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ORIGINAL ARTICLE

First detection and biological characterization of an avian metaavulavirus 8 isolated from a migratory swan goose in Qinghai Lake, Northwest China

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Abstract

Avian metaavulavirus 8 (AMAV-8), formerly known as avian paramyxovirus 8 (APMV-8), has been detected sporadically in wild birds worldwide since it was first identified in a Canadian goose in 1976. However, the presence of AMAV-8 in birds has never been reported in China. To understand the epidemiological situation of AMAV-8 and its ability to infect chickens, we conducted a surveillance study and *in vivo* analysis of the AMAV-8 isolate identified in total of 14,909 clinical samples collected from wild and domestic birds from 2014 to 2022 in China. However, in 2017, only one AMAV-8 virus (Y7) was successful isolated from the fresh droppings of a migratory swan goose in Qinghai Lake in Northwest China. Thereafter, we report the complete genome sequence of the Y7 strain with a genome length of 15,342 nucleotides and the Y7 isolate was genetically closely-related to wild bird-origin AMAV-8 viruses previously circulated in the United States, Japan, and Kazakhstan. Furthermore, AMAV-8 infections of one-day-old specific pathogen-free (SPF) chicks did not induce any clinical signs over the entire observation

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Abbreviations: AIV, avian influenza virus; AMAV-8, Avian metaavulavirus 8; APMV-8, avian paramyxovirus 8; CPE, cytopathic effects; ICPI, intracerebral pathogenicity index; ICTV, International Committee on Taxonomy of Viruses; MDT, mean death time; ND, Newcastle disease; NDV, Newcastle Disease Virus; SPF, specific pathogen free; TPCK, tosyl phenylalanyl chloromethyl ketone; VTM, viral-transport medium; WOAH, World Organization for Animal Health.

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period but was associated with viral shedding for up to 8 days. Interestingly, although all birds infected with the Y7 strain seroconverted within the first week of infection, virus replication was only detected in the trachea but not in other tissues such as the brain, lung, or heart. Here, we report the complete genome, genetic and biological characterization, replication and pathogenicity analysis in vivo and first detection of AMAV-8 in China.

KEYWORDS

avian metaavulavirus 8, biological characterization, genetic relationships, migratory waterfowl, Qinghai lake

INTRODUCTION

Avulaviruses belong to the subfamily *Avulavirinae* of the family *Paramyxoviridae* is known to cause infection in a wide range of poultry and non-poultry avian species and wild birds worldwide.[1,2] International Committee on Taxonomy of Viruses (ICTV) currently defines 22 different avulavirus species, divided into three recently created genera, named *avian orthoavulavirus* (including species AOAV-1, −9, −12, −13, −16, −17, −18, −19, and −21), *avian metaavulavirus* (including species AMAV-2, −5, −6, −7, −8, −10, −11, −14, −15, −20, and −22), and *avian paraavulavirus* (including species APAV-3 and −4).[1,3] The most studied specie of *Avulaviruses* is AOAV-1 (commonly known as Newcastle Disease Virus, NDV), as the virulent NDV can cause 100% mortality in infected birds resulting in severe economic losses for the global poultry industry.[4,5]

Prior to this study, there were very limited reports of AMAV-8 strains from wild birds across the globe, where AMAV-8 first detected in 1976 in the feces of a Canadian goose from the United States and then sporadically isolated from a pintail duck in Japan in 1978, and was recently isolated from wild migratory waterfowl in Kazakhstan and Mongolia.[6–10] To date, there is no report of AMAV-8 in China. The potential for spread and transmission of AMAV-8 is high as several bird species particularly wild migratory waterfowl, shorebirds and gull species, are native to China during the breeding season, where several migratory routes are connecting Asia, Australia, Africa, the Americas, and Europe. Meanwhile, very little is known about the genetic and biological information on AMAV-8 in domestic and wild birds and the disease significance of AMAV-8 in chickens. Systemically and continuous monitoring of AMAV-8 isolates in wild and domestic birds is important to provide data on viruses as well as emerging viruses in the field. Therefore, we conducted an epidemiological study and *in vivo* analysis of AMAV-8 strain isolated from wild bird in China.

MATERIALS AND METHODS

Ethics statement

All animal experimental protocols used in this study was approved by the Institutional Animal Care and Use Committee of Jilin University, China (approval number: 201803036, Date of approval: 8 March 2018).

