Two Novel Avian Influenza Virus Subtypes Isolated from

Domestic Ducks in North of Iran

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Abstract

Avian influenza viruses (AIV) are the causative agents of AI which is a contagious and zoonotic disease of birds. Among birds, wild waterfowl and ducks are the primary and natural reservoirs of low pathogenic avian influenza viruses (LPAIV). This study was aimed to identify and differentiate AIV HA and NA subtypes from domestic ducks by hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) assays. 962 cloacal swabs were collected from domestic ducks being sold at different Iranian live bird markets (LBM) in Gilan, Mazandaran and Golestan provinces of Iran located at the southern coast of the Caspian Sea. The samples were inoculated in 10-day-old embryonated specific pathogen-free (SPF) chicken eggs and harvested allantoic fluids were subjected to Agar gel immunodiffusion (AGID), HI and NI assays. Five positive samples, including two H4N2 and three of H3N2 AIV subtypes were identified. Isolation of H4N2 and H3N2 viruses has never been reported from Iranian domestic ducks before. This finding further suggests the diversity of LPAIV viruses in Iranian ducks and also shows that the HI and NI assays are highly efficient in determining AIV subtypes.
Keywords: Avian Influenza Virus, Domestic Duck, Isolation, HI, NI, Iran.

Introduction

Avian influenza viruses (AIVs) cause a drastic economic loss to the poultry industry and are considered as a threat to global public health (1). AIVs are classified on the basis of two surface glycoproteins namely the hemagglutinin (HA) and neuraminidase (NA) (2), (3). Currently, influenza A viruses are classified into 18 HA (H1–H18) and eleven NA (N1–N11) subtypes based on the antigenic differences of the HA and NA proteins. Two new subtypes (H17, H18) and (N10, N11) were proposed for influenza A viruses from bats (4).

Based on their ability to cause disease in chickens, the AI viruses are divided into two main groups of a) very virulent viruses, termed highly pathogenic avian influenza [HPAI], and b) milder viruses, that primarily cause respiratory disease, thus designated as low pathogenic avian influenza viruses [LPAI](2), (5).

Influenza viruses can infect variety of the birds including free-living and captive caged birds, domestic ducks, chickens, turkeys and other domestic poultry (2). Duck is considered as the primary reservoir for AI viruses (3). It has been shown that the influenza A viruses can replicate in the intestines of duck without any evident clinical sign, and shed the virus in high concentrations in the feces (6). Therefore, domestic ducks could be infected by HPAI viruses and shed large quantities of a virus without obvious clinical signs of the disease, while the owners or governing livestock officials are not aware (2), (6). Also domestic ducks could play a significant role to introduce and spread of the novel viruses in the poultry system by reassortment with other subtypes, resulting in further infections in live birds market (LBM) (7).

Based on surface glycoproteins, 16 different HA and 9 NA subtypes and strains of AIVs have been isolated from ducks in diverse geographic regions of the world (8). Most AIVs isolated from ducks
are from H3, H4, and H6 subtypes (9); however, the other subtypes of virus such as H5, H7, H8, and H9 are generally reported at low prevalence rates from ducks (10). Furthermore, three subtypes of NA including N2, N6, N8 are prevalent in ducks (6). Ducks have not been confirmed as a reservoirs of H1, H10, N5 and N7 viruses(9).

Several methods such as ELISA, RT-PCR, HI and NI assays can be used in diagnosis subtypes of AIVs (6). The HI assay described by Hirst 1942 and then modified by Salk 1944. The HI assay is extremely reliable and it is used for global influenza surveillance and determination of the antigenic characteristics for influenza viral isolates (6). The NA assay was described by Warren et al, 1959 and modified by Webster & Laver 1967. The NI assay has been introduced for many years and it is used in reference laboratories (6). In addition, this assay could detect the NA antigen to determine the subtypes of the viral samples from serum.

This study aimed to identify and differentiate AIV HA and NA subtypes from domestic ducks of Iran’s Northern provinces by PCR, HI and NI assays.

**Material and Methods**

**Sample collection**

A total of 962 cloacal swabs were collected from domestic ducks in multiple live bird markets (LBMs) from Gilan, Mazandaran and Golestan provinces, Iran. The swabs were placed in transport medium (2-3.5 ml of sterile phosphate-buffered saline (PBS) containing 1% bovine serum albumin and antibiotic(Penicillin+Gentamicin+Streptomycin) (0.1%) with pH 7.0–7.4) transferred at 4 °C (11), to the laboratory (11).

**Virus Isolation**
Isolation of the virus was carried out according to Pearson et al 2014. Briefly, suspension of swab contents was inoculated into the allantoic sac of three to five 9-11 days old embryonated specific pathogen free (SPF) chicken eggs and incubated at 37 °C for 2–7 days (12).

