Complete Genome Sequencing of an HPAI H5N8 (Clade 2.3.4.4b) Vaccine Seed Candidate from the Razi Vaccine and Serum Research Institute

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Abstract:

Introduction: With the inclusion of inactivated H5 vaccines in the national immunization program and the current reliance on imported products, the Razi Vaccine and Serum Research Institute has prioritized the development of a domestic vaccine seed candidate. This study aimed to provide a comprehensive genomic characterization of RHPAIV-4, an H5N8 avian influenza virus isolated in 2018 and considered as a potential vaccine seed candidate.

Material and Methods: Next-generation sequencing was performed, followed by molecular and phylogenetic analyses to characterize the genomic features of RHPAIV-4.

Result: Comparative genomic analysis revealed greater than 99.5% nucleotide sequence identity across all eight gene segments between RHPAIV-4 and A/crow/Aghakhan/2017 (H5N8). The RHPAIV-4 strain possessed the REKRRKR polybasic cleavage site motif, characteristic of highly pathogenic avian influenza H5N8 viruses belonging to clade 2.3.4.4b. Receptor-binding site analysis identified glutamine (Q) and glycine (G) at positions 226 and 228, respectively. Furthermore, no known antiviral resistance-associated markers were detected in the neuraminidase (NA) and matrix 2 (M2) proteins. The presence of glutamic acid (E) at position 627 of the PB2 protein was also consistent with the molecular characteristics of other circulating H5N8 strains.

Conclusion: Collectively, the genomic data presented here will serve as a valuable resource for the development of both monovalent and multivalent vaccine formulations. Future research will focus on generating a plasmid-based recombinant version of this virus to enhance production efficiency and diversify the product portfolio.

Keywords: Avian influenza virus, genetic characterization, next-generation sequencing, vaccine candidate, subtype H5N8.

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1. Introduction

Avian influenza viruses (AIVs) are a major global threat to both poultry production and public health, causing substantial economic losses worldwide. Aquatic birds, particularly ducks and geese, serve as the primary natural reservoirs, from which these viruses can spill over into highly susceptible domestic poultry, such as turkeys and chickens [1]. Belonging to the species *Alphainfluenzavirus influenzae* and the family *Orthomyxoviridae*, AIVs possess a segmented, negative-sense, single-stranded RNA genome that encodes at least 11 proteins [2, 3]. This species is further subdivided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), of which 19 and 11 have been identified, respectively [4]. Pathologically, AIVs cause two distinct disease forms in poultry: low-pathogenic (LPAI) and highly pathogenic avian influenza (HPAI), with the latter primarily associated with H5 and H7 subtypes [1].

The H5N8 subtype, a notable HPAI virus, was first isolated in China in 2010 [5]. Its subsequent evolution has unfolded in two major waves. The first, involving Group A (Buan-like) viruses, spread from eastern China across Asia and into Europe in 2014 [5]. Although this wave subsided, a second wave emerged in 2016, characterized by Group B (Gochang-like) reassortants, which circulated extensively across Europe, Asia, and Africa, causing one of the largest recorded HPAI epidemics to date [5, 6]. Despite intensive control efforts, including vaccination and culling, H5N8 has persisted, particularly in China, with active surveillance detecting the virus in wild birds and poultry from 2020 to 2023 [7, 8]. Critically, the host range of this virus is expanding; in 2020, a zoonotic event was documented in which poultry workers in Russia contracted novel H5N8 strains, highlighting its pandemic potential [9]. This risk is compounded by the virus's propensity for reassortment with co-circulating LPAI viruses in mixed domestic-wild bird interfaces, generating novel variants with unpredictable virulence and transmissibility [9].

A key factor driving the evolution of AIVs, particularly the HA gene, is immune selection pressure, which can be exacerbated by vaccination [10]. While vaccination remains a cornerstone of avian influenza control, evidence suggests that it can inadvertently accelerate viral evolution by favoring antigenic variants, enhancing genetic diversity, and increasing HA genetic distance, a phenomenon observed across multiple viral clades and datasets [10-12]. This underscores the critical need for continuous genomic surveillance to ensure that vaccine seed strains remain antigenically matched to circulating field viruses.

