Characterization of the low-pathogenic H7N7 avian influenza virus in Shanghai, China

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ABSTRACT H7N7 avian influenza virus (**AIV**) can divided into low-pathogenic AIV and high-pathogenic AIV groups. It has been shown to infect humans and animals. Its prevalence state in wild birds in China remains largely unclear. In this study, a new strain of H7N7 AIV, designated CM1216, isolated from wild birds in Shanghai, China, was characterized. Phylogenetic and nucleotide sequence analyses of CM1216 revealed that HA, NA, PB1, NP, and M genes shared the highest nucleotide identity with the Japan H7 subtype AIV circulated in 2019; the PB2 and PA genes shared the highest nucleotide identity with the Korea H7 subtype AIV circulated in wild birds in 2018, while NS gene of CM1216 was 98.93% identical to that of the duck AIV circulating in Bangladesh, and they all belong to the Eurasian lineage. A Bayesian phylogenetic reconstruction of the 2 surface genes of CM1216 showed that multiple reassortments might have occurred in 2015. Mutations were found in HA (A135 T, T136S, and T160 A [H3 numbering]), M1 (N30D and T215 A), NS1 (P42S and D97 E), PB2 (R389 K), and PA (N383D) proteins; these mutations have been shown to be related to mammalian adaptation and changes in virulence of AIVs. Infection studies demonstrated that CM1216 could infect mice and cause symptoms characteristic of influenza virus infection and proliferate in the lungs without prior adaption. This study demonstrates the need for routine surveillance of AIVs in wild birds and detection of their evolution to become a virus with high pathogenicity and ability to infect humans.

Key words: H7N7, avian influenza virus, phylogenetics, pathogenicity, mouse

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INTRODUCTION

Avian influenza viruses (**AIV**) have a segmented, single-stranded, negative-sense RNA genome. Wild birds are their natural hosts. Sixteen hemagglutinin (**HA**) and 9 neuraminidase (**NA**) subtypes of AIVs have been found, and strains of almost all possible combinations of HA and NA subtypes have been detected (Yoon et al., 1992; Fouchier et al., 2005). AIVs are divided into low-pathogenic avian influenza virus (**LPAIV**) and high-pathogenic avian influenza virus (**HPAIV**) groups. Only H5 and H7 subtypes have been associated with outbreaks of HPAIVs (Swayne, 2012). Some LPAIVs have the potential to evolve into HPAIVs through a series of mutations and recombination (Alexander, 2000). The H7 subtype of AIVs has caused outbreaks in poultry for decades and posed a great threat to humans (Belser et al., 2009; Swayne, 2012). The H7N9 AIV was first found to cause fatal infections in humans in 2013 in Eastern China (Gao et al., 2013; Zeng et al., 2013). Analysis of the origin and source of this H7N9 AIV revealed that it was derived from wild birds and had infected domestic ducks, leading to the 2013 H7N9 AIV outbreak in China (Lam et al., 2013). In the same year, another strain of H7N9 AIV was isolated from an apparently healthy tree sparrow in Shanghai. The entire gene composition of this virus was found to be similar to that of the isolates from humans (Zhao et al., 2014). These observations warrant the need to monitor H7 subtype influenza virus in wild birds.

The H7N7 AIV was first detected in chickens in Italy in 1902 (Horimoto and Kawaoka, 2001) and has been widely found in wild birds and domestic poultry around the world (Campitelli et al., 2008; Smietanka et al., 2011). In 2003, one case of fatal acute respiratory distress syndrome was reported in a patient with H7N7 AIV infection during an outbreak in the Netherlands (Fouchier et al., 2004). In this outbreak, 86 individuals involved in the culling operation of farm animals and 3

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of their family members who did not have direct contact with infected poultry were confirmed to be infected with this H7N7 AIV, suggesting that limited human-to-human transmission had occurred (Koopmans et al., 2004). Subsequently, another H7N7 AIV strain was found in poultry farms in several European countries. A highly pathogenic H7N7 AIV strain was also detected in poultry farms in the UK in 2008. Phylogenetic analysis showed that this HPAIV H7N7 was derived from an LPAIV H7N7 (Seekings et al., 2018).

In China, H7N7 AIV was first detected in a live poultry market in Zhejiang Province in 2013. It was shown that this AIV could infect and cause severe pneumonia in ferrets, indicating its ability to cross the interspecies barrier. In addition, 2 H7N7 AIV strains were detected in migratory birds in China in 2013 (Liu et al., 2018). Several genes of these 2 H7N7 strains were found to be closely related to those of the circulating H7N7 AIV in domestic poultry, suggesting gene exchanges between migratory birds and poultry. However, the information on H7N7 AIV in wild birds in China is very limited. In the database of Global Initiative on Sharing All Influenza Data, only 14 H7N7 AIVs from wild birds in China are found to date. These AIVs were distributed in Hubei, Hunan, Jiangxi, and Hong Kong from 2009 to 2014. Shanghai is located in the Yangtze River Delta and is an important stopover place for many migratory birds in the East Asia-Australia migratory flyway. To determine the prevalence of H7N7 AIV in Shanghai, we conducted a surveillance in wild birds in Chongming island in 2018. One novel H7N7 AIV with multiple genomic reassortments was isolated from wild birds. The phylogenetics and pathogenicity of this virus were investigated.

MATERIALS AND METHODS

Sampling

From April 2017 to December 2018, 751 tracheal and cloacal swab samples were collected from wild birds in Chongming island, Shanghai, China. Each of these samples was placed in a 5-mL Eppendorf tube containing 2 mL of viral transport media and then stored at −80°C until used. Wild birds were captured and sampled with the permission and supervision of the Shanghai Wild Life Conservation and Management Office.

