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Evidence of an emerging triple-reassortant H3N3 avian influenza virus in China

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Abstract

The H3 subtype of avian influenza virus (AIV) stands out as one of the most prevalent subtypes, posing a significant threat to public health. In this study, a novel triple-reassortant H3N3 AIV designated A/chicken/China/16/2023 (H3N3), was isolated from a sick chicken in northern China. The complete genome of the isolate was determined using next-generation sequencing, and the AIV-like particles were confirmed via transmission electron microscopy. Subsequent phylogenetic analyses revealed that HA and NA genes of the H3N3 isolate clustered within the Eurasian lineage of AIVs, exhibiting the closest genetic relationship with other H3N3 AIVs identified in China during 2023. Interestingly, the HA and NA genes of the nove H3N3 isolate were originated from H3N8 and H10N3 AIVs, respectively, and the six internal genes originated from prevalent H9N2 AIVs. These findings indicated the novel H3N3 isolate possesses a complex genetic constellation, likely arising from multiple reassortment events involving H3N8, H9N2, and H10N3 subtype influenza viruses. Additionally, the presence of Q226 and T228 in the HA protein suggests the H3N3 virus preferentially binds to α-2,3-linked sialic acid receptors. The HA cleavage site motif (PEKQTR/GIF) and the absence of E627K and D701N mutations in PB2 protein classify the virus as a characteristic low pathogenicity AIV. However, several mutations in internal genes raise concerns about potential increases in viral resistance, virulence, and transmission in mammalian hosts. Overall, this study provides valuable insights into the molecular and genetic characterization of the emerging triple-reassortant H3N3 AIVs, and continued surveillance of domestic poultry is essential for monitoring the H3N3 subtype evolution and potential spread.

Keywords Avian influenza virus, H3N3, Reassortment, Phylogenetics, Molecular characterization

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Introduction

Avian influenza viruses (AIVs) are considered significant pathogens affecting a wide range of species, primarily birds and mammals [1–3]. AIVs belong to the Alphainfluenzavirus genus of the *Orthomyxoviridae* family. As segmented negative-sense RNA viruses, AIVs exhibit high genetic diversity due to reassortment, enabling the emergence of novel subtypes. Currently, AIVs are classified into 16 HA (H1-H16) and 9 NA (N1-N9) subtypes based on the antigenicity diversity and genetic evolution of the HA and NA proteins, additionally, H17, H18, N9, and N10 were also described for influenza from bats [4, 5]. Intersubtypic reassortment between different AIVs facilitates gene exchange, leading to increased genetic diversity and the potential for antigenic shift. Furthermore, multiple reassortment events may lead to the generation of novel AIVs with increased capacity to replicate and transmit in mammals [6, 7]. The novel H3N8 AIV, originating from chickens is a zoonotic virus capable of infecting humans. The virus is a typical triple reassortant strain, combining the Eurasian avian H3 gene, the North American avian N8 gene, and the dynamic internal genes of the H9N2 virus [7].

Although waterfowls and shorebirds are well known as the natural reservoirs of AIVs, these viruses have also established themselves in domestic poultry populations [5, 8]. Based on their pathogenicity in chickens, AIVs were categorized into highly pathogenic avian influenza viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) by the World Organization for Animal Health (WOAH) [9]. While HPAIVs, primarily H5 and H7 subtypes, pose severe threats to poultry and public health, LPAIVs often cause mild or asymptomatic infections. However, LPAIVs play a crucial role in the evolution of AIVs, as they can serve as a reservoir for donating gene segments that can contribute to the emergence of novel and more pathogenic strains [2, 10, 11]. Specifically, novel H7N2 AIVs have emerged through reassortment between H9N2 LPAIV and H7N9 HPAIV. The H9N2 LPAIV contributed the PB2, PB1, PA, NP, and NA genes, while the H7N9 HPAIV provided the HA, M, and NS genes. This reassortment event has significantly increased the threat to poultry populations [12].

Among LPAIVs, the H3 subtype is common in birds, causing a range of symptoms from mild illness to severe disease in domestic poultry [13]. While most H3 subtype AIV infections in poultry are asymptomatic, their potential for cross-species transmission to humans poses a significant public health threat [13, 14]. Historically, H3 subtype AIVs have played a significant role in influenza pandemics, notably the 1968 Hong Kong H3N2 pandemic resulting from reassortment with human H2N2 influenza viruses [15, 16]. More recently, the emergence of H3N8 AIVs capable of infecting humans has raised concerns about the zoonotic potential of this subtype [17]. Three reported human cases in China, including a fatal case, have highlighted the severity of these infections [7, 17, 18]. Furthermore, H3 subtype AIVs exhibit a broad host range, encompassing wild and domestic birds, and mammals such as humans, swine, and seals [5, 19]. This extensive host diversity facilitates rapid viral evolution through antigenic drift and shift [20, 21]. Moreover, recent studies have documented the generation of novel H3 subtype AIVs through reassortment with H5, H7, and H10 subtypes, increasing genetic diversity and the potential for cross-species transmission [22–24].

Notably over the past decades, H3 subtypes AIVs have established lineages in poultry worldwide [13]. While H3N3 AIVs were historically uncommon, primarily isolated from ducks, a recent surge in detections in chickens from China, South Korea, and Vietnam has raised significant concerns [10, 25, 26]. These newly detected H3N3 AIVs are pathogenic to chickens, causing reduced egg production, respiratory symptoms including discharge from the eyes, nostrils and mouth, and severe dyspnea. Pathologically, the chickens infected with the novel H3N3 AIVs showed histopathological damage to vital organs including an increase in fat vacuoles between alveoli and mass erythrocytes in the alveolar interstitium [10]. Unfortunately, limited genetic data and inadequate monitoring have hindered our understanding of the evolutionary relationships and molecular characteristics of these novel H3N3 AIVs.

This study isolated and sequenced a novel H3N3 avian influenza virus (AIV), designated A/chicken/China/16/2022 (H3N3), from poultry in Liaoning Province, China. A systematic investigation of its molecular and genetic characterization was carried out to enrich our understanding of the emerging H3N3 AIVs, which are also essential in the prevention and control of the H3N3 avian influenza in domestic poultry.

Results

Virus isolation and transmission electron microscopy

The oropharyngeal and cloacal samples from one sick chicken were identified as positive for influenza virus by reverse transcription-PCR (RT-PCR). Further characterization revealed the novel influenza virus as H3N3 AIV, as confirmed by Sanger sequencing of the HA and NA genes. Moreover, Transmission electron microscopy (TEM) analysis of the allantoic fluid samples from infected specific pathogen-free (SPF) chicken embryos revealed the presence of influenza virus-like particles, characterized by their typical vesicular and spiked morphology (Fig. 1).

