Original Article

A Novel Reassortant H3N8 Influenza Virus Isolated from Drinking Water for Duck in a Domestic Duck Farm in Poyang Lake Area*

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Abstract

Objective To conduct a full genome sequence analysis for genetic characterization of an H3N8 influenza virus isolated from drinking water of a domestic duck farm in Poyang Lake area in 2011.

Methods The virus was cultivated by specific pathogen free (SPF) chicken embryo eggs and was subtyped into hemagglutinin (HA) and neuraminidase (NA) by real-time PCR method. Eight gene segments were sequenced and phylogenetic analysis was conducted.

Results The NA gene of this virus belongs to North American lineage; other seven genes belong to Eurasian lineage. Compared with the viruses containing NA gene, the PB2 and PB1 gene came from different clades. And this indicates that the virus was a novel reassortant genotype. The HA receptor binding preference was avian-like and the cleavage site sequence showed a low pathogenic feature. There was no drug resistance mutation of M2 protein. The mutations of Asn30Asp, and Thr215Ala of the M1 protein implied the potential of pathogenicity increase in mice.

Conclusion The finding of novel genotype of H3N8 virus in drinking water in this duck farm near Poyang Lake highlighted the importance of strengthening the surveillance of avian influenza in this region, which could contribute to pinpointing the influenza ecological relations among avian, swine, and human.

Key words: Avian influenza; H3N8; Sequence analysis

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INTRODUCTION

Wild aquatic birds are regarded as the natural hosts of influenza A viruses. 16 subtypes of HA and 9 subtypes of NA have been identified from them[1-2]. The similar biological characters of domestic and wild ducks make them to be possibly infected with the same kinds of influenza viruses[3]. Duck has been playing important roles in the transmission of avian

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influenza virus (AIV) from aquatic bird to terrestrial poultry[4]. Surveillance in North American, Europe and Japan showed the findings of subtypes H1-H16 in wild ducks and frequently detected subtypes H3, H4 and H6[1,5-7]. There were at least 9 HA subtypes and 6 NA subtypes reported in domestic ducks, including H1, H3-H6, and H8-H11 subtypes in eastern China[8].

H3N8 virus is a common subtype of virus detected in wild and domestic ducks[9,6], and it has been obtained high attention because of its species transmission from avian to mammals[10]. An equine influenza outbreak caused by H3N8 virus was firstly reported in1963 in Miami[11-12]. Since then, horse infected avian H3N8 virus was reported in China[13], and dog infected equine H3N8 virus was reported in Florida[14]. Recently a notable outbreak in seals caused by H3N8 virus was reported, with the result of 162 seals death[15]. And more importantly, a duck-origin H3N8 virus was supposed to be the donor of HA and PB1 gene segments that caused Hong Kong H3N2 avian influenza pandemic in 1968[16-17].

Jiangxi Poyang Lake is located in the East Asian-Australian migratory birds' flyway and it is the stopover and winter site for migratory birds[18]. Every year, a large number of migratory birds congregate in this location and share the same water source with the grazing poultry, which can promote the transmission of avian influenza virus between wild birds and poultry[3]. Therefore, it is very important to launch the AIV surveillance work in this lake area. And the data from the surveillance from 2002 to 2007 showed that the most common HA subtypes isolated from migratory birds were H4, H6, H10, H3; and H3, H4, H10, H11 were the most common HA subtypes in domestic ducks[19].

In March 2011, a study was initiated to survey the avian influenza virus at Poyang Lake. In this study, we isolated an H3N8 avian influenza virus (AIV) from drinking water for ducks in a duck farm. Results from the previous sequence analysis of the H3N8 viruses isolated from ducks in China showed a multiple reassortant from different origins[20-21], and it is therefore important for us to understand the relationship between this virus and other H3N8 viruses isolated in China and in other parts of the world. Results from the sequence and phylogenetic analysis showed that this H3N8 virus isolated in our study is a novel reassortant genotype virus emerged in Poyang Lake region.