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In Iran, the first HPAI H5N8 outbreak was confirmed on a commercial layer farm in Tehran province in November 2016, with the virus identified as clade 2.3.4.4 via real-time RT-PCR and sequencing [13]. Given Iran's location along major migratory bird routes, the country faces an ongoing risk of virus introduction and endemic establishment [14]. Consequently, developing an indigenous H5N8 vaccine is a strategic imperative to reduce dependency on imported products and ensure rapid response capabilities. However, the efficacy of a vaccine is predicated on comprehensive genetic characterization of the seed strain to confirm its antigenic relevance. Therefore, the present study was designed to provide a complete genetic and phylogenetic characterization of the H5N8 vaccine seed candidate (RHPAIV-4), establishing a foundational dataset for subsequent vaccine development and production.

2. Materials and Methods

2.1 Virus Isolation and Sample Preparation

The virus (RHPAIV-4) used in this study was originally isolated from tracheal samples collected from backyard poultry in Tehran Province, Iran, in March 2018. Isolation was performed at the Influenza Reference Laboratory of the Razi Vaccine and Serum Research Institute (RVSRI). Prior to RNA extraction, the virus was inactivated using a guanidine isothiocyanate-containing lysis buffer (Buffer RLT, RNeasy Mini Kit; Qiagen, Hilden, Germany) according to the protocol described previously [15]. Total RNA was extracted using the High Pure Viral RNA Kit (Roche Life Science, Mannheim, Germany) following the manufacturer's instructions. The extracted RNA was stored at -70°C until further use.

2.2 Reverse Transcription and Subtype Confirmation

Reverse transcription was carried out using the RevertAid cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA) with the universal primer Uni12 (5′-AGCRAAAGCAGG-3′). Preliminary confirmation of the H5 subtype was performed by PCR using subtype-specific primers as described previously [16].

2.3 Multisegment RT-PCR and Library Preparation

The complete genome of the H5N8 virus was amplified using a multisegment RT-PCR approach with the primer pair 5′-ACGCGTGATCAGCAAAAGCAGG-3′ and 5′-

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ACGCGTGATCAGTAGAAACAAGG-3′ [17]. Amplification was performed using the Titan One Tube RT-PCR System Kit (Roche, Mannheim, Germany). The resulting PCR products were purified, and DNA concentration and quality were assessed using a NanoDrop spectrophotometer. Purified amplicons were subsequently sent to Macrogen Inc. (Seoul, South Korea) for whole-genome sequencing.

2.4 Quality Control and Genome Assembly

Raw Illumina paired-end sequencing data were obtained in FASTQ format and initially assessed for sequence quality using FastQC v0.12.1. Adapter sequences and low-quality bases were removed using fastp v1.3.4, with bases having a Phred quality score (Q) below 30 excluded from downstream analyses. Reads that did not meet the predefined quality and length criteria after filtering were also removed. The quality of the filtered reads was subsequently reassessed using FastQC to confirm the effectiveness of adapter removal and quality filtering.

Genome reconstruction was performed using both de novo and reference-guided approaches. For de novo assembly, the quality-filtered paired-end reads were assembled without a reference genome using SPAdes v4.2.0. For reference-guided assembly, the filtered paired-end reads were aligned to A/crow/Aghakhan/2017 reference sequences using Minimap2 v2.28. The resulting alignments were converted to BAM format, sorted, and indexed using SAMtools v1.24. PCR duplicate removal was performed where appropriate, based on the sequencing library preparation protocol. Mapping statistics and sequencing depth were assessed using Qualimap v2.3 to evaluate the quality and completeness of the genome reconstruction.

Sequence variants were identified from the reference-guided alignments using BCFtools v1.24. Variant calls were filtered according to predefined quality and sequencing depth criteria. A consensus sequence was generated from the filtered variant calls and the reference sequence using BCFtools. High-quality consensus genomes were subsequently used for genome annotation and downstream comparative and phylogenetic analyses.

