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Evolution and biological characterization of H5N1 influenza viruses bearing the clade 2.3.2.1 hemagglutinin gene

Xin Xing[a]*, Jianzhong Shi [a,b,c]*, Pengfei Cui[a], Cheng Yan[a], Yaping Zhang[a], Yuancheng Zhang[a], Congcong Wang[a], Yuan Chen[a], Xianying Zeng[a], Guobin Tian[a], Liling Liu[a], Yuntao Guan[a,d], Chengjun Li [a,b], Yasuo Suzuki[e], Guohua Deng[a] and Hualan Chen [a,b,d]

[a]State Key Laboratory for Animal Disease Control and Prevention, Harbin Veterinary Research Institute, CAAS, Harbin, People's Republic of China; [b]Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, People's Republic of China; [c]Institute of Western Agriculture, CAAS, Changji, People's Republic of China; [d]National Poultry Laboratory Animal Resource Center, Harbin Veterinary Research Institute, CAAS, Harbin, People's Republic of China; [e]Department of Medical Biochemistry, University of Shizuoka School of Pharmaceutical Sciences, Shizuoka, Japan

ABSTRACT

H5N1 avian influenza viruses bearing the clade 2.3.2.1 hemagglutinin (HA) gene have been widely detected in birds and poultry in several countries. During our routine surveillance, we isolated 28 H5N1 viruses between January 2017 and October 2020. To investigate the genetic relationship of the globally circulating H5N1 viruses and the biological properties of those detected in China, we performed a detailed phylogenic analysis of 274 representative H5N1 strains and analyzed the antigenic properties, receptor-binding preference, and virulence in mice of the H5N1 viruses isolated in China. The phylogenic analysis indicated that the HA genes of the 274 viruses belonged to six subclades, namely clades 2.3.2.1a to 2.3.2.1f; these viruses acquired gene mutations and underwent complicated reassortment to form 58 genotypes, with G43 being the dominant genotype detected in eight Asian and African countries. The 28 H5N1 viruses detected in this study carried the HA of clade 2.3.2.1c (two strains), 2.3.2.1d (three strains), or 2.3.2.1f (23 strains), and formed eight genotypes. These viruses were antigenically well-matched with the H5-Re12 vaccine strain used in China. Animal studies showed that the pathogenicity of the H5N1 viruses ranged from non-lethal to highly lethal in mice. Moreover, the viruses exclusively bound to avian-type receptors and have not acquired the ability to bind to human-type receptors. Our study reveals the overall picture of the evolution of clade 2.3.2.1 H5N1 viruses and provides insights into the control of these viruses.

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KEYWORDS Avian influenza virus; H5N1; clade 2.3.2.1; evolution; pathogenicity; antigenicity

Introduction

Influenza A virus is a single-stranded negative-sense RNA virus. The viral genome consists of eight gene segments that mainly encode the basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural (NS) proteins. Based on the antigenic differences of the HA and NA surface glycoproteins, the viruses are divided into different subtypes, of which 16 different HA subtypes and nine different NA subtypes have been identified in aquatic birds; the H17N10 and H18N11 subtypes have been detected in bats [1,2].

The H5N1 highly pathogenic avian influenza virus was first detected in chickens in Scotland in 1959 [3]. In 1991, an H5N1 virus caused a disease outbreak in turkeys in England [4]. H5N1 virus was first detected in China on a goose farm in 1996 [5–7]. After long-term circulation in wild birds and domestic poultry in nature, H5 viruses have undergone extensive evolution by accumulating mutations and reassorting with other influenza virus subtypes [8,9]. The HA gene of the H5 viruses detected over the past two decades has evolved into 10 distinct clades, ranging from clade 0 to clade 9, and some of these clades are further categorized into different subclades [10].

The H5N1 viruses bearing the clade 2.3.2.1 HA gene (clade 2.3.2.1 viruses) were detected in wild birds in Hong Kong in 2007 [11]. Subsequently, the virus was detected from different wild birds in Japan in 2008 [12], in Mongolia in 2009 [13], in China in 2009 [14], in Bulgaria in 2010 [15], in Korea in 2011 [16], and in the United Arab Emirates in 2014 [17]. Moreover, disease outbreaks in domestic poultry

CONTACT Guohua Deng dengguohua01@caas.cn; Hualan Chen chenhualan@caas.cn
*These authors contributed equally to this manuscript.
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caused by the clade 2.3.2.1 viruses have been reported in Bangladesh, China, Indonesia, India, Japan, Nepal, and Vietnam, which have caused disastrous consequences for the poultry industry [18,19].

Although the clade 2.3.2.1 viruses are widely detected in birds around the globe and in humans in Asia, the genetic relationship of the strains detected in different countries is largely unknown, and their biological properties are rarely evaluated. In this study, we performed a detailed genetic analysis of 274 H5N1 representative viruses detected in different countries since 2007. We further evaluated the pathogenicity in mice, receptor binding, and antigenicity of the H5N1 representative viruses detected in recent years in China. Our study provides important information about the evolution and dissemination of different clade 2.3.2.1 viruses and provides insights into their control.

Materials and methods

Ethical statements

The 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 China. The protocol for the animal studies was approved by the Committee on the Ethics of Animal Experiments of the HVRI, CAAS.

Virus isolation and identification

Swab and fecal samples collected during active surveillance were processed in the enhanced biosafety level 2 (BSL2+) facility at our institute. Organ samples collected from dead birds during passive surveillance were processed in the animal biosafety level 3 (ABSL3) facility at our institute. Oropharyngeal and cloacal swabs from the same bird were put in the same sample collection tube and counted as one sample. All samples were individually inoculated into 10-day-old embryonated chicken eggs and incubated for 48 h at 37 °C. The HA subtype was identified by using the hemagglutinin inhibition (HI) test and the NA subtype was confirmed by direct sequence analysis. The H5N1 viruses sequenced in this study were biologically cloned three times by limiting dilution in embryonated specific-pathogen-free (SPF) chicken eggs, and the viral stocks were grown in SPF chicken eggs and maintained at −70°C.

