The Journal of Veterinary Medical Science

FULL PAPER

Virology

Molecular evolution and amino acid characteristics of newly isolated H9N2 avian influenza viruses from Liaoning Province, China

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ABSTRACT. H9N2 is widespread among poultry and humans. Though this subtype is not lethal to either species, it can cause considerable financial losses for farmers and threaten human health. In this study, 10 new H9N2 avian influenza viruses (AIVs) produced by reassortment were isolated from domestic birds in Liaoning Province between March 2012 and October 2014. Nucleotide sequence comparisons indicate that the internal genes of one of these strains are highly similar to those of human H7N9 viruses. Amino acid substitutions and deletions occurred in the HA and NA proteins separately, indicating that all 10 of these isolates may have an enhanced ability to infect mammals. A cross-hemagglutinin inhibition assay conducted with two vaccine strains that are broadly used in China suggests that antisera against vaccine candidates cannot completely inhibit the new isolates. Two of the 10 newly isolated viruses could replicate in respiratory organs of infected BALB/c mice without adaption, suggesting that these isolates can potentially infect mammals. The continued surveillance of poultry is important to provide early warning and control of AIV outbreaks. Our results highlight the high genetic diversity of AIV and the need for more extensive AIV surveillance.

KEY WORDS: H9N2, influenza, pathogenicity, phylogenetic, poultry

J. Vet. Med. Sci.
82(1): 101–108, 2020
doi: 10.1292/jvms.19-0421

Received: 31 July 2019
Accepted: 9 November 2019
Advanced Epub:
4 December 2019

Avian influenza virus (AIV) is a leading cause of zoonotic disease and bird flu outbreaks can lead to serious economic losses in industrial poultry farms, infectious disease outbreaks in humans, and a high risk of mortality [12]. Two categories of AIV, high pathogenic AIV (HPAIV) and low pathogenic AIV (LPAIV), have been identified based on virulence. Although LPAIV is less harmful, it has been widely detected in poultry and wild birds with highly variable prevalence. In addition, the rearrangement of the LPAIV genome inside an infectious vector may generate a novel strain of HPAIV, potentially leading to a bird flu epidemic [16]. This finding indicates that the potential hazards of LPAIV infection warrant extensive concern.

The H9N2 subtype was first isolated from turkeys in North America in 1966 and is categorized as an LPAIV. The first infectious case of H9N2 was reported at a chicken farm in Guangdong in 1994 in China [1]. This subtype likely possesses a wide range of poultry and waterfowl hosts like chickens, ducks, turkeys, pigeons, and mammalian hosts like pigs, dogs, cats, and humans. Host infection-induced H9N2 gene recombination can generate novel HPAIV subtypes that post an increased risk of influenza outbreak [2]. Increasing evidence has demonstrated that some gene fragments of the H5N1 subtype from Hong Kong are derived from the H9N2 subtype. A similar pattern of derivation has been observed for the newly isolated H7N9 influenza A virus from 2013 cases in Shanghai and the Anhui Province of China and the H10N8 human-infecting subtype isolated in 2014 [7, 18]. Researchers have studied the H9N2 strain for decades. If H9N2 viral pathogenesis were to be altered via gene mutations and recombination, the resulting pandemic outbreak would result in an enormous loss. The H9N2 subtype is capable of binding to human cell membrane receptors and being processed for cell entry [10], indicating an enhanced risk of human infections by this subtype and thus a

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potential threat to human health.

In this study, the virulence of the H9N2 AIV strain isolated in northern China from 2012 to 2014 was analyzed using different mammalian hosts. We successfully acquired ten H9N2 isolates, one of which harbors high identity to human H7N9. Our results provide baseline information about the prevalence of H9N2 AIV in poultry. This work sheds light on the characterization of H9N2 pathogenesis in animals and provides support for the prevention of potential infectious outbreaks.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with 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. All animal experiments were performed using enhanced animal biosafety level 2+ (ABSL 2+) protocols in the Harbin Veterinary Research Institute of the Chinese Academy of Agriculture Science.

Sample collection

In an epidemic investigation of avian influenza in chickens on poultry farms in the Liaoning Province between March 2012 and October 2014, we obtained 17,940 clinical swab samples and 315 clinical tissue and organ samples from the farms and fecal samples through normal surveillance. The chickens had no clinical signs and appeared healthy. The collected samples were placed in a phosphate-buffered solution (pH 7.0) containing penicillin, streptomycin, and 10% glycerin and stored at 4°C for transport.

