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Short Communication

Detection and Isolation of H9N2 Subtype of Avian Influenza Virus in House Sparrows (*Passer domesticus*) of Ahvaz, Iran

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ABSTRACT

Avian influenza (AI) is an acute infectious disease with worldwide significance causing extensive economic losses in the poultry industry. Avian influenza viruses (AIVs) belong to the family Orthomyxoviridae and categorized in the genus influenza virus A. These viruses have been isolated from more than 100 species of free-living birds. Migratory birds are considered as reservoirs for AIVs and are the major agents responsible for global outbreaks. The Passeriformes are found in most parts of the world and cover a variety of habitats from rural to urban areas. House sparrows are members of the family Passeridae and due to their free flying, are strongly associated with seabirds, indigenous, and industrial poultry. The aim of this study was to determine the role of house sparrows in AIV (H9N2) circulation in the Ahvaz region. The intestinal and tracheal samples were taken from 200 sparrows around Ahvaz during 2017. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using specific primers in order to detect M and H9 genes of AIVs. The positive specimens in the PCR for the M gene were inoculated into 9-11-day-old embryonated chicken eggs via the allantoic fluid. The results showed that 11 out of 200 samples were positive for the two genes of M and H9. According to the findings of the present study, house sparrows are infected with H9N2 and pose a threat to commercial poultry. These birds may play a significant role in the transmission of AIV between wildlife and domestic animals. Therefore, this issue is important to be considered in preventive measurements.

Keywords: Ahvaz, Avian influenza, House sparrows, Iran, Molecular detection

Détection et Isolement du Virus de l'Influenza aviaire du Sous-type H9N2 chez le Moineau Domestique (*Passer domesticus*) d'Ahvaz, en Iran

Résumé: L'influenza aviaire (IA) est une maladie infectieuse aiguë de portée mondiale qui entraîne des pertes économiques considérables dans l'industrie de la volaille. Les virus de l'influenza aviaire appartiennent à la famille des Orthomyxoviridae et appartiennent au genre A du virus de l'influenza. Ces virus ont été isolés chez plus de 100 espèces d'oiseaux vivant en liberté. Les oiseaux migrateurs sont des réservoirs des virus de la grippe aviaire et des agents majeurs responsables des épidémies mondiales. Les Passériformes sont présents dans la plupart des régions du monde et couvrent divers habitats des zones rurales aux zones urbaines. Les moineaux domestiques sont une variété de la famille des Passeridae. En raison de leur liberté de vol, ils sont fortement associés aux oiseaux de mer, aux volailles indigènes et industrielles. Le but de cette étude était de déterminer le rôle des moineaux domestiques dans la circulation du virus de l'influenza aviaire (H9N2) dans la région d'Ahvaz. Les échantillons intestinaux et trachéaux ont été extraits à partir 200 moineaux autour d'Ahvaz en 2017. Un test de réaction en chaîne par polymérase de la transcriptase inverse (RT-PCR) a été réalisée à l'aide d'amorces spécifiques afin de détecter les gènes M et H9 des virus de l'influenza aviaire. Les échantillons positifs issus de

la PCR du gène M ont été inoculés à des œufs d'embryons de liquide allantoïdien. Les résultats ont montré que 11 échantillons sur 200 étaient positifs pour les deux gènes (M et H9). Selon les résultats de cette étude, les moineaux domestiques peuvent être infectés par le virus H9N2 et constituent une menace pour les volailles commerciales. Par conséquent, il est important de tenir compte de ce risque potentiel dans les mesures préventives. Ces oiseaux peuvent également jouer un rôle important dans la transmission du virus de la grippe entre la faune et les animaux domestiques.

Mots-clés: Ahvaz, Grippe aviaire, Moineaux domestiques, Iran, Détection moléculaire

