Relationship between some H5 commercial vaccines and the highly pathogenic H5N8 avian influenza virus that is recently circulating in Egypt

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ABSTRACT

The prevalence of avian influenza viruses (AIVs) in Egypt has had a devastating effect on the poultry sector. Here, we isolated two HPAI (H5N8) viruses from broiler poultry farms in Menoufia and Dakahlia governorates during the period from November 2020 to December 2021. The phylogenetic profiling of the two isolates showed a relationship to clade 2.3.4.4b viruses. The percentage of identity between the sequences of nucleotides of the haemagglutinin gene (HA) between our two isolates and the two commercial vaccinogenic viruses; A/chicken/Egypt/18-H/2009/H5N1 that belongs to clade 2.2.1.1, and A/duck/postdam/1402/1986 (H5N2) that belongs to classical strains, is 84% and 87%, respectively, while the percentage of amino acid similarity is 88% and 86%, respectively. In addition, we compared the 3D protein structure of the HA gene from our recently isolated H5N8 viruses with the identical commercial vaccines previously mentioned, and we found that the newly emerging H5N8 viruses have some mutations in antigenic sites A, C, D, and E that are not found in the commercial vaccines. This antigenic mismatching leads to the vaccinal failure of avian influenza and the appearance of escape mutant strains.

Key words: AIVs, HA, vaccine

1. INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) is a highly infectious avian disease that generates significant financial damages for the poultry trade and threatens human health (Neumann, 2015). H5N8 was acquired from wild birds and has replaced the prevalent H5N1 virus in poultry (Amer *et al*. 2021). 2.3.4.4b H5N8 viruses were detected in Egypt in 2016 (Selim *et al*. 2017). Since that time, several H5N8 infections in poultry were found in backyard flocks, live poultry markets, and intensive raising farms in various Egyptian regions. At least five genotypes (Gt1-Gt5) of Clade 2.3.4.4b H5N8 virus were subgrouped; Gt-1 and Gt-2 were revealed in 2016, Gt-3 and -4 in 2017, Gt-5 since 2017 (Hassan *et al*. 2020).

The entire genetic structure of AIVs is eight segments, single-stranded, RNAs that are tied by both the vRdRp and the nucleoprotein (NP) (Bouvier and Plese, 2008). The primary surface glycoprotein, Hemagglutinin (HA), is encoded by segment number four of

the AIV genome. The HA is essential for IAV replication and host-specific adaptability as it is liable for the receptor attachment of AIV to the cellular membrane of the host by the receptor binding residues which are found in the domain of HA head (Mair *et al*. 2014). Numerous point mutations in the HA affect pathogenicity, host range, replication efficiency, antigenicity, and receptor binding selectivity (Ayora-Talavera *et al*. 2009; Myers et al. 2013; Kim *et al*. 2014).

In Egypt, vaccination is considered a crucial component of control measures. Despite the application of several vaccine strategies against AIVs in Egyptian chicken farms, newly evolving virus mutants are still evolving. The genetic and antigenic mismatching between most of the used vaccines and the circulating viruses is a principal cause of this failure, increasing the need to reevaluating the genetic and antigenic structure of spreading viruses during vaccine design (Kandeil *et al*. 2018). Moreover, previous research showed that the best control methods of viral shedding and lethality comes from increased antigenic identity between vaccinogenic and challenge viruses (Pica and Palse, 2013). This study characterizes recently isolated avian influenza viruses and determining the genetic and antigenic similarity between newly distributing avian influenza viruses and two routinely used commercial killed vaccines.

2. METHODOLOGY

2.1. Sample Collection

A total of 50 oropharyngeal swabs were taken from broiler poultry flocks in the Menoufia and Dakhlia governorates during the period from November 2020 to December 2021 that were exhibiting respiratory symptoms, such as sneezing, coughing and tracheal rales.

2.2. Isolation of Viral RNA

After that, isolation of RNA was carried out as stated by the company's instructions for the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany).

