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Unique phenomenon of H5 highly pathogenic avian influenza virus in China: co-circulation of Clade 2.3.4.4b H5N1 and H5N6 results in diversity of H5 Virus

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ABSTRACT

Recently, Clade 2.3.4.4b H5N1 virus has been widely prevalent globally. Although no outbreaks of Avian Influenza have occurred in poultry in China recently, Clade 2.3.4.4b H5 virus can still be isolated from wild birds, live poultry markets and environment, indicating the ongoing co-circulation of H5N1 and H5N6 viruses. In this study, phylogenetic analysis of global Clade 2.3.4.4b viruses and 20 laboratory-isolated H5 strains revealed that Chinese H5N1 and H5N6 viruses since 2021 cluster into two distinct groups, G-I and G-II. Bayesian phylodynamic analysis reveals that G-I H5N6 virus has become an endemic virus in China. In contrast, G-II H5N1 virus, with South China as its main epicentre, has been disseminated in China and its surrounding countries, with its transmission more reliant on the connections of wild birds and waterfowl. Reassortment analysis indicates that since 2023, Clade 2.3.4.4b H5 viruses isolated in China have formed seven genotypes. The genome of H5 viruses has undergone changes compared to those previously prevalent in China. Animal experiments have shown that prevalent H5 viruses exhibit significant lethality in chickens. Additionally, certain H5 viruses have shown the capability of systemic replication in mice. It is noted that H5N6 viruses with HA genes derived from H5N1 viruses demonstrate stronger virulence and pathogenicity in chickens and mice compared to G-I H5N6 viruses. Our study indicates that the co-circulation of H5N1 and H5N6 viruses in China has increased the diversity of H5 viruses, making continuous surveillance of H5 viruses essential.

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Introduction

H5 Highly Pathogenic Avian Influenza (HPAI) is a highly contagious avian disease caused by influenza A virus. The virus can continuously evolve through antigenic drift and antigenic shift, giving rise to new subtypes and genotypes [1]. Since the first emergence of the Gs/GD lineage of H5N1 virus in China in 1996, H5 subtype Avian Influenza (AI) has led to multiple outbreaks worldwide [2]. These outbreaks have caused substantial economic losses for the poultry industry and posed serious threats to human health, raising widespread concern [3,4].

In China, the HPAI H5N6 virus was first isolated from sick ducks in Guangdong Province in March 2014, and the first human case of H5N6 AI was confirmed in Sichuan Province in May of the same year [5,6]. Since then, H5N6 viruses have continued to circulate in China and other Southeast Asian countries. They have gradually evolved into the predominant strain of the Clade 2.3.4.4, becoming the most widely distributed and harmful H5 AIV in the region [7–9]. Since 2014, Clade 2.3.4.4 H5Nx viruses have spread to multiple regions beyond Asia and have been successively detected in wild birds across multiple continents worldwide [10,11]. Subsequently, the HA gene of this clade has further diverged into several subclades, namely 2.3.4.4a to 2.3.4.4 h [12]. In 2020, a novel Clade 2.3.4.4b H5N8 virus emerged in Europe, Asia, and Africa, widely infecting poultry and wild birds [13,14]. It then spread to China, where it underwent genetic reassortment with the locally prevalent H5N6 virus, resulting in the emergence of Clade 2.3.4.4b H5N6 virus and triggering multiple human infection cases [15–17]. Since the autumn

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of 2021, Clade 2.3.4.4b H5N1 virus, which evolved through reassortment of the H5N8 virus, has been prevalent and gradually replaced H5N8 as the new dominant strain, spreading across continents via wild bird migration [18,19]. Afterwards, the Clade 2.3.4.4b H5N1 virus also entered China and, together with the previous Clade 2.3.4.4b H5N6 virus, became the predominant strains of H5 AI in the country, initiating new epidemic trends [20,21]. Furthermore, H5N1 viruses have raised significant concern due to their frequent spillover into mammals. In 2023, there was a sharp increase in global AI cases among mammals, including both terrestrial and aquatic species, as well as companion animals [22–24]. In 2024, the Clade 2.3.4.4b H5N1 viruses were first detected in dairy cows in the United States, resulting in ongoing outbreaks across multiple states [25]. The rapid evolution of AIV, along with changes in its transmission patterns, has increased the likelihood of the virus spreading to new species, thus posing a significant threat to both global biodiversity and human health [26,27].

The coexistence of Clade 2.3.4.4b H5N1 and H5N6 viruses in China, which have distinct evolutionary histories and biological characteristics, has posed new challenges for the development of prevention and control measures [28]. Although some research has studied the biological characteristics of H5N1 and H5N6 viruses, there has been a lack of systematic research on the epidemic pattern of their coexistence in China in recent years. This study aims to explore the differences in genetic evolution, transmission patterns, and genome of the currently prevalent H5N1 and H5N6 viruses in China, reveal the impact of their co-epidemic situation on H5 virus diversity, and investigate the adaptive changes that genetic reassortment may bring about. In addition, this study also assesses the antigenic match between these two viruses and the current vaccine strains used in China, so as to provide a scientific basis for the optimization of AI prevention and control policies.

Materials and methods

Ethics statements and facility

Organ tissues, oropharyngeal and cloacal swabs collected during routine monitoring were processed in the biosafety level 2 facility of South China Agricultural University (SCAU). All experiments using H5 influenza virus were conducted in in an animal biosafety level 3 laboratory and animal facility under SCAU (CNAS BL0011) protocols. The animal research protocol is reviewed and approved by the Institution Animal Care and Use Committee at SCAU.

Virus isolation and genome sequencing

From January 2023 to June 2024, our laboratory conducted routine monitoring on wild bird habitats, farms, veterinary clinics and live poultry markets (LPMs) in different regions of China. A total of 10,077 samples were collected from poultry, including organ tissues and oropharyngeal and cloacal swabs obtained at LPMs. The samples were placed with PBS solution containing 5000U/mL penicillin and streptomycin, inoculated into 9–11 day old specific-pathogen-free (SPF) chicken embryos. After incubation at 37°C for 48–72 hours, allantoic fluids were harvested and haemagglutinin (HA) assays were performed using 1% chicken red blood cells. Viral RNA was extracted using Fastgene Kit (Shanghai Feijie Biotechnology, Shanghai, China), and the whole genome sequence of the virus was amplified by two-step RT–PCR and specific primers. PCR products were purified using the QIAamp Gel Extraction Kit (Qiagen) and sequenced on an ABI 3730XL DNA Analyser (Applied Biosystems). Nucleotide sequences were edited using the Seqman module of the DNAStar software package.