INTRODUCTION

Avian Influenza (AI) is one of the most important respiratory contagious diseases of domestic poultry and other bird species that can spread rapidly (Swayne et al., 2013). The causative agent of AI is a member of the genus influenza virus from the family Orthomixoviridea and has an eight-segment genome with a negative sense. Based on the antigenicity of the nucleoprotein and the matrix protein, AI viruses (AIVs) are divided into three genera, namely A, B, and C. However, only genus A viruses are known to cause a natural infection in birds. The strains of AIV genus A have been classified into several different subtypes according to the antigenicity of the two surface glycoproteins of hemagglutinin and neuraminidase. In terms of virus pathogenicity in domesticated poultry, these viruses can be divided into two pathotypes, including highly pathogenic avian influenza (HPAI) viruses and low pathogenic avian influenza (LPAI) viruses. The HPAI viruses are able to cause severe disease and high mortality, whereas LPAI viruses are not very pathogenic and do not lead to mortality (Swayne et al., 2013). The LPAI viruses typically result in mild respiratory symptoms and decreased egg production. The mortality rate is usually low unless accompanied by other microorganisms or inappropriate environmental conditions. Wild aquatic birds, especially wild ducks, are thought to be the natural reservoirs of the virus. It should be noted that the wild aquatic birds are highly resistant to the disease. On the other hand, domestic birds, such as chickens are particularly sensitive to the infection by this virus (Elmberg et al., 2017). The eradication program of this disease should be based on the confirmation of AIV presence and the carriers of the virus. Some species of Passeriformes are found in most parts of the world and often cover a variety of habitats from rural to urban areas. House sparrows are a large and widely varied member of the family Passeridae. Due to their free flying, they are strongly associated with seabirds, indigenous, and industrial poultry (Hadipour et al., 2011). As a result, they may contribute to the transmission and survival of AIV in nature. In order to identify the possible role of the house sparrow in AIV transmission to poultry flocks and free-living birds, some information about AIV shedding in passerines is essential. With this background in mind, this study was conducted to investigate the role of house sparrows in the spread of AIVs through virus detection in the respiratory and gut samples by RT-PCR.

MATERIAL AND METHODS

All observations, practices, and work on birds were approved by the Animal Ethics Research Committee of Shahid Chamran University of Ahwaz (EE/97.24.3. 49872/scu.ac.ir). The tracheal and intestinal samples of a total of 200 sparrows from different parts of Ahvaz were taken during summer to winter 2017. The studied sparrows did not show any clinical symptoms. In the Khuzestan region, local people hunt and sell wild birds at local markets (after killing by head separating). For this research, these birds were bought from local markets.

Virus Detection. The tissue samples were prepared from the respiratory and digestive tracts. Next, RNA extraction was performed using RNX-plus commercial solution (Sinaclon, Iran) and cDNA synthesis was completed applying a general and specific M protein primer as described in the RT PreMixAccupowerR (Bionier, Korea). A 450-bp fragment of the M gene of AIV was amplified utilizing a pair of specific primers, known as CN1 and CN2 (Table 1) (Seifi et al., 2009). The PCR process was carried out in a 20 µl reaction volume containing 10 µl 2X PCR master mix (Ampliqon, Denmark) with 1.5 mmol Mgcl2, 0.5 µl of each primer (10 Pmol/µl), 6 µl of ddH_2O, and 3 µl cDNA. Moreover, the thermocycler (Quanta Biotech, Germany) was configured as summarized in Table 2.

Virus Isolation. The positive specimens in M gene PCR were inoculated into 9-11-day-old embryonated chicken eggs via the allantoic fluid (Wang et al., 1997; Swayne et al., 1998). The samples of allantoic fluid were harvested 48 h post-inoculation (PI) and were subjected to the detection of the H9 influenza gene using specific primers (Table 1). The materials and quantities required for the reaction were similar to M gene detection with the temperature program shown in Table 2. In all the stages of PCR, negative control (ddH_2O) and positive control (RNA from A/chicken/ Iran/AIa/2013H9) were used. The PCR products (450 and 487 bp) were separated by electrophoresis using 1% agarose gel.

RESULTS AND DISCUSSION

This research was conducted during one year from summer to winter of 2017. Two hundred sparrows were sampled from the different parts of Ahvaz, Iran. The RT-PCR was performed to detect the M gene of influenza A virus in the respiratory and intestinal tissue samples of the 200 house sparrows. The results of RT-PCR showed that eleven intestinal samples (5.5%) were positive for avian influenza A virus (Figure 1). We did not detect AIV in the trachea samples. The infected birds presented no clinical signs. The positive intestinal samples were inoculated into 9-11-day embryonated chicken eggs, followed by harvesting the samples of allantoic fluid after 48 h. Afterwards, the samples were subjected to the detection of the H9 gene by RT- PCR. The findings demonstrated that all the allantoic fluid samples were positive for the H9 gene (Figure 2). The AI is an acute and highly contagious disease with a wide spread among the various species of birds. The AIVs are distributed throughout the world with diverse reports from Africa, Asia, Australia, Europe, North America, and South America (Swayne et al., 2013).

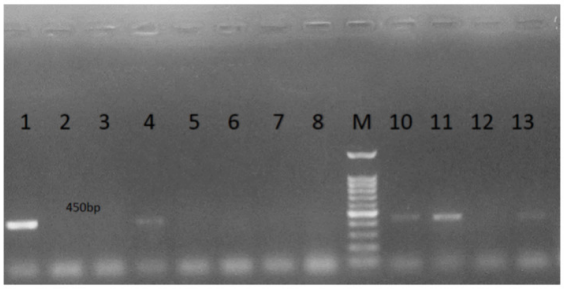

Figure 1. Electrophoresis analysis (1% agarose gel) of the M gene of avian influenza virus (AIV): Lane M = marker 100 base pair, No. 1: positive control (450 bp), No. 2: negative control, No. 4, 10, 11, and 13: positive samples.