2.3. Real-time RT- PCR for detection of M gene

Real-time polymerase chain reaction was used to detect the M gene in the samples (Spackman *et al*. 2003) by using QuantiTect probe RT-PCR catalogue No. 204443 that affords precise concurrent quantification of viral RNA (Table 1).

2.4. Viral isolation:

The M gene positive samples were individually injected into the allantoic cavity of specified pathogen-free embryonated hens' eggs aged 10 days. After the injection, 48 hours were spent incubating at 37^{o}C, and then refrigerated for 4 hours. Then, allantoic fluid was gathered and underwent subtyping by RT-PCR (Lee et al. 2001; Tsukamoto *et al*. 2009).

2.5. Subtyping:

Viral RNA was isolated from the harvested allantoic fluids of the isolated samples by applying of QIAamp Viral RNA Mini Kit (Qiagen,Valencia, Calif., USA, Cat. No.52904). RT-PCR was conducted for subtyping of HA and NA genes by utilizing primers in table (2).

2.6. DNA Sequencing and Analysis

Two isolates were submitted for complete sequencing of the HA gene by using of Next Generation Sequencing (IonTorrent System). Raw data was analyzed by ion reporter software then obtained Sequences were deposited in GenBank by the aid of the web tool BankIt of Genbank http:// www.ncbi.nlm.nih.gov/ WebSub/?tool=genBank.

Alignment of sequences was achieved by using Bio Edit software version 7.2.5. A nucleic acid BLASTN homology analysis was carried out, and international linked sequences were taken. Multiple alignments were carried out by BioEdit software 7.2.5 (BioEdit Company, Manchester, UK). Next, using the MEGA 5.03 programme, a phylogenetic tree was constructed from aligned sequences (Microsoft Windows, Redmond, WA, USA) using the aid of 1000 bootstrap replicates, the Kimura two-parameter distance model, and a neighbor-joining tool. The genetic signature markers connected with antigenic sites were defined using aligned amino acid sequences of each protein. The Bio Edit software version 7.2.5 was used to compare the resemblance of aligned nucleotide and protein sequences.

The 3D protein structure of the Egyptian H5N8 virus and the commercial vaccines were displayed by the SWISS-MODEL modeling online server (Arnold *et al*. 2006) and visualized by PyMOL 1.1 software (DeLano Scientific LLC).

2.7. Commercial vaccines:

The sequences of the HA genes of the two inactive commercial vaccines were supplied from the gene Bank. Information about the two vaccines is shown in table (3).

3. RESULTS

3.1. Detection of positive samples:

In the present study, 12 out of 50 oropharyngeal swabs were positive for AIV by qRT-PCR as shown in Figure (1). The CT values were ranged from 15 to 21 as shown in Table (4).

3.2. Results of subtyping:

The thirteen samples were related to H5N8 subtype by conventional RT-PCR.

3.3. Accession IDs of the obtained nucleotide sequences:

Two isolates were submitted for complete HA gene sequencing. The identified sequences were added to GenBank with the accession numbers in table (5).

3.4. Genetic and Antigenic analysis:

The HA proteins of our two H5N8 isolates under study had 103H, 222Q and 224G which are pointing to a preference for avian-like receptors over human-like receptors in the binding process. The two isolates had the "PLREKRRKR/ GLF" multiple basic amino acid motif at the cleavage site. Moreover, comparing the 3D protein structure of the HA gene of our isolate A/chicken/Egypt/Dakahlia and the two commercial vaccines A/chicken/Egypt/18-H/2009/H5N1 and A/duck/postdam/1402/1986(H5N2) showed the presence of many mutations in the antigenic site of our detected isolate that are not found in the two vaccinal strains as shown in Figures 2,3 and 4.

3.5. Phylogenetic profiling:

Based on the nucleotide sequence of the HA gene, phylogenetic profiling of identified isolates and a few other HPAI H5N8 isolated from Egypt and other nations was carried out (Fig. 5). The two isolates grouped with other Egyptian H5N8 isolates in clade 2.3.4.4b.