Virus isolation and identification

Samples were vortexed, centrifuged, and used to inoculate 9-day-old specific-pathogen-free (SPF) chicken embryos. After 72 hr of culturing, the allantoic fluid was harvested and its hemagglutination (HA) activity evaluated using an HA test. The influenza virus and HA subtypes were then identified using an HA inhibition (HI) test involving H1 to H16 subtype serum (stored by the State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China).

Viral RNA was extracted from HA-positive samples (except for the Newcastle disease-positive samples, which were analyzed using HA-HI tests) according to standard methods involving TRIzol mixed with 250 μl of allantoic fluid sample. cDNA was reverse-transcribed using the resulting RNA with 12 bp primers and the influenza virus segments amplified using H9N2-specific primers. PCR products were purified using a PCR purification kit (TianGen, China) and sequenced by GENEWIZ (China). NA subtypes were directly analyzed by subtype-specific RT-PCR and sequencing. All sequences were deposited into GenBank. Virus subtyping was confirmed by comparing sequences to those of isolates in GenBank and identifying the subtype with the highest percent similarity.

Sequence data collection and alignment

Sequencing data used to construct the phylogenetic tree was downloaded from the NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The coding sequence of each gene segment was aligned using MUSCLE (PB2, 2,277 bp; PB1, 2,271 bp; PA, 2,148 bp; HA, 1,698 bp; NP, 1,494 bp; NA, 1,407 bp; MP, 756 bp; and NS, 690 bp).

Genetic and phylogenetic analyses

Nucleotide sequences were edited using the SeqMan module of the DNAstar package and phylogenetic analyses performed using MEGA 6.0 maximum likelihood trees with bootstrap values of 1,000. Multiple sequence alignments were compiled using ClustalW in MegAlign of DNAstar. The sequences in the phylogenetic tree were downloaded from GenBank database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database).

Mouse infection experiments

Six-week-old female BALB/c mice were purchased from the Experimental Animal Center of Vital River (Beijing, China). These mice were randomly divided into three infection groups (eight mice/group) and one control group (five mice/group). Animals in the infection groups were mildly anesthetized with CO_2 then intranasally inoculated with viruses at a dose of 10^6 EID_{50} (50 μl/mouse) [4]. Three days later, three mice were randomly selected from each infected group and euthanized with CO_2. Mouse organs, including the brain, turbinate, spleen, lungs, and kidneys, were collected and separately homogenized. Homogenates were then used for virus titration in 9–11-day-old chick embryos. Virus titers were calculated using the method from Reed and Muench (Logarithm of viral dilution greater than 50% of deaths–(more than half of the whole number–50%)/(more than half of the whole number–less than half of the whole number)* diluted multiples). The behavior and the body weight changes of the mice were observed and recorded for 14 consecutive days.

RESULTS

Influenza virus isolation and subtype identification

Based on HA testing, ten H9 subtype influenza viruses were identified. We configured the viruses into 4-unit antigen HA

inhibition (HI) tests using H1 to H16 subtype mono-factor serum. In total, ten H9N2 AIVs were isolated from 18,255 samples collected from chickens in Liaoning Province, China, from 2012–2014. Among the 10 isolated strains, one virus named LN/LN14 possessed internal genes matching H7N9 subtypes. In this paper, we analyzed the complete genome sequences of these ten H9N2 virus isolates and submitted the resulting sequence to the GenBank database (MK979285-MK979364).

Phylogenetic analysis of genes encoding surface proteins

Phylogenetic analysis revealed that the HA genes of the ten H9 isolates all appear to belong to the Eurasian lineage based on nucleic acid identities of >95% within each group (Fig. 1A). The homology within groups was 95.5–99.8% at the nucleic acid level, and all HA genes belonged to one group (Fig. 1A).

The N2 NA genes exhibited greater diversity than the HA genes and clustered into three groups based on nucleic identities of >95%. The N2 NA genes of viruses in these three groups all belong to the Eurasian lineage. There were two viruses in group 1, one virus in group 2, which had the highest identity with A/chicken/Yantai/2243/2013 (H9N2), and seven viruses in group 3. The homology of nucleotides and amino acids was 91.7–100% and 92.7–100% among these ten isolates, respectively (Fig. 1B).