Sample collection, and virus isolation

A total of 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 year 2014–2022, as part of the avian influenza virus (AIV) and AOAV −1 surveillance project. In total of 13,221 fresh fecal drops were collected from wild birds, while 1688 clinical samples, including 703 fresh fecal drops, and 985 oropharyngeal and cloacal swabs were obtained from domestic poultry (Table 1). The collected samples were then soaked in 2 ml EP tubes with 1.5 ml of viral transport medium (VTM) that consisted of 2000U/ml penicillin, 2 mg/ml streptomycin, 50 μg/ml gentamycin, 50U/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.

Then 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) using World Organization for Animal Health (WOAH) standard manual to detect Newcastle disease (ND).[11] In brief, the eggs were incubated at 37°C with 60% humidity for 2–7 days, and embryos viability was monitored daily. After checking that the embryos have died or dying embryos as they arise, the allantoic fluids were harvested upon embryo death or at the end of the incubation period, and then tested with both hemagglutination (HA) test and a semi-nested RT-PCR to detect Paramyxoviruses specific nucleic acid signatures.

Hemagglutination (HA), and hemagglutination inhibition (HI) tests

The HA and HI tests used in this study were conducted using the WOAH standard manual to detect Newcastle disease (ND) without any modification.[11] Briefly, the procedure for the HA test, serially two-fold diluting the infective allantoic fluid (25 μl) with 25 μl of phosphate-buffered saline (PBS) in a plastic V-bottomed microwell plate at first, and then 25 μl of 1% (packed cell v/v) chicken red blood cells (RBCs) suspension were added to each well. Subsequently, the plates were incubated at room temperature, and the evaluation of hemagglutination titers determined after a 40-min incubation

TABLE 1 Summary of sample collection for wild and domestic birds.

Sample type	Collection year	Collection location	Number of samples
Wild birds	2014	Hubei	1202
	2015	Hubei	1301
	2016	Hubei	1303
	2017	Qinghai	1102
	2018	Hubei	1103
		Hunan	113
		Qinghai	771
	2021	Henan	1121
		Hubei	2208
		Qinghai	1121
	2022	Hubei	1631
		Heilongjiang	245
Domestic birds	2019	Anhui	112
		Heilongjiang	86
		Inner Mongolia	112
		Jilin	221
	2020	Anhui	77
		Heilongjiang	89
		Inner Mongolia	92
		Shandong	88
	2021	Inner Mongolia	89
		Jilin	224
		Shandong	78
	2022	Jilin	278
		Shandong	142

period. The HI assay was performed using four HAU of the Y7 virus, Y7-specific chicken sera were treated with receptor destroying enzyme (RDE, Denka Seiken) at 37°C for 18 h firstly and then heat inactivated at 56°C for 30 min. Starting with the sera were serially diluted twofold, mixed with the Y7 virus, and incubated at room temperature for 30 min. Subsequently, 1% RBCs suspension were added and settled for an additional 30 min at room temperature, and the HI titer was defined as the highest dilution of serum causing complete inhibition of four HAU of Y7 virus.

RNA extraction, semi-nested RT-PCR, whole genome sequencing, and phylogenetic analysis

The information on RNA extraction, RT-PCR, and whole genome sequencing have been previously described in detail in our studies without any modification.[12–16] In brief, viral RNA from the harvested infectious allantoic fluids was extracted using Trizol (Sigma, Shanghai, China) according to the manufacturer 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.[17] Purified semi-nested RT-PCR product were sequenced using an ABI 3730XL automated DNA analyzer (Applied Biosystems, Massachusetts, USA). A BLAST similarity search confirmed that the viruses related to negative sense single strand RNA virus that we named AMAV −8. The AMAV-8 related samples were then sequenced for complete viral genome sequences.

Regarding the whole genome sequencing, 2 ml of infectious allantoic fluid of each sample containing AMAV-8 were used for RNA isolation using Trizol (Sigma, Shanghai, China) 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.[18] 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.[13,18] Representative consensus sequences for *Avulavirus* types 1 through 21 were used for reference input, with reads mapping to AMAV-8. The alignment was visualized in DNAStar SeqMan Pro v14. The raw reads were remapped with the consensus obtained to confirm the final sequence.

Phylogenetic trees were constructed using MEGA 11 software[19,20] based on the alignment of either the whole genome or full-length F gene sequences of of Y7 strain in this study and other reported AMAV-8 reference strains. Specifically, the phylogenetic trees were generated using the neighbor-joining method, with the Kimura 2-parameter model employed for the analysis. A discrete gamma distribution was applied to model the rate variation among sites. Statistical support for the tree topology was assessed using 1000 bootstrap replicates, as implemented in MEGA 11. The trees were drawn to scale, with branch lengths representing the number of substitutions per site. For all analyses, codon positions included were 1st+2nd +3rd+Noncoding, and sites with gaps or missing data were eliminated. In the final dataset, a total of 15,726 positions for the whole genome and 1714 positions for the complete F gene were analyzed.

