



Letter

Characterization of novel highly pathogenic avian influenza A(H5N6) clade 2.3.4.4b virus in wild birds, East China, 2024



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Dear Editor,

The highly pathogenic avian influenza viruses (HPAIVs) are important epizootic and zoonotic pathogens that cause significant economic losses to the poultry industry and pose a serious risk to veterinary and public health. Wild birds have been recognized as the primary reservoirs for influenza A virus, and some species show little sign of clinical disease or even can be asymptomatic during long distance carriers of the virus (Lycett et al., 2019). Since it was first discovered in 1959, the H5Nx HPAIVs have spread globally and cause outbreaks in wild birds, poultry and sporadic human and other mammalian infections (Lycett et al., 2019). Due to the reassortant events of diverse strains facilitated by migratory waterfowl, the clade 2.3.4.4 of H5Nx viruses acquiring neuraminidase (NA) gene from other low pathogenicity avian influenza viruses (LPAIVs) emerged in 2014 and gradually became the dominant sub-clade (Lee et al., 2017). The genetic diversity of clade 2.3.4.4 of H5Nx hemagglutinin (HA) has further evolved into eight subclades (2.3.4.4a to 2.3.4.4h) according to a unified nomenclature (Graziosi et al., 2024). H5N6 of clades 2.3.4.4d–h were predominantly identified in China from 2014 to early 2020 until the occurrence of a novel H5N6 derived the clade 2.3.4.4b of H5N8 in December 2020 (Gu et al., 2022). Subsequently, the preponderant clade of the H5N6 subtype HPAIV in China switched into 2.3.4.4b. Recently, novel H5N6 HPAIVs containing HA gene from clade 2.3.4.4b H5N1 virus entered R. O. Korea, with disease outbreaks in poultry and wild bird mortality events (Cho et al., 2024).

In June 2024, the case of H5Nx HPAIV infection emerged in Xiangshan County, Ningbo City, Zhejiang Province, which caused forty-three deaths of migratory wild birds belonging to the family *Laridae*. We received eleven clinical tissue samples from dead birds, nine fecal swabs and six environmental swabs. The HA subtypes and NA subtypes were identified by qRT-PCR using commercial kit (QIAGEN Shenzhen Co., Ltd., No.1066846; Shanghai ZJ Bio-Tech Co., Ltd., REF.RR-0376-02), and the results were validated using WHO recommended qRT-PCR conditions

with the primers as previously described (Li et al., 2010; Fan et al., 2015; Bi et al., 2016). Apart from environmental swabs, all other samples tested positive for both the H5Nx and HxN6, and four viruses were isolated. To investigate the potential origin, genetic characteristics and evolution of these viruses, we conducted next-generation sequencing (NGS) and phylogenetic analysis. We determined the whole genome sequences of four isolated H5N6 viruses. The genome sequence between viruses possessed high homology with each other (99.99%–100%). All H5N6 isolates contained the characteristic of HPAIVs according to the presence of multiple basic amino acids motif at the HA proteolytic cleavage site (REKRRKR/GLF). As a representative virus, the sequence of A/wild bird/Zhejiang/NBXS-1/2024 (H5N6) was performed BLAST researching against the database of NCBI and GISAID EpiFlu database. All eight segments showed a high nucleotide sequence identity (99.08%–99.80%) with the respective genes of clade 2.3.4.4b H5N1 or H5N6 viruses isolated from wild birds and poultry in Japan and R. O. Korea during 2022–2023 (Supplementary Table S1). Further analysis revealed that the nucleotide homology across eight segments of NBXS-1 and recent H5N6 viruses from Japan and R. O. Korea ranged from 90.57% to 99.57%. Notably, PB2, NP, and NS genes exhibited low sequence similarity (<96.00%) to H5N6 viruses from Japan and R. O. Korea. Extending our comparison to H5N6 viruses from China during 2020–2024, we found that all genes of NBXS-1 showed distinct diversity, with identities ranging from 56.10% to 99.51%. The nucleotide sequences of the PB2 and NP genes in particular exhibited distinct differences (<95.00% homologies), when compared to China's H5N6 viruses since 2020. A/HeFei/04171/2024, identified subsequent to NBXS-1, is a newly recognized H5N6 virus from a human infection case. It had relatively higher nucleotide sequence similarity with NBXS-1's eight genes (98.53%–99.49%) as compared to the homology observed between NBXS-1 and other H5N6 viruses in China. Additionally, the NS1 protein of NBXS-1, which lacked thirteen amino acid residues at the C-terminus, presented a significant deviation from recent H5N6 viruses of Japan and

