GENETICS AND MOLECULAR BIOLOGY

Genetic characterization of fowl aviadenovirus 4 isolates from Guangxi, China, during 2017–2019

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ABSTRACT Hepatitis-hydropericardium syndrome (**HHS**) is a severe disease that causes 20 to 80% mortality in chickens aged 3 to 6 wk. Fowl aviadenovirus serotype 4 (**FAdV-4**) plays an important role in the etiology of HHS. Since 2015, outbreaks of HHS have been reported in several provinces of China; however, details regarding the FAdV-4 genome properties are lacking. In the present study, the complete genomes of 9 isolates responsible for these outbreaks in Guangxi Province, China, were sequenced. To investigate the molecular characteristics of these FAdV-4 isolates, we compared their genomes with those of other reported pathogenic and nonpathogenic FAdV-4 isolates. A variable number of GA repeats were observed in the isolates of this study. Each of the isolates GX2017-01, GX2017-02, GX2018-08, and GX2019-09 had 11 GA repeats; GX2017-03, GX2017-04, and GX2017-05 each had 10 GA repeats, while GX2017-06 and GX2018-07 each had 8 GA repeats. We observed several deletions and distinct amino acid mutations in the major structural genes of these isolates when compared with non-Chinese isolates. We found 2 novel putative genetic markers in the hexon protein, one present in GX2017-02, in which aspartic acid (D) was changed to tyrosine (Y), and another present in each of isolates GX2018-08 and GX2019-09, in which serine (S) was changed to arginine (R), when compared with selected Chinese and some non-Chinese isolates. Moreover, the phylogenetic analysis revealed that all the isolates of this study were clustered within FAdV-C. We found that these isolates were closely related to other recently isolated Chinese strains. The data presented in this study will not only increase the understanding of the molecular epidemiology and genetic diversity of FAdV-4 isolates in China but also has an important reference value of the major factors that determine the virulence of FAdV-4 strains.

Key words: fowl adenovirus 4, genetic characterization, amino acid change, outbreak

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INTRODUCTION

Fowl aviadenoviruses (**FAdV**) have been classified into 5 species, A–E, and have 12 serotypes (Liu et al., 2016). Fowl aviadenoviruses cause mainly inclusion body hepatitis, hepatitis-hydropericardium syndrome (**HHS**), and gizzard erosion in chickens (Anjum et al., 1989; Ono et al., 2003). Research has shown that inclusion body hepatitis is caused by all 12 serotypes of FAdV, gizzard erosion is caused by FAdV-1, and HHS is caused by mainly FAdV-4 (Jiang et al., 2019).

Hepatitis-hydropericardium syndrome was first reported in Pakistan in 1987 (Li et al., 2017), and subsequent outbreaks have been recorded in other countries such as India, Japan, Korea, Iraq, Mexico, and Canada and in those in South and Central America (Anjum et al., 1989; Abdul-Aziz and Al-Attar, 1991; Asrani et al., 1997; Abe et al., 1998; Hess et al., 1999; Toro et al., 1999; Kim et al., 2008). The virus affects mainly 3- to 6-week-old broiler chickens, causing 20 to 80% mortality. The disease is characterized by the accumulation of clear and straw-colored fluid in the pericardial sac. Other signs included an enlarged and discolored liver with hemorrhage or necrotic foci of hemorrhage and necrosis (Ren et al., 2019).

Several studies have found that recent Chinese FAdV-4 isolates have significant deletions and substitutions in their genome compared with those of FAdV-4 isolates reported from other countries around the world (Liu et al.,

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2016; Ye et al., 2016). However, further evaluation of the complete genome of new isolates is needed to better understand the pathogenesis of these viruses.

The important viral structural proteins of FAdV are the penton, hexon, and fiber proteins (Echavarria, 2008). The penton protein is responsible for virus internalization during the infectious cycle; the hexon protein contains the main neutralizing epitope and therefore can be used for serotyping, while the fiber protein is responsible for virus–host interactions (Steer et al., 2009; Uusi-Kerttula et al., 2015; Meng et al., 2019). Moreover, recent findings suggested that hexon and fiber 2 are associated with the virulence of emerging and highly pathogenic FAdV-4 isolates (Zhang et al., 2018).

Since May 2015, outbreaks of HHS have increased in several provinces of China, including Guangxi, causing a massive economic loss to the country. For proper understanding of the disease, molecular characterization of the complete genome of FAdV-4 is indispensable. We isolated, sequenced, and characterized the whole genome of 9 different isolates of FAdV-4 from different broiler flocks in Guangxi Province, China. To better understand their genome characteristics, we compared them with some of the previously reported pathogenic and nonpathogenic FAdV-4 strains from different countries, including China. Our study presented additional detailed information on the molecular characteristics of FAdV-4 isolates.

