
Genetic and Antigenic Relatedness of Highly Pathogenic Avian Influenza H5Nx Viruses Currently Circulating in Egypt and Some H5 Commercial Vaccines

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ABSTRACT

In the present study, samples were collected from wild and domestic waterfowls to detect currently circulating HPAI H5Nx viruses. All H5Nx detected in migratory ducks were characterized as HPAIV H5N8, clade 2.3.4.4b, while domestic waterfowls viruses belonged to HPAIV H5N1, clade 2.2.1.2. Partial HA gene sequencing followed by phylogenetic analysis of the obtained sequences of five selected isolates was performed. Phylogenetic analysis revealed clustering of the three H5N8 isolates in clade 2.3.4.4b, with other Egyptian H5N8 isolates with amino acid identity percent with each other ranging from (96.9% - 99.3%). The two H5N1 isolates clustered in clade 2.2.1.2 with other current Egyptian isolates. The amino acid identity percent between vaccinal strains and H5N8 field isolates ranged from (88.2% - 89.6%) while identity with H5N1 field isolates was (96.5% - 99%). The amino acid identity percent between H5N8 and H5N1 field isolates was (86.3% - 87.8%). Antigenic analysis of currently circulating HPAI H5Nx of different clades were conducted using cross HI test using hyperimmune sera raised against (A/Teal/Egypt/Damietta-1/2016/H5N8 and A/Geese/Egypt/Men-21/2016/H5N1) and three commonly used commercial vaccinal strains. The antigenic relatedness between the two HPAI local isolates (H5N1 and H5N8) was low (R value 24.8%) indicating major antigenic difference between the two subtypes. Also, a major subtype difference in antigenicity (R value 20.7-31.0%) was also detected between the local HPAI H5N8 isolate and the three vaccinal strains. While, a minor subtype difference (R value 38.7-68.7%) was detected among the examined H5N1 local isolate and the three vaccinal strains. Our results support that, HPAI H5N8 virus is genetically and antigenically distinct from HPAI H5N1 virus. Also, some commercial vaccines which are used to control avian influenza in Egypt are genetically and antigenically distinct from HPAI H5N8 virus currently circulating in Egypt. These findings necessitate updating the HA of H5 Vaccine to be genetically and antigenically closer to the field viruses together with, regular monitoring of wild birds to predict and prevent possible AIV outbreaks.

Key words: Antigenic, Egypt, Genetic, HPAI, H5Nx.

INTRODUCTION:

Highly Pathogenic Avian Influenza (HPAI) is a highly contagious avian disease that causes serious economic losses in poultry industry and has a potential threat to public health (Neumann, 2015). Migratory birds were considered to be the main source for introduction and re-introduction of AIV into many countries in Asia,

Europe, and Africa (Prosser *et al.*, 2011). The outbreak of highly pathogenic avian influenza of the H5N1 subtype in Asia, which has subsequently spread to Russia, the Middle East, Europe, and Africa, has put increased focus on the role of wild birds in the persistence of influenza viruses (Olsen *et al.*, 2006). In 2010, strains of HPAI H5N8 viruses of clade 2.3.4.4 were detected among wild birds in Asia and later

spread to domestic birds across China, South Korea, and Japan (Lee *et al.*, 2014). Recently, a novel reassortant virus of subtype H5N8 clade 2.3.4.4b was reported in Russia and further spread to many countries in Europe, Asia, and the Middle East and the spread has been linked to the overlapping flyways of migratory wild birds (Lee *et al.*, 2017). Egypt lies at the crossroads of 2 major spatially overlapping migration flyways therefore; attention has been paid to the role of wild birds in the introduction of different pathogens including AIVs (El-Zoghby *et al.*, 2013). The HPAIV H5N1 of clade 2.2.1 has been introduced into Egypt in early 2006 (Aly *et al.*, 2008) probably via infected wild ducks (Saad *et al.*, 2007). Despite control efforts, the virus had become endemic in poultry in Egypt since 2008 (Abdelwhab *et al.*, 2011). Another distinct cluster of HPAIV H5N1 clade 2.2.1.2 has emerged since 2012 (El-Shesheny *et al.*, 2014) and gained predominance since summer 2014 and caused an upsurge of outbreaks in poultry holdings in late 2014 (Arafa *et al.*, 2015). Recently, HPAI H5N8 virus of clade 2.3.4.4 has been introduced to Egypt through migratory birds in 2016 (Kandeil *et al.*, 2017; Selim *et al.*, 2017). Later on, Yehia *et al.*, (2018) documented the incursion of HPAI H5N8 viruses in both commercial farm and backyard sectors in Egypt. Strains of HPAIV H5N8 have been involved in multiple independent reassortment events with other AIV subtypes found in wild birds in China, South Korea, the United States, and recently in Russia (Lee *et al.*, 2017). The introduction of HPAIV H5N8 to poultry populations in Egypt may further complicate the current situation, especially if HPAI H5N1 of clade 2.2.1.2 and LPAI H9N2 strains are enzootic in poultry (Naguib *et al.*, 2015).

