Acta virologica **64**: 67 – 77, 2020 doi: 10.4149/av_2020_109

Two genetically similar H9N2 influenza viruses isolated from different species show similar virulence in minks but different virulence in mice

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Received May 03, 2019; revised August 27, 2019; accepted February 5, 2020

Summary. – The H9N2 influenza virus has been frequently endemic in poultry, infected mammals and humans and has threatened public health. It is therefore imperative to understand the molecular mechanism enabling this virus to jump from avian to mammalian species. In this study, two H9N2 influenza viruses were isolated from the same region in eastern China but from different hosts; one was isolated from mink and named A/Mink/Shandong/WM01/2014(H9N2)(WM01), while the other was isolated from chicken and named A/Chicken/Shandong/LX830/2014(H9N2)(LX830). Sequencing and phylogenetic analysis showed that both H9N2 influenza viruses had similar genetic backgrounds. The results of infection in minks suggested that both viruses caused significant weight loss and pathological changes in the lungs. Mouse infection showed that LX830 was nonpathogenic in mice, but WM01 resulted in 25% mortality and pathological changes in the lungs, such as severe edema and diffused inflammation of the interalveolar septa. Comparison of the full genomes of both H9N2 influenza viruses showed 52-nucleotide-synonym mutations in 8 gene segments and 7-nucleotide-antonym mutations, resulting in 7 amino acid (AA) substitutions distributed in the PB1, PA, NA and M gene segments. None of these mutations did affect splicing of the M and NS gene segments at the nucleotide level or minor open reading frames (ORFs), such as PB1-F2 and PA-X. Phylogenetic analysis showed that both H9N2 influenza viruses belong to the prevalent epidemic genotype in Asia.

Keywords: H9N2 influenza virus; chicken; minks; pathogenicity; phylogenetic

Introduction

Currently, H9N2 influenza virus has been circulating worldwide and represents a significant threat not only to the poultry industry but also to public health, owing to the trend of expanding from avian to mammalian species (Ali *et al*., 2019; Butt *et al*., 2005; Peiris *et al*., 1999; Sun *et al*., 2013a). Constant evolution dynamics are crucial for the survival of the influenza virus. There are two common mechanisms adapted by influenza viruses in evolution: reassortment and mutation. Influenza virus has a segmented genome that allows for gene reassortment. For instance, the 1957 H2N2, 1968 H3N2, and 2009 H1N1 influenza viruses are typically thought to be the results of reassortment between human, avian or swine influenza viruses (Belshe, 2005; Scholtissek, 1995). On the other hand, gene mutation, referred to as antigen drift, occurs frequently in the evolution of influenza virus due to the error-prone nature of the RNA-dependent RNA polymerase (RdRP) and the lack of proofreading mechanisms during replication. These changes in RNA fragments coding amino acids allow the emergence of new influenza viruses able to infect humans (Anhlan

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Abbreviations: LX830 = A/Chicken/Shandong/LX830/2014 (H9N2); WM01 = A/Mink/Shandong/WM01/2014(H9N2); AA = amino acid; EID_{50} = egg infectious dose 50; AA = amino acid; p.i. = post infection

et al., 2011). Studies indicate that 1918 pandemic H1N1 influenza virus originated from a swine H1N1 influenza virus, which might be derived from a H1N1 avian precursor carrying accumulated mutations, and distinguished from the bulk of other avian influenza viruses (Cheung *et al*., 2014; Dulin *et al*., 2015). Similar to all other avian influenza viruses, the H9N2 influenza virus is prone to genetic changes that can affect virulence, pathogenicity and host specificity. Due to constant mutations in the genome, multiple novel genotypes of H9N2 influenza viruses emerged in China (Li *et al*., 2017a). Exchange of gene segments was also detected frequently among H9N2 influenza virus and other subtypes. For example, a recent outbreak of the H7N9 influenza virus confirmed that the H7N9 influenza virus was a reassortant of the H9N2 and H7 subtype influenza viruses and increased concern about the ability of H9N2 influenza viruses to jump the host species barrier (Liu *et al*., 2013; Pu *et al*., 2015). Additionally, multiple sequence alignment and phylogenetic analysis indicated that six internal gene segments of a human H10N8 also originated from H9N2 chicken influenza virus (Qi *et al*., 2014). Naguib and colleagues reported that natural reassortants of H5N1 and H9N2 avian influenza viruses showed obvious pathogenicity in experimental ferrets (Naguib *et al*., 2017). More importantly, an increasing number of H9N2 influenza viruses carrying markers of human influenza virus have been reported, which indicates that H9N2 influenza viruses can infect mammals and humans without preadaptation (Cong *et al*., 2008; Li *et al*., 2014; Yu *et al*., 2008; Zhang *et al*., 2009). In total, these data suggest that it is necessary to enforce the surveillance of H9N2 influenza viruses through global collaborative efforts.

Minks, as economically important animals, are raised worldwide. The scale of mink farming has been expanding consistently in China. Similar to ferrets, minks are susceptible to influenza viruses (Okazaki *et al*., 1983). There are several subtypes of influenza viruses that were isolated from naturally infected minks (Akerstedt *et al*., 2012; Berg *et al*., 1990; Yoon *et al*., 2012). H9N2 influenza viruses have also recently been isolated from minks in China (Peng *et al*., 2015; Xue *et al*., 2018). Furthermore, recombinant cases of different influenza viruses in minks have also been reported (Gagnon *et al*., 2009; Tremblay *et al*., 2011). According to published reports, the seroprevalence of H9N2 influenza virus in minks reached 20% in 2013 but rose to approximately 50% in 2015 in China (Peng *et al*., 2015; Zhang *et al*., 2015). Notably, serological surveillance of poultry workers showed that a significant number had H9N2-specific antibodies and experienced subclinical symptoms. This finding indicates that human infection with H9N2 avian influenza virus is widely spread in China (Ge *et al*., 2018; Li *et al*., 2017b; Ma *et al*., 2018).

To date, molecular determinants of H9N2 influenza virus naturally infecting mammals and humans are highly limited. In this study, two influenza viruses, named LX830 and WM01, were isolated from mink and chicken from different farms. Virulence of both viruses was tested in minks and BALB/c mice. Furthermore, the full genomes of both H9N2 isolates were sequenced, and phylogenetic analysis was conducted.

Materials and Methods

Virus isolation and cells. Lung samples were collected from reported sick minks and cloacal swabs from chickens. The minks and chickens were raised on different farms in eastern China. For virus isolation, 1 g of lung samples was suspended in 1 ml PBS supplemented with 2,000 unit/ml penicillin and 2,000 μg/ml streptomycin, homogenized and centrifuged at 12,000 x g for 10 min at 4°C. Then, 200 μl supernatant from each sample was inoculated into 9-day-old specific pathogen-free (SPF) embryonated chicken eggs (Melia, Beijing), with 3 eggs being employed for each sample. After a 72-hour incubation at 37°C, allantoic fluids were harvested and tested by hemagglutination assay with 1% SPF chicken red blood cells (Zhang *et al*., 2015). Allantoic fluids with titers >2^4 were combined and stored at -80°C. Madin-Darby canine kidney (MDCK) (ATCC) cells were cultured in modified Eagle's medium (MEM) supplemented with gentamycin, sodium pyruvate and 5% fetal bovine serum. Viral titers were determined by calculating the egg infectious dose 50 (EID_{50})/ml by the method of Reed and Muench (Reed and Muench, 1938).

Virus identification. Hemagglutination assays and RT-PCR were performed to detect influenza virus in inoculated eggs. The primer sequences used are listed in Table S1. Briefly, RNA was extracted from the allantoic fluid with a hemagglutination assay titer >4 by using TRIzol reagent (ThermoFisher). cDNAs were synthesized with the universal primer U12, and RNA served as the template. Reverse transcription was conducted to amplify hemagglutinin (HA) and neuraminidase (NA) gene segments using 20 μl reaction mixture containing 11 μl RNA extraction solution, 1 μl 10 pmol backward primer, 4 μl 5 × MLV buffer, 2 μl dNTPs (2.5 mM), 1 μl RNase inhibitor (40 U/μl) and 1 μl reverse transcriptase XL (MLV, 100 U). PCR was performed under the following procedure in a thermal cycler: 94°C for 4 min followed by 30 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 7 min and holding at 12°C, and product presence was confirmed by agarose gel electrophoresis.

