Molecular characterization and phylogenetic analysis of highly pathogenic avian influenza A(H5N8) viruses isolated from chicken flocks during the 2016 outbreak in Iran

Kiarash Roohani Shahrestani[1], Safieh Vatandour[2*], Abdolhamid Shoushtari[3], Saeid Charkhkar[2], Mohammad Hossein Fallah Mehrabadi[3]

1. *Department of Clinical Sciences, Faculty of Veterinary Medicine, Islamic Azad University, Science and Research Branch, Tehran, Iran.*
2. *Department of Clinical Sciences, SR.C., Science and Research Branch, Tehran, Iran.*
3. *Department of Avian Diseases Research and Diagnostics, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.*

**** Corresponding Author:*** Safieh Vatandour***, PhD. Address:*** *Department of Clinical Sciences, SR.C., Science and Research Branch, Tehran, Iran Tel**: +989121327588** E-mail: Safiehvatandour@iau.ac.ir*

Orcid ID:

Kiarash Roohani Shahrestani: 0009-0002-3120-6282

Safieh Vatandour: 0000-0001-5470-9873

Abdolhamid Shoushtari: 0000-0002-3581-0834

Saeid Charkhkar: 0009-0005-2365-3463

Mohammad Hossein Fallah Mehrabadi: 0000-0002-1221-7771

Abstract

Introduction: The objective of this study was to investigate the genetic diversity, evolutionary relationships, and molecular characteristics of highly pathogenic avian influenza A(H5N8) viruses isolated from Iranian poultry during the 2016 outbreak through sequencing and phylogenetic analysis of the hemagglutinin (HA), neuraminidase (NA), and matrix (M) gene segments.
Materials & Methods: Four H5-positive isolates were characterized using RT-PCR, Sanger sequencing, comparative sequence analysis, and maximum-likelihood phylogenetic reconstruction, followed by evaluation of molecular markers associated with pathogenicity, host adaptation, antiviral susceptibility, and viral fitness.
Results: Phylogenetic analyses placed all four isolates within HPAI clade 2.3.4.4b and demonstrated close genetic relationships with contemporary strains from Egypt, Nigeria, and Cameroon, while molecular characterization identified a conserved polybasic HA cleavage site, mutations linked to enhanced binding to human-type receptors, the NA I314V substitution associated with reduced oseltamivir susceptibility, and the absence of major M2 antiviral resistance markers.
Conclusion: These findings provide an important molecular baseline for H5N8 viruses circulating in Iran, emphasize the value of continuous genomic surveillance for early detection of viral

evolution and transboundary spread, and support strengthened veterinary biosecurity and integrated One Health surveillance to reduce risks to poultry production, animal health, and potential zoonotic transmission.

Keywords: Highly Pathogenic Avian Influenza Viruses (HPAIV), Iran, Outbreak, Phylogenetic, Poultry

1. Introduction

Since its emergence in 2014, highly pathogenic avian influenza (HPAI) H5N8, a descendant of the A/goose/Guangdong/1/1996 lineage, has caused outbreaks across Asia and Europe[1,2]. Its spread is facilitated by migratory bird flyways, adaptation to diverse environments, and reassortment with local avian influenza viruses, posing substantial threats to poultry production, animal health, food security, and potentially public health[3].

In 2016, Iran experienced major HPAI H5N8 outbreaks across 12 provinces, affecting both wild and domestic birds[1,4]. Phylogenetic analyses placed these Iranian isolates within clade 2.3.4.4b, indicating multiple introductions via the West Asia/East Africa migratory flyway[2,4]. Risk factors such as live bird markets, poor biosecurity, and poultry movement highlighted the vulnerability of Iran's poultry sector and the need for enhanced biosecurity and surveillance.

The rapid evolution of H5N8 through the reassortment of previously known avian influenza viruses has resulted in their genetic diversity and adaptability [1,5].The role migratory birds play in the long-distance dispersal of H5N8 is quite significant, while domestic ducks act as reservoirs, both amplifying and transmitting H5N8 to other poultry [1,3,6]. Based on phylogenetic studies conducted in Iran, strains of H5N8 have also been introduced through the avian migratory pathways and reassorted with low-pathogenic influenza viruses found in East Asia, complicating control measures [1,4]. A comprehensive understanding of the dynamics of H5N8 transmission will provide insight into future outbreaks, allowing for the appropriate preparation and prevention.

Molecular epidemiology is a critical factor in providing insight into the genetic evolution, transmission pathway, and zoonotic potential of HPAI H5N8. Techniques such as complete genomic sequencing and phylogenetic analysis have been used to assist in the identification of reassortments, track the movement of the virus and assess its pathogenicity. Early detection and containment of outbreaks depend on surveilling wild birds, poultry, and live-bird [2,5,7]. The One Health approach is required for effective pandemic preparedness and disease management and includes animal health, environmental health, and public health [1,3,5].

The emergence and circulation of HPAI H5N8 viruses threaten poultry production, avian biodiversity, and food security through their high transmissibility and pathogenicity. Although zoonotic transmission remains limited, their persistent evolution underscores the need for continuous surveillance and molecular characterization. The aim of this study was to investigate the genetic diversity and evolutionary relationships of highly pathogenic avian influenza viruses isolated from poultry in Iran. Sequencing of the HA, NA, and M gene segments was performed to characterize the genetic features of the isolates, followed by phylogenetic analyses to determine

their evolutionary relationships with representative regional and global HPAI strains. Furthermore, key molecular markers and functional domains associated with pathogenicity, host adaptation, and viral fitness were examined. Collectively, these analyses provide valuable insights into the genetic diversity, phylogenetic relationships and molecular determinants of HPAI viruses circulating in Iran.

2. Materials and Methods

2.1. RT-PCR amplification and sequencing of HA, NA, and M gene segments from avian influenza A (H5) virus isolates

Virus isolates previously confirmed as H5 avian influenza viruses were selected for further molecular characterization. The isolates were obtained from infected poultry samples collected during the 2016 avian influenza outbreak investigation in Iran. Following initial virus detection, isolation, and subtype confirmation, representative H5-positive isolates were used for downstream molecular analyses, including amplification and sequencing of the hemagglutinin (HA), neuraminidase (NA), and matrix (M) gene segments.

