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Phylogenetic Analysis of Duck and Turkey H5N1 Ai Strains Isolated in 2014 in Egypt

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Abstract: Highly pathogenic avian influenza (HPAI) caused by the influenza A H5N1 virus, represent significant threat to the poultry industry and humans in Egypt. Silently infected free ranging ducks and geese as well as mixed species in backyard holdings are suspected to play essential role in spread of virus and most of human cases occur in this sector. In this study two samples; one from turkey and another one from ducks recently isolated from backyard were processed in Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP) from January 2014 till March 2014 and they were examined by real time reverse transcriptase PCR for M and H5 genes of AI and the results revealed that both samples were positive for M and H5 genes. The same isolates had been examined by conventional RT-PCR for H5 Gene and exhibited the same findings. Isolation of the two samples through inoculation on SPF-ECE via allantoic route and resulted in chicken embryo death within 48h and gave positive HA activity for both samples. The 300bp fragment of the PCR product had been subjected to sequencing procedures. Sequencing of H5 gene of isolates revealed that both isolates were highly pathogenic contain multiple basic amino acids cleavage site and belong to sub clade 2.2.1.These results indicate that AIVs continue to circulate in backyard between turkey and ducks with no great variation and mutate leading to antigenic drift but mutations are the same as that firstly appeared in 2006, 2007. 2008 indicating that no drastic changes recently acquired to PCS (proteolytic cleavage site).

Key words: Highly Pathogenic Avian Influenza (HPAI) · H5N1 · HA · Proteolytic Cleavage Site (PCS) · Real-Time RT-PCR · Conventional RT-PCR · Aivs (Avian Influenza Viruses) · Sequencing · Phylogenetic Analysis

INTRODUCTION

Influenza A virus is the only orthomyxo virus known to infect birds. It's negative sense single stranded enveloped virus contain genome composed of eight separate ribonucleic acids (RNA) segments encode for at least 11 viral proteins; Two surface glycoprotein haemagglutinin (HA1,HA2) and neuraminidase (NA) are playing a vital role in attachment and release of the virus respectively [1].

The World Organization for Animal Health (OIE) that codifies sanitary and health standards and is affiliated to the World Trade Organization (WTO), has included HPAI as a List A reportable Disease [2]. Countries with representation in the WTO are obliged to report outbreaks of HPAI. Any AI virus that fits into either one of the following criteria is considered an HPAI virus: Lethal for six, seven, or eight 4 to 6 week-old chickens within 10 days following intravenous inoculation with 0.2 ml of a 1:10 dilution of virus in a bacteria-free, allantoic fluid, If It has a polybasic amino acid region at the hemagglutinin cleavage site and if it is of the H5 or H7 subtype or if it is not an H5 or H7 virus but kills one to five chickens and grows in cell culture in the absence of trypsin [3].

In Egypt, by 17th of February, 2006, severe outbreak of H5N1 HPAI, has officially reported and the disease has spread in several governorates and associated with drastic mortality up to 100% in infected chickens [4-6]. Extensive surveillance and genetic studies have revealed that, H5N1 viruses had become endemic in poultry in many countries including Egypt [7].

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The aims of this work is to determine the difference in proteolytic cleavage site (pcs) in HPAI from Egyptian isolates recently isolated in 2014 in Egypt and correlation with older ones by sequencing of HA gene cleavage site (HACS) (300bp)of Egyptian AI H5N1 isolates among backyard.

MATERIALS AND METHODS

Sampling: In this study 2 suspected cases were sampled from backyard system from Fayoum Governorate in Egypt, Specimens from poultry included 2 cases (one from Turkey and another one from duck), each sample was represented by 5 tracheal and 5 cloacal swabs and were processed in Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute during the period from January 2014 to march 2014.