Phylogenetic analysis of the internal protein-coding genes

To better understand the evolution of the H9 AIVs examined in this study, phylogenetic analysis of all six internal genes of the four novel genotypes identified in this survey was performed. All internal genes are of Eurasian lineage and exhibit diversity (Table 1).

Based on nucleic acid identity, the PB1, NP, and M genes belong to one group while the PB2, PA, and NS genes divide into two groups. The respective nucleotide and amino acid homologies from the ten H9 isolates in this study were 94.3–100% and 97.5–100% for the PB2 genes, 96.4–99.9% and 98.2–99.9% for the PB1 genes, 94.7–100% and 97.2–99.9% for the PA genes,

Fig. 1. Phylogenetic analysis by maximum likelihood of the HA and NA genes of H9N2 subtype avian influenza viruses (AIVs) isolated in 2012–2014. Phylogenetic trees were generated using Mega 6.0. Evolutionary history was inferred using a maximum likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood (−10 541.1792) is shown. The percentage of trees in which the associated taxa cluster together is shown next to the branches. The initial tree(s) for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. This tree is drawn to scale, with branch lengths indicating the number of substitutions per site. The phylogenetic trees of HA (A) and NA (B) genes use no root tree. The virus sequences listed in black were downloaded from available databases while viruses listed in red, purple, and pink were sequenced in this study. CK, chicken; DK, duck; EN, environment; GS, goose; ML, mallard; SW, swine; WCK, wild chicken; WDK, wild duck.

Table 1. Information about viruses examined in this study

Number	Virus name	Abbreviation	Location of isolation in Liaoning Province	Host	Date
1	A/Chicken/LiaoNing/PJ03/2012 (H9N2)	LN/PJ03	Panjin	Chicken	2012
2	A/Chicken/LiaoNing/SN02/2012 (H9N2)	LN/SN02	Shenyang	Chicken	2012
3	A/Chicken/LiaoNing/LY04/2012 (H9N2)	LN/LY04	Liaoyang	Chicken	2012
4	A/Chicken/LiaoNing/SY03/2012 (H9N2)	LN/SY03	Shenyang	Chicken	2012
5	A/Chicken/LiaoNing/FX04/2012 (H9N2)	LN/FX04	Fuxin	Chicken	2012
6	A/Chicken/LiaoNing/TL01/2013 (H9N2)	LN/TL01	Tieling	Chicken	2013
7	A/Chicken/LiaoNing/SZ02/2013 (H9N2)	LN/SZ02	Suizhong	Chicken	2013
8	A/Chicken/LiaoNing/CY09/2014 (H9N2)	LN/CY09	Chaoyang	Chicken	2014
9	A/Chicken/LiaoNing/A10/2014 (H9N2)	LN/A10	LiaoNing	Chicken	2014
10	A/Chicken/LiaoNing/LN14/2014 (H9N2)	LN/LN14	LiaoNing	Chicken	2014

95.1–100% and 98.2–100% for the NP genes, 97.7–100% and 98.2–100% for the M genes, and 94.3–99.9% and 90.3–100% for the NS genes (Fig. 2A–C, 2E and 2F).

Molecular characterization of viral genes

To determine whether the H9N2 viruses in this study have acquired genetic markers associated with mammalian pathogenicity, virulence, and adaptation to new hosts, we analyzed the whole-genome sequences of all 10 viruses.

The amino acid substitutions Q226L and G228S (H3 numbering, which is used throughout the manuscript) increase the affinity of influenza viruses for human receptors [9–11]. In the 10 viruses we examined, Q226L, but not G228S, is present. All 10 viruses had NA gene stalk deletions from amino acids 63–65, which confers enhanced virus lethality in mice [17]. In addition, E119G, R152K, H274Y, and R292K mutations at NA active sites confer resistance by the influenza virus to neuraminidase inhibitors, however, we did not find any mutations at these NA active sites in isolates we examined.

The influenza virus PB2 protein has several possible mutations that contribute to virulence and adaptation in mammalian hosts, including 89V, 271A, 627K, and 701N [8, 14]. All 10 viruses showed the 89V mutation in their PB2 proteins but lacked 271T, 627E, and 701D mutations. The amino acid 292V/I is conserved in human and avian isolates, therefore the presence of the 292V mutation in a PB2 protein may be another important for the mammalian adaptation of AIVs. Four viruses we examined, LN/SN02, LN/TL01, LN/A10, and LN/LN14, had the 292V variant in their PB2 proteins.

