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RAPID COMMUNICATION

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Biological and phylogenetic characterization of a novel hemagglutination-negative avian avulavirus 6 isolated from wild waterfowl in China

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Abstract

Up to now only nine whole genome sequences of avian avulavirus 6 (AAvV-6) had been documented in the world since the first discovery of AAvV-6 (AAvV-6/duck/HongKong/18/199/77) at a domestic duck in 1977 from Hong Kong of China. Very limited information is known about the regularities of transmission, genetic and biological characteristics of AAvV-6 because of the lower isolation rate and mild losses for poultry industry. To better further explore the relationships among above factors, an AAvV-6 epidemiological surveillance of domestic poultry and wild birds in six provinces of China suspected of sites of inter-species transmission and being intercontinental flyways during the year 2013–2017 was conducted. Therefore, 9,872 faecal samples from wild birds and 1,642 cloacal and tracheal swab samples from clinically healthy poultry of live bird market (LBM) were collected respectively. However, only one novel hemagglutination-negative AAvV-6 isolate (AAvV-6/mallard/Hubei/2015) was isolated from a fresh faecal sample obtained from mallard at a wetland of Hubei province. Sequencing and phylogenetic analyses of this AAvV-6 isolate (AAvV-6/mallard/Hubei/2015) indicated that this isolate grouping to genotype I were epidemiological intercontinentally linked with viruses from the wild birds in Europe and America. Meanwhile, at least two genotypes (I and II) are existed within serotype AAvV-6. In additional, this novel hemagglutination-negative AAvV-6 isolate in chicken embryos restored its hemagglutination when pre-treated with trypsin. These findings, together with data from other AAvV-6, suggest potential epidemiological intercontinental spreads among AAvV-6 transmission by wild migratory birds, and reveal potential threats to wild birds and domestic poultry worldwide.

KEYWORDS
AAvV-6, domestic poultry, genotype, intercontinental transmission, wild bird

1 | INTRODUCTION

Over the last 40 years, many viruses from the Paramyxoviridae family isolated from not only human or animal but also in birds have been newly identified (Kolakofsky & Roux, 1987; Samal, 2011). Paramyxoviruses are enveloped, non-segmented, pleomorphic RNA viruses containing a single stranded, negative-sense genome. Avian paramyxoviruses that have been isolated from birds; however, due to changes in taxonomy is now referred to as avian avulavirus (AAvV) (Amarasinghe et al., 2017). There are 13 described AAvV

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serotypes (AAvV-1 to -13) based on neuraminidase inhibition tests and hemagglutination inhibition (HI), and eight another putative serotypes have been recently isolated (AAvV-14 to -21) (Jeong et al., 2017; Lee et al., 2017; Neira et al., 2017; Thampaisarn et al., 2017; Thomazelli et al., 2017; Yamamoto, Ito, & Ito, 2016). While very limited information is known about the biological and molecular characteristics of AAvV-2 to -21, extensive study has been mainly conducted on AAvV-1 (Newcastle disease virus, NDV) (Cardenas-Garcia et al., 2015; Umali, Ito, Katoh, & Ito, 2014).

Newcastle disease (ND), caused by the virulent AAvV-1, a well-characterized AAvV serotype, is a highly contagious devastating viral disease to the domestic poultry worldwide because of its high mortality and heavy losses for economy (Saif & Barnes, 2008). Other serotypes AAvV, such as AAVV-2, -3 and -7, are also known to cause reproductive and respiratory diseases in turkeys and chickens, sometimes resulting in death of the infected birds (Samuel, Subbiah, Shive, Collins, & Samal, 2011; Warke, Stallknecht, Williams, Pritchard, & Mundt, 2008). Meanwhile, some serotypes AAvV strains display their specific host restriction, such as AAvV-5 cause diarrhoea and high mortality in budgerigars but not in chickens and ducks (Briand, Henry, Massin, & Jestin, 2012). However, AAvV-6 was first identified at a domestic duck in 1977 from Hong Kong (duck/Hong Kong/18/199/77) and then was found to cause drop in egg production and mild respiratory disease in turkeys, but was avirulent in chickens (Chang et al., 2001; Tian et al., 2012; Xiao et al., 2010). But recent serosurveillance of commercial chickens in the USA showed the likely prevalence of all serotypes AAvV including AAvV-6, excepted with AAvV-5 (Warke, Appleby, & Mundt, 2008).

