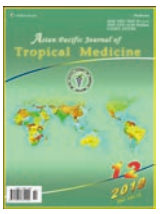

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Prevalence and risk factors of avian influenza H9N2 among backyard birds in Iran in 2015

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ABSTRACT

Objective: To investigate the prevalence and the risk factors of H9N2 avian influenza among backyard birds in Iran between October and November 2015.

Methods: In this study, a total of 15 500 blood samples and 2 884 cloacal swab samples of backyard birds were collected in villages of Iran between October and November 2015. Then, serum samples were examined with the hemagluttination inhibition test and cloacal swab samples were pooled together and examined by RT-PCR. The samples that had serological titer ≥ 4 ($\log^2$) and villages that had at least one seropositive sample were considered positive.

Results: Out of 559 villages, 526 (94.10%) were seropositive for the infection. Among 15 500 serum samples, 7 468 (48.18%) samples were seropositive for the infection. The seroprevalence according to species was 54.02% among chickens, 17.59 % among ducks, 18.73% among turkeys, 84.21% among pigeons and 12.15% among ostriches, partridges and pheasants. Based on molecular test, 3.04% villages were positive. The seroprevalence in hot and humid area was less than that in cold and humid area (P<0.05).

Conclusions: H9N2 avian influenza has high seroprevalence among backyard birds of Iran. Therefore, preventive measures such as biosecurity Practices and monitoring should be applied to reduce the prevalence.

1. Introduction

Backyard poultry breeding for their eggs and meat are considered one of the main sources of inexpensive protein, an important source of improving rural family income, a reserve of pure native breeds, and an essential component of village ecology in many developing countries[1,2]. Evidently, it is not practical to implement many improved biosecurity measures in backyard poultry flocks;

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resultantly, backyard poultry have become a constant source of avian influenza virus transmission and pose a threat to the commercial poultry industry, and indeed to public health[1].

Iran is one of the developing countries in which, based on data from the Iran Veterinary Organization database, there are an estimated 50 million backyard birds. In almost all villages in Iran backyard birds (especially chickens) are kept for egg and meat production. Due to the annual migration of wild migratory birds towards Iran, subtypes of avian influenza virus are transmitted to the country every year. Since backyard birds are in direct contact with migratory birds, they can be infected and harbor the virus and cause mortality in susceptible population. Moreover, backyard birds can act as an intermediate host and cause genetic mutation in the virus and therefore causes new epidemic among commercial birds[3].

Avian influenza virus subtypes H9N2, which during the second half of the 1990s has noticeably increased worldwide, for the first time was reported in 1998 in Iran and since then is endemic among backyard and commercial birds in the country. Although the subtypes is low pathogenic, its presence is a major problem for the poultry industry in Iran and has caused economic losses in poultry industry in Iran[3]. In addition to H9N2 subtype, H5N1 and H5N8 are highly pathogenic subtypes of the virus that were reported and caused several outbreaks among bird population in the country[4].

Therefore, identifying risk factors of avian influenza H9N2 (including management, environmental and host factors) among backyard birds in Iran is very important and leads to better control of the disease in them. Consequently, that leads to the better prevention of disease spread from these birds to commercial birds[1,5]. Thus, this study conducted with the aims to determine the prevalence and risk factors of AI H9N2 among backyard birds in rural areas of Iran.

2. Materials and methods

2.1. Ethic statement

This study was approved by the ethics committee of Razi Vaccine and Serum Research Institute, Karaj, Iran (No. 2-18-18-056-96023-960683).

