Illumina high-throughput sequencing for the genome of emerging fowl adenovirus D species and C species simultaneously

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ABSTRACT

In recent years, clinical cases of inclusion body hepatitis (IBH) and hydropericardium syndrome (HPS) have been emerging and increasing in chicken flocks worldwide. Mixed infections with 2 or more fowl adenovirus (FAdV) serotypes were common in these cases. Herein, we collected a clinical sample that was positive for FAdV from 40-day-old broilers with IBH and HPS symptoms in Shandong province of China and determined the complete genome of FAdVs on the Illumina HiSeq4000 platform. The results showed that the sample contained 2 FAdV strains of D species and C species and named SD1763-1 and SD1763-2 respectively. The genome of SD1763-1 strain was 43,913 nt in length, with a G+C content of 53.51%, whereas SD1763-2 strain was 43,721 nt in length, with a G+C content of 54.87%. Sequence alignment and phylogenetic analysis revealed that strain SD1763-1 was clustered together with serotype 2/11 of FAdV-D, and SD1763-2 was clustered together with FAdV-4. There is no recombination between the genomes of the 2 viruses of FAdV-D and FAdV-C in the present study. This is the first report of obtaining 2 genomic sequences of FAdV strains simultaneously by direct use of deep sequencing in one clinical individual chicken sample, which provided direct evidence for mixed infections of adenovirus serotypes in the clinic and enriched the genome data to explore the geographic biomarkers and virulence signatures of the genus Aviadenovirus.

Key words: fowl adenovirus, mix infection, high-throughput, genome, evolution

INTRODUCTION

The family Adenoviridae is divided into 6 genera Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, Siadenovirus and Testadenovirus, and can cause infectious diseases in a broad spectrum of vertebrate hosts (Benkó et al., 2022). Fowl adenoviruses (FAdVs) belong to the genus Aviadenovirus and are grouped into 5 species (FAdV-A to E) based on restriction fragment length polymorphism (RFLP) and molecular structure. They were further divided into 12 serotypes (FAdV-1 to 8a and -8b to 11) as a result of serum cross-neutralization tests within the 5 species (Hess, 2000). FAdVs are reported in the poultry industry worldwide and are mainly responsible for naturally acquired outbreaks of inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), and gizzard erosions (GE) in chickens, causing substantial economic losses. Previously published documents showed that most commonly FAdVs isolated from IBH cases belong to various serotypes of species FAdV-D and FAdV-E, whereas new emerging pathogenic FAdV-4 strains from FAdV-C are directly connected to HPS outbreak, and GE cases are mostly caused by FAdV-A infection (Schachner et al., 2018). However, some FAdVs may be isolated from asymptomatic chickens with no or mild clinical signs, and are considered ubiquitous in poultry populations (Schachner et al., 2021). Mixed infections with 2 or more adenovirus serotypes were also common in IBH and HPS cases, thus aggravating the infection caused by a single nonpathogenic virus (Meulemans et al., 2001). Natural recombination likely occurs in these co-infected clinical samples as reported in some HAdV and FAdV species, which

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served as the primary source of adenovirus evolution (Schachner et al., 2019).

FAdVs are icosahedral nonenveloped viruses containing double-stranded linear DNA genomes encoding proteins on both strands (Hess, 2000). Avianadenoviruses have the largest genome (43–45 kb) among adeno-
noviruses (Benkő et al., 2022). Previously, we and other teams have employed high-throughput sequencing technology to obtain the genome of different FAdV strains (Huang et al., 2019; Schachner et al., 2019). In the present study, we collected a clinical sample that was positive for FAdV from 40-day-old commercial broilers with IBH and HPS symptoms in Shandong province of China and obtained 2 genomic sequences of FAdV strains simultaneously by direct use of high-throughput sequencing in one clinical individual chicken sample.