The genome size of AAvV range from 14.9 to 17.4 kb that is transcribed into at least six genes, which separately encode for up to nine different proteins (Saif & Barnes, 2008). However, AAvV-6 has an RNA genome consists of seven genes in the order of 3′-NP (56–1,626)-P(1,634–3,119)-M(3,122–4,526)-F(4,586–6,420 or 4,586–6,416)-SH(6,470–7,061 or 6,464–7,037)-HN(7,072–9,102 or 7,066–9,096)-L(9,166–16,182 or 9,160–16,176)-5′ in length with 16,230 or 16,236 nucleotides (Xiao et al., 2010; Yamamoto, Ito, Tomioka, & Ito, 2015). Six major proteins are encoded, including the nucleocapsid protein (NP, 128–1,525, 1,398 nt), phosphoprotein (P, 1,687–2,979, 1,293 nt), matrix protein (M, 3,235–4,335, 1,101 nt), fusion protein (F, 4,598–6,265, 1,668 nt or 4,628–6,265, 1,638 nt), haemagglutinin-neuraminidase (HN, 7,122–8,963 or 7,116–8,957, 1,842 nt) and large polymerase protein (L, 9,278–16,003 or 9,272–15,997, 6,726 nt). In addition, the small hydrophobic protein (SH, 6,542–6,970 or 6,536–6,964, 429 nt) that AAvV-6 has, is not found in the other serotypes (Sobolev et al., 2016; Warke et al., 2008).

The few reports on the incidence of AAvV-6 in commercial and domestic poultry from different parts of the world have shown a notable presence of several of this virus (Chang et al., 2001; Warke et al., 2008). Despite this, knowledge about the regularities of transmission, genetic and biological characteristics of AAvV-6 viruses in commercial poultry and wild birds in the China recent years remains limited. Therefore, in this study, an AAvV-6 surveillance of domestic poultry and wild birds in six provinces of China suspected of sites of inter-species transmission and being intercontinental flyways from December 2013 to June 2017 was conducted.

2 | MATERIALS AND METHODS

2.1 | Ethical states

All experimental protocols (Approval ID: 20130113–1, approval date: 15th Jan 2013) used in this work were reviewed and approved by the Experimental Animal Council of Jilin University, China.

2.2 | Sample collection

Nine thousand eight hundred and seventy-two faecal samples were obtained from wild birds of wetlands and 1,642 cloacal and tracheal swab samples were collected from clinically healthy domestic poultry of live bird market (LBM) in China for AAvV-6 epidemiological surveillances from December 2013 to June 2017. The samples were obtained in one province in north China (Neimenggu), two in central China (Hubei and Hunan), one in east China (Anhui), one in northeast China (Jilin) and one in northwest China (Qinghai).

2.3 | Virus identification and isolation

Presence and identification of AAvV-6 in each individual collected specimen was performed through allantoic cavities inoculation of 9 to 10-day-old specific-pathogen-free (SPF) chicken embryos (Merial, Beijing, China) (Kim, King, Suarez, Wong, & Afonso, 2007; Yin et al., 2017). The presence of the AAvV-6 in allantoic fluid was identified by RT-PCR and sequencing for paramyxoviruses (Tong, Chern, Li, Pallansch, & Anderson, 2008).

2.4 | Cell culture and virus infection of cells

The chicken fibroblast cell line DF-1 and the chicken bone marrow macrophages cell line HD11 were grown in DMEM containing 10% foetal bovine serum (FBS) (Gibco, Life Technologies) and complete DMEM/F12 containing 10% FBS respectively. Cells were planted into a 24-well cell culture plate at a viable cell density (determined by Trypan blue exclusion, Sigma, Shanghai, China) of 3×10^5 cells per well at 37°C under 5% CO_2 for 8 hr. Cells then were washed three times with phosphate buffered saline and supernatant was changed into fresh medium supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin without FBS. Thereafter, cells were absorbed with virus at 100 μl allantoic fluid containing the Hubei isolate for 1 hr in the presence or absence of TPCK-trypsin (Sigma, Shanghai, China) and fresh medium was added into the well and then incubated with 72 hr post infection (hpi). Subsequent to infection, virus titre in the supernatants was

measured using a micro-hemagglutination assay (HA) method (Zhang et al., 2018).