MATERIALS AND METHODS

Sample Collection and Virus Isolation

All isolates were collected from commercial broiler farms that were experiencing HHS in Nanning city, Guangxi province, China, between 2017 and 2019. The mortality due to FAdV-4 recorded in different commercial broiler farms was from 60 to 80%. The complete information of these isolates is given (Table 1). The virus was isolated from the livers of infected birds and propagated in primary cultures of chicken embryo liver cells (Vera-Hernández et al., 2016). Medium 199 (Invitrogen, CA) was used, which was supplemented with 10% fetal bovine serum (Invitrogen, CA). Antibiotics, 100 U/mL of penicillin (Sigma-Aldrich, Rockville Pike, Bethesda, MD) and 100 μg/mL of streptomycin (Sigma-Aldrich), were added to the medium and were used for chicken embryo liver cell culture. After isolation, virus dilutions were made for plaque purification in the chicken liver cell line (LMH) (ATCC, Manassas, VA). Each virus was plaque purified. In brief, virus dilutions from 10^{-2} to 10^{-7} were inoculated onto LMH monolayers. One h after infection, the cell layer was overlaid with Medium 199 containing 2% agarose (Sigma-Aldrich) and incubated for 3 to 5 D at 37°C in a 5% CO_2 humidified atmosphere. Isolated plaques were then picked and transferred to a new fresh culture of LMH cells (Ren et al., 2019). The detection of virus in the samples was performed by PCR using the primers specific for the FAdV hexon gene (GenBank: GU188428), forward: 5′-CAA CTA CAT CGG GTT CAG GG-3′and reverse: 5′-TGG CGT TTC TCA GCA TCA-3′. The PCR profile used was as follows: 95°C for 5 min, followed by 34 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a final elongation step for 10 min at 72°C.

Complete Genome Sequencing

The viral DNA of all the isolates used in this study was extracted from the livers of affected broiler chickens using an Easy Pure Viral DNA/RNA kit (cat no.: ER201-01; Transgene, Guangzhou, China) according to the manufacturer's instructions. Specific primers for the complete genome sequencing, which were designed on the basis of the sequence of the FAdV-4 ON1 strain, were used (Griffin and Nagy, 2011). However, new primers were also designed where necessary to further ensure the sequencing results (Supplementary Table). PCR products with expected lengths were sequenced directly or cloned into the pEASY-Blunt Cloning vector (TransGen, Beijing, China) according to the manufacturer's instructions. The complete sequence was manually assembled using the Seqman program in the DNAstar software package (version 5.01; Madison, WI).

Sequencing Analysis

The nucleotide and deduced amino acid sequences of all the FAdV-4 isolates and certain reference strains used in this study were edited, aligned, and analyzed using the MegAlign program, a part of the Lasergene software package (DNASTAR, Madison, WI). The major structural genes, penton base, hexon, and fiber, were translated into amino acid sequences using EditSeq in the Lasergene software package. The amino acid sequences from these isolates were compared with the

Table 1. Complete information of all isolates.

S.No.	Name of the isolate	GenBank number	Sampling time	Sampling place	Sampling tissue	Age of sampling chicken (days)
1	GX2017-01	MN577977	2,017.7	Guangxi	Liver	40
2	GX2017-02	MN577978	2,017.7	Guangxi	Liver	63
3	GX2017-03	MN577979	2,017.11	Guangxi	Liver	56
4	GX2017-04	MN577980	2,017.11	Guangxi	Liver	65
5	GX2017-05	MN577981	2,017.7	Guangxi	Liver	96
6	GX2017-06	MN577982	2,018.5	Guangxi	Liver	75
7	GX2018-07	MN577983	2,018.1	Guangxi	Liver	63
8	GX2018-08	MN577984	2,018.5	Guangxi	Liver	96
9	GX2019-09	MN577985	2,019.4	Guangxi	Liver	50

amino acid sequences of these proteins of the HNJZ, ON1, and SH95 strains. A phylogenetic tree of the complete genomes of these 9 isolates and additional isolates from China and other parts of the world was produced using the neighbor-joining method and bootstrap test of 1,000 replicates in MEGA 5.0 software (MEGA, Pennsylvania State University, University Park, PA).