Genotypic and phylogenetic analysis

This study downloaded complete coding region sequences of AIV genomes from GISAID (http://www.gisaid.org) and NBCI (https://www.ncbi.nlm.nih.gov). These included HA gene sequences of global Clade 2.3.4.4b H5 viruses from 2020 to 2024, and NA, PB2, PB1, PA, NP, MP, and NS gene sequences of various virus subtypes from 2005 to 2024. The full-genome sequences of the strains were aligned using MAFFT v7.471 [29]. The best-fitted nucleotide substitution model was identified using the ModelFinder function within the PhyloSuite v1.2.2, and maximum-likelihood (ML) phylogenetic trees for the eight gene segments were constructed using the IQ-TREE [30]. Visualization was carried out on the iTOL v6(https://itol.embl.de).

TempEst v1.5.3 was utilized to perform regression analysis on the root-to-tip distances of phylogenetic trees [31], thereby assessing the temporal signal of the sequence data ($R^2 = 0.8021$, Correlation Coefficient = 0.8956). BEAST v1.10.4 was used to construct the maximum clade credibility (MCC) tree for the HA gene of H5 viruses within Clade 2.3.4.4b, with the General Time Reversible (GTR) substitution model, an uncorrelated lognormal relaxed molecular clock, and a Bayesian SkyGrid coalescent tree prior [32]. Markov Chain Monte Carlo (MCMC) chains were executed for 200,000,000 iterations, with samples taken every 20,000 steps. Tracer v1.7.1 was utilized to assess the convergence and effective sample size of the results from the runs (Effective sample size values > 200) [33]. After a burn-in of 10%, MCC tree was generated for each dataset using TreeAnnotator. To investigate the transmission dynamics of H5 viruses, we conducted a Bayesian stochastic search variable selection (BSSVS) model with asymmetric substitution in

BEAST v1.10.4. The available sequences were categorized into different geographical and host categories, with detailed classifications provided in the supplementary materials. Subsequently, we used SpreaD3 v0.9.6 to calculate Bayes Factor (BF) in order to assess the support for individual transitions between different geographic locations and hosts [34].

Animal experiments

Based on the isolation time, location, and internal gene of the strains, this study selected four representative isolates (B450-4, B14-9, B1338, and B583) for animal experiments. These isolates represent the major genotypes (N1.1, N1.2, N6.1, and N6.4) of the prevailing viruses and cover different geographical origins and sampling time periods, to evaluate the virulence characteristics of current H5 viruses in China and the differences in virulence and pathogenicity among different H5 virus genotypes in chickens and mice.

Four-week to six-week-old SPF chickens (purchased from Jinan SPF Poultry Co., Ltd.) were randomly divided into four groups, with nine chickens in each group. The chickens were intranasally inoculated with a volume of 200 μL of virus solution with a concentration of 10^6 EID_{50}. At 1 day post-inoculation (dpi), six contact chickens were placed in the same cage with the inoculated group. Three of the nine infected chickens were randomly selected at 2 dpi, euthanized, and their organs (heart, liver, spleen, kidney, duodenum, bursa of Fabricius, thymus, brain, trachea and lung) were collected for viral titration assays. The remaining six infected chickens and six contact chickens were observed for two weeks to monitor mortality and viral shedding from the oropharynx and cloaca. At 14 dpi, the serum of surviving chickens was collected for determination of seroconversion by haemagglutination inhibition (HI) assay.

Twenty six-week-old BALB/c mice (Purchased from Guangdong Medical Laboratory Animal Center) were allocated into four groups. Following mild anaesthesia with CO_2, they were intranasally inoculated with a volume of 50 μL of virus solution with a concentration of 10^6 EID_{50}. Three days post-infection (dpi), three mice were randomly selected for necropsy, the following tissues were collected in the order listed and tested for viral titres: mammary fat pad, muscle, brain, liver, spleen, intestine, kidney, heart, nasal concha and lung. In order to assess 50% mouse lethal dose (MLD_{50}) for infection with the four representative strains, four groups of five mice were intranasally inoculated with virus containing doses ranging from 10^1 to 10^8 EID_{50}. Weight loss was monitored, and mice were euthanized when their weight dropped below 75% of the initial weight, in accordance with animal welfare guidelines, and the mortality rate was recorded over a 14-day period.

All surviving animals were euthanized in accordance with ethical requirements at the end of the experiment.

Antigenic analysis

This study selected certain strains from isolated epidemic strains for the preparation of antiserum, including three strains of H5N1 virus and three strains of H5N6 virus. The selection of strains for antiserum preparation was based on a comprehensive consideration of viral genotypes, isolation times, and geographical locations, to ensure that the selected strains could represent the antigenic characteristics of different genotypes and time periods of the viruses. The inactivated viral allantoic fluid was emulsified with Freund's incomplete adjuvant, and these preparations were used to vaccinate 4-week-old SPF chickens. Serum was collected after 3 weeks to evaluate the antibody titers. Additionally, antiserum was prepared by inoculating a trivalent H5/H7 vaccine at a dosage of 0.5 mL. The antigens and antisera for Re-13 and Re-14, as well as the trivalent H5/H7 vaccine (H5-Re13 + H5-Re14 + H7-Re4), were all purchased from Harbin HARVAC Biotechnology Co., Ltd. (http://www.hvriwk.com/). The antigens and antisera for rGD59 and rHN5801, as well as the trivalent H5/H7 vaccine (H5N2 rHN5801 + rGD59 + H7N9 rHN7903), were all purchased from Guangzhou South China Biologic Medicine Co., Ltd. (http://www.gzscbm.com/). HI tests were conducted according to standard procedures of the World Organisation for Animal Health (WOAH) to perform antigenic analysis of the viruses.

