Clinical Cancer Investigation Journal-ISSN: 2278-0513

Phylogenetic analysis of Hemagglutinin gene of H9N2 Avian Influenza virus circulated In Iran during 2019-2020.

Abstract

H9N2 avian influenza viruses are now internationally prevalent in poultry over the last couple of decades, posing a significant threat to the global poultry industry and humans due to their high zoonotic infection and pandemic risk. The haemagglutinin (HA) protein is AIV's primary surface antigen important for viral infection. This research was conducted to investigate the avian Influenza virus H9N2 in six provinces of Iran. First, among 60 broiler flocks with respiratory symptoms, tracheal samples were obtained during 2019-2020. Subsequently, RNA was extracted, then RT-PCR was performed by targeting the Hemagglutinin gene. This study sequenced and described partial Haemagglutinin genes from 13 Iranian H9N2 viruses. According to phylogenetic studies, all Iranian viruses belong to the G1 sub-lineage. Considering the virus's profound influence on the poultry industry, individuals, and public health, more epidemiological investigations are necessary to assess the success of the approaches and practices used to control the H9N2 virus.

Keywords: *H9N2, Iran, Avian Influenza, Phylogenetic Study, Hemagglutinin*

Peyman Younesi[1], Arash Ghalyanchi Langroudi[2], Hossein Hosseini[3] Kaveh Parvandar Asadollahi[1]

1- Department of Clinical Sciences, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran ; younesi1978@gmail.com
2- Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran ; ghalyana@ut.ac.ir
3- Department of Clinical Sciences, Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Alborz, Iran. ; Hosseini.ho@gmail.com
Corresponding Author
Kaveh Parvandar Asadollahi
Department of Clinical Sciences, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran
Email: kaveh_parvandar@yahoo.com
Cell number: +989121078908

1. Introduction

Avian influenza, as a viral infection in birds, leads to economic loss in the poultry industry throughout the world. In the last two decades, the infection of the avian influenza virus (AIV) has become a frequent concern for poultry production in numerous countries in Asia, including Iran. The low pathogenicity of H9N2 infections in poultry allows the virus to acclimatize and grow while also promoting the evolution of numerous antigenic changes in the circulating virus strains. (1)
Avian influenza virus (AIV) belongs to Influenza A virus, a genus of the Orthomyxoviridae family with a negative-sense, single-stranded, segmented RNA genome. The influenza virus's negative-sense RNA genome is made up of eight segments that encode 10 to 12 proteins, the most significant of which are hemagglutinin (HA) and neuraminidase (NA). AIV type A is classified into subtypes based on their two surface glycoproteins, HA and NA. 16 HA (H1-H16) and 9 NA (N1-N9) were isolated from wild birds, primarily aquatic ones serving as the primary natural reservoirs. (2, 3)
The HA surface glycoprotein is responsible for the virus attaching to the sialic acid receptor on the cell surface in the early stage of the disease. Sialic acid promotes viral and endosomal membrane fusion, which leads to viral entry into the host cell. To enable the budding of offspring virions from cells, the NA eliminates sialic acid from cell surface glycoconjugates and freshly generated viral protein. (4)
Furthermore, earlier research has shown that some H9N2 avian influenza viruses have acquired receptor-binding properties similar to human strains (SA-α-2,6-Gal)occasionally transferred from poultry to mammalian species such as humans and pigs. (5) H9N2 AIV infections typically cause mild respiratory symptoms and decreased egg production. When co-infected with other diseases, such as Escherichia coli, this can cause severe illness and death in chicken flocks. (6)
The H9N2 virus was isolated from chickens in Iran for the first time in 1998 in Qazvin province, and it is now the most common subtype of influenza virus in the poultry industry. Some broiler farms in Iran have mortality rates of up to 65 percent. (7, 8) The co-circulation of H9N2 and H5N1 viruses in the field increases the possibility of gene reassortment between the viruses. (9)
Since its discovery in turkeys in 1966 in Wisconsin, the avian influenza virus (AIV) subtype H9N2 has been primarily found in shorebirds and wild ducks(10). Many bird species were infected with H9N2 influenza viruses in Germany, Ireland, Italy, South Africa, the United States, Korea, and China in the mid-1990s. H9N2 infections were observed in Middle Eastern nations after 1998, resulting in significant commercial poultry epidemics (11).
According to antigenic and genetic research of the H9N2 AIV, two different lineages, Eurasian and North American, were identified. The former is divided into main groups of G1- like, Y280-like, and Y439-like viruses, which are represented respectively by A/Quail/Hong Kong/G1/97(G1), A/Duck/HongKong/Y280/97(Y280), and A/Duck/Hong Kong/Y439/97(Y439). (12)
A phylogenetic study of H9N2 Iranian isolates' HA genes revealed that all Iranian isolates belonged to the G1 sublineage. (13, 14) Iranian H9N2 influenza viruses were found to replicate in organs such as the spleen, kidney, and other organs, which was previously unheard of for a low pathogenicity avian