2.2. Study design and sampling

This cross sectional study was conducted among backyard birds in villages of 30 provinces of Iran during the October to November, 2015 (Table 1). All premises where birds are kept are registered in the veterinary disease database in Iran. There are approximately 65 000 registered villages in the database and in the study we selected 559 of them using stratified random sampling. Indeed, in each province we prepared a list of villages and randomly selected a number of villages given the number of registered villages in the provinces. Then in overall 15 500 blood samples and 2 884 cloacal swab samples were collected. The sampling was carried out with the aim of finding at least one infected bird, assuming a 5% prevalence using a molecular test, with 95% confidence level (*CI*)[6]. One mililiter of blood was collected from the wing vein of the selected birds and placed into a 1.5 mL micro tube. The blood and cloacal swab samples were transferred to the Razi Institute Poultry Laboratory with cold chain. Moreover, a questionnaire (The questionnaire is available from the senior author upon request) was prepared and the data related to the birds and villages were collected. Questions were constructed using previously known potential risk factors associated with avian influenza infection[7, 8]. Descriptive data was collected on the location and size of villages, type and number of species present, and the climate type (based on the climate classifications in Iran).

2.3. Laboratory tests

Serological testing was carried out for detection of antibodies against H9N2, based on the guidelines of the World Organization for Animal Health and the Iranian Veterinary Organization[9]. Sera with titers ≥4 (*i.e.* $\log^2$) were considered as positive and villages with at least one positive bird were considered as a positive unit. For the molecular test, 5 cloacal swab samples were pooled together (a maximum 12 pooled samples for each village) and the RNA extracted using High Pure Viral RNA Kit (Roche, Germany). Real time RT-PCR was conducted using viral subtype H9-specific primer and probe sets for conserved regions in the HA2 subunit of the *H9 HA* gene sequences. The following primers were used for sequencing and confirmation of *H9 HA* gene: *H9*- For, 5´- ATG GGG TTT GCT GCC-3´ and *H9*-Rev, 5´- TTA TAT ACA AAT GTT GCA C(T)CT G-3´ and *H9* probe, 5´- TTC TGG GCC ATG TCC AAT GG-3´. Also, The succeeding protocol was used for all primer/probe sets: 20 min at 50 ℃ and 15 min at 95 ℃, followed by 40 cycles at 94 ℃ for 45 s and 54 ℃ for 45 s[10].

2.4. Data analysis

Eventually, the data was prepared in an Excel spreadsheet and entered into SPSS statistical software (version 22) for analysis. Absolute and relative frequencies with 95% *CI* were used to describe dependent variable (serological status and molecular status). *Chi*-square tests were applied to determine the association between each of the independent variables and serological status of each villages (as the unit of the study) and odds ratios with 95% *CI* were calculated[11,12]. A *P*-value of 0.05 or less was considered statistically significant.

3. Results

In this study, a total of 15 500 blood samples of backyard birds and fowl and a total of 2 884 cloacal swab samples were collected in Iran during October to November 2015.

Table 1. Seroprevalence of avian influenza H9N2 among backyard birds in village of Iran according to Province (October-November 2015).

Province	Number of blood samples	Number of positive blood samples [*n*(%)]	Number of sampled village	Number of positive village [*n*(%)]
Alborz	502	379 (75.50)	16	16 (100.00)
Ardabil	312	182 (58.33)	13	13 (100.00)
East Azerbaijan	1513	721 (47.65)	57	53 (92.98)
West Azerbaijan	1193	410 (34.37)	49	47 (95.92)
Bushehr	176	169 (96.02)	8	8 (100.00)
Charmahal	264	244 (92.42)	10	10 (100.00)
Fars	484	364 (75.21)	20	20 (100.00)
Gilan	806	174 (21.59)	19	17 (89.47)
Golestan	642	200 (31.15)	19	17 (89.47)
Hamadan	225	133 (59.11)	9	9 (100.00)
Hormozgan	240	0 (0.00)	10	0 (0.00)
Ilam	302	249 (82.45)	12	12 (100.00)
Isfahan	373	259 (69.44)	16	14 (87.50)
Kerman	480	285 (59.38)	20	20 (100.00)
Kermanshah	358	205 (57.26)	15	15 (100.00)
Razavi Khorasan	396	184 (46.46)	16	16 (100.00)
South Khorasan	244	83 (34.02)	9	7 (77.78)
North Khorasan	264	105 (39.77)	11	11 (100.00)
Khuzestan	541	336 (62.11)	15	15 (100.00)
Kohgiluyeh-Boyerahmad	240	174 (72.50)	10	10 (100.00)
Kurdistan	236	171 (72.46)	10	10 (100.00)
Lorestan	164	103 (62.80)	7	7 (100.00)
Markazi	250	176 (70.40)	10	10 (100.00)
Mazandaran	1 807	691 (38.24)	58	54 (93.10)
Qazvin	2 003	670 (33.45)	58	55 (94.83)
Semnan	247	78 (31.58)	10	9 (90.00)
Sistan	366	159 (43.44)	15	14 (93.33)
Tehran	343	231 (67.35)	15	15 (100.00)
Yazd	288	195 (67.71)	12	12 (100.00)
Zanjan	241	138 (57.26)	10	10 (100.00)
Total	15 500	7 468 (48.18%)	559	526 (94.10)

