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Research Article

Continued evolution of H10N3 influenza virus with adaptive mutations poses an increased threat to mammals

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ABSTRACT

The H10 subtype avian influenza virus (AIV) poses an ongoing threat to both birds and humans. Notably, fatal human cases of H10N3 and H10N8 infections have drawn public attention. In 2022, we isolated two H10N3 viruses (A/chicken/Shandong/0101/2022 and A/chicken/Shandong/0603/2022) from diseased chickens in China. Genome analysis revealed that these viruses were genetically associated with human-origin H10N3 virus, with internal genes originating from local H9N2 viruses. Compared to the H10N8 virus (A/chicken/Jiangxi/102/2013), the H10N3 viruses exhibited enhanced thermostability, increased viral release from erythrocytes, and accumulation of hemagglutinin (HA) protein. Additionally, we evaluated the pathogenicity of both H10N3 and H10N8 viruses in mice. We found that viral titers could be detected in the lungs and nasal turbinates of mice infected with the two H10N3 viruses, whereas H10N8 virus titers were detectable in the lungs and brains of mice. Notably, the proportion of double HA Q222R and G228S mutations in H10N3 viruses has increased since 2019. However, the functional roles of the Q222R and G228S double mutations in the *HA* gene of H10N3 viruses remain unknown and warrant further investigation. Our study highlights the potential public health risk posed by the H10N3 virus. A spillover event of AIV to humans could be a foretaste of a looming pandemic. Therefore, it is imperative to continuously monitor the evolution of the H10N3 influenza virus to ensure targeted prevention and control measures against influenza outbreaks.

1. Introduction

Avian influenza A virus is a zoonotic pathogen that causes intermittent pandemics. The first step for avian influenza A virus to cause a pandemic is to spread widely among host species and spill over into humans (Lipsitch et al., 2016). Over the years, several subtypes avian influenza viruses have caused human infections and fatalities, posing a significant threat to human health. These subtypes include H7N9, H5N6, H10N8, H7N4, H5N8, H10N3, H10N5, and H3N8 (Bi et al., 2016; Liu et al., 2013; Pyankova et al., 2021; Yang et al., 2022; Zhang J, et al., 2020, 2022a). Genetic mutation and recombination are the primary mechanisms of influenza virus evolution. Novel influenza viruses accumulate adaptive mutations to infect humans, leading to pandemics (Long et al., 2019). Consequently, continuous surveillance of influenza viruses is imperative.

Avian influenza viruses can be divided into highly pathogenic avian influenza virus (HPAIV) and low pathogenic avian influenza virus (LPAIV) based on their pathogenicity in chickens (Rebel et al., 2011). Most H5 and H7 subtype HPAIVs can cause high morbidity and mortality in poultry flocks (Parums, 2023; Zhu et al., 2019). The H7N9 virus has

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resulted in millions of poultry deaths in China and human infections with a mortality rate of about 39.7% (Jiang et al., 2022; Zhu et al., 2019). Additionally, H5N1 has led to over 800 cases and 400 deaths worldwide since its first appearance (Philippon et al., 2020). These findings indicate that both H5 and H7 subtype HPAIVs pose a threat to birds and humans. In addition to HPAIVs, LPAIVs should not be ignored (Li et al., 2017; Zhang et al., 2023a). LPAIVs (i.e., H3, H9, and H10 subtypes) are generally low pathogenic to birds. However, they can still cause human infections and deaths (Zhang et al., 2023b), posing serious public health concerns.

In recent years, the emergence of H3 and H10 AIVs in China has been of particular concern (Chen et al., 2023; Liu et al., 2024; Sun et al., 2023). Both have caused human deaths. The first H10 subtype AIV was isolated from chickens in Germany in 1949. Since then, more than 2000 H10 subtype AIVs have been isolated in wild waterfowl, poultry, and mammals around the world. The continued prevalence of H10Nx subtype AIVs in birds has raised concerns about the risk of cross-species infection with H10 viruses. Since 1984, more than 60 H10Nx viruses have been isolated from mink, seals, and pigs (Wang et al., 2022). Although rare, some cases of human infection with H10 subtype AIVs have been reported. In December 2013, the first human case of H10N8 infection was reported in China, where a total of three people were infected with H10N8, two of whom died (Chen et al., 2014; Qi et al., 2014; To et al., 2014). From 2011 to 2015, H10N8 was the predominant subtype of influenza virus in China. However, from 2016 to 2021, H10N3 became the most prevalent subtype (Zhang et al., 2021). H10N3 viruses isolated by Wu et al. in live poultry markets (LPM) in 2016 and 2017 exhibited reduced pathogenicity in mice (Wu et al., 2019). However, recent studies have demonstrated that H10N3 viruses isolated from chickens exhibit high pathogenicity in mice and can be efficiently transmitted to Guinea pigs via aerosol route (Guo et al., 2022; Liu et al., 2022). Notably, these novel H10N3 viruses exhibit a preference for both avian and human receptors, indicating a growing public health threat (Liu et al., 2022). In 2021, a case of human infection with H10N3 influenza virus was identified in China. The male patient infected with the H10N3 virus exhibited fever, fatigue, severe pneumonia, acute respiratory distress syndrome, septic shock, and other physiological characteristics (Qi et al., 2022). These findings suggest that H10N3 AIV has the potential to adapt to mammals, which could pose a serious threat to human health. Therefore, it is essential to strengthen routine surveillance and risk assessment for avian-origin H10N3 influenza virus in China, to ensure that early warning, prevention, and control measures can be put in place to effectively combat AIVs.

Previous studies have shown that H10N3 and H10N8 are the two most abundant subtypes of H10 subtype viruses in China, and the dominant H10 subtype viruses in China shifted from H10N8 to H10N3 during 2016–2021. Therefore, we performed evolutionary analyses of H10N3 viruses and compared the biological properties of H10N3 and H10N8 viruses *in vivo* and *in vitro*, with the intention of indicating whether H10N3 viruses became the dominant viruses among the H10 subtypes of viruses for the prevention and control of avian influenza of the H10 subtype. We found that H10N3 viruses increased thermostability, viral release from erythrocytes, and expression of HA protein in mammalian cells, suggesting that H10N3 viruses pose an increasingly threat to public health in recent years. Our results provide novel insights into the risk assessment of H10N3 viruses and provide an early warning of influenza pandemic potential.

2. Materials and methods

2.1. Virus isolation

Two H10N3 influenza viruses were isolated from chicken tracheal samples from a farm in Shandong Province of China, which were designated as A/chicken/Shandong/0101/2022 (H10N3) and A/chicken/Shandong/0603/2022 (H10N3), respectively. The whole genomes of two H10N3 influenza viruses were amplified using Hoffmann's primer (Hoffmann et al., 2001). In December 2013, the first human infection with influenza A (H10N8) virus was detected in China. To better understand the origin of the influenza A H10N8 virus, which has caused human deaths, an epidemiologic investigation was conducted at the live poultry market that the patient visited a few days before the onset of symptoms. Our laboratory successfully isolated the H10N8 virus from a chicken swab sample named A/chicken/Jiangxi/102/2013 (H10N8) (EPI530539–EPI530546, hereafter 102/H10N8). In the 102/H10N8 isolate, the cleavage site of HA has only one basic amino acid (arginine, R). In addition, amino acid residues 226Q and 228G (H3 numbering) of the HA protein show avian-like receptor binding preferences (Qi et al., 2014). Mice in the 102/H10N8 virus-infected group survived infection without significant weight loss, indicating low pathogenicity in mice (Chen et al., 2015). The three viruses were propagated in 10 days SPF embryonated chicken eggs and stored at −80 °C.

2.2. Phylogenetic analysis of H10 subtype influenza viruses

All available gene sequences of H10N3 AIVs were downloaded from GISAID. We first performed quality control analysis to remove sequences with 100% nucleotide homology. Subsequently, multiple sequence alignment of these sequences was conducted using MAFFT in PhyloSuite (v1.2.2) (Zhang D. et al., 2020). Subsequently, IQ-tree (v1.6.12) was used to construct a maximum likelihood (ML) tree of eight gene segments, in which the HKY nucleotide substitution model was selected, and the operation was repeated 1000 times. Finally, the generated trees were illustrated by FigTree (v1.4.4) and iTOL (https://itol.embl.de).

2.3. Geographical distribution analysis of subtype H10 influenza viruses

The *HA* gene sequences of H10 subtype influenza viruses from China between 1978 and 2023 were downloaded from EpiFlu's database of GISAID. Then, the number of H10 subtype influenza viruses in China was counted, and the distribution of H10 subtype influenza viruses in China was plotted using the R package ggplot2 (v 3.4.4) and Adobe Illustrator 2023.

