Cleavage site stability of Egyptian highly pathogenic avian influenza viruses in backyard chickens during 2009–2011

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Purpose: Two distinguishable subclades of H5N1 (classic and variant strains) are cocirculating among the poultry populations in Egypt despite the intensive vaccination programs. A study to investigate the genetic relationship between avian influenza virus (AIV) isolates from backyard chickens in Sharkia (2009–2011), subclades, and commercially available vaccines was carried out.

Methods: Forty-eight suspected AIV infected birds were clinically examined and used for virus isolation followed by reverse transcription-polymerase chain reaction. Four H5N1 virus isolates were sequenced and analyzed. The intravenous pathogenicity index (IVPI) of three AIV isolates was determined.

Results: Thirty-four hemagglutinating viral agents (30 AIV subtype H5N1 and 4 Newcastle disease virus) were detected. Both the nucleotide and amino acid sequence identities of four H5N1 virus isolates (SHZA-0412/2009, SHZA-0801/2010, SHMK-1903/2010, and SHAH-1403/2011) were high—98.4–99.7% and 100%, respectively—indicative of their genetic homogeneity. The hemagglutinin cleavage site characterization revealed the presence of multiple basic amino acids (–PQRERRRKR/GL–) of the highly pathogenic phenotype. These results were
Introduction

Avian influenza is a contagious disease caused by influenza A virus (AIV) that causes great economic losses in the poultry industry and threatens human health. The avian influenza virus (AIV) is an envelope, negative-sense single-stranded RNA virus belonging to the Orthomyxoviridae family. Surface glycoproteins, hemagglutinin (H), and neuraminidase (N) are used to classify the AIV into 16 H and 9 N subtypes, respectively. Subtype H9 is a low pathogenic virus, whereas subtypes H5 and H7 can be differentiated as low pathogenic or high pathogenic forms that can be distinguished on the basis of their genetic sequence subsequently determining the severity of disease in poultry. The high pathogenic forms of H5 and H7 strains evolved because of mutations, and these mutations resulted in multiple basic amino acids in the connecting peptide between the HA1 and HA2 domains of the HA0 precursor protein. The H5N1 subtype was first confirmed in poultry in Egypt on 17 February 2006, and the virus has been reported in 21 out of 26 Governorates of Egypt. The reemergence of H5N1 resulted in severe outbreaks in vaccinated chickens in the province of Sharkia, Egypt, in October 2007. Despite intensive attempts to eradicate the virus, the endemic status of AIV is reported in Egypt. Continuous viral circulation likely increases the risks of sporadic human infections. In Egypt, the HPAI-H5N1 virus of "clade 2.2.1" first emerged in February 2006, possibly originating from wild ducks. Until April 8, 2011, the epidemic status of AIV has resulted in the culling of more than 30 million birds.

There are two subclades of H5N1 cocirculating in Egypt: the "Classic" 2.2.1 strains, present mainly in backyard birds, and "Variant" 2.2.1 strains, circulating mainly in vaccinated commercial farms since late 2007. These viruses are considered antigenic drift variants that limit the efficiency of the currently used vaccines. The envelope proteins are continuously changing through the processes of shift and drift, giving rise to antigenic variants. The sequence analysis of the endoproteolytic cleavage site within the hemagglutinin (HA) precursor protein HA0 is fundamental for studies of the molecular biology of influenza A viruses. There is an urgent need to investigate the genetic relationship between the Egyptian AIV H5N1 and similar subtypes that cocirculate in neighboring geographic areas.

This study not only characterizes the AIV sequences isolated from backyard chickens reared in different localities in Sharkia, Egypt, during 2009–2011, but also recognizes the genetic relationship between these sequences and that of the two subclades of H5N1 (classic and variant) that currently circulate in Egypt.

Materials and methods

Clinical and postmortem examination

A total number of 48 chickens of different ages (from 3 weeks to 12 months) and breeds suspected to be affected by avian influenza (AI) at different localities in Sharkia between 2009 and 2011 were submitted to clinical and postmortem (PM) examination.

Virus isolation

Tissue samples were collected from affected birds for AI viral isolation and identification. Samples were inoculated in 9–11-day-old SPF embryonated chicken eggs according to the recommendations of the Office International des Epizooties (International Office of Epizootics). At least three successive embryo passages were applied for each sample to be negative. The collected allantoic fluid was screened by slide HA.

Influenza A Rapid Kit and RNA extraction

The HA positive allantoic fluids were tested for the presence of influenza A virus using the Antigen Rapid AIV Ag Test Kit (Synobiotics Corporation, Lyon, France) following the manufacturer’s instructions. Viral RNA from allantoic fluid (HA positive) was purified using the GeneJET RNA purification kit (Fermentas Inc., Maryland, USA) according to the manufacturer’s instructions.