Virus Identification and Isolation

After centrifugation of the tube containing the swab, the supernatant was collected. Virus RNAs were extracted from each supernatant using the MagMAX Pathogen RNA/DNA Kit (Applied Biosystems, Waltham, MA) according to manufacturer's protocol. AIVs were identified by real-time reverse-transcription PCR with the matrix (**M**) gene primer and probe set (WHO, 2002). Subtypes of AIVs were determined by nucleotide sequencing using universal primers described in the WHO (2002). To propagate the virus, selected AIV-positive samples were inoculated into 9- to 10-day-old specific pathogen-free chicken embryos. The inoculated chicken embryos were incubated for 72 h at 37°C under humid conditions and then chilled at 4°C for 6 to 8 h. The allantoic fluids from inoculated chicken embryos were checked for successful AIV growth using the HA test with 1% chicken red blood cells as previously described (WHO, 2002). HA-positive allantoic fluids were collected and stored at −80°C until used.

RT-PCR and Genome Sequencing

Viral RNAs were extracted from allantoic fluids using an RNeasy Mini kit (Qiagen, Hilden, Germany) and transcribed into cDNAs using the Uni12 primer (5′-AGC AAA AGC AGG-3′) and PrimScript II first Strand cDNA Synthesis Kit (Takara, Japan). The 8 segments of the H7 subtype AIV genomic RNA were amplified using universal primers. Each PCR reaction contained 1 μL of cDNA, 1 μL each of forward and reverse primers, 12.5 μL of Taq HS Perfect Mix (Takara, Mountain View, CA), and 10.5 μL of Rnase-free water in a final volume of 25 μL. PCR products were sequenced using a BigDye termination kit (Applied Biosystems, Foster City, CA) on an ABI 3730 sequence analyzer.

Sequence Analysis and Phylogenetic Trees

Nucleotide sequences were analyzed and aligned using the DNAMAN program (version 6.0). Phylogenetic trees were generated using the neighbor-joining algorithm and the 2-parameter model with bootstrap analysis (1,000 replicates) in the MEGA-X (https://www.megasoftware.net/) software. Other sequences used for phylogenetic analysis were obtained from GenBank and the Global Initiative on Sharing All Influenza Data EpiFlu database.

Phylodynamic Analysis

According to published workflow of Bayesian evolution analysis (Wei et al., 2017), the evolution rates of the 2 surface genes were analyzed. TempEst (version 1.5.3; http://tree.bio.ed.ac.uk/) was used to conduct a regression analysis of genetic distance and sampling dates in this study. The Bayesian Evolutionary Analysis Sampling Trees (**BEAST**) v1.10.4 was used to estimate the time of the most recent common ancestor (**tMRCA**) and nucleotide substitution rates. We used the Hasegawa-Kishino-Yano nucleotide substitution model with a gamma-distributed among-site rate variation and an uncorrelated relaxed clock. All chains were run in 50,000,000 generations, and the effective sample size (**ESS**) values in the results were greater than 200. Moreover, Tracer v1.6 (http://tree.bio.ed.ac.uk/) was used to confirm the reliability of the results. All trees were annotated by Tree Annotator with 10% burn-in cutoffs, and the maximum clade credibility of trees was visualized and decorated by FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 1. Homology analyses of CM1216 with isolates in NCBI.

Gene	Segment-ID	Virus	Homology (%)
PB2	MN483236.1	A/White-fronted Goose/South Korea/KNU18-119/2018(H7N7)	99.82
PB1	LC496342.1	A/mallard/Tottori/31 C/2019(H7N7)	99.78
PA	MN602509.1	A/White-fronted Goose/South Korea/KNU18-119/2018(H7N7)	99.76
HA	LC496344.1	A/mallard/Tottori/31 C/2019(H7N7)	99.57
NP	LC496345.1	A/mallard/Tottori/31 C/2019(H7N7)	99.87
NA	LC496346.1	A/mallard/Tottori/31 C/2019(H7N7)	99.63
M	LC496347.1	A/mallard/Tottori/31 C/2019(H7N7)	99.90
NS	MT090541.1	A/duck/Bangladesh/38292/2019(H2N2)	98.93

Animals

A total of 42 six- to eight-week-old specific pathogen-free BALB/c female mice, purchased from Shanghai Jiesijie Experimental Animal Co., Ltd. (Shanghai, China), were used in this study. Mice were provided with food and water ad libitum and kept in a 12-hour light and 12-hour dark cycle.

Infection Studies in Mice

To investigate AIV infection in animals, 26 mice were randomly divided into 2 groups with 20 in the experimental group and 6 in the control group. After being anesthetized with ether, the mice in the experimental group were intranasally inoculated with H7N7 AIV (50 μL of 10^6 EID_{50}/100 μL), and those in the control group were intranasally inoculated with an equal amount of noninfectious allantoic fluid. To evaluate viral replication in the lungs, 3 mice each from the experimental group were euthanized at 3, 5, and 7 d post infection (**d.p.i.**), and their lungs were collected. The weight of each lung was determined, and the lung index was calculated according to the following formula: lung index (%) = total lung mass/the mean control body mass (day 0) × 100%. A portion of each lung was fixed in formalin and processed for histological examinations and immunohistochemistry (**IHC**). Briefly, the lung samples were fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin. An anti-influenza H7N7 (CM1216) antibody against the nucleoprotein (**NP**) was used for IHC. The remaining lung tissues were used for subsequent viral titer determination on Madin-Darby canine kidney cells as described previously (Price et al., 2000). The remaining mice were monitored daily for clinical symptoms, body weight, and survival time for 14 d after infection.