Genetic and phylogenetic analyses

The genome of the H5N1 viruses was sequenced on an Applied Biosystems DNA Analyzer (3500xL Genetic Analyzer, USA). The nucleotide sequences were edited with the SeqMan module of the DNAStar package. Phylogenetic analysis of the HA gene of the H5N1 representative viruses was performed by using an asymmetric continuous-time Markov chain with Bayesian stochastic search variable selection implemented in BEAST (v1.10.4). A preliminary check using TempEst (v1.5.1) confirmed a significant temporal signal in our dataset, which was proven by linear regression of the root-to-tip distance against the sampling date (R^2 = 0.859, correlation coefficient = 0.927). The GTR + F + I + G4 substitution model, which was selected by using the Bayesian information criterion by ModelFinder in IQ-TREE, was used along with an uncorrelated lognormal relaxed molecular clock and a Bayesian Skygrid coalescent tree prior. Markov Chain Monte Carlo (MCMC) chains were run enough iterations to ensure that all parameters converged (effective sample size values greater than 200), and the first 10% of samples was discarded as burn-in. The maximum clade credibility (MCC) tree was generated and summarized by TreeAnnotator (v1.10.4). Then, the Ggtree package in R was used to visualize and annotate the tree. Phylogenetic analysis of the NA gene and the six internal genes was performed by using the MEGA 7.0.14 software package, implementing the neighbor-joining method. The tree topology was evaluated by 1,000 bootstrap analyses. We used 95% sequence identity cutoffs to categorize the gene groups in the phylogenetic trees.

Receptor-binding analysis

The receptor binding specificity of the clade 2.3.2.1 viruses was determined by using a solid-phase direct binding test, with two glycopolymers: α−2, 3-sialylglycopolymer [Neu5Acα2-3Galb1-4GlcNAcb1-pAP (para-aminophenyl)-alpha-polyglutamic acid (α-PGA)] and α−2, 6-sialylglycopolymer [Neu5Acα2-6Galb1-4GlcNAcb1-pAP (para-aminophenyl)-alpha-polyglutamic acid (α-PGA)]. In this study, chicken antiserum against the clade 2.3.2.1 virus was generated by using the H5N1 vaccine seed strain H5-Re12 and was used as the primary antibody. Horseradish peroxidase (HRP)-conjugated goat-anti-chicken antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as the secondary antibody. The absorbance was determined at a wavelength of 490 nm.

Replication and virulence of H5N1 viruses in mice

Groups of three 6-week-old female BALB/c mice (Beijing Experimental Animal Center, Beijing, China) were lightly anesthetized with CO_2 and then inoculated intranasally (i.n.) with 10^6 50% egg infective dose (EID_{50}) of the H5N1 virus in a volume of 50 μl. Their organs, including the nasal turbinate, lungs, spleen, kidneys, and brain, were collected on Day 3 post-inoculation (p.i.) for virus titration in chicken eggs. The virus titers were calculated by

using the method of Reed and Muench. To evaluate the 50% mouse lethal dose (MLD_{50}) of the H5N1 viruses, groups of five mice were inoculated i.n. with 10-fold serial dilutions of the test virus containing 10^1–10^6 EID_{50}. The control group of five mice was inoculated i.n. with phosphate-buffered saline (PBS) in a volume of 50 µl. The body weight loss and mortality of the mice in each group were monitored for 14 days.

Antigenic analysis

As we reported in previous studies, the vaccines used in China for highly pathogenic avian influenza control are updated regularly to ensure that the vaccine strain antigenically matches the circulating strains [20–25]. The H5 vaccine seed viruses were generated by reverse genetics as previously reported [26,27]. All of the vaccine seed viruses bear the internal gene of the A/Puerto Rico/8/1934 (H1N1) (PR8) virus, but their HA and NA genes are derived from different H5 viruses [21–24]. H5-Re6 bears surface genes derived from A/duck/Guangdong/S1322/2010 (H5N1) (a clade 2.3.2 virus) and was used between June 2012 and September 2017 to control the clade 2.3.2 viruses; H5-Re8 bears surface genes derived from A/chicken/Guizhou/4/2013 (H5N1) (a clade 2.3.4.4 g virus) and was used between December 2015 and December 2018 to control the clade 2.3.4.4 g viruses; and H5-Re11 bears surface genes derived from A/duck/Guizhou/S4184/2017(H5N6) (a clade 2.3.4.4 h virus) and was used between December 2018 and December 2021 to control the clade 2.3.4.4 h viruses. H5-Re12 bears surface genes derived from A/chicken/Liaoning/SD007/2017(H5N1) (a clade 2.3.2.1f virus) and was used between December 2018 and December 2021 to control the clade 2.3.2.1f viruses [25].

The chicken antisera used in this assay were generated in six-week-old SPF White Leghorn chickens (Harbin Experimental Animal Center, Harbin, China). Chickens were inoculated with 0.5 mL of oil-emulsified inactivated H5-Re6 vaccine, H5-Re8 vaccine, H5-Re11 vaccine, or H5-Re12 vaccine, and their sera were collected three weeks later. Antigenic analysis of the H5N1 viruses was performed by using cross hemagglutinin inhibition (HI) with 1.0% chicken erythrocytes.

Protective efficacy of H5-Re12 vaccine against clade 2.3.2.1f virus.

The H5-Re12 oil-adjuvanted, whole virus inactivated vaccine was produced by following the procedure described previously [27,28]. Groups of 3-week-old SPF chickens were injected intramuscularly with 0.3 ml of vaccine preparations containing 2.8 µg of HA antigen. Sera were collected from all of the chickens three weeks after vaccination, and HI antibody titers were determined by using 1.0% chicken red blood cells. Chickens were challenged intranasally with 10^5EID_{50} of the indicated H5N1 viruses. Oropharyngeal and cloacal swabs of the chickens were collected on Days 3 and 5 post-challenges for virus titration in eggs. Chickens were observed for two weeks after challenge.

Results

H5N1 viruses bearing the clade 2.3.2.1 HA gene isolated in China

From the 264,282 samples collected between January 2017 and December 2022, 7,313 avian influenza viruses of different subtypes were isolated, of which 28 were H5N1 viruses bearing the clade 2.3.2.1 HA gene. Of note, 24 viruses were isolated from samples collected from live poultry markets, three viruses were isolated from poultry farms, and one virus was isolated from the cloacal swab of a dead turtle dove collected in the wetland (Table 1). Importantly, the clade 2.3.2.1 virus was not detected in any live poultry markets or poultry farms after October 2019 during our surveillance (Table 1).

