Iranian Journal of Virology 2020;14(2): 29-37
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Original Article

Phylogenetic Study of the Recent Outbreak of Avian Influenza H5 Subtype in Turkeys of East Azerbaijan in 2018

Fallah Mehrabadi MH [1], Hosseini H [2], Ghafouri SA [3], Sadri N [4], Ziafati Kafi Z [4], Aghajantabar S [4], Modiri Hamdan A [4], Tolooe A [4], Haghbin Nazarpak H [2], Parvandar Asadollahi K[5], Hojabr Rajenoni A [4], Ghalyanchi Langeroudi A [4*]

1. Department of Avian Diseases Research and Diagnostics, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.
2. Department of Clinical Sciences, Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Alborz, Iran.
3. Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
4. Department of Microbiology and Immunology, University of Tehran, Faculty of Veterinary Medicine, Tehran, Iran.
5. Department of Clinical Sciences, Faculty of Veterinary Medicine, Islamic azad university, Science and research branch,Tehran, Iran.

Abstract

Background and Aims: Global epizootic distribution of HPAI H5N8 (Clade 2.3.4.4) in poultry and wild birds was demonstrated after 2010. HPAI virus is a major concern in the birds and poultry industry and global human health. Wild migratory birds and their link to backyard birds play a critical role in spreading HPAI and creating genetic reassortment.

Materials and Methods: In this study in 2018, HPAI H5N8 was isolated from Backyard poultry (turkey) in East Azerbaijan Province, Iran. Three reports of outbreaks were submitted. The first outbreak was in a village, Sholebaran, and the second was in a Livestock market in Bahman, and the third was in another Village, Yaghbastloo. Tracheal and pancreas tissue samples were obtained from 10 dead birds, and 300 susceptible domestic birds include in the turkey and chickens. Samples' diagnosis was based on real-time reverse transcriptase PCR (RRT-PCR) and partial HA gene sequencing. Turkey samples were positive and characterized as H5N8.

Results: Phylogenetic analysis result based on a partial HA gene revealed that the Iranian HPAI H5N8 virus in our study, belong to the subgroup clade 2.3.4.4 and cluster within group B.

Conclusion: These findings indicate that it provides new insights into the evolution and spread of H5N8 in Iran; based on these results, we have to recognize an improper monitoring protocol for reducing the reassortment of them. Therefore, we could prevent HPAI from circulating.

Keywords: Phylogenetic tree; H5N8; Avian influenza; Backyard poultry; HAPI; Iran

*** Corresponding author:** Arash Ghalyanchi Langeroudi. Email: ghalyana@ut.ac.ir

Introduction

Avian influenza (AI) is an acute zoonotic infectious disease in birds and humans. Avian influenza viruses (AIV) are the viruses with segmented genomes placed in the genus influenzavirus A of the family Orthomyxoviridae. Many birds, such as poultry, waterfowl, and wild birds, are susceptible to AI (1-4). Based on their haemagglutinin (H1–H16) and neuraminidase (N1–N9) nucleocapsid antigens, AIVs are classified into subtypes (1, 2, 5). To date, AIV subtypes that produce acute disease in birds of economic importance are H5 and H7 subtypes. The H5 and H7 subtypes isolated from birds included low pathogenicity (LPAI) and high pathogenicity (HPAI). These subtypes are notifiable to the OIE (2).

Global epizootic distribution of HPAI (2013-2018) in poultry and wild birds was in 63 countries where HPAI subtypes in Asia were included H5N1, H5N2, H5N3, H5N6, H5N8, H7N9 (6). HPAI H5N1 viruses, A/goose/ Guangdong/1/1996-lineage (Gs/Gd), were identified in live bird markets in China in 1996; after that, it became enzootic in some countries and spread to other Asian countries; H5N8 subtype is one of the NA gene reassortments, that occurred among GsGd clade 2.3.4.4 viruses after spreading. H5N1viruses association with HA gene included ten phylogenetic clades (clades 0–9), subdivided into subclades based on the NA gene (1, 3, 4).

HPAI H5N1 prevalences to be reported from Asia and Africa in poultry and wild birds (3, 6). The HPAI H5N1 lineage has become enzootic by incidence in poultry and sporadic human infections. Direct contact with infected live or dead birds or contaminated environments is the main reason for H5N1 infection in humans (6). Ongoing H5N1 outbreaks are the major veterinary and public health threats in the world (1). HPAI H5N6 viruses first reported in china and then some other Asian countries.

A new variant strain of HPAI H5N6 emerged in Asia and Europe. This strain differs from previous H5N6, cause of human infections in China (6). The origin of H5N2 HPAI outbreaks was in 2012 from Chinese Taipei. H5N2 has varying levels of pathogenicity, leading to significant losses in poultry (6).