2.5 | RNA extraction, RT-PCR and sequencing

Viral RNA was isolated from allantoic fluid using the AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Shanghai, China) according to the manufacturer's instructions. Following extraction, cDNA synthesis was performed by using GoScript™ Reverse Transcription System (Promega, Shanghai, China) following the manufacturer's instructions using random primer. Then samples were measured by seminested PCR for L gene of paramyxoviruses using 2×EasyTaq PCR kit (TransGen Biotech, Beijing, China) (Tong et al., 2008). The first amplification in the seminested PCR assay consists of 10 μl 2×EasyTaq PCR supermix, 2 μl cDNA, 10 μM PAR-F1 primer, 10 μM PAR-R primer and H_2O to achieve a final volume of 20 μl. The cycling reactions consisted of a cycle of 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 48–50°C for 30 s and 72°C for 30 s. For the second amplification in the seminested PCR assay, we used 2 μl aliquot from the first PCR reaction, 10 μl 2×EasyTaq PCR supermix,10 μM PAR-F2 primer, 10 μM PAR-R primer and H_2O to achieve a final volume of 20 μl. The cycling conditions consisted of an initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 48–50°C for 30 s and 72°C for 30 s. After that, the PAR-F2 and PAR-R primers were used for PCR amplicons sequencing (Sangon Biotech, Shanghai, China).

The BLAST search identified the relatedness of the isolated viruses with other reported AAvV-6 strains and therefore this Hubei stain was designated as AAvV-6/mallard/Hubei/2015. After that this AAvV-6 in this study were amplified for the entire genome using 16 primer pairs (Table 1). The cycling reactions consisted of a cycle of 95°C for 3 min followed by 40 cycles of 95°C for 1 min, 45–57°C for 45 s and 72°C for 150 s. PCR amplicons sequencing was performed by Major-bio Company (Beijing, China).

TABLE 1 Primers used in this study

Name	Primer sequence (5′-3′)	Length (bp)
1-F	GCAGCCCCTTTGTAAATAGAGAC	494
1-R	AAATGCCATGTAGGGTCCATC	
2-F	AACACGACGCGATATTATGCC	1049
2-R	TCCGTGTTGCCCTTACTGTC	
3-F	ACACCCCTCAGAGAGATCCAA	979
3-R	TAATCAGCGTCAAGAGTGTCCA	
4-F	CTCACCCCGTACTCTGACA	2455
4-R	CGCTTGAAGTTGCATAGATGTACC	
5-F	AGAATAATCTAACAGCCCAACCAA	2392
5-R	GGCGATCCTCCAGTCTCC	
6-F	TAGCAGCCACAGAATCAGGT	2387
6-R	TTACTGCCCCGATTAGCCT	
7-F	AAGCAGCATACTCATTAACCAC	1977
7-R	CGCTCAGATCTTCAACTAAGTCA	
8-F	CTCCCGCGTCTCTAGCAAGG	2396
8-R	TCCCTGGATTCCCTTACGTG	
9-F	GGAATACAAACTCTCGAGGCTA	536
9-R	TCAATAGTCATGTCAGGCTAGTGT	
10-F	ATATGCTTGGGGAATTTACGAGA	486
10-R	CATACATCTGGCGTGCTCT	
11-F	AGGAAACCATATGCTTGGGGA	724
11-R	GTACTCCGGATCACTCTGTTT	
12-F	CTGCATCACCCTTGGCAGCAT	977
12-R	CTAAGGAAGGAATAGTTAGGAAG	
13-F	CAGGGTTATGGCCAAGTGTCA	1458
13-R	GAGATGGTTCAGGCTCCAAGG	
14-F	TTTTACACCTATTAAGGCGAAC	1759
14-R	AGCACCTGCATGATTACCTG	
15-F	CCATAACCGGAAGTATTGCTG	1844
15-R	TTCAGGCAGAACACTAAGGA	
16-F	TGCTCTCTGATTAAGATCTCG	780
16-R	TCAGATTATTAATTGCCGGTA	

2.6 | Pathogenicity test

The pathogenicity of the AAvV-6 isolate was determined by (a) mean death time (MDT) in 9 to 10-day-old embryonated SPF eggs, (b) Intra-cerebral pathogenicity index (ICPI) tests in 1-day-old SPF chickens (Merial, Beijing, China) and (c) the intravenous pathogenicity index (IVPI) in 6-week-old chickens (Merial, Beijing, China) according to the Office International des Epizooties (OIE) manual of standards (Newcastle disease 2018).