RESULTS

Genome Sequencing and Sequence Analysis

The complete genomic sequences of all 9 isolates, FAdV-4-GX2017-001 (GX2017-01), FAdV-4-GX2017-002 (GX2017-02), FAdV-4-GX2017-003 (GX2017-03), FAdV-4-GX2017-004 (GX2017-04), FAdV-4-GX2017-005 (GX2017-05), FAdV-4-GX2017-006 (GX2017-06), FAdV-4-GX2018-007 (GX2018-07), FAdV-4-GX2018-008 (GX2018-08), and FAdV-4-GX2019-009 (GX2019-09), all of which were from Guangxi Province, China, were deposited in GenBank with accession numbers MN577977, MN577978, MN577979, MN577980, MN577981, MN577982, MN577983, MN577984, and MN577985, respectively.

The genome sizes of all these isolates were similar to those of previously reported Chinese isolates; however, they differed in size from those of the isolates from other countries, including nonpathogenic isolates (Griffin and Nagy, 2011; Liu et al., 2016; Vera-Hernández et al., 2016; Ye et al., 2016; Mo et al., 2019; Ren et al., 2019). The full genome sizes of isolates GX2017-01, GX2017-02, GX2017-03, GX2017-04, GX2017-05, GX2017-06, GX2018-07, GX2018-08, and GX2019-09 were 43,725, 43,723, 43,723, 43,722, 43,718, 43,719, 43,726, and 43,726 bp, respectively. The G + C content was found to be 54.85% for GX2017-02, and for all other isolates, the G + C content was 54.87%.

There was a GA repeat region between the Px and pVI genes. The longest GA repeats were found for GX2017-01, GX2017-02, GX2018-08, and GX2019-09 (11 GA repeats each), followed by those for GX2017-03, GX2017-04, and GX2017-05 (10 GA repeats), whereas the least number of GA repeats were found for GX2017-06 and GX2018-07 (8 GA repeats). However, each of these 9 isolates had a longer GA repeat region than the nonpathogenic ON1 strain which has 5 GA repeats. When comparing the GA repeats of all the 9 isolates of the present study with the previously reported Chinese and Mexico isolates, we found that each of the isolates GX2017-06 and GX2018-07 is 3 GA repeats less than HNJZ and MX-SHP95 (Figure 1). Moreover, each of the isolates, GX2017-03, GX2017-04, and GX2017-05 is 1 GA repeat less than HNJZ and MX-SHP95 (Figure 1), whereas GX2017-01, GX2017-02, GX2018-08, and GX2019-09 had the same number of GA repeats as HNJZ and MX-SHP95. All 9 isolates in our study and the previously reported MX-SHP95 and HNJZ isolates had longer TC repeats than the ON1 strain. A deletion of 6 bp at positions 28,784 to 28,789 was also observed in all 9 isolates, as well as in the previous Chinese isolate HNJZ, when compared with the ON1 genome. This deletion was only observed in isolates from China. We found that all 9 isolates had a 1,966-bp deletion in the 3′-end region of the genome when compared with that of ON1. This kind of deletion was also observed in all the Chinese isolates yet studied (Ye et al., 2016). The deletion was located at 35,389 bp to 37,354 bp compared