Virus Isolation: Specific pathogen free eggs (SPF) ECE 9-11 day were used for isolation and propagation of virus isolates. They were obtained from (SPF production project, Fayoum, Egypt).Inoculation of specific pathogen free (SPF) embryonated chicken eggs (ECG) via allantoic route(200ul/egg): Procedures were performed according to OIE [10], then Haemagglutination test to determine ability of this virus to make agglutination to chicken RBCs 1%. Procedures were performed according to OIE [10].

Molecular Characterization: RNA was extracted from pools of tracheal and cloacal swabs by using QIAamp Viral RNA Mini Kit(Qiagen, Germany, procedure was performed according to the kit's instruction. Then master mix was prepared by using quantitect kit for amplification of the extracted product. Published Sequence of AI common type A primers& probe were used in one step real time PCR acc. to Spackman *et al*. [8].Also published Sequence of AI H5 primers& probe were used in one step real time PCR acc. to Slomka *et al*. [9]. Gel electrophoresis was used to determine proteolytic cleavage site (PCS) by the use of specific primers for PCS (Kh1 and Kh3). The amplified products were run on 1.5% agarose gel and the appearance of specific band at 300 bp confirmed the presence of the virus.

Sequencing: The amplified PCR products from gel electrophoresis were purified using the Qiagen PCR purification kit and then submitted for sequencing to gene analysis unit in RLQP Egypt, Animl Health Research Institute, the sequencing was done in both directions using the same primers as used in RT-PCR reactions (gel electrophoresis). Forward and reverse sequences were aligned together using sequencher software (www.msi.umn.edu).followed by BLAST analysis (www.ncbi.nlm.nih.gov) acc. to Altschul *et al*. [11]. The nucleotide sequences thus obtained were aligned by the clustal w method using MEGA 6.0 software. A phylogenetic tree of aligned sequences was constructed by the neighbor-joining method.

GenBank Accession Numbers: Partial nucleotide sequences of the Egyptian HPAI HACS of (turkey and ducks) were submitted to GenBank to get accession numbers.

RESULTS

Results of Isolation, Detection, Identification and Phylogenetic Analysis for Recent HPAI H5N1 Viruses Circulating in Fayoum Governorate

The Study Included 3 Stages

First Stage: Confirmation of avian influenza isolates by isolation on SPF ECE (9-11) days and HA titer then Real Time RT PCR (RRT-PCR) and gel electrophoresis.

Second Stage: Sequencing of HA gene (proteolytic cleavage site) of HPAI H5N1 isolates.

Third Stage: Analysis of sequencing data.

Confirmation of Avian Influenza Isolates: Two Egyptian strains were tested (one duck and one turkey) from household poultry sector from Fayoum Governorate. These strains represent recent Egyptian HPAI H5N1 viruses in 2014 in this governorate. Isolation of the virus on SPF ECE at (9-11) days old chick via allantoic route resulted in embryo death within first 48hr followed by determination of HA activity and Real time PCR for detection of type A and H5 AI as shown in Table (1). both isolates were positive by reverse transcriptase RT-PCR using specific primer (Kha-1/ Kha-3) and the specified band appeared at 300 pb.

Results of Sequence Analysis: Both samples had the same nucleotide sequence with one silent nucleotide mutation coding the same AA without any change in AA sequence between them. Both have the same SPQGEKRRKKRGLF amino acid pattern for cleavage site.

By converting nucleic acid into protein through MegAlign software which changed nucleic acid into AA. The proteolytic cleavage site amino acid pattern was SPQGEKRRKKRGLF for both samples.