2.4. Amino acid frequency analysis of different amino acid residues

Amino acid frequencies at positions 222 to 228 of HA protein were analyzed using Weblogo3.4 (http://weblogo.threeplusone.com/). All available genome sequences of H10N3 viruses isolated in China were downloaded from GISAID and aligned using MAFFT (v7.3). Amino acid frequencies at positions 222 to 228 of HA protein were visually represented through WebLogo3.4.

2.5. Plaque assay

MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) and seeded on six-well plates, confluent monolayers were washed twice with PBS. MDCK cells were infected with 10-fold serially diluted viruses and cultured at 37 °C in a 5% CO_2 atmosphere. After 2 h post-infection (hpi), the cells were washed twice with PBS and overlaid with 2% agar in 2 × concentrated DMEM with 0.2% bovine serum albumin (BSA) and 0.5 μg/mL TPCK-trypsin. After 48 hpi, the agarose was removed, and the cells were fixed in 4% paraformaldehyde fix solution and stained with 0.5% crystal violet. Plaques were visualized and manually counted.

2.6. Thermal stability

To determine the thermal stability of H10 subtype AIVs, the HA units of each virus were measured and then diluted to a uniform HA potency using PBS and the two-fold dilution method. The diluted virus was subjected to incubation at 55 °C for different time intervals, including 15

min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, and 5 h (Zhang et al., 2022b). The titers of heat-treated H10N3 and H10N8 viruses were determined using the HA assay. Three independent experiments were conducted.

2.7. Virus release assay

To evaluate the HA-NA functional balance of the viruses, a virus elution assay was performed. A 96-well hemagglutination plate was taken, and 50 μL of PBS was added to each well. Next, 50 μL of virus solution was added to the first vertical well. Then a two-fold gradient dilution was made to the 11th well, and the 12th well as a control well. After that, 50 μL of chicken erythrocytes were added to each well. The plate was then incubated for 1 h at 4 °C, and the initial hemagglutination potency was recorded (Sekine et al., 2022). The microtiter plate was stored at 37 °C, and the decrease in HA titer was recorded every hour until 10 h had passed.

2.8. Growth kinetics of the virus in MDCK cells

MDCK cells were infected with 10-fold serially diluted viruses and cultured at 37 °C in a 5% CO_2 atmosphere for 48 h to determine the 50% tissue culture infective dose ($TCID_{50}$). Virus-infected cells were examined by detection of viral M1 using immunocytochemistry (ICC). $TCID_{50}$ value was calculated using the Reed-Muench method. Multistep replication kinetics were determined by inoculating MDCK cells with viruses at a multiplicity of infection (MOI) of 0.001. Following 1 h incubation at 37 °C, the cells were washed two times and incubated in DMEM containing 0.25 μg/mL TPCK-trypsin and 0.2% BSA. Supernatants were collected at 12, 24, 36, 48, and 60 hpi and titrated on MDCK cells.

2.9. Determination of viral protein expression at different time points

MDCK and A549 cells in 12-well plates were infected with H10 subtype AIVs at an MOI of 1 and maintained in DMEM containing 0.25 μg/mL TPCK-trypsin and 0.2% BSA. Cell samples were collected at 3, 6, 9, and 12 hpi. The expression of HA and NP protein was determined by Western blotting. Briefly, cell lysates were prepared from infected cells using cell lysis buffer, heated at 100 °C for 10 min, and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, protein samples were electroblotted onto nitrocellulose filter (NC) membranes (Millipore) and blocked for 2 h in phosphate-buffered saline (PBS) containing 5% (w/v) non-fat dry milk. The membranes were incubated with the primary antibodies overnight at 4 °C, followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The presence of HRP was detected by using a Western Lightning chemiluminescence kit (Beyotime) according to the manufacturer's protocol. Anti-H10N3 IAV HA antibody (11693-RP01) was purchased from Sino Biological. Anti-IAV NP antibody (11675-R707) was purchased from Sino Biological. Anti-GAPDH antibody (60004-1-Ig) was purchased from Proteintech.

2.10. Mouse experiment

First, the 50% egg infection dose (EID_{50}) values of the three H10 influenza viruses in 10-day-old normal chicken embryos were determined using the Reed-Muench method. Six-week-old female BALB/c mice (Dean Genetic Technologies Ltd., Guangzhou, China) were intranasally inoculated with 50 μL of 10^5 EID_{50} of each virus diluted in DMEM, and six mice from each group were monitored daily for 14 days to record the body weight and clinical signs. The mice that lost more than 25% of their original body weight were humanely euthanized. Three mice from each group were euthanized at day 4 post-infection (dpi) for virus titration and histology. Lungs, brains, and nasal turbinate were collected and homogenized in cold PBS. Viral titers were determined by EID_{50} assays (Kong et al., 2021). In addition, lungs were collected at 4 dpi, fixed with tissue fixation for 48 h, and paraffin sections were performed to observe histopathological changes of the lungs. After dewaxing and hydration, the slides were stained with hematoxylin and eosin (H&E) (Feldman and Wolfe, 2014).

2.11. Quantification and statistical analysis

All statistical analyses were performed using GraphPad Prism (v8.0.2). Data are presented as the mean ± the standard errors of means (SEMs). One-way and two-way ANOVA were used to compare the differences between different groups. Statistical significance was indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3. Results

3.1. Evolution of H10N3 avian influenza viruses

To elucidate the distribution and evolution of H10 subtype influenza viruses, we performed a phylogenetic analysis of global H10 subtype influenza viruses based on *HA* gene. Our findings indicate that H10 subtype influenza viruses can be divided into two lineages, including North American lineage and Eurasian lineage. We found that wild birds are the main hosts of H10 subtype AIVs. In addition, the number of H10N3 viruses increased during 2021–2023, and H10N3 influenza viruses occurred in North America and Eurasia (Fig. 1A), indicating that H10N3 viruses had been dominant in recent years.

Two strains of the H10N3 subtype AIVs were gisolated in tracheal samples of chicken from the Shandong Province of China, which were designated as A/chicken/Shandong/0101/2022 (H10N3) (hereafter 0101/H10N3) and A/chicken/Shandong/0603/2022 (H10N3) (hereafter 0603/H10N3), respectively. We found that the *HA* and *NA* genes of the two H10N3 subtype AIVs were closely related to human-origin H10N3 isolate A/Jiangsu/428/2021 (H10N3) (Fig. 1B and C). However, the internal genes of H10N3 viruses were more complex. Phylogenetic analysis of internal genes showed that all internal genes (PB2, PB1, PA, NP, M, NS) of H10N3 AIVs were closely related to H9N2 subtype AIVs (Supplementary Fig. S1). Notably, except for the surface genes (*HA*, *NA*), the PB2, M and NS genomes of H10N3 isolates did not cluster together with the human-origin H10N3 viruses (Supplementary Fig. S1), suggesting that H10N3 viruses continuously evolved in 2022 and were undergoing reassortment with local H9N2 viruses.

In addition, we analyzed the geographic distribution of H10 subtype influenza viruses in China. We found that H10N3 subtype viruses were mainly distributed in Jiangxi, Jiangsu, and Zhejiang (Supplementary Fig. S2A), while H10N8 subtype viruses were primarily distributed in Jiangxi (Fig. 1D), indicating that H10 subtype influenza viruses were widespread in eastern China. To investigate the genetic characteristics of two avian-origin H10N3 viruses, nucleotide homology analysis of the complete coding sequence of each gene fragment was performed using the Cluster Omega online website. Genomic analysis showed that both avian isolates had seven gene sequences with >95% homology at the nucleotide level when compared to A/Jiangsu/428/2021 (H10N3) isolated from the human patient, indicating that several genes of the two avian-origin H10N3 isolates had high homology with human-origin isolates (Table 1).

We then analyzed influenza viruses with the highest nucleotide homology to the two H10N3 isolates in the NCBI. BLAST search results showed that the *HA* and *NA* genes of two avian-origin H10N3 isolates were related to the avian-origin H10N3 subtype. The majority of the internal genes were derived from H9N2 (Table 2). It has been reported that internal genes derived from H9N2 viruses are critical in the cross-species transmission of AIVs to humans (Gu et al., 2017). As shown in Table 2, both viruses had 98.07%–99.56% nucleotide homology compared to sequences from NCBI for all eight genes.

