Original research

Influenza A (H6N6) viruses isolated from chickens replicate in mice and human lungs without prior adaptation

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ABSTRACT

The H6H6 subtype avian influenza virus (AIV) is currently prevalent in wild birds and poultry. Its host range has gradually expanded to mammals, such as swines. Some strains have even acquired the ability to bind to human-like SAα-2,6 Gal receptors, thus increasing the risk of animal to human transmission. To investigate whether the H6N6 AIV can overcome interspecies barriers from poultry to mammals and even to humans, we have assessed the molecular characteristics, receptor-binding preference, replication in mice and human lungs of three chicken-originated H6N6 strains. Among these, the A/CK/Zhangzhou/346/2014 (ZZ346) virus with the P186T, H156R, and S263G mutations of the hemagglutinin molecule showed the ability to bind to avian-like SAα-2,3 Gal and human-like SAα-2,6 Gal receptors. Moreover, H6N6 viruses, especially the ZZ346 strain, could replicate and infect mice and human lungs. Our study showed the H6N6 virus binding to both avian-like and human-like receptors, confirming its ability to cross the species barrier to infect mice and human lungs without prior adaptation. This study emphasizes the importance of continuous and intense monitoring of the H6N6 evolution in terrestrial birds.

1. Introduction

The global outbreak of coronavirus disease 2019 (COVID-19) has seriously threatened human health and public health security.[1] However, one cannot overlook the importance of the influenza virus as highly pathogenic avian influenza viruses (AIV) such as the H5N1, H7N9, and H5N6 strains have crossed the species barrier from poultry to humans in the past two decades.[2–4] The H6 subtype influenza virus of low pathogenicity was also isolated from a patient diagnosed with lower respiratory tract infection in 2013 [5], and a very close H6 subtype was subsequently isolated from a dog in 2014 [6], indicating that the H6 subtype could cross the species barrier to mammals as well as posing a potential threat to human health.

The H6 subtype influenza A virus was first isolated in turkeys in 1965.[7] Since then, it has spread around the world. It is mainly divided into North American and Eurasian lineages, the Eurasian lineage being further divided into Group I (ST339-like), Group II (ST2853-like), Group

III (HN573-like), and WT312-like minor poultry groups.[8–10] In China's live poultry markets, H6 viruses (including H6N1, H6N2, H6N5, H6N6, H6N8, and others.) have been transmitted to poultry and been prevalent for a long time.[11] From 2000 to 2005, the ST339-like Group I H6N2 was the most frequently detected H6 subtype in live poultry markets in southern China.[12] However, since the emergence of the H6N6 virus in 2005, the ST2853-like Group II H6N6 has gradually replaced the H6N2 subtype.[11] A similar group replacement scenario has also been observed in eastern China, but generally 2–4 years later than in southern China. More importantly, the H6N6 virus transmitted from waterfowl to land poultry is causing an endemic disease in poultry.[13,14]

The specific recognition and binding of the hemagglutinin (HA) molecule to sialic acid (SA) receptors on host cells play a crucial role in determining the host range of the influenza virus. Most AIV binds to avian-like SAα-2,3 galactose (Gal) receptors, while the human influenza virus recognizes human-like SAα-2,6 Gal receptors. For an effective human-to-human transmission, AIV must first acquire the ability to bind

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to human-like receptors. Some H6N2 and H6N6 strains isolated in live poultry markets during 2009–2011 and 2016–2019 in China have acquired the ability to recognize human-like receptors.[13] Molecular epidemiological investigations have found that the reassortment between the H6 and non-H6 subtypes (especially the H5N6 prevalent in Southern China) may occur easily.[15] When considering the cases of human infection by the H5N6 virus that occurred in 2014, it was found that the H5N6 virus was generated by the reassortment of H6N6, H7N9/H9N2, and H5 viruses,[16] which indicated that H6N6 acted as the progenitor for H5N6, a novel AIV infecting human beings. Meanwhile, some H7N9 viruses able to infect humans have altered their nucleoprotein (NP) and NS gene fragments by reassortment with the H6N6 and H5N6 genes,[17] which indicates that the AIV H6N6 is currently in a dynamic state of evolution and prone to reassortment with other viral subtypes, thus increasing viral genetic diversity. In 2011, the swine-originated H6N6 virus A/SW/GD/K6/2010 (GDK6) was isolated in Southern China.[18] Gene sequencing analysis has indicated that it had originated from domestic ducks, and that its HA molecule belonged to the Group II virus of H6 Eurasian lineages. Animal experiments have shown that the virus has limited transmissibility between ferrets,[19] suggesting that the H6N6 virus could cross the interspecific barriers and infect humans. Seroepidemiological investigations found that the serum of exposed people in turkey farms in the United States was positive for H6 [20], with the presence of H6 antibody in the serum of 15,689 exposed people in China, resulting in a positive rate of 0.4%.[21] Therefore, the possibility for human infection by the H6N6 virus is increasing, so is the risk for an epidemic.

The H6N6 subtype AIV is widely prevalent in wild waterfowl and poultry in Eurasia, and its host range has gradually expanded to mammals, such as swines. Some viruses have even acquired the ability to recognize human-like SAα-2, 6 Gal receptors.[13,22] Following this ongoing evolution, it remains unclear how readily it can develop the ability to cross interspecific barriers and affect humans. In this study the H6N6 subtype strains isolated from chickens were sequenced, phylogenetically analyzed, and the viral receptor-binding preference was analyzed. Then, mice in *vivo* and human lung tissue in *vitro* were inoculated with viruses to observe the replication in mice and human lung tissue, as well as the viral molecular characteristics that can infect mice and replicate in humans. Furthermore, we have evaluated the potential of H6N6 viruses to infect humans and the key molecules that support binding to the human-like SAα-2,6 Gal receptors and replication in humans.

2. Materials and methods

2.1. Viruses

Three strains of the H6N6 subtype AIV isolated from chickens were selected in this study, including the A/CK/Jiangxi/20490/2014 (JX20490), the A/CK/Zhangzhou/1923/2015(ZZ1923) and the A/CK/Zhangzhou/346/2014(ZZ346) ones. The avian influenza virus H9N2 A/DK/Guangxi/767/2010(GX767), which had been previously confirmed to bind to avian-like SAα-2,3 Gal receptors, was used as positive control for receptor-binding analysis. The human virus H3N2 A/Shantou/602/2005(ST602), which can effectively replicate in humans and bind to human-like SAα-2,6 Gal receptors, was used as a positive control for the *in vitro* human lung infection and receptor-binding analysis. The swine influenza virus H1N1 A/SW/Guangxi/3843/2011(GX3843), which was previously confirmed as able to infect mice, was used as positive control for the *in vivo* mice infection study.

Three strains of the H6N6 virus and one strain of the H9N2 virus were passaged in 10-day-old embryonated chicken eggs; the swine influenza virus H1N1 and human virus H3N2 were cultured in Madin-Darby canine kidney (MDCK) cells. The HA subtype was identified by the haemagglutination inhibition (HI) test, and the NA subtype identified by direct sequencing.

