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Original Article

Serological Survey, Molecular Identification and Phylogenetic Analysis of Avian Influenza Virus H9N2 in Backyard Chickens in Rural Areas of Fars Province, Iran

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Abstract

Background and Aims: Avian influenza virus belongs to genus A of Orthomyxoviridae family and only this genus is pathogenic in birds. Backyard chickens all over the world, especially in the Middle East, play an important role in feeding people. These birds can also play a significant role in the epidemiology of AI and the spread of the virus to commercial poultry flocks. Therefore, the aim of this study was to investigate H9N2 influenza virus in backyard chickens of rural areas of 30 counties of Fars province, Iran using serological and molecular methods.

Materials and Methods: In this study, from July 2019 to January 2020, tracheal and cloacal swabs and blood samples were taken from backyard chickens of rural areas of Fars province, Iran for virus isolation and HI test in order to detect H9N2 infection and identification of possible isolates.

Results: 54% of the serum samples were HI test positive also 14 out of the 30 surveyed counties showed positive results in terms of H9N2 influenza virus serum antibodies and also average HI titer of 5.18 and 74% as the rate of seroprevalance detected in these cities, respectively. In RT-PCR test with H9 specific primer, only one isolate was positive demonstrating the band of 488bp which confirmed by sequencing. According to comparative alignment and phylogenetic analysis this strain was closely related to the strain D1 in the gene bank.

Conclusion: Due to the positive serum titer in a large number of backyard chickens in the rural areas, constant monitoring of these birds in terms of avian influenza infection and implementing more effective control programs seem necessary.

Keywords: Commercial poultry flock; blood sample; tracheal and cloacal swabs; HI; molecular method

Introduction

Influenza virus belongs to Orthomyxoviridae family, which includes 7 genera influenza A, influenza B, influenza C, influenza D, Togotovirus, Quranjavirus and Isavirus. Avian influenza belongs to genus A and only this serotype is pathogenic in birds. Genus A is divided into several subtypes based on two surface antigens, hemagglutinin and neuraminidase, including 16 subtypes of hemagglutinin (H1-16) and 9 subtypes of neuraminidase (N1-9) (1-3). Avian influenza virus in terms of pathotype, is divided into highly-pathogenic avian influenza (HPAI) and low-pathogenic avian influenza (LPAI), H9N2 virus is in the category of low-pathogenic avian influenza (1)(4, 5). The H9N2 virus was first isolated from turkey flocks in Wisconsin in the USA(4) (6). The virus was first isolated from chicken in China in the early 1990s, and in the following decades the viruses associated with this Chinese progenitor have become endemic in poultry farms in the most parts of Asia, the Middle East, North and West of Africa (5). The virus, especially in the Middle East countries, has caused serious damage to

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the poultry industry (6). Endemicity of H9N2 virus in rural chickens of these countries plays a crucial role in its epidemiology and transmission to commercial poultry farms, therefore; considering the importance of H9N2 influenza virus in Iran, the aim of this study was to investigate the presence of H9N2 influenza infection in rural chickens in 30 counties of Fars province, Iran using serological (Hemagglutination inhibition test) and molecular methods.

Methods

Sampling: In this study, from July 2019 to January 2020, backyard chickens were sampled from 60 villages in 30 counties of Fars province (2 villages in each counties were selected based on the existence of commercial poultry farms around them). (Fig. 1).



Fig. 1. Location of Fars province and its counties in Iran, where sampling was done.

According to the calculations of Commission Decision 2010/367 / EU, 60 villages were randomly chosen for collecting the samples via entire the Fars province. In each village, 30 backyard chickens randomly selected and two samples, including tracheal and cloacal swaps were collected. Finally 60 swabs were gathered from each village and totally 3600 swap samples transferred under standard condition to the laboratory. At the same time, 20 blood samples were taken from the wing vein.

A total of 1200 blood samples was collected from unvaccinated backyard chickens to carry out serum HI test in order to detect any previous H9N2 infection (7).

