SHORT COMMUNICATION

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Serological prevalence of avian H9N2 influenza virus in dogs by hemagglutination inhibition assay in Kerman, southeast of Iran

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Abstract

Influenza is a highly contagious zoonotic disease in the world. Avian H9N2 influenza virus is a significant pandemic pathogen widely distributed throughout the world. Pet ownership has been documented as a risk factor for infection transmission to human. Considering major public health concern, the prevalence of antibodies against avian H9N2 influenza virus was evaluated in 170 serum samples of dogs by hemagglutination inhibition assay. This study is the first survey to assess the epidemiology of avian H9N2 influenza virus infection in dogs in Kerman, southeast of Iran. Out of 170 samples, 65 (38.23%) were positive for H9N2. Antibodies were higher in farm dogs that were kept with other animals and also in dogs were fed a raw diet. These findings emphasize the importance of close attention to these populations for control and prevention programs. It is important to reduce infection burden, especially in regions with widespread distribution of H9N2.

Introduction

Influenza, a highly contagious infectious disease, is caused by influenza viruses belonging to *Orthomyxoviridae* family.[1,2] Influenza viruses are divided into three types including A, B and C, of which type A is the most virulent one.[3] Various species including humans, horses, birds, pigs, dogs and cats can be infected by influenza A virus.[4,5] Dogs often demonstrate respiratory disease with high morbidity and low mortality; nevertheless, no clinical signs to death can also be observed.[6] Subtypes of influenza A virus are identified by the antigenic properties of hemagglutinin (H1-H18) and neuraminidase (N1-N11) surface glycoproteins.[6] Avian H9N2 influenza virus is widely distributed throughout the world, especially in Asia[7] and causes high mortality in the poultry industry, decline in farm yield and significant economic losses.[1] The dogs susceptibility to avian H9N2 virus was recently reported.[7,8]

Serology is one of the outstanding figures of diagnostic methods for influenza infections.[9] Dogs may have an important role in interspecies transmission and creation of reassortant influenza viruses,[10] therefore, pets should be considered as significant sources of this zoonotic pathogen with pandemic potential for humans.[11]

Role and pathogenic importance of avian H9N2 influenza virus in dogs as well as its transmission and distribution remain inconclusive. The avian H9N2 influenza virus is widely distributed in Kerman, southeast of Iran, especially in the poultry industry. Despite the great importance, no epidemiological study has yet been done regarding influenza disease (caused by any subtypes) in dogs in the southeast region of Iran. Thus, the present study was designed to assess avian H9N2 influenza virus prevalence among dogs in this region.

Materials and Methods

Sample collection. Serum samples were collected from 170 apparently healthy dogs referred to Veterinary Teaching Hospital of Shahid Bahonar University of Kerman, Kerman, Iran for vaccination or check-up from September 2012 to February 2013. This study was

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approved by the Animal Care Committee of Veterinary Faculty of Shahid Bahonar University of Kerman (No: 940120). History taking was done by completing a questionnaire to record different variables including age, gender, diet (cooked or raw), housing type (indoor or outdoor) and contact with other animals. Of 170 dogs, 58 (34.11%) were under 10 months and 112 (65.88%) were more than 10 months. Then, general clinical examination was performed and 3.00 mL blood sample was collected via cephalic vein of each dog. Serum was separated by blood centrifugation at 327 *g* for 15 min. Sera were stored at –20 ˚C until assessing the presence of antibodies against avian H9N2 influenza virus.

Hemagglutination inhibition (HI) assay. Serum samples were first treated to remove non-specific inhibitors. For this purpose, 150 µL of serum was mixed with 50.00 µL of 1.00% chicken red blood cells (RBCs) and incubated at room temperature for 30 min, followed by heating of the specimens at 56.00 ˚C for 30 min. Then, the supernatant serum was separated through centrifugation at 800 *g* for 2 to 5 min. The HI assay was carried out according to the World Health Organization (WHO) guidelines. Briefly, 25.00 µL of serial two-fold dilutions of treated samples were mixed with four hemagglutinin units of virus (Batch No: 01/14; Pasouflu; H9N2 subtype of avian influenza Ag; Pasouk, Mahdasht, Iran) in the microtiter plates, incubated at room temperature for 30 min, added with 25.00 µL of 1.00% chicken RBCs and finally incubated at room temperature for 30 min. Positive and negative control samples were also included. The H9N2 Ag (Pasouk) and phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, USA) were considered as the positive and negative controls respectively. The highest serum dilution that could completely inhibit hemagglutination reaction was determined as a HI antibody titer. The HI antibody titers above 16 were considered positive in the present study.[12]

Statistical analysis. Statistical analysis was carried out using the chi-square test in SPSS (version 21.00; SPSS Inc., Chicago, USA). Additionally, logistic regression test was used to assess the association between seropositivity and predisposing factors including age, gender, diet, housing type and contact with other animals. A p value of < 0.05 was considered significant for all analyses.

