Virus Genes (2014) 48:391–396
DOI 10.1007/s11262-014-1033-4

Sequence and phylogenetic analysis of H2N7 avian influenza viruses isolated from domestic ducks in Zhejiang Province, Eastern China, 2013

Xiaorong Peng · Haibo Wu · Changzhong Jin · Hangping Yao · Xiangyun Lu · Linfang Cheng · Nanping Wu

Received: 25 October 2013 / Accepted: 6 January 2014 / Published online: 18 January 2014

Abstract Two H2N7 avian influenza viruses (AIVs) were isolated from domestic ducks in live poultry markets in Zhejiang Province, Eastern China, 2013. All viruses were characterized by whole-genome sequencing with subsequent phylogenetic analysis and genetic comparison. Phylogenetic analysis of all eight viral genes showed that the viruses clustered in the Eurasian lineage of AIVs and originated from genes reassortment among different viruses co-circulating in domestic ducks in Eastern China. The hemagglutinin cleavage site of all viruses indicated that the two strains were low-pathogenic avian influenza viruses. Considering the important role of the domestic ducks in the dissemination and reassortment of AIVs, continued surveillance of circulating H2 subtype AIVs in domestic ducks in live poultry markets is needed.

Keywords Avian influenza viruses (AIV) · Domestic ducks · Live poultry markets (LPM) · Surveillance · H2N7

Introduction

Avian influenza viruses (AIVs) are members of the *Orthomyxoviridae* family and contain eight segments of single-stranded RNA with negative polarity [1]. AIVs are classified into subtypes based on their envelope proteins, hemagglutinin (HA) and neuraminidase (NA). Currently, 16 HA and 9 NA subtypes were isolated from aquatic birds, such as ducks and geese, which were considered as the natural reservoir of AIVs [2]. Since AIVs have segmented genomes, reassortment is an important mechanism for producing diversity very rapidly [3]. AIVs can be preserved in the wild reservoir in evolutionary stasis for many years [4]. It is recognized that the influenza virus pool in aquatic birds is a never-ending source of genetic and antigenic material [5].

The H2N2 subtype influenza viruses were responsible for the 1957 pandemic, ''Asian Influenza'' [6]. After 1968, influenza viruses of the H2 subtype have not circulated in human population, but were continuously to be detected and isolated from avian population [7, 8]. In mainland China, the information concerning the molecular characteristics of H2 AIVs was limited. There were only eight H2 AIV strains, including H2N9, H2N8, and H2N3 had been isolated from avian species [9]. In 2013, two H2N7 AIVs, A/duck/Zhejiang/465/2013 and A/duck/Zhejiang/468/2013, were isolated from domestic ducks during our surveillance of AIVs in live poultry markets (LPMs) in Zhejiang Province, Eastern China. In order to have a better understanding of the genetic relations between these H2 AIVs from China and from wild birds in Asia, all gene

Electronic supplementary material The online version of this article (doi:10.1007/s11262-014-1033-4) contains supplementary material, which is available to authorized users.

X. Peng · H. Wu · C. Jin · H. Yao · X. Lu · L. Cheng · N. Wu (✉)
State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang, China
e-mail: flwnp2013@163.com

X. Peng
e-mail: 699xiaorong@163.com

X. Peng · H. Wu · C. Jin · H. Yao · X. Lu · L. Cheng · N. Wu
Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, China

segments of the two H2N7 AIVs were sequenced and compared with sequences available in GenBank.

