

Original Article

Investigation of Avian Influenza Viruses (H9N2-H5nx) in Pigeons during Highly Pathogenic Avian Influenza Outbreaks in Iran, in 2016

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ABSTRACT

Avian influenza (AI) virus (H9N2 and H5 subtypes) infections in birds cause major concerns around the world. The majority of the avian species, such as domestic, pet, and wild birds, are natural and experimental hosts of avian influenza viruses. There are global concerns about members of the Columbidae family, namely pigeons or doves, for their role as the potential interspecies bridge in influenza A viruses ecology. The acquired scientific data in this regard is still not clear since there are doubts about whether or not they transmit viruses between susceptible populations, and spread viruses among farms during outbreaks. To monitor H5 and H9 influenza virus infection status in the rural, backyard, and domestic birds, an annual active surveillance program was performed from September to October 2016. In December 2016, an outbreak of highly pathogenic avian influenza (HPAI) virus subtype H5N8 was detected in a layer farm in Tehran province, Iran. The present research was conducted to study H9N2 or H5 infections in pigeons within HPAI H5N8 2016 outbreaks and annual national AI surveillance in Iran. For this purpose, cloacal swabs and tissue samples (trachea, lung, brain, liver, heart, pancreas, and cecal tonsil) were collected and examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) method and virus isolation. Results of the tests performed on the swab and tissue samples were negative for H5 nor H9N2 viruses. The samples in real-time RT-PCR that after three passages still showed negative results in HA and molecular tests were considered negative. Moreover, the Newcastle disease virus was isolated in most of the samples taken from dead pigeons, after inoculation in embryonated chicken eggs.

Keywords: H5 outbreak, Pigeon, Real-time RT-PCR, Surveillance

Enquête sur les Virus de la Grippe Aviaire (H9n2-H5nx) chez les Pigeons Lors d'Épidémies de Grippe Aviaire Hautement Pathogènes en Iran, en 2016

Résumé: Les infections par le virus de la grippe aviaire (sous-types H9N2 et H5) chez les oiseaux suscitent des préoccupations majeures dans le monde. La majorité des espèces aviaires, telles que les oiseaux domestiques, de compagnie et sauvages, sont des hôtes naturels et expérimentaux des virus de la grippe aviaire. Le rôle des membres de la famille des Columbidae, à savoir les pigeons ou les colombes, en tant que pont interspécifique potentiel dans l'écologie des virus de la grippe A est une préoccupation mondiale, pour. Les données scientifiques acquises à cet égard ne sont toujours pas claires, car il existe des doutes sur leur capacité à transmettre ou non des virus entre les populations sensibles et s'ils propagent des virus parmi les fermes pendant les épidémies. Afin d'évaluer la présence d'infection par le virus de la grippe H5 et H9 chez les oiseaux élevés en zone rurale, de basse-cour et domestiques, un programme annuel de surveillance active a été mené de septembre à octobre 2016. En décembre 2016, une épidémie du sous-type H5N8 du virus de l'influenza aviaire hautement pathogène (IAHP) a été détectée dans une ferme de poule pondeuses dans la province de Téhéran, en Iran. Cette étude a été menée afin d'évaluer la présence d'infections aux virus H9N2 ou H5 chez les pigeons

dans l'épidémie de l'IAHP H5N8 2016 dans le contexte de la surveillance nationale annuelle de la grippe aviaire en Iran. À cette fin, des écouvillons cloacaux et des échantillons de tissus (trachée, poumon, cerveau, foie, cœur, pancréas et amygdales caecales) ont été collectés et examinés en temps réel par la méthode d'amplification en chaîne par polymérase couplée à une transcription inverse (RT-PCR) suivi de l'isolement du virus. Les résultats des tests effectués sur l'écouvillon et les échantillons de tissus étaient négatifs pour les virus H5 et H9N2. Les échantillons en RT-PCR en temps réel qui, après trois passages, montraient toujours des résultats négatifs en HA et des tests moléculaires étaient considérés comme négatifs. De plus, le virus de la maladie de Newcastle a été isolé dans la plupart des échantillons prélevés sur des pigeons morts, après inoculation dans des œufs de poule embryonnés.

