Preplanned Studies

Phylogenetic and Molecular Characteristics of An H3N8 Avian Influenza Virus Detected in Wild Birds — Beijing, China, September 2024

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Summary

What is already known about this topic?

The H3N8 avian influenza virus (AIV) demonstrates considerable capacity for interspecies transmission and has been documented in multiple mammalian hosts, including equine and canine species. During 2022–2023, three laboratory-confirmed human infections with H3N8 were reported in China, heightening public health concerns about the zoonotic spillover potential of H3 subtype AIVs.

What is added by this report?

This study reports the isolation of a genetically reassorted, low-pathogenicity H3N8 avian influenza virus (AIV) from an islet in Niukouyu Wetland Park, Beijing Municipality — the first detection of this viral strain in a wild environment within the city. Throat swabs collected from park staff tested negative for influenza viruses. Phylogenetic analysis demonstrated that the viral hemagglutinin gene originated from the Eurasian lineage, while the neuraminidase gene was derived from the North American lineage. Although no direct evidence of human infection has been documented, multiple mutations identified in the virus's internal genes are associated with enhanced replication capacity, increased virulence, and improved adaptation to mammalian hosts. These molecular features indicate a potential risk for cross-species transmission to humans.

What are the implications for public health practice?

Given the potential threat that H3N8 AIVs pose to mammalian species, including humans, this study emphasizes the critical need to strengthen influenza surveillance networks and broaden monitoring efforts specifically targeting H3 subtype AIVs.

ABSTRACT

Introduction: The H3N8 avian influenza virus (AIV) is recognized for its capacity for interspecies transmission and has been detected in multiple mammalian hosts. Between 2022 and 2023, three human infections with H3N8 were documented in China, raising significant concerns about its zoonotic spillover potential. In this study, we characterized an H3N8 isolate from Niukouyu Wetland Park in Beijing Municipality to elucidate the genetic variability and evolutionary dynamics of this AIV subtype.

Methods: The virus underwent whole-genome sequencing followed by comprehensive molecular and phylogenetic characterization.

Results: We identified a genetically reassorted, low-pathogenicity H3N8 AIV, marking the first detection of this subtype in a wild environment in Beijing. Throat swabs from the park staff tested negative for influenza viruses. Phylogenetic analyses demonstrated that the viral hemagglutinin and neuraminidase genes originated from Eurasian and North American lineages, respectively. Nucleotide sequence comparisons revealed 97.57%–99.06% similarity between the eight gene segments of this virus and those of reference strains. Multiple internal gene mutations were identified, including PB2-K318R and PB1-F2-N66S, which are associated with enhanced polymerase activity, increased virulence, and improved mammalian adaptation.

Conclusions: The molecular characteristics of this H3N8 virus indicate a potential risk for cross-species transmission to humans, emphasizing the critical need to strengthen influenza surveillance networks and expand monitoring efforts targeting H3 subtype AIVs.

Avian influenza viruses (AIVs) pose a persistent

threat to poultry, mammals, and humans due to their high mutation rates, complex reassortment mechanisms, and capacity for interspecies transmission. Among all influenza A virus subtypes, those belonging to the H3Ny group demonstrate the broadest host range. Notably, the H3N8 subtype has been detected in diverse mammalian hosts, including dogs (*1*), donkeys (*2*), horses (*3*), pigs (*4*), harbor seals (*5*), and humans (*6*). To date, three laboratory-confirmed human infections with H3N8 AIV have been reported globally, all occurring in China (*6*–*7*). Given this epidemiological context, active surveillance of AIVs in regions with high spillover potential remains crucial for public health preparedness. Beijing Municipality, situated within the East Asian-Australasian Flyway, harbors wetland ecosystems that serve as critical stopover and breeding sites for migratory birds. These areas represent high-risk interfaces for viral reassortment and cross-species transmission. In this study, we performed whole-genome sequencing of an H3N8 virus identified from environmental surveillance samples collected in Beijing during September 2024. We conducted subsequent phylogenetic and molecular analyses to characterize the genetic evolution of this virus and identify key mutations. This investigation aimed to elucidate the genetic variability and evolutionary dynamics of H3N8 AIVs circulating in Beijing, China.