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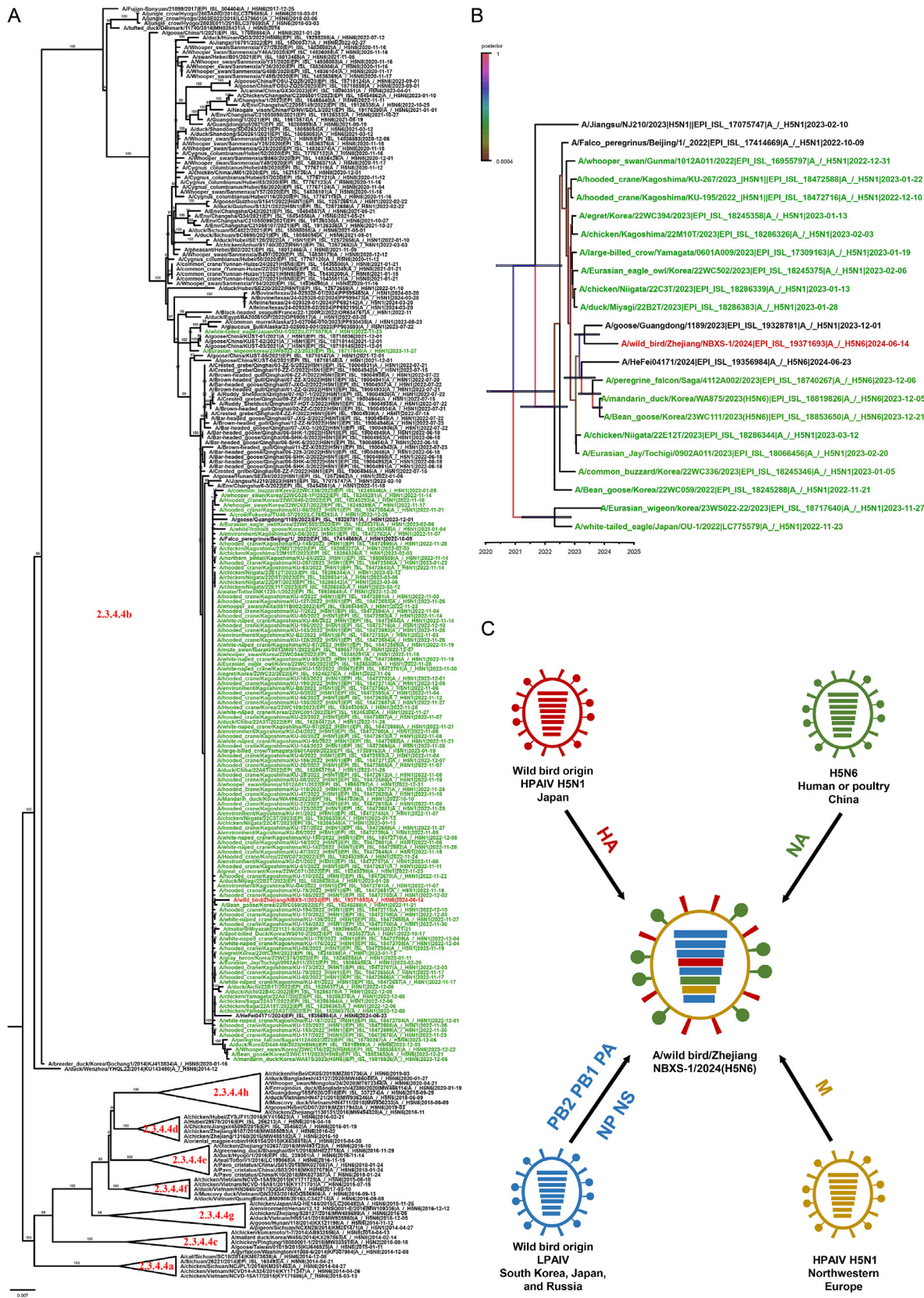


Fig. 1. Phylogenetic analysis of the NBXS-1. **A**, **B** The ML phylogenetic tree and the MCC tree of HA gene of NBXS-1. NBXS-1 is highlighted in red, recent H5N6 and H5N1 viruses from Japan and R. O. Korea are shown in green. **C** Schematic illustration of the potential genetic origins of NBXS-1 for each gene segment. Eight bars represent the eight segments (PB2, PB1, PA, HA, NP, NA, M, NS from top to bottom), with the color of each bar indicating the closest ancestral donor for that segment. HPAIV, high pathogenicity avian influenza virus; LPAIV, low pathogenicity avian influenza virus.

R. O. Korea and was also uncommonly observed in the H5N6 viruses circulating in China during 2020–2024. These results demonstrated that NBXS-1 was distinguish from recent H5N6 viruses of China, Japan and R. O. Korea on homology analysis, implying that it was a novel strain introduced via migratory birds.