Table 1: The name of antibiotics-antimycotic mix

Antibiotic	Dose
Streptomycin sulfate	1mg/ml
Gentamycin	1mg/ml
Penicillin G	10,000 IU/ml
Nystatin	20 IU/mL
Kanamycin sulfate	650 µg/mL

Swap samples were placed in 2xPBS Falcon tubs at pH 7.4 and sent to the laboratory. After centrifugation, they were stored at -70 °c (8). Blood samples centrifuged for 10 minutes at 3500 rpm to collect the sera and HI test (9).

Swap samples from each village were pooled together and passed through syringe filters (Biofil^{TM}, China) with the size of 0.44 micrometer and then 0.22 micrometer and an antibiotic mixture was added to prevent the growth of bacteria and fungi (10, 11). (Table 1) Then the specimens were inoculated into 9 to 10 day old SPF embryonated chicken eggs.

The inoculated eggs were transferred to the refrigerator (4°C) 48 to 72 hours after inoculation. Allantoic fluid of the samples was then extracted under sterile conditions and the presence of virus was determined by hemagglutination test using 0.5% chicken red blood cells. Positive samples were stored in the -70 °C freezer for confirming the diagnosis by molecular tests (8).

Serology: HA and HI tests were performed according to the OIE (2004) guideline using reference antigen (A / chicken / Tehran / ZMT-173/99 (H9N2)) for subtype H9 of avian influenza. HI test was performed using 96 U-

shaped microtiter plates, dilution ½ with phosphate buffer solution, 1% red blood cell and influenza antigen with 4 HA units. According to OIE guidelines, titers equal to or more than log2 4 or 24 or dilution of 1:16 were considered positive for infection (12).

RT-PCR: Confirmation and subtyping of HA positive isolates were done by RT-PCR method. RNA extraction was done by Viral Gene-spin™ RNA extraction kit (iNtRON Biotechnology, Korea) according to kit protocol. RT-PCR was done using One step RT-PCR premix (iNtRON, Korea) and the pair of forward and reverse primers of H9 based on previous study with the sequences of 184F 5'CTYCACACAGARCACAATGG3' and 652 R 5'GTCACACTTGTTGTTGTRTC3' respectively (13, 14).

About 1-2 µg of each extracted RNA template was added to the mixture containing 8µl of premix, 0.5 µM of each primer, 5 units RNase inhibitor and DEPC-treated water to final volume of 20 µl. The PCR tests were carried out in Eppendorf gradient thermocycler (Germany). Cycling program was consisted of one cycle of 45 ºC for 30 min to perform the reverse transcription reaction, followed by the step of denaturation of RNA: DNA hybrid plus inactivation of reverse transcriptase in 94 ºC for 5 min. then 35 cycles including three steps of denaturation, annealing and extension set at 94, 50 and 72 ºC for 40, 30 and 50 sec, respectively. The program was ended by the final extension of 72 ºC for 5 min.

PCR products were loaded on 1.5% agarose gel in TBE buffer and subjected to electrophoresis (Cleaver, England) and visualized under UV light in gel documentation of Kodak GL200 imaging system (15).

Sequencing and Phylogenetic Analysis: The PCR product was purified using PCR product purification kit (Roche Diagnostic, Germany). The Purified DNA fragment was sequenced in both directions through Sanger method with ABI 3500 Genetic analyzer system (Thermo fisher scientific, USA) using the same primers as for RT-PCR. All data related to the nucleotide sequence were edited by Bioedit software and submitted to a search for similarity in GenBank using the Blast program (16, 17). Multiple sequence alignment was performed using Bioedit version 7.2.5. and shown in Esprit sever (18, 19). The phylo-genetic tree was drawn using the MEGA X program through Maximum Likelihood (ML) phylogenetic trees based on the Tamura-Nei model (20). The GenBank data of mostly related species were also included in the phylogenetic analysis.

Statistical analysis: The data table of HI tests of counties were statistically analyzed with SPSS software version 24.