Phylogenic analysis of H5N1 viruses globally circulating since 2007

To understand the genetic relationship of the clade 2.3.2.1 viruses, we sequenced the whole genome of the 28 H5N1 viruses detected in this study and two H5N1 viruses, A/duck/Guangdong/S1322/2010 and A/duck/Anhui/S1246/2015, previously detected in China [the sequence data have been deposited in the Global Initiative on Sharing All Influenza Data databases (GISAID; https://www.gisaid.org) and the accession numbers are EPI2794232-EPI2794471], and compared the sequences with the genome sequences of the 244 clade 2.3.2.1 virus representative strains of different genotypes from different host species at different times and in different countries since 2007. The reference sequences were downloaded from the GISAID databases and the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov).

The HA genes of the 274 clade 2.3.2.1 viruses shared 91%–100% identity at the nucleotide level. In the Bayesian time-resolved phylogenetic tree, and the HA genes of these viruses formed seven different branches, which matched the previously reported seven subclades [11,29], namely clade 2.3.2.1, and 2.3.2.1a to 2.3.2.1f (Figure 1, Figure S1). The viruses bearing the clade 2.3.2.1a HA gene were mainly detected in South Asia and Southeast Asia; the viruses bearing the clade 2.3.2.1b HA genes were mainly detected in China; the viruses bearing the clade 2.3.2.1c HA genes were mainly detected in Southeast Asia; the viruses bearing the clade 2.3.2.1d HA genes

Table 1. H5N1 viruses bearing the clade 2.3.2.1 HA gene isolated in this study.

	Virus			Sample information	
Full name	Abbreviation	The clade of HA	Genotype	Collected date	Location
A/chicken/Jiangsu/SE2228/2017	CK/JS/SE2228/17	2.3.2.1f	G43	February 09, 2017	Poultry market
A/duck/Hunan/SE905/2017	DK/HuN/SE905/17	2.3.2.1c	G53	February 22, 2017	Poultry market
A/chicken/Liaoning/SD007/2017	CK/LN/SD007/17	2.3.2.1f	G54	May 23, 2017	Farm
A/duck/Zhejiang/S4091/2017	DK/ZJ/S4091/17	2.3.2.1f	G43	October 30, 2017	Poultry market
A/chicken/Shanghai/S4030/2017	CK/SH/S4030/17	2.3.2.1f	G43	November 03, 2017	Poultry market
A/duck/Jiangsu/S4439/2017	DK/JS/S4439/17	2.3.2.1f	G43	November 17, 2017	Poultry market
A/duck/Jiangsu/SE1128/2018	DK/JS/SE1128/18	2.3.2.1f	G43	January 12, 2018	Poultry market
A/chicken/Hunan/S10029/2018	CK/HuN/S10029/18	2.3.2.1c	G35	March 19, 2018	Poultry market
A/environment/Hunan/S11301/2018	EN/HuN/S11301/18	2.3.2.1d	G45	March 22, 2018	Poultry market
A/environment/Jiangsu/S1349/2018	EN/JS/S1349/18	2.3.2.1f	G56	March 28, 2018	Poultry market
A/duck/Fujian/S1233/2018	DK/FJ/S1233/18	2.3.2.1f	G57	April 17, 2018	Poultry market
A/duck/Fujian/S1273/2018	DK/FJ/S1273/18	2.3.2.1f	G58	April 17, 2018	Poultry market
A/chicken/Liaoning/SD035/2018	CK/LN/SD035/18	2.3.2.1f	G56	December 13, 2018	Farm
A/environment/Hunan/S10232/2019	EN/HuN/S10232/19	2.3.2.1d	G45	March 14, 2019	Poultry market
A/chicken/Jilin/S1271/2019	CK/JL/S1271/19	2.3.2.1f	G56	April 09, 2019	Poultry market
A/duck/Jilin/S1261/2019	DK/JL/S1261/19	2.3.2.1f	G56	April 09, 2019	Poultry market
A/chicken/Jilin/S1255/2019	CK/JL/S1255/19	2.3.2.1f	G56	April 09, 2019	Poultry market
A/duck/Xinjiang/S1126/2019	DK/XJ/S1126/19	2.3.2.1f	G43	May 06, 2019	Poultry market
A/chicken/Xinjiang/S1016/2019	CK/XJ/S1016/19	2.3.2.1f	G43	May 06, 2019	Poultry market
A/chicken/Xinjiang/S1170/2019	CK/XJ/S1170/19	2.3.2.1f	G43	May 06, 2019	Poultry market
A/duck/Xinjiang/S1066/2019	DK/XJ/S1066/19	2.3.2.1f	G43	May 06, 2019	Poultry market
A/chicken/Xinjiang/S1081/2019	CK/XJ/S1081/19	2.3.2.1f	G43	May 06, 2019	Poultry market
A/chicken/Shanghai/S1123/2019	CK/SH/S1123/19	2.3.2.1f	G43	May 06, 2019	Poultry market
A/goose/Jilin/S4034/2019	GS/JL/S4034/19	2.3.2.1f	G56	October 22, 2019	Farm
A/duck/Jilin/S4152/2019	DK/JL/S4152/19	2.3.2.1f	G56	October 22, 2019	Poultry market
A/duck/Jilin/S4171/2019	DK/JL/S4171/19	2.3.2.1f	G56	October 22, 2019	Poultry market
A/pigeon/Jilin/S4176/2019	PG/JL/S4176/19	2.3.2.1f	G56	October 22, 2019	Poultry market
A/turtle dove/Hunan/S4004/2020	TD/HuN/S4004/20	2.3.2.1d	G45	October 27, 2020	Wetland

were mainly detected in China; the viruses bearing the clade 2.3.2.1e HA genes were mainly detected in Japan and Korea; and the viruses bearing the clade 2.3.2.1f HA genes were mainly detected in Africa and China (Figure 1, Figure S1).

