

## RESEARCH ARTICLE

### Pathogenesis and Cross-species Potential of Emerging H3N3 Influenza Viruses with Mammalian-adaptive Mutations

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#### ABSTRACT

The emergence of triple-reassortant H3N3 avian influenza viruses (AIVs) poses a threat to poultry and public health. This study characterized three novel H3N3 isolates (SQ2049, HN0120, and GT2125) from chickens in China, evaluating their evolution, pathogenicity, and vaccine efficacy. Phylogenetic analysis revealed these isolates originated from H10N3 and H3N8 lineages, carrying an H9N2-derived internal gene backbone. Although identified as low pathogenic AIVs (IVPI=0), they exhibited distinct replicative fitness. GT2125 showed reduced efficiency in embryos, and carried unique PB2 substitutions (V598I and K702). Pathogenicity trials in SPF chickens demonstrated that SQ2049 achieves efficient horizontal transmission in SPF chickens without clinical signs. Notably, cloacal viral shedding persisted for 11 days, whereas oral viral shedding only lasted 2 to 3 day, with mild histopathological lesions observed in the trachea and immune organs. Importantly, the inactivated SQ2049 vaccine elicited high cross-reactive antibody titers ( $HI \geq 8\log_2$ ) and provided 100% protection in SPF chickens. Mouse infection studies identified two HA substitutions (G174E and N209D) after viral replication in mice. These two mutations have previously been associated with mammalian adaptation and enhanced binding to human-type  $\alpha$ -2,6 receptors, though their functional effects in the H3N3 context were not evaluated in this study. These findings expand our understanding of H3N3 AIV evolutionary dynamics and highlight the importance of surveillance for reassortant influenza viruses. The demonstrated vaccine efficacy provides a foundation for future H3N3 prevention and control strategies.

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#### INTRODUCTION

The influenza virus is a negative-sense, single-stranded RNA virus that belongs to the Orthomyxoviridae family. Influenza A, B, C, and D types are further classified by antigenicity. Among them, influenza A viruses are responsible for most zoonotic infections and influenza pandemics (Jester *et al.*, 2020). Influenza A virus possesses a genome consisting of eight RNA segments, and is classified by the antigenicity and/ or genome sequences of its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), into HA subtypes H1–H19 and NA subtypes N1–N11. Wild aquatic birds are recognized as the primary natural reservoir of most influenza A viruses, whereas H17/H18 and N10/N11 subtypes have been identified exclusively in

bats (Karakus *et al.*, 2024). Influenza A viruses have also been detected in a wide range of domestic and wild mammals through cross-species transmission. The H3 subtype of influenza virus is recognized for its extensive host range. In 1968, it was identified as the causative agent of seasonal influenza in humans, and even today, its antigenicity continues to evolve on an annual basis (Bao *et al.*, 2022). Moreover, there have been reports of H3 AIV infecting mammals, including dogs, horses, and seals (Castleman *et al.*, 2010).

Genetic reassortment is a major driving force in influenza A virus evolution and contributes to the emergence of novel viruses with altered pathogenicity and host range (Yan *et al.*, 2025). While highly pathogenic AIV H5 and H7 subtypes have caused severe outbreaks in poultry and numerous human infections (Huang *et al.*,

2025), low pathogenic avian influenza viruses (LPAIVs) are often overlooked because they usually cause asymptomatic infections in birds. Nevertheless, several LPAIV subtypes, including H10N1 and H6N1, have infected humans (Arzey *et al.*, 2012), highlighting the potential public health risk posed by the continued evolution.

H9N2 subtype AIV plays a central role in the emergence of reassortant AIV by frequently donating internal genes to novel subtypes. Recent human infections with H10N3 and H3N8 subtype influenza viruses in China were caused by reassortant viruses carrying H9N2-derived internal genes (Bao *et al.*, 2022; Qi *et al.*, 2022; Wang *et al.*, 2025). In 2023, a novel H3N3 virus was identified in chickens, with H3N8-derived HA, H10N3-derived NA, and H9N2-derived internal genes (He *et al.*, 2024). Given the zoonotic history of its parental viruses, systematic characterization of H3N3 viruses is warranted. This study aimed to characterize the genetic features and biological properties of three reassortant H3N3 avian influenza viruses (derived from H10N3, H3N8 and H9N2 subtypes), with particular emphasis on their pathogenicity, vaccine efficacy in chickens, and potential for mammalian adaptation.

## MATERIALS AND METHODS

**Host Tropism and Infection Risk of H3Ny Subtype AIVs:** The published HA and NA sequences of H3Ny viruses were downloaded from the GISAID database (<https://www.gisaid.org>) updated to June 8, 2025. The numbers of H3Ny isolates were classified according to their isolation time and host, and analyzed with GraphPad Prism (Version 9.0; La Jolla, CA, USA).

**Viruses and Animals:** Three H3N3 avian influenza virus isolates named A/chicken/Henan Shangqiu/SQ2049/2023(H3N3) (GenBank No.: PP758466-PP758473), A/chicken/China/GT2125/2023(H3N3) (GenBank No.: PP077078-PP077085), and A/chicken/China/HN0120/2023(H3N3) (GenBank No.: PP838576-PP838583), abbreviated as SQ2049, GT2125, and HN0120 respectively, were isolated from symptomatic chickens in Henan and Hebei Provinces, China, in 2023. Specific-pathogen-free (SPF) White Leghorn embryos, SPF chickens, and 6-week-old female SPF BALB/c mice were used for all infection and immunogenicity studies.

**Virus Isolation and Genetic Characterization:** Tissue homogenates were inoculated into 10-day-old SPF embryonated chicken eggs for virus propagation and purification (Philippon *et al.*, 2020). Viral RNA was extracted (TaKaRa, China) and reverse-transcribed into cDNA using Uni12 primers (Vazyme, China). The HA, NA and other six internal genes (PB2, PB1, PA, NP, M, and NS) of three isolates were amplified with universal primer sets (Hoffmann *et al.*, 2001) then sequenced by Sangon Biotech (China). Phylogenetic trees were constructed using the Neighbor-Joining method with 1000 bootstrap replicates in MEGA 11.0.

