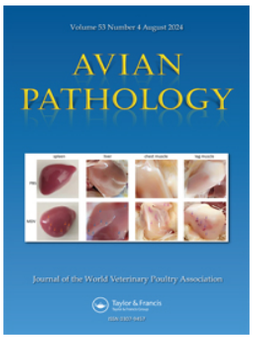

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ORIGINAL ARTICLE

The first report and biological characterization of *Avian Orthoavulavirus 16* in wild migratory waterfowl and domestic poultry in China reveal a potential threat to birds

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ABSTRACT

The *Avulavirus* within the family *Paramyxoviridae* includes at least 22 different species, and is known to cause different types of infections and even be fatal in multiple avian species. There is limited knowledge of the genetic and biological information of *Avulavirus* species 2 to 22 in domestic and wild birds and the disease significance of these viruses in birds is not fully determined, although as many as 10 new distinct species have been identified from wild birds and domestic poultry around the world in the last decade. This study aimed to use PCR, virus isolation, and sequencing to genetically and biologically characterize *Avian Orthoavulavirus 16* (AOAV-16) in wild birds and domestic poultry collected from different locations in China between 2014 and 2022. Of five isolated AOAV-16 strains (Y1 to Y5), only the Y4 strain had a haemagglutination (HA)-negative result. All of these isolates were low virulent viruses for chickens, except Y3 which was detected simultaneously with avian influenza virus (AIV) of H9N2 subtype. Furthermore, at least four different types of intergenic sequences (IGS) between the HN and L genes junction, and recombination events, as well as interspecific transmission by wild migratory birds, existed within the species AOAV-16. These findings and results of other reported AOAV-16 strains recommend strict control measures to limit contact between wild migratory birds and domestic poultry and imply potential threats to commercial poultry and even public health challenges worldwide.

RESEARCH HIGHLIGHTS

- First confirmation of AOAV-16 in domestic and wild birds in China.
- AOAV-16 are low virulent viruses for chickens.
- Co-circulation/co-infection of AOAV-16 and H9N2 subtype AIV enhanced pathogenicity.
- Different intergenic sequences and recombination events exist within AOAV-16.

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AOAV-16; domestic poultry; recombination event; co-infection; intergenic sequence; H9N2 AIV

Introduction

Paramyxoviruses, a family of single-stranded negative-sense-enveloped RNA viruses belonging to the family *Paramyxoviridae*, are known to cause different types of infections and even fatal in a broad range of vertebrates, including mammals, fish, reptiles, and birds (Rima *et al.*, 2019). Paramyxoviruses consist of four subfamilies based on the genetic distances of complete L protein amino acid sequences: avulaviruses, metaparamyxoviruses, orthoparamyxoviruses, and rubulaviruses (Rima *et al.*, 2019).

Avulavirus, a new subfamily within the family *Paramyxoviridae*, contains three recently created genera, named *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus*, according to the virus taxonomy and taxon nomenclature that was approved and ratified by the International Committee on Taxonomy of Virus (ICTV) (Amarasinghe *et al.*, 2019). The genus

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Orthoavulavirus is comprised of *Avian Orthoavulavirus* (AOAV)-1, -9, -12, -13, -16, -17, -18, -19, and -21, the genus *Metaavulavirus* is comprised of *Avian Metaavulavirus* (AMAV)-2, -5, -6, -7, -8, -10, -11, -14, -15, -20, and -22, and *Avian Paraavulavirus* (APAV)-3, and -4 are classified as the genus *Paraavulavirus* (Jeong *et al*., 2018; Rima *et al*., 2018).

So far, despite the few reports on the biological and genetic studies of species 2 to 22 of *Avulavirus*, AOAV-1 (also known as Newcastle disease virus, NDV) has been extensively investigated, because of the huge mortality and morbidity as well as economic significance associated with the global poultry industry (Gogoi *et al*., 2017). Meanwhile, previous research suggested that AOAV-1 and AOAV-16 may have a common evolutionary origin, since AOAV-16 is phylogenetically very close to AOAV-1 in its genomic characteristics, as compared to other species of the *Avulavirinae* subfamily (Lee *et al*., 2017; Karamendin *et al*., 2020). Prior to this study, there were published reports of only two AOAV-16 strains from wild birds in Asia, where AOAV-16 was first identified in faeces of wild geese from South Korea in 2014 and then sporadically isolated from archived wild bird samples from Kazakhstan in 2018 (Lee *et al*., 2017; Karamendin *et al*., 2020). In addition, scientists found that the geographic distribution of this virus extends beyond Asia, where an AOAV-16 isolate was recovered from emperor goose (*Anser canagicus*) faeces collected in Alaska in 2019 (Reeves *et al*., 2021). However, the genetic and biological information on AOAV-16 in domestic and wild birds in China, and the disease significance of AOAV-16 in birds, are not fully determined.