None of the ten H9 viruses had Y436H substitutions in their PB1 proteins or T515A substitutions in their PA proteins, which suggests low pathogenicity towards mammalian and avian hosts. An N66S substitution was found in the PB1-F2 proteins of H9 strains associated with the increased virulence of the 1918 pandemic virus and the highly pathogenic AI H5N1 virus in mice and ferrets [5, 9, 13]. All 10 viruses in our study did not have N66S substitutions in the PB1-F2 protein. Additionally, N30D and T215A mutations in the M1 protein, P42S in the NS1 protein, and L98V in the PB2 protein suggest that viruses exhibit increased virulence in mammals. All 10 virus strains we examined possessed the L89V mutation in PB2 but did not contain the N30D and T215A mutations in M1 or P42S in NS1.

No S31N amino acid substitutions were found in the M2 transmembrane domains, suggesting that this virus strain is sensitive to M2 ion channel inhibitors [6]. However, the S31N amino acid substitution in M2 was present in all 10 viruses in this study, indicating that these viral strains are resistant to amantadine inhibitors. The virulence of influenza viruses in humans is related to their resistance to the antiviral effects of cytokines such as interferon (IFN), and the D92E mutation in NS1 protein resistance to IFN [15]. However, no mutations at residue 92 of NS1 were observed in this study.

Mouse infection experiments

To evaluate the replication capacity and virulence of the three virus strains in mammals, we used conventional methods to perform mouse infection experiments in a biosecurity level 2+ laboratory. After infection, mouse body weights transiently decreased or stabilized then immediately returned to normal levels of growth. Mice maintained stable body weights after infection with strain LN/A10. The body weights of mice infected with strain LN/FX04 transiently decreased at days 0–3 after and then returned to normal levels of growth, however, mice infected with LN/LN04 showed less weight after infection (Fig. 3A). All mice survived throughout observation days. Virus titrations of mouse organs showed that none of the test strains replicated in the brain, spleen, or kidneys. The viruses CK/LN/FX04/12 and CK/LN/A10/14 are able to replicate in the lungs and turbinate, however, no CK/LN/A10/14 virus was present in the lungs and turbinate of mice, indicating differential replication (Fig. 3B). Virus titers were markedly higher in the lungs than in the turbinate.

DISCUSSION

In 1994, the H9N2 subtype was first identified in chicken farms in Guangdong Province in South China and has since become widespread in chickens, causing great economic loss from reduced egg production and highly lethal co-infections. H9N2 vaccines