2.2. Genetic, phylogenetic and structural analyses

Viral RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed. PCR reaction amplification was performed by using segment-specific primers, and the products were purified. The whole virus gene was sequenced by the Illumina Solexa system. Reference sequences were retrieved from GenBank, https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database. The sequence dataset was aligned by the Muscle program followed by manual adjustment.[23] The best-fit nucleotides substitution model was selected from the 286 model candidates based on the Bayesian information criterion (BIC) by using ModelFinder.[24] The maximum likelihood method was used to construct phylogenetic trees implanted in the IQ-TREE 2.1.2 .[25] The phylogeny topological structure was supported by 10,000 times Ultrafast Bootstrap (UFBoot), 10,000 times Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT), and the approximate Bayesian-like test (aBayes) .[26,27,28] The receptor-binding site (RBS) structure of ZZ346 (H6N6) virus was simulated based on H6 HA structure (PDB accession code: 5BR0) with molecular replacement and manual refinement using PyMOL 2.4.2 (Schrödinger, LLC).

2.3. Viral growth kinetics

MDCK and A549 cells (human lung carcinoma cells) were infected with ZZ346, ZZ1923 and JX20490 strains at a multiplicity of infection (MOI) of 0.01, overlaid with serum-free DMEM containing 2 μg/mL TPCK-trypsin (Sigma-Aldrich, USA). One hour post-inoculation (hpi), cells were washed with phosphate-buffered saline (PBS) to remove nonbound virus particles and incubated at 37 °C with 5% CO_2. Samples of culture supernatant were harvested at 12, 24, 48 and 72 hpi and titrated in MDCK cells by using the Medium Tissue Culture Infectious Dose ($TCID_{50}$) assay.

2.4. Receptor-binding analysis by the HA assay

An α-2,3-specific sialidase can effectively eliminate SAα-2,3Gal, but retain SAα-2,6 Gal. With α-2,3-sialidase-untreated turkey red blood cells (TRBCs) and α-2,3-sialidase-treated TRBCs, the receptor-binding preference of the virus could be detected by the HA's change titer. The HA assays using resialylated TRBCs were performed as described previously with some minor modifications.[29] Briefly, 1% TRBCs solution was divided into the α-2,3-sialidase treated group and untreated group. The α-2,3-sialidase treated group was incubated with 1 Uα-2,3-specific sialidase (Takara, Japan) at 37 °C for 12h, and the untreated one with PBS as a mock control. TRBCs were washed three times with PBS. After centrifugation, two TRBCs groups were reconstituted to a 0.6% concentration and the virus receptor-binding preference was detected using the HA assay. Complete elimination of the SA α-2,3 Gal receptors on sialidase-treated TRBCs was confirmed by receptor staining and flow cytometry. The human influenza A virus ST602 (H3N2) and avian influenza virus GX767 (H9N2) strains were used as controls in the HA assay.

2.5. Receptor-binding analysis using a solid-phase direct-binding assay

The viral receptor-binding specificity was determined using the solid-phase direct binding assay as described previously with minor modifications.[30] Briefly, 96-well microtiter plates were coated with biotinylated glycans SAα-2,3 Gal (Neu5Acα2-3Galβ1-4GlcNAcβ-C3-PAA-biotin, 3′SLN) and SAα-2,6 Gal receptors (Neu5Acα2-6Galβ1-4GlcNAcβ-C3-PAA-biotin, 6′SLN) (GlycoNZ Corporation, MD, USA). Viral dilutions containing 64 HA units were then added and plates incubated at 4 °C for 12 h. The virus-receptor binding reaction was detected with human antisera against influenza A viruses HA and HRP-linked rabbit-*anti*-human antibody (Beyotime Biotechnology). The

reaction was stopped with 100 µl Stop Solution for TMB Substrate, and the absorbance was determined at 450 nm. The cutoff value for the glycan-binding assays was the background value of the well with 100 ng of glycopolymer in the absence of added virus. The human influenza A ST602 (H3N2) and avian influenza GX767 (H9N2) strains were used as controls for the solid-phase direct binding assay.

2.6. Animal experiments

In this study, sixty 6-week-old BALB/c mice were obtained through the Vital River Laboratories, Beijing, China. Nasal swabs of all animals were inoculated in 10-day old embryonated chicken eggs and MDCK cells to confirm that mice were free of influenza viruses; The HI assays were used to confirm that animals were negative for serum influenza virus antibody. This study was approved by the Animal Ethics Committee of Guangxi Medical University. Animal experiments were conducted in biosafety level 2^+ containment facilities in strict compliance with the Institute's guidelines for the care and use of laboratory animals.

The replication and infectivity of H6N6 AIV in mice were investigated with 60 BALB/c mice divided into 5 groups (n = 12) by random number generator. Each animal was inoculated with one strain. The mock-infected group was inoculated with PBS. After anesthesia, each animal was intranasally inoculated with one strain at a dose of 10^6 egg infective dose at 50% (EID_{50}) in 0.2 mL of PBS or with 0.2 mL of PBS. The animal survival rate, weight change, temperature and symptoms were recorded daily over 14 days. Animal nasal swabs were collected at 1, 3, 5, and 7 days post-inoculation (dpi), isolated and cultured in 10-day-old embryonated chicken eggs and MDCK cells. Three mice in each group were euthanized at 3, 5, and 7 dpi, respectively. Tracheal and lung tissues were collected and divided into two parts. One part was ground and centrifuged to obtain a supernatant and then isolated and cultured in 10-day-old embryonated chicken eggs and MDCK cells. The other part was fixed in 10% formalin solution at room temperature for 24 h for pathology examination and virus protein detection. Sera were collected at 14 dpi, and HI tests were used to detect serum antibodies in the recovery period.

2.7. H6N6 viral replication in human respiratory tissue in vitro

This study was approved by the Medical Ethics Committee of Guangxi Medical University. Five samples of lung tissue, collected during surgery, were provided by the First Affiliated Hospital of Guangxi Medical University. The selected specimens had no related respiratory tract infectious diseases. Specimens were dispatched to the laboratory immediately upon collection, and any suspected cancerous and/or other abnormal tissues were removed. Bronchial and lung tissues were cut into $0.2 \times 0.2 \times 0.2$ cm^3. Two blocks of human bronchial and lung tissues were selected to detect whether samples were infected by influenza viruses. One of the blocks was ground to isolate and culture the virus, the other one was used to detect viral antigens by immunohistochemistry and to ensure that specimens were free from influenza virus infection.

Bronchial and lung tissues were placed into a 6-well cell culture plate, rinsed with F–12K tissue culture medium containing antibiotics and L-glutamate, inoculated with 10^6 TCID$_{50}$ of the virus in a volume of 500 µL medium, and then cultured at 37 °C and 5% CO$_2$ for 1 h. Addition of 500 µL of PBS into one well was used as a mock control. Tissue blocks were then rinsed with F–12K culture medium containing 0.2% TPCK-trypsin and 1%BSA and further incubated with the above medium. Two tissue blocks were collected at 12h, 24h, and 48 hpi, respectively, one ground in cold PBS and homogenated, the supernatant then collected and inoculated into 10-day-old embryonated chicken eggs and MDCK cells. The TCID$_{50}$ was used to determine viral titer. The other one was fixed in 10% formalin for 24 h for pathology examination and detection of viral proteins.