Vaccination has been used as an essential control strategy in Egypt. The genetic and antigenic matching between the circulating viruses and commercial vaccine strains influence the efficacy of vaccine (Wong and Webby, 2013). Previous studies demonstrated that matching antigenic similarity of the HA between the vaccine and challenge virus provides the best protection against mortality and virus shedding (Pica and Palse, 2013 & Romer-Oberdorfer *et al.*, 2008).

Although some of the commercial vaccines protected chickens from mortalities after H5N8 challenge, they failed to prevent shedding of the virus (Kandeil *et al.*, 2018). The aim of this study is to determine the genetic and antigenic relatedness within recently circulating HPAI H5Nx and between three commonly used inactivated oil adjuvant commercial vaccines.

MATERIAL AND METHODS:

Clinical Sample:

Thirty-two tracheal samples were collected from waterfowls (wild and domestic) during the period from March 2016 till March 2017. Twenty-five samples were collected from different localities in Damietta governorate from four different species of wild ducks (teal, pintail, mallard and coot). Other 7 samples were collected from domestic waterfowls from Menofiya and Giza governorate. Samples were homogenized with saline containing antibiotics in a sterile mortars and pestles. Freezing and thawing of the homogenate was done three times and then clarified by centrifugation. The supernatants were collected and kept at -80°C until used for RNA extraction.

Molecular detection of AIV:

RNA from tracheal samples homogenates was extracted using viral Gene-SpinTM viral DNA/RNA extraction kit (iNtRON Biotechnology, Inc.) according to the manufacturer instructions. H5-HA1-813FP (forward primer 5': TGTC AAGAAAGGGGACTCAACA) and H5-HA2-1123 RP (reverse primer 3': GCTCGTTGCTATGGTGGTAC) were used for partial amplification of HA gene (kindly provided by Dr Chang-Won Lee, The Ohio State University, OH, USA). These primers overlapping the HA gene cleavage site and the expected band size is 310 bp. The RNA of all identified positive samples were shipped to Prof. Dr. Timm C. Harder (OIE reference Lab., Germany) for subtyping by real time RT-qPCR Linage typing. The purified RT-PCR product of five selected positive samples (three wild and two domestic) were shipped to Germany through Biovision Egypt company for sequencing of the amplified part (310bp) of the HA gene.

Genetic analysis:

A BLAST analyses (<http://www.ncbi.nlm.nih.gov/BLAST>) were conducted on each sequence to identify related reference viruses. The nucleotide sequences were analyzed with the BIOEDIT program using the Clustal W alignment algorithm to determine nucleotide and amino acid sequence similarity. Phylogenetic trees were constructed by the maximum likelihood method using MEGA 7 software. The phylogeny test options used to construct the trees were 1000 bootstrap replicates, complete deletion of gaps/missing data and nearest neighbor interchange for the heuristic method. The Hasegawa–Kishino–Yano nucleotide substitution model was used as selected by MEGA software as the best model. Sequence submission was conducted following the instructions offered by the web tool **BankIt** of Genbank <http://www.ncbi.nlm.nih.gov/WebSub/?tool=genBank>.

Antigenic analysis:

Vaccines and their antigens: Two types of inactivated oil-emulsion AIV experimental vaccines (H5N8 and H5N1) and their homologous antigens were prepared from two local isolates (**A/Geese/Egypt/Men-21/2016/H5N1** and **A/Teal/Egypt/Damietta-1/2016/H5N8**) kindly prepared by local company (MEVAC). Selected viruses were sent to MEVAC where it were propagated and titrated in specific pathogen free (SPF) embryonated chicken eggs then suspensions were inactivated using 0.2% formalin (Sigma Chemical Co., St. Louis, MO) for 36 hr. at 37°C. Complete inactivation was assured by passaging the inactivated suspensions in SPF-ECE for three successive passages via the allantoic sac route. The haemagglutination activities were calculated and 350 HAU/dose were mixed with Montanide™ ISA 70 VG adjuvant (SEPPIC SA, Puteaux, France) at a ratio of 30:70 (w/w) at

3000 rpm using the Silverson L5M high-shear Laboratory mixer (Silverson Machines, Inc., Buckinghamshire, United Kingdom).