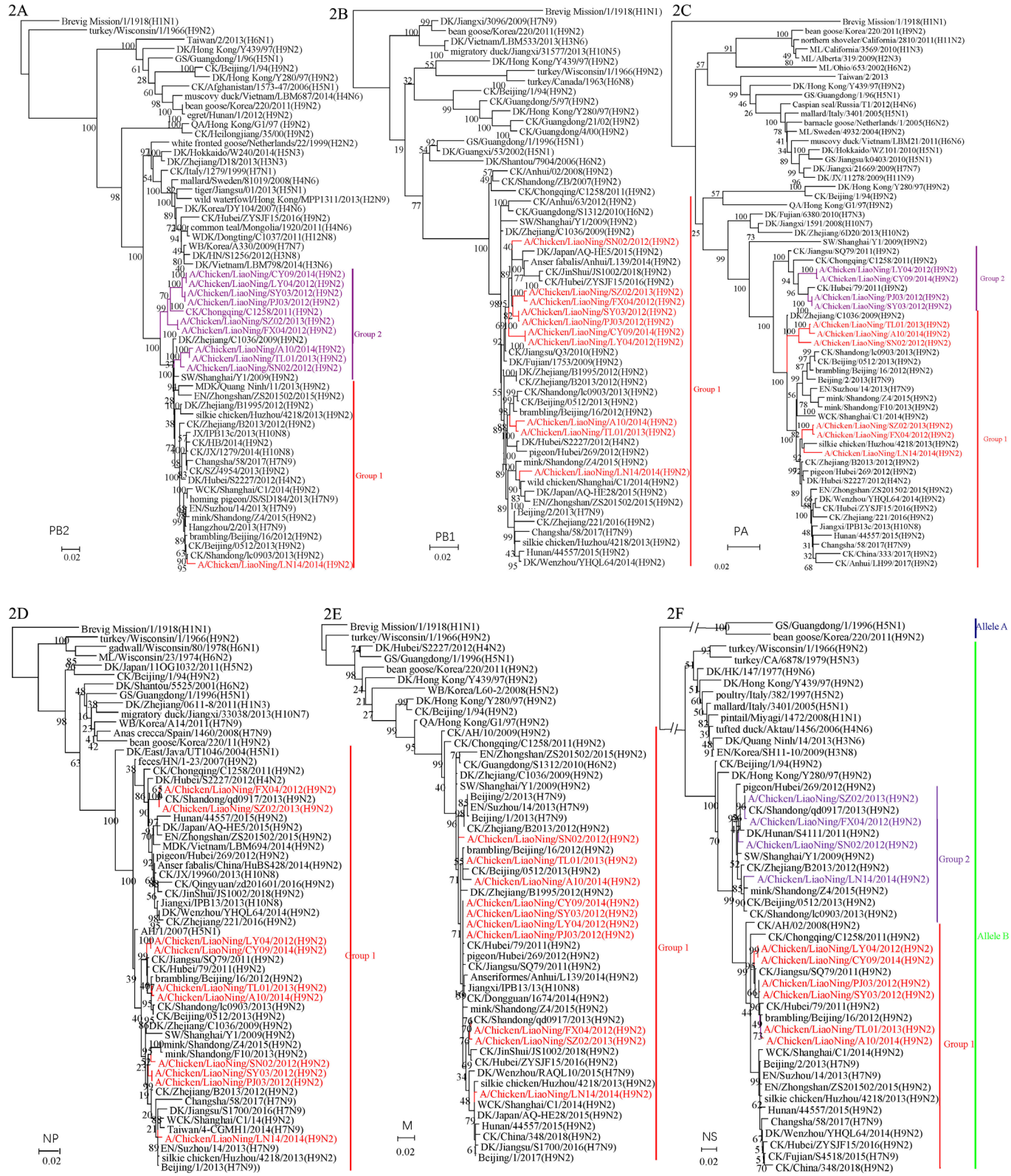

Fig. 2. Phylogenetic analysis using maximum likelihood of the internal genes of H9N2 subtype avian influenza viruses (AIVs) isolated in 2012–2014. Phylogenetic trees for (A) PB2, (B) PB1, (C) PA, (D) NP, (E) M, and (F) NS. The colors of the virus names in the PB2, PB1, PA, NP, M, and NS trees match those used in the genotype table. The sequences of viruses listed in black were downloaded from GenBank while viruses listed in red and purple were sequenced in this study. CK, chicken; DK, duck; EN, environment; GS, goose; ML, mallard; SW, swine; WDK, wild duck.

initially limited viral outbreaks and spread, however, despite the application of multiple doses, H9N2 vaccines became less effective, especially after 2007, and H9N2 influenza virus has since been detected in chicken flocks.

There are few viruses in the GISAD and NCBI influenza virus databases that have been isolated from Liaoning Province in China. Until April 30, 2009, there were twelve such strains in the NCBI influenza virus database, including 11 strains of H9N2 from chickens, three H5N1 strains from chickens, six H9 strains from chickens, one H5 strain from a duck, and one H5N1 strain from a wild duck.

The viruses isolated in this study showed genetic diversity. These 10 viruses formed four genotypes (Fig. 4). Genotype 1 contained LN/LN14, which is a two-reassortment virus containing six internal genes and an HA gene from an A/brambling/Beijing/16/2012 (H9N2)-like virus gene pool. Genotype 2 included two viruses, LN/A10 and LN/TL01, which are tri-reassortment viruses. Genotype 2 PB2 genes and NS genes belong to the A/duck/Ganzhou/GZ188/2016 (H9N2)-like virus gene pool while their PB1, PA, NP, M and HA genes belong to the A/brambling/Beijing/16/2012 (H9N2)-like virus gene pool and their NA genes originate from a different gene pool. Genotype 3 includes four viruses, LN/CY09, LN/SY03, LN/LY04, and LN/PJ03, which are