Descriptive statistics were used for mean, standard devia-tion, skewness, Kurtosis, minimum and maximum score, frequency, percentage and cumulative percentage.

Also, t-test and binomial test were used to know the difference between the results of HI titers among 30 counties.

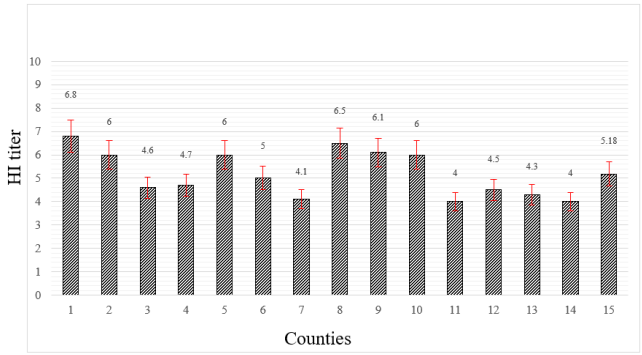


Chart 1: mean of HI titer in Positive counties:
1. Shiraz 2.Marvdasht 3.Sepidan 4.Abadeh 5.Bavanat 6.Zarin Dasht 7.Estahban 8.Farashband 9.Mosiri 10.Jahrom 11.khonj 12.Mohr 13.Lamerd 14.Kavar 15.Total Mean

Results

The results of HI test based on log2 4 or higher as the positive serum titer showed that 648 (54%) out of the 1200 serum samples were HI test positive also 14 out of the 30 surveyed counties were positive in terms of the presence of influenza virus antibodies. Total mean of serum titer of positive counties was 5.18. Also, 4 and 6.8 were the lowest and highest total mean serum titers in these positive counties, respectively. (Chart 1) 16 counties out of 30 counties were considered as the negative counties with a total mean of 2.66. Also, 1.6 and 3.5 were the lowest and highest total mean

serum titers in these negative counties, respectively. (Chart 2) Significant Variation (P<0.05) in H9N2 avian influenza virus antibody titers were found among 30 counties.

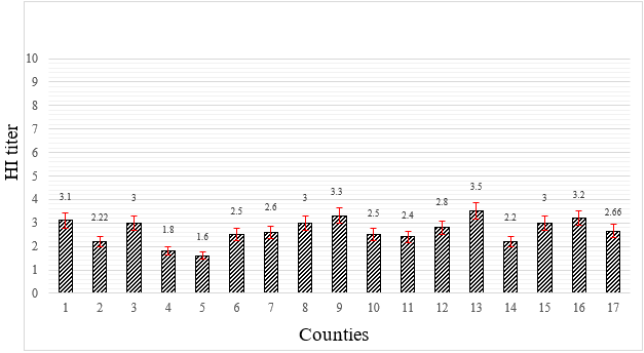


Chart 2: mean of HI titer in Negative counties:
1. Safashahr 2.Zarghan 3.Eghlid 4.Darab 5.Fasa 6.Arsenjan 7.Kharameh 8.Sarvestan 9.Neyriz 10.Ghirokarzin 11.Firozabad 12.Kazeron 13.Noorabad 14.Gerash 15.Lar 16.Saadat Shahr 17.Total Mean

It must be noted that no clinical signs of avian influenza were observed in any of the studied birds, particularly birds with high serum titers. Also, the study of seroprevalence showed that total mean of seroprevalence of the positive and negative counties were 74% and 41.5%, respectively. Also, the lowest and highest seroprevalence in the positive counties were 42.6% and 100%, respectively, and in the negative counties were 0% and 80%, respectively (Chart 3) (Chart 4).

