Molecular genotyping of highly pathogenic avian influenza H5N1 in Egypt

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Abstract: Avian influenza virus usually refers to influenza A viruses found chiefly in birds, but infections can occur in humans. The risk is generally low to most people, because the viruses do not usually infect humans. However, confirmed cases of human infection have been reported since 1997by H5N1 subtype. In the current study, a highly pathogenic avian influenza subtype H5N1 was isolated and confirmed by PCR and sequencing. Sequence analysis revealed some degree of heterogeneity withers on the level of HA or HI. The poly basic amino acid sequence of the isolated virus was similar to the highly pathogenic strains of H5N1 (PQGE (R/G/K)RRKKR↓GLF) with some strains having differences in the 5th amino acid were the R residues were substituted with either G or K. phylogenetic tree revealed that 3 out of the 7 studied samples were greatly homologues while the other 4 samples were widely heterologous.
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Key words: avian influenza; haemagglutinin gene; neuraminidase gene; highly pathogenic; cleavage site.

1. Introduction

Influenza is a highly contagious, acute, viral respiratory diseasewhich causes significant morbidity and mortality worldwide eachyear (**Gambotto et al., 2008**). Global outbreaks of human or poultry influenza (pandemics) arisefrom influenza A viruses with novel hemagglutinin (HA) and/orneuraminidase (NA) molecules to which the subjects have no immunity (**Skehel and Wiley, 2000**). Specifically, the structural changes and antigenic variationsin the HA molecule are the main obstacle to the control ofviral transmission (**Doherty et al., 2006**).

Influenza A viruses are enveloped, pleomorphic and negativesense segmented RNA viruses belonging to the familyOrthomyxoviridae. These viruses have been isolated froma wide range of hosts including humans, pigs, birds, horsesand sea mammals, and are classified as types A, B and Cbased on the antigenic differences in two of their geneproducts, nucleoprotein (NP) and matrix (M1). Avian influenzais caused by type A influenza virus, which is furtherclassified into subtypes on the basis of two surface glycoproteins,haemagglutinin (HA) and neuraminidase(NA). Accordingly, 16 HA (HA1–HA16) and nine NA(NA1–NA9) subtypes have been identified (**Fouchier et al., 2005*)***

Highly pathogenic avian influenza H5N1(HPAIV)was first identified in 1996 as the causative agent of thegeese outbreak in the Guangdong Province, China (**Xu et al., 1999**);in 1997, the same subtype caused outbreaks in chickens, with 18human infections reported in Hong Kong.

Recent outbreaks of avian influenza A virus in poultryand humans have raised concerns that an influenzapandemic will occur in the near future (**Horimoto et al., 2004**). Avian influenza viruses include highlypathogenic strains (HPAI) and low pathogenic strains(LPAI); H5N1 virus is the most common HPAI that caninfect both humans and poultry. According to the latestreported data of the World Health Organization inmid-January 2009, mortality of H5N1 in humans is greater than 60% (**WHO, 2009**).

To eradicatepathogenic avian influenza viruses and prevent relateddiseases, researchers have emphasized the study ofpathogenesis, epidemiology, therapy, and vaccines beside a deep investigation on molecular level (**Yang et al., 2010**).

This study was undertaken for molecular investigation of the field isolates of HPAI H5N1 strains from Egypt.

2. Material and Methods:

Virus isolation:

Trachealswabs were taken from recently dead chickens from poultry farms showing sever clinical signs of influenza infection table (1), the swabs were soaked in phosphate buffered saline (pH7.2) containing 100 U/ml penicillin and 100µg/ml streptomycin. The solution was then centrifuged at 5000rpm/10min/4 °C, the supernatant was collected and filtrated using 0.22µ syringemilliporefilter before inoculation in 9 days old SPF chicken (**Xu et al., 1999**)eggs via the Allantoic route. The eggs were incubated at 37°C in a humid champerstill embryo death (usually within 24 hours).