3.6. Similarity percent:

Between our two isolates and other H5N8 isolates, the nucleotide sequence identity percentage was from (90%-98%) while the amino acid identity percent ranged from (92% -99%). The nucleotide sequence identity percent between our two isolates and the two vaccinal strains used in the current study including A/chicken/Egypt/18-H/2009/H5N1 that belongs to

clade2.2.1.1, and A/duck/postdam/1402/1986(H5N2) that belongs to classical strains is 84% and 87% respectively. While, the percentage of amino acid similarity is 88% and 86% respectively as shown in table (6).

4. DISCUSSION

2.3.4.4b H5N8 viruses were first reported in China and South Korea in 2013 in domesticated birds then after that, this category was found in wild birds in 2016 (Kwon *et al*. 2018). The first virological evidence of a zoonotic transmission of highly pathogenic avian influenza A (H5N8) virus from birds to humans was reported in Russia in 2021, but there had been no evidence of transmission from person to person (WHO, 2021).

The H5N8 viruses have undergone ongoing reassortment and evolution, resulting in the diversification of six genotypes (Hassan *et al*. 2020; Hagag *et al*. 2022). Generally, H5N8 has supplanted the endemic H5N1 virus in poultry and is distributed widely in commercial farms. H5N8 was acquired from untamed birds which play a fundamental role in the distribution of avian influenza viruses. Due to its location at the confluence of two migratory bird flyways, the East Africa-West Asia Flyway and the Black Sea-Mediterranean Flyway, Egypt is thought to be a prime location for influenza virus evolution (Amer *et al*. 2021; Kandeil *et al*. 2022).

Our study reported two H5N8 viruses that were isolated from broiler poultry farms through 2021. The HA amino acid of our two H5N8 Egyptian viruses maintained 103H, 222Q and 224G residues, which are indicative of preferential attachment to avian like α2, 3-sialic acid receptors rather than the humanlike α2, 6-sialic acid receptors as reported (Cai *et al*. 2012; Mair *et al*. 2014; Kandeil *et al*. 2017; Yehia *et al*. 2018; Hassan *et al*. 2020).

The HA amino acid had polybasic amino acid at the cleavage site "PLREKRRKR/ GLF" which is characteristic of HPAIV, clade 2.3.4.4b and is similar to previously characterized Egyptian isolates (Selim *et al*. 2017; Anis *et al*. 2018; Salaheldin *et al*. 2018 ; Shehata *et al*. 2019 ; Hassan *et al*. 2020). In addition, the presence of polybasic sequences at HA1/HA2 cleavage site indicates the virulence of these isolates as reported (Kandeil *et al*. 2017; Yehia *et al*. 2018; Hassan *et al*. 2020). A previous study showed that four mutations (Q222L, G224S, T156A and H103Y) in HA glycoprotein of H5N1 virus can render it transmissible between ferrets via respiratory droplets (Herfst *et al*. 2012). Our two Egyptian H5N8 isolates have the T156A mutation which would increase its transmissibility to mammals.

According to Cai *et al*. (2012), five antigenic sites are recognized in highly pathogenic H5 influenza viruses (A, B, C, D and E).Those antigenic sites control antibody binding efficacy and therefore the vaccinal response. In the present study, comparison between the 3D protein structure of HA gene of our isolate A/chicken/Egypt/Dakahlia and HA proteins of two commercial vaccines displayed that our isolate had three mutations (A140T, S141P and S133A) in antigenic site A and D45N substitution in antigenic site C. another mutation, V174I was found in antigenic site D. Moreover, A83D mutation was found in antigenic site E as shown in Figure (1). The 3D protein structure of A/chicken/Egypt/18-H/2009 revealed the presence of only two mutations, one in the antigenic site B, A184E and one mutation in the antigenic site E, A83I as shown in Figure (2) while all the other antigenic sites are different from the detected

mutations in our H5N8 isolates. The 3D protein structure of A/duck/postdam/1402/1986 displayed the presence of three mutations; one at antigenic site C, D45N, I71L mutation was detected in antigenic site E and V174I mutation in antigenic site D. These observations show that the newly developing H5 viruses differ in their antigens from the commonly applied vaccines that lead to vaccinal failure. These results confirm previous studies that reported the high antigenic difference between clade 2.3.4.4b viruses and viruses from other clades (Ohkawara *et al*. 2017).

