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Research Article

Molecular Surveillance of Avian Influenza Virus Based on HA Gene Isolated from Commercial Poultry in Pakistan

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Abstract

Avian influenza virus (AIV) continues to pose a major risk to the global poultry industry and human health on account of its high mutation rate, segmented genome, and its ability to undergo genetic reassortment. H9N2 is among the low-pathogenic types that are particularly important due to being highly endemic in the poultry, causing severe economic losses, and is a possible source of internal genes of the highly pathogenic and zoonotic influenza viruses. Continuous molecular surveillance of circulating H9N2 strains is essential to monitor the evolution of the virus, its reassortment potential, and its effectiveness in vaccines. The aim of the study was to examine the molecular prevalence and partial genetic characterization of low-pathogenic subtype of AIV H9N2 in commercial poultry in Punjab, Pakistan, in 2024. One hundred pooled oropharyngeal, tracheal, lungs, and cloacal samples were collected from symptomatic flocks with respiratory symptoms and low egg production and 40 samples were processed to isolate the virus in specific antibody-negative embryonated chicken eggs and tested with hemagglutination (HA) and hemagglutination inhibition (HI) assays. Molecular confirmation was done through SYBR Green based real-time RT-PCR targeting a 765bp fragment of the hemagglutinin (HA) gene. PCR-positive samples were sequenced and analyzed through BLAST, multiple sequence alignment, and phylogenetic analysis. 10 isolates were classified as H9N2, showing nucleotide similarity of 87.8% to 98.3% with previously reported Pakistani isolates. Mutation analysis revealed various deletions and nucleotide substitutions, indicating that there has been continuous genetic evolution. All indigenous isolates were clustered within the G1-like lineage of Eurasian H9N2 viruses in phylogenetic analysis. In conclusion, the study confirms the continuation of H9N2 AIV circulation and genetic diversification in Pakistani Poultry and highlights the importance of continued molecular surveillance to support effective control and vaccination strategies.

Keywords

Avian Influenza H9N2 Virus, RT-PCR, Phylogenetic Tree, Mutation Analysis

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1. Introduction

Avian influenza is a transmittable viral disease of birds and mammals caused by Influenza A viruses, which are a family of Orthomyxoviridae. These are single-stranded, negative-sense, segmented RNA viruses belonging to five genera Influenza A, B, C, Thogotovirus, and Isavirus [1]. The most concerning among them is the Influenza A viruses due to its wide host, high mutation rate, and its potential to cause epidemics and pandemics [2]. Avian influenza viruses (AIVs) are further classified according to the pathogenicity into high pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses [3]. Strains of HPAI, commonly of H5 and H7, have a multi-basic cleavage site at hemagglutinin (HA), which permits systemic infection and mortality in poultry. Conversely, LPAI strains, such as H9N2, are usually known to cause mild respiratory disease, reduced egg production, moderate mortality but can still have sustainable economic impact [4, 5].

Despite its low pathogenicity, H9N2 is considered important due to its potential to contribute genetic segments to other viruses via reassortment, its circulation in vaccinated flocks due to antigenic drift, and its occasional zoonotic transmission, as strains with mammalian-adaptive markers have been isolated from humans [6]. Influenza A viruses encode ten proteins, including surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are critical for viral entry and release [7]. HA determines host specificity, pathogenicity, and antigenicity, and frequent variation in HA enables persistent circulation and immune evasion. H9N2 infections in chickens are characterized by mild to moderate respiratory symptoms, head and face edema, reduced egg production, and soft-shelled eggs, and by sudden mortality caused by airways obstruction by necrotic debris [8, 9].

Conventional diagnosis methods such as molecular techniques including Real time polymerase chain reaction (qPCR) provide rapid, sensitive, and specific detection and genetic characterization. It is thus necessary to continuously monitor the evolution of the viruses, determine the effectiveness of vaccines and the risks to the health of the population through continuous molecular surveillance.

This study aims to determine the prevalence and partial genetic characteristics of the circulating low-pathogenic H9N2 avian influenza viruses in poultry by using molecular and serological techniques (qPCR, HA/HI), to aid in surveillance, disease control, and vaccination approaches.

2. Materials and Methods

2.1. Study Design

The present study was designed to carry out molecular surveillance and genetic characterization of circulating low-pathogenic avian influenza virus subtype H9N2 in poultry during the year 2024. The genetic variation of field isolates was determined by examining partial hemagglutinin (HA) gene analysis, especially mutation patterns and evolutionary trends by analyzing the database-verified sequences at the National Center of Biotechnology (NCBI).

2.2. Sample Collection

100 samples comprising of oropharyngeal, tracheal, lung and cloacal swabs were taken from suspected morbid poultry flocks likely to be infected with avian influenza virus. The respiratory symptoms in the flocks were mild to moderate, and there was reduced egg production and the mortality rate was less than 10%, consistent with low-pathogenic avian influenza infection.

From each farm, 10 individual oropharyngeal and cloacal swabs were pooled and treated as a single sample to improve the effectiveness of surveillance. Samples were collected from different regions of Punjab, placed in sterile containers with ice packs, and transported under cold-chain conditions to the Ottoman Pharma R&D Molecular Laboratory for further processing.