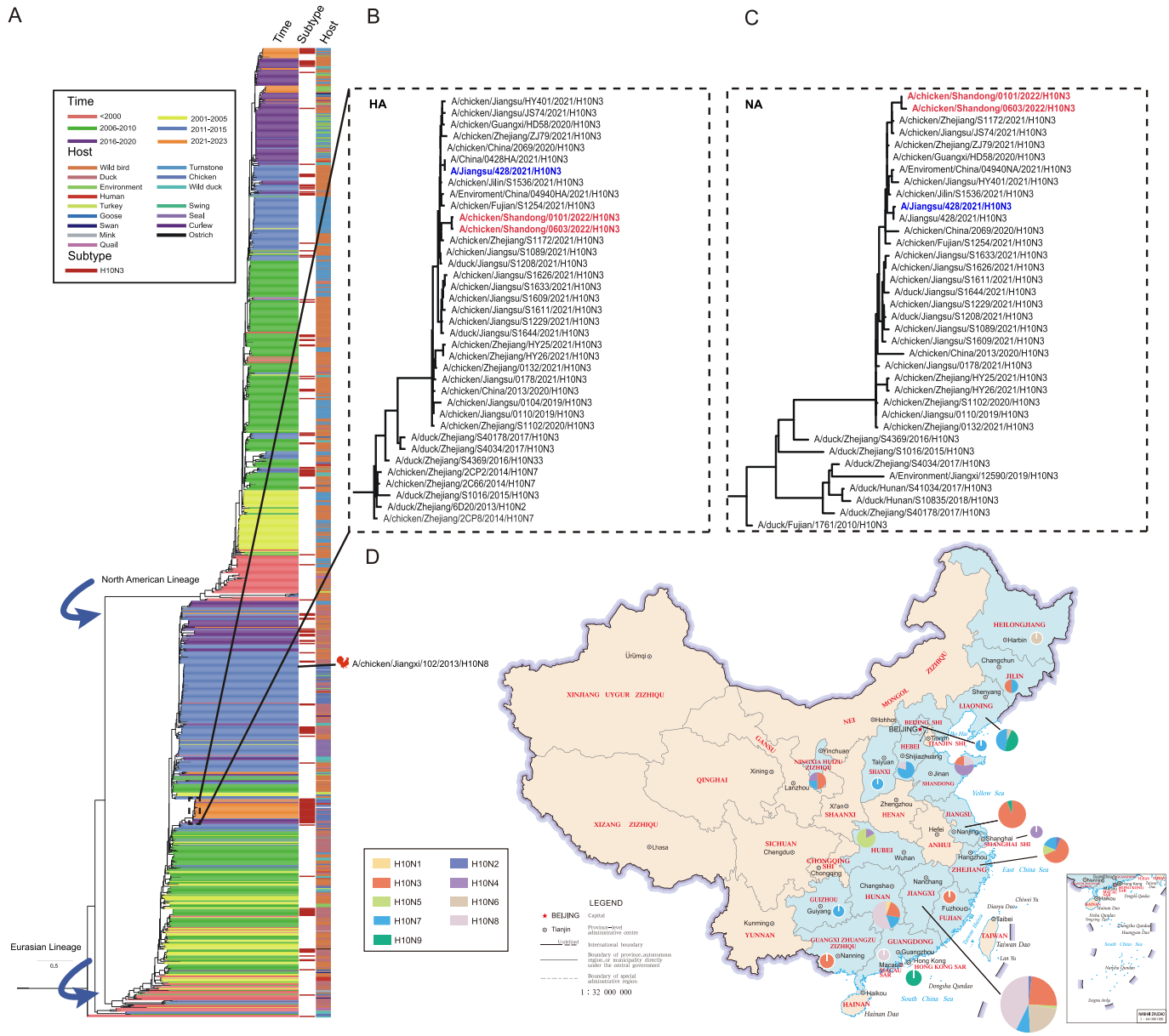

Fig. 1. Phylogenetic analysis and geographic distribution of H10 subtype influenza viruses. **A** Maximum likelihood (ML) phylogenetic tree of the *HA* gene of H10 viruses. Target sequences are labeled in red; the human isolate is labeled in blue. Different subtypes, hosts, and years are indicated by different markers. The full gene segments used IQ-TREE to construct an ML phylogenetic tree, in which the HKY nucleotide substitution model was selected, and the operation was repeated 1000 times. **B** Phylogenetic clustering of ML tree of *HA* gene of H10N3 viruses. **C** Phylogenetic clustering of ML tree of *NA* gene of H10N3 viruses. **D** The geographic distribution of H10 subtype viruses in China. The size of the pie chart represents the number of H10 subtypes in China, and different colors represent H10 subtypes with different *NA* genes. Data were obtained from the Epiflu database of GISAID. The A/chicken/Jiangxi/102/2013/H10N8 virus is labeled in Fig. 1A.

Table 1
Comparative analysis of the nucleotide sequence homology of two avian-origin H10N3 viruses and one human-origin H10N3 virus.

Strain	Nucleotide identify (%)							
	HA	NA	PB2	PB1	PA	NP	M	NS
A/Jiangsu/428/2021	100	100	100	100	100	100	100	100
A/chicken/Shandong/0101/2022	98.7	99.01	95.35	97.14	94.65	98.73	97.89	96.64
A/chicken/Shandong/0603/2022	98.7	98.79	95.31	97.19	94.75	98.73	97.89	96.94

PB, basic polymerase; PA, acidic polymerase; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix protein; NS, nonstructural protein.

3.2. Mutations in residues 222 and 228 within the 220-loop of the receptor binding region of H10N3 viruses

During transmission, viruses have the potential to produce new viruses that are more infectious and pathogenic through genetic recombination and mutation. The important adaptive mutations in these two H10N3 isolates were analyzed (Table 3). Mutations in the polymerase complexes (PB1, PB2 and PA) of AIVs are necessary for viral adaptation to new hosts. The two H10N3 isolates contain the I292V and A588V mutations in PB2, which may increase polymerase activity and virulence in mice (Suttie et al., 2019). Both H10N3 isolates have an I368V mutation in PB1, which also enhances polymerase activity and virulence in mammals (Guo et al., 2022). We also

Table 2
Influenza viruses with the highest nucleotide identity to each gene of these two H10N3 viruses isolated in this study.

Gene segment	Highly homologous strains in GenBank	Homology (%)	
		0101/H10N3	0603/H10N3
PB2	MK053853.1:A/chicken/Shanghai/06/2018(H9N2)	98.11%	98.07%
PB1	OL636798.1:A/chicken/China/146-2/2021(H10N3)	99.47%	99.56%
PA	OM055793.1:A/chicken/Shandong/303/2021(H9N2)	99.26%	99.30%
HA	OL636800.1:A/chicken/China/146-4/2021(H10N3)	98.93%	98.93%
NP	ON368084.1:A/chicken/China/2088/2020(H9N2)	99.20%	99.20%
NA	OL638128.1:A/chicken/China/189-6/2021(H10N3)	99.36%	99.15%
M	MW100642.1:A/chicken/Hunan/6.27_YYGK36S2-OC/2017(H9N2)	99.19%	99.19%
NS	MH667576.1:A/chicken/Shandong/3424/2016(H9N2)	98.21%	98.45%

PB, basic polymerase; PA, acidic polymerase; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix protein; NS, nonstructural protein. 0101/H10N3, A/chicken/Shandong/0101/2022; 0603/H10N3, A/chicken/Shandong/0603/2022.

Table 3
Adaptive mutations of H10N3 viruses were used in this study.

Protein	Mutation	Function	0101	0603	0428
HA	Q222R	Unknown	R	R	R
(H3 numbering)	G228S	Increased virus binding to α2–6	S	G	S
	Q226L	Increased virus binding to α2–6	Q	Q	Q
Cleavage site		Only one basic amino acid at the HA cleavage site, which is a characteristic of low-pathogenicity AIV in chicken	PEIIQGRG	PEIIQGRG	PEIIQGRG
PB2	E627K	Mammalian adaptive markers	E	E	E
	D701N		D	D	D
	I292V		V	V	V
	A588V		V	V	V
PB1	I368V	Improved the polymerase activity and virulence in mammals	V	V	V
	S375N		S	S	N
PA	A343S	Increased the replication of viruses in mammalian cells and pathogenicity in mice	A	A	S
	K356R		R	R	R
	N409S		N	N	N
M1	N30D	Increased virulence in mice	D	D	D
	T215A		A	A	A
M2	S31N	Resistance to adamantanes	N	N	N

Specific references are in the Supplementary Table S1. 0101, A/chicken/Shandong/0101/2022; 0603, A/chicken/Shandong/0603/2022; 0428, A/Jiangsu/0428/2021.

identified K356R mutation in PA, which may increase viral replication in mammalian cells and pathogenicity in mice (Xu et al., 2016). Both H10N3 AIVs have N30D and T215A mutations in M1, which are associated with increased virulence in mice (Fan et al., 2009). Two H10N3 viruses have the S31N mutation in M2, a change that would confer resistance to adamantanes in influenza viruses (Cheung et al., 2006). In conclusion, these data suggest that H10N3 viruses contain multiple mammalian adaptive mutations and may pose an increased threat to human health.