Statistical Analysis

Data are expressed as mean ± SD. Differences between groups were analyzed by one-way ANOVA. The differences between 2 groups were analyzed by the Student t test using SPSS 19 (SPSS Inc., Chicago, IL). Graphs were generated using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA).

Accession Numbers

Nucleotide sequences were deposited in the GenBank with accession numbers MK554564 - MK55571.

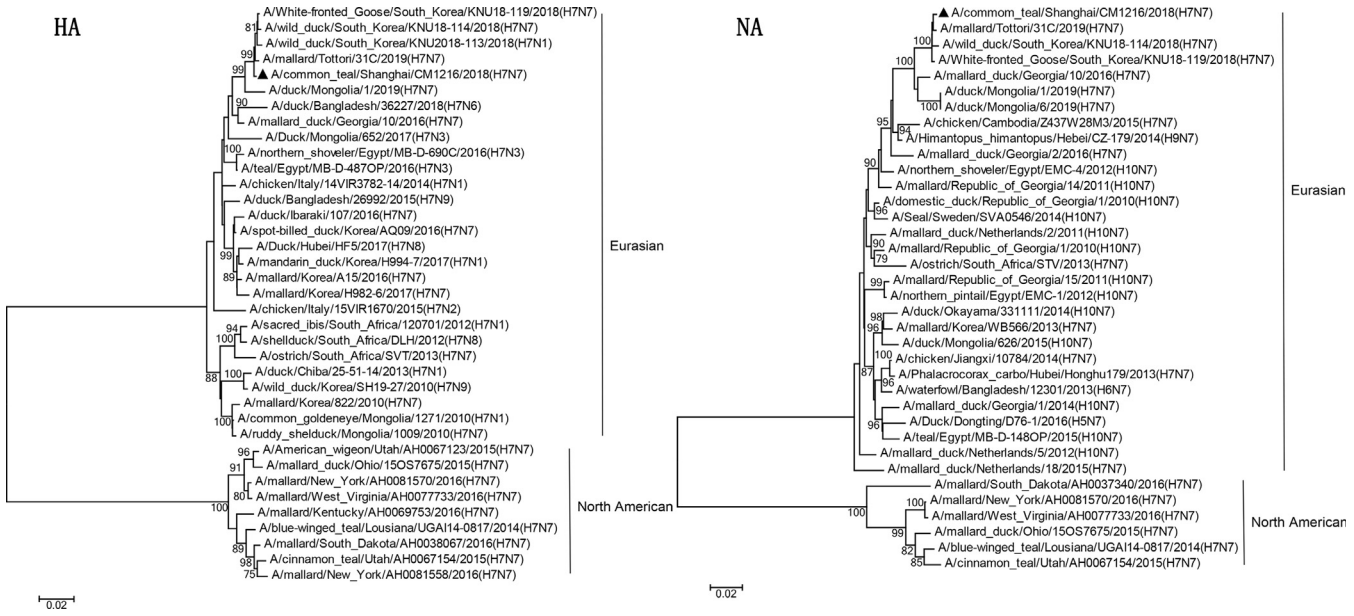

Figure 1. Phylogenetic tree of HA and NA genes of CM1216. The neighbor-joining tree was constructed using the Kimura 2-parameter model in the MEGA-X software (http://www.megasoftware.net/). Bootstrap values were calculated for 1,000 replicates; values less than 75% are not shown. Numbers indicate neighbor-joining bootstrap values. The virus (CM1216) characterized in this study is indicated by a filled triangle, the Korean H7N7 virus is indicated by a filled circle.