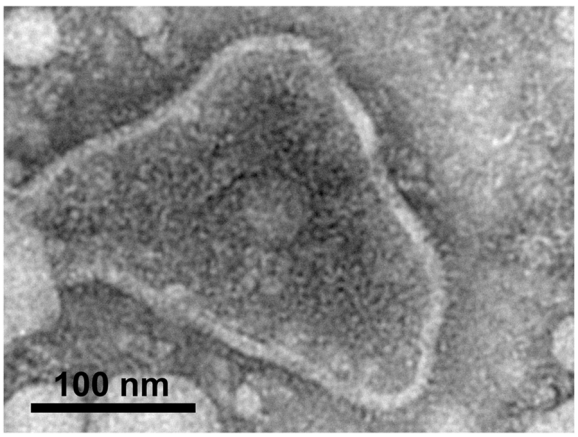

Fig. 1 Transmission electron microscopy of Influenza Virus-Like Particles. Transmission electron micrograph of negatively stained allantoic fluid from SPF chicken embryos infected with the AIV isolate. Bar = 100 nm

In this study, we comprehensively analyzed all available influenza virus strain information from the GenBank and GISAID EpiFlu™ database (Information to be collected by August 2024). Despite a relatively small total number (n = 67) of H3N3 influenza virus isolates recorded since its initial detection in 1972, the virus exhibited a broad host range, including swine, humans, seals, and avian species (Fig. 2A). Furthermore, avian species, particularly chickens, ducks, geese and other birds were the primary hosts for H3N3 viruses. Notably, ducks constituted the largest host group, representing over half (49.3%) of all the H3N3 virus hosts, followed by the environment isolates (13.4%). In contrast, the isolation rate from chickens was relatively low (9.0%) (Fig. 2B). Moreover, these data also imply that the novel H3N3 AIVs isolated from chickens appeared in China in 2023.

Genomic structure and comparative analysis

The complete genome of A/chicken/China/16/2023 (H3N3) was sequenced by next-generation sequencing (NGS). It comprised eight open reading frames (ORFs) encoding PB2 (2280 nucleotides, nt), PB1 (2274 nt), PA (2151 nt), HA (1707 nt), NP (1497 nt), NA (1410 nt), M (982 nt), and NS (876 nt). The genome organization was consistent with other members of the Influenzavirus A genus (Table 1). The amino acid sequences of the

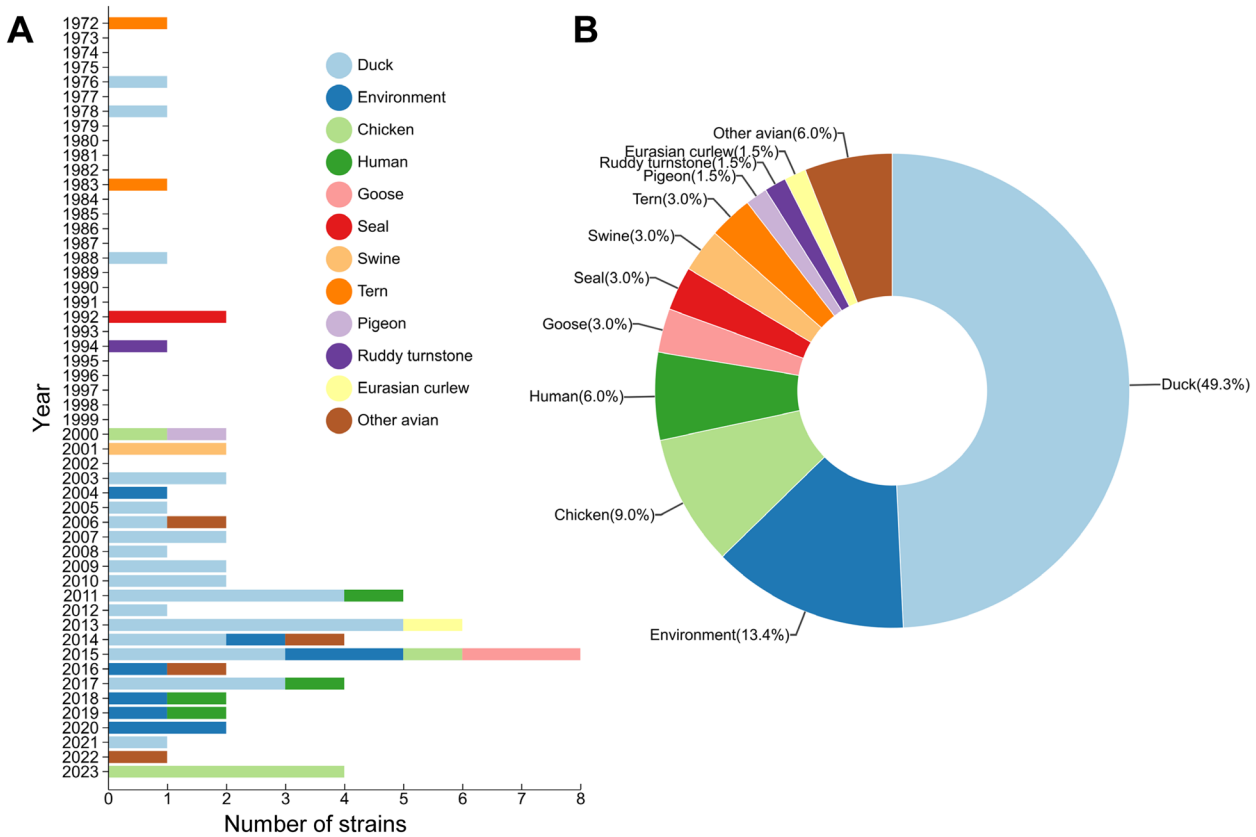

Fig. 2 Distribution of H3N3 Influenza Viruses available in GenBank and GISAID EpiFlu™ Databases. **A** Temporal distribution of the H3N3 influenza virus isolates based on year of isolation. **B** Host species distribution of the H3N3 influenza viruses represented as a percentage of the total

Table 1 Genomic features of A/chicken/China/16/2023(H3N3) strain

Segment number	Gene	Abbreviation	Coding sequence (from-to)[a]	Total length/nt	Protein length/aa[b]
1	Polymerase PB2	PB2	1–2280	2280	759
2	Polymerase PB1 and PB1-F2 protein	PB1	1–2274; PB1-F2: 95–367	2274	847
3	Polymerase PA and PA-X protein	PA	1–2151; PA-X: 1–570, 572–760	2151	968
4	Hemagglutinin	HA	1–1701	1707	566
5	Nucleocapsid protein	NP	1–1497	1497	498
6	Neuraminidase	NA	1–1410	1410	469
7	Matrix protein 2 and matrix protein 1	M	M1: 1–759; M2: 1–26, 715–982	982	97
8	Nuclear export protein and nonstructural protein 1	NS	NS1: 21–674; NS2: 21–50, 523–858	876	338

[a] Including stop codon

[b] Without stop codon

encoded proteins were similar in length and number to those of other AIVs. Additionally, the complete coding sequence (CDS) of this novel H3N3 AIV isolate was annotated (Table 1).

Comparative nucleotide sequence analysis of each gene was performed using BLAST against the GenBank database. As shown in Table 2, comparative nucleotide sequence analysis revealed high identity between the H3N3 AIV genome segments and their closest influenza virus counterparts: 98.82% (PB2), 99.16% (PB1), 99.30% (PA), 99.06% (HA), 99.20% (NP), 99.57% (NA), 99.19% (M), and 99.20% (NS), respectively. Intriguingly, five of the H3N3 AIV genes identified in this study exhibited the highest nucleotide sequence similarity to the novel H3N3 AIVs isolated in China in 2023. The remaining three genes showed the highest homology to H3N8 AIVs isolated in China in 2022. In addition, three genes (PB1, PA, and NP) also shared a high sequence similarity with H9N2 subtype AIVs, while the NA gene had a high degree of homology to H10N3 AIVs. These viruses with the highest homology to the novel H3N3 isolate were widely distributed in China (Fig. 3A). These findings strongly suggest that the novel H3N3 AIV had close relationships with the H9N2, H3N8, and H10N3 influenza viruses that were isolated from various regions of China.