Result

Phylogenetic analysis of Clade 2.3.4.4b H5 viruses

During routine surveillance from January 2023 to June 2024, a total of 20 H5 viruses were isolated, comprising 11 H5N1 and 9 H5N6 strains (Table S1). To investigate the origins of these viruses and understand their genetic relationships with strains from other countries, we conducted phylogenetic analyses using the 20 isolates and an additional 423 Clade 2.3.4.4b H5 virus sequences retrieved from public databases. MCC tree constructed using the HA gene demonstrates that during the course of this study, all H5 viruses isolated by our laboratory are affiliated with two phylogenetic clusters of Clade 2.3.4.4b, namely G-I and G-II (Figure 1, Figure S1). The H5N6 viruses isolated in this study, except for B583 and B450-2 which belong to G-II, are distributed in G-I, clustering with the HA genes of H5N6 and H5N8 viruses isolated in China since 2020. All 11 H5N1 strains isolated are distributed in G-II, exhibiting a close genetic

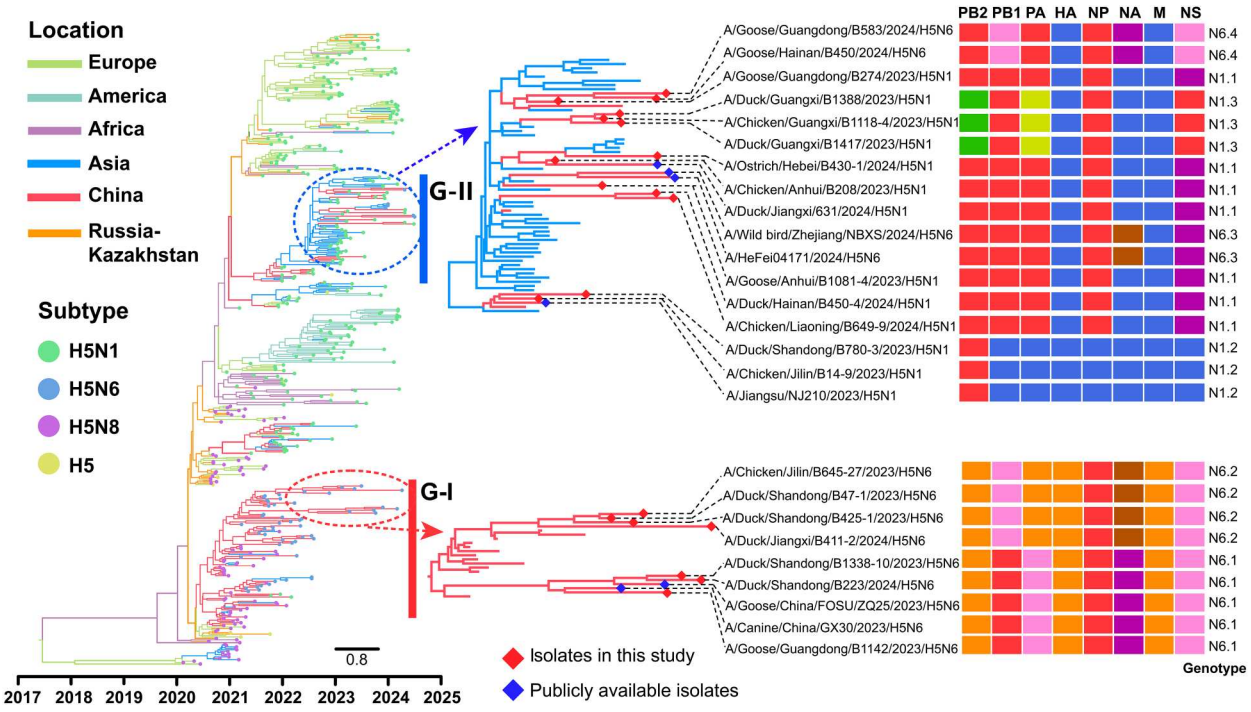

Figure 1. The maximum clade credibility tree for the HA sequences of 443 Clade 2.3.4.4b H5 viruses. HA sequences from different geographical locations are indicated by different branch colours. Different coloured dots at the branch ends represent different subtypes. The full names and positions in the tree of the 26 H5 viruses detected in China from 2023 to the present are shown in the figure. Bars represent the gene segments, and the colours of the bars indicate the donor strains closely related to the gene segments.

relationship with the H5N1 viruses that have been prevalent in Asia, since 2021. The HA genes of the 20 H5 viruses exhibit nucleotide identity ranging from 96.0% to 99.7%. Phylogenetic tracing analysis indicated that the common origin of the two groups can be traced back to the H5N8 viruses prevalent in Russia and Kazakhstan in the second half of 2020(Figure 1, Figure S1). The most recent common ancestor of the G-I viruses is the H5N8 virus that circulated in Europe in 2020, with the Time to the Most Recent Common Ancestor (TMRCA) dating back to June 2020(95% HPD: February 2020 to October 2020). The most recent common ancestor of the G-II viruses is the H5N1 virus that circulated in Africa in 2021, with the TMRCA dating back to April 2021(95% HPD: January 2021 to July 2021). Consequently, while the HA genes of the H5N1 and H5N6 viruses that have been circulating in China since 2021 share a common origin, they may have traversed divergent evolutionary pathways during the course of viral evolution. This divergence could subsequently give rise to differences in various aspects of the viruses' biological characteristics.

Transmission dynamics of H5N6 and H5N1 viruses in China

The HA genes of G-I H5N6 viruses and G-II H5N1 viruses were subjected to a bayesian discrete phylogeographic analysis in this study, and their transmission dynamics were reconstructed across various geographical regions of China and surrounding countries. The analysis of the G-I H5N6 viruses indicates that since 2021, the Southwest China has been a central node for the transmission of the H5N6 viruses. The transmission pathway from the Central China to the Southwest China was decisively supported with BF > 1000. Other transmission pathways, such as from the Southwest China to the Northeast and East China, and from the South China to the Central China, also showed strongly support. Additionally, the spread of the H5N6 viruses from Laos to the Northeast China was supported. Overall, the transmission of G-I H5N6 viruses is primarily concentrated within China (Figure 2(a), Table S2). As for G-II H5N1 viruses, the South China is a central node for the transmission. Within the domestic transmission pathways, routes from South China to East China, as well as from Northwest and Central China to South China, are supported, suggesting that South China plays a significant role as a transit hub in the spread of the H5N1 viruses. In terms of international transmission pathways, the route from Bangladesh to Vietnam received decisively support. The routes from South China to Bangladesh and from South Korea to Northeast China also showed strong support. Additionally, the routes from North China to Bangladesh and Japan, from South China to South Korea, and from Central China to Bangladesh all received support. Overall, South China is the primary epicentre of the H5N1 viruses, while Central and North China

