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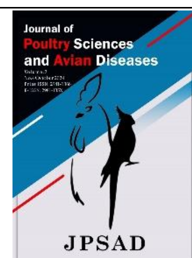

Molecular and phylogenetic study of H9N2 Avian Influenza virus in 2020 in six provinces of Iran

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A B S T R A C T

Avian Influenza viruses (AIVs) of the H9N2 subtype have become prevalent among birds in recent decades. Due to the high degree of shared infection between humans and animals and the risk of a pandemic, they pose a significant threat to the global poultry industry and human health. Hemagglutinin (HA) protein is the main surface antigen of avian influenza and is crucial for infectivity. This research was conducted to study the molecular characteristics of the H9N2 avian influenza virus in six provinces of Iran. Initially, samples were collected from 60 broiler flocks showing respiratory symptoms from September to December 2020. RNA extraction and cDNA synthesis by RT-PCR was performed. Sequencing and characterizing the hemagglutinin genes led to identifying 13 H9N2 viruses. Phylogenetic analysis and comparison with selected strains from the Middle East and the Indian subcontinent revealed that the isolated strains in this study formed three distinct subgroups in the phylogenetic tree. According to our study results and previous studies over the past twenty years, no significant genetic changes were observed in the isolated viruses from Iran, and the 13 studied strains showed a high similarity with recently isolated strains from Iran, Pakistan, Bangladesh, Saudi Arabia, and Kuwait. Considering the impact of the virus on the poultry industry and public health, further research is necessary to overcome its spread, along with more efficient control measures for the H9N2 virus.

Keywords: *H9N2, Iran, Avian influenza, molecular study, hemagglutinin gene*

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1 Introduction

As a viral bird infection, Avian Influenza leads to economic losses in the poultry industry worldwide. In the past two decades, Avian Influenza virus (AIV) contamination has become a recurrent concern for the poultry industry in many Asian countries, including Iran. The low pathogenicity of H9N2 infections in birds allows the virus to adapt and increase with the host while inducing multiple antigenic changes in circulating strains (1). Avian influenza viruses belong to *Orthomyxoviridae*, genus *Influenza A*, with a single-stranded negative-sense RNA genome. The RNA genome of influenza virus consists of eight segments encoding 10 to 12 proteins, with the most important ones being hemagglutinin (HA) and neuraminidase (NA). *Influenza A* viruses are classified based on the surface glycoproteins' serological reactions, HA and NA, into hemagglutinin (H1-H16) and neuraminidase (N1-N9) subtypes. These subtypes have been separated from wild birds, primarily aquatic, as their primary natural reservoirs (2). The surface glycoprotein HA mediates viral membrane fusion and endocytosis, allowing the virus to enter the host cell. The NA glycoprotein facilitates the budding of progeny virions from the cell surface and their subsequent release (3). Some H9N2 avian influenza viruses tend to bind to human receptors, occasionally crossing from birds to mammalian species such as humans and pigs (4). The H9N2 virus was first isolated in 1998 from layer farms in Qazvin province, Iran, and is currently the most prevalent subtype of influenza virus in the poultry industry (5). Some broiler farms in Iran have experienced up to 65% mortality rates (6). Simultaneous circulation of H9N2 and H5N1 viruses in the host increases the possibility of genetic reassortment between viruses (7). However, in recent years, H9N2 has often been identified in vaccinated poultry. This indicates that the virus has undergone surface antigenic changes due to various conditions, leading to reduced efficacy of existing vaccines (8). Therefore, surveillance and characterization of viruses are vital for a better understanding of public health concerns. The aim of the study was to investigate the molecular and phylogenetic characteristics of the HA gene of the H9N2 influenza virus from broiler farms in six provinces of Iran to compare genetic variations in new strains in these six provinces of Iran.

2 Materials and Methods

2.1 *Sample Collection*

Tracheal samples were collected from 60 broiler farms exhibiting respiratory symptoms from September to December 2020 in six provinces of Iran, including Isfahan, Khuzestan, Khorasan Razavi, East Azerbaijan, Qazvin, and Golestan. Tracheal samples were obtained from broiler chickens aged 25 to 35 days, showing clinical signs such as depression, rhinitis, coughing, swollen sinuses, ocular discharge, general weakness, and diarrhea. Fifteen tracheal samples were taken from each farm, with every five samples from the same farm pooled together. It is worth mentioning that all farms had been vaccinated with inactivated H9N2 avian influenza vaccine. The samples were collected in phosphate-buffered saline (PBS, pH 7.4) on ice packs and sent to the molecular laboratory.