Replication of H9N2 influenza viruses in mice. To test the replication of both H9N2 influenza viruses in mice, 12 six-week-old female BALB/c mice purchased from Shandong Experimental Animal Center were divided randomly into 4 groups. Each mouse was intraperitoneally anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) in PBS and intranasally

challenged with 10^6 EID_{50} of LX830 or WM01 in a 50-µl volume. At days 3 and 5 post infection (p.i.), mice were sacrificed with CO_2. Then, 1 g of each lungs, heart, liver and brain was collected in 1 ml of PBS supplemented with 2,000 unit/ml penicillin and 2,000 µg/ml streptomycin for each sample, homogenized and centrifuged at 12,000 x g for 10 min at 4°C. Then, 200 µl of 10-fold serially diluted supernatant of each sample was inoculated into 9-day-old SPF embryonated chicken eggs. After a 72-hour incubation at 37°C, allantoic fluids were harvested and tested by hemagglutination assay (HA) (Zhang *et al*., 2015). Finally, viral titers were expressed as 50% (median) embryo infectious doses.

Animal pathogenesis experiments. Twelve six-week-old female minks without infection by H9N2 influenza virus were purchased from a local mink farm and divided into 3 groups. Each mink was weighed and anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and then intranasally inoculated with 10^6 EID_{50} of LX830 or WM01 H9N2 influenza virus in a 200-µl volume. Minks were treated with PBS as controls. Subsequently, minks were weighed and observed daily for clinical symptoms. Forty six-week-old female BALB/c mice were randomly divided into 5 groups. Each mouse was weighed and anesthetized with ketamine and xylazine (same dose as previously described). Subsequently, mice were intranasally inoculated with 10^5 or 10^6 EID_{50} of LX830 or WM01 H9N2 influenza virus in a volume of 50 µl. Mice were treated with PBS as a naive control. All mice were weighed and observed daily for clinical symptoms and survival. Mice recorded with > 25% weight loss were scored as dead for ethical reasons. Animal use was approved by the animal welfare committee of Qingdao Agricultural University (permission number #30). All animal samples were collected in accordance with the 2016 standards of laboratory animal in China and other related regulations in Animal Welfare Act.

Histopathological analysis. Mice or minks were anesthetized with ketamine and xylazine as described above and infected intranasally with 10^6 EID_{50} of LX830 or WM01 H9N2 influenza virus per animal. Four days after infection, challenged animals were sacrificed, their lungs were collected, fixed in 10% neutral buffered formalin, and embedded in paraffin wax. Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) and evaluated for histopathological alterations.

Sequencing. Isolated viruses were plaque-purified by 3 rounds in MDCK cells propagated in SPF embryonated chicken eggs. Each virus was picked with 3 plaques. Harvested allantoic fluid was tested with HA, and each viral gene segment was amplified by RT-PCR as described above. Then, PCR products were visualized on 1.0% agarose gel electrophoresis. PCR bands were cut and purified using a gel extraction kit (Takara, Dalian) according to the manufacturer's instructions. Finally, the PCR product was resolved in 50 µl of deionized water, quantified and ligated into the pMD-18T easy vector (Takara, Dalian). Three positive clones for each gene segment were identified and sequenced using an ABI3730 DNA sequencer (Sangon Biotech, Shanghai). In parallel, RNA extracted from initially harvested allantoic fluid was sequenced by next-generation sequencing-based virome assessment (Tanpu Biotek, Shanghai). The nucleotide sequences were compiled, edited by using ApE software. Furthermore, representative reference sequences of each gene segment of H9N2 influenza virus were downloaded from the Influenza Research Database and aligned with those of LX830 and WM01. Phylogenetic analyses were conducted using the Megalign program (MEGA 6.0). Estimates of the phylogenetic relationships were calculated by performing 1000 neighbor-joining bootstrap replicates.

Statistical analysis. Comparisons between groups were performed by using a nonparametric one-way ANOVA with the Tukey multiple comparison test and the Fisher exact test, and survival dates were analyzed using the log-rank test. The analyses were performed using GraphPad Prism version 6.0 for Windows (GrahPad Software). P-values <0.05 were considered to be significant.

Results

Virus isolation and sequencing

In this report, two H9N2 influenza viruses were isolated from different farms in the same region in eastern China; the virus isolated from chickens was named A/Chicken/Shandong/LX830/2014(H9N2)(LX830), and the mink virus was named A/mink/Shandong/WM01/2014(H9N2) (WM01). WM01 was isolated from the lungs of sick mink, which showed fever, cough and nasal discharge. Infection with canine distemper virus (CDV) and mink enteritis (MEV) virus was excluded. Both influenza viruses were confirmed by RT-PCR to be H9N2, and the full virus genomes were successfully sequenced. Two sequencing methods were utilized to sequence the genomes of both H9N2 influenza viruses. Nucleotide sequence alignment of each gene segment sequenced by two methods showed no difference.

Replication of two H9N2 influenza viruses in mice

To test the replication potential of both H9N2 influenza viruses *in vivo*, each BALB/c mouse was infected with 10^6 EID_{50} of the respective H9N2 virus. Viral titers in the lungs of infected mice are indicated in Fig. 1. At 3 days p.i., the viral titer of both H9N2 influenza viruses was approximately 10^7 EID_{50}, but the titer of LX830 decreased 10^4-fold at 5 days p.i. (P-value <0.05). Interestingly, neither

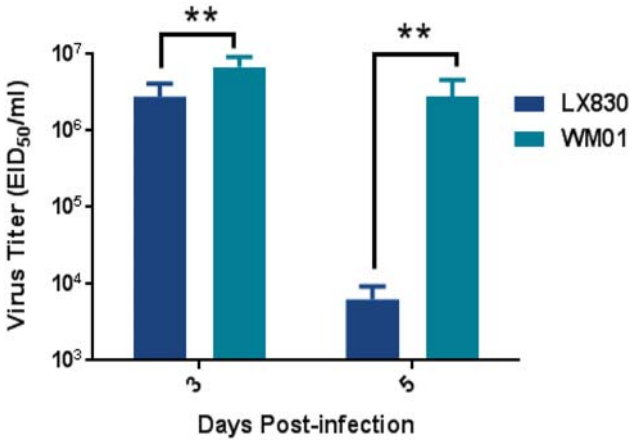

Fig. 1

Replication of H9N2 influenza virus in mice
Six-week-old female BALB/c mice were inoculated intranasally with 10^6 EID_{50} of LX830 or WM01 per mouse in 50 µl. At 3 and 5 days p.i., infected mice were sacrificed, and the lungs were collected and homogenized. LX830 or WM01 virus derived from the lungs of infected mice were titrated in eggs, and the titers are presented as EID_{50}/ml (**P-value <0.05).

LX830 nor WM01 virus was recovered from hearts, brains or livers.

Pathogenicity in mice and minks

To test the pathogenicity of both H9N2 isolates in mammals, minks and mice were intranasally infected. As shown in Fig. 2, when mice were infected with 10^5 EID_{50} of LX830 or WM01 per mouse, no obvious clinical symptoms of infection were observed, but such clinical symptoms as decreased activity, huddling, ruffled fur, labored breathing, hunched posture, and weight loss (P-value <0.05) were observed in mice inoculated with 10^6 EID_{50} of WM01. Moreover, two death cases occurred in the group of mice infected with 10^6 EID_{50} of WM01 per mouse (shown in Fig. 2b). When minks were inoculated with 10^6 EID_{50} LX830 or WM01 H9N2 influenza virus, clinical symptoms of infection, including lethargy, dullness, nasal discharge, cough, sneezing, and weight loss, were observed in both groups. No weight loss was observed in the PBS group (P-value <0.05) (Fig. 2c).

