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ORIGINAL ARTICLE

Analysis of the phylogeny of Chinese H9N2 avian influenza viruses and their pathogenicity in mice

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Abstract We isolated nineteen strains of H9N2 influenza virus from farms across five northern Chinese provinces between 2001 and 2012. Sequence analysis of the genes for the two surface glycoproteins revealed that residue 226 of the hemagglutinin (HA) of eight isolates was a leucine. A T300I mutation in three strains resulted in the loss of a potential glycosylation site. The P315S mutation in seven strains added a potential glycosylation site in HA. The isolates CK/HN/323/08 and CK/HN/321/08 had a full-length neuraminidase (NA) that differed from those seen in other isolates. Phylogenetic and molecular analysis revealed that the nineteen strains shared common ancestry with strains BJ/94 and G1. We examined eight gene sequences in the present study and concluded that the HA and NS genes appeared to be derived directly from BJ/94. The remaining six genes evolved from different reference strains. Specifically, the NA and PA genes of CK/HN/321/08 and CK/HN/323/08 clustered with the G9 and Y439 branch, respectively, and the PB2 genes of CK/SD/513/11 and CK/GS/419/12 were in an unknown lineage. We found evidence that seven new genotypes had undergone intra-subtype reassortment. A mouse infection experiment with six selected isolates showed that five of these isolates were able to replicate in mouse lungs without adaptation. Viral replication in infected mice resulted in minimal weight loss, suggesting that these H9N2 avian influenza viruses had low virulence in mammals. Our findings highlight the genetic and biological diversity of H9N2 avian influenza viruses circulating in China and emphasize the importance in continuing surveillance of these viruses so as to better understand the potential risks they pose to humans.

Abbreviations

EID_{50}	50 % egg infectious dose
HI	Hemagglutination-inhibition
HPAIV	Highly pathogenic avian influenza virus
LPAIV	Low pathogenic avian influenza virus
RBS	Receptor-binding site
RNP	Ribonucleoprotein
SA	Sialic acid
SPF	Specific-pathogen-free

Introduction

Sixteen different subtypes of hemagglutinin (HA) and nine different subtypes of neuraminidase (NA) glycoproteins have been identified on the surface of avian influenza viruses isolated from wild birds worldwide [16]. Recently, a distinct subtype of influenza A virus was isolated from bats in which HA and NA exhibited divergent biological activities [51, 65, 66]. Avian influenza virus (AIV) can be classified as highly pathogenic (HPAIV) or with low pathogenicity (LPAIV). The H5 and H7 subtypes appear to be responsible for the most severe HPAI outbreaks in

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poultry. Although HPAIVs have been extensively investigated since 1996, when the first H5N1 avian influenza virus was isolated from geese in Guangdong Province, China [6, 58], information pertaining to phylogenetic and biological properties of LPAIVs is limited.

In domestic birds, H9N2 is one of the most common subtypes among LPAIVs and does not normally induce obvious respiratory signs or symptomatic infections in animals. However, infection of chickens with H9N2 AIV results in reduced egg production and variable rates of morbidity and mortality when a co-infection with another agent exists [30]. H9N2 viruses are able to acquire receptor specificity for human influenza viruses and can occasionally be transmitted to humans [37]. More than 11 human cases of H9N2 infection in southern China have been reported since 1999. Humans presenting with influenza are more likely to be designated as seasonal flu cases, and therefore the number of reported H9N2 cases is lower than their actual incidence [4, 8, 28, 40]. H9N2 AIV was first isolated from turkeys in the United States in 1966 [24], and it has been continually reported to be isolated from birds of different species [5, 22, 61]. In 1994, an H9N2 AIV was isolated for the first time from a bird in Guangdong Province. Vaccination programs are carried out in widely China to reduce the economic impact of H9N2 infection in poultry, since H9N2 is the most prevalent subtype in poultry from mainland China [5, 47].

Influenza virus genomes are composed of eight independent segments, with a tendency to reassort with each other frequently, thereby altering lineages. The result of this is the emergence of new genotypes, such as H7N9, a triple reassortant virus with an HA gene from H7N3, an NA gene from H7N9 and internal genes from H9N2 [17, 63]. The rearrangement of genes in these new genotypes can confer the ability of interspecies virus transmission. The H9N2 subtype of AIVs in Eurasia can be divided among three lineages: A/Chicken/Beijing/1/1994(BJ/94-like); A/Quail/Hong Kong/G1/1997 (G1-like); and A/Duck/Hong Kong/Y439/1997(Y439-like) [20, 22, 57]. In China, another two prevalent H9N2 representatives are the strain A/Chicken/Hong Kong/G9/1997(G9-like), a reassortant of the G1, BJ/94 lineages and a new NA gene [34], and A/Chicken/Shanghai/F/1998(SH/F-like), in which the RNP of the BJ/94 lineage was replaced by new segments [61].

Systematic surveillance for the evolution of H9N2 virus in northern China has been performed previously [25, 47]. However, information on H9N2 virus evolution and pathogenicity still need to be updated frequently, and we therefore sought to confirm recent results related to the circulation of H9N2 AIVs across northern China. We investigated the relationships among nineteen isolates taken from farms in northern China and attempted to determine whether these H9N2 viruses exhibited enhanced cross-species infection in mice.

Materials and methods

Viruses, cells, and animals

From 2001 to 2012, we isolated influenza viruses from samples that were taken from farms across five Chinese provinces (Table 1). Fecal samples were collected in 1 ml of cold phosphate-buffered saline containing 1000 U/ml penicillin and 1000 μg/ml streptomycin. Specific-pathogen-free (SPF) chicken embryos (10 days old) were inoculated with 100 μl of fecal sample supernatants and incubated at 37 °C for 48-72 h. The allantoic fluid was harvested and tested using hemagglutination and hemagglutination-inhibition (HI) assays. The HI assays were performed as described previously [38]. Viruses were isolated by three rounds of limited-dilution purification in SPF chicken eggs. Allantoic fluids with the highest hemagglutination titer were used as the virus stock. The 50 % egg infectious dose (EID_{50}) was determined by propagating 10-fold serial dilutions of viruses in SPF chicken eggs and calculated by the method of Reed and Muench [43]. The H9N2 influenza viruses used in this study and their EID_{50} values are listed in Table 1. SPF chicken embryos were purchased from Beijing Merial Vital Laboratory Animal Technology Co. Ltd. BALB/c mice were bred at the Experimental Animal Center of Lanzhou Veterinary Research Institute. Our animal study was authorized by the biosafety and ethics committee of Lanzhou Veterinary Research Institute (SYXK (G) 2010-0003). MDCK cells were purchased from the Chinese Academy of Sciences (Shanghai, China).

