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Characterization of fowl adenovirus serotype 4 circulating in chickens in China

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ABSTRACT

Outbreaks of fowl adenovirus (FAdV) has resulted in huge economic losses in poultry industry in China since 2015. This study detected the pathogens from diseased chickens and determined that fowl adenovirus serotype 4 (FAdV-4) and co-infection of immunosuppressive pathogens were the causes of the outbreaks. Phylogenetic analysis results indicated that these pandemic strains originated from previously FAdV-4 predecessor in China and had obtain gene mutations that might contribute to enhanced pathogenicity of these strains. Compared with early strains, the pathogenicity of novel FAdV-4 strains significantly increased, which led to systemic infections and injuries to multiple organs in the infected chickens. Our study could provide useful information for understanding of the FAdV-4 and favorable theory basis for clinical prevention and control of the disease.

1. Introduction

FAdV is a common pathogenic agent in chickens and wild birds worldwide. According to the hexon gene and cross-neutralization tests, FAdVs are divided into five species (FAdV-A to FAdV-E) and 12 serotypes (FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11) (Meulemans et al., 2004). Inclusion body hepatitis (IBH) in chickens is often associated with FAdV-8a, -8b, or -11 (Schachner et al., 2016). However, only FAdV-4 has been reported to cause hydropericardium-hepatitis syndrome (HHS), characterized by pericardial effusion and hepatitis, which always induced high mortality rates (20%–80%) and caused severe economic losses in the global poultry industry (Lim et al., 2011). The first outbreak of HHS that raised concern happened in the Angara region' chicken farms in Pakistan in 1987, and caused huge economic losses, which is why the virus is also referred to as "Angara virus" (abdul-Aziz and al-Attar, 1991; Anjum et al., 1989). Since then, the FAdV-4 has become prevalent in many countries, such as Australia (Christensen and Saifuddin, 1989), India (Dahiya et al., 2002), Japan (Nakamura et al., 2011), Canada (Grgic et al., 2013), Korea (Choi et al., 2012; Kim et al., 2008), and South Africa (Joubert et al., 2014).

Prior to 2014, FAdV had been isolated sporadically in poultry in China (Sun et al., 2008; Wang et al., 2012; Xie et al., 2013). The isolates were genetically related to FAdV-4, FAdV-8a, FAdV-8b and FAdV-11, FAdV-11 (Changjing et al., 2016; Wang et al., 2012). Although multiple serotypes of FAdV were isolated in the field, no widespread epidemics happened until the outbreak of FAdV-4 in the chicken farms of China in 2015 (Niu et al., 2016; Zhang et al., 2016). Due to the robust transmission and high pathogenicity of the novel FAdV-4, many poultry farms have been affected in a short period and suffered from huge economic loss.

In order to understand the characteristics of FAdV epidemics, an epidemiological study was performed. Further characteristics of the virus was determined by whole genome sequence analysis and pathogenicity experiments. In addition, we also investigated the co-infection with immunosuppressive pathogens in the diseased chicken. In summary, we presented a comprehensive study to better understand the epidemic characteristics of the novel FAdV-4 in China for control and prevention of HHS.

2. Materials and methods

2.1. Ethics statement

All animal research was approved by the Beijing Association for Science and Technology (approval ID SYXK [Beijing] 2007-0023) and

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conducted in accordance with the Beijing Laboratory Animal Welfare and Ethics guidelines, as issued by the Beijing Administration Committee of Laboratory Animals, and in accordance with the China Agricultural University (CAU) Institutional Animal Care and Use Committee guidelines (ID: SKLAB-B-2010-005). The animal experiment protocol was approved by the Animal Welfare Committee of the CAU.