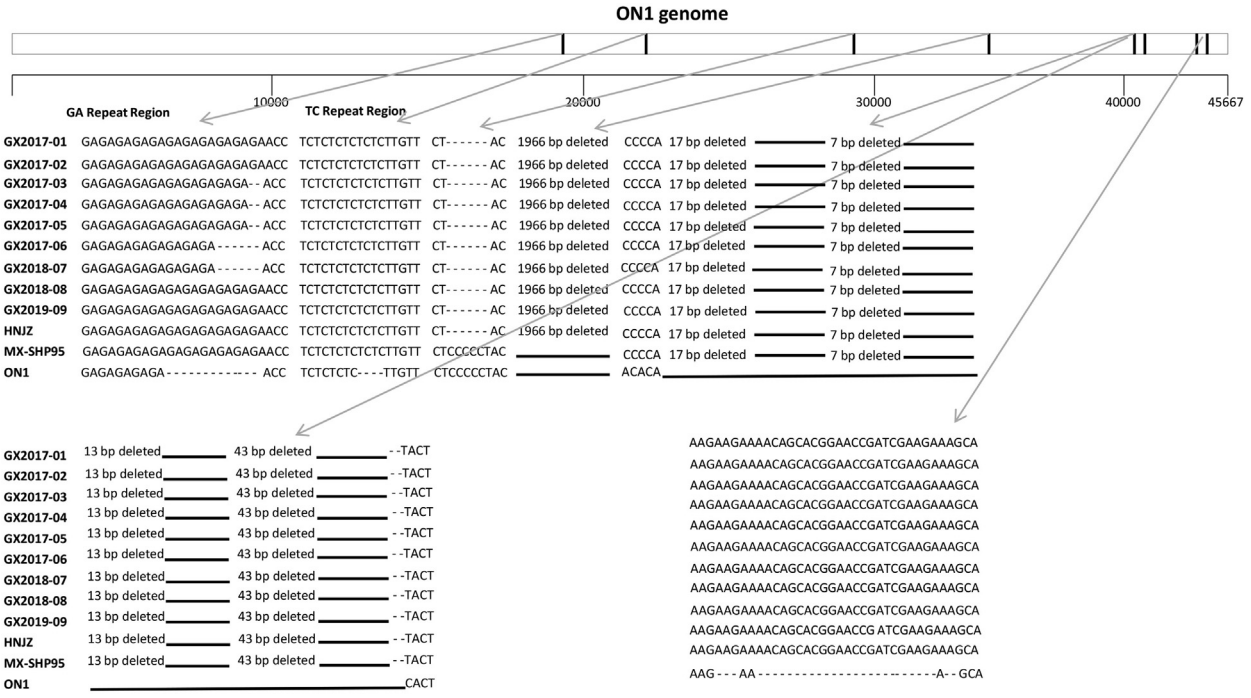

Figure 1. A comparison of the complete genomes of the isolates used in this study and the previously reported sequences of highly pathogenic and nonpathogenic FAdV-4 strains. Major insertions and deletions are shown. Abbreviation: FAdV-4, fowl aviadenovirus serotype 4.

with the nucleotide positions within the ON1 genome. Moreover, deletions from 41,675 bp to 41,691 bp, 41,704 bp to 41,710 bp, 41,748 bp to 41,760 bp, and 41,766 bp to 41,809 bp were also observed in all 9 isolates compared with the nucleotide sequence positions within the ON1 genome. These deletions were observed in all pathogenic strains. An insertion of 27 bp in ORF19 A in all 9 isolates at the position 44,376 compared with the nucleotide sequence positions within the ON1 genome was also observed. The same insertion was also found for the HNJZ and MX-SHP95 strains (Figure 1).

All of these 9 isolates had high nucleotide similarities with other recently reported Chinese FAdV-4 isolates. After comparison, we found that GX2017-01 has 99.88 to 99.99%, GX2017-02 has 99.85 to 99.96%, GX2017-03 has 99.88 to 99.99%, GX2017-04 and GX2018-07 have 99.89 to 100%, GX2017-05 has 99.99 to 99.88%, GX2017-06 has 99.99 to 99.95%, and GX2018-08 and GX2019-09 have 99.81 to 99.92% similarity with other reported Chinese isolates. However, when we compared the nucleotide identities with non-Chinese isolates, we found that GX2017-01 has 98.46 to 98.73%, GX2017-02 has 98.41 to 98.73%, GX2017-03 has 98.46 to 98.78%, GX2017-04 has 98.46 to 98.78%, GX2017-05 has 98.77 to 98.45%, GX2017-06 has 98.78 to 98.46%, GX2018-07 has 98.47 to 98.79%, and GX2018-08 and GX2019-09 have 98.43 to 98.75% similarity with non-Chinese isolates (Table 2).

The Major Structural Proteins of FAdV-4

The FAdV capsid consists of 3 main exposed structural proteins, the hexon, the fiber, and the penton base. Hexon is the major capsid protein that contains group-, type-, and subtype-specific antigenic determinants against which antibodies are produced. Moreover, the molecular classification of FAdV is based on the hexon gene loop 1 region and fiber gene (Hess, 2000; Schachner et al., 2016). The sizes of penton base, hexon, fiber 1, and fiber 2 genes are 1,578, 2,814, 1,296, and 1,440 bp, respectively. Comparison of the amino acid sequences of each of the penton base, hexon, and fiber proteins revealed the presence of several amino acid mutations and deletions with respect to those of the pathogenic strain MX-SHP95 and nonpathogenic strain ON1. We also compared the amino acid sequences of all 9 isolates with the pathogenic isolate HNJZ recently isolated from China. The size of the penton protein for all the 9 isolates was 525 amino acids, the same as HNJZ, ON1, and MX-SHP95. In the penton base, the substitution from S to P was observed at position 42 in all 9 isolates and in the previously isolates HNJZ and MX-SHP95 (Liu et al., 2016; Vera-

Table 2. Nucleotide similarity with some Chinese and non-Chinese isolates.

