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Molecular Characterization and Phylogenetic Analysis of Avian Influenza Virus Circulating in Kafrelsheikh Governorate, Egypt

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Abstract: Avian influenza (AI) is a viral disease affecting the respiratory, digestive and/or the nervous system of many species of birds. The present study was designed for isolation and molecular characterization of AI viruses circulating among different regions in Kafrelsheikh, Egypt. The results revealed 14/25 (56%) samples were positive for type A influenza (M gene) from which only one sample (4%) showed identity for both M and H5. Sequence analysis of the HA gene of A/Chicken/Egypt (KFS)/2013/H5, revealed genetic homology to almost all the Egyptian strains isolated during 2010-2012.While analysis of the nucleotide sequences of the M gene of the A/Chicken/Egypt (KFS)/2013/H9N2 isolate showed its close relationship with A/chicken/Egypt/ BSU-CU/2011 and a group of recent Israeli strains. The presence of H9N2 viruses, which are co-circulating with H5N1, make thegenetic characterization of influenza viruses circulating in Egypt of great importance for continuous monitoring of genetic and antigenic changes in AI viruses as an early detection system for new variants.

Key words: Avian Influenza · H5 · H9 · Egypt

INTRODUCTION

Poultry sector is one of the most important segments of the agriculture sector in Egypt, where investment in this industry is about 20 billion Egyptian pounds [1]. This sector has faced serious challenges through Avian Influenza viral infection [2]. Avian influenza is a viral disease affecting the respiratory, digestive and/or the nervous system of many species of birds, both domestic and wild [3]. It is a highly contagious notifiable disease of birds, which is caused by a highly heterogeneous group of viruses with varying pathogenicity in different species [4]. Avian Influenza viruses are a public health threat worldwide because they are usually associated with severe illness and consequently a high risk of death [5]. The viruses are enveloped with an 8-segment single stranded RNA genome with high potential for in situ recombination. Two segments code for haemagglutinin (H) and neuraminidase (N) antigens used for host cell entry. At present 16 (H) and 9 (N) subtypes are known, for a total of 144 possible different influenza subtypes [6]. Avian influenza viruses are divided into two pathotypes: high pathogenicity avian influenza (HPAI) viruses, which cause severe and fatal infections in chickens and the low pathogenicity avian influenza (LPAI) viruses, which are generally much less virulent in these birds. LPAI viruses can contain any haemagglutinin, but to date all HPAI viruses have contained either H5 or H7 [7].

In mid February 2006 a devasting HPAI H5N1 infected the commercial poultry production sectors and backyards in Egypt. The outbreak caused great socioeconomic losses in poultry industry and still considered a renewable problem with a possible endemic [8]. The virus has been detected in wild, feral and zoo

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birds and recently was found in donkeys and pigs. Most of the outbreaks in poultry and humans occurred in the highly populated Nile Delta [2].

Laboratory diagnosis of AIV is routinely performed by isolation and identification of virus either in embryonated chicken eggs or in cell cultures followed by typing of isolated virus by haemagglutination inhibition test and /or RT-PCR assays [9]. PCR assay is the most sensitive and rapid test for the detection of the virus from clinical samples, which might appear negative due to inappropriate sampling, or loss of infectivity during shipment [10]. In this study we report the isolation, molecular characterization and phylogenetic analysis of AI viruses isolated from field samples collected from broiler and layer chicken flocks in Kafrelsheikh Governorate (North of Egypt) during the period from October 2011 till February 2012.

MATERIALS AND METHODS

Source and Collection of Samples: A total of 25 fresh postmortem tissue samples (lung, trachea) were collectedfrom clinically diseased birds in 25 different commercial poultry flocks located in Kafrelsheikh governorateand experiencing severe respiratory manifestations and relatively high mortality rates during the period from October 2011 till February 2012. The samples were immediately transported on ice to the lab and stored at -80°C till used.

