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Isolation and characterization of a novel H7N8 avian influenza virus from domestic ducks in Central China in 2017

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Abstract

In 2017, an H7N8 avian influenza virus (AIV) was isolated from a domestic duck from a farm in Central China. Sequences analysis showed that this strain received its genes from H7, H1, H2, H3, H5, and H6 AIVs of domestic poultry and wild birds in Asia. It exhibited low pathogenicity in chickens and mild pathogenicity in mice. These results suggest the importance of continued surveillance of the H7N8 virus to better understand the ecology and evolution of the AIVs in poultry and wild birds and the potential threat to human health.

Keywords Avian influenza virus · H7N8 · Phylogenetic analysis · Reassortment · Pathogenicity

Avian influenza viruses (AIVs) belong to the family *Orthomyxoviridae*, specified as influenza A virus. Based on the antigenic properties of the predominant viral surface glycoproteins, the hemagglutinin (HA), and neuraminidase (NA) glycoproteins, AIVs are further classified into 16 HA and 9 NA subtypes [1]. Aquatic birds are considered a natural reservoir for AIVs, in which all subtypes of avian influenza viruses have been detected [2].

Most subtypes of AIVs are low pathogenic; however, they also are very important. On the one hand, low pathogenic avian influenza viruses (LPAIVs) could potentially infect domestic poultry and humans if they undergo reassortment to produce pathogenic forms. This was demonstrated in 2013 by the unexpected emergence of the LPAI H7N9 virus in humans in China. On the other hand, LPAIVs could serve as progenitors of highly pathogenic avian influenza viruses (HPAIVs) [3]. This was demonstrated in 2017, a novel H7N9 AIV associated with highly pathogenicity in chickens has emerged in China, which likely evolved from the preexisting H7N9 LPAIVs.

During routine surveillance for avian influenza in Hubei Province, Central China, in April 2017, we collected 2099 swab samples from healthy chickens, ducks and environment from 62 of live bird markets (LBM), poultry farms, and slaughtering houses in eleven counties. Thirty swab samples were collected from each source. For virus isolation, the trachea and cloacal swab samples were centrifuged at 10,000×*g* for 5 min at 4 °C, and the supernatants were inoculated into the allantoic cavities of 10-day-old specific-pathogen-free (SPF) chicken embryos. The embryos were incubated at 35 °C for four days and checked daily. The dead ones were picked out and stored in a refrigerator. After the incubation period, the live embryos were killed at 4 °C and the allantoic fluids were collected and tested by using the hemagglutination assay. All the hemagglutination-positive samples and the allantoic fluid of the dead embryos were confirmed as influenza A virus isolates by RT-PCR, with the primers designed in previous studies which cover the entire length of the HA and NA genes [4].

We have isolated 166 strains of AIVs, subtypes H3 ($n=1$), H6 ($n=1$), H7 ($n=58$) and H9 ($n=106$) (Table S1). Of those, 25 H7N8 AIVs were isolated from apparently

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healthy domestic ducks in one farm. The entire viral genomes of these H7N8 viruses were amplified with primers as described elsewhere by RT-PCR assay [4]. RT-PCR products were purified with an agarose gel DNA extraction kit (Sangon, Shanghai, China) and sequenced using the ABI 3730xl DNA Analyzer. Their sequences are very similar (data not shown). One strain named as A/duck/Hubei/HF5/2017(H7N8) (DK/HF5) was selected for further study. The nucleotide sequences of DK/HF5were deposited in GenBank under accession nos. MH458919-MH458926. To better understand the genetic relationship between the H7N8 strain from Central China and from birds in the world, the phylogenetic analyses were carried out with the software MEGA 6.05 of the ClustalW software package (http://www.megasoftware.net) using a neighbor-joining algorithm. Nucleotide substitutions were set under the Kimura 2-parameter model, and substitution rates among sites were set in gamma distribution. The gaps were handled by pairwise deletion. A bootstrap analysis was conducted using 1000 replicates.

Based on the deduced amino acid sequence of the hemagglutinin (HA), the DK/HF5 virus has two basic amino acid residues (PELPKGR/GLF) at the cleavage site, which is typical for low-pathogenicity AIVs [5]. The amino acid residues at the receptor binding site in the HA protein are Q235 and G237, which indicates its avian-like receptor binding preference [6]. The strain has five potential *N*-glycosylation sites at positions 30, 46, 249, 421, and 493 in the HA protein.

