

Research Note: Molecular relationship of the fowl adenovirus serotype 4 isolated from the contaminated live vaccine and wild strains isolated in China, 2013-2018

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ABSTRACT Since June 2013, hydropericardium-hepatitis syndrome caused by putative novel fowl adenovirus 4 (FAdV-4) infection has spread all over China, leading to great economic losses. Previous study found that the use of attenuated vaccines contaminated with FAdV-4 is likely to be an important cause of such large-scale transmission. Here, we sequenced the whole genome of this strain through the next-generation sequencing and carried out a retrospective analysis of the FAdV-4 strains that have been determined in China recently. Results show the vaccine strain was almost

100% identical with wild virus strains, especially with 4 strains considering the difference of the GA repeat region, further linking the relationship between vaccine contamination and FAdV-4 prevalence in China. Meanwhile, there is no time and regional preference for the emergence of FAdV-4 strains with different molecular characteristics in China, which indicates that there may be multiple routes of transmission of this virus, suggesting that we still need to pay more attention to and formulate correct prevention and control in the future.

Key words: FAdV-4, whole genome, inclusion body hepatitis-hydropericardium syndrome, vaccine contamination

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INTRODUCTION

Since June 2013, hydropericardium-hepatitis syndrome (HHS) caused by fowl adenovirus 4 (FAdV-4) has appeared in many provinces of China, posing a huge threat to poultry industry (Zhang et al., 2016; Pan et al., 2017; Chen et al., 2019). Fowl adenovirus 4 mainly infects 20- to 30-day-old broilers and causes a mortality rate ranging from 20 to 80% (Meng et al., 2019). Analysis showed that the pathogenicity of this epidemic strain was significantly higher than that of previous isolates, and there were distinct mutations in its genome, especially in the genes of *fiber-2* and *hexon*, which were subsequently identified as closely related to the increased virulence (Liu et al., 2016; Ye et al., 2016; Li et al., 2018; Zhang et al., 2018).

However, it is still not clear how the strain spread in China at an incredible fast speed. Previous studies have shown that the use of attenuated vaccines contaminated with FAdV-4 may be an important reason for the outbreak of HHS in some flocks (Su et al., 2018a,b). However, the complete genome of the vaccine-contaminated strain is not provided, which hinders the further determination of its relationship with other identified strains. Therefore, this study sequenced the above-mentioned FAdV-4 isolate and compared it with other strains isolated from 2013 to 2018, which showed almost 100% consistency among them, verifying our previous reasoning again.

MATERIALS AND METHODS

Virus Background

The FAdV-4 strain (F-Vac) used in this study was isolated from a Newcastle disease virus attenuated vaccine and stored in our laboratory at -80°C , and it can induce obvious HHS in specific-pathogen-free chicken (Su et al., 2018a,b). Detailed isolation and identification

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Table 1. Background information and characteristics of FAdV-4 strains in China.

Strain name	GenBank No.	Time	Location	Identity ¹ (%)	GA repeat	TC repeat
F-Vac	MT119964	2015	Hebei		GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
JSJ13	KM096544	2013	Jiangsu	99.89	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HLJFAd15	KU991797	2015	Heilongjiang	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
ZJ2015	MF521611	2015	Zhejiang	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
NIVD2	MG547384	2017		99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HLJDAD15	KX538980	2015	Heilongjiang	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHSXCZ2015	KU558762	2015	Sichuan	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHAHMC2015	MG148335	2015	Anhui	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
SD1511	MF496037	2015	Shandong	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHAHBZ2015	KU569295	2015	Anhui	99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HN1501	KX421403	2015	Henan	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
SD1501	KX421404	2015	Shandong	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HB1502	KX421401	2015	Hebei	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HB1510	KU587519	2016	Hubei	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HLJ160826	KY569422	2016	Heilongjiang	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
SCnj1601	KY927938	2016	Sichuan	99.96	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
GX-1	MH454598	2018	Guangxi	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHJSTCZHP2015	MG824745	2015	Jiangsu	99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHJSXZ2015	KU569296	2015	Jiangsu	99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
SDSX1	KY636400	2015	Shandong	99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HLJ151118	KX061750	2015	Heilongjiang	99.96	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HN151025	KU245540	2015	Henan	99.96	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
GDMZ	MG569454	2016	Guangdong	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
SD1601/FADV-4	MH006602	2016	Shandong	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
AQ	KY436520	2016		99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
JS7	KY436519	2016	Jiangsu	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HN	KY379035	2016	Henan	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHGDYF201706	MK387062	2017	Guangdong	99.99	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
SCDY	MK629523	2018	Sichuan	99.57	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHSDDZ2015	KU558761	2015	Shandong	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
CHHNJZ2015	KU558760	2015	Henan	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
HN151029	KX090424	2015	Henan	99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
ZZ	MN337322	2016	Henan	99.98	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
AH712	KY436522	2016	Anhui	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG
AH726	KY436523	2016	Anhui	99.97	GAGAGAGAGAGAGAGAACC	TCTCTCTCTCTTTG

Abbreviation: FAdV-4, fowl adenovirus 4.

¹Indicates the identity between F-Vac and reference.

procedures can be found in the previous report (Su et al., 2018a). Phylogenetic analysis based on the partial *hexon* gene has demonstrated that this isolate belongs to FAdV-4 (Su et al., 2018a).