It is well known that wild and migratory bird populations are considered natural reservoirs and the main route of transmission of avulaviruses (Gogoi *et al*., 2017). China is home to many different bird species, especially wild and migratory waterfowl, shorebirds, and gulls, where intercontinental flyways are connecting Europe, Asia, Australia, Africa, and America. Therefore, all species of avulaviruses, including AOAV-16, can spread rapidly throughout the world through waterfowl migration and poultry trade. However, there have been no reports of AOAV-16 in China to date. The goal of this study was to explore the molecular and biological characterizations of AOAV-16 viruses in wild migratory birds and domestic poultry in China. For this purpose, we performed an epidemiological surveillance study and molecular analysis of AOAV-16 viruses detected in wild birds and poultry in China between 2014 and 2022. Our data highlight the importance of continuous surveillance of AOAV-16 in China to stay abreast of the potential introduction of new genetic variants from other hosts and geographic regions.

Materials and methods

Ethical statement

All experimental protocols used in this work were reviewed and approved by the Institutional Animal Care and Use Committee of Jilin University, China (approval number: 201803036).

Sample collection

In total 14,909 clinical samples were collected from the nine provinces of China located at the intersection of several wild bird migration flyways across Asia (Hubei, Hunan, Henan, Shandong, Anhui, Jilin, Qinghai, Inner Mongolia, and Heilongjiang) during the years 2014–2022, as part of the avian influenza virus (AIV) and AOAV-1 surveillance project. The information on sample collection, transportation, and handling has been described in detail in our previous studies (Zhang *et al*., 2016; Yin *et al*., 2017). Briefly, the collected samples were transported using sterile swabs soaked in 2 ml EP tubes with 1.5 ml viral-transport medium (VTM) that consisted of 2000 U/ml penicillin, 2 mg/ml streptomycin, 50 μg/ml gentamycin, 50 U/ml nystatin, and 0.5% bovine serum albumin. Samples were maintained in liquid nitrogen on the sampling site and were kept at −80°C after return to the laboratory.

Virus isolation

Virus isolation was carried out using previously described protocols (Zhang *et al*., 2016; Yin *et al*., 2017). Briefly, each individually collected swab specimen was inoculated into 9- to 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) according to the World Organization for Animal Health (WOAH) standard manual to detect Newcastle disease (ND) (The World Organisation for Animal Health, 2023). Haemagglutination (HA) assay with AOAV-16 antigen was performed according to standard WOAH protocols for detection of ND.

RNA extraction, semi-nested RT–PCR, and Sanger sequencing

The detailed information on RNA extraction from infectious allantoic fluids, semi-nested RT–PCR for *Paramyxoviruses*, and Sanger sequencing for the PCR product were described in our previous studies (Zhang *et al*., 2016; Yin *et al*., 2017). In brief, viral RNA from the harvested infectious allantoic fluids was extracted using Trizol (Sigma, Shanghai, China) according to the manufacturer's instructions.

Following viral RNA extraction, samples were assayed for the L gene of paramyxoviruses by semi-nested RT–PCR using previously described protocols without any modification (Tong *et al.*, 2008). The short genome fragments were sequenced using an ABI 3730XL automated DNA analyser (Applied Biosystems, Bedford, MA, USA). A BLAST similarity search confirmed that there are viruses related to the negative-sense single-strand RNA virus that we named AOAV-16 (all viruses in this study shared at least 96% nucleotide sequence identity with other reported AOAV-16). The AOAV-16-related samples were then sequenced to determine the complete viral genome sequences.

Whole genome sequencing for AOAV-16

To investigate the genetic characteristics of the five AOAV-16 isolates, the complete viral genome sequence was determined by next-generation sequencing (NGS), based on the random sequencing of total RNA using the *de novo* RNA-seq assembly method (Dimitrov *et al.*, 2017). Briefly, 2 ml of infectious allantoic fluid of each sample containing AOAV-16 was used for RNA isolation using Trizol (Sigma) according to the manufacturer's instructions, and then reverse transcription reactions as well as cDNA purification was performed using the protocols described in a previous study (Dimitrov *et al.*, 2017). Following cDNA purification, the DNA sequencing library (using 1.5 ng of purified cDNA) with an insert size of 200 bp was prepared by end-repairing, dA-tailing, adaptor ligation, and PCR amplification. Then the DNA libraries were sequenced on the Illumina HiSeq 4000 platform, and the complete viral genome was assembled through the Galaxy platform interface (Dimitrov *et al.*, 2017; Fei *et al.*, 2019). Representative consensus sequences for *Avulavirus* serotypes 1 to 21 were used for reference input, with reads mapping to AOAV-16, specifically Kazakhstan/1971/2006. The alignment was visualized in DNAStar SeqMan Pro v14. The raw reads were remapped with the consensus obtained to confirm the final sequence.

