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Newly Emergent Highly Pathogenic H5N9 Subtype Avian Influenza A Virus

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ABSTRACT

The novel H7N9 avian influenza virus (AIV) was demonstrated to cause severe human respiratory infections in China. Here, we examined poultry specimens from live bird markets linked to human H7N9 infection in Hangzhou, China. Metagenomic sequencing revealed mixed subtypes (H5, H7, H9, N1, N2, and N9). Subsequently, AIV subtypes H5N9, H7N9, and H9N2 were isolated. Evolutionary analysis showed that the hemagglutinin gene of the novel H5N9 virus originated from A/Muscovy duck/Vietnam/LBM227/2012 (H5N1), which belongs to clade 2.3.2.1. The neuraminidase gene of the novel H5N9 virus originated from human-infective A/Hangzhou/1/2013 (H7N9). The six internal genes were similar to those of other H5N1, H7N9, and H9N2 virus strains. The virus harbored the PQRERRRKR/GL motif characteristic of highly pathogenic AIVs at the HA cleavage site. Receptor-binding experiments demonstrated that the virus binds α-2,3 sialic acid but not α-2,6 sialic acid. Identically, pathogenicity experiments also showed that the virus caused low mortality rates in mice. This newly isolated H5N9 virus is a highly pathogenic reassortant virus originating from H5N1, H7N9, and H9N2 subtypes. Live bird markets represent a potential transmission risk to public health and the poultry industry.

IMPORTANCE

This investigation confirms that the novel H5N9 subtype avian influenza A virus is a reassortant strain originating from H5N1, H7N9, and H9N2 subtypes and is totally different from the H5N9 viruses reported before. The novel H5N9 virus acquired a highly pathogenic H5 gene and an N9 gene from human-infecting subtype H7N9 but caused low mortality rates in mice. Whether this novel H5N9 virus will cause human infections from its avian host and become a pandemic subtype is not known yet. It is therefore imperative to assess the risk of emergence of this novel reassortant virus with potential transmissibility to public health.

Five dramatic instances of pandemic influenza were reported worldwide during the 20th century: the 1918 H1N1 Spanish influenza, the 1957 H2N2 Asian influenza, the 1968 H3N2 Hong Kong influenza, the 2003 H5N1 avian influenza, and the 2009 H1N1 pandemic Mexico influenza (1–3). Global epidemic and pandemic influenza has caused devastating catastrophes among humans and animals, and undoubtedly, influenza A virus continues to pose a serious threat to public health.

A wide range of host adaptions and continuous evolution facilitate the emergence of novel influenza viruses. For example, infection of little yellow-shouldered bats with influenza A viruses H17N10 and H18N11 poses a risk of zoonotic spread to humans and the generation of pandemic or panzootic viruses (4, 5). Influenza A virus possesses a characteristically segmented genome, which allows for exchange of eight gene segments between different virus strains. Genetic reassortment among different coinfected viruses may generate novel, human-adapted virus with drastic antigenic change or antigenic shifts (6). In February 2013, a novel H7N9 avian influenza virus "jumped" from chickens to humans, with fatal consequences (7). Late in 2013, a novel reassortant avian influenza virus H10N8 was identified as the causative agent of a fatal case in Nanchang, China (8), and the first human H6N1 influenza virus infection was confirmed in Taiwan (9). The newly emergent subtypes have again sounded the alarm signaling the potential risk to global public health.

Live bird markets (LBMs) are considered to be the source of human H7N9 infections. Little is known about whether the different subtypes of avian influenza viruses (AIV) coexist in poultry, although Yu et al. reported the detection of genomic segments from various AIV in specimens from LBMs epidemiologically linked to human H7N9 cases (10). Here, we investigated the coexistence of AIV subtypes related to human-infecting H7N9 virus in poultry at LBM by next-generation sequencing (NGS), virus isolation, and biological characterization. We identified a novel,

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TABLE 1 Identification of different subtypes of avian influenza A virus from live poultry samples[a]

Source and date of collection[b] and original sample no.	Animal	Subtypes analyzed by NGS[c]	Isolated and designated virus name	Time (h) of chicken embryonated egg death[d]
Binsheng, 4 April 2013				
35	Quail	H9, N2	A/Quail/Hangzhou/1/2013(H9N2)	45
Wuhang, 10 April 2013				
42	Chicken	H5, N1	A/Chicken/Yuhang/1/2013(H5N1)	38
43	Chicken	H7, H9, N2, N9	A/Chicken/Yuhang/3/2013(H7N9)	72
			A/Chicken/Yuhang/2/2013(H9N2)	72
44	Chicken	H5, H7, N9	A/Chicken/Yuhang/2/2013(H5N9)	39
			A/Chicken/Yuhang/5/2013(H7N9)	47
46	Chicken	H5, H7, N1, N9	A/Chicken/Yuhang/4/2013(H7N9)	72
47	Chicken	H5, H9, N1, N2	A/Chicken/Yuhang/3/2013(H9N2)	72
48	Chicken	H5, H7, H9, N2, N9	A/Chicken/Yuhang/1/2013(H7N9)	72
			A/Chicken/Yuhang/1/2013(H5N9)	36
50	Chicken	H7, H9, N2, N9	A/Chicken/Yuhang/2/2013(H7N9)	72
			A/Chicken/Yuhang/1/2013(H9N2)	72
62	Duck	H7, N9	A/Duck/Yuhang/1/2013(H7N9)	72

[a] Five environmental specimens had no hemagglutinin activity, and 1 chicken pharyngeal swab and 3 chicken cloacal swabs were identified as carrying Newcastle disease virus (data not shown). Of 18 specimens, only 9 were identified as influenza A virus-positive samples, and they were subjected to NGS.
[b] Live poultry markets located in Hangzhou, the capital of Zhejiang province, China.
[c] Reads for different subtypes of influenza A virus by Illumina HiSeq Sequencer.
[d] Viruses in these specimens could cause chicken embryonated eggs death within different times.

highly pathogenic (HP) H5N9 avian influenza virus from live poultry.