Genetic and phylogenetic analysis

Viral RNA was extracted from infected allantoic fluids and purified using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription followed by polymerase chain reaction (PCR) was conducted using specific primers for each gene segment (primer sequences available upon request). Amplicons were purified using a gel extraction kit (Omega, Norcross, GA, USA) and sequenced directly or cloned into the pMD18-T plasmid vector (Takara, Dalian, China) following the manufacturer's instructions. Sequencing was completed by GENEWIZ (Beijing, China). All sequences were assembled and edited using the Lasergene

Table 1 H9N2 AIV isolates used in this study

Virus	Abbreviated name	Isolation date	Location of isolation	50 % Egg infectious dose	Accession no.
A/Chicken/Shandong/241/01(H9N2)	CK/SD/241/01	07/2001	Liaocheng	3.16×10^{9}	KF746823-KF746830
A/Chicken/Shandong/244/02(H9N2)	CK/SD/244/02	04/2002	Lianshan	3.16×10^{9}	KF746807-KF746814
A/Chicken/Hebei/274/04(H9N2)	CK/HB/274/04	07/2004	Hebei	3.16×10^{9}	KF746831-KF746838
A/Chicken/Beijing/243/05(H9N2)	CK/BJ/243/05	04/2005	Beijing	1.78×10^{9}	KF746753-57,89,90,43
A/Chicken/Shandong/317/07(H9N2)	CK/SD/317/07	09/2007	Linyi	3.16×10^{10}	KF715233-KF715240
A/Chicken/Shandong/237/07(H9N2)	CK/SD/237/07	11/2007	Zichuan	5.62×10^{9}	KF746857-62,746855
A/Chicken/Shandong/251/07(H9N2)	CK/SD/251/07	07/2007	Zibo	3.16×10^{9}	KF746791-KF746798
A/Chicken/Henan/321/08(H9N2)	CK/HN/321/08	07/2008	Henan	3.16×10^{9}	KF746864-69,71-72
A/Chicken/Henan/323/08(H9N2)	CK/HN/323/08	07/2008	Henan	3.16×10^{9}	KF746774-KF746781
A/Chicken/Hebei/274/09(H9N2)	CK/SD/363/09	07/2009	Hebei	3.16×10^{10}	KF746759-KF746766
A/Chicken/Shandong/363/09(H9N2)	CK/HN/323/09	11/2009	Shouguang	3.16×10^{10}	KF715225-KF715232
A/Chicken/Henan/323/09(H9N2)	CK/HN/323/09	09/2009	Henan	5.62×10^{9}	KF746839-KF746846
A/Chicken/Shandong/377/09(H9N2)	CK/SD/377/09	08/2009	Feixian	3.16×10^{9}	KF746815-KF746822
A/Chicken/Shandong/382/09(H9N2)	CK/SD/382/09	10/2009	Pingdu	5.62×10^{8}	KF746767-73,746744
A/Chicken/Shandong/397/10(H9N2)	CK/SD/397/10	09/2010	Huiming	1.78×10^{10}	KF746847-KF746854
A/Chicken/Beijing/243/10(H9N2)	CK/BJ/243/10	05/2010	Beijing	3.16×10^{9}	KF746873-78,56,70
A/Chicken/Shandong/384/11(H9N2)	CK/SD/384/11	08/2011	Shouguang	5.62×10^{8}	KF746745-KF746752
A/Chicken/Shandong/513/11(H9N2)	CK/SD/513/11	08/2011	Cangle	1.78×10^{10}	KF746799-KF746806
A/Chicken/Gansu/419/12(H9N2)	CK/GS/419/12	03/2012	Xigu	5.62×10^{9}	KF715241-KF715248

software package (DNASTAR, Madison, WI, USA) [64]. Phylogenetic trees were generated using the MEGA software suite (version5.0) [49]. Phylogenetic and molecular evolutionary analyses were conducted using the neighbor-joining method with 1000 bootstrap replicates. Values lower than 70 % were hidden. The sequences obtained in this study were available from GenBank under accession numbers KF715225-715248 and KF746743-746878 (Table 1). BJ/94, G1, Y439, G9 and SH/F were chosen as lineage representatives. Another 22 sequences of Chinese H9N2 avian influenza viruses isolated in northern provinces in GenBank were used in the study for phylogenetic analysis.

Mouse infection studies

To evaluate the ability of H9N2 virus to replicate in BALB/c mice, we selected six H9N2 isolates (A/CK/SD/244/02, A/CK/BJ/243/05, A/CK/HN/323/08, A/CK/HN/323/09, A/CK/SD/363/09 and A/CK/GS/419/12) for animal infection experiments. Groups of 6-week-old female BALB/c mice ($n = 11$ mice per group) were anesthetized with dry ice and inoculated intranasally (i.n.) with 10^{6} EID_{50} of each strain in a volume of 50 µl [64]. Three mice in each group were euthanized on day 3 postinfection, with another three mice euthanized on day 6 postinfection. Lungs were collected for virus titration in MDCK cells. Weight loss and mortality of the remaining five mice in each group were monitored daily until 14 days postinfection.

Results and discussion

H9N2 viruses have been reported in many regions worldwide, including North American, South America, Europe, Africa, Asia, and the Pacific [12, 19, 32, 33, 50, 52, 56]. The characteristics of influenza viruses mean they are prone to antigenic drift (mutations in genes) and shift (reassortment of segments), and therefore the host range has gradually expanded [13]. In early studies, H9N2 was only isolated from turkeys and ducks [21, 24, 45]; however, over the years, these AIVs have been isolated from chickens, other birds, pigs, and humans [4, 10, 59]. These viruses can donate segments of their genes to other subtypes, as is the case with H9N2, which is regarded as the donor of certain genes to the H5N1 isolate found in Hong Kong in 1997 [20]. Hence, continuous surveillance of influenza viruses and their evolution is critical to public health. The findings from our current study provide an insight into the evolution of nineteen H9N2 isolates from northern China between 2001 and 2012.