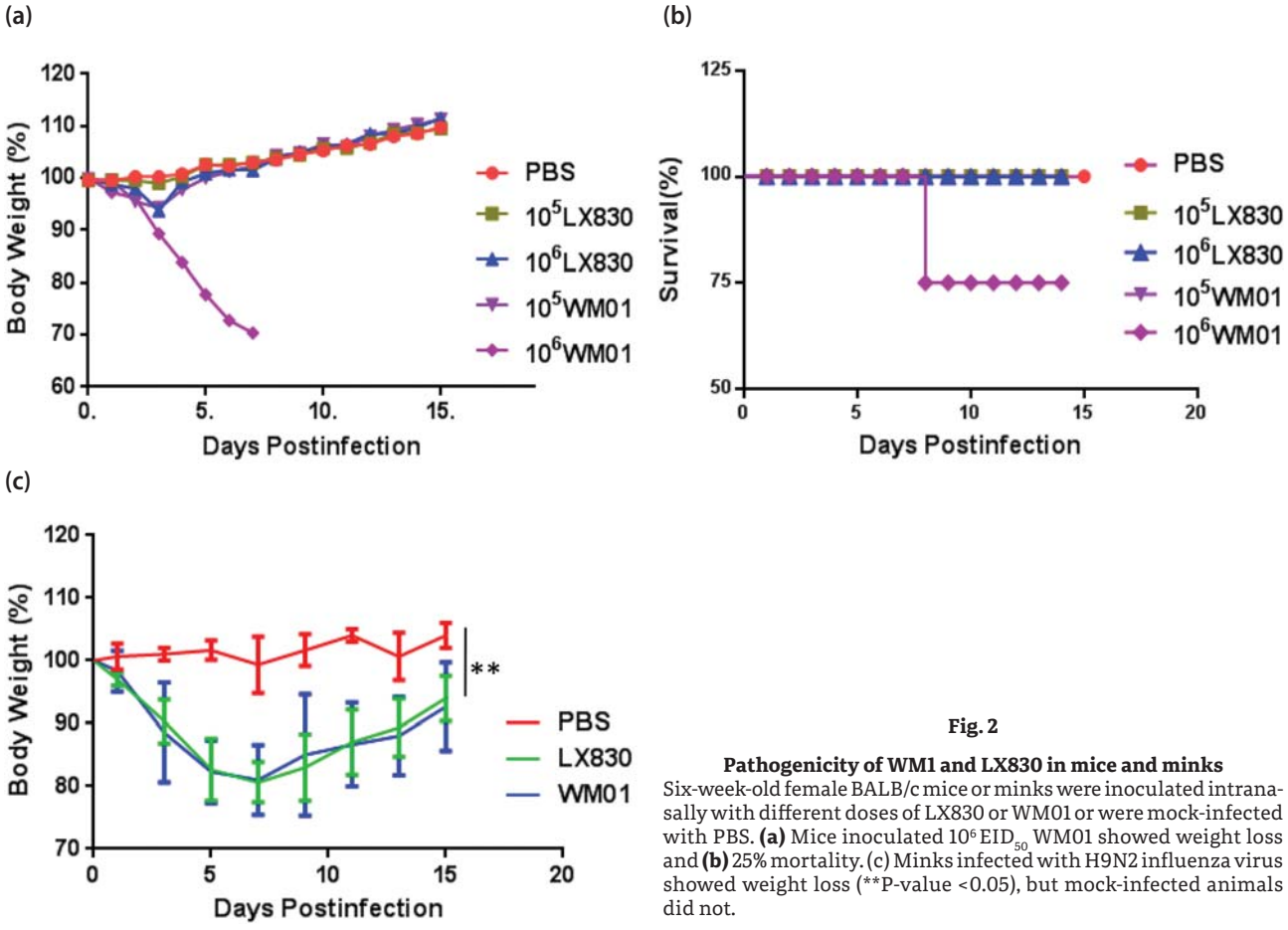

Fig. 2

Pathogenicity of WM1 and LX830 in mice and minks
Six-week-old female BALB/c mice or minks were inoculated intranasally with different doses of LX830 or WM01 or were mock-infected with PBS. **(a)** Mice inoculated 10^6 EID_{50} WM01 showed weight loss and **(b)** 25% mortality. (c) Minks infected with H9N2 influenza virus showed weight loss (**P-value <0.05), but mock-infected animals did not.

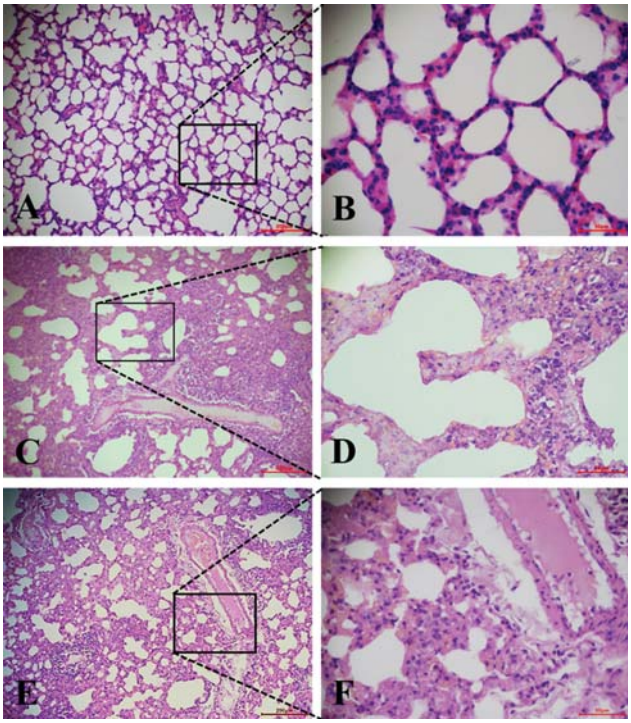

Fig. 3

Histopathological changes in lungs of minks infected by H9N2 viruses

Six-week-old female minks were inoculated intranasally with 10^6 EID_{50} of LX830 or WM01 or mock-infected with PBS. Four days after infection, the animals were sacrificed, and the lungs were collected for histopathological analysis. **(a, b)** Mink lungs of the PBS control group revealed no obvious pathological changes. **(c, d)** Thickened alveolar walls of the lungs of minks infected with 10^6 EID_{50} of LX830. **(e, f)** Alveolar walls of minks infected with 10^6 EID_{50} of WM01 were markedly thicker and contained many exudates resulting from inflammation. Substantial amounts of serous fluid had seeped out of veins, and infiltration of inflammatory cells into lung tissue was observed.

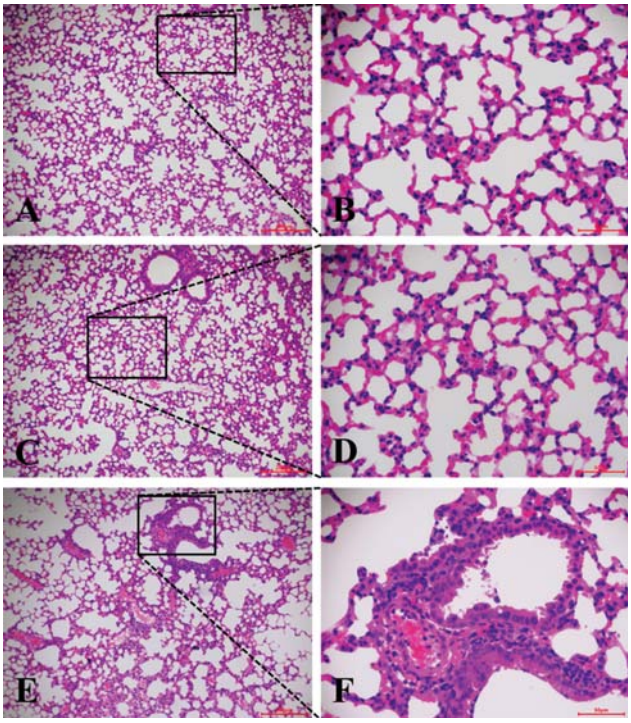

Fig. 4

Histopathological changes in lungs of mice infected by H9N2 viruses

BALB/c mice were inoculated intranasally with 10^6 EID_{50} of LX830 or WM01 or mock-infected with PBS. At 4 days after infection, the animals were sacrificed, and the lungs were collected for H&E staining and histopathological analysis. **(a, b)** Lung tissue of control mice **(c, d)**. Representative histopathological findings in LX830-infected lungs of BALB/c mice. Almost no pathological changes were observed. **(e, f)** Representative histopathological findings of WM01-infected lungs of BALB/c mice. Inflammatory cell infiltrates around the bronchus, edema and thickening of alveolar walls, and desquamation of epithelial cells in bronchial lumens was observed.

Histopathological lesions

To determine the histopathological changes of both H9N2 influenza viruses in mammals, mink and BALB/c mice were intranasally infected with 10^6 $EID_{50\ of}$ LX830 or WM01 H9N2 influenza virus. Histopathological images in naive mink control are shown in Fig. 3a and 3b (enlarged square region of 3A). Infiltration of macrophages and neutrophils was observed in alveolar walls of minks infected with LX830 or WM01 H9N2 virus isolates. In addition, alveolar walls were markedly thickened, as shown in Figs. 3c, 3d (enlarged square region of 3c), 3e and 3f (enlarged square region of 3e). Lung tissue of a naive control mouse is shown in Fig. 4a and 4b (enlarged square region of 4a). In contrast to mice infected with 10^6 EID_{50} of LX830 (shown in Fig. 3c and 3d (enlarged square region of 3c)), mice infected with 10^6 EID_{50} of WM01 exhibited severe lesions in lungs, including severe edema, congestion, inflammatory cell infiltration around the bronchus, thickening of alveolar walls and desquamation of epithelial cells in bronchial lumens, as shown in Fig. 4e and 4f (enlarged square region of 4e).

