Characterization of H10-H12 subtypes avian influenza virus isolated from wild birds in Shanghai, China, 2016 to 2019

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July 1, 2020

Abstract

The H10-H12 subtypes are designated as the "waterfowl-associated" subtypes and are not frequently detected in nature, but these viruses can highly reassort with other subtypes of AIVs. It has shown that very few H10-H12 subtypes were isolated from wild birds in China, it is essential to conduct the extensive surveillance of these rare subtypes in wild birds to narrow this knowledge gap in this region. In this study, 12 AIVs of H10-H12 subtypes were identified with the routine surveillance in wild birds in Shanghai, China from 2016 to 2019. There were 2 H10 subtypes, 3 H11 subtypes and 7 H12 subtypes, and the HA-NA combinations were H10N4, H11N2, H11Nx, H11N9, H12N2, H12N5 and H12N8. Sequence and phylogenetic analysis showed these gene segments of the 12 strains had high levels of genetic diversity among them, and most of them were closely related to the Eurasian lineage, and shared high sequence identity with those isolated from wild birds and domestic ducks in Japan, Korea, Bangladesh, Vietnam and China located at the East Asian-Australasian Flyway route. However, part of the gene segments of the two H12N2 strains (NH112319-H12N2 and NH101807-H12N2) were belonged to the North American lineage, which indicated that the gene flow and reassortment had occured between the Eurasian and American lineages in H12 subtypes. To better understand the ecological and phylodynamic features of these H10-H12 subtypes in wild birds, it is necessary to continuously conduct large-scale surveillance of wild birds in future.

Keywords

Avian influenza virus, H10-H12 subtypes, phylogenetic analysis, surveillance, wild birds

Introduction

Avian influenza viruses (AIVs) in birds can be divided into H1-H16 subtypes and N1-N9 subtypes according to the antigenic characteristics of their two surface proteins, Haemagglutinin (HA) and Neuraminidase (NA) (Webster et al., 1992). Wild birds are considered to be the natural hosts of a variety of AIVs of different subtypes (Kawaoka et al., 1988), especially *Charadriiformes* and *Anseriformes* distributed all over the world (Brown et al., 2007; Guan et al., 2019). Some HA subtypes of AIVs are potentially associated with species susceptibility, such as H5, H7 and H9 subtypes are most endemic in poultry (Lee et al., 2010), while the H4, H11 and H13 subtypes are mostly from shorebirds and gulls (Krauss et al., 2004; Latorre-Margalef et al., 2014), H3 and H6 subtypes are prevalent in waterfowl (Munster et al., 2007).

The H10-H12 subtypes are also usually associated with "waterfowl-associated" subtypes and are detected very infrequently in nature, but as part of the influenza A virus pool, they can highly reassort with other subtypes of AIVs (Wille et al., 2018). It has been reported that the H10 viruses can spillover to humans and mammal animals (Vachieri et al., 2014; Zohari et al., 2014). The H10N4 virus was detected from farmed mink in Sweden in 1984 (Klingeborn et al., 1985), which was the first example of a disease in mammals

caused by the H10 subtype. The H10N4 virus was closely to the circulating avian influenza virus and the migratory waterfowls probably could spread the subtype of virus (Koehler et al., 2008; Wille et al., 2018). Subsequently, H10N7 virus was observed to cause mass deaths among harbor seals in Sweden in 2014 (Zohari et al., 2014). In late 2013, the human infections with a novel reassortant avian influenza A H10N8 virus was identified in Jiangxi, China (Chen et al., 2014). All of these indicate that the H10 subtype viruses could cause disease in a broad host range. Unlike the H10 subtype, there was no evidence demonstrate that the H11 subtype viruses could directly cause the human infection at present, but it could provide gene donors for other subtype viruses, such as the recent human infections with H7N9 virus revealed that H11N9 virus was the NA gene donors for them (Lam et al., 2013). H12N1 was first reported in Canada in 1983 (Velarde et al., 2010) and then H12 subtype viruses were occasionally reported in wild birds (Bui et al., 2015; Wongphatcharachai et al., 2012). According to current research, there was little research has been done on the H12 subtypes (Latorre-Margalef et al., 2014; Wilcox et al., 2011), and their ecology and phylogenetic analysis were largely unknown.