During September 2024, a total of 3,110 environmental specimens from wild birds and domestic poultry (including feces, water samples, and environmental surface swabs) were collected from 8 districts of Beijing: Daxing, Huairou, Shunyi, Miyun, Tongzhou, Fangshan, Xicheng, and Yanqing. Real-time PCR was performed using the Influenza A Virus Nucleic Acid Detection Kit (PCR-Fluorescence Probing) (Londe Medical Co., Ltd., Cat. No. V2.1) to identify influenza A virus in the collected environmental samples. A multiplex real-time PCR method was performed using the AIVs Typing Test Kit (MABSKY BIO-TECH CO., LTD., SKY-8908F) to detect the subtypes of positive samples. Following AIV identification, throat swab tests were performed on staff members from an islet in Niukouyu Wetland Park, Fangshan District.

The genome of the A/environment/Beijing/03/2024 (H3N8) (BJ03) virus was amplified using a SuperScript® III One Step RT-PCR Platinum Taq HiFi kit (Invitrogen, USA), and sequencing libraries were prepared using a Nextera XT DNA sample preparation kit. Whole-genome sequencing was performed on the Illumina MiniSeq platform with a 2×150 bp paired-end sequencing kit. Raw sequencing data were processed and assembled using CLC Workbench (version 10, QIAGEN, Germany), yielding complete genome sequences for all segments with an average coverage depth exceeding 800×, thereby ensuring that 100% of genomic regions were covered by at least 100 reads. The eight gene segments of the assembled BJ03 genome were submitted to the NCBI BLAST online tool for homology comparison. For phylogenetic analysis, reference strains included 1) representative classical H3N8 strains, 2) strains exhibiting high genetic similarity to the BJ03 isolate, and 3) known human-infecting H3N8 strains. Reference sequences were downloaded from the NCBI and GISAID databases for multiple sequence alignment (performed using MEGA 6.0) and phylogenetic tree construction. Phylogenetic trees were constructed using the neighbor-joining method in MEGA 6.0 with 1,000 bootstrap replicates. Glycosylation site prediction of the BJ03 HA and NA amino acid sequences was performed using NetNGlyc (version 1.0, Lyngby, Denmark).

Analysis of collected specimens revealed AIV positivity in 43 samples: 3 for H3N8, 35 for H7N1 (all detected in Niukouyu Wetland Park in March 2024), and 3 for H5N1. Additionally, 2 specimens tested positive for mixed H5N1 and H9 AIVs. The identification of H3N8 AIV from the Niukouyu Wetland Park sample represents the first detection of this subtype in a wild environment in Beijing. One of the three H3N8-positive specimens, designated A/environment/Beijing/03/2024(H3N8) (EPI_ISL_20063625) and hereafter referred to as BJ03, was successfully sequenced. Following detection of H3N8 viruses in the environmental sample, staff members working on the wetland park islet underwent influenza testing. All staff members tested negative for influenza viruses and remained asymptomatic throughout the surveillance period.

BLAST analysis revealed that the *HA* and *NA* genes of BJ03 exhibited the highest nucleotide sequence similarity with the corresponding genes of A/Wild duck/South Korea/KNU2020-104/2020(H3N8) and A/Muscovy duck/Vietnam/HN5257/2019(H4N8), respectively (Table 1). The *PB2* gene demonstrated 99.06% nucleotide identity with that of an H4N6 AIV isolated from chickens in Vietnam, whereas the *PB1* gene showed the greatest similarity to that of an H7N6 AIV from wild birds in China. The *PA*, *NP*, and *M* genes of BJ03 exhibited the highest sequence similarity

TABLE 1. Nucleotide sequence similarity of BJ03 gene segments, an H3N8 AIV identified in Beijing in 2024, compared with those of other AIVs.

