Phylogenetic Analysis of Avian Influenza Viruses in the Iran-Pakistan Region

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Abstract

Avian influenza viruses (AIVs) are a constant threat to the poultry industry and public health. The H9N2 subtype has been endemic in the Middle East since the 1990s, acting as a genetic segment donor and facilitating the emergence of new viruses through reassortment. This study provides a phylogenetic analysis of the evolutionary relationships and genetic reassortments among influenza virus sequences from the high-risk region of Iran and Pakistan and their neighboring countries. In this study, sequences of all six internal genes (PB2, PB1, PA, NP, M, and NS) of avian-origin influenza A viruses from Iran, Pakistan, Iraq, Turkey, and Afghanistan were analyzed. The sequences were obtained from the BV-BRC public database. Phylogenetic reconstruction was performed for each gene segment using the maximum likelihood method in RAxML (version 8.2.12) with the GTR+GAMMA model; topological robustness was assessed with 1000 bootstrap replicates. Examination and analysis of phylogenetic trees revealed distinct viral dynamics. The H9N2 subtype was detected in diverse avian hosts, with sequences from domestic chickens, ducks, and pigeons forming intertwined clusters within the phylogenetic tree, confirming the virus's active circulation and adaptation across multiple avian species. Phylogenetic analysis of H9N2 primarily revealed distinct country-specific clusters for Iranian and Pakistani virus isolates, suggesting independent evolution of local viruses within the poultry populations of each country. However, Phylogenetic analysis of H9N2 primarily showed distinct country-specific clusters for Iranian and Pakistani virus isolates, suggesting independent evolution of local viruses within the poultry populations of each country. However, some regions of the tree exhibited shared phylogenetic branches with closely related sequences from both countries, providing evidence of cross-border transmission.

Evidence also suggested a possible reassortment event between a 2009 Iranian H9N2 virus and a 2004 Pakistani H7N3 virus involving the PB1 gene, indicating genetic exchange between this highly pathogenic strain and H9N2. Overall, phylogenetic analysis revealed that viral circulation in these two countries has led to diverse and independent lineages, alongside evidence of viral interaction between them.

Consequently, this study emphasizes the urgent need for a coordinated regional surveillance framework based on the One Health approach. Such a system, focused on monitoring genetic changes in influenza viruses, would enable early warning of emerging threats and support the development of more effective strategies to protect the poultry industry and mitigate public health risks.

Keywords: Avian Influenza; Phylogenetics; Reassortment; Poultry; One Health; Iran; Pakistan; Middle East.

1. **INTRODUCTION**

Avian influenza viruses, with characteristics such as genetic reassortment and rapid evolution, are a constant threat to the poultry industry and human health worldwide, as they can lead to the emergence of new strains with altered pathogenicity and antigenic properties in diverse hosts. The H9N2 subtype is the most prevalent AIV subtype in birds worldwide, with varying disease severity, and has been reported to be endemic in the Middle East since the 1990s (1). Although this virus often causes mild disease in poultry, its role as a "gene donor" in reassortment events that have contributed to the emergence of zoonotic viruses such as H5N1 and H7N9 is prominent (2). Serological evidence of H9N2-specific antibodies in Iranian poultry workers, despite poultry vaccination, highlights its zoonotic transmission potential(3).

The epicenters of highly pathogenic avian influenza (HPAI) are shifting towards the Middle East and Africa (4) and This shift, combined with the presence of Eurasian migratory bird routes and inadequate surveillance in many neighboring countries, creates favorable conditions for viral gene exchange between influenza subtypes(5).

In addition, industrial poultry production and cross-border trade are increasing the spread of endemic avian influenza viruses (6). The outbreak of H5N8 avian influenza in Pakistan (2018), which was genetically related to a virus isolated from Saudi Arabia (2017), and the outbreak of H5N1 avian influenza in Europe (2021–2022), which resulted from reassortment with low-pathogenic viruses, highlight the importance of continuous surveillance of all circulating strains (4, 7). At the same time, low-pathogenic avian influenza viruses (LPAIVs) such as H9N2 and H3N2 are continuously circulating in live bird markets (LBMs). These markets act as convenient hubs where different subtypes co-circulate, allowing for frequent reassortment events(8).

Given the characteristics and potential of avian influenza viruses to harm the poultry industry and human health, monitoring circulating strains and analyzing key epidemiological events are crucial.

Objectives

This study aimed to investigate phylogenetic relationships and reassortment in avian influenza viruses in Iran, Pakistan, and neighboring countries.

