

## Sequence Analysis of H5N1 Isolates from Backyard Poultry in Upper Egypt

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**Abstract:** Highly pathogenic avian influenza (HPAI) subtype H5N1 represents a threat to the poultry industry and human health worldwide. Fifteen samples from apparently healthy birds in backyard holdings H5N1 isolates were subjected to sequencing of the proteolytic cleavage site of the haemagglutinin (HA) gene. PQGEKRRRKRGL/FGA sequence was present in 11/15 of the strains. In 2014, five selected H5N1 positive isolates were subjected to HA1 sequencing and the sequences revealed that the five isolates are belonging to the newly recorded subclade 2.2.1.2. and the amino acid identities showed 97-99% amino acid homology to the lineage 2.2.1.2. Four N-glycosylation sites were detected in the HA1 of the isolated strains and also in many other H5N1 strains. A conspicuous deletion in amino acid 129 and critical amino acid substitutions in potential sites responsible for receptor binding sites, were found in the five isolates.

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**Keywords:** Highly Pathogenic Avian Influenza (HPAI), H5N1, HA, HA1, Proteolytic Cleavage Site, N-glycosylation sites.

### 1. Introduction :

Avian influenza (AI) has been firstly reported in 1878 by Perroncito in Italy since more than 130 years [1]. It causes a range of disease symptoms that range from subclinical to highly fatal disease with up to 100% mortality with subsequent drastic economic losses [2]. AI viruses also possess wide host range, including mammals and represent a zoonotic risk. The main reservoir for the AI viruses is the wild aquatic birds; therefore, complete eradication is problematic [3,4].

The epidemiological role of backyard flocks in the spread of HPAI H5N1 in Egypt can be summarized in three main aspects. First, backyard flocks are raised with multiple and different species of birds and animals (chickens, ducks, geese, turkeys, pigeons, rabbits and small ruminants) and birds of various ages. On the one hand, this could be an indicator for circulation of the virus in the field, but tracing back of positive cases under these conditions is impossible. Second, keeping of different avian species together provides suitable conditions for inter- and intraspecies transmission, the existence of different gene pools of the virus, notable mutational changes and selection of variants of a potential pandemicity. Third, backyard flocks spread the virus, or newly emergent variants, to poultry and humans in a large extended area, especially to women and children in rural areas [5].

The aims of this work are; i) Screening the presence of sequence diversity in the cleavage site of 15 selected strains (2009-2014) and ii) Sequence analysis of the HA1 of the most recent Egyptian H5N1 strains.

### 2. Material and Methods

#### Sampling:

The samples were collected from backyard poultry admitted by the householders in the villages and from apparently healthy birds in LBMs. A total of fifteen H5N1 positive samples, were collected from three governorates (Beni-Suef, Fayoum, and Minia governorates) for isolation and molecular characterization.

#### Molecular detection and screening the presence of H5N1 in different avian species:

Detection of AIV-M, H5 and N1 genes in the tested samples using rRT-PCR. RNA was extracted from pools of tracheal and cloacal swabs by using QIAamp Viral RNA Mini Kit(Qiagen, Germany), procedures were 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 according to Spackman *et al.* [6]. Also published Sequence of AI H5 primers& probe were used in one step real time PCR according to Slomka *et al.* [7]. But published sequence of AI N1 primers and probe were used according to Agüero *et al.* [8].

#### 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 [9], then Haemagglutination test to determine ability of this

virus to make agglutination to chicken RBCs 1%. Procedures were performed according to OIE [9].

#### Molecular characterization:

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 of HA gene (15 isolates) and HA1 (five recent isolates of 2014). The sequencing was done in both directions using the same primers as used in rRT-PCR reactions (gel electrophoresis). Sequences were initially analyzed by BLAST analysis ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) according to Altschul *et al.* [10]. The nucleotide sequences thus obtained were aligned by the Clustal W method using MEGA 3.1 software. A phylogenetic tree of aligned sequences was constructed by the neighbor-joining method.