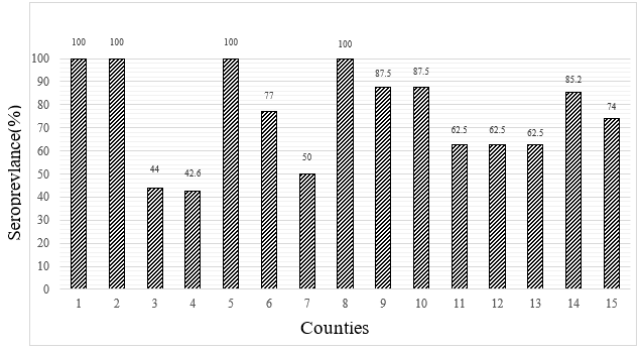


Chart 3: Seroprevalence of Positive Counties:
1. Shiraz 2. Marvdasht 3. Sepidan 4. Abadeh 5. Bavanat 6. Zarin Dasht 7. Estahban 8. Farashband 9. Mosiri 10. Jahrom 11. khonj 12. Mohr 13. Lamerd 14. Kavar 15.Total Mean

RT-PCR: Among the egg cultures, 27 samples were positive by hemagglutination test. In PCR test with H9 specific primer, only one of them, which was related to Sangbor village in Bavanat counties, was confirmed (Fig. 2).
Sequencing analysis: The sequence obtained in this study was deposited in GenBank with the accession number of MW066473 with the definition (A/chicken/Iran/sh1/2019(H9N2).
The length of this sequence was 474 bp.The results of the nucleotide BLAST of this isolate showed a 99.79% similarity to the isolate of A/Chicken/Iran/D1/1998. Based on the alignment results the difference was only in one bp at the position of 471 of our sequence (567 of D1) which the nucleotide C was replaced with T .

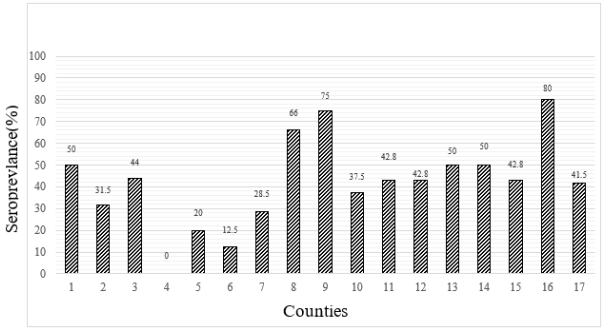


Chart 4: Seroprevalence of Negative counties:
1. Safashahr 2.Zarghan 3.Eghlid 4.Darab 5.Fasa 6.Arsenjan 7.Kharameh 8.Sarvestan 9.Neyriz 10.Ghirokarzin 11.Firozabad 12.Kazeron 13.Noorabad 14.Gerash 15.Lar 16.Saadat Shahr 17.Total Mean

The phylogenetic tree, based on nucleotide sequence analysis, by Maximum Likelihood method for evolutionary analysis was drawn. (Fig. 3).



Fig. 2. RT-PCR analysis. The 448bp specific fragment from H9-151f / H9-638r primers: Lane M: 100 bp DNA molecular weight marker (Cinnagen, Iran.) Lane C+: positive control (Influenza H9N2, laboratory approved isolate). Lane C-: negative control. Lanes 6,7,8: HA positive samples.

The tree with the highest log likelihood (-4307.67) is shown. Initial tree for the heuristic

search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value.
The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 nucleotide sequences. There were a total of 1743 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (21) (23).