MATERIALS AND METHODS

Sample collection. After the first H7N9 virus infection case was identified in Zhejiang Province, China, bird and environmental specimens were collected in two live poultry markets in the Binjiang (BJ) and Yuhang (YH) districts of Hangzhou City, the capital of Zhejiang Province. A total of 18 specimens were collected, comprising five environmental specimens, one quail pharyngeal swab, one duck cloacal swab, eight chicken pharyngeal swabs, and three chicken cloacal swabs (Table 1).

RNA extraction and NGS. Viral RNA was extracted from the specimens using the RNeasy minikit (Qiagen, Germany) and checked using an Agilent Technologies 2100 Bioanalyzer for quality. First- and second-strand cDNAs were obtained by reverse transcription and amplification using the influenza A-specific primers MBTuni-12 and MBTuni-13 (Invitrogen) (11). cDNA libraries with an insert size of 200 bp were prepared by end reparation, A tailing, adapter ligation, DNA size selection, amplification, and product purification according to the manufacturer's instructions (Illumina). The cDNA library was then sequenced by 90-bp paired-end sequencing on an Illumina HiSeq Sequencer.

Virus isolation and identification. Virus isolation and identification were performed as described previously (12, 13). In order to isolate one subtype of avian influenza A virus, all the samples were mixed simultaneously with the antisera to two AIV subtypes (1:1:1, vol/vol/vol), including chicken anti-H5N1 serum (hemagglutinin inhibition [HI] titer, 2^7; our unpublished data), anti-H9N2 serum (HI titer, 2^8; unpublished), and human anti-H7N9 serum (HI titer, 2^8) provided by Hangzhou Center of Disease Control. Subsequently, the mixture was neutralized for 2 h at 4°C and was inoculated individually into 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs with a dose of 0.6 ml for virus propagation and isolation. The following tests were conducted to identify these specimens: hemagglutinin (HA) test, HI test, subtype-specific primers for real-time reverse transcriptase (RT)-PCR), and high-throughput next-generation sequencing. All the viruses were isolated in a biosafety level 3 laboratory.

Sequencing data analysis. Sequencing reads were assembled and analyzed using a previously described method (10). The raw NGS reads were processed by removing low-quality reads (eight reads with quality of <66), duplication, >8 terminal unknown bases (Ns), adaptor-contaminated reads (>15 bp matched to the adapter sequence), and reads mapped to the host (short oligonucleotide analysis package [SOAP]; 14) of <5 mismatches. The remaining high-quality reads were first assembled *de novo* using SOAPdenovo (version 1.06) (15) and Edena (v3.121122) (16). Based on the references chosen by mapping the clean reads to the Influenza database, we used MAQ (17) to perform reference-based assembly. To correct some incorrect indels and mismatches, the *de novo* contigs (>200 bp) were aligned to the reference-based assembly sequences. The improved sequence was used as a reference to reassemble the high-quality reads to generate the final reference-based assembly sequences.

Sequence alignment and phylogenetic analysis. All the sequences used (coding regions) in this study were downloaded from the Influenza Virus Resource in NCBI or the Global Initiative in Sharing All Influenza Data (GISAID) database. The Clustal W function of MEGA 5.2 was used for alignment and editing of sequences, and we constructed maximum likelihood phylogenetic trees for all eight gene segments with the GTR+I+c4 model of MEGA 5.2 (18). The BEAST 1.8.0 software was used to construct temporal phylogenies using the Bayesian Markov Chain Monte Carlo (MCMC) method. We used SRD06 and an uncorrelated log-normal-distributed model. Bayesian MCMC sampling was run up to 10,000,000 times and sampled every 1,000 steps.

Mouse study. To determine the 50% mouse lethal dose (MLD_{50}) value of this novel virus in mice, groups of five mice under light CO_2 anesthesia were inoculated intranasally with 10^5 to 10^8 50% egg lethal dose (ELD_{50}) of tested virus in a volume of 50 μl. Meanwhile, a group of five mice were inoculated with an equal volume of phosphate-buffered saline (PBS) as a negative control. After 24 h, three naive mice were placed in direct contact with the inoculated mice to investigate the virus transmission ability. All the mice were monitored for body temperature, weight loss, and mortality daily for 14 days. For virus infectivity and replication testing, groups of three mice were lightly anesthetized with CO_2 and inoculated intranasally with 10^6 ELD_{50} of tested virus in a volume of 50 μl, the three inoculated mice were euthanized at 2, 4, 6, 8, 10, and 14 days postinoculation, and their organs were collected for virus reisolation, immunohistochemistry (IHC), and hematoxylin and eosin (H&E) staining. Virus reisolation was performed in 9-day-old SPF embryonated chicken eggs. The procedures for IHC and H&E staining have been described previously (12, 13).

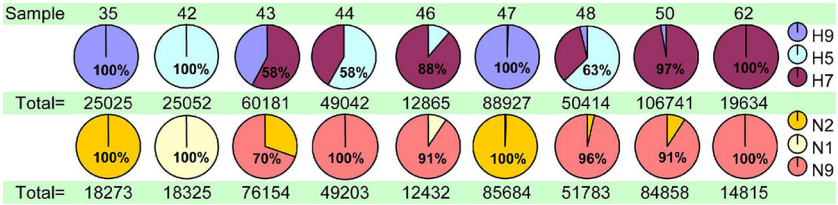

FIG 1 Subtypes of influenza A virus detected in live poultry samples. The numbers under the circles represent the total reads of NGS mapping to H9, H5, and H7 or N2, N1, and N9.