#### GenBank accession numbers:

The HA and HA1 sequences of the Egyptian HPAI H5N1 of our study were submitted to GenBank to get accession numbers.

### 3. Results

The samples that gave positive results for H5 and N1 genes (Fig. 1) were subjected for sequencing of the proteolytic cleavage site of the H5N1 strains after isolation and confirmation. RT-PCR of the cleavage site resulted in 300bp-amplions (Fig. 2). The sequence of the cleavage site amplicon revealed that PQGEKRRKKRGL/FGA multiple basic amino acid containing cleavage site is present in 11/15 of the strains sequenced in the current study (Table 1). K to R substitution (PQGERRRKKRGLFGA), was detected in A/duck/Egypt/BSU-NLQP-MN-1/2009, A/chicken/Egypt/BSU-NLQP-MN-2/2010 and A/goose/Egypt/BSU-NLQP-FY-1/2009 strains while K to G substitution (PQGEGRRKKRGLFGA) was detected in A/chicken/Egypt/BSU-NLQP-BS-1/2010 HA1 sequence analysis of the five recent 2014 Egyptian strains in the current study, revealed that they are related to sublineage 2.2.1.2 (Fig. 3, Table 2). L to F amino acid substitution was detected in 1/5 of the recent 2014 strains in the signal peptide. The four N-glycosylation sites detected in the HA1 of strains of the current study and also in other H5N1 strains in different Egyptian lineages of subclade 2.2.1.2. A conspicuous deletion of S129 in the HA protein (H5 numbering) and many mutations were detected.

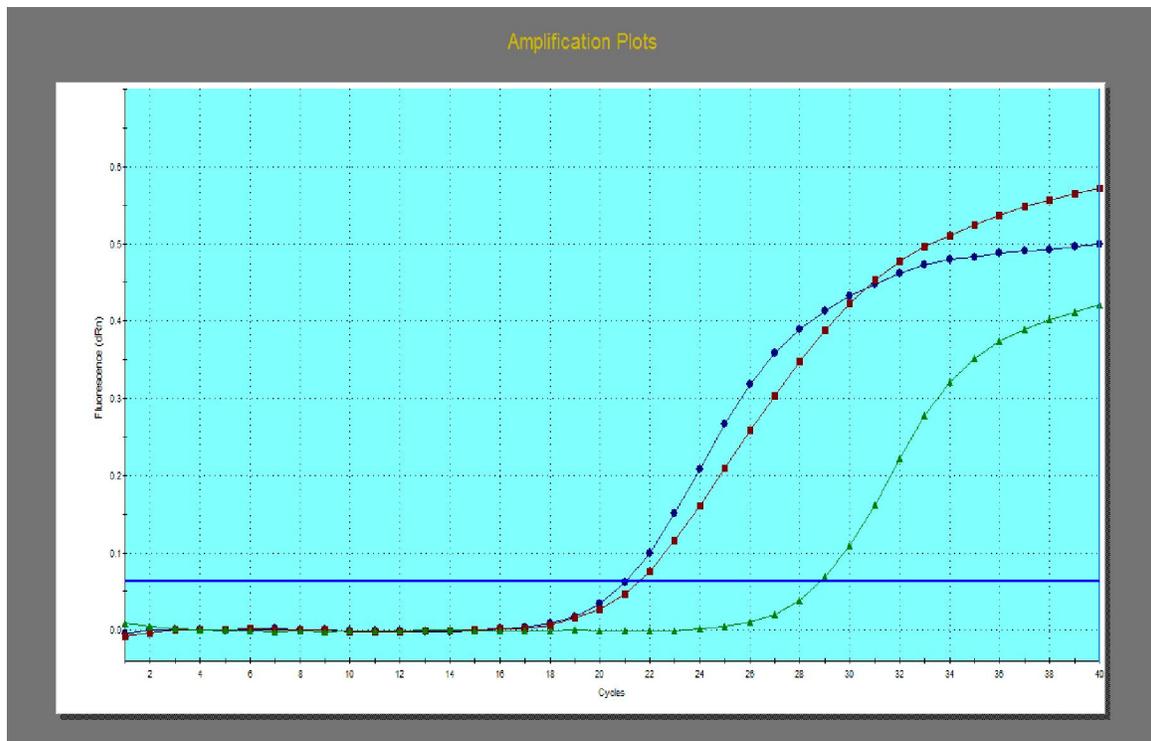
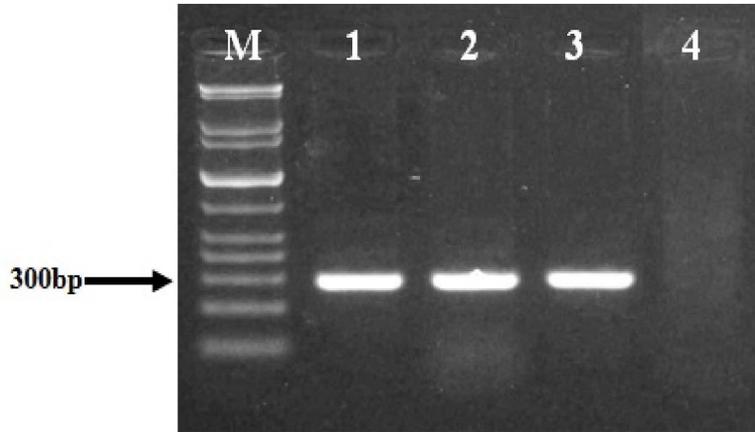


