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Original Article

Investigation of seroepidemiology of H9N2 influenza in native poultry of different regions of Khuzestan province

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Summary
Bird flu is one of the most critical and dangerous viral diseases that have been reported all over the world. Although H9N2 viruses are considered a threat by themselves, they are known to be very zoonotic as donors of gene segments to other viruses, so to prevent the emergence of new zoonotic viruses, better control of viruses should be done and H9N2 should be noted. The current cross-sectional study was conducted from March 2017 to June 2018. In total, 480 birds, including 222 chickens and roosters, 84 turkeys, 120 ducks, and 54 pigeons, were randomly sampled from 16 villages. Then, the ELISA screening test was performed first, followed by the HA and HI tests. Out of the 480 samples collected, 461 (96.04%) were positive for ELISA and 451 (93.95%) were positive for serum titers. Since the H9N2 influenza viruses are not acute viruses and the conflict with them does not lead to the bird's death, much effort is not made to eradicate them. This issue leads to the widespread circulation of this virus in poultry flocks. It is especially endemic to poultry and gives this group of viruses a chance to become acute viruses due to the accumulation of point mutations. It is considering that there is no extermination policy regarding H9N2 influenza in Iran. Effective vaccines can reduce the infection of local and rural poultry with this virus.
Keywords: Influenza, H9N2, Native poultry, Khuzestan province

Introduction

Avian influenza is one of the most critical and dangerous viral diseases reported from all over the world (Alexander, 2007). Influenza viruses are members of the Orthomyxoviridae family and contain an RNA genome with harmful division, code for ten core proteins, and a variable number of accessory proteins (Peacock et al., 2019). Based on surface proteins, influenza viruses have three types: A, B, and C (Lee and Song, 2013). To date, all strains isolated from birds have belonged to type A. Influenza type A has 16 subtypes of hemagglutinin HA and nine subtypes of neuramidase NA (Bouvier and Palese, 2008). Today, two new subtypes, H17N10 and H18N11 have been isolated and reported (Brown et al., 2010; Yang et al., 2021). The recent human infection with the avian influenza virus revealed that the H9N2 influenza virus is the gene donor for H7N9 and H10N8 viruses infecting humans (Sun and Liu, 2015). Influenza is an acute infectious viral disease shared between humans and animals, and wild birds (mainly ducks, geese, and swans) and Charadriiformes (mainly shrews, terns, and terns) constitute the natural reservoir of low pathogenic avian influenza (LPAI) viruses (Naguib et al., 2019). The H9N2 viruses of the LPAIV subtype are found worldwide in wild birds and are

endemic in poultry in many regions of Eurasia and Africa. Compared to H5 and H7 viruses, they have been somewhat neglected. However, recent evidence, summarized in this review, suggests that they could potentially play an important role in influenza pandemics (Peacock et al., 2019). The occurrence of avian influenza epidemics caused by the H9N2 subtype during the past years in industrial and native poultry of some provinces has raised the possibility of contamination and circulation of the avian influenza virus from different subtypes in rural and wild birds of the country (Norouzian et al., 2012). Although H9N2 is considered a threat in itself, it has recently been known to be very zoonotic as a donor of gene segments to other viruses, so it is suggested to pay attention to better control of H9N2 to prevent the emergence of new zoonotic viruses (Di and Gao, 2014). Influenza is an infectious viral disease that affects both the upper and lower respiratory tracts. This disease is caused by a wide range of influenza viruses. Some of these viruses can infect humans, and others are species-specific. These viruses can be transmitted through respiratory droplets that come from the mouth and respiratory system during coughing, talking, and sneezing. Avian influenza virus infection in Iran's poultry industry was first detected by subtype H9N2 in Qazvin province in 1998, and since then, it has become endemic in the country. In the past years, in the industrial and native poultry of some provinces, the possibility of contamination and circulation of avian influenza virus under different types in rural and wild birds of the country, including Khuzestan province, has increased (Norouzian et al., 2012). In this research, which was conducted simultaneously throughout the province of Khuzestan, the status of the native poultry of this province was investigated in terms of serotype H9N2 influenza, to determine the seroprevalence of this subtype.