Full-length HA, NA, and M gene segments were amplified using the SuperScript III One-Step RT-PCR System (Invitrogen, USA). Each 25 μL reaction contained 12.5 μL of 2× Reaction Mix, 5 μL of RNA, 1 μL of each primer (10 μM), and 1 μL of enzyme mix. Thermocycling conditions were: 45°C for 30 min, 94°C for 2 min; 40 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 3 min; and a final extension at 68°C for 5 min[8]. PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Germany) and Sanger sequenced for downstream genetic, molecular marker, and phylogenetic analyses.

2.2. Sequence assembly, alignment, and phylogenetic analysis

Sanger sequencing reads were quality-assessed, trimmed, and assembled into consensus sequences via reference-based mapping using UGENE v53.0. The consensus sequences were aligned using ClustalW in MEGA v12.0. The NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the closest related sequences and assess genetic similarity. The consensus sequences were subsequently compared with the most closely related virus strains available in the NCBI GenBank databases.

Maximum-likelihood phylogenetic trees were constructed separately for each gene segment using IQ-TREE version 3 (https://iqtree.github.io). The best-fitting nucleotide substitution models were selected according to the Bayesian information criterion (BIC), as follows: GTR + F + I + G4 for HA, GTR + F + I + R2 for NA, and TVM + F + I + G4 for M. Tree robustness was evaluated using ultrafast bootstrap resampling analysis with 1000 replicates. The resulting phylogenetic trees were visualized and edited using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

2.3. Molecular Marker and Functional Domain Analysis

The hemagglutinin (HA), neuraminidase (NA), and matrix (M) gene segments of Iranian H5N8 influenza A viruses were analyzed using comprehensive in silico approaches to identify nucleotide and amino acid variations. The genetic characteristics of the studied isolates were curated and subjected to multiple sequence alignment and comparative analyses at both nucleotide and deduced amino acid levels using Unipro UGENE. Sequence homology analyses were performed to evaluate genetic relatedness between the Iranian isolates and selected reference strains.

2.3.1. Prediction of N-linked glycosylation sites

Potential N-linked glycosylation sites in the HA and NA proteins were predicted using the NetNGlyc 1.0 Server (Technical University of Denmark, Lyngby, Denmark; http://www.cbs.dtu.dk/services/NetNGlyc/). Glycosylation motifs were identified based on the consensus sequence Asn-X-Ser/Thr, where X represents any amino acid except proline, together with the prediction scores provided by the server.

2.3.2. Analysis of functional domains in HA, NA, and M proteins

Functionally important regions of the HA, NA, and M proteins were systematically examined. These included the HA cleavage site, receptor-binding residues, antigenic regions, NA catalytic and framework residues, and conserved structural motifs in the M1 and M2 proteins. Amino acid substitutions were identified by aligning the Iranian isolates with established influenza reference sequences and representative H5N8 strains.

2.4. Screening and annotation of influenza-associated mutations

Amino acid residues were annotated using standardized reference-based numbering systems. HA and NA substitutions were reported using H3 and N2 numbering, respectively, relative to A/Aichi/2/1968 (H3N2), whereas M1 and M2 residues were numbered relative to A/goose/Guangdong/1/1996 (H5N1). Substitutions were cross-referenced with the CDC inventory of influenza-associated mutations to identify previously reported associations with antiviral resistance, virulence, host adaptation, receptor-binding specificity, and other phenotypic traits.

3. Results

3.1. RT-PCR amplification and sequencing of HA, NA, and M gene segments

Full-length HA, NA, and M gene segments from representative H5-positive avian influenza virus isolates from Iran were successfully amplified and Sanger sequenced. High-quality consensus sequences were assembled using Unipro UGENE v53.0 and deposited in GenBank under the accession numbers listed in Table 1. The sequences were subsequently used for genetic, molecular marker, and phylogenetic analyses.

Table 1. GenBank accession numbers of HA, NA, and M gene segment sequences obtained from Iranian highly pathogenic avian influenza A(H5N8) virus isolates used in this study.

Isolate name	Accession number	Identifier	Gene
RVSRI_2	PX457184	A/chicken/Chaharmahal_and_Bakhtiari/RVSRI_2/2016	HA

RVSRI_16	PX457189	A/chicken/Kurdistan/RVSRI_16/2016	
RVSRI_104	PX464114	A/chicken/Qazvin/RVSRI_104/2016	
RVSRI_127	PX457194	A/chicken/Alborz/RVSRI_127/2016	
RVSRI_2	PX457183	A/chicken/Chaharmahal_and_Bakhtiari/RVSRI_2/2016	NA
RVSRI_16	PX457188	A/chicken/Kurdistan/RVSRI_16/2016	
RVSRI_104	PX457192	A/chicken/Qazvin/RVSRI_104/2016	
RVSRI_127	PX457193	A/chicken/Alborz/RVSRI_127/2016	
RVSRI_2	PX464110	A/chicken/Chaharmahal_and_Bakhtiari/RVSRI_2/2016	M
RVSRI_16	PX464111	A/chicken/Kurdistan/RVSRI_16/2016	
RVSRI_104	PX464115	A/chicken/Qazvin/RVSRI_104/2016	
RVSRI_127	PX464116	A/chicken/Alborz/RVSRI_127/2016	

3.2. Phylogenetic and Sequence Similarity Analysis

Phylogenetic analysis of the HA gene (Figure 1) clustered all Iranian isolates within subclade 2.3.4.4b. Specifically, isolate RVSRI_127 was most similar to A/Crow/Aghakhan/2017. RVSRI_16 grouped with Egyptian and Nigerian strains, showing closest relation to A/green-winged_teal/Egypt/871/2016. Isolates RVSRI_2 and RVSRI_104 clustered together, grouping with Israeli and Egyptian strains, and shared the highest similarity with A/chicken/Egypt/N13717E/2017 and A/duck/Egypt/N13733B/2017.

Based on the NA gene tree (Figure 2), isolates RVSRI_2 and RVSRI_104 clustered with Egyptian strains and exhibited the closest genetic relationship to A/chicken/Egypt/H13795A/2017 and A/goose/Egypt/A13779B/2017_H5N8. Isolate RVSRI_127 clustered most closely with A/Crow/Iran/2017_H5N8, A/chicken/Cameroon/17RS1661-1/2017_H5N8, A/pigeon/Cameroon/17RS1661-4/2017_H5N8, A/duck/Nigeria/VRD19-068DC019T_19RS1081-12/2019_H5N8, and A/chicken/Nigeria/VRD-19-023_19RS1081-1/2019_H5N8. Meanwhile, isolate RVSRI_16 was most closely similar to A/little_grebe/Egypt/MB-D-1056C/2016_H5N8, A/little_grebe/Egypt/1056OP/2016_H5N8, and A/green-winged_teal/Egypt/871/2016_H5N8, in that order.