**Pathogenicity Assessment:** The median embryo infective dose (EID<sub>50</sub>) and median embryo lethal dose (ELD<sub>50</sub>), and mean death time (MDT) were determined in SPF embryos

using the Reed and Muench method. The intravenous pathogenicity index (IVPI) was calculated according to WOAHA guidelines 23 (Manual, 2018). For systemic pathogenicity, 3-week-old SPF chickens (n=10/group) were infected ocularly and intranasally with 10<sup>6.0</sup>EID<sub>50</sub> SQ2049 or GT2125 strain in 0.2mL, while chickens in control group received 0.2mL PBS via the same routes (n=10/group). Body weight was recorded daily until 14dpi. At 3dpi, three chickens per group were euthanized for tissue collection, histopathological examination, and virus titration. Formalin-fixed tissues were paraffin-embedded, sectioned (5µm), and stained with H&E. Tissue homogenates were inoculated into SPF embryonated chicken eggs by three blind passages, and allantoic fluids were subsequently harvested and tested for virus presence by HA assay; a HA titer ≥5log<sub>2</sub> was considered positive.

**Transmission and Immunogenicity in SPF Chickens:** A total of fifteen 3-week-old SPF chickens were used to evaluate the transmission potential of SQ2049 strain. Ten SPF chickens were infected ocularly and intranasally with 10<sup>6.0</sup>EID<sub>50</sub> SQ2049 strain in 0.2mL. At 24h post-infection, other five same age SPF chickens were introduced into the same enclosure as cohabitation group to assess direct-contact transmission. Oral and cloacal swabs were randomly collected at 1, 3, 5, 7, 9, 11, and 13dpi (n=6/challenge group, n=3/cohabitation group) and subjected to viral detection as described above. Serum samples (n=5/group) collected at 14dpi were tested for SQ2049-specific antibodies using HI assay (Wang *et al.*, 2025).

Allantoic fluid of SQ2049 isolate (10<sup>8.52</sup>EID<sub>50</sub>/0.1mL) was inactivated with 0.3% formaldehyde and emulsified with Montanide ISA 78 VG adjuvant (3:8.5 v/v). Viral inactivation was verified via blind passage in eggs. Twenty 3-week-old SPF chickens were immunized intramuscularly (0.3mL/chick), and ten same age chickens in control group received 0.3mL PBS via the same route. At 21 days post-vaccination, half of chickens in each group were challenged ocularly and intranasally with 10<sup>6.0</sup>EID<sub>50</sub> SQ2049 in 0.2mL. Oral and cloacal swabs were collected from all chickens at 3dpi for virus detection as described above. According to the evaluation criteria for avian influenza vaccine efficacy described in the *Chinese Veterinary Pharmacopoeia*, serum HI titers against SQ2049, GT2125, and HN0120 strains were measured weekly from 0 to 5 weeks post-vaccination (wpv). A serum HI titer of ≥6log<sub>2</sub> in vaccinated chickens was considered to meet the efficacy requirement, whereas the HI titer in the unvaccinated control group should not exceed 2log<sub>2</sub>.

**Mouse Infection:** To assess zoonotic potential, 6-week-old SPF BALB/c mice (n=6/group) were intranasally challenged with 10<sup>6.0</sup>EID<sub>50</sub> in 0.05mL, while mice in control group received 0.05mL PBS via the same routes (n=6/group). At 3 dpi, mice were euthanized and tissues (nasal turbinate, lung, etc.) were collected from 3 mice per group. Tissue samples were homogenized in PBS (1:10, w/v), clarified by centrifugation, and the supernatants were subjected to viral detection as described above. The HA gene of positive samples was sequenced to identify amino acid substitutions.

**Animal ethics and experimental conditions:** All chickens and mice were randomly divided into groups. Animals were humanely euthanized at each time point. All sampling and testing were blinded. All animal experiments were carried out in negative-pressure ABSL-2 isolators under biosecurity protocols.

**Statistical Analysis:** Statistical analyses (mean  $\pm$  SD) were performed using GraphPad Prism 9.0. One-way ANOVA with Bonferroni correction and log-rank tests were used, with  $P < 0.05$  considered significant.

## RESULTS

**Epidemiological Characteristics and Host Range of H3Ny:** Influenza A viruses spanning subtypes H3N1 to H3N9 have all been identified in natural environments according to comprehensive data retrieved from the GISAID database. Among these, H3N2 remains the most prevalent subtype, followed by H3N8. While H3N2 viruses have been predominantly isolated from humans and swine and have also been detected in avian species, indicating a broad host range and a substantial public health risk. Similarly, H3N8 viruses, primarily found in ducks and equines, have been linked to several human cases, underscoring their zoonotic potential (Bean *et al.*, 1992). In contrast, subtypes H3N3, H3N4, H3N5, H3N7, and H3N9 are relatively rare, with fewer than 100 isolates each. Notably, H3N3 exhibits a broader host range than other rare H3Ny subtypes, having been identified in humans, avian species, mammals, and environmental samples, whereas H3N4, H3N5, H3N7, and H3N9 have been isolated exclusively from humans or birds (Fig. 1A). Historically, the earliest documented H3N3 isolation occurred in 1972 in terns, followed by detections in ducks and seals. By 2002, H3N3 had been identified in terrestrial poultry, including chickens and pigeons. While early circulation was largely associated with aquatic

environments, strains detected after 2002 appear to exhibit adaptation to terrestrial hosts, demonstrating an evolving ecological niche (Fig. 1B).

**Genetic Characterization of Novel H3N3 AIV isolates :** In 2023, tissue samples from chickens suspected of H3N3 subtype infection were inoculated into embryonated eggs, and virus isolation was confirmed via HA assay and whole-genome sequencing. Comparative genomic analyses of the SQ2049, GT2125, and HN0120 strains revealed that these three isolates are novel reassortants. They possess HA genes originating from H3N8, NA genes derived from H10N3 viruses, and a complete set of six internal genes originating from the H9N2 AIV lineage that is widely prevalent in Chinese poultry. Sequence homology analysis demonstrated that the HA and NA genes shared over 98% nucleotide identity with representative H3N8 and H10N3 strains, respectively, while the six internal segments exhibited over 97% identity with H9N2 AIVs (Table 1). These findings indicate that the long-term, endemic circulation of H9N2 viruses in chicken populations has provided a substantial genetic reservoir facilitating the emergence of such triple-reassortant H3N3 viruses (Table 1 and Fig. 1C). Phylogenetic analysis further showed that the HA genes clustered within the Eurasian lineage (Fig. 2A), closely related to the human-derived H3N8 strain ZMD22-2, suggesting a recent common ancestor. The NA genes formed a distinct monophyletic cluster within the Asian lineage, showing a close evolutionary relationship with H10N3 viruses isolated from poultry in 2021 (Fig. 2B). For the internal genes, the M and PB2 genes grouped within the G1-like lineage, whereas the NS, PB1, PA, and NP genes clustered within the F/98-like lineage (Fig. 3). While SQ2049 and HN0120 exhibited high genetic similarity, GT2125 displayed a distinct reassortment pattern, with several internal genes positioned on separate phylogenetic branches.