In Iran, the HPAI H5N1 was first isolated from wild birds, Swan corpes, in 2006. Most of the outbreaks of the H5N1 in Iran took place in backyard poultry in the Mazandaran Province (1, 3, 7). The Asian lineage of HPAI H5N8 A/duck/Jiangsu/k1203/2010 virus was the first to report in eastern China in 2010, isolated from mallard ducks (3, 8). H5N8 prevalences to be reported from Europe, Africa, the Middle East, and Asia in poultry and wild birds; The countries of HPAI H5N8 subtypes outbreaks are located migratory route, so H5N8 spread along the route and contaminated migratory waterfowls (3, 6). Therefore, the Poultry trade can cause the virus spread, and it has affected poultry production and public health (3, 9).

In Iran, HPAI H5N8 (2006) first was identified in a commercial layer farm in Tehran Province (3, 4). Phylogenetic analysis of the HA gene of Iranian lineage HPAI H5N1 and H5N8 indicated that these viruses belong to the clade (2.2.1, 2.2.2 and 2.3.2.1c) and 2.3.4.4, respecti-vely (1, 3, 4). In 2017, four outbreaks of H5N8 was documented in Isfahan Province (3, 6). In our study, new H5N8 viruses were isolated from turkey and chickens in East Azerbaijan, Iran.

We sequenced the partial HA viral genome, performed the phylogenetic analysis, and determined the virus's molecular characteristics.

Methods

Sample history. The outbreak locations of the HPAI H5N8 were in East Azerbaijan. It happened among 300 susceptible terrestrial birds, including turkey and chickens (Table.1).

Among 300 susceptible birds, 158 deaths were observed. The first outbreak was in a village, Sholebaran (38.48559; 47.09883); the second was in a Livestock market in Bahman (38.474 76742; 47.072252); and the third was in another Village, Yaghbastloo (38.50463;47. 05185) (Fig. 1).



Fig. 1. The location of outbreaks in Iran

samples. The thermo-cycler protocol of reaction was Initial denaturation 95 ºC for 3 minutes, followed by 35 cycles (denaturation 95 ºC for 30 seconds, Connection 52 ºC for 30 seconds, expansion 68 ºC for 30 seconds) and a final extension step at 68 ºC for 10 minutes.

Five µL of the PCR products were loaded by 1 µL EvaGreen (Jena Bioscience, Germany) loading buffer on 1.5% agarose gel near 100bp leader in 1x TBE buffer were electrophoresed.

Table. 1. Report reference: Reference OIE: 29122, Report Date: 05/01/2019, Country: Iran

Susceptible	Cases	Deaths	Killed and disposed of		Location	Date
Total 300	Total 274	Total 158	Total 142		East Azerbaijan	
215	199	145	70	Village	Sholebaran	15-18/11/2018
53	53	3	50	Livestock market	Bahman	18-21/11/2018
32	22	10	22	Village	Yaghbastloo	19-22/11/2018

Ten pancreas samples were collected from dead turkey, according to the standard method. The samples were transferred to the University of Tehran by biosecurity protocols; and were stored at -70 ℃ until examined. Based on partial HA gene sequences, these samples were analyzed.

RNA extraction. RNA extraction of pancreas samples was performed with an RNA extraction kit (Sinaclone Column kit) according to the manufacturer's instructions. The total RNA extracted was stored at -70°C until used (10).

RT-PCR for gene amplification. The amplification of reverse transcription was performed using random hexamers with a cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instruction (2). Real-time PCR for H5 detection, based on the partial HA gene, was implemented with the Qiagen Kit, according to using specific primers and the method described by Slomka et al. (1). The H5N8 subtype isolates were determined by PCR assay using specific H5N8 primers (3, 4). The PCR amplification was carried out in a 25 µL total volume reaction; containing 1 µL Forward Primer, 1 µL Reverse Primer, 8 µL distilled water, 12.5 µL master mix, and 2.5 µL cDNA

Sequencing and bioinformatics analysis. After purification of PCR products, one of the positive samples sent to Bioneer Co., Korea, for DNA sequencing; After that, the results have been check through National Center for Biotechnology Information (NCBI) nBLAST. Our study used the AccuPrep® PCR purification Kit (Bioneer Co., Korea) for purification of the PCR products. Genetic Analyzer ABI 3100 (Applied Biosystems, USA) was used for sequencing PCR products with the primers (Both directions) (Bioneer Co., Korea).