Phylogenetic profiling revealed that the two isolates were grouped into clade 2.3.4.4b H5N8 viruses. These results also agreed with Hassan *et al*. (2020), who reported the circulation of clade 2.3.4.4b H5N8 viruses in Egyptian domesticated birds sectors since 2017. The nucleotide sequence similarity between the HA gene sequences of our two Egyptian H5N8 isolates and the vaccinal strains including A/chicken/Egypt/18-H/2009/H5N1 which belongs to clade 2.2.1.1 and A/duck/Potsdam/1402/1986 (H5N2) which belongs to classical strains was 87% and 84% respectively. While the amino acid sequence similarity was 88% and 86% respectively. These findings are in agreement with Kandeil *et al*. (2018), who reported that the genetic mismatch between most of the commercial poultry H5 vaccines and the currently circulating H5N8 viruses results in ineffective vaccination.

5. CONCLUSION

In the present study, two HPAI H5N8 viruses were isolated from broiler poultry farms in Egypt during 2021. The two isolates were correlated with clade 2.3.4.4b. The comparison between the antigenic sites of the HA gene of one detected isolate and two commercial H5 vaccines revealed that there are antigenic variation amongst the H5 viruses that are now circulating in Egypt and the applied commercial vaccines and highlights the urgent need for the genetic and immunological update of avian influenza vaccines.

6. REFERENCES

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Table (1): Sequences of primers and probe used in the qRT-PCR for detection of M gene.

Virus	Gene	Primer/ probe sequence 5'-3'	Reference
AI	M	**Sep1** AGATGAGTCTTCTAA CCGAGGTCG	**Spackman *et al*. 2003**
		Sep 2 TGCAAAAACATCTTC AAGTCTCTG	
		SEPRO [FAM]TCAGGCCCC CTCAAAGCCGA [TAMRA]	

Table (2): Sequences of Primers used in the subtyping of avian influenza virus.

Target	Primer	Sequence (5′ to 3′)	Product size(bp)	Reference
H5	H5–155f	ACACATGCYCARGACATACT	545	**Lee *et al*., 2001**
	H5–699r	CTYTGRTTYAGTGTTGATGT		
H9	H9–151f	CTYCACACAGARCACAATGG	488	
	H9–638r	GTCACACTTGTTGTTGTRTC		
N2	N2-59F	TYTCTMTAACYATTGCRWCARTATG	278	**Tsukamoto *et al*., 2009**
	N2-336R	GARTT GTCYT TRGAR AAVGG		
N8	N8-93F	CATRTVGTBAGYATYAYARTAAC	137	
	N8-209R	ACAYTRGYATTGTRCCATTG		

Table (3): Vaccines used for detection of antigenic characters.

Vaccine name	Virus used	Lineage
The Egy Flu	A/chicken/Egypt/18-H/2009/H5N1	2.2.1.1
Nobilis	A/duck/Potsdam/1402/1986(H5N2)	Classical

Table (4): CT values of the positive samples.

Sample	AI Matrix	
	Result	CT
1	+	16
2	+	15
3	+	16
4	+	15
5	+	16
6	+	16
7	+	16
8	+	15
9	+	18
10	+	17
11	+	21
12	+	15

Table (5): Accession numbers of submitted HA genes of detected isolates.