Ethics statement. The animal experiment was conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China. The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang University, permission number SYXK 2012-0178.

HA receptor-binding assay. A solid-phase binding assay (19, 20) was conducted to determine the direct receptor-binding capacity of the viruses isolated from infected chickens (YH1) and infected mice ($YH1^m$). All viruses were purified through sucrose density gradient centrifugation. Two biotinylated glycans used in this assay, α-2,6 glycan 6′sialy LacNAc-PAA-biotin and α-2,3 glycan 3′sialy LacNAc-PAA-biotin, were purchased from GlycoTech Corporation (USA). In brief, a streptavidin-coated high-binding-capacity 96-well plate (Pierce) was incubated with 100 μl of PBS containing different concentrations (2-fold serial dilutions starting from 0.24 μM) of biotinylated glycans at 4°C overnight; PBS was used as a negative control. The glycan solutions were then removed, and the plates were washed 4 times with ice-cold PBS and incubated at 4°C overnight with 100 μl of PBS containing 64 HA units of purified influenza virus. After washing 4 times, the plate was incubated for 4 h at 4°C with chicken antiserum against YH1 virus (our unpublished data) as a primary antibody and with horseradish peroxidase (HRP)-conjugated goat anti-chicken antibody (Sigma) as a secondary antibody. The plate was then incubated with 200 μl tetramethylbenzidine (TMB; Sigma) solutions for 15 min at room temperature. Finally, the reaction was stopped with 100 μl of 0.5 M H_2SO_4. Absorbance was determined using a plate reader (Biotech) at 450 nm.

Statistical analysis. The statistical significance of differences between groups was determined using the Student *t* test. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The genomes of the YH1 and YH2 viruses in this study have been deposited in GenBank (accession numbers KP793720 to KP793735).

RESULTS

Coexistence of multiple avian influenza A virus subtypes. To quantitatively analyze the coexistent influenza A virus infections, the samples obtained from LBMs were analyzed by high-throughput NGS using the specimens positive for H5, H7, and H9 in RT-PCR. We generated a total of 3,563,960 paired-end clean reads from one quail pharyngeal swab, one duck cloacal swab, and seven chicken pharyngeal swabs and cloacal swabs after removing adaptor-contaminated or low-quality reads. The coexistence of different subtypes, including H9, H5, and H7 and N2, N1, and N9, was detected in most of the collected samples (Fig. 1). Surprisingly, large amounts of N9 genes were coexistent with H5 and H7 in specimen number 44, and the coexistence of H9, H5, and H7 with N2 and N9 was detected in specimen number 48. To further confirm the NGS data, virus isolation was performed from 18 specimens neutralized with anti-H5, anti-H9, and anti-H7 sera. Of these 18 specimens, influenza viruses were isolated from 9 samples, with 13 different viruses identified from these samples collected in April 2013 (Table 1). Subtype analysis showed one H5N1 virus isolate, two H5N9 virus isolates, four H9N2 virus isolates, and six H7N9 virus isolates. Two H5N9 viruses were designated A/Chicken/Yuhang/1/2013 (H5N9) (YH1 virus) and A/Chicken/Yuhang/2/2013 (H5N9) (YH2 virus). These data confirmed the coexistence of different subtypes of AIV in chickens *in vivo*.

Genome diversity of the isolated H5N9 viruses. To analyze the origin of H5N9 viruses isolated from chickens, their complete genomes were sequenced and deposited in NCBI and GISAID databases. The maximum likelihood phylogenetic trees were constructed with sequences available in public databases. Molecular clock analysis (21) was used to investigate the source of the eight gene segments of these novel H5N9 viruses. Homological analysis showed that two viruses shared 100% nucleotide identities with HA, NS, NP, and PA genes, 99.93% with NA gene, 99.9% with M gene, 98.55% with PB2 gene, and 96.48% with PB1 gene. In comparisons of nucleotide sequences with those of other influenza A viruses available from public databases, the highest homologies of the isolated H5N9 genomes were as follows: 96.95% homology with the HA gene of A/Muscovy duck/Vietnam/LBM227/2012 (H5N1) belonging to clade 2.3.2.1, 99.79% with the NA gene of A/Hangzhou/1/2013 (H7N9), 97.95% with the PA gene of A/wild duck/Jilin/HF/2011 (H5N1), 98.86% with the NP gene of A/duck/Vietnam/NCVD-672/2011 (H5N1), 98.07% with the M gene of A/chicken/Zhejiang/329/2011 (H9N2), and 97.06% with the NS gene of A/wild duck/Jilin/HF/2011 (H5N1). Interestingly, the PB1 (99.74%) and PB2 (99.91%) segments of YH2 virus shared the greatest identity with A/Changsha/1/2013 (H7N9), while the highest similarities of segments PB1 and PB2 of the YH1 virus were found to be 99.56% with A/Hangzhou/3/2013 (H7N9) and 99.17% with A/Quail/Hangzhou/35/2013 (H9N2). Phylogenetic analysis (Fig. 2 and 3; see also Fig. S1 in the supplemental material) revealed that the HA gene of the isolated H5N9 virus belongs to clade 2.3.2.1 of the H5N1 virus, which circulates mainly in chickens and waterfowl in the southern provinces of China and Southeast Asia, but not the LPAIV H5N9 subtype, circulating in migrating wild birds, which was clustered mainly in another sublineage. However, the NA genes of the isolated H5N9 viruses were clustered with human-infecting H7N9 viruses. Notably, those previously reported H5N9 viruses were disseminated in another branch with H7N9 viruses circulating in waterfowl in North America. Similarly, in evolutionary trees of the six internal genes, the PA, NP, and NS genes of the isolated H5N9 viruses belonged to the H5N1 lineage. The PB1 and PB2 genes of YH1 virus belonged to the H9N2 and H7N9 lineages, respectively, whereas the PB1 and PB2 genes of YH2 virus belonged to the H7N9 lineage, and the M gene of the isolated H5N9 viruses originated from H9N2 subtype AIV. These data indicated that the isolated H5N9 viruses were