2.7 | Hemagglutination (HA) and Hemagglutination Inhibition (HI) assay

HA and HI assay were carried out according to the OIE guidelines (Newcastle disease 2018). In HI tests, anti-sera against avian influenza virus (AIV) H1, H5 and H9 (Weike Biotechnology, Harbin, China) and NDV LaSota strain (Weike Biotechnology, Harbin, China), AAvV-4 (prepared by our lab) were used as references.

2.8 | Phylogenetic analysis

Nucleotide sequences of AAvV-6 in this study were aligned through Mega X software with the sequences of representative AAvV-6 strains retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/GenBank). The homology analysis was carried out using the maximum likelihood method through MegAlign (DNASTAR). The phylogenetic consensus tree of complete F gene and viral whole genome sequence were generated a Maximum Likelihood method based on the General Time Reversible model through MEGA X software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

3 | RESULTS AND DISCUSSION

Only one sample from mallard in 2015 at a wetland of Hubei province, China, produced suspect AAvV-6 positive result for the L gene by seminested PCR for paramyxoviruses, and then genome sequencing and phylogenetic analysis was conducted on this Hubei isolate (AAvV-6/mallard/Hubei/2015, GenBank accession number MH551526). Surveillance data were also observed by researchers who performed similar monitoring researches of wild birds and domestic poultry and identified AAvV-6 viruses (Shortridge, Alexander, & Collins, 1980; Sobolev et al., 2016), such as the isolate rate of AAvV-6 in shorebirds was 2.4% in Germany during the year 2001–2002 (Hlinak et al., 2006) and in free-living wild ducks was 0.76‰ in South Korea during the winter season (November to February) between 2010 and 2014 (Choi et al., 2018). However, our current data clearly indicate that AAvV-6 is a lower isolated virus (the isolate rate was 0.1‱, 1/9, 872) in wild birds in China from the year 2013–2017 as compared with other AAvVs, such as AAvV-1 and -4 (Yin et al., 2017; Zhang et al., 2015). Meanwhile, no AAvV-6 isolate was obtained from domestic poultry. To our best knowledge, most of reported AAvV-6 viruses were isolated from waterfowl species, such as geese, teal, mallard and so on (Figure 1). Therefore, future AAvV-6 monitoring studies in domestic poultry should include more samples from LBMs as well as their susceptibility to AAvV-6 viruses may differ from that of wild bird species.

The complete sequence obtained for the genome of this Hubei isolate consisted of 16,236 nucleotides, which is the same in size as the other reported AAvV-6 strains excepted with two isolates that is AAvV-6/duck/Italy/4524-2/07 and AAvV-6/red-necked_stint/Japan/8KS0813/2008 (16,230 nucleotides genome length). As same as other reported AAvV-6 isolates, the genome of this Hubei isolate also encoding seven structural proteins (NP, P, M, F, SH, HN and L) in the order 3′ leader-N-P-M-F-SH-HN- L -trailer 5′.

To further determine the virulence of the AAvV-6/mallard/Hubei/2015, the cleavage site in the fusion (F) protein, MDT, ICPI and IVPI were determined. For AAvV-6/mallard/Hubei/2015, deduced amino acid sequence of the cleavage site of the F protein, ^{112}PAPEPR*L^{117}, that contains a monobasic aa residue (arginine, R), is identical to most of AAvV-6 strains (Table 2). However, there are several differences in the cleavage site of AAvV-6/duck/Italy/4524-2/07 and AAvV-6/red-necked stint/Japan/8KS0813/2008 (^{112}SIREPR*L^{117}) strains. In addition, this Hubei isolate was able to grown in chicken cell lines HD11 and DF-1 only with the addition of trypsin. The MDT score was more than 168 hr, with no mortality after 7 days, as well as both ICPI and IVPI were 0, suggesting this Hubei isolate is a low virulent virus for chickens.