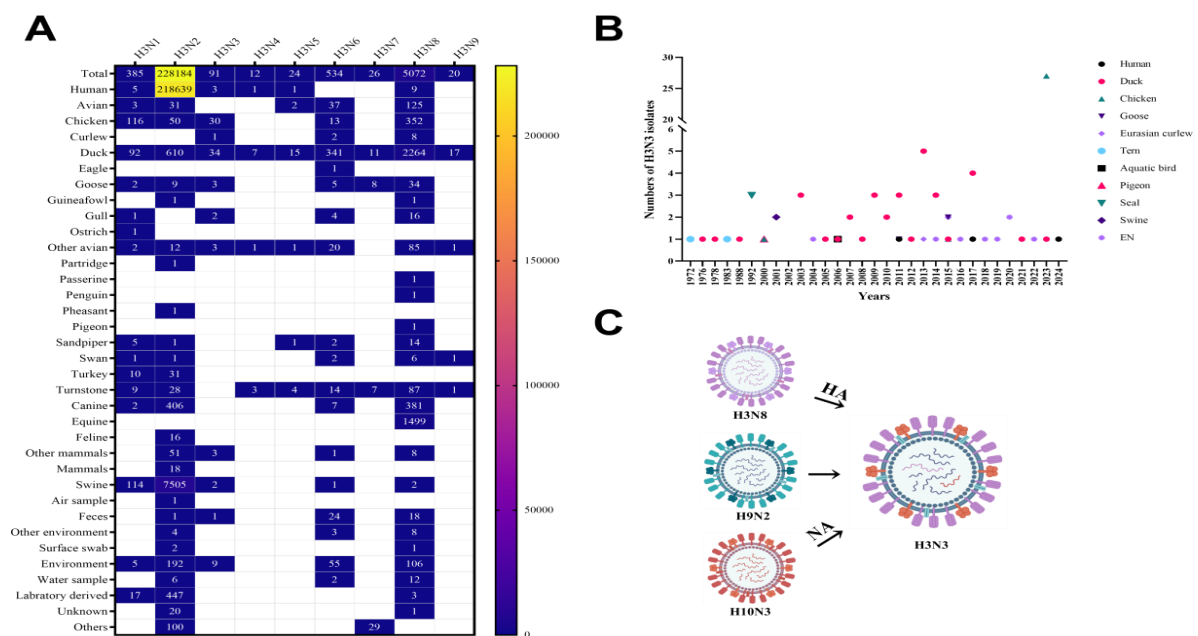


**Fig. 1:** Epidemiological characteristics and host range of H3Ny. A: Global host distribution of H3Ny avian influenza virus subtypes (1972–2025). B: Temporal and host distribution of H3N3 AIV isolates highlighting terrestrial adaptation post-1972. C: Schematic Diagram of the Triple Reassortment Event in Novel H3N3 Isolates. (HA, NA, and internal gene origins are indicated. Created with BioRender.)

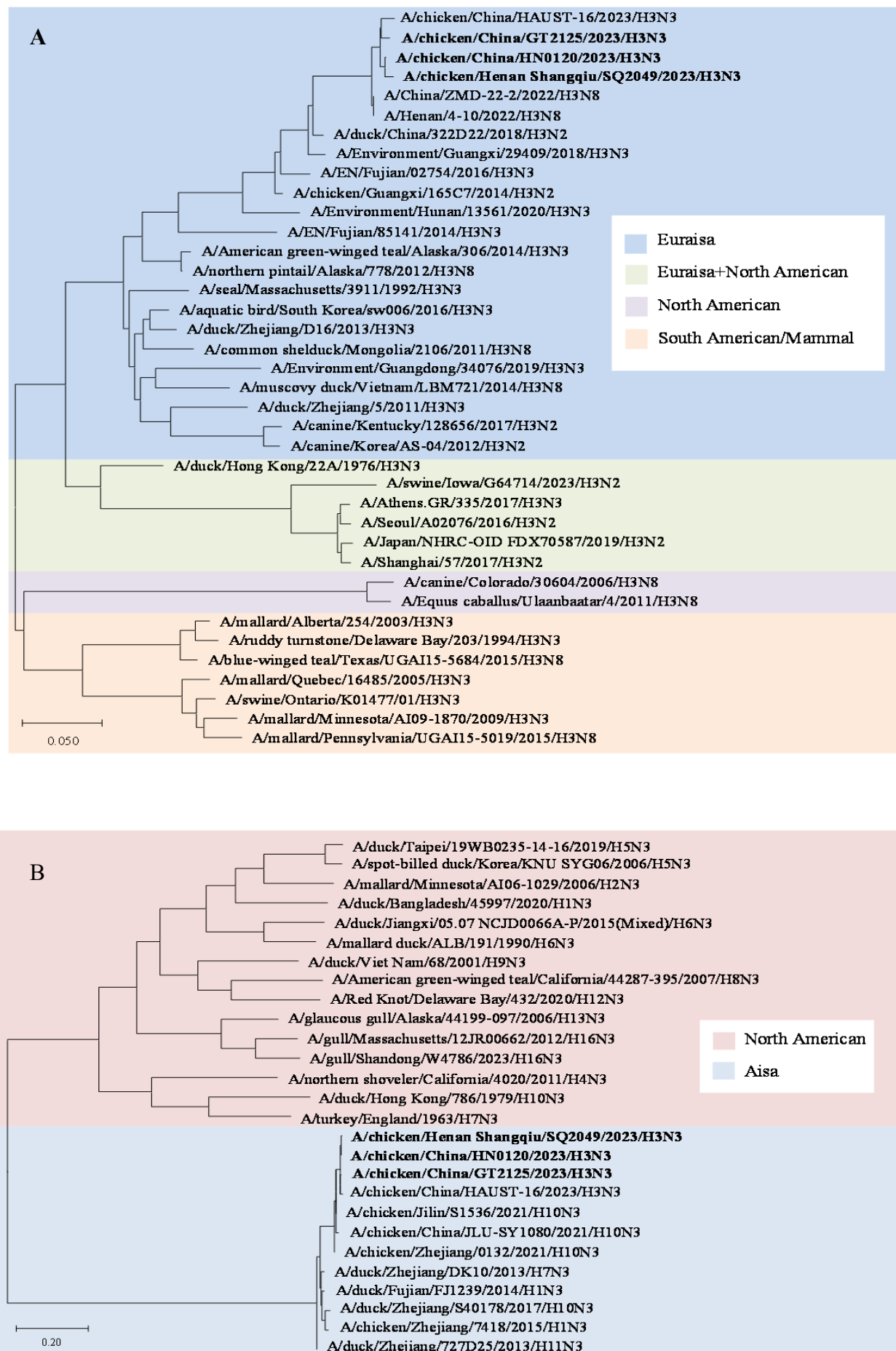


**Fig. 2:** Phylogenetic tree for HA (A) and NA (B) gene of three H3N3 subtype isolates using MEGA software. Bold text indicates isolated strains.