Virus Isolation: Organ types were pooled and blended to prepare 10% suspension in PBS (pH 7.2) solutioncontaining Penicillin (1 million IU/L), Gentamycin (250 mg/L), Streptomycin (200 mg/L) and Enrofloxacin (200 mg/L). It was centrifuged at 800 xg for 10 min at 10°C.The supernatant fluid of each sample was inoculated into three 9-11 days old embryonated chicken eggs via the allantoic cavity route. The eggs were incubated at 37°C for 4-6 days and were examined daily. Before harvesting, the eggs were chilled at 4°C and the allantoic fluids were used for further evaluation by haemagglutination test.

Haemagglutination Test: In a V-bottomed microtiteration plate, 50 µl of PBS were added to all wells.50 µl of the infected allantoic fluidwere added to the first well only to make two-fold serial dilution across the plate. 50 µl of 1% washed chicken RBCs suspension were added to all wells. The plate was incubated at 4°C until the control RBCs shows a clear dark red button shape in the bottom of the well. The HA titers were recorded as the reciprocal of the highest dilution of virus suspensions showing complete haemagglutination.

RNA Extraction: RNA was extracted using Trizol Easy-Red™ Total RNA extraction Kit (Intron Biotechnology, Korea) as per manufacturer'sprotocol. Briefly, 500 µlof infected allantoic fluid were mixed with 750 µl of Trizol reagent followed by centrifugation at 10,000 rpm for 10 min. The supernatant was transferred to a fresh DEPC treated tube and added with 200 µl of chilled chloroform. The mixture was left at room temperature for15 min. The same was later centrifuged at 10,000 rpm for15 min at 4°C. The upper aqueous phase was thentransferred to a fresh tube and 500 µl of chilled isopropanolwas added followed by centrifugation at 10,000 rpm for 10min at 4°C. The supernatant was discarded and the pellet was firstwashed with 100% followed by 70% ice-cold ethanol. Finally the pellet was re-suspended in 30 µl RNAse free water and stored at -80°C for further analysis. Moreover, theextracted RNA was analyzed for its quality and quantityusing Nano-drop method. Briefly, the equipment was first activated with 2 µlof RNA/DNA free water. Later the same amount of samplewas placed on the machine pedestal and the setting for"RNA" selected. The system measures the quality andquantity of RNA in the given sample.

Reverse Transcription of Extracted RNA to cDNA: The reverse transcription (RT) reaction was performed using Maxime™ RT PreMix Kit (GeneON, GmbH, Germany) with oligo (dt) 18 primers.Briefly, a mixture of 5 µl of extracted RNA,1 µl of OligodT and 2 µl of Sterile RNAs free water were prepared and incubated at 70 °C for 10 min.Then placed on ice for 5 min. Another mixture of 4 µl 5X reaction buffer, 1 µl dNTP mix (10 mM of each =40mM), 1 µl RNAs inhibitor, 1 µl MMLV Reverase (200µ/ µl) and 13 µl sterile RNAse free water was prepared. Mix I and Mix II were combined, gently vortexed and incubated in heat block at 55°C for 90 min, then at 70°C for 10 min for inactivation of the enzyme. cDNA was stored at -20°C for further applications.

Multiplex PCR Reaction: Two sets of oligonucleotide primers that specifically amplify type A influenza virus (Matrix protein gene) and the haemagglutinin (HA gene) of H5 subtype were commercially synthesized (Metabion international AG, Germany) and listed in Table 1. The mPCR reaction employed in this study was performed in

Table 1: Oligonucleotide primers used in PCR reaction

Primer name	Oligonucleotide sequence	Target gene	bp	Reference
AIM-F	CTTCTAACCGAGGTCGAAACG	M gene		
AIM-R	AGGGCATTTTGGACAAAGCGTCTA	type A	244bp	[11]
H5-F	ACTATGAAGAATTGAAACACCT	H gene		
H5-R	GCAATGAAATTTCCATTACTCTC	H5 subtype	456bp	[12]

AIM-F = AI forward primer; AIM-R= AI reverse primer; H5-F = H5 forward primer; H5-R = H5 reverse primer; M gene = matrix protein gene; H gene = haemagglutinin gene