MATERIALS AND METHODS

Virus Isolation

One H3N8 influenza virus was isolated from drinking water for duck in a domestic duck farm in Nanchang County and the initial samples were inoculated in 9-day-old specific pathogen free (SPF) embryonated chicken eggs at 35 °C for 48 h[22] (each egg for 0.2 mL initial sample). Allantoic fluids were collected and the influenza virus was identified by hemagglutination (HA) assay using 1% turkey and equine erythrocyte suspension[22]. The HA positive sample was further subtyped by real-time PCR method using specific primers and probes of HA and NA. The virus was named as A/Environment/ Poyang/521/2011(H3N8) abbreviated as Poyang -521.

RNA Extraction and RT-PCR

Viral RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany), and reverse transcription PCR (RT-PCR) was conducted by One-step RT-PCR Kit (QIAGEN, Germany), in accordance with the manufacturer's instructions. Primers and probes used for HA and NA subtyping and eight gene segments amplification were synthesized by TaKaRa Biotechnology (Dalian) company, and sequences for these primers and probes were as same as described in the influenza virus resource at NCBI[23].

Gene Sequencing

PCR products were purified with a QIA quick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's instructions. The purified PCR products were sequenced by the Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI) and analyzed with Sanger's method ABI 3730 DNA sequence (ABI3730XL; Applied Biosystems, Carlsbad, CA, USA).

Phylogenetic Analysis

The BALST software from NCBI website was used for the nucleic acid and amino acid homology analysis. The multiple sequences were aligned by Muscle program and phylogenetic tree was conducted with neighbor-joining method using MEGA5 software, and the bootstrap value was tested 1000 replications for each gene segment. Different clades of each gene were based on average 1.5% gene distance, and the genotype was constructed based on the results from the clade analysis.

RESULTS

Homology Comparison

The genome of Poyang-521 consists of eight segments, including PB2, PB1, PA, HA, NP, NA, M, and NS (Table 1). The lengths of the segments are 2341, 2341, 2233, 1701, 1559, 1413, 1027, and 890 nucleotides, respectively. The eight genes encode proteins with the following amino acid lengths: PB2, 759; PB1, 757; PA, 716; HA, 566; NP, 498; NA, 470; M1, 252; M2, 97; NS1, 230; and NS2, 121.

BLAST analysis revealed that the HA gene of Poyang-521 had 94% nucleotide identity with that of A/duck/Siberia/100/2001(H3N8). The highest amino acid similar virus was A/mallard/Oregon/44221-105/2006(H3N6), with the homology of 98%. The highest related virus of NA gene of Poyang-521 was A/swan/Shimane/42/1999(H7N8), with a nucleotide identity of 94% and amino acids similarity of 97%. The nucleotide homologies of internal genes were closely related to other influenza viruses subtypes isolated from Eurasian ducks or wild birds. The amino acids homologies were close to the other subtypes with similarity up to 99% and above (Table 1).

Phylogenetic Analysis

Results from phylogenetic analysis showed that all eight segments could be divided into either North American or Eurasian lineages, except for the NA gene of A/Environment/Poyang/521/2011 (H3N8) located in North American lineage and other genes were belong to the Eurasian lineage.

Different genes belonging to different lineages were further divided into different clades based on gene evolution distance. The HA gene and PA gene of Eurasian lineage fell into four clades, namely clade A, B, C, D, The NA gene of North American lineage was further divided into clade A, B, and C. The PB2, PB1, and NP genes of Eurasian lineage were divided into clade A, B, and C. The M gene of Eurasian lineage fell into clade A and B. NS gene, including allele A and allele B, could be divided into Eurasian and North American lineage [Figure 1, Figure S1 (see the website of this journal) and Figure 2A]. Poyang-521 was located in clade B of HA, clade A of NA, clade A of PB2, clade B of PB1, clade A of PA, clade C of NP, clade B of M, the allele A Eurasian lineage of NS gene (Figure 2B).

In order to compare with the viruses containing NA gene, which belongs to North American lineage, we selected five representative viruses as following: A/chicken/Vietnam/G14/2008 (H3N8), A/duck/Vietnam/G119/2006 (H3N8), A/mallard/Sanjiang/90/2006 (H3N8), A/avian/Japan/8K10162/2008 (H3N8) and A/duck/Nanchang/1681/1992 (H3N8). The PB2 and PB1 genes of Poyang-521 differed from the other representative viruses (Figure 2B). The results indicated that the H3N8 virus presented in this study is a novel reassortant between North American and Eurasian lineages, with the different internal gene segments origin.