Reverse transcription and polymerase chain reaction

Extracted RNA was transcribed to cDNA using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas Inc., Maryland, USA) following the manufacturer’s instructions. The primers used for HA gene amplification are forward H5-kha-1: 5’-CCTCCAGARTATGCMTAYAAAATTGTC-3’ and reverse

Conclusion

Genetic characterization and IVPI data of backyard H5N1 isolates are indicative of a highly pathogenic avian influenza virus with hemagglutinin cleavage site constancy and two amino acids substitutions with Egyptian classic and variant lineages, suggesting a beginning of antigenic drift.

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H5-kha-3: 5’-TACCAACGTCTACCATKCCY-TG-3’ to amplify a portion of the HA gene spanning the HA cleavage site.15

Sequencing

Four AIV isolates were selected to represent 3 successive years in backyard chickens. Amplicons of the proper molecular size were purified using the GeneJET Gel Extraction Kit (Fermentas Inc., Maryland, USA) following the manufacturer’s instructions, then sequenced (Solgent Co. Ltd., Daejeon, South Korea). The HA subtypes were identified by nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and submitted to GenBank with accession numbers JQ927213, JQ927214, JQ927215, and JQ927216.

Phylogenetic analysis

The nucleotide sequences obtained from polymerase chain reaction (PCR) products were aligned with other HA gene sequences available in GenBank by the Clustal W method, using the MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA). The phylogenetic tree was generated using the neighbor-joining method in MEGA version 5 (www.megasoftware.net). The tree topology was evaluated by 1000 bootstrap analyses.

Intravenous pathogenicity index

The intravenous pathogenicity index (IVPI) of three AIVs—A/chicken/Egypt/SHZA-0412/2009, A/chicken/Egypt/SHMK-1903/2010, and A/chicken/Egypt/SHAH-1403/2011—was determined according to the OIE.14

Results

Clinical and PM examination

The clinical signs of the examined birds included decreased feed and water consumption, ruffled feathers, sinusitis, and lacrimation. Most birds revealed cyanosis of the head, comb, and wattles. Subcutaneous edema of the head and neck with greenish diarrhea, as well as ecchymoses on the shanks and feet were commonly observed. The mortality rate reached 100% in five of the 48 examined farms. The

Figure 1. Clinical manifestation and PM lesion of chickens suspected to be affected by AI. (A) Cyanosis in comb and wattles. (B) Hemorrhage on the shank. (C) Submandibular edema. (D) Subcutaneous hemorrhages. (E) Petechial hemorrhages on coronary fat. (F) Trachea congested with exudates. (G) Petechial and ecchymotic hemorrhages on serosa of intestine. (H) Congested ovary and oviduct. (I) Congested testicles and hemorrhages in kidneys. AI = avian influenza; PM = postmortem.
handlers also observed edema with diffuse subcutaneous hemorrhages on the feet and shanks. Congestion, swelling, and hemorrhages of the conjunctivae as well as hemorrhagic tracheitis with excess mucoid exudates were seen. The lungs were hemorrhagic and congested and filled with exude fluid. Petechiae were noted throughout the serosal surfaces of the abdomen and on the peritoneum. Hemorrhages were also seen on the mucosa and in the glands of the proventriculus, beneath the lining of the gizzard, and in the intestinal mucosa. The kidneys were severely

<table>
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<th>Locality</th>
<th>Year</th>
<th>Breed</th>
<th>Clinical picture</th>
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<tr>
<td>Zagazig</td>
<td>2010</td>
<td>Baladi</td>
<td>Cyanosis in the head, picture of septicemia</td>
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<td>Petechial hemorrhage on coronary fat</td>
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<td>Kafr Sakr</td>
<td>2011</td>
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<td>Respiratory signs, picture of septicemia</td>
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<td>Petechial hemorrhage on coronary fat</td>
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<tr>
<td>Abu Hamad</td>
<td>2011</td>
<td>Cobb</td>
<td>Cyanosis in the head, picture of septicemia</td>
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<td>Hemorrhage on papillae of proventriculus</td>
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<td>Petechial hemorrhage on coronary fat</td>
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<td>Respiratory signs, nervous signs</td>
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<td>Greenish yellow diarrhea</td>
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<td>Severe congestion in pancreas, spleen, and lung</td>
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<td>Catarrhal enteritis</td>
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NDV = Newcastle disease virus.
congested and plugged with ureate deposits. The ovaries and testes were congested with hemorrhage (5/48). Peritonitis (1/48) and pancreatitis with congestion and hemorrhages were seen in some birds (9/48). Hearts were congested with petechial hemorrhages (Fig. 1).

