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Letter

Emergence of a novel H4N6 avian influenza virus with mammalian adaptation isolated from migratory birds in Zhejiang Province, China, 2024

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Dear Editor,

Avian influenza viruses (AIVs) continue to evolve at the interface of wild and domestic birds, posing ongoing threats to animal and public health (Bi et al., 2020; Uyeki et al., 2022). Wild migratory birds serve as natural reservoirs and facilitate viral gene exchange through long-distance migration, whereas dense poultry farming in East Asia creates opportunities for reassortment and spillover (Li P. et al., 2022; Verhagen et al., 2015). Historically, H4-subtype AIVs have been considered low-pathogenic, yet recent detections in poultry and swine suggest an expanding host range and genetic plasticity (Li et al., 2024; Song et al., 2024; Parsons et al., 2023). In this study, we identified and characterized a novel reassortant H4N6 virus isolated from a red-necked stint (*Calidris ruficollis*) during surveillance in Zhejiang Province, China, in May 2024 and evaluated its molecular signatures associated with mammalian adaptation.

During 2024, a total of 400 cloacal and oropharyngeal swabs from migratory shorebirds were collected in Hangzhou and Xuanmen Bays, which are key sites along the East Asian–Australasian Flyway (Supplementary Table S1). Among these, one sample tested positive for H4N6 AIV by qRT-PCR, and the isolated virus, designated A/red-necked stint/Zhejiang/G030/2024 (H4N6/G030), was propagated in 10-day-old embryonated chicken eggs. The harvested allantoic fluids exhibited a hemagglutination titer of 1:256 and caused mild embryonic hemorrhage, confirming efficient viral replication, with other potential factors ruled out (Supplementary Fig. S1). Full-genome sequencing using next-generation methods revealed a typical avian eight-segment structure. Subsequent BLAST and Bayesian phylogenetic analyses demonstrated that the hemagglutinin (*HA*) gene (1737 bp) clusters with duck-origin H4N6 viruses from Jiangxi, China (2023), while the neuraminidase (*NA*) gene (1427 bp) shares >98% identity with H3N6 strains from Bangladesh (2022) (Fig. 1A). Non-HA/NA segments, including PB2, PB1, PA, NP, M, and NS, originate from multiple avian lineages, including H6N8 and H9N2 isolates (Supplementary Fig. S2–S7). Consistent with this genetic diversity, H4N6 viruses have been widely detected across multiple geographic regions and host species, particularly among migratory waterfowl (Fig. 1B). This mosaic composition reflects ongoing reassortment between wild and domestic bird populations across Asia (Ding et al., 2024; Li Y. et al., 2022).

While the HA cleavage site sequence (PEKASR↓GLF) lacks multibasic motifs, confirming its low-pathogenic phenotype, several amino acid substitutions linked to mammalian adaptation were identified, including PB2 I504V, PA I550L, and NS1 P42S. Notably, the HA T160A mutation, which removes a potential glycosylation site adjacent the receptor-binding domain (RBD), is predicted to enhance affinity for human-type (α2,6-linked) sialic acids receptors (Supplementary Table S2) (Gao et al., 2018). This was experimentally validated by receptor binding assays, which confirmed a dual-binding affinity for both avian (α2,3-linked) and human (α2,6-linked) receptors (Fig. 1C and D), a pattern shared by recent zoonotic AIVs like H10N8 and H3N8 (Liu et al., 2024; Tian et al., 2023). Moreover, the virus demonstrated moderate thermostability and environmental persistence, with hemagglutination activity remaining stable at 55 °C for 4 h (Fig. 1E). These molecular features suggest enhanced fitness for cross-species maintenance.