Waterfowls play an important role in transmitting the viruses, since faeces from infected birds were excreted into the water, and the viruses could be further transmitted by migratory birds and even caused infection in other birds and mammals by cross the interspecies barrier (Appel et al., 1991; Huang et al.; Sharp et al., 1997). Shanghai as a city in Yangtze River estuary on China's east coast, is located on the East Asian-Australian Flyway and is an important stopover and wintering site for migratory birds. During our routine surveillance in wild birds in recent years, we found that a small amount of H10-H12 subtype viruses could be detected in this region. In order to expand our understanding of the ecological distribution and evolution of these rare AIV subtypes in China, we studied the characteristic of these H10-H12 subtypes AIVs in wild birds in Shanghai from 2016 to 2019.

Materials and Methods

Sample collection

Under the permission and supervision of the Shanghai wildlife protection and management office, a total of 6,944 throat and anal swab samples of wild birds were collected in the Nanhui Dongtan wetland of Pudong (30°51' to 31deg06' N, 121deg50' to 121deg51' E) and Jiuduansha Natural Reservation Zone (31deg06' to 31deg14' N, 121deg46' to 122deg15' E), Shanghai, China from 2016 to 2019. All wild birds were released after the samples were collected. The swabs were placed in a 5 ml cryopreserved tubes containing 2 ml virus carrier fluid and stored at -80 refrigerator for further analysis.

Virus identification and genome sequencing

All experiments were conducted under biosafety level (BSL)-2 conditions. The swab tubes were swirled, and the supernatants were collected after centrifugation. RNAs were extracted by referring to the manual of MagMAX^{TM} Pathogen RNA/DNA Kit (Applied Biosystems, USA). The extraction process was completed on the Magmax-96 Express instrument (Applied Biosystems). After extraction, positive samples were screened using a real-time reverse transcription PCR system with primers specific for the matrix gene primer and probe set (WHO, 2009) on a 7500 real-time PCR instrument (Applied Biosystems), and then positive samples were transcribed into cDNA using the Uni12 primer (5'-AGC AAA AGC AGG-3') and PrimScript II 1st Strand cDNA Synthesis Kit (Takara, Japan). The subtypes were determined using specific primers for HA and NA (Huang et al., 2013; Kim et al., 2019) and the eight segments of these H10-H12 subtypes were amplified using the universal primers (Hoffmann et al., 2001). The PCR reaction contained 1 μL of cDNA, 1 μL of forward and reverse primers, 12.5 μL of Taq HS Perfect Mix (Takara, Japan) and 10.5 μL Rnase-free water, with a final volume of 25 μL. All sequences were confirmed using a BigDye termination kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 sequence analyzer.

Sequence analysis

The obtained sequences were further spliced and analyzed by software DNAMAN 6.0. The other representative sequences were downloaded from NCBI and GISAID databases. The phylogenetic trees were construct

using the neighbor-joining method, the Kimura 2-parameter model with bootstrap analysis (1,000 replicates) was selected in MEGA version 6.0.

Results

Prevalence of H10-H12 subtypes in wild birds in Shanghai

During 2016-2019, 6,944 samples of throat and anal swabs from health wild birds were collected in Shanghai, China. 11.36% samples were tested positive for AIVs as assessed using qRT-PCR method. Among these positive samples, 12 H10-H12 subtypes were determined, including 2 H10 subtypes, 3 H11 subtypes and 7 H12 subtypes. Of these 12 viruses, one H11 virus was isolated from *Gruiformes* and designed as A/Eurasian coot/Shanghai/PD112440/2016 (H11N9, abbreviated as PD112440-H11N9), while the remaining 11 strains were isolated from *Anseriformes*, which were named as A/common teal/Shanghai/JDS120613/2018 (JDS120613-H10N4), A/mallard/Shanghai/JDS120662/2018 (JDS120662-H10N4), A/common teal/Shanghai/PD112452/2016 (PD112452-H11Nx), A/Eurasian wigeon/Shanghai/NH101834/2017 (NH101834-H11N2), A/common teal/Shanghai/NH101807/2017 (NH101807-H12N2), A/mallard/Shanghai/JDS110851/2017 (JDS110851-H12N5), A/common teal/Shanghai/NH102615/2018 (NH102615-H12N2), A/common teal/Shanghai/NH110165/2018 (NH110165-H12N2), A/common teal/Shanghai/NH112319/2018 (NH112319-H12N2), A/mallard/Shanghai/NH011204/2018 (NH011204-H12N5), and A/common teal/Shanghai/ JDS110203/2019 (JDS110203-H12N8), respectively. The full genomes of these 12 strains were sequenced and these sequences have been deposited in GenBank database, and their accession numbers were indicated in Table 1.