The NA genes of the 274 clade 2.3.2.1 viruses shared 81.8%–100% identity at the nucleotide level and formed four groups in the phylogenetic tree (Figure 2, Figure S2). The basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), nucleoprotein (NP), matrix (M), and nonstructural protein (NS) genes of the 274 viruses shared 86.6%–100%, 86.6%–100%, 87.2%–100%, 88.5%–100%, 87%–100%, and 83.4%–100% identity at the nucleotide level, respectively (Figure 2, Figure S2). The PB2 and NP genes each formed 11 groups in their phylogenetic trees; the PB1 gene of these viruses formed 15 groups in the phylogenetic tree; the PA and M genes each formed seven groups in their phylogenetic trees; and the NS gene of these viruses formed eight groups in the phylogenetic tree. These results indicate that the NA and internal genes of the H5N1 viruses are also clearly diverse.

Based on their genomic differences, the 274 clade 2.3.2.1 viruses detected in Asia, Europe, and Africa formed 58 different genotypes (G1 to G58) (Figure 1, Figure S1). Of note, the G43 viruses were the most widely distributed strains, being detected in two Asian countries and six African countries. The 28 clade 2.3.2.1 viruses detected in this study belonged to eight different genotypes: one G35 virus, 11 G43 viruses, three G45 viruses, one G53 virus, one G54 virus, nine G56 viruses, one G57 virus, and one G58 virus (Table 1).

Molecular characteristics of the H5N1 viruses

All 28 viruses in this study have the same amino acid motif (-PQRERRRKR/GLF-) at their HA cleavage site, indicating that they are highly pathogenic to chickens [30]. Several amino acid residues are known to increase human-type receptor binding or to promote the replication and virulence of avian influenza viruses in mammals [31–37]. We performed an amino acid substitution analysis with the FluSurver in the GISAID and found that some of these residues, including 137A and 225G in HA (H3 numbering), 89V and 309D in PB2, 3V and 622G in PB1, 30D, 43M, and 215A in M1, and 42S, 106M, and 138F in NS1 are highly conserved and present in all 28 of the viruses we analyzed (Table S1). Other residues, including 192I and 193R in HA, 383D in PA, and 103F in NS1 were also detected in some of these viruses. Lack of glycosylation at positions 158–160 in HA increases affinity for human-type receptors [38], and 25 of the 28 viruses in this study lack the glycosylation site at positions 158–160 in their HA (Table S1). These results suggest that the H5N1 avian influenza viruses bear multiple genetic changes that are purported to favor adaptation to mammals.

Cross-reactivity of the H5N1 virus with antisera induced by different vaccine seed viruses

H5-Re6, H5-Re8, and H5-Re11 have 10, 24, and 24 amino acid differences at the HA antigen epitopes, respectively, compared with H5-Re12 vaccine seed virus (Table S2). The three clade 2.3.2.1d viruses have up to 10 amino acid differences compared with

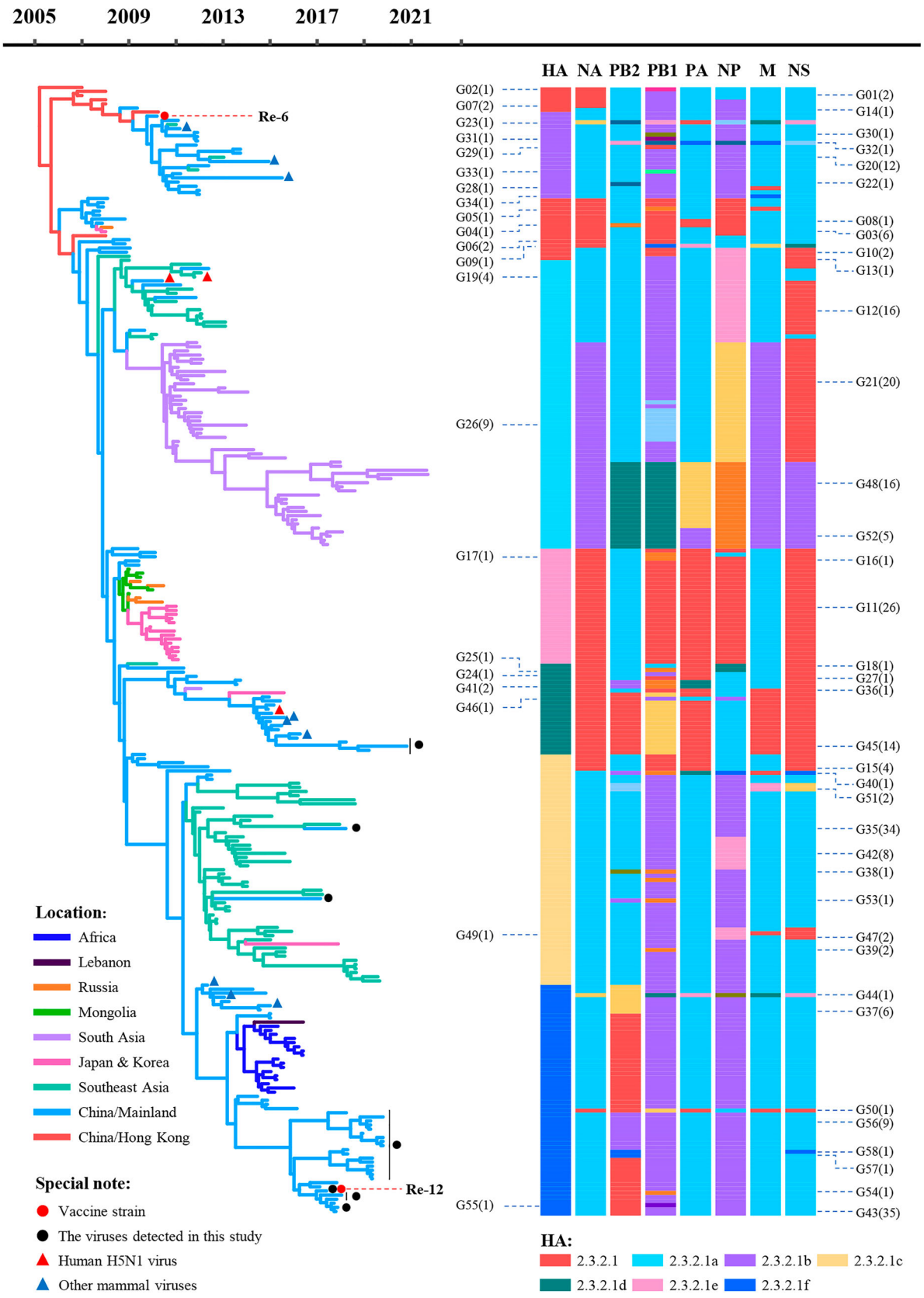

Figure 1. Phylogenetic analyses, genotypes, and distribution of H5N1 viruses. The Bayesian time-resolved phylogenetic tree was generated with the clade 2.3.2.1 HA gene of 274 H5N1 viruses. The 28 H5N1 strains reported in this study are marked with black dots. The two H5N1 viruses that provided surface genes for the H5-Re6 and H5-Re12 vaccine seed strains are marked with red dots. The eight bars represent the eight gene segments. The horizontal row corresponds to a virus, and the gene colors in columns 2–8 correspond to the colors of the corresponding genes on the evolutionary tree shown in Figure 2 and Supplementary Figure S2. A phylogenetic tree of the HA gene with more complete information is shown in Supplementary Figure S1.