There were no amino acid substitutions associated with resistance to oseltamivir (F119G, E275Y, N293K, and T295S) in the viral neuraminidase protein (NA) [7]. The DK/HF5 virus does not have mutations associated with high pathogenicity of the virus in mammals (T271A, E627K, and D701N) were identified in the viral PB2 protein [8]. No mutation of Y436H associated with decreased virulence in ducks and no mutation of N66S associated with increased virulence were found in PB1and PB1-F2 [9, 10]. A mutation of A515T occurred in the PA protein which could render a lethal virus in ducks [9]. No mutations associated with resistance to amantadines (V27A and S31N) were observed in the M2 protein [11]. However, a mutation of G149A occurred in the viral NS1 protein, which could increase the viral virulence in chickens [12].

Sequences analysis showed that the HA and NA genes showed the highest sequence identities (97.7% and 99.4%) with those of the virus strains A/duck/Bangladesh/27042/2015(H7N9) and A/duck/Hunan/S1256/2012(H3N8), respectively. The internal genes showed high nucleotide identity with those of the viruses circulating in wild birds and domestic poultry, including H1N1, H5N7, H6N2, and H2N8 (Table 1).

Phylogenetic analysis showed that the HA gene of DK/HF5 was very closely related to H7N9 viruses circulating in Southern Asia (Fig. 1a). The NA gene was very closely related to H3N8, H4N8, or H12N8 viruses circulating in Eastern Asia (Fig. 1b). According to the above-described analysis, DK/HF5 was a reassortant virus and received its genes from H7, H1, H2, H3, H5, and H6 subtype viruses of poultry and wild birds in Asia. Both high- and low-pathogenic H7N8 AIVs were previously reported in Midwestern USA [3–15]. The phylogeny of these viruses was analyzed and the results showed that the lineages in AIVS H7N8 in MIdwest USA and Central China are different (Fig. 1).

Table 1 Percentage identity of influenza viruses closely related to A/duck/Hubei/HF5/2017(H7N8) virus

Gene	Virus	Nucleotide identity (%)
PB2	A/hooded crane/Korea/1176/2016(H1N1)	98.9
PB1	A/hooded crane/Korea/1176/2016(H1N1)	99.0
PA	A/duck/Mongolia/520/2015(H1N1)	99.3
	A/hooded crane/Korea/1176/2016(H1N1)	99.2
HA	A/duck/Bangladesh/27042/2015(H7N9)	97.7
NP	A/Duck/Dongting/D76-1/2016(H5N7)	99.4
	A/duck/Bangladesh/31227/2016(H6N2)	99.4
NA	A/duck/Hunan/S1256/2012(H3N8)	99.4
M	A/duck/Bangladesh/31227/2016(H6N2)	99.6
NS	A/Pigeon/Longquan/LQ67/2016(H2N8)	99.5

PB basic polymerase, *PA* acidic polymerase, *HA* hemagglutinin, *NP* nucleoprotein, *NA* neuraminidase, *M* matrix, *NS* nonstructural

To determine the pathogenicity of DK/HF5 virus in chickens, ten 6-week-old specific-pathogen-free chickens were inoculated intravenously with 0.1 mL of a 1/10 dilution of the fresh infectious allantoic fluid (HA titer > 16), and ten chickens were inoculated with 0.01 M PBS as a control group. All of the chickens were examined daily over a 10-day period as described previously [16]. The pathogenicity index of the DK/HF5 was zero. The results show that this virus was of low pathogenicity to chickens, which is consistent with the results of molecular characteristics.