Pathogenicity test, and virus infection of cells

Viral virulence for Y7 isolate in the study was assessed using the intracerebral pathogenicity index (ICPI), according to the protocol standards established by the WOAH for ND.[11] Meanwhile, virulence of the virus was also evaluated by determining the mean death time (MDT) in 9- to 10-day-old SPF embryonated chicken eggs. For the test, allantoic fluid was diluted 10-fold in PBS and inoculated into - to 10-day-old SPF-embryonated chicken eggs. The eggs were candled two times per day for embryonic death and the results were recorded. The virus in allantoic fluids causing embryonic death (MDT) of up to 60 h, from 61 to 90 h, and more than 90 h were designated velogenic, mesogenic, and lentogenic, respectively.[21]

MDCK, HD 11, and DF-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's media (DMEM) (Life Technologies, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin 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 of infectious allantoic fluid containing AMAV-8 with a viral titer of 10^6 EID50/ml. 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 the presence or absence of 1 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, Shanghai, China). Following viral infection, cytopathic effect (CPE) of cells was observed every 12 h for up to 5 days.

Animal experiments

At least 20 one-day-old chicks from SPF eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) per group were challenged with a single dose of either 10^6 EID_{50} or 10^7 EID_{50} Y7 virus or PBS via intraocular-nasal drops. At indicated time intervals post infection either oropharyngeal and cloacal swabs were taken for virus detection. Meanwhile, the body weight and HI titers against AMAV-8 of the chickens were monitored for 14 days. In addition, on each of day 2, 4, 6, 8, and 14 dpi two inoculated chickens were sacrificed and all organs, including liver, kidneys, spleen, heart, lungs, brain, stomach, bursa of Fabricius, rectum, cecum, duodenum, and trachea were tested for AMAV-8 by virus isolation and semi-nested RT-PCR.

Accession numbers

The complete genome sequence of the AMAV-8 strain determined in this study was submitted to GenBank and is available under the accession number PP503728.

RESULTS AND DISCUSSION

As part of the avian influenza virus (AIV) and Newcastle disease virus (NDV) surveillance program, a total of 14,909 clinical samples were collected from both wild and domestic birds in nine provinces (Hubei, Hunan, Henan, Shandong, Anhui, Jilin, Qinghai, Inner Mongolia, and Heilongjiang) in China from 2014 to 2022 (Table 1). One AMAV-8 strain was successfully isolated from the fecal specimens of a migratory swan goose collected in 2017 at Qinghai Lake (longitude 100.18, latitude 36.844) in Qinghai Province, Northwest China. The isolate was designated as AMAV-8/Swan goose/China/Qinghai/Y7/2017 (Y7). In this study, no AMAV-8 strain was detected in 1688 domestic poultry samples. However, in the United States, the seroprevalence of AMAV-8 in commercial chickens has been reported to be 31%,[22] suggesting that the virus can be transmitted between wild birds and domestic poultry. To the best of our knowledge, this work represents AMAV-8 was detected for the first time in migratory wild bird at Qinghai Lake in China, one of the most important breeding and resting grounds for the migratory birds along the Central Asian flyway.

Y7 strain was successfully propagated in 9- to 10- day-old specified pathogen-free (SPF) embryonated chicken eggs, and the harvested infectious allantoic fluid tested positive by HA assay with titers between 64 and 128 per 25 μl. Furthermore, the virus caused no cytopathic effects (CPE) on the MDCK, HD11, and DF-1 cell lines with and without 1 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin exogenous proteases.

To assess the virulence and/or pathogenicity index of the Y7 strain, the F protein cleavage site motif and intracerebral pathogenicity index (ICPI) were determined, according to the protocol standards established by the WOAH for ND.[11] The putative cleavage site motif of the F protein of strain Y7 contained a single basic amino acid (^{98}TYPQR/L^{104}), typical of avirulent avulaviruses strains. The ICPI of day-old SPF chicks was 0 and the mean death time (MDT) score of 9- to 10-day-old SPF embryonated chicken eggs was more than 168 h, and all chicken embryos was not dead at 7 dpi, demonstrating that the Y7 isolate had low pathogenicity for chicks.