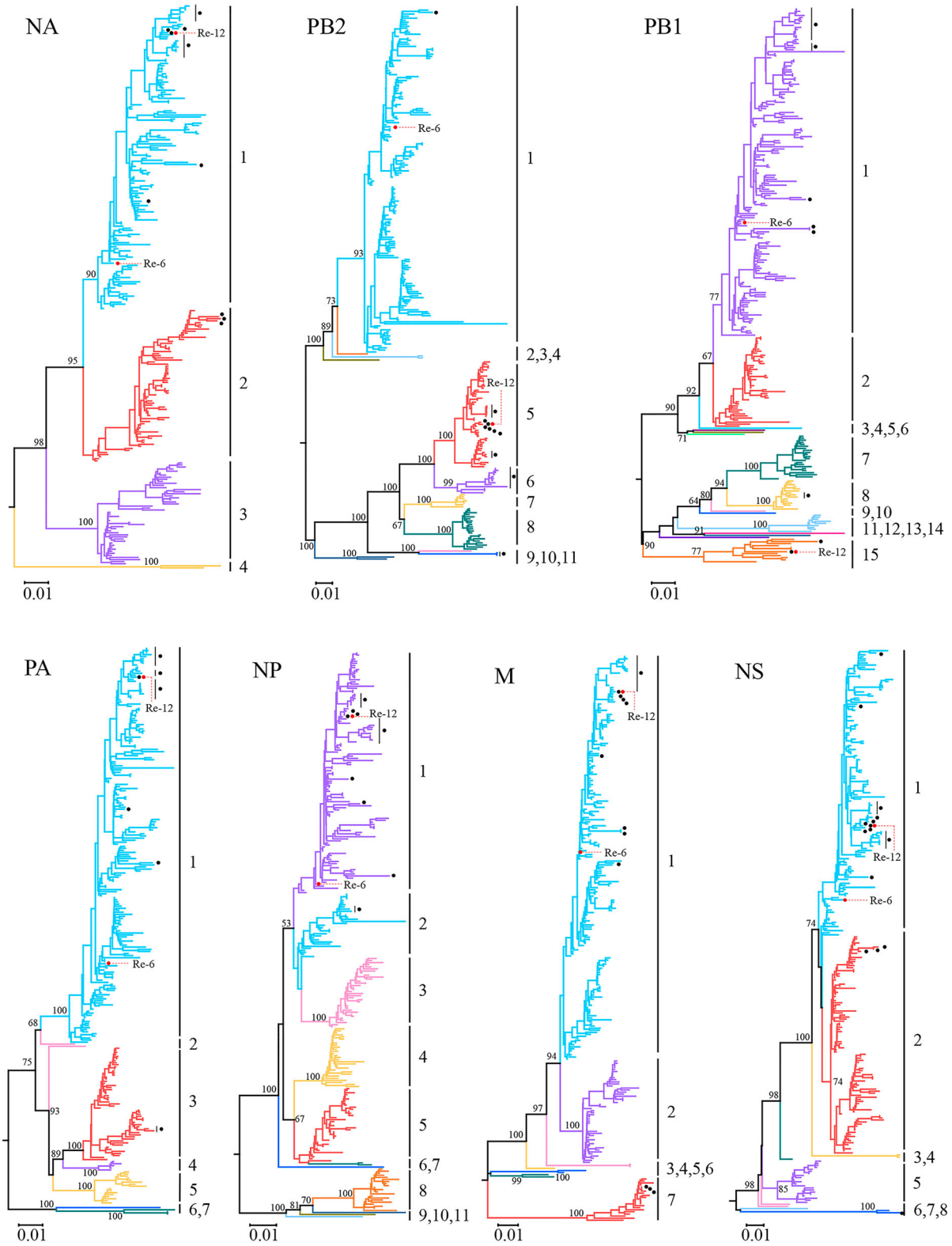

Figure 2. Phylogenetic analyses of the NA gene and the six internal genes of the H5N1 viruses. Phylogenetic analysis of the NA, PB2, PB1, PA, NP, M, and NS genes of 274 H5N1 viruses was performed using the MEGA 7.0.14 software package, implementing the neighbor-joining method. The tree topology was evaluated by 1,000 bootstrap analyses. The different colors represent the different genetic groups. The 28 H5N1 viruses reported in this study are marked with black dots. The two H5N1 viruses that provided surface genes for the H5-Re6 and H5-Re12 vaccine seed strains are marked with red dots. The more complete version of these trees is shown in Supplementary Figure S2.

the HA of H5-Re12 at the antigenic epitopes, whereas the remaining 25 viruses have up to five amino acid differences at the antigenic regions compared with the HA of H5-Re12 (Table S2).

To better understand the antigenic relationships between the H5N1 viruses and different vaccine strains, we evaluated the cross-reactivity of 28 viruses with antisera induced by the four different vaccines

described above. The HI antibody titers of H5-Re6, H5-Re8, H5-Re11, and H5-Re12 antisera against the homologous viruses were 512, 256, 1024, and 1024, respectively (Table 2). The HI titers of H5-Re8 antiserum against the 28 H5N1 viruses ranged from 8 to 64, which were 4- to 32-fold lower than that to the homologous titer. The HI titers of the H5-Re11 antiserum against the 28 H5N1 viruses ranged from 16 to 256, which were 4- to 64-fold lower than that to the homologous titer. The HI titers of the H5-Re6 antiserum against the 28 H5N1 viruses ranged from 8 to 256, which were 2- to 64-fold lower than that to the homologous titer. The HI titers of the H5-Re12 antiserum against the 26 of the 28 H5N1 viruses ranged from 128 to 1024, which were less than 8-fold lower than that to the homologous titer (Table 2). The monovalent H5 vaccine produced with the H5-Re12 seed virus provided complete protection against challenge with different clade 2.3.2.1f H5N1 viruses (Table 3). These results indicate that H5-Re12 was an ideal vaccine seed virus for clade 2.3.2.1 H5N1 virus control in China.