Mots-clés: Épidémie de H5, Pigeon, TI-RCP en temps réel, Surveillance en Iran

INTRODUCTION

Avian Influenza (AI) infection, caused by type A influenza virus, is one of the most important viral diseases in different avian and mammalian species. Influenza A viruses belong to the family of Orthomyxoviridae and are subtyped based on their two surface glycoproteins, namely hemagglutinin (HA) and neuraminidase (NA). Up to this time, 16 HA and 9 NA subtypes have been identified in birds. Based on their pathogenicity in poultries, they are divided into two pathotypes of highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Swayne et al., 2008). Most avian species, such as domestic, pet, and wild birds, are natural and experimental hosts of avian influenza (AI) viruses. Moreover, aquatic wild birds, such as ducks, are a natural reservoir of AI viruses (Swayne and Suarez, 2000). Before 2002, it was thought that HPAI viruses are not capable of causing disease or mortality in wild aquatic birds. However, after 2002, there were multiple reports on HPAI infection with high mortality rates and long-lasting viral shedding in wild birds (Perkins and Swayne, 2002; Ellis et al., 2004; Sturm-Ramirez et al., 2004). The ability of AI viruses to mutate, recombine, and adapt to new hosts and the risk they pose to public health make them important hazards (Boon et al.,

2007). This virus proliferates in gastrointestinal and respiratory tracts and is transmitted by respiratory aerosols or fecal-oral routes (OIE, 2015). Birds from various orders are susceptible to AI; however, susceptibility, severity, and symptoms of the disease are different among them. Terrestrial birds, including pigeons and doves, can freely fly and easily contact a wide range of domestic, nondomestic, and aquatic birds. Therefore, it can be said that Columbidae plays a substantial role in the ecology and interspecies transmission of HPAI viruses (Boon et al., 2007). According to previous studies, ducks, starlings, gulls, and pigeons are the least susceptible birds to this disease. Moreover, infection with AI in these birds is associated with few or no clinical signs (Perkins and Swayne, 2003). There are global concerns about members of the Columbidae family, namely pigeons and doves, regarding their potential role as the interspecies bridge in the ecology of type A influenza viruses. Usually, wild pigeons or doves dwell in places with water or/and food and nesting sites, which results in direct contact with people in cities and poultries on farms. Furthermore, they can fly long distances which increases the risk of the transmission of the virus. In some countries, such as Egypt, an increasing number of pigeons are raised in commercial farms as a source of meat for humans (Alqutatory et al., 2016). Moreover, pigeons are sometimes kept as companions or for the

purpose of racing which puts them in close contact with other birds and mammals. Pigeon racing is a popular sport that is growing in East Asia and is regarded as one of the multi-million dollar industries. Scientific information on pigeon susceptibility to AIV infection under natural conditions is scarce and there is a disagreement amongst researchers over it and its consequences. A total of 30 commercial, rural, and wild bird populations were involved in an H5N8 HPAI outbreak was reported in Iran, in 2016. In the present study, H5 or H9N2 infection was investigated in pigeons in H5N8 HPAI 2016 outbreaks and the annual national AI surveillance in Iran.

MATERIAL AND METHODS

To monitor the status of infection in H5 and H9 influenza viruses in the rural, backyard, and domestic birds, an annual active surveillance program was carried out from the start of the immigration season of wild aquatic birds, which was late summer or early autumn. Following this schedule, swab samples were collected from September to October 2016. In December 2016, a highly pathogenic AI outbreak by the H5N8 subtype was detected in a layer farm in Tehran province, Iran. Eventually, this infection resulted in many outbreaks in the next three months throughout the country. To track the infection in dead pigeons, which were in the same infected farm or village, samples were taken from trachea, lung, brain, liver, heart, pancreas, and cecal tonsil. In addition, following the zonal monitoring program of infected sites, swab samples were taken from pigeons within 3 KM of the infected areas.

Sample Preparation. Tissue and swab samples were prepared following the routine procedure (Hitchner et al., 1980). Each sample was divided into two parts for RNA extraction and inoculation. Viral RNA was extracted using high pure viral Nucleic Acid Kit (manufactured in Roche, Germany) according to the instruction provided by the manufacturer.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR). For molecular examination, two different real-time RT-PCRs were simultaneously conducted in two different tubes, targeting the Matrix gene (Capua and Alexander, 2009) of AI viruses and Hemagglutinin gene subtype H5 and H9 (Monne et al., 2008). If the result of the molecular detection test of a sample was negative, further investigation was performed by inoculating the second part of the sample in specific pathogen-free embryonated chicken eggs. Samples were considered negative after at least two other blind passages.

Hemagglutination Inhibition Test. Negative samples were investigated using Haemagglutination inhibition test for detection of possible Newcastle disease virus (NDV) or H9N2 infection based on OIE protocol, using antigens with 4HA units (antigens were provided by Razi Vaccine and Serum Research institute). Samples with an antibody titer of $4 \leq$ with \log_2 were considered as positive samples.

RESULTS

In the present study, cloacal swab samples were taken from 181 pigeons in nine provinces during active surveillance programs (a total of 6378 different bird species were sampled) (Table 1).

