Isolation and characterization of avian influenza from different species of ducks in delta region

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ABSTRACT

In this study, the epidemiological situation of avian influenza in duck species was investigated during the period from July 2013 to January 2015 in 3 governorates in Delta region. The study focused on domestic ducks which have been implicated in the spread of both LPAI and HPAI viruses to domestic poultry and terrestrial birds. In addition, the risk factor arises from the co-circulation of both subtypes H5N1 and H9N2 was considered among poultry populations in Egypt. The HA gene sequence of 6 selected isolates from 17 positive real time RT-PCR was evaluated. The results revealed that 5 isolates were H5 subtype and one isolate was H9 subtype. The phylogenetic analysis of H5 viruses indicates their relatedness to clade 2.2.1, while the H9 virus was related to G1-like lineage. The sequence analysis of H5 nucleotides and amino acids revealed that all isolates are closely related to each other and with identity percent slightly different (95.4 to 98.5%) from the first avian influenza 2006 isolate in Egypt, while H9 isolate showed slightly different (96.7%) identity from the first H9N2 avian influenza isolated in Egypt during 2011. Mutation analysis profile indicates difference among H5N1 isolates as the D70N was shown in one isolate while A25V, K98T, D113V, Y114S were shown in others. The H9N2 isolate showed one specific mutation (S5P and S16N) in comparison to the original 2011 virus. The results from this work indicate the importance of periodical monitoring of circulating viruses especially in endemic countries like Egypt to update the epidemiological situation and that will help the efforts to control.

Keywords: Avian influenza, H5N1, H9N2, duck, HA gene, HA protein, sequence analysis.

INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease affecting several species including birds, which classified into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), depending on the severity of the disease in susceptible birds. HPAI outbreaks in birds have been caused mainly by H5 and H7 subtypes, while H9 and some strains of the H5 and H7 subtypes have been characterized as LPAI (Zhou et al., 1999).

Avian influenza is caused by infection with viruses of the family Orthomyxoviridae placed in the genus Influenzavirus which categorized based on the antigenic properties of the two surface glycoproteins; hemagglutinin and neuraminidase into multiple subtypes, 18 subtypes of HA (H1–18) and 11 subtypes of NA (N1–11) have been found to circulate in avian and/or mammalian hosts. The recent discoveries of the H17N10 subtype of bat influenza A viruses (Tong et al., 2012). While H18N11 was discovered in a Peruvian bat in 2013 (Tong et al., 2013).

Avian influenza virus is enveloped, and pleomorphic with a size ranging from 80-120 nm. The genome of type A influenza is single-stranded, negative-sense RNA and contains eight genome segments that encode 11 proteins (Spackman, 2008). One of these gene products is HA protein which is the major surface antigen and an integral membrane glycoprotein. It is responsible for attachment to the host cell receptor as well as fusion between the virion envelope and host cell and plays a major role in determining
virus pathogenicity. The HA protein also influences host specificity by preferentially binding to one of two different sialic acid receptors on the host cell surface. As found for sialic acid-containing receptors of the epithelial cells in duck intestine (Ito, et al., 2001).

The duck species can shed and spread virus from both the respiratory and intestinal tracts while showing few or no disease signs. While the HP Asian H5N1 viruses are 100% lethal for chickens and other gallinaceous poultry, the absence of disease signs in some duck species has led to the concept that ducks are the “Trojan horses” of H5N1 in their surreptitious spread of virus. Ducks survive infection with highly pathogenic avian influenza strains they are able to fly for long distances, while simultaneously being carriers of HPAI H5N1 (Truszcynski and Samorek 2008).

The aim of this work is to study the epidemiological situation of avian influenza of both circulating H5 and H9 subtypes focusing on duck species in Egypt delta region after isolation and characterization of HA gene by using the standard methodologies.