The alignment analysis and phylo1genetic tree of the Multiple sequences were performed by the Neighbor-joining method and the Kimura 2-parameter model using MEGA 7 software (11). After sequencing, for confirmation assessment, H5N8 sequences were retrieved from NCBI and GISAID's EpiFluTM Database (http://platform.gisaid.org/epi3/frontend), all sequences of each sample were compounded; then used to NCBI BLAST. For the construction of the phylogenetic tree, 1000 bootstrap replicates were used to test the major phylogenetic groups' robustness. Nucleotide results of partial HA gene sequencing of the H5N8 virus in this study were submitted to GenBank of NCBI (BLAST)

with the following accession numbers; MT225015.

Results

Phylogenetic results. Turkey samples were positive and characterized as H5N8. Phylogenetic analysis result based on partial HA gene revealed that the Iranian HPAI H5N8 virus, UT-Sohrab (A/Chicken/UTSohrab/ 2019_(H5N8) (MT225015)) obtained in this study, have high similarity (95.78%) in nucleotides, with previous Iranian H5N8 viruses A/crow/Aghakhan/2017(H5N8) (MK1 68599.1) (Fig. 2).
In addition, the UT-Sohrab share a similarity of 98.95% with A/turkey/ Israel/1076 /2016 (H5N8)) (MF166578) and 98. 95% with A/ Sacred_ibis/South_Africa/009/ 2017 (H5N 8)) (MH165612.1) 98.80% with A/peregrine falcon/Israel/1086/2016(H5N8)) (MF166577.1) and A/turkey/Israel/1076/2016 (H5N8)) (MF 166578) isolates in NCBI BLAST (Fig. 2). In subgroup Russia 2016, the UT-Sohrab clustered clade 2.3.4.4, within-group B (Gochang-like) of HPAI H5 virus (12).
Although, based on the comparative alignment of HA sequences, the UT-Sohrab is not the same as the previous Iranian H5N8 A/chicken/Iran/ Tehran-F-2/2016(H5N8)) (KY701529.1) and A/chicken/Iran/Tehran-F-1/2016(H5N8)) (KY7 01528.1) but it is 97.62% similar to them (Table.2).

Discussion

Despite the widespread use of H9N2 vaccination for prophylaxis, different strains of AIV are circulating through domestic poultry and wild birds in Iran; hence it is critical to investigate the current strains of AIV circulating and transmission in Iran. Ghalyanchi-Langeroudi, et al. isolated AIV in different species Such as duck, swan, parrot, crow, pigeons, and chicken at the Tehran Bird park (13). Clade 2.3.4 HPAI is one of the most genotypes in Asia, including 2.3.4.1, 2.3.4.2, 2.3.4.3, and 2.3.4.4 subclades (4). Different NA subtypes such as H5N2, H5N5, and H5N8 are among the clade 2.3.4 (4). Asian H5N8 Goose/Guangdong/96 lineage, clade 2.3.4.4 group B, achieved to South Africa by June 2017; it disappears over the summer and then in 2018, H5N8 HPAI outbreaks resurged in the north of Africa. The virus was isolated from quails (Coturnix japonica) in the North West Province and another from comercial pullets in the Gauteng province in 2018 (14).