The complete genome of the Y7 strain obtained in this study was 15,342 nucleotides (nt) long and had a GC content of 41.3%. A comparison of the whole genome sequence of AMAV-8 strain with the sequences available in the GenBank database revealed that Y7 has a high degree of similarity (97.31%–97.78%) to AMAV-8 strains isolated in the United States, Japan, and Kazakhstan. To further characterized, the AMAV-8 strain isolated in this study had similar key protein sites in two glycoproteins, haemagglutinin-neuraminidase (HN) and fusion (F) with the previously described AMAV-8 viruses include deduced amino acid sequences at the F-protein cleavage site TYPQR/L (98-104), the conserved neuraminidase active site residue 176(R), 401(E), 416(R), 506(R), 534(Y) and 555 (E), and silicic acid binding sites 236 – 241 (N-R-K-S-CS) as well as the 11 conserved cysteine residue sites corresponding to the globular head structure of HN proteins(174, 188, 198, 240, 253, 346, 463, 469, 473, 539, and 550).

To explore potential epidemiological and intercontinental connections between AMAV-8 viruses and wild birds, we performed the phylogenetic analyses based on both complete genome and F gene sequences of Y7 strain in this study and other reported AMAV-8 reference strains (Figure 1a, b).

Phylogenetic relationship analysis based on complete genome sequences revealed that the AMAV-8 strains isolated in this study clustered within the same clade as previously reported AMAV-8 strains from China, Kazakhstan, the United States, and Japan, demonstrating a high degree of genetic homogeneity (Figure 1a). Interestingly, the F gene sequences of these Chinese AMAV-8 isolates, including Y7 virus in this study, were highly similar and clustered into the same sub-clade, whereas one AMAV-8 strain from Anhui of China grouped into another sub-clade, which is closer phylogenetically to isolates from the United States, Japan, and Kazakhstan (Figure 1b), suggesting the existence of multiple sub-clades/groups within AMAV-8.

Subsequently, the replication and pathogenicity of the Y7 strain was examined *in vivo* by inoculated one-day-old SPF chicks with a single dose of either 10^6 EID_{50} or 10^7 EID_{50} viruses via intraocular-nasal drops. Consistent with the previous study,[23] the Y7 virus infected chickens remained healthy throughout the observation period and developed like uninfected chicks, evidenced by the body weight loss (Figure 2a). However, virus shedding was detected in oropharyngeal swabs of the Y7 virus-inoculated chickens at 2, 4, 6, and 8 dpi rather than 14 dpi, positive by virus isolation and RT-PCR. Moreover, viral RNA was only detected in the upper respiratory tract of all infected chickens on day 6, and 8 dpi rather than day 2, and 14 dpi, and was not detected in other tissues, including brain, lung, heart, liver, stomach, cecum, rectum, kidneys, bursa, duodenum, and spleen. This result is partially consistent with previous studies in which viral RNA was detected in the trachea but not in the brain, spleen, and lungs of infected birds sampled at day 4 after infected with AMAV-8.[23,24]

All birds infected with Y7 strain seroconverted within the first week after infection. However, the HI titers of the harvested chicken blood samples at different stages of challenge exhibited time-dependent significant increases after 6 dpi, represented a significant increase from 6 dpi to a peak at 14 dpi, when chickens inoculated with 10^6 EID50 of AMAV-8 Y7 virus. By contrast, the HI titers was dramatically increased from 4 dpi to a peak at 7 dpi but not within the period from 7 to 14 dpi, when chickens infected with 10^7 EID50 of Y7 virus