MATERIALS AND METHODS

A clinical case with IBH and HPS symptoms occurred in 40-day-old broiler chickens with 3% mortality rate in Shandong province of China in 2019. Heart and liver tissue from each dead broiler chicken were collected and homogenized individually to obtain a 10% suspension. After low-speed centrifugation, the tissue suspensions were detected by PCR targeting a 507 nt fragment of the hexon gene using primer set FAdV-F: AATTTCGACCCCCATGACGCCGAGG and FAdV-R: TGGC GAAAGGCCTACCGAAGTAAGC. The FAdV-positive supernatants were propagated on the confluent monolayers of a chicken hepatoma cell line respectively. The collected supernatants were clarified by low-speed centrifugation and then ultracentrifuged to obtain the pelleted cell-free virions. Total DNA of FAdV strain for sequencing was extracted from ultracentrifuged virus suspensions obtained in the same branch on the entire genomic and some previously suspected FAdV-11 isolates were clustered in the same branch on the entire genomic and

RESULTS AND DISCUSSION

The SD1763 isolate contained the genome of 2 fowl adenovirus serotypes and named SD1763-1 and SD1763-2 respectively. The genome of FAdV-D strain SD1763-1 was 43,913 nt in length, with a G+C content of 53.51%, whereas FAdV-C strain SD1763-2 was 43,721 nt in length, with a G+C content of 54.87%. The whole genome sequences of SD1763-1 and SD1763-2 have been deposited to GenBank database under accession numbers ON260920 and ON260919. The PRKM ratio of the SD1763-1 to SD1763-2 is 1.417, which demonstrated that the SD1763-1 strain possessed more viral genomes than the SD1763-2 strain. This is the first report of obtaining two viral genomes by direct use of deep sequencing in one individual chicken sample co-infected with FAdVs.

Molecular characteristics of potentially devastating FAdVs like FAdV-D which has long been circulating in China are relatively lacking. The genome sequences of all FAdV-D members until now show high sequence conservation throughout the genome. Initially, FAdV typing was achieved with a cross-neutralization test and the SR48 strain was primarily considered as the FAdV-2 reference strain (Hess, 2000). However, there is a common evolutionary origin between the SR48 strains and FAdV-11 strain 380 as evidenced by hexon gene sequences. Further cross-neutralization results in more recent studies also support the proposed reclassification of the SR48 prototype into FAdV-11 (Meulemans et al., 2001). The evolution of the whole genome (Figure 1A) and adequate phylogenetic analysis of important antigenic proteins Hexon, Fiber and Penton (Figure 1C) in the present study systemically proved the close relationship of SR48 and 380, which supports the grouping of FAdV-2 and -11 into a single type. Prototype strain 685 from Northern Ireland has been proposed as an antigenic variant with prime relationships to FAdV-2/-11. The FAdV-D isolate SD1763-1 in the present study and some previously suspected FAdV-11 isolates were clustered in the same branch on the entire genomic and

PRKM = \frac{\text{total viral genome reads}}{\text{mapped reads (millions) \times viral genome length (KB)}}

Multiple sequence alignment with other FAdVs worldwide (Table 1) was carried out using the sequence analysis software Lasergene 1 (DNASTAR Inc., Madison, WI) and the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequenced strain was compared to all published whole genome sequences of avian FAdVs representing the different genera. The evolutionary history of FAdV genomes was inferred using Neighbor-joining method (MEGA 6). Phyloge-
netic analysis of the Hexon, Fiber, and Penton proteins of isolates from recent years was carried out. ORF identifi-
cation and gene prediction were carried out by Gene-MarkS v 4.28. Multiple genome alignments were analyzed with the suite of recombination detection algorithms using MEGA6 and SimPlot v 3.5.1 to detect possible recombination events among FAdV strains. Genetic features of geographical characteristics and genomic signatures associated with biological properties were explored based on the genomic analysis and patho-
genic properties of all document strains.
Table 1. FAdV strains used in comparison with the genome of SD1763-1 and SD1763-2.

