immunological analysis was further performed using amAb against DAdV-3 Fiber-2 proteins, whereby Fiber-2 proteins were observed to be highly expressed in the infected cells (Figure 1F). These results indicated that the newly isolated viruses belonged to the DAdV-3 class.

Figure 1. Isolation of DAdV-3 from the samples of dead domestic ducks. (A) Gross lesion on the liver of dead domestic ducklings demonstrating swelling and hemorrhage. (B) PCR identification of the liver samples with the primers against DEV, DAdV-4 NDPV, AIIV, DTMUV, DHAV-1, NDV, DAdV-3, FAdV-4, and DuCV. (C) Pathogenicity of the purified virus in SPF duck embryo eggs. (D) Cytopathogenic formation (CPE) of LMH cells caused by TZ193 virus. (E) Electron microscopic view of the LMH cells’ ultrastructure with accumulation of viral particles in the cytoplasm arranged in a crystal lattice (white arrow). (F) IFA analysis of TZ193 virus during infection in LMH cells.
Nucleotide Sequence Accession Number

In order to investigate the molecular pathogenicity of the isolates, the complete genomic sequences of isolates TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, and GDHS were obtained using a set of specific primers (Figure 2A), and the whole genome nucleotide sequences of the isolates were deposited in GenBank with the respective accession numbers (Table 3).

Phylogenetic Analysis and Homology Comparison

The phylogenetic tree for the complete genomes was constructed in MEGA7, using the maximum-likelihood method with 1,000 bootstrap replicates (Figure 2B). The complete phylogenetic tree included the isolates TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, and GDHS.

Sequence comparisons indicated that the newly isolated DAdVs shared nearly 100% genomic sequence identity as well as 100% amino acid identity in the Hexon, Fiber-1, and Fiber-2 genes with all 5 DAdV-3 isolates previously published, that is, CH-GD-12-2014, FJGT01, AHAQ13, ZJJH07, and GDMM10. However, compared to the complete genomes of the previous isolates from NCBI, the ORF67 gene of TZ193 virus at 3’ terminal was truncated due to a point mutation, as compared to the CH-GD-2014, ZJJH07, and GDMM10 isolates.

Table 3. The data of compete sequences of 7 DAdV-3 isolates.

| Strain    | Location  | Duck type         | Collection date | Accession Number |
|-----------|-----------|-------------------|-----------------|------------------|
| TZ193     | Jiangsu   | Shelduck          | 2019.12         | MT934842         |
| FJPT20161124 | Fujian    | Muscovy ducks     | 2016.11         | MW645349         |
| GX20170519 | Guangxi   | Muscovy ducks     | 2017.5          | MW667580         |
| FJZZ      | Fujian    | Muscovy ducks     | 2017.11         | MW667581         |
| GDMM      | Guangdong | Muscovy ducks     | 2017.12         | MW667582         |
| AHAQ      | Anhui     | Muscovy ducks     | 2018.3          | MW677005         |
| GDHS      | Guangdong | Muscovy ducks     | 2018.3          | MW677006         |

TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, GDHS.
caused the ORF67 genome to be truncated (Figure 2C and Table 4). To further confirm the potential molecular variations of the isolates in this study, the purified PCR products of the original samples as well as the genome of TZ193 strain amplified from LMH cells or duck embryo eggs were all observed to possess the same mutation in the ORF67 gene (Figure 2C).

Pathogenicity of TZ193 Strain in Muscovy Ducklings

In the infection group, the TZ193 virus was highly virulent in Muscovy ducklings, with a mortality rate of 100% at 4 dpi (Figure 3A). Tissue samples from the heart, liver, spleen, lung, kidney, bursa of Fabricius, duodenum, cecum, thymus, trachea, and brain of dead Muscovy ducklings were collected for histological examination. The livers of infected ducklings showed significant signs of yellowing and hemorrhage; the gallbladder was distinctly enlarged, and there were hemorrhagic spots present in the heart tissues. Additionally, the kidneys of infected ducklings were pale and fragile. The cecum showed signs of severe hemorrhaging as well (Figure 3B). Contrastingly, all ducklings in the control group remained healthy.