Reference antigens and their antisera: Three vaccinal strains used as AIV haemagglutinating antigens (two different H5N1 and one H5N3) and there antisera were obtained from the local agencies to be used in HI test (**Table 1**).

Ducks: Muscovy ducklings were obtained from a commercial hatchery. They were floor reared under hygienic conditions in previously cleaned and disinfected experimental rooms; water and feed were provided ad-libitum. They were used for production of hyperimmune serum. Ducklings were inoculated with the experimental vaccines 1 ml/bird subcutaneously at 14th and 28th day and the antisera were collected at day 56 and tested for antibodies using HI test. The summary of the laboratory experiment is shown in (**Table 2**).

Haemagglutination Inhibition (HI) test (OIE, 2008): HI test was carried out according to the OIE manual using a 1% suspension of duck red blood cells. The HI antibody titer was determined as the reciprocal of the highest serum dilution that had complete inhibition of hemagglutination.

Determination of antigenic relatedness: The antigenic relatedness among different isolates was expressed as an R-value based on the Archetti and Horsfall formula; $r = \sqrt{r_1 \times r_2}$ (Archetti and Horsfall, 1950), using the cross HI results. The resulting R values were expressed as percentage relatedness and the interpretation of the results was done according to **Brooksby, (1967)** as follows:

(R) value between 0 – 10% = a serotype difference, (R) value between 11 – 32% = a major subtype differences, (R) value between 33 – 70% = a minor subtype differences and (R) value greater than 70% = a little or no differences.

Table (1): Vaccine antigen used in the study:

Vaccine trade name	Virus used	Lineage	Manufacturer, Country
EgyFlu	RG A/chicken/Egypt/18-H/2009/H5N1	Clade 2.2.1.1	Harbin Veterinary Research Institute, China
ME Flu VAC	A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1	Clade 2.2.1.2	ME-VAC, Egypt
Zoetis H5N3	RG A/chicken/Vietnam/C58/2004/H5N3	Clade 1	Zoetis, USA
*experimental H5N8	A/Teal/Egypt/Damietta-1/2016/H5N8	Clade 2.2.3.4	-
*experimental H5N1	A/Geese/Egypt/Men-21/2016/H5N1	Clade 2.2.1.2.	-

Table (2): Experimental plan for determination of antigenic relatedness between two (H5N8 and H5N1) field isolates and three commercial vaccines

Group no.	Duck no.	Vaccination Regime			Seroconversion
		Age/day	Type of vaccine	Dose/ml	
1	10	14	Inactivated	1	Seroconversion by cross HI test at 56 day using different antigens (H5N1 ^{3,4} , H5N3 ⁵ and local antigens H5N1 ⁶ and H5N8 ⁷).
		28	H5N8 ¹		
2	10	14	Inactivated	1	
		28	H5N1 ²		
3	10	None vaccinated			

- 1- local H5N8 inactivated oil emulsion vaccine
- 2- local H5N1 inactivated oil emulsion vaccine
- 3- A/chicken/Egypt/18-H/2009/H5N1
- 4- A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1
- 5- A/chicken/Vietnam/C58/04/H5N3
- 6- A/Geese/Egypt/Men-21/2016/H5N1 local isolate
- 7- A/Teal/Egypt/Damietta-1/2016/H5N8 local isolate

HI: Hemagglutination inhibition

no.: Number

RESULTS:

Molecular findings: In the present study most of investigated wild ducks were apparently healthy and showed no clinical signs or PM lesions. Fourteen samples from wild ducks (14/25) and two samples from domestic waterfowls (2/7) were H5 positive by RT-PCR. The RNA of all positive samples was shipped to Prof. Dr. Timm C. Harder (OIE reference Lab Germany) for subtyping. All H5Nx strains detected in migratory ducks were characterized as HPAIV H5N8, clade 2.3.4.4. The H5Nx detected in domestic waterfowls were characterized as HPAIV H5N1, clade 2.2.1.2.