2.2 *Viral Detection and RNA Extraction*

Tracheal samples were thoroughly homogenized under sterile conditions. Following the manufacturer's protocol, RNA was extracted from the homogenized samples using the SinaClon RNA extraction kit (SinaClon, Iran).

2.3 *RT-PCR and PCR Reactions*

cDNA synthesis was conducted according to the manufacturer's instructions using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and random hexamer primers (SinaClon, Iran). Specific primers targeting the matrix gene were used for the initial PCR reaction to identify avian *influenza A* viruses, following the method described by Wu et al. (9). The PCR reaction mixture consisted of 2.5 µL of 10x buffer, 17µL of distilled water, 0.5 µL of dNTPs, 1 µL of forward primer (AF: 5'AGGTCGAAACGTAYGTTCTCTAT13'), 1 µL of reverse primer (AR3: 5'GGTCTTGTCTTTAGCCAYTCCAT3'), 2 µL of MgCl2, 0.25 µL of Taq DNA polymerase, 0.5 µL of cDNA and finally 0.25 µL of RNase Inhibitor. The PCR thermal cycling conditions included an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 10 seconds, annealing at 58°C for 20 seconds, extension at 72°C for 20 seconds, and a final extension step at 72°C for 5 minutes.

For the second PCR reaction targeting H9 subtype identification, specific primers designed by Lee et al. (10) to amplify a portion of the HA gene (488 base pairs) were utilized. The final reaction volume of 25 µL included 2.5 µL

of 10x buffer, 17 µL of distilled water, 0.5 µL of dNTPs, 0.5 µL of Taq DNA polymerase, 0.3 µL of RNase Inhibitor, 0.2 µL of RT enzyme, 1 µL of forward primer (H9-151f: 5'-CTYCACACAGARCACAATGG),1µL of reverse primer (H9-638r: 5'-GTCACACTTGTTGTTGTRTC), and finally, 2 µL of cDNA. The PCR thermal cycling conditions were as follows: an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 40 seconds, extension at 72°C for 40 seconds, and a final elongation step at 72°C for 10 minutes.

2.4 *Detection of amplified PCR products:*

After completion of PCR, the products of both PCR reactions were separately loaded onto agarose gel (1%) in TBE buffer. Following electrophoresis, the gel image was visualized using a transilluminator. The presence or absence of the expected specific band (133 bp) was recorded in the first PCR reaction. The presence or absence of the expected specific band (488 bp) was anticipated in the second PCR reaction.

2.5 *Sequencing and Bioinformatics Analysis*

Samples that tested positive in the second PCR electrophoresis were sent to Codon Genetics Company (Iran) for sequencing using forward and reverse primers. Upon receiving the sequencing results, the quality of the reads was examined using Chromas 2.5 software. If the quality was deemed suitable, each read was individually compared with sequences available in the gene bank, and the initial similarity level was investigated. MEGA7 software was utilized with the Neighbor-Joining method using the CLUSTAL-W model to construct the phylogenetic tree. Additionally, the P-distance method was employed to compare the nucleotide distance between sequences from the gene bank and those relevant to this study.

The nucleotide sequences of identified HA in this study were deposited in the National Center for Biotechnology Information (NCBI) gene bank with accession numbers MZ442351, MZ442353, MZ442354, MZ433242, MZ433262, MZ413299, MZ4334301, MZ4334301, MZ413299, MZ4334301, MZ433301, MZ413299, MZ4334301, MZ413299, and MZ4334301. Then, each sequence was individually compared with sequences available in the gene bank, and the initial similarity level was investigated.

3 Results

In the present study, 13 strains of H9N2 were identified and sequenced from September to December 2020 from broiler chicken flocks. The sequenced viruses in this study showed 68% to 100% similarity. Additionally, these viruses exhibited significant similarity with H9N2 viruses isolated in Iran, Kuwait, Pakistan, Bangladesh, and Saudi Arabia the previous year (Figure 1).

Alongside the branches, the percentage of replication is indicated, where the relevant species are determined by bootstrap test (1000 repetitions). Evolutionary distances were calculated using the p-distance method and measured based on the fundamental differences at each site. Bootstrap values less than 70 were disregarded. MEGA7 software was used for evolutionary analysis. (Black circles: Strains studied in Iran). Table 1 shows the genetic comparison of sequences from a portion of the HA gene of H9N2 viruses in this study.