Notably, the HA and PA gene of the H3N3 isolate exhibited high nucleotide homology with A/China/ZMD-22–2/2022(H3N8) strain, isolated from a human [27], while the NS gene was similar to A/Duck/Jiangxi/447/2022(H3N8) isolated from a duck. The PB1 gene showed high homology to A/ swine/ Shandong/ TA009/ 2019/ (H9N2) isolated from a pig. These results indicate that the H3N3 virus is likely to be closely associated with AIVs from multiple host species, including chickens, humans, ducks, and pigs.

Phylogenetic analysis of HA and NA genes

To explore the evolutionary relationships of the novel H3N3 AIV, phylogenetic trees were constructed by using the full-length nucleotide sequences of the surface genes (HA gene and NA gene). The best-fit models for the phylogenetic trees based on HA and NA genes were determined by using MEGA software. As listed in supplementary Table S1, the HA genetic tree was performed by using the GTR + G + I model, and the T92 + G model was utilized for the phylogenetic tree of the NA gene. Phylogenetic analyses of the HA and NA genes revealed a clear division of the reference AIV isolates into Eurasian and North American lineages. The newly characterized A/chicken/China/16/2023(H3N3) strain was confidently assigned to the Eurasian lineage (Fig. 4).

H3 subtype influenza viruses are typically classified based on their host range, including human, avian, swine and equine. Phylogenetic analysis of the HA gene further supports this classification. As shown in Fig. 4A, the maximum likelihood (ML) tree of the HA gene definitively places the novel H3N3 strain identified in this study within the Eurasian lineage of avian-origin H3 AIVs, and confirms its derivation from H3N8 AIVs. Within the Eurasian lineage of the HA gene-based evolutionary tree, the novel H3N3 strain (A/chicken/China/16/2023(H3N3)) was situated within a subclade, alongside other H3N3 AIVs isolated from China in 2023, including A/chicken/China/GT2125/2023(H3N3), A/chicken/China/YC01/2023(H3N3), A/chicken/Henan Shangqiu/SQ2049/2023(2023), and A/chicken/China/HN0120/2023(H3N3), indicating that the emerging H3N3 avian influenza viruses have similar phylogenetic links. Specifically, the novel H3N3 strain had the closest genetic evolutionary relationship to A/chicken/China/

Table 2 Sequence identity of novel H3N3 AIV with closest homologous Influenza Viruses

Gene	Viruses with greatest homology	Nucleotide identity (%)	GenBank Accession No.
PB2	A/chicken/China/YC01/2023(H3N3)	99.82	PP998321
	A/chicken/Jiangsu/CKJS612/2022(H3N8)	99.25	OP225627
	A/chicken/China/GT2125/2023(H3N3)	99.12	PP077085
	A/chicken/Jiangsu/CKJS03/2022(H3N8)	99.12	OP024214
PB1	A/chicken/Jiangsu/CKJS03/2022(H3N8)	99.16	OP024215
	A/chicken/Jiangsu/CKJS64/2022(H3N8)	99.08	OP225636
	A/environment-air/Kunshan/NIOSH-BL20/2018(H9N2)	98.24	MN606218
	A/swine/Shandong/TA009/2019(H9N2)	98.01	MT265024
PA	A/chicken/China/GT2125/2023(H3N3)	99.30	PP077083
	A/chicken/Jiangsu/CKJS612/2022(H3N8)	98.23	OP225629
	A/chicken/Jiangsu/CKJS610/2022(H3N8)	98.23	OP225621
	A/chicken/Anhui/CKAH02/2022(H3N8)	98.19	OP024208
	A/chicken/China/2-3-1/2022(H9N2)	98.19	OR528343
	A/China/ZMD-22-2/2022(H3N8)	98.19	ON342808
HA	A/chicken/China/YC01/2023(H3N3)	99.06	PP977193
	A/chicken/China/HN0120/2023(H3N3)	99.06	PP838576
	A/China/ZMD-22-2/2022(H3N8)	98.77	ON342803
	A/chicken/Anhui/CKAH01/2022(H3N8)	98.77	OP024201
	A/chicken/Anhui/CKAH02/2022(H3N8)	98.77	OP024209
NP	A/chicken/Jiangsu/CKJS610/2022(H3N8)	99.20	OP225623
	A/chicken/Zhejiang/CKZJ04/2022(H3N8)	99.13	OP024226
	A/chicken/Anhui/12.25_YHZGS004-O/2018(H9N2)	98.33	MW102497
	A/chicken/China/HP-1/2018(H9N2)	98.33	OR528592
NA	A/chicken/China/YC01/2023(H3N3)	99.57	PP977197
	A/chicken/Henan Shangqiu/SQ2049/2023(H3N3)	99.57	PP758467
	A/chicken/China/HN0120/2023(H3N3)	99.57	PP838577
	A/chicken/China/GT2125/2023(H3N3)	99.15	PP077080
	A/Enviroment/China/04940NA/2021(H10N3)	98.72	OM802522
M	A/chicken/Dongguan/364/2022(H3N8)	99.19	OQ291664
	A/chicken/Jiangsu/CKJS03/2022(H3N8)	99.19	OP024220
	A/chicken/Huizhou/104/2022(H3N8)	99.19	OQ291992
NS	A/chicken/China/GT2125/2023(H3N3)	99.20	PP077082
	A/chicken/Huizhou/231/2022(H3N8)	98.69	OQ292065
	A/Duck/Jiangxi/447/2022(H3N8)	98.40	OQ826115
	A/chicken/Hong Kong/21-17040/2021(H3N8)	98.25	ON909101

GT2125/2023(H3N3) isolated from chicken, boasting robust bootstrap support (Fig. 4A). Notably, a human-derived H3N8 strain, A/China/ZMD-22–2/2022 (H3N8), had a relatively close genetic evolutionary link to the novel H3N3 AIV identified in this study, suggested a potential evolutionary link between the HA genes of human H3N8 virus and novel H3N3 AIVs, which likely originated from a common ancestor.

Phylogenetic analysis of the NA gene, as depicted in Fig. 4B, revealed a clear division into north American and Eurasian lineages. The novel H3N3 isolate clustered within the Eurasian lineage of H3 subtype AIVs. Similar to the phylogenetic analysis of the HA gene, The ML tree also showed that the NA gene of these emerging H3N3 AIVs formed a distinct subclade. Within the subclade, the novel H3N3 virus isolated in this study exhibited the closest relationship to A/chicken/China/GT2125/2023(H3N3). Additionally, a human-derived H10N3 strain (A/China/0428NA/2021 (H10N3)) was genetically related to the novel H3N3 virus, exhibits a close relationship between the novel H3N3 AIVs and the human-derived H10N3 strain. These findings strongly support a common ancestral origin for the H3N3 and these other H3N3 AIVs, further solidifying the phylogenetic placement of the H3N3 isolate based on the NA gene.