2.5 Phylogenetic and Bioinformatics Analysis

For comparative analysis, related gene sequences were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) database. Additionally, the most closely related sequences to the studied virus were identified using BLAST searches against the NCBI

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GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment of deduced amino acid sequences was performed using the CLUSTAL W algorithm implemented in MEGA12 software [18]. Phylogenetic trees were constructed for each of the eight gene segments using the Neighbor-Joining (NJ) method with the Maximum Composite Likelihood (MCL) model in MEGA12 [18]. The robustness of the tree topology was assessed by bootstrap analysis with 1,000 replicates. Potential N-linked glycosylation sites in the HA and NA proteins were predicted using the NetNGlyc 1.0 server.

3. Result

3.1 PCR and Multisegment RT-PCR

The results of PCR confirmation of H5 subtype and multisegment RT-PCR of RHPAIV-4 are shown in Figure 2. Each gene was amplified denoted in the photo (Figure 1).

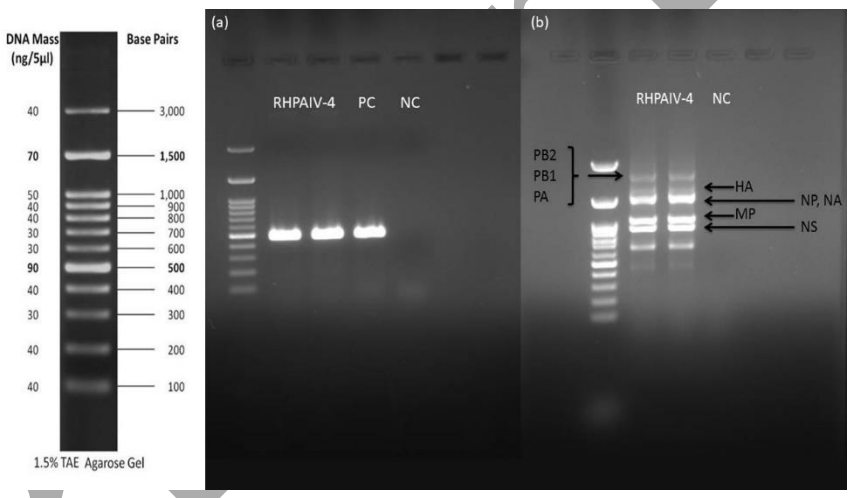


Figure 1. Agarose gel electrophoresis results. (a) RT-PCR confirmation of the H5 subtype using specific primers. (b) Whole-genome amplification products of all eight influenza A gene segments separated by size.

3.2 Phylogenetic analysis

BLAST analysis was performed against the NCBI GenBank database, and the most closely related sequences to the studied virus were retrieved from this database (Table 1). The RHPAIV-4 isolate showed approximately 100% nucleotide identity with an H5N8 strain isolated from a crow (A/crow/Aghakhan/2017). The phylogenetic tree based on the HA gene is shown in Figures 2 and 3. The studied virus clusters within clade 2.3.4.4b based on the HA

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sequence (Figures 2 and 3). For the remaining gene segments (NA, PB2, PB1, PA, NP, MP, and NS), phylogenetic trees are presented in Figure 4.

Table 1. Top BLAST hits for each of the eight gene segments of the studied influenza A virus isolate (RHPAIV-4) against the GenBank nucleotide database. For each segment, the closest matching reference sequence (highest percent identity) is shown.

Gene	Closely Related Strain 1	Identity [a]	Closely Related Strain 2	Identity [a]
PB2	A/crow/Aghakhan/2017 (MK168596) [b]	99.56	A/green-winged teal/Egypt/877/2016 (MF037863)	99.12
PB1	A/crow/Aghakhan/2017 (MK168597)	99.60	A/little grebe/Egypt/1056OP/2016 (MH498573)	99.21
PA	A/crow/Aghakhan/2017 (MK168598)	99.91	A/common teal/Korea/W548/2016 (KY576102)	99.49
HA	A/crow/Aghakhan/2017 (MK168599)	99.65	A/chicken/Alborz/RVSRI 127/2016 (PX457194)	99.59
NP	A/crow/Aghakhan/2017 (MK168600)	99.80	A/teal/Egypt/MB-D-487OP/2016 (H7N3) (MN208011)	99.33
NA	A/crow/Aghakhan/2017 (MK168601)	99.86	A/chicken/Alborz/RVSRI 127/2016 (PX457193)	99.79
MP	A/crow/Aghakhan/2017 (MK168602)	100	A/chicken/Alborz/RVSRI 127/2016 (PX464116)	100
NS	A/crow/Aghakhan/2017 (MK168603)	99.71	A/little grebe/Egypt/MB-D-1056C/2016 (OQ793924)	99.42