2.8. Pathological examination and virus protein antigen detection

Mice respiratory tract tissues and human lung tissues were dehydrated, embedded, and serially sectioned with a thickness of 4 µm. Sections were stained with HE, and abnormal changes were observed under microscope. Immunohistochemistry for the detection of viral proteins was conducted as previously described.[31] After antigen retrieval, the primary NP antibody (1:500) (kindly provided by the National Institute of Diagnostics and Vaccine Development in Infectious Diseases, Xiamen University) was added in sections. Sections were incubated overnight at 4 °C, followed by the addition of goat anti-mouse IgG-specific biotin conjugate (Calbiochem) (1:50), development by DAB stain, and counterstaining by hematoxylin. Human lung tissue sections infected with the H5N1 influenza virus were used as positive controls, with 10% normal mouse serum used as a MOCK control. Positive results were judged by the light brown color of the nucleus and of the cytoplasm around the nucleus.

3. Results

3.1. Molecular features and viral phylogenetic analysis

Sequencing results showed that the HA cleavage site sequence of the three H6N6 strains was PQIETR/GL, with only a single basic amino acid belonging to the low pathogenic AIV. No mutation occurred at the 224, 226, 228, 137, 138, and 190 sites (H3 numbering) of the main receptor binding sites. The P186T, H156R, S263G mutations, and an amino acid deletion at the HA position 158 were found in the ZZ346 virus; these sites were not mutated in the other two viruses (Table 1 and Fig. 1). Whether mutations at these HA sites would switch receptor specificity requires furher evaluation.

The amino acid sequences of NA, PB2, and PB1–F2 of the three viruses were analyzed. The 11aa deletion in the neurominidase (NA) stalk region located at positions 59–69 was found in the ZZ346 virus strain; this deletion was not found in the other two JX20490 and ZZ1923 viruses. In this study, three strains of H6N6 viruses, including 627E, 271T, 701D of PB2-23, 66 N of PB1–F2, 38I of PA did not show mutations but still replication and prevalence in poultry animals (Table 1) .[32,33,34]

Results from the gene evolution analysis showed that three H6N6 AIV subtypes were reassortment viruses, and their gene segments derived from group-II (ST2853-like) of the Eurasian lineages (Fig. 2). For the ZZ346 virus, the HA gene was 99.3% genetically identical to the Fujian strain (duck-originated H6N6 subtype in Fujian province in 2007), which can be inferred to be from the same strain. The ZZ1923 HA gene came from a clade of the H6N2 subtype AIV in Jiangxi province in 2007. The JX20490 HA gene also came from the clade of the duck-originated A/Duck/Jiangxi/7510/2007(H6N2) strain in Jiangxi province. Sequence and phylogenetic analyses further indicate that the ZZ1923 and JX20490 strains were within the same clade.

3.2. Viral growth kinetics

The *in vitro* growth properties of the ZZ346, ZZ1923 and JX20490 strains were characterized in MDCK and A549 cells. Though three strains of H6N6 influenza A virus reached a maximum at 48 hpi in MDCK cells, the ZZ346 strain grew to significantly higher virus titer than ZZ1923 and JX20490 strains at 24, 48 and 72 hpi (p < 0.05) (Fig. 3A). In A549 cells, the ZZ346 virus reached a maximum at 72 hpi, and the ZZ1923, JX20490 ones a maximum at 24 hpi. Similarly, the ZZ346 virus grew to significantly higher virus titers than the ZZ1923 and JX20490 ones at 48, 72 hpi (P < 0.05) in A549 cells (Fig. 3B). These results suggest that three viruses, especially the ZZ346 one, have good replication capacity in mammalian cells.

Table 1
Important amino acid sequences in HA, NA, PB2, PB1–F2 and PA of viruses.

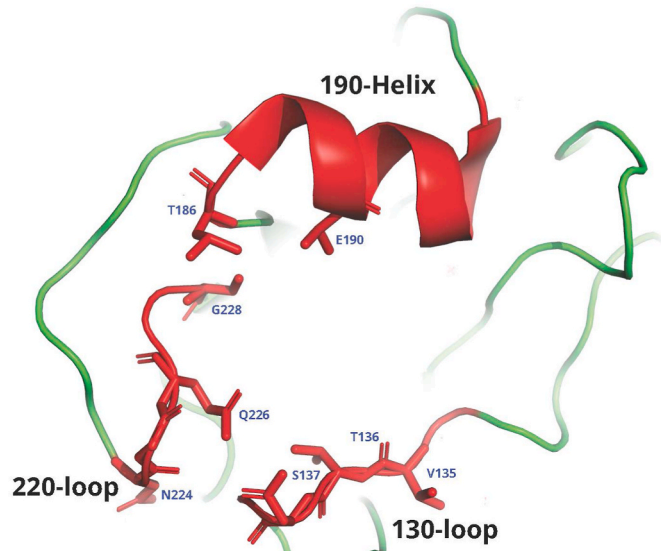| Virus	HA											HA-connecting	NA deletion	PB2			PB1–F2 66	PA 38
	224	226	228	186	190	158	137	138	156	263	318			627	271	701		
ZZ346	N	Q	G	T	E	DEL	C	P	R	G	L	PQIETR/GL	11AA(59–69)	E	T	D	N	I
JX20490	N	Q	G	P	E	T	C	P	H	K	L	PQIETR/GL	NO	E	T	D	N	I
ZZ1923	N	Q	G	P	E	T	C	P	H	K	L	PQIETR/GL	NO	E	T	D	N	I

Fig. 1. The predicted receptor-binding site (RBS) structure of H6N6 ZZ346 virus. The 130-loop, 190-helix and 220-loop were highlighted in red. The important amino acids in the RBS pocket were labeled individually. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. H6N6 virus receptor-binding specificity

A screening assay for receptor switching of avian influenza viruses was recently developed.[29] The SAα-2,6 or α-2,3-binding preference could be distinguished by the change in HA titer in reaction with TRBCs and SAα-2,3 Gal-specific sialidase-treated TRBCs. Our results showed that the H9N2 avian influenza virus GX767 and H3N2 human virus ST602 were preferentially bound to SAα-2,3 Gal and SAα-2,6 Gal receptors, respectively. Compared to the HA titer of untreated TRBC of 1:128, the ZZ346 strain's HA titer was 1:8 in SAα-2,3Gal-specific sialidase-treated TRBCs, which only had α-2,6Gal receptors. Nevertheless, the ZZ1923 and JX20490 strains could not agglutinate SAα-2,3 Gal-specific sialidase-treated TRBCs (Fig. 4). Our results indicate that the H6N6 ZZ346 strain could bind to avian-like SAα-2,3 Gal and human-like SAα-2,6Gal receptors.We further confirmed that H6N6 viruses preferentially bind to SAα-2,3 Gal or SAα-2,6 Gal receptors by using a solid-phase binding assay. The ZZ346 strain bound to both SAα-2,3 Gal or SAα-2,6 Gal receptors, although its affinity for the SAα-2,3 Gal receptors was higher than that for the SAα-2,6 Gal receptors. The ZZ1923 and JX20490 strains were found to bind only to the SAα-2,3 Gal receptors (Fig. 5). Results of the two assays were in agreement, confirming that the ZZ346 strain has acquired the ability to recognize human-like receptors.