Phylogenetic analysis of HA, NA, M, and NS genes

Phylogenetic analysis of HA genes revealed that all strains from chickens contained HA genes similar to those in the BJ/94 lineage (Fig. 1a), sharing 91.6–96.9 % nucleotide sequence identity. Nucleotide sequence analysis of nineteen HA gene from 2001 to 2012 showed that they were closely related (90.4–100 %). The NA genes of all viruses

exhibited homology of 87.6–100 % and belonged to the BJ/94 and G9 lineages, with a bootstrap value ≥70 % indicating a common origin (Fig. 1b). All isolates possessed a deletion of three amino acids in the stalk region, but the strains CK/HN/321/08 and CK/HN/323/08 differentially clustered into the G9 lineage, indicating that they might evolve at a slower rate compared with the other isolated strains in the study (Table 2). The M genes of thirteen viruses could be grouped with BJ/94-like viruses, while the remaining viruses were of the G1 lineage (Fig. 1g). The NS genes in the nineteen isolates from 2001 to 2012 were most similar to BJ/94-like H9N2 viruses, which corresponded to their HA gene lineages and exhibited less diversity (Fig. 1h), with nucleotide homologies of 92.8–99.9 %. Our analyses clearly show that all of the HA and NS genes and seventeen NA genes belonged to the BJ/94 lineage, suggesting that BJ/94-like viruses still are predominant in northern China and relatively conserved in recent years. However, phylogenetic analysis of M genes showed that some of these strains clustered with the BJ/94 lineage, while others were more similar to those found in the G1 lineage. More specifically, no M genes from H9N2

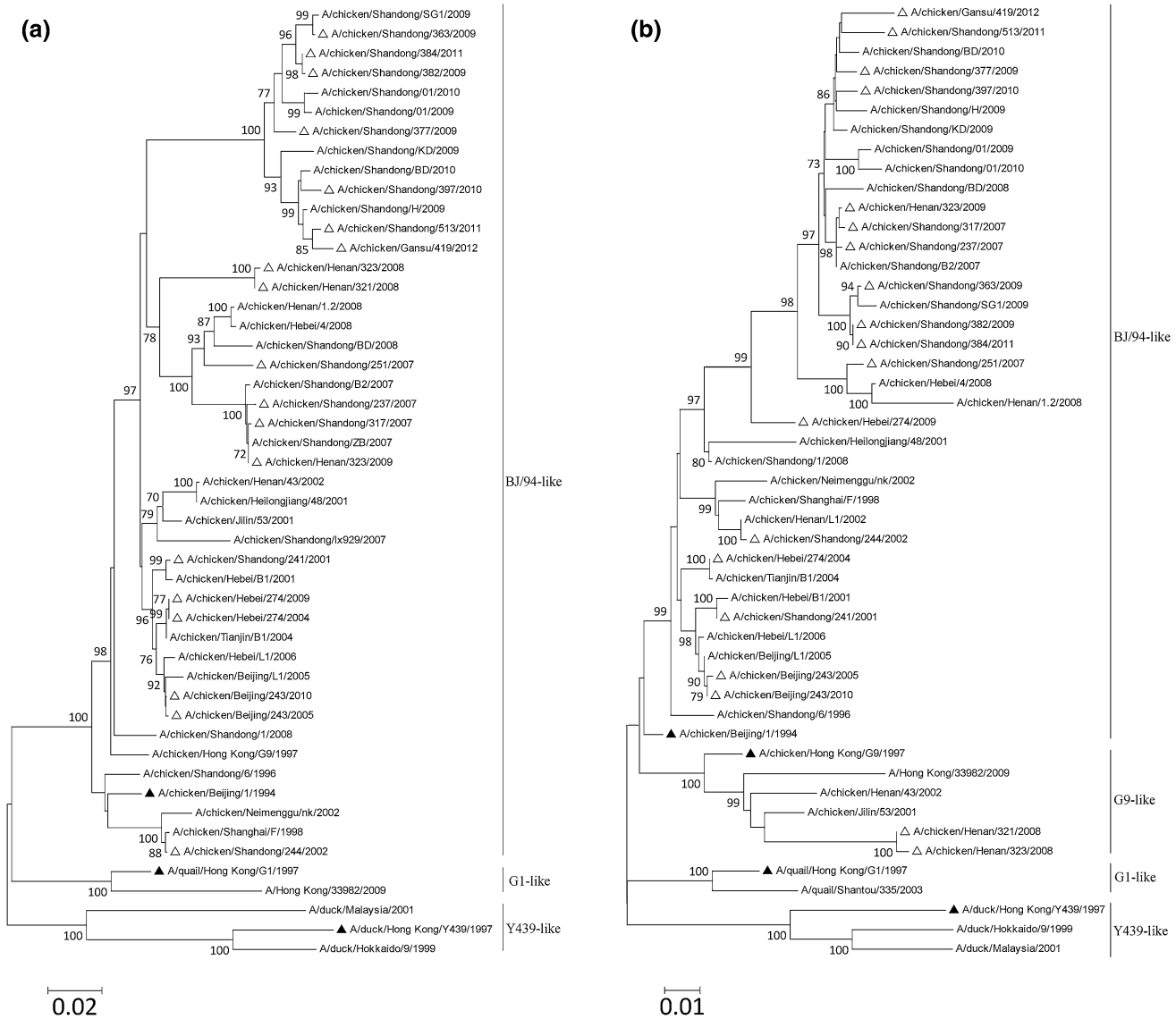

Fig. 1 Phylogenetic trees for HA (**a**), NA (**b**), PB1 (**c**), PB2 (**d**), PA (**e**), NP (**f**), M (**g**), and NS (**h**) of AIVs collected from mainland China between 2001 and 2012. Representative strains are indicated by *solid triangles*; the *empty triangles* indicate strains isolated in this study. The unrooted phylogenetic tree was generated by the distance-based neighbor-joining method using the MEGA5 software suite. The reliability of the tree was assessed through bootstrap analysis with 1000 replications, and values lower than 70 % were hidden. Analysis was based on the following nucleotides: 13–1061 for the HA gene, 1–1393 for the NA gene, 95–2180 for PB1, 40–2262 for PB2, 27–2127 for PA, 1–1259 for NP; 25–884 for M, and 34–707 for the NS gene