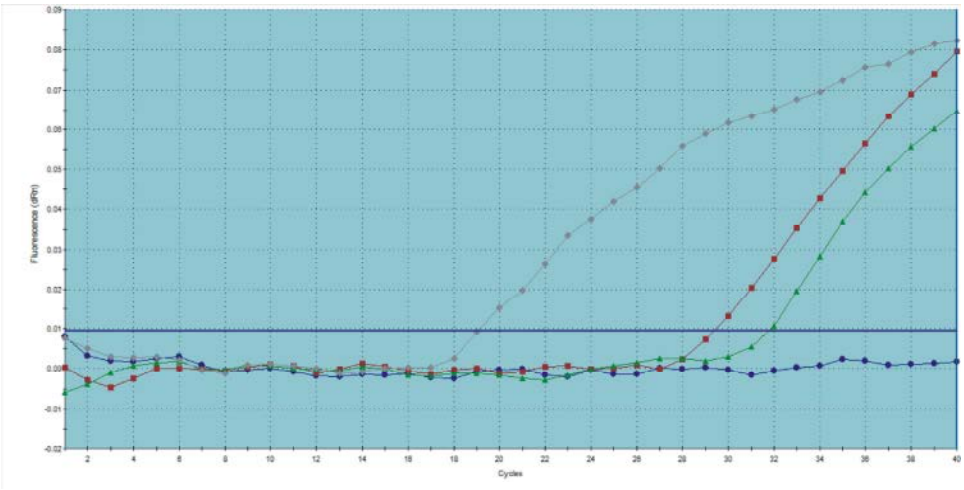

Fig. 1: Amplification curve for AIV matrix gene type A RRT-PCR

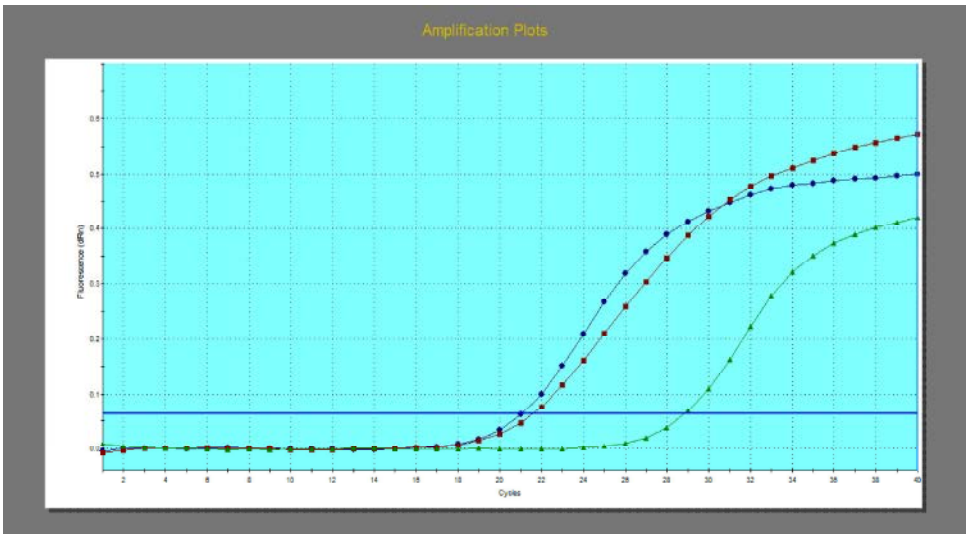

Fig. 2: Amplification curve for AIV H5 gene RRT-PCR Reverse transcriptase PCR using gel electrophoresis.

Table 1: Results for RRT-PCR for matrix and H5 genes and HA activity :

	Results of M gene		Results of H5 gene		
Sample no.	Result	CT value	Result	CT value	HA activity
Neg. control	Neg.	No CT	Neg.	No CT	7 HAU for turkey
1-turkey	Pos.	29.45	Pos.	21.5	8 HAU for ducks
2-duck	Pos.	32	Pos.	29	
Pos. control	Pos.	19	Pos.	21	

CT: Threshold cycle
Pos.: Positive
Neg.:Negative

Table 2: Samples data with accession numbers

Isolate	Host	Collection date	Location	Breeding	Accession no.
A/turkey/Fay-1/2014(H5N1)	Turkey	25-Feb-2014	Fayoum	HH	KM070505
A/duck/Fay-5/2014(H5N1)	Duck	15-Mar-2014	Fayoum	HH	KM070506

Analysis of sequence data:

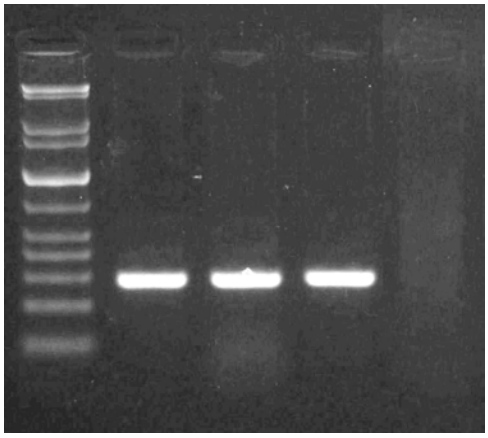

Fig. 3: Agarose gel electrophoresis of some positive isolates showing 100 bp molecular weight marker (M); Negative control (Lane. 1), Positive control (lane. 2).

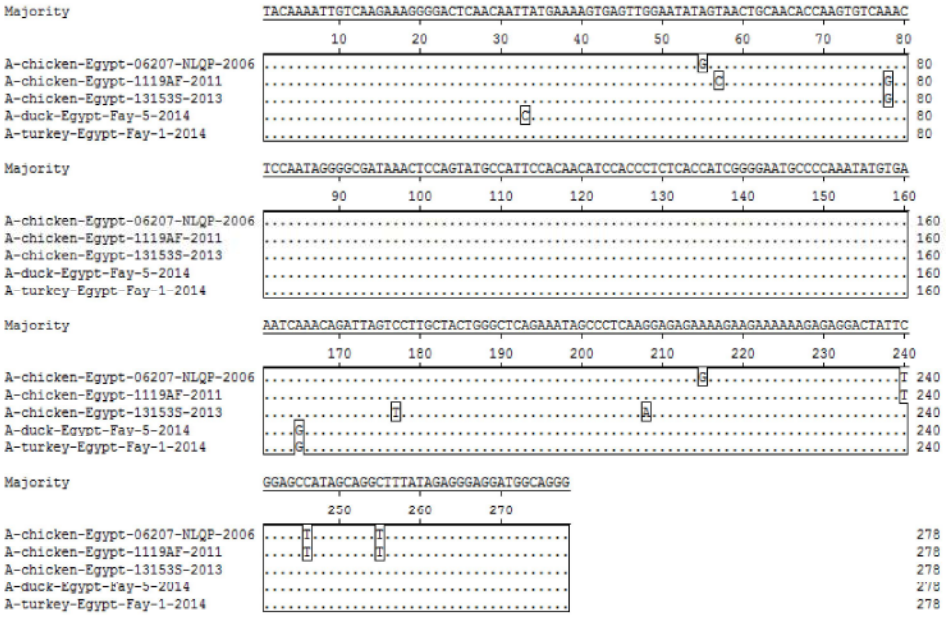

Fig. 4: H5 nucleotide identities and divergence of Egyptian H5N1 strains (2014) in comparison with A/Chicken/Egypt/06207/NLQP/2006 strain.

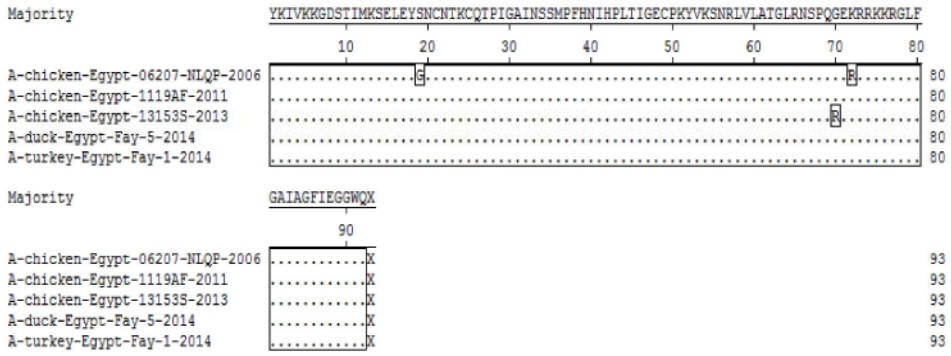

Fig. 5: H5 AA identities and divergence of Egyptian H5N1 strains (2014) in comparison with A/Chicken/Egypt/06207/NLQP/2006 strain.