ON739168	(A/chicken/Egypt/Dakahlia segment 4 HA gene/2021(H5N8)) segment 4 hemagglutinin (HA) gene, complete cds
OP413016	(A/chicken/Egypt/Menoufia/2021(H5N8)) segment 4 hemagglutinin (HA) gene, complete cds

Table (6): Nucleotide and amino acid sequences identity of HA gene of HPAI H5N8 isolates with other strains that are in circulation in Egypt and other nations.

Amino acid Identity / Nucleotide Identity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
1		99%	97%	97%	98%	99%	91%	92%	90%	90%	88%	90%	98%	98%	97%	98%	98%	98%	98%	98%	98%	98%	91%	87%	84%	1
2	99%		99%	97%	97%	97%	98%	91%	92%	90%	90%	88%	90%	98%	98%	98%	98%	98%	98%	98%	98%	98%	91%	87%	84%	2
3	99%	98%		100 %	97%	98%	90%	92%	90%	90%	88%	90%	97%	97%	97%	97%	97%	98%	98%	98%	98%	98%	90%	87%	85%	3
4	99%	98%	100 %		97%	98%	90%	92%	90%	90%	88%	90%	97%	97%	97%	97%	97%	98%	98%	98%	98%	98%	90%	87%	85%	4
5	99%	98%	98%	98%		99%	92%	93%	92%	92%	89%	92%	98%	98%	98%	99%	99%	99%	99%	99%	99%	99%	92%	87%	85%	5
6	99%	99%	99%	99%	99%		92%	94%	92%	91%	89%	91%	99%	99%	99%	99%	99%	99%	93%	99%	99%	99%	92%	87%	85%	6
7	93%	93%	92%	92%	94%	93%		98%	97%	97%	95%	98%	92%	92%	92%	93%	93%	93%	93%	93%	93%	93%	96%	91%	88%	7
8	94%	94%	94%	94%	94%	95%	98%		97%	97%	94%	96%	94%	93%	93%	93%	93%	93%	92%	93%	93%	94%	97%	91%	88%	8
9	92%	92%	92%	92%	93%	93%	98%	98%		93%	96%	92%	96%	91%	91%	92%	92%	92%	92%	93%	92%	93%	95%	90%	87%	9
10	93%	92%	92%	92%	93%	93%	98%	98%	98%		96%	92%	96%	91%	91%	92%	92%	92%	92%	92%	92%	92%	95%	90%	87%	10
11	90%	90%	99%	90%	99%	91%	96%	95%	95%	95%		96%	94%	89%	89%	92%	92%	92%	99%	93%	92%	93%	95%	91%	87%	11
12	93%	92%	92%	92%	99%	93%	98%	97%	97%	97%	93%		96%	91%	91%	99%	99%	98%	99%	99%	99%	99%	92%	88%	85%	12
13	99%	99%	99%	99%	99%	99%	93%	95%	93%	93%	93%	93%		99%	99%	99%	99%	98%	99%	99%	99%	99%	92%	87%	85%	13
14	99%	90%	99%	99%	99%	99%	93%	95%	93%	93%	93%	93%	100 %		99%	99%	99%	98%	99%	99%	99%	99%	92%	87%	84%	14
15	99%	99%	98%	98%	99%	99%	93%	94%	93%	93%	93%	93%	99%	99%		99%	99%	98%	99%	99%	99%	99%	92%	87%	84%	15
16	99%	99%	99%	98%	98%	99%	93%	94%	93%	93%	93%	93%	99%	99%	100 %		99%	98%	99%	99%	99%	99%	92%	87%	84%	16
17	99%	99%	98%	98%	98%	99%	93%	93%	93%	93%	94%	93%	99%	99%	100 %	100 %		98%	99%	99%	99%	99%	92%	87%	84%	17
18	98%	98%	97%	97%	98%	98%	94%	93%	93%	93%	93%	94%	99%	98%	98%	99%	99%		98%	98%	98%	98%	91%	87%	85%	18
19	97%	97%	97%	97%	99%	98%	94%	93%	93%	93%	93%	94%	98%	98%	98%	98%	98%	99%		99%	99%	99%	92%	87%	85%	19
20	97%	97%	97%	97%	99%	98%	93%	93%	93%	93%	94%	93%	98%	98%	97%	97%	97%	98%	98%		99%	99%	92%	87%	84%	20
21	98%	98%	98%	98%	98%	98%	94%	93%	92%	93%	93%	94%	98%	98%	98%	98%	98%	99%	99%	98%		98%	91%	87%	85%	21
22	98%	98%	98%	98%	99%	98%	94%	93%	93%	93%	93%	94%	98%	98%	98%	99%	98%	99%	99%	98%	99%		92%	87%	85%	22
23	92%	92%	92%	92%	92%	92%	95%	96%	95%	95%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%		92%	88%	23
24	88%	88%	88%	88%	89%	89%	91%	91%	91%	91%	89%	89%	88%	88%	88%	88%	88%	88%	89%	88%	89%	91%	92%		88%	24
25	86%	86%	86%	86%	86%	86%	89%	89%	88%	88%	86%	86%	86%	86%	86%	86%	86%	86%	86%	86%	86%	89%	88%	92%		25
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	