[a] Percentage of nucleotide sequence identity based on the complete coding sequences (CDS) of the eight influenza A virus gene segments: PB2 (positions 28–2307), PB1 (25–2298), PA (25–2175), HA (29–1732), NP (46–1542), NA (21–1433), MP (26–784), and NS (27–719), relative to the reference strain.

[b] Viruses without a subtype designation are H5N8 unless otherwise specified.

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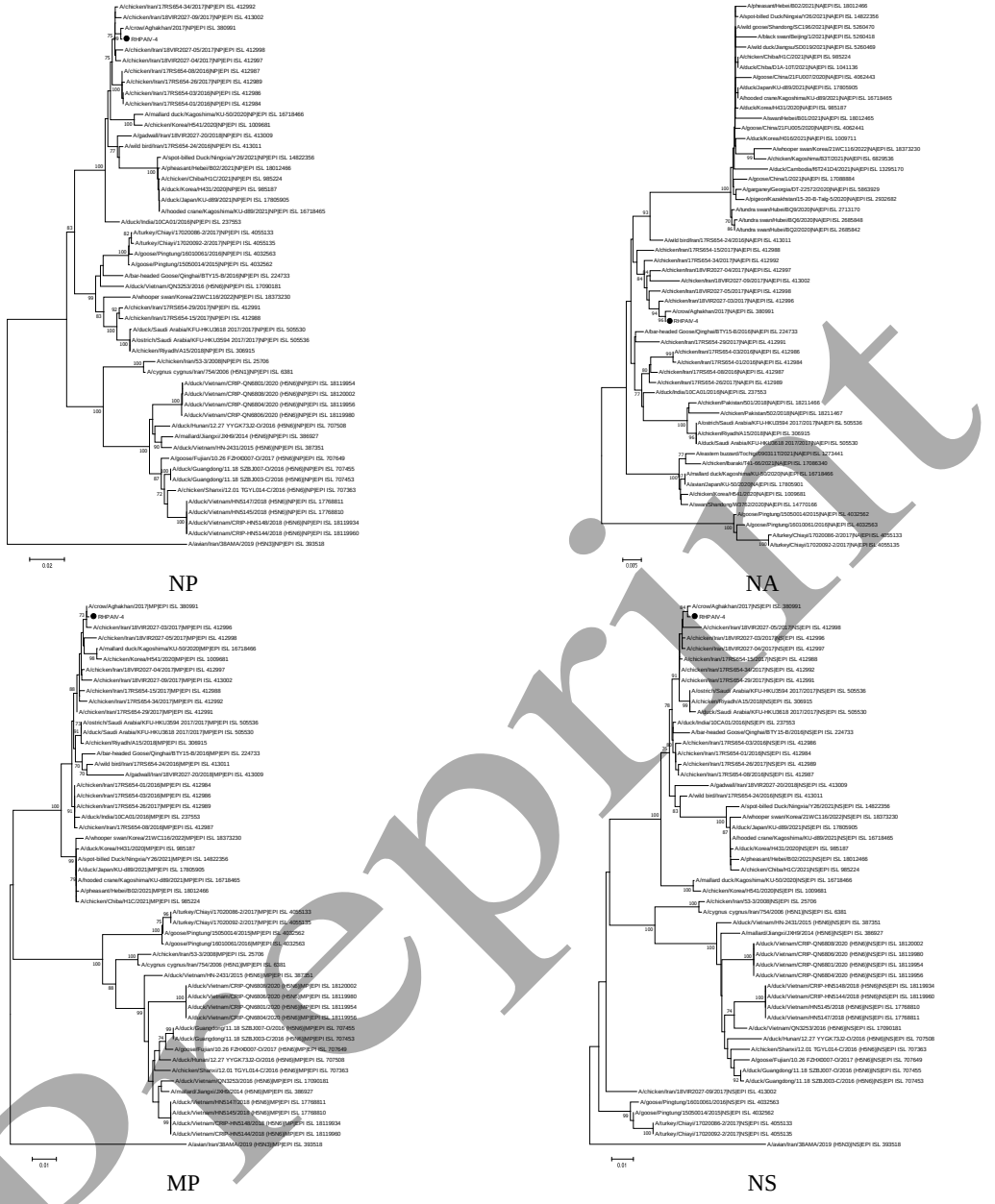
Figure 2. Phylogenetic tree based on the nucleotide sequences of the HA gene. The tree includes the study strain (indicated by a filled circle, ●) and reference sequences representing major global clades, retrieved from GISAID. Bootstrap support values (≥70%) are shown at the branch nodes. Clades are indicated by vertical bars on the right, with corresponding clade designations (e.g., clades 1–9). The scale bar indicates the number of nucleotide substitutions per site.