Materials and methods

The current cross-sectional study was conducted from March 2017 to June 2018. Four hundred eighty native birds (including 222 chickens and roosters, 84 turkeys, 120 ducks, and 54 pigeons) were randomly sampled from 16 villages in different regions of Khuzestan province and analyzed. Then, the clinical symptoms and blood samples of the birds were recorded. In this research, the number of villages required for sampling was calculated based on the prevalence rate of 50% the accuracy of 5%, and 95% confidence. Also, the number of birds required for sampling for serodiagnosis was chosen assuming a seroprevalence equal to or greater than 30%. With 95% confidence, at least one cheerful bird could be identified.

For species diversity, sampling was done from all species of birds in a village; the studied species included chickens, roosters, turkeys, ducks, and pigeons. 1 mL of blood was taken from the birds with a 2.5 mL syringe from the wing vein, which was inserted at an angle of 25 degrees. The collected blood was Fallah Mehrabadi M. kept at room temperature for one hour to separate the serum, and after transferring the samples to the laboratory, their serum was separated and placed in a 1.5 mL microtube. After coding and recording the information related to the sampling location and the characteristics of the bird's gender and species, the sample was placed in a freezer at -20 °C to be subjected to ELISA, HA, and HI tests in the following stages. After serum separation, the blood samples were first tested for screening with the AI-Type A ELISA method using the relevant kit (Bio check Company) for chickens and roosters. Due to the existence of indirect ELISA kits for other birds (ducks, ostriches, quails, etc.), ELISA tests were performed on the samples taken from these birds in a centralized manner using a dedicated ELISA kit. In this method, we added 200 microliters of the dilution solution to all wells, then added 200 microliters of serum standards and controls to the wells. At this stage, we added 20 microliters of serum samples to the wells. They were incubated for 20 minutes. After the incubation stage, we removed the samples and washed and dried the

wells 5 times with a washing solution. In this step, we added 100 microliters of the ready-to-use conjugate solution to all the wells and incubated for 20 minutes, after which the samples were washed 5 times as in the previous step and dried. Then, 100 microliters of the ready-to-use substrate were added. We added it to all the wells and incubated for 10 minutes, then we added 50 microliters of the stop solution to the wells, and finally, we read the samples with an ELISA reader device at a wavelength of 450 nm. The cases that were positive for ELISA were subjected to hemagglutination inhibition test using H9N2 antigen (antigen of Pasouk company) to check the level of anti-virus antibody (HI). After initial evaluations, antigens by HA test and preparation of appropriate dilution, before each HI test, to ensure the accuracy of antigens, a reverse titration test was performed in this regard. Considering the necessity of validation of the said test in the laboratory, a positive serum sample with a certain titer and a negative serum sample such as the samples obtained from chickens (SPF) were used at the end of each row to evaluate the validity of the test. To ensure the accepted answers and prevent false results, the results were reviewed as soon as possible. In the HI test, the red blood cells of these birds were exclusively used to evaluate the serum response of turkey, pigeon, and chicken. HI, a trial to assess the serum response of the bird's immune system to some infections, is the ability to agglutinate red blood cells. One of the most common uses of this test is to calculate the antibody titer against influenza viruses based on $\log_2$ of the tested dilutions. A serum titer of 3 and above (1.8 titer) was considered the cut-off point and positive titer. Birds with a serum titer of 3 or higher were considered positive (Easterday and Tumova, 1972). The SPSS software version 16 was used to prepare the data table and analyze the data analysis. Based on the positive and negative results of the tests, the relationship between the ranked qualitative and quantitative variables and influenza infection was analyzed using the Chi-square statistical test, at the level of $P<0.05$ as a statistically significant level. Variance analysis chart, standard deviation, and comparison of averages in different geographical areas are also displayed using Excel 2016 software.