Sequence and phylogenetic analysis

Nucleic acid sequence alignment analysis of LX830 and WM0 indicated a difference in 59 nucleotides found in the entire genome of both H9N2 influenza viruses. Among these nucleotide discrepancies, as shown in Table 1, there were 52 silent mutations distributed in all eight gene segments. Interestingly, nucleotide substitutions were found in the 5' and 3' terminal noncoding regions (NCRs)

Table 1. Amino acid difference between LX830 and WM01 H9N2 influenza viruses

	PB1	PA	NA				M2
	322	614	19	51	325	425	47
LX830	I	N	I	M	N	G	L
WM01	M	S	T	V	S	R	F
Avian	I(1256/1260) M(1/126 0) T(1/1260) V(2/1260)	N(1376/1420) S(8/1420) D(2/1420) T(34/1420)	I(957/1779) T(652/1779) A(112/1779) M(7/1779) S(19/1779) V(32/1779)	M(414/1779) V(1281/1779) A(44/1779) E(3/1779) I(20/1779) L(3/1779) S(3/1779) T(5/1779) X(6/1779)	N(689/1779) S(1/1779) D(283/1779) G(10/1779) H(2/1779) I(1/1779) P(1/1779) S(2/1779) T(790/1779)	G(3/1779) R(983/1779) E(790/1779) I(1/1779) K(2/1779)	L(3/1300) F(1295/1300) V(1/1300) Y(1/1300)
Mammal	I(30/31) M(1/31)	N(32/33) S(1/33)	I(17/35) T(14/35) A(3/35) V(1/35)	M(2/35) V(27/35) A(4/35) C(2/35)	N(17/35) S(1/35) D(7/35) R(2/35) T(8/35)	R(25/35) E(8/35) I(2/35)	F(33/33)
Human	I(13/13)	N(13/13)	I(6/17) T(11/17)	M(6/17) V(8/17) C(3/17)	N(9/17) T(5/17) R(3/17)	R(9/17) E(5/17) I(3/17)	F(13/13)

Table 2. Nucleic acid differences between the LX830 and WM01 H9N2 influenza viruses

		Nucleic acids at indicated position									
PB2		267	273	336	693	804	1059	1096	1171	1764	1797
	LX830	A	C	A	A	G	A	C	A	C	C
	WM01	G	T	G	G	A	G	A	G	T	T
		2025	2128								
	LX830	G	G								
	WM01	A	A								
PB1		65	75	927	990	1080	1302	1647	1947		
	LX830	C	C	A	A	G	C	G	C		
	WM01	T	T	G	G	A	T	A	T		
PA		261	1618	1865	2013						
	LX830	T	C	A	A						
	WM01	C	T	G	G						
HA		93	501	604	626	652	729	858	915	1653	
	LX830	G	C	A	T	A	T	G	A	A	
	WM01	A	T	C	C	G	C	A	G	G	
NP		294	306	444	507						
	LX830	T	A	C	C						
	WM01	C	G	T	T						
NA		**16**	75	170	250	550	580	583	993	1105	1198
	LX830	**G**	T	A	A	T	G	T	A	G	C
	WM01	**A**	C	G	T	C	A	C	G	A	T
		1292	1366	1423							
	LX830	G	A	**C**							
	WM01	A	G	**T**							
M		162	830	854							
	LX830	T	C	A							
	WM01	C	A	C							
NS		**25**	32	38	359	506	513	861			
	LX830	**T**	T	T	A	C	T	T			
	WM01	**C**	C	C	G	T	G	C			

The nucleic acids highlighted in bold are located in the non-coding region.

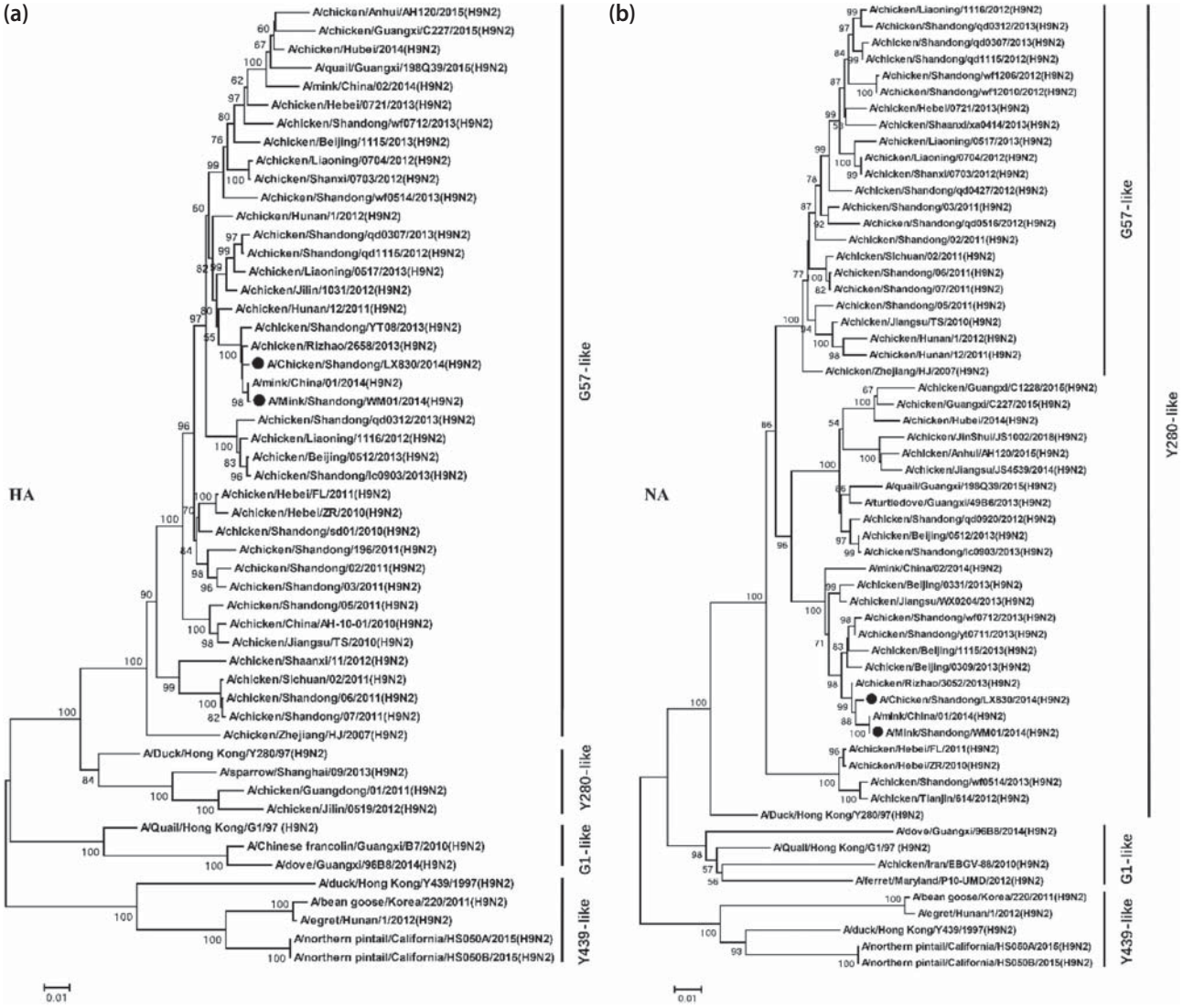

Fig. 5

Phylogenetic trees of full-length hemagglutinin (HA) (a) and neuraminidase (NA) (b) genes of H9N2 influenza viruses
The trees were generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic trees were based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus published in GenBank.

of the NA and 5' NCRs of the NS gene segment. The amino acid sequences of each gene product of both isolates were compared. Our findings showed that both isolates had a PSRSSR/GL AA motif at the cleavage site between HA1 and HA2, which is a low pathogenicity influenza virus marker. Alignment results revealed 7 amino acid differences between the two virus genomes, which were mapped to amino acid (AA) 322 in PB1, AA 614 in PA AA 19, 51, 325, 425 in NA, and AA 47 in M2. No amino acid differences were observed in PB2, HA, NP, M1, NS1 and NS2. To investigate the amino acid bias of H9N2 influenza virus isolated from different host species, we analyzed the H9N2 sequences deposited in the Influenza Research Database (IRD). Data are presented in Table 2. The number between parentheses is the ratio of the indicated amino acid to the total amino acid cited. Most avian and mammalian influenza viruses possessed 322I in PB1, 614N in PA, and 47F in M2. Amino acids in NA showed a high diversity among avian, mammal and human influenza viruses. All human influenza viruses possessed PB1-322I, PA-614N and M2-47F. The substitution of PB1-I322M did not influence the amino acid sequence of PB1-F2, and substitution of PA-N614S did not affect the PA-X amino acid sequence. Therefore, the results demonstrate the unique features

of the nucleotide and amino acid sequences of LX830 and WM01 H9N2 influenza viruses.