Fig. (1): Amplification curve for AIV H5 gene by rRT-PCR.



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

**Table (1): Diversity in the cleavage site in different selected Egyptian H5N1 strains of our study**

Isolate	Cleavage Site sequence
A/goose/Egypt/BSU-NLQP-FY-1/2009	PQGE <u>R</u> RRRKKRGLFGA
A/chicken/Egypt/BSU-NLQP-FY-2/2011	PQGEKRRRKKRGLFGA
A/duck/Egypt/BSU-NLQP-FY-3/2012	PQGEKRRRKKRGLFGA
A/duck/Egypt/BSU-NLQP-FY-4/2013	PQGEKRRRKKRGLFGA
A/duck/Egypt/BSU-NLQP-FY-5/2013	PQGEKRRRKKRGLFGA
A/Chicken/Egypt/BSU-NLQP-FY-6/2014	PQGEKRRRKKRGLFGA
A/Chicken/Egypt/BSU-NLQP-FY-7/2014	PQGEKRRRKKRGLFGA
A/chicken/Egypt/BSU-NLQP BS-1/2010	PQGE <u>G</u> RRRKKRGLFGA
A/chicken/Egypt/BSU-NLQP-BS-2/11	PQGEKRRRKKRGLFGA
A/Duck/Egypt/BSU-NLQP-BS-3/2014	PQGEKRRRKKRGLFGA
A/Duck/Egypt/BSU-NLQP-BS-4/2014	PQGEKRRRKKRGLFGA
A/duck/Egypt/BSU-NLQP-MN-1/2009	PQGE <u>R</u> RRRKKRGLFGA
A/chicken/Egypt/BSU-NLQP-MN-2/2010	PQGE <u>R</u> RRRKKRGLFGA
A/chicken/Egypt/BSU-NLQP-MN-3/2012	PQGEKRRRKKRGLFGA
A/Chicken/Egypt/BSU-NLQP-MN-4/2014	PQGEKRRRKKRGLFGA