Materials and methods

One hundred and six cloacal swabs were collected from apparently healthy ducks (*Anas platyrhyncha var. domestica*, from commercial farms), in LPMs ($n = 3$) in Hangzhou, Zhejiang Province, in April, 2013. Each sterilized swab sample was inoculated into the allantoic cavities of 9-day-old specific-pathogen-free embryonated eggs as described elsewhere [10]. Viral RNA from hemagglutination positive allantoic fluid was extracted using the Viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription-PCR was performed using Uni12 primer: 5′-AGCAAAAGCAGG-3′ and the One-Step RNA PCR Kit (AMV-optimized Taq) (TaKaRa, China). All the segments were amplified with segment-specific primers as described elsewhere [11]. The appropriate PCR products were sequenced using the Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction kit (ABI, Foster City, CA). The sequences were analyzed using BioEdit version 7.2.0 (DNA analysis software) [12]. The nucleotide sequences were deposited into GenBank under the accession numbers: KF357777–KF357792. Phylogenetic trees were constructed by molecular evolutionary genetics analysis (MEGA) software version 5.2.0, applying the neighbor-joining method and bootstrap analysis (1,000 replicates) [13].

Results

We have isolated 14 strains of AIVs, subtypes H1 ($n = 4$), H2 ($n = 2$), H3 ($n = 4$), and H9 ($n = 4$), in LPMs in Zhejiang Province, Eastern China, in 2013. In this study, all the eight gene segments of the subtype H2 isolates were sequenced. Detailed phylogenetic analysis and molecular characterization of eight gene segments of the H2N7 AIVs were carried out in order to track the evolutionary status of the viruses.

The results of phylogenetic analysis showed that all of the genes (HA, PB2, PB1, PA, NP, NA, M, and NS) of the H2 AIVs were clustered in the Eurasian lineage (Fig. 1; Fig. S2). In 2000, H2N8 and H2N9 viruses were found in domestic ducks from LPMs in Guangdong Province and Nanchang (a city of Jiangxi Province), suggesting a prevalence of H2 subtype of AIVs in China at this period. In 2009, one H2N3 subtype AIV was indentified in environment of Guangdong Province. In 2013, the two H2N7 viruses were isolated from domestic ducks in LBMs in Zhejiang during our surveillance. Detection of the H2 viruses with different NA subtypes in the areas indicated the H2 viruses were still preserved in the domestic ducks and reassorted frequently with other viruses. In the NA (N7) phylogenetic tree, a N7 genetic group was co-circulating in Zhejiang and Guangdong Province. According to phylogenetic analysis of the rest genes, the internal genes belonged to a sub-lineage of the wild bird viral gene pool. Interestingly, the internal genes of AIVs circulating in Zhejiang, such as H7N9 and H7N7, also belonged to this sub-lineage [14].

The whole genomes of two H2N7 AIVs were compared to the nucleotide sequences available in the GenBank database. As they were identical, we chose the isolate A/duck/Zhejiang/465/2013 to represent the H2N7 AIVs. The HA gene shows the highest nucleotide sequence similarity to A/duck/Guangdong/707/2000 (H2N9) at 93 %. The NA gene shows the highest nucleotide sequence identity (96 %) with A/duck/Wenzhou/47/2013 (H7N7). The PB2, PB1, PA, NP, M, and NS gene of this virus has the highest nucleotide sequence similarity to A/duck/Wenzhou/775/2013 (H7N2), A/duck/Wenzhou/771/2013 (H7N3), A/duck/Wenzhou/775/2013 (H7N2), A/duck/Zhejiang/0611-8/2011 (H1N3), A/pigeon/Wenzhou/559/2013 (H7N7), and A/duck/Jiangsu/10-d4/2011 (H11N3), respectively (Table 1). These phylogenetic relationship of the viral genome indicated that the A/duck/Zhejiang/465/2013 (H2N7) strain was a reassortant virus and derived its genes from H2 subtype and H7 subtype AIVs from aquatic birds in Eastern China.

