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Original Article

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 Avian Influenza (AI), which is a contagious and zoonotic disease in birds. Among birds, wild waterfowls and ducks are the primary and natural reservoirs of low pathogenic avian influenza viruses (LPAI). This study aimed to identify and differentiate between two AIV subtypes (i.e., hemagglutinin and neuraminidase from domestic ducks by hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) assays. To this end, 962 cloacal swabs were collected from domestic ducks being sold at different Iranian Live Bird Markets in Gilan, Mazandaran, and Golestan provinces, located at the southern coast of the Caspian Sea. The samples were inoculated in 10-day-old embryonated specific pathogen-free chicken eggs, and subsequently, harvested allantoic fluids were subjected to agar gel immunodiffusion, HI, and NI assays. In total, five positive samples, including two H4N2 and three H3N2 AIV subtypes were identified. Isolation of H4N2 and H3N2 viruses has never been reported from Iranian domestic ducks previously. This finding further suggests the diversity of LPAI 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

1. Introduction

Avian Influenza Viruses (AIVs) cause a drastic economic loss to the poultry industry and are considered a threat to global public health (1). AIVs are classified on the basis of two surface glycoproteins, namely hemagglutinin (HA) and neuraminidase (NA) (2, 3). Currently, influenza A viruses are classified into 18 HA (i.e., H1 to H18) and 11 NA (i.e., N1 to N11) subtypes based on the antigenic differences of the HA and NA proteins. Furthermore, two new HA (i.e., H17 and H18) and NA (i.e., N10 and N11) subtypes were proposed for influenza A viruses from bats (4).

Based on their potential to cause disease in chickens, the AI viruses are divided into two main groups of very virulent viruses, termed Highly Pathogenic Avian Influenza (HPAI) and milder viruses, which primarily cause respiratory disease, and therefore, designated as Low Pathogenic Avian Influenza viruses (LPAI) (2, 5).

Influenza viruses can infect a variety of birds, including free-living and captive-caged birds, domestic ducks, chickens, turkeys, and other domestic poultry (2). Ducks are considered the primary reservoir for AIVs (3). It has been shown that the AIVs can replicate in the intestines of ducks without any evident clinical signs and shed the virus in high concentrations in the feces (6). Therefore, domestic ducks could be infected by HPAI viruses and spread large quantities of a virus without obvious clinical signs of the disease, while the

owners or governing livestock officials are not aware (2, 6). Additionally, domestic ducks could play a significant role in introducing and spreading novel viruses in the poultry system by reassorting with other subtypes, resulting in further infections in Live Bird Markets (LBMs) (7).

Based on surface glycoproteins, 16 different HA and 9 different NA subtypes, as well as strains of AIVs have been isolated from ducks in diverse geographic regions of the world (8). Most AIVs isolated from ducks belong to H3, H4, and H6 subtype (9), while the other subtypes of the virus, such as H5, H7, H8, and H9 are generally reported to be rarely prevalent among ducks (10). Regarding NA subtypes, three of them, including N2, N6, and N8, are prevalent among ducks (6). Ducks have not been confirmed as reservoirs of H1, H10, N5, and N7 virus subtypes (9). Several methods, such as the enzyme-linked immunosorbent assay, reverse transcription polymerase chain reaction (RT-PCR), hemagglutinin inhibition (HI), and neuraminidase inhibition (NI) assays can be used in diagnosing the subtypes of AIVs (6). The HI assay was first described by Hirst (11) and then modified by Salk (12). It is extremely reliable, and it is mostly used for global influenza surveillance and determining the antigenic characteristics for influenza viral isolates. The NA assay was described by Warren (13) and modified by Webster and Laver (14).The NI assay has been introduced for many years, and it is used in reference laboratories.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 among domestic ducks from the Northern provinces of Iran by PCR, HI, and NI assays.

2. Materials and Methods

2.1. Sample Collection

A total of 962 cloacal swabs were collected from domestic ducks in multiple LBMs from Gilan, Mazandaran, and Golestan provinces in Iran. The swabs were placed in a transport medium with 2 to 3.5 ml of sterile phosphate-buffered saline (PBS) containing 1% bovine serum albumin and antibiotic (0.1% combination of Penicillin, Gentamicin, and Streptomycin), with pH 7.0 to 7.4, transferred at 4˚C to the laboratory (15).