50 µl volumes, in which the reaction mixture consisted of 10 µl of 5X Master Mix (Taq/High Yield-Jena Bioscience, Jena, Germany) (5X Conc. of thermostable DNA Polymerase, dATP, dCTP, dGTP, dTTP, $(NH4)SO_4$, MgCl, Tween 20, Noniodet P-40, stabilizers), 1 µl of forward and reverse primers of M gene and H5 subtype, 5 µl of cDNA and 31 µl of PCR grade water. The mPCR was performed in (Bio Rad T100 thermal cycler). The cycling protocol consisted of initial denaturation at 96°C for 5 min followed by 40 cycles of denaturation at 95°C /1min, annealing at 53°C /1min and extension at 70°C /1min with a final extension at 72 °C /10 min. A negative control containing only PCR master mix, primers and PCR grade water was also included.

PCR products were analyzed by agarose gel electrophoresis using 2% agarose. Amplified products were visualized by ultraviolet light trans illumination after staining with 0.1µg/ml-ethidium bromides. A 100bp ladder (GeneON, GmbH, Germany) was used as a molecular weight marker.

Sequencing and Phylogenetic Analysis: To identify the AIV isolates sequences of the M andHA genes of the isolates were compared with the published AIV sequences deposited in the Gene Bank database using a BLAST search via the National Center of Biotechnology Information (USA). Sequence identities by BLAST analysis were included in alignment and phylogenetic construction.

RESULTS

Virus Isolation and Haemagglutination Test: The inoculated ECE were daily examined and the embryo mortality was recorded. Embryonic deaths occurred between 2-5 days post inoculation in 20 out of 25 inoculated samples (Fig. 1). Most of the isolated samples were recovered from the first egg passage. All samples resulting in embryonic death showed complete haemagglutination reaction.

Identification and Subtyping of the Isolated Viruses Using Multiplex PCR: The collected allantoic fluids were tested for the presence of influenza A virus by detection of the matrix protein (M) gene and subtyped for H5haemagglutinin using specific (M) and (H5) gene primers that are directed to the most conserved regions in the (M) and (H5) genes.

A multiplex PCR reaction was applied on 20 samples showing positive reactivity in virus isolation and haemagglutination test. The results revealed that 14 (56%) samples were positive for type A influenza (M gene) as they show amplification of only one specific band (244 bp), while only one sample (4%) showed identity for both M and H5 gene by amplification of two specific bands (244 bp and 456 bp respectively) as shown in (Fig. 2).

Sequencing and Phylogenetic Analysis: The viruses isolated in this study were designated as A/Chicken/KFS/2013/H9N2 and A/Chicken/KFS/2013/H5. Nucleotide sequence analysis of the M gene of A/Chicken/KFS/2013/H9N2 revealed the closest genetic similarity to the Egyptian isolate A/chicken/Egypt/BSU-CU/2011(H9N2) with a nucleotide identity of 99% and was also closelyrelated to the other Middle East H9N2 strains as it shows 97% homology with the Israeli strains A/chicken/Israel/1167/2010(H9N2), A/chicken/Israel/951/2007(H9N2), A/chicken/Israel/702/2008(H9N2), A/chicken/Israel/184/2009(H9N2) and A/turkey/Israel/1608/2006(H9N2).In addition it revealed 96% identity with other Asian and Egyptian strains as (A/chicken/India/IVRI-0011/2011(H9N2), A/chicken/Iran/AGH-B4/2012(H9N2), A/chicken/El-Fayoum/CAI25/2011(H9N2) and A/chicken/Egypt/S4456B/2011(H9N2) as shown in Fig. 3 and Fig. 5A.

Concerning the other isolate, nucleotide sequence analysis of the HA gene of A/Chicken/KFS/2013/H5 revealed 97- 99% genetic homology to almost all the