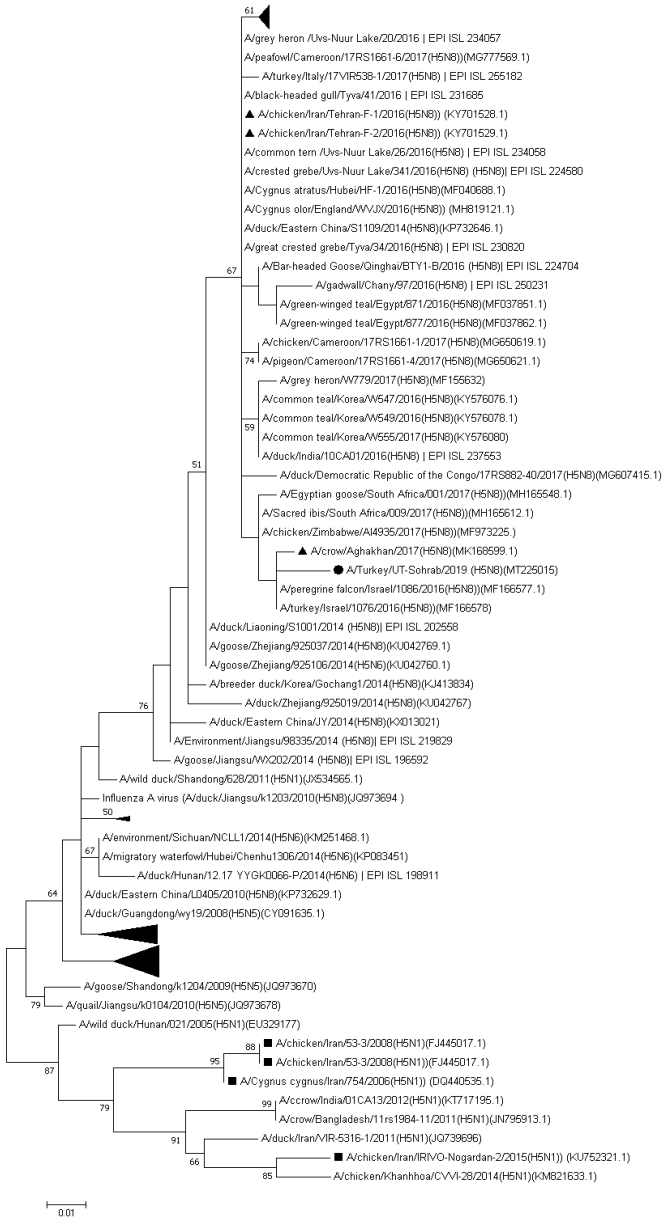


Fig. 2. Phylogenetic tree of the partial HA gene of the H5N8 AI detected in East Azerbaijan, Iran, 2019; The Tree construction was by MEGA version 7, with the neighbor-joining method with 1000 bootstrap replicates (bootstrap values are shown on the tree). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (black triangle: Iranian H5N8 samples, reported 2016,2016, 2017; black circle: Iranian H5N8 samples, reported 2019; black square: Iranian H5N1 samples, reported 2008, 2008,2006,2015).

Abolnik, C. *et al*. (2019) reported that H5N8 clade 2.3.4.4 had entered the avian populations of white-winged terns (*Chlidonias leucopterus*) of the African Rift Valley, Lake Victoria in Uganda, in January 2017; also in June 2017, an outbreak of H5N8 in commercial breeder chickens near Harare in Zimbabwe and another outbreak in a broiler breeder located near Villiers, Mpumalanga Province, South Africa were reported.

humans. Recommendations for antiviral drugs and vaccination was varied across countries (17).

The Eurasian lineage outbreak clade 2.3.4.4 H5 in the U.S. infected backyard flocks with minor gallinaceous poultry, and large commercial poultry (chickens and turkeys), in 2014-2015.

Bertran, K. *et al*. (2019), investigated that both viruses H5N8 and H5N2 caused 80–100% mortality in all gallinaceous species include

Table. 2: Nucleotide identity of the partial HA gene of the Iranian H5N8 AI detected 2019, in comparison to some other H5N1 and H5N8 strains

		1	2	3	4	5	6	7	8	9	10
1	A/Chicken/UT-Sohrab/2019_(H5N8)(MT225015)										
2	A/Crow/Aghakhan/2017(H5N8)(MK168599.1)	95.78									
3	A/Turkey/Israel/1076/2016(H5N8))(MF166578)	98.95	99.25								
4	A/Sacred_ibis/South_Africa/009/2017(H5N8))(MH165612.1)	98.95	99.25	99.70							
5	A/peregrine_falcon/Israel/1088/2016(H5N8))(MF166577.1)	99.80	99.10	99.85	99.55						
6	A/chicken/Iran/Tehran-F-2/2016(H5N8))_(KY701529.1)	97.62	98.31	98.66	99.00	98.66					
7	A/chicken/Iran/Tehran-F-1/2016(H5N8))_(KY701528.1)	97.62	98.31	98.66	99.00	98.66	100.00				
8	A/Cygnus_cygnus/Iran/754/2006(H5N1))_(DQ440535.1)	87.65	88.92	90.77	91.17	90.57	88.64	88.64			
9	A/chicken/Iran/53-3/2008(H5N8)(FJ445017.1)	87.25	88.51	90.39	90.79	90.18	87.73	87.73	99.04		
10	A/chicken/Iran/IRIVO-Nogardan-2/2015(H5N1))_(KU752321.1)	82.51	82.35	83.14	83.91	83.14	84.66	84.66	93.17	92.09	

The virus rapidly spread to all nine South African provinces. This study demonstrated that wild birds had a critical role in the incursion and spread of H5N8 in Africa (15). In 2019, Valley-Omar, Z. *et al*. (2019), in a study, decided to demonstrate the transmission of AI H5N8 to humans working with infected birds during the 2017 outbreak in South Africa. People's symptoms were coughing, coryza, fever, sore throat, conjunctivitis, and shortness of breath in some cases. Some of them wore overalls, boots, masks, gloves, and goggles. The samples were negative for AI H5 viruses. 3% of samples were positive for seasonal human influenza H3N2 viruses. They have found no human H5N8 infection, and sequence analyses have shown no H5N8 adaptation for mammalian infection(16).