1 A/chicken/Egypt/Menoufia/2021
2 A/chicken/Egypt/Dakahlia
3 A/duck/Egypt/Ismailia/2021
4 A/Turkey/Egypt/Giza/2021
5 A/great egret/Israel/1084/2016
6 A/Teal/Dakahlia/VRLCU/2019
7 A/shrike/Tibet/13/2006
8 A/chicken/Sichuan/81/2005
9 A/Hunan/1/2009
10 A/Hunan/2/2009
11 A/chicken/Vietnam/NCVD-20
12 A/Vietnam/UT31412II/2008
13 A/chicken/ Egypt/N13732A
14 A/duck/Egypt/N13736E/2017
15 A/chicken/Egypt/H13795A/2017
16 A/goose/Egypt/A13779B/2017
17 A/teal/Egypt/1202C/2017
18 A/duck/Egypt/SS19/2017
19 A/chicken/Egypt/FL6/2018
20 A/green-winged teal/Egypt/877/
21 A/Teal/Dakahlia/VRLCU/2019
22 A/chicken/Egypt/S18182C/2020
23 A/chicken/Vietnam/C58/04(H5N3)
24 A/chicen/Egypt/18-H/2009(H5N1)
25 A/duck/Potsdam/1402-6/1986(H5N2)

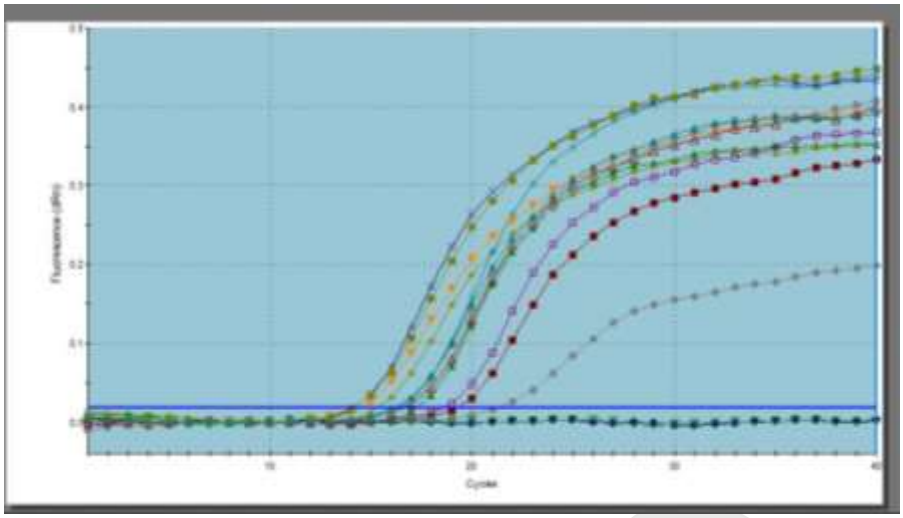


Fig (1): Amplification curves of positive samples including negative and positive controls.