Sequence and phylogenetic analysis of H10 subtypes

Two H10N4 strains (JDS120613-H10N4 and JDS120662-H10N4) were isolated from Common teal(*Anas crecca*)and Mallard (*Anas platyrhynchos*) at Jiuduansha Natural Reservation Zone in 2018. Except for one polymerase acidic (PA) gene, the whole genome sequences of the two strains were obtained, and the sequence homology analysis showed that they shared 92.9% to 99.3% nucleotide sequence identity among the seven gene segments. A BLAST search (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) revealed that the two hemagglutinin (HA) genes of these H10N4 strains were most closely related to that of A/duck/Mongolia/709/2015(H10N7), while their neuraminidase (NA) genes were highly related to those genes in A/duck/Mongolia/258/2011(H8N4) and A/garganey/Bangladesh/38920/2019(H7N4). The matrix (M) gene of JDS120662-H10N4 had the most similarity with H6N2 virus isolated in Hubei, and the other internal genes of the two H10N4 strains shared most (>98.36%) similarities with H10, H7, H11, H8, H12 and H3 subtypes isolated in Hokkaido, Mongolia, Egypt, Bangladesh, Georgia and Tottori (Table 2).

In the phylogenetic trees, the HA and NA genes of JDS120613-H10N4 and JDS120662-H10N4 strains were grouped together and clustered in the Eurasian Lineage. The HA genes showed a close relationship with the H10N7 strains circulating in ducks in Cambodia and Mongolia, and NA genes were associated with H7N4 and H8N4 viruses isolated in garganey and ducks in Bangladesh (Figure 1). PB1 polymerase (PB1) and M genes of the two H10N4 strains were all grouped together in a small sublineage, and were closely related to H3N2, H5N2 and H6N2 viruses circulating in Japan and China. Nucleoprotein(NP)gene of JDS120662-H10N4 was closed to H7N7 and H7N1 viruses isolated from Tottori and South Korea, and the NP gene of JDS120613-H10N4 was clustered with them in a small group. PB2 polymerase (PB2) and nonstructural (NS) genes of the two strains were all clustered in two different groups and were genetically close to those of viruses isolated from ducks circulating in Tomsk, Mongolia, Japan, Bangladesh and China located along the Eurasian-Australian Migration Flyway (Figure S1).

Sequence and phylogenetic analysis of H11 subtypes

In the three strains of the H11 subtype, two 2016 strains (PD112440-H11N9 and PD112452-H11Nx) were isolated from Eurasian coot (*Fulica atra*) and Common teal (*Anas crecca*), respectively, and the other 2017 strain (NH101834-H11N2) was isolated from Eurasian wigeon (*Anas penelope*) at Nanhui Dongtan wetland

of Pudong. Except for the NA gene of PD112452-H11Nx, the whole genome sequences of the three strains were obtained. The two 2016 isolates (PD112440-H11N9 and PD112452-H11Nx) were almost genetically identical and the six gene nucleotide sequences identity were 99.1% to 100%, whereas their PA genes shared 93.7% identity; accordingly, their closest relative gene segments were the same, and showed in Table 2. Except for NA gene, the 2017 isolate NH101834-H11N2 shared a relative low nucleotide identity (91.0%-97.6%) with the two 2016 isolates in other 7 gene segments and had the most similarity with the duck and wild bird viruses circulating in Vietnam, Japan, Korea, South Africa and China (Table 2).

In the phylogenetic tree, HA genes of the three strains were clustered in the Eurasian Lineage, and grouped into 2 sublineages. The two 2016 strains grouped together in a small sublineage, and more closely related to H11N9 virus circulating in ducks in Ibaraki (A/duck/Ibaraki/99/2016). The 2017 isolate NH101834-H11N2 was grouped in another sublineage and showed a close relationship with the H11N3 and H11N9 strains circulating in ducks and mandarin ducks in Bangladesh and South Korea (Figure 2). Phylogenetic analyses of the NA genes showed that 2016 strain PD112440-H11N9 was closely related to H11N9 virus circulating in ducks in Japan, and 2017 strain NH101834-H11N2 showed a close relationship with the H6N2 and H5N2 viruses circulating in wild ducks in Korea and China (Figure 2). Phylogenetic relationships among the six internal genes of the three strains revealed the topologic structure were similar to the HA gene tree, that is the two 2016 strains closely clustered together in the same sublineage, and the 2017 strain clustered into another sister sublineage. They were all genetically closed to those of viruses isolated from domestic ducks or wild birds circulating in Japan, Korea, Bangladesh, Mongolia, Vietnam, Cambodia and China (Figure S2).