The phylogenetic tree based on the M gene (Figure 3) showed that isolates RVSRI_104 and RVSRI_2 were closely related to each other and displayed the highest similarity to A/duck/Egypt/H13797E/2017_H5N8, A/goose/Egypt/A13779B/2017_H5N8, and A/teal/Egypt/1198C/2017_H5N8. Isolates RVSRI_16 and RVSRI_127 fell within the same large major clade. However, RVSRI_16 grouped more specifically with strains from Cameroon, South Korea, Egypt, and Nigeria, showing the closest affinity to A/chicken/Cameroon/17RS1661-1/2017_H5N8 and A/pigeon/Cameroon/17RS1661-4/2017_H5N8. In contrast, RVSRI_127 was most similar to A/Crow/Iran/2017_H5N8.

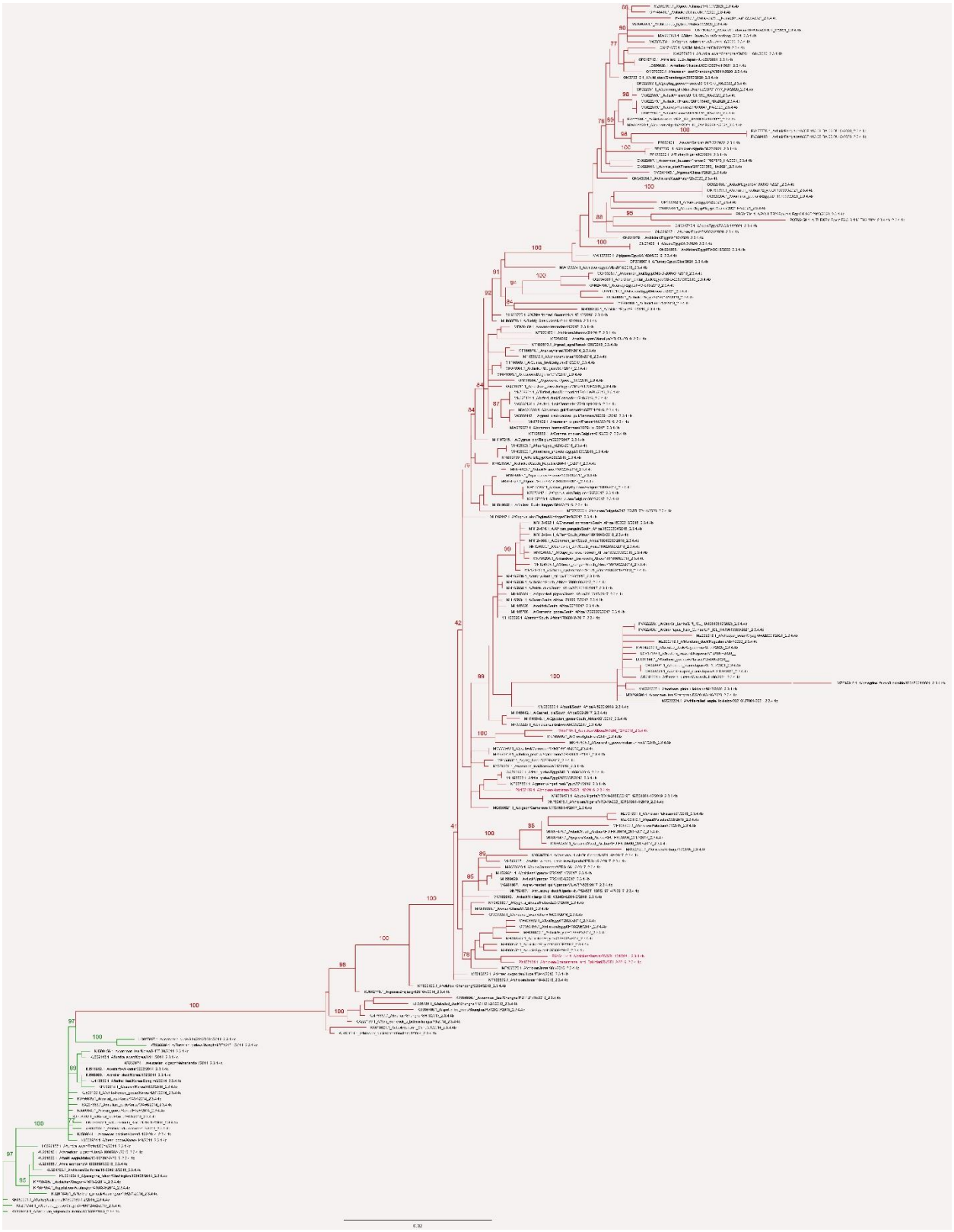

Figure 1. Maximum-likelihood phylogenetic tree of H5N8 HA gene sequences showing the placement of Iranian isolates within clade 2.3.4.4b. The tree was constructed in IQ-TREE v3 using the GTR + F + I + G4 nucleotide substitution model. Branch support was estimated using ultrafast bootstrap analysis (1000 replicates) and visualized using FigTree v1.4.4.