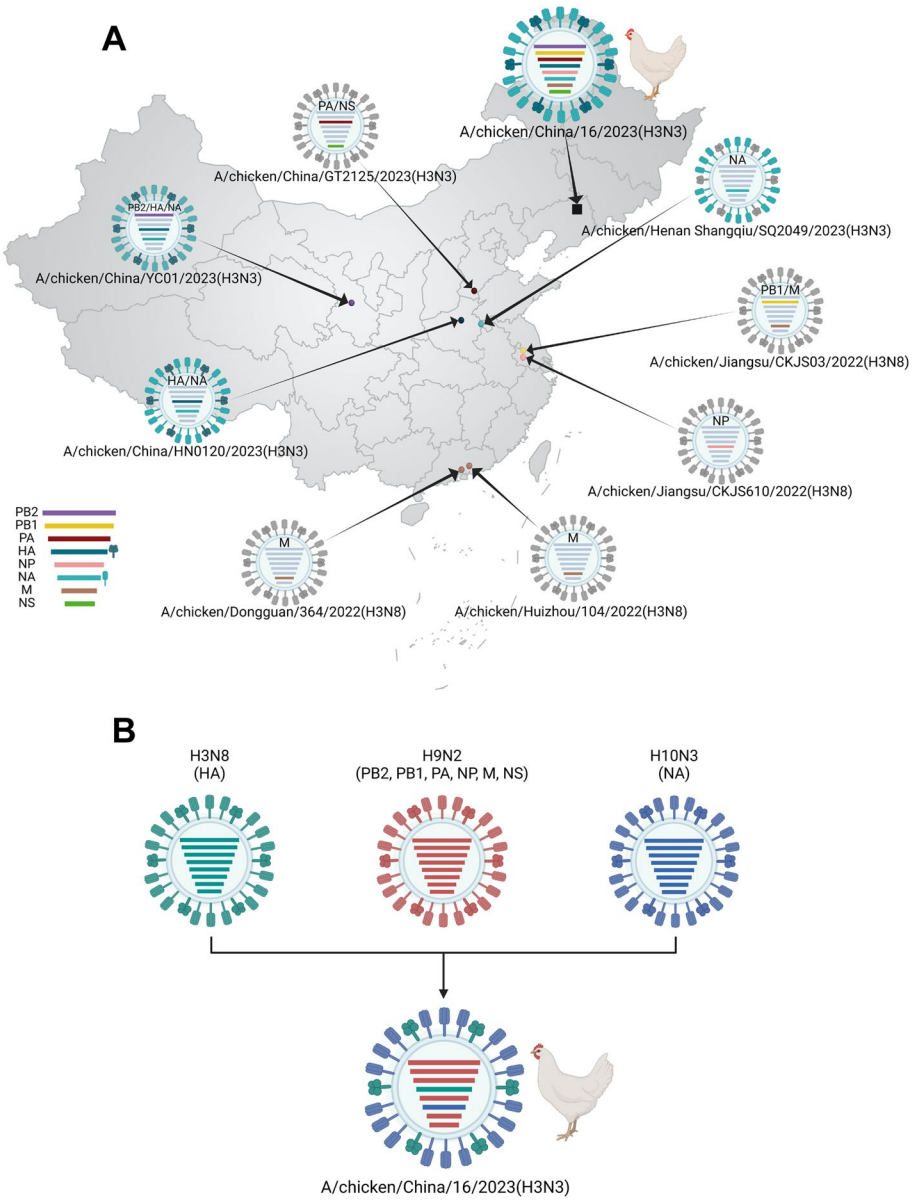

Fig. 3 The related virus's distribution and reassortment event of the novel H3N3 isolate. Distribution of the viruses and reassortment events associated with novel H3N3 virus isolate (A/chicken/China/16/2023(H3N3)). **A** Geographic distribution of viruses with the highest homology of the novel H3N3 isolate. The map represents China. If the collection region of the virus was not available, the city of the institution submitting to the GenBank and GISAID EpiFlu™ databases is reported. **B** Schematic representation of the hypothesized reassortment events that led to the H3N3 virus identified in this study. The viral genome segments are depicted in descending order: PB2, PB1, PA, HA, NP, NA, M, and NS. The two illustrations were created with BioRender.com

Phylogenetic analysis of internal genes

To further elucidate the evolutionary history of this novel H3N3 AIV, phylogenetic analyses were conducted on the full-length nucleotide sequences of the remaining six internal genes. The optimal substitution model and its corresponding parameters for tree construction are detailed in Supplementary Table S1. The GTR + G + I model was used for the phylogenetic analysis of PB2, NP and M genes, and the phylogenetic trees based on PB1, PA and NS genes were performed using the GTR + G model. Overall, the internal genes of the novel H3N3 isolate were classified in the lineage of H9N2 AIVs. Among