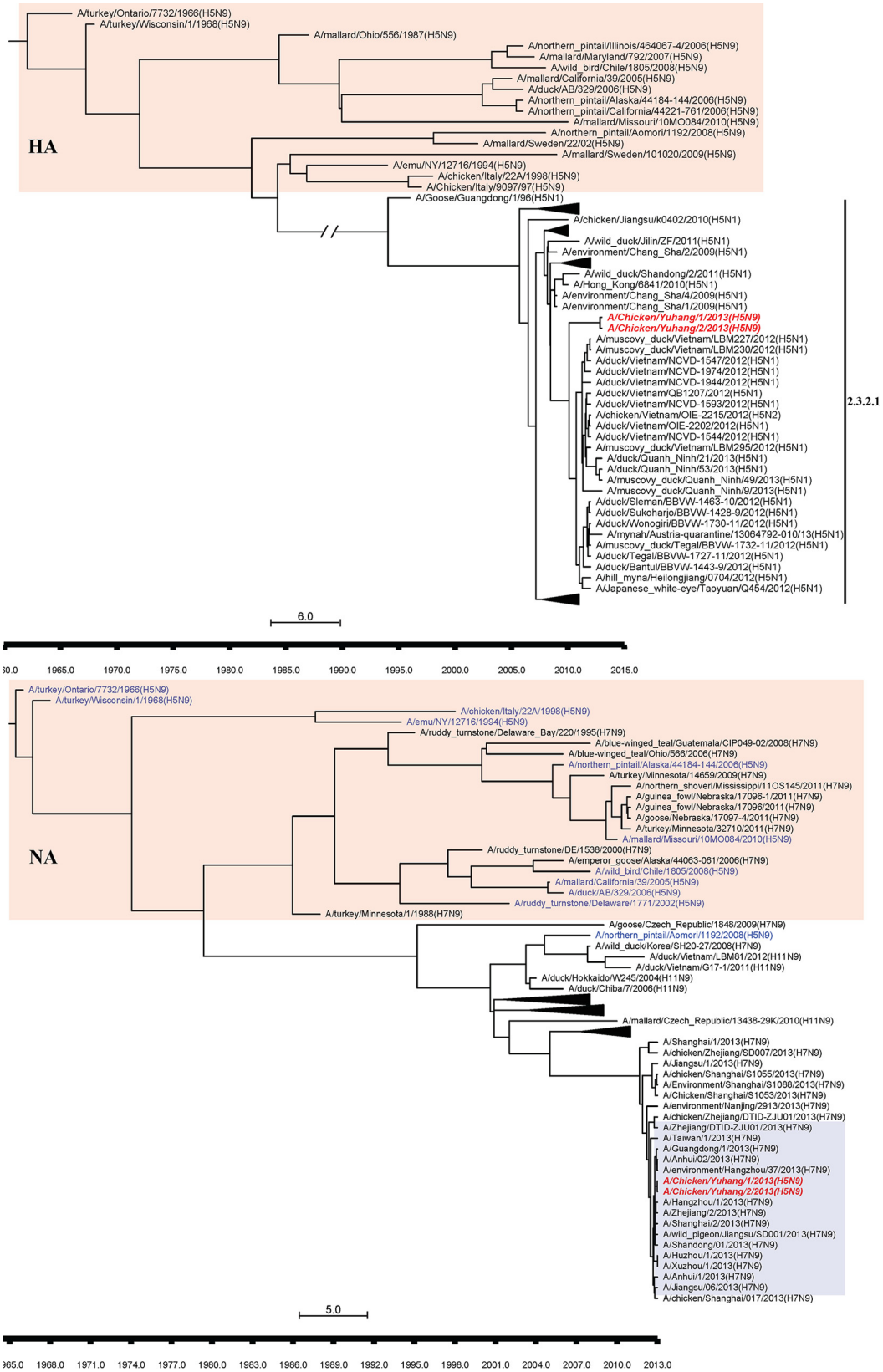

FIG 2 Phylogenetic analysis of hemagglutinin and neuraminidase of the isolated H5N9 viruses. The HA genes of YH1 and YH2 are distributed in clade 2.3.2.1 of HPAIV H5N1 virus. Those LPAIV H5N9 viruses are clustered in another branch (highlighted in orange). The gap in the branch indicates that there is no close relationship between the two sublineages. The NA genes of YH1 and YH2 are clustered with human-infecting H7N9 viruses (highlighted in blue). Previously reported H5N9 viruses are marked in blue and disseminated in another branch with H7N9 viruses of waterfowl origin (highlighted in orange).