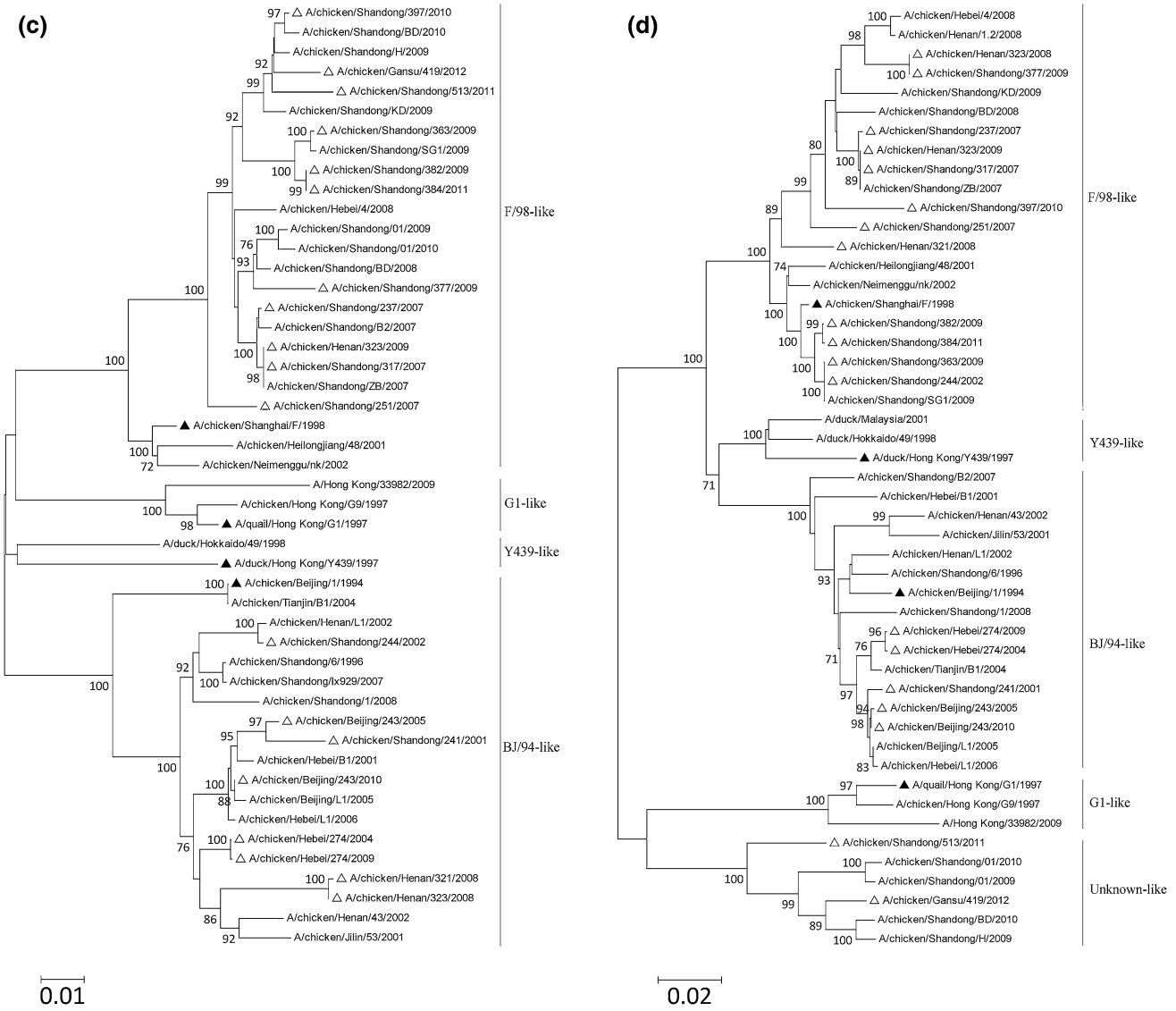

Fig. 1 continued

AIVs isolated before 2008 were seen in the G1 branch. This finding indicates that (i) a reassortment event specific for the M gene was often seen during H9N2 virus evolution, supporting the view that reassortment frequently occurs in this endemically circulating group of viruses, and (ii) G1-like strains have a potential to spread up to northern areas in China, suggesting that continuous work needs to be done for multiple-genotype H9N2 influenza control.

Phylogenetic analysis of PA, PB1, PB2, and NP genes

Compared with genes that encode viral surface proteins, phylogenetic analysis of the four ribonucleoprotein (RNP) genes (PA, PB1, PB2, and NP) revealed different levels of diversity (Fig. 1c–f). For the PA gene, the strains clustered into three different lineages: ten strains belonged to the SH/F group and seven strains belonged to the BJ/94 group, but CK/SD/317/07 seems to be in another small branch far from BJ94, while CK/HN/321/08 and CK/HN/323/08 clustered into the Y439 branch, implying that these two isolates might be recombinants, as they contain the Y439-like PA gene, an indicator of reassortment with the H5N1 virus [57, 62] (Fig. 1e). The PB2 genes were predominantly divided among the SH/F and BJ/94 branches, except that the CK/SD/513/11 and CK/GS/419/12 strains clustered within an unknown branch although they were in a large branch of G1 lineage, revealing that a new genotype emerged in Shandong Province in 2011 and spread to Gansu Province in 2012 (Fig. 1d). However, analysis of the PB1 and NP genes indicated that all H9N2 chicken isolates