3.4. BALB/c mice infected with the H6N6 subtype AIV

To investigate the H6N6 virus replication in mice, we have inoculated groups of twelve 6-week-old BALB/c mice with 10^6 EID$_{50}$ of each strain. Three mice in each group were sacrificed at 3, 5, 7 dpi, with virus

detected in trachea and lung. The remaining three mice in each group were used to analyze weight changes, symptoms, and death after 14 days. After inoculation of the H6N6 subtype AIV, BALB/c mice showed decreased activity, diet decline, coarse and disordered hair, but no disease symptoms such as weight loss or death. Trachea and lung tissues were ground into homogenates and isolated in embryonated chicken eggs and MDCK cells, respectively. In embryonated chicken eggs, virus was detected in the mice tracheas and lungs inoculated with the ZZ346 strain; HA titers at 3, 5 dpi were 1:32 and 1:16, respectively (JX20490 and ZZ1923 were not detected). Similarly, for MDCK cell isolation, the ZZ346 virus was detected in mice lungs; HA titers at 3, 5 dpi were 1:16 and 1:8, respectively (JX20490 and ZZ1923 were not detected).

At 14 dpi, BALB/c mice serum was collected, and the HI test used to detect influenza virus antibody in serum. Results showed that although the ZZ1923 and JX20490 strains were not isolated, the convalescent period serum was positive for the antibody. The antibody level induced by the ZZ346 strain was higher than that of the JX20490 and ZZ1923 strains.

No obvious gross pathological changes were observed in mice inoculated with the JX20490 and ZZ1923 strains, but a zone of hyperemia of $1 \times 1.2\,cm^2$ appeared in the left lung inoculated with the ZZ346 strain at 5 dpi. Mice trachea inoculated with the ZZ346 and JX20490 strains showed various degrees of tracheal mucosal congestion, edema, mucosal epithelial necrosis, and a small amount of inflammatory cell infiltration. However, no obvious pathological changes were found in the trachea of the ZZ1923 strain-inoculated mice (Fig. 6). In mice inoculated with the ZZ346 strain, dilatation and congestion of small blood vessels, red blood cell exudate, inflammatory cell infiltrate, alveolar wall widening, and interlobular septum in lung tissue were observed. Still, there were no obvious typical lesions in the mice lung tissue inoculated with the JX20490 and ZZ1923 strains (Fig. 6). Immunohistochemical detection of the viral NP protein in tissue showed that the number of cells infected by the ZZ346 strain was higher than in those infected by the JX20490 and ZZ1923 ones (Table 2 and Fig. 6). At 3, 5, and 7 dpi of the ZZ346 strain, the NP protein was detected in the trachea, bronchi, and lung tissues of some mice. A small amount of the protein could be detected in the mice trachea and bronchi inoculated with the JX20490 strain, but none in the lung. The NP protein was not detected in the trachea, bronchi, and lung tissues of mice inoculated with the ZZ1923 strain. These results indicate that the H6N6 viruses, especially the ZZ346 strain, can replicate in the mice respiratory system without prior adaptation.

3.5. H6N6 subtype AIV replication in human lung tissue

Human lung and bronchial tissues were collected at 12h, 24h, and 48 hpi of H6N6 viruses, after which they were ground. The supernatant was then inoculated into 10-day-old embryonated chicken eggs and MDCK cells. In embryonated chicken eggs, the virus was detected in human lungs inoculated with the ZZ346 strain with HA titers at 3 dpi at 1:8; this was not observed for the ZZ1923 and JX20490 viruses. For the MDCK cell isolation, three strains were not detected. The lung tissue inoculated with the ZZ346 strain showed its structure destroyed, inflammatory cells such as lymphocytes and monocytes infiltrating the lung interstitial and bronchiolar mucosal and epithelium tissue with necrosis However, obvious pathological changes in lung tissue inoculated with the ZZ1923 and JX20490 strains were not observed. There were no obvious histopathological changes in bronchial tissue inoculated with three H6N6
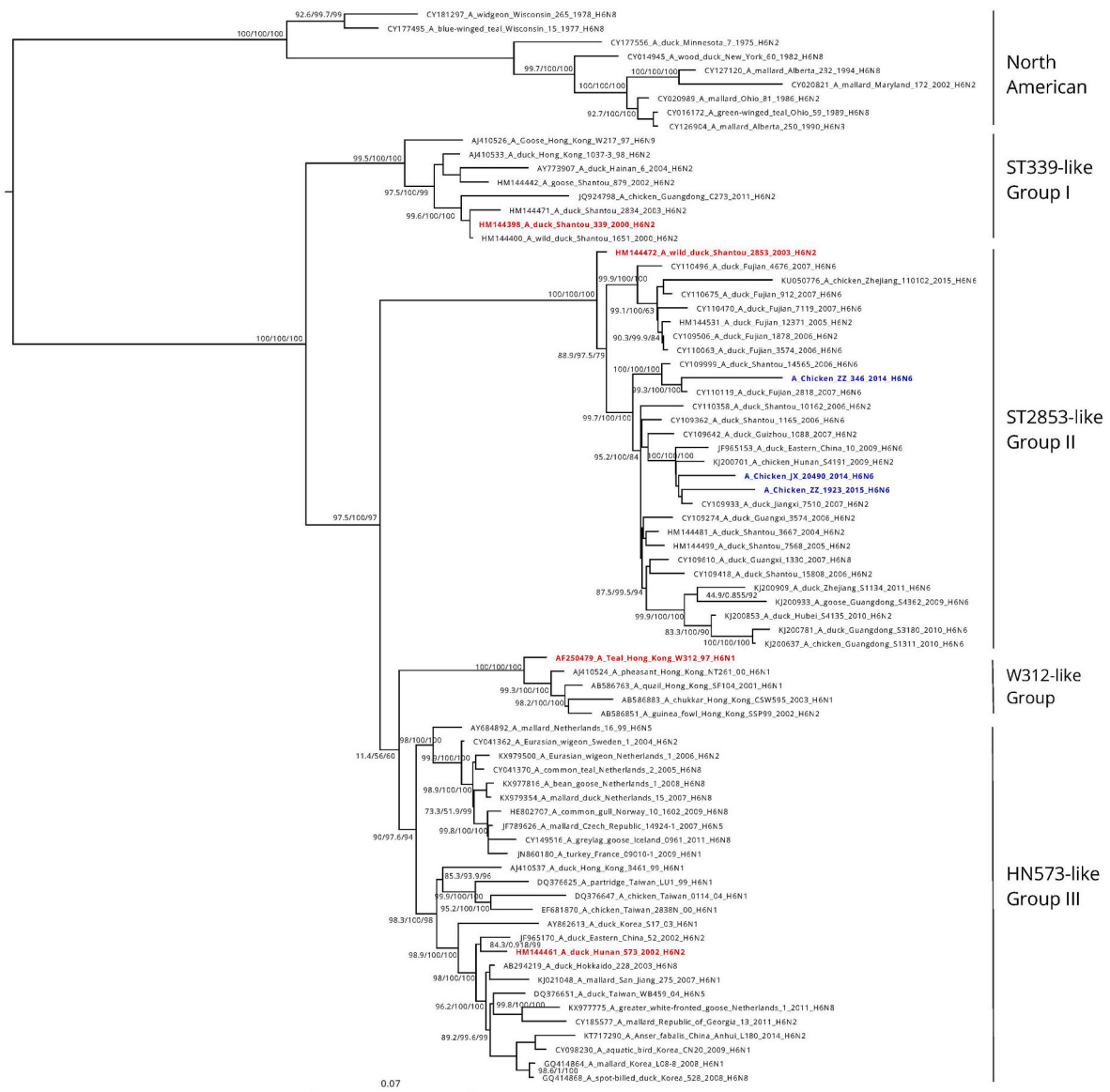
North American
- CY181297_A_widgeon_Wisconsin_265_1978_H6N8
- CY177495_A_blue-winged_teal_Wisconsin_15_1977_H6N8
- CY177556_A_duck_Minnesota_7_1975_H6N2
- CY014945_A_wood_duck_New_York_60_1982_H6N8
- CY127120_A_mallard_Alberta_232_1994_H6N8
- CY020821_A_mallard_Maryland_172_2002_H6N2
- CY020989_A_mallard_Ohio_81_1986_H6N2
- CY016172_A_green-winged_teal_Ohio_59_1989_H6N8
- CY126904_A_mallard_Alberta_250_1990_H6N3