Pathogenicity test

Virulence of four AOAV-16 strains, excluding the Y3 isolate (because of co-circulation of H9N2 AIV), in the study was assessed using the intracerebral pathogenicity index (ICPI), according to the protocol standards established by the WOAH for ND (The World Organisation for Animal Health, 2023). Briefly, 10 1-day-old SPF chickens in each group were inoculated intracerebrally with a single dose of 10^7 EID_{50}/0.05 ml virus per chicken, and clinical signs were scored every 24 h for 8 consecutive days. For each observation, normal chickens were scored as 0, sick chickens as 1, and dead chickens as 2. The ICPI refers to the average number of all observed scores of each chicken within 8 days. Any virus having an ICPI score of $\geq$ 0.7 was considered a virulent virus, whereas lentogenic and asymptomatic enteric viruses gave scores close to 0.0.

Virus infection of cells

The detailed information on MDCK, HD11, and DF-1 cells infected with AOAV-16 viruses in this work as previously described (Chen *et al.*, 2018; Fei *et al.*, 2019). In brief, cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Woburn, MA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Biological Industries, Cromwell, CT, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C with 5% CO_2. The cell suspension was cultured in the growth medium in 48-well plates at a concentration of 60,000 cells per well under standard conditions. Then the next day, the cell monolayer was washed twice with Hanks' solution and infected with 50 µl infectious allantoic fluid containing AOAV-16. Following viral absorption for 2 h, the unattached viruses were removed, the cells were washed three times with Hanks' solution, and the cell culture medium was replaced with FBS-free DMEM with or without 1 µg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). Following viral infection, the cytopathic effect (CPE) of cells was observed every 12 h for up to 5 days.

Phylogenetic analysis

Phylogenetic trees were constructed by MEGA 11 software (Stecher *et al.*, 2020; Tamura *et al.*, 2021) based on either the whole genome or full-length *F* gene sequence alignment of representative viruses of the *Avulavirinae* subfamily. Specifically, the phylogenetic trees were constructed using the maximum-likelihood method based on the Tamura–Nei model with a discrete gamma distribution (+G) and allowing for invariant sites (+I) with statistical analysis based on 1000 bootstrap replicates, as implemented in MEGA 11 (Stecher *et al.*, 2020; Tamura *et al.*, 2021). The tree was drawn to scale, with branch lengths measured in the number of substitutions at each site. For all analyses, the codon positions included were 1st+2nd+3rd +Noncoding, and all p locations that contain blank and missing data are eliminated. In total, 35,726 and 1890 positions were included in the final dataset of the whole genome and complete F gene, respectively.

The complete *F* gene data set used for the phylogenetic analysis was also used to estimate the average evolutionary distances comparing Chinese AOAV-16 strains to other relative strains. Pairwise analysis was conducted using the maximum composite likelihood model using MEGA 11 (Stecher *et al.*, 2020; Tamura

et al., 2021). The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). Codon positions included were 1st+2nd+3rd +Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). A total of 1890 positions were included in the final dataset.

Recombination event analysis

The whole genome sequences of all AOAV-16 isolates were alignmented and input into the Recombination Detection Program (RDP)4 software (version 4.101). Recombination event analysis of isolates was performed using eight algorithms implemented in the RDP4 package, including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, Phylpro, and 3Seq, and the software was run according to the default conditions of the program (Martin *et al.*, 2015). Thereafter, if five or more detection methods in the RDP4 software showed a $P < 0.05$, this was identified as a recombination event.

Accession numbers

The complete genome sequences of the AOAV-16 strains and partial genome sequences of the H9N2 AIV strain determined in this study were submitted to GenBank and are available under the accession numbers OR270139 to OR270143 (AOAV-16) and OR272281 to OR272288 (the accession numbers for each influenza virus gene segment PB2, PB1, PA, HA, NS1, NA, M1, and NS2 of AIV, respectively).

