

Isolation and genetic characterization of avian influenza virus H4N6 from ducks in China

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Abstract An avian influenza virus (AIV) strain belonging to the H4 subtype and provisionally designated as A/duck/China/J1/2012(H4N6) was isolated from diseased ducks with respiratory disease at a commercial poultry farm in Shandong, China, in 2012. The genomic coding sequences of all eight segments of this J1 isolate were determined and used for subsequent analysis. Phylogenetic analysis of all eight segments showed that this duck H4N6 virus was of Eurasian lineage and not American lineage. The results show that the virus probably emerged because of a reassortment event involving other avian H4N6 and H6N1 viruses. Interestingly, this H4N6 virus had all the conserved features common to low-pathogenic AIVs, including the HA cleavage sequence, receptor-binding sequences for the 2,3-linked sialic acid receptor in avian species, and the PB2 627E motif. These results suggest that the duck H4N6 isolate could not cross the species barrier to

infect and replicate in mammals, including humans. In addition, screening of the duck serum samples showed that only 0.57 % (2/352) of the individuals had weak but measurable hemagglutination inhibition (HI) antibody titers. The low antibody prevalence data were also supported by the failure to detect H4N6 virus (0/56) in clinical nasal swabs of the ducks. These data indicate an alternate reservoir for the H4N6 virus.

Introduction

Avian influenza is an infectious disease caused by influenza A viruses, which mainly infect poultry and affect the systemic and respiratory organs of chickens, turkeys, ducks, and geese [1–3]. Classification of the AIVs is based on hemagglutinin (HA) and neuraminidase (NA) genes, and influenza viruses from poultry have 16 types of HA and 9 types of NA. Various combinations of HA and NA genes have resulted in numerous subtypes. Some subtypes, such as H9N2 and H4N6, are low-pathogenic AIVs (LPAIVs), whereas some subtypes, such as H5N1, are highly-pathogenic AIVs (HPAIVs) to poultry [4, 5].

H4N6-subtype AIVs belong to the LPAIVs group and have been reported previously in southern China [6]. This subtype has also been reported in other countries, including South Korea and Russia [7, 8]. In 1999, an H4N6 strain was isolated from a pig in Canada; genome sequence analysis showed that the HA gene carried a characteristic Q226L mutation, which provided the virus with a higher affinity for the sialic acid α 2,6 galactose receptor, with better replication fitness for swine and human cells [9]. This single mutation was thought to have enabled the virus to cross species barriers to infect and replicate in pigs,

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indicating that avian H4N6 is gradually adapting to mammalian hosts. In the current study, we isolated an H4N6 AIV from diseased domestic ducks with influenza-like illness and examined its genetic and pathogenic properties.

Materials and methods

Viral isolation

Nasal swabs were collected from 90-week-old ducks exhibiting influenza-like illness at a commercial poultry production facility in Shandong, China, in April 2012. Virus isolation was performed by inoculation of 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Institute of Shandong Poultry Science, Jinan, China). Embryonic death occurred 96 h after inoculation. After the allantoic fluid was collected, hemagglutination assays were run with 0.5 % packed chicken red blood cells.

Genome sequencing and analysis

The virus was concentrated from collected allantoic fluids by ultracentrifugation and subsequently purified through a 20 % sucrose cushion by ultracentrifugation. Viral RNA was isolated using a QIAGEN Viral RNA Isolation Kit and converted to cDNA at 45 °C for 50 min using random primers included in a GoScript Reverse Transcription Kit (Promega), and PCR was performed using an LA PCR Amplification Kit (TaKaRa). Primer Premier 6.0 software was used to design sets of H4N6-specific primers according to the whole genome sequence of H4N6 AIV in GenBank [10]. The PCR reaction procedure was as follows: pre-denaturation at 95 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 90 s, annealing at 50 °C–55 °C for 90 s, extension at 72 °C for 90 s; and final extension at 72 °C for 5 min. Reaction mixtures were loaded onto agarose gels for electrophoresis after amplification, and the expected fragment sizes were observed.