ST339-like Group I
- AJ410526_A_Goose_Hong_Kong_W217_97_H6N9
- AJ410533_A_duck_Hong_Kong_1037-3_98_H6N2
- AY773907_A_duck_Hainan_6_2004_H6N2
- HM144442_A_goose_Shantou_879_2002_H6N2
- JQ924798_A_chicken_Guangdong_C273_2011_H6N2
- HM144471_A_duck_Shantou_2834_2003_H6N2
- HM144398_A_duck_Shantou_339_2000_H6N2
- HM144400_A_wild_duck_Shantou_1651_2000_H6N2

ST2853-like Group II
- HM144472_A_wild_duck_Shantou_2853_2003_H6N2
- CY110496_A_duck_Fujian_4676_2007_H6N6
- KU050776_A_chicken_Zhejiang_110102_2015_H6N6
- CY110675_A_duck_Fujian_912_2007_H6N6
- CY110470_A_duck_Fujian_7119_2007_H6N6
- HM144531_A_duck_Fujian_12371_2005_H6N2
- CY109506_A_duck_Fujian_1878_2006_H6N2
- CY110063_A_duck_Fujian_3574_2006_H6N6
- CY109999_A_duck_Shantou_14565_2006_H6N6
- A_Chicken_ZZ_346_2014_H6N6
- CY110119_A_duck_Shantou_2818_2007_H6N6
- CY110358_A_duck_Shantou_10162_2006_H6N2
- CY109362_A_duck_Shantou_1165_2006_H6N6
- CY109642_A_duck_Guizhou_1088_2007_H6N2
- JF965153_A_duck_Eastern_China_10_2009_H6N6
- KJ200701_A_chicken_Hunan_S4191_2009_H6N2
- A_Chicken_JX_20490_2014_H6N6
- A_Chicken_ZZ_1923_2015_H6N6
- CY109933_A_duck_Jiangxi_7510_2007_H6N2
- CY109274_A_duck_Guangxi_3574_2006_H6N2
- HM144481_A_duck_Shantou_3667_2004_H6N2
- HM144499_A_duck_Shantou_7568_2005_H6N2
- CY109610_A_duck_Guangxi_1330_2007_H6N8
- CY109418_A_duck_Shantou_15808_2006_H6N2
- KJ200909_A_duck_Zhejiang_S1134_2011_H6N6
- KJ200933_A_goose_Guangdong_S4362_2009_H6N6
- KJ200853_A_duck_Hubei_S4135_2010_H6N6
- KJ200781_A_duck_Guangdong_53180_2010_H6N6
- KJ200637_A_chicken_Guangdong_51311_2010_H6N6

W312-like Group
- AF250479_A_Teal_Hong_Kong_W312_97_H6N1
- AJ410524_A_pheasant_Hong_Kong_NT261_00_H6N1
- AB586763_A_quail_Hong_Kong_SF104_2001_H6N1
- AB586883_A_chukkar_Hong_Kong_CSW595_2003_H6N1
- AB586851_A_guinea_fowl_Hong_Kong_SSP99_2002_H6N2

HN573-like Group III
- AY684892_A_mallard_Netherlands_16_99_H6N5
- CY041362_A_Eurasian_wigeon_Sweden_1_2004_H6N2
- KX979500_A_Eurasian_wigeon_Netherlands_1_2006_H6N2
- CY041370_A_common_teal_Netherlands_2_2005_H6N8
- KX977816_A_bean_goose_Netherlands_1_2008_H6N8
- KX979354_A_mallard_duck_Netherlands_15_2007_H6N8
- HE802707_A_common_gull_Norway_10_1602_2009_H6N8
- JF789626_A_mallard_Czech_Republic_14924-1_2007_H6N5
- CY149516_A_greylag_goose_Iceland_0961_2011_H6N8
- JN860180_A_turkey_France_09010-1_2009_H6N1
- AJ410537_A_duck_Hong_Kong_3461_99_H6N1
- DQ376625_A_partridge_Taiwan_LU1_99_H6N1
- DQ376647_A_chicken_Taiwan_0114_04_H6N1
- EF681870_A_chicken_Taiwan_2838N_00_H6N1
- AY862613_A_duck_Korea_S17_03_H6N1
- JF965170_A_duck_Eastern_China_52_2002_H6N2
- HM144461_A_duck_Hunan_573_2002_H6N2
- AB294219_A_duck_Hokkaido_228_2003_H6N8
- KJ021048_A_mallard_San_Jiang_275_2007_H6N1
- DQ376651_A_duck_Taiwan_WB459_04_H6N5
- KX977775_A_greater_white-fronted_goose_Netherlands_1_2011_H6N8
- CY185577_A_mallard_Republic_of_Georgia_13_2011_H6N2
- KT717290_A_Anser_fabalis_China_Anhui_L180_2014_H6N2
- CY098230_A_aquatic_bird_China_CN20_2009_H6N1
- GQ414864_A_mallard_Korea_LC8-8_2008_H6N1
- GQ414868_A_spot-billed_duck_Korea_528_2008_H6N8

0.07

Fig. 2. The phylogenetic tree based on the HA gene of H6 influenza viruses. The viruses obtained in this study were highlighted in blue while the representative strains were highlighted in red. Each group was labeled to the right of the phylogeny. The length of scale bar represents the nucleotide substitutions per site. The values for the nodes are the consensus percentage support of SH-aLRT, aBayes, and UFBoot, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

virus strains.

Immunohistochemistry was used to detect the NP protein of influenza virus in bronchial and lung tissue inoculated with three H6N6 strains. The NP protein was detected in lung alveolar cells inoculated with the ZZ346 virus strain, but not in human lung tissues for the JX20490 and ZZ1923 strains (Fig. 7). The NP protein was not detected in bronchi or bronchioles inoculated with three H6N6 strains. Therefore, our study suggests that the H6N6 ZZ346 strain, with binding to avian-like SAα-2,3 Gal and human-like SAα-2,6Gal receptors, was effectively replicating in human lungs without prior adaptation.