influenza virus. These researches have revealed that the Iranian H9N2 virus can undergo structural alterations. (15)

In latest years, however, H9N2 AIV has frequently been detected in vaccinated hens. It suggests that the virus experienced antigenic drift as a result of various circumstances, resulting in decreased efficiency of existing vaccinations. (16) As a result, viral surveillance and characterization are critical for better understanding any ongoing public health concern in Iran.

This research aimed to investigate the evolution and phylogenetics of the H9N2 influenza virus from the poultry farms of the six Iranian provinces with major broiler farms and provides epidemiological data about phylogenetic and genetic analyses based on partial sequences of the H9N2 LPAI viruses' hemagglutinin (HA) genes.

2. Materials and methods

2.1. History and sample collection

Tracheal samples were collected using the standard technique from 60 broiler farms with respiratory symptoms from September 2019 to November 2020 in six provinces of Iran, including Isfahan, Khuzestan, Razavi Khorasan, East Azerbaijan, Qazvin, and Golestan. Fifteen trachea samples were taken from each farm, and Five tracheae from each farm were pooled together. In broilers aged 25 to 35 days, clinical signs included depression, rhinitis, cough, conjunctivitis, ocular discharge, weakness, and diarrhea. In addition, all farms were vaccinated with the inactivated oil emulsion avian influenza H9N2 vaccine. The samples were gathered in phosphate-buffered saline (PBS, pH 7.4). All samples were transferred to the laboratory on ice packs and stored at -70 ° C until used for subsequent steps.

2.2. RNA extraction

The trachea samples were completely homogenized under sterile conditions. Then, according to the kit protocol, RNAs were extracted by the SinaClon RNA extraction kit (SinaClon, Iran). The RNA extracted was kept at -70 °C until used for cDNA synthesis.

2.3. RT and PCR reaction

For the first step, extracted RNAs were utilized in reverse transcription (RT) reaction to synthesize cDNA. Based on the manufacturer's instruction, the cDNA synthesis was performed by the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) and random hexamer primers (SinaClon, Iran).

Specific primers of the M gene were used for recognizing all avian influenza A viruses via PCR. For this purpose, PCR reaction was carried on in a volume of 20 µl reaction mixture encompassing 2.5 µl 10X buffer, 10.2 µl of distilled water, 0.5 µl of dNTP, 1 µl of forwarding primer (AF: 5'AGGTCGAAACGTAYGTTCTCTCTAT3'), 1µl of reverse primer (AR3:5'GGTCTTGTCTTTAGCCAYTCCAT3'), 2 µl Mgcl2, 0.25 µl Taq DNA polymerase. Finally, 2.5 µl of cDNA was added to the mixture. PCR amplification was performed at 94 ◦C for 2 min for the first denaturation, followed by 40 cycles for 10 s at 94 ◦C, for 20 s at 58°C, for 20 s at 72°C, and the last extension for 5 min at 72◦C. The primers were used in this reaction described previously by Wu et al. (17).

To identify subtype H9, a particular primer was applied to amplify part of the 488 bp HA gene. The required materials followed by the total volume of 25 µl with 12.5 µl 2x master mix (SinaClon, Iran), 1µl forward primer: H9-151f 5'-CTYCACACAGARCACAATGG, 1 µl reverse primer: H9-638r 5'-GTCACACTTGTTGTTGTRTC,2.5 µl cDNA and 8 µl distilled water. The PCR condition for the amplification was for 3 min at 95°C,35 cycles for 30 s at 95°C, for 40 s at 50°C, and 40 s at 72°C, followed by 10 min at 72°C. The primers were used for this reaction previously designed by Lee *et al*. (18).