Receptor-binding specificity of the clade 2.3.2.1 viruses

Receptor-binding preference is a very important determinant for the replication and transmission of influenza virus. In general, binding to α2,6-link sialic acids (SAs) (human-type receptor) is a prerequisite for an influenza virus to transmit efficiently among humans [39]. To investigate their receptor-binding properties, five representative viruses carrying different amino acid substitutions associated with receptor-binding properties were evaluated for their binding capability to α−2,3-sialylglycopolymer (avian-type receptor) and α−2,6-sialylglycopolymer by using a solid-phase binding assay as described previously [40]. We found that all five viruses exclusively bound to α−2,3-sialylglycopolymer (Figure 3). These results indicate that the clade 2.3.2.1 viruses have retained their ability to bind to avian-type receptors and have not yet acquired the ability to bind to human-type receptors.

Table 2. Cross-reactive HI antibody titers of H5N1 viruses with the antisera induced by different vaccines*.

Virus (HA clade)	Antiserum induced by			
	H5-Re6	H5-Re8	H5-Re11	H5-Re12
H5-Re6 (2.3.2.1b)	**512**	16	32	512
H5-Re8 (2.3.4.4 g)	64	**256**	256	64
H5-Re11 (2.3.4.4 h)	16	64	**1024**	16
H5-Re12 (2.3.2.1f)	64	16	32	**1024**
DK/HuN/SE905/17 (2.3.2.1c)	128	32	64	256
CK/HuN/S10029/18 (2.3.2.1c)	128	64	256	256
EN/HuN/S11301/18 (2.3.2.1d)	64	32	128	256
EN/HuN/S10232/19 (2.3.2.1d)	64	32	64	128
TD/HuN/S4004/20 (2.3.2.1d)	8	8	16	32
CK/JS/SE2228/17 (2.3.2.1f)	128	32	64	256
CK/LN/SD007/17 (2.3.2.1f)	64	16	32	1024
CK/SH/S4030/17 (2.3.2.1f)	32	16	64	256
DK/JS/S4439/17 (2.3.2.1f)	32	16	64	256
DK/ZJ/S4091/17 (2.3.2.1f)	8	16	64	64
DK/FJ/S1233/18 (2.3.2.1f)	64	8	16	256
DK/FJ/S1273/18 (2.3.2.1f)	64	16	32	512
DK/JS/SE1128/18 (2.3.2.1f)	32	16	32	256
EN/JS/S1349/18 (2.3.2.1f)	32	16	32	256
CK/LN/SD035/18 (2.3.2.1f)	32	16	32	256
CK/JL/S1271/19 (2.3.2.1f)	16	8	32	128
DK/JL/S1261/19 (2.3.2.1f)	32	8	32	256
CK/JL/S1255/19 (2.3.2.1f)	32	16	32	256
DK/XJ/S1126/19 (2.3.2.1f)	64	16	64	256
CK/XJ/S1016/19 (2.3.2.1f)	256	64	64	1024
CK/XJ/S1170/19 (2.3.2.1f)	64	8	64	256
DK/XJ/S1066/19 (2.3.2.1f)	64	32	64	512
CK/XJ/S1081/19 (2.3.2.1f)	64	16	64	512
CK/SH/S1123/19 (2.3.2.1f)	128	16	64	512
GS/JL/S4034/19 (2.3.2.1f)	32	16	32	256
DK/JL/S4152/19 (2.3.2.1f)	32	16	32	256
DK/JL/S4171/19 (2.3.2.1f)	64	16	32	512
PG/JL/S4176/19 (2.3.2.1f)	32	16	32	128

*, Homologous titers were shown in bold face and underlined.

Replication and virulence of the H5N1 viruses in mice

Mice have been widely used as model animals for evaluating the virulence of avian influenza viruses in mammals [41–48]. To investigate the virulence of the H5N1 viruses detected in this study, we selected 12 H5N1 viruses from different genotypes in different years and evaluated their replication and virulence in BALB/c mice. Groups of eight mice were intranasally inoculated with 10^6 EID_{50} of the test virus. Three mice in each group were euthanized on day 3 p.i. and their organs were collected for virus titration, while the other five mice in each group were observed for two weeks for body weight loss and death. We found that four viruses, CK/SH/S1123/19, DK/JL/S1261/19, EN/HuN/S11301/18, and EN/HuN/S10232/19, were only detected in the nasal turbinate and lungs of mice, and they were not lethal to any mice they infected (Figure 4(a)). DK/HuN/SE905/17 was detected in the nasal turbinate, lungs, and brain of mice, but was not detected in the spleen or kidneys of any mice, whereas TD/HuN/S4004/20 was detected in the nasal turbinate, lungs, spleen, and kidneys of mice, but was not detected in the brain of any mice. Only one of the five mice infected with DK/HuN/SE905/17 and one of the five mice infected with TD/HuN/S4004/20 died during the observation period (Figure 4(a)). Six viruses, CK/JS/SE2228/17, DK/JS/SE1128/18, CK/LN/SD035/18, CK/HuN/S10029/18, DK/FJ/S1233/18, and DK/FJ/S1273/18, replicated systemically and were detected in all five organs tested (Figure 4(a)); the mice inoculated with these viruses lost over 20% of their body weight and all died within the two-week observation period (Figure 4(a, b)). We then tested the MLD_{50} of these six viruses, and found that their MLD_{50} values ranged from 0.8 $\log_{10}$ EID_{50} to 4.3 $\log_{10}$ EID_{50} (Figure 4(c)). These results indicate that H5N1 viruses bearing the clade 2.3.2.1 HA detected in nature have distinct pathotypes in mice, with some strains replicating systemically and being highly lethal in mice. The pathogenicity of

Table 3. Protective efficacy of the H5-Re12 vaccine against challenge with clade 2.3.2.1f H5N1 avian influenza viruses in chickens[a].

