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Clade 2.3.2.1 H5N1 avian influenza viruses circulate at the interface of migratory and domestic birds around Qinghai Lake in China

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ABSTRACT

During 2012-2015, six H5N1 avian influenza viruses were isolated from domestic birds and the environment around Qinghai Lake. Phylogenetic analysis of HA genes revealed that A/chicken/Gansu/XG2/2012 (CK/GS/XG2/12) belonged to clade 2.3.2.1a, while A/environment/Qinghai/1/2013 (EN/QH/1/13), A/chicken/Qinghai/QH1/2015 (CK/QH/QH1/15), A/chicken/Qinghai/QH2/2015 (CK/QH/QH2/15), A/chicken/Qinghai/QH3/2015 (CK/QH/QH3/15), and A/goose/Qinghai/QH6/2015 (GS/QH/QH6/15) belonged to clade 2.3.2.1c. Further analysis of the internal genes of the isolates found that the PB2 gene of EN/QH/1/13 had 99.6% nucleotide identity with that of A/tiger/Jiangsu/1/2013 (H5N1), which clustered into an independent branch with PB2 from multiple subtypes. PB2, PB1, and M genes of CK/QH/QH3/15 were from H9N2, suggesting it was a reassortant of H5N1 and H9N2. Animal studies of three selected viruses revealed that CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 were highly lethal to chickens, with intravenous pathogenicity indexes (IVPIs) of 2.97, 2.81, and 3.00, respectively, and systemically replicated in chickens. In a mouse study, three selected H5N1 viruses were highly pathogenic to mice and readily replicated in the lungs, nasal turbinates, kidneys, spleens, and brains. Therefore, isolates in this study appear to be novel reassortants that were circulating at the interface of wild and domestic birds around Qinghai Lake and are lethal to chickens and mice. These data suggest that more extensive surveillance should be implemented, and matched vaccines should be chosen for the domestic birds in this area.

1. Introduction

As a major zoonosis worldwide for many years, influenza A virus infection has posed a threat to wild and domestic birds, other livestock, and humans. Among the various influenza viral subtypes, H5N1 is a causative agent for the highly pathogenic avian influenza (HPAI) that was first isolated from geese in Guangdong Province of China in 1996 (Xu et al., 1999). Subsequently, H5N1 cross-species transmission from birds to humans was first reported in 1997, resulting in 18 human infections and 6 deaths (Claas et al., 1998; Subbarao et al., 1998). From 2003 to 2016, H5N1 virus caused 854 human infections all over the world, among which 450 were lethal (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/).

Migratory birds are natural reservoirs of influenza A viruses. Sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes have been isolated from migratory birds. Among these, HPAI H5N1 viruses have been classified into 10 distinct clades (0-9) due to their significant genetic variation and antigenic drift (WHO, 2012), and each clade is further classified into different subclades based on divergence through phylogenetic analysis (Smith et al., 2015; World Health Organization/World Organisation for Animal and Agriculture Organization, 2014). Infected migratory birds, as mobile carriers, are able to transmit viruses to different areas during migration and affect domestic animals. Qinghai Province and its adjacent Gansu Province, located in northwestern China, are in a vital position for three flyways of migratory birds, including the Central Asia migratory flyway, the East Africa West Asia flyway, and the East Asian Australian flyway (Olsen et al., 2006). In particular, Qinghai Lake is a natural habitat for migratory birds for stopovers and breeding. However, an outbreak of H5N1 occurred in Qinghai Lake, causing more than 6000 bird deaths in May of 2005 (Chen et al., 2006, 2005; Liu et al., 2005); the responsible virus was subsequently classified as clade 2.2. Clade 2.2 HPAI H5N1 virus from Qinghai Lake was found to have a close genetic relationship with those isolated from other countries on the migratory flyways of wild birds. The virus eventually spread into other parts of Asia, Europe, and Africa, resulting in the deaths of millions of birds and hundreds of cases in humans (Chen et al., 2006; Fan et al., 2015; Pearce et al., 2017).

Clade 2.3.2 virus was first isolated in wild birds during 2008 in

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Japan, and then viruses of the same clade were isolated in wild birds in Shanghai, China (Fan et al., 2015). This clade was detected in migratory birds from May to June 2009 in Qinghai Lake (Hu et al., 2011; Li et al., 2011). Further phylogenetic analysis of the isolates of Qinghai Lake subsequently classified these strains into clade 2.3.2.1. In 2009, Zhou et al. reported that the wild pikas dwelling in Qinghai were carrying the same clade of virus (Zhou et al., 2009). Currently, clade 2.3.2.1 has evolved into clades 2.3.2.1a, 2.3.2.1b, and 2.3.2.1c in migratory birds (Jiang et al., 2017; Pearce et al., 2017) and is prevalent in domestic birds in many provinces of China.

During 2012-2015, six H5N1 influenza viruses were isolated in domestic birds and the environment around Qinghai Lake. We first investigated the evolutionary relationship of the six viruses from the interface of wild and domestic birds, and then their replication and pathogenicity in cells and animals were tested to determine the potential threat of these H5N1 viruses to poultry and public health.

2. Materials and methods

2.1. Facilities and ethics statement

All of the experiments with live H5N1 viruses were conducted within biosafety level 3 (BSL3) facilities. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal studies were approved by the Committee on the Ethics of Animal Experiments of the institutions.