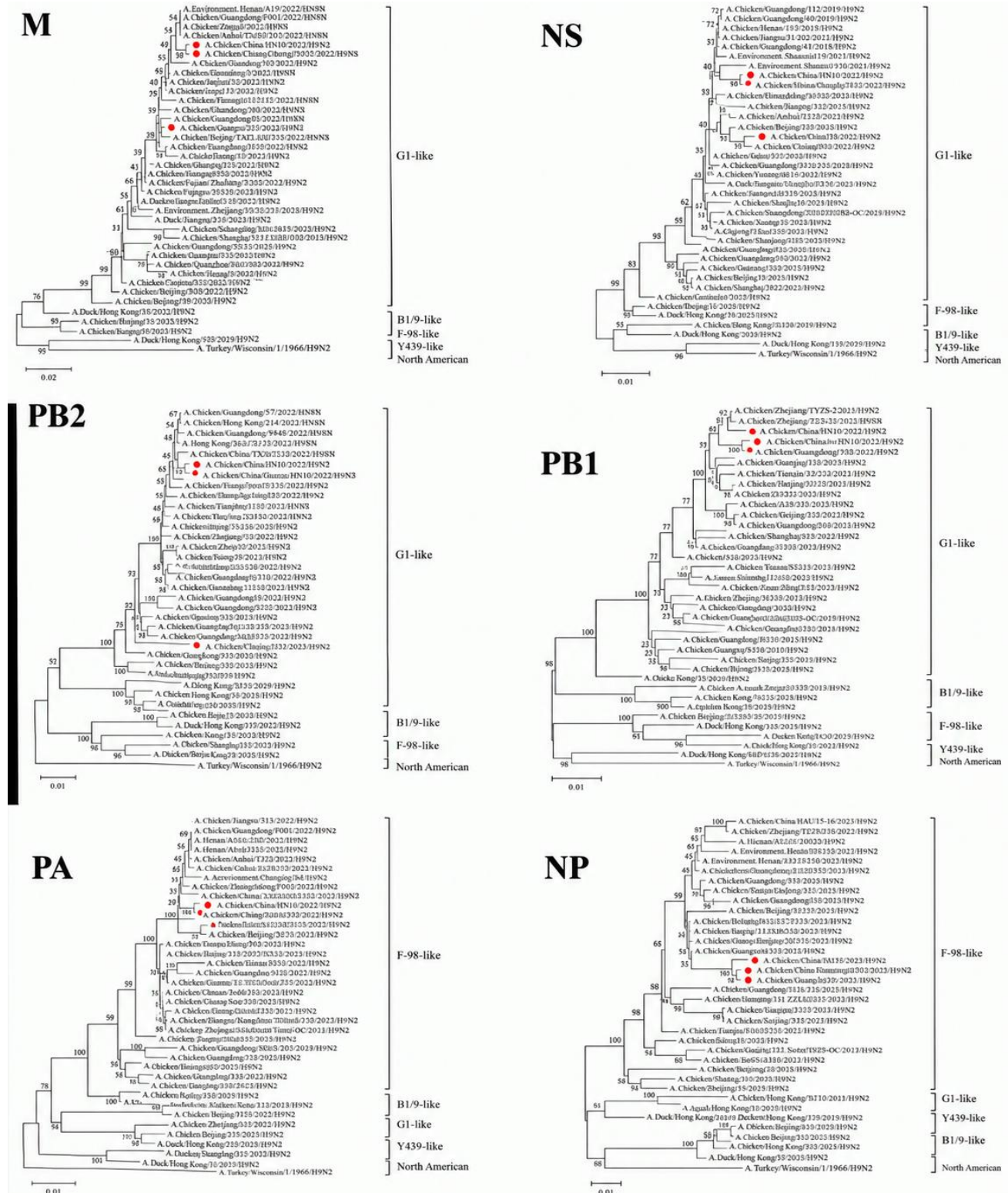


**Fig. 3:** Genetic evolution tree of M, NS, PB2, PB1, PA, NP were constructed using MEGA software. ● indicates isolated strains.

**Table 1:** Nucleotide identity of H3N3 SQ2049 gene segments to most closely related avian influenza reference strains.

| Gene | SQ2049—Closest reference strain (GenBank accession)  | Homology (%) |        |        |
|------|------------------------------------------------------|--------------|--------|--------|
|      |                                                      | SQ2049       | GT2125 | HN0120 |
| PB2  | A/chicken/China/YL22/2022(H9N2)(PP091193)            | 98.50%       | 97.94% | 93.86% |
| PB1  | A/chicken/China/3-1/2022(H9N2)(OR528462)             | 98.95%       | 98.28% | 98.59% |
| PA   | A/chicken/China/2-3-1/2022(H9N2)(OR528343)           | 98.84%       | 98.09% | 98.93% |
| HA   | A/China/ZMD-22-2/2022(H3N8)(ON342803)                | 98.58%       | 98.47% | 99.00% |
| NP   | A/chicken/Shandong/097/2020(H9N2)(MZ703075)          | 98.93%       | 98.60% | 98.93% |
| NA   | A/Environment/China/04940NA/2021(H10N3)(OM802522)    | 98.58%       | 98.16% | 98.72% |
| M    | A/pigeon/Fujian/3.15_FZHX0008-C/2018(H9N2)(MW101305) | 99.96%       | 99.39% | 98.37% |
| NSI  | A/chicken/Shandong/3424/2016(H9N2)(MH667576)         | 98.76%       | 97.83% | 98.65% |

Note: Nucleotide identity was determined by pairwise alignment of each gene segment against sequences available in GenBank.