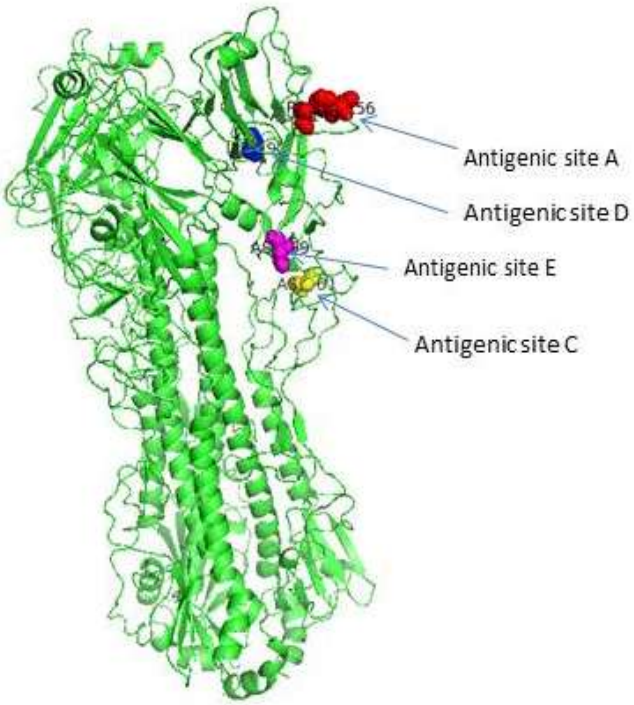


Fig (2): 3D protein structure of HA protein of HPAI H5N8 A/chicken/Egypt/Dakahlia isolate showing mutations in the antigenic sites A, C, D and E. most of the mutations were in antigenic site A

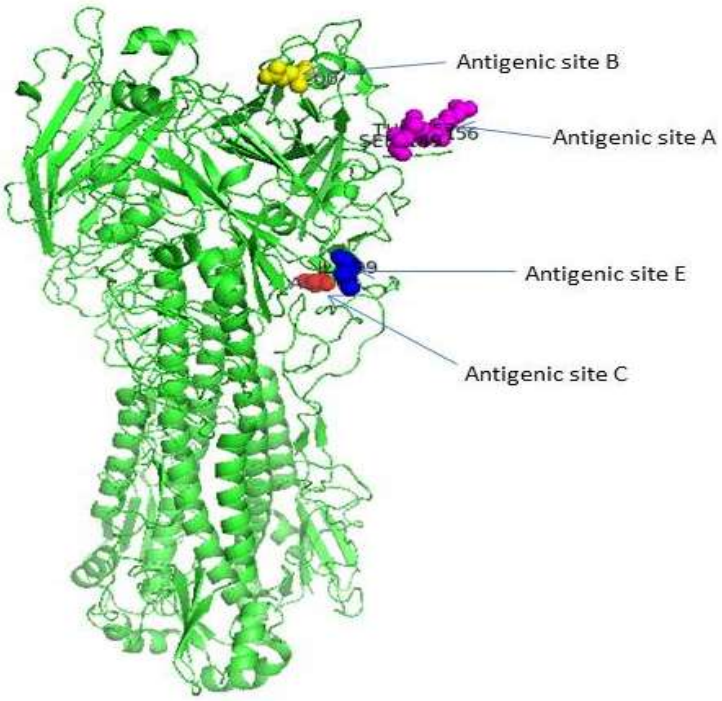


Fig (3): 3D protein structure of HA protein of A/chicken/Egypt/18-H/2009 vaccine showing the antigenic sites A, B, C and E. Two mutations were found in antigenic site B and E.