Results

In this study, sixty-five out of 170 (38.23%) samples were positive for H9N2 antibody, while 105 samples (61.76%) were negative. Frequency and percentage of HI antibody titers against the avian H9N2 influenza virus are presented in Table 1.

Out of 65 positive samples, 26 (40.00%) were obtained from dogs younger than 10 months and 39 (60.00%) were related to dogs older than 10 months. No statistically significant difference was seen among groups (p = 0.20). Forty-six out of 124 (37.09%) male dogs were positive for avian H9N2 influenza virus, while 19 out of 46 (41.30%) female dogs were positive. There was also no statistically significant difference with respect to the gender (p = 0.70).

Table 1. Frequency and percentage of detected hemagglutination inhibition antibody titers against the avian H9N2 influenza virus.

HI antibody titer	Frequency	Percentage
Negative (< 16)	105	61.76
Positive (16)	21	12.35
Positive (32)	27	15.88
Positive (≥ 64)	17	10.00

HI: Hemagglutination inhibition.

Additionally, five out of 96 (5.20%) dogs consumed the cooked diet were positive in comparison with 60 out of 74 (81.08%) dogs fed a raw diet. There was a statistically significant correlation between these groups (p = 0.00), and the dogs fed raw diet had a higher prevalence of avian H9N2 influenza virus. Five out of 48 (10.41%) and 60 out of 122 (49.18%) dogs kept indoor and outdoor were seropositive to avian H9N2 influenza virus, respectively. Regarding housing status, the difference between groups was significant (p = 0.00). Higher prevalence of avian H9N2 influenza virus was detected in farm dogs compared to pet dogs. Moreover, antibodies against the avian H9N2 influenza viruses were detected in 48 out of 119 (40.33%) dogs kept with other animals, while 17 out of 61 (27.86%) dogs separated from other animals were positive for H9N2 virus. According to HI assay, a statistically significant difference was seen among groups (p = 0.02). The associations between seropositivity against avian H9N2 influenza virus in dogs and various factors are shown in Table 2.

Discussion

Influenza is a ubiquitous and common zoonosis in the world.[1] Avian H9N2 influenza virus as a significant pandemic pathogen is widely distributed in many Asian countries and causes significant economic losses every year.[7,13] Pet ownership and contact with infected animals such as dogs and cats have been documented as risk factors for influenza virus transmission to humans.[8] We found that 65 out of 170 dog samples (38.23%) were positive for avian H9N2 influenza virus.

Characteristics of influenza A virus and the host affect the susceptibility to influenza infection.[2] In the past, it was thought that dogs are resistant to influenza virus infections.[10] In Florida, Crawford *et al*. have firstly reported equine H3N8 influenza viruses transmission to greyhound dogs.[14] After that, natural and experimental infections by various subtypes of influenza A virus including H1N1,[2,5] H3N2[4,11,15] and H5N1[16] from dogs were reported in multiple countries throughout the world. In a study conducted by Amirsalehy *et al*., dogs were

Table 2. Association between seropositivity against avian H9N2 influenza virus in dogs and various factors.

Variables	Total		Positive titer		Chi-square	*p*-value
	Frequency	Percentage	Frequency	Percentage		
Age						
< 10 months	58	34.11	26	44.82	1.62	0.20
> 10 months	112	65.88	39	34.82		
Gender						
Male	124	72.94	46	37.09	0.13	0.70
Female	46	27.05	19	41.30		
Diet						
Cooked	96	56.47	5	5.20	101	0.00
Raw	74	43.52	60	81.08		
Housing						
Indoor	48	28.23	5	10.41	21.90	0.00
Outdoor	122	71.78	60	49.18		
Contact with other animals						
Yes	119	70.00	48	40.33	5.92	0.02
No	61	35.88	17	27.86		

experimentally infected with avian H9N2 virus for the first time.[7] Consequently, H9N2 influenza virus that is closely related to avian H9N2 virus was firstly isolated in dogs by Sun *et al*. [13] Similar to our findings, the susceptibility of dogs to avian H9N2 virus has been also demonstrated by other researchers.[8,16]

In the present study, 65 out of 170 (38.23%) dogs were positive (titers ≥ 16) for avian H9N2 influenza virus by HI assay. In comparison with our results, the seroprevalences of avian H9N2 virus in dogs were 20.87% (95/455) in 2010, 28.98% (273/942) in 2011 and 44.85% (410/914) in 2012 by using HI assay (positive titers ≥ 40) suggesting an increased virus distribution among dogs in southern China.[13] In another study conducted in Shiraz, Iran, sero-positivity for antibodies against avian influenza A viruses was found in 82 out of 182 samples (45.05%) using enzyme-linked immunosorbent assay (ELISA) method.[9] High prevalence of avian H9N2 virus is related to the enlargement of host range and adaptation characteristics of this pathogen causing prevention and control measurements difficulties.[13] One of the major properties of the influenza virus is mutation, reassortment and interspecies transmission facilitating the host expansion.[1] Based on previous literature, transmission of influenza A virus from horses,[14] humans[4] and birds[13] to dogs has been reported. Additionally, it has been demonstrated that dogs can transmit this virus to cats, chickens[10] and humans[5]. Therefore, the role of dogs in the ecology and epidemiology of influenza virus should not be overlooked.