2.2. Cells and animals

Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% fetal bovine serum plus with 100 UI/mL penicillin and 100 μg/mL streptomycin. A549 cells were maintained in F-12 K Nutrient Mixture (Gibco) supplemented with 10% fetal bovine serum plus with 100 UI/mL penicillin and 100 μg/mL streptomycin. All cells were incubated at 37 °C with 5% CO_2.

Specific-pathogen free (SPF) chicken eggs and chickens were purchased from Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China). Six-week-old female BALB/c mice were purchased from Beijing Vital River Co. Ltd. (Beijing, China). All of the animals were housed in individual isolators or ventilated cages and provided food and water *ad libitum*.

2.3. Sample collection and virus isolation

In 2012, a total of 30 nasal swabs were collected from chickens with influenza-like symptoms in a local farm in Gansu Province. Another 30 fecal and water samples were collected from the shore of Qinghai Lake in 2013. In 2015, tissues (trachea, lung, spleen, and pancreas) of four dead chickens and three sick geese were sampled from a local poultry farm in Golmud city near Qinghai Lake and immediately sent to the laboratory for virus isolation and identification. All of the individual samples were inoculated into 10-day-old embryonated chicken eggs for 48 h at 37 °C. The allantoic fluid samples were collected and tested for HA activity with 1% chicken red blood cells. Where HA assays were positive, hemagglutinin inhibition (HI) assays were performed to determine the HA subtype of the isolated avian influenza virus (AIV) and Newcastle disease virus (NDV). HA subtypes were first determined by chicken serum anti-HA of each subtype, then specific PCR was used to further identify HA and NA subtypes (primer sequences are available on request). The isolates were purified as virus stocks and designated as [A/chicken/Gansu/XG2/2012 (H5N1) (CK/GS/XG2/12)], [A/environment/Qinghai/1/2013 (H5N1) (EN/QH/1/13)], and [A/chicken/Qinghai/QH1/2015 (H5N1) (CK/QH/QH1/15), A/chicken/Qinghai/QH2/2015 (H5N1) (CK/QH/QH2/15), A/chicken/Qinghai/QH3/2015 (H5N1) (CK/QH/QH3/15), and A/goose/Qinghai/QH6/2015 (H5N1) (GS/QH/QH6/15)]. CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 were selected among them for the cell, chicken, and mouse studies.

2.4. DNA sequencing and phylogenetic analysis

Viral RNA extraction and RT-PCR amplification were described previously (Li et al., 2016) (primer sequences are available on request), and the PCR products of eight segments of six viruses were sequenced using specific sequencing primers by BGI (Beijing, China). The GISAID accession numbers for the genome sequences of six H5N1 reassortant AIVs are from EPI-ISL-350912 to EPI-ISL-350917. Sequence data were aligned by SEQMAN, and key amino acid sites were identified by MEGALIGN (DNASTAR, Madison, WI, USA). Reference sequences were downloaded from GenBank and GISAID databases. Phylogenetic analysis was conducted by using the neighbor-joining algorithm (MEGA 6.0 software). Bootstrap values of 1000 were used in this study.

2.5. Multi-cycle replication curves in cells

CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 viruses were inoculated into MDCK and A549 monolayers with 0.001 multiplicities of infection (MOI). One hour after infection, the cultures were replaced with fresh OPTI-MEM and incubated at 37 °C. Virus-containing culture supernatants were collected at 12, 24, 48, and 72 h post-infection (hpi) and titrated in 10-day-old chicken embryos. The growth data shown are the averages of three independent experiments.

2.6. Chicken study

As described elsewhere (Zhu et al., 2008), three groups of six-week-old SPF chickens (n = 10) were inoculated intravenously (iv) with 0.2 mL of 1:10 PBS-diluted virus. Chickens were monitored each day until 10 days after inoculation. Chickens with no clinical symptoms were given 0 points, those with clinical signs of diarrhea and labored breathing were marked with 1 point, and those with paralysis and death were given 2 and 3 points, respectively. Another three chickens were intranasally inoculated with 10^6 EID_{50} in a volume of 100 μL. Nasal swabs, cloacal swabs, brains, tracheae, lungs, spleens, kidneys, intestines, pancreases, and Fabricius bursae were collected on day 2 post-infection (dpi). The viral load was titrated in 10-day-old chicken embryos.

2.7. Mouse study

Following a previous study (Wang et al., 2018), eighteen groups of BALB/c mice (six-week-old female mice, n = 5 or 11) were lightly anesthetized with CO_2 and inoculated intranasally (i.n.) with 10^1-10^6 EID_{50}/50 μ L to investigate the pathogenicity and viral replication of three H5N1 viruses in mammals. Six mice in the 10^6 EID_{50} groups were killed on days 3 and 5 post-infection. Half of the various tissues were saved for virus titration (lungs, nasal turbinates, brains, kidneys, and spleens), and the rest were collected and fixed in formalin for hematoxylin and eosin (H&E) staining. The remaining five mice in each group were monitored daily for body weight change until 14 days, and mice were sacrificed when the body weight loss was greater than 25%, which was considered death.

3. Results

3.1. Clade 2.3.2.1a and 2.3.2.1c H5N1 avian influenza viruses were isolated from migratory and domestic birds around Qinghai Lake

From 2012 to 2015, six H5N1 influenza viruses were isolated from sick domestic birds and the environment around Qinghai Lake. Of these

Table 1
The highest nucleotide identity (%) of three isolates of representative influenza viruses with three reference strains in GenBank.