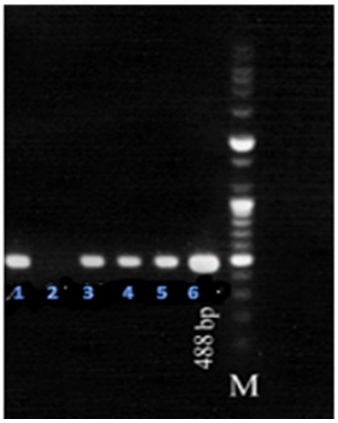

Figure 2. Electrophoresis analysis (1% agarose gel) of the H9 gene of avian influenza virus (AIV): Lane M = marker 100 base pair, No. 1: positive control (488 bp), No. 2: negative control, No. 3 to 6: positive samples.

Based on the pathogenicity of the virus, AIVs are divided into the two groups of highly pathogenic avian influenza virus (HPAI) and less pathogenic avian influenza virus (LPAI) (Spickler et al., 2008). Among the LPAI, the H9N2 subtype gets the most attention in

the studies mainly due to the probability of a pandemic occurrence, effective transfer to humans, and the fact that it has the internal genes needed to transfer H5N1 subtype to humans (Yuanji, 2002; Tweed et al., 2004). Since the 1990s, there have been many reports about the infection of poultry flocks all over the world with this type of influenza that causes significant economic losses in the poultry industry (Hadipour et al., 2011). In addition, there are many reports of human infection with the virus, which increased the importance of this type in terms of public health (Spickler et al., 2008). The AIV genus A (H9N2 subtype) was prevalent in the poultry farms of Tehran and Qazvin provinces of Iran in June 1998. Therefore, from that time the poultry farms of Iran are being screened for the disease. Although this virus is in the non-highly pathogenic group, it caused a mortality rate of up to 65% and reduced egg production up to 75% in the poultry farms of the different parts of Iran (Nili and Asasi, 2003). The presence of H9N2 AIV in 11 out of 200 (5.5%) examined samples may result from the free-ranging properties of house sparrows, which are founded in the various habitats throughout the world, or because of their long contacts with other wild birds. These features may help the spread and conservation of AIV in the environment. In the current study, all the positive samples were obtained from the intestine specimens showing these birds could transmit AIV through the cloaca. Some researchers have reported that the amount of cloacal shedding was higher than tracheal shedding in ducks and chickens (Lu and Castro, 2004; Mundt et al., 2009; Jourdain et al., 2010). Forrest et al. (2010) detected the H5N1 viral shedding and infection susceptibility in sparrows. These authors stated that along with the presence of sparrows in the chicken houses, they could spread the virus between chickens and wild birds in H5N1 occurrences. In spite of the detection of H9N2 AIV in sparrows, no clinical signs were observed. Furthermore, Hadipour et al. (2011) reported the serological evidence of H9N2 infection in house sparrows from Fars province of Iran. They believed that the lack of clinical symptoms of influenza in sparrows, despite the high level of antibodies in the blood, might be due to the resistance of these birds to the AIV as the result of continuous exposure making these birds naturally vaccinated. Sparrows may play a significant role in spreading the AIV as a typical carrier. Wild migratory aquatic birds can play an important role in the spread of influenza A virus. Moreover, these viruses have been isolated from wild aquatic birds frequently in Asia, Europe, and Africa (Chen et al., 2005; Liu et al., 2005). Some of the HPAI viruses have been identified or isolated by RT-PCR in at least 24 species of free-flying passerines, although

Table 1. Sequences and positions of the oligonucleotide primers used in RT-PCR

Oligonucleotide	Gene	Sequence	Product size (base pair)	Reference
CN1 Forward	M	GGGAAGAACACAGATCTTGAGG	450	Seifi et al., 2009
CN2 Reverse	M	TGCTGGCTAGCACCATTCTC		
H9 Forward	H9	CTY CAC ACA GAR CAC AAT GG	488	Lee et al., 2001
H9 Reverse	H9	GTC ACA CTT GTT GTT GTR TC		

Table 2. RT-PCR program of M and H9 genes for 35 cycle

Phase	Temperature (°C)		Time	
	M gene	H9 gene	M gene	H9 gene
Early denaturation	94	94	2 min	2 min
Denaturation	94	94	35 s	30 s
Annealing	54.9	50	45s	60 s
Elongation	72	72	45s	60 s
Final elongation	72	72	5 min	5 min