Nucleotide sequence similarities in the HA genome segment of strains in the present study, with a length of 488 base pairs, were aligned and compared with other relevant strains whose sequences were registered in the gene bank (Table 2)

YAHG 8150N11 2020 (MZ442353)
YAHG 3226 2020(MZ413328)
YAHG 8150F5 2020 (MZ442351)
A/chicken/Yazd/RIV-6/2018 (MN319509.1)
A/chicken/Kermanshah/RIV-10/2018(MN319513.1)
YAHG 974 2020(MZ433242)
YAHG 8125 2020(MZ413327)
A/chicken/Iran/H2965-16/2019(MN960171.1)
YAHG 982 2020(MZ433262)
A/chicken/Iran/H2965-8/2019(MN960169.1)
YAHG 8186 2020(MZ413326)
A/chicken/Pakistan/660BYP/2015(MG720814.1)
UT-BARIN (MK159013.1)
A/chicken/Pakistan/871CF/2015(MG720815.1)
YAHG 9292 2020(MZ433301)
A/Aves/Pakistan/332/2018(MK348243.1)
A/chicken/Pakistan/304/2018(MH105306.1)
A/chicken/Pakistan/289-186/2010(KF975469.1)
A/chicken/Iran/189/2011(KF800940.1)
A/chicken/Pakistan/287-98/2010(KF975467.1)
A/chicken/Iran/Markazi/2014(KJ696534.1)
A/chicken/Iran/B76/2004(EF063731)
A/chicken/Karaj/GHA/2017(MH360730.1)
A/chicken/Iran/B99/2005(EF063732)
YAHG M2 2020(MZ413325)
A/chicken/Iran/119/2009(JQ419722.1)
A/chicken/Pakistan/UDL-01/2006(CY038426)
A/chicken/Pakistan/UDL-02/2006(CY038434)
A/chicken/Saudi Arabia/582/2005(JX273556.1)
A/chicken/Israel/184/2009(GQ120550)
A/chicken/India/99321/2009(MH045822.1)
A/chicken/Orissa/2317/2004(CY068651.1)
A/chicken/Bangladesh/FDIL(M)112/2007(JX273540.1)
A/chicken/India/3/2003(JX273542)
YAHG 22248 2020 (MZ442354)
YAHG 22247 2020 (MZ437001)
A/chicken/Bangladesh/627/2007 (JX273539.1)
A/chicken/Bangladesh/VP01/2006(KC986294.1)
YAHG 903 2020 (MZ413329)
A/chicken/Kuwait/9/2004(JX273545)
A/chicken/Iran/SS2/2008(FJ794818)
A/chicken/Iran/THLBM864/2007(GU071980)
A/chicken/Dubai/339/2001(KF188352)
A/Chicken/Iran/TH81/2002(EU477246)
A/chicken/Iran/L252/2003(EF063725)
A/Chicken/Iran/TH79/2000(EU477248)
A/Chicken/Iran/TH78/1999(EU477247)
A/chicken/Iran/233/2001(GQ497123)
A/chicken/Israel/90658/2000(EF492221)
A/Chicken/Iran/TH77/1998(EU477241)
YAHG MixR2 2020(MZ413299)
A/chicken/Saudi Arabia/CP7/1998(CY081264)
A/chicken/Beijing/1/1994(KF188294)
A/chicken/Guangdong/V/2008(JQ639786)
A/duck/Hong Kong/Y439/1997(KF188265)
A/chicken/Korea/S21/04(DQ299829)
0.05

Figure 1. The neighbor-joining method was utilized to infer the evolutionary history.

Table 1. Detected strains and their geographical isolation sites and identified subgroups in the present study.

Subgroup	H9N2 Virus in Iran	Province
First	YAHG 8250N11 2020	Isfahan
	YAHG 3226 2020	Isfahan
	YAHG 8150F5 2020	Isfahan
	YAHG 974 2020	Khuzestan
	YAHG 8125 2020	Khuzestan
	YAHG 982 2020	East Azerbaijan
	YAHG 8186 2020	Qazvin
	YAHG 9292 2020	Khorasan
Second	YAHG M2 2020	Khorasan
	YAHG 22248 2020	Golestan
	YAHG 22247 2020	Golestan
	YAHG 903 2020	East Azerbaijan
Third	YAHG MixR2 2020	East Azerbaijan