Results and discussion

First molecular confirmation of AOAV-16 from healthy domestic poultry and wild migratory waterfowl in China

In total, five AOAV-16 strains were isolated during the surveillance programme of avian influenza and Newcastle disease in wild and domestic birds in nine provinces of China from 2014 to 2022. Among the AOAV-16 isolates, one was obtained from cloacal and oropharyngeal swabs of a healthy domestic duck at Honghu live bird market (LBM) of Hubei province (longitude 113.47, latitude 30.305), and four from the fresh faecal droppings of wild migratory swan geese in either Qinghai Lake of Qinghai province (longitude 100.18, latitude 36.844) or Chenhu Lake Wetland of Hubei province (longitude 113.87, latitude 30.305) or Poyang Lake of Jiangxi province (longitude 29.18, latitude 116.20). AOAV-16 strains were confirmed by a conventional semi-nested RT–PCR previously described for the detection of the *Paramyxoviridae* family viruses, followed by Sanger sequencing (Tong *et al.*, 2008). The strains were designated as AOAV-16/swan goose/Hubei/Y1/2015 (Y1), AOAV-16/swan goose/Qinghai/Y2/2017 (Y2), AOAV-16/duck/Hubei/Y3/2016 (Y3), AOAV-16/swan goose/Hubei/Y4/2014 (Y4), and AOAV-16/swan goose/Jiangxi/Y5/2021 (Y5), respectively. In addition, the genome of the H9N2 AIV was detected and sequenced only from the Y3 sample. To the best of our knowledge and available literature, this study represents the first molecular confirmation of AOAV-16 in wild and domestic birds in China, and the first reported coinfection of AOAV-16 and AIV in domestic ducks.

Molecular characterizations of five AOAV-16 strains

Next, the complete genome sequences of the AOAV-16 strains were obtained by a combination of second-generation sequencing, and Sanger sequencing for ambiguous regions, and 5′/3′ rapid amplification of cDNA ends (RACE). The genome length of Y3 and Y5 strains was 15,198 nucleotides (nt), which was 6 nt longer than that of Y2 and Y4 (15,192 nt) and 12 nt longer than that of Y1 (15,186 nt). They had six non-overlapping open reading frames (ORFs) with sequence identity to the NP, P, M, F, HN, and L proteins of other reported AOAV-16 isolates in the following order: 3′ leader-NP-P/V-M-F-HN-L-trailer 5′, with intergenic regions of 0–30 nt (Lee *et al.*, 2017).

Consistent with previous studies (Lee *et al.*, 2017; Karamendin *et al.*, 2020; Reeves *et al.*, 2021), the intergenic sequence (IGS) of all AOAV-16 strains in this study was identical to the IGS of other reported AOAV-16 strains except the IGS between the HN gene and L gene junction, where the length varied between 33 and 51 nt. Interestingly, one to four or five tandem nucleotide repeats (TRs), "AAAAAT", were present in the 5′-end trailer region of the genome of AOAV-13 (Fei *et al.*, 2019), and the IGS between the HN and L genes of AOAV-16 also had the same TRs of "AAAAAT" (Table 1). In comparison, Y2 and Y4 had three TRs of "AAAAAT", while Y1 had two TRs, Y3, and Y5 in this study and strains from Kazakhstan and America have four TRs and the isolate from Korea has only one TR (Table 1). There is increasing evidence that the TRs, a type of consecutive sequence duplication that abundantly reside in viral noncoding and coding genomic sequences, regulate gene expression, genome replication, lifecycle, and pathogenicity of viruses, such as Marek's disease virus, adeno-associated virus, and African swine fever virus (Almazan *et al.*, 1995; Stik *et al.*, 2010; Hirsch, 2015; Jilani & Ali, 2022). However, all reported AOAV-16 viruses are avirulent, implying that the number of TRs of "AAAAAT" may be unrelated to the virulence of AOAV-16. Nevertheless, further work should be carried out in the future on the

Table 1. Alignment of the intergenic sequence region (IGS) between the HN gene and L gene junction of all reported AOAV 16 isolates worldwide.

Strain	Number of TRs	IGS start	Sequences for tandem nucleotide repeats (TRs) of "AAAAAT"	IGS end
Kazakhstan	4	8386	ACAAAAAATAAAAATAAAAATAAAAATAAAATC	8417
America	4	8365	ACAAAAAATAAAAATAAAAATAAAAATAAAATC	8396
Korea	1	8386	ACA————————AAAAATAAAATC	8399
Y1	2	8386	ACG————AAAAATAAAAATAAAATC	8405
Y2	3	8386	ACA——AAAAATAAAAATAAAAATAAAATC	8411
Y3	4	8386	ACAAAAAATAAAAATAAAAATAAAAATAAAATC	8417
Y4	3	8386	ACA——AAAAATAAAAATAAAAATAAAATC	8411
Y5	4	8386	ACAAAAAATAAAAATAAAAATAAAAATAAAATC	8417

potential functions of TRs "AAAAAT" existing in IGS between the HN gene and L gene junction in the AOAV-16 lifecycle.