Virus	Gene segment	Virus with the highest nucleotide identity	Identity
A/chicken/Gansu/XG2/2012 (H5N1)	PB2	A/duck/Hunan/S4030/2011 (H5N1)	99.8%
	PB1	A/duck/Hunan/S4030/2011 (H5N1)	99.7%
	PA	A/duck/Hunan/S4030/2011 (H5N1)	99.6%
	HA	A/duck/Hunan/S4030/2011 (H5N1)	99.5%
	NP	A/duck/Hunan/S4030/2011 (H5N1)	99.5%
	NA	A/duck/Hunan/S4030/2011 (H5N1)	99.8%
	M	A/duck/Hunan/S4030/2011 (H5N1)	100.0%
	NS	A/duck/Hunan/S4030/2011 (H5N1)	99.8%
A/environment/Qinghai/1/2013 (H5N1)	PB2	A/tiger/Jiangsu/1/2013 (H5N1)	99.6%
	PB1	A/tiger/Jiangsu/1/2013 (H5N1)	99.5%
	PA	A/tiger/Jiangsu/1/2013 (H5N1)	99.6%
	HA	A/tiger/Jiangsu/1/2013 (H5N1)	99.5%
	NP	A/tiger/Jiangsu/1/2013 (H5N1)	99.9%
	NA	A/tiger/Jiangsu/1/2013 (H5N1)	99.7%
	M	A/tiger/Jiangsu/1/2013 (H5N1)	99.6%
	NS	A/tiger/Jiangsu/1/2013 (H5N1)	99.5%
A/chicken/Qinghai/QH3/2015 (H5N1)	PB2	A/peacock/Yunnan/1/2015 (H5N1)	99.0%
	PB1	A/peacock/Yunnan/1/2015 (H5N1)	99.4%
	PA	A/peacock/Yunnan/1/2015 (H5N1)	99.3%
	HA	A/peacock/Yunnan/1/2015 (H5N1)	99.0%
	NP	A/peacock/Yunnan/1/2015 (H5N1)	97.7%
	NA	A/peacock/Yunnan/1/2015 (H5N1)	99.3%
	M	A/peacock/Yunnan/1/2015 (H5N1)	99.3%
	NS	A/peacock/Yunnan/1/2015 (H5N1)	99.4%

six strains, CK/GS/XG2/12 was an earlier one from chickens in Gansu Province, (EN/QH/1/13) was from the water of Qinghai Lake in 2013, and the other four viruses (CK/QH/QH1/15, CK/QH/QH2/15, CK/QH/QH3/15, and GS/QH/QH6/15) were isolated from dead chickens and geese with influenza-like symptoms in Golmud city near Qinghai Lake in 2015.

To analyze the genetic relationships between these AIVs circulating around Qinghai Lake, we fully sequenced the eight segments (HA, NA, PB2, PB1, PA, NP, M, and NS genes) of the six strains. Among them, three selected isolates shared 97.7-100% identity with the representative H5N1 strains (A/duck/Hunan/S4030/2011, A/tiger/Jiangsu/1/2013, and A/peacock/Yunnan/1/2015) at the nucleotide level (Table 1). HA of CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 respectively had 99.5%, 99.5%, and 99% identity with that of the reference strains isolated from Hunan, Jiangsu, and Yunnan in China, while the HA gene of CK/GS/XG2/12 clustered with clade 2.3.2.1a, and the other five isolates from Qinghai Province belonged to clade 2.3.2.1c (Table 1 and Fig. 1).

Generally, the HA, NA, PB2, PB1, or NP genes formed three distinct groups in their phylogenetic trees, while the PA and M genes clustered into two branches in the trees. The NS genes of the six strains were shown to form one branch in the tree (Fig. S1).

To further observe the evolutionary relationships, as shown in Table 1 and Fig. 1, CK/GS/XG2/12 shared the highest homology with A/duck/Hunan/S4030/2011 (H5N1). However, EN/QH/1/13 from the environment around Qinghai Lake appeared to be a close genetic relative with the tiger-originating H5N1 influenza virus, A/tiger/Jiangsu/1/2013 (H5N1), that was derived from birds. Notably, the PB2 gene of EN/QH/1/13 clustered into an independent branch with that of H3N8, H4N6, and H10N8, in addition to that of H5N1. The other four H5N1 viruses isolated from chickens and geese possessed a similar phylogenetic character with a zoo-emerging H5N1, A/peacock/Yunnan/1/2015 (H5N1). Among the four H5N1 strains, the PB2 gene of CK/QH/QH1/15 and the PB1 genes of CK/QH/QH1/15 and CK/QH/QH2/15 had a closer relationship with A/chicken/Hunan/1/2012(H9N2), while the M gene of these four strains had a close correlation with A/peacock/Yunnan/1/2015 (H5N1), which may have evolved from H9N2 viruses. Therefore, H5N1 viruses circulating in wild or domestic birds around Qinghai Lake are designated as clades 2.3.2.1a and 2.3.2.1c, in which novel reassortants have appeared in this area.