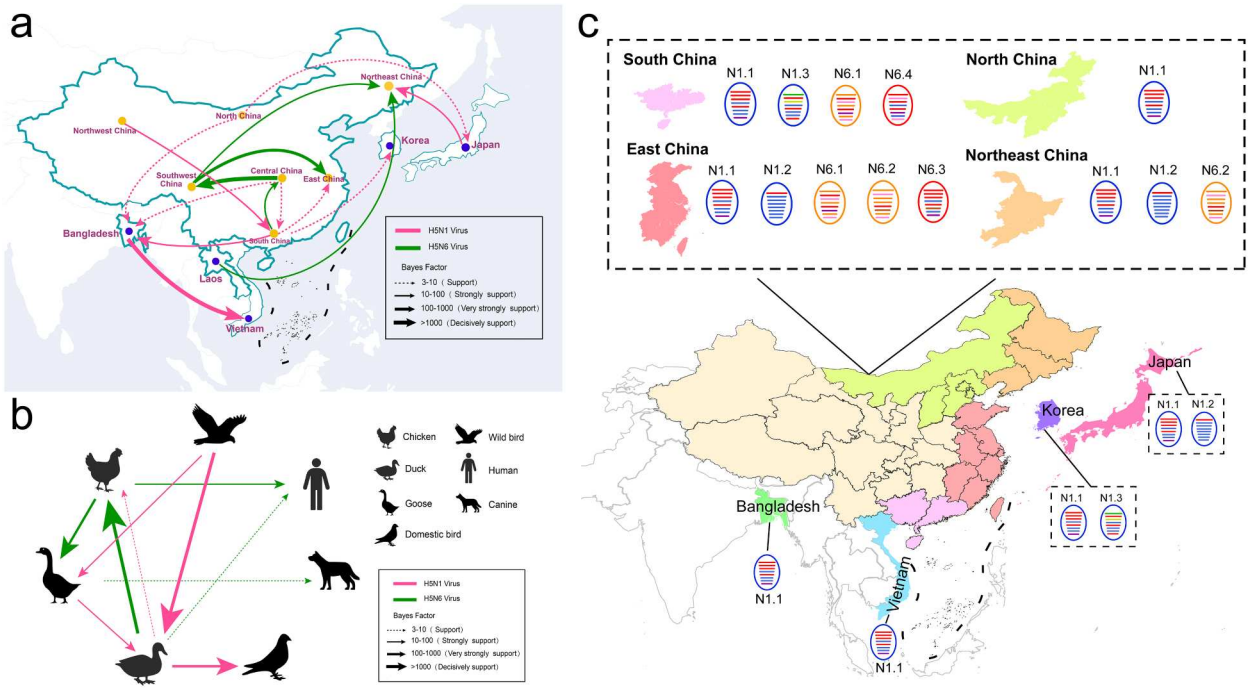

Figure 2. Bayesian phylodynamic analysis of the HA genes of G-I H5N6 and G-II H5N1 viruses. (a) Transmission patterns between different geographical locations for G-I H5N6 and G-II H5N1 viruses. (b) Transmission patterns between different hosts for G-I H5N6 and G-II H5N1 viruses. The thickest solid lines indicate diffusion with decisive support (BF > 1000); the medium solid lines indicate diffusion with very strong support (100 < BF < 1000); the thinnest solid lines indicate diffusion with strong support (10 < BF < 100); the dashed lines indicate diffusion with support (3 < BF < 10). (c) Schematic diagram of the distribution of seven genotypes of H5 viruses in China and surrounding countries.

serve as important transmission nodes, facilitating the diffusion of the H5N1 virus in China and surrounding countries (Figure 2(a), Table S4).

The transmission patterns of the G-I H5N6 viruses and the G-II H5N1 viruses among different host species were evaluated. For the G-I H5N6 viruses, analysis indicates that the transmission pathway from domestic ducks to chickens is decisively supported, and transmission from chickens to geese is also strongly supported. Additionally, the spillover pathway of the H5N6 viruses from poultry to humans and canine received support (Figure 2(b), Table S3). In regard to the transmission pattern of the G-II group H5N1 viruses, wild birds transmit the viruses to ducks and geese. Waterfowl (domestic ducks and geese) act as intermediate hosts, spreading the H5N1 viruses to other poultry hosts, including pigeons and other farmed birds (Figure 2(b), Table S5). In summary, these results suggest that the H5N6 viruses is primarily transmitted among domestic poultry through ducks, while the transmission of the H5N1 viruses relies more on the complex interactions between wild birds and waterfowl.

Complex gene reassortment of H5 viruses in China

We have conducted a phylogenetic analysis of the NA gene and internal genes of H5 viruses in China and its surrounding countries since 2023. Based on the tree topology, we have grouped these genes accordingly. The NA gene of 11 H5N1 viruses has 98.1%−99.8% nucleotide identity, while the NA gene of 9 H5N6 viruses has 83.3%−99.6% nucleotide similarity, and cluster into two groups in the phylogenetic tree (Figure S2). The nucleotide similarity of the PB2, PB1, PA, NP, M, and NS genes among 20 H5 viruses is 90.3%−99.9%, 88.3%−99.9%, 88.3%−99.7%, 92.7%−99.9%, 97.9%−100%, and 88.5%−100%, respectively. Apart from the M gene segment, the other internal gene segments exhibit considerable variability in sequence similarity, resulting in the formation of multiple groups in the phylogenetic tree, indicating significant diversity (Figure S3). Based on genomic differences, this study has categorized the H5 viruses isolated in China since 2023 into 7 genotypes (Figure 3(a), Table S6). The dominant genotype of the H5N1 viruses is N1.1. Its PB1, PB2, PA, NP, and NS genes are closely related to those of LPAI viruses in Asia from 2018 to 2022, with the NS gene particularly related to the H6N6 viruses. Additionally, the HA, NA, and M genes are derived from the H5N1 viruses that were prevalent from 2021 to 2023. PB2 of the N1.2 viruses is closely related to the LPAI viruses in Asia in recent years, while the remaining seven gene segments are derived from the H5N1 viruses. The N1.3 viruses share five genes (PB1, HA, NP, NA, and M) with the N1.1 viruses, while the