Adlhoch, C. *et al*. (2018), in a study, investigated national approaches for managing human health risks during outbreaks of infection with the H5N8 virus during 2016–2017. They inspected 23 countries in the Union/European Economic Area and Israel. They evaluated the risk to the public as low and medium in 18 and 1 countries, respectively. All person samples were negative for the H5N8 virus. They identified no transmission of this virus to

chickens, Japanese quail, Bobwhite quail, Pearl guinea fowl, Chukar partridges, and Ring-necked pheasants; except for H5N2 virus that caused 60% mortality in chickens. The birds that survived had no clinical disease and seroconversion, so they remained uninfected. In early clinical stages, chickens and Japanese quail were asymptomatic and listless and had no histopathologic lesions; in later clinical stages, all species of birds had histopathologic lesions and systemic virus replication HPAI virus infection. These birds indicated widespread multifocal areas of necrosis and sometimes had to infiltrate with heterophilic or lymphoplasmacytic inflammatory and viral antigen in parenchymal cells of most tissues. Regardless of virus and bird species, lesions and antigen distribution were similar generally.

Nevertheless, the most significant difference among species was endotheliotropic; and only Pearl guinea fowl showed widespread replication of both viruses in most tissues' endothelial cells. They demonstrated that in later clinical stages compared to asymptomatic birds, expression of IFN-γ, and IL-10 in Japanese quail and IL-6 in chickens were regulated (18).

Kouam, M. K., *et al*. (2019) tried to collect some information on the epidemiology of HPAI in Cameroon. The epidemy of HPAI H5N1 and H5N8 in Cameroon was indistinct. They were collected from the second HPAI outbreaks reported to the OIE by Cameroon's veterinary health officials. In 2006, the first outbreak of H5N1 happened in the Mvog-Betsi poultry complex in the Center region and then spread to other regions. HPAI virus strain H5N1 occurred in the Center, South, West, Adamawa, and H5N8 outbreak was included the only Far North. Laying hens, backyard chickens, turkeys, guinea fowls, ducks, broiler and layer breeders, and geese were affected with H5N1, and Indian peafowl (*Pavo cristatus*), pigeon, ducks, back-yard chickens, and guinea fowls were affected with H5N8. They had shown no human cases. The risk factors for distribution of disease on the field were delays of poultry and eggs moving from farms to another place without any preventive care, poor biosecurity measures on farms, and live poultry markets. In 2016-2017, the second HPAI outbreak occurred in Cameroon, including two virus strains (H5N1 and H5N8). That was a concern for the poultry industry (19).

The ability to pass easily from wild Anseri-formes to commercial Galliformes and spread to galliforms populations is an important charac-teristic of exotic origin AI. HPAI H5N8 clade 2.3.4.4 has spread globally via migratory waterfowl, such as ducks (20). In 2017-2018, HPAI H5N8 Clade 2.3.4.4b was spread to Africa and limited to outbreaks in poultry, while also circulating in Europe. Interestingly, some previous study indicated the successful transmission of Korean origin HPAI H5N8 from infected chickens to chickens, but chickens infected with North American-origin H5N8 did not; which may reflect differences among these H5N8 viruses (21).

Puranik, A. *et al*. (2020) implemented a study to attempt transmission of a UK virus H5N8 from Pekin ducks infected to chickens and turkeys. The transmission was efficient, and all of the turkeys becoming infected, but not among chickens; the faster mortality rate may have influenced it among chickens compared to a lower mortality rate among the turkeys. Pathogenesis and mortality in chickens and turkeys were typical for these galliform species as described previously, contrasting with 5% mortality among ducks (20), with high viral titers in their systemic organs such as lungs, proventriculus, intestine, Bursa, cecal tonsil, kidney, spleen, pancreas. Systemic propagation in infected turkeys with H5N8 was with endothelial tropism in the tissues. In this study, mean death time (MDT) was from at least 6.7 to greater than 4.4 and 5.5-days post-contact. Virus-specific immunohistochemical (IHC) was observed in the samples of infected turkeys with extensive endothelial tropism. Turkeys may be a more susceptible host than chickens and retained the ability to transmit back the infection to ducks. This study demonstrated the importance of contacting wild birds and poultry to HPAI, particularly H5N8 clade 2.3.4.4 circulating among them. Transmission from Anseriformes to Galliformes leads to economi-cally damaging outbreaks in chickens and turkeys and more adaption in different species based on manifestation viral genetic polymer-phisms, including transmission by influencing the viral epidemiology. Characteristic of viral phenotype such as host range and transmissi-bility between different species is invaluable for outbreaks management. Amino-acid changes did not show, but D296N polymorphism was observed in the PB2 gene in turkey swab; it was suggested that H5N8-2014 could infect the host species without requiring any significant additional changes in the viral genome (20).