Table 3. Detection rate of respiratory viruses by RT-PCR in backyard chickens (n=100) of Ahvaz, Iran

Type of virus	NDV	AIV	IBV	NDV+AIV	NDV+IBV	AIV +IBV	NDV + AIV + IBV	Total
Positive birds (n)	17	10	21	13	23	4	7	95

these detections are moderately unusual (Mase et al., 2005; Peterson et al., 2008). Researches in different parts of the world show that a large number of free-living passerines may be infected with LPAI viruses. Consequently, it could be concluded that these birds probably have an important role in the spread of AIV (Hadipour et al., 2011). In conclusion, AIV has been easily isolated from house sparrows. As a result, in epidemiological studies, these birds may be of a significant role in the transmission of AIVs between wildlife and domestic animals. According to the findings of the present study, house sparrows may have an effect on the circulation of AIV (H9N2) as natural carriers and have a role in the transmission of the infection to other animals, such as other wild birds.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Chen, H., Smith, G.J., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., *et al.*, 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436, 191-192.

Elmberg, J., Berg, C., Lerner, H., Waldenstrom, J., Hessel, R., 2017. Potential disease transmission from wild geese and swans to livestock, poultry and humans: a review of the scientific literature from a One Health perspective. Infect Ecol Epidemiol 7, 1300450.

Forrest, H.L., Kim, J.K., Webster, R.G., 2010. Virus shedding and potential for interspecies waterborne transmission of highly pathogenic H5N1 influenza virus in sparrows and chickens. J Virol 84, 3718-3720.

Hadipour, M., Vosoughi, A., Fakhrabadipour, M., Azad, F., Khademi, I., 2011. Serological Evaluation for Supporting the Potential Role of House Sparrows in LPAIV (H9N2) Transmission. Int J Anim Vet Adv 3, 189-192.

Jourdain, E., Gunnarsson, G., Wahlgren, J., Latorre-Margalef, N., Brojer, C., Sahlin, S., *et al.*, 2010. Influenza virus in a natural host, the mallard: experimental infection data. PLoS One 5, e8935.

Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X.W., *et al.*, 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309, 1206.

Lu, H., Castro, A.E., 2004. Evaluation of the infectivity, length of infection, and immune response of a low-pathogenicity H7N2 avian influenza virus in specific-pathogen-free chickens. Avian Dis 48, 263-270.

Mase, M., Tsukamoto, K., Imada, T., Imai, K., Tanimura, N., Nakamura, K., *et al.*, 2005. Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. Virol 332, 167-176.

Mundt, E., Gay, L., Jones, L., Saavedra, G., Tompkins, S.M., Tripp, R.A., 2009. Replication and pathogenesis associated with H5N1, H5N2, and H5N3 low-pathogenic avian influenza virus infection in chickens and ducks. Arch Virol 154, 1241-1248.

Nili, H., Asasi, K., 2003. Avian influenza (H9N2) outbreak in Iran. Avian Dis 47, 828-831.

Peterson, A.T., Bush, S.E., Spackman, E., Swayne, D.E., Ip, H.S., 2008. Influenza A virus infections in land birds, People's Republic of China. Emerg Infect Dis 14, 1644-1646.

Seifi, S., Asasi, K., Mohammadi, A., 2009. A study of co-infection caused by avian influenza (H9 subtype) and infection bronchitis virus in broiler chicken farms showing respiratory signs. OJVR 13, 53-62.

Spickler, A.R., Trampel, D.W., Roth, J.A., 2008. The onset of virus shedding and clinical signs in chickens infected with high-pathogenicity and low-pathogenicity avian influenza viruses. Avian Pathol 37, 555-577.

Swayne, D.E., Glisson , J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M., 1998. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, American Association of Avian Pathologists, College Station, TX, pp. 169-174.

Swayne, D.R., Suarez, D.L., Sims, L.D., 2013. Influenza. In: Swayne, D.E., Glisson, J.R., McDougald, L.R., Nolan, L.K., Suarez, D.L., Nair, V. (Eds.), Diseases of Poultry, Ames: A John Wiley & Sons, Inc., PublicationIowa 50010, USA: Wiley - Blackwell pp. 181-218.

Tweed, S.A., Skowronski, D.M., David, S.T., Larder, A., Petric, M., Lees, W., *et al.*, 2004. Human illness from avian

influenza H7N3, British Columbia. Emerg Infect Dis 10, 2196-2199.

Wang, H.N., Wu, Q.Z., Huang, Y., Liu, P., 1997. Isolation and identification of infectious bronchitis virus from chickens in Sichuan, China. Avian Dis 41, 279-282.

Yuanji, G., 2002. Influenza activity in China: 1998–1999. Vaccine 20, S28-S35.