Virus isolation and hemagglutination

The infected embryonated chicken eggs revealed embryo mortality within 24–48 hours. Based on embryo lesions and pattern of mortality, 38 samples showed evidence of AIV infection. The hemagglutinating viruses were detected in 34 out of 48 samples (70.8%). Influenza A virus and Newcastle disease virus (NDV) were detected in 30 samples and four out of 48 birds, representing 62.5% and 8.33%, respectively. Four NDV were isolated from samples collected from Zagazig (n = 1), Abu-Hamad (n = 1), and Kafr Sakr (n = 2), with clinical findings described in Table 1. The HA unit of infective AIV allantoic fluids ranged from 1/32 to 1/1024.

Molecular epidemiology of AIV

The positive HA allantoic fluids were submitted to reverse transcription (RT)-PCR for the detection of the H5 gene. The AIV subtype H5N1 was detected in 30 out of 48 chickens (62.5%) using specific H5N1 primer. The highest percentage of AI virus isolation was detected in Zagazig (41.67%; 20/48) followed by Minet Elkamh (8.33%; 4/48), and in the remaining localities, the rate was 2.08% (1/48). However, there was no virus detection in Awlad Sakr, because clinical signs appeared only in one flock during the period of this study. The trend of AIV isolation in the different localities of Sharkia and the case number analyzed (n/n) are shown in Fig. 2. Among the examined chicken breeds, the baladi (native breed) in the backyard system was the most affected, with a percentage of 100%, 55.1%, and 37.5% in 2009, 2010, and 2011, respectively.

Sequence and phylogenetic analysis

The isolates were determined to be of the H5N1 subtype by PCR assay using specific H5N1 primer and were further characterized by genomic sequencing and the nucleotide BLAST analysis. Sequencing of the HA gene segment revealed that it contained multiple basic amino acids at the cleavage site (– PQRERRRKR/GL –), which were identical to most of the HPAI H5N1 viruses. The HA genome sequence comparison of the A/chicken/Egypt/SHZA-0412/2009, A/chicken/Egypt/SHZA-0801/2010, A/chicken/Egypt/SHMA-1903/2010, and A/chicken/Egypt/SHAH-1403/2011 showed that they share 98.4–99.7% and 100% homology at the nucleotide and amino acid levels, respectively.

Comparative alignment of HA sequences showed that HA genes from four viruses share a similarity of 95.8–99.9% with Egyptian AIV H5N1 subtypes (2006–2011) and 95.5–97.7% with neighboring isolates from Gaza, Israel, Niger, Nigeria, Sudan, Kuwait, and Saudi Arabia. A comparison of the HA gene of the AIV subtype H5N1 isolated in our study was done with some available vaccine strains. The highest similarity for vaccine H5N1 AIV was observed with A/Vietnam/1194/2004 (NIBRG-14/H5N1) at 94.5–95.1%, followed by 93.9–94.5% for A/Goose/Guangdong/1/1996. However, a lower similarity was observed for H5N2 vaccine strains: 89.5–91.9% for A/chicken/Italy/8/1998 (Italy/H5N2), 87.2–87.8% for A/duck/Potsdam/1402-6/1986 (Potsdam/H5N2), and 77.8–78.2% for A/chicken/Mexico/232/94. 1194/2004 (NIBRG-14/H5N1) at 94.5–95.1%, followed by 93.9–94.5% for A/Goose/Guangdong/1/1996. However, a lower similarity was observed for H5N2 vaccine strains: 89.5–91.9% for A/chicken/Italy/8/1998 (Italy/H5N2), 87.2–87.8% for A/duck/Potsdam/1402-6/1986 (Potsdam/H5N2), and 77.8–78.2% for A/chicken/Mexico/232/94.
which showed the lowest similarity. The phylogenetic tree (Fig. 3) based on the 300-bp sequence of the HA gene showed that our AIV isolates are closely related to A/chicken/Egypt/1029/2010, A/chicken/Egypt/NLQP-0918/2009, and A/chicken/Egypt/1012sf/2010. Interestingly, the A/chicken/Egypt/1029/2010 and A/chicken/Egypt/1012sf/2010 were also isolated from backyard chickens in Monofia and Fayoum, respectively. The four sequences had a nucleotide similarity of 97.1–97.7% and 97.4–98.1% with parent, variant and classic lineages, respectively. There were two amino acid mutations of the sequenced part of HA of the viruses with parent, classic and variant strains at positions 249 and 251 (Fig. 4).

IVPI

The three AIVs—A/chicken/Egypt/SHZA-0412/2009, A/chicken/Egypt/SHMK-1903/2010, and A/chicken/Egypt/SHAH-1403/2011—had an IVPI of 2.74, 2.90, and 2.69, respectively, a feature of highly pathogenic avian influenza viruses (HPAIVs).