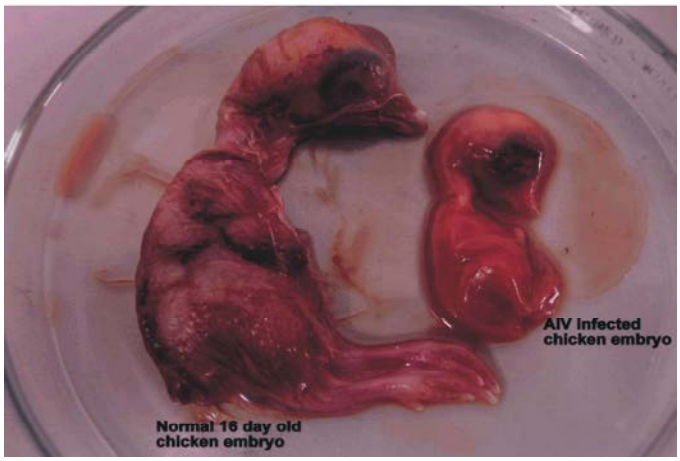

Fig. 1: The of embryo infected with AIV compared to a normal non inoculated 16-day-old embryo

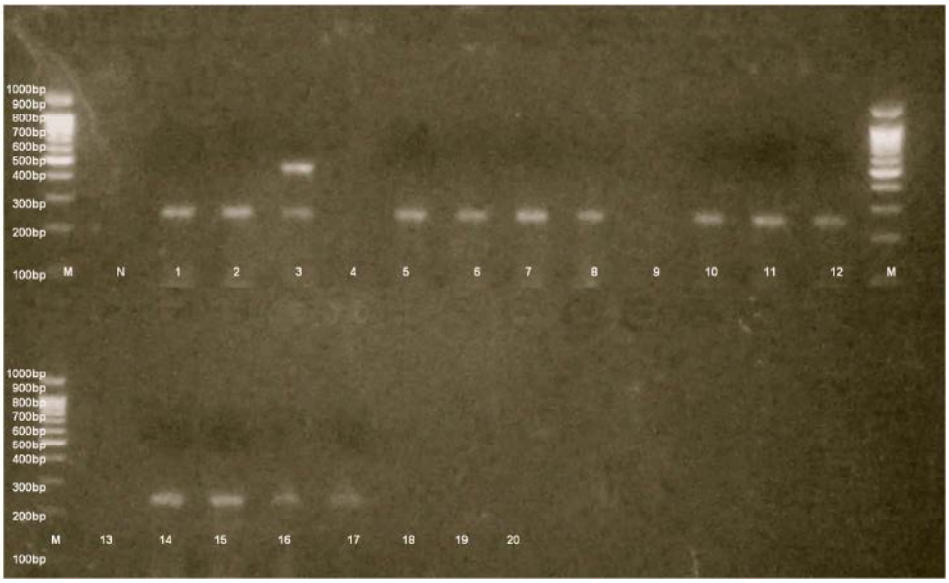

Fig. 2: Agarose gel electrophoresis of multiplex PCR amplified products Lane M; 100bp DNA size marker. Lane N; Negative control. Lanes 1, 2, 5, 6, 7, 8, 10, 11, 12, 14, 15, 16 and 17 showed positive amplification of M gene only (244 bp). Lane 3; showed positive amplification of both M (244 bp) and H5 (456 bp) genes. Lanes 4, 9, 13, 18, 19 and 20 showed negative samples with no amplification

Egyptian strainsisolated from different poultry species during 2010-2012 e.g. A/pigeon/Egypt/SHAH-5803/2011(H5), A/quail/Egypt/1171SG/2011(H5N1) A/duck/Egypt/Q4596C/2012(H5N1), A/harrier/Israel/531/2011(H5N1), A/chicken/Zagazig/C6/2011(H5), A/ostrich/Egypt/11139F/2011(H5N1), A/chicken/Egypt/10347SF/2010(H5N1), A/goose/Egypt/11162S/2011(H5N1) and A/duck/Egypt/11106SF/2011(H5N1, in addition to the Israeli isolate A/harrier/Israel/531/2011/H5N1 as shown in Fig. 4 and Fig. 5B.

DISCUSSION

Avian influenza viruses are the major cause of acute respiratory tract infection of chickens and every year bring about high morbidity and mortality worldwide [10]. Therefore, the early detection and identification of AI viruses are paramount in their surveillance in a situation [13]. PCR assay is the most sensitive and rapid test for the detection of viruses from clinical samples, which might appear negative due to inappropriate sampling or loss infectivity during shipment [14].