2.2. Virus Isolation

Isolation of the virus was carried out according to Pearson (16). Briefly, the suspension of swab contents was inoculated into the allantoic sac of three to five embryonated specific-pathogen-free (SPF) chicken eggs which were 9 to 11 days old and incubated at 37˚C for 2 to 7 days (16).

2.3. Agar Gel Immunodiffusion Assay

Agar gel immunodiffusion (AGID) assay can detect the presence of the AIVs by detecting 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 (pH 7.4 without calcium and magnesium). The mixture was autoclaved at 121˚C for 5 min, stored at room temperature, and melted again as required. Ten ml of dissolved agar was poured into 100×15 mm Petri dishes. The wells were then punched in the solid agar in a way that one central and six peripheral 5.3-mm diameter wells were formed, which were 2.4 mm apart in a hexagonal pattern. The peripheral well of each pattern, which was the closest to the perimeter of the dish, was identified as well No. 1. Following that, wells 2 to 6 were counted clockwise according to well No 1.

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 had to be loaded into wells 2, 4, and 6 (5).

2.4. Molecular Analysis

RNA was extracted from allantoic fluids using the High Pure Viral RNA Extraction kit (Roche, Germany) and screened for AIV using RT-PCR primer pair targeting the matrix (M) gene (17) (Table 1). An H5-specific Real-Time RT-PCR was performed on the AIV-positive samples using Rotor Gen 3000 (Corbett, Australia) and a one-step kit (Quanti Tec Multiplex

RT-PCR Kit, Germany) to detect if the H5 subtype was present (18) (Table 2).

Table 1. Primer sequences used for RT-PCR amplification of the *M* gene of AIV

Primers	Sequence (5 ´to 3´)
Forward Primer	AGCAAAAGCAGGTAGATATTGAAAGA
Reverse Primer	AGTAGAAACAAGGTAGTTTTTTACTC

Table 2. Primers and probe sequences for Real-Time RT-PCR for detecting H5

Primer/probe	Sequence (5 ´to 3´)
Forward Primer	TTATTCAACAGTGGCGAG
Reverse Primer	CCAG(T)AAAGATAGACCAGC
Probe	CCCTAGCACTGGCAATCATG

2.5. Hemagglutination Assay and Hemagglutination Inhibition Assay

Antigenic subtyping can be accomplished by monospecific antisera which are prepared against purified or recombinant H and N subtype-specific proteins and are used in HI and NI assays. The HA assay was performed by the conventional technique in microplates using 1% chicken erythrocytes and incubating at room temperature by a standard method, as presented by OIE (5). The HI assay was conducted using 96 'U'-well microplates, doubling dilution in PBS, 1% v/v red blood cells, and four HA units of AIV antigen in 25 µl amounts as described by OIE (5).

2.6. Neuraminidase Inhibition Assay

The NI assay was conducted in glass tubes as described previously by Motamed, Shoushtar (19) using all the nine reference anti neuraminidase antibodies with Fetuin. In each run, virus control and blank tubes (just PBS and Fetuin) were considered the reaction controls. At the end of the reaction process, the virus control tube should become pink, indicating the presence of the NA activity in the sample antigen, which reduces Fetuin. A positive tube should become clear or only develop a slight color, which indicates that the NA activity has been inhibited by the specific reference antiserum. The absence or significant reduction of color in the test tubes containing virus samples and reference NA subtype-specific antiserum indicates certain subtypes of NA (19).

3. Results

In the present study, five influenza viruses were isolated from 962 collected swab samples as described earlier. There were no characteristic lesions in the inoculated embryos and the dead eggs. The HI results demonstrated that among 962 swab samples collected from domestic docks, five samples were HA positive. The isolates were further investigated through AGID assay to detect the nucleocapsid or matrix antigens, specific for AIVs. Results of the *M* gene-specific RT-PCR on the allantoic fluid further confirmed the presence of IV (Figure 1).