The positive products were purified using a MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa). The purified fragments were ligated into T-vector pMD-20 (TaKaRa) and used to transform competent *Escherichia coli* DH5 α cells. Two positive clones were selected for each sample and sequenced in the forward and reverse directions by the HuaDa Biotechnology Company using the Sanger sequencing methodology with M13F/R primers. Nucleotide sequence editing and analysis and deduction of amino acid sequences were performed using the EditSeq program in the DNASTAR package (DNASTAR Inc, USA). Sequences of all eight segments were subsequently submitted to the NCBI database with accession numbers KJ690578–KJ690585.

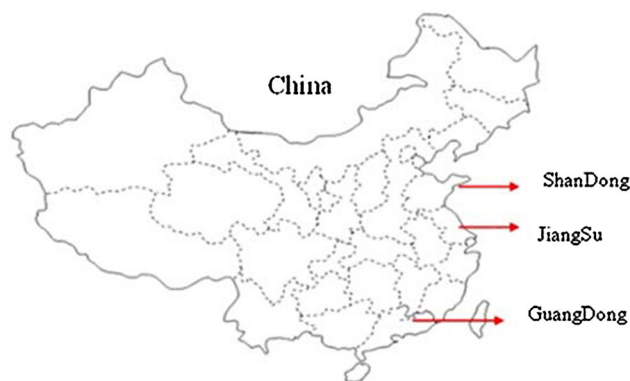


Fig. 1 Locations and numbers of collected swab and serum samples as follows: 185 sera and 35 nasal swabs from ShanDong, 21 sera and 8 nasal swabs from JiangSu and 146 sera and 13 nasal swabs from GuangDong

Serology and clinical surveillance

Duck sera were treated with receptor-destroying enzyme overnight at 37 °C and heat inactivated at 56 °C for 30 min. Hemagglutination inhibition (HI) assays were run with 0.5 % packed chicken red blood cells as described in the WHO's *Manual on Animal Influenza Diagnosis and Surveillance*. A total of 352 duck sera were collected between 2012 and 2013 from 20 poultry farms located in Shandong, Jiangsu, and Guangdong provinces (Fig. 1). To detect the H4N6 genome, 56 nasal swabs derived from some of these farms were analyzed by RT-PCR.

Results

Isolation of H4N6 virus in ducks

In April 2012, nasal swabs from 90-week-old ducks exhibiting influenza-like illness were submitted to the Shandong Poultry Research Institute for viral isolation. RT-PCR analysis showed that the swabs were positive for H4 but negative for other HA subtypes, such as H5 and H9. In 9-day-old SPF chicken embryonic eggs, the viruses caused a lethal infection by 96 h. The allantoic fluids that agglutinated chicken red blood cells were again tested by RT-PCR, and identical results were obtained. The hemagglutination titer of the harvested virus reached 64 after three more passages in eggs. A cross-HI assay using sera prepared against AIVs (H1, H4, H5, and H9), Newcastle disease virus, and avian infectious bronchitis virus confirmed that the virus was H4 subtype AIV.