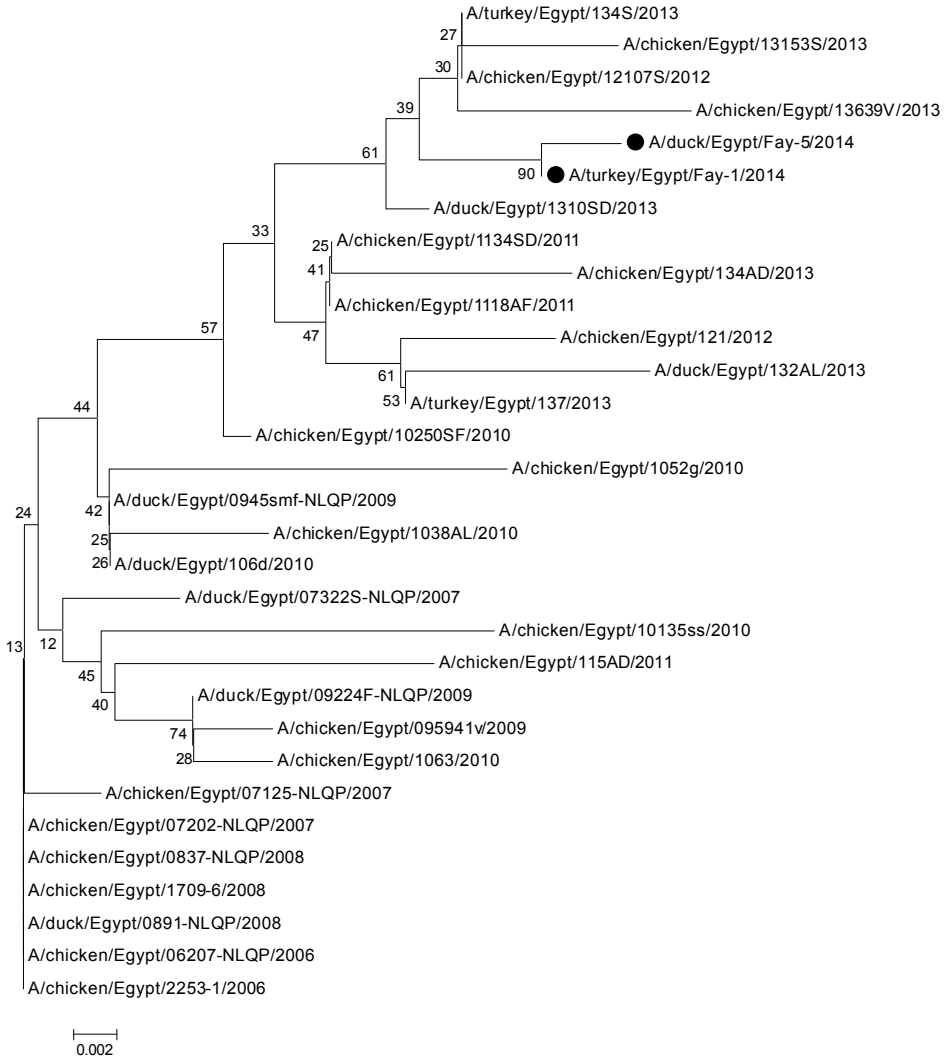

Fig. 6: Phylogenetic tree of HA nucleotide sequences (300pb) of Egyptian isolates compared with other representative H5N1 strains.The tree was produced with Meg Align software using neighbor joining method withA/chicken/Egypt/2253-1/ 2006. The viruses were related to 2.2.1 sub clade H5N1