Results

Out of the 480 samples collected, 461 (96.04%) were positive for ELISA and 451 (93.95%) were positive in terms of serum titer. The statistics are as follows.

Poultry group (chickens and roosters)

Out of 222 samples (Table 1), 213 samples were positive (94.95%), and nine samples were negative (06.4%) in the chickens. According to the analysis of variance, between the averages in the regions, there was a significant difference at the 0.05 level in different geographical areas, and the average in the northern region was higher than other regions (figure1-A) $p\leq0.05$.

Pigeon group

According to Table 2, out of the 54 pigeons that were tested, 51 were positive, representing 44.94 percent, and 3 were negative, representing 56.5 percent. (Graph 2). According to the result of the analysis of variance, there was a significant difference between the averages in different geographical areas at the 0.05 level, and the average in the center area was higher than other areas, (figure1-B) $p\leq0.05$.

Turkey group

According to Table 3, out of 84 samples taken from turkey, 75 samples were positive (89.28%), and 9 samples were negative (10.71%). According to the analysis of variance in graph 3, there was a significant difference between the averages in different geographical areas at the 0.05 level. According to the figure, the average in the northern region is higher than in other regions, (figure1-B) $p\leq0.05$.

Duck group

As shown in Table 4, out of 120 samples taken from ducks, 112 samples were positive (93.33%), and 8 samples were negative (6.66%). According to the result of the analysis of variance, there was a

significant difference between the averages in different geographical regions at the 0.05 level, and the average in the northern region was higher than in other regions, (figure1-D) $p \leq 0.05$.

Table 1- Average, number, standard deviation, the minimum, maximum, and variance of information in each geographical area

Geographical region	Average headline	number of samples	standard deviation	minimum	maximum	Variance	positive number	negative number
Center	5.01	44	2.166	2	10	4.691	39	5
East	5.67	52	2.154	3	10	4.639	52	0
North	8.54	47	1.275	7	10	1.625	47	0
West	6.30	40	2.046	4	8	4.186	40	0
South	4.16	39	1.095	2	6	1.199	35	4
Total	6.42	222	2.197	2	10	4.826	213	9

Table 2- Average, number, standard deviation, the minimum, maximum, and variance of information in each geographical area

Geographical region	Average headline	number of samples	standard deviation	minimum	maximum	Variance	positive number	negative number
Center	6.27	12	1.095	5	8	0.199	11	0
East	2.94	11	0.837	1	4	0.702	9	2
North	4.02	10	0.637	3	5	0.407	10	0
West	3.77	10	0.551	4	8	0.304	9	0
South	5.86	11	1.080	2	6	1.166	11	1
Total	5.14	54	1.715	2	10	2.942	51	3

Table 3- Average, number, standard deviation, the minimum, maximum, and variance of information in each geographical area

Geographical region	Average headline	number of samples	standard deviation	minimum	maximum	Variance	positive number	negative number
Center	6.76	15	1.092	5	8	1.192	15	0
East	4.09	18	1.746	2	7	3.048	18	2
North	9.44	19	0.589	9	10	0.348	19	0
West	4.63	17	2.614	2	7	6.832	12	3
South	3.86	15	0.975	2	5	0.950	11	4
Total	5.72	84	2.564	2	10	6.574	75	9

Table 4- Average, number, standard deviation, the minimum, maximum, and variance of information in each geographical area

Geographical region	Average headline	number of samples	standard deviation	minimum	maximum	Variance	positive number	negative number
Center	4.71	21	1.612	2	6	2.598	18	3
East	5.20	28	2.130	2	7	4.536	27	1
North	6.42	24	2.321	3	10	5.387	24	0
West	5.84	25	2.240	3	10	5.017	25	0
South	4.23	22	1.083	2	6	1.172	18	4
Total	5.42	120	1.723	2	10	2.698	112	8