3.2. Three selected H5N1 strains display molecular characteristics of highly pathogenic avian influenza viruses

Due to having the highest genetic divergence and being from different phylogenetic branches of the six strains, CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 viruses were selected for further analysis of their molecular characteristics. As shown in Table 2, a multiple basic amino acid motif, PQRERRRKR/GLF, appearing in the HA cleavage sites of the three isolates met the characteristic of highly pathogenic AIV in chickens; furthermore, the amino acids in HA of the three isolates at positions 226 and 228 were Q and G, suggesting their preferable affinity to the α2, 3-type receptor, which is predominant in avian species (Vines et al., 1998). However, CK/GS/XG2/12 and EN/QH/1/13 possessed A at position 160, possibly indicative of increased affinity to the human α2, 6-type receptor (Gao et al., 2009). All three H5N1 viruses retained H275 and N294 in NA, indicating sensitivity to oseltamivir (Govorkova et al., 2013). Mutations at positions 627 K and 701 N of the PB2 protein were not detected in the three selected viruses; these are important for adaptation of avian influenza viruses to mammals (Hatta et al., 2001; Li et al., 2005). The three isolated viruses had no specific mutations in the NP, PB1, PA, or PB2 proteins, which are associated with increased virulence or polymerase activity. In addition, amino acids M1 30D and 215A, and NS1 42S in the three isolates indicated the possibility of increased virulence in mice (Fan et al., 2009; Jiao et al., 2008). Unlike NA of the three strains, the M2 of CK/QH/QH3/15 harboring 31 N seems to be resistant to amantadine drugs (Lee et al., 2008).

3.3. Three H5N1 avian influenza viruses efficiently replicate in mammalian cells

To test viral replication *in vitro*, CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 strains were chosen to infect MDCK and A549 cells with an MOI of 0.001. The three viruses readily replicated in MDCK cells, which are a classical cell line for the propagation of influenza viruses, starting from 12 hpi, and basically reached a peak at 48 hpi. However, EN/QH/1/13 virus replicated slightly less than the other two strains (Fig. 2A). In human A549 cells, which are an epithelial cell line targeted by influenza virus, the viral replication of the three strains had a similar trend with that in MDCK cells from 12 to 72 hpi, although the

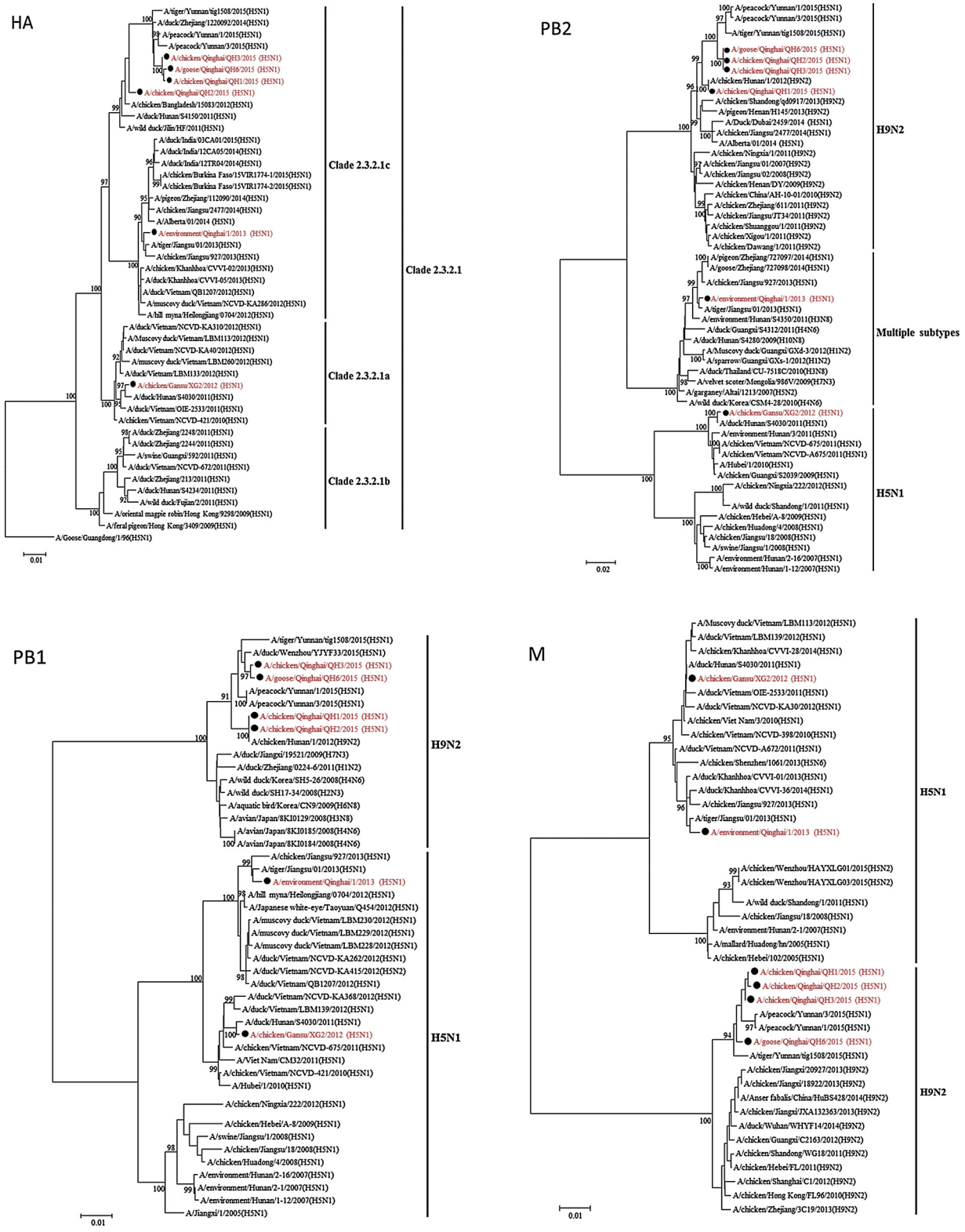

Fig. 1. Genetic relationships among the HA, PB2, PB1, and M genes of six H5N1 avian influenza viruses. The tree was based on nucleotides (nt) 22-1728, 28-2307, 25-2298, and 26-1007. Viruses with names in red and black solid circles were sequenced in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

three viruses had much lower titers at the initial time point (Fig. 2B). The results of viral replication kinetics indicated that the three selected AIVs from wild and domestic birds were capable of replicating well in mammalian cells.