**Table (2): Nucleotide and amino acid identities of the isolated H5N1 strains of our study in comparison to different Egyptian strains**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1		97	98	97	97	97	97	97	97	97	97	96	95	95	96	96	96	95	94	94	95	95	1	KR088162A Chicken/Egypt/BSU-NLQP-FY-6/2014
2	99		97	99	97	99	97	97	97	97	97	96	95	95	96	96	95	94	93	93	95	95	2	KR088163A Chicken/Egypt/BSU-NLQP-FY-7/2014
3	98	99		97	98	97	97	97	97	97	97	96	95	96	96	97	96	95	94	96	96	96	3	KR088164A Duck/Egypt/BSU-NLQP-BS-3/2014
4	99	100	99		97	99	97	96	97	97	96	95	95	95	96	96	96	94	94	93	95	95	4	KR088165A Duck/Egypt/BSU-NLQP-BS-4/2014
5	98	99	98	99		97	99	97	97	97	96	96	95	96	96	96	95	94	94	95	95	5	KR088166A Chicken/Egypt/BSU-NLQP-MN-4/2014	
6	98	98	98	99	98		97	97	97	97	96	95	95	96	96	96	95	94	94	95	95	6	2.2.1.KP202165A Egypt/MOH-NRC-7271/2014	
7	98	99	99	99	98		97	97	97	97	96	96	95	96	96	96	95	94	94	95	95	7	2.2.1.KP209299A Chicken/Egypt/14126S/2014	
8	97	98	98	98	97	98		97	97	97	96	96	96	96	96	96	95	95	95	95	96	8	2.2.1.KJ52273 A Turkey/Egypt/137/2013	
9	96	97	96	97	96	96	97	96		99	98	97	97	98	98	98	98	97	96	96	97	97	9	2.2.1.A1JF357720 A Chicken/Egypt/17/2009
10	97	98	97	98	97	97	98	97	98		98	97	97	98	98	98	98	97	96	96	97	10	2.2.1.A1JG1026296 A Duck/Egypt/D980SM-NLQP/2009	
11	96	97	96	97	96	96	96	96	98	98		98	98	99	98	99	99	97	97	97	98	11	2.2.1.A4IEF463650 A Chicken/Egypt/1129N3-HK9/2007	
12	95	96	95	96	95	95	95	95	97	96	98		97	98	98	98	98	97	96	96	97	12	2.2.1.A4[GQ184220 A Chicken/Egypt/10813-NLQP/2008	
13	93	93	93	93	93	92	93	93	95	94	96	95		98	97	98	98	97	96	96	97	13	2.2.1.A3/EU371912 A Duck/Egypt/9387NAMRU3-CLEVB148/07	
14	94	95	94	95	94	94	95	95	97	96	98	96	96		98	98	99	97	96	97	98	14	2.2.1.A3/EU183326 A Chicken/Egypt/F6/2007	
15	96	97	96	97	96	96	96	96	97	97	98	96	94	96		99	98	97	96	96	97	15	2.2.1.A5/EU371901 A Duck/Egypt/3047NAMRU3-CLEVB63/07	
16	95	96	95	96	95	95	95	95	97	97	98	96	94	96	98		98	97	97	97	98	16	2.2.1.A5/EU371898 A Duck/Egypt/3044NAMRU3-CLEVB59/07	
17	95	96	96	96	95	96	96	98	97	99	97	95	97	98	98		98	96	97	98	98	17	2.2.1.A2/EU496392 A Duck/Egypt/07322S-NLQP/2007	
18	93	94	93	94	93	93	94	93	94	94	95	94	92	94	94	94	95		95	96	96	18	2.2.1.A2.JF357722 A Chicken/Egypt/F9/2009	
19	93	92	92	92	92	91	92	92	93	93	94	92	92	92	93	93	93	91		99	98	19	2.2.1.1.JF357723 A Chicken/Egypt/F10/2009	
20	91	91	92	91	91	90	91	91	92	92	93	92	91	92	92	92	92	90	97		98	20	2.2.1.1.FJ686831 A Duck/Egypt/10NLQP-CLEVB331/08	
21	93	94	93	94	93	93	94	94	95	94	96	95	94	95	95	95	96	92	97	96		99	21	2.2.1.1.FJ686839 A Duck/Egypt/10NLQP-CLEVB299/08
22	94	94	94	94	93	94	94	94	95	95	96	95	94	95	95	95	96	93	96	96		99	22	2.2.1.1.FJ686843 A Duck/Egypt/16NLQP-CLEVB242/08