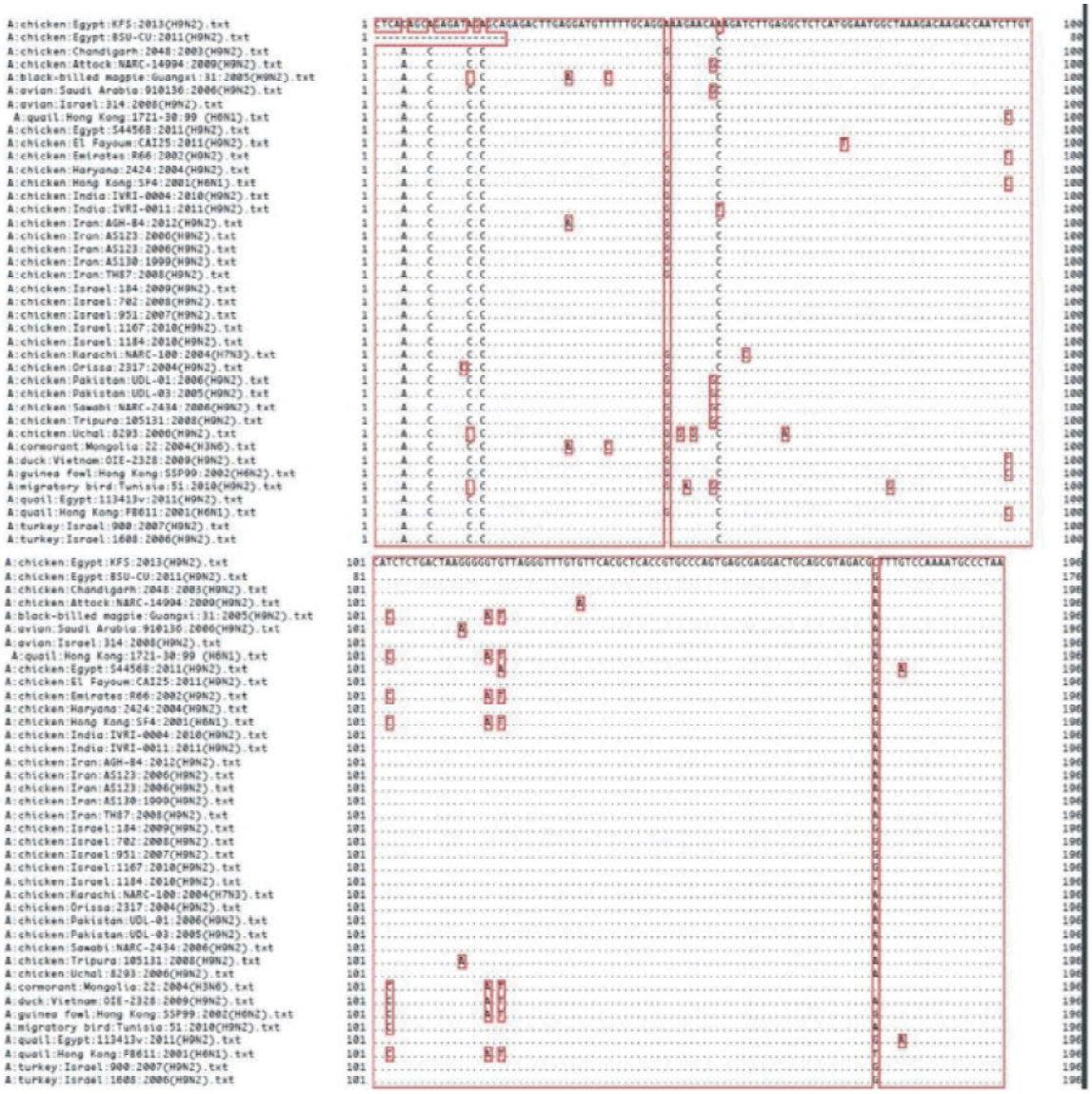

Fig. 3: Nucleotide sequence alignment of M gene sequences of A/chicken/KFS/2013/H9N2 isolate in comparison with that of consensus sequence made with earlier isolates (Similarities shown as dots)