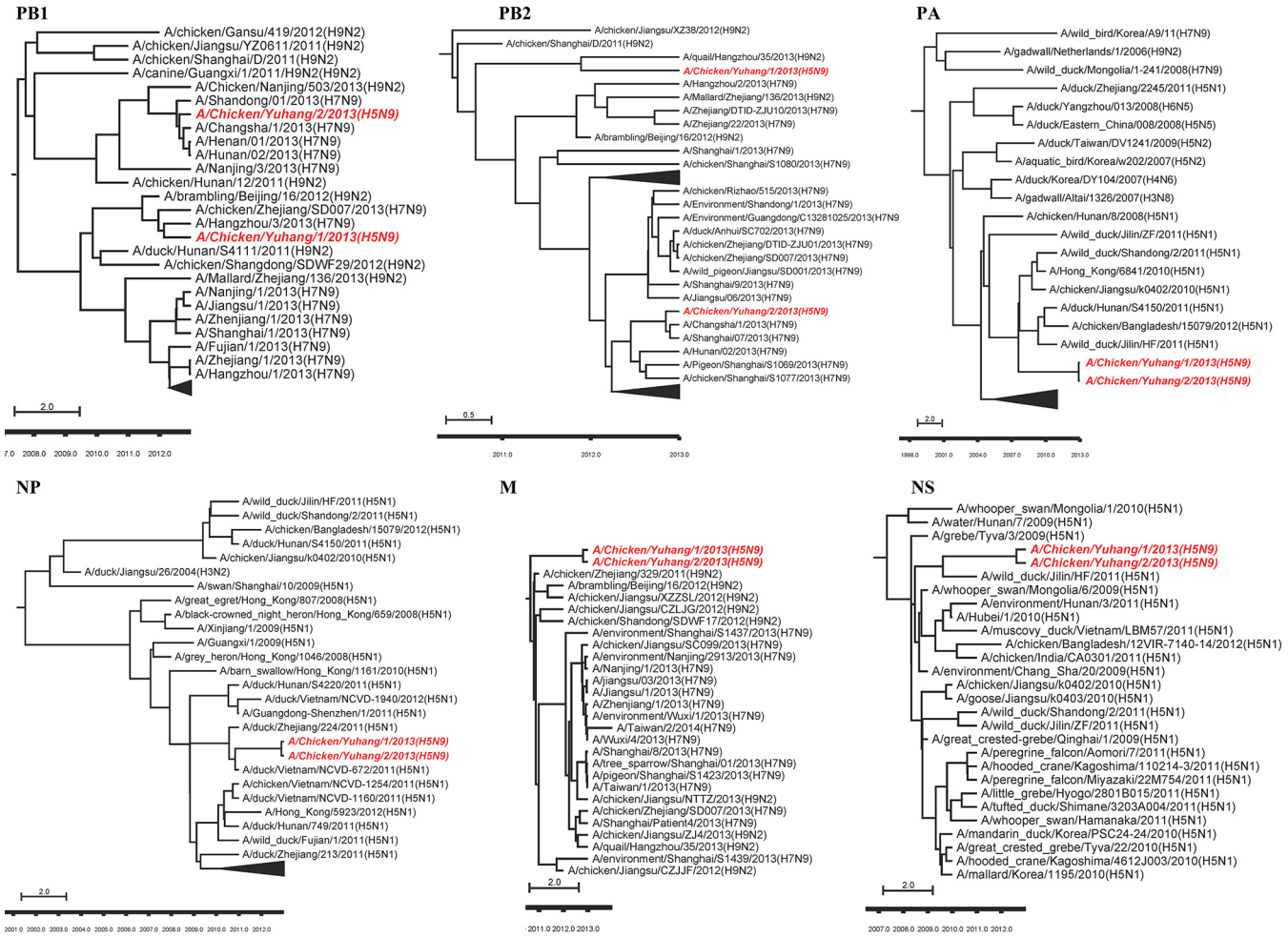

FIG 3 Phylogenetic evolution of six internal genes of the isolated H5N9 viruses. Phylogenetic trees were constructed for six internal gene segments using the limited homologous viruses, which is in accordance with the timeline. YH1 and YH2 are marked in red.

reassortant viruses originating from H5N1, H7N9, and H9N2 subtypes of influenza A virus and that the origins of the PB2 and PB1 genes of the YH1 virus were different from those of the YH2 virus.

Molecular characterization. Molecular analysis indicated that the PQRERRRKR/GL motif of multiple basic amino acids present at the cleavage site of HA protein of the newly isolated H5N9 viruses (Table 2) is identical to that of A/Muscovy duck/Vietnam/LBM227/2012 (H5N1). This motif is characteristic of HPAIV. However, it differed from the PQRETR/GL motif absent in A/Turkey/Ontario/7732/1966 (H5N9) (22). These observations suggested that the newly isolated H5N9 viruses originated from the highly pathogenic H5N1 virus circulating mainly in southeastern Asia. Analysis of the receptor-binding site revealed the characteristics of the avian-like receptor present at the 210-amino-acid loop of HA protein of the newly isolated YH1 virus (23). The neuraminidase stalk deletion (amino acids 69 to 73) was detected in the YH1 virus but not in the seashore bird H5N9 viruses (Table 2). Interestingly, the M2 and PB1 proteins of the YH1 virus and A/Hangzhou/1/2013 (H7N9) exhibited an S31N mutation, which confers resistance to the antiviral drug adamantane (24, 25), and an I368V substitution, which renders H5 subtype virus transmissible in ferrets (7). However, the mutations/substitutions involved in drug resistance and transmission to mammals were not observed in the NA and PB2 proteins of YH1 virus (Table 2). A canonical avian PDZ ligand motif ESEV in the NS1 protein enables YH1 to interact with cellular proteins, interfere with cellular signaling, and defeat host defense (26, 27). These data suggest that the newly isolated H5N9 viruses possess a unique characteristic compared with the previously reported H5N1, H5N9, and H7N9 viruses.

Receptor-binding properties of H5N9 viruses. Human-infective influenza viruses preferentially recognize a receptor with saccharides terminating in α-2,6-galactose sialic acids (SA α-2,6 Gal), whereas avian-infective influenza viruses preferentially recognize α-2,3-galactose sialic acids (SA α-2,3 Gal) (19). To investigate the novel H5N9 HA receptor-binding properties, we determined the receptor-binding capacities of chicken-originating YH1 virus and mouse-originating $YH1^m$ virus. The YH1 and $YH1^m$ viruses bound avidly to SA α-2,3 Gal but not to SA α-2,6 Gal. However, YH1 showed higher affinity for SA α-2,3 Gal than did $YH1^m$ virus (Fig. 4). These data indicated that the novel H5N9 virus recognized a receptor with SA α-2,3 Gal.