3.4. Three H5N1 avian influenza viruses are highly pathogenic to chickens

To investigate the virulence of three H5N1 viruses in SPF chickens, we tested the intravenous pathogenicity index (IVPI) and viral replication in chickens. IVPIs of CK/GS/XG2/12, EN/QH/1/13, and CK/

Table 2
Molecular characteristics of three isolates presented in this study.

Protein	Amino acid	CK/GS/XG2/12	EN/QH/1/13	CK/QH/QH3/15	Changes of phenotypic impacts
HA	Cleavage site	PQRERRRKR/GLF	PQRERRRKR/GLF	PQRERRRKR/GLF	HA cleavage site
	H107Y	H	H	H	Increased transmissibility in ferrets
	I155T	I	I	I	Increased affinity to human-type receptor
	T160A	A	A	T	
	Q226L	Q	Q	Q	
	G228S	G	G	G	
NA	H274Y	H	H	H	Reduced oseltamivir susceptibility
	N294S	N	N	N	
PB2	E158G	E	E	E	Enhanced polymerase activity and virulence
	Q591K	Q	Q	Q	Enhanced replication efficiency and virulence in mice
	E627 K	E	E	E	Mammalian host adaption
	D701 N	D	D	D	Increased virulence in mice
PB1	H99Y	H	H	H	Increased transmissibility in ferrets
	I368V	I	I	I	
	G622D	G	G	G	Increased virulence in mice
PB1-F2	N66S	N	N	N	Increased virulence in mice
PA	T97I	T	T	T	Enhanced polymerase activity and virulence
NP	N319K	N	N	N	Increased replication efficiency
M1	N30D	D	D	D	Increased virulence in mice
	T215A	A	A	A	
M2	S31 N	S	S	N	Reduced susceptibility to amantadine and rimantadine
NS1	P42S	S	S	S	Increased virulence in mice

QH/QH3/15 were 2.97, 2.81, and 3.00, respectively, indicating that these are highly pathogenic isolates in chickens (Table 3). Then we tested viral replication in different organs of chickens and found that all three strains were recovered from brains, spleens, kidneys, trachea, lungs, pancreases, intestines, and Fabricius bursae with high viral loads, suggesting that the viruses systematically replicated in chickens. Moreover, high virus titers were detected from throat and cloacal swabs at 2 dpi, suggesting that chickens shed viruses from the respiratory tract and feces and may transmit the viruses to other birds directly. The chicken study indicated that the selected strains were highly pathogenic and could systemically replicate in chickens.

3.5. Three H5N1 avian influenza viruses are lethal to BALB/c mice

To evaluate the virulence of three H5N1 viruses to mammals, we tested their replication and lethality in mice. On 3 dpi and 5 dpi, three mice in each group were sacrificed to collect nasal turbinates, lungs, kidneys, livers, and brains for the evaluation of viral replication. CK/GS/XG2/12 and CK/QH/QH3/15 viruses were detected in all collected organs with high viral loads at the indicated time points; however, EN/QH/1/13 only efficiently replicated in lungs and nasal turbinates, but not in brain, spleen, or kidneys (Fig. 3A). We collected a half lung sample from each mouse in the group of the 10^6 EID_{50} dose to perform pathological studies. The three viral strains caused pulmonary interstitial and alveolar edema and inflammatory cell infiltration. However, CK/GS/XG2/12 induced the most severe damage in the lungs at 5 dpi (Fig. 3B). As shown in Fig. 4, the CK/GS/XG2/12 virus was the most lethal to mice and caused 100% death at a minimum of 10^2 EID_{50} dose. CK/QH/QH3/15 virus infection with 10^4 or 10^5 EID_{50} led to a 40% survival rate in mice, while EN/QH/1/13 virus was comparatively mild and did not cause the death of mice infected with a 10^4 EID_{50} dose. Taken together, the data from the mouse study indicated that the three H5N1 viruses were highly lethal to mice and could replicate in them.

4. Discussion

Qinghai Lake in northwestern China is a protected natural reserve for wild birds and is a major breeding site for migratory birds whose flyways extend to southeast Asia, India, Siberia, Australia, and New Zealand (Chen et al., 2006). Domestic poultry farms in this region are poorly built with no biosecurity, making this region an excellent ecosystem for AIV transmission *via* cohabitation of domestic and migratory birds. This pattern of breeding and environment facilitates interspecies transmission and viral gene reassortment. In the present study, three locations for sampling collection were within the range of the flyways of wild birds, given that Xigu of Gansu Province and Golmud of Qinghai Province are within 400 km of Qinghai Lake. Six H5N1 influenza viruses were collected and identified from sick domestic birds and the environment at these three sites during 2012-2015. Phylogenetic analysis revealed that six viruses were in clade 2.3.2.1 with a novel gene