2.2. Pathogen detection

From 2015 to 2017, an epidemic survey for FAdVs in domestic poultry was carried out in China. The 77 liver samples from 77 chicken farms suspected FAdVs infection were collected and maintained in transport medium containing antibiotics and kept at 4℃ till transported to the laboratory. In addition, if the pericardial cavity of diseased chickens was filled with pale yellow transparent jelly-like liquid, we would also collect the heart to inoculate the chicken eggs to isolate the FAdV. To detect the FAdV, chicken infectious anemia virus (CIAV), A, B and *J* subtypes of avian leukemia virus (ALV-A, ALV-B, ALV-*J*), Reticuloendotheliosis virus (REV), Marek's disease virus (MDV), the DNA of collected samples were directly extracted using DNeasy mini-kit (Aidlab Biotechnologies, Beijing, China) and further identified by polymerase chain reaction (PCR). For avian infectious bronchitis virus (IBDV) and Reoviridae (REO) virus, the RNA of collected samples was directly extracted from infected allantoic fluid using RNeasy mini-kit (Qiagen, Chatsworth, CA), and reverse transcription was performed using an Oligo dt. PCR was conducted using corresponding primers (Table S1). PCR products were gel purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA, US) and then sequenced at the Beijing Genomics Institute, China. The phylogenetic and molecular evolutionary analyses were conducted using the neighbor-joining method with 1000 bootstrap replicates using MEGA (version 6.0), where a distinct phylogenetic lineage with bootstrap support of ≥70% indicated a common origin.

2.3. The FAdV-4 isolation

The FAdV positive samples were homogenized and centrifuged 10 min at 3000 *g* at 4℃. 0.2 ml of the supernatant was inoculated into the allantoic cavities of 9 to 11-day-old specific pathogen free (SPF) embryonated chicken eggs, and incubated at 35℃ for 72 h. The allantoic fluids were then harvested, and stored at -80℃ for sequencing and pathogenicity analysis.

2.4. Deep sequencing

Viral DNA was extracted from allantoic fluids using DNeasy mini-kit (Aidlab Biotechnologies, Beijing, China). DNA was subjected to PCR using primer sets that cover the entire viral genome. These primer sets were designed according to the genome sequences of FAdV-4 and by using Primer Premier 5.0 software. The fragments, approximately 600 to 800 nucleotides in length, were sequenced using the Illumina HiSeq2000 sequencing platform in the Chinese National Human Genome Center, Shanghai. Briefly, a library was constructed with TruSeq DNA sample prep kit set A. The DNA library was diluted and hybridized to the paired-end sequencing flow cells. DNA clusters were generated on a cBot cluster generation system with the TruSeq PE cluster generation kit v2, followed by sequencing on a HiSeq 2000 system with the TruSeq SBS kit v2.

2.5. Viruses and cells

FAdV-4 strain AV211, isolated in 1992 in China, was purchased from the China Veterinary Culture Collection Center (CVCC). SD1504, SD1530 and SD1560 were isolated from the infected chickens in 2015 and kept in our lab. GenBank accession numbers are as follows for SD1504 (BankIt2236677 Seq1MN091376), SD1530 (BankIt2236677 Seq2MN091377) and SD1560 (BankIt2236677 Seq2MN091378). All viruses used in the study were detected without extraneous virus. These viruses were propagated in chicken hepatoma (LMH) cells and calculated by 50% tissue culture infective dose ($TCID_{50}$). LMH cells were maintained in Way mouth MB752/1 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 ˚C in a 5% CO2 atmosphere.

2.6. Chicken studies

To identify the pathogenicity of prevalent FAdV-4 isolates, 3-week-old SPF chickens (15 per group) (Beijing Experimental Animal Center, Beijing) were challenged with 10^6 $TCID_{50}$ of SD1504 or AV211 by peritoneal injection in 0.2 ml volume. All inoculated chickens were monitored daily for 10 days to investigate the survival rate. Oropharyngeal and cloacal swabs of infected chickens were collected to detect virus shedding on 3, 5 and 7 days post inoculated (dpi) by PCR. In addition, three chickens from each group were euthanized on 5 dpi, liver, brain, thymus, lung, heart, spleen, pancreas, glandular stomach, kidney, duodenum and blood were collected for the virus detection. These corresponding tissues were also collected for histopathology.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA, USA). The survival data from different ages, different scale and different breeds were analyzed using Chi-squared statistics. Only significant differences that existed between one group and all other groups were indicated (Chi2); Kaplan-Meier method was employed for survival analysis. $P < 0.05$ were considered statistically significant.