Pathogenicity of the H5N9 virus in mice. When mice were

TABLE 2 Molecular analysis comparison of A/Chicken/Yuhang/1/2013(H5N9) (YH1) with three different viruses harboring an H5 or N9 gene

Gene and function/effect	Mutation[a]	Molecular analysis finding			
		A/muscovy duck/Vietnam/LBM227/2012 (H5N1)	A/turkey/Ontario/7732/1966 (H5N9)	A/Chicken/Yuhang/1/2013(H5N9)	A/Hangzhou/1/2013 (H7N9)
HA gene					
HA cleavage site		PQRERRRKR/GL	PQRRKKR/GL	PQRERRRKR/GL	PEIPKGR/GL
Receptor binding site (H3 numbering)	Q226L	Q	Q	Q	I
	G228S	G	G	G	G
NA gene					
Stalk 69–73 deletion		Yes	QISNT	Yes	Yes
Resistant to oseltamivir (N2 numbering)	R294K	R	R	R	R
PB2 gene					
Increased virulence in mice	E627K	E	E	E	K
Enhanced transmission in mammals	D701N	D	D	D	D
PB1 gene					
H5 virus transmissible among ferrets	H99Y	H	H	H	H
	I368V	I	I	V	V
PB1-F2 gene					
Full-length increased virulence in mice (no. of amino acids)		57	90	90	90
M1 gene					
Increased virulence in mice	N30D	D	D	D	D
	T215A	A	A	A	A
M2 gene					
Resistance to adamantane	S31N	S	S	N	N
NS1 gene					
Increased virulence in mice	P42S	S	S	S	S
PDZ motif deletion		ESEV	ESEV	ESEV	Yes

[a] The mutation site was numbered from the start codon (Met).

inoculated intranasally with the H5N9 virus, no signs of disease or death were observed in those animals dosed with 10^5 to 10^6 ELD_{50}. In contrast, the mice inoculated with 10^7 to 10^8 ELD_{50} exhibited signs of illness, anorexia, and dyspnea. The MLD_{50} of H5N9 virus was 7.22 $\log_{10}$ ELD_{50}. The mice infected with 10^8 ELD_{50} presented with decreased body temperature, which reached its lowest level at 3 days postinfection (dpi) (Fig. 5A). These mice had lost over 20% of their body weights by 7 dpi (Fig. 5B) and died during the observation period (Fig. 5C). Among the mice infected with YH1, virus was successfully reisolated from lung and turbinate tissues at 2, 4, and 6 dpi. However, viruses were not isolated from any of the organs collected 1 week later. Virus replication was not detected in any of the direct-contact mice. H&E staining showed that inflammatory exudates and congestion filled the alveolar space and alveolar septum of inoculated mice, and viral antigen was detected in alveolar epithelial cells and macrophages by IHC staining (Fig. 6).

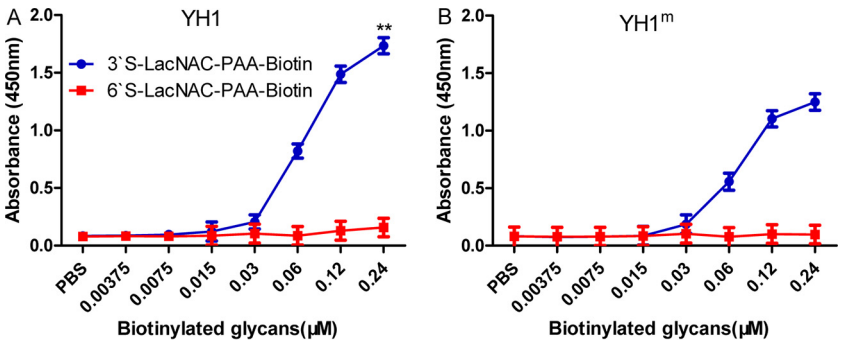

FIG 4 Hemagglutinin receptor-binding capacity of H5N9 virus. Two biotinylated labeled glycans, α-2,6 glycan and α-2,3 glycan, were bound to 96-well microplates in gradient concentrations. (A) YH1 virus; (B) $YH1^m$ virus. Data shown are the means of results from three repeats; the error bars indicate the standard deviations. **, $P < 0.01$ compared with the corresponding value of $YH1^m$.

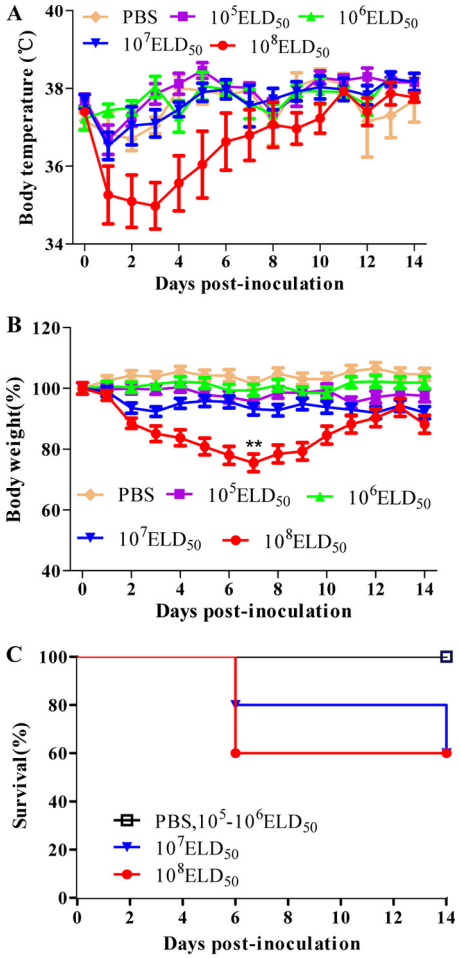

FIG 5 Virulence of YH1 virus in mice. (A) Body temperature. Groups of five mice were inoculated with the indicated dose virus; an equal volume of PBS was used as negative control. Body temperature was measured daily for 14 days. (B) Body weight (%). The mice in each group were measured for body weight changes daily for 14 days. Data represent the average percent changes. (C) Survival (%). Data shown are the means ± standard deviations (SD) for each group. **, $P < 0.01$ compared with the corresponding value of negative control.