To evaluate replication efficiency, H4N6/G030 was inoculated into avian (DF-1) and mammalian (MDCK, MAC-T, MDBK, 293T) cell lines. The virus reached peak titers of $10^{3.5}$ $TCID_{50}$/mL in MDCK and 10^{4} $TCID_{50}$/mL in DF-1 cells at 48 h post-infection (hpi), demonstrating efficient replication in mammalian epithelial cells without prior adaptation and indicating broad cell tropism (Fig. 1F). When BALB/c mice were intranasally inoculated with 10^{6} $TCID_{50}$ virus, no mortality was

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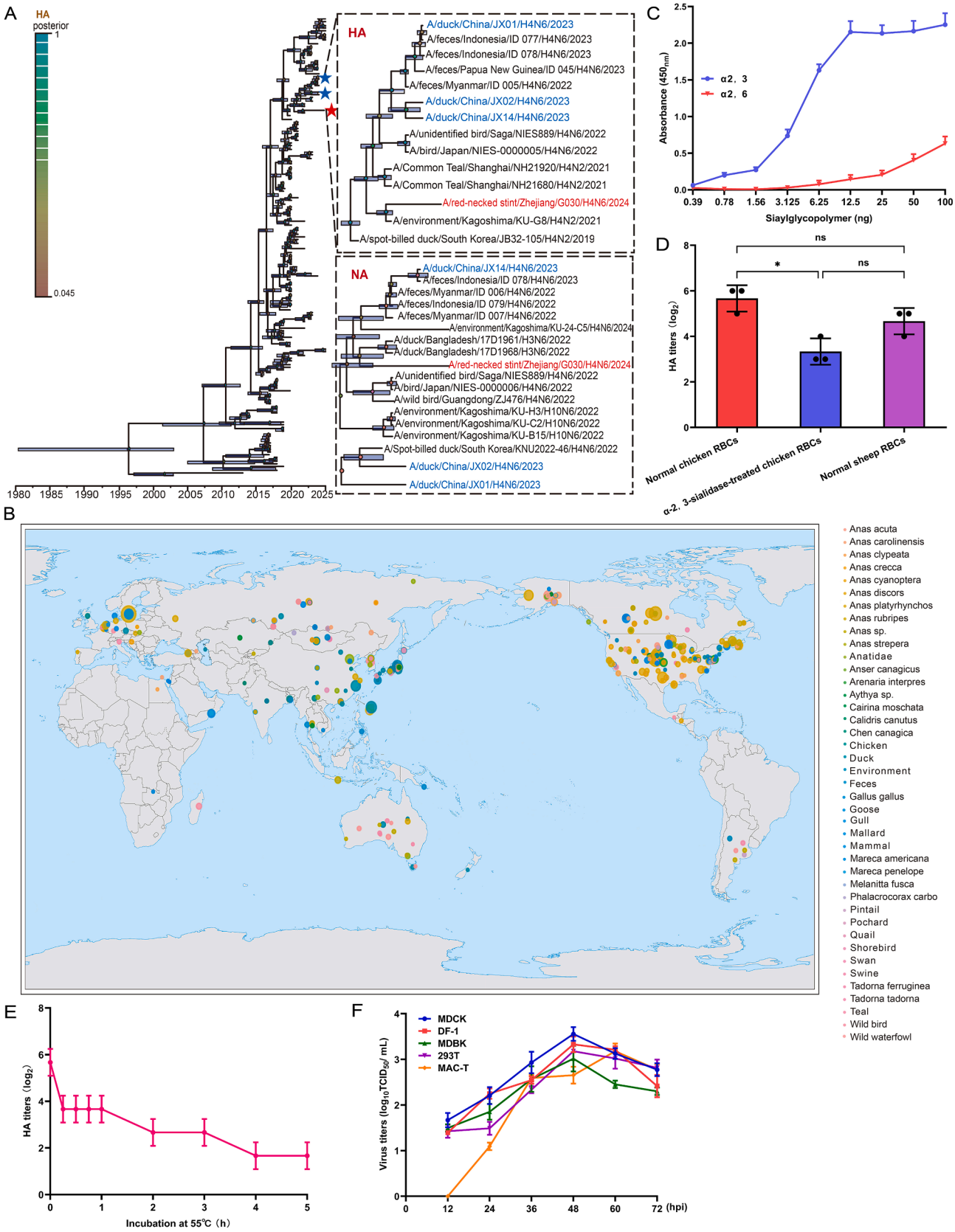