3. Result

3.1. Epidemiology of HHS outbreaks in chickens

Since 2015, an HHS disease emerged in Chinese poultry farms and has posed a great threat to the poultry industry. The disease is characterized by depression, fluffy feathers, diarrhea, anemia, and weight loss. Necropsy findings showed that the pericardial cavity was filled with pale yellow transparent jelly-like liquid. Liver was observed with hemorrhagic spots and necrotic lesions in various sizes. Swollen and crisp kidneys, pancreatic necrosis and glandular stomach bleeding could also be seen in dead chickens. The incubation period of the disease was 5–7 days, and the mortality of infected poultry was 20%–80%.

During the outbreak, 77 farms with infected chickens were investigated, including local breeder chicken, such as 817 broiler chickens, Ma chickens and three-yellow chickens, as well as commercial layer and broiler (Table S2). Statistical analyses showed that a higher average mortality (35%) was observed in the young poultry (≤20 day) compared to adult chickens (> 50 day) ($p < 0.001$) (Table S3). Furthermore, the average mortality was higher (29% or 31%) in smaller scale chicken farm (< 5000) ($p < 0.05$) (Table S3). And the mortality in the local breeds is slightly higher than that in the commercial breeds in the surveyed poultry farms (Table S3). Collectively, our findings indicated that the prevalent FAdV-4 in 2015 in China has higher infectivity to multiple species of chickens and higher lethality to young chickens.

3.2. Pathogen identification and virus isolation

77 liver samples were randomly collected from infected poultries and screened for the hexon gene of FAdV by PCR. The results showed that 71 out of 77 samples tested were FAdV positive, a positive rate of 92.2% (Table 1). Phylogenetic analysis of the hexon gene identified 68 strains of FAdV-4, 2 strains of FAdV-10 and 1 strain of FAdV-7 among

Table 1
Virus identification in collected liver samples.

Pathogeny	no. of positive samples/ no. of samples	Virus detection rate (%)
FAdV	71/77	92.2
CIAV	27/71	38
REV	4/71	5.6
REO	1/71	1.4
MDV	6/71	8.4
IBDV	0/71	0
ALV-J	30/71	42.2
ALV-A	0/71	0
ALV-B	0/71	0

the 71 positive samples, suggesting that FAdV-4 was the causative agent of the HHS outbreaks in Chinese poultry farms.

Previous studies reported that the co-infection of FAdV with immunosuppressive pathogens could increase the morbidity and mortality (Rosenberger et al., 1975). Thus, we screened immunosuppressive pathogens, including ALV-A, ALV-B, ALV-J, CIAV, REO, IBDV, REV, and MDV from the liver samples. Our results showed among the 71 FAdV-positive samples, the infection rates of ALV-J, CIAV, MDV, REV and REO were 42.3% (30/71), 38% (27/71), 8.4% (6/71), 5.6% (4/71), and 1.4% (1/71), respectively (Table 1), while no IBDV, ALV-A, or ALV-B were detected in the tested samples (Table 1). Moreover, the co-infection of FAdV-4 with multiple immunosuppressive pathogens also was observed in the same liver samples, even five pathogens positive in a same sample (Table 2). Overall, our results revealed that the co-infection of FAdV-4 with immunosuppressive pathogens was widespread, especially with ALV-J and CIAV, which might contribute to the high infection rate and mortality in FAdV-4 outbreak in 2015.

3.3. Genome sequence analysis of FAdV-4 isolates

To further verify the potential molecular variations in the pandemic FAdV-4 isolated during the outbreak, the whole genome sequence of three FAdV-4 isolates (SD1504, SD1530 and SD1560) were obtained by the deep sequencing. The three virus genome sequences are 43,719 bp, with 54.85% G + C content. Genomic alignment results suggested that the isolates were closely related to the FAdV-4 strains isolated in China and shared 99.6%–100% nucleotide homology (Fig. 1). This confirmed that the pandemic FAdV-4 was originated from the early FAdV-4 strain in China. The deletions of different sizes in the 3′ end of the genome is usually associated with elevated virulence of FAdV-4 isolates (Park et al., 2017). Notably, genetic deletions in the 3′ end were also observed in the three isolates, which might determine the high infectivity and pathogenicity to chicken. In addition, deletion of 144 bp in ORF29 in the genome of the three isolates were also identified by sequence alignment with FAdV-4 viruses isolated in 2013 in China (Fig. 2). In conclusion, these results suggested that the FAdV-4 strains prevalent in China were originated from early strains circulating here and had obtained gene mutations that might contribute to enhanced pathogenicity to poultry.