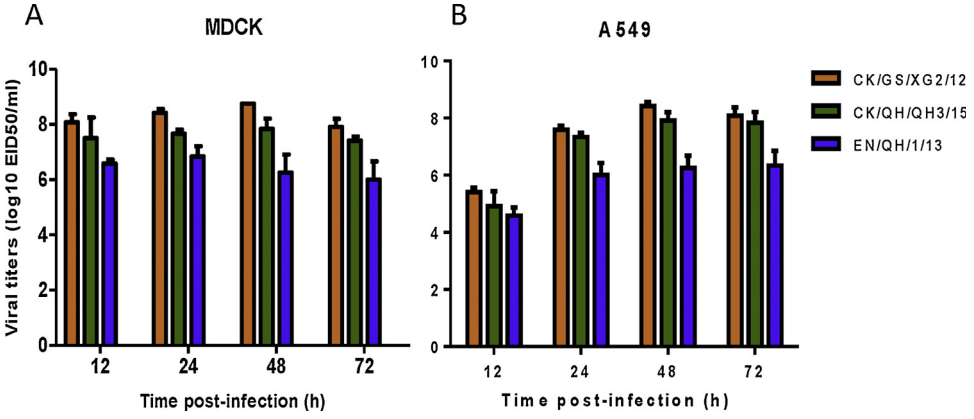

Fig. 2. Replication curves of three selected avian influenza viruses in mammalian cells. MDCK and A549 cells were inoculated with CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 avian influenza viruses (0.001 MOI), and supernatants were collected for titration at the indicated time points. (A) Viral growth curves in MDCK cells. (B) Viral growth curves in A549 cells. Data shown are viral titers (log_{10} EID_{50}/mL), and the dashed lines indicate lower limits of detection.

Table 3
Pathogenicity of the selected H5N1 viruses in chickens.

Virus	dpi	Viral titers in different tissues ($log_{10}EID_{50}$/mL)									IVPI
		Brain	Spleen	Kidney	Trachea	Lung	Pancreas	Intestine	Fabricius Bursae	Cloacal Swab	Throat Swab	
CK/GS/XG2/12	2	6.67 ± 0.52	8.33 ± 0.14	9.17 ± 0.38	7.75 ± 0	7.25 ± 0.66	8.33 ± 0.14	7.92 ± 0.29	8.67 ± 0.88	5.25 ± 0.66	6.08 ± 0.58	2.97
EN/QH/1/13	2	7.08 ± 0.72	7.92 ± 0.52	8.42 ± 1.13	7.42 ± 0.14	7.96 ± 0.69	8.17 ± 0.80	7.42 ± 1.53	7.42 ± 0.76	3.83 ± 0.38	5.58 ± 0.58	2.81
CK/QH/QH3/15	2	7.5 ± 0	8.58 ± 0.14	8.67 ± 0.14	7.75 ± 0.43	7.42 ± 0.29	7.42 ± 0.29	7.67 ± 0.52	9 ± 0.43	6.67 ± 0.14	6.58 ± 0.58	3.00

reassortment in which PB2, PB1, or M genes may have originated from H9N2 or other subtypes. Animal studies showed that three selected H5N1 viruses were lethal to chickens and mice and showed systemic replication in different tissues. The present study suggests that novel avian influenza virus variants exist at the interface of migratory and domestic bird populations around Qinghai Lake in China and pose a threat to poultry breeding in this area.

Transmission and replication of influenza viruses across host barriers require adaptation, as influenza viruses overcome host barriers through antigenic shift and drift in order to increase replication and pathogenicity in a new host. Glycoprotein HA of influenza virus plays an important role during this process. Generally, avian influenza viruses prefer to bind with NeuA(α2,3), while human or mammalian influenza viruses prefer to bind with NeuA(α2,6). Multiple amino acids of HA are the determinants of whether viruses prefer to bind different types of sialic acid receptors. Q226 L and G228S (H3 numbering) change the binding properties of influenza viruses from NeuA(α2, 3) to NeuA(α2, 6) (Liu et al., 2009; Vines et al., 1998; Viswanathan et al., 2010). In addition to HA, positions E627 K and D701 N of PB2 are two hallmarks for virulence that increase and enhance polymerase activity and mammalian host adaptation (Chen et al., 2007; Hatta et al., 2001; Li et al., 2005; Shinya et al., 2004; Subbarao et al., 1993). In the present study, the main molecular characteristics of the H5N1 isolates were avian-like features including Q226 and G228 of HA, and E627 and D701 of PB2. However, while on the one hand the *in vitro* study indicated that three selected H5N1 viruses replicated efficiently in MDCK and A549 cells, on the other hand the mouse study showed that these viruses were lethal and systemically replicated in mice without prior adaptation, suggesting that other unknown factors may play important roles in replication and pathogenicity in mice. Notably, both EN/QH/1/13 and CK/QH/QH3/15 isolates were genetically highly homologous with two zoo-emerging strains that cause lethal infections in tigers (He et al., 2015; Hu et al., 2016). This confirms that the selected viruses might have acquired the ability to infect mammalian hosts, even without prior adaptation.