In contrast to our findings, a 0.80% (19/2357) prevalence of influenza virus was reported in dogs using HI assay; however, 3.94% (93/2357) of nasal swab samples from the same dogs were positive by reverse transcription polymerase chain reaction method. Interestingly, no antibodies were detected in the serum of dogs by HI assay in China and monoclonal antibody-based competitive ELISA in Italy regardless of active distribution of avian influenza A virus between the birds in these regions.[16] In another study conducted in Germany, no antibody titers were also found by either ELISA or HI assays.[17] It seems that the difference between prevalence rates in various studies may be related to variation between evaluated populations, geographical and environmental conditions and identification methods. Moreover, the cut-off levels of HI antibody titers should be considered as one of the underlying reasons for this difference.

Dogs are among the most common pet animals in Iran and can pose a potential threat to public health following close contact with humans due to the emergence of new viruses with pandemic potential.[1,4] This issue is particularly important regarding H9N2 influenza virus as one of the most significant pandemic pathogens in the WHO list.[13] Compared to our results, antibodies against avian H9N2 influenza virus were detected by HI assay in poultry-farm workers, slaughter-house workers, veterinarians, patients with respiratory signs and normal individuals and the seroprevalences of these five groups were 87.00, 76.20, 72.50, 35.60 and 23.00%, respectively.[3]

We found that the influence of age and gender on H9N2 virus infection was statistically insignificant. Similar to our findings, other researchers did not report any significant relationship between these factors and influenza virus infection.[5,9] It demonstrates that dogs with different age can be infected by influenza viruses. In contrary, Sun *et al*. have reported a significant influence of age on the seroprevalence of canine H3N2 influenza virus that dogs aged 2 to 5 years showed a higher prevalence, while effects of age and gender on H1N1 and human H3N2 influenza viruses were not significant in this study.[15]

The results of our study showed that dogs with raw diet had a higher prevalence of avian H9N2 influenza virus compared to the animals consumed the cooked diet. In this region, the avian H9N2 influenza virus is widely distributed and causes high mortality in the poultry industry.

Unfortunately, commercial or home-made diets are infrequently used for feeding dogs and most dogs commonly feed on chicken carcasses. Our finding is in accordance with Thiry *et al*., who found that feeding a raw diet can lead to influenza infection.[18] The H5N1 influenza virus infection following eating an H5N1-infected duck was also described in a dog.[18] In another study, H5N1 virus infection was reported in a domestic cat infected by pigeon carcass ingestion.[19]

In this study, the higher prevalence was detected in farm dogs compared to pet dogs. Furthermore, antibodies against the avian H9N2 influenza viruses were higher in dogs kept with other animals. Similar to our results, other authors have revealed that farm dogs which had contact with other animals show a higher seroprevalence.[11,16] Outdoor housing provides ample opportunity for influenza A virus transmission from other animals to dogs. Susceptibility of feral dogs to acquire the pathogens is higher due to poor diet, improper environment, and immunocompromised conditions. Stray dogs also eat bird carcasses more than pet dogs.[16] In contrast to our findings, other researchers did not detect any relation regarding housing status.[5] Interestingly, Yin *et al*. have found that antibodies against H1N1 are higher among pet dogs due to the exposure to human influenza A viruses.[2]

Considering the fact that the present research is the first epidemiological study in dogs in this region, HI assay was carried out as a screening test; thus, cross-reactivity between other subtypes of virus may affect the findings.[2] Thus, performing confirmatory assays such as neutralization test and isolation of H9N2 virus and/or virus gene segments from these dogs are postponed to the future studies. Since our results have been limited to only one subtype, it is not possible to comment on the predominance of subtypes in the studied dogs.

Based on our results, it is determined that avian H9N2 influenza virus is present as a common pathogen in dogs in Kerman, southeast of Iran. Moreover, antibodies against the avian H9N2 influenza virus were higher in farm dogs kept with other animals and fed a raw diet. These findings emphasize the importance of close attention to these populations for control and prevention programs like vaccination. Therefore, it is important to reduce the infection burden, especially in this region with widespread distribution of H9N2. It is difficult to discuss the role of dogs in the ecology of influenza A and it remains to be further investigated. Our findings will help to inform the presence of avian H9N2 influenza virus in dogs from Kerman, southeast of Iran. No epidemiological studies regarding humans have been previously performed in this region. The zoonotic potential of influenza viruses has been well established, but the role of dogs in the influenza ecology has still remained unclear. To determine whether the virus can be transmitted directly from dogs to humans, further in-depth studies are necessary.

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

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