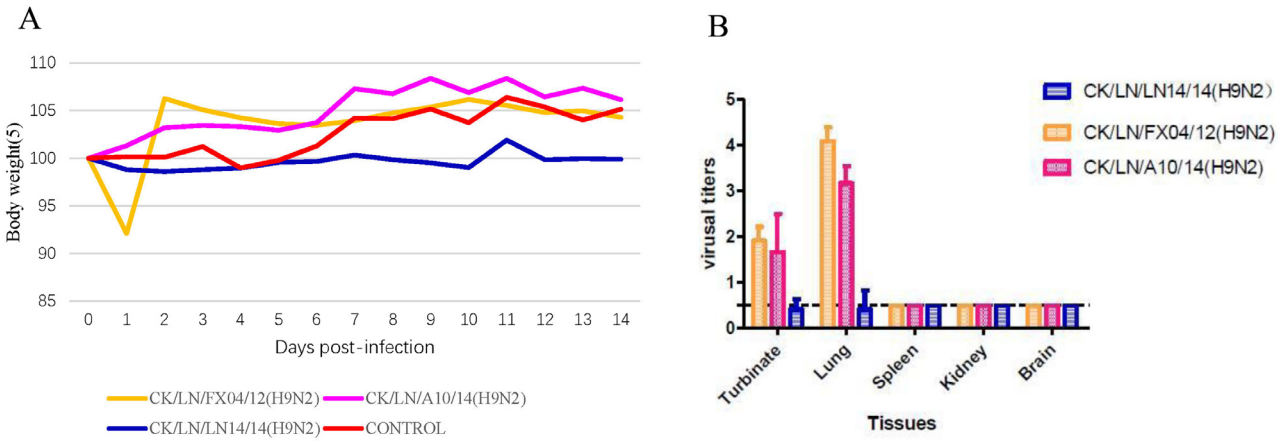

Fig. 3. Mouse infection experiments. (A) Post-inoculation weight changes of mice until 14 days post-inoculation; (B) Viral titers in tissues of mice 3 days post-infection with H9N2.

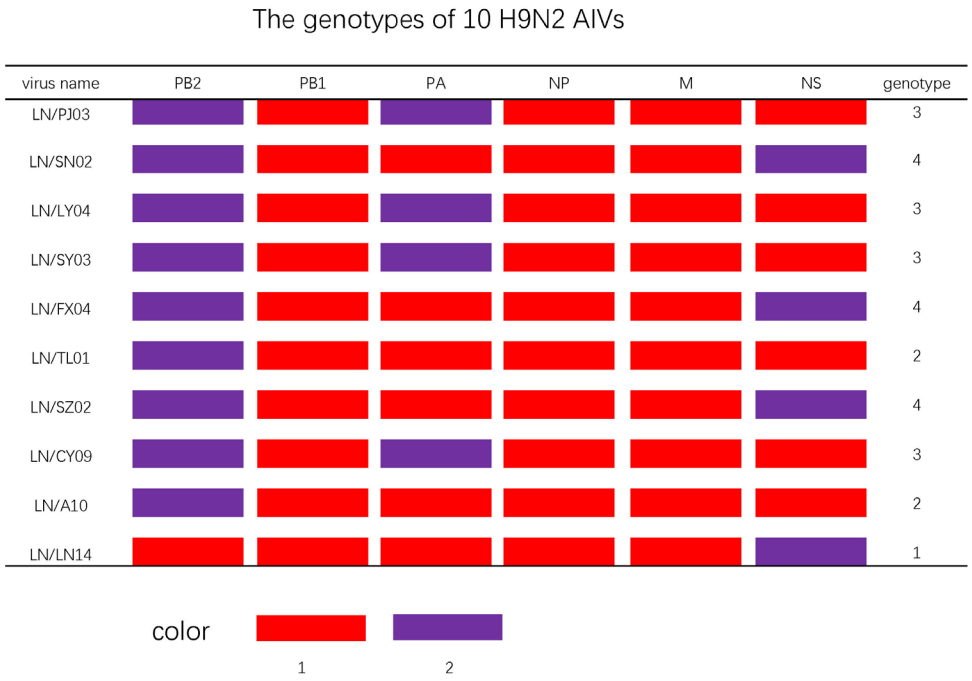

Fig. 4. Simplified schematics showing the putative genomic composition of the novel H9N2 avian influenza viruses (AIVs) produced by reassortment that are described in this study. The six gene segments (from top to bottom) in each virus are PB2, PB1, PA, NP, M, and NS.