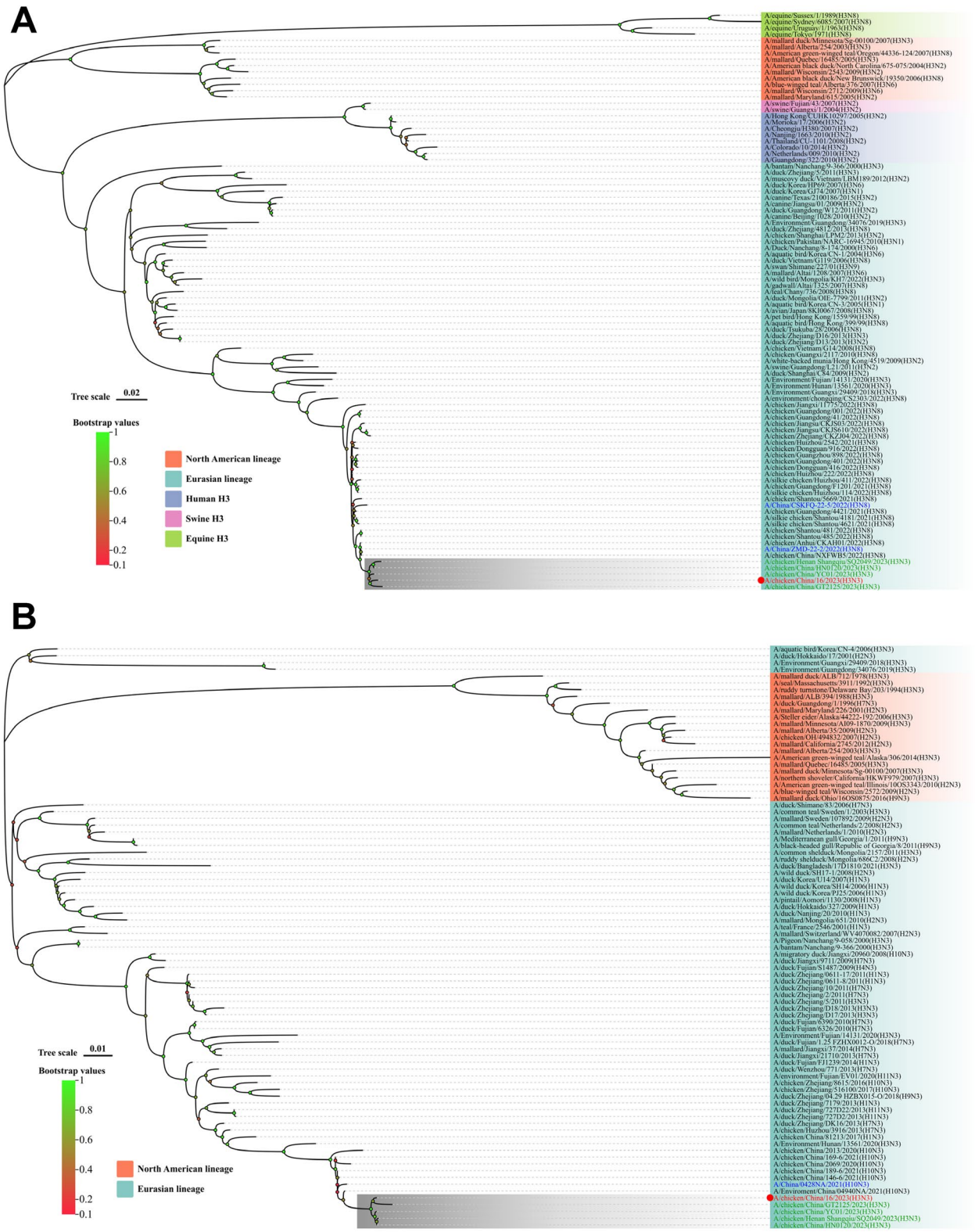

Fig. 4 Phylogenetic relationships of HA (**A**) and NA (**B**) genes of the novel H3N3 AIV isolated in this study. The maximum likelihood (ML) trees were constructed from multiple alignments of the nucleotide sequences using MEGA software (Version 11.0.11) with 1000 bootstrap replicates. The H3N3 virus from this study was highlighted using red text, the human-derived viruses in the Eurasian lineage of AIV were marked in blue, and the other H3N3 AIVs identified in 2023 were marked in green

them, four genes (PB1, PA, NP, NS) were further categorised into the F/98-like lineage of H9N2 AIVs, and the other two genes (PB2, M) were categorised within the G1-like lineage of H9N2 AIVs.

In the ML tree of the PB2 gene (Supplementary Fig. S1), the H3N3 AIV isolated in this study were closely related to prevalent H3N3, H9N2 and H3N8 AIVs. Specifically, the PB2 gene of the novel H3N3 virus exhibited the closest relationship to the other H3N3 AIV (A/chicken/China/YC01/2023(H3N3)). These two H3N3 viruses formed a subclade with these H3N8 AIVs identified in China in 2022, suggesting that based on the PB2 gene, implying these viruses are genetically related, and likely share a common ancestor.

In contrast, analysis of the PB1 gene (Supplementary Fig. S2) indicated a closer genetic relationship between the H3N3 strain and two H3N8 AIVs (A/chicken/Jiangsu/CKJS64/2022(H3N8), A/chicken/Jiangsu/CKJS03/2022(H3N8)) isolated in Jiangsu province, China in 2022. Strong bootstrap support for these relationships suggests a common ancestor for these viruses. Importantly, a human-derived H7N9 influenza virus (A/Changsha/41/2017(H7N9)) isolated in 2017 and a chicken-derived H7N9 influenza virus (A/chicken/Shandong/SD216/2016(H7N9)) isolated in 2016 exhibited the genetically related to the novel H3N3 strain. These phylogenetic results suggest that the origin of the PB1 gene may be similar in these H7N9 HPAIVs and the novel H3N3 strains.

In the H9N2 F/98-like lineage of the PA gene-based phylogenetic tree (Supplementary Fig. S3), the novel H3N3 strain exhibits the closest relationships with a novel chicken-derived H3N3 AIV (A/chicken/China/GT2125/2023) with robust bootstrap support. The two novel H3N3 viruses also formed a subclade, which was similar to the results of the phylogenetic analysis of the HA and NA genes. The ML tree of the NP gene reveals that the novel H3N3 strain had the closest evolutionary affiliation with two H3N8 AIVs (A/chicken/Zhejiang/CKZJ04/2022(H3N8) and A/chicken/Jiangsu/CKJS610/2022(H3N8)) isolated from China in 2022 (Supplementary Fig. S4). Notably, the other four novel H3N3 AIVs identified in 2023 were classified into a distinct subclade, which was distant from the H3N3 virus isolated in this study, indicating that there was a genetic difference between the novel H3N3 strain in this study and the other H3N3 AIVs based on the NP gene.

The M gene of the newly isolated H3N3 strain was clustered within the G1-lile lineage of H9N2 AIVs, sharing the closest genetic linkage with A/chicken/Jiangsu/CKJS03/2022(H3N8) identified in China (Supplementary Fig. S5). Moreover, the novel H3N3 virus also showed a closer evolutionary lineage to the chicken-derived H3N8 AIVs isolated from China, suggesting that they may shared a common ancestor. For the phylogenetic analysis of the NS gene, the novel H3N3 strain demonstrated its closest genetic connection with A/chicken/China/GT2125/2023(H3N3), which was similar to the results of the evolutionary analysis based on the PA, HA and NA genes. (Supplementary Fig. S6). Additonally, the novel H3N3 virus in this study formed a subclade with the A/chicken/China/GT2125/2023(H3N3), which was distinct from the subclade composed of another novel H3N3 AIVs (A/chicken/China/YC01/2023(H3N3), A/chicken/China/HN01/2023(H3N3), and A/chicken/China/Henan Shangqiu/ SQ2049/2023(H3N3)) isolated in 2023, implying that these novel H3N3 AIVs were genetically distinct at the NS gene level.

Overall, comprehensive phylogenetic analysis of all the novel H3N3 AIV isolate's genes revealed a

Table 3 Amino acid comparison of cleavage and receptor binding sites in the HA protein of the novel H3N3 isolate and reference influenza viruses

Strains	Cleavage sites	Receptor-binding sites (H3 numbering)									
		98	138	153	155	183	190	194	195	226	228
A/chicken/China/16/2023(H3N3)	PEKQTR/GIF	E	T	S	C	T	F	Y	I	Q	T
A/China/ZMD-22–2/2022(H3N8)[a]	PEKQTR/GLF	E	T	S	C	T	F	Y	I	Q	T
A/China/CSKFQ-22–5/2022(H3N8)[a]	PEKQTR/GLF	E	T	S	C	T	F	Y	I	Q	T
A/Iran/Clinical Sample/2019(H3N3)[a]	PEKQTR/GIF	K	N	S	C	T	F	Y	I	Q	A
A/bantam/Nanchang/9–366/2000(H3N3)	PEKQTR/GLF	E	T	G	C	T	F	Y	I	Q	T
A/duck/Zhejiang/D16/2013(H3N3)	PEKQTR/GLF	E	T	N	C	T	F	Y	I	Q	T
A/chicken/China/NXFWB5/2022(H3N8)	PEKQTR/GLF	E	T	S	C	T	F	Y	I	Q	T
A/chicken/Shantou/481/2022(H3N8)	PEKQTR/GLF	E	T	S	C	T	F	Y	I	Q	T
A/duck/Hunan/199/2014(H3N8)	PEKQTR/GLF	E	T	G	C	T	F	Y	I	Q	T

[a] The strain was isolated from human

complex reassortment history. This novel H3N3 virus was detected from the reassortment of H3N8, H9N2, and H10N3 subtypes of influenza virus. These donor viruses were likely to be widely distributed in China, highlighting the intricate reassortment events leading to the novel H3N3 AIV isolate (Fig. 3B).