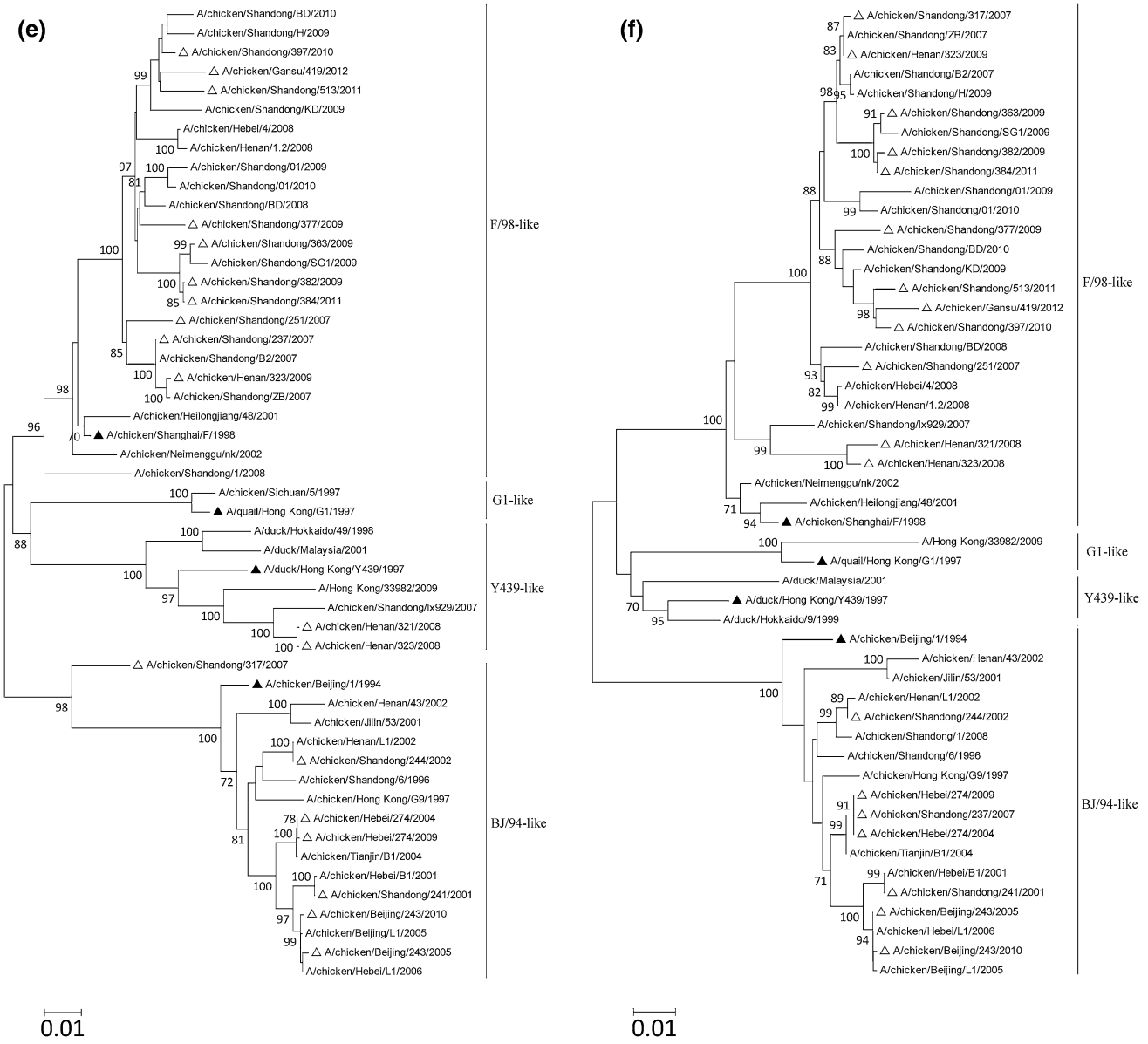

Fig. 1 continued

formed two distinct BJ/94 and SH/F lineages, respectively (Fig. 1c, f), and shared 88.6–99.9 % and 91.7–100 % nucleotide sequence identity, respectively. Our findings demonstrated that the RNP genes were mainly derived from the two distinct SH/F and BJ/94 lineages, which are the dominant strains that have been circulating north of the Yangtze River since 1998 [47].

Genotyping

Many genotyping methods for AIVs have been proposed by laboratories worldwide [35, 42, 47, 57]. It is necessary to establish a robust molecular genetic classification system to gain a better understanding of virus evolution and disease outbreaks. In the current study, we used a genotyping technique described by Li et al. [34]. The evolution of H9N2 influenza virus genotypes in China from 2001–2012 is presented in Fig. 2. On the basis of genomic diversity, five (CK/SD/241/01, CK/HB/274/04, CK/BJ/243/05, CK/SD/274/09, and CK/SD/243/10) of the nineteen analyzed viruses could be classified as genotype A viruses and were genetically close to BJ/94. The four strains (CK/SD/251/07, CK/SD/363/09, CK/HN/323/09, and CK/SD/397/10) in genotype H group were similar to the SH/F virus. The remaining seven genotypes (Fig. 2) are new genotypes that we are reporting here for the first time. The polymerase segments in the J, K, and L genotypes contained partial substitutions from the SH/F polymerase genes. Three strains (CK/SD/382/09, CK/SD/