3.4. Pathogenicity of the FAdV-4 isolate in chickens

To identify the pathogenicity of prevalent FAdV-4, SD1504 virus was used to infect SPF chicken, and early FAdV-4 strain AV211 was used as control. 3-week-old SPF chickens (15 per group) were challenged with 10^6 $TCID_{50}$ viruses in 0.2 ml volume. The survival rate of the infected chickens was monitored daily for 10 days. Three chickens from each group were euthanized at 5 dpi for pathological and virologic examination. Virus shedding was tested on 3, 5 and 7 dpi. The results showed that the mortality was 50% in the SD1504 group on day 5 after virus infection, whereas no chicken infected with AV211 virus died during 10 days observation (Fig. 3A). By necropsy, severe hepatitis and hydropericardium was observed in chickens infected with SD1504 (Fig. 3B). Additionally, kidney swollenness, glandular stomach intima and pancreatic hemorrhage were also presented in the SD1504 group (Fig. 3B). However, no obvious lesion was found in chickens infected the AV211 virus (Fig. 3B). Histopathology results revealed that the chickens in SD1504 group displayed a more severe necrosis and disintegration of hepatocytes, including the extensive eosinophilic and basophilic inclusions. Pulmonary congestion, renal tubular epithelial cell degeneration, pancreatic acinar necrosis was also observed (Fig. 3C). While only focal necrosis and a small amount of basophilic inclusions in liver were observed in AV211 infection group, no obvious pathological changes were observed in other tissues (Fig. 3C).

Furthermore, we also measured the viral infection in tissues by PCR. SD1504 viruses could be detected in many tissues including the liver, brain, thymus, lung, heart, spleen, pancreas, glandular stomach kidney, duodenum and blood (Table 3). However, AV211 virus could only be detected in liver, duodenum and blood (Table 3). Virus shedding in the oropharynx and cloaca were tested by PCR on 3, 5 and 7 dpi. The results showed that both SD1504 and AV211 could be detected in the oropharynx and cloacal until 7 dpi, and the detection rate of virus collected from cloacal swabs was higher than that from oropharyngel swabs (Table 4), indicating that fecal transmission may be the major way of transmission of FAdV-4. Collectively, these experiments indicated that the prevalent FAdV-4 in China obtained stronger pathogenicity and wider tissues infectivity to poultry compared with early FAdV-4 strains.

4. Discussion

Since 2015, there have been outbreaks of HHS in Chinese poultry farms. Epidemiological data of the current investigation revealed that FAdV-4 is epidemic in chicken flocks in China. Phylogenetic analysis suggested that the prevalent FAdV-4 is originated from early FAdV-4 strain circulating in chickens in China. Genome sequence alignment revealed that the prevalent strains have already obtained the molecular characteristics of virulent strains. Moreover, as compared with parental strains isolated in China, the pandemic viruses show higher pathogenicity in chickens, causing severe visceral organ damage and systemic infections. It is noteworthy that co-infection with immunosuppressive pathogens played an important synergistic role in the adenovirus epidemic, especially the co-infection with ALV-J and CIAV.

Table 2
Co-infection of FAdV with other immunosuppressive pathogens in clinical samples.