Hemida, M. G., *et al*. (2019) investigated an outbreak of H5N8 in 10 local backyard flocks in Al Ahsa, Eastern Saudi Arabia, in 2017-2018. Poultry, including chickens, ostriches, ducks, pigeons, and turkeys, was affected clinically. Various species of infected birds revealed typical AI clinical signs and postmortem lesions. Some cases, especially in chickens and turkeys, infectious were peracute form, which in this case, sudden mortality up to 100 percent and no obvious pathognomonic signs were observed. In other cases, a less acute form of infection was observed.

The infected birds reduced food and water consumption associated with respiratory and nervous manifestations such as paralysis of the

wings and legs. Some birds indicated edema and depression. Cyanosis of combs, wattles, and non-feathered parts of the skin was observed. Coughing, gasping, and diarrhea, and ecchymosis of the shanks and feet were sometimes observed. In native breed chicken, postmortem inspection indicated congestion and hemorrhage in the internal organs, particularly in the ovaries and oviducts, and indicated petechial hemorrhage in the epicardial, congestion and ulceration in the caecal tonsils and intestinal mucosa, and congestion and enlargement of the spleen. In the native ducks, depression and paralysis were observed. In pigeons, anorexia, depression, greenish diarrhea, and respiratory and neurological signs such as paresis and paralysis of wings, torticollis, opisthotonos, circling, congestion, and hemorrhage of the brain were observed. Infected waterfowls such as ducks and geese indicated depression, anorexia, nasal discharges, and diarrhea; some showed neurological manifestations such as paralysis, incoordination, head shaking, and torticollis. In native breed, ducks were showed necrosis in the pancreas. Some affected turkeys also demonstrated the congestion and hemorrhage in the pancreas and duodenal blood vessels (22).

In Hemida, M. G. *et al.* (2019), Phylogenetic analysis of the M gene of AI was indicated that H5N8 was isolated from birds, including ostriches, pigeons, and ducks. Full genome sequencing of these isolates indicated that these were firmly related to H5N8 viruses reported previously in Riyadh elsewhere in Saudi Arabia in 2017. H5N8 of their study was similar to H5N8 of Riyadh, ranging from 99.87to 99.98%. The HA gene analysis showed H5N8 in their study was a cluster in H5 clade 2.3.4.4 group B. (22).

H5N1 and H9N2 viruses are endemic in Egypt. Kandeil, A. *et al.* (2019) isolated AI viruses from 329 samples during 2016-2018 in Egypt. 37.1% of samples were H5N8; 7.6% of samples were H5N1; 48% of samples were H9N2, and 7.3% of samples were co-infections with 2 of the three subtypes. The sequencing of the HA gene of H5N1 viruses was lineage within clade 2.2.1.2, and the Sequencing of the HA gene of H5N8 viruses belonged to the 2.3.4.4 subclade. The outbreak of H5N8 in ducks was higher than in chickens, 2.4 %, and 0.94%, respectively. H5N8 indicated a higher replication rate than 2 other genotypes. H5N8 was more common in Southern Egypt, H9N2 in the Nile Delta, and H5N1 in both areas (23).

Mortalities of HPAI strains (H5N1) of AI were observed in wild swans, in the north of Iran, in 2006. H5N1 was also detected again in northern Iran in 2011; this isolate phylogenetically was closely related to H5N1 isolated in Mongolia in 2010 (7). In 2010, HPAI H5N8 was spread to poultry and wild birds in Asia, Europe, and North America; after that, in 2016, H5N8 was detected in a commercial layer farm in Tehran province (4).

In 2017, the HPAI H5N8 epidemics, demonstrated in black crow in Isfahan Province, in our country and cause mortality (2). To date, any human cases of H5N8 infected have not been reported, although based on results of some recent reports, some mammals such as mice, ferrets, dogs, and cats could be infected and having mild clinical disease (4).

The first report of H5N8, 2015 in Iran, was A/chicken/Iran/Tehran-F-1/2016(H5N8)) (KY701528.1) and A/chicken/Iran/Tehran-F-2/2016 (H5N8)) (KY701529.1) in commercial poultry in Tehran, and the second report, 2016, was A/crow/Aghakhan/2017(H5N8)(MK168599.1) in Hooded Crow (Corvus cornix) in Isfahan province (3). Most of the H5N1 outbreaks in Iran were reported from Tehran, except one of them in Isfahan Province. Iranian H5N8 occurred in 2015 is one of the clade 2.3.2.1c. The previous report of H5N8 in Iran, 2017, (A/crow/Aghakhan/2017) demonstrated that the Aghakhan virus is clustered in clade 2.3.4.4 of H5 viruses, subgroup b of group B, like other H5N8 isolates from Korea, Egypt, and Cameron in Russian 2016 (3).