Interestingly, infective allantoic fluid of AAvV-6/mallard/Hubei/2015 only produced HA positive results after five passages in 9 to 10-day-old SPF embryonated chicken eggs. Meanwhile, the HA-negative allantoic fluid of this Hubei isolate was negative for avian coronavirus (infectious bronchitis virus), avian avastrovirus and avian adenovirus serotype 4. Furthermore, the HA-positive allantoic fluid from AAvV-6/mallard/Hubei/2015 was negative for AIV, NDV and AAvV-4 based on the HI test and PCR. However, the HA-negative allantoic fluid of AAvV-6/mallard/Hubei/2015 restored the HA-positive results when the virus pre-treated with 1% trypsin for 30 min. But the nucleotide and aa (amino acid) sequences homologies of the HN gene between this novel HA negative Hubei isolate and a reported HA-positive isolate AAvV-6/teal/Novosibirsk region/455/2009 (128 hemagglutination units (HAU) per 50 μl, GenBank No: KT962980) (Sobolev et al., 2016) was 100%. Therefore, more research is needed to elucidate the molecular mechanisms of HA-negative AAvV-6 isolates in the field.

HA and HI assays are the classical methods to identify AAvV worldwide. Novel non-hemagglutination AAvV-6 isolates that are not detected by traditional HI assay, suggesting it is critical to continuously update surveillance systems, comprising biosecurity measures, research and diagnostic assays, to protect domestic poultry across the globe.

To further study the genetic characteristics of this AAvV-6 virus, phylogenetic trees were generated based on the genome sequence and the complete F gene sequence respectively. However, the complete F gene sequence is considered as the main target for molecular epidemiological investigations and genotyping of AAvV. Meanwhile, a unified nomenclature and classification system of the NDV (AAvV-1) genotyping method based on the mean inter-populational evolutionary distances of the complete F gene sequence, with cut-off values more than 10% to assign new genotypes (Diel et al., 2012), will provide a more rational and scientific genotyping method for epidemiological studies of other serotypes AAvV. Therefore, 24 reported AAvV-6 isolates, including this Hubei isolate, were classified into two genotypes (I and II, with the mean inter-populational evolutionary distances between groups varying of 0.476 [47.6%] (Figure 1b; Table 3a). Furthermore, the isolates within genotype I and II were grouped into two subgenotypes (Ia and Ib, with the mean inter-populational evolutionary distances between groups was 0.0485 [4.85%] and IIa and IIb, with the mean inter-populational evolutionary distances between groups was 0.0438 [4.385%]) respectively (Figure 1b; Table 3b). To our interestingly, no similar genotype II isolates were detected in domestic poultry and wild birds of this study during the year 2015–2017, suggesting that no genotype II viruses were introduced into China from nearby country Japan and south Korea and limited virus circulation. Taken together, at least two genotypes (I and II) is existed within AAvV-6, based on the evolutionary distances of the complete F gene. Meanwhile, highly similar genotype I and II AAvV-6 isolates from distinct bird species in different regions of America, Asian and Europe clearly demonstrate that AAvV-6 can be intercontinental and inter-species transmitted by wild migratory birds.

A tremendous amount of information about AAvV-1 is available on the characteristics and genetic relationships because of the severe disease it causes in poultries worldwide (Shittu, Joannis, Odaibo, & Olaleye, 2016; Xue et al., 2017, 2017; Zhang et al., 2015). By comparison, the pathological phenomenon which AAvV-6 causes are relatively weak, just manifested in decreased egg production and mild respiratory disease in turkeys and was avirulent in chickens