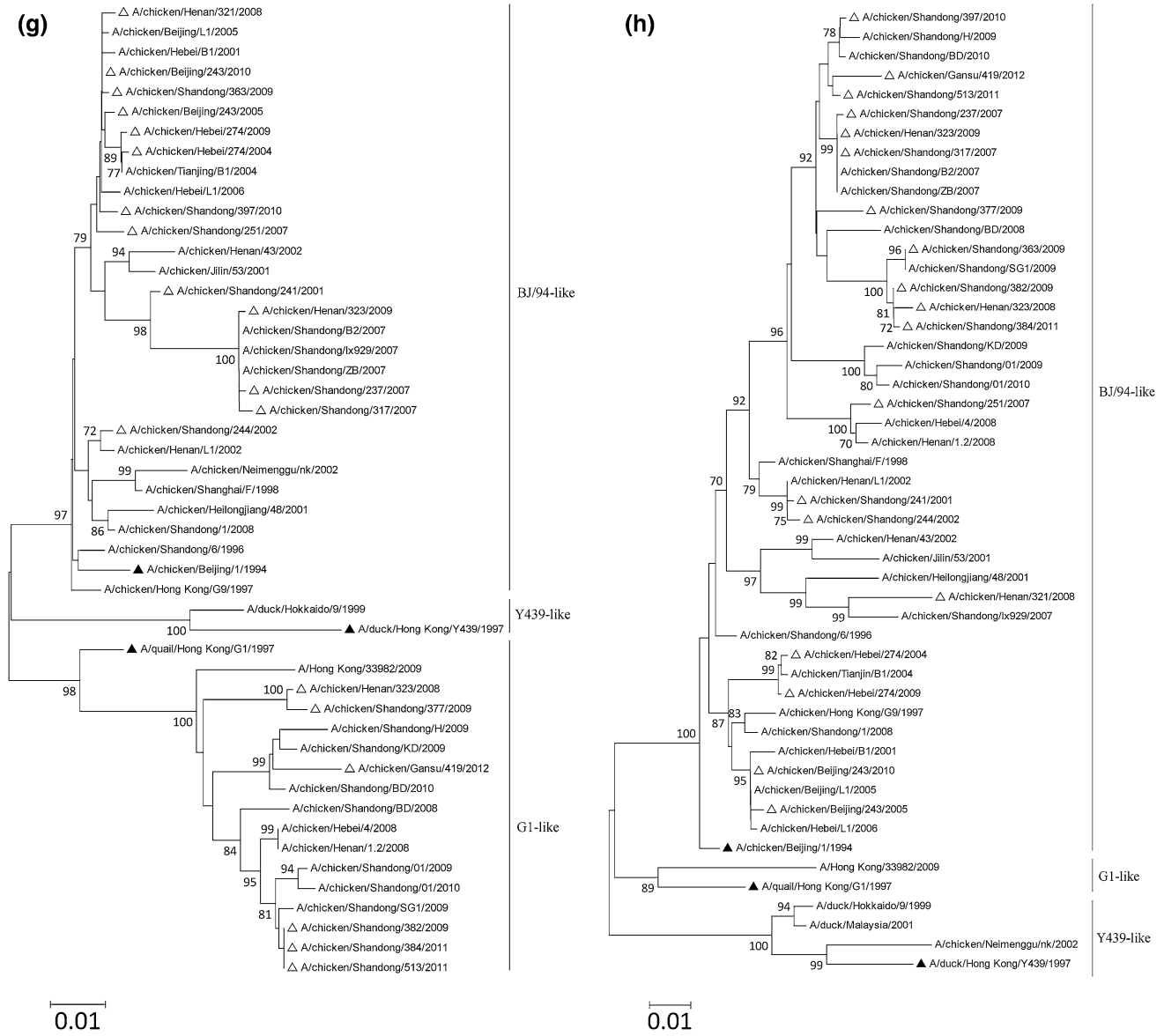

Fig. 1 continued

377/09, and CK/SD/384/11) containing G1-like M genes have been found in genotype O viruses since 2009. The G1 or Y439 lineages were mainly prevalent in southern China, while the BJ/94 lineage predominantly circulated in northern China [47]. Our findings showed that the dominant H9N2 lineage in China has changed as a result of reassortment. Genotypes M and N appear to be derived from the Y439-like lineage that became evident in 2008 in Henan Province. The new genotype P, which emerged in 2011–2012, contains a PB2 gene of unknown ancestry. Taken together, our phylogenetic analyses show that all genotypes were originally from the CK/BJ/1/94 strain, a prevalent H9N2 virus that has been circulating in northern China. H9N2 viruses are evolving in chicken farms in northern China. An SH/F PB2 gene (genotype J) was first found in 2002, and during 2009, a BJ/94, G1, and SH/F triple reassortant (genotype O) that had established a stable lineage was isolated in Shandong Province. However, genotypes M and N were possibly unstable lineages, as these two genotypes were not seen again after 2008. The new genotype P emerged in 2011–2012, with the PB2 gene originating from an unknown influenza virus [3]. Whether this new genotype continues to evolve in birds requires long-term surveillance and further basic study.

Molecular characterization

To investigate the molecular characteristics of nineteen H9N2 isolates, the deduced amino acid sequences of the

Table 2 Molecular characteristics of virus isolates investigated in this study

Virus	Amino acid sequence at cleavage site of HA	Receptor-binding sites in HA			NA deletion	M2 residue at amantadine resistance mutation position	PB2 enhanced polymerase activity and increased virulence in mice	
		191	198	234-236		31	627	701
CK/SD/241/01	PARSSRG/LF	N	V	LQG	62-64	S	E	D
CK/SD/244/02	PSRSSRG/LF	N	V	QQG	62-64	S	E	E
CK/HB/274/04	PSRSSRG/LF	N	V	QQG	62-64	S	E	E
CK/BJ/243/05	PARSSRG/LF	N	T	QQG	62-64	S	E	D
CK/SD/251/07	PARSSRG/LF	N	T	QQG	62-64	N	E	D
CK/SD/237/07	PARSSRG/LF	N	A	QQG	62-64	S	E	D
CK/SD/317/07	PSRSSRG/LF	N	V	QQG	62-64	S	E	D
CK/HN/323/08	PARSSRG/LF	S	V	QQG	No deletion	S	E	D
CK/HN/321/08	PSRSSRG/LF	S	V	QQG	No deletion	S	E	D
CK/SD/382/09	PSRSSRG/LF	N	V	LQG	62-64	N	E	E
CK/HB/274/09	PARSSRG/LF	N	V	QQG	62-64	S	E	D
CK/SD/377/09	PSRSSRG/LF	N	V	LQG	62-64	S	E	D
CK/HN/323/09	PARSSRG/LF	N	T	QQG	62-64	S	E	D
CK/SD/363/09	PSRSSRG/LF	N	T	LQG	62-64	S	E	E
CK/SD/397/10	PSRSSRG/LF	N	V	LQG	62-64	R	E	D
CK/BJ/243/10	PARSSRG/LF	N	T	QQG	62-64	S	E	D
CK/SD/384/11	PSRSSRG/LF	N	V	LQG	62-64	N	E	E
CK/SD/513/11	PSRSSRG/LF	N	T	LMG	62-64	N	E	D
CK/GS/419/12	PSRSSRG/LF	N	T	LMG	62-64	N	E	D