2.4. Sequence and phylogenetic analysis

Thirteen positive PCR products obtained from the HA gene amplifying were sent for sequencing (Codon Order, Iran) in forward and reversed directions. After receiving the sequencing data, chromatograms were evaluated by Chromas Pro (Chromas Pro version 2.5). The nucleotide sequences of HA genes were compared to other similar sequence data in GenBank to establish a phylogenetic link. CLUSTALW with MEGA7 software was used to align all of the sequences. The Tamura-Nei model was used to build distance-based neighbor-joining trees. The robustness of the phylogenetic trees was evaluated using 1000 bootstrap repetitions. Also, the alignments were subsequently used to construct distance matrices using the General Time Reversible (GTR) model Rates and Patterns implemented in the Mega software version 7.

2.5. GenBank accession numbers

The partial HA nucleotides sequences identified in the present research are accessible in NCBI with the accession numbers MZ442351, MZ442353, MZ442354, MZ433242, MZ433262, MZ413299, MZ433301, MZ437001, and MZ413325 to MZ413329.

3. Results

In the current research, 13 H9N2 LPAI strains were detected from broiler flocks from September 2019 to November 2020, approved for AIV by RT-PCR and sequencing.

A phylogenetic study of the partial nucleotides of the HA gene showed that numerous H9N2 genetic lineages had disseminated in the South Asian and Middle Eastern poultry populations over the last 20 years. Also, phylogenetic tree

analysis showed that Iranian viruses detected in this research were clustered into the G1 sub-lineage (Figure 1).

A comparison of the HA gene sequences of Iranian strains with the Asian H9N2 subtype gives an outline of the linkages. The viruses sequenced in this study had 68 to 100% similarity. Also, these viruses have high similarity with H9N2 viruses isolated last years in Iran, Kuwait, Pakistan, Bangladesh, and Saudi Arabia (Table 1).

4. Discussion

H9N2 AIVs have been isolated from poultry, including many vaccinated flocks, and spread in Iranian domestic poultry for more than 20 years. Even though H9N2 viruses are classified as low pathogenic avian influenza (LPAI), they can cause significant morbidity and mortality. (1, 19) According to our research, explicit clinical symptoms in chickens seen at impacted farms comprised acute respiratory signs and a 14–35% mortality percentage in the outbreaks. H9N2 viruses have been observed to cause substantial mortality in the field due to environmental stress and subsequent bacterial (Escherichia coli), Mycoplasma gallisepticum infections, infectious bronchitis virus and Ornithobacterium rhinotracheale.(6, 20). Furthermore, Kishida et al. discovered that co-infection with Staphylococcus aureus or Haemophilus paragallinarum increases the risk of H9N2 influenza A virus infection in hens(21). These studies suggested that concurrent infections or environmental factors may cause clinical symptoms and increase mortality rates.

Previous research proposed that isolates from Iran, Germany, Pakistan, and Saudi Arabia shared a range of similarities and may have originated from the same source. However, based on the phylogenetic study, Iranian isolates, including isolates in this study, belong to a well-defined group associated with the G1 sublineage, leading to a single ancestor for all Iranian isolates. (13, 22)

Karimi et al. published one of the first scientific papers on the phylogenetic research of Iranian isolates of H9N2. Comparisons of the HA gene's nucleotide sequence from Iranian isolates revealed 97-99 percent group identity and 98 percent homology with the two isolates from Pakistani parakeets transported to Japan (23). Another study reported the Iranian isolates' amino acid sequences in 2000-2001 to be very similar to those in Germany, Saudi Arabia, and Pakistan; it's also suggested that Iranian isolates' pathogenicity is close to Eurasian isolates, except for some Chinese isolates (24).

According to Ghalyanchi et al., low-pathogenic Influenza H9N2 viruses have circulated continuously during the last decade in the Middle East area, particularly in Iran. Based on phylogenetic data, it is hypothesized that the development of H9N2 avian influenza in Iran was introduced from Pakistan due to low international border quarantine rules (25).