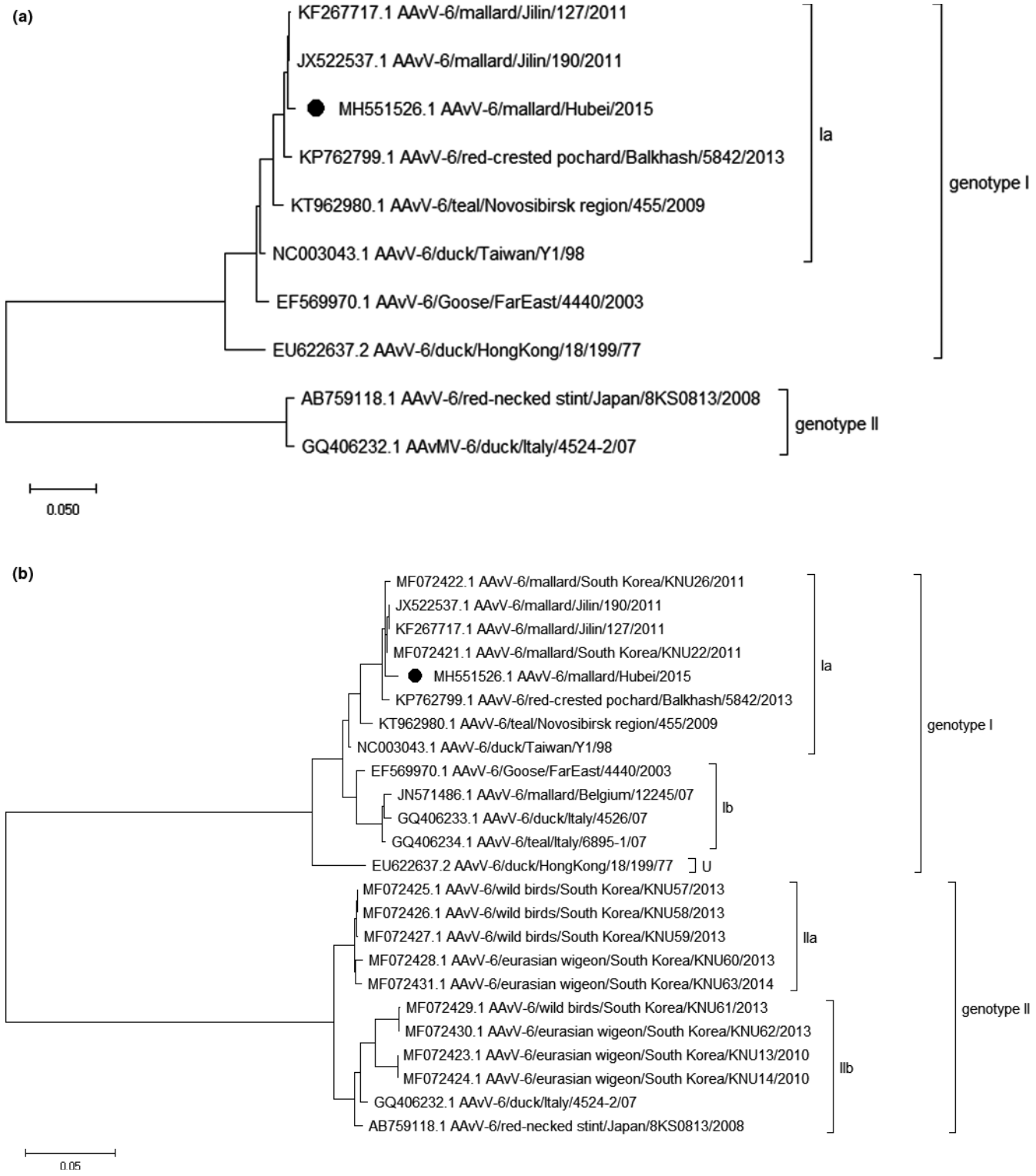

FIGURE 1 Phylogenetic analysis of whole genome sequence (a) and complete F gene sequences (b) of AAvV-6. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (−48402.50) (a) and (−5766.82) (b) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 (a) and 24 (b) nucleotide sequences. Codon positions included were 1st+2nd+3rd+Non-coding. All positions containing gaps and missing data were eliminated. There were a total of 16,192 (a) and 1,638 (b) positions in the final dataset. Evolutionary analyses were conducted in MEGA X