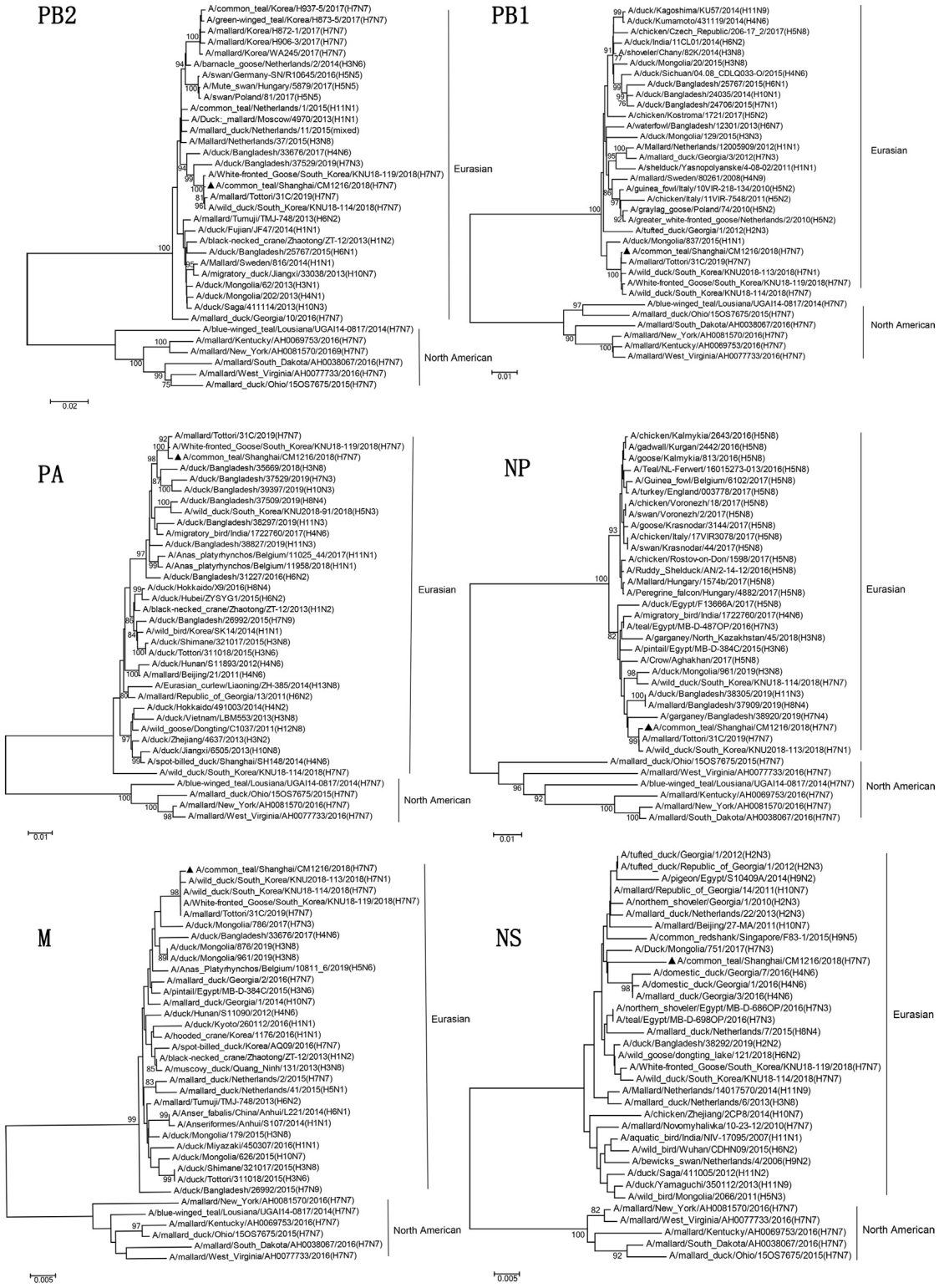

Figure 2. Phylogenetic tree of other 6 internal genes of CM1216.

Biosafety and Animal Handling

All experiments were conducted under biosafety level-2 conditions. Animals were maintained according to the National Institutes of Health guidelines for the Care and Use of Experimental Animals. All experimental protocols were reviewed and approved by the Animal Investigation Committee (no. BRDW-XBS-19-02).

RESULTS

Nucleotide Sequence and Phylogenetic Analyses of CM1216

On December 16, 2018, 2 isolates of H7N7 AIVs were obtained from common teals (*Anas crecca*) in Chongming Island, Shanghai. The nucleotide sequences of these 2

Table 2. Amino acid substitutions in various CM1216 proteins.

Protein	Mutation sites (aa)	Amino acid residue in CM1216	Possible function
HA		PEKLPKGR	HA cleavage site
	Q226 L	Q	Receptor binding shift (RBS) positions (H3 numbering), altered receptor specificity
	G228S	G	Increased affinity for the human α-2, 6 linked sialic acid receptors
	V186 N	G	Human receptor binding preference of H13 subtype
	T160 A	A	The binding affinity to human-like (α2-6-SA) receptor may be increased (Gao et al., 2018)
	A135 T	T	Receptor binding specificity
	T136S	S	
	E190D	E	Human receptor binding preference(H5)
		30GNT, 46NAT, 423NWT, 495NNT, 249NDT	Glycosylation motifs
NA	69-73	No deletion	Stalk
	R292 K	R	Antiviral resistance R292 K (oseltamivir) (Song et al., 2015)
	E119 V	P	Neuraminidase inhibitor resistance mutation (Song et al., 2015)
	R152 W	T	
	H274Y	H	
	D293 N	D	
	E276D	E	
		32NVS, 87NKS, 401NWS, 67NNTT, 145NGT, 200NAT, 234NGT, 47NLT, 56NNTT	Glycosylation motifs
M1	N30D	D	Increased pathogenicity of H5N1 to mice
	T215 A	A	Increased pathogenicity
	V15I	V	Increased pathogenicity
M2	S31 N	S	Adamantine resistance mutation/Antiviral resistance S31 N(amantadine)
NS	218-230	No deletion	
	P42S	S	Increased virulence in mice
	D97 E	E	Increased pathogenicity to mice
	L89 V	Y	Enhanced polymerase activity and increased virulence in mice
PB1	13	P	
	H99Y	H	H5 virus transmission among ferrets
	198	K	
	I368 V	I	H5 virus transmission among ferrets
	R118I	R	
PB2	E627 K	E	Enhanced polymerase activity and increased virulence in mice/Mammalian adaption mutations (Jong et al., 2013; Li et al., 2017)
	T271 A	T	
	D701 N	D	Enhances the replication, pathogenicity, and transmission of the H1N1 virus (Zhou et al., 2013)
	R389 K	K	Mammalian adaption mutations
	T271 A	T	Increased adaption in mammals
	S224P	S	Increased pathogenicity of H5N1 to ducks
PA	N383D	D	Increased pathogenicity of H5N1 to ducks
	V100 A	V	
	K356 R	K	
	S409 N	S	
	L550 M	L	

isolates were found to be identical. The virus was named A/common teal/Shanghai/CM1216/2018, referred to as CM1216 hereafter.

Analyses of genomic sequences revealed that the HA and NA genes of CM1216 were 99.57 and 99.63%, respectively, homologous to the Japan wild bird H7N7 AIV (A/mallard/Tottori/31 C/2019) in 2019. The 3 internal genes, polymerase basic 1, NP, and matrix genes, also shared 99.78 to 99.90% nucleotide identity with the Japan wild bird H7N7 AIV. The polymerase basic 2 (**PB2**) and polymerase acidic (**PA**) genes shared 99.82 and 99.76% nucleotide identity with that of the H7N7 goose AIV circulating in South Korea, while the nonstructural (**NS**) gene of CM1216 was 98.93% identical to that of the duck AIV circulating in Bangladesh (Table 1).