Figure 3. Phylogenetic trees based on the complete coding sequences of all eight gene segments (PB2, PB1, PA, HA, NP, NA, MP, and NS) of the studied H5N8 virus (RHPAIV-4) and related H5Nx reference sequences from different geographic regions, retrieved from GISAID. The study isolate is marked with a black filled circle (●). Bootstrap support values (≥70%) are shown at branch nodes. For the HA tree, major sub-clades are indicated by vertical bars on the right, with corresponding clade designations. The scale bar represents the number of nucleotide substitutions per site.

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Figure 3 (continued)



3.3 Genetic characteristics

The amino acid sequences of the eight genes of RHPAIV-4 were deduced and compared with those of Iranian H5N8 viruses retrieved from GenBank. Amino acid numbering of the HA protein in this study follows the H3 subtype numbering convention (Table 2) [19]. Molecular markers affecting biological characteristics were evaluated using a comprehensive inventory list. From this list, only those markers that exhibited mutations associated with virulence or pathogenicity are presented in Table 2. Analysis revealed that RHPAIV-4 possesses the cleavage site motif PLREKRRKR*GLF. The vaccine reference strain was predicted to have

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five and two potential N-linked glycosylation sites (N-X-S/T, where X is any amino acid except proline) in the HA1 and HA2 subunits, respectively. At the receptor binding site, the studied virus contained the following amino acids at key positions: E190, G225, Q226, R227, and G228. The presence of Q226 and G228 is characteristic of avian-type receptor specificity.

Analysis of the NA stalk region of RHPAIV-4 revealed no deletions. Prediction of potential N-linked glycosylation sites identified six putative sites at positions 56, 69, 86, 146, 294, and 402. All residues within the enzymatic active site were conserved, and no mutations associated with antiviral resistance were detected (data not shown).

Amino acids at positions 26, 27, 30, 31, and 34 in the M2 protein are associated with resistance or sensitivity to M2-blocking antiviral drugs (adamantane derivatives). Sequence analysis of the M2 protein showed that the studied virus does not have any mutations at these sites. Analysis of the amino acid sequence of the NS1 protein showed that all studied viruses had 230 amino acids and did not show any deletions at positions 80–84. The NS1 protein of the isolates in this study possessed a PDZ domain motif of GSEV.

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Table 2. Amino acid sequence comparison of key molecular markers in eight gene segments of the Iranian isolate RHPAIV-4 versus reference H5N8 strains from GISAID.

Virus	Key Molecular characteristics																			
	PB2	**PB1**		**PA**			**HA**					**NP**		**NA**	**M1**				**NS1**	
Segment position	627	3	622	383	137	158	160	226	228	Cleavage site	Glycosylation sites	105	184	Glycosylation sites	30	43	215	42	138	149	PDZ domain
RHPAIV-4	E	V	G	D	A	N	A	Q	G	PLREKRRKR	20, 21, 33, 169, 289 /* 154, 212	V	K	56, 69, 86, 146, 294, 402	D	M	A	S	F	A	GSEV
Iranian H5N8 reference strains	—†			Same HA profile as RHPAIV-4								—		44‡, 56, 69, 86, 146, 294, 402	—						

* Slash (/) separates glycosylation sites located in HA1 (N-terminal side) from those in HA2 (C-terminal side).