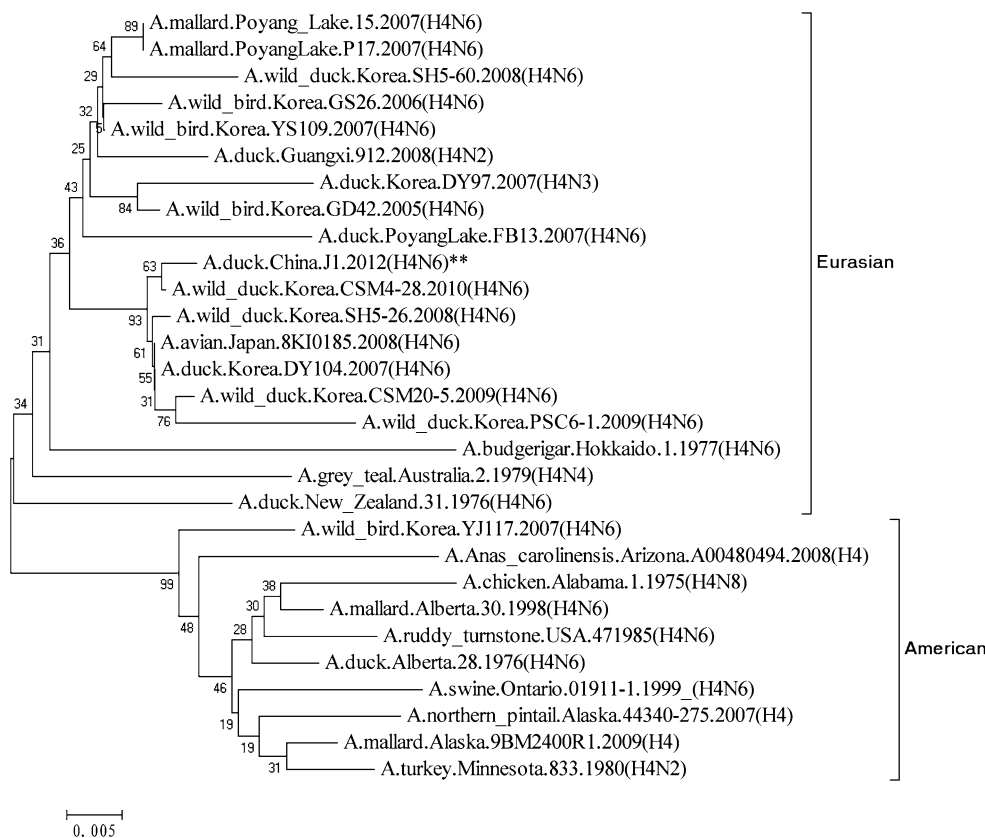
Genome sequencing and phylogenetic analysis

Sanger sequencing was employed to determine the protein-coding sequences of all eight segments. BlastP searches of

Table 1 Sequence homology of each gene from J1 with reference virus sequences from GenBank

Gene (nucleotide positions of J1)	Virus with highest homology	Amino acid sequence identity (%)
PB2(13-2292)	Avian/Japan/8KI0185/2008(H4N6)(CY079219)	99.7 %
PB1(25-2298)	Pintail/Alaska/44500-126/2009(H4N6)(AFM09126)	99.6 %
PA(8-2158)	Avian/Japan/8KI0185/2008(H4N6)(CY079219)	99.1 %
HA(8-1702)	Wild duck/Korea/CSM4-28/2010(H4N6)(JX454690)	99.6 %
NP(37-1497)	Spot-billed duck/Korea/546/2008(H6N1)(GQ414896)	99.8 %
NA(7-1419)	Avian/Japan/8KI0185/2008(H4N6)(CY079219)	98.1 %
M(1-982)	A/teal/Chany/494/2009(H4N6)(KF462353)	99.5 %
NS(15-707)	Avian/Japan/8KI0185/2008(H4N6)(CY079219)	99.9 %

Fig. 2 Phylogenetic tree of HA genes from H4 AIVs. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.0. The robustness of groupings was assessed using bootstrap resampling of 1000 replicate trees. The H4N6 J1 isolate is indicated by **



the individual proteins revealed the highest homology to AIV H4N6 viruses (Table 1), which suggests that the proteins belonged to the H4N6 subtype. Consequently, the virus was provisionally designated as A/duck/China/J1/2012(H4N6). The genomic coding sequences of all eight segments were used for subsequent genetic and phylogenetic analyses.