2.3. Virus Isolation

Specific antibody negative (SAN) embryonated chicken eggs were used in virus isolation. The Ottoman Pharma hatchery supplied 400 SAN eggs. Every pooled sample was prepared in 5 mL of normal saline with Penstrep (Penicillin+Streptomycin) (200-ug/mL) and centrifuged at 4000 rpm for 2 minutes.

The supernatant was filtered through a 0.22 µm membrane filter (Hy Docs, UK), and 0.1 mL was inoculated into 10-day-old embryonated chicken eggs via the allantoic sac route. Inoculated eggs were incubated at 37°C for 48–72 hours, followed by chilling at 4°C for 2 hours prior to harvesting.

Allantoic fluid was harvested and subjected to hemagglutination (HA) and hemagglutination inhibition (HI) assays with subtype-specific antisera as the preliminary serological confirmation of the presence of avian influenza virus. Three serial passages were performed before declaring samples negative, as described by Edwards (2006). Serologically confirmed samples were further processed for molecular analysis.

2.4. Molecular Characterization

The FAVORGEN Nucleic Acid Extraction Kit (Taiwan) was used to extract viral RNA in HA-positive allantoic fluid based on the instructions of the manufacturer. The synthesis of the complementary DNA (cDNA) was done with a reverse transcription kit of Transgene S. A., containing RNA template, RNase-free water, and the H9-specific primers.

The RT-PCR amplification of the partial hemagglutinin

(HA) gene (~765 bp) was carried out using SYBR green-based RT-PCR in a total reaction volume of 25 µL, which included antiQ qPCR SYBR mix, cDNA template, RNase-free water and forward (5'-AGCAAAATCACGGGAAYWWC-3') and reverse (5'-CGATACGATGGGGCAAATAG-3') primers.

The thermal cycling conditions included an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation, annealing, and extension at a temperature of 55–72°C for 30 seconds, with a final extension at 72°C for 10 minutes performed on a Genetier 96E Real-Time PCR system (TIAN LONG, USA). The instrument has an integrated software that analyzed amplification curves.

2.5. Nucleotide Sequence Accession Numbers

The partial sequences of hemagglutinin genes of 10 indigenous H9N2 isolates generated in this study were submitted to the NCBI GeneBank database.

2.6. Phylogenetic Analysis

The PCR-positive samples were purified and submitted to Apical Scientific SDN. BHD., Malaysia to be sequenced. Basic Local Alignment Search Tool (BLAST) was used to identify the similarity of sequences with reference strains present in the NCBI GeneBank database. The CLUSTAL W algorithm was used to perform multiple sequence alignment and phylogenetic trees were constructed using the maximum likelihood method under the MEGA X software. The resulting trees were visualized and further refined with the help of Interactive Tree of Life (ITOL) to determine the evolutionary relationship among the indigenous, national, and international H9N2 isolates.

3. Results

The morbid poultry flocks from which samples were collected exhibited clinical signs consistent with low-pathogenic avian influenza virus (LPAI) infection. These included mild to moderate respiratory distress characterized by gasping, anorexia, lethargy, and a noticeable decline in egg production. These were mild to moderate respiratory distress which was gasping with anorexia and lethargy with a significant reduction in egg production. An average mortality rate of less than 10% was recorded in the affected flocks. Such clinical manifestations are commonly associated with infection by avian influenza virus subtype H9N2.