Gene	Length (bp)	Strain with the highest similarity	NCBI ID	Similarity (%)
PB2	2,341	A/chicken/Viet Nam/LBFecal200LC/2021(H4N6)	PV410220.1	99.06
PB1	2,297	A/wild bird/China/SUB12913325.2/2021(H7N6)	OQ509912.1	98.48
PA	2,199	A/duck/Tottori/311215/2020(H5N2)	LC656332.1	98.54
HA	1,701	A/wild duck/South Korea/KNU2020-104/2020(H3N8)	OK236003.1	98.41
NP	1,551	A/duck/Bangladesh/58751/2023(H6N2)	PP680426.1	98.64
NA	1,433	A/Muscovy duck/Vietnam/HN5257/2019(H4N8)	MW935581.1	97.56
M	982	A/duck/Akita/51019/2017(H5N3)	MK592464.1	98.78
NS	855	A/environment/Bangladesh/42635/2020(H10N7)	MW466161.1	98.60

Abbreviation: AIV=Avian Influenza Virus; PB2=Polymerase Basic 2; PB1=Polymerase Basic 1; PA=Polymerase Acidic; HA=Hemagglutinin; NP=Nucleoprotein; NA=Neuraminidase; M=Matrix; NS=Non-Structural; NCBI=National Center for Biotechnology Information.

to the corresponding genes from H5N2, H6N2, and H5N3 AIVs isolated from ducks in Tottori (Japan), Bangladesh, and Akita (Japan), respectively. The *NS* gene was most similar to that of an H10N7 AIV detected in an environmental sample from Bangladesh.

Phylogenetic analysis of the *HA* gene from BJ03 demonstrated that this virus belongs to the Eurasian lineage (Figure 1A). The amino acid sequence at the HA cleavage site (PEKQTR ↓ GLF) contains only one basic residue, consistent with low-pathogenicity AIV characteristics. Amino acid mutations identified in this and other genes (*6*,*8*), compared with those from reference viruses, are summarized in Table 2. The α-2,3 sialic acid receptor-binding sites of BJ03 retained the avian-origin residues Q242 and G244 without mutations (*6*). Additional receptor-binding motifs, including ^{151}GSG and ^{206}EQTN, also remained unchanged (*6*). Analysis of the BJ03 HA amino acid sequence identified six potential N-linked glycosylation sites: ^{24}NSTA, ^{38}NGTI, ^{54}NATE, ^{181}NVTM, ^{301}NGSI, and ^{499}NGTY. All six sites exhibited high conservation when compared with those of the reference strains.

The full-length *NA* gene of BJ03 spans 1,433 bp, encoding 476 amino acids, and phylogenetically clusters within the North American lineage (Figure 1B). Examination of antiviral resistance sites revealed an I312V substitution, suggesting potential alterations in oseltamivir susceptibility. Furthermore, four putative N-linked glycosylation sites were identified within the *NA* sequence: ^{46}NETV, ^{54}NETV, ^{144}NGTV, and ^{293}NWTG.

Analysis of the internal genes of BJ03 revealed multiple key mutations: K318R, K389R, and V598T in *PB2*; L13P and L473V in *PB1*; N66S in *PB1-F2*; K26E and V160D in *PA*; K398Q in *NP*; N30D and T215A in *M1*; V27I in *M2*; and P42S in *NS1* (Table 2). These mutations are associated with enhanced virulence, pathogenicity, and mammalian host adaptation.

DISCUSSION

The *H3* and *N8* genes of BJ03 exhibited high nucleotide similarity with H3 subtypes circulating in wild ducks in Korea and N8 subtypes prevalent in Muscovy ducks in Vietnam, respectively. The internal genes demonstrated high nucleotide similarity with those of low-pathogenicity avian influenza virus (LPAIV) subtypes *H4*, *H5*, *H6*, *H7*, and *H10* previously identified in northeastern China, Vietnam, Japan, and Bangladesh. These findings suggest that BJ03 represents a reassortant virus generated through co-infection of a single avian host by multiple AIV subtypes along migratory flyways. Genetic exchange and reassortment among different LPAIV subtypes occur frequently in nature and can generate novel highly pathogenic strains when highly pathogenic AIVs undergo reassortment within the host (*9*). Consequently, LPAIVs not only carry an intrinsic risk of genetic evolution but also pose a substantial threat to public health.