Phylogenetic analysis indicated that the HA and NA segments of J1 and A/duck/China/J1/2012(H4N6) were clustered in the Eurasian lineage (Fig. 2 and 3). H4N6 strains identified previously in China and some H4N6 AIVs from Japan and Korea were also grouped into the

Eurasian lineage. Phylogenetic analysis of the HA genes of H4N6 strains from the USA indicated that these strains were of American lineage and closely related to A/avian/Korea/YJ117/2007(H4N6) (Fig. 2). The J1 HA gene had the highest amino acid homology (99.6 %) with its counterparts in the wild duck/Korea/CSM4-28/2010(H4N6) strain, whereas the NA, PB2, PB1, PA, M, and NS genes had the highest amino acid homology with those in H4N6 AIVs. Interestingly, the NP gene of J1 virus had highest homology with that of the H6N1 subtype, Spot-billed duck/Korea/546/2008(H6N1), as shown in Table 1.

Fig. 3 Phylogenetic tree of NA genes from H4N6 AIVs. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.0. The robustness of groupings was assessed using bootstrap resampling of 1000 replicate trees. The H4N6 J1 isolate is indicated by **

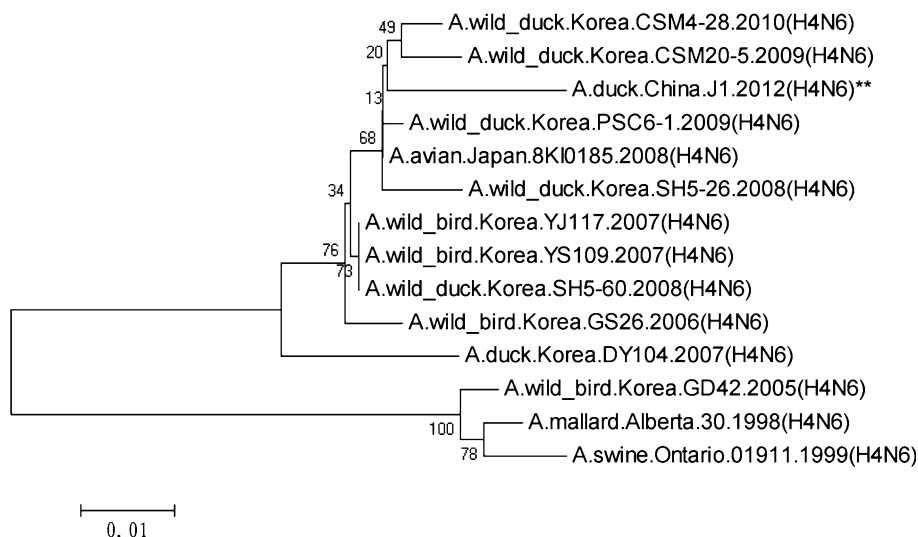


Table 2 Amino acids of receptor-binding sites in the HA gene of H4N6 AIVs

Strain	Receptor-binding sites (amino acid position)							
	98	134–137	153–155	183	190	194–195	226	228
Duck/China/J1	I	AEEF	RAN	N	L	GV	V	P
Mallard/PoyangLake/P17	V	AEEF	RAN	N	L	GV	V	P
Avian/Japan/8KI0185	I	AEEF	RAN	N	L	GV	V	P
Wild duck/Korea/SH5-60	I	AEEF	RAN	N	L	GV	V	P
Duck/Poyang Lake/FB13	V	AEEF	RAN	N	L	GV	V	P
Duck/New Zealand/31	V	AEEF	RAN	N	L	GV	V	P
Duck/Alberta/28	V	AEEF	RAN	N	L	GV	V	P

Genetic characterization

The J1 strain possessed the amino acid sequence PEKASR↓GLF (338–346 aa) at the HA cleavage site, which is specific for avirulent or low-virulent strains of AIV. All reference H4 AIVs studied so far have the cleavage motif PEKAXR↓GLF in the cleavage site. X can be a T, S, or A residue, depending on the different viral isolates.