The topologies of CM1216 8 genes on the phylogenetic trees were similar, and they all belonged to the Eurasian lineage (Figures 1 and 2). The HA, NA (Figure 1), PB2, PB1, PA, NS, and M (Figure 2) genes of CM1216 were combined with Japan and Korea H7 subtypes into a small sublineage and then clustered with the different subtype AIVs circulating in poultry and wild birds in Mongolia and Bangladesh, while the NS gene (Figure 2) was separate in a small sublineage, which was close to the Georgia AIVs.

Table 3. Evolution rates and the most recent common ancestors (tMRCA) of HA and NA genes of CM1216 strain.

	Substitution rate (subs/site/year)		tMRCA (calendar year)	
Gene	Mean	95% HPD interval	tMRCA	95% HPD
HA	5.9766E-3	[4.7247E-3, 7.2532E-3]	2015.6257	[2015.2334, 2016.7161]
NA	4.4501E-3	[3.4735E-3, 5.3627E-3]	2015.8924	[2013.8419, 2018.1656]

Abbreviation: HPD 95%, highest posterior density interval (95%).

Molecular Characterization and Genetic Analysis of CM1216

CM1216 was found to possess a monobasic cleavage site (PEKLPKGR) located between HA1 and HA2 genes, implying that it is low pathogenic in chickens (Table 2). The receptor-binding motif of CM1216 was found to be QRG, located at amino acids 226-228 (H3 numbering), suggesting that CM1216 preferentially binds to avian receptors (sialic acid alpha-2,3-galactose, SA α-2, 3 Gal) (Chutinimitkul et al., 2010). The HA protein of CM1216 was found to have the T160 A mutation, which has been shown to increase the binding affinity of AIV to human-like (SA α-2, 6 Gal) receptors (Stevens et al., 2008). Analyses of CM1216 HA amino acid sequence revealed 5 possible glycosylation motifs (30GNT, 46NAT, 423NWT, 495NNT, 249NDT). No amino acid deletions in the NA stalk region (positions 69-73) were observed, and the NA amino acid sequence of CM1216 was found to have 9 potential glycosylation sites (32NVS, 87NKS, 401NWS, 67NNTT, 145NGT, 200NAT, 234NGT, 47NLT, 56NNTT). Mutations involved in drug resistance (NA inhibitors and M2 inhibitors) were not found in CM1216, suggesting that it may be sensitive to NA inhibitors and M2 ion channel blockers. N30D and T215 A substitutions, which have been shown to increase pathogenicity to mammals, in the M1 protein were found (Table 2). The presence of E627 and D701 in PB2 suggests that the isolate was not mammal-adapted AIVs, but the existence of K389 in PB2 is suggestive of mammalian adaption. E97 and S42 residues in the NS gene and the D383 residue in the PA gene were also found; these residues have been shown to be associated with increased pathogenicity of AIV in mice (Song et al., 2015).

Estimation of Rates of Evolution of CM1216 Genes

Phylogenetic and Bayesian coalescent analyses were carried out to estimate the evolution rates and the tMRCAs of the 2 surface gens of CM1216 strain. There