One of the key factors in the interspecies transmission of influenza A viruses is a shift in viral receptor binding specificity, which is largely determined by mutations in viral hemagglutinin (Shi et al., 2014). Amino acid substitutions in HA are major determinants of preferential targeting of human receptors. Here, we downloaded the *HA* genes of H10N3 AIVs in China from the GISAID database and analyzed the amino acid conservation in the 220-loop of the receptor binding site of H10N3 AIVs. We found that amino acids in loop 220 (amino acid position 221–228) of the HA receptor-binding region of H10N3 viruses isolated in China were conserved until 2018. However, amino acids at 222 and 228 sites were more diverse from 2019 to 2023 (Fig. 2A). In H10N3 isolates, the *HA* gene of 0101/H10N3 virus harbors Q222R and G228S double mutations. A previous study demonstrated that G228S substitution may increase the binding affinity of the virus to the human receptor and decrease the binding affinity to the avian receptor. Influenza viruses bearing *HA* Q222R and G228S double mutations have dual receptor binding properties and increased pathogenicity in mice (Zhang Y.C. et al., 2023), indicating that the H10N3 avian influenza virus poses a continuing threat to human health. We found that the amino acid 222 site was mainly prevalent amino acid Q before 2019, while the proportion of amino acid R gradually increased after 2018. Amino acid 228 was predominantly prevalent in amino acid G until 2018, while the proportion of amino acid S gradually increased after 2018 (Fig. 2B), indicating that the dominant shift of receptor binding regions in the *HA* gene of H10N3 viruses occurred after 2018. We analyzed the *HA* gene's 222 and 228 sites among AIVs of the H10 subtype worldwide. Our findings indicate that regular mutations at these two sites were observed only in H10N3 viruses isolated in China. Since the isolation of the novel H10N3 virus in 2019, these novel H10N3 viruses (n = 29) were initially isolated in Jiangsu and concentrated in Jiangsu (n = 15) and Zhejiang (n = 5) provinces (Supplementary Fig. S2B).

3.3. Plaque assay of H10N3 subtype avian influenza viruses

The plaque phenotypes of two avian-origin H10N3 isolates (0101/H10N3 and 0603/H10N3) and H10N8 virus (102/H10N8) kept in our laboratory were analyzed using a standard plaque assay on MDCK cells (Fig. 3A). There was no significant difference in plaque diameters between 0101/H10N3 and 0603/H10N3 viruses. However, the plaque diameters of the two H10N3 viruses were slightly larger than those of the H10N8 virus (Fig. 3B). This suggests that the aggressive ability of H10N3 viruses is slightly higher than that of H10N8 virus.

3.4. Thermostability and elution of H10N3 subtype avian influenza viruses

The stability of the HA protein is a critical factor in determining the host range, transmissibility, and pandemic potential of influenza viruses (Russell et al., 2018). To test the thermostability of HA protein, 64 hemagglutinating units (HAUs) of each virus were incubated at a temperature of 55 °C for 5 h, during which the HA titers were measured every hour. The results showed that the thermostability of 0101/H10N3 virus was similar to that of 0603/H10N3 virus. However, the 102/H10N8 virus was completely inactivated within 15 min (Fig. 3C). This indicates that H10N3 viruses were more thermally stable than the

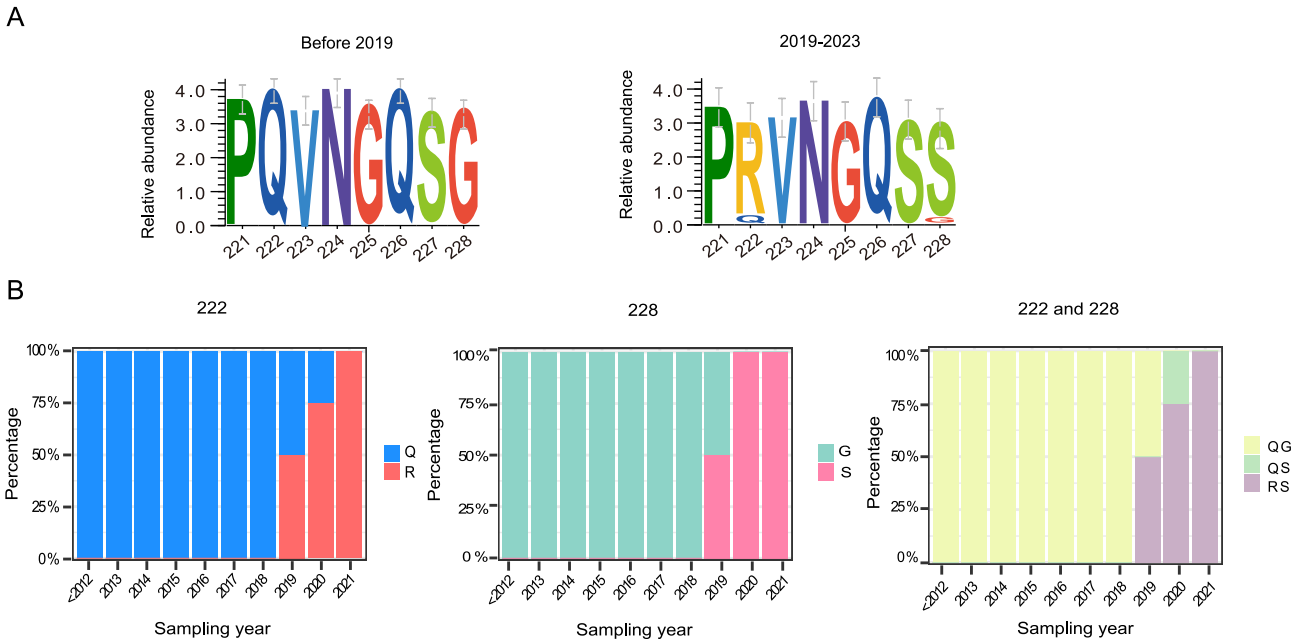

Fig. 2. Amino acids frequency of different amino acid residues of HA protein of influenza A (H10N3) viruses. **A** Frequencies of each position of the HA protein 220 loop of influenza A (H10N3) viruses, including 221, 222, 223, 224, 225, 226, 227, 228, are illustrated using WebLogo3.4. The Y-axis represents relative abundance, and the X-axis represents amino acid position. **B** Prevalence of codons encoding amino acid residues 222 and 228 in the HA protein of H10N3 influenza viruses. Sequences from 2012 to 2023 in GISAID's EpiFlu Database were garthered, with no strains documented for 2022 and 2023.

H10N8 virus, which was very poorly thermally stable. The high temperature was not conducive to the survival of the H10N8 virus.

HA-NA functional balance is crucial for AIV growth, pathogenicity, and host range. The viral release assay can demonstrate this balance, which involves the receptor binding activity for HA and receptor disruption activity for NA (Sekine et al., 2022). We compared the elution of 0101/H10N3, 0603/H10N3 and 102/H10N8. The results showed that 0101/H10N3 virus was completely eluted from chicken erythrocytes after 9 h incubation, while 0603/H10N3 virus was only partially eluted after 3 h incubation. Both the 0603/H10N3 and 102/H10N8 viruses remained incompletely eluted from erythrocytes until 10 h (Fig. 3D). In conclusion, the results indicate differences in the virus release ability of these three viruses. The 0101/H10N3 virus exhibits slightly more efficient elution than the 102/H10N8 virus, while the 0603/H10N3 virus showed weaker elution ability (Fig. 3D).

3.5. *Replication of H10N3 subtype avian influenza viruses in mammalian cells*

To assess the replication ability of H10 subtype AIV in mammalian cells, MDCK cells were infected with H10 subtype AIV with an MOI of 0.001 for 12, 24, 36, 48 and 60 hpi. The supernatants were collected, and viral titers were determined by $TCID_{50}$ assays on MDCK cells. We found that only at 36 hpi, the viral titer of 0101/H10N3 was slightly higher than that of 102/H10N8. Viral titers of the three viruses could not be measured until 12 hpi (Fig. 3E), indicating poor replication of the three viruses in MDCK cells. In addition, viral protein expression in MDCK and A549 cells infected with H10 subtype virus was analyzed at 3, 6, 9 and 12 hpi. At each time point, the HA protein expression of 0101/H10N3 virus was higher than that of 0603/H10N3 and 102/H10N8 viruses (Fig. 3F). In addition, no viral protein was detectable in A549 cells at 3 hpi (Fig. 3G). These findings suggest that 0101/H10N3 viruses are more dominant in early viral expression.

3.6. *Pathogenicity of H10N3 and H10N8 isolates in mice*

To compare the pathogenicity of the H10N3 and H10N8 viruses in mammals, BALB/c mice were inoculated intranasally with each virus at a dose of 10^5 EID_{50}. Mice were then observed over 14 days to assess clinical signs, weight loss and survival rates. The body weights of all infected mice reached their lowest point on day 8 post-infection (Fig. 4A). There were no significant clinical signs of disease or mortality. To further investigate the replication capacity of the three isolates in mouse organs, three BALB/c mice from each group were euthanized on day 4 post-infection. Subsequently, the viral titers in the lungs, nasal turbinate, and brains of mice were determined. All three viruses could replicate efficiently in the lungs of the mice, with no significant difference in replication titers (Fig. 4B). However, 102/H10N8 virus was undetectable in the nasal turbinates of mice, suggesting inefficient replication in this tissue (Fig. 4C). Surprisingly, the 0101/H10N3 and 0603/H10N3 viruses were undetectable in the brain tissues of mice, whereas the 102/H10N8 virus was detected in the brain tissue of a single mouse (Fig. 4D). The findings indicate that the H10N3 virus is not a neurotropic virus.