Challenge virus	Group	Mean HI antibody titer to (log_2)[b]		No. of chickens shedding virus/total No. chickens on day *p.c.* (viral titer, $log_{10}EID_{50}$/ml)				Survival /total
				Day 3		Day 5		
		H5-Re12	challenge virus	Oropharynx	Cloaca	Oropharynx	Cloaca	
CK/LN/SD007/17	Vaccinated	8.1	7.7	0/10	0/10	0/10	0/10	10/10
	Control	<1	<1	10/10 (5.2 ± 0.7)	10/10 (4.3 ± 0.5)	/[c]	/[c]	0/10
CK/JL/S1271/19	Vaccinated	7.9	5.2	0/10	0/10	0/10	0/10	10/10
	Control	<1	<1	10/10 (4.9 ± 0.8)	10/10 (3.8 ± 0.6)	/[c]	/[c]	0/10

[a]Groups of three-week-old specific pathogen-free chickens were intramuscularly vaccinated with 0.3 ml of H5N1 inactivated vaccine and then challenged intranasally with 10^5EID_{50} of the indicated virus three weeks post-vaccination. Oropharyngeal and cloacal swabs were collected on days 3 and 5 post-challenge (*p.c.*) and titrated in eggs.
[b]The HI titers were measured by using the vaccine seed virus (H5-Re12) and the challenge viruses with the chicken antisera that were collected three weeks after vaccination.
[c]All of the chickens in this group died before day 5 *p.c.*

influenza virus can be affected by gene constellations or certain amino acid substitutions, and the underlying mechanism of the pathogenicity differences among H5N1 viruses identified in this study deserves further investigation.

Discussion

The H5 influenza viruses are mainly spread by migratory wild birds and have caused serious damage and huge economic losses for the poultry industry across multiple continents [25]. Our analysis indicates that the clade 2.3.2.1 viruses are widely detectable in wild birds in Russia, Africa and Asia, and have caused disease outbreaks in domestic poultry in Russia and Asia. Unlike the currently circulating H5N1 viruses carrying the clade 2.3.4.4b HA gene that have been widely detected in Europe, Africa, Asia, North America, and South America [49], the clade 2.3.2.1 viruses were not detected in other European countries or in North America.

Cui et al. previously detected the clade 2.3.2.1 viruses in brown-headed gulls in Tibet in 2010, H5N1 viruses bearing the clade 2.3.2.1d HA gene in several different kinds of wild birds in Jiangsu province

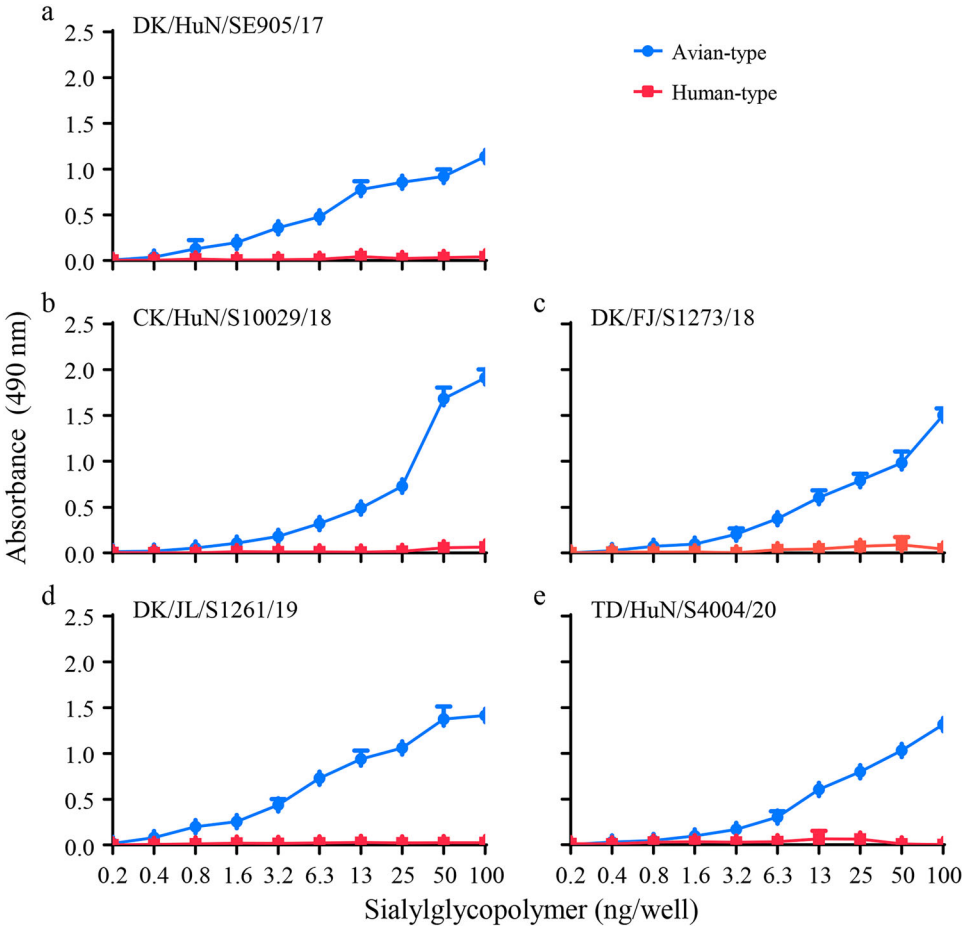

Figure 3. Receptor-binding properties of H5N1 viruses bearing the clade 2.3.2.1 HA gene. Binding of the indicated viruses to sialylglycopolymers (α−2,3-sialylglycopolymer, blue; α−2,6-sialylglycopolymer, red). The data shown are the means of three repeats; the error bars indicate standard deviations.

