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

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 Ahvaz (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.

Mots-clés: Ahvaz, Grippe aviaire, Moineaux domestiques, Iran, Détection moléculaire
**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 ddH2O, 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 (ddH2O) 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](image1.png)

**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](image2.png)

**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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Gene</th>
<th>Sequence</th>
<th>Product size (base pair)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN1 Forward</td>
<td>M</td>
<td>GGGAAGAACACAGATCTTGAGG</td>
<td>450</td>
<td>Seifi et al., 2009</td>
</tr>
<tr>
<td>CN2 Reverse</td>
<td>M</td>
<td>TGCTGGCTAGCACCATTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9 Forward</td>
<td>H9</td>
<td>CTY CAC ACA GAR CAC AAT G</td>
<td>488</td>
<td>Lee et al., 2001</td>
</tr>
<tr>
<td>H9 Reverse</td>
<td>H9</td>
<td>GTC ACA CTT GTT GTT GTR TC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Phase</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early denaturation</td>
<td>94</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>2 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>54.9</td>
<td>45 s</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>60 s</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72</td>
<td>5 min</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Type of virus</th>
<th>Positive birds (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDV</td>
<td>17</td>
</tr>
<tr>
<td>AIV</td>
<td>10</td>
</tr>
<tr>
<td>IBV</td>
<td>21</td>
</tr>
<tr>
<td>NDV+AIV</td>
<td>13</td>
</tr>
<tr>
<td>NDV+IBV</td>
<td>23</td>
</tr>
<tr>
<td>AIV+IBV</td>
<td>4</td>
</tr>
<tr>
<td>NDV + AIV + IBV</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
</tr>
</tbody>
</table>
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.

Acknowledgment

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