Table 2. Seroprevalence of avian influenza H9N2 among backyard birds in village of Iran according to species of birds (October–November 2015).

Bird species	Number of blood samples	Number of positive blood samples [*n*(%)]
Chicken	12 976	7 010 (54.02)
Duck and goose	1 768	311 (17.59%)
Turkey	630	118 (18.73)
Pigeon	19	16 (84.21)
Ostrich, partridge and pheasant	107	13 (12.15)
Total	15 500	7 468 (48.18)

Serological tests were conducted on 15 500 blood samples and revealed that 7 468 out of 15 500 (48.18%, *CI* 95%; 47.39% - 48.97%) serum samples were positive for H9N2. The seroprevalence according to species of birds was respectively the highest in pigeons (84.21%, *CI* 95%; 62.43%-94.48%) and chickens (54.02%, *CI* 95%; 53.16%-54.88%) species (Table 2). The seroprevalence according to province was the highest in Bushehr province which is located in southern of Iran (96.02%, *CI* 95%; 92.02%-98.06%). Also, the seroprevalence at village level was 94.10 % (*CI* 95%; 91.83%-95.77%) (526 out of 559 villages were seropositive) (Table 2).

Molecular tests conducted on the 550 pooled samples identified 43 pooled samples on 17 villages (3.04%, *CI* 95%; 1.78%-4.82 %) positive for H9. Univariable analysis found a statistically significant association between the climate type with H9N2 infection. Hot and humid climate (*OR*=0.11, 95% *CI* 0.03–0.37) was protective factors for H9N2. No statistically significant associations were discovered between H9N2 infection and the other investigated variables (Table 3, Table 4).

4. Discussion

Backyard birds play an important role in the economy of rural households in Iran, especially in Northern provinces of the country. Indeed, breeding of the birds not only supply required food of the households but also is a source of income. Breeding of the backyard birds is a traditional way of life in Iran, and consequently biosecurity levels of the breeding premises are low. Moreover, high density of the birds and diversity of species increases possibility of transmission and spreading of diseases between these birds. Among the diseases, avian influenza H9N2 that is the most prevalent low pathogenic avian Influenza is a major challenge for poultry industry in Iran and every year causes severe economic losses in the country[3,13]. For example, based on the data available in Iranian Veterinary Organization mortality of the broiler poultry due to H9N2

was approximately 5 800 000 and 4 140 000 in 2013 and 2014 in Iran. Backyard birds are an important host for H9N2 virus. The dynamics of transmissions and movements of backyard birds in Iran causes these birds to be mixed with other birds in different premises, especially in the live bird markets. Meanwhile, backyard birds play an important role in spreading the virus to different area and other species, especially commercial birds in the country.

According to the findings of this study, approximately 48 % of backyard birds were seropositive for H9N2, which was different among provinces. Likewise, seroprevalence of the H9N2 at village level was 94.10%, which indicates that the seroprevalence is high among backyard birds in Iran. Other related studies in Iran also concluded that seroprevalence was high both in bird level and village level. For example, two separated studies in 2010 in Northern provinces (Gillan, Mazandaran, and Golestan) and Fars province showed that 100 percent of the studied villages in these provinces

Table 3. Univariate analysis of qualitative independent variables for H9N2 seropositivity in villages in Iran (October-November 2015).