4. Discussion

The H6N6 subtype AIV is widely prevalent in poultry, and its host range has expanded to mammals. Undoubtedly, it has become an endemic disease of domestic fowl and animals. Here, three chicken-originated H6N6 subtypes of AIV were of multiple reassortment viruses, with gene segments derived from the Group-II (ST2853-like) of Eurasian lineages. Terrestrial birds may be an intermediate host in the cross-species transmission of the influenza virus from birds to humans.[35,36] At the same time, molecular epidemiological investigations have shown that the H6N6 subtype AIV is prevalent in terrestrial chickens.[14] Therefore, the H6H6 virus in chickens may acquire the potential to infect humans. The switch of the receptor-binding preference from the avian-like SAα-2,3 Gal to the human-like SAα-2,6 Gal receptor is a key factor in AIV crossing interspecies barriers and efficiently transmitting to humans. The receptor-binding domains in the head of the influenza virus HA can specifically recognize and bind to the avian-like SAα-2,3 Gal and/or human-like SAα-2,6 Gal receptors; yet, the molecular mechanism of receptor-binding preference switch in different avian influenza virus subtypes needs to be further elucidated. The H5N1 HA with the N224K/Q226L mutations has a key role in switching the receptor-binding preference from the avian-like SAα-2,3 Gal to the human-like SAα-2,6 Gal receptor.[37] The HA with the Q226L and G186V mutations in the H7N9 virus could result in virus binding to the human-like receptors, and the H6N6 HA with the S137 N, E190V,
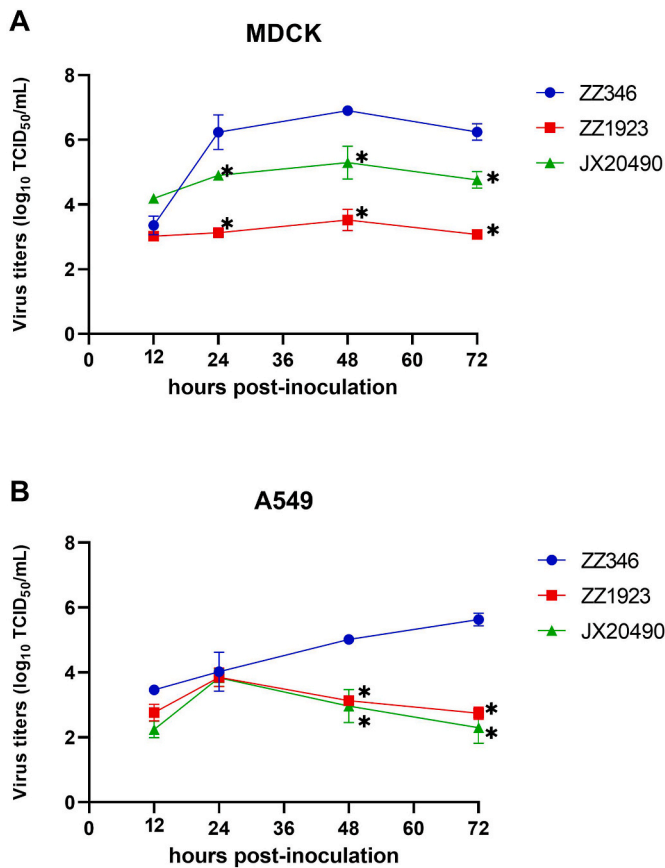
A

MDCK

B

A549

Fig. 3. In *vitro* replication kinetics of the ZZ346, ZZ1923 and JX20490 viruses. MDCK (A), A549 (B) cells were infected with ZZ346, ZZ1923 and JX20490 viruses at a MOI of 0.01. Virus titers were determined at the indicated time points in MDCK cells by using TCID$_{50}$ assays. The detection limit was 2 log$_{10}$ TCID$_{50}$/ml. Graphs are representative of three independent experiments. Asterisks indicate *P* values of <0.01. Error bars show standard deviations.
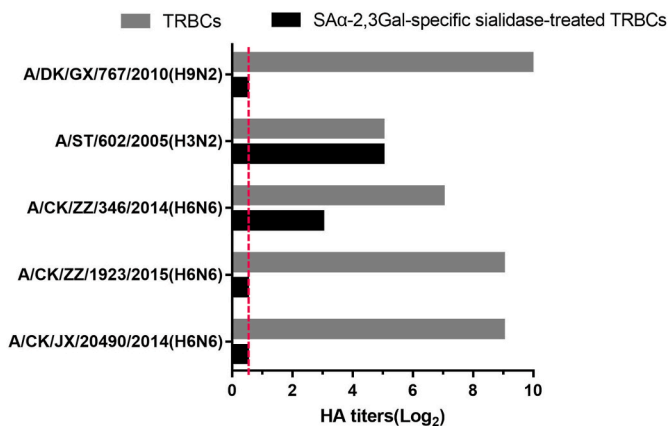

Fig. 4. Characterization of the receptor-binding properties of influenza viruses was determined by Hemagglutination test with 0.6% TRBCs treated and untreated with SAα-2,3 Gal-specific sialidase. The dashed line indicates the detection limit.

and G228S mutations is essential in the process of acquiring the ability to recognize human-like virus receptors.[38–40]

In this study, three H6N6 strains, the 224, 226, 228, 137,138, and 190 of the HA receptor binding domains, have no mutations. However, the P186T, H156R, S263G mutations, an amino acid deletion at position 158 of the HA, were found in the ZZ346 strain. Interestingly, the

receptor-binding analysis indicated that the H6N6 ZZ346 strain could bind to the avian-like SAα-2,3 Gal and human-like SAα-2,6 Gal receptors. Some studies have reported that the HA with the G186V mutation in the H7N9 strain was able to bind to human-like receptors.[38] A combination of the HA (H156 N, S263R) and PA (I38 M) mutations might enhance viral virulence in mice,[34] suggesting that substitution (186, 156, 263) in the HA protein might be related to the binding to human-like SAα-2,6 Gal receptors to effectively replicate in mammals. Therefore, we have speculated that the chicken-originated H6N6 ZZ346 virus strain, with the HA protein P186T, H156R, S263G mutations, could bind to the avian-like SAα-2,3 Gal and human-like SAα-2,6 Gal receptors. Mutation or deletion of NA was observed during viral adaptation to a new host, thus suggesting that it can cross host restriction.[41] Recently, an amino acid deletion was found in the NA stalk region in some H6N6 subtype AIV.[15] In this study, the 11aa deletion in the NA stalk region located at positions 59–69 was found in the ZZ346 strain but not in the JX20490 and ZZ1923 ones. The functional balance between the HA and NA is crucial to viral survival. The HA protein affects virus binding to host cells, and the NA progeny virus particle release from host cells. Only when the two cooperate to reach a balanced state can the virus effectively replicate in host cells.[42] The T271K, E627K, D701 N of PB2 can enhance the polymerase activity, which increases H6N6 pathogenicity and transmission in mammals. However, no substitution at these sites in the PB2 of the H6N6 virus was observed in this study, which is consistent with previously published papers.[22]

It remains unclear whether the H6N6 ZZ346 strain that can bind to the avian-like SAα-2,3 Gal and human-like SAα-2,6 Gal receptors could replicate and infect mammals and humans. Accordingly, we have selected the three chicken-originated H6N6 strains to inoculate mammalian cells, BALB/c mice and human lung tissues. Three H6N6 viruses, especially the ZZ346 strain, have good replicative kinetics in mammalian cells *in vitro*. In the mice infection experiment, some H6N6 strains were able to infect mice. After inoculating mice with the ZZ346 strain, the virus and viral NP antigen were detected in trachea and lung. In addition, serum antibodies were detected at 14 dpi, indicating that effective viral replication and infection in mice. The JX20490 strain NP protein was detected in trachea and bronchial epithelial cells but not in lungs. In addition, no virus was detected in trachea, bronchi, and alveolar tissues. Results show that the H6N6 chicken-originated ZZ346 strain could directly infect mice without prior adaptation, which is consistent with a previous study.[14] Human lung tissue was inoculated with three strains of the H6N6 chicken-originated subtype AIV *in vitro*. Lung tissue inoculated with the ZZ346 strain showed local cell necrosis with the virus and viral NP antigen detected in tissues. In contrast, the virus and the NP protein were not detected in lung tissue after inoculation with the JX20490 and ZZ1923 strains. Therefore, these data suggest that the ZZ346 strain effectively replicates in human lung without prior adaptation. It also provides evidence that the P186T, H156R, S263G mutations and amino acid deletion at position 158 of the HA protein were instrumental in the switch of the H6N6 virus binding from the avian-like SAα-2,3 Gal receptor to the human-like SAα-2,6 Gal and avian-like SAα-2,3 Gal receptors. Based on serum H6 antibody of the exposed population,[20,21] our results indicate that the H6N6 strain has acquired the potential to infect humans. The 11aa deletion (59–69) in the NA stalk region was found in the ZZ346 strain, which also occurred in H5N6 virus-infected with human beings, suggesting that the deletion at positions 59–69 in the NA stalk region may be related to the H6N6 infection in mammals, and especially in humans.[17] In this study, the ZZ346 strain with HA variation (P186T, H156R, S263G mutations, and amino acid deletion at position 158) and the 11aa (59–69) deletion in the NA stalk region directly infected mice and effectively replicated in human lung tissue without prior adaption.