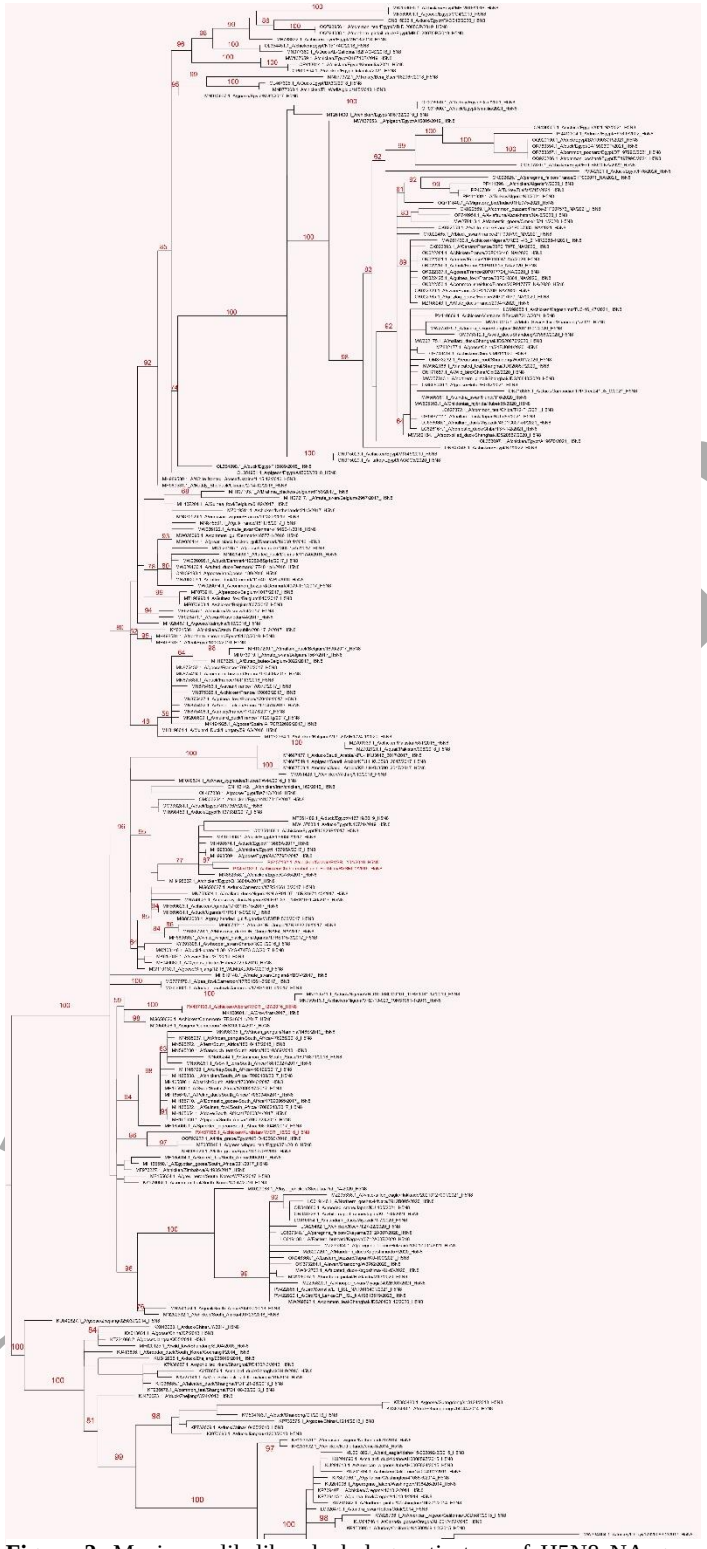

Figure 2. Maximum-likelihood phylogenetic tree of H5N8 NA gene sequences showing the phylogenetic placement of Iranian isolates among global reference strains. The tree was constructed in IQ-TREE v3 using the GTR + F + I + R2 model. Branch support was estimated using ultrafast bootstrap analysis (1000 replicates) and visualized using FigTree v1.4.4.

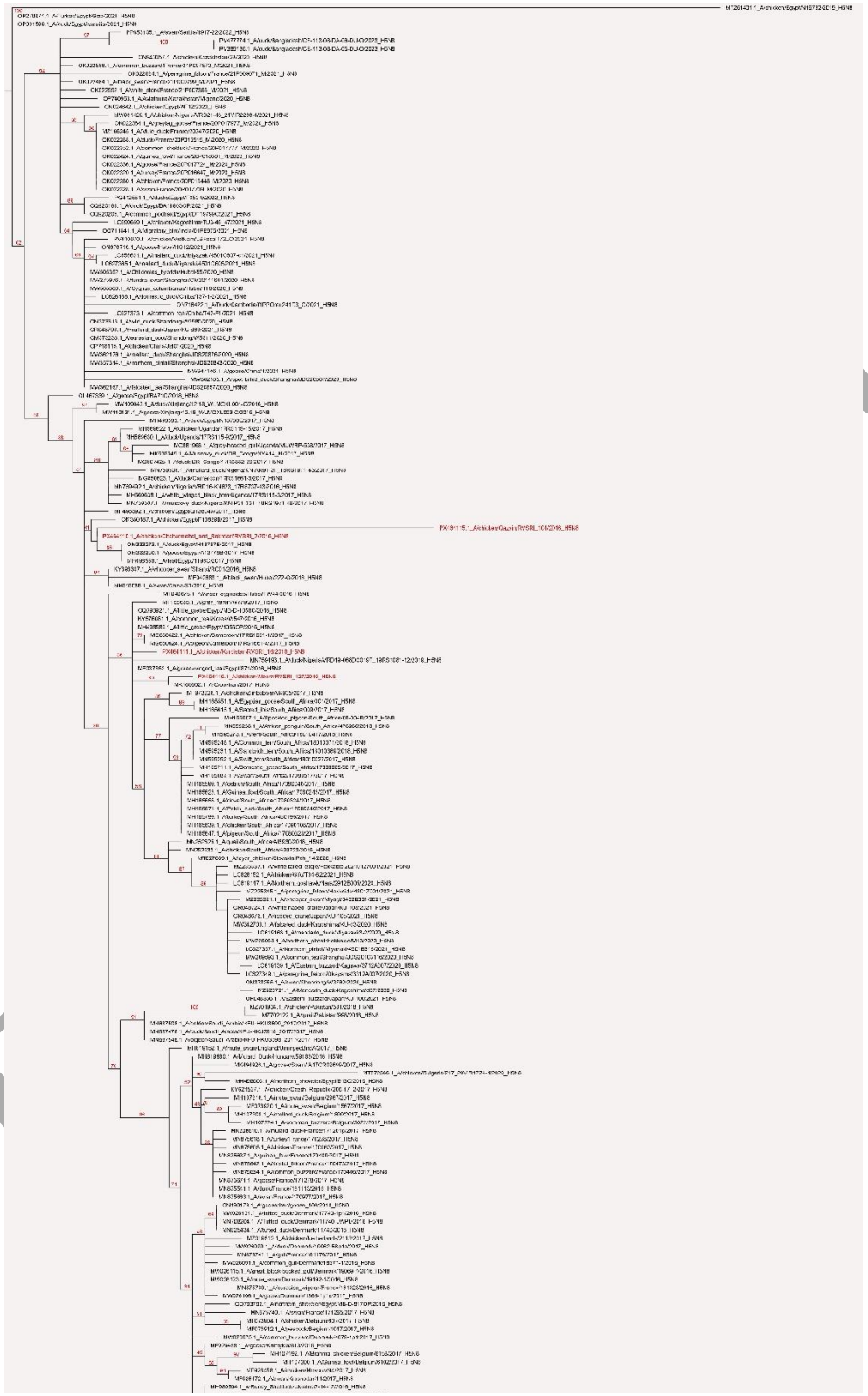

Figure 3. Maximum-likelihood phylogenetic tree of H5N8 M gene sequences showing the phylogenetic placement of Iranian isolates among global reference strains. The tree was constructed in IQ-TREE v3 using the TVM + F + I + G4 model. Branch support was estimated using ultrafast bootstrap analysis (1000 replicates) and visualized using FigTree v1.4.4.