Hemaglutination test:

Virus clarified from the Allantoic fluid were used in the HA assay(**Killian 2008**). Freshly prepared 1%washed erythrocytes of SPF chicken blood was used to conduct the HA assay. Virus titration was then calculated using the **Reed and Munch1938**.

Hemaglutination inhibition test:

It was done according to **Katz et al., 2009** and **Potter and Oxford 1979**. Monospecific antisera against H5N1 (prepared from **A/ Gs/Gd /1/96 H5N1**) was used to determine the Hemaglutination inhibition of 4 HA units of egg propagated virus. Monospecific antisera against other Hemaglutination agent as Newcastle virus (standard Newcastle Anti- serum obtained from CVL weybridge) and egg drop syndrome were used to evaluate the purity of the isolate.

RT-PCR amplification

Viral RNA was extracted from the clarified Allantoic fluid(**Sambrook et al., 1989*),*** byQIAamp Viral RNA Mini Kit (Qiagen Germany, cat #52904), according to themanufacturer's instructions.One step RT-PCR was done using AffinityScript One-Step RT-PCR Kit (Agilent USA , cat # 600188) using the primers that target either the cleavage site of HA gene or the full N1 gene (Table 2) These primers were designed using Lasergene DNASTAR software Version 10.windows platform.Theamplicons were electrophoresed on a 1% agarose and the size of the amplicons were determine using BiometraBioDocAnalyze software version 2.66.3.1.

Sequencing:

The complete nucleotide sequences of the cleavage site of the H5 gene of the 7 isolated strains were performed in (Macrogen USA). For preparation of the gene for sequencing, the PCR product was separated on 1%low melting agarose. The bands were sliced off and purified with the biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacture. Briefly, the gel slices were melted at 60°C for 5 min, mixed with 500 ml of gel extraction buffer and placed on the biospin column provided with the kit, centrifuged at 4000 rpm/2min/4°C and washed twice with the washing solution. Finally the amplicon was eluted in 50 µL of the elution buffer and stored at -20 °C till sequenced. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Analysis:

The nucleotide sequence analyses and the phylogenetic trees were performed using Lasergene DNASTAR software Version 10.windows platform. The deduced amino acid sequence analysis was performed using CLC main work bench Version 6.6 Windows platform developed by CLC bio A/S.

Table (1) the H5N1 viruses used in this study with the date and province of isolation

Sample ID	Virus mane	Date of sampling	breed of the birds	province
4	A/chicken/ Kalyobia /ch1.12.3/2010(H5N1)	Dec-10	Cobb	Kalyobia
5	A/chicken/ Kalyobia /ch1.12.5/2010(H5N1)	Dec-10	Baladi	Kalyobia
6	A/chicken/ Sharkia /ch2.1.6/2011(H5N1)	Jan-11	White Lohmann	Sharkia
7	A/chicken/ Kalyobia /ch2.1.7/2011(H5N1)	Jan-11	Cobb	Kalyobia
9	A/chicken/ Mansoura /ch2.2.12/2011(H5N1)	Feb-11	Arbo	Mansoura
10	A/chicken/ Kalyobia /ch2.1.10/2011(H5N1)	Jan-10	White Lohmann	Kalyobia
18	A/chicken/ Kalyobia /ch2.2.18/2011(H5N1)	Feb-11	White Lohmann	Kalyobia

Table (2) primer sequences used for identification of HAPI isolates:

Primer name	Target gene	sequenceprimer (5'- 3')	Expected amplicon size
CsH5-f	Cleavage site of H5 gene of H5N1 HPAI	5'–CCT-CCA-GAA-TAT-GCG-TAG -3'	315 bp
CsH5-r		5' - TAC-CAA- CCG- TCT- ACC- ATG- CCG -3'.	
N1-f	N1 gene of H5N1 HPAI	5' - AGCAAAAGCAGGAGGTTAAAAGGA-3'.	1400 bp
N1-r		5' - TAGCAACAAGGAGGTTTTTGAACAACC -3'.	