The first case of human infection with the H6N1 avian influenza virus was reported on May 20, 2013, in Taiwan.[5] The emergence of human cases infected with H6N1 shows the unpredictability of influenza virus transmission and the potential threat from novel viruses. Some
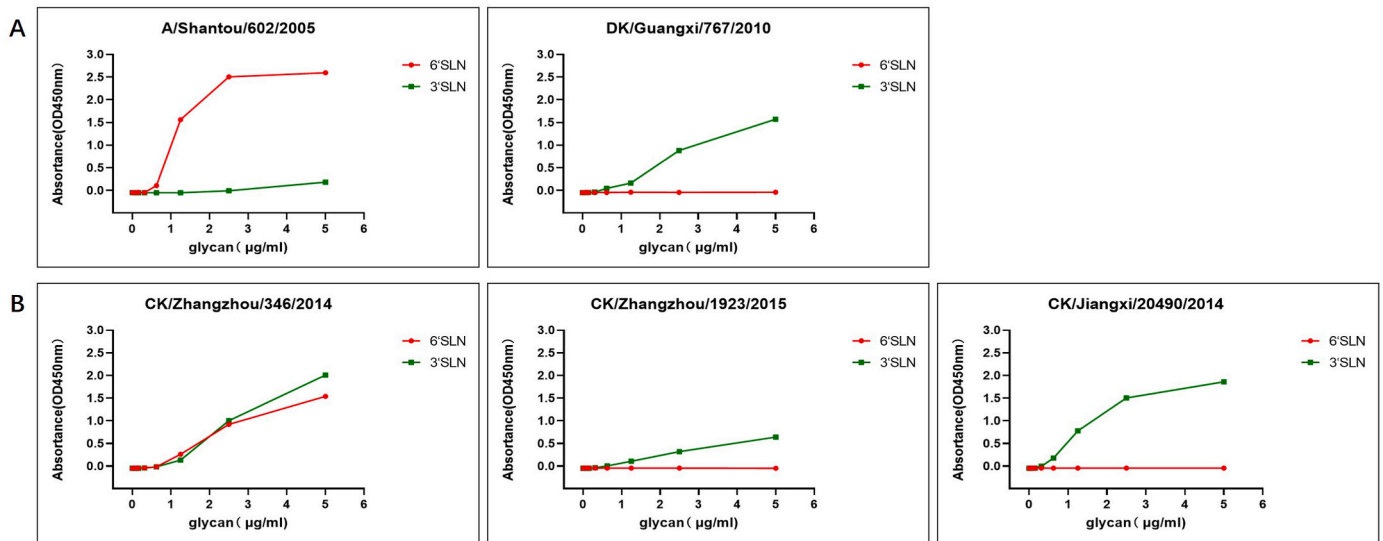
Fig. 5. Receptor-binding specificity of H6N6 viruses using a solid-phase direct-binding assay. A: The human influenza A virus A/Shangtou/602/2005(H3N2) and avian influenza virus DK/Guangxi/767/2010(H9N2) were used as controls. B: Receptor-binding properties of the representative AIV strains to the human-like SAα2-6 and avian-like SAα2-3 receptors were tested using the solid-phase direct binding assay with trisaccharide receptors. Red and green lines represent human-like and avian-like receptors, respectively. Two replications presented similar results with mean values shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
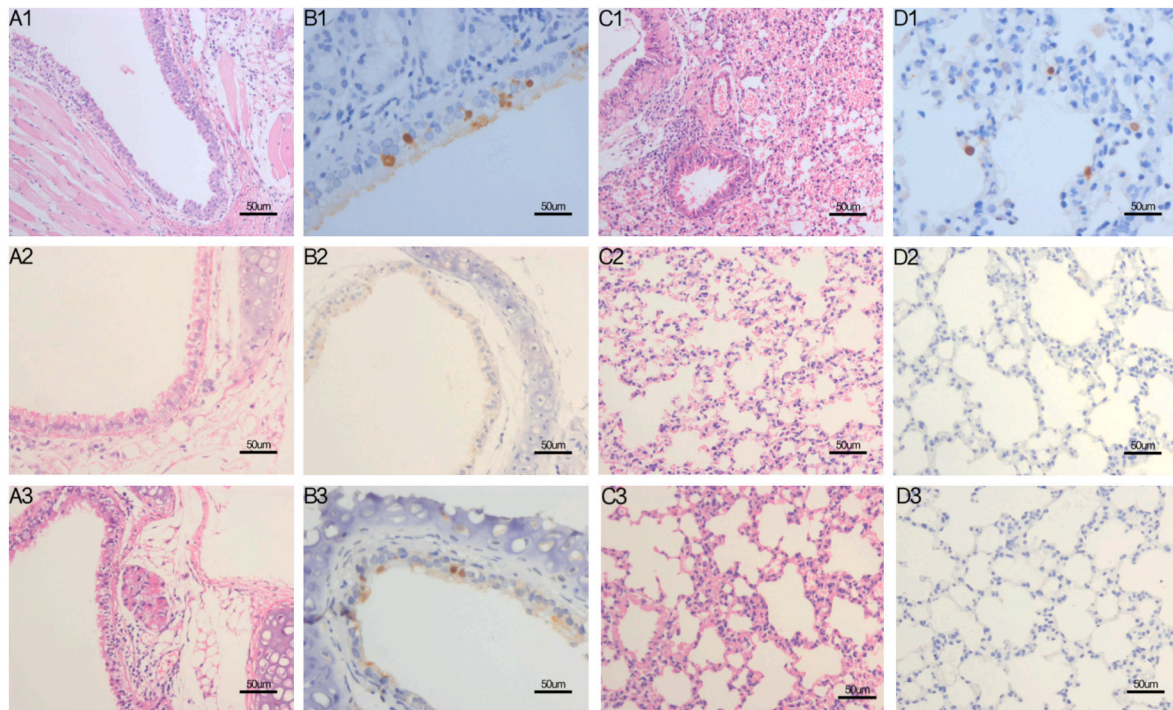
Fig. 6. Pathological changes and virus replication in mice trachea and lungs inoculated with H6N6 viruses. Hematoxylin and eosin staining of the trachea (A1 to A3) and lungs (C1 to C3), immunohistochemical staining of the trachea (B1 to B3) and lungs (D1 to D3). The mice trachea and lungs were inoculated with the ZZ346 (A1 to D1), ZZ1923 (A2 to D2) and JX20490 strains (A3 and D3). The influenza NP antigen staining appeared in brown (B1, B3 and D1). Scale bars, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