3.3. Molecular Marker and Functional Domain Characterization

3.3.1. HA Cleavage Site and N-Linked Glycosylation Profiles

All four HA sequences contained the characteristic polybasic cleavage site motif PLREKRRKR/GLF. Analysis of predicted N-linked glycosylation motifs (N-X-T/S) in neuraminidase (NA) and hemagglutinin (HA) showed a largely conserved glycosylation profile among the four studied isolates, with NA positions interpreted according to N2 numbering.

In the NA protein, five glycosylation sites were conserved in all isolates at positions 54, 67, 84, 114, and 293. Variation was limited to three sites: position 42 was present in RVSRI_2 and RVSRI_104, position 364 was detected only in RVSRI_16, and position 398 only in RVSRI_2. Overall, NA glycosylation differences were minor and restricted to a small number of isolate-specific sites (Table 2). In contrast, the HA protein showed complete conservation of glycosylation motifs across all four isolates. Six N-linked glycosylation sites were consistently identified at positions 27, 39, 181, 302, 499, and 558, with no gain or loss of motifs among the analyzed sequences (Table 2).

Overall, the isolates exhibited a highly conserved HA glycosylation pattern and a predominantly conserved NA glycosylation profile with only minor isolate-specific variation.

Table 2. Predicted N-linked glycosylation sites in the hemagglutinin (HA) and neuraminidase (NA) proteins

Neuraminidase (NA) Glycosylation sites								
Isolate name	Location							
	42	54	67	84	114	293	364	398
RVSRI_2	NGTC	NETV	NTSV	NNTE	NGTV	NWTG	-	NWSG
RVSRI_16	-	NETV	NTSV	NNTE	NGTV	NWTG	NRTS	-
RVSRI_104	NGTC	NETV	NTSV	NNTE	NGTV	NWTG	-	-
RVSRI_127	-	NETV	NTSV	NNTE	NGTV	NWTG	-	-

Hemagglutinin (HA) Glycosylation sites						
Isolate name	Location					
	27	39	181	302	499	558
RVSRI_2	NSTE	NVTV	NNTN	NSSM	NGTY	NGSL
RVSRI_16	NSTE	NVTV	NNTN	NSSM	NGTY	NGSL
RVSRI_104	NSTE	NVTV	NNTN	NSSM	NGTY	NGSL
RVSRI_127	NSTE	NVTV	NNTN	NSSM	NGTY	NGSL

3.3.2. HA receptor-binding site and antigenic region features

Receptor-binding site (RBS) analysis of the hemagglutinin (HA) globular head, based on established H3 numbering structural motifs (130-loop, 190-helix, and 220-loop), showed that all four isolates (RVSRI_2, RVSRI_16, RVSRI_104, and RVSRI_127) share identical sequences in these regions (130-loop: HETSLGVSAACP; 190-helix: EQTNLYKNPT; 220-loop: RSQVNGQRGRM). These elements form the shallow receptor-binding pocket responsible for

sialic acid engagement on host cells, and variations in their amino-acid composition are known to influence receptor specificity and immune recognition across influenza subtypes. Compared with the reference sequence from A/Goose/Guangdong/1/1996 used for comparison, the isolates display multiple substitutions in each loop/helix region, suggesting divergence from that reference while maintaining a conserved RBS architecture within the isolate set (Table 3).

Consistent with this pattern of localized variability, antigenic site analysis revealed shared mutations among the isolates in the major HA1 immunodominant regions flanking the RBS. In HA1 head site 1 (residues 140–145) and site 2 (residues 156–157), all isolates possess the motif PYQGTP and either KK or KR, respectively, differing from the reference sequence's PYHGRS and KK. The adjacent F' subdomain of HA1 (HA1 residues 129–133) is also altered to NHETS in all isolates compared with NHDAS in the reference, while the HA1 stem region (residues 275–307) remains conserved. These antigenic site substitutions occur in surface-exposed loops and helices that surround the RBS and are frequently implicated in antigenic drift and immune escape, consistent with patterns observed in other influenza variants where substitutions near the RBS contribute to altered antigenicity and potential host adaptation (Table 3).

Table 3. Antigenic sites and receptor binding sites of the hemagglutinin (HA) protein in four Iranian isolates compared with A/goose/Guangdong/1/1996 (H5N1)

Receptor Binding Sites in HA based on H3 numbering				
Receptor Binding Sites	130-Loop	190-Helix		220-Loop
Sample	130-140 Residue	190-199 Residue		220-230 Residue
A/Goose/Guangdong/1/1996	H**DAS**SGVS**S**ACP	EQT**K**LY**Q**NPT		R**PK**VNGQ**S**GRM
RVSRI-2	H**ETSL**GVS**A**ACP	EQT**N**LY**K**NPT		R**SQ**VNGQ**R**GRM
RVSRI-16	H**ETSL**GVS**A**ACP	EQT**N**LY**K**NPT		R**SQ**VNGQ**R**GRM
RVSRI-104	H**ETSL**GVS**A**ACP	EQT**N**LY**K**NPT		R**SQ**VNGQ**R**GRM
RVSRI-127	H**ETSL**GVS**A**ACP	EQT**N**LY**K**NPT		R**SQ**VNGQ**R**GRM
Antigenic Sites in HA based on H3 numbering				
Antigenic Sites	HA1 Head (site 1)	HA1 Head (site 2)		F' subdomain of HA1
Sample	140-145 Residue	156-157 Residue Subunit 1	129-133 Residue Subunit 2	HA1 stem
A/Goose/Guangdong/1/1996	PY**H**G**RS**	KK	NH**DA**S	275-307 Residue
RVSRI-2	PY**Q**G**TP**	KK	NH**ET**S	275-307 Residue
RVSRI-16	PY**Q**G**TP**	K**R**	NH**ET**S	275-307 Residue
RVSRI-104	PY**Q**G**TP**	KK	NH**ET**S	275-307 Residue
RVSRI-127	PY**Q**G**TP**	KK	NH**ET**S	275-307 Residue

3.4. Amino acid substitutions and phenotype-associated molecular markers

Mutation analysis identified several amino acid substitutions previously associated with altered receptor-binding profiles or antiviral susceptibility (Table 4). In the hemagglutinin (HA) protein,

all four isolates shared the substitutions S128P, S137A, and T160A, which are associated with enhanced binding to human-type (α2–6) sialic acid receptors and, in the case of T160A, increased mammalian transmissibility. Additionally, all isolates carried K222Q and S227R, mutations linked to altered affinity for both avian-type (α2–3) and human-type (α2–6) receptors.