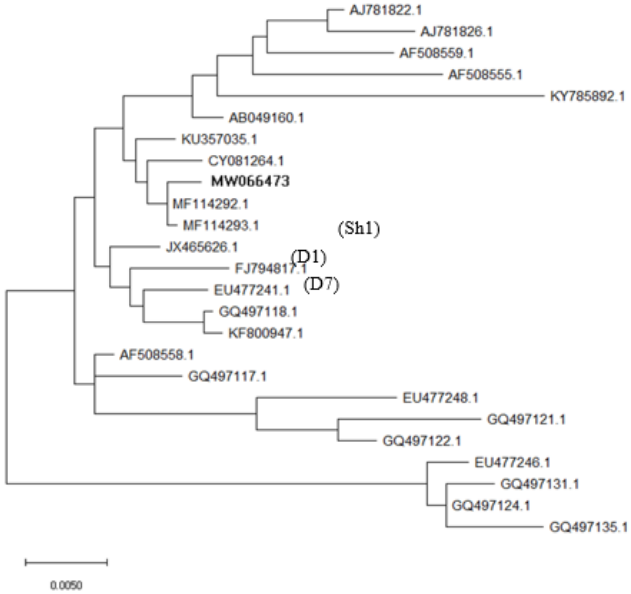


Fig. 3. Phylogenetic tree for the H9N2 subtype HA gene of our isolate (Sh1) in this study and 24 selected subtypes. The strains of Sh1 and D1 are highly related and belong to a sister group. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model.

The results of the phylogenetic analysis of our sample and 24 populations of influenza virus from different countries with more similarity showed that they clustered in 4 clades. One clade included our sh1 sample (MW066473) and some Iranian isolates from the year 1998. An isolate from Saudi Arabia shown by CY081264 isolated in 1998 were included in this clade as well. The strains of Sh1 and D1 (MF114292) are highly related and belong to a sister group.

Discussion

The H9N2 influenza virus has been prevalent in wild birds, backyard chickens and industrial poultry in the recent years (22-24). The virus has become endemic in industrial poultry farms in some countries and it has caused many problems in commercial poultry flocks in countries such as Iran, Pakistan, Saudi Arabia and the United Arab Emirates. Endemicity of this virus in backyard chickens in rural areas can play an important role in its transmission to commercial poultry flocks (1, 6, 25). Therefore, continuous monitoring of infection in backyard chickens is crucial to prevent transmission of the virus to commercial poultry flocks. Serological studies as well as a case of isolation and confirmation by RT-PCR in this study, like previous studies, confirm the endemicity of H9N2 virus in backyard chickens in Iran (26).
In this study, in terms of serum mean titer, 46% of counties in Fars province with a seroprevalence of 74% were positive for H9N2 virus infection.
Also total mean of seroprevalence in negative counties was 41.5%. These indicate a high frequency of infection with the virus among rural chickens in rural areas of the province.
This high rate of infection in backyard chickens, in addition to the potential risk to public health, can also be significant in transmitting the infection to commercial poultry flocks. Also, the absence of clinical signs and mortality among the infected birds indicates that these birds can play an important role in the epidemiology and spread of the disease as a reservoir. In the study of positive and negative counties, it shows that some factors such as geographical location, more communication and keeping a large number of chickens have important roles in the incidence of infection in positive counties (27, 28, 5).
While 27 egg culture samples were positive for HA test, the PCR test results were positive only in one case, which could be due to infection of the birds with other viruses with haemagglutinating activity such as Newcastle disease virus and egg drop syndrome virus or the RT-PCR test materials and enzymes may

have not had the quality and sensitivity needed to detect (29).

In two studies by Hadipoor et al. (2010 and 2011) on avian influenza (H9N2) infection in unvaccinated chickens in several villages around the Caspian Sea and also in several villages around the Maharloo lake in Fars province, total mean of HI titer was above 6.5 and the seroprevalence was over 70% and all the villages were positive in terms of mean serum titers. This study also confirmed the high rate of infection in backyard chickens in rural areas of the country. However, the reason for the higher rate of infection in this study compared to the current study could be due to the geographical location of the studied villages and their proximity to the sea and lake and consequently more contact with migratory waterfowl (30, 31).

Also, in the study of Saadat et al. (2014) in Bushehr province in Iran in unvaccinated backyard chickens, all studied cities were positive in terms of serum titer of avian influenza (H9N2) by HI test with a seroprevalence 39%. This study also shows a significant presence of H9N2 infection. In Saadat et al. study, despite the lower seroprevalence compared to the current study, all cities were positive in terms of serum mean titer, which indicates a high titer of H9N2 influenza antibody in the positive birds due to high exposure to the virus, which can be owing to the proximity of the studied areas of this province to the coastal areas and consequently more contact of domestic birds with migratory water fowl (32).