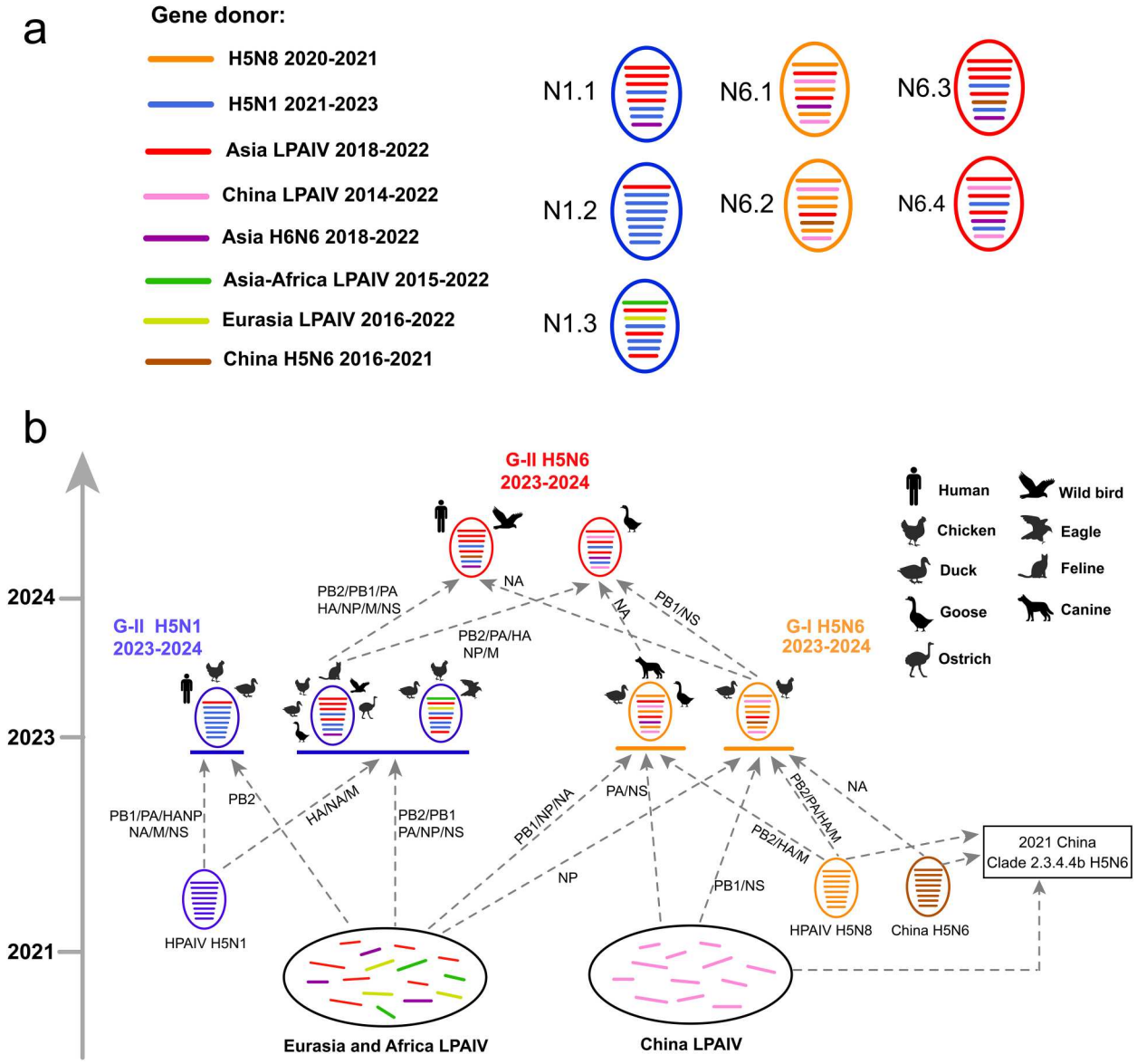

Figure 3. Schematic diagram of the origins and reassortment history of the gene segments of seven H5 virus genotypes in China. (a) Classification of the seven H5 genotypes. The eight gene segments of each genotype are represented by horizontal bars within each ellipse (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, and NS), with gene segments coloured according to their respective viral origins. (b) Schematic diagram of the reassortment of the seven H5 genotypes and the hosts in which these genotypes were detected.

other three genes (PB2, PA, and NS) are similar to those found in various LPAI viruses circulating among wild birds in Eurasia and Africa (Figure 3). Our research findings indicate that the internal genes of H5N1 viruses circulating in China are no longer limited to a single subtype of AIV. Instead, they have undergone extensive and complex genetic reassortment with multiple subtypes of LPAI viruses.

Compared to H5N1 viruses, the dominant genotype of H5N6 viruses, N6.1, has a more complex internal gene component. Its PA and NS genes are closely related to LPAI viruses isolated in China, while the HA, PB2, and M genes are derived from the H5N8 viruses that were prevalent between 2020 and 2021. The PB1 and NP genes are closely related to LPAI viruses in Asia, and the NA gene is closely associated with the H6N6 viruses. The N6.2 virus has four gene segments (PB2, PA, HA, M) derived from the H5N8 viruses that were prevalent between 2020 and 2021. The PB1 and NS genes are closely related to LPAI viruses in China, the NA gene originates from H5N6 viruses in Chinese poultry, and the NP gene is closely related to LPAI viruses in Asia. Notably, N6.3 viruses share seven genes with the N1.1 viruses, except for the NA gene, which is similar to that of the H5N6 viruses circulating in poultry in China. Additionally, the N6.4 viruses have five genes (PB2, PA, HA, NP, M) similar to those of the N1.1 viruses, while the PB1 and NS genes are similar to those of the N6.2 viruses, and the NA gene shows similarity with that of the N6.1 viruses (Figure 3). This phenomenon indicates the co-circulation pattern

of H5N1 and H5N6 viruses in China, suggesting that some gene segments of H5N6 viruses may have directly originated from H5N1 viruses. Such genetic exchange could potentially increase the genetic complexity and adaptability of H5N6 viruses.

The prevalence of H5 viruses with different genotypes

To further understand the epidemiological characteristics of H5 viruses in China between 2023 and the first half of 2024, this study analysed the geographical distribution of different genotypes of H5 viruses in China and surrounding countries. The results indicate that the dominant genotype N1.1 of H5N1 viruses is not only widely distributed across various regions in China, including South China, East China, North China, and Northeast China, but also prevalent in other Asian countries such as South Korea, Japan, Bangladesh, and Vietnam, suggesting that this genotype has strong transmission capabilities within Asia. Additionally, the N1.2 genotype is predominantly found in China and Japan, while the N1.3 genotype has been isolated in China and South Korea. This further reflects that there is a certain transmission link between the H5N1 viruses prevalent in China and those in surrounding countries. Unlike the extensive geographical distribution of H5N1 viruses, G-I H5N6 viruses exhibit a strong regional specificity. The main genotypes of H5N6, N6.1 and N6.2, have only been isolated in China, indicating that G-I H5N6 viruses are endemic AIV in China. Additionally, genotypes N6.3 and N6.4 of G-II H5N6 viruses were isolated in the East China and South China, respectively, during the spring and summer of 2024. Between 2023 and 2024, H5 viruses in South China and East China showed significant genotype diversity (Figure 2(c)). In conjunction with the epidemiological characteristics of genotypes, the co-circulation of H5N1 and H5N6 viruses in South China and East China provided the prerequisite conditions for the emergence of new genotypes N6.3 and N6.4.