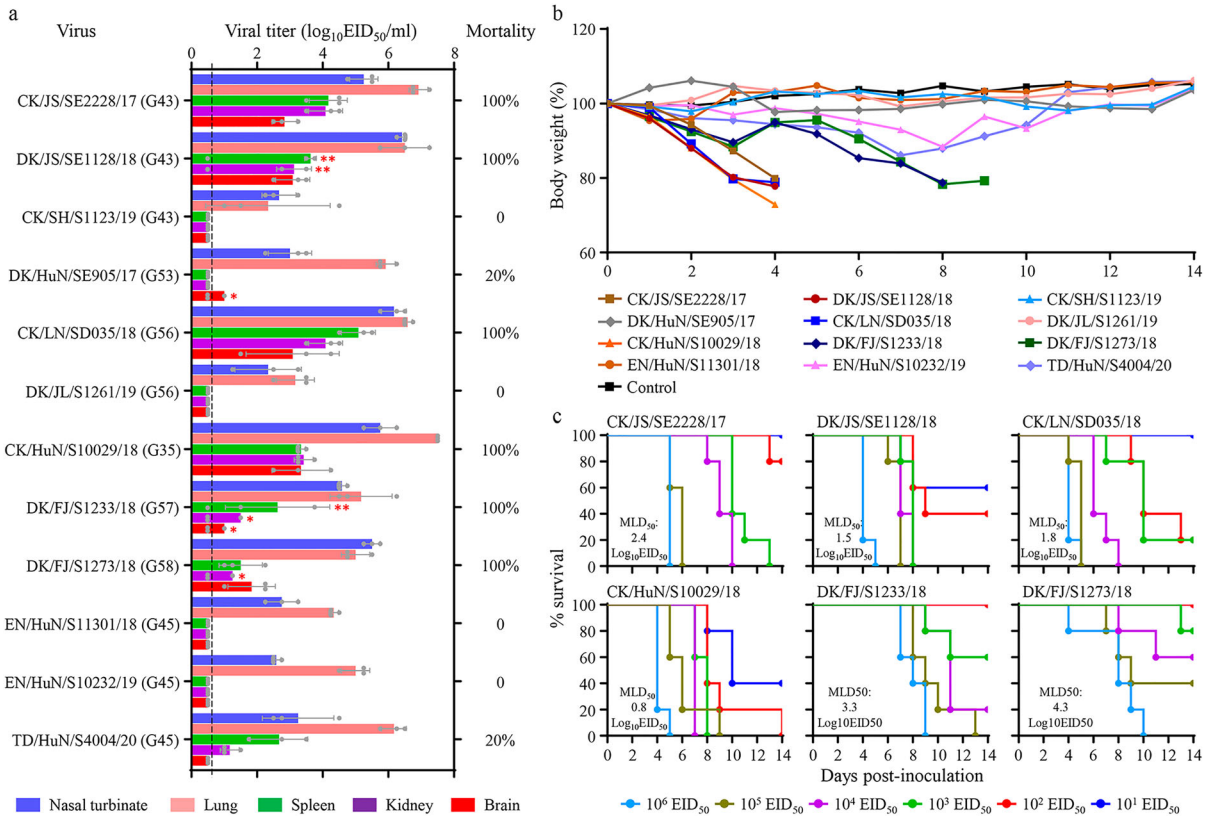

Figure 4. Replication and virulence of H5N1 viruses in mice. (a) Virus titers in organs of mice inoculated intranasally with 10^6 EID_{50} of different H5N1 viruses. Three mice from each group were euthanized and their organs were collected on day 3 post-inoculation for virus titration in eggs. Data shown are means ± standard deviations. The values labeled with one red star indicate that the virus was only detected in the organ of one mouse and two red stars indicate that the virus was detected in the organ of two mice. The dashed lines indicate the lower limit of detection. (b) Changes in body weight in the groups of five mice after inoculation with 10^6 EID_{50} of virus. (c) The death pattern and MLD_{50} of the indicated viruses.

in 2015, and H5N1 viruses bearing the clade 2.3.2.1f HA gene in several kinds of wild birds in Tibet, Inner Mongolia, and Henan provinces in 2015 [29]. Our analysis in this study indicates that viruses bearing the clade 2.3.2.1b, 2.3.2.1d, or 2.3.2.1f were circulating in domestic birds in multiple provinces (mainly in live poultry markets) in China, and that the viruses bearing the 2.3.2.1f HA gene predominated in the live poultry markets from 2017 to 2019 (Table 1).

Vaccination is a major strategy for highly pathogenic avian influenza control in China, and different vaccine seed viruses have been developed and used to prevent and control viruses bearing the HA gene of different clades or subclades [21–23,27]. The vaccine seed virus H5-Re6, which bears the HA gene of DK/GD/S1322/2010(H5N1), was developed and used to control the clade 2.3.2 viruses between June 2012 and September 2017, and the H5-Re6 vaccine has been proven to be protective against clade 2.3.2.1b, 2.3.2.1c, and early clade 2.3.2.1f viruses [22]. The vaccine seed virus H5-Re12 was developed and first used in December 2018 for the control of clade 2.3.2.1f viruses [23]. Of note, the H5-Re12 seed virus was mainly used to produce a trivalent vaccine along with H5-Re11 and an H7N9 vaccine seed virus. As China has eliminated such H5N1 viruses from poultry, the H5-Re12 vaccine was discontinued in December 2021. Since the clade 2.3.2.1 viruses are still circulating in some other countries [50,51], the potential re-introduction of such viruses into China must be carefully monitored.

In summary, our study revealed the genetic properties of the clade 2.3.2.1 viruses circulating around the world, and found that viruses in different regions bear the HA gene of different subclades. Clade 2.3.2.1 viruses detected in domestic poultry in China from 2009 to 2019 carry the clade 2.3.2.1b, 2.3.2.1d, or 2.3.2.1f HA genes. The viruses have different pathotypes in mice and lack the ability to bind to human-type receptors. Our study reveals the overall picture of H5N1 virus evolution and provides insights into the control of these viruses.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

X.X., J.S., P.C., C.Y., YP.Z., YC.Z., C.W., Y.C., X.Z., G.T., L.L., Y.G., C.L., and G.D. conducted the experiments; X.X., J.S., G.D., and H.C. analyzed the data; Y.S. contributed reagents. J.S. and H.C. wrote the manuscript.

ORCID

Jianzhong Shi http://orcid.org/0000-0003-4580-9455
Chengjun Li http://orcid.org/0000-0001-8460-8619
Hualan Chen http://orcid.org/0000-0001-8910-898X

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