Phylogenetic analysis (Fig. 5a and Fig. S1) showed that the PA, HA, PB1, NP, M and NS genes of both H9N2 influenza viruses belong to the G57-like genotype, which has been predominant since 2010 in China (Pu *et al*., 2015), but the NAs (Fig. 5b) belong to the Y280-like genotype, and the PB2 genes belong to the G1-like genotype, respectively (Gu *et al*., 2017). Overall, both H9N2 influenza viruses represent the prevalent genotype that is associated with epidemics in Asia, and it is possible for these viruses to exchange internal gene segments with H7N9 influenza viruses (Pu *et al*., 2015; Zhu *et al*., 2018).

Nucleotide sequence accession numbers

The nucleotide sequences of each gene segment were submitted to GenBank, and Acc. Nos. were assigned as follows: PB2, PB1, PA, HA, NP, NA, M, and NS of LX830 were assigned Nos. from MK072966 to MK072973, respectively, while PB2, PB1, PA, HA, NP, NA, M, and NS of WM01 were assigned Nos. from MK078567 to MK078574, respectively.

Discussion

H9N2 influenza virus possesses a wide animal host range and is believed to be able to jump to new host species. However, when new hosts are infected, H9N2 is rarely capable of transmitting well among the new hosts (Wan *et al*., 2008). The ability of H9N2 to infect ferrets, minks and humans without changes in receptor binding affinity increases the likelihood of virus transmission from birds to mammals, especially humans, providing an opportunity for genetic reassortment between avian and human influenza viruses (Butt *et al*., 2005; Wan and Perez, 2007). To date, H9N2 influenza viruses have been isolated from a variety of mammalian species, including swine, dogs, and minks, but the molecular determinant of H9N2 influenza virus enabling it to infect mammals has not been elucidated (Sun *et al*., 2013a; Zhou *et al*., 2014; Peng *et al*., 2015; Zhang *et al*., 2015; Xue *et al*., 2018). Previous data by Xue *et al*. (2018) showed that N at the position 701 (701N) in PB2 enhanced the virulence of H9N2 influenza viruses in minks. Another study showed substitutions of amino acids D253N and Q591K in the PB2 protein of the H9N2 influenza virus, which increased replication and pathogenicity in mammals (Mok *et al*., 2011; Wang *et al*., 2016; Zhang *et al*., 2018). Investigations by Xiao *et al*. (2016) showed that A588V in PB2 of the H9N2 influenza virus promoted mammalian adaptation not only of H10N8 and H7N9 but also of H9N2. Kamiki and colleagues showed that the PB1-K577E mutation in PB1 of H9N2 influenza virus increased polymerase activity and virus pathogenicity in mice (Kamiki *et al*., 2018). The K356R mutation in PA of avian H9N2 influenza virus also increased replication and pathogenicity in mammalian species (Xu *et al*., 2016).

Receptor binding is a crucial step in influenza virus infection. Several studies reported that field isolates of H9N2 influenza viruses possess human virus-like receptor-specificity that allows preferential binding to sialic acids attached to galactose via an alpha (2,6) linkage, a major type of sialic acid presenting on human respiratory epithelial cells; otherwise, the classic avian virus-like receptor specificity allows for preferential binding to 2-3-linked sialic acid (SAa2-3) receptors, which are found on ciliated epithelial cells, a minor population within the human respiratory tract, and on some epithelial cells in the lower tract (Matrosovich *et al*., 2001; Choi *et al*., 2004; Wan and Perez, 2006). Several amino acid residues on HA have been reported to be related to the receptor binding-specificity of H9N2 influenza virus in mammals. Teng and colleagues reported that single mutations at position 190 in HA enhanced binding to the human-type sialic acid receptor and enhanced also the replication in mice (2016). Substitution of I155T or Q226L in the HA of H9N2 influenza virus determines human cell tropism, replication in human airway epithelial cells and aerosol transmission among ferrets (Matrosovich *et al*., 2001; Wan and Perez, 2007; Wan *et al*., 2008; Li *et al*., 2017b; Obadan *et al*., 2019). Investigations by Sang *et al*. (2015) showed that substitution N313D and N496S in HA of H9N2 influenza virus increased virus affinity with to both human and avian sialic acid receptors. Substitution of A316S, which increased HA cleavage efficiency, combined with length alteration enhanced the virulence of H9N2 influenza virus in chickens and mice (Sun *et al*., 2013b). These investigations indicate that polygenic factors contribute to the virulence of H9N2 influenza viruses in mammalian hosts.

However, in the present study, 627K and 701N in PB2 of both H9N2 isolates were not observed elsewhere. Additionally, AA 226 and 228 in HA of LX830 and WM01 were glycine (G) and arginine (R), respectively, not the prevalent amino acid glutamine (Q) and leucine (L) of human A type influenza viruses (Pusch and Suarez, 2018). The nucleotide sequences of both H9N2 influenza viruses were aligned. The results showed difference in 59 nucleotides. The amino acid sequences of both H9N2 influenza viruses were also compared, and differences in seven amino acids were recorded; these amino acid changes were present in only 4 of the 10 viral proteins. None of these AAs have been related to the pathogenicity of H9N2 influenza virus in mammals or humans. This result indicated that no single amino acid dominated the infectivity of H9N2 influenza virus in mice. Furthermore, 52 silent mutations

were found in all eight gene segments, with 3 located in the NCRs. To date, we do not know whether mutation in NCR affected the function and stability of gene segments. Further investigation is needed.

To date, mammals are not acknowledged as the H9N2 influenza reservoir; however, the serological surveillance of farmed mink in eastern China revealed that the H9N2 influenza virus infection rate reaches 50% (Zhang *et al.*, 2015). These data confirm a high susceptibility of minks to H9N2 influenza virus. The role of mink in the adaptation of H9N2 influenza virus in mammals or humans has not been determined to date. It is not known whether mink-adapted H9N2 influenza viruses can directly infect humans. Circulation of H9N2 influenza viruses in minks may promote H9N2 influenza virus adaptation in mammals, including humans. Reducing human exposure to H9N2 influenza virus would help to investigate the location where the exposure occurred. In this study, H9N2 viruses LX830 and WM01 infected minks without inducing severe outcomes; however, minks may spread H9N2 influenza virus directly or may serve as a viral mixer, similar to the role played by pigs. Vaccination of minks is not currently planned to prevent H9N2 influenza virus infection, but it would be important to reinforce the serological surveillance of minks to monitor the spread of potentially dangerous H9N2 viruses.

Conclusion

In this study, two H9N2 influenza viruses were isolated from minks and chickens. Animal experiments showed that WM01 replicated better and was more virulent in mice. Sequence analysis identified differences in 59 nucleotides in the entire genome and 7 amino acid mutations distributed in the PB1, PA, NA and M2 proteins. Comparison with the corresponding amino acids in the reference protein suggested a unique feature in the nucleic acid and AA sequences of the LX830 and WM01 H9N2 influenza viruses. Phylogenetic analysis showed that both H9N2 influenza viruses belonged to the prevalent epidemic genotype in Asia.

Supplementary information is available in the online version of the paper.