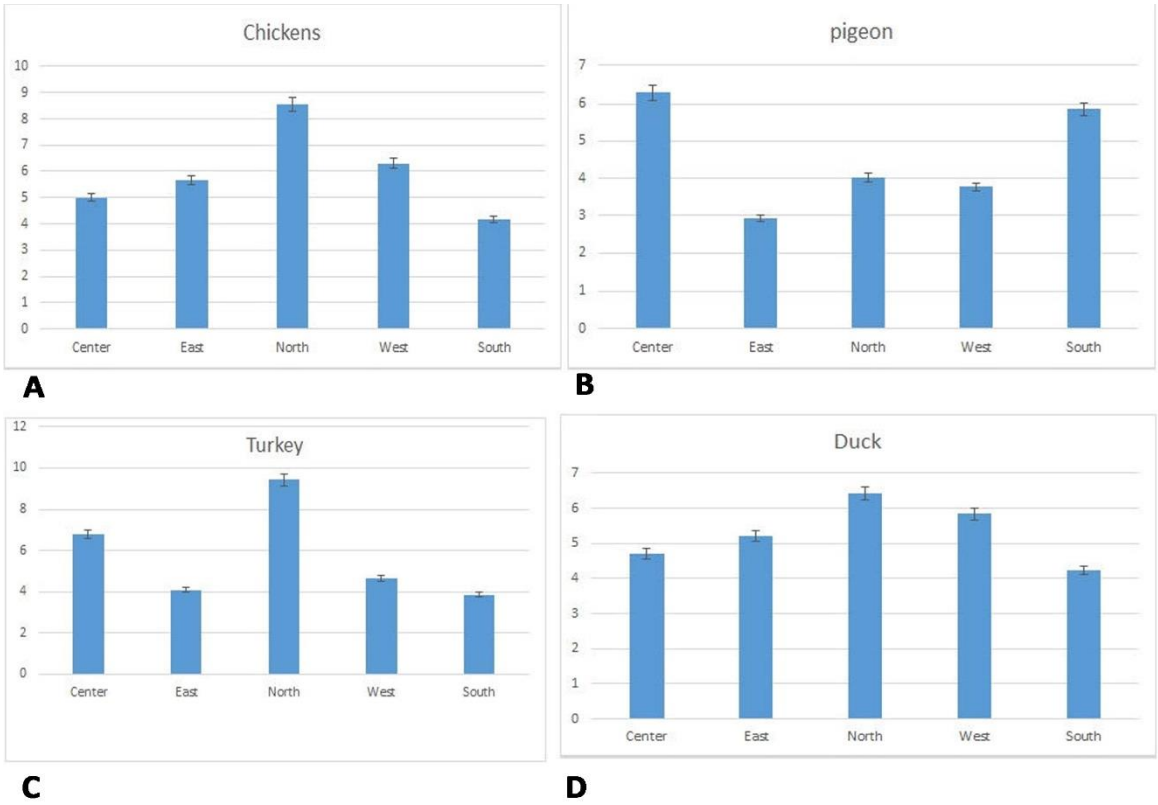

Fig. 1. Variance analysis test, standard deviation and comparison of averages in different geographical areas in various hosts. A: chicken, B: pigeon, C: Turkey, D: Duck.

Discussion

H9N2 influenza has been reported in different countries, including Iran (Norouzian et al., 2012). The H9N2 virus does not have high pathogenicity for poultry. Still, various outbreaks and epidemics of this virus in the past years in different parts of the world, including Iran, have caused severe damage to the poultry industry. Serum tests such as ELISA, HI, NI, and AGID are used to determine and identify and care for this disease (Brown et al., 2010). The HI test is more specific and is used in diagnostic laboratories to assess infection. The results of the current study, which was conducted to detect the influenza virus in native birds of Khuzestan province, indicate a high percentage of this virus infection in native birds of the city. Out of 480 samples, 461 samples (96.04%) were positive for ELISA, and 451 samples (93.95%) were positive in terms of serum titer.