In the neuraminidase (NA) protein, the isolates carried the I314V substitution, which is associated with reduced oseltamivir susceptibility. However, no major NA inhibitor resistance markers (including E119, D198, H274, R292, and N294 variants) were detected, indicating a lack of principal markers for high-level resistance to neuraminidase inhibitors. Similarly, analysis of the M2 protein revealed no adamantane resistance markers, with wild-type residues conserved at positions 26, 27, 30, 31, and 34.

Overall, the isolates consistently carried HA substitutions associated with enhanced receptor binding, one NA substitution linked to reduced oseltamivir susceptibility, and no major NA or M2 antiviral resistance markers.

Table 4. Amino acid markers and motifs identified in the genomes of HPAI H5N8 viruses from Iran sequenced in the current study, with associated phenotypic effects as reported in the literature[9]. The phenotypic effects presented in this table were inferred from associations reported in the published literature and were not experimentally evaluated in the present study.

4. Discussion

The study demonstrated that Iranian highly pathogenic avian influenza A (HPAI) H5N8 virus isolates from the 2016 outbreak belong to clade 2.3.4.4b and show close genetic relationships with contemporary strains from Egypt, Nigeria, and Cameroon. Molecular analyses revealed a conserved polybasic HA cleavage site motif characteristic of high pathogenicity, along with amino acid substitutions in the HA protein associated with enhanced binding to human-type receptors, raising concerns about zoonotic potential. The neuraminidase (NA) gene carried the I314V substitution linked to reduce oseltamivir susceptibility, while no major antiviral resistance markers were detected in NA or M2 proteins. Phylogenetic data indicate multiple introductions into Iran, facilitated by migratory bird pathways, with ongoing viral evolution through reassortment and local adaptation. The isolates exhibited conserved glycosylation profiles and antigenic site mutations suggestive of immune escape potential. These findings establish a molecular baseline for H5N8 viruses circulating in Iran and underscore the importance of continuous genomic surveillance.

Phylogenetic analyses based on the hemagglutinin (HA), neuraminidase (NA), and matrix (M) genes revealed that all four Iranian isolates belong to clade 2.3.4.4, subgroup B (2.3.4.4b), a lineage that has been responsible for multiple waves of H5N8 outbreaks across Eurasia and Africa since 2014[10,11]. The clustering of Iranian isolates with Egyptian, Nigerian, and Cameroonian strains-particularly in the HA and NA trees-suggests that these viruses share a common evolutionary origin and that migratory routes have facilitated their intercontinental dissemination[2,11,12]. For instance, the HA gene of isolate A/chicken/Kurdistan/RVSRI_16/2016 showed closest similarity to A/green-winged_teal/Egypt/871/2016, and the NA gene of the same isolate grouped with Egyptian little grebe isolates, reinforcing the role of wild waterfowl as reservoirs and vectors

[1,11]. In contrast, the M gene analysis placed isolates A/chicken/Alborz/RVSRI_127/2016 and A/chicken/Kurdistan/RVSRI_16/2016 in a major clade containing strains from Cameroon, South Korea, and Nigeria, indicating that multiple introduction events may have occurred into Iran (18).

Hemagglutinin Protein (HA)			
Mutation/motif H3 numbering	Existence	Current study	Phenotype
S128P	YES	P (4/4)	Increased virus binding to α2–6
S137A	YES	A (4/4)	Increased pseudovirus binding to α2–6
T160A	YES	A (4/4)	Increased virus binding to α2–6, increased transmission in guinea pigs
K222Q	YES	Q (4/4)	Increased virus binding to α2–3 and α2–6
S227R	YES	R (4/4)	Increased virus binding to α2–3 and α2–6
Neuraminidase Protein (NA)			
Mutation/motif N2 numbering	Existence	Current study	Phenotype
E119A/D/G/V	NO	E (4/4)	Reduced susceptibility to oseltamivir, zanamivir, peramivir, and laninamivir
I314V	YES	V (4/4)	Reduced susceptibility to oseltamivir
D198G	NO	D (4/4)	Reduced susceptibility to oseltamivir and zanamivir
H274Y	NO	H (4/4)	Reduced susceptibility to oseltamivir and peramivir
R292K	NO	R (4/4)	Reduced susceptibility to oseltamivir, zanamivir, peramivir, and laninamivir
N294S	NO	N (4/4)	Reduced susceptibility to oseltamivir, zanamivir, and peramivir
Matrix Protein 2 (M2)			
Mutation/motif	Existence	Current study	Phenotype
L26F	NO	L (4/4)	Increased resistance to amantadine and rimantadine
I/V27A/T/S	NO	V (4/4)	Increased resistance to amantadine and rimantadine
A30V/T/S	NO	A (4/4)	Increased resistance to amantadine and rimantadine
S31N/G	NO	S (4/4)	Increased resistance to amantadine and rimantadine
G34E	NO	G (4/4)	Increased resistance to amantadine and rimantadine

These observations are consistent with previous reports that H5N8 clade 2.3.4.4b viruses have undergone extensive reassortment and geographic expansion, leading to the emergence of distinct local lineages[1,2,4,10].

A defining feature of highly pathogenic avian influenza viruses is the presence of a polybasic cleavage site in the HA protein, which allows furin-like proteases to cleave HA0 into HA1 and HA2 in a wide range of host tissues, enabling systemic spread and high mortality[5,13]. All four Iranian isolates carried the motif PLREKRRKR/GLF at the HA cleavage site. This motif contains multiple basic amino acids (arginine and lysine) and is identical to the canonical HPAI H5 cleavage site described in A/goose/Guangdong/1/1996 and subsequent H5Nx viruses[13]. The presence of this signature confirms the highly pathogenic phenotype of the isolates and is consistent with the severe clinical outcomes observed in the affected poultry farms[1,4,10].