The first subgroup virus was primarily isolated between 1998 and 2004, while the second subgroup virus was primarily isolated after 2004, with minor exceptions for both groupings. The sequenced isolates were more similar to several recent Pakistan isolates than previous Iranian isolates (2005-2008). In the phylogenetic tree, they were shown to be more closely related to new Pakistan isolates than to other Iranian isolates. These findings could point to a new sublineage of AI viruses in Iran and Pakistan (26, 27)

Analysis of the Hemagglutinin gene showed that the Iraqi strain shared a high degree of identity with isolates from Iran, Jordan, Saudi Arabia, Israel, and the UA E. Iraqi isolates, 95.2% to 96.7%, were identical to Pakistani isolates (Pak-294, P.UDL-01, P.UDL-02, Karachi-03). These findings could reveal that these countries had a close geographical relationship, which raises the possibility of the outbreak observed originating from a significant epizootic as a result of disseminating a single virus. Also, recently, trade-in poultry and their products with bordering countries, including Turkey, Iran, and Saudi Arabia, have eased the spread of the virus to Iraq and such countries. This trade plays a significant role in spreading H9N2 viruses in the Middle East and Central Asia (28).

Another study was carried out in Afghanistan, which tried to develop its poultry industry. The strains identified had similarities between 85% and 91% to those previously identified in Afghanistan in 2008 and 2009. On the other hand, they shared 97% similarity to the 2015 Pakistani strains and showed a similarity of 91%,95%, and 92% with the Iranian strains from 2011, 2015, and 2016. Such resemblances may have arisen as a result of the large volume of poultry products and equipment traded from Iran and Pakistan to Afghanistan, particularly recently, in conjunction with advancements in the Afghan poultry industry (29).

Inactivated vaccines (A/chicken/Iran/101/1998/H9N2, A/chicken/Iran/1999/H9N2) have been broadly applied to minimize infection impact on the poultry industry. These vaccines were initially successful, but their efficacy has declined over time. Despite frequent vaccinations with inactivated vaccines, H9N2 LPAI viruses spread in both vaccinated and unvaccinated flocks, resulting in multiple outbreaks. The viral strain varied from the vaccinal strain and the earliest Iranian isolate may be determined by post-vaccinal specific immunity as a selective factor. In addition, vaccine-induced immune pressure can cause antigenic changes in viruses, such as antigenic shift and drift .(19, 30, 31)

Following the virus was identified, an inactivated vaccine prepared from an initial isolate (A/chicken/Iran/101/1998/H9N2) was used for several years. Fallah Mehrabadi et al. declared that the pressure of vaccination and the resulting immune response in vaccinated

poultry could accelerate genetic alterations in the H9N2 virus, leading to a lack of adequate response to vaccines derived from the primary (early) virus. This result is supported by a phylogenetic and genetic study of surface glycoproteins from current and early H9N2 isolates from Iran (19, 32). The result confirmed that at least two groups of influenza viruses are circulating in Iran. In line with other recent studies, the virus is experiencing rapid genetic changes, which could be caused by unsuccessful vaccines, causing the virus to circulate in the poultry population.

Regarding the possibility that imported vaccines use older strains and do not match the circulating strains in Iran, Razi Vaccine and Serum Research Institute, (AREEO), recently used a strain isolated from poultry three years earlier as a vaccine seed. However, it does not seem to be sufficient. In addition to the up-to-datedness of vaccine seeds, multivalency of vaccine seeds also seems necessary, as, in human influenza vaccines, updated and multivalent seeds in vaccine production seem effective. On the other hand, a lack of research on the live bird market, one of the most significant sources of virus circulation among industrial and backyard birds, is one of the other problems in controlling and preventing avian influenza in Iran.

The illegal trading of ornamental birds from neighboring countries is another important source of the virus's dissemination. Smuggling parakeets from Pakistan with well-adapted viruses, in particular, could result in the virus spreading to commercial birds in Iran. The H9N2 virus has spread between Iran and Iraq due to the illegal trading of day-old chicks, poultry feed, and equipment. Studies on the virus in Iraq have revealed high genetic similarities between Iranian and Iraqi strains(28). The resistance of some strains of influenza viruses to the amantadine used for prevention and treatment in industrial poultry allows the viruses to evolve and circulate in Iranian flocks (33).