	Similarity with some Chinese isolates at the nucleotide level (%)			Similarity with some non-Chinese isolates at the nucleotide level (%)		
Isolates	Strain	Accession number	Similarity	Strain	Accession number	Similarity
GX2017-01	SD1601	MH006602	99.99	KR5	HE608152	98.78
	AHHQ	MG148334	99.99	SH95	KP295475	98.69
	TCZHP	MG824745	99.99	B1-7	KU342001	98.58
	JSJ13	KM096544	99.88	ON1	GU188428	98.46
GX2017-02	SD1601	MH006602	99.96	KR5	HE608152	98.73
	AHHQ	MG148334	99.96	SH95	KP295475	98.64
	TCZHP	MG824745	99.96	B1-7	KU342001	98.53
	JSJ13	KM096544	99.85	ON1	GU188428	98.41
GX2017-03	SD1601	MH006602	99.99	KR5	HE608152	98.78
	AHHQ	MG148334	99.99	SH95	KP295475	98.69
	TCZHP	MG824745	99.99	B1-7	KU342001	98.58
	JSJ13	KM096544	99.88	ON1	GU188428	98.46
GX2017-04	SD1601	MH006602	100	KR5	HE608152	98.78
	AHHQ	MG148334	100	SH95	KP295475	98.69
	TCZHP	MG824745	100	B1-7	KU342001	98.58
	JSJ13	KM096544	99.89	ON1	GU188428	98.46
GX2017-05	SD1601	MH006602	99.99	KR5	HE608152	98.77
	HB1510	KU587519	99.99	SH95	KP295475	98.69
	TCZHP	MG824745	99.99	B1-7	KU342001	98.57
	JSJ13	KM096544	99.88	ON1	GU188428	98.45
GX2017-06	NIVD2	MG547384	99.99	KR5	HE608152	98.78
	HB1510	KU587519	99.99	SH95	KP295475	98.69
	GX-1	MH45498	99.99	B1-7	KU342001	98.58
	HN/151025	KU245540	99.95	ON1	GU188428	98.46
GX2018-07	NIVD2	MG547384	100	KR5	HE608152	98.79
	AHHQ	MG148334	99.99	SH95	KP295475	98.70
	TCZHP	MG824745	99.98	B1-7	KU342001	98.59
	JSJ13	KM096544	99.89	ON1	GU188428	98.47
GX2018-08	SD1601	MH006602	99.92	KR5	HE608152	98.75
	AHHQ	MG148334	99.92	SH95	KP295475	98.67
	TCZHP	MG824745	99.92	B1-7	KU342001	98.55
	JSJ13	KM096544	99.81	ON1	GU188428	98.43
GX2019-09	SD1601	MH006602	99.92	KR5	HE608152	98.75
	AHHQ	MG148334	99.92	SH95	KP295475	98.68
	TCZHP	MG824745	99.92	B1-7	KU342001	98.57
	JSJ13	KM096544	99.81	ON1	GU188428	98.43

Hernández et al., 2016). In the hexon protein of the isolate GX2017-02, the position 691 amino acid was changed from aspartic acid (D) to tyrosine (Y), and the position 372 amino acid in GX2018-08 and GX2019-09 was changed from serine (S) to arginine (R). The size of the hexon protein of all 9 isolates in this study was 937 amino acids, which is the same as that in HNJZ, ON1, and MX-SHP95. The fiber protein is related to virus neutralization, cellular receptor binding, and virulence variation (Pallister et al., 1996). Recombinant FAdV-4 fiber 2 protein has been proven to be a protective immunogen against HHS (Schachner et al., 2014). The fiber gene can also be used to differentiate HHS-inducing FAdV-4 isolates from other FAdV-4 strains (Mase et al., 2010). In the present study, the fiber 1 and fiber 2 proteins of all 9 isolates were aligned with those of the HNJZ Chinese isolate, ON1 Canadian isolate, and MX-SHP95 Mexican strain, which enabled us to identify multiple amino acid substitutions. The fiber 1 protein was 431 amino acids long in all 9 isolates, which was the same length as that in HNJZ, but it was 1 amino acid shorter in both the ON1 and MX-SHP95 strains. This 1 amino acid was deleted at position 428. The fiber 2 protein was 479 amino acids long in all 9 isolates, which was the same length as that in HNJZ, but it was 5 amino acids shorter in both the ON1 and MX-SHP95 isolates. These 5 amino acid residues were deleted at position 11. These results indicated that the sizes of the penton, hexon, and fiber proteins were conserved in isolates from China (Table 3).