2. MATERIAL AND METHODS

2.1. Collection of recorded sequences

In this study, genomic sequences of avian-origin influenza A viruses from Iran, Pakistan, Iraq, Turkey, and Afghanistan were retrieved from the BV-BRC (Bacterial and Viral Bioinformatics Resource Center) database. The data were screened using the BV-BRC search interface with filters applied for host (avian species), virus type (influenza A), and country of origin.

For all sequences, metadata including host species (e.g., chicken, duck, pigeon, wild bird) and subtype (e.g., H9N2, H5N1, H7N3) was collected. Sequences of short length were removed to minimize potential biases in phylogenetic reconstruction.

Gene segments: All internal genes were included: polymerase basic (base) 2 (PB2), polymerase basic 1 (PB1), polymerase acid (PA), nucleoprotein (NP), matrix (M) , and non-structural (NS) genes.

2.2. Data preparation and alignment

Extracted sequences for each gene segment (PB2, PB1, PA, NP, M, NS) were aligned using the multiple sequence alignment tool MUSCLE. The resulting alignments were then visually inspected in MEGA X software to identify and correct obvious misalignments or excessive gaps, thereby improving the reliability of the phylogenetic tree topology.

2.3. Phylogenetic tree construction

Phylogenetic reconstruction for each gene segment was performed using the "Gene/Protein Tree" tool within the BV-BRC database, which employs RAxML (version 8.2.12) under the maximum likelihood (ML) framework. The GTR+GAMMA nucleotide substitution model was applied to all trees, and model parameters were optimized using the Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm as implemented in RAxML.

Node support was assessed with 1000 bootstrap replicates. Separate maximum likelihood trees were constructed for the PB2, PB1, PA, NP, M, and NS gene segments. The resulting trees were exported in both Newick and graphic formats for downstream interpretation and figure preparation. (9).

3. RESULTS

Phylogenetic trees were constructed using sequences extracted from the datasets using RAxML (version 8.2.12), and different subtypes in the tree were distinguished by color -coded for clarity.

3.1 .H9N2 in different bird species

Analysis of available sequences and phylogenetic relationships revealed that circulating H9N2 viruses have been isolated from multiple bird species. For instance, H9N2 sequences from Pakistan (2015) obtained from diverse hosts, including chickens, ducks, and pigeons, formed tight phylogenetic clusters. This clustering pattern suggests that H9N2 viruses, despite being endemic in a region, can readily transmit among different avian species. Circulation among different avian hosts increases the potential for viral reassortment and the emergence of novel viruses.

3.2 .Host and Geographic Phylogenetic Patterns

The phylogenetic trees for the genes showed a tendency to form distinct clusters for sequences of Iranian and Pakistani origin, indicating intra-country and endemic evolution within the poultry production networks of each country. However, some sequences from the two countries were closely related in some branches of the trees, suggesting the possibility of transboundary transmission.

3.3 .Reassortment between subtypes

Sequences from waterfowl hosts tended to cluster in relatively separate branches from those of chickens. However, there were key points of common ancestry in the trees, with sequences from ducks and chickens being closely related, suggesting the circulation of some subtypes between waterfowl and domestic poultry in the region.

3.4. Genetic Diversity and Reassortment Potential of Avian Influenza Viruses in Iran and Pakistan

Analysis of sequenced samples and phylogenetic trees reveals significant diversity of avian influenza subtypes and evidence of genetic reassortment in Iran and Pakistan, particularly among waterfowl. In Iran, sequenced subtypes to date include H3N2, H5N1, H5N3, H5N8, and H9N2. Notably, H9N2 viruses isolated from ducks in Iran form a distinct phylogenetic branch separate from endemic viruses. In Pakistan, studies have documented the presence of various subtypes, including H14N13 and H4N6, co-circulating with highly pathogenic strains.