Recombination occurs in many families of viruses, and has a major impact on their epidemiology, emergence, and evolution, such as increases in virulence, the expansion of the viral host range, the evolution of resistance to antivirals, and the evasion of host immunity (Perez-Losada *et al.*, 2015). Systematic phylogenetic analyses have suggested a low rate of recombination in negative-sense genome RNA viruses (NSVs) (Chare *et al.*, 2003). However, with the growing availability of viral genome sequences, more and more recombination events have been observed in numerous NSVs recently (Simon-Loriere & Holmes, 2011), such as AOAV-1 (Chong *et al.*, 2010; Rahman *et al.*, 2019; Bahoussi *et al.*, 2023), human parainfluenza virus (Yang *et al.*, 2011), measles virus (Schierup *et al.*, 2005), respiratory syncytial virus (Zheng *et al.*, 1999; Spann *et al.*, 2003), and canine distemper virus (Piewbang *et al.*, 2019). Among the AOAV-16 viruses from this study and other reported strains with probably parental isolates, the Y2 isolate was verified as a recombinant virus, and the Y4 isolate was confirmed as the major parent (Table 2, and Figure 1) by recombination event analysis using RDP4 software. In addition, the putative recombination breakpoint of this recombinant was verified for the region located at nt positions 3946–8266 (numbering based on alignment) using the indicated methods (*P*-values ranged from 1.791×10^{-2} to 8.600×10^{-6}) (Table 2, and Figure 1). Meanwhile, the presence of the recombination breakpoint at nt positions 3946–8266 of Y2 and Y4 was also confirmed by Sanger sequencing to ensure the accuracy of the sequences. Therefore, to our knowledge, a natural homologous recombination event of AOAV-16 has been described for the first time, contributing to our understanding of the genetic diversity of recently discovered AOAV-16 around the world. However, further studies on the role of natural recombination in AOAV-16 evolution should be conducted in the future.

To elucidate the genetic relationship of AOAV-16 viruses in this study, phylogenetic trees were constructed based on the whole genome and full-length *F* gene sequence alignments of representative viruses of the *Avulavirinae* subfamily (Figure 2(A,B)), respectively. According to the phylogenetic trees here, constructed using the maximum-likelihood method based on the Tamura–Nei model, all AOAV-16 strains in this study grouped together with other reported AOAV-16 strains from Kazakhstan, South Korea, and Alaska in the same clade, whose sister clade had different strains of AOAV-1 (Figure 2(A,B), parts highlighted with light grey and dark grey, respectively). However, neither genotypes nor sub-genotypes have been proposed within the species AOAV-16, since the cut-off value for the nucleotide distance between these *F* genes was below 0.05 (5%) (Table 3). We made this proposition according to an updated unified phylogenetic classification system and a revised nomenclature for AOAV-1 based on the mean nucleotide evolutionary distances of the full F protein, with cut-off values (> 5% of the mean nucleotide evolutionary distance) to assign new sub-genotypes (Dimitrov *et al.*, 2019). The isolation of such highly similar viruses from different bird species in different regions of Asia and America suggests that the AOAV-16 virus could be intercontinental and transmitted between species in certain ways, such as through wild migratory birds (Karamendin *et al.*, 2020; Reeves *et al.*, 2021). Since Asia, Alaska, the Black Sea/Mediterranean, eastern Mongolia, eastern Siberia, and the Russian Far East are connected by

Table 2. Identification of a putative 4320 nt-long sequence in the Y2 genome highlighted by RDP4 software between nt 3946 and nt 8266.

Recombinant	Potential parents		Recombination breakpoint	Average *P*-values in detecting algorithms[a]						
	Major	Minor		R	M	C	S	G	T	B
Y2	Y4	Unknown	3946–8266	–	5.139×10^{-5}	8.600×10^{-6}	3.591×10^{-4}	1.791×10^{-2}	6.140×10^{-4}	7.807×10^{-4}

[a]R: RDP, M: MaxChi, C: Chimaera, S: SiScan, G: GENECONV, T: 3Seq, B: BootScan.