Pathogenicity and transmissibility in SPF chicken

Pathogenicity experiments in SPF chickens showed that all chickens in the inoculated groups died after being infected with four genotypes of H5 viruses. The mean death time (MDT) for B450-4, B14-9, B1338-10, and B583 was 48, 56, 60, and 48 hours, respectively. In the contact groups, the mortality rate was 50% for B14-9 and B583, and 33% for B450-4 and B1338-10 (Figure 4(a)). In the inoculated groups, significant viral shedding in both the throat and cloaca samples was observed for all four viruses at 1 dpi. In the contact groups, the viral shedding of throat samples from B1338-10 persisted until 11 days post-contact (dpc), while the viral shedding of the other three viruses ceased by 7 dpc. By 14 dpc, only one surviving chicken showed seroconversion (Table S7). At 2 dpi, all four viruses replicated efficiently in SPF chickens, and high viral titers were detected in multiple organs, including the heart, liver, spleen, kidney, duodenum, bursa of Fabricius, thymus, brain, trachea and lung (Figure 4(b)). These results demonstrate that the prevalent H5 viruses caused significant lethality in SPF chickens, leading to systemic infections rapidly and facilitating effective transmission. B1338-10 caused delayed mortality in the inoculated group compared to the other three viruses but showed prolonged oropharyngeal and cloacal viral shedding in the contact group. Additionally, the novel reassortant H5N6 virus B583 caused earlier and more pronounced lethal effects in SPF chickens with higher viral titres compared to the G-I group H5N6 viruses.

Replication and virulence of H5 viruses in mice

The results of MLD_{50} test showed that these viruses exhibited different lethal rates after inoculation in mice. The MLD_{50} of B450-4, B14-9, B1338-10 and B583 was 5.63 $\log_{10}EID_{50}$, 3.00 $\log_{10}EID_{50}$, 6.63 $\log_{10}EID_{50}$ and 5.17 $\log_{10}EID_{50}$, respectively (Figure 5(a)). The results of viral titres in organs of mice indicate that, after the third day of inoculation, the viral titre in lungs was significantly higher than in other organs, ranging from 3.25 to 5.50 $\log_{10}EID_{50}$/0.1 mL. Both B14-9 and B583 demonstrated systemic replication capabilities in mice, with detectable virus in heart, liver, spleen, lungs, kidneys, brain, and nasal concha. Notably, B583 also exhibited low-level replication in the mammary fat pad. B450-4 showed relatively poor replication capability in mice, with detectable virus in the liver, spleen, lungs, kidneys and nasal concha. In contrast, B1338-10 was only detectable in four organs: the liver, spleen, lungs, and nasal concha, and the viral titres in organs other than the lungs were relatively low (Figure 5(b)). These results indicate that there are differences in virulence among different genotypes of H5 viruses in mice. The N1.2 virus B14-9 exhibited the strongest virulence in mice, significantly impacting their survival rate with only a low dose of the virus. Although both the N6.1 virus B1338-10 and the N6.4 virus B583 are H5N6 viruses, the N6.1 virus B1338-10 had a much weaker impact on mice compared to the N6.4 virus B583, showing the weakest replication and virulence in mice.

Cross-reactivity of H5 viruses with different antisera

In order to evaluate the protective efficacy of commercial vaccines against H5 epidemic strains and the