| Species | Prototype | Isolate | Accession no. | Year | Country of origin |
|---------|-----------|---------|---------------|------|-------------------|
| A       | 1         | CELO    | NC_001720     | 1957 | USA               |
| B       | 5         | 340     | KF493646      | 1970s| Northern Ireland  |
| C       | 4         | KR5     | HE608152      | 1950s/1960s | Japan    |
|         | 10        | C2-B    | MK572851      | —    | USA               |
|         | ON1       |         | GU188428      | 2004 | Canada            |
|         | JP/LVP-1-96|         | LC628937      | 1996 | Japan             |
|         | BI-7      |         | KU342001      | 2011 | India             |
|         | MX-SHP95  |         | KP235475      | 1995 | Mexico            |
|         | AG234-CORR|         | MK572849      | 1995 | Mexico            |
|         | INT4-ATTENUATED-AG234| MK572850 | 1995 | Mexico            |
|         | SCDY      |         | MK629523      | 2018 | China             |
|         | AH-F19    |         | MN781666      | 2019 | China             |
|         | JSJ13     |         | KM096544      | 2013 | China             |
|         | CH/AHMGC/2018|         | MN603933      | 2018 | China             |
|         | AQ        |         | KY343520      | 2016 | China             |
|         | SCnij1601 |         | KY3927938     | 2016 | China             |
|         | ZJ2015    |         | MF521611      | 2015 | China             |
|         | D9        |         | KY379035      | 2016 | China             |
|         | JS7       |         | KY435619      | 2015 | China             |
|         | AH726     |         | KY435621      | 2016 | China             |
|         | AH712     |         | KY435622      | 2016 | China             |
|         | HLJ-J160826|         | KY59422       | 2016 | China             |
|         | CH/S/JCN/2015|         | MG924745      | 2015 | China             |
|         | GX-1      |         | MH454598      | 2017 | China             |
|         | D2004737  |         | MT813039      | 2020 | USA               |
|         | HLJDAd15  |         | KX538980      | 2015 | China             |
|         | SDX1      |         | KY636140      | 2015 | China             |
|         | HLJFA15   |         | KU991797      | 2015 | China             |
|         | CH/AHW/2018|         | MN66092       | 2018 | China             |
|         | SDTA2     |         | MW349185      | 2019 | China             |
|         | AH-F18    |         | MN781665      | 2018 | China             |
|         | CH/AHBZ/2015|         | KU569295      | 2015 | China             |
|         | CH SDDZ 2015|         | KU569276      | 2015 | China             |
|         | CH/SXZ/2015|         | KU569276      | 2015 | China             |
|         | CH/HSZ/2015|         | KU569276      | 2015 | China             |
|         | CH/SXZ/2015|         | KU569276      | 2015 | China             |
|         | ZZ        |         | MN337322      | 2016 | China             |
|         | CH/GDFY/201706|         | MK387062      | 2017 | China             |
|         | HB1502    |         | KX424101      | 2015 | China             |
|         | SD1501    |         | KX424101      | 2015 | China             |
|         | HN1501    |         | KX424103      | 2015 | China             |
|         | SD1511    |         | MF496037      | 2015 | China             |
|         | FAdv-n22  |         | MT119964      | 2019 | China             |
|         | NIVD2     |         | MG547384      | 2017 | China             |
|         | SD1601    |         | MH00602       | 2016 | China             |
|         | HN/151092 |         | KX090424      | 2015 | China             |
|         | CH/AHMC/2015|         | MG143335      | 2015 | China             |
|         | HB1510    |         | KU585719      | 2015 | China             |
|         | GDMZ      |         | MG556954      | 2016 | China             |
|         | HLJ/151118|         | KX067150      | 2015 | China             |
|         | HN/151025 |         | KU245450      | 2015 | China             |
|         | AHFY19    |         | MN54222       | 2019 | China             |
|         | SR48      |         | KT862806      | 1950s/1960s | Japan   |
|         | SR49      |         | KT862807      | 1950s/1960s | Japan   |
|         | A-2A      |         | NC_008909     | 1999 | Canada            |
|         | 380       |         | KT862812      | 1950s/1960s | United Kingdom |
|         | 389-CORR  |         | MK572873      | 1971 | United Kingdom    |
|         | P7-A      |         | MK572866      | —    | USA               |
|         | GB528     |         | MK572867      | 1998 | Switzerland       |
|         | 13-11324  |         | MK572872      | 2012 | Austria           |
|         | 685-CORR  |         | MK572874      | 1950s/1960s | United Kingdom |
|         | 685       |         | KRT862805     | 1950s/1960s | United Kingdom |
|         | 08-9513   |         | MK572870      | 2008 | Germany           |
|         | 08-8872   |         | MK572869      | 2008 | Germany           |
|         | GB591     |         | MK572868      | 1998 | Germany           |
|         | FAdv-D    |         | MN509168      | 2018 | Australia         |
|         | GA-1358/1995|        | MN711799      | 1995 | USA               |
|         | Iran/UT-Kiaae/2018|     | MK757569      | 2018 | Iran              |
|         | PKFAd18   |         | MN428137      | 2018 | Pakistan          |
|         | 08-18926  |         | MK572871      | 2008 | Austria           |
|         | MX05-S11  |         | KU746935      | 1995 | Mexico            |
|         | ON P2     |         | KU310942      | 2005 | Canada            |
|         | ON NP2    |         | KP231537      | 2005 | Canada            |
|         | FAdv-D    |         | KM696546      | 2014 | China             |