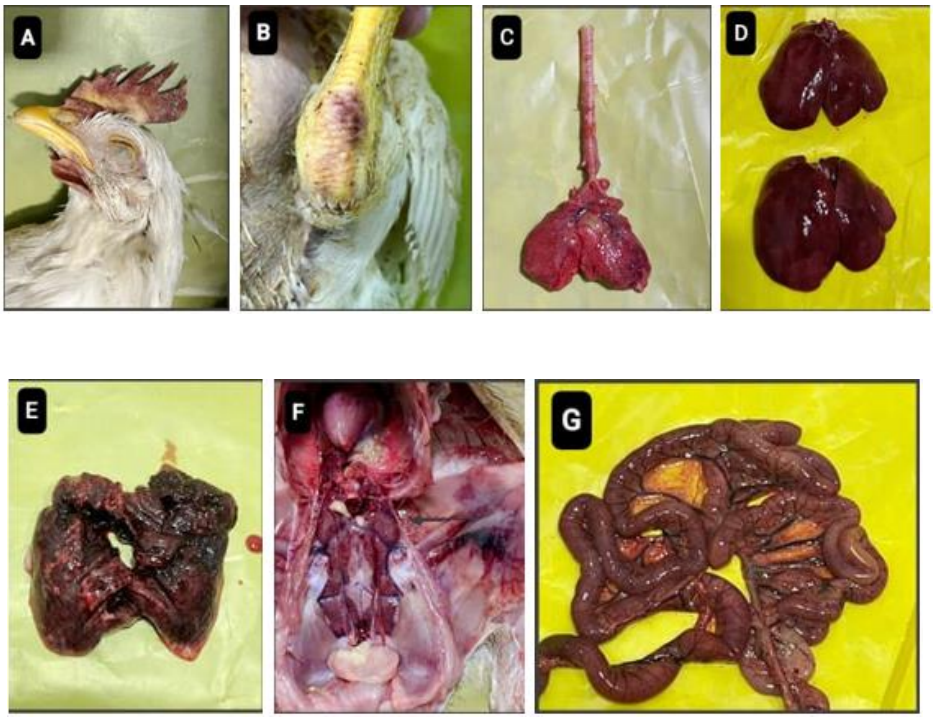


Figure 1. *Clinical signs and Postmortem lesions caused by Avian Influenza virus (AIV H9N2) shows Upper respiratory tract (URT) and Lower respiratory tract (LRT) infection: (A) Hemorrhagic comb (B) Hemorrhages on shanks (C) Severely hemorrhagic and necrosed respiratory tract (D) Hemorrhagic enlarged liver (E) Necrosis of lungs (F) Molted texture and petechial hemorrhages on kidneys (G) Hemorrhagic mesentery.*

40 samples were selected for virus cultivation and subsequent serological and molecular analysis. Among these, 20 samples tested positive by spot agglutination assay, indicating seroconversion against avian influenza virus. In the hemagglutination (HA) assay, all 20 samples showed positive activity with titers up to a 1:512 dilutions, while in the hemagglu-

tination inhibition (HI) test, 15 samples inhibited agglutination at a dilution of 1:128, confirming subtype-specific antibodies against avian influenza virus H9.

For molecular confirmation, Real-time polymerase chain reaction (qPCR) was performed using H9-specific primers targeting the partial hemagglutinin (HA) gene. Ten samples were PCR-positive, generating the expected 765 bp amplicon, as shown in Figure 2.

The NCBI analysis of the partial Hemagglutinin protein gene of these isolates led to the interpretation that OP53 under the accession number PV269864 showed 98.30% similarity with Gene Bank accession number PQ803294, which belongs to Pakistan. Similarly, 5 isolates that included OP44 (PV018512), OP45 (PV018567), OP47 (PV018632), OP50 (PV018703) & OP52 (PV022338) showed 96.82%, 97.46%, 97.44%, 97.675, and 96.95% similarity, respectively, to the already submitted sequences to NCBI under the accession numbers MW358029 & MW386860. In addition, OP46 (PV018629) and OP51 (PV022333) showed 97.56% and 87.80% similarity to the sequences under accession numbers MT912793 and MT912810. OP48 (PV18666) and OP49 (PV018689) depicted 97.77% and 97.64% similarity to the accession number MZ067008 of Pakistan, as shown in Table 1.

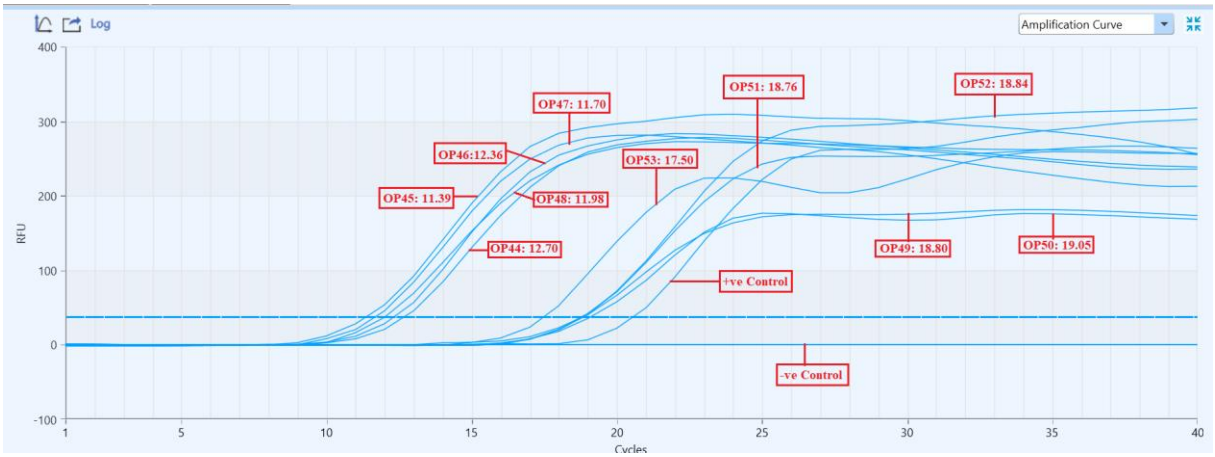


Figure 2. *Real time PCR results blue channel FAM for AIV-H9 positive samples through Heamagglutination inhibition assay with positive and negative control.*

On the other hand, amongst these isolates, OP44, OP45, OP47, OP50, and OP52 were identical to the strain UVAS-LHR-19, and OP46, OP51 resembled the strain GP-LHR-19. Likewise, OP48 and OP49 were similar to the strain UDL106/21, and OP53 to the strain Pak/AIVH9/24 (Table 1).