Table 2. Sequence similarities of H9N2 separated viruses in this study and other related H9N2 viruses based on the HA gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	YAHG_8150F5_2020_(MZ442351)																				
2	YAHG_8150N11_2020_(MZ442353)	100.00																			
3	YAHG_982_2020(MZ433262)	97.84	97.84																		
4	YAHG_974_2020(MZ433242)	99.06	99.06	97.08																	
5	YAHG_9292_2020(MZ433301)	93.33	93.33	96.00	93.94																
6	YAHG_903_2020_(MZ413329)	85.46	85.46	85.56	86.53	87.30															
7	YAHG_3226_2020(MZ413328)	100.00	100.00	97.08	98.96	92.11	85.92														
8	YAHG_8125_2020(MZ413327)	94.58	94.58	93.99	93.93	84.93	78.43	93.92													
9	YAHG_8186_2020(MZ413326)	97.00	97.00	99.53	97.36	94.78	88.97	97.14	91.31												
10	YAHG_M2_2020(MZ413325)	86.26	86.26	88.44	85.98	86.02	91.41	86.46	78.72	89.57											
11	YAHG_MixR2_2020(MZ413299)	79.85	79.85	78.95	78.12	81.25	88.38	77.03	68.34	81.28	86.70										
12	YAHG_22248_2020_(MZ442354)	90.32	90.32	88.44	90.15	89.01	96.36	88.13	79.75	91.59	92.36	89.06									
13	YAHG_22247_2020_(MZ437001)	90.32	90.32	88.44	90.15	89.01	96.36	88.13	79.75	91.59	92.36	89.06	100.00								
14	A/chicken/Yazd/RIV-6/2018_(MN319509.1)	99.78	99.78	96.60	99.06	93.79	84.62	100.00	94.03	97.00	86.76	82.97	90.34	90.34							
15	A/chicken/Bangladesh/627/2007_(JX273539.1)	87.93	87.93	86.72	88.31	88.14	98.50	86.80	79.62	90.11	91.67	88.31	99.50	99.50	88.17						
16	A/chicken/Iran/H2965-16/2019(MN960171.1)	97.87	97.87	99.75	96.86	94.55	86.58	97.27	94.50	99.25	91.16	82.60	90.28	90.28	97.01	87.91					
17	A/Aves/Pakistan/332/2018(MK348243.1)	93.22	93.22	93.63	93.51	99.76	84.62	92.28	86.97	94.83	86.42	81.71	89.35	89.35	92.96	86.27	93.65				
18	A/chicken/Iran/119/2009(JQ419722.1)	85.09	85.09	86.39	85.07	86.26	91.36	85.28	80.07	88.37	100.00	86.70	92.42	92.42	85.79	91.95	87.95	84.92			
19	A/chicken/Pakistan/304/2018(MH105306.1)	93.50	93.50	93.94	93.89	100.00	84.96	92.72	87.29	95.20	86.02	81.25	89.01	89.01	93.33	86.27	93.93	99.72	84.92		
20	A/chicken/Kuwait/9/2004(JX273545)	85.36	85.36	86.20	86.99	86.39	100.00	85.84	84.51	89.10	91.13	87.71	96.64	96.64	85.75	98.83	86.98	85.29	91.83	85.75	
21	A/chicken/Saudi_Arabia/CP7/1998(CY081264)	81.02	81.02	82.94	82.25	76.95	90.41	80.93	80.18	85.84	86.44	100.00	89.34	89.34	81.55	90.72	83.89	80.51	89.57	81.03	90.41

4 Discussion

The H9N2 virus has been isolated from numerous vaccinated poultry flocks and has circulated in industrial poultry in Iran for over twenty years. Although H9N2 viruses are classified as low-pathogenic avian influenza (LPAI), they have the potential to cause significant mortality (11). Clinical signs observed in affected broiler flocks include acute respiratory symptoms, with mortality rates ranging from 20% to occasionally 65% based on previous reports. It has been observed that H9N2 viruses, due to environmental stress and subsequent bacterial (*Escherichia coli*) infections, *Mycoplasma gallisepticum* infections, infectious bronchitis virus, and *Ornithobacterium rhinotracheale* infections, lead to significant mortality in the farm (12, 13).

Based on phylogenetic analysis and sequencing of H9N2 virus genes, viruses of this subtype are classified into major lineages of North America and Euro-Asian. The Euro-Asian lineage is further divided into three sub-lineages: G1, identified with virus A/quail/Hongkong/G1/97; Y280-Like, identified with virus A/duck/Hongkong/Y280/1997; and Korean-like, identified with virus A/chicken/Korea/38344-pa6323/96 (14).