Table 1. Comparisons of A/Environment/Poyang/521/2011(H3N8) with Isolates in GenBank of Highest Nucleotide and Amino Acid Identity (%)

Gene	Sequence Length	Nucleotide Acid Sequence Isolate with the Highest Homology▲	Homology (%)	Amino Acid Sequence Isolate with the Highest Homology▲	Homology (%)
HA	1765	A/duck/Siberia/100/2001 (H3N8)	94	A/mallard/Oregon/44221-105/2006 (H3N6)	98
NA	1432	A/swan/Shimane/42/1999 (H7N8)	94	A/swan/Shimane/42/1999 (H7N8)	97
M	1027	A/duck/Eastern China/166/2004 (H4N6)	99	A/duck/Korea/S17/03 (H6N1)*	100
				A/mallard/New York/6750/1978 (H2N2)#	100
NS	890	A/duck/Nanchang/1904/1992 (H7N1)	97	A/northern shoveler/California/44363-062/2007 (H9N2)&	99
				A/Quail/Arkansas/16309-7/94 (H7N3)$	99
NP	1559	A/duck/Shimane/188/1999 (H1N1)	99	A/swine/Fujian/1/2003 (H5N1)	99
PA	2233	A/duck/Shiga/8/2004 (H4N6)	99	A/whooper swan/Mongolia/1-23/2007 (H3N8)	99
PB1	2341	A/aquatic bird/Korea/CN9/2009 (H6N8)	99	A/mallard/Netherlands/22/2007 (H7N1)	99
PB2	2341	A/environment/Mongolia/1-33/2007 (H4N6)	99	A/duck/Hokkaido/Vac-3/2007 (H5N1)	100

Note. *Amino acid sequence of M1 protein was compared. #Amino acid sequence of M2 protein was compared. $Amino acid sequence of NS2 protein was compared. &Amino acid sequence of NS1 protein was compared. ▲the listed virus is one of the highest homology viruses.