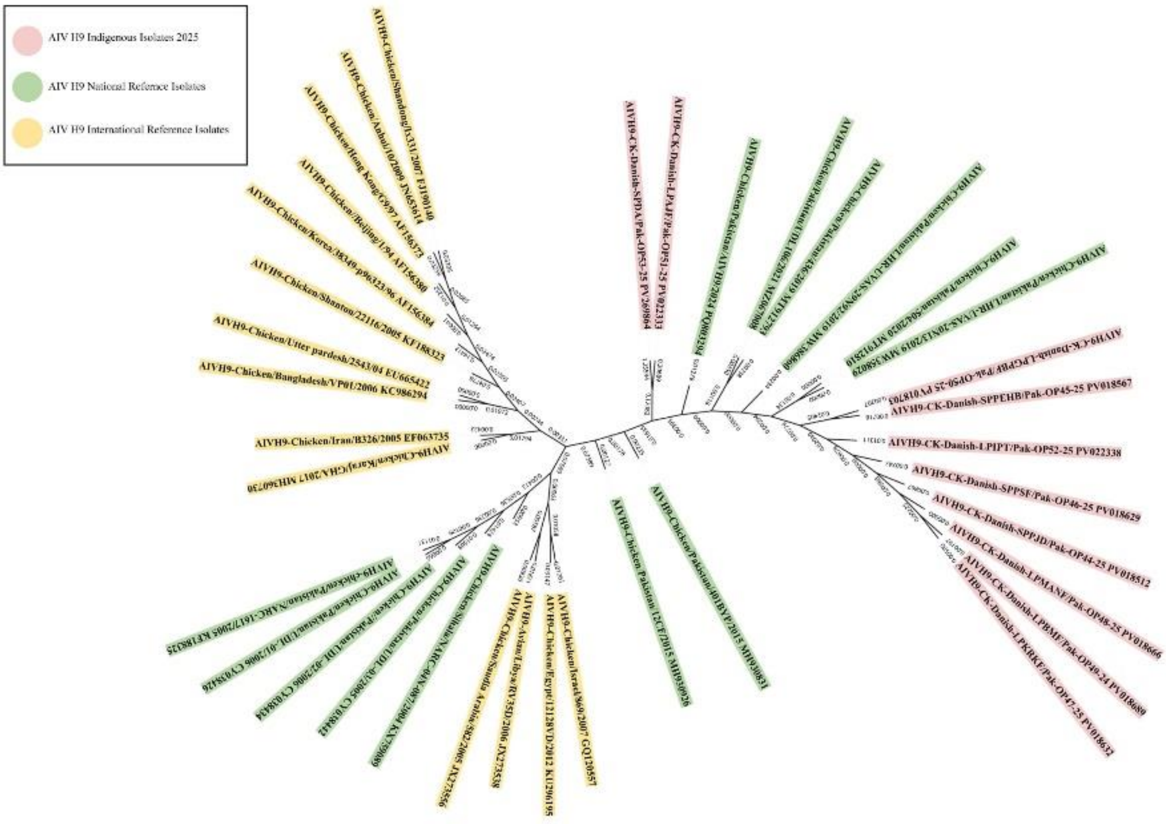
Table 1. *Shows a complete description of the Avian Influenza low-pathogenic serotype H9, including the date and location of sample collection, sample IDs, similarity rate, isolate accession numbers, and similar sequences from NCBI.*

Sr#	Title	Date	Location	Age (Days)	Mortality rate (%)	NCBI% similarity	NCBI BLAST A#	Strain	NCBI OP A#
1	AIVH9-CK-Danish-Pak-OP44-24	10-12-24	Multan	30	7%	96.82%	MW358029	UVAS-LHR-19	PV018512
2	AIVH9-CK-Danish-Pak-OP45-24	4-12-24	Lahore	28	8%	97.46%	MW386860	UVAs-LHR-19	PV018567
3	AIVH9-CK-Danish-Pak-OP46-25	9-01-25	Sheikhupura	34	5%	97.56%	MT912793	GP-LHR-19	PV018629
4	AIVH9-CK-Danish-Pak-OP47-25	31-12-24	Kot Radha Kishan	32	3%	97.44%	MW358029	UVAS-LHR-19	PV018632
5	AIVH9-CK-Danish-Pak-OP48-25	31-12-24	Mandianwala	36	4%	97.77%	MZ067008	UDL106/21	PV018666
6	AIVH9-CK-Danish-Pak-OP49-25	31-12-24	Badu Murady	32	8%	97.64%	MZ067008	UDL106/21	PV018689