Phylogenetic analysis confirmed that the *HA* gene of NBXS-1 belonged to clade 2.3.4.4b and was closely associated with major H5N1 HPAIVs, as well as few H5N6 HPAIVs, recently isolated from migratory birds and poultry in R. O. Korea and Japan. With the exception of two H5N1 HPAIVs and a human H5N6 virus (shown black within a green cluster) isolated from China, our findings revealed that the *HA* gene of NBXS-1 were genetically distant from other H5Nx viruses of clade 2.3.4.4b circulating in China during 2020–2024. A combined analysis of the maximum likelihood (ML) tree and the maximum clade credibility (MCC) tree (selecting representative sequences from the ML tree) suggested that the *H5* gene of NBXS-1 likely originated from an HPAIV H5N1 ancestor in wild birds from Japan in 2022 (Fig. 1A and B, Supplementary Fig. S1). Interestingly, the *NA* gene of NBXS-1 clustered closely with H5N6 HPAIVs lately identified in R. O. Korea, Japan, and the human infection H5N6 strain (A/HeFei/04171/2024). Notably, these viruses shared a common ancestor of unknown origin but were most closely related to a H5N6 HPAIV from poultry or human found in China between late 2021 and 2022 (Supplementary Fig. S2A and Fig. S3), which might raise concerns regarding their potential for zoonotic transmission. The remaining six internal genes clustered closely with recent H5N1 viruses from wild birds and poultry in R. O. Korea and Japan (Supplementary Fig. S2B–G). The majority of these internal genes likely originated from the LPAIVs ancestors: the *PB2*, *PB1*, and *PA* genes from those of R. O. Korea, the *NP* gene from those of Japan, and the *NS* gene from those of Russia, while the *M* gene could trace back to an ancestor that closely related to H5N1 HPAIVs from northwestern Europe (Fig. 1C, Supplementary Fig. S2B–G). By employing the TempEst v1.5.3 software for estimation of the time to the most recent common ancestor (tMRCA), we estimated that NBXS-1 approximately emerged between May 2023 and February 2024 and spread to eastern China probably via East Asian-Australasian migratory flyway (Supplementary Table S2). Moreover, it is important to note that temporal bias of virus sequences from wild birds and sampling bias in reference virus sequences might potentially affect the estimation of genetic origin and tMRCA.

To understand its virulence in mammals and avian hosts and capability of cross-species transmission, we conducted the analysis of the key amino acid substitutions of viral proteins (Supplementary Table S3). We identified the S133A and T156A mutations in the *HA* protein of NBXS-1, which were known to enhance binding affinity to α -2,6-linked sialic acid (human-type receptor), and were consistent with those observed in H5N6 strains circulating in Japan and R. O. Korea (Yang et al., 2007; Suttie et al., 2019; Cho et al., 2024). For the *NA* protein, NBXS-1, similar to the Japan and R. O. Korea strains, exhibited a deletion in the stalk region, which was associated with high pathogenicity in avian hosts and the adaptation of viruses from wild birds to domestic poultry (Suttie et al., 2019; Cui et al., 2020). In the *PB1*, *PB1-F2*, *M1* and *NS1* protein, NBXS-1 and these strains shared the multiple molecular markers which correlate with previously reported phenotypic traits associated with enhanced replication in avian cells and increased virulence in mice, chickens and ducks (Suttie et al., 2019). Notably, in the *NP* protein, the Japan and R. O. Korea strains lacked the M105V mutation found in NBXS-1, while both possessed the A184K mutation (Wasilenko et al., 2009; Tada et al., 2011; Suttie et al., 2019). Moreover, no adamantane-resistance mutations were observed in the *M2* protein of NBXS-1.

Given that frequent reassortment events with clade 2.3.4.4 H5Nx viruses in wild birds, rapidly and accurately obtaining whole genome sequences is important and more helpful for us to understand high genetic diversity of viruses and identify the possible origins. Sanger sequencing deeply relies on specific primers for each of eight segments of AIV, which is time-consuming and can lead to a lack of information on co-infections. NGS is a powerful and cost-effective tool that does not depend

on known sequences and can obtain AIV complete genome sequences through one reaction. In this study, we reported novel reassortant clade 2.3.4.4b H5N6 HPAIs isolated from wild birds in eastern China in June 2024, and rapidly acquired the whole genome sequence by targeting NGS method. Our phylogenetic analysis suggested that novel H5N6 HPAIVs in this study most likely descended from clade 2.3.4.4b H5N1 viruses circulating in Japan during 2022–2023, evolved from reassortment with other LPAIVs from wild birds and H5 HPAIVs, and were introduced into eastern China by migratory flyway. Furthermore, H5N1 of clade 2.3.4.4b was lately detected in dairy cattle, domestic cats and unpasteurized bovine milk in the United States (Burrough et al., 2024). In 2023, clade 2.3.4.4b H5N1 viruses were isolated from felines in R. O. Korea, and they were extremely originated from migratory birds travelled from Japan and R. O. Korea (Lee et al., 2024). Here, we compared key molecular markers between NBXS-1 and H5N6 viruses from wild birds circulating in these regions. The similarities and differences between these viruses provide insights into the potential for cross-species transmission and increased virulence, warranting further investigation into the epidemiological and pathogenic implications.

Collectively, this report provides valuable genetic insights into evolution of H5Nx clade 2.3.4.4b viruses in wild birds. The winter migration season has arrived, it is necessary to strengthen effective genomic surveillance in both wild birds and poultry, with additional attention to felines and dairy cattle, in order to monitor the spread and further evolution of H5 influenza viruses.

FOOTNOTES

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