To investigate pathological changes in the lung tissue of mice infected with three H10 influenza viruses, an H&E staining assay was performed. Mice infected with 0101/H10N3 virus developed granulocytic infiltration in the walls of the extra alveoli, accompanied by mild thickening of alveolar walls and septa widening. Mice infected with the 0603/H10N3 virus also exhibited symptoms such as mild fine bronchial mucosal epithelial cell detachment, slight lymphocytic infiltration, and mild perivascular hemorrhage. Mice infected with 102/H10N8 virus exhibited localized hemorrhage in the lungs, characterized by the presence of erythrocytes around blood vessels and slight infiltrations of granulocytes and lymphocytes (Fig. 4E). Pathological studies revealed that mice infected with either the 0603/H10N3 or 102/H10N8 virus exhibited slightly more pronounced interstitial pneumonia and associated symptoms, whereas the 0101/H10N3-infected group displayed mild lesions. In summary, the mice experiments showed no significant weight loss in mice infected with the H10N3 or H10N8 viruses. Mice infected with 0101/H10N3 virus exhibited the least significant weight loss and mild lung lesions. Furthermore, the H10N3 viruses exhibited efficient replication in the lungs and nasal turbinates of mice, in contrast to the H10N8 virus.

4. Discussion

The H10 subtype AIVs are primarily found in wild waterfowl, but can also infect a wide range of poultry and mammals (Wu et al., 2019).

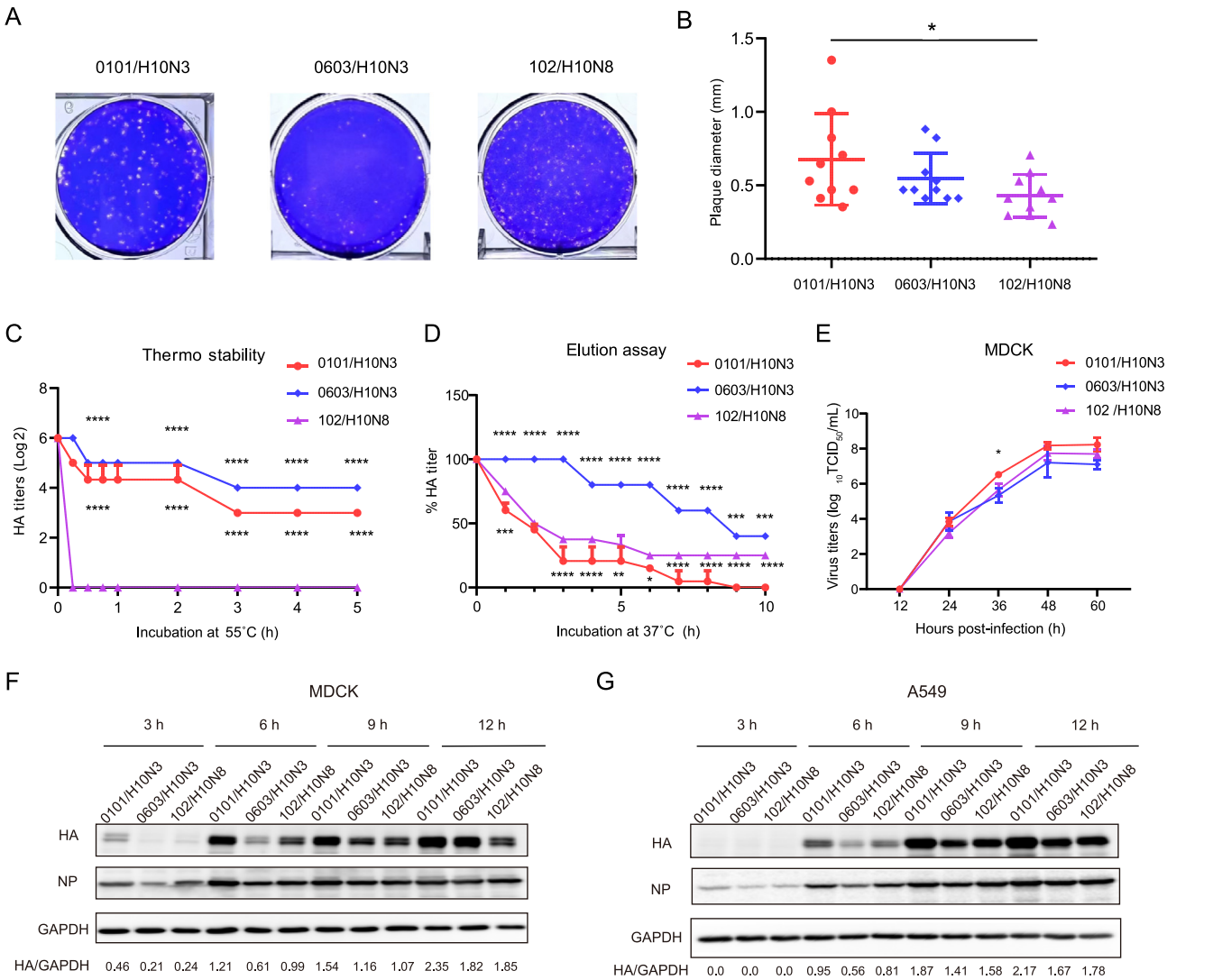

Fig. 3. Biological characteristics of H10 viruses. **A** Plaque-forming ability of H10N3 and H10N8 viruses in MDCK cell monolayers. Cells were infected with H10N3 and H10N8 viruses. After 2 hpi, cells were washed twice with PBS and overlaid with DMEM containing 1% agarose and 0.5 μg/mL TPCK-trypsin. After 48 hpi, the cells were stained with crystal violet. **B** Plaque diameters of H10N3 and H10N8 viruses in MDCK cells. Plaque assays were produced under standard conditions and stained with 0.5% crystal violet. The diameters of random plaques were measured for each virus. **C** Thermostability of the H10N3 and H10N8 viruses. The H10N3 and H10N8 viruses were incubated for 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h and 5 h at 55 °C. The titers of heat-treated H10N3 and H10N8 viruses were determined by HA assay. **D** Virus release assay results in chicken erythrocytes. Different H10N3 and H10N8 viruses were adsorbed to a 1% suspension of chicken at 4 °C for 1 h, and the HA titers at 37 °C representing virus elution from chicken erythrocyte were monitored each hour for 10 h. **E** Growth kinetics analysis of H10N3 and H10N8 viruses in MDCK cells at an MOI of 0.001. Supernatants were collected at the indicated time points, and virus titers in MDCK cells were monitored using $TCID_{50}$. Western blots of viral protein expression in MDCK **(F)** and A549 cells **(G)**. The cells were infected with H10N3 and H10N8 viruses at an MOI of 1. Western blots were performed to detect HA and NP protein expression in MDCK and A549 cells at 3, 6, 9 and 12 hpi. Viral protein was detected with specific antibodies against HA and NP. Relative HA protein expression was calculated according to the equation HA/GAPDH. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Occasional human infections with H10 subtype viruses have been reported. In 2010, multiple cases of H10N7 infection were reported in Australia (Arzey et al., 2012). In 2013, the first human case of H10N8 infection resulting in human death was reported in China (Chen et al., 2014). Recently, WHO (https://www.who.int/) reported human infections with H10N3 and H10N5. Therefore, H10 subtype AIVs pose threats to both avian and human health.

A recent study by Gao et al. revealed that the H10N3 influenza virus infecting humans originated from avian-origin H10N3 viruses. The virus has formed a distinct sublineage, significantly differing from other H10 viruses and posing a potential threat to public health (Gao et al., 2021). From 2011 to 2015, H10N8 viruses were the predominant subtype of H10 viruses in China. However, the dominant subtype switch from H10N8 to H10N3 occurred between 2016 and 2021 (Zhang et al., 2021). In December 2019, two H10N3 viruses were isolated from chickens during routine AIV surveillance in eastern China. These viruses exhibited high pathogenicity in mice and were capable of effective aerosol transmission in guinea pigs (Guo et al., 2022; Liu et al., 2022). In particular, these novel H10N3 viruses are preferred to both avian and human receptors (Liu et al., 2022), suggesting that H10N3 viruses pose an increasing risk of infecting humans. Although the H10N3 viruses isolated in this study did not result in significant weight loss or mortality in mice, they can replicate effectively in the lungs and induce lung lesions. Additionally, the two avian-origin isolates shared high nucleotide sequence homology with the human-origin isolate (A/Jiangsu/428/2021) and had mutations with mammalian adaptation (Tables 1 and 2). Based on these findings, it is evident that the potential threat posed by the H10N3 influenza virus to humans cannot be overlooked.