In the phylogenetic analysis conducted by Banks et al. in 1998 on 35 H9 influenza viruses over 33 years from various parts of the world, Iranian H9N2 viruses and selected viruses from other parts of the world were categorized into three distinct groups. The first group includes viruses from Europe and America, the second group comprises viruses from Korea and China, the third group contains viruses from Iran, Saudi Arabia, and Pakistan, and viruses isolated from humans in Hong Kong, Qa/HK/G1/97. Iranian isolated viruses are placed in the G1 lineage (15).

In other phylogenetic studies by Toroghi et al. (16) in 2003 and Karimi et al. (17) In 2004, viruses from Iran, Saudi Arabia, Germany, and Pakistan share a common lineage, a finding corroborated by Ghalyanchi Langeroudi et al. in 2008 (18) and Soltani Alvar et al. in 2010 (19). In the present study, Iranian strains showed high similarity with recently separated Iranian strains and strains from Pakistan, Bangladesh, Saudi Arabia, and Kuwait, all of which clustered within the G1 lineage. No change in lineage has been observed over these 20 years, and the strains in this study do not fit into other lineages.

Karimi et al. (2004)suggested that Pakistan originates from Iranian strains due to the high similarity between Iranian and Pakistani viruses. They attribute the delay in diagnosing this virus in Pakistan compared to Iran to negligence in investigating the virus in Pakistan. They consider the reports of H9N2 virus isolation from Pakistani parrots exported to Japan in 1997 and 1998, preceding the report of Pakistan's contamination by the virus by Naeem et al. (1999), as evidence of Pakistan's contamination before Iran. (17). In this study, similar to the results of Karimi et al., which suggested Pakistan as the origin of Iranian strains, 13 identified strains showed similarity ranging from 81.17% to 99.76% with Pakistani strain A/Aves/Pakistan/322/2018, with the least to the highest similarity observed with strains YAHG MixR2 2020 and YAHG 9292 2020, respectively. Another Pakistani strain, A/Chicken/Pakistan/304/2018, showed similarity ranging from 25.81% to 100% with 13 studied strains, with the least similarity to strain YAHG MixR2 2020 and the highest to strain YAHG 9292 2020. Strain YAHG 9292 2020, identified in Khorasan Province, showed similarity ranging from 76.99% to 100% with two Pakistani strains, A/Aves/Pakistan/322/2018 and A/Chicken/Pakistan/304/2018, suggesting a common origin between Pakistani and Eastern Iranian strains. The trade of livestock, illegal import of ornamental birds, and proximity between Pakistan and Iran indicate similarities between these viruses. Additionally, in this study, two strains, YAHG 2247 2020 and YAGH 2248 2020, both isolated from Golestan Province, showed 95.5% homology with strain A/Chicken/Bangladesh/627/2007 in the HA gene, suggesting viral circulation with a common origin in Iran and countries of the Indian subcontinent, possibly through Pakistan .

In Ghalyanchi Langeroudi et al.'s study (2008), out of nine studied viruses, five from the years 1998 to 2002 (TH77, TH78, TH79, TH80, TH81) were clustered together (showing similarity with strains from Saudi Arabia and Kuwait). Four identified viruses from the years 2006 and 2007 (TH85, TH186, TH286, TH386) formed a separate cluster. However, no change in lineage was observed. The recent four viruses out of nine studied (from the years 2006 and 2007) formed a separate cluster with two viruses from India and two from Pakistan, which could signify viral changes due to population shifts in birds and the extensive spread of the virus in Asia. Since these strains (from the years 2006 and 2007) were simultaneously separated and are similar to strains from Pakistan and India, it is possible to suggest a viral transmission between these two countries, perhaps due to trade exchanges from India or indirectly from Pakistan to Iran.

In the third subgroup of the present study, the YAHG Mix R2 2020 strain, separated from East Azerbaijan, is placed alongside strains TH77, TH78, TH79, and TH81 (studied by Ghalyanchi et al., 2008), as well as a strain from Israel and one from Saudi Arabia, with no strains from India or Pakistan in this subgroup. This finding may indicate that the Mix R2 strain is grouped with the primary Iranian strains in the same subgroup. The lack of grouping with Pakistani and Indian strains suggests no common origin between the Mix R2 virus and viruses from India and Pakistan or recent genetic changes in the Mix R2 virus.