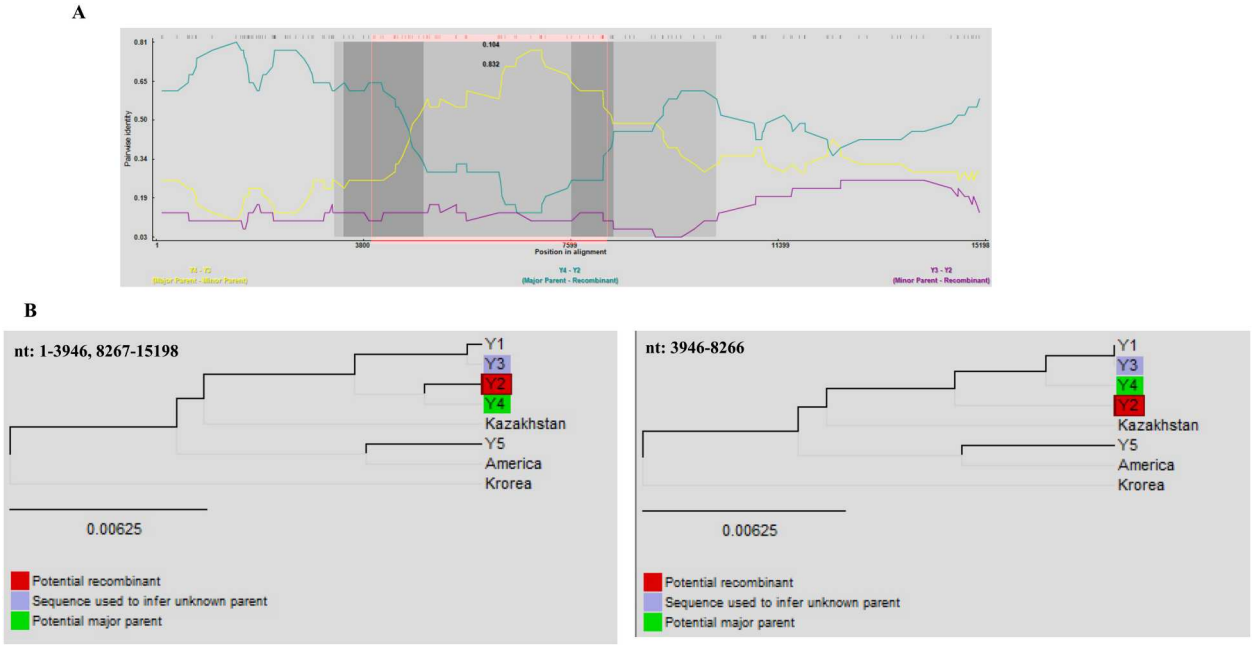

Figure 1. Recombination analysis of AOAV-16 strains. (A) RDP analysis result of the recombinant strain Y2. The horizontal axis represents position in the alignment, the vertical axis represents pairwise identity. (B) The phylogenies of the regions 1–3945, 8267–15,198 (left), and 3946–8266 (right) of the recombinant strain Y2.

the migratory routes of the species analysed here, our data should generally apply to Asian AOAV-16 viruses. Therefore, further testing of samples from domestic and wild migratory birds for AOAV-16 on a global level is necessary to better identify the evolution and distribution of this viral pathogen.

Biological characterizations of five AOAV-16 strains

All of the AOAV-16 strains were successfully propagated in 9- to 10-day-old SPF chicken embryos, and the harvested infectious allantoic fluids tested positive by a HA assay with titres between 256 and 1024 per 25 µl except the Y4 strain obtained from the year 2014. As described in our previous study, HA-positivity was restored to an HA-negative *Avian Metaavulavirus 6* virus (AMAV-6/mallard/Hubei/2015) isolated from mallard when the virus was either pre-treated with 1% trypsin for 30 min or propagated in 9- to 10-day-old SPF embryonated chicken eggs for at least five passages (Chen *et al.*, 2018). However, the Y4 virus in this work was still unable to produce an HA-positive result at the seventh passage in 9- to 10-day-old SPF embryonated chicken eggs, although the HA-negative allantoic fluid of Y4 could generate an HA titre of 64 per 25 µl when the virus was pre-treated with 1% trypsin for 30 min. But the nucleotide and amino acid (aa) sequence homologies of the HN gene among this HA-negative Y4 virus and other all reported HA-positive AOAV-16 isolates was 97.58% identical, and the potential sialic acid binding motif NRKSCS is located at 236–241 aa in the Y4 virus HN protein, the same as all other reported AOAV-16 strains. Therefore, further studies are needed to unveil the molecular mechanisms of HA-negative viruses from the subfamily of *Avulavirinae*, such as AMAV-6/mallard/Hubei/2015, and AOAV-16/swan goose/Hubei/Y4/2014 (Y4) strain in this study. HA and HI assays are the classical diagnostic methods to test haemagglutinating agents, including *Avulavirus*, in many laboratories. Identifying novel HA-negative viruses, such as Y4 in this work, that are not detected by traditional assays is essential to continuously update monitoring regulations, comprising biosecurity measures, diagnostic assays, and research, to safeguard domestic and wild birds across the globe.