H9N2 viruses usually silently circulate in avian species due to their low pathogenicity; however, they can undergo host adaptation and gene reassortment with other subtypes, resulting in a potential threat to public health. The H9N2 subtype has acquired the ability to bind both avian and mammalian receptors and to transmit among ferrets (Li et al., 2014). In March of 2013, the avian-originating reassortant H7N9 caused three human deaths in China, with six internal genes having come from H9N2 (Gao et al., 2013). In this study, our data showed that the PB2, PB1, and M genes of CK/QH/QH3/15 came from H9N2 originally, suggesting that this reassortant is evolutionarily different from EN/QH/1/13. It is worth noting that the novel reassortants of clade 2.3.2.1c have internal genes (PB2, PB1, or M) from H9N2. However, EN/QH/1/13 and CK/QH/QH3/15 belong to clade 2.3.2.1c as well, in which the phylogenetic tree showed that the PB2 gene of EN/QH/1/13 had high homology with different PB2 genes from H5N1, H3N8, H4N6, and H10N8, leading to difficulty in clarifying the actual origin of PB2; but in other studies, the PB2 gene is readily classified into a corresponding group (Deng et al., 2015, 2013; Kang et al., 2013; Liang et al., 2016), suggesting viral complexity in domestic and wild birds. Therefore, it is necessary to strengthen the surveillance of novel variants of animal influenza viruses in different species, including wild birds, in these flyways with DNA barcoding techniques (Lee et al., 2010a, b; Tavares and Baker, 2008).

In addition to Qinghai Province, clade 2.3.2.1c has recently been identified in many regions across the world. Human infection by H5N1 was reported in Alberta, Canada, in 2014. Phylogenetic analysis revealed that this human H5N1 virus belongs to clade 2.3.2.1c; furthermore, the PB2 gene was found to have originated from H9N2 (Pabbaraju et al., 2014). In the spring of 2015, clade 2.3.2.1c H5N1 bearing H9N2 PB2 was also isolated from rooks in Western Siberia (Marchenko et al., 2016), ducks in India (Tosh et al., 2016), and falcons