The simultaneous circulation of the H9N2 and H5 viruses in commercial poultry could lead to the appearance of new viruses due to the rearrangement of the virus genome and the possibility of internal gene exchange. As a result, Iran's influenza control and prevention plan should include isolation and assessment of pathogenicity and the sequence of internal genes.

Authors' Contribution

Study concept and design experiment: A. G.L., H. H., K.P.A.

Sample Collection and technical and material support: A. G. L., P. Y., H. H., K. P. A.

Data analysis: A. G.L., H. H., K.P.A., P. Y.

Drafting of the manuscript: A. G. L., H. H., K. P. A.

Ethics

All the procedures have been done according to the instructor's guide and ethical standards of the University of Tehran's animals.

Conflict of Interest

No conflict of interest was declared.

Grant Support

The current project was performed without funding support.

Acknowledgment

The authors would like to thank PCR Lab and Ghalyanchi Lab Experts.

References

1. Peacock TTP, James J, Sealy JE, Iqbal M. A global perspective on H9N2 avian influenza virus. Viruses. 2019;11(7):620.
2. Shao W, Li X, Goraya MU, Wang S, Chen J-L. Evolution of influenza a virus by mutation and re-assortment. International journal of molecular sciences. 2017;18(8):1650.
3. Naguib MM, Verhagen JH, Mostafa A, Wille M, Li R, Graaf A, et al. Global patterns of avian influenza A (H7): virus evolution and zoonotic threats. FEMS Microbiology Reviews. 2019;43(6):608-21.
4. Kosik I, Yewdell JW. Influenza hemagglutinin and neuraminidase: Yin–Yang proteins coevolving to thwart immunity. Viruses. 2019;11(4):346.
5. Song W, Qin K. Human-infecting influenza A (H9N2) virus: A forgotten potential pandemic strain? Zoonoses and public health. 2020;67(3):203-12.
6. Samy A, Naguib MM. Avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: field and experimental findings. Veterinary sciences. 2018;5(1):23.
7. VASFI MM, BOZORG MM. Isolation of H9N2 subtype of avian influenza viruses during an outbreak in chickens in Iran. 2002.
8. Nili H, Asasi K. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathology. 2002;31(3):247-52.
9. Arai Y, Ibrahim MS, Elgendy EM, Daidoji T, Ono T, Suzuki Y, et al. Genetic compatibility of reassortants between avian H5N1 and H9N2 influenza viruses with higher pathogenicity in mammals. Journal of Virology. 2019;93(4):e01969-18.
10. Reeves AB, Hall JS, Poulson RL, Donnelly T, Stallknecht DE, Ramey AM. Influenza A virus recovery, diversity, and intercontinental exchange: a multi-year assessment of wild bird sampling at Izembek National Wildlife Refuge, Alaska. PLoS One. 2018;13(4):e0195327.
11. Alexander DJ. Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian diseases. 2007;51(s1):161-6.
12. Matrosovich MN, Krauss S, Webster RG. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology. 2001;281(2):156-62.
13. Toroghi R, Momayez R. Biological and molecular characterization of Avian influenza virus (H9N2) isolates from Iran. Acta virologica. 2006;50(3):163-8.
14. Ghorbani A, Moosakhani F, Marandi MV. Phylogenetic analysis of the hemagglutinin gene of recent H9N2 avian influenza viruses isolated from broiler flocks in Iran. Veterinarski arhiv. 2016;86(1):95-109.
15. Mosleh N, Dadras H, Mohammadi A. Evaluation of H9N2 avian influenza virus dissemination in various organs of experimentally infected broiler chickens using RT-PCR. Iranian Journal of Veterinary Research. 2009;10(1):12-20.
16. Cong Y, Zhong Y, Sun Y, Ding Z. Advance on epidemiology and vaccine of H9N2 avian influenza virus. Chin J Vet Sci. 2017;37:386-92.

17. Wu C, Cheng X, He J, Lv X, Wang J, Deng R, et al. A multiplex real-time RT-PCR for detection and identification of influenza virus types A and B and subtypes H5 and N1. Journal of virological methods. 2008;148(1-2):81-8.

18. Lee M-S, Chang P-C, Shien J-H, Cheng M-C, Shieh HK. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. Journal of virological methods. 2001;97(1-2):13-22.