N-linked glycosylation of the HA and NA proteins plays a critical role in viral fitness, antigenicity, and host adaptation[14]. In this study, the HA protein of all four isolates exhibited six conserved glycosylation sites (positions 27, 39, 181, 302, 499, and 558) with no isolate-specific variation. This uniform HA glycosylation pattern suggests strong selective pressure to maintain these glycan shields, which may stabilize the HA structure and protect antigenic epitopes from antibody recognition[14,15]. Conversely, the NA protein displayed minor variability at positions 42, 364, and 398. Five sites (54, 67, 84, 114, and 293) were conserved across all isolates and are located within the enzymatic head domain, where they are thought to contribute to proper folding and catalytic activity[16]. The loss of the site at position 42 in isolates RVSRI_16 and RVSRI_127, and the unique presence of sites at 364 and 398 in RVSRI_16 and RVSRI_2, respectively, may reflect recent evolutionary drift. Although the functional consequences of these NA glycosylation changes are not fully understood, similar variations in other H5N8 strains have been associated with altered neuraminidase activity and immune evasion[17]. Further studies are needed to assess whether these differences affect viral replication or transmissibility.

The receptor-binding site (RBS) of the HA globular head, comprising the 130-loop, 190-helix, and 220-loop, was identical among all Iranian isolates (130-loop: HETSLGVSAACP; 190-helix: EQTNLYKNPT; 220-loop: RSQVNGQRGRM). This sequence conservation indicates that the RBS architecture is maintained despite the multiple substitutions relative to the reference strain A/goose/Guangdong/1/1996 (H5N1). The observed substitutions (e.g., in the 130-loop: H**ET**SLGVS**A**ACP vs. H**DAS**SGVS**S**ACP; in the 190-helix: EQT**N**LY**K**NPT vs. EQT**K**LY**Q**NPT; in the 220-loop: R**SQ**VNGQ**R**GRM vs. R**PK**VNGQ**S**GRM) may reflect adaptation to avian receptors (α2-3-linked sialic acids) while preserving the core binding pocket[18,19]. Importantly, the antigenic sites surrounding the RBS specifically HA1 head sites 1 (140–145) and 2 (156–157), and the F' subdomain of HA1 contained mutations distinct from the reference. The motifs PYQGTP (site 1) and KK or KR (site 2), along with NHETS in the F' subdomain of HA1 have been previously identified in diverse H5N8 isolates and are associated with altered antigenicity[4,20]. These changes may facilitate immune escape from pre-existing antibodies, complicating vaccination strategies[21].

Several key amino acid substitutions were uniformly present in the HA of all Iranian isolates: S128P, S137A, T160A, K222Q, and S227R. Each of these mutations has been experimentally shown to enhance binding to human-type (α2-6) sialic acid receptors, thereby increasing the potential for zoonotic transmission[22,9]. Specifically, T160A is known to increase binding to α2-6 receptors and increased transmission in guinea pigs[9]. S137A have been associated with increased pseudovirus binding to α2-6 receptors and are considered markers of mammalian adaptation[9]. K222Q and S227R further augment binding to both α2-3 and α2-6 receptors, potentially bridging the gap between avian and human host specificity[9]. The simultaneous presence of these mutations in all Iranian isolates raises concern about their potential for human infection, although no human cases of H5N8 have been reported in Iran to date[10]. Continuous surveillance of these molecular markers is imperative.

Analysis of the NA gene revealed the presence of the I314V substitution in all four isolates. This mutation has been linked to reduced susceptibility to oseltamivir, although with a moderate effect[9]. The absence of other well-established NA resistance mutations (E119A/D/G/V, D198G, H274Y, R292K, N294S) indicates that the viruses remain largely susceptible to neuraminidase inhibitors, including zanamivir, peramivir, and laninamivir[9,23]. Notably, all isolates retained

wild-type residues at positions 119, 198, 274, 292, and 294, suggesting that oseltamivir resistance is not yet fixed in this Iranian lineage. In the M2 protein, none of the adamantane resistance mutations (L26F, I/V27A/T/S, A30V/T/S, S31N/G, G34E) were detected; the conserved residues L26, V27, A30, S31, and G34 correspond to a drug-sensitive phenotype[9,24]. This is in contrast to many currently circulating influenza A viruses, which have acquired the S31N mutation and are resistant to amantadine and rimantadine[9,24]. The absence of M2 resistance in the Iranian H5N8 isolates suggests that these viruses have not been exposed to adamantane selection pressure, likely because these drugs are not routinely used in poultry.

5. Conclusion

In conclusion, the Iranian HPAI H5N8 isolates analyzed in this study belonged to clade 2.3.4.4b and were genetically related to contemporary viruses reported in Africa and the Middle East. All isolates contained the polybasic HA cleavage site motif and shared amino acid substitutions previously reported to be associated with altered receptor-binding properties. The NA I314V substitution was also detected, whereas major NA inhibitor and M2 adamantane-resistance markers were absent. Given the limited sample size and sequence-based nature of this study, the phenotypic implications of these molecular markers require experimental confirmation. Nevertheless, these findings provide baseline molecular data for H5N8 viruses in Iran and support the need for continued genomic surveillance and functional characterization.

Acknowledgment
The authors extend their gratitude to all the authors of the research paper included in this research paper. Also, we would acknowledge the Directorate of Health and Management of Poultry Disease of the Iran Veterinary Organization for their support.

Compliance with ethical guidelines
No animal or human samples were used. All methods were carried out according to the relevant guidelines and regulations.

Data availability
All the data associated with this project is presented in this manuscript.