Table 3: H5 percent of nucleotide identities and divergence of Egyptian H5N1 strains (2014) in comparison with A/Chicken/ Egypt/06207/ NLQP/2006 strain

Percent Identity

Divergence	1	2	3	4	5		
1		98.2	97.1	97.5	97.8	1	A-chicken-Egypt-06207-NLQP-2006
2	1.5		97.8	97.5	97.8	2	A-chicken-Egypt-1119AF-2011
3	3.0	2.2		98.2	98.6	3	A-chicken-Egypt-13153S-2013
4	2.6	2.6	1.8		99.6	4	A-duck-Egypt-Fay-5-2014
5	2.2	2.2	1.5	0.4		5	A-turkey-Egypt-Fay-1-2014
	1	2	3	4	5		

NT identity

Table 4: H5 AA percent of identities and divergence of Egyptian H5N1 strains (2014) in comparison with A/Chicken/Egypt/06207/NLQP/2006 strain.

Percent Identity

Divergence	1	2	3	4	5		
1		97.8	96.7	97.8	97.8	1	A-chicken-Egypt-06207-NLQP-2006
2	2.2		98.9	100.0	100.0	2	A-chicken-Egypt-1119AF-2011
3	3.3	1.1		98.9	98.9	3	A-chicken-Egypt-13153S-2013
4	2.2	0.0	1.1		100.0	4	A-duck-Egypt-Fay-5-2014
5	2.2	0.0	1.1	0.0		5	A-turkey-Egypt-Fay-1-2014
	1	2	3	4	5		

AA identit

DISCUSSION

Influenza A virus possesses a continuing threat to the health of humans and farmed animals and predictions of impending pandemics are commonplace [12].

Despite what is known about the virulence of influenza viruses in mammals, the role of the genes important for host tropism and the virulence of AIVs in avian species remain largely undetermined. Currently, pathogenesis studies of AIVs in chickens clearly indicate that the proteolytic cleavage site of HA [13-15] and changes in the NS1 protein [16] are important determinants of virulence. Recently it was reported that PA and PB1 gene mutations were responsible for differences between nonpathogenic and highly pathogenic viral clones in ducks [17].

It was established that the cleavage site and glycosylation patterns in the hemagglutinin (HA) play important roles in determining the pathogenicity of H5 avian influenza viruses.

In this study, the molecular characterization of the viral proteins of the Egyptian isolates was carried out for pathogenicity markers in the HACS. The HA of the original Egyptian isolate had the same cleavage site like Eurasian strain of H5N1 (GERRRRKR) that was first reported in three whooper swan isolates in Mongolia in 2005 and also in the recent Nigerian isolates. Most influenza virus strains contain an HA cleavage site (HACS) with only a single basic amino acid residue which is cleaved by tissue-restricted proteases only [18], thereby limiting spread in the infected host. In contrast, HPAIV carry a polybasic HA cleavage site leading to proteolytic activation by the ubiquitous protease fur in Stieneke-Grober *et al*. and Stech *et al*. [19, 20].

The presence of a polybasic HA cleavage site is not the sole determinant for virulence in chicken. And beyond the polybasic HACS the virulence of HPAIV in chicken is based on additional pathogenicity determinants within the HA itself or other viral proteins [20].

By changing one amino acid in the cleavage site of HA, the pathogenicity of the viruses can be modulated. The HA of the MP virus contained one more basic amino acid than did that of the HP virus. When lysine at position 338, in the cleavage site of the HA of the MP virus, was replaced with glutamic acid, as found in the HA of the HP virus, the MP virus became more pathogenic. The opposite was also true: when glutamic acid has been replaced with lysine in the HP virus, pathogenicity was reduced. These results confirmed the importance of the composition of the cleavage site in pathogenicity, a finding similar to those described in previous reports [21-23] and also suggested that the presence of an acidic residue within an otherwise polybasic region has great influence on the pathogenic phenotype of the virus [24]. Viruses contain multiple basic amino acids found to be associated with highly pathogenic avian influenza viruses [25] and the GERRRKKR cleavage site found in the majority of the Egyptian viruses of 2006 [7]. Glutamine and glycine occupied amino acids 222 and 224 (H5 numbering) Respectively, which is suggestive of preference for avian alpha-2,3 receptor binding [26]. Amino acid 97 of HA of some viruses contained an aspartic acid (D) residue had been identified in other highly pathogenic AIVs [27]. Previously, P251S was determined to be a signature mutation in HA for the geographic region of Egypt [28]. However, the Egypt/08 virus maintained a proline residue at 251, while Egypt/07 displayed the signature serine at 251, along with the remaining Egyptian viruses and Israeli viruses [29].