(continued)
segmental phylogenetic trees, which is closer to FAdV-2 prototype strain 685 than SR48 and FAdV-11 strain 380. Before re-genotyping the FAdV-D group, we consider these isolates more likely to belong to the FAdV-2 type, which remains to be precisely determined by the systemic cross-neutralization test. FAdV-D isolates are global distribution and not grouped phylogenetically according to geographic regions, but all Chinese isolates were grouped in the same branch with three North American isolates ON NP2, ON P2, and MX95-S11.

Table 1 (Continued)

| Species | Prototype | Isolate | Accession no. | Year | Country of origin |
|---------|-----------|---------|---------------|------|------------------|
| HBQ12   | JL/1407   | KM096545| 2012          | China|
| LN/1507 |           | KU497449| 2015          | China|
| E       | 6         | CR119   | KT862808      | 1950s/1960s | Japan |
| 7       | YR36      | KT862809| 1950s/1960s   | Japan |
| 8a      | TR59      | KT862810| 1950s/1960s   | Japan |
| 8b      | 764       | KT862811| 1950s/1960s   | United Kingdom |
| HG      |           | GU734104| 2011          | Canada |

— indicated that the information was not queried.

Figure 1. Phylogenetic analysis and sequence alignment of Chinese FAdV strains SD1763-1 and SD1763-2 based on the whole genome sequences of FAdVs. (A) Phylogenetic analysis of the entire genome of all FAdV-D strains with full-length sequences available and comparison of the longer repeat region (TR-2) present in the FAdV-D genomes. FAdV-9 prototype strain A-2A was used as a reference strain. (B) Phylogenetic analysis of all FAdV-C strains with full-length sequences available and comparison of all FAdV-4 complete genomes. FAdV-4 prototype strain KR5 was used as a reference strain. The repeated region subunit is marked by a separated blue box and the box numbers represent the repeats numbers. Red box represents deletion and other blue box represents insertion at the indicated position. Open reading frames (ORFs). (C) Phylogenetic trees based on amino acid sequences of hexon, fiber and penton genes of SD1763-1, SD1763-2 and other FAdVs. For each gene, deduced amino acid sequences from SD1763-1 and 1763-2 were aligned with other representative FAdV isolates of different serotypes for which full-length sequences were available in GenBank using DNASTAR (version 7.1.3). All phylogeny trees in Figure 1 were created by the neighbor-joining method with MEGA (version 5.05). The numbers at the branch points show the bootstrap values calculated from 1000 bootstrap replicates, and the scale bars indicate the numbers of nucleotide/amino acid substitutions per site. The blue branches represent Chinese isolates, and the red branches represent isolates from other countries. The red triangle represents FAdV prototype strains and the blue circle represents SD1763-1 and SD1763-2 strains.
Genomes of FADV-D and FADV-C Species

According to the genome evolution, indicating a close evolutionary relationship between these isolates.

