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

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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 (Benkő 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). Aviadenoviruses have the largest genome (43−45 kb) among adenoviruses (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 d 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 507nt fragment of the hexon gene using primer set FAdV-F: AATTTCGACCCCATGACGCGCCAGG and FAdV-R: TGGCGAAAGGCGTACGGAAGTAAGC. The FAdV-positive supernatants were propagated on the confluent monolayers of a chicken hepatomacell 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 using the TIANamp Genomic DNA Kit according to the manufacturer's instructions.

Sequencing of the FAdV whole genome was performed using Next-Generation Sequencing on an Illumina platform (HiSeq4000). Paired-end (PE) libraries with 300 bp paired-end distance were generated and sequenced. Clean sequence reads were obtained after quality control and filtration, and then mapped against the available genome of G. gallus v. 5.0 to remove the contamination of G. gallus genome. Newbler v2.8 was employed for de novo assembly of the genome sequence by comparison with already available complete FAdV genomes. Gaps between the contigs and sequence ambiguities generated in the high-throughput sequencing were resolved by PCR and classic Sanger sequencing in order to finish the whole genome assembly. A number of clean reads obtained in each viral genome were counted and converted into RPKM by the following formula.

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

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). Phylogenetic analysis of the Hexon, Fiber, and Penton proteins of isolates from recent years was carried out. ORF identification and gene prediction were carried out by GeneMarkS 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 pathogenic properties of all document strains.

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

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	KC493646	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
		B1-7	KU342001	2011	India
		MX-SHP95	KP295475	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/AHMG/2018	MN606303	2018	China
		AQ	KY436520	2016	China
		SCnj1601	KY927938	2016	China
		ZJ2015	MF521611	2015	China
		HN	KY379035	2016	China
		JS7	KY436519	2015	China
		AH726	KY436521	2016	China
		AH712	KY436522	2016	China
		HLJ/160826	KY569422	2016	China
		CH/JS/TCZHP/2015	MG824745	2015	China
		GX-1	MH454598	2017	China
		D2004737	MT813039	2020	USA
		HLJDAd15	KX538980	2015	China
		SDSX1	KY636400	2015	China
		HLJFAd15	KU991797	2015	China
		CH/AHWH/2018	MN606302	2018	China
		SDTA2	MW349185	2019	China
		AH-F18	MN781665	2018	China
		CH/AHBZ/2015	KU569295	2015	China
		CH SDDZ 2015	KU558761	2015	China
		CH/SXCZ/2015	KU558762	2015	China
		CH/HNJZ/2015	KU558760	2016	China
		CH/JSXZ/2015	KU569296	2015	China
		ZZ	MN337322	2016	China
		CH/GDYF/201706	MK387062	2017	China
		HB1502	KX421401	2015	China
		SD1501	KX421404	2015	China
		HN1501	KX421403	2015	China
		SD1511	MF496037	2015	China
		FAdV-n22	MT119964	2019	China
		NIVD2	MG547384	2017	China
		SD1601	MH006602	2016	China
		HN/151029	KX090424	2015	China
		CH/AHMC/2015	MG148335	2015	China
		HB1510	KU587519	2015	China
		GDMZ	MG856954	2016	China
		HLJ/151118	KX061750	2015	China
		HN/151025	KU245540	2015	China
		AHFY19	MN542422	2019	China
D	2	SR48	KT862806	1950s/1960s	Japan
	3	SR49	KT862807	1950s/1960s	Japan
	9	A-2A	NC_000899	1999	Canada
	11	380	KT862812	1950s/1960s	United Kingdom
		380-CORR	MK572873	1971	United Kingdom
		P7-A	MK572866	—	USA
		GB528	MK572867	1998	Switzerland
		12-11324	MK572872	2012	Austria
		685-CORR	MK572874	1950s/1960s	United Kingdom
		685	KT862805	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	MN711789	1995	USA
		Iran/UT-Kiaee/2018	MK757569	2018	Iran
		PKFAd18	MN428137	2018	Pakistan
		08-18926	MK572871	2008	Austria
		MX95-S11	KU746335	1995	Mexico
		ON P2	KU310942	2005	Canada
		ON NP2	KP231537	2005	Canada
		FAdV-D	KM096546	2014	China

(*continued*)

Table 1 (*Continued*)

Species	Prototype	Isolate	Accession no.	Year	Country of origin
		HBQ12	KM096545	2012	China
		JL/1407	KY012057	2014	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.