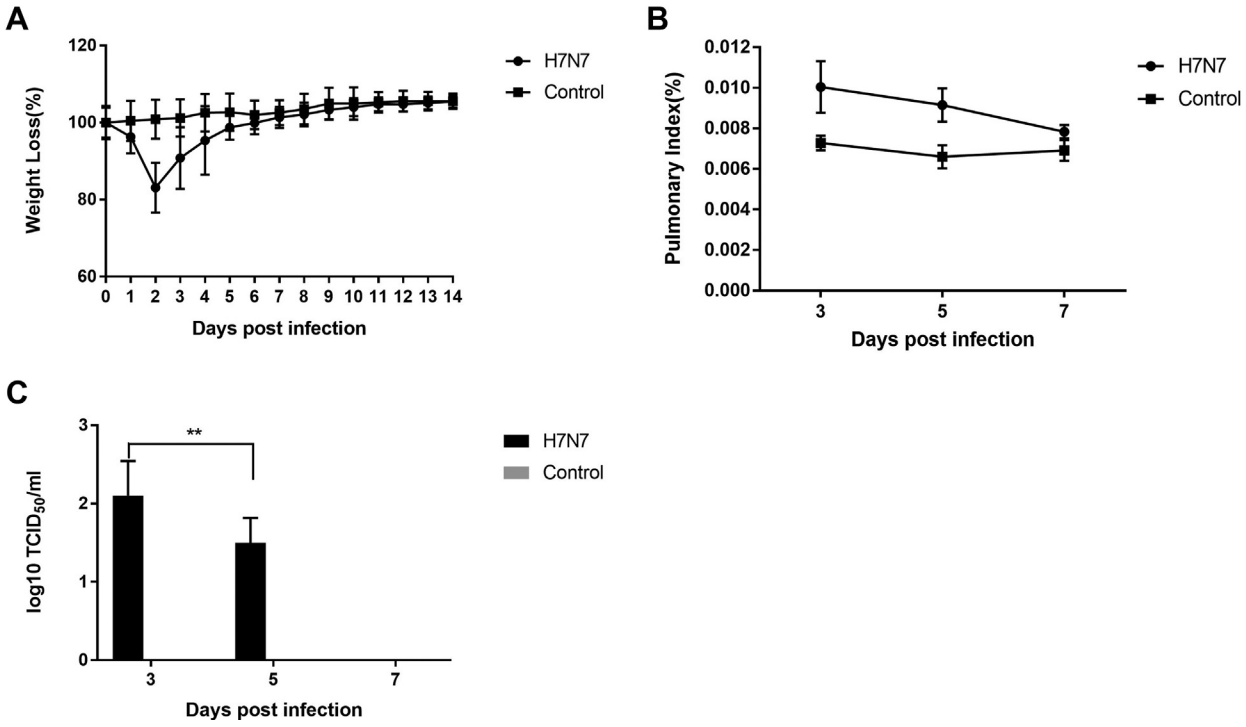

Figure 3. Weight loss, pulmonary index, and virus titer in mice. (A) Weight loss of infected mice (filled circle) and control mice (filled square). (B) Pulmonary index of infected mice (filled circle) and control mice (filled square). (C) The titers of virus replication in lungs at 3, 5, and 7 d.p.i. of infected mice (black bars) and control mice (gray bars). Mice were infected intranasally with H7N7 virus (50 μL of 10^6 EID50/100 μL). Samples were collected at corresponding days after infection, and viral titers were determined by TCID50. $^*P < 0.05$, $^{**}P < 0.01$.

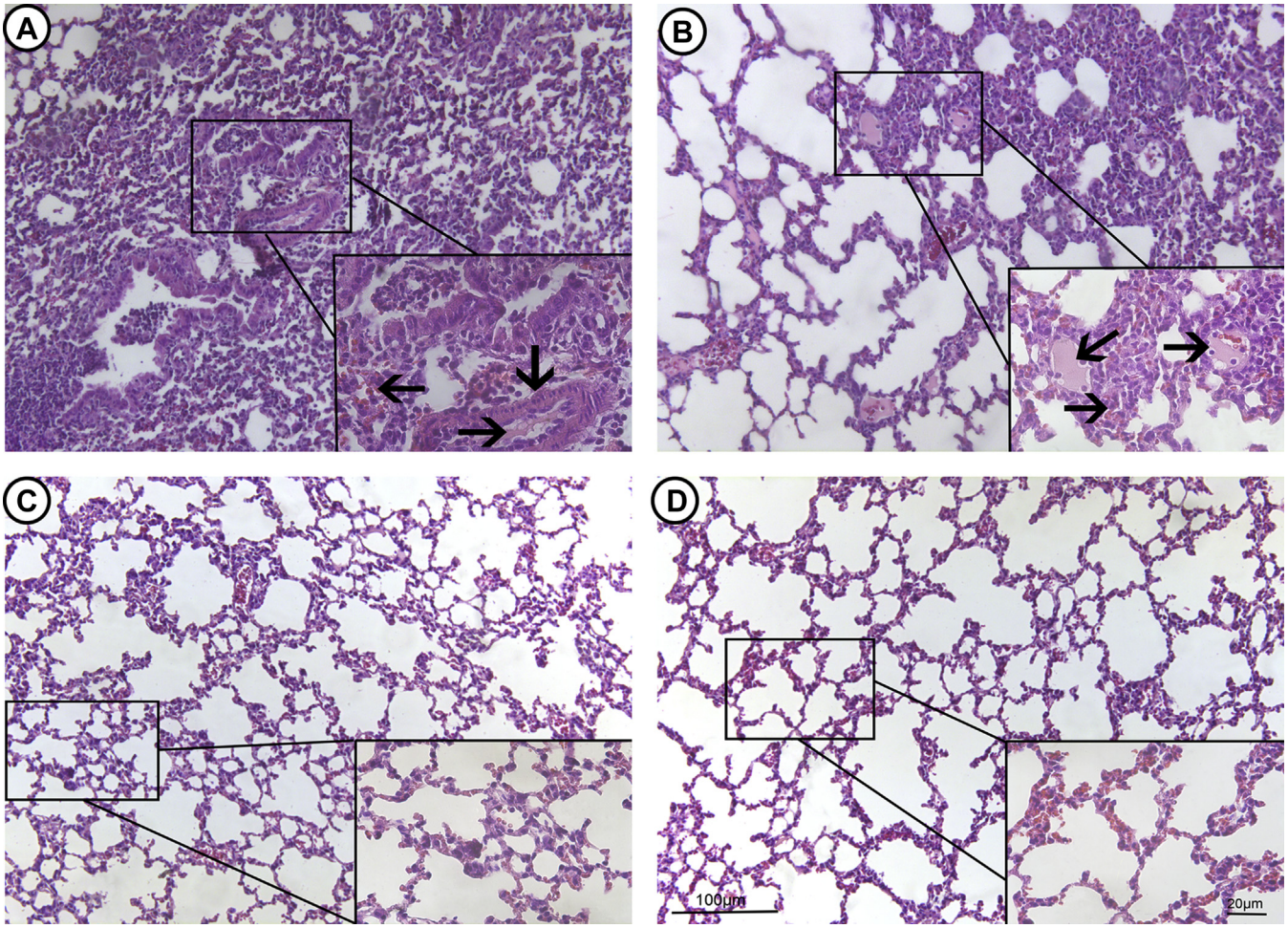

Figure 4. Histopathologic analyses of lung tissue from CM1216 infected mice. Histology of lung sections stained with hematoxylin and eosin (H&E) from inoculated mice at 3 (A), 5 (B), 7 (C) d.p.i., and control mice (D). Black arrows indicate representative areas with infiltration of inflammatory cells, edema, and mild detachment of bronchial epithelial cells.

were strong association between the genetic distance and sampling sites in HA (n = 78; correlation coefficient = 0.9257; R^2 = 0.8568) and NA (n = 97; correlation coefficient = 0.9857; R^2 = 0.97) genes, which indicated that the data sets were available to perform the phylogenetic molecular clock analysis in BEAST. The substitution rates (in the unit of nucleotide substitutions per site per year) of HA and NA genes were estimated to be 5.9766E-3 (95%HPD: 4.724E-3-7.2532E-3) and 4.4501E-3 (95%HPD: 3.4735E-3-5.3627E-3), respectively (Table 3). Using the relaxed molecular clock model, we conducted independent Bayesian Markov chain Monte Carlo estimation for the 2 surface genes. The tMRCAs of the 2 genes were found to cluster in 2015.6257 and 2015.8924 (Table 3). Thus, genomic reassortment of CM1216 might have occurred in 2015.