Some other substitutions were also observed that were unique to pathogenic strains only. Notably, all 9 isolates and the virulent strains from China, HNJZ, and Mexico, MX-SHP95, had amino acid substitutions at position 188 (I to R) in the hexon protein and at 196 (T to V) and at 431 (S to G) in the fiber 1 protein. In the fiber 2 protein, at position 219 (214 in ON1 and MX-SHP95), the nonpathogenic strain ON1 has a glycine (G), whereas in all 9 isolates, HNJZ and MX-SHP95 have an aspartic acid (D). At position 380 (375 in ON1 and MX-SHP95), the nonpathogenic strain ON1 has an alanine (A), whereas in all 9 isolates, HNJZ and MX-SHP95 have a threonine (T). Moreover, we also observed substitutions at positions 232 (E to Q), 300 (I to T), 305 (S to A), 307 (P to A), 319 (V to I), 329 (V to L), 378 (A to T), 405 (P to S), 435 (T to S), and 453 (S to A) in the fiber 2 protein (Table 3). Based on the information that we obtained in this study, fiber 2 may serve as a virulence factor protein. Two other putative genetic markers in the hexon protein of the isolates GX2017-02, GX2018-08, and GX2019-09 need to be investigated further. Moreover, to identify critical sites responsible for the high pathogenicity of FAdV-4 isolates, further molecular analysis and reverse genetic systems are required.

Phylogenetic Analysis

Phylogenetic analysis using nucleotide sequences revealed that the complete genomes of the isolates from the present study clustered within FAdV-C together with other FAdV-4 isolates. The analysis revealed that all 9 isolates in our study were closely related to viruses previously isolated from China. Among these isolates, GX2017-05, GX2017-06, GX2018-07, GX2018-08, and GX2019-09 were closely related among themselves. The isolates GX2017-01 and GX2017-03 were also closely related. The phylogenetic analysis also showed that all the isolates of this study and previous Chines isolates were in close resemblance with the highly pathogenic MX-SHP95 strain of Mexico (Figure 2).

DISCUSSION

Fowl aviadenoviruses cause different diseases of special importance in broiler chickens; moreover, since 2015, the occurrence of these diseases displayed an increasing trend in China (Liu et al., 2016). Fowl adenovirus 4 has been isolated, and its complete genome has been sequenced from different parts of the world and China. To date, the complete genome of 44 isolates of FAdV-4 has been sequenced worldwide; so far, only one isolation has been made from Guangxi province, China (Ren et al., 2019). Therefore, more knowledge and study was required from this province, so the complete genome of 9 FAdV-4 isolates was sequenced and genetically characterized from this province. Moreover, to fully understand the mechanisms involved in the high pathogenesis of these viruses, information about their complete genome is needed. In this study, the whole-genome sequencing of 9 highly pathogenic FAdV-4 isolates has improved the existing knowledge of FAdV genomes.

The genomes of all 9 isolates varied in length from 43,718 bp to 43,726 bp, which was in accordance with those of previously reported Chinese isolates (Zhao et al., 2015; Liu et al., 2016). However, the genome size of these isolates varied from that of the previously reported highly pathogenic and nonpathogenic FAdV-4 isolates from other parts of the world (Griffin and Nagy, 2011; Marek et al., 2012; Vera-Hernández et al., 2016).

Our findings suggested that most of the insertions and deletions occurred on the right-hand side of the genome. All 9 isolates had a deletion of 1,966 bp on the 3′-end region of the genome compared with that of non-Chinese isolates (Figure 1) (Griffin and Nagy, 2011; Vera-Hernández et al., 2016). However, similar to our findings, all of the viruses previously isolated from China had a similar deletion of nucleotides (Liu et al., 2016). It was speculated that this deletion might have a role in the pathogenicity of these isolates, but recent studies using the clustered regularly interspaced short palindromic repeat (**CRISPR**) and CRISPR associated protein 9 (**CRISPR–Cas9**) system found that this deletion was dispensable for the high pathogenicity of such isolates (Pan et al., 2018). The 6-bp deletion in all 9 isolates at position 28,784, when compared with the ON1 genome, was also present in HNJZ, indicating this deletion was specific to Chinese isolates. Moreover, some characteristics were found that were unique to pathogenic strains only, that is, those with the longer GA repeats; GX2017-01, GX2017-02, GX2018-08, and

Table 3. Amino acid differences in the major structural genes from FAdV-4 isolates used in this study.