**Molecular Characterization of H3N3 Isolates:** HA receptor-binding and cleavage site features: All three H3N3 isolates (SQ2049, GT2125, HN0120) possessed a monobasic HA cleavage site (PEKQTR↓GLF or PEKQSR↓GLF), consistent with a low-pathogenic avian influenza (LPAI) phenotype (Sun *et al.*, 2013). Several amino acid substitutions associated with altered receptor-binding properties were identified, including T160S and G186S, which are shared with the human-infecting H3N8 strain ZMD-22-2 and may enhance binding avidity (Bao *et al.*, 2022). All isolates retained Q226 and G228 (H3 numbering), typically associated with a preference for avian-type  $\alpha$ -2,3-linked sialic acid receptors (Table 2) (Vines *et al.*, 1998; Song *et al.*, 2017).

Polymerase complex mutations (PB2, PB1, PA): Sequence analysis identified several PB2 substitutions that have previously been reported to be associated with enhanced replication or virulence in mammalian hosts. In the PB2 protein, I292V has been shown to increase polymerase activity and suppress IFN- $\beta$  responses in human cells (Gao *et al.*, 2019), A558V enhances virulence in mice (Xiao *et al.*, 2016), and K702R is associated with mammalian host adaptation (Finkelstein *et al.*, 2007). Intriguingly, GT2125 retained V598I and K702 in PB2, a combination reported to increase mammalian virulence while limiting human adaptability (Hu, 2016). In PB1, the D622G substitution and conserved residue V473 were detected, both known to enhance polymerase activity in mammalian cells (Xu *et al.*, 2012). The PA protein contained K356R and S409N, both associated with increased pathogenicity in mammalian models (Huang *et al.*, 2015; Xu *et al.*, 2016).

Virulence and drug resistance markers in other proteins: Additional markers were identified in structural and non-structural proteins. M1 protein harbored T215A and N30D mutations, which are known virulence markers in mammals and are also present in the H3N8 ZMD-22-2

strain (Fan *et al.*, 2009; Bao *et al.*, 2022). All isolates carried the M2 S31N mutation, conferring amantadine resistance (Wang *et al.*, 2013). In the NS1 protein, E172K and N205S substitutions, which enhance viral replication in mammalian cells, were present (Patil *et al.*, 2021). Notably, unlike many Chinese H9N2 strains, these isolates lacked the 13-amino-acid deletion (residues 218–230) in the NS1 effector domain (Abdelwhab *et al.*, 2016).

#### Viral Replication in SPF Embryonated Chicken Eggs:

HA assays revealed differences in replication efficiency among the H3N3 isolates. SQ2049 and HN0120 exhibited robust replication with HA titers of 9log<sub>2</sub>, whereas GT2125 showed lower activity at 7log<sub>2</sub>. Quantitative analysis confirmed efficient propagation for SQ2049 and HN0120, with EID<sub>50</sub> values of 10<sup>8.52</sup> and 10<sup>7.69</sup>/0.1mL, and MDT of 70 and 73h, respectively. Conversely, GT2125 exhibited limited replication, failing to replicate upon dilution or cause embryo mortality.

#### Pathogenicity and Tissue Tropism in SPF Chickens:

Throughout the 14-day observation period, neither SQ2049 nor GT2125 infected groups displayed no clinical signs or significant body weight loss ( $P > 0.05$ ) (Fig. 4A, B). However, necropsy at 3dpi revealed pathological alterations, including Harderian gland hemorrhage, renal congestion, and thymic pinpoint hemorrhages (Fig. 4C). Tracheal mucoid exudates and bursal hemorrhagic foci were also observed. Histopathological analysis (Fig. 5) confirmed strain-specific lesions; SQ2049 and GT2125 caused inflammatory cell infiltration in the Harderian gland and kidneys, cortical lymphocyte apoptosis in the thymus, and ciliated epithelial desquamation in the trachea. SQ2049 further induced follicular atrophy in the bursa, while GT2125 caused interstitial widening and follicular regression.

**Table 2:** Amino Acid Substitutions in Three H3N3 Isolates Associated with Host Adaptation, Virulence, and Antiviral Resistance.

| ProteinFunction |                                                                                               | Mutation                       |        |        |               |
|-----------------|-----------------------------------------------------------------------------------------------|--------------------------------|--------|--------|---------------|
|                 |                                                                                               | SQ2049                         | GT2125 | HN0120 | H3N8 ZMD-22-2 |
| HA              | HA cleavage site (Low pathogenic strain)                                                      | PEKQTR↓GLFPEKQSR↓GLFPEKQTR↓GLF |        |        |               |
|                 | Increased transmission in guinea pigs                                                         | T160A                          | S      | S      | S             |
|                 | Increased affinity to human-type receptor                                                     | Q226L                          | Q      | Q      | Q             |
|                 |                                                                                               | G228S                          | G      | G      | G             |
| PB2             | Conserved in the seasonal human influenza virus                                               | A199S                          | A      | A      | A             |
|                 | Increase viral polymerase function, attenuates IFN- $\beta$ induction in human cells          | I292V                          | V      | V      | V             |
|                 | Enhance polymerase activity, virus replication and virulence in mice                          | A588V                          | V      | V      | V             |
|                 | Enhanced replication efficiency and increased virulence in mice                               | Q591K                          | Q      | Q      | Q             |
|                 | Increase virulence in mammals                                                                 | V598T/I                        | V      | V      | V             |
|                 | Enhance polymerase activity and replication in mammalian cells                                | E627K                          | E      | E      | K             |
|                 | Enhance polymerase activity and replication in mammalian cells                                | D701N                          | D      | D      | D             |
|                 | adapt to human hosts                                                                          | K702R                          | R      | R      | R             |
| NP              | Enhanced replication efficiency                                                               | N319K                          | N      | N      | N             |
| M1              | Species-specific signature                                                                    | T215A                          | A      | A      | A             |
|                 | Enhanced virulent in mammals                                                                  | N30D                           | D      | D      | D             |
| M2              | Amantadine resistance                                                                         | S31N                           | N      | N      | N             |
| NS1             | Increased virulence in mice                                                                   | P42S                           | S      | S      | S             |
|                 | Enhance CPSF30 binding and replication in mice                                                | I106M                          | I      | I      | I             |
|                 | Increase virulence in mice                                                                    | N139D                          | N      | N      | N             |
|                 | Enhance virus replication in mammalian cells, while retain replication ability in avian cells | E172K                          | K      | K      | K             |
|                 | Enhance viral polymerase activity and virus replication in mammalian cells                    | N205S                          | S      | S      | S             |
| PB1             | Confer higher polymerase activity in mammalian cells                                          | 473V                           | V      | V      | V             |
|                 | Correlate with transmissibility in ferrets                                                    | I368V                          | V      | V      | V             |
| PA              | Increase mammalian replication and pathogenicity                                              | K356R                          | R      | R      | R             |
|                 | Species-specific signature                                                                    | S409N                          | N      | N      | N             |

Note: the different amino acids of three H3 subtype influenza isolates were colored with yellow.