† — indicates not done.

‡ Underlined positions indicate glycosylation sites that are present in some strains but absent in others (polymorphic).

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4. Discussion

HPAI viruses of the H5Nx subtype have posed a persistent threat to poultry production and wild bird populations worldwide. Iran has experienced repeated introductions and outbreaks of HPAI H5 viruses over the past two decades, with several distinct viral lineages and subtypes being reported in both wild and domestic birds. Understanding the genetic evolution of these viruses and developing vaccines based on epidemiologically relevant strains are therefore important components of HPAI prevention and control strategies in Iran.

The first major H5N1 outbreak in Iran was reported in wild swans in the Anzali wetland in northern Iran in early February 2006, resulting in substantial mortality among the affected birds [20]. Subsequently, outbreaks of H5N1 HPAI in domestic poultry were officially reported in Mazandaran Province in 2007 and 2011, according to records available through the World Organization for Animal Health (WOAH) World Animal Health Information System (WAHIS). Phylogenetic analyses demonstrated that the H5N1 viruses detected in 2011 were genetically distinct from the H5N1 viruses previously reported in Iran. Whereas the earlier Iranian viruses belonged to clade 2.2, the 2011 viruses clustered within subclade 2.3.2.1 and showed a closer genetic relationship to viruses isolated from Mongolia in 2010 [21].

According to the WOAH Influenza Situation Report (September 2026) and data available through the WAHIS interface, HPAI viruses belonging to H5N1, H5N8, H5N6, and H5N5 subtypes have been reported in wild birds, backyard poultry, and commercial poultry in different regions of Iran since 2011. The repeated detection of these viruses highlights the continuing risk of introduction, dissemination, and genetic evolution of HPAI H5 viruses within the country.

Until approximately mid-2018, the principal strategy for controlling HPAI H5 infection in Iran relied on stamping-out and culling of infected and epidemiologically linked poultry. However, the extensive H5N8 outbreaks occurring during 2016–2018 resulted in substantial economic losses and prompted a strategic reconsideration of disease-control measures. Consequently, the Iranian Veterinary Organization introduced targeted vaccination of pullet, layer, and breeder flocks in high-risk provinces, initially focusing on areas such as Alborz, Qazvin, Qom, and Tehran, where poultry populations are dense and the risk of virus introduction and transmission is relatively high. Subsequently, H5 vaccination was expanded to other provinces according to regional risk assessments.

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At the time, vaccination programs in Iran relied largely on imported H5 vaccines. The use of vaccines developed from heterologous or genetically and antigenically divergent strains may result in suboptimal protection when substantial differences exist between the vaccine strain and viruses circulating in the field. Furthermore, Iran had not previously established domestic production of an inactivated H5 vaccine based on an indigenous H5 virus. These circumstances provided an important rationale for the identification and characterization of an appropriate local H5 vaccine seed strain. Once characterized, a suitable candidate virus could be adapted for vaccine production by reverse genetics, using an internal-gene backbone such as A/Puerto Rico/8/1934 (H1N1) (PR8) and removing the multibasic sequence at the HA cleavage site linked to high pathogenicity.

An effective avian influenza vaccine should ideally provide strong protection against disease, mortality, and virus replication following exposure to circulating field viruses. In addition to antigenic compatibility with prevalent viruses, desirable characteristics of an avian influenza vaccine include the ability to induce protective immunity following practical vaccination regimens, applicability to relevant poultry production systems, suitability for mass administration, compatibility with the detection of infected animals in vaccinated populations (DIVA), and effectiveness in the presence of maternally derived antibodies. Additional desirable characteristics include suitability for administration at the hatchery, including at one day of age or in ovo, and affordability [22]. Although no vaccine necessarily possesses all of these characteristics simultaneously, antigenic compatibility between the vaccine strain and circulating field viruses remains a particularly important consideration.