In the study of Mehrabadi et al. (2020) on the serum titer of H9N2 influenza in commercial poultry flocks in several provinces of Iran, it was found that the prevalence of H9N2 infection in the commercial poultry flocks was high and confirms its endemicity in the country and it shows that in addition to being endemic in backyard chickens, this virus is also endemic in commercial poultry flocks in the country (33).

Human infection with avian H9N2 influenza A virus in Hong Kong and China has been reported previously in 1999 (34). This subtype can potentially undergo the gene combination for infecting human and other mammals (35). For this reason, continuous monitoring and subtyping of AIV viruses among different avian flocks and evaluation of viral genetic changes throughout the years is of high importance .

Based on the comparative alignment of HA nucleotide sequences, it is the first report of mutation of single nucleotide C to T in our sh1 isolate. This change in the nucleotide did not affect amino acid codon (substitution of AGC codon to AGT in position of 189 of D1) which both coded the amino acid serine. According to Burke Reference Sequence Alignment HA Subtype Numbering Conversion (beta) this amino acid was serine 193 (36). Based on protein sequence of 158 amino acid residue of sh1, the two isolates of D1 and sh1 share 100% identity with 30% coverage.

Phylogenetic analysis of hemagglutinin gene sequence in the present study was partial cds containing 474 bp nucleotides, in other words, containing 158 amino acids that does not include cleavage site and receptor binding site. Because strains D1 and D7 have the most similarity to our strain, they probably evolved from the A / Chicken / Iran / ZMT-101/1998 strain with JX465626 accession number. Our strain also has the most similarity (99.16%) among foreign strains to Saudi Arabia strain A/Chicken/Saudi Arabia/CP7/1998/ (H9N2) .

In Iran, the H9N2 subtype of influenza virus was isolated in 1998 at Razi Vaccine and Serum Research Institute and Veterinary College of Tehran University for the first time (37).

Mehrabanpour and colleagues conducted a survey in an outbreak of respiratory disease in broiler chicken in Fars province during the years 2009 until 2011 from 44 commercial broiler chicken flocks with high mortality. Their results of Molecular Subtyping showed that all of six AIV isolates were H9 and the serotypes of H5 or H7 were not found (11).

In the study by Fusaro et al (2011) on the phylogenetic analysis of H9N2 viruses isolated from nine Middle Eastern and Central Asian countries from 1998 to 2010, these viruses were classified into four groups A, B, C, D, and East Asia was identified as the main

reservoir of H9N2 gene segments. Group A included strains of some Middle Eastern countries and group C mostly included strains of Saudi Arabia, Qatar and UAE. Iranian strains were in groups B and D. Group B was more diverse and the strains of most Asian countries, including the Middle East, were classified in this group (19). Strain sh1 and similar strains including D1, D7 and ZMT are in group B.

According to the results of phylogenetic analysis of H9N2 by Fazel and colleagues, 4 distinct clades were introduced. Their two sequenced strains D1 and D7 were included in one of the clades along with Saudi Arabian strain and some other Iranian strains. Considering the close homology and relatedness of our strain with the strain of D1 our strain can be included in this clade (35).

Conclusion

The findings of this study indicate and confirm the endemicity of H9N2 influenza virus with a high infection prevalence in backyard chickens in rural areas of Fars province. This shows that bird flu control and prevention programs have not been carried out effectively.

Absence of clinical signs and mortality may indicate low virulence of the virus or resistance of the studied birds due to differences in genetic and breeding conditions as well as lack of production stress compared to commercial poultry flocks.

However, the presence of a high percentage of infection indicates the transmission and circulation of this virus among backyard chickens, which in addition to the possibility of transmission to commercial poultry flocks, could pose a risk of creating new more pathogenic strains for birds and even humans.

Therefore, it is necessary to implement more effective control programs for influenza in rural areas, including biosecurity programs, vaccination of exposed birds and training of villagers in relation to this disease.

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Conflict of interest

No conflict of interest is declared.

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