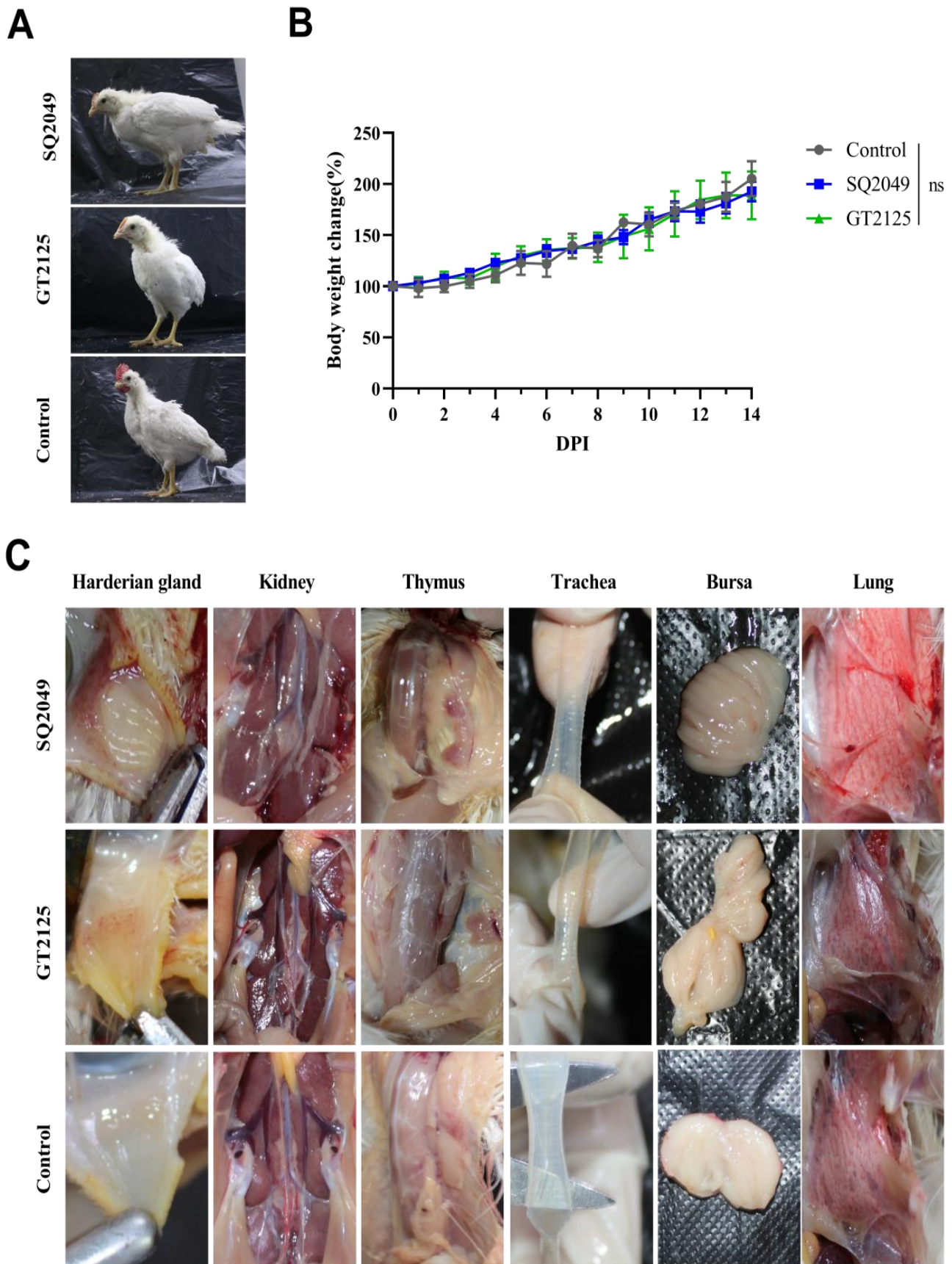


**Fig. 4:** The appearance (A), body weight dynamics (B) and gross lesions (C) observed in SPF chickens challenged with SQ2049, GT2125, and mock-inoculated controls at 3dpi.

Tissue distribution analysis at 3dpi showed consistent tropism patterns (Fig. 6). Both isolates were detected at 100% in the trachea and nasal turbinates,

while spleen and brain tissues remained negative. SQ2049 had a 100% detection rate in the Harderian gland and bursa, with intermediate detection in the

thymus and lungs. GT2125 had a 100% detection rate in the Harderian gland and lungs, but lower recovery rates in the bursa and other organs (Fig. 6A, B).

**Transmission and Immunogenicity in SPF Chickens:** In SQ2049-challenged chickens, oral shedding was transient (1-3dpi), while cloacal viral shedding lasted until 11dpi. Cohabitation chickens showed viral presence in both oral and cloacal swabs by 2 days post-contact, with cloacal shedding also lasting until 11dpi (Fig. 7A, B). Successful horizontal transmission was confirmed by 100% seroconversion at 14dpi, with HI titers reaching 8-9log<sub>2</sub> (Fig. 7C).

Regarding immunogenicity, 3-week-old chickens immunized with inactivated SQ2049 vaccine developed peak mean HI titers of 8log<sub>2</sub> by 3 wpv (Fig. 7D).

Furthermore, the vaccine elicited comparable HI titers against heterologous H3N3 isolates (GT2125 and HN0120), indicating high antigenic conservation. Consistent with HI titer, no viral detection in oral and cloacal swabs collected from SQ2049-vaccinated chickens at 3dpi, while 80% control chickens shed detectable virus (Fig. 7E).

**Identification of Mouse-Adaptive Mutations in HA Gene:** Intranasal inoculation of BALB/c mice with SQ2049 resulted in replication restricted to the nasal turbinates (3/3 mice positive) and lungs (1/3 mice positive) at 3dpi (Fig. 7F). Two non-synonymous mutations G174E and N209D were identified in HA gene of mouse-reisolated viruses (Fig. 8A). Structural mapping placed these substitutions at the HA oligomerization interface (Fig. 8B).