In the studies conducted, the high prevalence of H9N2 serum levels without clinical signs of the disease can be due to the constant exposure of native poultry to this virus and the acquisition of immunity in these birds as well as their natural vaccination, which can be caused by the circulation of this virus in the environment and between Native poultry and keep it as a reservoir and transfer it to industrial breeding centers (Rehman et al., 2022). The results of this study are similar to the results of a study conducted by(Hadipour et al.,2011) on native poultry, in which 2.78% of ducks and 9.62% of domestic fowl were positive in terms of serum titers. The findings showed that the circulation of the virus in the environment caused frequent exposure in the birds and their serum levels became positive (Hadipour,2011). Van Kammen (1982) reported the presence of serum levels in native rural birds. Al-Natour et al. (2005) have also reported a 71% prevalence of influenza

in broiler chickens in Jordan. In a study about the poultry slaughterhouse in Kashan reported by Nikkhah (2009), out of 20 samples collected from non-vaccinated flocks, 17 flocks were positive for H9N2 influenza. In another study conducted by Fathi et al. (2010) in Shahrekord on meat poultry, 33.53% of the flocks were reported to be H9N2 positive. In another study, the prevalence of H9N2 influenza in industrial chickens in Pakistan has been reported in the range of 10-20% (Naeem K et al., 2003). Another study aimed to determine the prevalence of H9N2 influenza in broiler farms at the time of slaughter in Iran, the samples taken from 74 farms were assessed using e hemagglutination inhibition (HI) test. They found that 57 farms (77%) were seropositive. In general, the prevalence of H9N2 was high, which indicates the continuous circulation of the virus in Iran (Fallah Mehrabadi et al., 2020). In agreement with the current study, other previous studies conducted in Iran (Soltani et al., 2010; Fallah Mehrabadi et al., 2015; Azizpour et al., 2016; Fallah Mehrabadi et al., 2017) have also reported high level of infection with the involvement of the influenza virus. The high prevalence of this disease can be the presence of genetic reservoirs of influenza in waterfowl and the wide spread of this virus. Serological methods, including HI, are used for detecting influenza virus in infected flocks (Swayne and Suarez, 2000). One of the most essential features of this method is its simplicity, which can be performed in most laboratories. We have also used the same testin the present study. In previous studies, this strain has been considered to be the most important cause of influenza in the country's poultry population (Fallah Mehrabadi et al., 2015), causing a lot of damage. The results of the present study also investigated the serology of the H9N2 influenza virus in native poultry and showed a high percentage of infection, reflecting that native poultry is one of the main reservoirs of contamination for the initial emergence of influenza virus in commercial poultry flocks (Conan et al., 2012). Given to the possible ability of H9N2 to transmit to human populations, this virus has great importance in terms of studies and monitoring (Webster and Govorkova, 2014).

Conclusion

Since the H9N2 influenza viruses are not acute viruses and the conflict with them does not lead to the bird's death, much effort is not made to eradicate them. This issue leads to the widespread circulation of this virus in poultry flocks, especially chickens, becoming endemic and giving this group of viruses a chance to become acute viruses due to the accumulation of point mutations. Therefore, the contamination of native poultry, especially chickens, is significant. Native birds can generally become the foci of reproduction and the emergence of new genotypes. In recent years, in addition to H9N2 viruses, other types, such as H5N1, H5N8, and H5N6, have been reported in Iran. Due to the simultaneous presence of these viruses in the country, the occurrence of the genetic market and, as a result, new serotypes in the country, especially in Khuzestan province, which is the place of passage of many different species of migratory water birds, is not far from expected. Therefore, continuous monitoring of rural poultry, bird markets, and waterfowl is necessary to check the influenza situation Also, the positivity of many areas in the province can be a warning for the poultry industry of the province, which will have destructive effects on this industry in the future if it is not controlled and prevented. Considering that there is no extermination policy regarding H9N2 influenza in Iran, effective vaccines can reduce the infection of local and rural poultry with this virus.

Acknowledgment

Not applicable.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethical approval

Not applicable.

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