Most Egyptian and Nigerian viruses at position 553 (537 H5 numbering) where 553F were found to be predominant [30]. Arginine at position 193 increased the affinity of HA for á2-6 sialosides, when combined with the receptor binding changes at 226 and 228 [31]. The S123P amino acid change has also been shown to increase alpha-2,6 receptor binding when combined with other amino acid changes [32].

The risk of HPAI H5N1 virus presence in countries like Egypt, Thailand and Vietnam has been associated with free-ranging duck numbers and the local abundance of both duck and geese [33], in addition to other risk factors such as chicken numbers, human population and topographical features [34]. There are many determinants of pathogenicity that vary between mammals and avian species and amino acid changes significant in one species does not necessarily correspond with pathogenicity of the virus in the other [17].

In this study two samples; one from duck and another one from turkey from backyard from Fayoum Governorate were tested in RLQP from January 2014 till March 2014 and they are examined by real time PCR for Matrix(M) type A and H5 genes of AI and the results reveled that both samples were positive for M and H5 genes.

Isolation of the two samples through inoculation on ECE via allantoic cavity indicated that both samples grew on the eggs causing chicken embryo death with hemorrhages within first 48 h and gave positive HA activity of 7 and 8 to turkey and duck respectively. Sequencing of isolates revealed that all isolates were highly pathogenic contain multiple basic amino acids in cleavage site and belong to sub clade 2.2.1.

The proteolytic cleavage site amino acid pattern was SPQGEKRRKKRGLF. This pattern was present also in some AI H5N1 viruses isolated in 2006, 2007, 2008 (29). also revealed that both AIVs isolated in 2014 were closely related to each other and show more than 99% nucleotide identity with each other's with one different silent nucleotide mutation and100% AA identity with each other's.

The proteolytic AA pattern for both isolates were the same and polybasic as following SPQGEKRRKKRGLF. These results match with Hagag,Hassan and El-sonoussi [29] who found this pattern in some isolates circulating in 2006, 2007, 2008. Also [29] denoted that the Consensus region of AA of cleavage site for the Egyptian isolates from 2006 to February 2010, was mainly (SPQGERRRKKRGLF) this was the same as the first introduced into Egypt in 2006 and the Eurasian viruses circulated in that time which were originated from Qinghai strain 2005. Finally these result indicate that both viruses are closely related to each other's and AI H5N1 circulate between duck and turkey in backyard in 2014 with minor or negligible changes. Also indicate that AI H5N1 isolates in 2014 are closely related to those in 2006 and firstly introduced to Egypt in 2006.Indicating that no drastic changes happened to PCS.

CONCLUSION

Both AI H5N1 viruses isolated in 2014 were closely related to each other's and circulate between duck and turkey in backyard in 2014 with minor or negligible changes. Also indicate that AI H5N1 isolates in 2014 are closely related to some isolated viruses in 2006, 2007, 2008 which were related to those firstly introduced to Egypt in 2006.Indicating that no drastic changes happened to PCS.

The Egyptian H5N1 viruses continue to mutate and evolve under the field conditions and these variations affected the most important part of the HA gene known to be responsible for virus pathogenicity and carry the main virulence markers in cleavage site (HACS) and that may reflect on the change of virus behavior toward pathogenicity and host infectivity.

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