In the current study, we described the isolation of AI viruses from chicken flocks suffering from respiratory distress with relatively high mortality rates. The viruses were isolated from Kafrelsheikh Governorate in the Northern part of Egypt. All the collected samples were inoculated into ECE and tested using HA test. The positive samples were confirmed as AIV and subtyped as H5 through mPCR assay. Only 14/25 (56%) of the samples were identified as Influenza A viruses, from which only one sample (4%) was subtyped as H5. The remaining AIV negative samples may possibly contain AIV similar respiratory viruses of potential haemagglutinating activity as Newcastle disease virus. The other 13 samples with negative H5 reaction were probably of the H9 subtype as shown from the sequencing results. Some earlier studies indicate that, low pathogenic Avian Influenza (LPAI) is more commonly isolated from poultry and the clinical signs are usually mild respiratory disease [15]. Although, the increased mortality that associated withthe disease described here, might be due to additional factors that affected the case severity and allowed the LPAIvirus to cause severe signs such as co-infection with other pathogens.

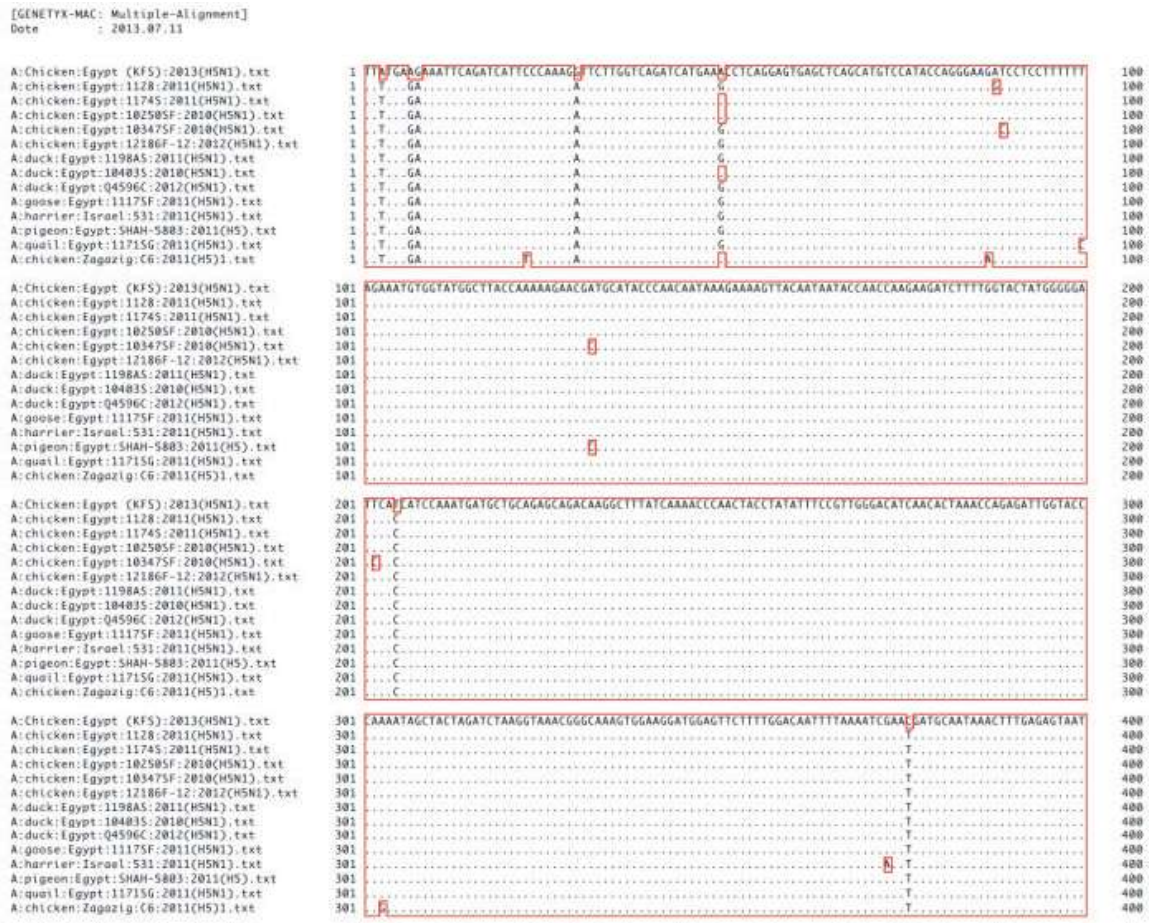

Fig. 4: Nucleotide sequence alignment of HA gene sequences of A/chicken/KFS/2013/H5 isolate in comparison with that of consensus sequence made with earlier isolates (Similarities shown as dots)

The high percentage of AIV of non H5 subtype (52%) reported in this study addresses the strong need for more aggressive monitoring and vaccination of the susceptible and already vaccinated poultry flocks, as Egypt embark mainly on inactivated H5N1 and H5N2 vaccines [16], to limit the spread of the virus and minimize its socioeconomic impacts as the vaccine should be effective against current local strains [5]. Recently, Influenza A subtype H9N2 were isolated from many H5N1 vaccinated flocks suffering from mild influenza like symptoms [17]. Inter-subtype reassortments have been detected between the co-circulating H9N2 and the highly pathogenic H5N1 viruses [18]. These finding together with the endemic nature of the H5N1 virus infection in Egypt [19], make the genetic characterization of other avian viruses circulating in Egypt important for screening the evolution of new strains with increased virulence to mammals.