**MATERIAL AND METHOD**

**Sampling:**

Specimens of 200 suspected avian influenza cases in duck species during the period between February 2013 to October 2014 among Delta region governorates including Damietta, Minufiyah, Dakahlia, Gharbia, and Kafr El-Sheikh was performed. Specimens were from different local duck breed (Mallard, Sudanese, Pekin, Muscovy and Sharman) collected by using tracheal and cloacal swabs loaded to 1-2 ml of phosphate buffer solution (PBS) containing antibiotics. Specimens of 150 suspected avian influenza caseswere collected from backyards while 50 Specimens were collected from commercial farms, all of the samples were chilled in an ice pack immediately after collection and submitted to NLQP (national laboratory quality control on poultry production).

**Real-Time PCR-Examination:**

RNA was extracted from a pool of five cloacal and five tracheal swabs by using a QiaAmp ViralRNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. The extracted RNA was used for RRT-PCR using primers and probe for H5 subtype and H9 subtype detection.

Primers used in RRT-PCR for H5 subtype detection were H5 LH1 (5' - ACG TAT GAC TAC CCG CAG TAT TCA - 3') and H5 RH1 (5’ – AGA CCA GCC ACC ATG ATT GC - 3’) while sequence of H5 Probe used was (5’-FAM-TCW ACA GCC GGT TTC CCT AGCA-TAMRA -3’) (slomka et al., 2007). Primers used in RRT-PCR for H9 subtype detection were forward H9F (GCC ACC TTT TTC AGT CTG ACA TT) and reverse H9R (GGA AGA ATT AAT TAT TAT TGG TCG GTA C) while sequence of H9 Probe used was (Joe- AAC CAG GCC AGA CAT TGC GAG TAA GAT CC- TAMRA) (Ben Shabat et al., 2010).

**Virus Isolation:**

Fertile Specific pathogen Free 9 - 11 dayold Embryonated chicken Eggs (SPF-ECE) were inoculated with 0.1 ml of pooled swab samples through the allantoicroute and the inoculated eggs were incubated at 37°C. Inoculated eggs were examined daily for embryo mortality. Amnio-allantoic fluid was harvested after five days and tested for hemagglutination activity according to therecommended protocol. The test was carried out using 1% chicken erythrocytes in 96-well V-shape plates(Nunc, Wiesbaden, Germany) (Alexander, D.J. 2009).

**HA gene sequencing:**

The amplification of partial HA gene was done by Reverse transcription at 50°C for 30
min, followed by an initial denaturation step at 95°C for 15 min. cDNA was then amplified with 40 cycles of 95°C for 30 seconds, annealing at 56°C for 1 minute and extension at 72°C for 2 minutes. A final extension step was at 72°C for 10 minutes.

Primers used in conventional RT-PCR for H5 subtype were forward HGGT (CTC TTC GAG CAA AAG CAG GGT) and reverse H5-KH3 (TAC CAA CCG TCT ACC ATK CCY TG) while Primers used in conventional RT-PCR for H9 subtype were forward F1-6 (TAG CAA AAG CAG GGG AAT TTC TT) and reverse R-1320 (ATC TTG TAT TTG GTC ATC AAT C) according to national laboratory quality control on poultry production (NLQP).

The amplified product (5µl) were loaded onto 1.5% agarose gel containing 0.5 µg/ml ethidium bromide for nucleic acid visualization. Electrophoresis was conducted using 1x TAE buffer and PCR products were visualized under UV transillumination according to the manufacturer’s instructions.

Gene sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an ABI PRISM® 3100. The primer used in sequencing of H5 gene was (H5-R16) and its sequence (ATG CTG AGC TCA CTC CTG ATG). While the primer used in sequencing of H5 gene was (F1-6) and its sequence (TAG CAA AAG CAG GGG AAT TTC TT) according to (NLQP).

Genetic and Phylogenetic analysis:
Bioedit software was used to make analysis for the sequence of HA gene, BLAST (Basic Local Alignment Search Tool) analysis was initially performed to establish sequence identity to GenBank accessions. For Phylogenetic analysis used MEGA 6 (Molecular Evolutionary Genetics Analysis Version 6.0) which is an integrated tool for conducting automatic and manual sequence alignment. Sequence Distances calculated to display the divergence and identity percent values of each sequence pair in the current alignment by DNA star software (Tamura, K. et al., 2011).