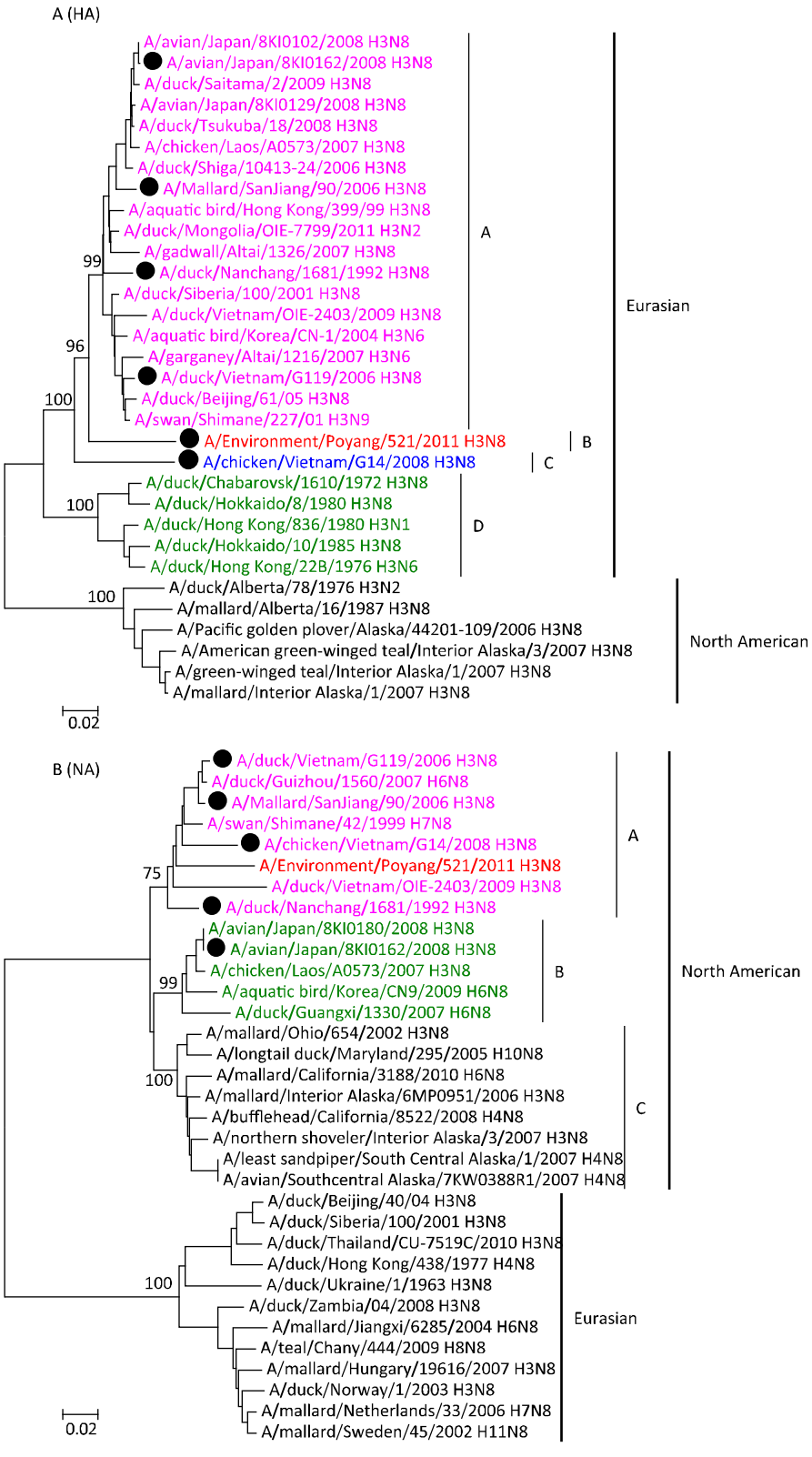

Figure 1. Phylogenetic tree of HA (A) and NA (B) of the H3N8 influenza viruses. Phylogenetic tree was conducted with neighbor-joining method using MEGA5 software, and the bootstrap value was tested by 1000 replications. The Poyang-521 virus was marked with red.