TABLE 2 Detailed information of AAvV-6 isolates obtained from GenBank

GenBank accession numbers	Strain	Cleavage site
KF267717.1	AAvV-6/mallard/Jilin/127/2011	PAPEPR↓L
JX522537.1	AAvV-6/mallard/Jilin/190/2011	PAPEPR↓L
KP762799.1	AAvV-6/red-crested pochard/Balkhash/5842/2013	PAPEPR↓L
KT962980.1	AAvV-6/teal/Novosibirsk region/455/2009	PAPEPR↓L
NC003043.1	AAvV-6/duck/Taiwan/Y1/98	PAPEPR↓L
EF569970.1	AAvV-6/Goose/Fareast/4440/2003	PAPEPR↓L
EU622637.2	AAvV-6/duck/Hong Kong/18/199/77	PAPEPR↓L
JN571486.1	AAvV6-/mallard/Belgium/12245/07	PAPEPR↓L
AB759118.1	AAvV-6/red-necked stint/Japan/8KS0813/2008	SIREPR↓L
GQ406232.1	AAvV-6/duck/Italy/4524-2/07	SIREPR↓L
MH551526	AAvV-6/mallard/Hubei/2015	PAPEPR↓L

under normal circumstances (Alexander, 2000; Saif & Barnes, 2008; Sobolev et al., 2016). As a low virulence virus for chickens and low separation rate, the potential harm of the AAvV-6 is easily overlooked. However, in a recent study of the pathogenicity of two AAvV-6 variant isolates, AAvV-6/red-necked stint/Japan/8KS0813/2008 and AAvV–6/duck/Hong Kong/18/199/1977, as representative isolate of genotype I and II respectively, could replicate in respiratory tissues of infected mice and induce respiratory disease, sometimes resulting in death of the infected mice (Bui et al., 2017). Further researches about the virulence and susceptibility of AAvV-6 should be include more isolates, since differences of viral propagation properties in same cells were observed between the two variant isolates, owing to the change of host from red-necked stint to duck and sites where the two variant isolates separated at such a distance to some extent (Bui et al., 2014). Therefore, the identification and isolation of Hubei isolate is beneficial for the further understanding of HA-negative AAvV-6 in this study for the high sequence identity (99.1%–99.2%) with two Jilin isolates (AAvV-6/mallard/Jilin/190/2011 and AAvV-6/mallard/Jilin/127/2011) and the same cleavage site with other AAvV-6 isolates.

In conclusion, our current data indicate that AAvV-6 is distributed sporadically in wild migratory birds, not in domestic birds, in China during the year 2013–2017. Because the Russian Far East, eastern Mongolia, eastern Siberia, Alaska, Black Sea/Mediterranean and Asian are linked by the migratory routes of the migratory wild bird species investigated here, our conclusion should commonly be applicable to other similar Asian AAvV-6 isolates. Bird shows, import and trade are other potential opportunities that easily allow the introduce of emerging isolates to susceptible populations, unless strict control measures are complied. The AAvV-6 Hubei isolate here is epidemiological connected to AAvV-6 from other geographical areas, such as FarEast, Siberia, Kazakhstan and South Korea, therefore their presence implies the potential risk of AAvV-6 being spread into the region and country, possibly causing to domestic poultry and wild birds infections. It also necessitates the demand for constant epidemiological surveillance for AAvV-6 isolates among domestic poultry and wild birds in China to discover the potential spread of novel variants from other countries and regions.

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TABLE 3 Estimates of evolutionary distances between AAvV-6 genotypes (A) and subgenotypes (B)

		No. of base substitutions per site or standard error estimate [a]	
Genotype		I	II
(A)			
I			(0.437)
II		0.476	

	No. of base substitutions per site or standard error estimate [b]			No. of base substitutions per site or standard error estimate [c]	
Subgenotype	Ia	Ib	Subgenotype	IIa	IIb
(B)					
Ia		(0.0102)	IIa		(0.0099)
Ib	0.0485		IIb	0.0438	

The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved (a) 24 nucleotide sequences (I, n = 13; II, n = 11), (b) 13 nucleotide sequences (Ia, n = 8; Ib, n = 4) and 11 nucleotide sequences (IIa, n = 5; IIb, n = 6). Codon positions included were 1st+2nd+3rd+Non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1,638 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

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AUTHOR CONTRIBUTIONS

RY, XL, YC and ZD designed and performed the study, drafted the manuscript and analyzed the data. All authors collected clinical samples. RY, XL, ZD and YC carried out experiments.

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