19. Mehrabadi MHF, Ghalyanchilangeroudi A, Ghafouri SA, Malekan M, Ziafati Z, Hosseini H, et al. Full-genome characterization and genetic analysis of a H9N2 virus in commercial broilers in Iran, 2017. Tropical animal health and production. 2019;51(6):1737-49.

20. Umar S, Guerin JL, Ducatez MF. Low pathogenic avian influenza and coinfecting pathogens: a review of experimental infections in avian models. Avian Diseases. 2017;61(1):3-15.

21. Kishida N, Sakoda Y, Eto M, Sunaga Y, Kida H. Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. Archives of virology. 2004;149(11):2095-104.

22. Homayounimehr AR, Dadras H, Shoushtari A, Pourbakhsh SA. Sequence and phylogenetic analysis of the haemagglutinin genes of H9N2 avian influenza viruses isolated from commercial chickens in Iran. Tropical animal health and production. 2010;42(6):1291-7.

23. Karimi V, Bozorgmehri Fard MH, Shahbazzadeh D, Esmaelizad M, Pourbakhsh SA. Sequence analysis and phylogenetic study of hemagglutinin gene of H9N2 subtype of avian influenza virus isolated during 1998-2002 in Iran. Iranian Biomedical Journal. 2004;8(4):167-72.

24. Kianizadeh M, TOROUGHI R, Pourbakhsh S, Momayez R. Pathogenicity and haemagglutinin gene sequence analysis of Iranian avian influenza H9N2 viruses isolated during (1998–2001). 2006.

25. Langeroudi AG, Karimi V, Kheiri MT, Barin A. Full-length characterization and phylogenetic analysis of hemagglutinin gene of H9N2 virus isolated from broilers in Iran during 1998–2007. Comparative clinical pathology. 2013;22(3):321-30.

26. Norouzian H, Gholami S, Vasfi MM. Phylogenetic analysis of hemagglutinin gene of H9N2 avian influenza viruses isolated from chicken in Iran in 2010-2011: emerging of a new subgroup. 2012.

27. Bashashati M, Mojahedi Z, Roudsari AA, Taghizadeh M, Molouki A, Motamed N, et al. Ongoing genetic evolution of H9N2 avian influenza viruses in Iranian industrial poultry farms. Acta Veterinaria Hungarica. 2020;68(3):328-35.

28. Kraidi QA, Madadgar O, Langeroudi AG, Karimi V. Genetic analysis of H9N2 avian influenza viruses circulated in broiler flocks: a case study in Iraq in 2014–2015. Virus genes. 2017;53(2):205-14.

29. Hosseini H, Ghalyanchilangeroudi A, Mehrabadi MHF, Sediqian MS, Shayeganmehr A, Ghafouri SA, et al. Phylogenetic analysis of H9N2 avian influenza viruses in Afghanistan (2016-2017). Archives of virology. 2017;162(10):3161-5.

30. Su H, Zhao Y, Zheng L, Wang S, Shi H, Liu X. Effect of the selection pressure of vaccine antibodies on evolution of H9N2 avian influenza virus in chickens. Amb Express. 2020;10(1):1-19.

31. Khalili I, Ghadimipour R, Eteghad SS, Najafi MF, Ebrahimi MM, Godsian N, et al. Evaluation of immune response against inactivated avian influenza (H9N2) vaccine, by using chitosan nanoparticles. Jundishapur Journal of microbiology. 2015;8(12).

32. Bashashati M, Chung DH, Fallah Mehrabadi MH, Lee DH. Evolution of H9N2 avian influenza viruses in Iran, 2017–2019. Transboundary and Emerging Diseases. 2021;68(6):3405-14.

33. Gholami-Ahangaran M, Salehi MH, Karimi-Dehkordi M, Azizi S. A Retrospective Phylogenetic Analysis of Matrix Gene and Amantadine Resistance in Avian Influenza (H9N2 subtype) During 2014-2015 in Isfahan, Iran. Avicenna Journal of Clinical Microbiology and Infection. 2020;7(3):85-9.