RESULTS

Real time RT-PCR:
Testing of 40 pooled specimens by real time RT-PCR revealed 17 positive cases for subtype H5 and/or H9 of avian influenza virus with percentage of 94.1% for H5 and 29.4% for H9 with mixed samples 23.5% and threshold cycle ranged for positive results from 22.25 to 39.99 for H5 gene and 21.04 to 34.81 for H9 gene.

Virus Isolation:
The positive 17 samples revealed by RRT-PCR were submitted for isolation. The isolated were tested for presence of haemagglutinating agent using the HA test; there were 4 of the tested samples negative while 13 were positive with HA titers ranged from 4-8 log2.

Reverse Transcriptase PCR:
From 17 positive samples revealed by real time RT-PCR we have selected 6 isolates, which are listed as shown in Table (1). As a result of Reverse Transcriptase PCR five isolates from six selected isolates were positive for H5 subtype while one isolate was positive for H9 subtype. The amplification of HA gene for the selected six isolates of study was conducted; the PCR products gave specific bands at 1100 pb in weight for H5 isolates. While H9 isolate gave specific band at 1320 pb in weight.
Table (1): Results of H5 and H9 RT-PCR sequencing

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>H5 subtype</th>
<th>H9 subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-duck-Egypt-1422F-Dak-2-2013</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>A-duck-Egypt-1422F-KFS-3-2013</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>A-duck-Egypt-1422F-GHR-5-2013</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>A-duck-Egypt-1422F-MNF-8-2014</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>A-duck-Egypt-1422F-KFS-2014</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>A-duck-Egypt-1422F-GHR-19-2014</td>
<td>Negative</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Sequence analysis results for HA gene:


Amino Acids identity among H5N1 isolates of delta region ducks obtained in this study was ranged from 96.2 to 100%%, while Amino Acids identity in compare with (A/Duck/Egypt/2253-3/2006) was ranged from 95.4to 98.5%.

Amino acids identity of H9N2 isolate of delta region duck obtained in this study in compare with (A/Quail/Egypt/113413v-2011) was 96.7%.

Phylogenetic analysis:

The phylogenetic relationships between the H5 genes of study isolates and those of selected H5N1 viruses isolated from Egypt and several other countries were analyzed. All the study isolates were closer to the viruses isolated from Egypt in the last few years and belonging to clade 2.2.1.

The phylogenetic relationship between the H9 gene of study isolate and those of selected H9N2 viruses isolated in Egypt and several other countries were analyzed. The study isolate were belonging to viruses from the so-called G1 like viruses.

Figure (1): Sequence alignment for the Amino Acids of HA protein for the selected 5 isolates of H5 gene in comparison with (A/Duck/Egypt/2253-3/2006)
Table (2): Amino Acids differences in H5 protein among the study isolates

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Amino Acid mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A-duck-Egypt-1422F-KFS-2014)</td>
<td>A26V, K99T, D113V, Y114S</td>
</tr>
<tr>
<td>(A-duck-Egypt-1422F-Dak-2-2013)</td>
<td>D70N</td>
</tr>
<tr>
<td>(A-duck-Egypt-1422F-KFS-3-2013)</td>
<td></td>
</tr>
<tr>
<td>(A-duck-Egypt-1422F-GHR-5-2013)</td>
<td></td>
</tr>
<tr>
<td>(A-duck-Egypt-1422F-MNF-8-2014)</td>
<td>There wasn’t found amino acids mutation in the sequenced part.</td>
</tr>
</tbody>
</table>

This table shows that isolate (A-duck-Egypt-1422F-KFS-2014) contain the highest mutation rate among the study isolates.