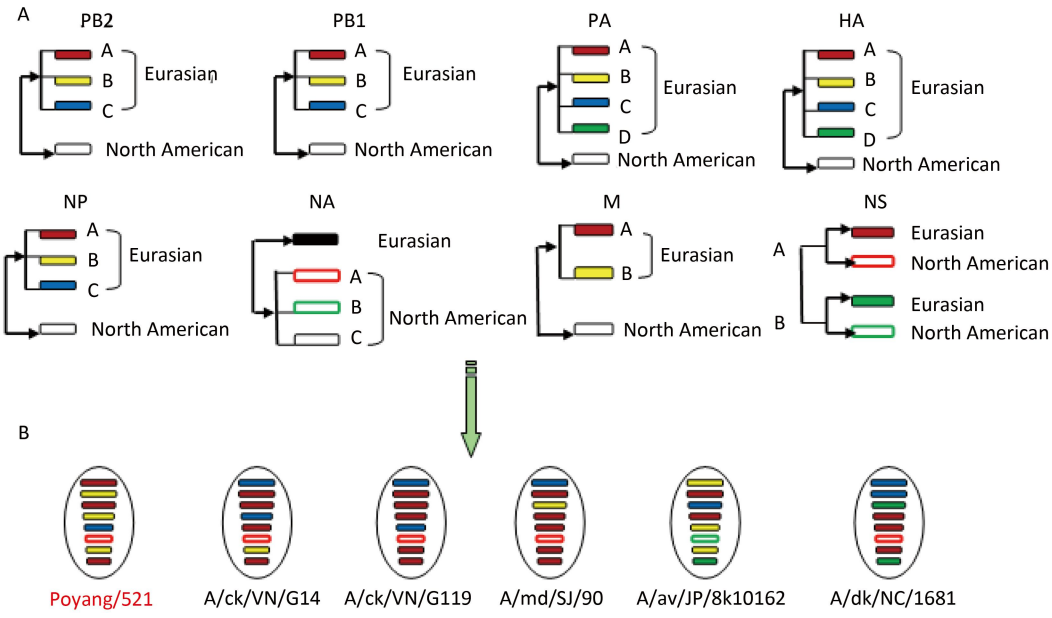

Figure 2. The clades designation diagram of the 8 gene segments is based on the results from the phylogenetic analysis of H3N8 viruses. Phylogenetic tree was conducted with neighbor-joining method using MEGA5 software, and the bootstrap value was tested by 1000 replications. The solid rectangle is Eurasian lineage and the hollow rectangle indicated North American lineage. The different clades were marked in different colors: red rectangle is clade A, yellow one is clade B, blue one is clade C, and green one is clade D, (A). The eight gene segments (Horizontal bar starting at the top downward) were PB2, PB1, PA, HA, NP, NA, M, and NS, (B) respectively. Abbreviations: A/Ck/VN/G14(A/chicken/Vietnam/G14/2008 H3N8), A/dk/VN/G119 (A/duck/Vietnam/G119/2006 H3N8), A/md/SJ/90 (A/mallard/Sanjiang/90/2006 H3N8), A/av/JP/8K10162 (A/avian/Japan/8K10162/2008 H3N8), A/dk/NC/1681 (A/duck/Nanchang/1681/1992 H3N8).

Molecular Analysis

The amino acid sequence at cleavage site in HA protein is PEKQTR/GL, with characteristic of low pathogenic avian influenza virus[24] as other H3 subtypes. Six potential N-glycosylation sites (PGS) of HA proteins were sited at positions 6, 22, 38, 165, 285, 483 respectively, with five PGS which were similar with the H3N8 viruses isolated in Beijing 2005[20]. The main positions of the HA receptor binding sites were 98(Y), 134-138(GGSGA), 183(H), 190(E), 194(L), 224-228(RGQSG). The amino acids of 226Q and 228G indicated that Poyang-521 receptor binding sites are still avian-like[25].

No deletion was detected in NA proteins, and it contained six potential glycosylation sites at amino acid positions 46, 54, 84 144, 293, 398 respectively.

There was mammalian-adapted mutation at positions E627K and D701N of the PB2 protein[26-27]. There were mutations at positions Asn30Asp, and Thr215Ala of the M1 protein, which could increase the virulence of avian influenza in mice[28]. There was no gene substitution in adamantine resistance positions 26, 27, 30, 31, 34 of M2 protein[29], indicating that the virus may be sensitive to adamantine drugs.

DISCUSSION

The reassortant among different influenza viruses was considered as the main mechanism for the emergence of novel virus, which could lead to the influenza pandemic. H3N8 subtype viruses could infect avian and mammalian hosts. The avian origin H3N8 virus caused thousands of horses suffering from the infection and died in China in 1989 and 1990[13], and lead to 162 seals death recently in New England[15]. In addition, H3N8 viruses have been continually detected in ducks in China[20].

The whole genome sequence of Poyang-521, the H3N8 influenza virus which was isolated in this study showed that it is still avian-like virus but with a novel genotype.

The phylogenic tree of the NA gene of Poyang-521 was located in Asian sub-lineage of North American lineage. The transmission pattern of this Asian sub-lineage mixing into South Eastern Asian influenza viruses is still unclear. It may have occurred by poultry trade or wild birds migration.

The lineage including a mixture of viruses from poultry and wild birds indicated that viruses had been exchanged from one to another. It is therefore significantly important to monitor the ecology of AIV in migratory birds at this Poyang Lake region.

Still, viruses isolated previously in Vietnam and Japan showed a high similarity of internal gene with Poyang-521. This indicated that frequent exchange of influenza virus occurred among South-Eastern Asian countries, which might be due to, as we mentioned above, wild birds migration, or international poultry trade.

We strongly believe that the extensive reassortant of H3 subtype posed a threat to avian and human health. The ecological background and the poultry farming and trade provided a crucial and suitable condition for the virus reassortant. It is therefore important to strengthen the AIV surveillance work in different hosts such as avian, swine, equine and in humans.

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