Based on the deduced amino acid sequences of the HA genes of two H2N7 strains (Table 2), the HA cleavage site pattern is monobasic PCIESK/GL indicated a feature of low pathogenic AIVs [15, 16]. Whether these H2N7 viruses were low pathogenic AIVs requires further study. In this study, the amino acids in 220 loop (at positions 220–229) and 130 loop (at positions 134–138) (H3 numbering system) of the two H2N7 AIVs were "RPKVNGQGGR" and "GGSRA", respectively. Q226 and G228 (H3 numbering system) at the receptor-binding sites (RBS) of the H2N7 were similar to all H2 AIVs in both Eurasian and North American lineages [except for A/Japan/305/1957 (H2N2)], suggesting that these viruses would preferentially bind to α-2,3-linked sialic acid receptors, which are predominant in avian species [15]. 192 (R) (H3 numbering system) at the RBS were highly conserved without any variations in H2 AIVs. Interestingly, some of the other amino acids at the RBS of H2N7 AIVs were similar to those of seasonal H2 influenza viruses that circulated in human, such as HA 156 (E), 189 (T),193 (A), and 222 (K) (H3 numbering system) [16] (Table 2). Whether H2N7 viruses with such characteristic amino acids at RBS bear potential threats to infect humans require more studies in future.

The specific polypeptide for *N*-linked glycosylation is defined as Asn-X-Ser/Thr, where X can be any amino