Table 1. Number of sampled pigeons in 2016 national surveillance program

| Province | Number of sampled pigeons in the surveillance program in 2016 |
|-----------------|---|
| Qazvin | 4 |
| Hormozgan | 29 |
| Fars | 9 |
| Lorestan | 12 |
| Khuzestan | 26 |
| Bushehr | 23 |
| Alborz | 38 |
| Tehran | 20 |
| Khorasan Razavi | 20 |
| Total | 181 |

Tissue samples from 36 dead pigeons and cloacal swab samples from 45 alive pigeons around the infected farms were collected during H5N8 highly pathogenic AI outbreaks (Table 2). The result of molecular tests for H5 infection in all swab and tissue samples were negative. The samples that showed negative results in HA and molecular tests for H5 and H9 after three passages were considered negative. No other influenza virus, including H9N2, was detected after three blind passages in embryonated eggs. In most samples of dead pigeons, NDV was isolated after inoculation in embryonated chicken eggs.

DISCUSSION

Members of the Columbidae family are usually kept as companions or pet birds, thereby have direct contact

2006). Based on the results of a serological and virological surveillance program that was conducted by Siengsan-Lamont et al. (2011), on 44 wild bird species, no H5N1 HPAI virus was isolated from tracheal and cloacal swabs in pigeons. Moreover, some other researchers reported the absence of clinical disease signs, gross and histological lesions in all tissues and relative resistance of experimentally infected pigeons (Panigrahy et al., 1996; Perkins and Swayne, 2002, 2003). In addition, the minimal role of pigeons in ecology and epidemiology of HPAI avian influenza viruses has been reported previously (Perkins and Swayne, 2002; Kaleta and Honnicke, 2004; Boon et al., 2007; Brown et al., 2009; Yamamoto et al., 2012). Pigeons that are easily infected, efficiently replicate the virus, and shed the virus in high quantities through the

Table 2. Number of sampled pigeons in infected areas and their surrounding in the H5N8 outbreak

| Province | Number of sampled pigeons in infected areas | Number of sampled pigeon in the surrounding areas |
|------------|---|---|
| Isfahan | 13 | 19 |
| Mazandaran | 7 | 13 |
| Kermanshah | 3 | 0 |
| Gillan | 6 | 6 |
| Qazvin | 2 | 1 |
| Markazi | 6 | 5 |
| Total | 36 | 45 |

with humans. Besides, wild and domestic pigeons can fly for long distances, move between different regions, and contact wild birds and poultry. Moreover, pigeon and doves are sold in live bird markets; therefore, they may have an important role in the ecology of influenza virus among various species. It must be noticed that AI H5N8 HPAI outbreaks caused mortality and morbidity of various commercial and domestic poultry species and free-flying wild birds including magpies in Iran in 2016 (OIE, 2017). In this study, the results of tests for H5 or H9N2 in all samples, which were taken from suspected dead or alive pigeons in infected areas and the ones in annual surveillance programs, were negative. Previously, it was reported that the pigeons are resistant or have an innate resistance to AI infection and are not transmission hosts, despite their immune dysfunction (Perkins and Swayne, 2002; Fang et al.,

oropharyngeal or fecal routes are considered significant agents in the ecology of the virus. In several experimental studies on infection, there was either no or low viral shedding in uninfected susceptible contact birds after HPAI infection (Kaleta and Honnicke, 2004; Boon et al., 2007; Brown et al., 2009; Pantin-Jackwood and Swayne, 2009). Liu *et al.* (2007) in their study reported the resistance of pigeons to 5 Asian H5N1 viruses and no contact bird infection. It has been suggested by Brown *et al.* (2009) that pigeons are resistant to an HPAI virus; therefore a high concentration of a virus is needed to cause death or infection. Furthermore, when infection occurs, the duration of viral shedding is brief and viral titer is low (Brown et al., 2009). Moreover, according to the results of another study performed by Smietanka et al. (2011), the pigeons were resistant to H5N1 infection and there