Infection status	Mixed infection pathogens	no. of positive samples/ no. of samples	Positive rate (%)
Double infection	FAdV + CIAV	9/71	12.7
	FAdV + ALV-J	12/71	16.9
	FAdV + MDV	2/71	2.8
Triple infection	FAdV + CIAV + ALV-J	11/71	15.5
Fourfold infection	FAdV + CIAV + ALV-J + REV	3/71	4.2
	FAdV + CIAV + ALV-J + MDV	2/71	2.8
Fivefold infection	FAdV + CIAV + ALV-J + REV + MDV	1/71	1.4
	FAdV + CIAV + ALV-J + REO + MDV	1/71	1.4

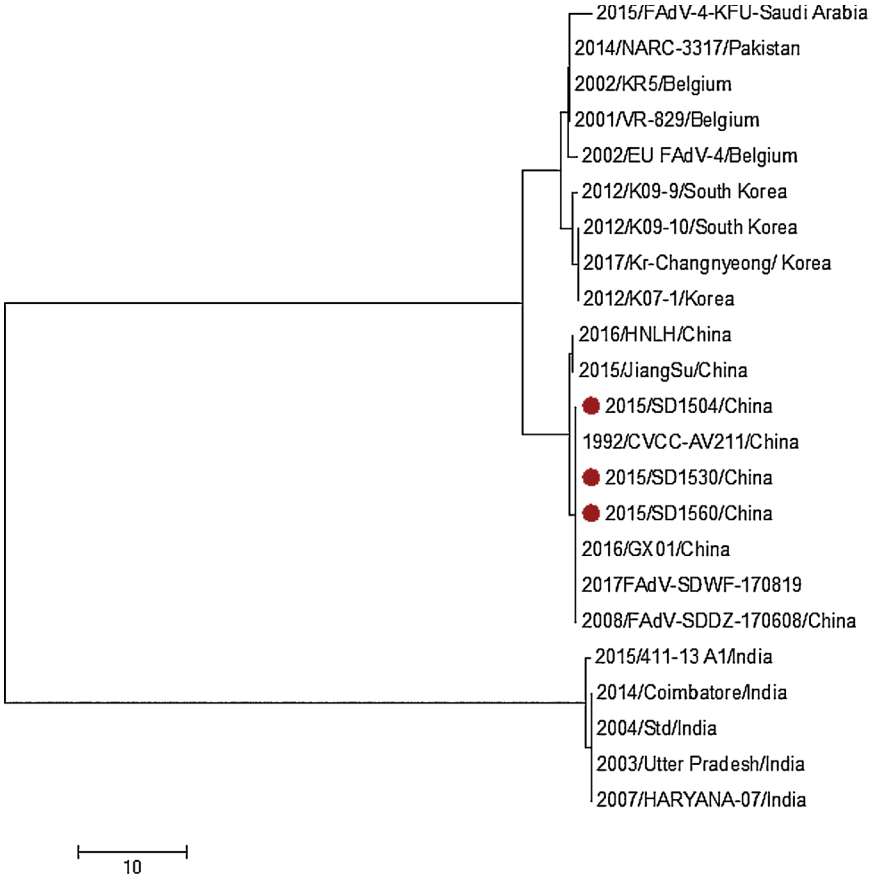

Fig. 1. Phylogenetic trees of hexon genes of representative FAdV-4 viruses. Gene sequences of the SD1504, SD1530 and SD1560 strains isolated from test samples and representative adenovirus strains constructed by the maximum likelihood method in MEGA 6.0. Bootstrap majority consensus values based on 1000 replicates are indicated at each branch point as a percentage.

Prior to 2014, several serotypes of FAdVs, including FAdV-4 had been isolated from diseased chickens with IBH and HHS, but had rarely caused outbreaks. Since 2015, the FAdV-4 has caused huge economic losses to the Chinese poultry industry, which has raised a concern about the origin of the virus. Li et al isolated a FADV-4 strain from live Newcastle disease virus vaccine, which suggests that vaccine contamination may be an important cause of the outbreak of HHS (Li et al., 2017). Zhang et al found that the prevalent FAdV-4 strains in China had high identity to the strains isolated in India, suggesting that the strains most likely originated from India (Zhang et al., 2016). However, the whole genome comparison analysis in our study revealed that the prevalent FAdV-4 strains in 2015 are highly homologous to the early FAdV-4 strains in China, indicating that the prevalent FAdV-4 strains were originated from early strains circulating here.