studies have reported that the influenza virus responsible for a pandemic is generated by avian-human (or-swine) influenza A virus reassortments,[43] but the AIV involved in the reassortment is not necessarily a highly pathogenic one. Moreover, mild symptoms caused by low pathogenic viruses can be easily overlooked, increasing the chances of virus spread, adaptive mutation, and reassortment. Currently, prevention and control of the influenza pandemic are mainly focusing on the H5N1 and H7N9 subtypes which cause severe human disease and deaths. However, due to the unpredictability and gaps in knowledge about influenza, we cannot predict which subtype of the influenza A virus will cause the next pandemic. Although the H6N6 virus has low pathogenicity, it is widely prevalent in poultry. It has repeatedly infected swines, with the potential to evolve into a novel influenza virus infecting human beings. Therefore, this study has confirmed that some chicken-originated H6N6 viruses might acquire the ability to recognize and bind to human-like receptors, thus increasing risk to humans. Our study emphasizes the importance of

Table 2
Viral protein distribution in the respiratory tract of BALB/c mice inoculated with H6N6 influenza A viruses.

Virus and mice	Trachea				Bronchus				Lung			
	+++	++	+	−	+++	++	+	−	+++	++	+	−
ZZ1923	0	0	0	9	0	0	0	9	0	0	0	9
JX20490	0	0	1	8	0	0	2	7	0	0	0	9
ZZ346	0	2	1	6	0	2	3	4	0	4	3	2

The symbols -, +, ++, and +++ indicate that the numbers of cells with viral NP positive signal were 0, 1 to 20, 20 to 100, and >100, respectively, in each section.
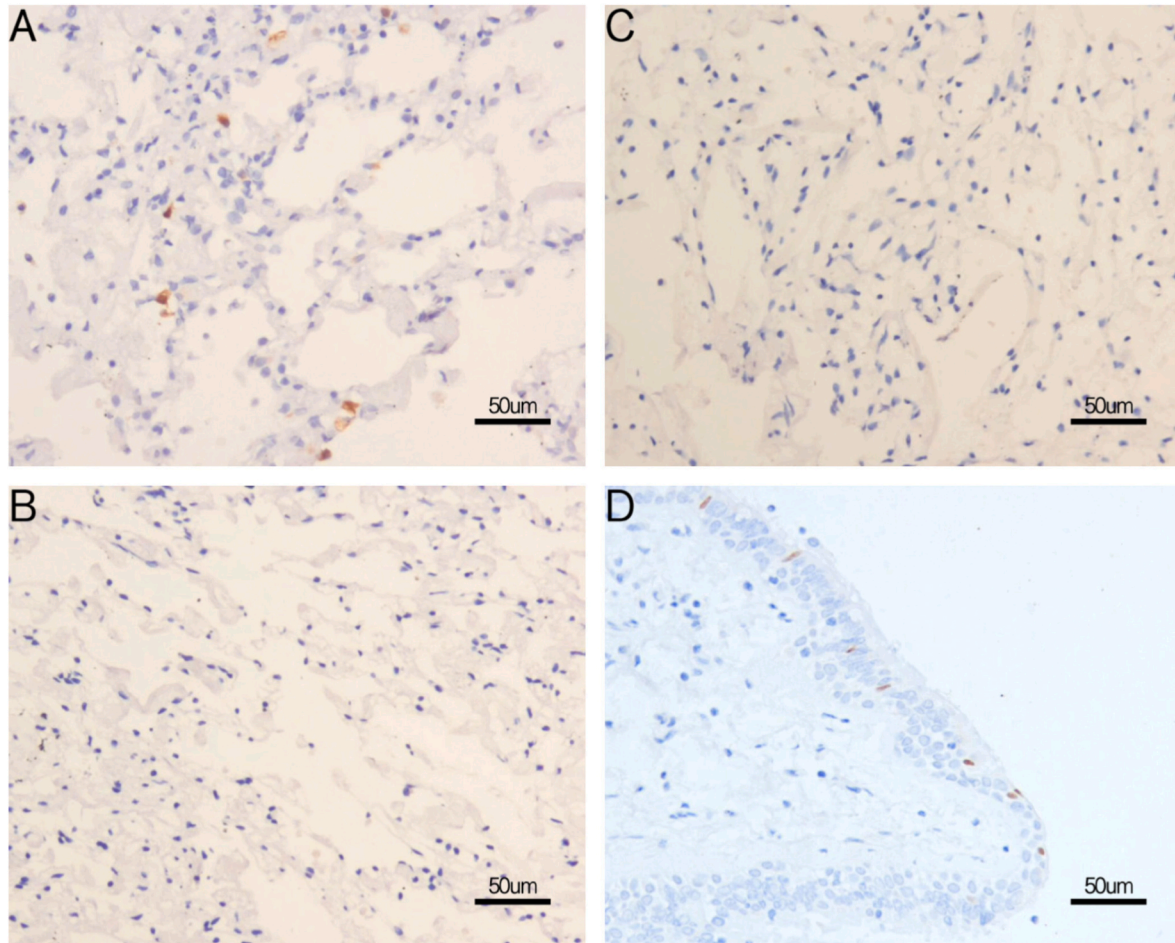
Fig. 7. The H6N6 viruses replication in the lungs of human inoculated with H6N6 viruses *in vitro*. A immunohistochemical method was used to detect the virus NP protein in lungs inoculated with the ZZ346 (A), ZZ1923 (B2), JX20490 (C) and ST602 strains (D). The influenza NP antigen staining appeared in brown (A and D). Scale bars, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

continuous and intensive monitoring of these viruses evolution to prevent transmission to humans.

Institutional review board statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Guangxi Medical University. The patients/participants provided their written informed consent to participate in this study. All animal experiments were conducted in strict compliance with the regulations set by the Animal Ethics Committee of Guangxi Medical University.

Author contributions

Weijuan Zhong, Lingxi Gao, Xijing Wang, Shanggui Su,Yigui Lin performed the experiments, Siyu Zhou, Kai Huang, Xiaohui Fan and Zengfeng Zhang analyzed the data, wrote the manuscript and prepared the figures and tables. Zengfeng Zhang revised the manuscript, organized collaborations and directed the project. All authors read and approved the final version of the manuscript.

Data availability

Genome sequences generated in this study are publicly available in the GenBank database under the accession numbers: MW494902-

MW494909; MW494931-MW494938; MW495024-MW495031.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

AIV	avian influenza virus
HA	Hemagglutinin
COVID-19	Coronavirus disease 2019
SA	sialic acid
Gal	galactose
MDCK	Madin-Darby canine kidney
BIC	Bayesian information criterion
UFBoot	Ultrafast Bootstrap
SH-aLRT	Shimodaira-Hasegawa approximate likelihood ratio test
aBayes	approximate Bayesian-like test
RBS	receptor-binding site
A549	human lung carcinoma cells
MOI	multiplicity of infection
hpi	hours post-inoculation
TRBCs	turkey red blood cells
PBS	phosphate-buffered saline
$TCID_{50}$	tissue culture infective dose 50%
EID_{50}	egg infective dose 50%
dpi	days post-inoculation
NP	nucleoprotein

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