was a lack of transmission of the virus to susceptible contact birds were reported. In a study conducted on the experimental infection of pigeons with H5N8, Kwon et al. (2017) observed the lack of clinical signs, no transmission to contact birds, and short duration of viral shedding only in a few infected pigeons. From four continents, a total of 2046 apparently healthy pigeons were sampled for antibodies against AIV and only the results of 164 (8.01%) blood sample tests were positive. Moreover, H5 seropositive samples were concurrent with H5 outbreaks in poultry (Abolnik, 2014). However, according to a study conducted by Fallah Mehrabadi et al. (2016) footprint of H9N2 was observed in some pigeon samples. Nevertheless, in the above mentioned research, only 4 samples were seropositive from the total 19 (21%) samples; moreover, the results of molecular tests were negative for all samples (Fallah Mehrabadi et al., 2016). Regardless of the aforementioned studies, some other researchers believe in the susceptibility of pigeons and their role in the transmission and distribution of AI viruses. Ellis et al. (2004) in their study, reported the isolation of the H5N1 HPAI virus from a dead pigeon at Kowloon park during the H5N1 outbreak in Hong Kong, in 2002, and declared the susceptibility of these species to AI infection. Furthermore, Klopffleisch et al. (2006); (Werner et al., 2007) reported the susceptibility of pigeons to H5N1 infection. However, they did not observe the transmission to contact birds or obvious clinical signs in the majority of infected pigeons (Klopffleisch et al., 2006; Werner et al., 2007). Mortality and neurotropism of the Indonesian HPAI H5N1 virus in experimentally and naturally infected pigeons were observed by Klopffleisch et al. (2006). Besides Jia et al. (2008) in their study, described the neurotropism of the virus following the infection of pigeons. The findings of another study carried out by Mansour et al. (2014) indicated the mortality, nervous signs, and nasal and cloacal swab isolation following natural and experimental HPAI H5N1 infection of pigeon in Egypt. Isolated pigeon viruses in Egypt had

similarities with Chinese viruses which caused concerns about the origin of the virus. Petersen et al. (2012) studied the replication of LPAI viruses in multiple avian species and concluded that the resistance of Columbidae is due to the difference in type and distribution pattern of AIV receptors. Expression of different types of sialic acid receptors of the influenza virus in tissue is thought to be one of the major determinants of its host range and tropism (Petersen et al., 2012). Franca et al. (2013) in their study showed that there was moderate to strong expression of $\alpha 2, 6$ receptors in the respiratory tract of rock pigeon and mourning dove. Moreover, according to another research conducted by Liu *et al.*, SAa2,6 Gal was the major receptor in the airway of pigeons, while in chickens SAa2,3Gal was the main receptor, which may be partly due to inefficient virus attachment and replication, and consequently resistance of pigeons to AIVs (Liu et al., 2007). Since there is a direct association between virus replication and disease virulence, the lack of replication fitness involving host-specific co-factors and viral proteins would contribute to a decrease of replication efficiency and resistance of pigeons (Perkins and Swayne, 2003). It has been pointed out that variations in the genes, strains, and clades of HPAI viruses are other influential factors on the incidence or absence of clinical signs in pigeons. Cytokine responses in pigeons have been monitored with HPAI H5N1 infection by Hayashi et al. (2011). They declared that the virus replicates in the lungs efficiently; however, it does not make excessive expressions of inflammatory-related and/or innate immune genes in the lungs of the infected pigeons. They discussed that pigeons tolerate infection due to moderate cytokine response after infection. Ducks and pigeons, unlike chickens, have innate immune systems, such as retinoic acid-inducible gene I (RIG-I), an RNA sensor in the cytoplasm that plays an important role in clearing the influenza infection. Therefore, it can be concluded that pigeons are susceptible to AI infection, while the virus cannot replicate or extend in this

species. Moreover, pigeons are dead-end hosts, which means that despite the replication of the virus in them, it does not result in clinical diseases, shedding, and transmission through contact with other birds. If there is any nasal or cloacal shedding, the duration will be short with lower titer than that in other species (Boon et al., 2007; Brown et al., 2009; Pantin-Jackwood and Swayne, 2009).

In Conclusion, despite the fact that no influenza virus infection was detected in pigeons in the recent H5N8 HPAI outbreak in Iran, the role of pigeons in ecology and survival of influenza A viruses is still questionable. Therefore, further natural and experimental studies are required to elucidate whether or not Columbidae is involved in the transmission or spread of avian influenza viruses. However, it should not be ignored that pigeons can fly for long distances, their carcasses are accidentally consumed by other animals, and AI viruses are mechanically transmitted by their foot and feathers.

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.

Authors' Contribution

Study concept and design: Motamed, N.; Shoushtari, A.; Fallah Mehrabadi, M. H.

Acquisition of data: Motamed, N.; Shoushtari, A.; Fallah Mehrabadi, M. H.

Analysis and interpretation of data: Motamed, N.; Shoushtari, A.; Fallah Mehrabadi, M. H.

Drafting of the manuscript: Motamed, N.

Critical revision of the manuscript for important intellectual content: Motamed, N.; Shoushtari, A.; Fallah Mehrabadi, M. H.

Statistical analysis: Fallah Mehrabadi, M. H.

Administrative, technical, and material support: Shoushtari, A.

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