**Agar gel immunodiffusion assay (AGID)**

Agar gel immunodiffusion assay (AGID) can detect the presence of the AI virus by detection of the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses. Briefly, the agar gel was prepared with 1 % Noble agar (Difco Laboratories, Detroit, Michigan, USA) containing 8% NaCl in PBS (pH7.4, without calcium and magnesium). The mixture was autoclaved at 121°C for 5 min, stored at room temperature, and melted again as needed. Ten ml of dissolved agar were poured into 100 × 15 mm Petri dishes. Wells were punched in the solid agar such that one central and six peripheral 5.3 mm diameter wells were formed, 2.4 mm apart and in a hexagonal pattern. The peripheral well of each pattern that is, the closest to the perimeter of the dish, was identified as well No. 1 and wells 2–6 counted clockwise from it. Using 50 µL of reagent per well, load reference antigen in the central well and reference serum in wells 1, 3 and 5. Specimens to be tested for antigen should be loaded into wells 2, 4 and 6 (5).

**Molecular analyses**

RNA was extracted from alantoic fluids using High Pure Viral RNA Extraction kit (Roche, Germany) and screened for AIV using RT-PCR primer pair targeting the matrix (M) gene (13). An H5-specific Real time RT-PCR was performed on the AIV-positive samples using Rotor Gen 3000 (Corbett, Australia) and one-step kit (Quanti Tec Multiplex RT-PCR Kit, Germany) to detect if H5 subtype was present.

**Hemagglutination assay (HA) and Hemagglutination Inhibition assay (HI)**
Antigenic subtyping can be accomplished by monospecific antisera prepared against purified or recombinant H and N subtype-specific proteins, used in HI and NI assays. The HA assay was performed by conventional technique in microplates using 1% chicken erythrocytes and incubating at room temperature by a standard method according to (5).

The HI assay was done using 96 ‘U’-well microplates, doubling dilution in PBS, 1% v/v red blood cells (RBC) and 4 HA units of AIV antigen in 25 µl amounts as described by (5).

**Neuraminidase Inhibition assay (NI)**

The NI assay was conducted in glass tubes as described previously Motamed et al., 2015 using all the 9 reference anti Neuraminidase antibodies with Fetuin. In each run, a virus control and blank tubes (just PBS and Fetuin) were considered as reaction controls. At the end of reaction the virus control tube should appear pink, indicating the presence of the NA activity in the sample antigen which reduces Fetuin. A positive tube should appear clear or have only slightly developed color, indicating that the NA activity has been inhibited by the specific reference antiserum. Absence or significant reduction of color in the test tubes containing virus sample and reference NA subtype specific antiserum indicates the certain subtype of NA (15).

**Results**

In the present study, five influenza viruses were isolated from 962 collected swab samples as described further; there were no characteristic lesions in inoculated embryos and the dead eggs. The HI results demonstrated that among the 962 swab samples collected from domestic docks, 5 samples were HA positive. Using AGID assay detecting the nucleocapsid or matrix antigens, specific for avian influenza viruses, the isolates were further investigated. Results of the M gene specific RT-PCR on the allantoic fluid further confirmed the presence of influenza virus (Figure 1). Moreover, Real time RT-PCR using H5 gene-specific primers, came back negative.
Among the 5 isolations which were positive in HA assay, 3 isolates were H3 subtype and 2 were H4 according to HI assay (Figure 2 and 3). However, all the 5 isolates were identified as N2 subtype in NI assay (Figure 4). Therefore, according to the HI and NI assay, two new subtypes of AIV i.e. H3N2 and H4N2 were identified in domestic ducks of Northern provinces of Iran for the first time.

**Discussion**

Although two subtypes of AIVs (H9 and H5) have been previously reported from different Iranian bird species, including the poultry, (16), (17), (18), (19), the current work was the first report of isolation and detection of two different strains of AIVs, i.e. H3N2 and H4N2, from Iranian domestic ducks using only the HI and NI assays.

Domestic ducks have been considered as natural reservoirs of AIV and usually do not show any symptoms of *influenza* disease (20). Ducks could be infected by several different subtypes of AIV simultaneously (21). In other words, AIVs could reassort in ducks to create a new subtype (22).

On the other hand, different species of birds are housed together in LBM, giving the opportunity to viruses to circulate between them. This will automatically result in further reassortment and cross-species transfer (23). According to the results of this study, diagnosis of two new subtypes of AIVs from ducks in LBMs further indicate the possibility of the cross-species virus transfer and reassortment in different bird species, or even to humans.