Sequence and phylogenetic analysis of H12 subtypes

To better understand the evolutionary relationship of these H12 subtypes influenza viruses with other influenza viruses, we performed phylogenetic analysis. Of the seven H12 subtypes, four were of the H12N2 subtype, two were of the H12N5 subtype and one was of the H12N8 subtype. Five strains were isolated from Common teal (*Anas crecca*) and the other two strains were from Mallard (*Anas platyrhynchos*) at Jiuduansha Natural Reservation Zone and Nanhui Dongtan wetland of Pudong during 2017-2019. The whole genome sequences of these H12 strains were sequenced and analyzed. The homological analysis showed that they shared a low similarity in nucleotide sequence identity among the eight gene segments, such as 77.9% to 99.9% for the HA gene, 51.0% to 98.8% for the NA gene, 83.7% to 96.9% for PB2 gene, 87.9% to 99.0% for PB1 gene, 92.1% to 99.6% for PA gene, 92.1% to 98.9% for NP gene and 95.0% to 98.7% for M gene, 70.0% to 99.7% for the NS gene, indicated that these H12 subtypes were highly divergent. BLAST (https://www.ncbi.nlm.nih.gov/blast/) search showed that the HA, PB2 and PB1 genes of NH112319-H12N2, and PB1 gene of NH101807-H12N2 shared the highest sequence identity with A/Mallard/Alaska/AH0029066S.1.A/2016 (H12N5) and A/mallard/California/3070/2012 (H11N2) viruses, respectively, which were circulating in North American sublineage. The remaining of other gene segments of these H12 subtypes shared >97% identity with those AIVs were from wild birds or ducks along the East Asian-Australasian Flyway route, such as in Japan, Korea, Mongolia, Bangladesh, Vietnam, Netherlands and China (Table 2).

Phylogenetic analysis of HA genes showed that the seven HA genes of these H12 subtypes belonged to two sublineages: NH112319-H12N2 was clustered into the North American lineage, while the other six strains clustered into the Eurasian lineages (Figure 3). Two H12N2 strains (NH102615-H12N2 and NH110165-H12N2) were highly similarity and closely clustered together, and grouped with another H12N2 virus (NH101807-H12N2) and other duck viruses from Japan into a small sublineage, and then with two H12N5 strains formed a sister sublineage. The H12N8 strain (JDS110203-H12N8) shared high homology with A/mallard/Novosibirsk region/964k/2018 (H12N5) and formed another clade in the Eurasian lineages.

Phylogenetic analysis showed that NA gene of these H12 strains were all clustered into the Eurasian lineage. Likewise, two H12N2 strains of NH102615-H12N2 and NH110165-H12N2 were closely clustered together, and then grouped with the other two H12N2 strains and other Southeast Asian viruses, such as Vietnamese and Indian, in two small clades (Figure 3). The N5 and N8 genes of these H12 subtypes grouped with H6N5,

H12N5 and H3N8 viruses circulating in Mongolia, China and Japan, respectively (Figure 3).

Phylogenetic analysis of the internal genes showed that PB2 and PB1 genes of NH112319-H12N2, and PB1 gene of NH101807-H12N2 were clustered into the North American lineage, and in contrast the other internal genes all belonged to the Eurasian lineage and most of them formed a relatively unique clade with other strains isolated form migratory waterfowls, domestic aquatic birds and chickens in Japan, Korea, Mongolia, Bangladesh, Vietnam, and China (Figure S3). The results indicated that two H12N2 strains (NH112319-H12N2 and NH101807-H12N2) were generated through genetic reassortment between viruses belonging to the Eurasian and North American virus lineage.

Discussion

Wild birds are the natural hosts of AIVs (Globig et al., 2009), and recently with the improvement to people's ecological awareness, more and more data have been obtained in the monitoring of avian influenza in wild birds. It has shown that the H10-H12 subtypes could be isolated almost every year worldwide and the number of these strains began to increase gradually since 2000 (Figure 4a). Up to Apr 20, 2020, about 3000 H10-H12 HA sequences were available in GenBank database, and more than 80% of them were from wild birds (Figure 4b). However, compared with the total numbers, relatively few H10-H12 subtypes were isolated from wild birds in China. There are about 50 H10-H12 subtype viruses were detected from wild birds, and the majority of them were from Jiangxi and Hong Kong. Especially for H12 subtype, only 1 strain could be found in the database in China.

