A molecular survey of Avian Influenza among captive birds in the city of Tehran between November 2008 and February 2009

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ABSTRACT

Background and objectives: To determine the potential circulation of avian influenza viruses among different captive bird species, molecular surveillance was conducted at Tehran Zoo, Saiee Park and Pardisan Park of Tehran, Iran. These places are at risk for spread and transmission of influenza virus because of bird species diversity and close contact of birds with humans.

Materials & Methods: During the influenza season in Tehran, in the cold weather (November 2008-February 2009), 76 cloacae samples were collected from 5 orders of Anseriformes, Galliformes, Columbiformes, Pelicaniformes and Phoenicopteriformes, including 13 bird species plus 5 hybrid species of ducks. Presence of avian influenza genome was monitored with RT-PCR as a sensitive and specific assay. The assay targeted a 132 bp fragment of the conserved M gene of influenza type A.

Results: Influenza type A virus was not detected in samples collected from November 2008 to February 2009. The sensitivity of RT-PCR based on M primers was 0.1ng total RNA. Interestingly, during the study period, there was no report of death or clinical signs of disease among the captive birds, whereas the birds did not have vaccinated history against influenza A virus.

Conclusion: Although the results could be attributed at least partially to the presence of an undetectable amount of genomic RNA, based upon the sensitivity of the test our findings suggest that no RNA genome of influenza A viruses was present in the samples under study.

Keywords: Avian influenza viruses, RT-PCR, Captive birds, Tehran.

INTRODUCTION

Avian influenza viruses (AIVs) belong to influenza virus A and the family of Orthomyxoviridae. Antigenic subtypes of virus A are based on the diversity of surface glycoproteins, Haemagglutinin (HA) and Neuraminidase (NA). There are 16 HA and 9 NA based on the amino-acid variations. Each virus has a combination of one HA and also one NA (1).

AIVs can infect a variety of bird species and also can transmit easily among birds. Avian influenza in poultry depending on its ability to cause disease symptoms and fatality can be divided into low pathogenic avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV). LPAIV causes minimal clinical signs of the disease although some mutations can cause low pathogenic subtypes like H5 and H7 to emerge as highly pathogenic (2, 3). The greatest concern is typically with HPAIVs (H5 and H7) because of their severe signs of disease and sometimes death rate of 100% in birds. Although the wild birds, especially waterfowls, are the natural reservoir of avian influenza, viral infection in these birds is usually unapparent or nonclinical (4). Close contact of wild birds and poultry can cause virus transmission from wild bird to poultry and also from poultry to wild birds.

One of the ecological characteristics of AIVs includes interspecies spills into new hosts such as humans. For example, in the 20th century, the Asian influenza pandemic of 1957 was caused by a reassortment of H2N2 virus genes with some genes coming from avian viruses and H3N2 virus that caused...
The Asian influenza pandemic of 1957 had genes originating from reassortment of human and avian influenza genes (5, 6). In recent years, direct transmission of H5N1 (7, 8), H7N7 and H9N2 (9) viruses from birds to humans have been reported. Although these infections seem to have had a limited human to human transmission, if people infected with an ‘avian’ virus become also infected with a ‘human’ influenza virus, reassortment could potentially occur and result in a virus which could spread from human to human.

In such a scenario, the human population could not have any immunological memory against such reassorted avian-human influenza virus.

Zoological collections are man-made systems where captive birds have close contact with free-flying birds, staff and visitors to parks and zoos. Due to a variety of host bird species in these new niches, the ecology and epidemiology of avian influenza viruses should be reviewed again. The viruses could be transmitted interspecies and in this way could become adapted in new hosts (1). Therefore, it is essential to monitor such places to track the influenza viruses.

The present study was undertaken to assess the presence of avian influenza virus genome among captive birds of the Tehran Zoo and two additional parks in the city of Tehran.

**MATERIALS AND METHODS**

**Sample collection.** From November 2008 to February 2009, 76 cloacal samples were collected from birds which were healthy at time of sampling, according to the local authorized veterinarians from Tehran Zoo, Saiee Park and Pardisan Park located in Tehran. Table 1 shows different captive birds which were studied. Samples taken using cloacal swabs with Dacron tip were transferred into viral transport medium (VTM) followed by vortexing of the tubes. VTM was prepared with sterile glycerol-PBS (1:1) solution (pH 7.2) containing benzyl-penicillin, streptomycin and amphotericin B. The harvested supernatant was collected after centrifugation at 3000 ×g for 10 min, and stored at -20°C for further process (10).

**RNA extraction.** RNA extraction from cloacal specimens was performed using RNX™-Plus solution (CinnaGen, Tehran, Iran). In brief 250 μl of cloacal sample were mixed with 750 μl of RNX™-Plus solution and incubated for 5 minutes at room temperature. The mixture was then extracted by adding 0.2 ml chloroform and centrifuged at 12000 ×g for 15 minutes (4°C). RNA on the upper layer was collected and precipitated by the same volume of isopropyl alcohol followed by incubation for 15 min on cold ice and centrifugation at 12000 ×g for 15 minutes (4°C). The pellet was washed with 75% ethanol, centrifuged (7600 ×g, 8 minutes at 4°C) and, finally the RNA pellet was dissolved in DEPC-treated water for use in cDNA synthesis.