Molecular characterization

To assess the potential risk of the novel H3N3 strain spilling over to mammals, we conducted a molecular characterization of its amino acid sequences. The H3N3 AIV cleavage site between HA1 and HA2 contains the amino acid motif PEKQTR/GIF, typical of avian H3 subtype influenza viruses. This monobasic cleavage site characteristic classifies the novel isolate as a LPAIV. It is well known that the receptor binding sites (RBSs) motif of HA protein are critically crucial for cellular receptor specificity and determine the host range of influenza viruses [28, 29]. We analyzed the amino acid sequences at the RBSs of the HA protein in the novel H3N3 strain and other referenced H3N3 and H3N8 influenza viruses. According to Table 3, the amino acids at the RBSs of the novel H3N3 strain were well conserved and did not show variation compared to the related H3N8 viruses isolated from humans and chickens. Consistent with other H3N3 AIVs, the conserved amino acids Q226 and T228 (H3 numbering) were identified in the RBS of this novel H3N3 isolate (Table 3), suggesting that this H3N3 isolate also binds to the α-2,3-linked sialic acid receptor, which is generally considered to be the primary receptor in avian species.

The specific polypeptide sequence for N-linked potential glycosylation was defined by the amino acid configuration of Asn-X-Ser/Thr, where X can represent any amino acid apart from proline [30]. The N-linked potential glycosylation of HA protein is associated with viral pathogenicity and affinity for the influenza virus receptor [31, 32]. For the novel H3N3 isolated in this study, the HA protein shared six N-linked potential glycosylation sites (PGSs) at positions 38, 54, 61, 181, 301 and 499, which were highly conserved with these referenced H3N8 viruses isolated from chickens and humans (Supplementary Table S2). Notably, eight PGSs of NA protein were detected at the positions of 14, 57, 66, 72, 146, 308, 387, and 401 (Supplementary Table S3). Comparative to the most similar H10N3 isolate at the NA gene level, the novel H3N3 strain isolated in this study has two additional PGSs at positions 387 and 401, respectively; whether this will alter the viral characteristic needs to be further investigated.

Furthermore, a detailed amino acid analysis of the gene segment of the novel H3N3 virus isolated in this study was carried out. As shown in Table 4, several mutations were observed including in the NA protein (M26I), PB2 protein (L89V, K251R, G309D, T339K, Q368R, R389K, H447Q, R477G and I495V), PB1 protein (D/A3V, L13P, R207K, K328N, S375N/T, H436Y, L473V and D622G), PA protein (H266R, K356R, N383D, S409N and S/A515T), NP protein (I353V), M1 protein (V15I, N30D and T215A), and NS1 protein (P/A42S, D97E and V149A). These mutations were reported to be related to an increase in the viral replication ability and virulence of AIVs in mice or mammalian cells (Table 4). Besides, mutations of PB1 protein (I368V) and PA protein (F277S, C278Q and L653P), which may facilitate the adaptation of AIVs to mammalian hosts, were also found (Table 4).

Moreover, the detection of a T160A substitution in the HA protein suggests enhanced binding affinity of the H3N3 isolate to human-type influenza receptors [33]. In contrast, the absence of Q591K, E627K, and D701N mutations in the PB2 protein indicates a low potential for cross-species transmission to mammals [41, 42, 47–49]. The NA protein's I117T mutation may reduce susceptibility to oseltamivir and zanamivir [37], while P/A42S and V149A substitutions in the NS1 protein could enhance interferon resistance [69, 71]. Additionally, the S31N mutation in the M2 protein confers resistance to amantadine and rimantadine [66, 67].

Overall, molecular characterization reveals that the novel H3N3 isolate predominantly binds to avian-type receptors despite possessing several mutations in internal genes that could potentially increase viral resistance, virulence, and transmission in mammalian hosts.

Discussion

This study conducted a comprehensive molecular and genetic analysis of a newly detected, triple-reassortant H3N3 AIV isolated from one poultry in China. Since the initial identification of the H3N3 influenza virus strain in 1972, there are currently more than 60 H3N3 isolates documented in the GenBank and GISAID EpiFlu™ databases. While the majority of these isolates originated from ducks, only six have been identified in chickens (Fig. 2A and 2B) [73]. Recent surveillance in Chinese chicken flocks has revealed an increasing prevalence of H3N3 subtype AIV infections [10, 74]. However, limited whole genome sequence data and a lack of understanding of their evolutionary relationship with other subtypes hinder our comprehensive characterization of this novel reassortant H3N3 strain [10, 75].

In this study, the whole genome sequence of the novel H3N3 isolate was determined by utilizing next-generation sequencing, and the genome was annotated based on the sequence characterization of the reported AIV isolates. The results showed that the genome structure of the novel H3N3 isolate is closely similar to other known AIVs in the GenBank and GISAID EpiFlu™ databases [73]. However,

Table 4 Molecular characterization of the novel H3N3 isolate presented in this study

Viral protein	Amino acid	A/chicken/China /16/2023(H3N3)	Comments	References
HA	T160A	A	Increased binding to human-type influenza receptor	[33]
	Q226L	Q	Increased binding to human-type influenza receptor	[34]
NA	M26I	I	Increased virulence in mice	[35]
	V116A	V	Resistance to neuraminidase inhibitors	[36]
	I117T	T	Reduces susceptibility to oseltamivir and zanamivir	[37]
	E119V	E	Resistance to neuraminidase inhibitors	[3, 38]
	Q136L	Q	Resistance to neuraminidase inhibitors	[39]
	R152W	R	Resistance to neuraminidase inhibitors	[38, 40]
PB2	L89V	V	Enhanced polymerase activity and virulence in mammals	[41]
	E158G	E	Enhanced polymerase activity and virulence in mice	[42]
	K251R	R	Increased virulence in mice	[43]
	G309D	D	Enhanced polymerase activity and virulence in mice	[41]
	T339K	K	Enhanced polymerase activity and virulence in mice	[41]
	Q368R	R	Increased polymerase activity and virulence in mice	[44, 45]
	R389K	K	Enhanced polymerase activity and virulence in mice/Mammalian host adaptation	[46]
	H447Q	Q	Increased polymerase activity and virulence in mammals	[44, 45]
	R477G	G	Enhanced polymerase activity and virulence in mice	[41]
	I495V	V	Enhanced polymerase activity and virulence in mice	[41]
	Q591K	Q	Enhanced replication efficiency and virulence in mice	[42]
	E627K	E	Mammalian host adaptation	[41, 47, 48]
	D701N	D	Increased polymerase activity and viral replication in mammalian	[49]
PB1	D/A3V	V	Increased polymerase activity and virulence in mice	[45]
	L13P	P	Increased polymerase activity and virulence in mammals, Mammalian host marker	[50]
	H99Y	H	Increased transmissibility in ferrets	[42, 46]
	R207K	K	Increased polymerase activity in mammalian cells	[51]
	K328N	N	Increased polymerase activity and virulence in mammals	[45]
	I368V	V	Mammalian host adaptation	[42]
	S375N/T	N	Increased polymerase activity and virulence in mammals, human host marker	[45]
	H436Y	Y	Increased polymerase activity and virulence in mallards, ferrets and mice	[51]
	L473V	V	Increased polymerase activity and replication efficiency	[2]
	D622G	G	Increased polymerase activity and virulence in mice	[52]
PB1-F2	N66S	N	Increased replication, virulence and antiviral response in mice/Increased virulence in mammals	[53]
	T68I	T	Increased virulence in mice	[54]
PA	S37A	S	Increased polymerase activity and viral replication in mammalian cells	[55]
	T97I	T	Enhances polymerase and virulence	[3, 42]
	V100A	V	Contributed to the virulence and mammalian adaptation	[56, 57]
	H266R	R	Increased polymerase activity and virulence in mammals and birds	[44]
	F277S	S	Mammalian host adaptation	[43]
	C278Q	Q	Mammalian host adaptation	[44]
	K356R	R	Enhanced virulence and mammalian adaptation	[58]
	N383D	D	Increased polymerase activity and mammalian adaptation	[59]
	A404S	A	Human host marker	[60]
	S409N	N	Increased polymerase activity, viral replication and virulence to mammalian; enhanced transmission	[61]
	S/A515T	T	Increased polymerase activity and virulence in mammals and birds	[51]
	L653P	P	Mammalian host adaptation	[44]