The Eurasian lineage of the *H3* gene and North American lineage of the *N8* gene in BJ03 are consistent with patterns observed in human-infecting H3N8 AIV strains, including A/Changsha/1000/2022(H3N8), A/Henan/4-10CNIC/2022(H3N8), and A/Guangdong/ZS-2023SF005/2023(H3N8). Studies in southern China have demonstrated that reassortment between Eurasian and North American lineage genes is common among H3Ny subtypes (*10*). Migratory birds can harbor viruses carrying this *N8*

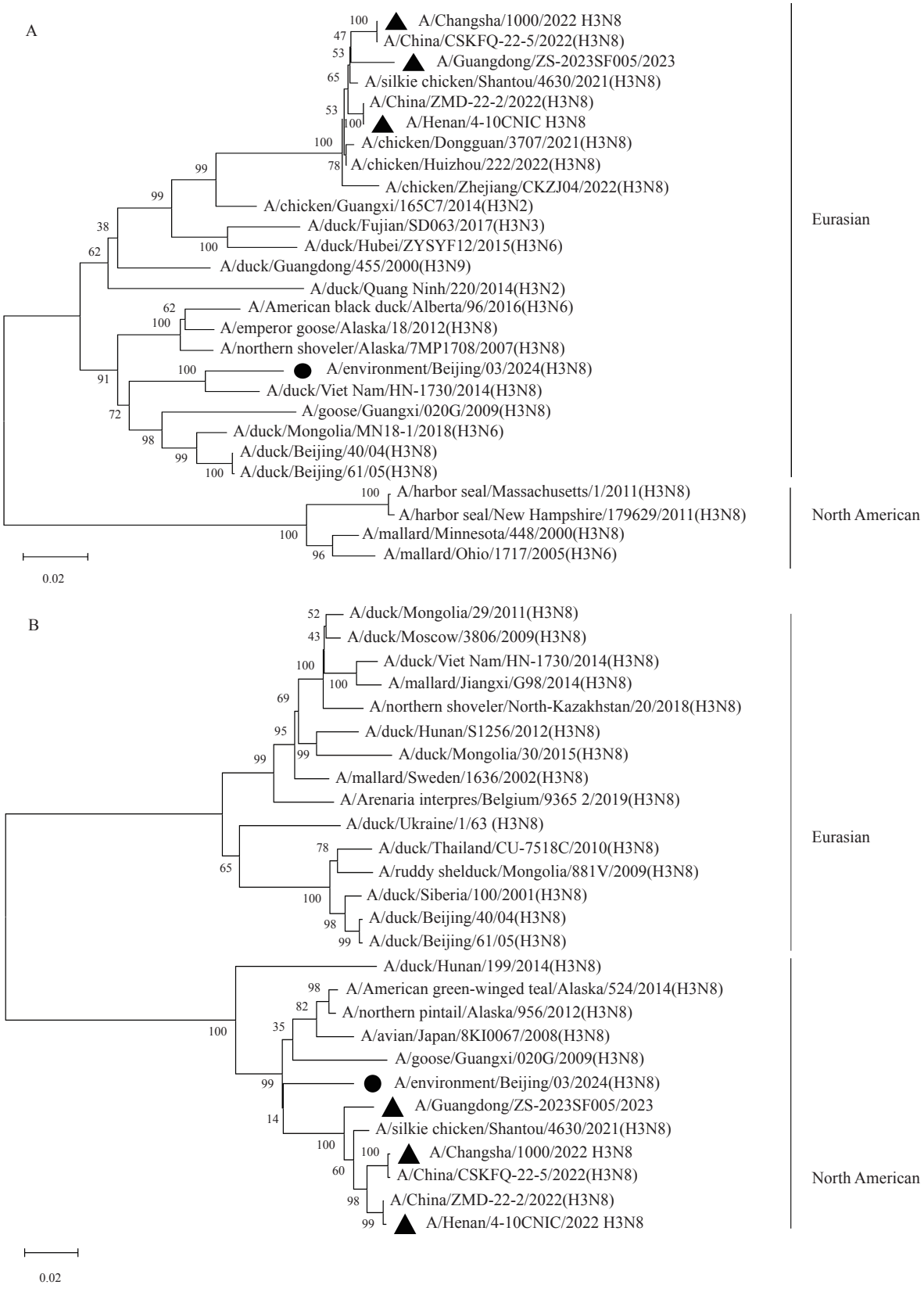

FIGURE 1. Phylogenetic trees based on (A) HA and (B) NA genes of A/environment/Beijing/03/2024(H3N8).
Note: ● means BJ03 specimen from this study; ▲ means reference H3N8 avian influenza virus strains previously associated with human infections.
Abbreviation: HA=hemagglutinin; NA=neuraminidase.