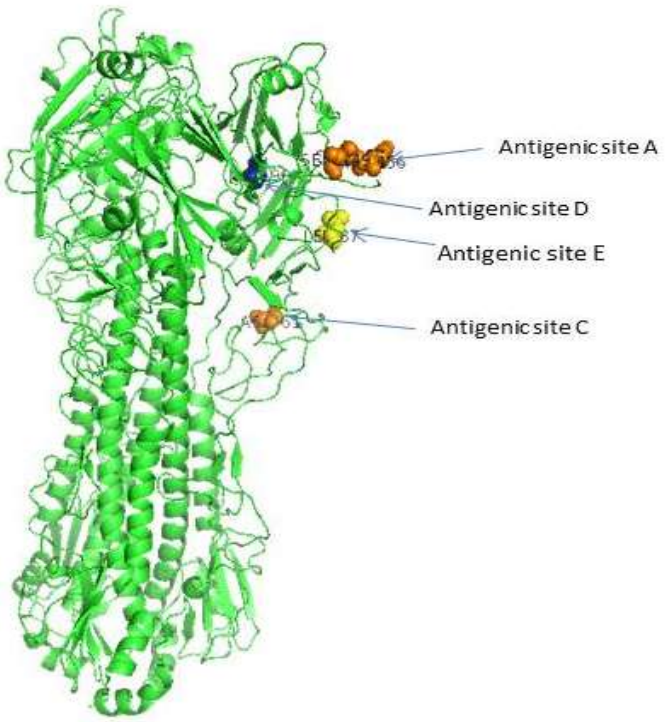
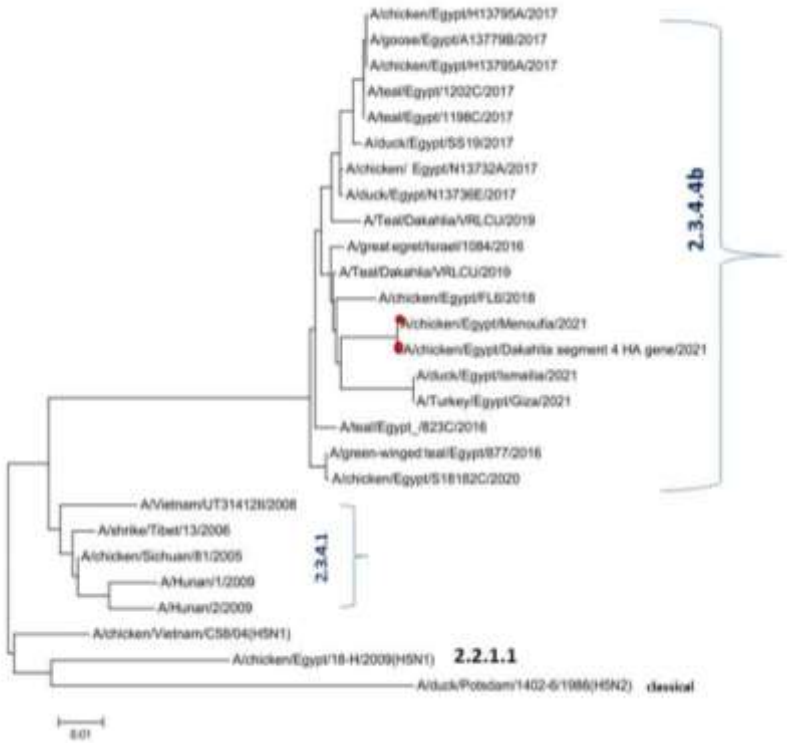


Fig (4): 3D protein structure of HA protein of A/duck/postdam/1402/1986 vaccine showing the antigenic sites A, D, C and E. Three mutations were found in antigenic sites C, D and E.



Fig(5): Phylogenetic relationship of the two H5N8 AIV local field isolates to other selected AIV isolates based on nucleotide sequence of the (HA) gene. Our isolates are identified by circular red marks.