3. Results:

Virus isolation and characterization:

The clinical signs of AI infection in poultry farms were characteristics ranging from drop in egg production, hemorrhagic spots on the leg shafts, swollen cyanotic combs. In the P/M there was severe muscular hemorrhage, with petechial hemorrhagic on the peritoneum and coronary fat. The mortality rate begins in drastic increasing fusion that reached to ~ 70% within 3 days. Samples from the recently died birds were transported on ice to the lab were individual swabs were taken from the trachea and inoculated in the SPF embryonated chicken eggs.

Hemaglutination test.

Chorio-allantoic fluid obtained from all inoculated eggs was subjected first for rapid (slide) HA assay to test for the presence of haemagglutinating agents. those gave negative results were excluded from the study (usually no embryonic deaths were observed with these samples till 72 h post inoculation) As shown in table (3) the samples gave HA ranged from 9 -12 which is mainly depends on virus concentration in the tested samples.

Hemaglutination inhibition test.

The chorio-allantoic fluid of the samples were diluted to obtain 4HA unit which was used for HI assay using anti H5N1 monospecific antisera, NDV antisera or adeno monospecific antisera. As shown in table (3) all the test samples gave no reaction with the monospecific antisera against NDV or Adeno, on the other hand, all samples gave positive inhibition to the agglutination using the monospecific antisera against H5N1. The titer vary from 6 - 9.

Table (3) the results of haemagglutination and haemagglutination inhibition assay showing that most samples gave a very high HA and HI titer in comparison the negative control (Chorio-allantoic fluid obtained from sterile PBS inoculated eggs). No detectable titer was seen with other antisera

Sample ID	HA titer	HI titer		
		H5N1	NDV	Adeno
4	11	7	0	0
5	12	8	0	0
6	11	5	0	0
7	11	7	0	0
9	11	6	0	0
10	9	6	0	0
18	11	7	0	0
Negative control	0	0	0	0

Reverse transcriptase polymerase chain reaction (RT-PCR):

SPF eggs showed embryo mortalities within 24 h and gave positive HA and HI were subjected to further analysis by RT-PCR using specific primers directed towards the H5 cleavage site and the N1 gene of the HPAI. As shown in Fig 1, clear visible band with molecular size of ~ 315 bp having high intensity was seen for the cleavage site and the amplified N1 amplicons gave a product with 1400 bp in length.

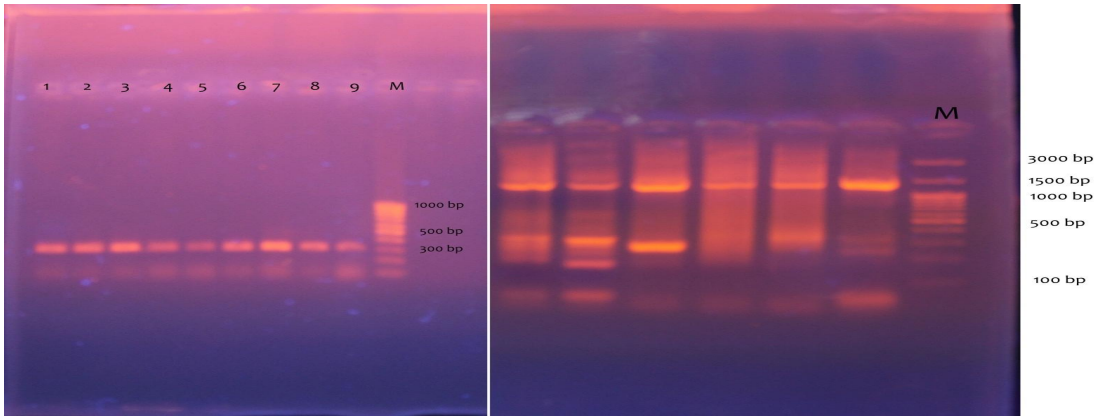

Fig 1: The PCR amplification of 315 bp cleavage site of H5 gene of the 9 isolated H5N1 virus purified from the Allantoic fluid (left)and the 1200 bp full N1 gene (right) of some represented samples , note that some non specific low size amplifications were seen during the amplification of the full N1 gene. M is DNA ladder