To analyse the biological characteristics of the AOAV-16 strains in the study, virulence and/or pathogenicity markers were characterized for Y1, Y2, Y4, and Y5. All strains in the study, including Y3, had the same cleavage site motif of the *F* gene, 110 LVQAR↓L115, which is identical to all other reported AOAV-16 viruses (Lee *et al.*, 2017). Meanwhile, the ICPI was zero and the mean death time (MDT) score was more than 168 h, and all chicken embryos were alive at 7 days post-infection with either Y1, Y2, Y4, or Y5 strain, suggesting all these isolates in this study are low virulent viruses for chickens. In addition, all of the Y1, Y2, Y4, and Y5 viruses did not cause any cytopathic effect without exogenous protease in DF-1, HD 11, and MDCK cell lines (data not shown). However, increasing studies have demonstrated that co-infection of low pathogenic avian influenza H9N2 viruses with other viruses or bacteria can cause severe morbidity and mortality (Kishida

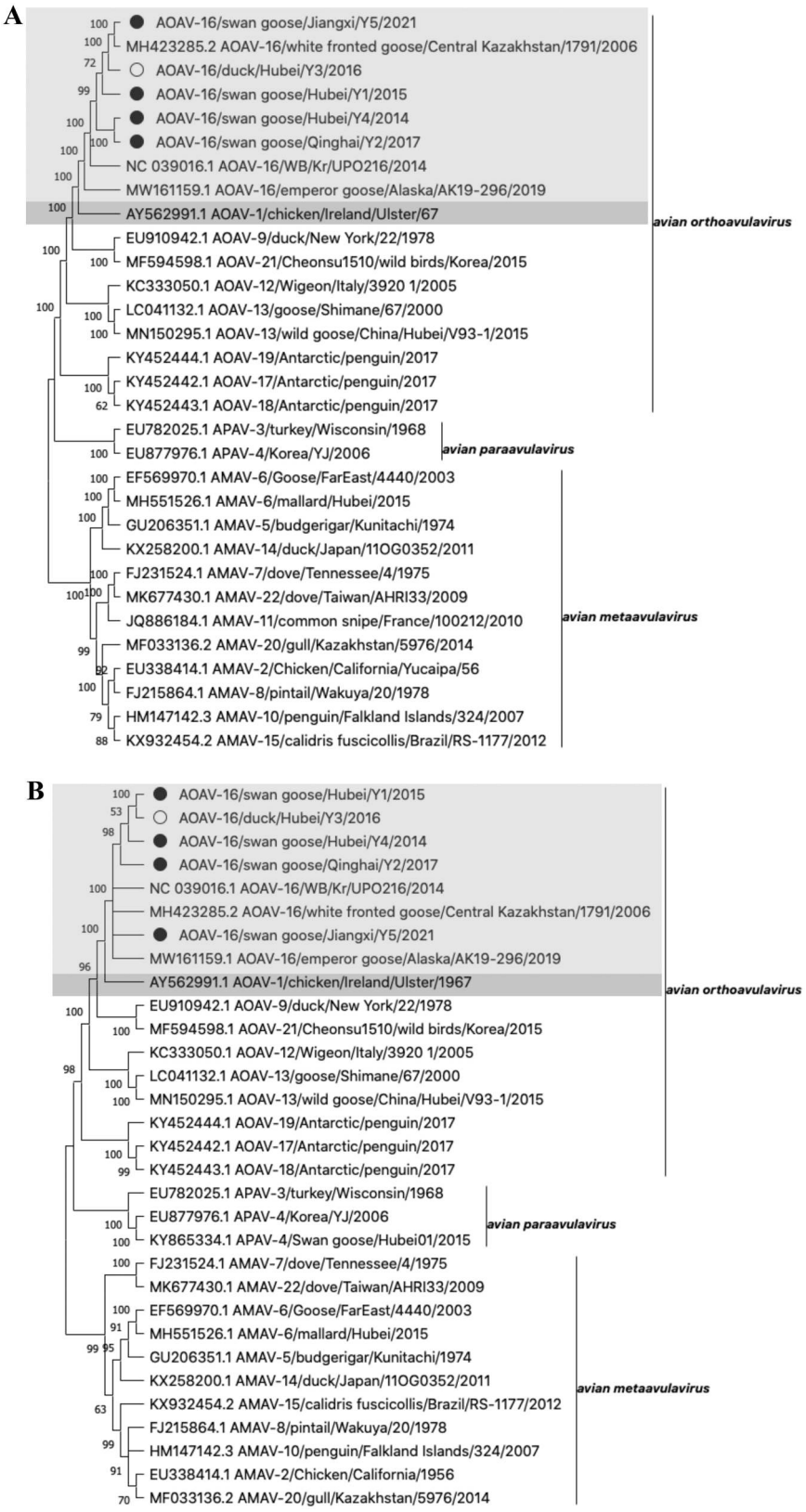

Figure 2. Phylogenetic analysis of the whole genome sequence (A) and complete F gene sequences (B) of AOAV16 and other serotype viruses belonging to *Avulavirinae*. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura–Nei model. The trees with the highest log likelihood (−983,774.81) (A) and (−39,327.35) (B) are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura–Nei model and then selecting the topology with superior log likelihood value. This analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were totals of 35,726 (A) and 1890 (B) positions in the final datasets. Evolutionary analyses were conducted in MEGA11. The solid circles represent the isolates obtained from migrant birds, and the open circles represent the isolate obtained from a domestic bird in this study. Isolates highlighted with light grey shading are AOAV-16 strains, and the dark grey shading highlights the AOAV-1 (NDV) strain.

Table 3. Estimates of evolutionary divergence between sequences of AOAV-16[a].

	1	2	3	4	5	6	7	8
1. NC_039016.1_AOAV-16/WB/Kr/UPO216/2014		0.0340	0.0305	0.0299	0.0312	0.0285	0.0300	0.0299
2. MW161159.1_AOAV-16/emperor_goose/Alaska/AK19-296/2019			0.0181	0.0207	0.0194	0.0168	0.0049	0.0207
3. MH423285.2_AOAV-16/white_fronted_goose/Central_Kazakhstan/1791/2006				0.0174	0.0161	0.0136	0.0143	0.0174
4. AOAV-16/swan_goose/Hubei/Y1/2015					0.0098	0.0061	0.0168	0.0000
5. AOAV-16/swan_goose/Qinghai/Y2/2017						0.0049	0.0156	0.0098
6. AOAV-16/swan_goose/Hubei/Y4/2014							0.0130	0.0061
7. AOAV-16/swan_goose/Jiangxi/Y5/2021								0.0168
8. AOAV-16/duck/Hubei/Y3/2016								

[a]Inferred from the complete nucleotide F gene sequences of AOAV-16. The number of base substitutions per site between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). This analysis involved eight nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were 1890 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