Fig. 1. Key features of A/red-necked stint/Zhejiang/G030/2024 (H4N6). **A** Bayesian phylogenetic trees of *HA* and *NA* genes. The *HA* gene tree is shown in the upper-right panel and the *NA* gene tree in the lower-right panel. The H4N6/G030 strain is indicated by red pentagrams, while a reference duck strain from Jiangxi, China, is marked with blue pentagrams. **B** Geographic and host distribution of H4N6 AIVs based on NCBI and GISAID data. Circle size indicates the number of H4N6 isolates detected in each host species. The map generated using R and Adobe Illustrator. **C** Dual-receptor solid-phase binding assay was performed to analyze the interaction of H4N6/G030 with α2,3- and α2,6-linked sialic acid receptors. **D** Receptor binding property tested using normal chicken RBCs (expressing both α2,3 and α2,6

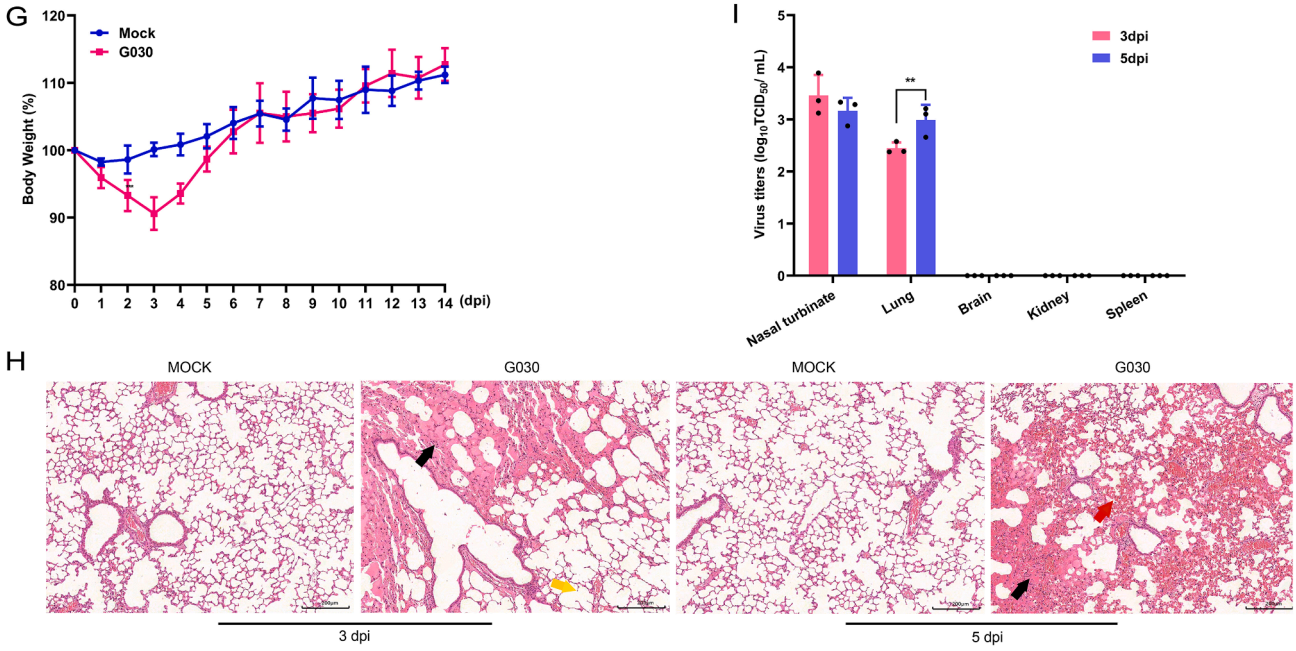

Fig. 1. (*continued*).

Table 1
H4 serological positivity rates in chicken farms near wild bird sampling sites[a].