HA protein were aligned and compared. The cleavage site motif in all nineteen HA proteins was PS(A)RSSR/GLF, which is recognized by tissue-specific proteases, thereby limiting spread in the infected host. This cleavage sequence is identical to that in reference strains such as BJ/94 and SH/F and suggests the presence of a LPAIV in poultry. A potential glycosylation site (N-X-T/S, where X could be any amino acid except for P) was reported to be related to virulence [1, 25, 33]. The HA proteins of nine H9N2 strains contained seven potential glycosylation sites. The HA proteins of CK/HN/323/08, CK/BJ/243/05, and CK/BJ/243/10 contained the T300I mutation, which resulted in the loss of one potential glycosylation site. However, seven strains contained an additional potential glycosylation site (P315S). Whether this change has an impact on antigenic variation or pathogenicity requires further investigation in animals. Gln226 is in the receptor-binding site (RBS) of the HA1 region and confers high binding affinity to 2, 3-linked sialic acid (SA) moieties typically found in birds and horses but low binding affinity to the 2, 6-linked SA moieties found in most mammals [26, 46]. In the present study, amino acid 234 in eight strains (amino acid 226 in H3) was a part of a human RBS motif that is of great importance for H9N2 viral replication in airway epithelial cells *in vitro* and for transmission in ferrets [53]. In accordance with previous reports, five of the seven amino acid residues (residues 109, 161, 163, 202, 203) in HA1 that were involved in the RBS were highly conserved, while the remaining two (191 and 198) contained variations (Table 2) [25, 34]. The HA proteins of nineteen isolates contained an asparagine at position 191, whereas this was a serine in strains CK/HN/323/08 and CK/HN/321/08. However, the amino acid residue at position 198 of the HA could be a V, A, or T. The NA protein in viruses of the BJ/94 lineage contains a previously described deletion of three amino acids (residues 62–64) in the stalk region. This resulted in increased NA activity and release of virus from erythrocytes [48]. The two H9N2 isolates, CK/HN/323/08 and CK/HN/321/08, possessed the full-length NA protein (469 amino acids), indicating that these three amino acids may not be required for NA activity and virus release, and it is possible that some other sites in the NA segment might compensate this function very well in virus evolution.

In previous studies, it was reported that a single E627K mutation in PB2 of an H5N1 virus contributed to systemic infection and impaired T-cell activation in mice, while the D701N mutation caused H5N1 to replicate at higher levels and increased its lethality in mice [23, 35, 41, 55]. None of the PB2 proteins of the nineteen H9N2 isolates contained

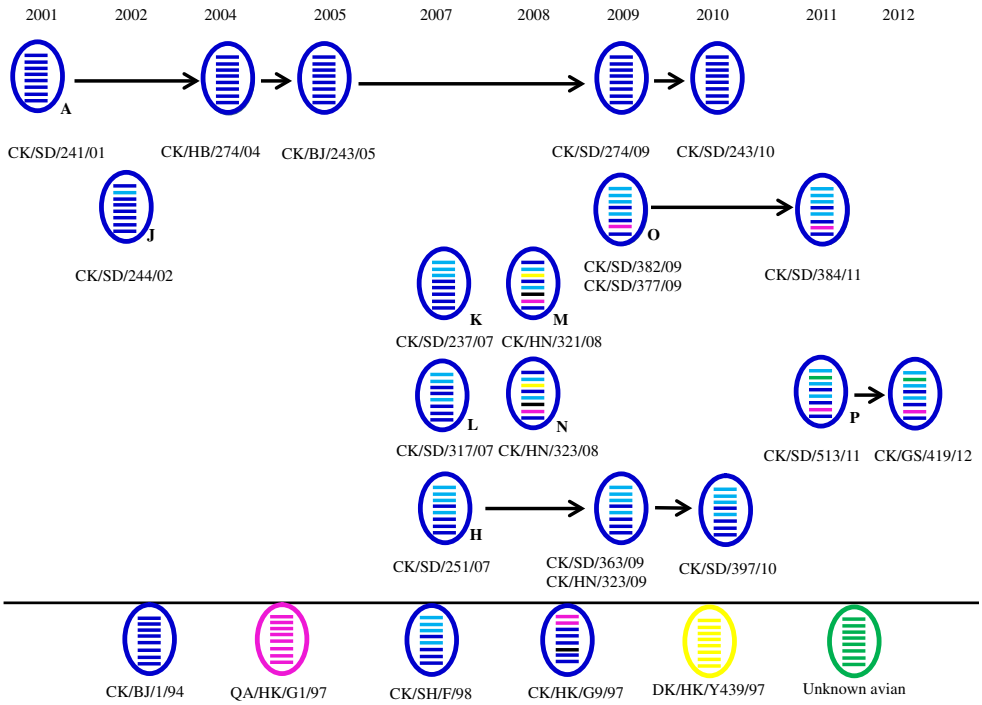

Fig. 2 Genotype evolution of Chinese H9N2 influenza viruses isolated from 2001 to 2012. The eight gene segments are (horizontal bars from the top) PB1, PB2, PA, HA, NP, NA, M, and NS. Each color represents a virus lineage. Genotypes were previously defined by Li et al. [33] (color figure online)

these mutations, suggesting that current strains may not possess the ability to infect mammals, or that these two sites are not important determinants for host range and pathogenicity in the H9N2 subtype. Analysis of the M2 protein revealed an S31N mutation in six isolates (Table 2). The S31N mutation confers resistance to the anti-influenza drug amantadine [54]. In the current study, the proportion of drug-resistant mutant strains was only 30 %, partly because antiviral drugs are not widely administered to chickens.

The NS1 protein antagonizes interferon (IFN) by inhibiting its production and interfering with IFN-induced antiviral processes [2, 18, 31, 36]. The NS1 proteins of all nineteen isolates comprised 217 amino acids. Compared with the full-length NS1 protein (230 amino acids), the NS1 proteins in our isolates lacked a C-terminal PDZ domain ligand (PDZ-L) [39]. The PDZ-L of the full-length avian NS1 protein is located between residues 227 and 230 and can increase the virulence of AIVs [15, 27]. The F103L and M106I mutations in the NS protein were also found to affect viral replication and virulence [11]. In this study, the strains we investigated contained F103L and M106I mutations, indicating that some H9N2 viruses in chickens are prone to variations in virulence. In addition, the presence of the P42S and V149A mutations in the NS1 protein increased virulence in mice and correlated with the ability of H5N1 viruses to antagonize IFN induction in chicken embryonic fibroblast cells [29, 36, 64]. All nineteen H9N2 isolates we examined in this work carried these two mutations in the NS1 protein, implying that these viruses had increased virulence in mice or other susceptible mammals.