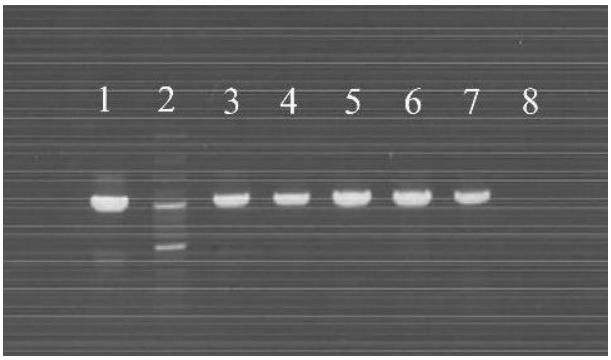

Figure 1. Analysis of the specific RT-PCR for the detection of the *M* gene. Lane 1: Positive control of the *M* gene AIV (1027 bp band). Lane 2: Marker (100bp DNA ladder). Lane 3-7 are the AIV isolates of this study, and lane 8 is the negative control

Moreover, Real-Time RT-PCR using H5 gene-specific primers came back negative. Among the five isolations which were positive in the HA assay, three were H3 subtype, and 2 were H4 according to HI assay (Figures 2 and 3). However, all the five 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 among domestic ducks from the Northern provinces of Iran most probably for the first time.

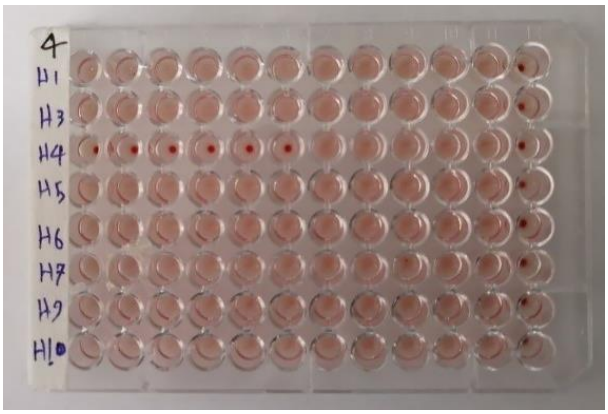

Figure 2. Hemagglutination inhibition assay for detecting H4

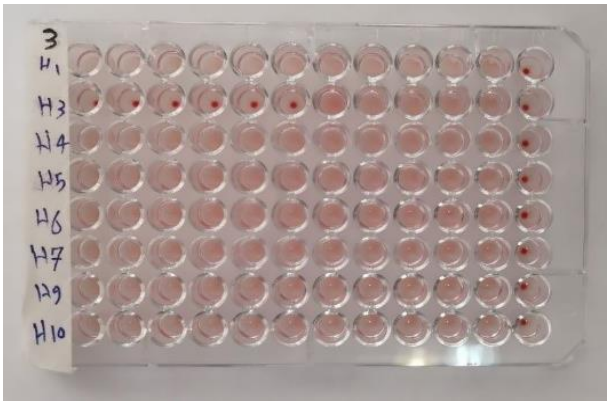

Figure 3. Hemagglutination inhibition assay for detecting H3

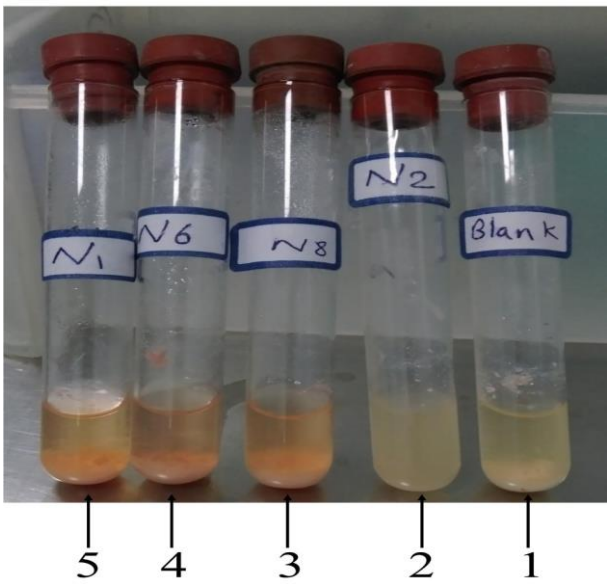

Figure 4. Neuraminidase inhibition assay. From right to left, Tube1: Blank, Tube2: N2 Antiserum, Tube3: N8 Antiserum, Tube 4: N6 Antiserum, and Tube 5: N1 Antiserum