Pathogenicity of CM1216 in Mice

To evaluate the pathogenicity of CM1216, 26 BALB/c mice were randomly divided into experiment and control groups. All mice infected with CM1216 survived the entire 2 wk of the observation period, but they began to show clinical signs 1 d after infection, including inappetence, inactivity, and ruffled fur (data not shown). None of the mice in the control group showed any clinical signs or died during the observation period. At 2 d.p.i., the average body weight of infected mice dropped to the lowest level (83%) and then gradually recovered (Figure 3A). At 3 and 5 d.p.i., the pulmonary index of infected mice was significantly increased compared with that of the control mice (Figure 3B). However, there was no significant difference in pulmonary index between the 2 groups at 7 d.p.i., indicating that infected mice have recovered at that time. High viral titer in the lungs of infected mice was observed at 3 and 5 d.p.i., and no viral titer was detected at 7 d.p.i. (Figure 3C). Severe diffuse pneumonia, characterized by infiltration of neutrophils, damage of the alveolar epithelium, bronchial epithelial cell desquamation, congestion, and hemorrhage, was observed in infected mice at 3 and 5 d.p.i. (Figures 4A and 4B, solid arrows). There were no histopathological changes in the lungs of infected mice at 7 d.p.i and control mice (Figures 4C and 4D). These data suggest that CM1216 can effectively proliferate and cause pulmonary edema in mice. These results were corroborated by IHC (Figure 5). The NP antigen was observed through IHC staining of lung sections

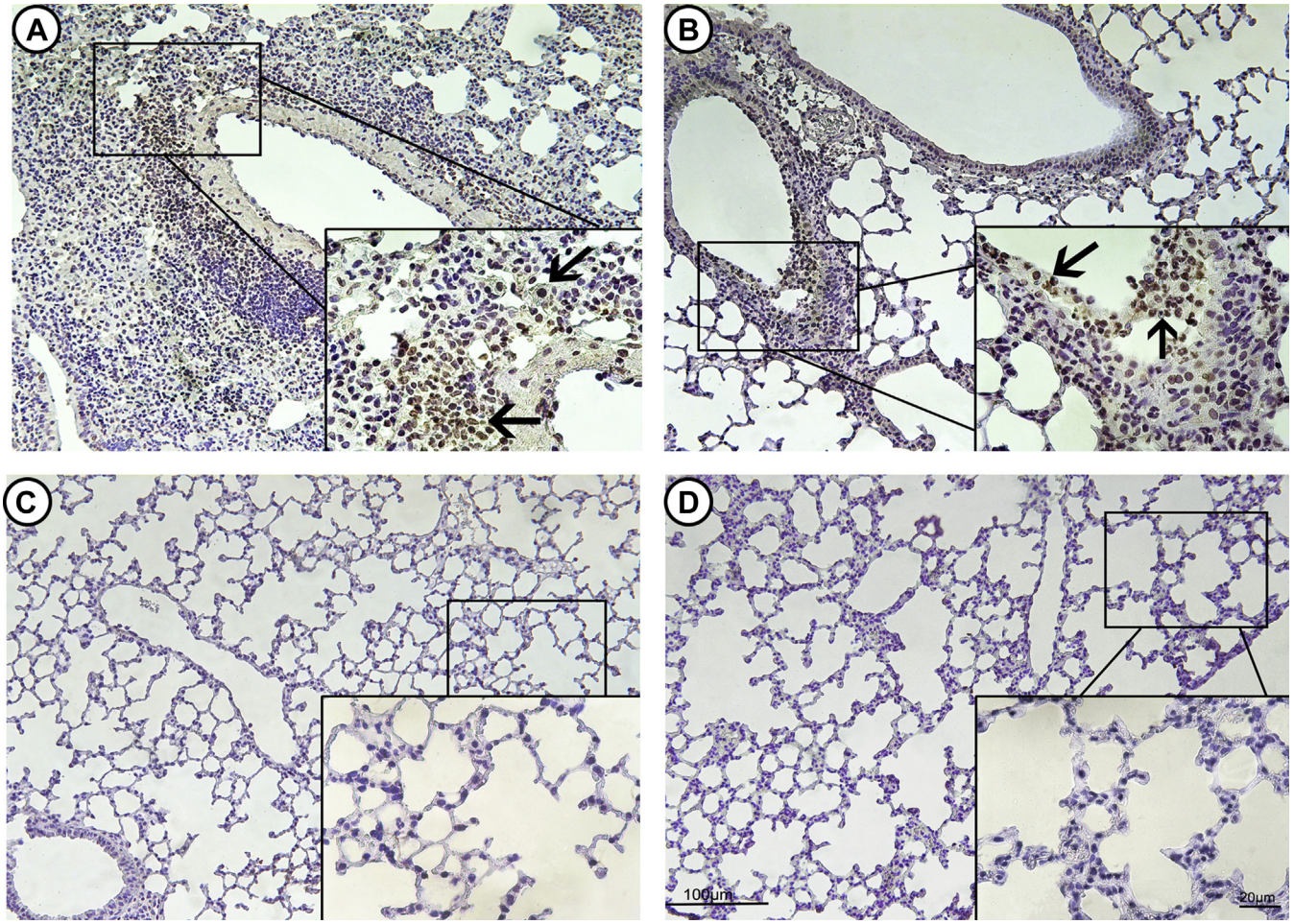

Figure 5. Immunohistochemical (IHC) analysis of lung tissue from CM1216 infected mice. IHC staining was performed on lung sections of 3 (A), 5 (B), 7 (C) d.p.i., and control mice (D). The black arrow indicates the virus area.

of mice after infection (Figures 5A and 5B), suggesting that CM1216 viruses replicated in mouse lungs.