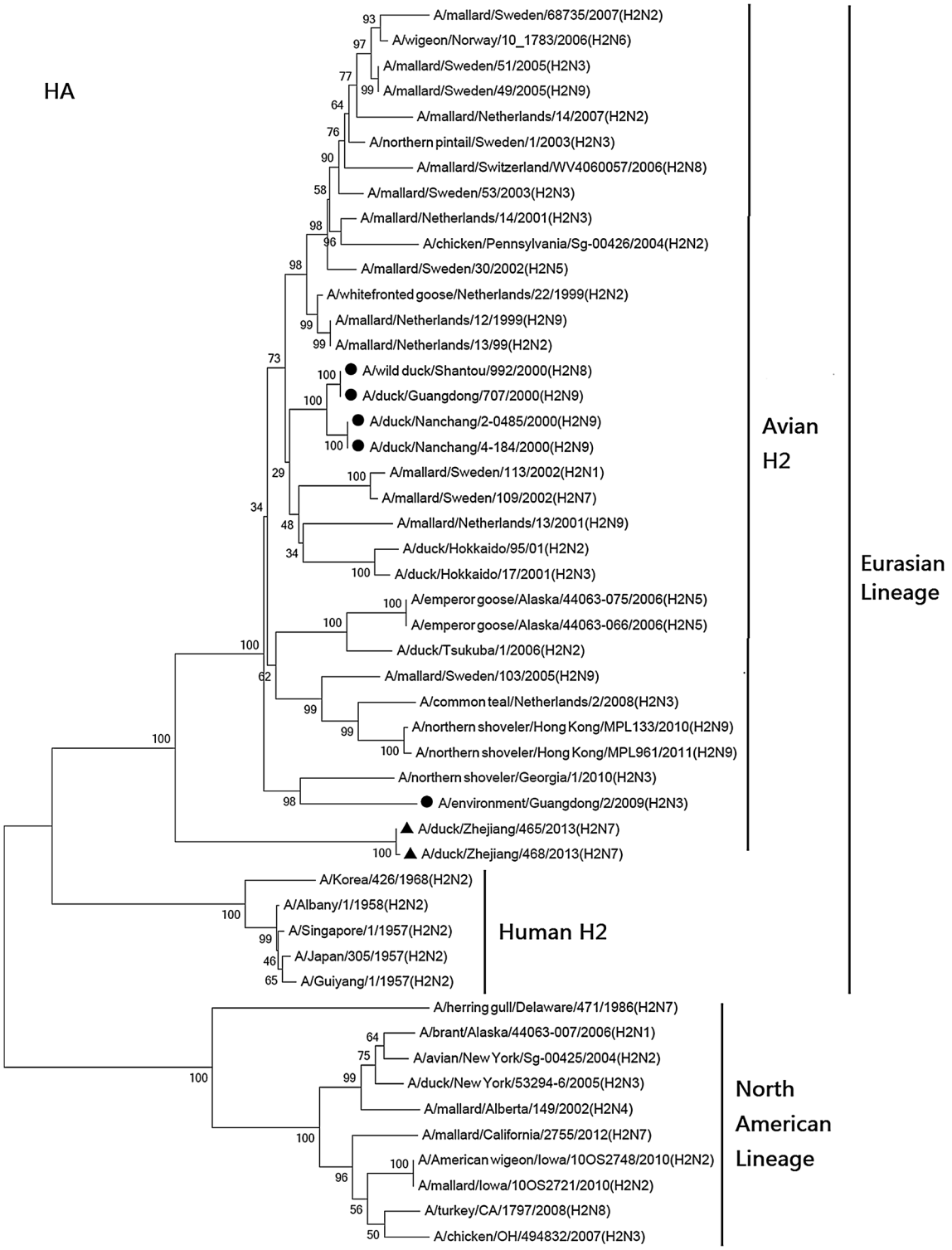

Fig. 1 Phylogenic analysis of HA and NA genes of these H2N7 influenza viruses isolates. The trees were created by the neighbor-joining method and bootstrapped with 1,000 replicates using the MEGA 5.2.0 package. The H2N7 AIVs characterized are *highlighted by a triangle*. The H2 subtype viruses isolated in China mainland are *highlighted by a circle*. The *scale bar* represents the distance unit between sequence pairs