Variable	Category	AI serology result		*OR*	95% *CI*	*P*
		Positive	Negative			
Climate type	Cold and humid	241	7			
	Caspian (moderate and humid)	45	4	0.32	0.91-1.16	0.08
	Mild	89	7	0.36	0.12-1.08	0.06
	Hot and humid	24	6	0.11	0.03-0.37	0.000
	Hot and dry	127	9	0.40	0.14-1.12	0.08
Status of bird keeping	Free range	419	27			
	Enclosed	107	6	1.14	0.46-2.85	0.76
Existence of road around	Yes	232	17	0.74	0.36-1.50	0.40
	No	294	16			
The highest number of offered birds	Domestic duck and geese	88	9	0.53	0.24-1.19	0.12
	Other birds	438	24			
Variety of offered birds	Domestic duck and geese	125	11	0.62	0.29-1.32	0.21
	Other birds	401	22			
Action for dead birds	Release the carcasses	353	23			
	Not released the carcasses	173	10	1.12	0.52-2.42	0.74
Distance of nearest live bird market to epidemiological unit	<1 km	51	2	1.79	0.41-7.80	0.43
	1 to 3 km	20	0	-	-	-
	>3 km	355	25			
Distance of nearest pond to epidemiological unit	<1 km	77	1	1.71	0.77-43.50	0.08
	1 to 3 km	14	0	-	-	-
	>3 km	331	25			
Distance of nearest main road to epidemiological unit	<1 km	137	7	1.14	0.45-2.86	0.77
	1 to 3 km	84	5	0.98	0.34-2.77	0.97
	>3 km	257	15			
Distance of nearest lake to epidemiological unit	<1 km	93	5	1.17	0.43-3.22	0.75
	1 to 3 km	7	0	-	-	-
	>3 km	316	20			
Distance of nearest river to epidemiological unit	<1 km	130	7	1.25	0.51-3.07	0.62
	1 to 3 km	57	2	1.92	0.43-8.51	0.39
	>3 km	267	18			
Distance of nearest dam to epidemiological unit	<1 km	68	1	4.40	0.58-33.10	0.15
	1 to 3 km	14	3	0.30	0.08-1.12	0.07
	>3 km	355	23			
Distance of nearest place of residence or temporary season for migratory birds to the epidemiological unit	<1 km	82	1	5.76	0.76-43.30	0.08
	1 to 3 km	25	2	0.87	0.19- 3.94	0.86
	>3 km	327	23			
Distance of nearest commercial bird farm to epidemiological unit	<1 km	71	5	0.86	0.30-2.39	0.77
	1 to 3 km	92	4	1.39	0.46-4.22	0.55
	> 3 km	297	18			
Distance of nearest bird slaughterhouse to the epidemiological unit	<1 km	31	0	-	-	-
	1 to 3 km	7	2	0.22	0.04-1.12	0.06
	>3 km	395	25			
Source of water supply	Industrial	152	13	0.62	0.30-1.28	0.20
	Natural source	374	20			
Source of bird feed supply	Manual Feeding	280	15			
	Grazing	246	18	0.73	0.36-1.48	0.38
bird source status	From other provinces	519	31			
	From the same province	7	2	0.20	0.04-1.04	0.05
Status of vaccination against influenza	Yes	39	3	0.80	0.23-2.74	0.72
	No	487	30			

AI: Avian influenza

Table 4. Univariate analysis of quantitative independent variables for H9N2 seropositivity in villages in Iran (October-November 2015).

Variable	Positive		Negative		*OR*	95% *CI*	*P*
	Median	Interquartile range	Median	Interquartile range			
Number of family that keeping birds in village	25	50	23	50	0.99	0.997-1.000	0.08
Number of family living in village	40	75	50	114	0.99	0.998-1.001	0.94

were seropositive to H9[14,15]. Another study in 2013 in Iran indicated that seroprevalence of H9 among backyard birds of the country was 88%; in another study conducted in Iran in 2014, the seroprevalence was 90%[3,16]. Although most of the studies in other countries concerning H9 have been carried out in commercial birds, some of them have reported a high seroprevalence of H9 among backyard birds; for instance, in a research that was carried out in Oman in 2012, the seroprevalence was 84% and high exposure to wild birds, continued introduction of new birds to villages, co-mixing of the birds among neighbor and feeding of uncooked poultry waste are mentioned as the causes of the high seroprevalence[17]. However, in some other countries, a lower seroprevalence has been recorded; for example, in a study in Egypt, the seroprevalence was 24%, which was higher in chicken than ducks[18]. Considering that in Iran there isn't any vaccination program for backyard birds against influenza, the high seroprevalence represents ongoing exposure of backyard birds to the virus.