Bashashati et al. (2013) conducted phylogenetic analysis and sequence alignment on eight genes of strains IR-10 (ck/IR/EBCV-88/10) and IR-98 (ck/IR/ZMT-101/98) (primary strains isolated from Iran). They found that these two strains have a 91.7 % similarity in the HA gene. The results of this study showed that the IR-10 virus is placed in a different subgroup from the subgroup where the IR-98 virus is located. Considering that the primary vaccine strain IR-98 shares approximately 91% similarity with strain IR-10 in the HA gene and the placement of these two strains in different phylogenetic tree subgroups of surface glycoproteins, it was inferred that one of the main reasons for strain changes (seed) of the influenza vaccine in Iran (20).

In another study by Bashashati et al. (2020), ML phylogenetic analysis was performed on the HA gene of fifteen Iranian strains between 2017 and 2019 in 7 provinces of Iran. Sequence alignment and phylogenetic analysis of the HA gene among these fifteen strains and previous Iranian strains extracted from the gene bank from 1998 to 2019 showed that four genetic groups of the H9N2 influenza virus have been introduced to Iran during these years and have undergone rotation. Iran-1 group includes H9N2 strains separated from 1998 to 2007 and belongs to genetic group D. Iran-2 group consists of strains between 2003 and 2009. The Iran-3 group includes strains from 2009 to 2013 in Iran, and the Iran-4 group includes strains between 2011 and 2019 and belongs to genetic group B. According to this study, the Iran-2 and Iran-3 groups disappeared, and the Iran-4 group has formed a separate subgroup 1-4, including recent strains (2017 to 2019). (21).

Consistent with the two studies mentioned above, the strains in the third subgroup of our study are grouped with the primary strains isolated from Iran and the primary vaccine strain (ZMT-101) in the study by Bashashati et al. (2020). The virus of this subgroup is similar to the Iran-1 group in the HA gene, as reported by Bashashati et al. (2020), and belongs to genetic group D. The other two subgroups are similar to the Iran-4 group identified by the same researcher, which belongs to genetic group B and has shown high similarity in the HA gene in recent years. Given these findings, placing the strains in this study in two separate genetic groups emphasizes the importance of revising the vaccine strains.

In the first subgroup of the present study, the strains separated from Iran (8 strains) are grouped with strains separated from Iran between 2018 and 2019 and Pakistani strains from 2015 and 2018. Iranian strains showed a high similarity to Pakistani strains. In the second subgroup, the YAHG 903 2020 strain separated from East Azerbaijan showed 100% similarity in sequence alignment of a part of the HA genome with strain A/Chicken/Kuwait/9/2004. Three other strains in this subgroup shared high similarity with Bangladeshi strains from 2006-2007, and strains from Pakistan (2006), India, and Saudi Arabia were included. In the present study, the strain separated from East Azerbaijan, YAHG Mix R2 2020, showed 100% concordance with strain A/Chicken/Saudi Arabia/CP7/1998 in the third subgroup of the present study.

The Mix R2 strain and the Saudi Arabia strain are placed in the third subgroup of the present study. As mentioned, in this subgroup, the primary strains of Iran and strains TH77, TH78, TH79, and TH81, as well as a strain from Israel (A/chicken/Israel/90658/2000) and a strain from the United Arab Emirates (A/chicken/Dubai/339/2001), are included.

5 Conclusion

Based on the results of this study and past studies over the past twenty years, no taxonomic changes have been observed in the strains separated from Iran. The 13 strains studied here are highly similar to strains separated from Iran, Pakistan, Bangladesh, Saudi Arabia, and Kuwait. Based on the data of this study, the similarity of 11 strains of this study with strains from Pakistan and the Indian subcontinent and the similarity of the other two strains of this study with strains from Saudi Arabia and Kuwait, it can be inferred that the major origin of Iranian strains is from Pakistan and the Indian subcontinent through border exchanges and trafficking of ornamental birds from Pakistan to Iran and even to neighboring countries. On the other hand, another origin for strains circulating in Iran may be from countries around the Persian Gulf, such as Saudi Arabia and Kuwait,

The simultaneous circulation of H9, H5, and H7 viruses in commercial birds can lead to the emergence of new

viruses due to genome rearrangements and the possibility of internal gene exchange. As a result, the control and prevention program for influenza in Iran should include the isolation and evaluation of pathogenicity, sequencing of all genes, and the use of up-to-date vaccines to combat this disease effectively.

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Conflict of Interest

The authors declared no conflicts of interest.

Author Contributions

All the authors contributed to all parts of this research.

Data Availability Statement

Data are available from the first author upon reasonable request.

Ethical Considerations

There is no ethical consideration.

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