4. Discussion

Although two subtypes of AIVs (i.e., H9 and H5) have been previously reported from different Iranian bird species, including the poultry (20-23), the present study was the first report of the 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 natural reservoirs of AIV, which usually do not show any symptoms of influenza (24). Ducks could be infected by several different subtypes of AIV simultaneously (25). In other words, AIVs could reassort in ducks to create a new subtype (26). On the other hand, different species of birds are housed together in LBM, making it possible for viruses to circulate among them. This will automatically result in further reassortment and cross-species transfer (27). According to the results of this study, the diagnosis of two new subtypes of AIVs among ducks in LBMs further indicates the possibility of cross-species virus transfer and reassortment in different bird species or even virus transfer to humans.

Although inoculation in the embryonated chicken eggs is a time-consuming process, it is the gold standard test, as it has been widely used in many laboratories for the diagnosis of AIV. The AGID assay is an inexpensive and simple test, which does not need any extraordinary supplies or expensive equipment. However, its sensitivity is not as much as molecular methods, such as PCR. In this study, PCR was also used simultaneously for ensuring the reliability of the findings. Therefore, it is revealed that HI and NI assays can be used for identifying 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 previous studies, the H3 and H4 subtypes were identified in aquatic birds by HI and NI assays (8, 28). Suss, Schafer (8) demonstrated that two AIV subtypes (i.e., H4N6 and H6N1) were predominant in aquatic birds, and they also isolated H3 AIV from sentinel and domestic ducks. In another study, Slemons,

Johnson (29) isolated and detected H3N2 from ducks in California. They used HI and NI assays for the subtyping of AIVs and demonstrated an antigenic relationship between the two subtypes of H3N2 and H7N2. Afterward, Shortridge, Butterfield (30) isolated and identified both H3N2 and H4N2 subtypes from ducks in Hong Kong through a similar methodology. Furthermore, Fereidouni, Werner (31) detected H3N8 AIV from wild wintering waterbirds, which visited the southern coast of the Caspian Sea by RT-PCR. Nearly, 1,000 swabs were collected in this study, and 0.9% of the samples were positive. Based on the findings, H3 and H4 subtypes could be currently predominant among duck species on the southern coast of the Caspian Sea. The isolation of H3N2 AIV could be quite important because this subtype of AIV could circulate among different birds and transmit to mammalian species, including humans, thereby threatening public health. In addition, domestic ducks are the reservoir of this pathogen, and they are in close contact with backyard poultry, animals, and even humans. Since AIVs can recombine and cross the interspecies barrier, the surveillance of AIV subtypes among ducks is really essential, especially in the LBMs.

Several studies demonstrated a close relationship between H3N2 AIV and H5 or H7 AIVs (29, 30, 32). Shi, Cui (33) showed that the H4 subtypes of the AIV could undergo mutations and become more virulent and transmissible during replication in mammals. The H4 subtypes can infect people who are working with chickens, thereby indicating that they can cross the species barriers.

In conclusion, in this study, two new strains of AIVs (i.e., H3N2 and H4N2) were detected among domestic ducks most probably for the first time in Iran. The isolation of AIV using SPF embryonated chicken eggs and their detection by HI and NI assays are useful, simple, and cheap methods for identifying AIVs, though molecular methods can be used for more accurate identification, characterization, and determination of the origin 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 controlling and treating influenza.

Abbreviation

AIV: avian influenza virus, LBM: live bird markets, AGID: Agar gel immunodiffusion, LPAIV: low pathogenic avian influenza viruses, NI: neuraminidase inhibition, HI: hemagglutinin inhibition, SPF: specific pathogen-free.

Authors' Contribution

A. A. performed the experiments and wrote the manuscript. A. Sh. and M. H. F. M. revised the manuscript and supervised the study.

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.

Conflict of Interest

The authors declare that they have no conflict of interest.

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