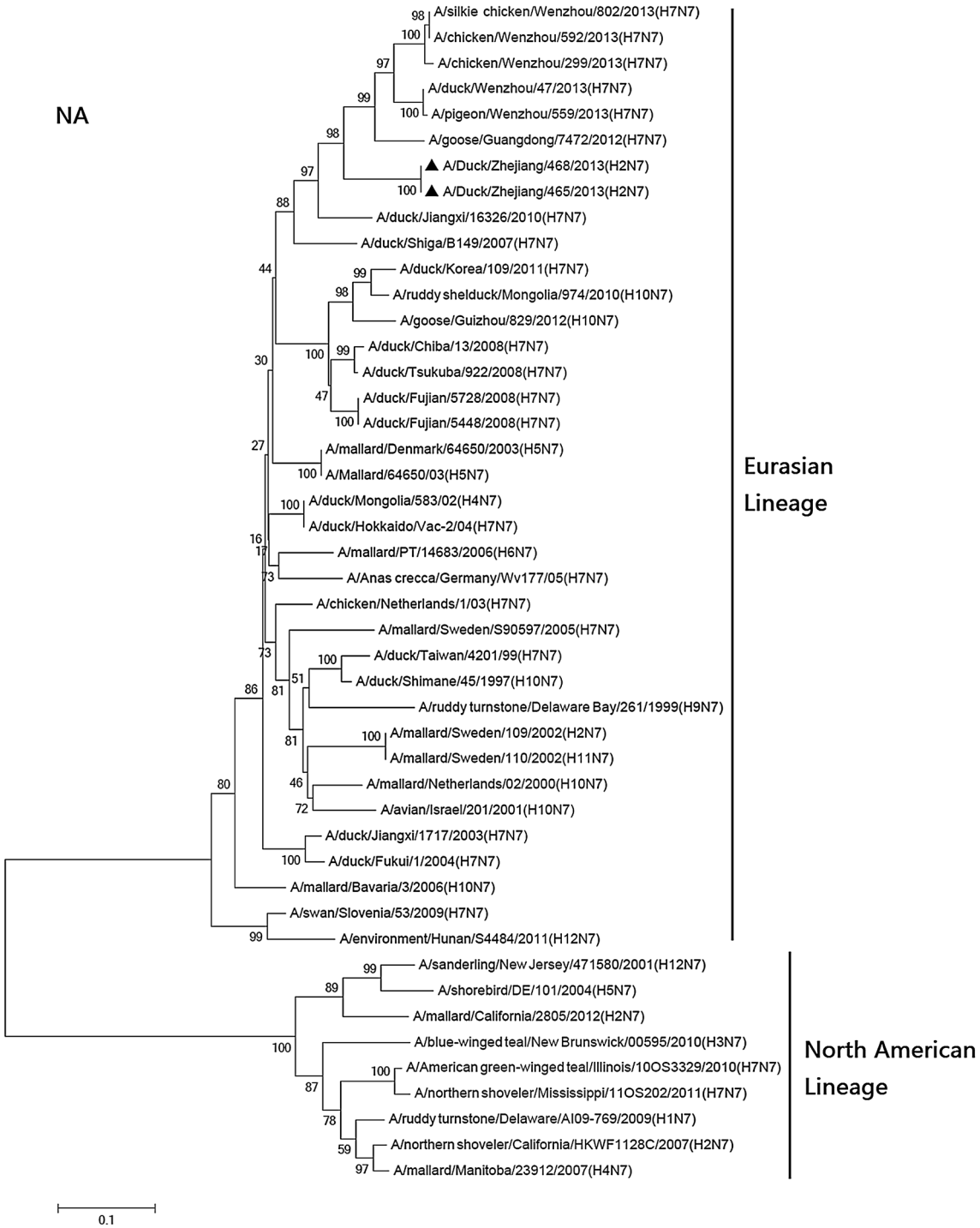

Fig. 1 continued

acid except proline or aspartic acid [17]. In HA, seven potential glycosylation sites, sites 25, 38, 154,179, 300, 494, and 558 were detected in the two H2N7 AIVs (Fig. S1). There were no different potential glycosylation sites between the H2N7 viruses and other H2 viruses.

A PB2 E627K mutation enhances AIVs replication in mammalian cells at lower temperature 33 °C and is considered as one of the important human-adaptation markers for AIVs [18]. This mutation was not observed in PB2 of any of the viruses analyzed in this study, indicating that

Table 1 Sequence homology of whole genome of A/duck/Zhejiang/465/2013(H2N7) compared to nucleotide sequences available in GenBank database

Gene	Position	Virus with the highest percentage of nucleotide identity	Genbank accession number	Homology (%)
PB2	1–2280	A/duck/Wenzhou/775/2013(H7N2)	KF260937	99
PB1	18–2294	A/duck/Wenzhou/771/2013(H7N3)	KF260657	99
PA	1–2090	A/duck/Wenzhou/775/2013(H7N2)	KF260449	99
HA	1–1689	A/duck/Guangdong/707/2000(H2N9)	KF258945	93
NP	1–1497	A/duck/Zhejiang/0611-8/2011(H1N3)	JN605386	99
NA	1–1416	A/duck/Wenzhou/47/2013(H7N7)	KF259671	96
M	1–982	A/pigeon/Wenzhou/559/2013(H7N7)	KF259444	99
NS	1–864	A/duck/Jiangsu/10-d4/2011(H11N3)	CY125011	99

Table 2 Genetic analysis of amino acids at the HA cleavage site and receptor-binding sites of the HA gene of H2N7 strains and reference H2 viruses

	HA cleavage	Receptor-binding site			Left edge of receptor-binding site	Right edge of receptor-binding site
H3 numbering system	320–329	156	192	193	220–229	134–138
H2 position	335–340	166	202	203	230–239	144–148
A/duck/Zhejiang/465/2013(H2N7)	PCIES***K***	***E***	R	***A***	RPKVNGQGGR	GGSRA
A/duck/Zhejiang/468/2013(H2N7)	PCIES***K***	K	R	***A***	RPKVNGQGGR	GGSRA
A/mallard/Sweden/9/2003(H2N7)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/wild duck/Shantou/992/2000(H2N8)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/duck/Nanchang/2-0485/2000(H2N9)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/duck/Nanchang/2-0486/2000(H2N9)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/duck/Guangdong/707/2000(H2N9)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/mallard/Netherlands/11/1999(H2N9)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/mallard/Sweden/53/2003(H2N3)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/chicken/New York/29878/1991(H2N2)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/chicken/Potsdam/4705/1984(H2N2)	PCIESR	K	R	T	RP***E***VNGQGGR	GGS***QG***
A/pintail/Alberta/293/1977(H2N9)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/mallard/New York/6750/1978(H2N2)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/Albany/1/1958(H2N2)	PCIESR	K	R	T	RPKVNG***L***GGR	GGSRA
A/Albany/6/58(H2N2)	PCIESR	K	R	T	RPKVNG***L***G***S***R	GGSRA
A/Cottbus/1/1964(H2N2)	PCIESR	K	R	***A***	RP***E***VNG***L***G***S***R	GGS***K***A
A/Japan/305/1957(H2N2)	PCIESR	K	R	T	RPKVNGQGGR	GGSRA
A/Singapore/1/1957(H2N2)	PCIESR	K	R	T	RPKVNG***L***G***S***R	GGSRA
A/Taiwan/1964(H2N2)	PCIESR	***E***	R	***A***	RPKVNG***L***G***S***R	GGS***M***A