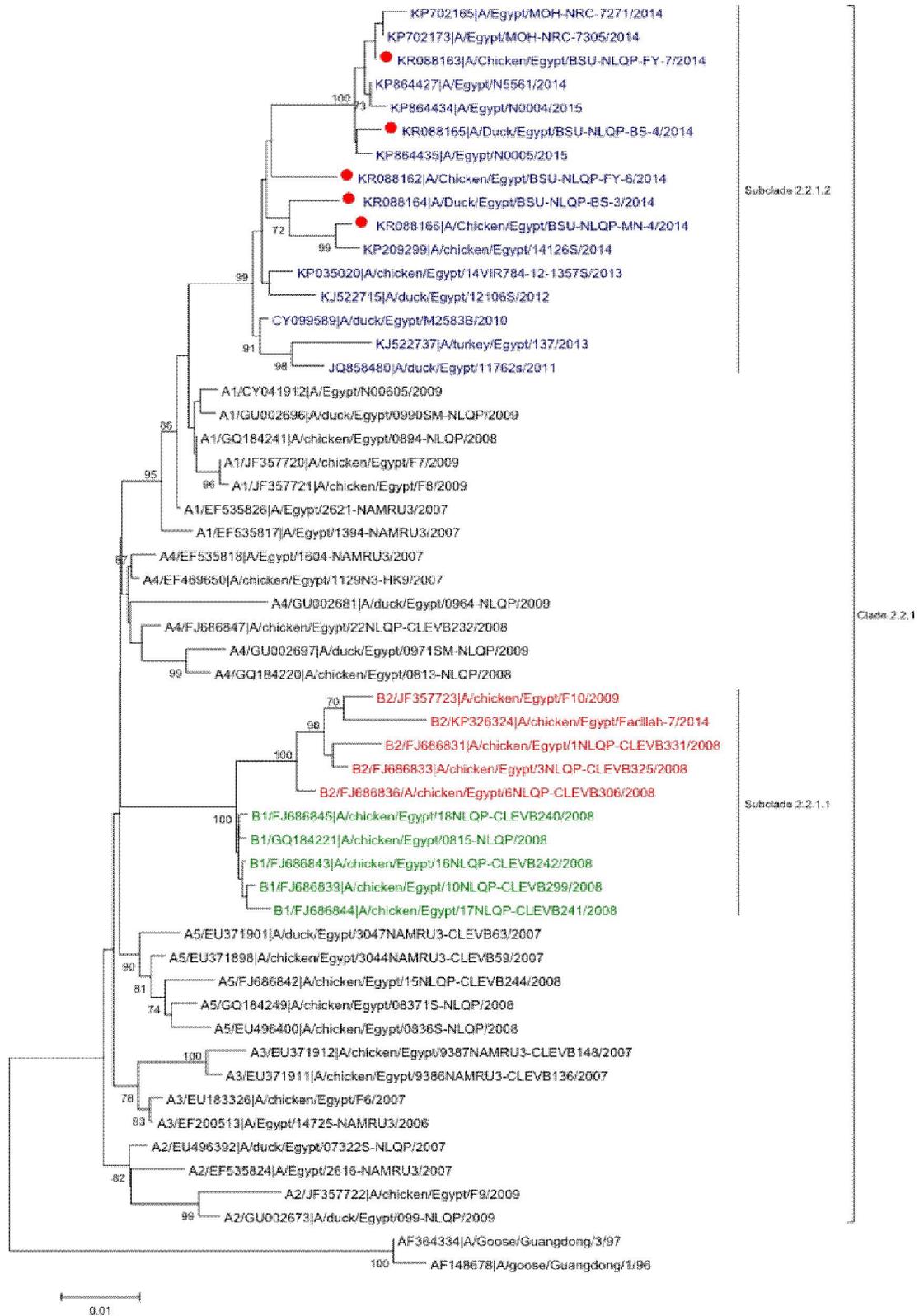


Fig (3): Phylogenetic tree of HA1 of the isolated strains and selected Egyptian strains. Isolates shown in blue belong to the newly recorded subclade 2.2.1.2, whereas 2.2.1.1 subclades are shown in red and green. Sequences of the isolated strains in the current study are indicated by red dots.

#### 4. Discussion

There are four sectors of poultry production in Egypt: sectors 1 and 2 include the grandparent and parent commercial production where there are good hygienic and biosafety measures. Sector 3 includes non regulated, small to medium-scale commercial activities, and sector 4 includes backyard, rural, in-house and rooftop-raised poultry [5].

Rearing backyard birds and livestock in the countryside is a complex cultural, social and ecological phenomenon in Egypt. It is known that the long-term endemic influenza virus infections in poultry increases the exposure risk to human and in turn, creates opportunities for the emergence of human-adapted strains with subsequent pandemic potential [3]. To date, there have been 342 confirmed human infections and 141 deaths from H5N1 from Egypt. More than 90% of the human cases in Egypt were linked to close contact with backyard birds [11]. On the other hand, only 3 workers in the commercial sector were infected and they were reported to be recovered [12,13]. Among the common sources of human infection is exposing to high H5N1 viral load during: slaughtering, defeathering, evisceration of birds, cleaning of infected premises and playing with infected birds.

The rearing of poultry outdoors or on range in areas with access to AIV infected free- birds is a major risk factor for transmission from wild birds to agriculture systems which resulted in infections [14]. Domestic ducks that are in contact with wild waterfowl and wild birds as well as other poultry species that can act as key