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

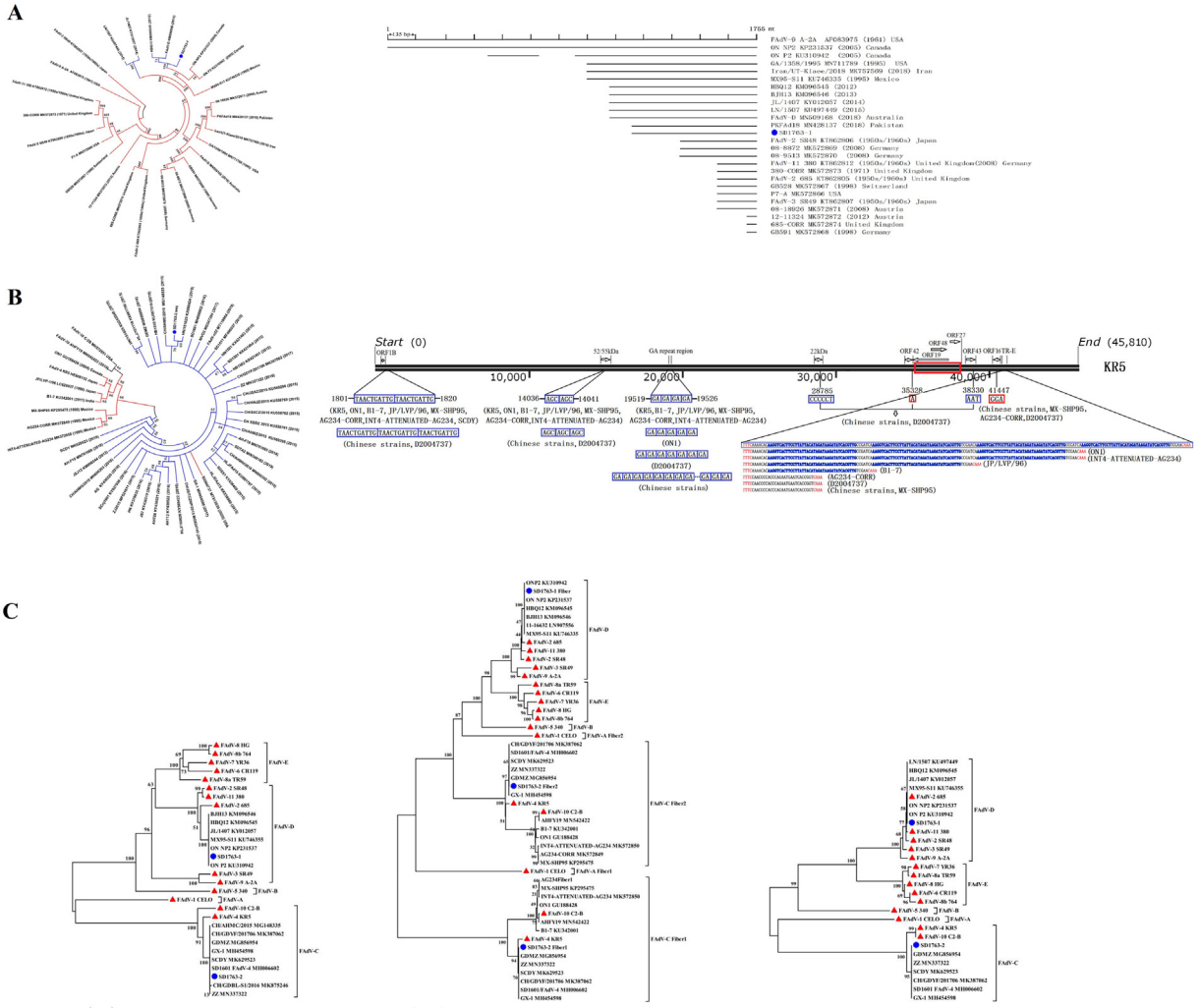

Figure 1. Phylogenetic analysis and sequence alignment of Chinese FAdV strains SD1763-1 and SD1763-2 based on the whole genome sequences of FAdVs. (A) Phylogenetic analysis of the entire genome of all FAdV-D strains with full-length sequences available and comparison of the longer repeat region (TR-2) 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.

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-D 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 (Ojkic 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 a relatively far branch with the American FAdV-4 strain D2004737 for an exception (Figure 1B). In the GA repeat region between gene pX and pVI, all the Chinese isolates had 8 to 11 repeats and the SD1763-2 in the present study had 9 repeats, whereas all of the isolates from other countries had less than 7 repeats. It is notable that conservative mutations were found in Fiber2 (459Asn, 479Leu) and Hexon (193Arg, 195Gln, 842Ala) of Chinese FAdV-4 isolates, which can also be used as genetic features. The American FAdV-4 strain D2004737 isolated in 2020 possessed a lot of genomic features the same as Chinese strains including three 10 nt-direct repeats in ORF1B, nucleotides insertion encoded an additional Gln in the 52/55kDa protein, conservative mutations in Fiber1 protein (28Ser, 157Arg, 314His, 378Ser) and ORF19A (849Gly). Similarly, these isolates also possessed conservative mutations on the right end region of the genome compared to the corresponding nucleotide positions within the KR5 reference strain. Deletion of CCCCCT at residue 28785 leads to the absence of two 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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