DNA Extraction and Sequencing

DNA was extracted from 1 mL of virus sample using a commercial kit (Bio-Tek, Norcross, GA). Total DNA was resuspended in 50 µL DNase-, RNase-, and proteinase-free water and then sequenced by next-generation sequencing (NGS) with the Illumina MiSeq. Then, all reads were processed through the quality trimming using FastQC to remove nontarget reads with similarities to chicken genome. First, the adapter-jointed reads were removed by the Trimmomatic with parameters of “forward-AGATCGGAAGAGCACACGTCTGAACT” and “reverse-AGATCGGAAGAGCGTCGTGTAGGGAA”. Then, all reads were searched for the similar sequences in the NCBI via BLASTN, and those sharing great similarity with chicken were considered as the nontarget and removed, while the rest of the clean reads were *de novo* assembled using CLC genomic workbench, V7.5, software (QIAGEN, Boston, MA). Gapfiller was used to make up gap for the contig, and PrinSeS-G was used to correct the clip errors and the insertion of small fragments.

Sequencing and analysis are performed twice independently to avoid any possible errors.

Genome Analysis

The genome of F-Vac isolated in China from 2013 was downloaded from GenBank, and the detailed information about these strains was recorded for analysis. Multiple alignments were carried out using ClustalX (BioEdit, version 7.0) and BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences obtained in this study were deposited to the GenBank, and the NGS raw data was deposited to the NCBI.

RESULTS

NGS and Raw Data Analysis

This study yielded 3,369 Mb of fastq format sequence data, which contain 47,355,126 reads. The Q30 bases ratio was 93.11%, demonstrating that the sequencing was effective and the data were reliable. After deleting nontarget reads, a total of 45,699,670 clean reads were finally generated and then used for *de novo* assembling. Meanwhile, contamination assessment showed that the data obtained do not contain any fragments of other

virus, which shows that the method we established before to isolate exogenous virus from contaminated vaccine is reliable (Su et al., 2018a).

Whole-Genome Sequence Analysis

After *de novo* assembly, the whole genome of F-Vac was successfully obtained, which is 43,723 in length and has been deposited in GenBank with the accession number MT119964. The C + G content for that is 54.87%, similar to other F-Vac (Liu et al., 2016). Meanwhile, in this study, 34 full-genome sequences of FAdV-4 isolated in China from 2013 to 2018 were retrieved from the NCBI database and used for the following analysis, and details about those strains can be found in Table 1.

Homology analysis showed that the F-Vac shares 99.57 to 99.99% identities with 34 references (Table 1), and the *fiber-2* and *Hexon* genes of all these strains are 100% identical (data not shown), indicating this strain isolated from the contaminated vaccine was highly consistent with that from the wild. However, alignment analysis of these whole genomes revealed the differences between these strains, which can be used for the classification of epidemic strains. Briefly, 4 different types (8–11 GAs) of GA repeats were found in these 35 strains, while F-vac and 4 reference strains [JSJ13/KM096544 (Zhao et al., 2015), HLJFAd15/KU991797 (Pan et al., 2017), ZJ2015/MF521611, and NIVD2/MG547384] belong to the same type (8 GAs), but these different types of strains have no clear regularity with the time and place of isolation (Table 1). Besides, 3 different types (5–7 TCs) of TC repeats were also found, but only 3 strains were different from the others that contain 6 TCs, including F-Vac (Table 1).

DISCUSSION

Fowl adenovirus 4 has been epidemic in China for a long time since June 2015, causing severe HHS in poultry and leading to great economic losses (Li et al., 2017). A lot of research works show that a novel type of FAdV-4 is the cause of this long-term infection, which contains various genomic deletions and multiple distinct amino-acid mutations in their major structural genes, such as *fiber-2* and *hexon* (Liu et al., 2016; Jiang et al., 2019). Furthermore, mutations in these 2 genes were identified as closely related with the increased pathogenicity (Zhang et al., 2018), leading to a 20 to 80% mortality rate (Ren et al., 2019; Wang and Zhao, 2019).

Meanwhile, previous studies identified that the using of live vaccines contaminated with FAdV-4 and chicken infectious anemia virus may be the cause of infection in certain flocks, and the copathogenicity of them should be responsible for up to 80% mortality (Su et al., 2018a). However, it has not provided the whole genome of the FAdV-4 contaminated in the vaccine, which makes it difficult to further identify the relationship between it and wild strains. Therefore, in this study, the whole genome of the strain was determined and analyzed with other strains isolated in China.

Analysis showed that the whole genome of all 35 F-Vac isolated from China is highly consistent, especially the *fiber-2* and *hexon* genes related to increased pathogenicity (Zhang et al., 2018), with 100% identity. However, it is worth noting that these strains are not exactly the same, and they can be divided into 4 types according to the differences of GA repeat fragments. Among them, 4 reference strains are consistent with the contaminated strains in the vaccine, indicating that they are more closely related. Above results suggested that we can preliminarily screen the possibility that whether the FAdV-4 comes from above vaccine contamination or related wild strains by sequencing such molecular marker when chickens are infected.

On the other hand, different types of FAdV-4 have been isolated in any year from 2013 to 2018, and these viruses have no regional preference, showing that the differences in the GA regions are not gradually emerging with evolution, but multiple types are spreading together (Table 1). Therefore, there may be multiple infectious sources and transmission routes in the prevalence of FAdV-4 in China, which deserves our attention and further study. Meanwhile, as the genome of FAdV-4 circulating in China is highly consistent, except for the GA repeat region, it is necessary to determine the effect of different lengths of the GA repeat on its biological characteristics, which is very important to understand the pathogenesis of FAdV-4.

In conclusion, this study provided the whole-genome sequence of an F-Vac isolated from contaminated attenuated vaccine and further linked it with 4 wild strains, showing a close relationship between them. Meanwhile, this study also reviewed all the F-Vac isolated in China from 2013 to 2018, which clarified the similarities and differences between them, laying a foundation for the formulation of reasonable prevention and control measures against it in the future.

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DISCLOSURES

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

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