PB2

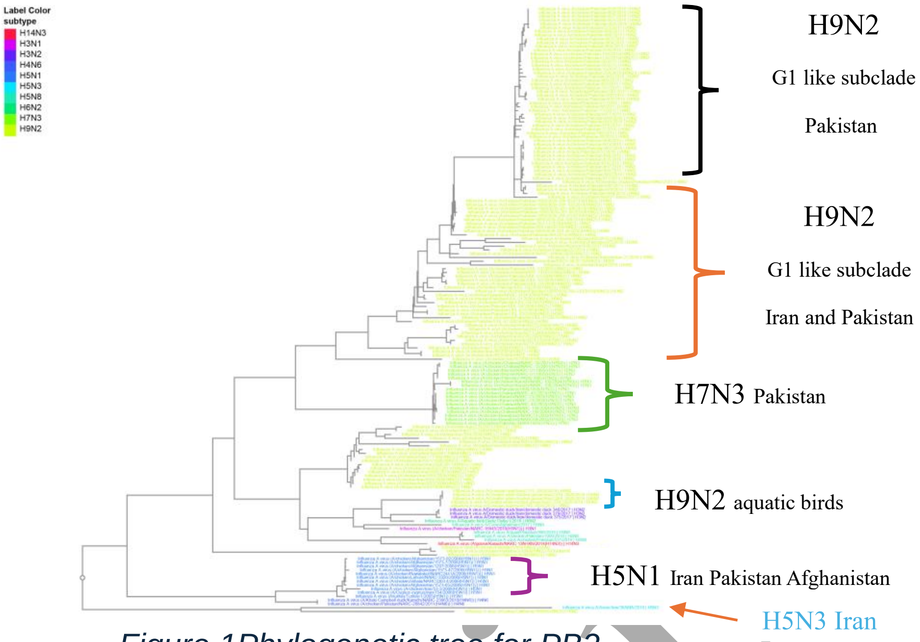

Figure 1Phylogenetic tree for PB2

PB1

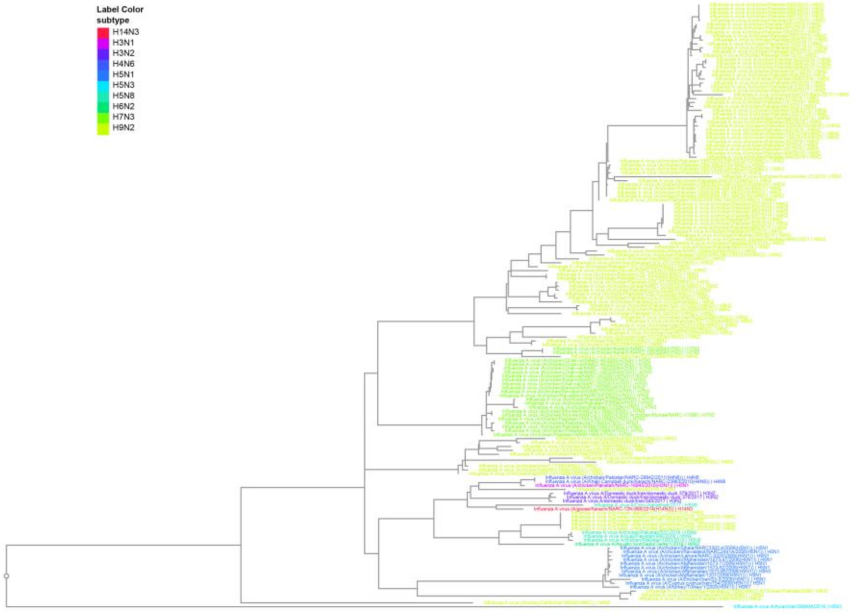

Figure 2 Phylogenetic tree for PB1

PA

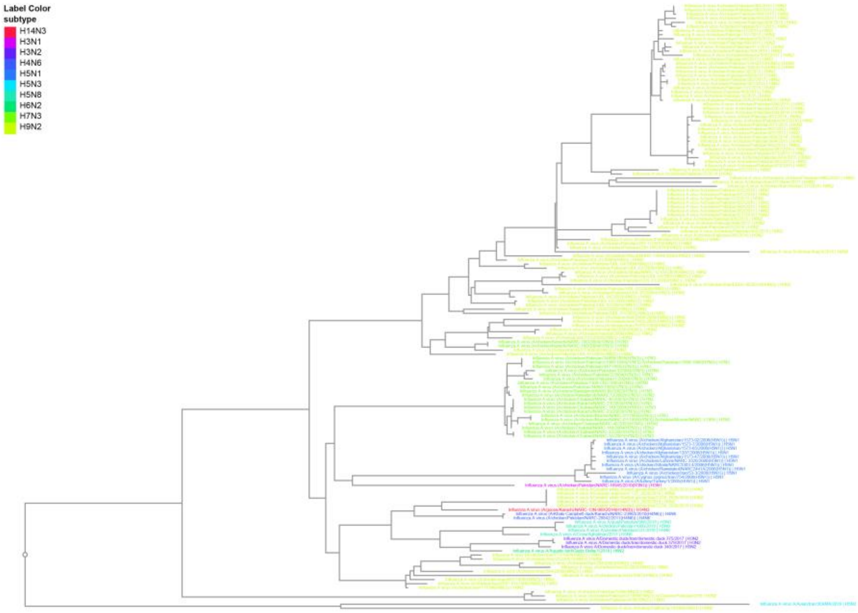

Figure 3Phylogenetic tree for PA

NP

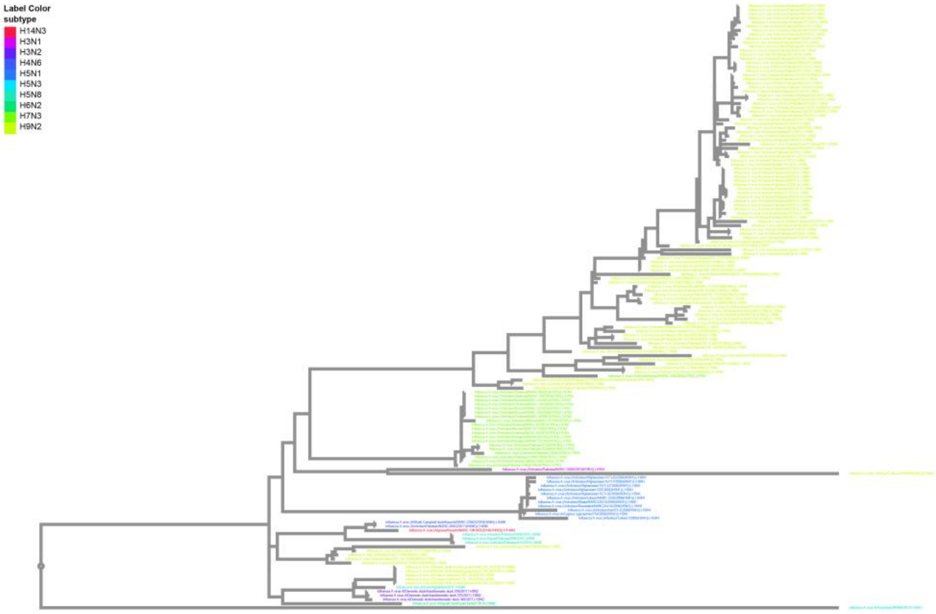

Figure 4Phylogenetic tree for NP

M

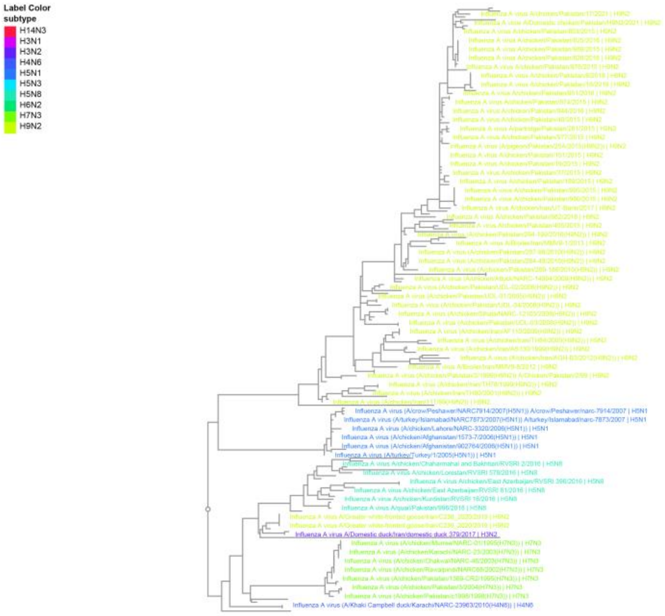

Figure 5Phylogenetic tree for M

NS

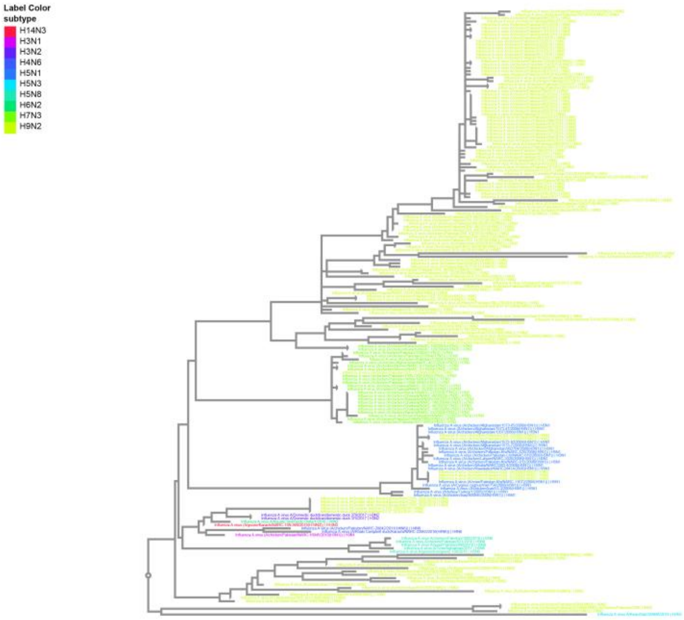

Figure 6Phylogenetic tree for NS

4. DISCUSSION

In this study, a distinct country-specific clustering was observed for H9N2 viruses from Iran and Pakistan, indicating independent circulation of these viruses in poultry populations, although shared branches were also identified in other parts of the phylogenetic tree. Poultry trade and geographic proximity facilitate the spread of avian influenza viruses in the region. As a result, influenza strains circulating in Iran and Pakistan can also spread to poultry in neighboring countries beyond the previously reported genetic exchanges between them. This is demonstrated by H9N2 isolates sequenced in Iraq that showed a close genetic relationship in their hemagglutinin (HA) gene to strains isolated in Iran and Pakistan (10, 11). However, in the absence of adequate surveillance, the local circulation of these endemic viruses plays a more significant role than bird migration in creating their viral population diversity.(12).

Recent H9N2 strains from 2015 to 2022 in both countries cluster in the G1-like lineage, indicating a common ancestor. The isolation of H9N2 from different species such as ducks, pigeons, and chickens suggests that these endemic viruses have high evolutionary dynamics and broad host adaptability, leading to increased evolutionary potential, as each host imposes a unique selective environment on the virus, favoring specific mutations. The circulation