Discussion

HPAIVs (H5N1) have become endemic in poultry in Egypt since 2006, posing a threat for both the local poultry industry and human health. Despite the intensive efforts to control AIV infection in Egypt, the virus persisted in poultry and evolved into phylogenetically distinguishable, cocirculating lineages.9,10,16 It is important to investigate the current situation of the circulating H5N1 viruses.

To better understand the extent of avian influenza infection among chickens in Sharkia, 48 birds were collected from different localities. The birds showed clinical features suspected to be caused by AIV with mortality rates of up to 100%. The recorded clinical disease and pattern of mortality confirmed the suspicion of AIV infection as previously mentioned by many authors.17–19

Thirty-eight samples succeeded to induce embryo deaths within 72 hours with hemorrhages in embryos that died within 24–48 hours. Hemagglutinating viral agents were detected in 34 allantoic fluids. Although AIVs are hemagglutinating viruses, the HA is not a specific assay for the detection of AIV. Thirty (62.5%) influenza A virus isolates were obtained using a rapid AIV antigen test kit and RT-PCR, but only four NDV isolates were obtained. Negative AIV detection was recorded in 18 samples (37.5%); these findings were consistent with the results of Swayne and Halvorson,20 who explained that AI could not be responsible for the changes observed. The clinical and PM findings induced by AIV are similar to those produced by other viral diseases such as Newcastle virus, avian pneumovirus, other paramyxoviruses, infectious bronchitis, chlamydia, mycoplasma, and other acute bacterial diseases including fowl cholera and Escherichia coli infections.21 Therefore, the four NDV isolates support the abovementioned rationale.

Avirulent influenza A virus can become virulent by the acquisition of certain genetic features, such as multibasic cleavage sites or glycosylation sites in the HA gene, as was seen in the outbreaks of disease in chickens in Pennsylvania 1983 and in Mexico in 1994.22,23 The HA gene was chosen for sequencing because this gene is a major determinant of virulence and pathogenicity of AIV isolates.24,25 In order to characterize the H5N1 virus circulating in Egypt, the ~300-bp HA sequence in our samples were analyzed and aligned with other HA sequences. The sequence analysis of HA revealed that all of the isolates were genetically closely related to each other (sharing a similarity of 95.8–99.9%), suggesting that these isolates share an immediate ancestor. The phylogenetic analysis showed that strains A/chicken/Egypt/SHZA-0412/2009, SHZA-0801/2010, SHMK-1903/2010, and SHAH-1403/2011 are closely related to the H5N1 viruses circulating in Gaza and Israel, suggesting a common virus progenitor, as demonstrated with the phylogenetic analysis of HA genes.26 It is well known that the molecular basis of the pathogenicity of HPAIVs for chickens depends mainly on the cleavability of the...
precursor HA0 into HA1 and HA2 subunits. The presence of multiple basic amino acids at the cleavage site of HA0 is characteristic of the HP viruses, and HA0 can be cleaved by ubiquitous intracellular proteases, such as furins and nontrypsin-like proteases. This enables the virus to disseminate systemically and to replicate in extrapulmonary organs, including the brain and the spleen, causing fatal disease and death of the birds. The HA protein of our four isolates harbor multiple basic amino acids (321PQGERRRKKR/G331) at the cleavage region, which is a characteristic feature of HPAIVs. The motif of multiple basic amino acids at the cleavage site of HA is seen in the HPAIV of the H5 subtype in Egypt.

Furthermore, HA sequence characterization besides the IVPI indicated that all the viruses were of the highly pathogenic phenotype with genetic stability of the HA cleavage site. A comparison of the representative subtype H5N1 viruses in 2009–2011 with Egyptian classic and variant lineages revealed two amino acid substitutions—alanine to proline (A249P) and asparagine to tyrosine (N251Y)—which could be indicative of the beginning of an antigenic drift.

Interestingly, the amino acid substitution N251Y in our isolates was similar to that of the vaccinal strains, either HA0 can be cleaved by ubiquitous intracellular proteases, such as furins and nontrypsin-like proteases. This enables the virus to disseminate systemically and to replicate in extrapulmonary organs, including the brain and the spleen, causing fatal disease and death of the birds. The HA protein of our four isolates harbor multiple basic amino acids (321PQGERRRKKR/G331) at the cleavage region, which is a characteristic feature of HPAIVs. The motif of multiple basic amino acids at the cleavage site of HA is seen in the HPAIV of the H5 subtype in Egypt.

Conflicts of interest
All authors declare that they have no conflicts of interest.

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