	Samples No.	HI antibody positive n (%)	Highest HI titer	MN antibody positive n (%)	Highest MN titer
Farm 1	28	28 (100)	>1:1024	28 (100)	>1:1280
Farm 2	19	18 (94.7)	>1:1024	18 (94.7)	>1:1280
Farm 3	18	15 (83.3)	1:128	14 (77.8)	1:320
Farm 4	27	11 (40.7)	1:256	10 (37)	>1:1280
Farm 5	20	15 (75)	>1:1024	13 (65)	>1:1280
Farm 6	18	15 (83.3)	>1:1024	16 (88.9)	1:640
Total	130	102 (78.5)	>1:1024	99 (76.2)	>1:1280

[a] HI titer ≥1:32 and MN titer ≥1:40 are considered seropositive, indicating previous infection. The H4N6/G030 virus strain, isolated from wild birds, was used as the antigen to detect HI and MN titers in chicken serum samples. HI, hemagglutination inhibition assay; MN, microneutralization assay.

observed, but body weight loss of 8%–10% occurred by day 3 post-infection (Fig. 1G). Histopathological analysis showed alveolar congestion and mild interstitial pneumonia, with viral antigens localized to bronchiolar epithelial cells (Fig. 1H). Consistent with these findings, viral loads in lungs reached 10^3 $TCID_{50}$/mL on day 5, confirming restricted replication to the respiratory tract (Fig. 1I). No virus was detected in brain or kidney samples, indicating absence of systemic spread. However, it should be noted that the mouse model has limitations in fully capturing virus transmission and human-like clinical symptoms. Therefore, future studies using ferret models will be crucial to fully assess the zoonotic potential and transmissibility risk of this virus.

To assess the field exposure risk, serological surveys were conducted on six chicken farms within a 20 km radius of sampling sites. Among 130 sera tested, 78.5% were positive for H4-specific antibodies by hemagglutination inhibition (HI) assay and 76.2% by microneutralization (MN) assay. Farm 1 showed 100% positivity with high titers (≥1:1024), suggesting recent or ongoing circulation (Table 1). Such co-circulation creates conditions for reassortment events that could enhance zoonotic potential.

The emergence of H4N6/G030 provides further evidence for active genetic reassortment at the wild-bird-poultry interface in China. Notably, although this virus is classified as a low pathogenic avian influenza virus (LPAIV), its dual receptor-binding affinity and efficient replication in mammalian cells represent a key step toward cross-species adaptation. This adaptive potential is well-established: previous studies show that single amino-acid changes in PB2 or HA can greatly enhance viral replication and transmissibility in mammals (Lin et al., 2024; Gao et al., 2019). Given the recent reports of H5N1 infection in cats and dairy cattle in North America (Peacock et al., 2025), enhanced surveillance of LPAIVs such as H4 has become increasingly important. The replication of H4N6 in bovine cells observed *in vitro* indicates that cross-species exposure cannot be ignored. Therefore, this enhanced surveillance that integrates genomic analysis, serology and biosecurity is crucial to identify emerging strains with potential public-health implications.

receptors), α2,3-sialidase-treated chicken RBCs (retaining only α2,6 receptor), and normal sheep RBCs (expressing α2,3 receptor). *$P < 0.05$; ns, not significant. **E** Thermostability of H4N6/G030 at 55 °C was assessed by HA assay. **F** Growth kinetics of H4N6/G030 in various cell lines were determined at MOI 0.01. **G** Body weight changes in mice infected with H4N6/G030 or PBS control (mock) over 14 days. H Lung histopathology of infected mice was examined by H&E staining. Black arrows point to necrosis of alveolar epithelial cells and disorganized alveolar structure, yellow arrows indicate ruptured alveolar septa, and red arrows denote capillary congestion. Scale bar: 200 μm. I Viral titers in organs of infected mice were determined by $TCID_{50}$ assay at 3 and 5 dpi. **$P < 0.01$.

In conclusion, we identified a novel H4N6 avian influenza virus from migratory birds, which exhibits mammalian adaptation markers, including dual receptor-binding affinity and a reassortant genome from wild-bird and poultry lineages. This, combined with the high seroprevalence in local chickens, compounds the risk and further highlights the urgent need for ongoing molecular and epidemiological surveillance along high-risk migratory flyways.

FOOTNOTES

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APPENDIX ASUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2026.03.005.

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