The J1 strain showed no changes in the receptor-binding sites in the HA protein (Table 2), suggesting that the virus preferentially binds to the 2,3-linked sialic acid receptor in avian species [11, 12]. Nonetheless, this virus strain had deletions in the NA gene stalk region, which is a typical adaptation of influenza viruses to terrestrial poultry. An E92D substitution in the NS1 gene, which is associated with severe pathology in mammals, was also noted in the J1 virus. Moreover, the PB2 627E residue, which is involved in the enhancement of virus replication in mammalian cells, was retained in the virus. In the M2 protein, the J1 virus had V and A residues at positions 27 and 30, respectively. These two mutations rendered influenza A virus to resist M2 protein inhibitor amantadine [13]. However, two characterized mutations (E119V and R292K) that confer resistance to NA protein inhibitors

were not present in J1, indicating that this virus was likely to be susceptible to suppression by NA protein inhibitors.

Prevalence of H4N6 virus in ducks

A total of 352 serum samples collected from 20 poultry farms throughout the eastern region of China (Shandong, Jiangsu, and Guangzhou) were tested by HI assays to detect H4N6 antibodies. Sera with HI antibody titers equal to or greater than 40 were scored as positive. All but two of these sera had undetectable H4N6 HI titers (≤ 10). Two had HI titers of 40. The low titers and number of positive samples (0.57 %) obtained were inconclusive in determining the circulation of H4N6 in domestic ducks. The data were also supported by our RT-PCR results in which 56 duck nasal swabs collected from some of these farms were negative for the H4N6 virus (data not shown).

Discussion

The AIV H4 subtype belongs to the LPAIV group, which can spread among poultry species without causing symptomatic infection. Its replication in poultry can lead to

antigenic drift. Reassortment between H4 and other subtypes can form new variants with increased virulence and transmission capability, which may pose a threat to humans as well as other agricultural animals. Therefore, good understanding and regular surveillance of this group of AIVs are important.

We identified an H4N6 virus from diseased ducks in Shandong, which is a major duck-producing province in China. The duck H4N6 virus investigated in this study is remarkably similar to those reported previously in Korea and other countries in terms of its receptor-binding sites and HA cleavage sequence. This observation is significant because it suggests that the H4N6 subtype is evolving relatively slowly. Detailed analysis of the genome of this H4N6 virus revealed no major mutations that could make it readily adapt to mammals. Therefore, the H4N6 virus we studied and all other reference AIV H4 strains remain likely to be confined to poultry and have not developed the ability to cross species barriers to infect humans and other animals.

Phylogenetic analysis indicated that the HA gene segment of the virus was clustered in the Eurasian lineage. Moreover, the HA, NA, NS, PA, PB1, and PB2 gene segments of our J1 isolate originated from the H4N6 subtype, whereas the NP gene was closely related to Spot-billed duck/Korea/546/2008(H6N1). These observations suggest that J1 (H4N6) possibly emerged from a novel genetic recombination of multiple influenza virus subtypes. Population diversity for improving viral replication fitness and antibody selective pressure are the leading causes of genetic recombination. AIVs mutate easily through antigenic drifts and shifts, indicating that genetic recombination among multiple influenza virus subtypes may be prevalent. In China, co-infections with different AIV subtypes have been reported in the area where the J1 virus was isolated. Similarly, ML/JX/1-4/10(H3N2) and ML/JX/1-19/10(H3N2) were found to be prevalent in the same farm of wild waterfowl from Poyang Lake, where a Korean H4N6 virus was isolated (ML/JX/1-15/10(H4N6)) [14]. Thus, emergence of the J1 strain (H4N6) may be due to the local mode of mixed feeding where chickens and ducks cohabitate.

Serological results show that approximately 0.57 % of individuals were positive for antibodies to H4N6. The failure to detect the H4N6 virus in 56 nasal swab samples seemed to challenge the theory that ducks are the primary host of H4N6 virus. Based on these results, we suspected an alternate reservoir for this virus, which will be investigated in the future.

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