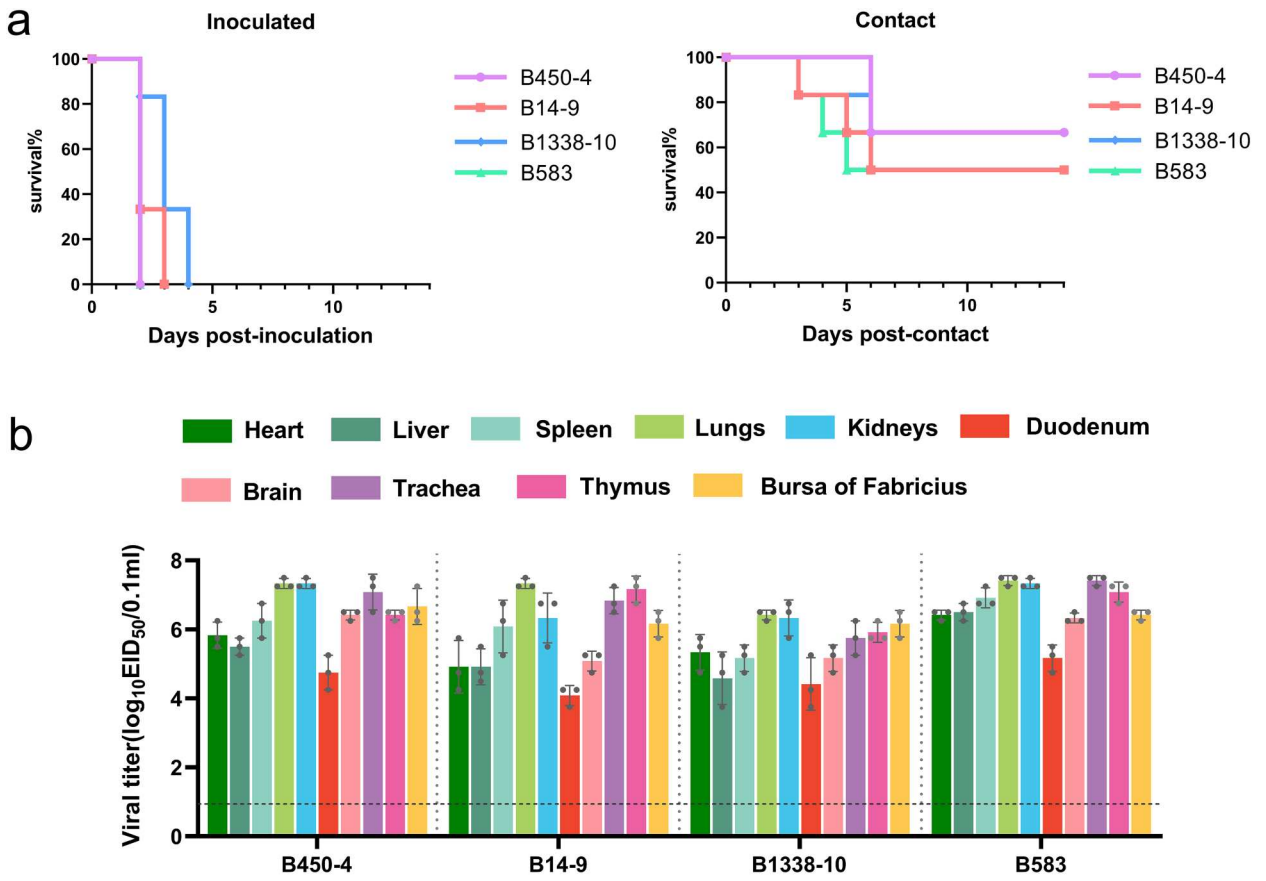

Figure 4. Pathogenicity and transmissibility of four representative H5 strains in SPF chickens. (a) Survival curves of the inoculated and contact groups of chickens for the four representative strains. (b) Viral titers in the organs of chickens euthanized at 2 dpi with 10^6 EID_{50} in 0.2 mL of the representative strains. Data are presented as means ± standard deviations. The dashed line indicates the lower limit of detection.

antigenic differences among H5 viruses within Clade 2.3.4.4b, we investigated the cross-reactivity of antisera derived from four commercial vaccines, six H5 epidemic strains, and two trivalent H5/H7 vaccine-induced antisera against 20 H5 viruses isolated in this study using the HI test. The results (Table S8) indicated that the HI titres of Re-14, rHN5801, Re13, and rGD59 antisera against their homologous viruses were all 1024. The HI titres of Re-14 and rHN5801 antisera against H5 epidemic strains ranged from 128 to 1024, which were either consistent with or 2 to 8 times lower than the homologous titres. The HI titres of Re13 and rGD59 antisera against the 20 H5 viruses ranged from 16 to 256, which were 4 to 64 times lower than the homologous titres. Compared to the H5 epidemic strains of 2023, the HI titres of Re14 and rHN5801 antisera against the H5 epidemic strains of 2024 were generally lower. This suggests that the virus may be accumulating mutations related to immune evasion. However, despite the decrease in titres, the antisera induced by the currently used H5/H7 trivalent vaccine in China still showed good cross-reactivity against the H5 epidemic strains of 2024, with HI titres ranging from 128 to 512. This indicates that the existing vaccine can still provide sufficient protection against H5 epidemic strains, but the efficacy of this protection may be at risk of diminishing. It is worth noting that the H5N1 virus antisera (B14-9, B1417, B1081-4) exhibited lower HI titres against H5N6 strains compared to the homologous subtypes. In contrast, H5N6 antisera (B1142, B47-1, B680-2) demonstrated good cross-reactivity with both H5N6 and H5N1 strains, suggesting that H5N6 viruses may have a high degree of antigenic consistency with strains within Clade 2.3.4.4b.

Discussion

Since the autumn of 2021, the Clade 2.3.4.4b H5N1 AIV has been widely circulating among wild birds and domestic poultry in various countries. Although the H5N1 subtype has become the predominant strain, other subtypes such as H5N2, H5N6, and H5N8 have also been detected in avian populations [35–37]. The novel H5N6 and H5N1 viruses from the Clade 2.3.4.4b emerged in China in 2021 and have since been co-circulating and spreading. This study elucidates the differences in transmission patterns and genotypic evolution of Clade 2.3.4.4b H5N1 and H5N6 viruses in China since 2021. The discrepancy in the spread of G-I H5N6 and G-II H5N1 viruses is closely related to the host ranges of the two viruses and the