of the virus in waterfowl populations, which are natural reservoirs of diverse subtypes, and subsequent transmission to poultry populations, creates a dangerous evolutionary cycle that can alter the virulence of local viruses or lead to the emergence of new strains (13, 14). Previous studies have reported highly pathogenic avian influenza (HPAI) viruses circulating in live bird markets and in wild bird carcasses in Iran and Pakistan, indicating their potential threat to the poultry industry (7, 15).

Low pathogenic avian influenza (LPAI) viruses, such as the endemic H9N2 in Iran and Pakistan and circulating H3N2 in bird markets, are capable of reassortment with highly pathogenic strains, leading to the exchange of genetic material (16). A notable example is the close phylogenetic relationship between the PB1 gene of a 2009 Iranian H9N2 virus and that of a 2004 Pakistani H7N3 virus, indicating a possible reassortment event. Also, in the phylogenetic tree for the NS gene, some H9N2 sequences cluster within the branch associated with H5N1, which is consistent with global events where H9N2 serves as a gene donor in reassortments with highly pathogenic strains such as H5 and H7(17).

Several circulating subtypes have been isolated from ducks and chickens and were found to be closely related. This genetic link, given the role of aquatic birds as suitable hosts for co-infection and reassortment, as well as the growing evidence highlighting the intermediary role of chickens in potentially facilitating bird-to-human transmission (18, 19)**,** along with reports such as the detection of H9N2 antibodies in poultry workers in Iran, underscores the need for continuous surveillance of these viruses.(3).

In Iran, the H5N3 virus sampled in Rasht in 2019 formed a distinct phylogenetic cluster based on its internal gene sequences, which may indicate a newly introduced virus to the existing avian influenza population in the country.