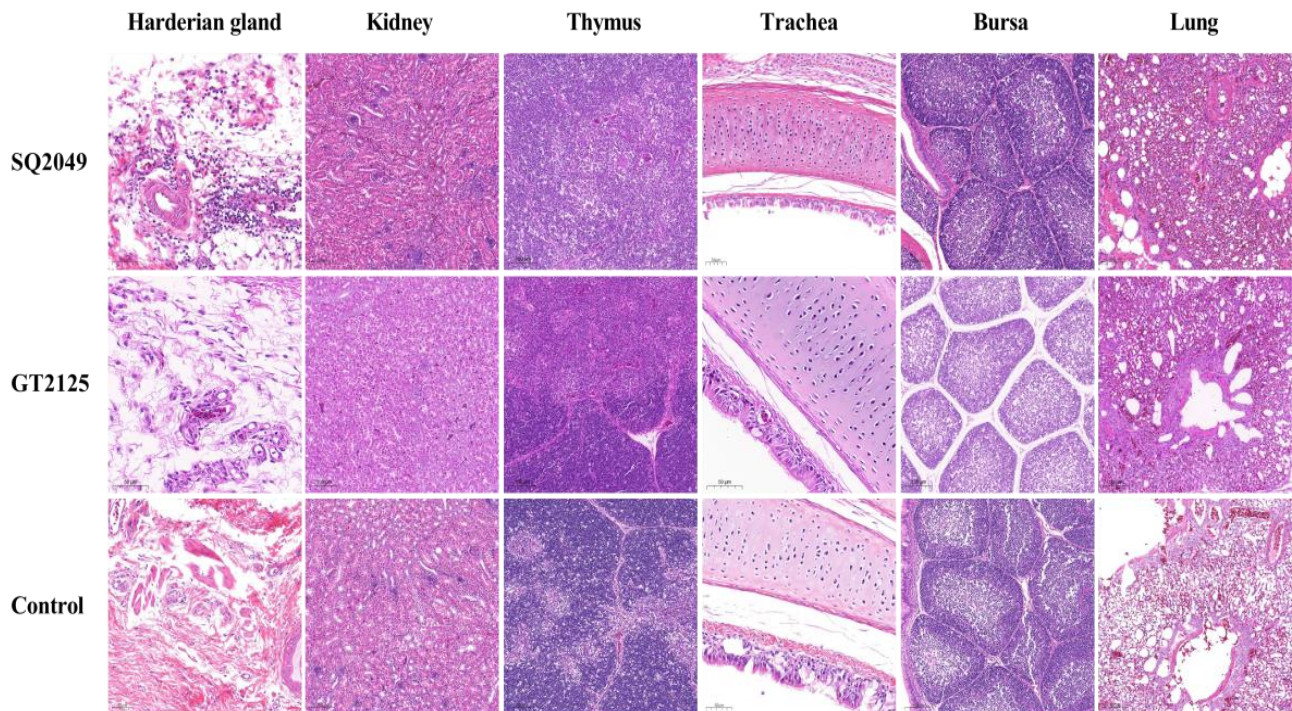


Fig. 5: Histopathological sections of chickens in H3N3 SQ2049 and GT2125 challenge and control group at 3dpi. Magnification: ×400.

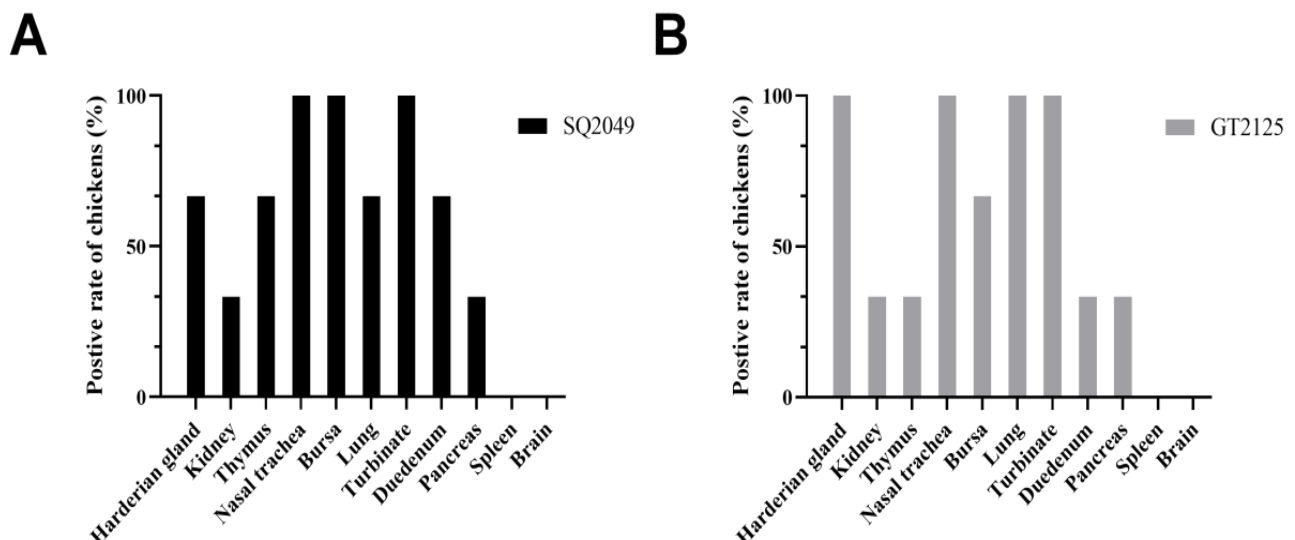
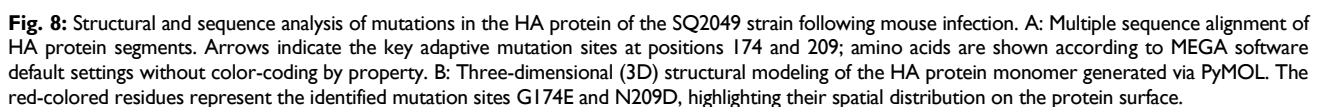
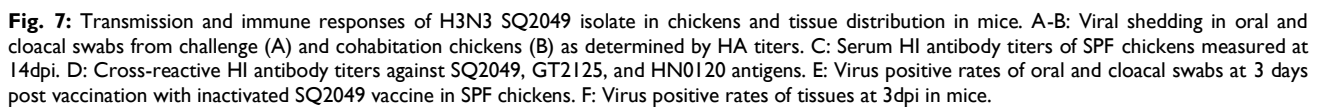


Fig. 6: Distinct tissue tropism of H3N3 SQ2049 (A) and GT2125 (B) isolates in SPF chickens.