The altered receptor binding specificity of influenza viruses is a key factor in interspecies transmission, which is largely determined by

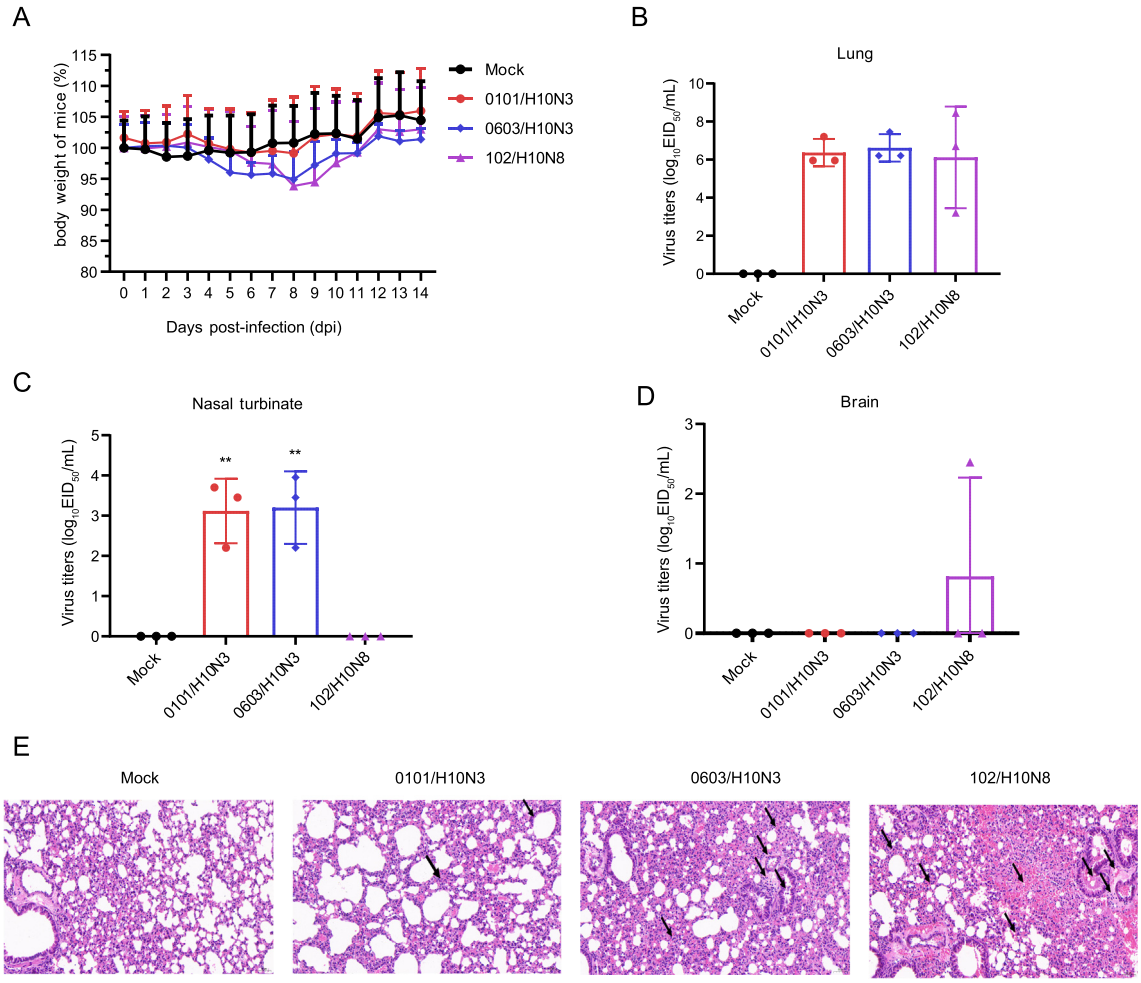

Fig. 4. Pathogenicity of H10 viruses in mice. **A** Body weight changes in six mice per group infected with 0101/H10N3, 0603/H10N3, and 102/H10N8 viruses (volume 10^5 EID_{50}/50 μL) for 14 days. Three mice per group infected with 10^5 EID_{50}/50 μL viruses was euthanized on 4 dpi. The viral titers of lungs **(B)** and nasal turbinate **(C)** and brains **(D)** from infected mice were determined by EID_{50} assay. **E** Analysis of pathological changes in the lungs of mice infected with H10N3 and H10N8 influenza viruses. Mice were euthanized on day 4 dpi, and lungs were collected and sectioned. Hematoxylin and eosin staining was performed. Scale bars, 50 μm; magnification, 20×. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

mutations in viral hemagglutinin. Previous studies have demonstrated that amino acids 225 and 190 of HA glycoproteins play a significant role in receptor binding specificity (Xu et al., 2012). The Q226L and G228S substitutions in the HA glycoprotein have the potential to modify the binding preference from the avian receptor to the human receptor (Matrosovich et al., 2000). The Q226L substitution resulted in a shift in binding preference from the avian to the human receptor (Lu et al., 2013). Furthermore, amino acid mutations at positions 186 and 226 of HA glycoprotein enhance binding affinity towards the human receptor (Shi et al., 2013). Here, we found that the continuous evolution of H10N3 viruses occurred in China in recent years, and the proportions of H10N3 viruses harboring the Q222R and G228S mutations in the HA protein increased. Zhang et al. reported that their H10N3 isolates carrying HA Q222R and G228S mutations, exhibited dual receptor-binding properties. Notably, certain isolates had a higher affinity for the human-type receptor compared to the avian-type receptor. This enhanced pathogenicity in mice suggests that these isolates have adapted to mammals (Zhang Y.C. et al., 2023). The functional roles of HA Q222R and G228S double mutations remain elusive and warrant further exploration.

We conducted a comprehensive biological investigation on H10N3 viruses isolated in China, including assessments of replicative capacity, thermal stability, virus release from erythrocytes, and HA protein accumulation. The stability of HA plays a crucial role in determining the influenza virus's adaptability and transmissibility across different species. Mutations in amino acid residues can affect HA stability, thus necessitating phenotypic assays for surveillance studies (Russell et al., 2018). Our research reveals that the 0101/H10N3 virus exhibits greater stability at high temperatures and expresses higher levels of HA protein compared to the 102/H10N8 virus. Conversely, the 102/H10N8 virus exhibits inferior characteristics in some respects, highlighting the ongoing evolution of H10N3 viruses and posing potential threats to public health security. Researchers have analyzed the binding affinity of H10N3 virus for avian and human-type receptors by using two different glycans (α 2, 6-siaylglycopolymer and α 2,3-siaylglycopolymer). The results showed that H10N3 has an affinity for both avian (SAα-2,3-Gal) and human (SAα-2,6-Gal) receptors (Zhang Y.C. et al., 2023). Liu et al. plotted the growth curves of H10N3 viruses on different cell types and observed significantly higher replication titers in MDCK and A549 cells compared to CEF cells (Liu et al., 2022), suggesting that better replication of the H10N3 viruses in mammalian-origin cells than in avian-origin cells.

5. Conclusion

In summary, we performed a phylogenetic analysis of the *HA* genes of global H10 subtype influenza viruses and analyzed the geographic distribution of H10 viruses in China. The results indicated that the number of H10N3 viruses increased during 2021–2023, and H10N3 viruses isolated in China are mainly concentrated in Jiangxi Province, Jiangsu

Province, and Zhejiang Province. However, Jiangsu Province had the highest number of novel H10N3 viruses isolated. We also found that recently isolated H10N3 viruses from China had a double mutation Q222R and G228S in loop 220 of the HA receptor binding region. In addition, we explored the biological properties of the H10N3 influenza viruses. Compared to H10N8, we found that H10N3 viruses were slightly more dominant. To some extent, H10N3 viruses exhibit increased heat stability, accelerated release of the virus from erythrocytes, increased accumulation of HA protein, and the ability to replicate efficiently in mice's lungs and nasal turbinate. Therefore, comprehensive influenza surveillance and control measures should be conducted to minimize the potential spread of influenza viruses in the future.

Data availability

All the data generated during the current study are included in the manuscript. The genomes of two H10N3 viruses were submitted to GISAID (https://www.gisaid.org/) with accession numbers EPI2840694–EPI2840710, and Science Data Bank (DOI:10.57760/sciencedb.08670).

Ethics statement

All animals involved in experiments were reviewed and approved by the Institution Animal Care and Use Committee at SCAU and treated in accordance with the guidelines (2017A002).