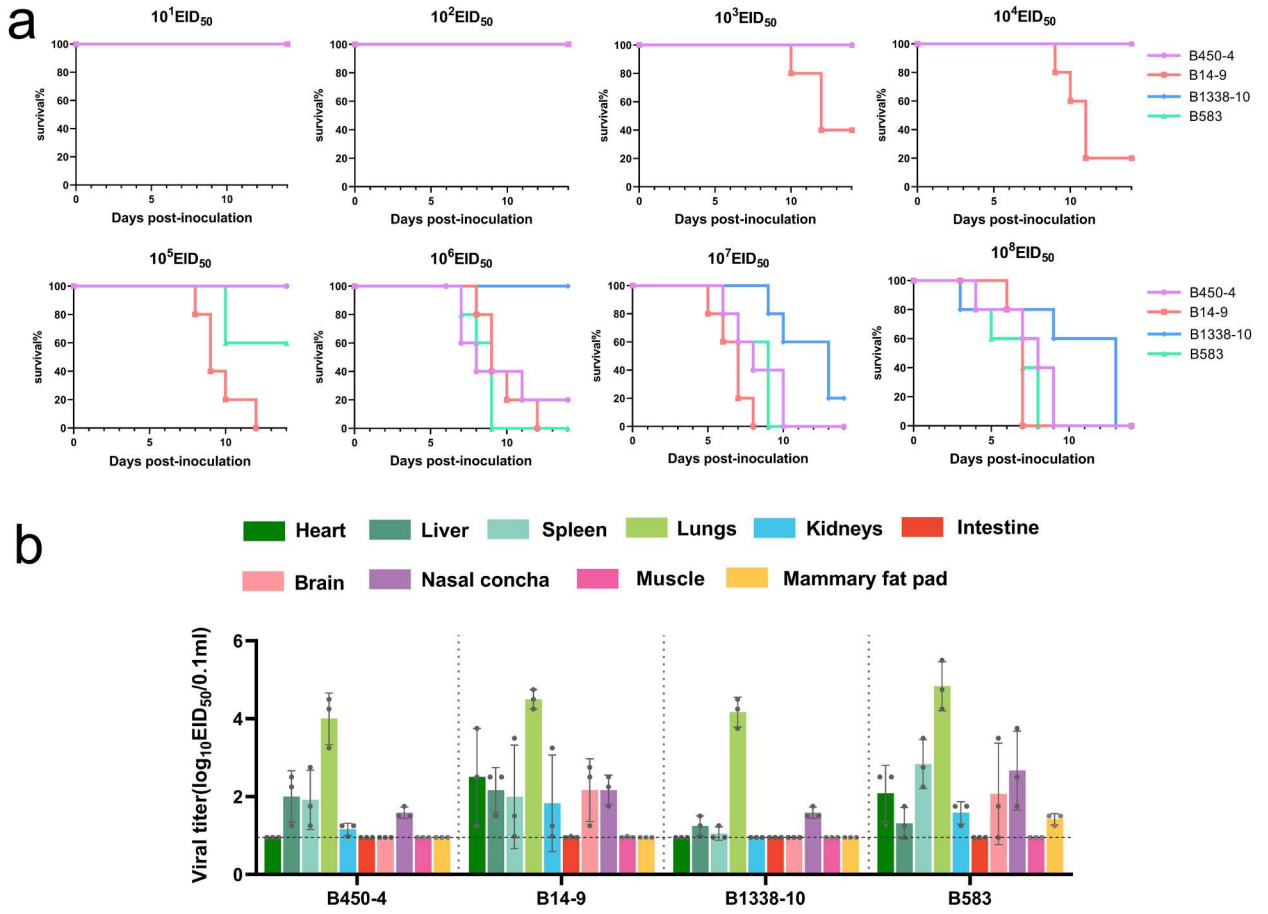

Figure 5. Replication and virulence in mice of representative strains of four H5 genotypes. (a) Survival curves of mice in challenge groups with 10^1 EID_{50} to 10^8 EID_{50}, which represent the MLD_{50} of the representative strains. (b) Viral titres in organs of mice humanely euthanized on day 3 after inoculation with 10^6 EID_{50} of representative strains. Data shown are means ± standard deviations. The dashed line indicates the lower limit of detection.

seasonal transmission patterns of AI in Asia [38]. H5N6 viruses in G-I groups mainly spread among poultry in China, and this host specificity may restrict their ability to spread widely geographically via wild bird migration. Studies have shown that once AIV adapt to domestic chickens, their adaptability in wild birds is reduced, making it difficult for them to re-enter the wild bird virus pool [1,39]. In contrast, the H5N1 virus exhibits a broader host range, with wild birds playing a pivotal role in its transmission. Wild birds, being the natural reservoirs of AIV, are diverse and widely distributed, driving the global spread of AIV through long-distance migration [40,41]. Moreover, the South China region, as an epicenter for H5 viruses in China, owes its geographical hub status to multiple ecological factors. The region is densely populated with poultry farms and live poultry markets, has frequent cross-regional poultry movement, and lies along the East Asian-Australasian Flyway, making it a crucial habitat for numerous migratory birds [42–45]. This unique combination of factors facilitates the coexistence and circulation of diverse viral genotypes in the region, thereby increasing the likelihood of genetic reassortment and further enhancing regional viral diversity.

Since 2023, the Clade 2.3.4.4b H5 virus in China has exhibited complex reassortment patterns, evolving into multiple genotypes with significant genomic differences from the earlier prevalent H5N1 and H5N6 viruses. During 2020–2021, the genes of H5N1 viruses prevalent in China mainly originated from H5 viruses in Europe and Russia, as well as from LPAI viruses. In contrast, H5N6 viruses were formed by the reassortment of the globally disseminated H5N8 viruses with local H5N6 and LPAI viruses in China [16,20]. Currently, the genes of H5N1 viruses mainly come from H5N1 viruses in Asia and LPAI viruses, while H5N6 viruses have more complex genetic origins than before. In the current context of co-prevalence of H5N1 and H5N6 viruses, a novel H5N6 genotype, N6.4, has been detected, whose HA gene and some internal gene fragments are directly derived from G-II H5N1 viruses. Animal experimental results further confirm that the N6.4 virus shows greater virulence and pathogenicity in chicken and mouse models than G-I H5N6 viruses, suggesting that it has enhanced adaptability and a heightened potential for cross-host transmission. This finding underscores the potential changes in biological properties that may arise from genetic reassortment

among H5 viruses. Such reassortment allows for rapid viral evolution, facilitating interspecies transmission and posing an ongoing threat to public health [46].

The viruses isolated in this study have Q226 and G228 at the HA receptor-binding site, indicating a preference for avian-type α-2,3 sialic acid receptors. However, the presence of amino acids 137A, 158N, 160A, 186N, and 192I in the HA protein also suggests a potential for binding to human-type α-2,6 sialic acid receptors (Table S9a) [47–49]. Notably, significant amino acid differences at positions 83, 154, 188, and 487 (H5 numbering) were observed in the HA between G-I and G-II group isolates (Table S10). These variations may be related to the differences in virulence and pathogenicity of the two types of viruses in animal models in this study, but the specific biological mechanisms still need further research. In addition, we identified the PB2-E627K substitution in one isolate – a well-documented mutation associated with enhanced viral polymerase activity and mammalian adaptation [50]. Furthermore, a series of amino acid substitutions previously reported to be associated with increased mouse virulence were identified in different proteins of the isolated strains, further supporting the potential risk of zoonotic transmission of these strains (Table S9) [48]. The mouse experiments in this study provide an early warning of the zoonotic potential of these viruses. Although the risk of human-to-human transmission of H5 viruses is still low now, their expanding host range and the outbreak of H5N1 in the US dairy cattle emphasize that Clade 2.3.4.4b viruses are constantly adapting to mammals [51,52].

Since 2023, seven H5 virus genotypes have emerged in China. However, overall genetic diversity remains lower than in Europe and the Americas [53]. This is partly attributable to inactivated vaccines are widely used in China, effectively curbing the spread of H5 viruses [54,55]. Also, H5N6 mainly spreads in poultry, restricting its gene pool expansion. Nevertheless, the emergence of new N6.3 and N6.4 genotypes in 2024 indicates that the co-circulation of H5N1 and H5N6 viruses is driving viral diversity evolution in China. Antigenic analysis shows that vaccine-induced antisera have good cross-reactivity with Clade 2.3.4.4b H5 isolates. Yet, given the ongoing genetic reassortment and evolution of H5 viruses, it is crucial to continue updating vaccine strains dynamically. In the future, monitoring of H5 viruses and vaccine development should be strengthened to provide solid scientific support for AI prevention and control in China, and contribute Chinese expertise to global AI prevention.

Disclosure statement

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