intermediaries in the transmission of avian influenza among birds [15]. The examined samples showed positivity for H5N1 although other AIVs rather than H5N1 (Non-H5N1) were recorded recently in Egypt [16,17]. Adoption of vaccination as a control measure in Egypt was mostly unsuccessful [18] and resulted in vaccination pressure and evolution of vaccine resistant H5N1 strains in Egypt [19,20]. Domestic ducks have been implicated in the dissemination and evolution of H5N1 HPAI viruses, and their inclusion in disease control programs is therefore important [15,21]. The role of backyard aquatic birds in evolution of viral mutants was evidenced in Egypt [22].

Viruses contain polybasic cleavage motif found to be associated with highly pathogenic AIVs [23], and the PQGERRRKKR/GLF cleavage site pattern was found in the majority of the Egyptian viruses of 2006 [24], however, sequence diversity in the cleavage site was recorded in the Egyptian stains [22]. In the current study, only 3/15 strains, showed the multibasic HA0 amino acid cleavage sequence characteristic for the highly pathogenic H5N1 strains clade 2.2 recorded in [24]. The rest of our isolates

(12/15) showed amino acid substitutions: PQGEGRRKKR/GLF in one strain while the remaining strains (11/15) possess PQGEKRRKKR/GLF cleavage site. The impact of the amino acid substitutions in the cleavage site is not known, however, all variations within the polybasic cleavage motif probably do not affect cleavage of the HA precursor molecule, since the RX(K/R)R consensus motif for cleavage by furin or subtilisin-like protein convertases [25] remains conserved.

The presence of a polybasic cleavage site is not the sole determinant for virulence in chicken and beyond the polybasic cleavage site, the virulence of HPAIV in chicken is based on additional pathogenicity determinants within the HA itself or other viral proteins [26].