Sequencing analysis of the cleavage site of the H5 gene:

The nucleotidesequence ofselected 7isolates were aligned using MegAlin suit of the DNASTAR software and the deduced amino acid sequence were aligned using CLC bio software. As seen in fig 2, several point mutations were seen between the isolates these mutations were mostly substitution mutations and resulted in changes in the amino acid sequence as seen in fig 3. At least 10 areas of amino acid substitutions were detected in the deduced amino acid but no more than 4 substitutions were detected for any analyzed strain. The poly basic amino acid sequence determinant for the HPAI was mainly GERRRKKR with the exceptionof strain5, 7 and 10 wherethe first R residue was substituted with G.

Cladogram created by *CLASTALW* with bootstrapping of 1000 (fig 4) revealed that strains 4, 6 and 18 were grouped together with 99.1% confidence , while other strains were widely differ with the most distant strains are 5 and 9

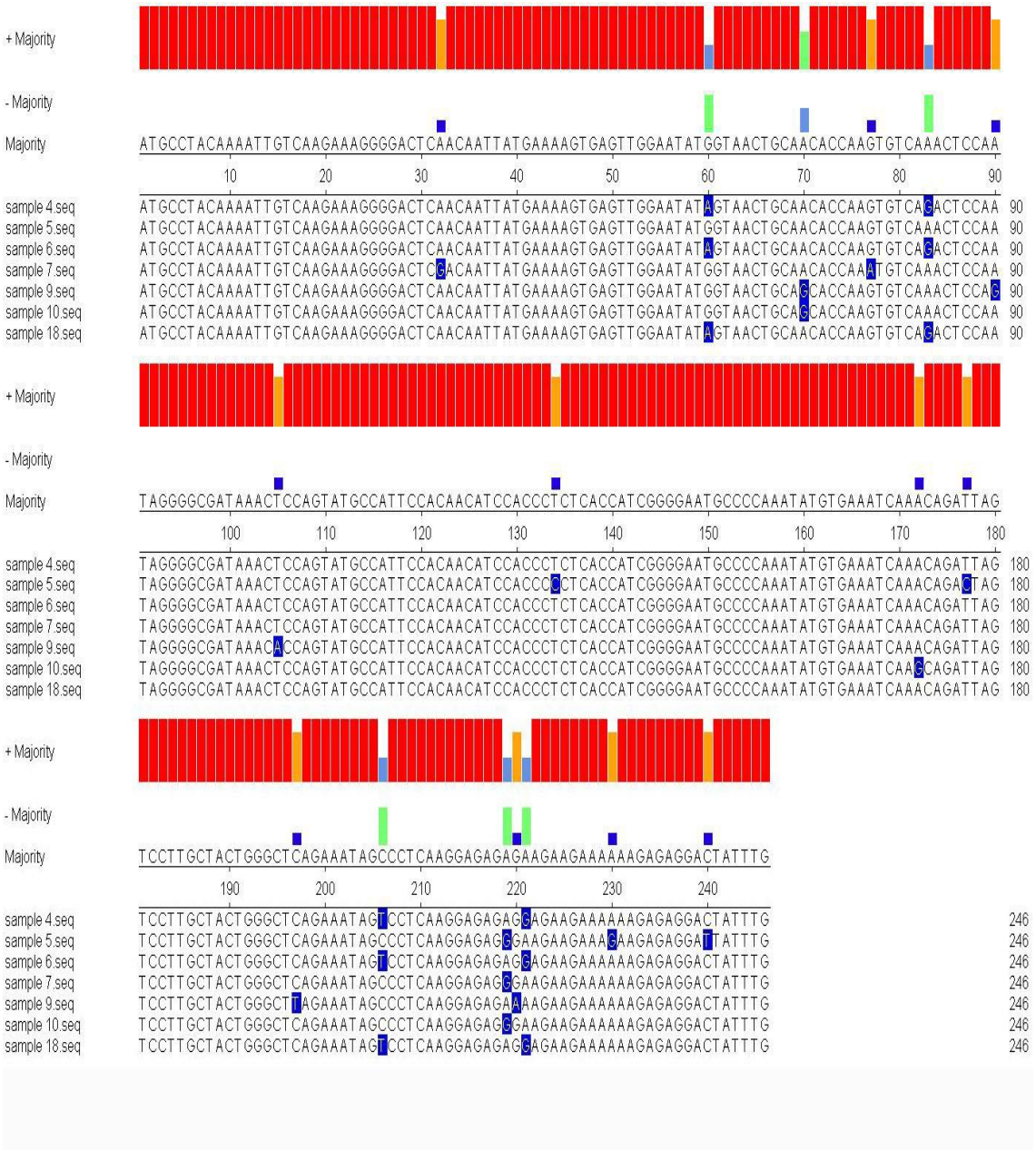

Fig 2. Alignment comparison of the nucleotide sequences of the cleavage site of the H5 gene showed the Egyptian AI virusesexhibited many point mutations.

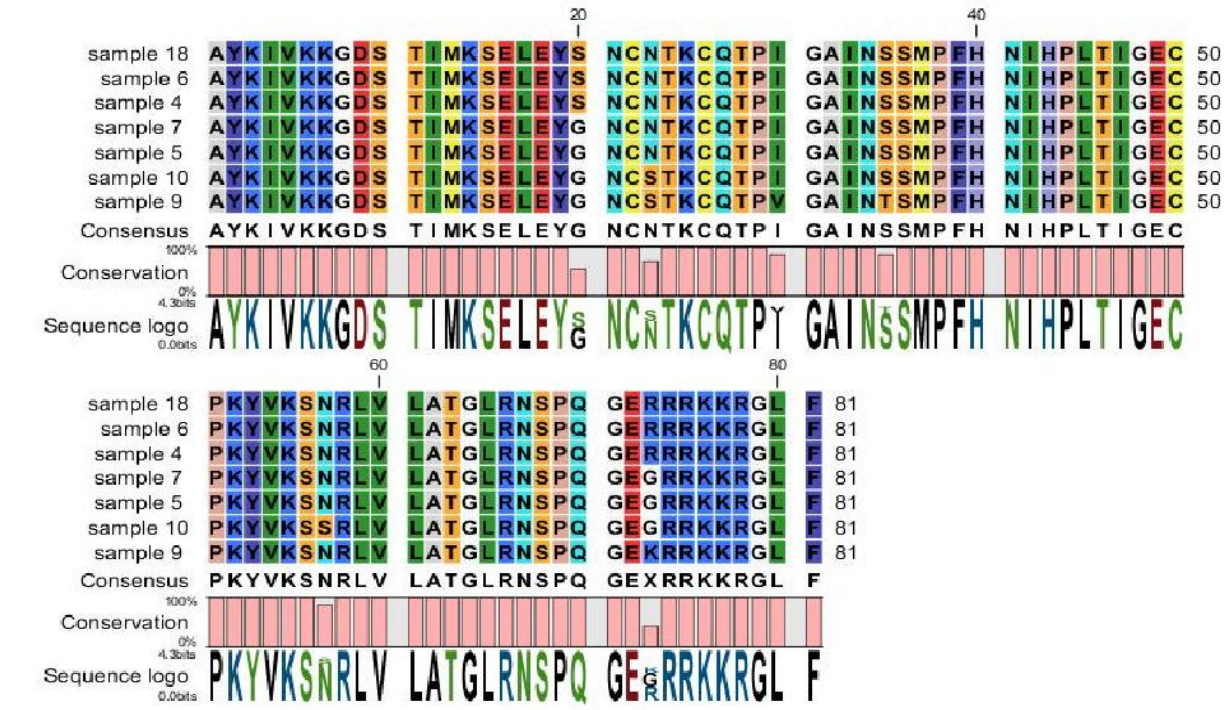

Fig 3. Alignment comparison of the deduced amino acid sequences of the cleavage site of the H5 gene showed the Egyptian AI viruses. Note the HA cleavage sequence was conserved in most sequence (GE*R*RRKKRG) with the exception of sample 5, 7 and 10 where the first R residue was substituted with G (GE*G*RRKKRG).