Table 4 (continued)

Viral protein	Amino acid	A/chicken/China /16/2023(H3N3)	Comments	References
NP	F253I	I	Results in attenuated pathogenicity of the virus in mice	[55]
	A286V	A	Affect the pathogenicity of the virus in mice	[62]
	N319K	N	Increased virulence in mammalian cells	[3, 63]
	I353V	V	Increased virulence in mice	[43]
	T437M	T	Affect the pathogenicity of the virus in mice	[62]
M1	V15I	I	Increased virulence in mammals	[2, 46, 64]
	N30D	D	Increased pathogenicity to mice	[65]
	A166V	A	Increased polymerase activity and virulence in mammals	[2]
	T215A	A	Increased virulence in mice	[65]
M2	L26P	L	Reduced susceptibility to amantadine and rimantadine	[66]
	V27A/I	V	Reduced susceptibility to amantadine and rimantadine	[66]
	A30T	A	Reduced susceptibility to amantadine and rimantadine	[66]
	S31N	N	Reduced susceptibility to amantadine and rimantadine	[66, 67]
	L55F	F	Mammalian host marker	[68]
NS1	P/A42S	S	Increased virulence in mice; Antagonism of IFN induction	[69]
	T/D92E	D	Increased virulence and antiviral response in mammals	[70]
	D97E	E	Increased pathogenicity to mice	[46]
	V149A	A	Pathogenicity in mice; Antagonism of IFN induction	[71]
NEP/NS2	M31I	M	Increased virulence in mice	[3, 72]

the novel H3N3 strain exhibited a distinct genomic composition compared to other H3 subtype AIVs, displaying high similarity to H3N8, H9N2, and H10N3 influenza viruses (Table 2). These findings classify the H3N3 isolate as a novel triple-reassortant AIV. Meanwhile, phylogenetic analysis revealed that all segments within the novel H3N3 virus genome were clustered in the Eurasian lineage of AIVs. The HA protein, crucial for viral entry into host cells and inducing the production of neutralizing antibodies production [76], exhibited a PEKQTR/GIF cleavage site characteristic of LPAIVs. Additionally, the presence of Q226 and T228 amino acids at the receptor-binding site suggested a preference for avian-like (α-2,3-linked sialic acid) receptors (Table 3) [76–78].

However, the results of both nucleotide sequence homology and evolutionary analyses based on the HA gene indicate that this newly H3N3 strain is closely genetically related to a human-derived H3N8 influenza virus (A/China/ZMD-22–2/2022(H3N8)), which was isolated in April 2022 from a four-year-old boy in China experiencing recurrent fever and severe pneumonia [27]. Further studies showed that this human-derived H3N8 influenza virus was likely generated by reassortment of the H3N2, H3N8 and H9N2 AIVs [7, 17]. The PB2 gene is known to significantly influence influenza virus pathogenicity, with the E627K mutation in the PB2 protein associated with zoonotic potential [47]. Previous studies have speculated that it is likely that H3N8 AIVs gained the ability to spill over to humans by recombining with H9N2 viruses with the amino acid mutation at position 627 (E627K) in the PB2 fragment [22, 27]. Notably, the absence of the E627K mutation in the PB2 protein of the novel H3N3 strain suggests a reduced risk to public health (Table 4).

The segment exchange by reassortment between different subtypes of influenza viruses is a critical feature driving the evolution of influenza viruses and contributing to the emergence of novel potential pandemic and zoonotic strains [79–81]. Reassortment between H3 subtype AIVs and other influenza viruses can generate new pathogenic influenza viruses that may lead to human infections [13, 17]. Notably, the 1968 Hong Kong pandemic was triggered by reassortment with avian-derived H3 subtype and human-derived H2N2 influenza viruses [82]. Herein, by comprehensively analyzing the homology and genetic evolution results of the segments constituting the novel H3N3 AIV genome sequenced in this study, we postulate that this virus was likely formed by combining these influenza viruses (H3N8, H9N2, and H10N3) (Fig. 3B). The reassortment results revealed that the novel H3N3 subtype AIVs was likely to emerge in Chinese chicken flocks, and remained circulating until now.

Strikingly, a novel reassortant H3N3 AIV was isolated from environmental samples collected in Hebei

Province, China, in 2022 [74]. In 2023, two novel H3N3 AIVs were also isolated from egg farms in Jiangsu Province, China, along with one novel H3N3 AIV from a live poultry market in the neighborhood [10]. In contrast, the novel H3N3 AIV identified in this study was sourced from broiler farms in Liaoning Province, northeastern China, in 2023. Considering these reports of novel H3N3 AIVs and the latest information on H3N3 strains in the NCBI and GISAID databases, it suggests that the novel H3N3 AIVs generated by reassortment of H3N8, H9N2, and H10N3 influenza viruses have already appeared in various regions of China, and have a tendency to become more prevalent.

Furthermore, AIVs are considered to be of high economic importance due to their significant impact on poultry production and reproduction globally, coupled with the looming threat of zoonotic diseases [83, 84]. Although the H3 subtype AIV was classified as LPAIV in poultry, recent epidemiological studies have demonstrated that this emerging H3N3 AIVs had enhanced pathogenicity in chickens, with the ability to cause massive reductions in egg production in infected flocks [10, 75]. Meanwhile, the presence of the novel H3N3 AIVs in poultry allows them to reassort with multiple subtypes of AIVs to generate various novel AIV strains that have the potential to cause pandemics and zoonotic diseases. Therefore, continued surveillance of H3N3 AIVs in domestic poultry is essential and effective control strategies, including vaccination, are urgently needed.

Conclusions

This paper presents a comprehensive genomic analysis of a newly dectected triple-reassortant H3N3 AIV strain, designated A/chicken/China/16/2023 (H3N3), isolated from a chicken in China during 2023. Comparative genomic studies and phylogenetic analysis revealed that this novel H3N3 virus originated from a triple reassortment event involving H3N8, H9N2, and H10N3 influenza viruses. Notably, several mutations in the internal genes of the novel H3N3 isolate raise concerns about potential increases in viral resistance, virulence, and transmission in mammalian hosts. This study expands our understanding of triple-reassortant H3N3 AIVs, and underscores the critical need for continued surveillance of these emerging viruses in domestic poultry.