These data indicated that the H5N9 virus caused infection and death in mice.

DISCUSSION

Since the appearance of the first three H7N9 human cases in eastern China in April 2013 (7), epidemiological monitoring shows that H7N9 human infections are still reported in China (28). Evidence of an epidemiological link with exposure to birds in markets has been found in some human cases (29, 30), thus implicating LBMs as a source of human infections. In this study, we investigated poultry specimens from two LBMs linked to the H7N9 human-infected cases reported in Hangzhou City of Zhejiang Province, China (10). NGS demonstrated the coexistence of H9, H5, and H7 and N2, N1, and N9 gene segments of influenza A viruses in chickens in one live bird market. Subsequently, two H5N9 viruses, one H5N1 virus, four H9N2 viruses, and six H7N9 viruses were identified in chicken samples (Table 1). Therefore, these data imply the coexistence of different subtypes of avian influenza virus in chickens in LBMs. China is a country in which all chickens are vaccinated actively with the inactivated vaccine against AIV (H5N1, H9N2) in all chicken farms. We consider that the chance of AIV infection ought to be low in chicken farms. Therefore, a possible cause is that chickens were infected with different subtypes of AIV in LBMs that contain different avian species of various sources.

H5N9 virus is an infrequently isolated subtype among influenza A viruses. Until 2013, most of the isolated H5N9 viruses were of low pathogenicity, with the exception of the pathogenic virus A/turkey/Ontario/7732/1966 (H5N9) (22) distributed in North America and Europe (Fig. 7A). In Asia, only low-pathogenicity H5N9 viruses have been isolated, at Aomori in Japan in 2008. The host range of H5N9 viruses developed gradually from turkey (Ontario, 1966) to mallards, northern pintails, emus, and occasionally chickens (Fig. 7B). However, to date, the highly pathogenic H5N9 subtype avian influenza virus has not been isolated in Eurasia. In our study, the novel H5N9 virus carrying HA protein with the PQRERRRKR/GL motif, which is characteristic of HPAIV, was first isolated from chickens with coexistent AIV subtypes in LBMs in China. This implies that a Eurasian HPAI H5N9 virus was possibly revealed and prompted vigilance in the poultry industry. More interestingly, the novel H5N9 virus belongs to clade 2.3.2.1 (Fig. 2) and matches the inactivated vaccine against clade 2.3.2.1

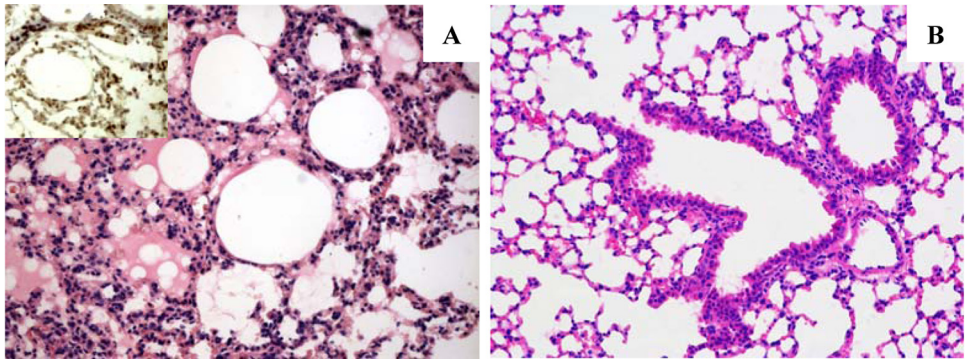

FIG 6 Microscopic observation of the lung of mice that were intranasally inoculated with the YH1 virus. Photomicrographs of hematoxylin and eosin-stained tissue sections (A and B) and sections stained by immunohistochemistry to demonstrate the presence of AIV. (A) Exudate-filled alveoli of the infected mouse. The top left corner in panel A indicates AIV antigen in alveolar epithelial cells of the same mouse. (B) Tissue section from the healthy control mouse.