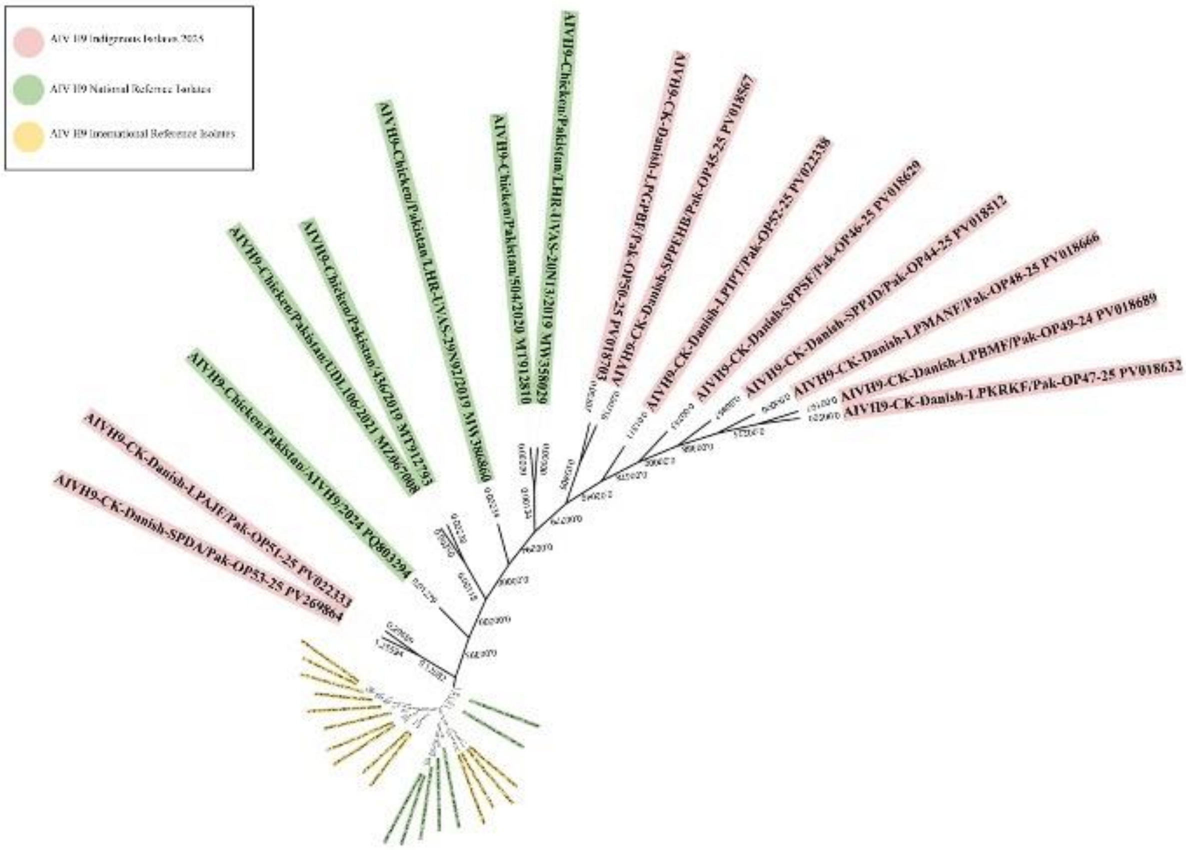
Sr#	Title	Date	Location	Age (Days)	Mortality rate (%)	NCBI% similarity	NCBI BLAST A#	Strain	NCBI OP A#
7	AIVH9-CK-Danish-Pak-OP50-25	2-6-22	Lahore	33	9%	97.67%	MW386860	UVAS-LHR-19	PV018703
8	AIVH9-CK-Danish-Pak-OP51-25	1-04-24	Ferozewala	25	5%	87.80%	MT912810	GP-LHR-19	PV022333
9	AIVH9-CK-Danish-Pak-OP52-25	27-12-24	Sahiwal	38	4%	96.95%	MW358029	UVAS-LHR-19	PV022338
10	AIVH9-CK-Danish-Pak-OP53-25	22-02-25	Gujranwala	27	6%	98.3%	PQ803294	PakA-IVH9/24	PV269864