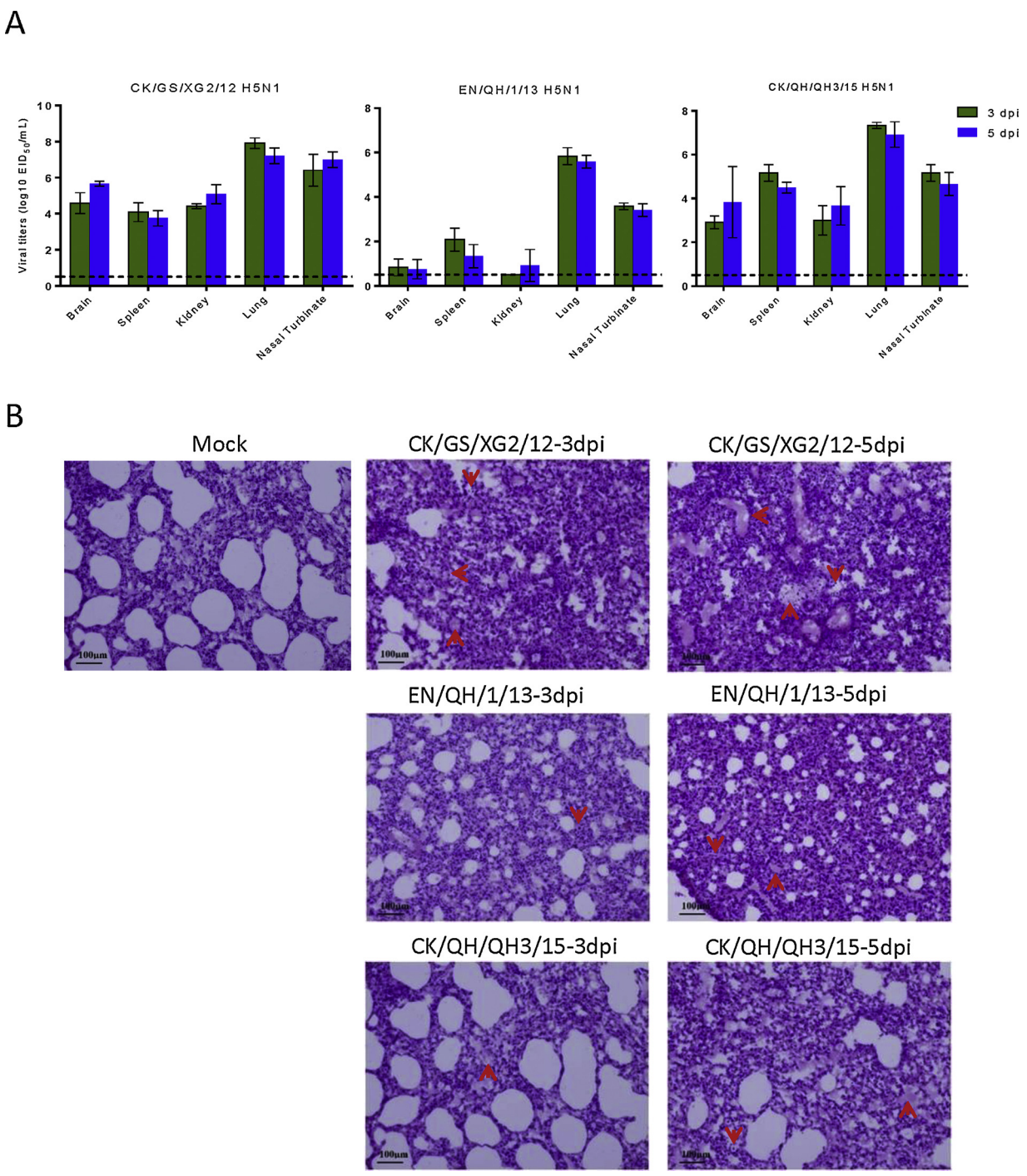

Fig. 3. Replication and histological lesions caused by three selected avian influenza viruses in the lungs of mice. (A) Virus replication in mice was assessed on 3 dpi and 5 dpi with 10^6 EID_{50} of CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 avian influenza viruses. Data shown are the mean titers from three mice; the error bars indicate the standard deviations. The dashed line indicates the lower limit of detection. (B) Mice were killed on 3 dpi and 5 dpi with 10^6 EID_{50} of the test viruses, and the lungs were collected for pathological study (the red arrow points to typical damage). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in Dubai (Naguib et al., 2015). Migratory birds in Inner Mongolia of China were also detected to have clade 2.3.2.1c H5N1 from 2014 to 2015 (Bi et al., 2016). Another two zoo outbreaks were reported in 2015 and 2016 that caused deaths of tigers and peacocks (He et al., 2015; Hu et al., 2016). Obviously, these sporadic occurrences in these regions might be closely related to that in Qinghai Lake, which is an important location for migratory bird breeding or stopovers.

Taken together, in the present study, we analyzed six avian influenza viruses isolated around Qinghai Lake at the interface of wild and domestic bird populations, an area that is an important world flyway. Genetic analyses indicated that all the viruses belong to clades 2.3.2.1a and 2.3.2.1c and that PB2, PB1, and M genes might have reassorted from H9N2 or other subtypes. Chicken and mouse studies suggested that the viruses are highly pathogenic and can replicate efficiently in animals. We presume that migratory birds stopping in Qinghai Lake contribute to the circulation of influenza viruses in domestic birds and across different countries. Therefore, it is of great importance to implement more extensive surveillance of AIVs in migratory and domestic

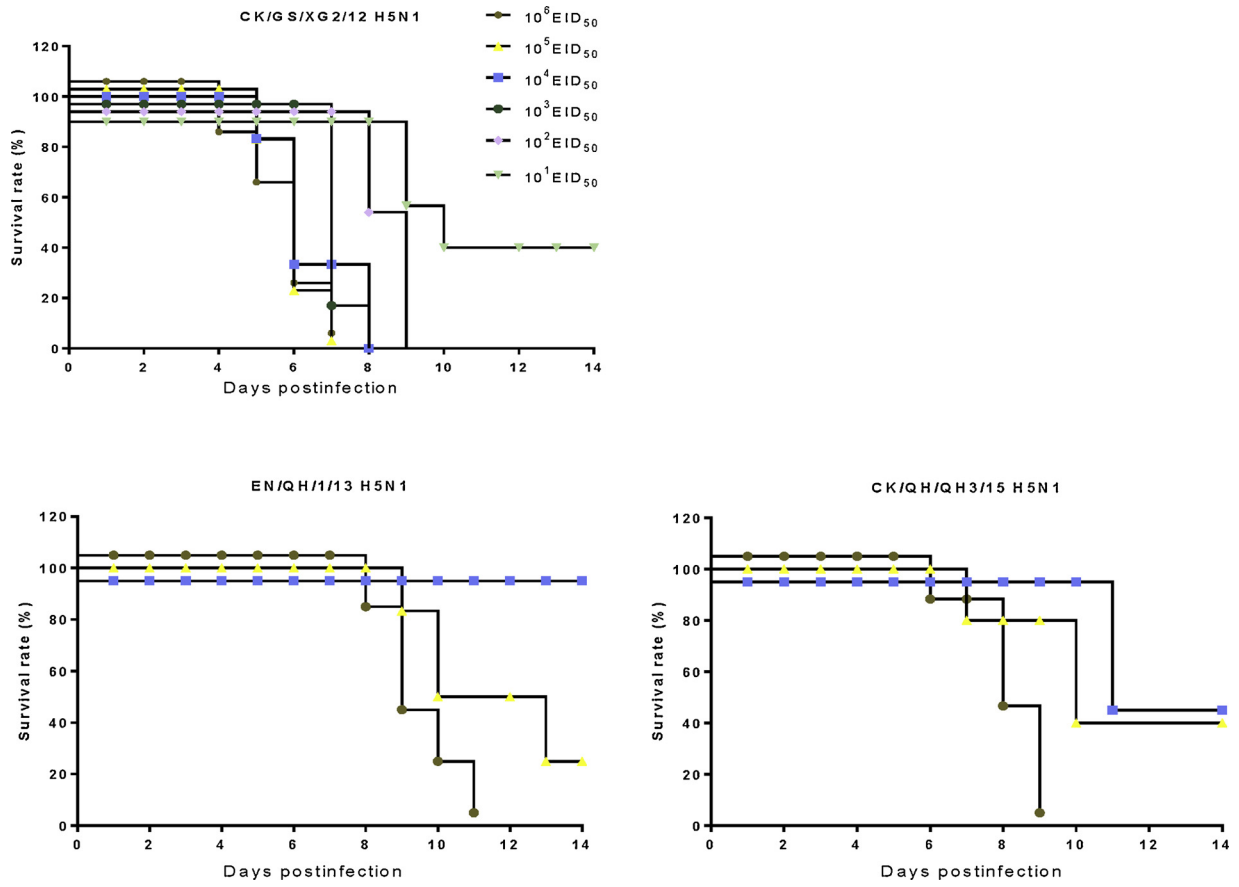

Fig. 4. Lethality of three selected avian influenza viruses in mice. Survival rates of mice infected with CK/GS/XG2/12, EN/QH/1/13, and CK/QH/QH3/15 avian influenza viruses are shown.

birds and to provide matched vaccines and enhance the vaccination coverage for domestic birds around Qinghai Lake in order to reduce the rates of infection and transmission.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.009.

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