TABLE 2. Comparison of key sites in the predicted amino acid sequences encoded by the genes of BJ03 (an H3N8 AIV identified in Beijing in 2024) with those of other isolates.

Gene	Amino acid sites	Phenotypic characteristics	Isolate					
			BJ03	AHTT41	HK110MA213	GD01	BJ40	GX020G
HA	^{151}GSG^{153}	Receptor binding site	GSG	GSS	GSN	GSG	GSN	QSA
	^{206}EQTN^{209}	Receptor binding site	EQTN	EQTN	EQTS	EQTN	EQTN	EQTN
	Q242L	Receptor binding site	Q	Q	L	Q	Q	Q
	G244S	Receptor binding site	G	G	S	G	G	G
	Cleavage sites		PEKQTR ↓GLF	PEKQTR ↓GLF	PEKQTR ↓GLF	PEKQTR ↓GLF	PEKQTR ↓GLF	PEKQTR ↓GLF
NA	I312V	Increased resistance to oseltamivir	V	S	V	V	V	V
PB2	I292V	Mammalian host adaptation	I	I	T	V	I	I
	K318R	Mammalian host adaptation	R	R	R	R	R	R
	K389R	Increased virus replication ability in mammals	R	R	R	K	R	R
	V598T	Increased virus replication ability in mammals	T	T	T	V	T	T
	E627K	Mammalian host adaptation	E	E	K	E	E	E
	D701N	Mammalian host adaptation	D	D	D	D	D	D
PB1	L13P	Mammalian host adaptation	P	P	P	P	P	P
	L473V	Mammalian host adaptation	V	V	V	V	V	V
PB1-F2	N66S	Increased virulence in mice	S	N	N	N	S	N
PA	K26E	Mammalian host adaptation	E	E	E	E	E	E
	V160D	Mammalian host adaptation	D	D	D	D	D	D
	K356R	Mammalian host adaptation	K	K	R	R	K	K
NP	K398Q	Mammalian host adaptation	Q	Q	Q	Q	Q	Q
M1	V15I	Mammalian host adaptation	V	V	V	I	V	V
	N30D	Increase pathogenicity and transmission in mammals	D	D	D	D	D	D
	T215A	Increased virulence in mammals	A	A	A	A	A	A
M2	V27A/I/T	Increased resistance to adamantane	I	V	V	V	V	V
	S31N	Increased resistance to adamantane	S	S	S	N	S	S
NS1	P42S	Increased virulence in mice	S	S	S	S	A	S

Note: BJ03 refers to specimen A/environment/Beijing/03/2024(H3N8), AHTT41 to reference strain A/Anseriformes/Anhui/TT41/2014(H3N8), HK110MA213 to reference strain A/Hong Kong/1-10-MA21-3/1968(H3N2), GD01 to reference strain A/chicken/China/Guangdong/01/2022(H3N8), BJ04 to reference strain A/duck/Beijing/40/04(H3N8), and GX020G to reference strain A/goose/Guangxi/020G/2009(H3N8).
Abbreviation: AIV=Avian Influenza Virus; PB2=Polymerase Basic 2; PB1=Polymerase Basic 1; PA=Polymerase Acidic; HA=Hemagglutinin; NP=Nucleoprotein; NA=Neuraminidase; M=Matrix; NS=Non-Structural.

gene for extended periods, introducing them into domestic regions along the East Asia–Australasia flyway (*11*). This flyway represents a major migratory corridor connecting the Arctic Circle to Australia and hosts the greatest diversity and abundance of migratory bird species among the world's nine major flyways. The route spans a coastal wetland network stretching from the Russian Far East to Australia, encompassing 22 countries and regions. Given Beijing's location along this flyway, similar reassortment events may occur in this region. However, further surveillance studies are required to confirm this hypothesis.