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Acta virologica **64**: 67 – 77, 2020 doi: 10.4149/av_2020_109

SUPPLEMENTARY INFORMATION

Two genetically similar H9N2 influenza viruses isolated from different species show similar virulence in minks but different virulence in mice

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Received May 03, 2019; revised August 27, 2019; accepted February 5, 2020

Table S1. Primers used for amplification of the eight gene segments of H9N2 influenza viruses

Gene	Forward primer	Reverse primer
PB2	Ba-PB2-1 : TATTGGTCTCAGGGAGCGAAAGCAGGTCAAATATA	Ba-PB2-2341R : ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTTTTA
PB1	Bm-PB1-1 : TATTCGTCTCAGGGAGCAAAAGCAGGCAAACCAT	Bm-PB1-2341R : ATATCGTCTCGTATTAGTAGAAACAAGGCATTTTTTC
PA	Bm-PA-1 : TATTCGTCTCAGGGAGCAAAAGCAGGTACTGATC	Bm-PA-2233R : ATATCGTCTCGTATTAGTAGAAACAAGGTACTTTTTTG
HA	Bm-HA-1 : TATTCGTCTCAGGGAGCAAAAGCAGGGGAATTTC	Bm-HA-1742R : ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTG
NP	Bm-NP-1 : TATTCGTCTCAGGGAGCGAAAGCAGGGTAGATAA	Bm-NP-1565R : ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTTC
NA	Ba-NA-1 : TATTGGTCTCAGGGAGCAAAAGCAGGAGTAAAAATG	Ba-NA-1458R : ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTTC
M	Bm-M-1 : TATTCGTCTCAGGGAGCAAAAGCAGGTAG	Bm-M-1027R : ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT
NS U12	Bm-NS-1 : TATTCGTCTCAGGGAGCAAAAGCAGGGTG AGCAAAAGCAGG	Bm-NS-890R : ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT

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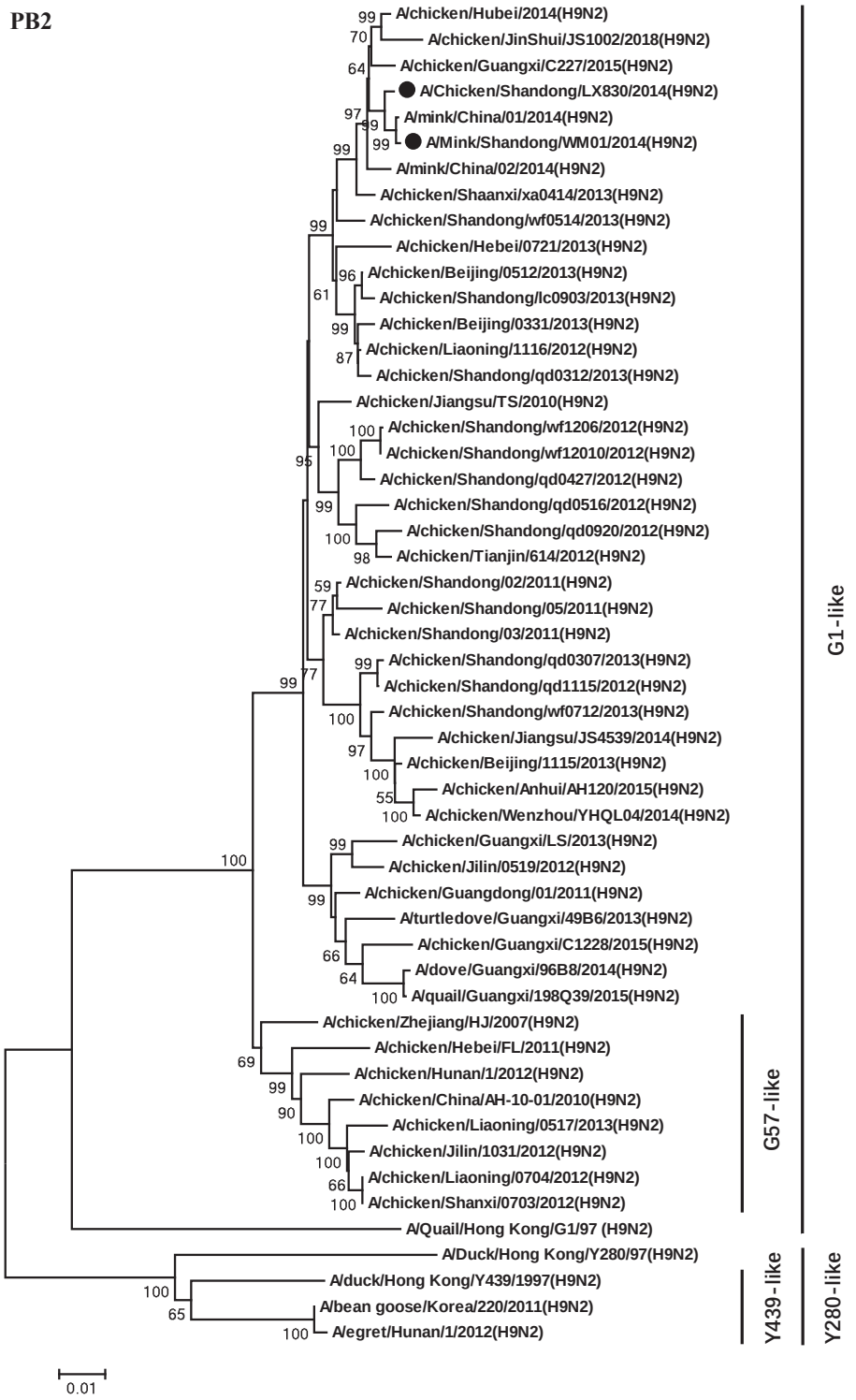

Fig. S1 Continued

Phylogenetic tree of *PB2* gene was generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic tree was based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus deposited in GenBank.

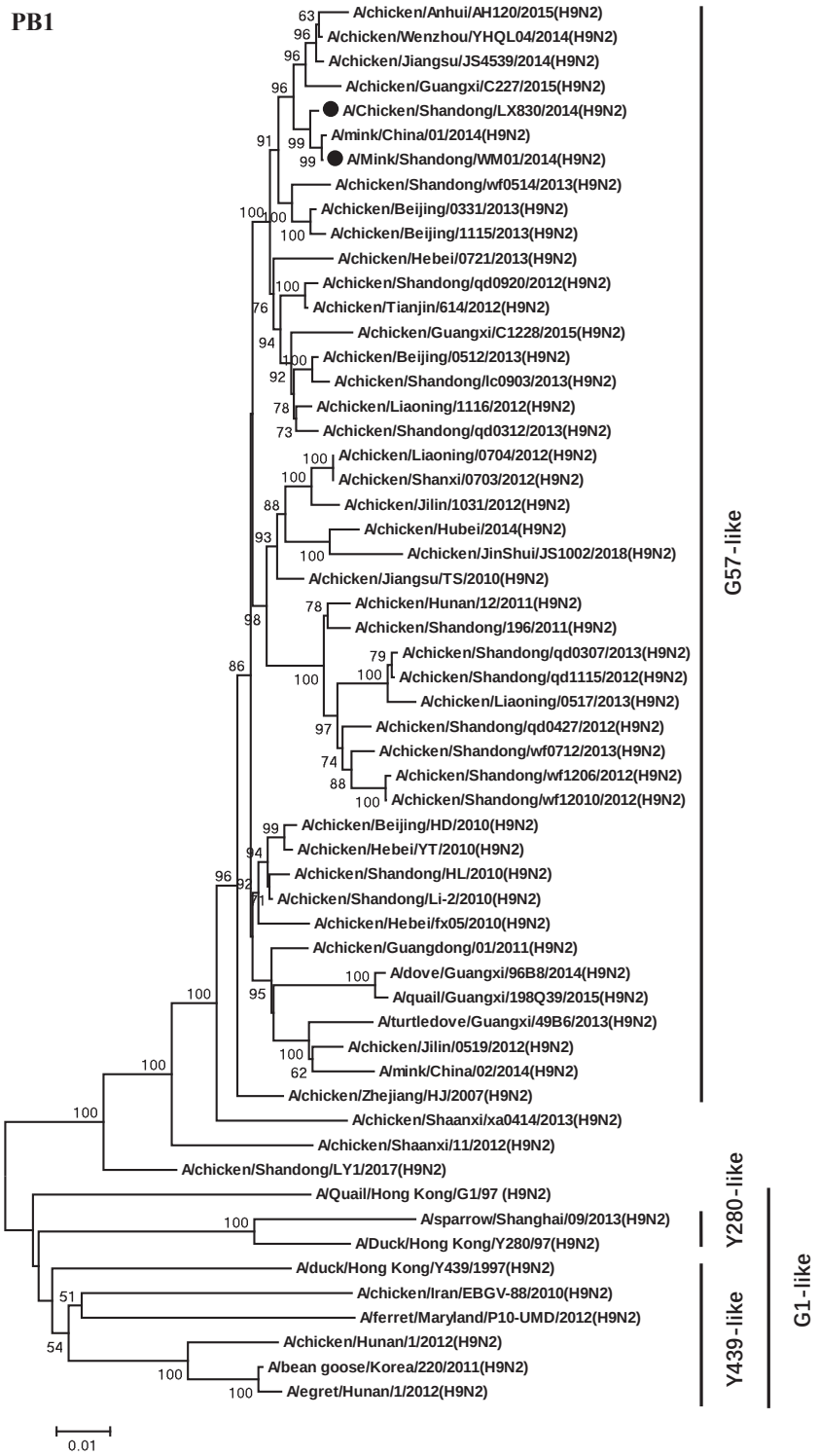

Fig. S1 Continued

Phylogenetic tree of *PB1* gene was generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic tree was based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus deposited in GenBank.