Although inoculation in embryonated chicken eggs is a time-consuming process, it is the gold standard test as it has widely been used in many laboratories for diagnosis of AIV. The AGID assay is inexpensive and simple test and does not need any out of the ordinary supplies or expensive equipment. However, its sensitivity is not as much as molecular methods such as PCR. In this study, PCR was used simultaneously for assurance of the results. Therefore, HI and NI
assays can be used for identifying of the HA and NA subtypes either for newly-isolated influenza viruses or antiserum samples. These assays are simple, cheap, easily interpretable and handy (5).

In several studies, the H3 and H4 subtypes have been identified in aquatic birds by HI and NI assays (24), (8). Suss et al.,1994 demonstrated that two AIV subtypes including H4N6 and H6N1 were predominant in aquatic birds (8). Also, they isolated H3 AIV from sentinel and domestic ducks. Furthermore, Richard et al., 1974 isolated and detected H3N2 from ducks in California (25). They used HI and NI assays for the subtyping of AIVs, and demonstrated that there was an antigenical relationship between two subtypes of H3N2 and H7N2. In another study, Shortridge et al., 1977 isolated and identified both the H3N2 and H4N2 subtypes from ducks in Hong Kong by similar method (26). Furthermore, Fereidouni et al., 2010 detected H3N8 AIV from wild wintering water birds that visited the southern coast of the Caspian Sea by using RT-PCR (27).

Nearly 1000 swabs were collected in this study and 0.9 % were positive. According to the results, H3 and H4 subtypes could be predominant among duck species in the southern coast of the Caspian Sea at the moment. Isolation of H3N2 AIV could be so important because this subtype of AIV could circulate among different birds and transmit to mammalian species including humans, this making it a threat to public health. Also, domestic ducks are the reservoir of this pathogen and these birds are in close contacts with backyard poultry, animals and even humans. Since AIVs can recombine and cross the interspecies barrier, surveillance of AIV subtypes in ducks especially in LBMs is really important.

Several studies demonstrated that there is close relationships between H3N2 AIV and H5 or H7 AIVs (28), (25), (26). Shi et al., 2016 showed that the H4 subtypes of the AIV can undergo mutations to become more virulent and transmissible during replication in mammals. H4 subtypes
can infect the people who are working with chicken, thus indicating that they can cross the species barriers (29).

In conclusion, in this study two new strains of AIVs H3N2 and H4N2 were detected from domestic ducks for the first time in Iran. Isolation of AIV using SPF embryonated chicken eggs and the detection by HI and NI assays are useful, simple and cheap methods for identifying AIVs, although molecular methods can be used for more accurate identification, characterization and determination of the origin and of these viruses. Annual monitoring of AIV in LBMs for diagnosing new strains and identifying centers of the disease outbreaks in the country are strongly recommended in order to plan a better strategy for control and treatment of influenza.

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Author contribution

AA performed the experiments and wrote the manuscript. AS and MHFM revised the manuscript and supervised the study.

Abbreviation


Conflict of interest

The authors declare that they have no competing interests.

Ethics
Animal handling procedures were performed in line with the national animal welfare regulations. The Institutional Animal Care and Use Committee (IACUC), Razi Vaccine and Serum Research Institute approved all animal experiments.

**Grant Support**

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**Reference**


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Table 1. The primers sequences used for RT-PCR amplification of M gene of AIV (WHO, 2018).

<table>
<thead>
<tr>
<th>Primers</th>
<th>sequence (5 ’ to 3’ )</th>
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<tr>
<td>Forward Primer</td>
<td>AGCAAAAGCAGGATAGATATTGAAAGA</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>AGTAGAAACAAGGTAGTTTTTTACTC</td>
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Table 2. The Primers and probe sequences for Real Time RT-PCR for detection of H5 (Coker et al., 2014).

<table>
<thead>
<tr>
<th>Primer/ probe</th>
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<tbody>
<tr>
<td>Forward Primer</td>
<td>TTATTCAACAGTGCGAG</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>CCAG(T)AAAGATAGACCAGC</td>
</tr>
<tr>
<td>Probe</td>
<td>CCCTAGCCTGGCCAATCATG</td>
</tr>
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Figure 1. Analysis of the specific RT-PCR for detection of M gene. Lane 1: Positive control of M gene AIV (1027 bp band). Lane 2: Marker (100bp DNA ladder). Lane 3-7 were the AIV isolates of this study and lane 8: Negative control.
Figure 2. Hemagglutination Inhibition assay (HI) for detecting of H4.

Figure 3. Hemagglutination Inhibition assay (HI) for detecting of H3.
Figure 4. Neuraminidase-Inhibition assay (NI). From right to left, Tube 1: Blank, Tube 2: N2 Antiserum, Tube 3: N8 Antiserum, Tube 4: N6 Antiserum, Tube 5: N1 Antiserum.