et al., 2004; Hassan *et al.*, 2017; Ismail *et al.*, 2018; Belkasmi *et al.*, 2020). As observed in our study, all chicken embryos were killed at 36 h after inoculation of the Y3 sample containing AOAV-16 virus and H9N2 AIV, which suggests that co-infection of AOAV-16 with other low pathogenic and/or low virulent viruses (such as H9N2 AIV in this work) or bacteria could be a potential threat to commercial poultry and public health. Therefore, additional studies are being conducted to uncover the molecular mechanisms of co-infection of AOAV-16 and other low pathogenic viruses or bacteria in birds, which may exacerbate disease burden and even alter disease transmission patterns.

Taken together, our study is the first to report that AOAV-16 is sporadically detected in clinically healthy domestic ducks and wild migratory swan geese in China. Our data in this work demonstrate that not only do the AOAV-16 viruses harbour at least four different intergenic sequences between the HN and L genes junction, as compared to other previously reported species within the subfamily of *Avulavirinae*, but also natural recombination events can occur within the species of AOAV-16. Furthermore, the AOAV-16 viruses that co-exist with other low pathogenic and/or low virulent viruses or bacteria, such as the H9N2 AIV in this study, could be a potential threat to commercial poultry and even a public health challenge. In addition, the detection of the AOAV-16 viruses in China is phylogenetically linked to the viruses from other geographical regions, such as Kazakhstan, South Korea, and Alaska. Therefore, strict control measures are needed to restrict contact between wild migratory birds and domestic poultry, as these hosts appear to continuously exchange AOAV-16 strains. Bird shows, trade, and import are other potential pathways that easily allow the introduction of new strains into susceptible hosts unless strict control actions are used. It also highlights a call for continuous active virological surveillance of AOAV-16 strains among wild migratory birds and domestic poultry in China and countermeasures to stay abreast of the potential introduction of new genetic variants from other populations and geographic regions.

Authorship contribution

RY, XinL, JC. ZD: investigation, conceptualization, formal analysis, writing – original draft, writing – review and editing, resources, Supervision, funding acquisition. WY, HC, HongjL: methodology, validation, formal analysis, Writing – original draft. MW, SJ, YS, SW, YR, HonglL, YL: methodology, validation, formal analysis. TS, AW, AD, CG, XX, CM, DA: formal analysis, writing – original draft, writing – review and editing, resources.

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