Shanghai is one of the most important overwintering and stopover site for wild birds on the East Asian-Australian Migration route. Every year, millions of migratory birds and hundreds of species pass through Shanghai. Nanhui Dongtan wetland of Pudong and Jiuduansha Natural Reservation Zone were two important wetlands for waterfowls in Shanghai. Given the importance of H10-H12 subtypes in the influenza viral ecosystem, it is essential that extensive surveillance of these rare subtypes in wild birds in this area should be implemented to narrow this knowledge gap.

It has shown that H11N9 virus could be transmitted directly from ducks to humans (Gill et al., 2006), and the recombination events could occur between H11 and H7 subtypes, such as the H11 subtype can provide internal gene fragments for the highly pathogenic H7 subtype (Shi et al., 2013). H10 subtype is frequency isolated from wild birds and domestic poultry. The NS1 gene of H10 AIVs mutates, leading to site substitution, which increases virulence and pathogenicity of the virus to mammals (El-Shesheny et al., 2018). The adaptation of virus to the host usually involves the reassortment of gene fragments of co-infected virus strains adaptive mutations in various virus genes (Ince et al., 2013). The H10 was reported to be associated with all possible NA subtypes, which contributed to the diversity of the HA lineage (Wille et al., 2018). In this study, we isolated 2 H10N4, 1 H11N9, 1 H11N2 and 1 H11Nx strains during 2016-2019 surveillance. The HA-NA recombination of H10 and H11 subtypes showed in this manuscript might be frequently detected in nature in wild birds. Interestingly, we identified 7 H12 subtype strains in this study, including 3 NA combinations: H12N2, H12N5 and H12N8. The H12 subtypes were relatively less than the other two subtypes and they had might an NA bias for N2, not for N5 (Wille et al., 2018). Among these positive samples, one strain was isolated from *Gruiformes* in 2016, while the remaining eleven strains were isolated from*Anseriformes* , so *Anseriformes* should play an important role in the maintenance of these rare subtypes (Wille et al., 2018).

Phylogenetic analysis of these H10-H12 strains showed that a frequent occurrence of reassortment could be identified among these subtypes. For example, two H10N4 strains were recovered from the same site in Jiuduansha Natural Reservation Zone in 2018, but parts of their internal gene segments were belonged to the different sublineages (Figure S1), suggesting that these two H10N4 viruses might share a different ancestor. The similar evolution patterns also could be found in H12N2 and H12N5 strains (Figure S3), indicative of frequent reassortment occurred among these subtypes in eastern China. Most of the eight gene segments of these 12 strains were clustered into the Eurasian lineage, and they shared high sequence identity with those isolated from wild birds and domestic ducks in Japan, Korea, Bangladesh, Vietnam and China where belong

to the East Asian-Australasian Flyway route. In addition, wild bird populations had been shown to be the main source of new reassortment, rather than poultry (Lu et al., 2014). Several evidences have showed that intercontinental transfer of highly pathogenic avian influenza viruses by migratory birds could be frequently occurred (Koehler et al., 2008), such as H5N8 viruses, but it is rare for low pathogenic avian influenza viruses via wild birds (Krauss et al., 2007; Winker et al., 2007). In this study, the intercontinental reassortment of avian influenza viruses between Eurasian and North American could be observed in two H12N2 strains (NH112319-H12N2 and NH101807-H12N2) (Figure S3), thus the gene flow between these sites were existed. In the same way, the study of AIV gene segments showed in different regions and recombination between different viral lineages, greatly increasing our understanding of the ecology of virus in this fragile and pristine environment (Hurt et al., 2016; Olsen et al., 2006). It is therefore wild birds play an essential role in the transmission of the influenza viruses.

In this study, multiple strains of H10-H12 subtypes were isolated from wild birds in Shanghai, the genetic analysis results of these strains indicated that avian influenza viruses in wild birds were diverse which may pose a threat to public health due to the frequent reassortment between wild birds and poultry. Shanghai plays an important role in the ecology of avian influenza virus in China and even the whole world, so monitoring and studying the rare subtypes in this region is of great help to improve our understanding of these virus subtypes. In future, more avian influenza virus surveillance in the migratory flyways should be strengthened, and which might provide a timely and effective method for understand the infection situation of AIVs and the avian influenza virus ecology in world.