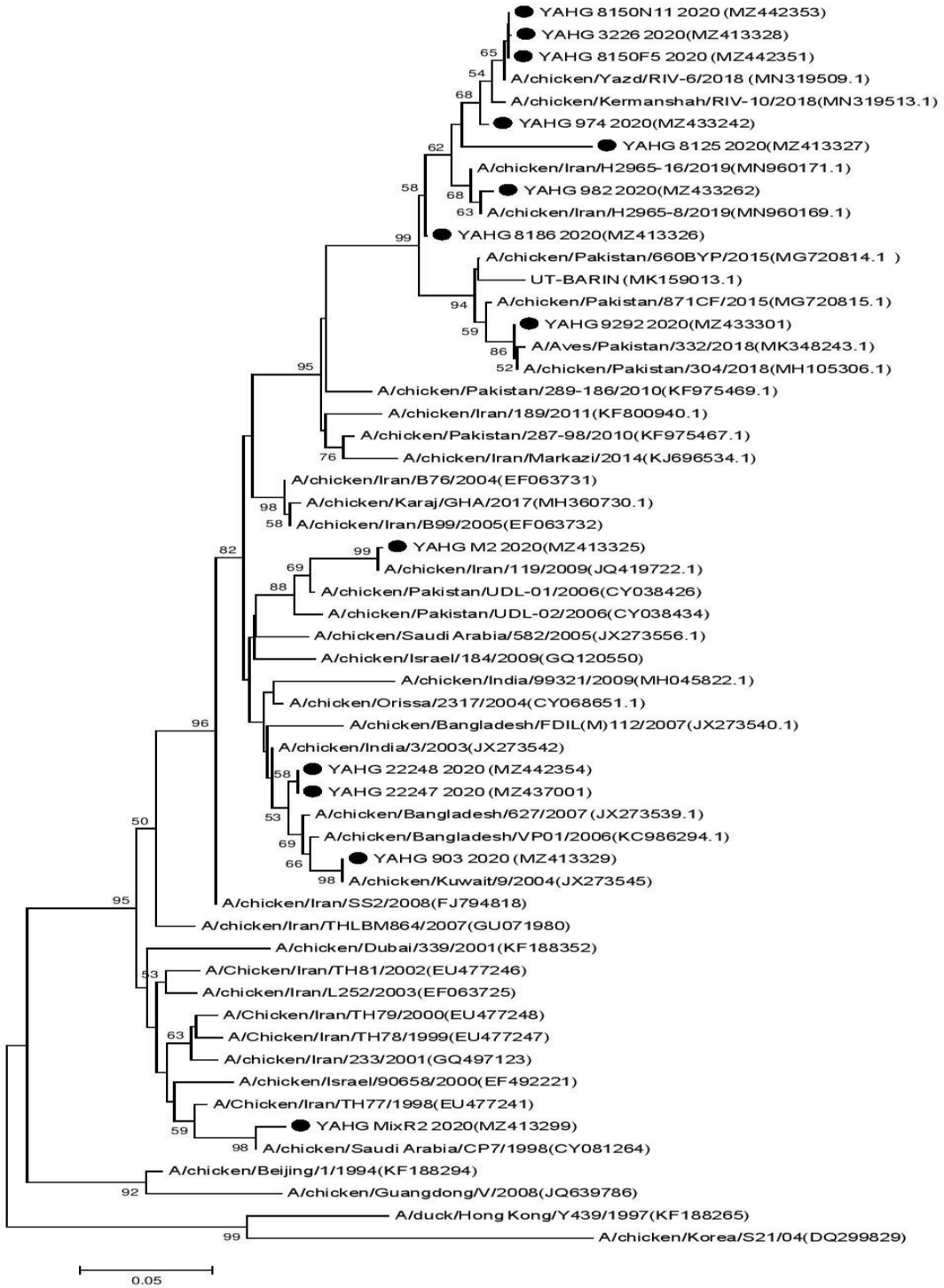

Figure 1: The Neighbor-Joining method was used to infer the evolutionary history. Next to the branches is the percentage of duplicate trees in which the related taxa clustered together in the bootstrap test (1000 repetitions). The tree is displayed to scale, with branch lengths measured in the same units as evolutionary distances used to estimate the phylogenetic tree. The evolutionary distances were calculated using the p distance method and are measured in base differences per site. Bootstrap values less than 70 were ignored. MEGA7 was used to perform evolutionary analysis (black circle: current study isolates in Iran).

Table 1: Sequence identity matrix for H9N2 isolated viruses in this study and other related H9N2 viruses, based on the partial 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	