Author contributions

Shiping Ding: data curation, formal analysis, investigation, methodology, resources, software, visualization, writing-original draft. Jiangtao Zhou: data curation, formal analysis, investigation, methodology, resources, software, visualization, writing-original draft. Junlong Xiong: formal analysis, investigation, methodology. Xiaowen Du: formal analysis, investigation, methodology. Wenzhuo Yang: formal analysis, investigation, methodology. Jinyu Huang: formal analysis, investigation, methodology. Yi Liu: formal analysis, investigation, methodology. Lihong Huang: formal analysis, investigation, methodology. Ming Liao: conceptualization, funding acquisition, project administration, supervision, visualization, writing-review & editing. Jiahao Zhang: conceptualization, funding acquisition, project administration, supervision, visualization, writing-review & editing. Wenbao Qi: conceptualization, funding acquisition, project administration, supervision, visualization, writing-review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.06.005.

References

Arzey, G.G., Kirkland, P.D., Arzey, K.E., Frost, M., Maywood, P., Conaty, S., Hurt, A.C., Deng, Y.M., Iannello, P., Barr, I., Dwyer, D.E., Ratnamohan, M., McPhie, K., Selleck, P., 2012. Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia. Emerg. Infect. Dis. 18, 814–816.

Bi, Y.H., Chen, Q.J., Wang, Q.L., Chen, J.J., Jin, T., Wong, G., Quan, C.S., Liu, J., Wu, J., Yin, R.F., Zhao, L.H., Li, M.X., Ding, Z., Zou, R.R., Xu, W., Li, H., Wang, H.J., Tian, K.G., Fu, G.H., Huang, Y., Shestopalov, A., Li, S.J., Xu, B., Yu, H.J., Luo, T.R., Lu, L., Xu, X., Luo, Y., Liu, Y.X., Shi, W.F., Liu, D., Gao, G.F., 2016. Genesis, evolution and prevalence of H5N6 avian influenza viruses in China. Cell Host Microbe 20, 810–821.

Chen, H.Y., Huang, L.H., Li, H., Zhou, X.F., Li, H.N., Sun, N., Qi, W.B., Xiao, C.C., Ni, X.S., Liu, M.B., Liao, M., 2015. High pathogenicity of influenza A (H10N8) virus in mice. Am. J. Trop. Med. Hyg. 93, 1360–1363.

Chen, H.Y., Yuan, H., Gao, R.B., Zhang, J.X., Wang, D.Y., Xiong, Y., Fan, G.Y., Yang, F., Li, X.D., Zhou, J.F., Zou, S.M., Yang, L., Chen, T., Dong, L.B., Bo, H., Zhao, X., Zhang, Y., Lan, Y., Bai, T., Dong, J., Li, Q., Wang, S.W., Zhang, Y.P., Li, H., Gong, T., Shi, Y., Ni, X.S., Li, J.X., Zhou, J., Fan, J.Y., Wu, J.W., Zhou, X.F., Hu, M.H., Wan, J.G., Yang, W.Z., Li, D.X., Wu, G.Z., Feng, Z.J., Gao, G.F., Wang, Y., Jin, Q., Liu, M.B., Shu, Y.L., 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection : a descriptive study. Lancet 383, 714–721.

Chen, M.Y., Lyu, Y., Wu, F., Zhang, Y., Li, H.K., Wang, R., Liu, Y., Yang, X.Y., Zhou, L.W., Zhang, M., Tong, Q., Sun, H.L., Pu, J., Liu, J.H., Sun, Y.P., 2023. Increased public health threat of avian-origin H3N2 influenza virus caused by its evolution in dogs. Elife 12, 21.

Cheung, C.L., Rayner, J.M., Smith, G.J.D., Wang, P., Naipospos, T.S.P., Zhang, J.X., Yuen, K.Y., Webster, R.G., Peiris, J.S.M., Guan, Y., Chen, H.L., 2006. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J. Infect. Dis. 193, 1626–1629.

Fan, S.F., Deng, G.H., Song, J.S., Tian, G.B., Suo, Y.B., Jiang, Y.P., Guan, Y.T., Bu, Z.G., Kawaola, Y., Chen, H.L., 2009. Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 avian influenza viruses in mice. Virology 384, 28–32.

Feldman, A.T., Wolfe, D., 2014. Tissue processing and hematoxylin and eosin staining. Methods Mol. Biol. 1180, 31–43.

Gao, R.Y., Zheng, H.F., Liu, K.T., Ji, Z.X., Cai, M., Gu, M., Hu, J., Liu, X.W., Hu, S.L., Wang, X.Q., Liu, X.F., 2021. Genesis, evolution and host species distribution of influenza A (H10N3) virus in China. J. Infect. 83, 633–635.

Gu, M., Xu, L.J., Wang, X.Q., Liu, X.F., 2017. Current situation of H9N2 subtype avian influenza in China. Vet. Res. 48, 10.

Guo, Y.N., Ding, P.Y., Li, Y.J., Zhang, Y.P., Zheng, Y.Q., Yu, M.Q., Suzuki, Y., Zhang, H.T., Ping, J.H., 2022. Genetic and biological properties of H10N3 avian influenza viruses: a potential pandemic candidate? Transbound. Emerg. Dis 69, E3171–E3182.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.

Jiang, W., Yin, X., Liu, S., Liang, S., Peng, C., Hou, G., Li, J., Yu, X., Li, Y., Wang, J., Liu, H., 2022. Continued antigenic variation of highly pathogenic avian influenza A (H7N9) virus in laying hens in China, 2020–2021. Virol. Sin. 37, 631–633.

Kong, X.T., Guan, L.Z., Shi, J.Z., Kong, H.H., Zhang, Y.P., Zeng, X.Y., Tian, G.B., Liu, L.L., Li, C.J., Kawaoka, Y., Deng, G.H., Chen, H.L., 2021. A single-amino-acid mutation at position 225 in hemagglutinin attenuates H5N6 influenza virus in mice. Emerg. Microb. Infect. 10, 2052–2061.

Li, C., Wang, S.G., Bing, G.X., Carter, R.A., Wang, Z.J., Wang, J.L., Wang, C.X., Wang, L., Wu, G., Webster, R.G., Wang, Y.Q., Sun, H.L., Sun, Y.P., Liu, J.H., Pu, J., 2017. Genetic evolution of influenza H9N2 viruses isolated from various hosts in China from 1994 to 2013. Emerg. Microb. Infect. 6, 11.

Lipsitch, M., Barclay, W., Raman, R., Russell, C.J., Belser, J.A., Cobey, S., Kasson, P.M., Lloyd-Smith, J.O., Maurer-Stroh, S., Riley, S., Beauchemin, C.A.A., Bedford, T., Friedrich, T.C., Handel, A., Herfst, S., Murcia, P.R., Roche, B., Wilke, C.O., Russell, C.A., 2016. Viral factors in influenza pandemic risk assessment. Elife 5, 38.

Liu, D., Shi, W.F., Shi, Y., Wang, D.Y., Xiao, H.X., Li, W., Bi, Y.H., Wu, Y., Li, X.B., Yan, J.H., Liu, W.J., Zhao, G.P., Yang, W.Z., Wang, Y., Ma, J.C., Shu, Y.L., Lei, F.M., Gao, G.F., 2013. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet 381, 1926–1932.

Liu, K., Qi, X., Bao, C., Wang, X., Liu, X., 2024. Novel H10N3 avian influenza viruses: a potential threat to publichealth. Lancet Microbe 5, e417.

Liu, K.T., Ding, P.Y., Pei, Y.R., Gao, R.Y., Han, W.W., Zheng, H.F., Ji, Z.X., Cai, M., Gu, J.Y., Li, X.L., Gu, M., Hu, J., Liu, X.W., Hu, S.L., Zhang, P.H., Wang, X.B., Wang, X.Q., Liu, X.F., 2022. Emergence of a novel reassortant avian influenza virus (H10N3) in Eastern China with high pathogenicity and respiratory droplet transmissibility to mammals. Sci. China Life Sci. 65, 1024–1035.

Long, J.S., Mistry, B., Haslam, S.M., Barclay, W.S., 2019. Host and viral determinants of influenza A virus species specificity. Nat. Rev. Microbiol. 17, 67–81.

Lu, X.S., Shi, Y., Zhang, W., Zhang, Y.F., Qi, J.X., Gao, G.F., 2013. Structure and receptor-binding properties of an airborne transmissible avian influenza A virus hemagglutinin H5 (VN1203mut). Protein Cell 4, 502–511.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M.R., Donatelli, I., Kawaoka, Y., 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. 74, 8502–8512.

Parums, D.V., 2023. Global surveillance of highly pathogenic avian influenza viruses in poultry, wild birds, and mammals to prevent a human influenza pandemic. Med. Sci. Mon. Int. Med. J. Exp. Clin. Res. 29, 3.