ACKNOWLEDGEMENTS

This work was funded by the Shanghai Committee of Science and Technology (grant No. 18DZ2293800), the Shanghai Wildlife-borne Infectious Disease Monitoring Program and Yangtze Delta Estuarine Wetland Ecosystem Observation and Research Station, Ministry of Education & Shanghai.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this article.

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Table 1. Virus information and GenBank serial number

Virus name	Abbreviation	Subtype	Accession numbers in GenBank
A/common teal/Shanghai/JDS120613/2018(H10N4)	JDS120613-H10N4	H10N4	MN049531-MN049537
A/mallard/Shanghai/JDS120662/2018(H10N4)	JDS120662-H10N4	H10N4	MN049523-MN049530
A/Eurasian coot/Shanghai/PD112440/2016(H11N9)	PD112440-H11N9	H11N9	MN049550-MN059557
A/common teal/Shanghai/PD112452/2016(H11Nx)	PD112452-H11Nx	H11Nx	MN044998-MN045004
A/Eurasian wigeon/Shanghai/NH101834/2017(H11N2)	NH101834-H11N2	H11N2	MN044910-MN044917

Virus name	Abbreviation	Subtype	Accession numbers in GenBank
A/common teal/Shanghai/NH101807/2017(H12N2)	NH101807-H12N2	H12N2	MN049563-MN049569
A/mallard/Shanghai/JDS110851/2017(H12N5)	JDS110851-H12N5	H12N5	MN049575-MN049581
A/common teal/Shanghai/NH102615/2018(H12N2)	NH102615-H12N2	H12N2	MN121558-MN121565
A/common teal/Shanghai/NH110165/2018(H12N2)	NH110165-H12N2	H12N2	MN122300-MN122307
A/common teal/Shanghai/NH112319/2018(H12N2)	NH112319-H12N2	H12N2	MN049593-MN049600
A/mallard/Shanghai/NH011204/2018(H12N5)	NH011204-H12N5	H12N5	MN049584-MN049591
A/common teal/Shanghai/ JDS110203/2019(H12N8)	JDS110203-H12N8	H12N8	MN795764-MN795771

Table 2. Virus of the highest homologs in GenBank database with H10-H12 subtype isolates in this study

Virus name
A/common teal/Shanghai/JDS120613/2018(H10N4)
A/mallard/Shanghai/JDS120662/2018(H10N4)
A/Eurasian coot/Shanghai/PD112440/2016(H11N9) A/common teal/Shanghai/PD112452/2016(H11Nx) A/Eurasian wigeo

Figure Legends

Figure 1. Phylogenetic trees of the HA and NA genes of the H10 subtype strains isolated in Shanghai, China.

The neighbor-joining tree was constructed using the Kimura 2-parameter model in MEGA software version 6 (http://www.megasoft ware net/). Bootstrap values were calculated for 1000 replicates, and the values less than 75% were not shown. Numbers indicate neighbor-joining bootstrap values. Virus characterized in this study were indicated by black circles.

Figure 2. Phylogenetic trees of the HA and NA genes of the H11 subtype strains isolated in Shanghai, China.

The analysis methods were the same as Figure 1.

Figure 3. Phylogenetic trees of the HA and NA genes of the H12 subtype strains isolated in Shanghai, China.

The analysis methods were the same as Figure 1.

Figure 4. Comparison of the number of H10-H12 subtypes in China and the world. (a) The number of H10-H12 subtypes isolated annually in China and the world since 1975. (b) The number of H10-H12 subtypes isolated in China and the world to date.

Figure S1. Phylogenetic analysis of the inner genes of H10 subtype strains isolated in Shanghai, China (the inner genes: PB2, PB1, PA, NP, M, NS).

The analysis methods were the same as Figure 1.

Figure S2. Phylogenetic analysis of the inner genes of H11 subtype strains isolated in Shanghai, China (the inner genes: PB2, PB1, PA, NP, M, NS).

The analysis methods were the same as Figure 1.

Figure S3. Phylogenetic analysis of the inner genes of H12 subtype strains isolated in Shanghai, China (the inner genes: PB2, PB1, PA, NP, M, NS).

The analysis methods were the same as Figure 1.