The importance of antigenic matching has become increasingly evident as HPAI viruses have continued to evolve. Unlike human seasonal influenza vaccines, which are routinely updated in response to antigenic drift, poultry H5 vaccines have historically provided relatively broad and long-lasting homosubtypic protection. Earlier studies demonstrated that several H5 LPAI vaccine strains and recombinant fowl pox virus expressing H5 could provide substantial protection against mortality following challenge with diverse H5 HPAI viruses isolated between 1959 and 1997, despite differences of up to approximately 12% in HA1 amino acid sequences between the vaccine and challenge viruses [23]. However, the increasing circulation of H5 viruses under vaccination pressure has highlighted the potential for antigenic divergence and reduced vaccine effectiveness. Since the mid-2000s, vaccine-resistant or vaccine-escape H5 viruses have emerged in several settings, particularly following prolonged vaccination campaigns or sustained circulation of H5 viruses in vaccinated or previously infected poultry

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populations [22, 23]. Consequently, continuous evaluation of vaccine seed strains against contemporary field viruses is essential to maintain protection against clinical disease, mortality, and virus shedding.

Evidence from Egypt further demonstrates the importance of vaccine-field virus matching [24]. In one study, eight commercially available H5 poultry vaccines were evaluated alongside an experimental vaccine based on an Egyptian H5N8 LPAI virus generated using reverse genetics. The experimental vaccine (clade 2.3.4.4) and a commercial Re-5 vaccine (clade 2.3.4) both provided complete protection against mortality and substantially reduced virus shedding following challenge. In contrast, several of the commercial vaccines evaluated in that study showed limited efficacy, which was associated with substantial genetic differences between their vaccine seed viruses and the H5N8 viruses circulating in Egypt. Importantly, some vaccines protected chickens against mortality but did not prevent virus shedding [24]. These findings illustrate that protection against clinical disease alone does not necessarily indicate effective control of virus replication and transmission, and further support the use of vaccine strains with appropriate genetic and antigenic relationships to currently circulating viruses.

The identification of HPAI H5Nx viruses in numerous wild bird species further complicates disease control because migratory birds can facilitate long-distance dissemination of these viruses and introduce them into domestic and commercial poultry populations [1]. Consequently, vaccination should be considered as one component of an integrated control strategy that also includes active surveillance, biosecurity, rapid detection, movement control, and appropriate outbreak management [22]. In this context, the continuous genetic and antigenic characterization of circulating H5 viruses is essential for identifying evolutionary changes that could compromise existing vaccines and for informing the selection or updating of vaccine seed strains.

5. Conclusion

Finally, the repeated occurrence of HPAI H5Nx outbreaks in wild birds and domestic poultry underscores the need for sustained national surveillance and periodic reassessment of vaccine effectiveness. Indigenous production of inactivated H5 vaccines based on appropriately characterized local strains could provide several potential advantages, including improved genetic and antigenic relevance to circulating viruses, greater flexibility in updating vaccine seed strains, reduced dependence on imported products, and potentially lower costs associated with vaccine procurement [22]. Nevertheless, genomic similarity alone is insufficient to

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establish vaccine efficacy. The H5N8 candidate characterized in this study should therefore undergo further evaluation, including antigenic characterization, immunogenicity studies, challenge experiments, assessment of virus shedding, and safety and production-quality evaluations, before its suitability as a vaccine seed is established.

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Authors' Contribution

Conceptualization, methodology, and supervision: Mohsen Bashashati. Virus amplification and molecular detection: Bahram Haerian Ardakani, Aida Chalesh, and Soroush Geramitabar. Formal analysis and investigation: Mohsen Bashashati and Fereshteh Sabouri. Drafting of the manuscript: Mohsen Bashashati, Fereshteh Sabouri, and Soroush Geramitabar. Writing, review and editing: All authors. Funding acquisition: Mohsen Bashashati. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no known conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

Not applicable. This study did not involve human participants, human samples, or animal subjects.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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