The BLAST study of indigenous isolates, compared with sequences already submitted to the Gene Bank of NCBI, revealed different mutation sites. The identical analysis (88%) of OP51 showed maximum substitution rate with MT912810 at positions30 (C→T), 39 (C→T), 43 (C→A), 44 (C→G), 49 (C→A), 52 (C→A), 54 (C→A), 58 (C→T), 62 (C→G), 71 (C→T), 82 (T→C), 92 (C→T), 104 (C→A), 105 (C→G), 121 (C→G), 129 (C→T), 138 (C→G), 156 (C→A), 161 (C→G), 199 (T→C), 252 (T→C), 261 (A→G), 264 (G→A), 275 (C→G), 301 (T→C), 339 (C→A), 340 (C→G), 341 (C→A), 342 (C→A), 343 (C→A), 358 (C→A), 359 (C→G), 366 (C→T), 379 (C→G), 391 (C→G), 400 (C→T), 402 (C→T), 407 (C→A), 424 (C→T), 446 (C→T), 462 (C→T), 467 (C→A), 468 (C→G), 469 (C→G), 497 (C→G), 508 (C→T), 513 (C→G), 516 (C→G) and base deletion at 37, 77, 89, 107, 108, 112, 119, 137, 245, 344, 408, 448 and 470. The comparison of OP44 and OP45 (97% identity) with accession numbers MW358029, MW386860 showed intermediate substitution at 19 (C→T), 40 (A→G), 49 (G→A), 51 (C→A), 53 (A→G), 57 (A→T), 66 (G→A), 81 (T→C), 129 (C→T), 138 (A→G), 159 (T→C), 204 (T→C), 270 (C→A), 315 (A→G), 416 (A→T), 585 (G→A), 693 (C→T), 711 (C→T), 791 (A→C) and25 (A→G), 39 (C→T), 76 (C→T), 115 (G→A), 202 (T→C), 214 (A→G), 305 (T→C), 316 (G→A), 373 (C→T), 523 (T→C), 544 (T→G), 584 (G→C), 622 (T→C), 628 (G→A), 649 (G→A), 688 (A→G), 705 (A→T), 787 (C→T), 789 (A→C) and deletion at 796, 794 respectively. Percentage similarity analysis (97%) of OP47 and OP52 with MW358029 also showed intermediate nucleotide substitution in query sequence at positions 17 (C→T), 47 (G→A), 49 (C→A), 51 (A→G), 55 (C→T), 79 (T→C), 127 (C→T), 136 (A→G), 157 (T→C), 204 (T→C), 268 (C→A), 313 (A→G), 415 (T→C), 437 (A→G), 475 (A→G), 559 (C→T), 691 (C→T), 709 (C→T) and at 49 (C→A), 51 (A→G), 55 (C→T), 79 (T→C), 127 (C→T), 136 (A→G), 192 (G→A), 202 (T→C), 265 (A→G), 273 (A→G), 313 (A→G), 341 (A→G), 415 (T→C), 418 (A→G), 419 (C→A), 475 (A→G), 559 (C→T), 613 (T→C), 658 (C→T), 691 (C→T), 709 (C→T), 757 (G→A), 789 (A→C), 791 (N→T) and base deletions at positions 788 respectively. 98% identity analysis of OP53, OP50, OP48 with PQ803294, MW386860, MZ067008 showed a minimum nucleotide substitution rate at site 123 (G→A), 138 (T→C), 165 (T→C) and26 (A→G), 40 (C→T), 77 (C→T), 116 (G→A), 203 (T→C), 215 (A→G), 233 (G→A), 306 (T→C), 317 (G→A), 374 (C→T), 524 (T→C), 545 (T→G), 585 (T→C), 623 (T→C), 629 (G→A), 650 (G→A), 689 (A→G), 706 (A→T) and at positions 47 (G→A), 49 (C→A), 51 (A→G), 55 (A→G), 79 (T→C), 127 (C→T), 136 (A→G), 157 (T→C), 202 (T→C), 214 (G A), 265 (A→G), 268 (C→A), 415 (T→C), 475 (A→G), 691 (C→T), 709 (C→T) and nucleotide deletion at 23 respectively. Besides the analysis of OP46 and OP49 with MT912793 and MZ067008 (98%) also suggested minimum nucleotide substitution at positions 43 (G→A), 45 (C→A), 47 (A→G), 51 (C→T), 75 (T→C), 123 (C→T), 132 (A→G), 153 (T →C), 158 (C→T), 198 (T→C), 261 (A→G), 264 (A→G), 309 (A→G), 411 (T→C), 471 (A→G), 586 (A→G), 687 (C→T), 705 (C→T), 783 (C→T)and49 (G→A), 51 (C→A), 53 (A→G), 57 (C→T), 81 (T→C), 129 (C→T), 138 (A→G), 159 (T→C), 204 (T→C), 216 (G→A), 267 (A→G), 270 (C→A), 417 (T→C), 477 (A→G), 693 (C→T), 711 (C→T).