Genes		Amino acids at position																														
Penton	Isolates	42	45	193	356	370	426	486																										
	GX2017-01	P	D	I	V	P	V	T																										
	GX2017-02	P	D	I	V	P	V	T																										
	GX2017-03	P	D	I	V	P	V	T																										
	GX2017-04	P	D	I	V	P	V	T																										
	GX2017-05	P	D	I	V	P	V	T																										
	GX2017-06	P	D	I	V	P	V	T																										
	GX2018-07	P	D	I	V	P	V	T																										
	GX2018-08	P	D	I	V	P	V	T																										
	GX2019-09	P	D	I	V	P	V	T																										
	HNJZ	P	D	I	V	P	V	T																										
	ON1	S	G	V	A	Q	I	S																										
	MX-SHP95	P	G	V	A	Q	I	S																										
Hexon		164	188	193	195	238	240	243	263	264	372	402	410	574	691	797	842																
	GX2017-01	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	GX2017-02	S	R	R	Q	D	T	N	I	V	S	A	A	I	Y	P	A																
	GX2017-03	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	GX2017-04	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	GX2017-05	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	GX2017-06	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	GX2018-07	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	GX2018-08	S	R	R	Q	D	T	N	I	V	R	A	A	I	D	P	A																
	GX2019-09	S	R	R	Q	D	T	N	I	V	R	A	A	I	D	P	A																
	HNJZ	S	R	R	Q	D	T	N	I	V	S	A	A	I	D	P	A																
	ON1	T	I	Q	E	N	A	E	M	I	S	A	T	V	D	A	G																
	MX-SHP95	T	R	Q	E	N	A	E	M	I	S	Q	T	V	D	A	G																
Fiber 1		14	28	44	69	70	119	126	153	186	196	204	251	262	263	310	329	331	374	383	401	428	431										
	GX2017-01	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2017-02	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2017-03	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2017-04	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2017-05	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2017-06	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2018-07	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2018-08	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	GX2019-09	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	HNJZ	A	S	R	G	S	N	A	R	D	V	G	L	H	D	H	H	R	S	I	N	–	G										
	ON1	v	i	P	S	G	D	V	H	N	T	G	I	Q	E	R	Q	K	P	L	Y	H	S										
	MX-SHP95	v	i	P	S	G	D	V	H	N	V	A	I	Q	E	R	Q	K	P	L	Y	N	G										
Fiber 2		11-15	22	29	114	144	219	232	261	300	305-307	319	324	329	334	338	343-344	346	378	380	391	400	403	405-406	413	435	439	453	459	478			
	GX2017-01	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2017-02	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2017-03	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2017-04	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2017-05	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2017-06	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2018-07	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2018-08	ENJKP	S	A	D		D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	GX2019-09	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	HNJZ	ENJKP	S	A	D	S	D	Q	T	T	ANA	I	V	L	A	N	LN	A	T	T	T	G	E	SI	S	S	E	A	N	L		
	ON1	–	S	p	D	S	G	E	S	I	SHP	V	F	V	T	T	NS	A	A	A	S	A	Q	PS	T	T	D	S	A	V		
	MX-SHP95	–	y	p	A	A	D	Q	N	T	AHA	I	F	L	T	T	NS	V	T	T	S	A	Q	SS	T	S	D	A	A	V		

Abbreviation: FAdV-4, fowl aviadenovirus serotype 4.