Phylogenetic analysis of the nucleotide sequences from the M gene of theA/Chicken/KFS/2013/H9N2 isolate showed that it was closely related (99%) to the recently isolated Egyptian strain A/chicken/Egypt/BSU-CU/2011, in addition toits high similarity with a group of recent Israeli strains (97 %) circulating from 2006–2010 which agreed with [17]. This isolate also shows identity to other H9N2 Middle East strains specially the A/chicken/India/IVRI 0011/2011 and A/chicken/Iran/AGH-B4/2012 strains with genetic homology (96%).

Nucleotide sequence analysis of the HA gene of the other isolate A/Chicken/KFS/2013/H5, revealed 99% genetic homology to the Egyptian strains A/chicken/ Egypt/1174S/2011(H5N1), A/chicken/ Egypt/10250SF/ 2010(H5N1) and (A/duck/Egypt/ 10403S/2010(H5N1). While it shows 98% homology toother strains as A/quail/ Egypt/1171SG/2011(H5N1), A/duck/Egypt/ 11106SF/ 2011 (H5N1), A/duck/ Egypt/ Q4596C/2012 (H5N1), A/chicken/

Fig. 5: A. Phylogenetic tree based on nucleotide sequences of the HA genes of the Egyptian isolate A/ Chicken/ KFS/ 2013/H9N2 in comparison to other strains B. Phylogenetic tree based on nucleotide sequences of the HA genes of the Egyptian isolate A/ Chicken/ KFS/ 2013/H5 in comparison to other strains

Zagazig/ C6/2011(H5) and A/ostrich/ Egypt/ 11139F/ 2011(H5N1) in addition to the Israeli isolate A/harrier/ Israel/ 531/2011 (H5N1). More dissimilarity was shown to A/goose/Egypt/ 11162S/2011(H5N1), A/pigeon/ Egypt/SHAH-5803/ 2011(H5) and A/chicken/ Egypt/10347SF/2010(H5N1) as they share 97% homology.

Circulation of AIV H5 subtypes in vaccinatedbirds continues to devastate the poultry industry in Egypt. Birds in backyards and live bird markets (LBM) remain the main potential source of H5N1 infection to both commercial poultry and humans in Egypt [16], despite repeated vaccination of chickens using a homologous H5N1 vaccine. Egyptian isolates emphasizes the need for continuous monitoring of genetic and antigenic changes in AI viruses as an early detection system for new variants.

In addition, to the above arguments it is also concluded that the primers sets tested in this study can be used successfully in Egypt and also endorses the homogeneity in genome and circulating nature of these viruses, which might had being transported through the migratory birds from other countries.

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