Figure (2): Alignment report for H9 protein Amino Acids of study isolate

Figure (3): H5 Amino Acids identities percent and divergence of isolates analyzed in this study in comparison with A/Duck/Egypt/2253-3/2006 strain

Figure (4): H9 Amino Acids identity percent and divergence of isolate analyzed in this study in comparison with (A/Quail/Egypt/113413v-2011) strain.
Figure (5): Phylogenetic tree for amino acids of H5 protein of the five AIV isolates analyzed in this study.
DISCUSSION
In this study, we have approached the epidemiological situation of avian influenza in duck species during the period between February 2013 to October 2014 among Delta region governorates including Damietta, Minufiyah, Dakahlia, Gharbia, and Kafr El-Sheikh revealing that avian influenza virus have been circulated in domestic ducks in delta region of Egypt in recent years and were still isolated from different duck breeds and were continuous to spread among different commercial flocks and backyard hold birds.

HA is encoded by RNA segment 4. The HA protein is an integral membrane protein and the major surface antigen of the influenza virus virion. It is responsible for
binding of virions to host cell receptors and for fusion between the virion envelope and the host cell (Webster et al., 1992). Segmentation nature of avian influenza viruses enhance the chance of reassortment between avian strains especially since reported of co-circulation of H9N2 and potentially other LPAI A viruses in Egypt (El-Zoghby et al., 2012 and Arafa et al., 2012).

The co-circulation of both subtypes H5N1 and H9N2 among poultry populations in Egypt enhances the high possibility of an occurrence of reassortment between the two subtypes in the future, where the new reassortant virus may cause serious outbreak in the poultry industry in Egypt with the risk of human infection that may occur by a new mutant virus that give us a great alert.

Domestic ducks that are in contact with wild waterfowl and also other poultry species can act as key intermediaries in the transmission of avian influenza among birds (Li KS et al., 2004). Avian influenza virus infections are constantly monitored in Egypt, not only because of their negative effects on poultry, but also because of the potential spread to humans.

Partial Sequence analysis performed for HA gene H5 and H9 subtypes after using the nucleic acid of selected 6 isolates in conventional Reverse Transcriptase PCR which resulting five isolates from six selected isolates were H5 subtype (A-duck-Egypt-1422F-Dak-2-2013, A-duck-Egypt-1422F-KFS-3-2013, A-duck-Egypt-1422F-GHR-5-2013, A-duck-Egypt-1422F-MNF-8-2014 and A-duck-Egypt-1422F-KFS-2014) while one isolate was H9 subtype (A-duck-Egypt-1422F-GHR-19-2014) as shown in (Table 1).

The investigation of the molecular characterization of H5 nucleotides and amino acids revealed that all study isolates are closely related to each other and slightly different (95.4 to 98.5%) identity from the first avian influenza isolate in Egypt while the sequence analysis of H9 nucleotides and amino acids revealed that study isolate is slightly different (96.7%) identity from the first H9N2 avian influenza isolated in Egypt during year 2011 which indicate continuous genetic evolution of H5N1 and H9N2 viruses.

The phylogenetic analysis of HA gene for duck isolates either based on nucleotide sequence or amino acid sequence showed all H5 isolates belong to clade 2.2.1 while H9 isolate belong to G1 like viruses. The Amino Acid alignment of sequenced part of the H5 gene revealed absence of amino acids variation in 3 isolates (A-duck-Egypt-1422F-KFS-3-2013), (A-duck-Egypt-1422F-GHR-5-2013) and (A-duck-Egypt-1422F-MNF-8-2014) while in (A-duck-Egypt-1422F-Dak-2-2013) there was one amino acid mutation D70N while highest variation rate among the study isolates was in isolate (A-duck-Egypt-1422F-KFS-2014) in positions A25V, K98T, D113V, Y114S as shown in (Figure 1). While the Amino Acid alignment of H9 gene revealed presence of two amino acids variations in isolate (A-duck-Egypt-1422F-GHR-19-2014) in compare with (A/Quail/Egypt/113413v-2011) which are (S5P and S16N) as in (Figure 2).

Aquatic birds including domestic ducks have a silent role in ecology of influenza viruses by its implication in the dissemination and evolution of H5N1 and H9N2 viruses in Egypt which enhancing the high possibility of an occurrence of reassortment between the two subtypes in the future.

REFERENCES

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