DISCUSSION

The H7 subtype of AIVs can infect a broad range of species, including wild birds, poultry, pigs, seals, and humans (Naeve and Webster, 1983; Capua and Alexander, 2004; Lewis et al., 2013; Zhou et al., 2014). Recently, a significant increase in the prevalence of the H7 subtype of AIVs and the evolution of H7 LPAIV to HPAIV were observed (Abdelwhab et al., 2013). Infection of free-range chickens in the UK by an H7N7 HPAIV derived from a wild bird LPAIV was also seen (Seekings et al., 2018). Therefore, it is necessary to monitor H7 subtype influenza virus in wild birds. In this study, a novel H7N7 AIV strain, referred to as CM1216, was isolated from common teals in Chongming Island, which is known as the "Gateway to the Yangtze River" and is the third largest island in China. Based on our annual surveillance data (not published), we found that H7 subtype of AIV was firstly identified from wild birds, indicating H7 subtype might be a rare AIV strain in this area. Common teals are one of the most abundant migratory waterfowls in this region and can carry many different subtypes of influenza viruses, which provide a potential condition for genomic reassortments among different influenza viruses. Results of this study indicated that CM1216 had multiple reassortments bearing genes from H7N7, H3N6, H6N2, and H3N8 viruses that had been isolated previously from wild birds and ducks in Asian, European, and American countries (Table 1). This observation suggests that the H7N7 AIV found in common teals in Shanghai might have undergone genomic reassortments with other influenza viruses.

Phylogenetic analysis showed that CM1216 are closely related to the viruses circulated in Japan and Korea H7 subtypes, and they all clustered into a small sublineage. We also found that this H7 subtype of virus has persisted in wild birds since 2018, suggesting they might have adapted to wild bird hosts in these countries. As we all known, Japan, Korea, and Shanghai are all situated along the East Asia-Australia migratory flyway, so migratory waterfowl in the dissemination of H7 subtype viruses along migratory flyways should play a crucial role. It can be inferred that there were complex interactions among various migratory birds resulting in changes in the gene pool of AIVs.

Phylogenetic and phylodynamic analyses of CM1216 revealed that the nucleotide substitution rates of its

HA and NA genes were higher than those of the internal genes previously reported (Xu et al., 2011); HA and NA proteins are known to undergo antigen shift, and the genomic fragments that carry these 2 genes reassort more frequently than the other segments (Rabadan et al., 2008), leading to continuous evolution and variation of the virus. The PB2 protein plays an important role in the pathogenicity of influenza virus in mammals. It has been shown that some mutations in the PB2 protein can promote the replication or enhance the pathogenicity of the virus (Jong et al., 2013; Zhou et al., 2013; Li et al., 2017). The tMRCA estimated for CM1216 was dated in 2015, which was 3 yr before the isolation of CM1216 in this study. Owing to the lack of information on CM1216 infection in wild birds in China during this period, we were unable to identify its predecessor.

Influenza viruses usually cause upper and lower respiratory tract manifestations in humans. In laboratory conditions, many AIVs including subtypes H5N1 and H7N9 also cause infections in both upper and lower respiratory tracts in mice (Dybing et al., 2000; Szretter et al., 2007; Belser et al., 2013). Therefore, mice have been used to study the pathogenesis of AIVs (Kawaoka, 1991; Kumar et al., 2015). In this study, we found that CM1216 could replicate efficiently in mice without prior adaptation (Figures 2 and 3); this replication ability of CM126 was similar to that of the Korean H7 isolates (Kang et al., 2014). However, the pathogenicity of CM1216 in mice is different from the 2 genetically diverse H7N7 AIVs (HH179/H7N7 and CH1288/H7N7) that are prevalent in central China as these 2 AIVs were avirulent in mice and could not replicate in any organs of infected mice (Liu et al., 2018). Virulence toward different hosts has been postulated to be due to mutations in multiple AIV proteins including HA, PB2, and NS1 (Jiao et al., 2008; Gao et al., 2009; Maines et al., 2011). In this study, no key amino acid mutations were found in PB2, especially the E627 K and D701 N mutations, which have been shown to increase adaptation of other AIVs to mammals (Vines et al., 1998; Shinya et al., 2004). Other mutations including A135 T, T136S, and T160 A in HA; N30D and T215 A in M1; P42S and D97 E in NS1; R389 K in PB2; and N383D in PA were found in CM1216. The T160 A substitution in HA has been shown to affect not only receptor-binding property but also transmissibility of H5N1 AIV clade 2.3.4 in guinea pigs (Gao et al., 2018). The HA protein of an H7N9 AIV isolated from humans in China has both T160 A and Q226 L (H3 numbering) substitutions, which may enhance binding of the virus to SA α-2, 6 Gal receptors, enabling transmission from birds. Whether the pathogenicity of CM1216 in mice is related to these mutations remains to be investigated. As CM1216 was isolated from wild birds, it is possible that it may disseminate to other areas by migrating birds or transporting poultry. Taken together, our results suggest the necessity to monitor the evolution of H7N7 AIVs with special focus on their potential pathogenicity to mammals.

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DISCLOSURES

The authors declare no conflict of interest.

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