In previous study, the FAdV can be isolated from the chickens with no obvious clinical symptoms (Dong et al., 2015; Kim et al., 2010). Several studies showed that FAdV is also a conditional pathogen. Currently, the immunosuppressive diseases show an increasing trend in Chinese poultry farms (Luan et al., 2016; Malhotra et al., 2015). Thus, the circulation of immunosuppressive diseases may be associated with

Fig. 2. Sequence alignment of ORF29 from FAdV-4 isolates. The ORF29 sequences of the Chinese FAdV-4, including three 2015 isolates from this study (SD1504, SD1530 and SD1560), one 2013 isolates (JSJ13) and two 2015 isolates (HB1502 and HN1501), were aligned using MEGA6.0.

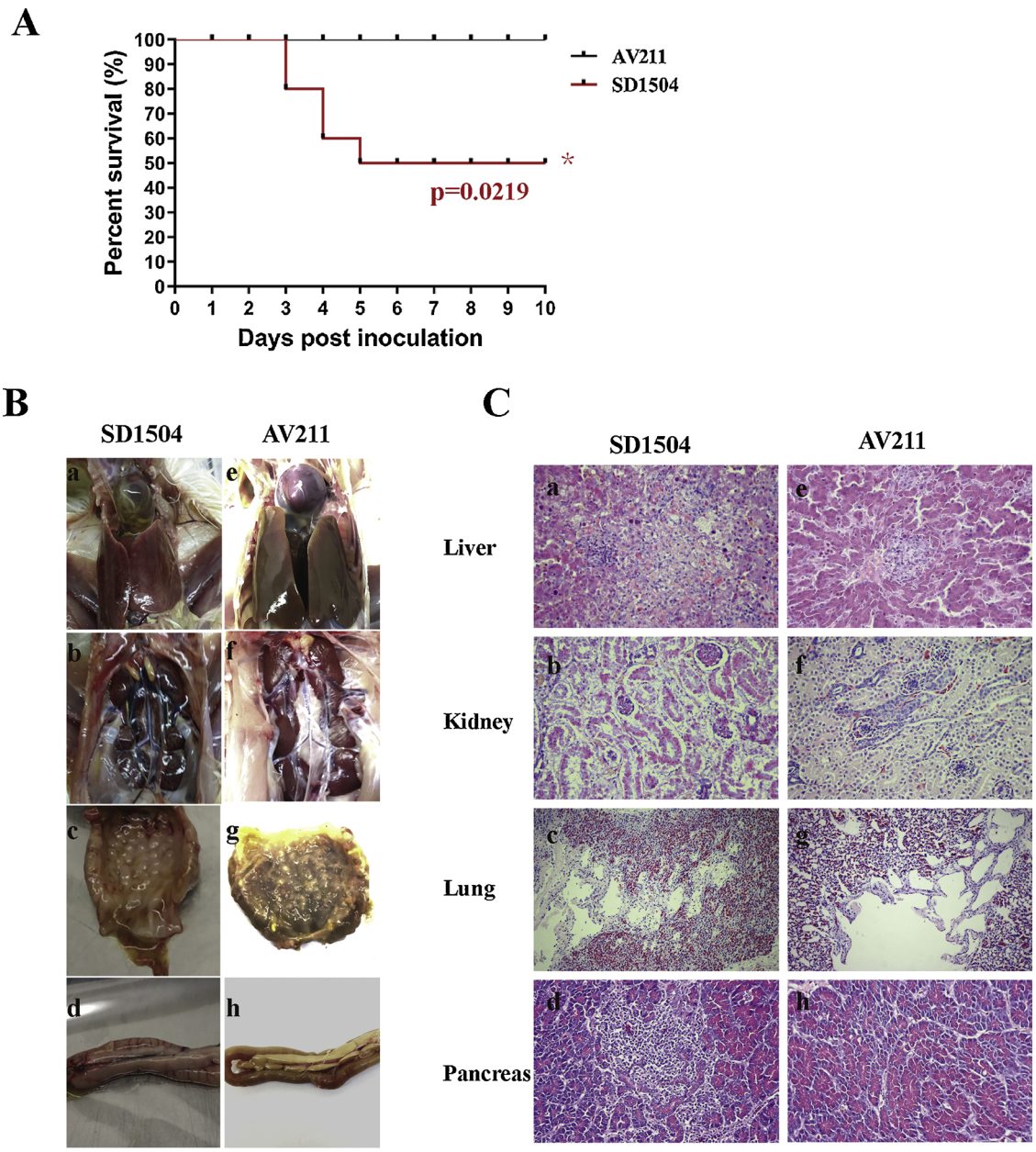

Fig. 3. Pathogenicity of the FAdV-4 in SPF chickens. (A) Survival rates of chickens after inoculation with SD1504 or AV211 virus. 15 3-week-old SPF chickens of each group were challenged with 0.2 ml of 10^6 $TCID_{50}$ of SD1504 or AV211. Kaplan-Meier method was employed for survival analysis. $p < 0.05$ were considered statistically significant (B) Gross lesion of Liver (a, e), kidney (b, f), lung (c, g) and glandular stomach (d, h) of chickens after inoculation with SD1504 or AV211 on 5dpi. (C) Histological changes in Liver (a, e), kidney (b, f), lung (c, g) and pancreas (d, h) of chickens after inoculation with SD1504 or AV211 on 5dpi (H&E stain, original magnification 400×).

Table 3
Virus detection in tissues of chickens after inoculation with FAdV.

Tissues	SD1504	AV211
liver	3/3[a]	1/3
brain	1/3	0/3
thymus	2/3	0/3
lungs	2/3	0/3
heart	1/3	0/3
spleen	3/3	0/3
pancreas	3/3	0/3
glandular stomach	2/3	0/3
kidney	2/3	0/3
duodenum	1/3	1/3
blood	2/3	1/3

[a] No. of positive tissues/total no. of analyzed tissues.

Table 4
Viral shedding of chickens after inoculation with FAdV-4.

	Days post-infection					
	3		5		7	
	oropharynx	cloaca	oropharynx	cloaca	oropharynx	cloaca
SD1504	2/5[a]	3/5	2/5	5/5	2/5	4/5
AV211	1/5	3/5	1/5	3/5	2/5	3/5

[a] No. of positive swabs/total no. of analyzed swabs.

the outbreak of HHS. Here, we found that there was a high proportion of co-infection of FAdV-4 with immunosuppressive pathogens. Immunosuppressive diseases can impair the immune system of host,