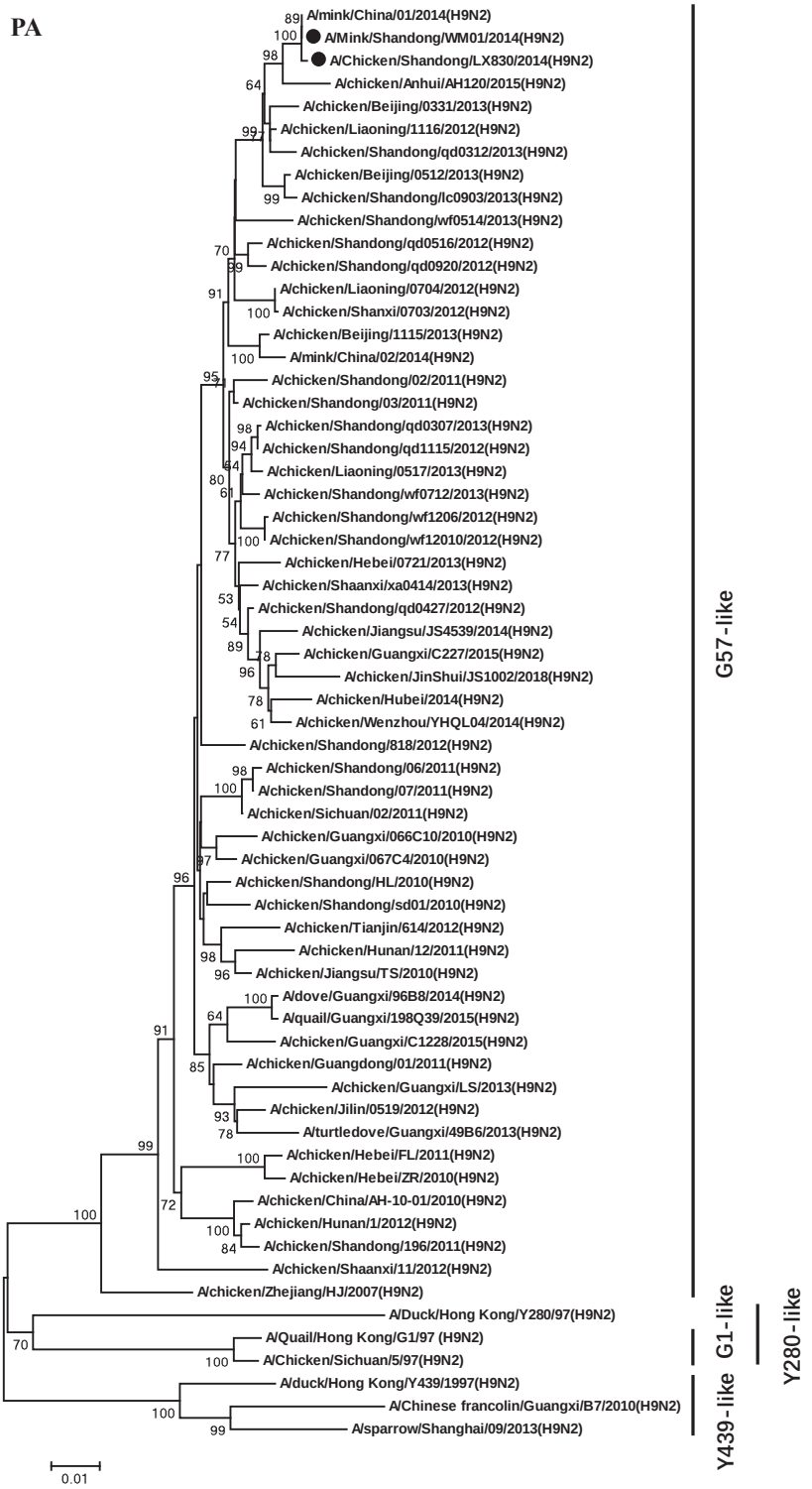

Fig. S1 Continued

Phylogenetic tree of *PA* gene was generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic tree was based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus deposited in GenBank.

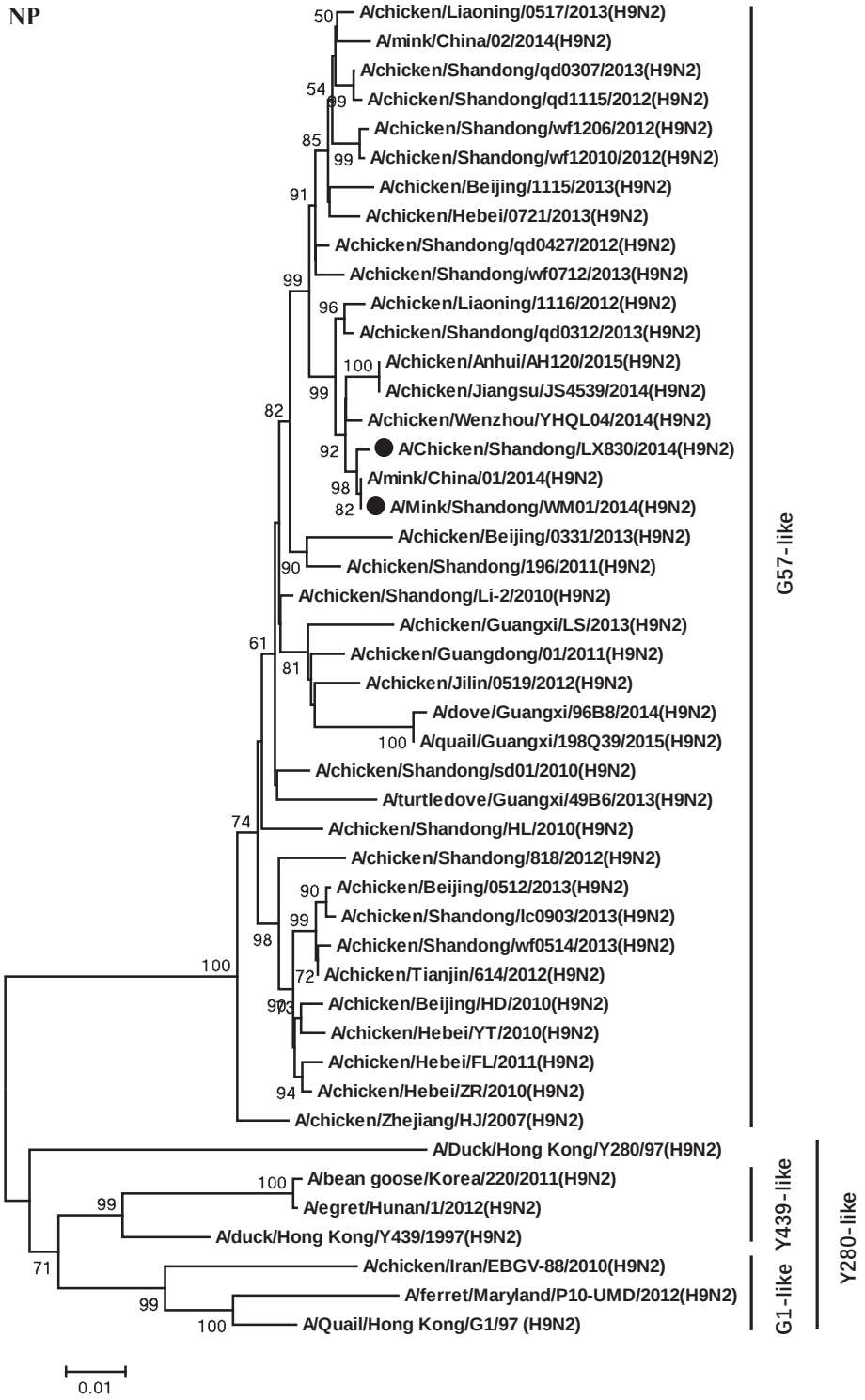

Fig. S1 Continued

Phylogenetic tree of *NP* gene was generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic tree was based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus deposited in GenBank.