Methods

Sampling

Obvious clinical symptoms such as swollen eyelids, decreased feed intake, and air sacculitis were observed in some chickens in broiler flocks in Tieling City, Liaoning Province, China (42°17′22.3″N, 123°50′22.2″E) in June 2023. The daily mortality within this broiler flock ranged from 0.5% to 2% (n = 5,000). Furthermore, Post-mortem examination of deceased chickens revealed excessive mucus accumulation on the tracheal mucosa, and the tracheal mucosa secretion samples were gently collected from a dead chicken using sterile swabs. These samples tested positive for AIV using a universal RT-qPCR assay, but negative for the specific H5, H7, and H9 subtypes by RT-qPCR. These samples were sent to the Key Laboratory of Animal Disease and Public Health, Henan University of Science and Technology for further subtype identification and pathogen investigation. Briefly, the samples were placed into a 2 mL microcentrifuge tube of cold phosphate-buffered saline (PBS) containing penicillin (2000 U/mL) and streptomycin (2000 μg/mL). The samples were centrifuged at 4000 xg for 20 min at 4℃, and the supernatant was filtered through 0.45-μm hydrophilic polyether sulfone membrane filters (Millipore, USA). The filtered solution was utilized for subsequent virus isolation and identification.

Virus isolation and identification

For virus isolation, 100 μL of the sample supernatant was inoculated onto the allantoic cavity of 10-day-old SPF chicken embryos obtained from the SPF Experimental Animal Center of Xinxing Dahua Agricultural, Poultry and Egg Co., Ltd. The inoculated SPF chicken embryos were incubated at 37 °C and monitored every 12 h. After 72 h of post-inoculation, allantoic fluids were harvested for testing. Viral RNA was extracted from allantoic fluids using a MiniBEST Viral RNA/DNA Extraction Kit (Takara, Beijing, China) according to the manufacturer's instructions. RT-PCR was performed using primers targeting the H3 subtypes' HA gene and NA gene (Primer sequences are available upon request) to confirm the presence of the virus.

The allantoic fluids of chicken embryos with positive RT-PCR results were subjected to a tenfold series of dilutions in sterile PBS, and five 10-day-old SPF chicken embryos were inoculated with each gradient dilution. The HA test was performed on the allantoic fluids of all chicken embryos at 72 h post-inoculation. The allantoic fluids with a positive HA test result and the highest dilution were harvested, again diluted in a tenfold series according to the above procedure and inoculated into SPF chick embryos, repeating three times. The allantoic fluids with the highest dilution and positive HA test result were collected. According to the above procedure, the allantoic fluid was again diluted in a tenfold series and inoculated into SPF chicken embryos, repeated three times to accomplish the isolation and purification of the virus for subsequent analysis.

Transmission electron microscopy

Viral particles in the purified AIV allantoic fluids were visualized by transmission electron microscope (Wuhan Servicebio Technology Co., Ltd, China). Briefly, 20 μL of the sample was placed onto 150 meshes cuprum grids with carbon film and settled at room temperature for 5 min. The excess fluid was removed using filter papers, and the dried grid was stained with 2% phosphotungstic acid for 60 s. After removing the excess stain, the grid was examined using an HT7800 transmission electron microscope (Hitachi, Japan) at 80 kV acceleration voltage to observe negatively stained viral particles.

RNA extraction and next-generation sequencing

Next-generation sequencing was undertaken by Shanghai Tanpu Biotechnology Co., Ltd (Shanghai, China) to obtain the whole genome sequence data of the novel AIV isolate. Briefly, Viral RNA was extracted from allantoic fluids containing purified virus using MiniBEST Viral RNA/DNA Extraction Kit (Takara, Beijing, China) according to the manufacturer's instructions for complete genome sequencing. Reverse transcription of fragmented RNA used random hexamers, and DNase treatment and cleanup were followed by second-strand synthesis. The sequencing libraries were prepared using TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA, USA) as recommended by the manufacturer. PCR amplification was performed after adapter ligation to sequence the target enrichment. The library was normalized and pooled in equimolar quantities, denatured and diluted to optimal concentration before sequencing. The Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) was used for sequencing with a read length of 150 nt paired-end.

Assembly protocol

The resulting 10,586,382 paired raw sequence reads from NovaSeq 6000 were used to obtain the whole genome of the novel AIV isolate. Initial quality control of all raw reads was generated, and the raw reads were processed by fastp (version 0.20.0) [85] for filtering to remove adapters, ambiguous bases, polyclonal reads and low-quality reads, including those reads scored below Q20. Reads trimmed of adaptor sequences shorter than 50 nt were also discarded. The trimmed sequence reads are filtered by read-mapping using the BBMap program (Version 38.51) to remove likely contamination from the host organism, ribosomal RNAs and bacteria genome [86]. Unmapped reads were used as input data for de novo assembly using SPAdes (Version 3.14.1) [87] and SOAPdenovo (Version 2.04) [88]. The results showed that the percentage of possible reads of rRNA, host reads, and bacterial reads were 31.77%, 31.25%, and 0.42%, respectively. Subsequently, twenty-one contigs (108–2328 bp) were generated corresponding to AIV sequences according to searches of the GenBank database by BLAST [89], and the average depth of contigs (> 1500 nt) was 153.51, with coverage ranging between 99.89% and 100%. Eventually, the complete genome sequence of the novel isolated AIV was obtained by splicing these contigs using Geneious Prime software (Version 10.2.2 Biomatters, Auckland, New Zealand).

Genome annotations, comparative and molecular analysis

The ORFs and CDS of the newly identified AIV strain were annotated based on the available AIV whole genome sequences in the GenBank database [73]. Subsequently, the AIV isolates showing the highest homology in nucleotide sequence to the individual genes of the novel AIV strain isolated in this study were obtained through the NCBI database with BLAST [89], and the multiple sequences alignment was performed using MAFFT software (Version 7.490) [90]. The amino acid sequences at the receptor binding site of HA protein were identified as described by Weis et al. [91]. Potential N-linked glycosylation sites of HA and NA proteins were predicted using the NetNGlyc 1.0 server [92].

Phylogenetic analysis

Phylogenetic analysis of the novel H3N3 AIV strain identified in this study was performed with other referenced AIV genome sequences available in the GenBank and GISAID databases [73]. The nucleotide sequences of each gene (PB2, PB1, PA, HA, NP, NA, M and NS) in the AIVs genome were aligned by MAFTT (Version 7.490) with default parameter (Gap open penalty 1.53; Offset value 0.123) [90]. Subsequently, the best-fit model testing of the AIV's gene for ML analysis was predicted in MEGA software (Version 11.0.11) [93]. Phylogenetic analysis for nucleotide sequences of eight genes of the novel AIV isolate was performed based on the best-fit model with 1000 bootstrap replicates in MEGA software (Version 11.0.11), and the phylogenetic trees were further modified by using tvBOT [94].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-11152-x.

Supplementary Material 1.

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Authors' contributions
HL, ZY and SC designed the study. ZY, KS and KS performed the experiments. YW and CY performed the bioinformatics and data analysis. HL and YZ wrote the initial manuscript. All authors read, edited and approved the final manuscript.

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Data availability
The newly A/chicken/China/16/2023 (H3N3) complete genome sequence and the associated data sets that were generated during this study were deposited in GenBank under the accession number OR497659-OR497666.

Declarations

Ethics approval and consent to participate
Samples collected were approved by the Ethics Committee of Henan University of Science and Technology (20230515032).

Consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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