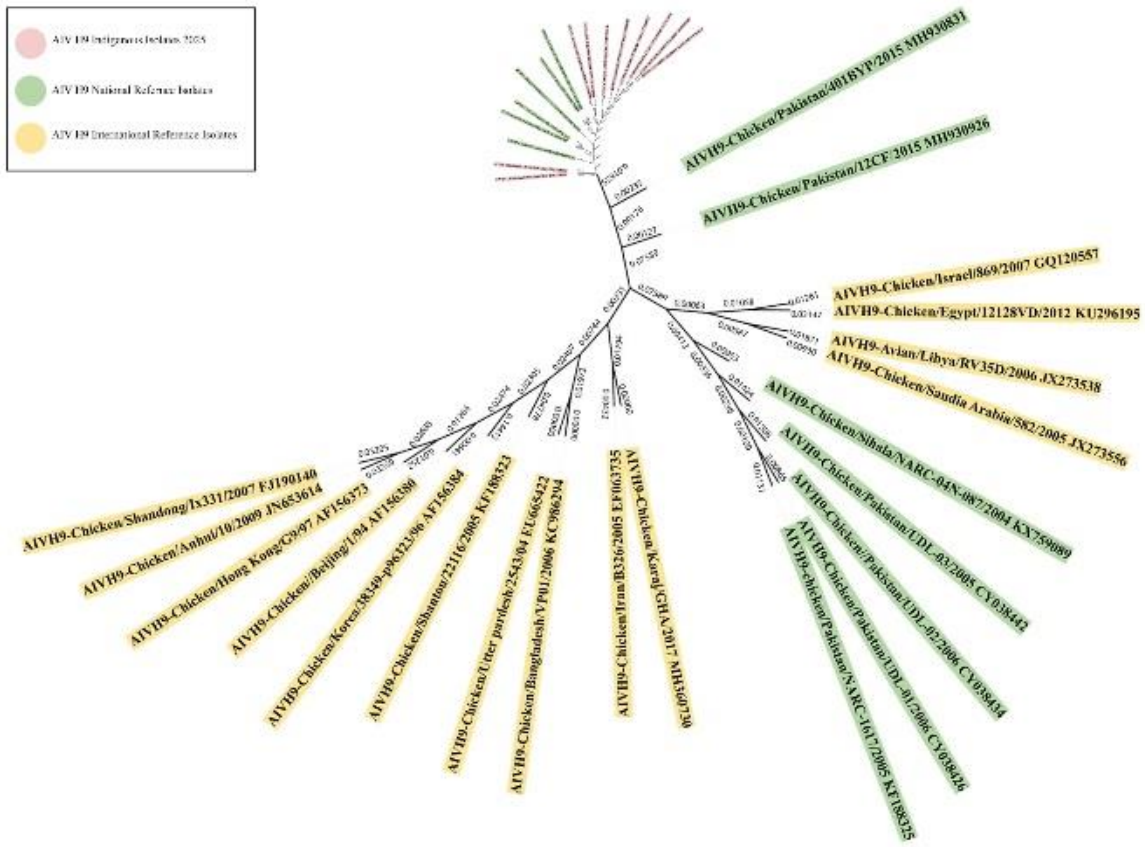
The phylogenetic tree was constructed by aligning 10 Indigenous, 13 National, and 14 International AIVH9 isolates in phylogenetic analysis. Indigenous isolates under accession numbers PV269864 and PV022333 fall with the Pakistani isolate PQ803294 with a difference of 0.00393, and PV018703, PV018567, PV022338, PV016829, PV018512, PV018666, PV018689 and PV018632 fall with the Pakistani isolates under accession numbers MW358029 and MT912810 with a length difference of 0.00779, 0.02049, 0.00678, 0.00000, 0.00388, 0.00225 respectively as shown in Figure 3 (a, b).


AIV H9 Indigenous Isolates 2025
AIV H9 National Reference Isolates
AIV H9 International Reference Isolates
AIVH9-Chicken/Iran/B326/2005 EF063735
AIVH9-Chicken/Bangladesh/VP01/2006 KC986294
AIVH9-CK-Danish-SPPEHB/Pak-OP45-25 PV018567
AIVH9-CK-Danish-LPIPT/Pak-OP52-25 PV022338

(A)


AIV H9 Indigenous Isolates 2025
AIV H9 National Reference Isolates
AIV H9 International Reference Isolates
AIVH9-CK-Danish-LPKRKF/Pak-OP47-25 PV018632

(B)



(C)

Figure 3. *Phylogenetic analysis of the Partial hemagglutinin gene 4th segment, showing the evolutionary relationship between the studied indigenous isolates (highlighted in pink color) to the National reference AIV sequence (Green color) and International Reference sequence (yellow color) in the Gene Bank database. The phylogenetic tree was constructed using the maximum likelihood (ML) method in Mega-11 software.*

4. Discussion

Low-pathogenic avian influenza (LPAI) is a well-documented viral disease responsible for major economic losses in the poultry industry due to reduced productivity, morbidity, and variable mortality. Although classified as low pathogenic, LPAI viruses particularly subtype H9N2 are known to affect multiple organ systems, predominantly the respiratory and reproductive tracts [3, 5].

In the present investigation, samples were collected from morbid poultry flocks exhibiting clinical signs typical of LPAI H9N2 infection, including respiratory distress, gasping, mild fever, anorexia, weight loss, raised heads, and a noticeable decline in egg production. The recorded mortality rate ranged less than 10%, which is consistent with previous reports of H9N2 outbreaks in commercial poultry under field conditions, particularly when secondary bacterial infections or management stressors are present [4, 9].

Postmortem examinations revealed gross pathological lesions, including congestion and inflammation of the trachea, hemorrhagic and necrotic changes in the upper and lower respiratory tract, and inflammatory exudate in the oviduct. Additional findings included hemorrhagic enlargement of the liver, necrosis of lung tissue, mottled kidneys with petechial hemorrhages, and hemorrhagic mesentery. Similar lesions have been described in previous studies on H9N2 LPAI, where dark-red lungs, congested kidneys, and proventricular mucosal epithelial necrosis were reported [9, 10].

Serological analysis through Hemagglutination (HA) and Hemagglutination Inhibition (HI) assays revealed that 50% of the 40 virus cultivation samples contained H9-specific antibodies, confirming exposure to avian influenza virus. Molecular detection using SYBR Green–based real-time RT-PCR targeting the partial hemagglutinin (HA) gene identified 10 positive samples, producing the expected 765bp amplification product. These results are in agreement with similar studies where HA and HI assays demonstrated titers ranging from 1:16 to 1:512, and molecular detection confirmed H9N2 presence in field samples [11, 12].