## DISCUSSION

In 2023, 27 H3N3 strains were isolated from chickens, accounting for one-third of all global reports to date. Although these isolates exhibit low pathogenicity in poultry, the history of human infections caused by H3N2 and H3N8 subtypes underscores the necessity for vigilant surveillance. Given its expanding host range, the public health risk of H3N3, which is amplified by sustained circulation in poultry, must not be underestimated.

This study molecularly characterized three novel H3N3 AIV isolates (SQ2049, GT2125, and HN0120) from China. Phylogenetic analysis revealed that their HA and NA genes originated from H3N8 and H10N3 lineages, respectively, while the six internal genes were derived from the H9N2 lineage (He *et al.*, 2024). Notably, H9N2-derived polymerase genes (PB1, PB2, and PA) have previously facilitated the emergence of significant zoonotic viruses, including H5N1, H7N9, and H10N8 (Hou *et al.*, 2024). Considering that 75% of emerging infectious diseases are zoonotic (Dong and Soong, 2021), and given the proven capacity of H3N8 and H10N3 to breach species barriers, systematic investigation into H3N3 pathogenicity and immunogenicity is imperative.

Phylogenetic data indicated that SQ2049 and HN0120 cluster within the same branch, whereas the internal genes (M, NS, PB2, and PA) of GT2125 belong to distinct sub-clades, suggesting an independent reassortment pathway. Molecular markers showed that SQ2049 and HN0120 share an identical HA cleavage site (PEKQTR↓GLF) and mutation profiles with the human-infecting H3N8 strain ZMD22-2. Conversely, GT2125 possesses unique substitutions, including a PEKQSR↓GLF cleavage site and PB2-V598I/K702. Biologically, GT2125 exhibited lower replicative fitness in chicken embryos. This divergence may be associated with the PB2 substitutions, and their specific roles in viral replication require further investigation. Thus, while recombinant H3N3 viruses are emerging, strains like GT2125 may require further genetic adjustment to achieve stable replication in hosts. Three isolates also share several accumulation of adaptive markers (I292V, A558V, K702R in PB2; K356R in PA; E172K in NS1) parallels patterns in zoonotic H3N8 (Bao *et al.*, 2022) and H5N1 viruses (Chen *et al.*, 2015). However, the absence of key human-adaptation markers, such as PB2-627K or 701N (Steel *et al.*, 2009), suggests that the isolates have not yet acquired established molecular signatures associated with human adaptation.

Despite being classified as LPAIV (IVPI=0), the infectivity and tissue tropism of SQ2049 and GT2125 were evaluated to identify potential strain-specific deviations. Both strains induced only mild histopathological changes without significant body weight loss. The Harderian gland, trachea, and nasal turbinates were primary targets, with replication also detectable in the kidneys and pancreas. The absence of viral recovery from the spleen or brain aligns with typical LPAI phenotypes (Pu *et al.*, 2015). SQ2049 was selected for transmission trials due to its efficient replication in embryonated eggs. Results demonstrated efficient horizontal transmission between chickens. Notably, cloacal viral shedding in SQ2049 persisted for up to 11

days, whereas oral viral shedding only last 2-3 days under the experimental conditions. The time of viral shedding and the absence of clinical signs and indicates that surveillance should prioritize environmental fecal sampling and serological monitoring. In the present study, detailed pathogenicity and transmission experiments were performed only with the SQ2049 isolate. Further characterization of GT2125 and HN0120 is needed to determine whether these biological properties are shared among contemporary H3N3 viruses.

Immunological experiments showed that the inactivated SQ2049 vaccine elicited broadly cross-reactive HI antibodies against heterologous H3N3 strains and provided 100% protection in SPF chickens. Despite amino acid variations, the high antigenic identity suggests that mutations in GT2125 may not substantially affect the major antigenic regions of HA. This antigenic conservation provides a theoretical basis for regional vaccination programs, yet relevant field efficacy, immunity duration and antigenic drift data remain absent to validate large-scale application.

Although H3N3 has not caused human epidemics, its HA gene shares over 85% homology with the 1968 pandemic H3N2 virus (Nakajima *et al.*, 1982; Jester *et al.*, 2020). Two HA substitutions (G174E and N209D) emerged during mouse infection, which merits further attention. Previous studies have associated G174E with enhanced viral infectivity, while N209D is known to strengthen binding affinity for human-type  $\alpha$ -2,6-linked sialic acid receptors (Yoshida *et al.*, 2009; Imai *et al.*, 2012). Similar adaptations were observed during the emergence of pandemic H2N2 (1957) and H3N2 (1968) viruses (Lindstrom *et al.*, 2004). These findings suggest that mammalian-adaptive substitutions may be selected during host adaptation. While the retention of Q226 and G228 residues currently limits human receptor preference, the identified HA substitutions and detectable viral replication in mice indicate a potential zoonotic concern that needs further investigation (Longdon *et al.*, 2014). However, the functional roles of H3N3 substitutions G174E and N209D in receptor binding and mammalian adaptation were not evaluated in this study. Future receptor-binding assays, reverse genetics, polymerase-activity assays, and airborne-transmission studies in mammals, will help clarify the contributions of these substitutions to H3N3 adaptation.

Some results presented here may overlap with previously published H3N3 data. Nevertheless, our findings provide additional information on the biological characteristics of these reassortant viruses. Further investigation is still needed to clarify their pathogenicity, host adaptation, and epidemiological significance.

**Conclusions:** Three novel triple-reassortant H3N3 avian influenza viruses carrying H3N8-derived HA, H10N3-derived NA, and H9N2-derived internal genes were characterized in this study. Although these viruses exhibited low pathogenicity in SPF chickens, SQ2049 showed efficient horizontal transmission and prolonged cloacal viral shedding. HA substitutions (G174E and N209D) were detected after replication in mice, indicating that further studies are warranted to determine their roles in mammalian adaptation. In addition, the inactivated

SQ2049 vaccine induced cross-reactive HI antibodies and provided complete protection against homologous virus challenge. These findings provide additional evidence for understanding the biological characteristics of emerging H3N3 avian influenza viruses and support continued surveillance of this reassortant lineage.

**Ethical declaration:** The animal experiments were approved by the HENAU Animal Ethics Committees were conducted in accordance with its guidelines. The approval number is HNND2023032218 (approved on 22 March 2023).

**Data Availability Statement:** Any supplementary data will be made available on request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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