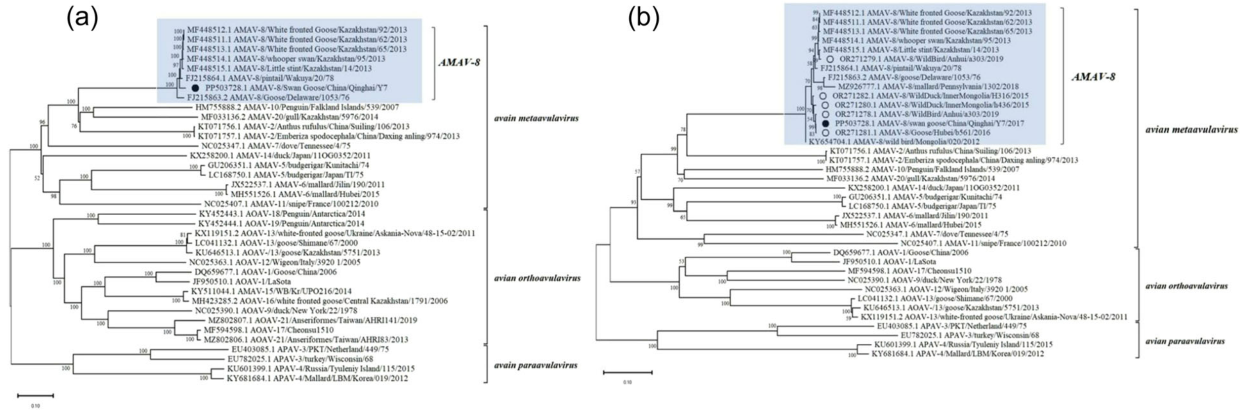

FIGURE 1 Phylogenetic relationship analysis of the whole genome sequence (a) and complete F gene sequences (b) of AMAV-8 and other type viruses belonging to Avulavirinae. This analysis involved 37 (a) and 38 (b) nucleotide sequences, with codon positions including 1st+2nd+3rd+Noncoding. The final datasets comprised 15,726 (a) and 1714 (b) positions, respectively. Evolutionary analyses were conducted using MEGA11. In the phylogenetic trees, solid circles represent isolate obtained in this study, while open circles denote previously reported isolates from China. AMAV-8 strains are highlighted in light blue.

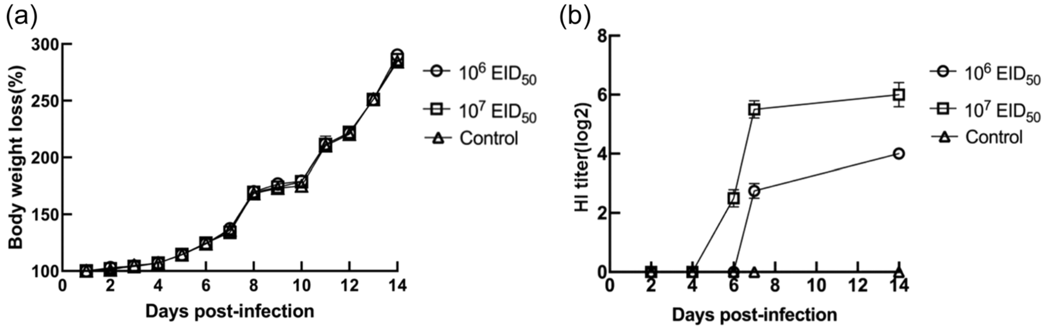

FIGURE 2 The biological information on AMAV-8 Y7 isolate in vivo. (a) Total body weight loss rates at different times after infection of chickens with either 10^6 EID_{50} or 10^7 EID_{50} Y7 virus. Body weight was recorded daily. (b) Antibody responses of chickens infected with Y7 virus. Serum samples were collected at 2, 4, 6, 7, and 14 dpi after infection of day-old SPF chickens. AMAV-8-specific antibodies using the hemagglutination inhibition (HI) assay according to standard World Organization for Animal Health (WOAH) protocols for detection of Newcastle disease (ND).

(Figure 2b). Consistent with previous studies,[23,24] Y7-specific chicken serum showed high HI titers (1:256) with homologous Y7 virus but low (less than 1:8) or no cross-reactivity with other serotypes of avulaviruses, including AOAV-1, APAV-4, AMAV-6, AOAV-13, and AOAV-16.

CONCLUSIONS

In summary, our study is the first to report the isolation of AMAV-8 from migratory wild bird in China. The AMAV-8 isolate in this study is genetically closely-related to viruses from other geographical regions, such as the United States, Japan, and Kazakhstan. In addition, AMAV-8 infections of one-day-old SPF chicks did not induce any clinical signs throughout the observation period but was associated with virus shedding for up to 8 days. All birds infected with the Y7 strain seroconverted within the first week of infection, virus replication was detected only in the trachea but not observed in other investigated tissues, such as. brain, lung, heart, liver, stomach, cecum, rectum, kidneys, bursa, duodenum, and spleen. However, further studies will be necessary to improve our understanding of AMAV-8 strains in the field.

AUTHOR CONTRIBUTIONS

R.Y., and Xin.L.: Investigation; conceptualization; formal analysis; writing—original draft; writing—review and editing; resources; supervision; funding acquisition. S.W., J.C., Yan. W., and Hongj.L.: methodology; validation; formal analysis; writing—original draft; W.Y., R.L., Xia.L., Y.Y., C.D., H.X., Hongl.L., and Y.L.: methodology; validation; formal analysis; T.S., A.W., A.D., C.G., C.M., and D.A.: writing—original draft; writing—review and editing; resources.

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DISCLOSURE

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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