Phylogenetic analyses revealed that the HA1 of the currently isolated strains of 2014 are related to the newly recorded sublineage, 2.2.1.2 [27]. Although the nucleotide identities did not clearly show clear segregation between different lineages of H5N1 circulated in Egypt, that probably due to the presence of variable number of silent mutations. The amino acid identities showed 97-99% amino acid homology to the lineage 2.2.1.2. L to F amino acid substitution was detected in 1/5 of the characterized strains in the signal peptide.

There are three highly conserved potential N-glycosylation sites in the stem of the HA1 protein: N10/11, N23 and N286 (H5 numbering) [28]. It has been previously reported that, loss of N-linked glycans in the stalk region at residue 11, leading to increased virulence [29]. Moreover, it has been demonstrated that glycans in the stalk and the length of the connecting peptide, determine the cleavability of the H5 influenza virus HA [30]. The Egyptian isolates in the current study possess four N-glycosylation sites of 11NST, 23NVT, 165NNT and 286NSS, that were similar to most of the Egyptian isolates. The H5N1 isolates in the current study own amino acids Q222 and G224 (H5 numbering) at the receptor-binding site in the binding pocket of the HA, denoting a preferential binding of  $\alpha$ -2,3 linkage, typical for the avian viruses [31,32]. Previously, it was identified that the majority of clade 2.2. HA proteins contained an arginine (R) residue at amino acid position 189 and lost the glycosylation site at amino acid 158. Arginine at position 189 increased the affinity of HA for  $\alpha$ 2-6 linked receptor [33], leading to increased affinity for humans with reduced affinity for poultry. All of the five Egyptian isolates in our study in 2014, lost the 158 glycosylation site and have arginine or lysine (K) at position 189, consistent with other clade 2.2. viruses.

Different amino acids that are implicated in receptor specificity, Y94, S132, W149, H179, E186, K189, L190, E212, P217, K218, G221, Q222, S223

and G224 (H5 influenza numbering), were tested [34]. Interestingly, 94 (Y to N), 212 (E to K) and 217(P to S) which were found in the five examined avian isolates have also been found in many other isolates from the Middle East in the flu database.

R to K189 substitution was recorded in 2/5 isolates: A/Duck/Egypt/BSU-NLQP-BS-3/2014, and A/Chicken/Egypt/ BSU-NLQP-MN-4/2014. Additionally, seven new sites (N94, S120, R162, E227, N252, T263, and I282) were allocated as antibody binding sites [35]. The currently isolated strains also had S120D and R162K.

A134V, N182K and S223N mutations were reported to confer  $\alpha$ -2,6-linked sialic acid binding to H5N1 virus [36,37]. None of such substitutions were found in the currently isolated strains. In addition, a lack of amino acid S129 was recorded.

Interestingly, AIVs with this deletion (S129) seem to evolve to a receptor usage that is similar to that of seasonal human H1N1 [38].

Specified six amino acid residues (86V, 124S, L/N138, T/S156, E/R212 and T263) are linked to the virulence of H5N1 in mammals [39]. T263 was found in all the examined strains in the current study while T156 was found only in one isolate.

H5N1 circulation in backyard poultry resulted in infection to mammals in a silent form [40, 41, 42], thus constituting a major risk to human.

## 5. Conclusion

Genetic analysis of the HA gene revealed different amino acid substitutions in the cleavage site and also along in the entire HA1 gene. Such findings reveals that vaccination pressure and raising mixed poultry populations could possess crucial roles in the evolution of multiple mutations. The Consensus sequence of cleavage site for our 15 Egyptian isolates in this study, from 2009 to 2014, was mainly PQGEKRRRKKR/GLF (11/15 isolates).

Phylogenetic analysis of the HA1, revealed that the isolated strains are belonging to the new subclade 2.2.1.2. Also, presence of many point mutations that may affect the pathogenicity of H5N1 viruses or their virulence affinity toward mammals (including humans) and poultry.

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