Funding
This article was extracted from the PhD dissertation of Kiarash Roohani Shahrestani, approved by the Department of Clinical Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran. This study was supported by Razi Vaccine and Rerearch Institute, Karaj, Iran (Grant No.: 020760-053-18-18-2)

Authors' Contribution

Study concept and design: A.S; Acquisition of data: A.S; Analysis and interpretation of data: K. R.S; Drafting of the manuscript: K.R.S; Critical revision of the manuscript for important intellectual content: S.V, A.S, M.H.F.M and S.C; Statistical analysis: K.R.S; Administrative, technical, and material support: S.V and A.S

Conflict of Interest

The authors declared no conflict of interest

References

1. Rafique S, Rashid F, Mushtaq S, Ali A, Li M, Luo S, et al. Global review of the H5N8 avian influenza virus subtype. Front Microbiol. 2023;14:1200681. https://doi.org/10.3389/fmicb.2023.1200681

2. Ghafouri SA, Fallah Mehrabadi MH, Talakesh SF, Hosseini H, Ziafati Z, Malekan M, et al. Full genome characterization of Iranian H5N8 highly pathogenic avian influenza virus from Hooded Crow (Corvus cornix), 2017: The first report. Comp Immunol Microbiol Infect Dis. 2019;64:73–80. https://doi.org/10.1016/j.cimid.2019.03.005

3. Yamaji R, Saad MD, Davis CT, Swayne DE, Wang D, Wong FYK, et al. Pandemic potential of highly pathogenic avian influenza clade 2.3.4.4 A(H5) viruses. Rev Med Virol. 2020;30:e2099. https://doi.org/10.1002/rmv.2099

4. Motahhar M, Keyvanfar H, Shoushtari A, Fallah Mehrabadi MH, Nikbakht Brujeni G. The arrival of highly pathogenic avian influenza viruses H5N8 in Iran through two windows, 2016. Virus Genes. 2022;58:527–39. https://doi.org/10.1007/s11262-022-01930-8

5. Chen J, Liang B, Hu J, Liu H, Sun J, Li M, et al. Circulation, Evolution and Transmission of H5N8 virus, 2016–2018. J Infect. 2019;79:363–72. https://doi.org/10.1016/j.jinf.2019.07.005

6. Kwon J, Bahl J, Swayne DE, Lee Y, Lee Y, Song C, et al. Domestic ducks play a major role in the maintenance and spread of H5N8 highly pathogenic avian influenza viruses in South Korea. Transbound Emerg Dis. 2020;67:844–51. https://doi.org/10.1111/tbed.13406

7. Wang R, Taubenberger JK. Methods for molecular surveillance of influenza. Expert Rev Anti Infect Ther. 2010;8:517–27. https://doi.org/10.1586/eri.10.24

8. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol. 2001;146:2275–89. https://doi.org/10.1007/s007050170002

9. Suttie A, Deng Y-M, Greenhill AR, Dussart P, Horwood PF, Karlsson EA. Inventory of molecular markers affecting biological characteristics of avian influenza A viruses. Virus Genes. 2019;55:739–68. https://doi.org/10.1007/s11262-019-01700-z

10. Ghafouri SA, GhalyanchiLangeroudi A, Maghsoudloo H, Kh Farahani R, Abdollahi H, Tehrani F, et al. Clade 2.3.4.4 avian influenza A (H5N8) outbreak in commercial poultry, Iran, 2016: the first report and update data. Trop Anim Health Prod. 2017;49:1089–93. https://doi.org/10.1007/s11250-017-1302-z

11. Tarek M, Naguib MM, Arafa A-S, Tantawy LA, Selim KM, Talaat S, et al. Epidemiology, Genetic Characterization, and Pathogenesis of Avian Influenza H5N8 Viruses Circulating in Northern and Southern Parts of Egypt, 2017–2019. Animals. 2021;11:2208. https://doi.org/10.3390/ani11082208

12. Kord E, Kaffashi A, Ghadakchi H, Eshratabadi F, Bameri Z, Shoushtari A. Molecular characterization of the surface glycoprotein genes of highly pathogenic H5N1 avian influenza viruses detected in Iran in 2011. Trop Anim Health Prod. 2014;46:549–54. https://doi.org/10.1007/s11250-013-0528-7

13. Tscherne DM, García-Sastre A. Virulence determinants of pandemic influenza viruses. J Clin Invest. 2011;121:6–13. https://doi.org/10.1172/JCI44947

14. Schulze IT. Effects of Glycosylation on the Properties and Functions of Influenza Virus Hemagglutinin. J Infect Dis. 1997;176:S24–8. https://doi.org/10.1086/514170

15. Tate M, Job E, Deng Y-M, Gunalan V, Maurer-Stroh S, Reading P. Playing Hide and Seek: How Glycosylation of the Influenza Virus Hemagglutinin Can Modulate the Immune Response to Infection. Viruses. 2014;6:1294–316. https://doi.org/10.3390/v6031294

16. Collins PJ, Haire LF, Lin YP, Liu J, Russell RJ, Walker PA, et al. Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants. Nature. 2008;453:1258–61. https://doi.org/10.1038/nature06956

17. Yen H-L, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, et al. Neuraminidase Inhibitor-Resistant Influenza Viruses May Differ Substantially in Fitness and Transmissibility. Antimicrob Agents Chemother. 2005;49:4075–84. https://doi.org/10.1128/AAC.49.10.4075-4084.2005

18. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, et al. Early Alterations of the Receptor-Binding Properties of H1, H2, and H3 Avian Influenza Virus Hemagglutinins after Their Introduction into Mammals. J Virol. 2000;74:8502–12. https://doi.org/10.1128/JVI.74.18.8502-8512.2000

19. Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. Structure and Receptor Specificity of the Hemagglutinin from an H5N1 Influenza Virus. Science. 2006;312:404–10. https://doi.org/10.1126/science.1124513

20. Lee D-H, Bahl J, Torchetti MK, Killian ML, Ip HS, DeLiberto TJ, et al. Highly Pathogenic Avian Influenza Viruses and Generation of Novel Reassortants, United States, 2014–2015. Emerg Infect Dis. 2016;22:1283–5. https://doi.org/10.3201/eid2207.160048

21. Zeng X, Shi J, Chen H. Control of highly pathogenic avian influenza through vaccination. J Integr Agric. 2024;23:1447–53. https://doi.org/10.1016/j.jia.2024.03.044

22. Dong Y, Bian Y, Huo C, Wu Y, Du Y, Yang R, et al. Adaptive mutations of H5 subtype avian influenza virus under the immune selection pressure in experimental conditions. J Integr Agric. 2026;S2095311926001450. https://doi.org/10.1016/j.jia.2026.04.004

23. McKimm-Breschkin JL. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. Influenza Other Respir Viruses. 2013;7:25–36. https://doi.org/10.1111/irv.12047

24. Bright RA. Adamantane Resistance Among Influenza A Viruses Isolated Early During the 2005-2006 Influenza Season in the United States. JAMA. 2006;295:891. https://doi.org/10.1001/jama.295.8.joc60020