Residues in bold italic indicate changes from the consensus alignment

they had a low level of pathogenicity and adaptation to human beings. The stalk region of NA protein of H2N7 viruses did not undergo the deletions, which is reported to modify replication of H2N2 AIVs in the respiratory tract of chickens [19].

NA inhibitors (oseltamivir and zanamivir) are effective antiviral drugs for the treatment and prophylaxis of influenza infections [20]. The His275Tyr(N1 numbering) mutation is the molecular marker of oseltamivir resistances [21]. Val27Ala and Ser31Asn mutations in the M2 protein are associated with amantadine resistance of influenza virus [22]. These mutations were not observed in the two H2N7 viruses.

Discussion

Previous surveillances revealed that H2 AIV viruses have emerged in China. However, to date, there were only eight H2 AIV isolates isolated from avian species in China, and

the information concerning the molecular characteristics of H2 AIVs was limited. This study reported the first cases of H2N7 subtype AIVs ever reported in poultry from China.

Periodic detections of H2 viruses with three NA subtypes in Eastern China after 2000 suggested that H2 might be maintained in the region for 13 years. Occasional reassortment among H2 viruses and other viruses had generated four different NA AIVs (H2N3, H2N8, H2N9, and H2N7). The internal genes of the H2N7 viruses are similar to H7 subtype (H7N9 and H7N7) AIVs that bear threat to infect human [14]. Some of amino acids at RBS of the two viruses are similar to those of H2 influenza viruses, including the H2N2 viruses that were responsible for "Asian Influenza." Further related studies and continued monitoring of the circulating H2 viruses may help to better understand the evolution of H2 subtype AIVs.

In this study, H1, H2, H3, and H9 subtype AIVs were detected from cloacal swabs of apparently healthy ducks. It suggested that domestic ducks provide an environment for the reassortment of low pathogenic AIVs, which could serve as progenitors of novel AIVs. In addition, LPMs bring together a number of hosts in a high-density setting, providing an ideal environment for viral reassortment and interspecies transfer [23, 24]. Previous studies have shown that LPMs in Hong Kong were closely linked to the infection of human with H5N1 AIVs in 1997 and H9N2 AIVs in 1999 [25, 26]. Recently, human infection with H7N9 AIVs emerged in Eastern China and has been associated with exposure to poultry from wet markets [27]. As LPMs play an important role in the dissemination of AIVs, active surveillance of H2 subtype AIVs in LPMs should be used in an early warning system for H2 evolution in the field.

In conclusion, two H2N7 AIVs were isolated from domestic ducks in LPMs Zhejiang Province, Eastern China, 2013. Whole genome sequences of the two H2N7 AIVs were determined. Phylogenetic analysis of all eight viral genes showed that the viruses clustered in the Eurasian lineage of influenza viruses. Genetic analysis showed that H2N7 AIVs displayed low pathogenic AIVs characteristics.

Acknowledgments This study was supported by the Special Grant for National Key Technologies R&D Programmer for the 12th Five-Year Plan of China (No. 2013ZX10004-904).

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