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ORIGINAL ARTICLE

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 (–PQRERRRKKR/GL–) of the highly pathogenic phenotype. These results were

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supported by IVPI in chickens of 2.69—2.90. The similarity of our isolates with H5N1 AIV vaccine strains (93.9—95.1%) was higher than that with H5N2 strains (77.8—91.9%). The divergence of four sequences with classic and variant lineages is 2—2.7% and 2.3—3%, respectively, with two amino acid substitutions (A249P and N251Y).
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.

Introduction

Avian influenza is a contagious disease caused by influenza A virus causing great economic losses in the poultry industry and threatening human health.[1,2] 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.[3] 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.[1]

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.[4] The reemergence of H5N1 resulted in severe outbreaks in vaccinated chickens in the province of Sharkia, Egypt, in October 2007.[5] 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.[6] In Egypt, the HPAI-H5N1 virus of "clade 2.2.1" first emerged in February 2006, possibly originating from wild ducks.[7] Until April 8, 2011, the epidemic status of AIV has resulted in the culling of more than 30 million birds.[8]

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.[9—11] These viruses are considered antigenic drift variants that limit the efficiency of the currently used vaccines.[12] 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.[13]

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 Épizooties (International Office of Epizootics).[14] 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