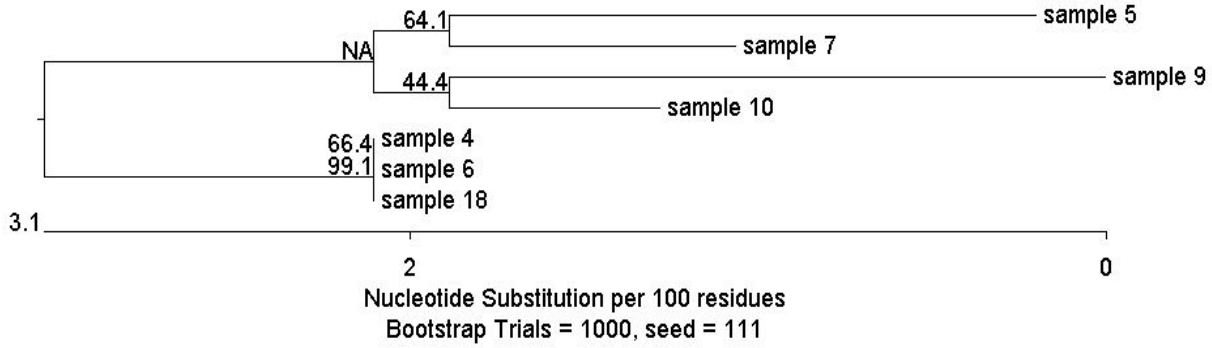

Fig 4: Phylogenic tree analysis of the cleavage site of HA gene for the 7 HPAI H5N1 viruses. Nucleotide sequences were analyzed with a CLASTALW. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join the nodes. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value.

4. Discussion:

Since re-emerging in 2003, avian influenza viruses of the H5N1 subtype have spread from Southeast Asia across central Asia and the Middle East into Europe and Africa by infecting wild birds and poultry. New influenza viruses and genotypes are emerging each year and are leading to significant genetic variation among H5N1 viruses (Wu , 2008).Avian influenza viruses (AIV) characterized by intravenous pathogenicity indices of greater than 1.2 are termed highly pathogenic (Hoffmann et al., 2007). Only representatives of subtypes H5 and H7 have been shown to exhibit highly pathogenic AIV (HPAIV) characteristics and to cause disastrous epidemic disease in poultry (Capua and Mutinelli, 2001). The presence of a polybasic, subtilisin-sensitive endoproteolytic cleavage site (CS) within the hemagglutinin (HA) precursor protein (HA_0) has

been identified as a reliable marker for HPAIV (Perdue and Suarez 2000). AIV strains of low pathogenicity, in contrast, reveal a monobasic composition at this site which is targeted by tissue-specific, trypsin-like proteases. Samples were taken from the recently died chickens were collected from representative governorates during the last couple of years (2010-2011) were subjected to extensive molecular characterization of the cleavage site of the H5 gene.

The farms from which the samples were taken showed a sever mortalities which in some cases reached to 100% with hardly no symptoms on the chicken in most cases, however some farms with relatively low mortalities showed the classical symptoms in the form of hemorrhagic spots on the shank and coronary tissue.These findings was also reflected on the virus titration either by HA or HI assays. Some of the isolates gave a very high HA titer which was further proofed by back titration of the virus, and the HI titer using 4HA units gave also a very high titer (table3). These findings may be attributed to the high viral load in the farms or the absence of appropriate vaccination programs; also some inadequate protocols for vaccinations mightprovokeimmune stressors which might result in vaccination induced mutations that allow the virus to escape the immune response(Cameron et al., 2008).