PB1-F2 is a nonstructural protein of influenza viruses, encoded by the PB1 gene segment, that contributes to viral pathogenicity [7, 9, 14, 44]. In our study, the deduced PB1-F2 proteins were 52, 79, 90, and 101 amino acids long. Of the nineteen strains, fourteen encoded a PB1-F2 protein longer than 78 amino acids, which is a prerequisite for it being functional [60]. The length of the PB1-F2 protein for the CK/SD/251/07 strain is 101 amino acids, which is rarely seen in AIVs [25]. Recent studies have shown that N66S may increase viral pathogenicity and replication in mice, as this mutation can increase the risk of co-infection with bacteria or block the innate immune response [9, 44]. We did not observe the N66S mutation in the PB1-F2 protein of virus strains in our analysis.

Viral replication and pathogenicity in mice

Chen et al. reported that H5N1 viruses that circulated in ducks of southern China have gradually acquired the ability to replicate in, and kill, mice [6]. Based on variations of in the HA, NA and PB1-F2 genes, we hypothesize that these variations could contribute to pathogenicity in mice. Therefore, we selected six H9N2 strains for a mouse infection experiment. In these strains, the amino acid at the receptor binding site of CK/SD/244/02 and CK/BJ/243/05 at position198 is V and T, respectively, and CK/SD/363/09 and CK/GS/419/12 have LQG and LMG, respectively, at position 234-236. CK/HN/323/09 has a deletion of aa

62-64 in the NA segment, but CK/HN/323/08 does not). Of the six viruses investigated, clinical signs of infection or death were not observed even when the viruses were recovered from the lungs of the mice, except for the CK/GS/419/12 strain on days 3 and/or 6 postinfection (Fig. 3a); however, the CK/BJ/243/05 strain, with 198T in the HA protein, caused the most weight loss (around 3.95 %) in these strains (Fig. 3b). The viruses only replicated in the lungs (Fig. 3a), and no virus was detected in the spleen, kidneys, liver and brain. CK/HN/323/08, without a 3-amino-acid deletion in the NA segment, replicated in the mouse lung, with the highest titer observed on day 3 postinfection. An amino acid difference in HA proteins of CK/SD/369/09 and CK/GS/19/12 (Q235M) resulted in a significant difference in viral replication on day 3 postinfection, presuming that the single mutation might contribute the receptor recognition of H9N2 AIVs. The recovered viruses from the lungs were completely sequenced, and we found that the amino acids at positions 627 and 701 of the PB2 protein still were glutamine and aspartic acid, respectively (data not shown). These results suggest that the strains we tested had not fully acquired the ability to infect mammals, and they further suggest that these two sites might not be determinants of host range and pathogenicity. However, it is still imperative to take strong measures to control the spread of H9N2 viruses among chickens in order to prevent threats to human health.

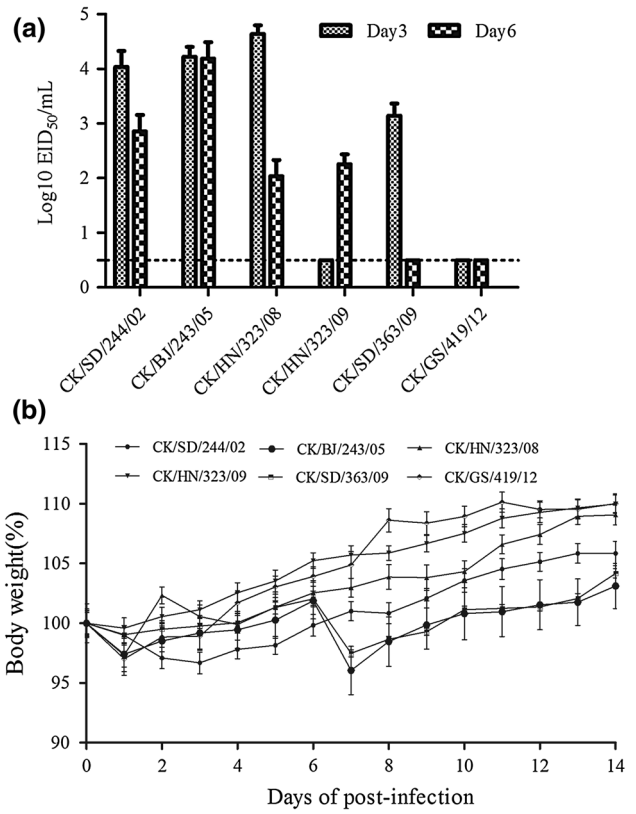

Fig. 3 **a** Titration of virus from the lungs of mice on days 3 and 6 postinfection in MDCK cells. **b** Weight changes in mice inoculated with H9N2 influenza viruses

In summary, the nineteen H9N2 viruses we investigated in this study are primarily re-assortments from BJ/94 and G1 common ancestors, and are prevalent in China. Our findings indicate that (i) All HA and NS genes were directly derived from BJ/94, (ii) CK/HN/321/08 and CK/HN/323/08 were recombinants of the SH/F, G9 and Y439 lineages, and (iii) M genes of CK/SD/513/11 and CK/GS/419/12 clustered in the G1 lineage, but the PB2 genes of these two strains were in an unknown branch. Seven new genotypes were identified in our current work. Two surface glycoproteins and three small proteins (M2, NS1, and PB1-F2) possessed typical point mutations or deletions. Whether these mutations affect pathogenicity, host range, or viral replication requires further investigation. The H9N2 viruses still appear to be a potentially high risk to poultry and humans, and therefore continued surveillance of domestic and migratory birds is necessary and would help to better understand virus evolution and the emergence of pandemic strains in China.

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Conflict of interest The authors have read the final manuscript and declare no conflict of interest.

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