Seroprevalence of H9N2 was different among provinces of Iran. It can be due to several reasons. First, density of backyard birds, species of the birds, the breeding system and the relations between birds that are determinants for spreading of the virus are different among provinces in Iran. Second, climatic conditions that play an important role in survival and circulation of the virus are different among provinces of Iran. Third, immune levels of backyard birds among provinces of Iran is different due to vaccination of birds in some provinces, also genetic resistance exist among some of the species of birds[3,13,16].

Result of this study revealed that prevalence of H9 based on molecular test at the village level was 3.04%. This result revealed that the virus exists and spreads among backyard birds in Iran. Some of the other related studies in other countries also revealed the molecular prevalence of the disease. For example, in one study among backyard birds in Mali, 3.6% of the tracheal and cloaca samples were positive[19]. In another study among backyard birds in China, 2% of the tracheal, cloaca, feces and feather samples were positive[20]. Molecular testing has been conducted to detect H9N2 influenza viruses' presence among backyard birds. Presence of these viruses indicates that active infection exists among the birds. Given that backyard birds are considered one of the most important reservoirs of these viruses and considering movements of the birds in Iran, the virus can transmit directly and indirectly by human and vehicle to other backyard birds and commercial birds.

Another finding of this study was that influenza H9N2 seroprevalence in cold and humid areas was higher than that in hot and humid areas. Some other studies confirm this finding[21,22]. This could be due to this fact that Influenza viruses are more durable in cold weather and hence the transmission of influenza viruses in cold weather occurs more effectively. The mechanism of weather impact has not yet been clearly understood. High temperatures can kill or disable the virus in two ways. In the other word, high temperature may alter the envelope or damage the proteins and nucleic acid. Enveloped viruses can be rendered harmless when their envelope is destroyed, because the virus no longer has the recognition sites necessary to identify and attach to host cells[23]. High temperatures also denature the proteins, and by damaging RNA can result in fatal mutations or can halt the process of protein synthesis[24]. In addition, colder weather can cause cold stress in the poultry and weaken the immune system of the poultry and make them susceptible to disease. Therefore, it is required to pay more attention to the control of the diseases in cold weather areas. This study did not disclose the relationship between the other independent variables studied and the occurrence of influenza H9. However, in other studies, the poor health status of the villages, high bird diversity in villages, high numbers of birds in a village, the lack of sanitation of dead birds have been considered as a risk factor for H9 influenza occurrence among backyard birds[16, 20].

Results of the current study indicate that the H9N2 influenza virus has a high seroprevalence among Iranian backyard birds, as it has been in recent years. Indeed, the seroprevalence has not decreased compared to previous years. This indicates that a specific control program over backyard birds of Iran has not been implemented or has not been effective. Due to the role of these birds in the survival and transmission of the H9N2 viruses, backyard birds should also be considered in influenza control programs. Promoting and improving the health status of backyard bird breeding systems, improving the status of biosecurity in backyard birds, and educating farmers about health issues and the importance of disease are among the most important measures in this regard. Epidemiological studies on the dynamics and transmission of poultry, cohort studies on risk factors, and the ongoing monitoring of the status of existing viruses are also other necessary measures to control the disease among backyard birds.

Conflict of interest statement

The authors declare no conflict of interest

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Authors' contributions

MHFM, AGL and MHR designed the study. Both MHR and FT collected the data. MHFR and MHR performed the analytic calculations and interpreted the results. MHFR, AGL and MHR drafted the article and conducted critical revision of the article. MHFR, AGL, MHR and FT contributed to the final version of the manuscript.

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