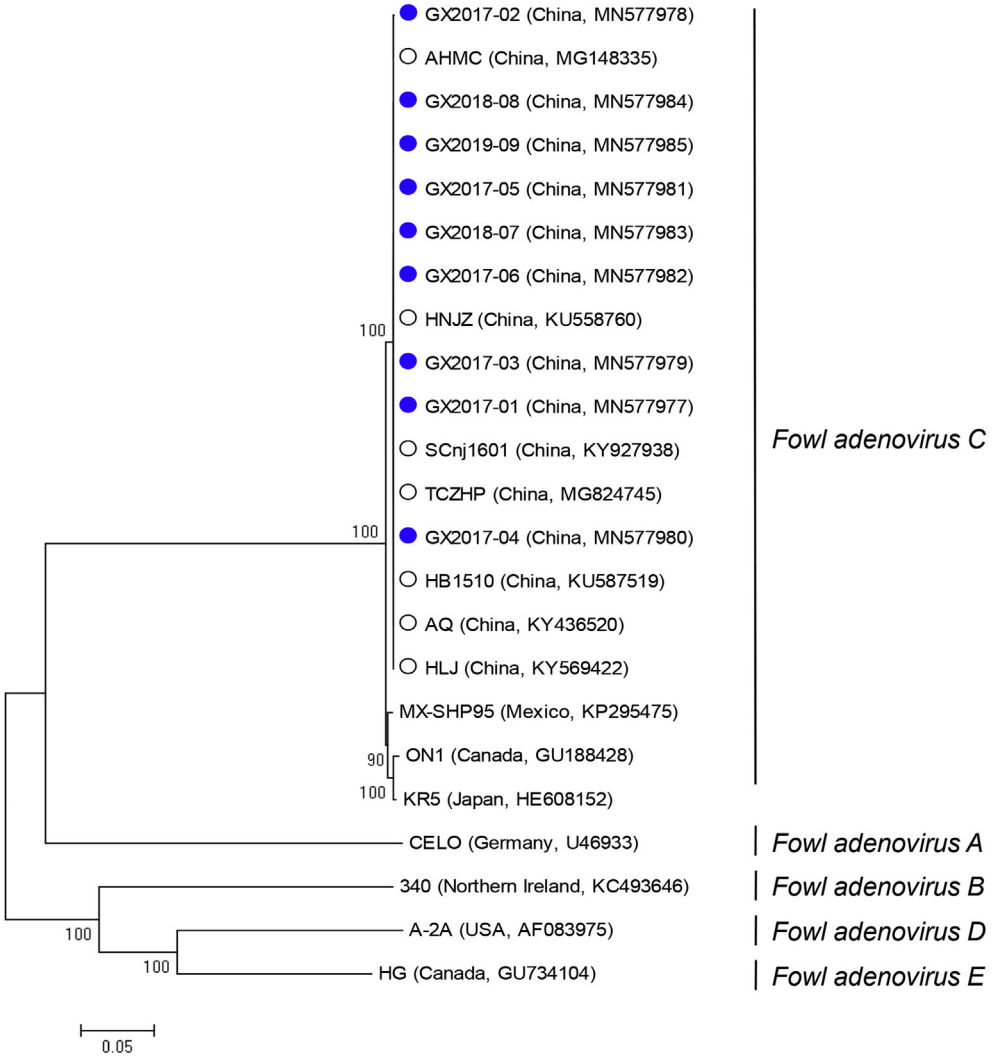

Figure 2. The results of the phylogenetic analysis based on the nucleotide sequences of selected complete genomes. The tree was generated by the neighbor-joining method with a bootstrap test of 1,000 replicates using MEGA 5.0. Blue circles represent the isolates of the present study. The empty circles represent Chinese isolates from previous studies.

GX2019-09 have 11 GA repeats each, GX2017-03, GX2017-04, and GX2017-05 have 10 GA repeats each, and the isolates GX2017-06 and GX2018-07 have 8 GA repeats each (Figure 1). All these isolates have longer GA repeats than the nonpathogenic ON1 strain. It will be interesting to investigate the role of GA repeats in terms of viral pathogenesis. Other characteristics of pathogenic strains are longer TC regions, the deletions between 41,675 and 41,809 and insertions of 27 bp in ORF19 A that were observed in HNJZ, MX-SHP95, and all the 9 isolates of the present study (Liu et al., 2016; Vera-Hernández et al., 2016).

The penton, hexon, and fiber proteins are the major structural proteins of the FAdV capsid (Hess, 2000; Schachner et al., 2016). Therefore, amino acid alignments were used to determine the differences and similarities in these structural proteins among the highly pathogenic and nonpathogenic FAdV isolates. Our findings suggested the presence of several amino acid mutations and deletions when compared with those of the MX-SHP95 and ON1 strains. In the penton protein, a total of 7 substitutions occurred compared with those of the MX-SHP95 and ON1 strains. Interestingly, all these substitutions were similar to those in the previously reported Chinese isolates (Table 3). The occurrence of the substitution from S to P at position 42 within this protein that is present even in the strain SHP95 may indicate that this mutation might be important for the virulence of this virus. The hexon protein showed unique amino acid substitutions at positions 691 (D to Y) in GX2017-02 and 372 (S to R) within each of GX2018-08 and GX2019-09 isolates with respect to those of the ON1 strain. The role of these 2 new putative genetic markers, each in 3 different isolates, may be further elucidated by using a reverse genetic system, such as that used for the hexon and fiber 2 protein, which were shown to be associated with the virulence of highly pathogenic FAdV (Zhang et al., 2018). However, all the other substitutions in the hexon protein were similar to those in a previous Chinese isolate (Liu et al., 2016). Moreover, in the fiber 1 and fiber 2 proteins of all 9 isolates, all the substitutions that were found were the same as those in previous Chinese isolates (Li et al., 2018). These findings suggested that most of the changes in all the major structural proteins of FAdV found in

China were evolutionarily conserved. However, in these 2 fiber proteins, several other amino acid substitutions founded were specific for pathogenic strains only. Moreover, the emergence of highly pathogenic strains of FAdV-4 with some genetic diversity demands additional preventive measures against FAdV-4 infections in poultry farms. The resemblance of all the 9 isolates of the present study and that of previously isolated Chinese isolates with the highly pathogenic MX-SHP95 strain from Mexico revealed a common conserved region which further needs to be investigated.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.06.003

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