To prevent future risks, a coordinated One Health surveillance framework is essential. Surveillance systems are fragmented and poorly integrated between the livestock, human and environmental sectors. Measures such as cross-country collaboration and building on successful models such as the Hong Kong surveillance model for H5N1 (20), which includes continuous virus genome sequencing, wild bird surveillance and cross-border data sharing, could be very effective. Close monitoring and surveillance of local bird markets, particularly in areas where migratory birds are present, effective vaccination strategies and education and awareness programs for poultry workers are essential to prevent the transmission of diseases shared between humans and livestock. In summary, The results of this study provide a snapshot of avian influenza viruses (AIV) in Iran and Pakistan, highlighting the need for an integrated approach to protect the poultry industry and prevent dangerous outbreaks.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICS STATEMENT

The study did not use any animals or biological samples. All analyses were performed with publicly available sequence data from online databases.

AUTHOR CONTRIBUTIONS

A.K.: Conceptualization, Investigation, Formal Analysis, Writing – Original Draft.
A.A.: Supervision, Writing – Review & Editing.

CONSENT FOR PUBLICATION

Informed consent for publication was obtained from all participants.

CONSENT TO PARTICIPATE

All authors contributed to the study conception, and approved the final manuscript.

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DATA AVAILABILITY STATEMENTS

Data sharing not applicable – no new data generated, or the article describes entirely theoretical research.

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