**RT-PCR.** Universal primer (Uni12) was used in reverse transcription process. Briefly, the mixture of 10μl RNA and 2μl Uni12 primer (10 pmol/μl) was incubated at 70°C for 5 min and immediately chilled on ice. Thereafter, the components of RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) including 5 × reaction buffer (4μl), Ribolock™ RNase Inhibitor 20U/ml (1μl), dNTP mix 10mM (2μl) and ReverAid™ M-MuLV Reverse Transcriptase 200U/ μl (1μl) was added to the tube containing RNA and primer. The tube was incubated for 5 min at 25°C followed by 42°C for 60 min and ultimately 70°C for 5 min according to the manufacturer’s instructions. M primers which targeted matrix gene of influenza type A viruses were used in PCR (Table 2). The PCR reaction mixture consisted of 6μl cDNA, 12.5μl Taq DNA Polymerase Master Mix RED kit (Ampliqon, Denmark) which contained Tris-HCl 150mM pH 9.0, MgCl₂ 3.0mM, dNTPs 10mM and Taq DNA polymerase.
8.5, (NH₄)₂SO₄ 40mM, MgCl₂ 1.5mM, 0.2% Tween 20, dNTPs 0.4mM, Ampliqon Taq DNA Polymerase (0.05Unit/ul), 1µl of each forward and reverse of M-primers and distilled water to the final volume of 25µl. The thermocycling conditions were 95°C for 5 min (primary denaturation), 35 cycles of 94°C for 40s (denaturation), 59°C for 40s (annealing) and 72°C for 40s (extension), and the final extension at 72°C for 5 min which terminated the PCR reaction. The PCR products were analyzed by 2% agarose gel and ethidium bromide staining followed by visualization with ultraviolet transillumination.

Sensitivity of RT-PCR. The sensitivity of RT-PCR was assessed by ten-fold serial dilution of extracted RNA (A/ Panama/ 2007/ 99, H3N2). Subsequently, RT-PCR was performed according to the procedure described above. The highest dilution of RNA with positive RT-PCR was considered as the sensitivity cut-off (11).

RESULTS

The sensitivity cut-off of RT-PCR was 0.1ng template RNA genome (Fig. 1). No avian influenza viruses were detected in any of 76 cloacal samples collected from Tehran Zoo, Saiee Park and Pardissan Park of Tehran between November 2008 and February 2009 (Fig. 2). This finding was consistent with absence of influenza specific clinical signs of disease and no report of death during the study period, according to the authorized veterinarians.

DISCUSSION

RT-PCR is a valid and universal molecular technique to detect and confirm the presence of influenza viruses genome even if they are present at a very low level in the laboratory specimens. Among the most frequently used primers to detect influenza type A viruses, M gene is a highly conserved region. These primers are type specific and can detect M segment of influenza type A viruses with origin of various hosts (12). This assay is more sensitive, more specific and less time consuming in comparison with other diagnostic assays (13). However, the absence of the expected RT-PCR products, i.e. a negative result, does not necessarily indicate absence of influenza A viruses. Results should be interpreted along with information about clinical signs and epidemiological data (14). In this study, local veterinarians confirmed that the birds had not been vaccinated against any avian influenza viruses and influenza clinical signs were not being reported in the tested birds during the study period. According to our findings, it could be concluded that no RNA genome of influenza A virus was present in the samples.

Our study is unique in that it is the first molecular surveillance of avian influenza virus among a variety of bird species in Tehran. Collection of samples was undertaken from Tehran Zoo, Saiee Park and Pardissan Park where several captive birds are living close to other bird species and also to humans. It is believed that these areas are highly susceptible to HPAIVs’ outbreaks due to the fact that in these places virus could transmit inter-species and intra-species via several routes: close contact to infected birds or contaminated food or water (usually in the same bird species that are kept in a cage), considerable movement of vehicles and people from cage to cage and airborne transmission of viruses (1). Transmission by flies and vermin is also possible (15).

Table 2. Sequence of M primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>MF</td>
<td>5’-GACTCAAATGTCAAGAACCCTTTA-3’</td>
<td>35-59</td>
<td>132 bp</td>
</tr>
<tr>
<td>MR</td>
<td>5’-CCACTTATTTTCTCCTGTTTAG-3’</td>
<td>145-167</td>
<td></td>
</tr>
<tr>
<td>Uni12</td>
<td>5’-AGC-AAA-AGC-AG-&lt;G&gt;-3’</td>
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Diversity of bird species in the studied zoo and parks can lead to a unique condition that changes ecology of viruses toward an easier adaptation to new hosts including other bird species and human. Aquatic birds including ducks, geese and swans, are the natural reservoir of influenza viruses in nature (16). In these places, avian influenza transmission to park staff, visitors and veterinarians can also occur.

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REFERENCES