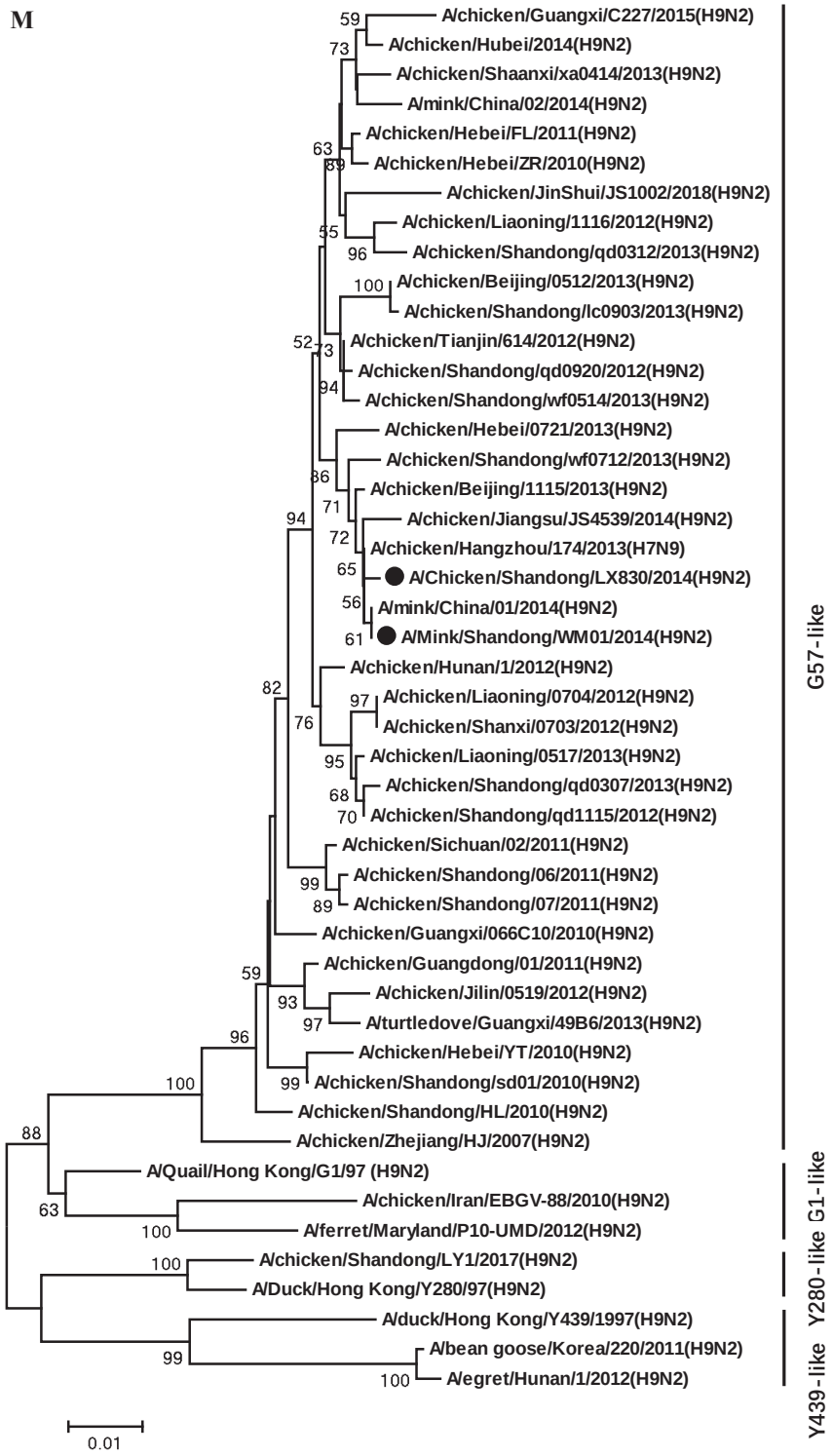

Fig. S1 Continued

Phylogenetic tree of *M* gene was generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic tree was based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus deposited in GenBank.

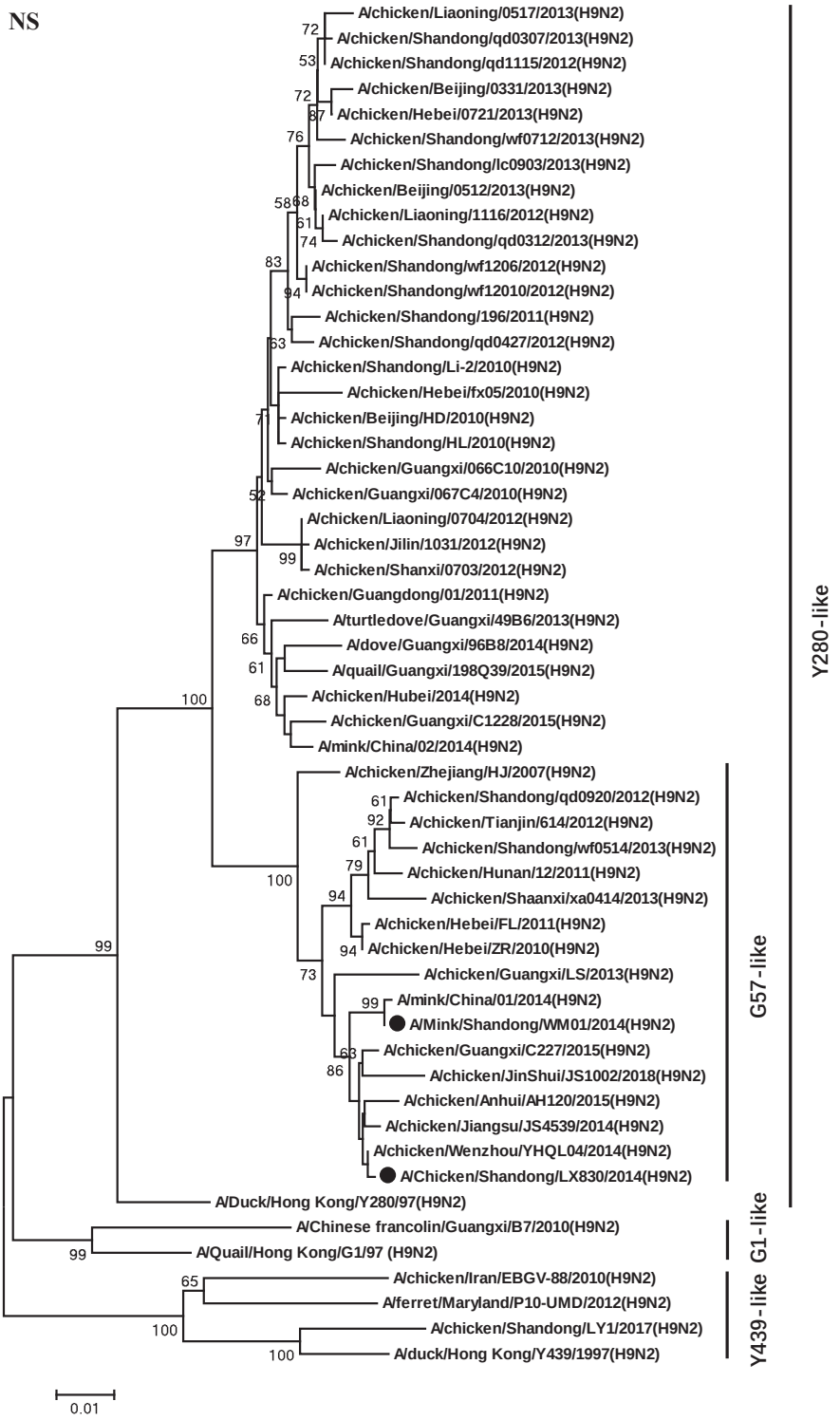

Fig. S1

Phylogenetic tree of *NS* gene was generated by the neighbor-joining method and bootstrapped with 1,000 replicates using MEGA software, version 6.05. Phylogenetic tree was based on the comparison of nucleotide sequences of the H9N2 avian influenza viruses isolated in this study (indicated by filled black circle) to the reference sequences of H9N2 influenza virus deposited in GenBank.