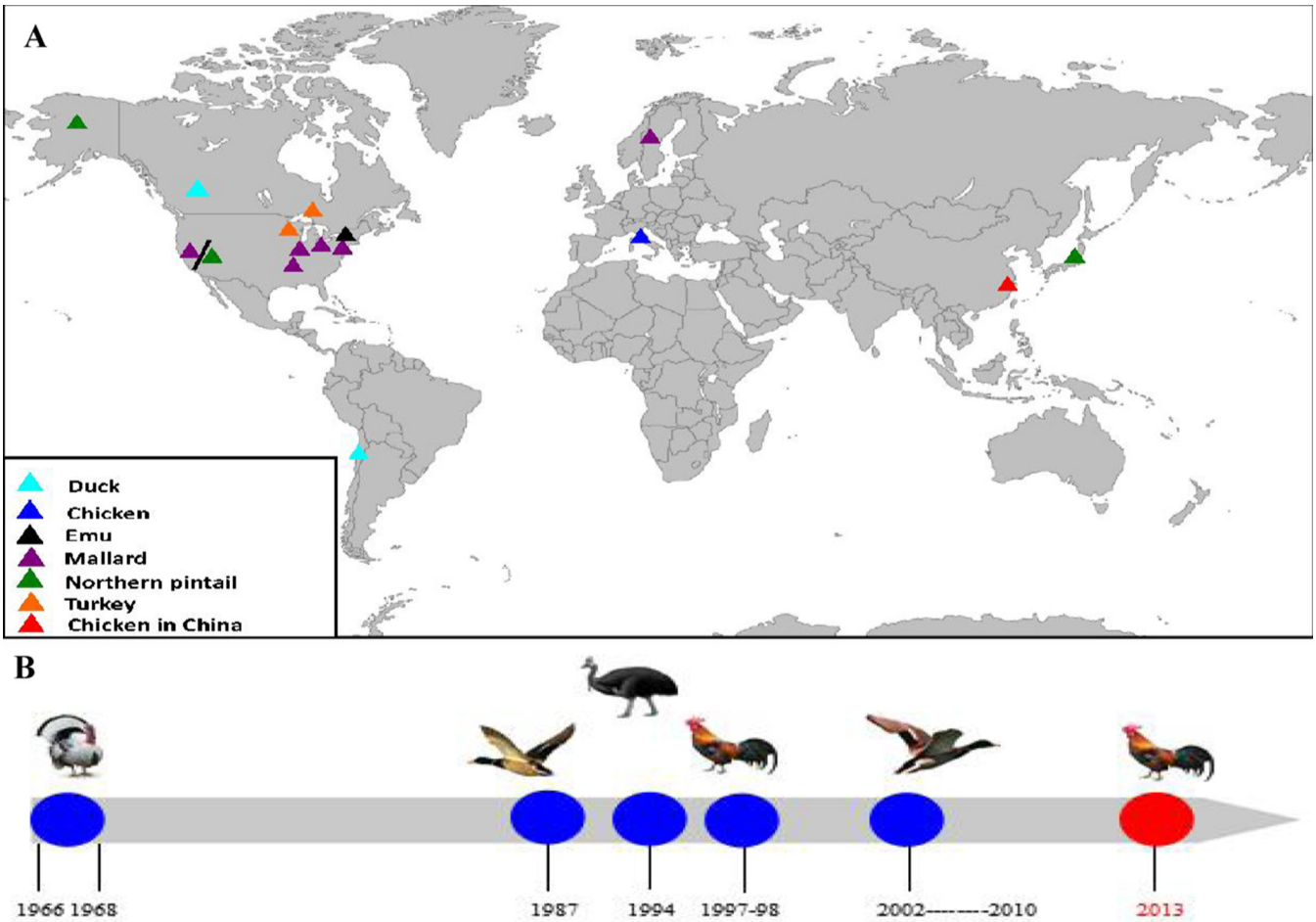

FIG 7 Emergence and distribution of H5N9 viruses. (A) Colored triangles indicate H5N9 viruses from different hosts. (B) Host and timeline of H5N9 virus emergence. High-pathogenicity viruses are marked with a red circle, and low-pathogenicity viruses are marked with a blue circle.

of the H5N1 virus circulating in China, which probably provides protection against H5N9 virus challenge.

Our results did not provide convincing evidence that the progenitor of the newly isolated H5N9 virus is the previously reported A/turkey/Ontario/7732/1966 (H5N9) virus. The HA and NA genes of the novel H5N9 virus have the greatest identity with A/Muscovy duck/Vietnam/LBM227/2012 (H5N1) and A/Hangzhou/1/2013 (H7N9) isolated in the human-infected case. As for six internal genes of the novel H5N9 virus, three genes originate from H5N1 virus, the M gene originates from H9N2 virus, and the PB1 gene originates from H7N9 virus (Fig. 2 and 3; see also Fig. S1 in the supplemental material). More interestingly, the NA and PB1 genes of the novel H5N9 YH1 virus originated from A/Hangzhou/1/2013 (H7N9) and A/Hangzhou/3/2013 (H7N9), respectively, and even the PB2 gene of the novel YH2 virus is closely related to the A/Changsha/1/2013 (H7N9) virus. Although recent reports considered that the human cases of H7N9 infection were possibly related to poultry and LBMs (30), explaining how and where the novel H5N9 viruses were assembled requires further investigation.

The novel H5N9 virus was generated by pairwise sequence alignment of an HPAI H5 gene with an N9 gene of human-infecting H7N9, thus raising the concern over the virulence of the novel virus in mammals. To address this concern, we actively conducted pathogenicity and transmission studies of the novel H5N9 virus in mice. The novel H5N9 virus caused partial mortality in the mice infected experimentally at a high dose of 10^8 ELD_{50}. Notably, the H5N1 virus with the PB2 gene encoding residue K627 in clade 2.3.2.1 and the human-infecting H7N9 virus have high mortality in mice (31, 32). However, the H5N9 virus isolated in this study has only low mortality in mice. Many research studies have shown that the amino acid residue K627 (lysine) encoded by the PB2 gene of HPAI virus, which recognizes a receptor with SA α-2,6 Gal, resulted in high mortality in mice (33). Therefore, a possible reason of low mortality in mice is that the newly isolated H5N9 virus recognizes a receptor with SA α-2,3 Gal and encodes the amino acid residue E627 (glutamic acid) in PB2 protein (Table 2 and Fig. 4).

In summary, two novel H5N9 subtype AIVs were isolated from the "hot spot" of the H7N9 emergence area in Hangzhou, China. These novel viruses were systematically characterized and analyzed genetically and phylogenetically. Our results indicated that the novel H5N9 viruses are HPAIVs and were reassortant from H5N1, H7N9, and H9N2 subtypes of influenza virus. The lack of proofreading among viral RNA polymerase, the segmented RNA genome that allows dynamic reassort-

ments within the large gene pool in live poultry markets, and the existence of multiple natural reservoirs all implicate influenza A virus as a noneradicable zoonosis. Therefore, a series of strategies should be implemented to further control influenza virus based on a combination of vaccination, extensive surveillance of poultry transportation, improved biosecurity, and an effective monitoring program.

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