suppress the humoral and cellular immunity response, and further promote the infectivity and pathogenicity of other pathogens (Craig et al., 2009). In China, eradication of immunosuppressive diseases is implemented only in commercial poultry but not in the local breed. Thus, higher mortality can be observed in local breeds due to high isolation rate of immunosuppressive diseases. This indicates that multiple infection of different immunosuppressive viruses, such as ALV-J and CIAV viruses is ever-present, and more attention should be paid in the diagnosis process.

Pathogenicity of the FAdV-4 isolate in chickens indicates that the prevalent virus possesses systemic infection ability and higher virulence to chicken compared with early FAdV-4 strains. Previous study found that gene-truncated isolates are more virulent than FAdV-4 isolates carrying the full-length gene (Vera-Hernandez et al., 2016). In our study, genome alignment analysis reveals a 144 bp deletion in ORF29 and deletions with different sizes in the 3′ end of the genome in three viruses isolated in 2015. Notably, genetic deletion in ORF29 and in the 3′ end of the genome have also been found in the FAdV-4 strains isolated in China after 2015 (Guan et al., 2018; Ye et al., 2016), which might be a common character of current FAdV-4 isolates. The high pathogenic strain MX-SHP95 isolated in Mexico also had the deletions with different sizes in the 3′ end of the genome (Park et al., 2017). The exact role of 144 bp deletion in ORF29 gene in these prevalent FAdV-4 still needs to be elucidated.

Collectively, our study shows that mutant FAdV-4 has been epidemic in chicken flocks in China since 2015. The pandemic viruses show higher pathogenicity in chickens, and co-infection with immunosuppressive pathogens plays an important synergistic role in the adenovirus epidemic. Our findings provide more information for further understanding the ecological characteristics of prevalent FAdV-4 in China. More studies still need be carried out to reveal their virulence determinants, monitor variants, and develop efficient antiviral strategies.

Declaration of Competing Interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108427.

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