Comparative alignment based on partial HA gene indicated that Iranian H5N8 2016 (Theran.F-1; Tehran.F-2) was similar to Russian viruses (A/great-crested grebe/Ubsu-NurLake/341/2016 & A/great-crested grebe/Tyva/34/2016) (4). The present study is the first official report of the H5N8 HPAI subclade 2.3.4.4 outbreak in turkeys in East Azerbaijan, Iran.

Based on the H5N8 AI virus's HA gene, the Iranian H5N8 100% looks like the Russian HPAI virus, which was identified in wild birds, but it differed from H5N8 isolated in Europe, Japan/South Korea, and North America in 2014. The Iranian H5N8 is located in group B (Gochang-like). The phylogenetic study suggested that originate of novel HPAI H5N8 viruses in China; Afterward, it spread to the westward by the migration of wild birds; Therefore, it created a global geographic distribution (12). The pathways of extension of migratory birds include Asia encompass Siberia, the Caspian Sea and the Persian Gulf, the former Soviet republics, and then flying to Alaska, Australia, and the Pacific's island countries. Every year in autumn, wild birds migrate from Russia to Iran; hence, Iran is an important migratory pathway (3, 4).

Epidemiological studies describe a migration route for wild bird reservoirs of H5N8 from East Asia to Russia (9). Ghafouri, SA, *et al*. (2019) indicated that infected crows are likely to be contacted with farmed turkeys' carcasses, which are abandoned by farm owners. They demonstrated that crows have an important role in transmitting viruses by free-flying and their displacement (3).

Wild birds played an important role in the spread of HPAI H5N8 from Siberia to Europe, Asia, and North America in 2014 and HPAI H5N1 from Qinghai Lake and circumpolar breeding areas to Europe and East Asia in 2005–2006; so it is suspected that wild birds have an important role in HPAI distribution (3).

Phylogenetic results indicated that migratory birds bring in HPAI viruses to Iran and other European and African counties. The most important migratory bird for the extension of H5N1 was aquatic birds (24). The migratory birds are the vectors of AIV and will carry AIV through Central Asia to the Caspian Sea area. Backyard poultry is an important reservoir for HPAI viruses and outbreaks of AI in humans and industrial poultry. It is a mixing vessel for the genetic mixing and spreading of AI viruses in different avian species (1).

In our country, people have kept backyard poultry; there is contact between migratory wild birds and domestic backyard poultry; it may be leading to virus transmission to the poultry industry. For example, it happened in 2016, and the HPAI H5N8 transmitted to the commercial layer farms. It took place by direct or indirect contact with contaminated material or workers that enter the farms (4). In addition to migratory wild birds, the displacement of backyard birds such as asymptomatic ducks and local wild birds could lead to the spread of the AIV to the poultry industry (3).

Because of Iran's location in the migratory birds' pathways, an inspection of HPAI viruses can help control the distribution of them; We can recognize a monitoring protocol to reduce the reassortment of the AIVs and then prevent the virus from circulating among different birds and mammals especially humans.

Conclusion

These findings of this study indicate that HPAI H5N8 isolated in Iran belongs to the subgroup clade 2.3.4.4 and cluster within-group B. these phylogenetic results provides new insights into the evolution and spread of H5N8 in Iran; based on these results, we have to recognize an improper monitoring protocol for reducing the reassortment of them. Therefore, we could prevent the HPAI from circulating.

Acknowledgment

The authors would like to thank the Ghalyanchi lab experts at the University of Tehran for their extensive technical supports.

Conflict of interest

No conflict of interest is declared.

Funding

This study was not financially supported by any individual, agency, or institution.