The isolates were subjected to further molecular investigations through amplification both the cleavage site of the HA gene or the N1 gene. All the tested strains gave a positive amplicons at 300bp and 1400bp for the CS and the full length N1 gene respectively denoting the H5N1 nature of all isolates.

All of the seven H5N1 isolates from Egyptbelongs to the highly pathogenic AI as they contained the amino acid sequencePQGE (R/G/K)RRKKR$^{\downarrow}$GLFat the cleavage site in the HA molecule,indicating their high virulence (Fig. 3) (Horimotoet al., 1995). This cleavage sequence was slightly differ from that of A/Hong Kong/156/97(H5N1) virusPQRERRRKKR$^{\downarrow}$G as the 3rd amino acid was substituted from R in hongkong strains to G in the Egyptian strains and also the 5th amino acid was R in all Hong Kong strains but it was either R, G or K in the Egyptian strains (Subbarao et al., 1998).Cleavage of the HA molecule (HA_0), by host-cell proteases,into two disulphide-linked HA_1 and HA_2 subunits isessential for viral infectivity. Avian influenza viruses withhigh and low levels of pathogenicity differ in their cleavagesequence, the former possess multiple basic amino acidresidues, while the latter do not. A cleavage sequencecontaining several basic amino acids is more readilyactivated by cellular proteases present in a variety of cellsdistributed throughout the body compared with a cleavagesequence containing only a single basic amino acid, whichcan be cleaved by a limited range of cellular proteases. It iswell accepted that influenza viruses containing multiplebasic amino acids have multiple sites of virus replicationand produce more severe infection in birds and mammals(Zambon, 2001).

In aquatic birds, normal influenza replication takes place in the intestinal tract and tends not to cause symptoms. In mammals like humans and swine, influenza replication is limited to epithelial cells of the upper and lower respiratory tract. This tissue tropism is controlled to some extent by the limited expression of the appropriate protease for viral activation by HA cleavage . In mammals, the suspected protease in the respiratory tract is tryptase Clara, a serine protease produced by nonciliated Clara cells of the bronchial and bronchiolar epithelia (*Rott et al., 1995*). Occasional avian influenza strains with an insertion mutation at the cledvavage site of HA, allowing HA to be cleaved by ubiquitously expressed proteases (furin and other subtilisin family proteases), as a consequence, the virus can replicate throughout the bird's body, producing necrotic foci in spleen, liver, lung, and kidney and encephaliticlesions in brain (Easterday and Tumova1978 and Kin et al., 2009).

These highly virulent strains have been observed in only two of the 14 described HA subtypes in birds that emerge only occasionally but can cause devastating mortality in poultry flocks (*Horimoto et al., 1995*). The insertion responsible for the ubiquitous cleavage adds additional basic amino acids at the cleavage site (Senne et al., 1996), with a minimal motif of R/L-X-R/L-R. Until recently, this mutation had been found only in avian viruses of the H5 and H7 subtypes, subtypes that were not thought to infect humans. This barrier was broken dramatically in 1997 in Hong Kong when 16 people were infected with an avian H5N1 influenza virus (Centers for Disease Control reports 1998).

Phylogenetic analysis of the 7 isolates reveled that samples 4, 6 and 18 were grouped together with 99 %confidence. These strains had the same amino acid sequence at the cleavage site PQGRRRKKR and also the same HA titer (11 Log $_2$) but HI titer was the same for sample 4 and 18 (7 Log $_2$) and much less for the sample 6 (5Log $_2$). Other samples were evolutionary distance, as sample 10 and 9 have 44% confidence while the last 2 samples (7 and 5) had 64%confidence.

In conclusion, Egyptian isolates from different governorates showed marked differences in either HA or HI titer and some degree of heterogeneity on the level nucleotide andamino acid sequence of the cleavage site of the HA gene .

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