The homology NCBI analysis of the hemagglutinin partial gene of these 10 isolates with other National strains revealed that the homology of gene sequences OP44, OP45, OP47, OP50, and OP52 with the Pakistani strain UVAS-LHR-19

ranged from 96.82%-97.67%. The similarity index of OP48 and OP49 with UDL106/21 was 97.77% and 97.64%. The homology between isolated stains OP46 and OP51 with GP-LHR-19 was 97.56% and 87.80%, respectively. Comparing the corresponding GeneBank submitted Pakistani strain PA-KAIVH9/24 with the isolated strain revealed gene homology exceeding 98%. The homology between the 35 isolated strains of virus based on HA nucleotide gene sequences was 92.1 to 99.7%, while the nucleotide homology between the 35 isolated strains and the Y280 vaccine strains was 86.1 to 92%, and the nucleotide homology of the 35 isolated strains with SY/97 strains was 86.4 to 91.8%. [13].

In the present study, nucleotide mutations were analyzed using the Basic Local Alignment Search Tool (BLAST) by comparing sequences of the partial hemagglutinin (HA) gene of indigenous H9N2 isolates. The analysis revealed maximum, intermediate, and minimum nucleotide variation, with sequence identities ranging from 88% to 98%. Mutations were observed as substitutions and deletions at multiple positions in the gene, as illustrated earlier. Some substitutions led to amino acid changes, which could influence viral antigenicity, but do not by themselves convert low-pathogenic strains into highly pathogenic strains. In different positions, the nucleotides like guanine (G) were substituted with adenine (A), cytosine (C), or thymine (T). The sequences of two isolates with low substitutions were relatively conserved, and the maximum number of mutations was observed in OP51, which means that it has more genetic diversity.

The same results have been reported previously. Zang sequenced 33 representative H9N2 viruses and identified homology of up to 93% to 96% HA nucleotide variation while [14]. Wu identified homology of between 90.8% and 92.0% similarity of HA sequences on comparison with the DK/H9N280/97 strain [15].

The evolutionary relationships and genetic homology of the native H9N2 isolates were evaluated by constructing a phylogenetic tree based on MEGA-11 and visualizing the topology with iTOL software. The analysis showed that isolates OP51 (PV022333) and OP53 (PV269864) were closely related to the Pakistani reference isolate PQ803294, indicating strong genetic relatedness within the regional lineage. The remaining indigenous isolates (OP44–OP50, OP52) exhibited high nucleotide homology (>90%) with previously reported Pakistani strains UVAS-LHR-19 and GP-LHR-19; [16].

Comparison with broader H9N2 reference sequences representing established Eurasian lineages such as G1, G9, Y280, and Y439 demonstrated that the studied isolates predominantly clustered within the G1-like lineage. This observation aligns with previous phylogenetic studies of H9N2 viruses from neighboring regions, where HA gene phylogeny indicated that H9N2 viruses from Iran, Pakistan, India, and other Middle Eastern countries group together within the G1-like sublineage of the Eurasian lineage [15, 16]. Specifically, earlier work on Iranian H9N2 isolates from 1998–2007 showed that all examined HA genes belonged to a single G1-like lineage with homology of approximately 89.4–93.9% with quail/G1/97 and parakeet/Narita/92A/98 strains, reflecting ongoing regional evolution of this lineage.

5. Conclusion

This study confirms the circulation of low-pathogenic H9N2 avian influenza viruses in commercial poultry in Pakistan, with partial HA gene analysis revealing high genetic similarity to regional G1-like strains. Molecular, serological, and phylogenetic findings highlight ongoing viral evolution and underscore the need for continuous surveillance to guide effective disease control and vaccine strategies.

Abbreviations

AIV	Avian Influenza Virus
H9N2	Hemagglutinin 9 Neuraminidase 2 Subtype
LPAI	Low Pathogenic Avian Influenza
HPAI	Highly Pathogenic Avian Influenza
RT-PCR	Reverse Transcription Polymerase Chain Reaction
qPCR	Real time Polymerase Chain Reaction
SYBR Green	SYBR Green Fluorescent Dye
HA	Hemagglutinin
HI	Hemagglutination Inhibition
RNA	Ribonucleic Acid
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
bp	Base Pair
µL	Microliter
rpm	Revolutions Per Minute
SAN	Specific Antibody Negative
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
CLUSTAL W	Cluster Alignment Tool W
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
ITOL	Interactive Tree Of Life
URT	Upper Respiratory Tract
LRT	Lower Respiratory Tract
NA	Neuraminidase
HA gene	Hemagglutinin Gene
A#	Accession Number

Author Contribution

Muhammad Danish Mehmood: Conceptualization & data Correction

Huma Anwar ul-Haq: Project Administration & Writing –

original draft

Romisa Sattar: Methodology

Mehak Aftab: Writing – review & editing

Nasir Abbas: Formal Analysis

Conflicts of Interest

There is no conflict of interest.

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