References

1. Ghafouri SA, Langeroudi AG, Maghsoudloo H, Tehrani F, Khaltabadifarahani R, Abdollahi H, et al. Phylogenetic study-based hemagglutinin (HA) gene of highly pathogenic avian influenza virus (H5N1) detected from backyard chickens in Iran, 2015. Virus Genes. 2017;53(1):117-20.
2. OIE W. Update on highly pathogenic avian influenza in animals (type H5 and H7). 2017.
3. Ghafouri SA, Mehrabadi MHF, Talakesh SF, Hosseini H, Ziafati Z, Malekan M, et al. Full genome characterization of Iranian H5N8 highly pathogenic avian influenza virus from Hooded Crow (Corvus cornix), 2017: The first report. Comp Immunol Microbiol Infect Dis. 2019; 64:73-80.
4. Ghafouri SA, GhalyanchiLangeroudi A, Maghsoudloo H, Farahani RK, Abdollahi H, Tehrani F, et al. Clade 2.3. 4.4 avian influenza A (H5N8) outbreak in comercial poultry, Iran, 2016: the first report and update data. Trop Anim Health Prod. 2017;49(5):1089-93.
5. Swayne D, Suarez D. Highly pathogenic avian influenza. Rev Sci Tech. 2000;19(2):463-75.
6. OIE W. Update on avian influenza in animals (types H5 and H7). 2018.
7. Kord E, Kaffashi A, Ghadakchi H, Eshratabadi F, Bameri Z, Shoushtari A. Molecular characterization of the surface glycoprotein genes of highly pathogenic H5N1 avian influenza viruses detected in Iran in 2011. Trop Anim Health Prod. 2014;46(3):549-54.
8. Li J, Gu M, Liu D, Liu B, Jiang K, Zhong L, et al. Phylogenetic and biological characterization of three K1203 (H5N8)-like avian influenza A virus reassortants in China in 2014. Arch Virol. 2016;161(2):289-302.
9. Kanehira K, Uchida Y, Takemae N, Hikono H, Tsunekuni R, Saito T. Characterization of an H5N8 influenza A virus isolated from chickens during an outbreak of severe avian influenza in Japan in April 2014. Arch Virol. 2015;160(7):1629-43.
10. Langeroudi AG, Karimi V, Kheiri T. Nucleotide and amino acid sequence analysis of hemagglutinin protein in cleavage site region of h9n2 isolated from broilers in tehran province during 1998-2007. J Anim Vet Adv. 2008;7(5):529-34.
11. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731-9.
12. Lee D-H, Torchetti MK, Winker K, Ip HS, Song C-S, Swayne DE. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. J Virol. 2015;89(12):6521-4.
13. Ghalyanchi-Langeroudi A, Soleimani M, Karimi V, Morovati A. Molecular Surveillance of Avian Influenza in Bird Parks of Tehran, Iran. Iran J Vet Med. 2012;6(3):165-9.
14. Abolnik C. Outbreaks of Clade 2.3. 4.4 H5N8 highly pathogenic avian influenza in 2018 in the northern regions of South Africa were unrelated to those of 2017. Transbound Emerg Dis.2020;67(3):1371-81.
15. Abolnik C, Pieterse R, Peyrot BM, Choma P, Phiri TP, Ebersohn K, et al. The incursion and spread of highly pathogenic avian influenza H5N8 clade 2.3. 4.4 within South Africa. Avian Dis. 2019;63(1s):149-56.
16. Valley-Omar Z, Cloete A, Pieterse R, Walaza S, Salie-Bassier Y, Smith M, et al. Human surveillance and phylogeny of highly pathogenic avian influenza A (H5N8) during an outbreak in poultry in South Africa, 2017. Influenza Other Respir Viruses. 2020;14(3):266-73.
17. Adlhoch C, Dabrera G, Penttinen P, Pebody R. Protective measures for humans against avian influenza A (H5N8) outbreaks in 22 European Union/European Economic Area Countries and Israel, 2016–17. Emerg Infect Dis. 2018;24(10):1-8.
18. Bertran K, Pantin-Jackwood MJ, Criado MF, Lee D-H, Balzli CL, Spackman E, et al. Pathobiology and innate immune responses of gallinaceous poultry to clade 2.3. 4.4 A H5Nx highly pathogenic avian influenza virus infection. Vet Res. 2019;50(1):89.
19. Kouam MK, Tchouankui HN, Ngapagna AN. Epidemiological Features of Highly Pathogenic Avian Influenza in Cameroon. Vet Med Int. 2019; 2019:1-5
20. Puranik A, Slomka MJ, Warren CJ, Thomas SS, Mahmood S, Byrne AM, et al. Transmission dynamics between infected waterfowl and terrestrial poultry: Differences between the transmission and tropism of H5N8 highly pathogenic avian influenza virus (clade 2.3. 4.4 a) among ducks, chickens and turkeys. Virology. 2020; 541:113-23.
21. Bertran K, Swayne DE, Pantin-Jackwood MJ, Kapczynski DR, Spackman E, Suarez DL. Lack of chicken adaptation of newly emergent Eurasian H5N8 and reassortant H5N2 high pathogenicity avian influenza viruses in the US is consistent with restricted poultry outbreaks in the Pacific flyway during 2014–2015. Virology. 2016; 494:190-7.
22. Hemida MG, Chu D, Abdelaziz A, Alnaeem A, Chan SMS, Peiris M. Molecular characterisation of an avian influenza (H5N8) outbreak in backyard flocks in Al Ahsa, Eastern Saudi Arabia, 2017–2018. Vet Rec Open. 2019; 6(1): e000362.
23. Kandeil A, Hicks JT, Young SG, El Taweel AN, Kayed AS, Moatasim Y, et al. Active surveillance and genetic evolution of avian influenza viruses in Egypt, 2016–2018. Emerg Microbes Infect. 2019;8(1):1370-82.
24. Webster RG, Peiris M, Chen H, Guan Y. H5N1 outbreaks and enzootic influenza. Biodiversity 2006;7 (1):51-5.