To investigate the replication and virulence of the virus in a mammalian host, fourteen 6-week-old BALB/c mice were inoculated intranasally with 10^6 EID_{50} of DK/HF5 virus. On 3, 4, and 5 days post-inoculation (dpi), three mice were euthanized to examine the virus activity in the organs, including nasal turbinate, lungs, spleen, brain, and liver; the other five mice were observed for a total of 14 days for body weight changes and death. On day 3, 4, and 5 post-inoculation, high titers of viruses were detected in the nasal turbinate, lungs, spleen, and liver but were not detected in the brain (Table S2). The body weights of mock mice gradually increased from 1 to 14 dpi. In contrast, there was slight weight loss in the mice infected with HF5 and had survival rate of 100% (5/5) (Fig. S2). These results suggested that the DK/HF5 virus was not

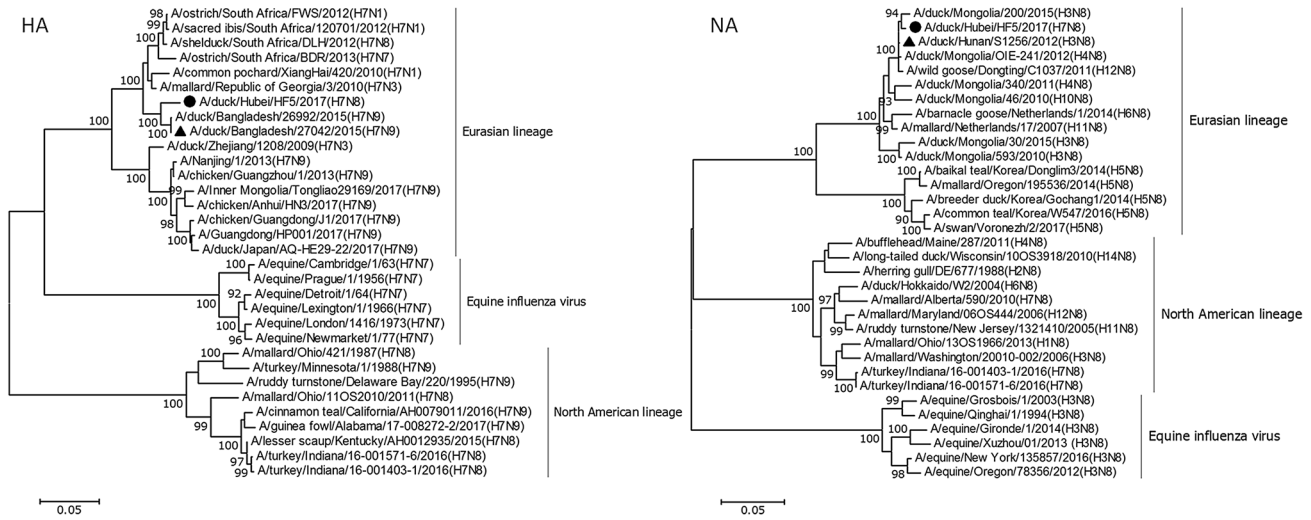

Fig. 1 Phylogenetic trees of hemagglutinin and neuraminidase genes of H7N8 subtype influenza viruses. Trees were constructed with MEGA6.05 software using the neighbor-joining method. Bootstrap analysis was performed with 1000 replications. "Filled circle" is for viruses obtained in this study, and "filled triangle" is for closely related viruses. Scale bars indicate nucleotide substitutions per site

capable of fatally infecting mice, but could effectively replicate in the nasal turbinate, lung, spleen, and liver without preadaptation.

We also performed the phylogenetic analyses of internal genes of these viruses including H7N9 emerged in 2013 and H9N2 endemic in China. The results showed that five internal genes (PB2, PB1, PA, NP, and M) of DK/H7N8 and H7N9 and H9N2 belong to different lineages (Fig. S1A–E). Except for NS genes, they belong to same lineage (Fig. S1F).

In conclusion, a novel H7N8 AIV was isolated from a domestic duck in Central China, in 2017. This novel H7N8 AIV underwent wide reassortment between the viruses circulating in domestic poultry and wild birds in Asia. This reassortant H7N8 virus was found to have low and mild pathogenicity in chickens and mice, respectively. Our results are useful for highlighting the genetic reassortment between viruses of different subtypes and the evolution of AIVs in China and such events may give rise to reassortants with significant pathogenic potential. Our results reinforce the importance of continued surveillance of the H7N8 virus to better understand the ecology and evolution of the AIVs in poultry and wild birds.

Author contributions JW designed the study. HG, LJ, and PC performed the experiments and analyzed the data together with CS and WS. YY and CJ wrote the initial draft of the manuscript, and JW revised the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of China Animal Health and Epidemiology Center (No. 2017-25). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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