The tissues were fixed and dissected into sections for Hematoxylin and Eosin (H & E) staining. Histopathological examination of the TZ193 infected group revealed congestion and dense populations of red blood cells in the liver. The myocardial fibers of the infected hearts were edematous, coarser, and significantly congested, while the kidneys showed signs of renal tubular epithelial cell degeneration and some epithelial cells were necrotic. In addition, mucosal exfoliation was evident in the intestinal epithelial cells. There were no distinct histological lesions in the spleen, lung, brain, or pancreas (data not shown). Contrastingly, no significant histopathological damage was observed in the ducks from the control group (Figure 3D). The IHC assay was performed on the liver sections of ducks that died of TZ193. The result showed that the TZ193 infected liver cells were remarkably stained with yellow color, in which Fiber-2 proteins were highly expressed when infection (Figure 3E).

In order to determine virus titers, 3 ducklings per group were euthanized at 3 dpi, and tissues of the heart, liver, spleen, lung, kidney, bursa of Fabricius, duodenum, cecum, thymus, trachea, as well as brain were collected. Viral DNA copies in sampled tissues were detected via qRT-PCR, which amplified a fragment of the fiber-2 gene using the primers pf2-TZ193F and pf2-TZ193R with FAM-pf2-Probe. The results, as summarized in Figure 3C, suggest that strain TZ193 was replicated and proliferated in all tissues of the ducks tested. Moreover, the highest viral loads were found in the cecum, followed by the duodenum, liver, and heart, whereas the lowest viral load was observed in the brain.

In summary, 6 variant strains of DAdV-3, TZ193, FJPT20161124, GX20170519, FJZZ, GDMM, AHAQ, and GDHS were isolated and identified, which have significant mutations in the ORF67 gene. The pathogenicity of the TZ193 strain in Muscovy ducklings indicated that the newly isolated strain TZ193 effected 100% pathogenicity and lethality in Muscovy ducklings, which was more virulent than the DAdV-3 isolates reported previously. This is heavily suggestive of the strain possessing the potential threat of circulating easily and widely among Muscovy ducks in China.

### DISCUSSION

In this study, GDMM and GDHS mutant strains were isolated in Guangdong in 2017 and 2018, respectively. The first DAdV-3 strain CH-GD-12-2014 was also isolated in Guangdong in 2014, while a previously reported GDMM10 strain had been isolated in Guangdong in 2015. Neither CH-GD-12-2014 nor GDMM10 strains had a mutation in the ORF67 gene, which indicates that the mutation likely occurred in Guangdong over time after 2015. In addition, prior to 2017, the isolated strains of DAdV-3 were mainly concentrated in the 3 provinces of Guangdong, Guangxi, and Fujian, all located in southern China. However, since 2017, DAdV-3 has gradually spread in the Anhui and Jiangsu provinces, located...
in central China. The TZ193 and AHAQ mutant strains in this experiment were isolated from Muscovy ducks in these 2 provinces in 2019 and 2018, respectively. The northward expansion of this disease area is possibly related to the higher pathogenicity of the mutant strain. Therefore, this study conducted in vivo animal experiments to assess the pathogenicity of the newly isolated strain TZ193 in 2019, and subsequently found that TZ193 was highly virulent in 5-day-old Muscovy, with a mortality rate of 100%. Contrastingly, Zhang et al. (2016) reported that the CH-GD-12-2014 strain was pathogenic to 1-day-old Muscovy ducks, but not fatal. FJGT01 was observed to be highly virulent in 10-day-old Muscovy ducklings, with a mortality rate of 60% (Shi et al., 2019; Shen et al., 2019). In the present study, the novel DAdV-3 variant, TZ193, exhibited more virulence than the CH-GD-12-2014 and FJGT01 strains. Although the specific reasons underlying the increase in fatality are still unknown, the ORF67 gene may potentially play a major role.

Histopathological examination of the TZ193 infected Muscovy ducklings indicated signs of severe hemorrhaging in the cecum and duodenum. The main lesions of ducks infected with DAdV-3 virus reported previously were liver hemorrhage and kidney congestion, whereas intestinal hemorrhage had not been mentioned. The results of qPCR detection of viral DNA content in various organs also demonstrated that the highest viral loads in the cecum were higher than those in the liver, suggesting that the TZ193 strain primarily invades the cecum, which might be related to the mutation in ORF67. Further studies must be conducted based on more detailed data on the transmission and pathogenicity of the variants by recombination strategy combined with the CRISPR/Cas9 system.
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Author’s contributions: HC and ZC conceived and designed the experiments. SX, CW, HS, and HC performed the experiments. SX, CW, HS, and ZC analyzed the data. HC and XW contributed to reagents/materials/analysis tools. SX, HS, ZC and HC wrote the manuscript.

DISCLOSURES

All the authors declared that there are no conflicts of interest with regard to this manuscript.

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