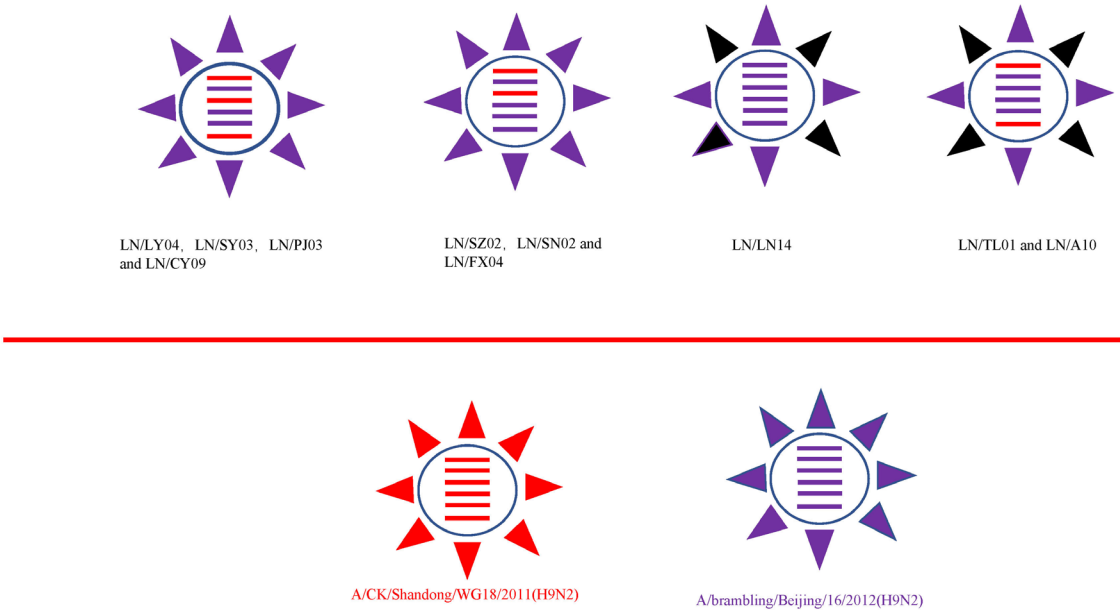

Fig. 5. The genotypes of ten H9N2 avian influenza viruses (AIVs). The triangles outside the circle represent the surface genes HA and NA. Each color represents a separate source background. This illustration is based on nucleotide distance comparison and phylogenetic analysis.

two-reassortment viruses. Their PB2, PA, and NS genes are from the A/duck/Ganzhou/GZ188/2016 (H9N2)-like virus gene pool while their other five genes belong to the A/brambling/Beijing/16/2012 (H9N2)-like virus gene pool. Genotype 4 includes three viruses, LN/SZ02, LN/FX04, and LN/SN02, which are two-reassortment viruses. Genotype 4 PB2 and NS genes are from the A/duck/Ganzhou/GZ188/2016 (H9N2)-like virus gene pool while their other six genes belong to the A/brambling/Beijing/16/2012 (H9N2)-like virus gene pool (Fig. 5).

In March and December 2013, China reported human deaths due to the novel AIVs H7N9 and H10N8. The segmented feature of influenza virus genomes allows for reassortment of segments from different viruses, generating novel influenza viruses with pandemic potential. H9N2 AIVs donated the internal genes for HP H5N1 AIV found in Hong Kong in 1997 [3, 5] and provided internal genes for the novel human AIVs H7N9 and H10N8 [18]. In this study, we investigated the roles of the evolution of H9N2 viruses in the creation of reassorted H7N9 viruses and identified one virus with an internal gene complex showing high identity with H7N9. This virus was isolated in 2014, close to the H7N9 outbreak in 2013. This is more evidence that H9N2 is the internal gene donor for virus reassortment yielding viruses examined in our study.

Our results revealed that H9N2 influenza viruses have evolved via reassortment and mutation over their 10 years of prevalence in chickens. These viruses have the ability to infect mammals and potentially threaten humans. We should therefore enhance the surveillance of chickens for warning and protection.

CONFLICT OF INTEREST. All authors do not have conflicts of interest.

ACKNOWLEDGMENTS. This study was supported by the Special Foundation for State Basic Research Program of China (2013FY113300-8) and the National Key R&D Program of China (2016YFD0500800). We also thank the State Key Laboratory of Veterinary Biotechnology, the Harbin Veterinary Research Institute, and the Chinese Academy of Agricultural Sciences.

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