Experimental trials to determine the genomic signatures associated with FAdV-D virulence have been conducted with inconsistent results. However, genomic signatures associated with FAdV-4 virulence are not clearly attributed to a specific gene until now (Schachner et al., 2021). The longer repeat region (TR-2) present at the right end of the genome has been considered a potential marker of pathogenic FAdV-D (Ojic and Nagy, 2001). The nonpathogenic ON-NP2 recovered from a healthy flock and the nonpathogenic FAdV-9 had 13 TR-2 repeats, while all pathogenic isolates contained 8 or fewer repeats in the TR-2 region. In contrast, FAdV-11 strain MX95-S11, which was also isolated from a healthy farm, contains only 6 repeats in the TR-2 region. The genome of SD1763-1 strain in the present study contained only four identical and contiguous 135 bp TR-2 repeats, as shown in Figure 1A. The TR-2 subunit repeats are required for efficient virus replication in vitro in FAdV-9. A large number of TR repeats at the 3’ end of gam-1 reduces the amount of GAM-1 protein, which increases in the late stage of infection and facilitates the spread of human adenovirus on the host tissue. The detailed function and its mechanism of the TR repeat in FAdV-D pathogenesis remains to be defined. Currently, only a few complete genomes of FAdV-D are available in the public database, and many of which have not been systematically studied for their pathogenicity. Further whole genome of FAdV-D and pathological characterization studies are needed to explore the genetic determinants of virulence.

Based on enlarged FAdV-4 cohort genome data, we were able to systematically explore the evolutionary relationship and genomic features among geographically distinct isolates worldwide. All Chinese FAdV-4 isolates were phylogenetically grouped in the same branch based on the entire genome, while the FAdV-4 isolates from other countries were clustered with FAdV-10 strains in the region of the genome compared to the corresponding nucleotide positions within the KR5 reference strain. Deletion of CCCCCCT at residue 28785 leads to the absence of a continuous Pro in 22KDa protein. Deletion of A at residue 35328 leads to the frameshift of ORF42 to encode a shorter protein. The 1966bp deletion resulted in the absence of ORF19, ORF27, and ORF48. Insertion of AAT at residue 38330 encoded an additional Ile in ORF43 (Figure 1B). These results indicate that there is a certain evolutionary relationship between D2004737 strain and the epidemic FAdV-4 isolates in China (Mete et al., 2021).

The emergence of high pathogenic FAdV-4 isolates is a concern for the poultry industry (Zhang et al., 2018). MX-SHP95 is a highly virulent FAdV that causes 100% mortality in 1-day-old chicks when challenged with a higher dosage of the virus, and leads to 40% mortality when administrated a lower dose of virus. The American isolate D2004737 recently reported induced IBH in adult chicken (Mete et al., 2021). However, some FAdV-4 strains are reportedly nonvirulent based on clinical observations or experimental infections, such as ON1 and KR5. B1-7 was also a nonvirulent strain isolated from healthy poultry birds in India reported in 2011. INT4-ATTENUATED-AG234 was generated by in vitro-attenuation of FAdV-4 isolate of AG234/INT4. Fiber2 and hexon genes play partial roles in the virulence of the emerging and highly pathogenic FAdV-4 isolates (Zhang et al., 2018). It is not clear whether there are other genetic features that enable a pathogenic FAdV-4 to cause a specific disease. The molecular differences in the entire genomes between highly virulent strains and nonvirulent strains of FAdV-4 were therefore further investigated (Figure 1B). The above nonpathogenic strains KR5, ON1, B1-7, and INT4-ATTENUATED-AG234 possessed 4, 3, 3, and 1 TR-E smallest units respectively, whereas the pathogenic strains including Chinese FAdV-4 isolates, AG234-CORR, D2004737, and MX-SHP95 lack the TR-E unit in the genome, it suggest that the region may also have a role in pathogenesis. The Japanese isolate JP/LVP/96 also contains 2 repeats of TR-E smallest unit suggesting that this strain is more likely to be a nonpathogenic virus. In addition, these virulent viruses contain a conservative Ser mutation at residue 66 and GGA insertion coding Gly at residue 56 in ORF16, the exact role of which remains to be determined.

It would be interesting to analyze the recombinants between whole genomes of isolate among different FAdV species. The homology of the entire genome sequence of FAdV-D strain SD1763-1 and FAdV-C strain SD1763-2 is relatively low, and there is no recombinant signal between the genomes of the two viruses of FAdV-D and FAdV-C in the present study, which is consistent with previous report (Schachner et al., 2019). However, it is worth noting that a single clinical specimen containing FAdV-D and FAdV-E cannot be sequenced and successfully assembled in a single next-generation sequencing reaction because recombinations are common among these specimens. The viral strains in such mixed infection samples should first be cloned for purification in order to obtain each accurate genome sequence.
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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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