Philippon, D.A.M., Wu, P., Cowling, B.J., Lau, E.H.Y., 2020. Avian influenza human infections at the human-animal interface. J. Infect. Dis. 222, 528–537.
Pyankova, O.G., Susloparov, I.M., Moiseeva, A.A., Kolosova, N.P., Onkhonova, G.S., Danilenko, A.V., Vakalova, E.V., Shendo, G.L., Nekeshina, N.N., Noskova, L.N., Demina, J.V., Frolova, N.V., Gavrilova, E.V., Maksyutov, R.A., Ryzhikov, A.B., 2021. Isolation of clade 2.3.4.4b A(H5N8), a highly pathogenic avian influenza virus, from a worker during an outbreak on a poultry farm, Russia, December 2020. Euro Surveill. 26, 2100439.
Qi, W., Zhou, X., Shi, W., Huang, L., Xia, W., Liu, D., Li, H., Chen, S., Lei, F., Cao, L., Wu, J., He, F., Song, W., Li, Q., Li, H., Liao, M., Liu, M., 2014. Genesis of the novel human-infecting influenza A(H10N8) virus and potential genetic diversity of the virus in poultry, China. Euro Surveill. 19, 50–62.
Qi, X., Qiu, H.B., Hao, S.X., Zhu, F.C., Huang, Y.Z., Xu, K., Yu, H.Y., Wang, D.W., Zhou, L., Dai, Q.G., Zhou, Y., Wang, S.J., Huang, H.D., Yu, S.Y., Huo, X., Chen, K., Liu, J., Hu, J.L., Wu, M., Bao, C.J., 2022. Human infection with an avian-origin influenza A (H10N3) virus. N. Engl. J. Med. 386, 1087–1088.
Rebel, J.M.J., Peeters, B., Fijten, H., Post, J., Cornelissen, J., Vervelde, L., 2011. Highly pathogenic or low pathogenic avian influenza virus subtype H7N1 infection in chicken lungs: small differences in general acute responses. Vet. Res. 42, 11.
Russell, C.J., Hu, M., Okda, F.A., 2018. Influenza hemagglutinin protein stability, activation, and pandemic risk. Trends Microbiol. 26, 841–853.
Sekine, W., Takenaka-Uema, A., Kamiki, H., Ishida, H., Matsugo, H., Murakami, S., Horimoto, T., 2022. Adaptation of the H7N2 feline influenza virus to human respiratory cell culture. Viruses-Basel 14, 12.
Shi, Y., Wu, Y., Zhang, W., Qi, J.X., Gao, G.F., 2014. Enabling the 'host jump': structural determinants of receptor-binding specificity in influenza A viruses. Nat. Rev. Microbiol. 12, 822–831.
Shi, Y., Zhang, W., Wang, F., Qi, J.X., Wu, Y., Song, H., Gao, F., Bi, Y.H., Zhang, Y.F., Fan, Z., Qin, C.F., Sun, H.L., Liu, J.H., Haywood, J., Liu, W.J., Gong, W.M., Wang, D.Y., Shu, Y.L., Wang, Y., Yan, J.H., Gao, G.F., 2013. Structures and receptor binding of hemagglutinins from human-infecting H7N9 influenza viruses. Science 342, 243–247.
Sun, H., Li, H., Tong, Q., Han, Q., Liu, J., Yu, H., Song, H., Qi, J., Li, J., Yang, J., Lan, R., Deng, G., Chang, H., Qu, Y., Pu, J., Sun, Y., Lan, Y., Wang, D., Shi, Y., Liu, W.J., Chang, K.-C., Gao, G.F., Liu, J., 2023. Airborne transmission of human-isolated avian H3N8 influenza virus between ferrets. Cell 186, 4074–4084.e4011.
Suttie, A., Deng, Y.M., Greenhill, A.R., Dussart, P., Horwood, P.F., Karlsson, E.A., 2019. Inventory of molecular markers affecting biological characteristics of avian influenza A viruses. Virus Gene. 55, 739–768.
To, K.K.W., Tsang, A.K.L., Chan, J.F.W., Cheng, V.C.C., Chen, H.L., Yuen, K.Y., 2014. Emergence in China of human disease due to avian influenza A(H10N8) - cause for concern? J. Infect. 68, 205–215.
Wang, Y.W., Wang, M.J., Zhang, H., Zhao, C.H., Zhang, Y.P., He, G.M., Deng, G.H., Cui, P.F., Li, Y.B., Liu, W.Q., Shen, J.Y., Sun, X.H., Wang, W.T., Zeng, X.Y., Li, Y.B., Chu, D., Peng, P., Guo, J., Chen, H.L., Li, X.Y., 2022. Emergence, evolution, and biological characteristics of H10N4 and H10N8 avian influenza viruses in migratory wild birds detected in eastern China in 2020. Microbiol. Spectr. 10, 15.
Wu, H.B., Yang, F., Liu, F.M., Peng, X.M., Chen, B., Cheng, L.F., Lu, X.Y., Yao, H.P., Wu, N.P., 2019. Molecular characterization of H10 subtype avian influenza viruses isolated from poultry in Eastern China. Arch. Virol. 164, 159–179.
Xu, G.L., Zhang, X.X., Gao, W.H., Wang, C.X., Wang, J.L., Sun, H.L., Sun, Y.P., Guo, L., Zhang, R., Chang, K.C., Liu, J.H., Pu, J., 2016. Prevailing PA mutation K356R in avian influenza H9N2 virus increases mammalian replication and pathogenicity. J. Virol. 90, 8105–8114.
Xu, R., McBride, R., Nycholat, C.M., Paulson, J.C., Wilson, I.A., 2012. Structural characterization of the hemagglutinin receptor specificity from the 2009 H1N1 influenza pandemic. J. Virol. 86, 982–990.
Yang, R.G., Sun, H.L., Gao, F., Luo, K.W., Huang, Z., Tong, Q., Song, H., Han, Q.Q., Liu, J.Y., Lan, Y., Qi, J.X., Li, H., Chen, S.L., Xu, M.Z., Qiu, J.S., Zeng, G., Zhang, X.X., Huang, C.Y., Pei, R.Q., Zhan, Z.F., Ye, B.W., Guo, Y.X., Zhou, Y.Z., Ye, W., Yao, D., Ren, M., Li, B., Yang, J.Z., Wang, Y.A., Pu, J., Sun, Y.P., Shi, Y., Liu, L., Ou, X.H., Gao, G.F., Gao, L.D., Liu, J.H., 2022. Human infection of avian influenza A H3N8 virus and the viral origins: a descriptive study. Lancet Microbe 3, E824–E834.
Zhang, D., Gao, F.L., Jakovlic, I., Zou, H., Zhang, J., Li, W.X., Wang, G.T., 2020. PhyloSuite: an integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Mol. Ecol. Resour 20, 348–355.
Zhang, J., Huang, L., Liao, M., Qi, W., 2023a. H9N2 avian influenza viruses: challenges and the way forward. Lancet Microbe 4, e70–e71.
Zhang, J., Wang, X., Chen, Y., Ye, H., Ding, S., Zhang, T., Liu, Y., Li, H., Huang, L., Qi, W., Liao, M., 2023b. Mutational antigenic landscape of prevailing H9N2 influenza virus hemagglutinin spectrum. Cell Rep. 42, 113409.
Zhang, J., Ye, H., Li, H., Ma, K., Qiu, W., Chen, Y., Qiu, Z., Li, B., Jia, W., Liang, Z., Liao, M., Qi, W., 2020. Evolution and antigenic drift of influenza A (H7N9) viruses, China, 2017-2019. Emerg. Infect. Dis. 26, 1906–1911.
Zhang, J., Ye, H., Liu, Y., Liao, M., Qi, W., 2022a. Resurgence of H5N6 avian influenza virus in 2021 poses new threat to public health. Lancet Microbe 3, e558.
Zhang, J.H., Huang, L.H., Chen, Y.Q., Wang, X.M., Liao, M., Qi, W.B., 2021. Avian influenza H10 subtype viruses continuously pose threat to public health in China. J. Infect. 83, 610–613.
Zhang, J.H., Wang, X.M., Ding, S.P., Ma, K.X., Jiang, Y.T., Guo, Y., Zhang, T., Li, H.A., Liu, Y., Liao, M., Qi, W.B., 2022b. Article Key amino acid position 272 in neuraminidase determines the replication and virulence of H5N6 avian influenza virus in mammals. iScience 25, 22.
Zhang, Y.C., Shi, J.Z., Cui, P.F., Zhang, Y.P., Chen, Y., Hou, Y.J., Liu, L.L., Jiang, Y.P., Guan, Y.T., Chen, H.L., Kong, H.H., Deng, G.H., 2023. Genetic analysis and biological characterization of H10N3 influenza A viruses isolated in China from 2014 to 2021. J. Med. Virol. 95, 12.
Zhu, W.F., Yang, L., Shu, Y.L., 2019. Did the highly pathogenic avian influenza A(H7N9) viruses emerged in China raise increased threat to public health? Vector Borne Zoonotic Dis. 19, 22–25.