Although BJ03 shares the same "Eurasian *H3*–North American *N8*" genomic framework with human-infecting H3N8 strains, its *H3* gene is phylogenetically distant from those strains, whereas its *N8* gene exhibits closer phylogenetic relatedness. This pattern highlights the complex ecological and evolutionary dynamics underlying H3N8 virus distribution. In China, H3 subtypes demonstrate a clear pattern of multiple co-circulating sublineages (*12*). The concurrent detection of H3N8 and H7N1 viruses in Niukouyu Wetland Park exemplifies the broader co-circulation pattern of multiple AIV subgroups and lineages observed throughout China. Although these sublineages share genetic homology, they have diverged through

geographical isolation and host adaptation. The regional coexistence of distinct subgroup viruses, such as H3N8 and H7N1, increases the probability of genetic reassortment due to overlapping host ranges — both subtypes readily infect ducks and other waterfowl. The N8 gene, which originated in North America and was introduced into Eurasia relatively recently by migratory birds, appears to have been maintained through avian transmission with minimal genetic variation. Nevertheless, the prolonged coexistence of this N8 gene with the locally prevalent H3 gene and other circulating subgroup viruses (such as H7N1) creates ongoing opportunities for adaptive mutation accumulation and genetic reassortment events.

The surface-expressed hemagglutinin (HA) glycoprotein plays a pivotal role in determining AIV pathogenicity and virulence, with its cleavage site sequence serving as a critical molecular marker. In BJ03, the predicted cleavage site sequence PEKQTR↓GLF contains a single basic amino acid residue, which is consistent with the molecular characteristics of HAs from LPAIVs. Residues Q242 and G244 on the HA protein represent canonical amino acids of avian origin and indicate a preferential binding affinity for avian-type receptors. Although glycosylation sites on the HA protein, particularly those near the receptor-binding domain, may influence viral virulence (*13*–*15*), no such glycosylation sites were identified near the receptor-binding domain in BJ03 (*13*). Nonetheless, the predicted proteins of BJ03 harbor several mutations associated with increased virulence in mammalian hosts, including N66S in PB1-F2, T215A and N30D in M1, and P42S in NS1 (*14*–*16*). These mutations have been linked to enhanced virulence in mice and increased pathogenicity and transmissibility in mammals (*14*–*16*). Additionally, several mammalian-adaptive mutations were detected, including K318R, K389R, and V598T in PB2; L13P and L473V in PB1; K26E and V160D in PA; and K398Q in NP (*17*). The presence of these mutations suggests that the BJ03 virus possesses considerable potential to cross the species barrier and infect humans, warranting heightened surveillance of H3 subtype AIVs. Furthermore, the mutations I312V in N8 and V27I in M2 suggest possible increased resistance to oseltamivir and amantadine, respectively; however, these findings require further experimental validation.

Although this study did not identify direct evidence of human infection with H3N8 AIVs, the molecular features of BJ03 suggesting mammalian host adaptation underscore a potential risk for zoonotic transmission. To date, all confirmed human infections with H3N8 AIVs have occurred in China, including two pediatric cases in 2022 and one adult female case in 2023 — the latter representing the first fatal H3N8 infection reported globally (*6*,*18*). All three cases involved prior exposure to live poultry, and in two instances, wild birds were reportedly active near the patients' residences. Although human-to-human transmission has not been documented, the propensity for genetic reassortment and the complexity of internal genes in H3N8 AIVs raise significant concerns regarding their pandemic potential (*19*). Consequently, sustained surveillance for pandemic risk remains critical. We recommend implementing a reinforced surveillance strategy that includes: 1) enhancing monitoring and information feedback systems in key areas with high wild bird activity; 2) establishing robust surveillance networks to ensure timely detection and reporting; and 3) strengthening multi-sectoral collaboration across public health, veterinary, and wildlife management agencies. A notable limitation of this study is the absence of *in vitro* or *in vivo* pathogenicity assessments. While our phylogenetic and molecular analyses provide compelling genetic evidence for the virus's potential mammalian adaptation and associated risks, these findings require functional validation through future experimental studies.

In conclusion, the H3N8 virus identified in this study exhibits genetic characteristics indicative of potential cross-species transmission to humans. Continuous surveillance and comprehensive risk assessment remain essential to mitigate the threat of emerging public health emergencies posed by avian influenza viruses.

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