Genetic analysis: Five H5 positive samples (three H5N8 and two H5N1) were sequenced, the amino acid sequences of HA cleavage site of the isolated strains revealed polybasic amino

acid indicating that they are highly pathogenic (PLREKRRKR/GLF in H5N8 viruses & PQGEKRRKRR/GLF in H5N1 viruses). The obtained sequences were assembled using BIOEDIT software and blasted to the National center for Biotechnology Information (NCBI) on the internet (www.ncbi.nlm.nih.gov) (**Table 3**).

Phylogenetic analysis of locally detected field isolates and some other HPAI H5N8 and H5N1 AIV isolates from Egypt and other countries was performed based on nucleotide sequence of the HA gene (**Fig. 1**). The three HPAI H5N8 isolates clustered in clade 2.3.4.4b with other Egyptian H5N8 isolates with amino acid identity percent with each other ranging from (96.9% - 99.3%) as shown in (**Table 4**). The three HPAI H5N8 isolates shared 99.3% similarity with the Russian HPAI H5N8 virus

A/great_crested_grebe/Uvs- Nuur_Lake /341/2016. The two HPAI H5N1 isolates clustered in clade 2.2.1.2 with other recent Egyptian isolates.

Vaccinal strain used in the current study including A/chicken/Vietnam/C58/04/H5N3, belong to clade 1, While vaccinal strains A/chicken/Egypt/18-H/2009/H5N1 belong to clade 2.2.1.1, and A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1 belong to Clade 2.2.1.2. The amino acid identity percent between vaccinal strains was ranging from (88.2% - 89.6%) with H5N8 field isolates and (96.5% - 99%) with H5N1 field isolates. The amino acid identity percent between HPAI H5N8 and HPAI H5N1 field isolates ranging from (86.3% - 87.8%).

Antigenic analysis:

Five antigens and their antisera were used in the antigenic analysis. The two local field isolates including (H5N8*) A/Teal/Egypt/Damietta-1/2016/ H5N8 and (H5N1*) A/Geese/Egypt/Men-21/2016/H5N1 were used with three commercial vaccinal strains including

A/chicken/Egypt/18-H/2009/ H5N1 (V1), (A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1 (V2) and A/chicken/Vietnam/C58/04/H5N3 (V3). The mean HI titers are shown in (Table 5).

The local isolate antigen (H5N8*) homologous titer was 6.5-Log₂. It cross reacted with V1, V2, V3 and local (H5N1*) antisera with (3.2, 2.7, 3 & 4.5) Log₂ differences, respectively. The local isolate antigen (H5N1*) homologous titer was 9.0-Log₂. It cross reacted with V1, V2, V3 and (H5N8*) antisera with (3.0, 0.5, 0.8 & 7.2) Log₂ differences, respectively.

The antigenic relatedness between the two local isolates (H5N1*) and (H5N8*) was low with R value 24.8% indicating major antigenic difference between the two subtypes. A major subtype difference in antigenicity was also detected between the local isolate (H5N8*) and the three vaccinal strains V1, V2 and V3 with R value (20.7, 31.0, 22.5), respectively. A minor subtype difference in antigenicity was detected between the local isolate (H5N1*) and the three vaccinal strains V1, V2 and V3 with R value (53.0, 68.7, 38.7), respectively as shown in (table 6).

Table (3): Designation and accession number of the identified isolates:

Designation	subtype	province	Host	Cleavage site of HA protien	GenBank Accession no.
A/Teal/Egypt/Damietta-1/2016/ H5N8	H5N8	Damietta	Teal duck	PLREKRRKR/GLF	MF215354
A/Teal/Egypt/Damietta-2/2016/ H5N8	H5N8	Damietta	Teal duck	PLREKRRKR/GLF	MF215355
A/Pintail/Egypt/Damietta-12/2016/ H5N8	H5N8	Damietta	Pintail duck	PLREKRRKR/GLF	MF215356
A/Geese/Egypt/Men-21/2016/H5N1	H5N1	Menofia	Native Geese	PQGEKRRKKR/GLF	MF2153568
A/Duck/Egypt/Giza-8/2016/H5N1	H5N1	Giza	Native duck	PQGEKRRKKR/GLF	MF2153571

(N.B.): the five isolates have not been published yet.

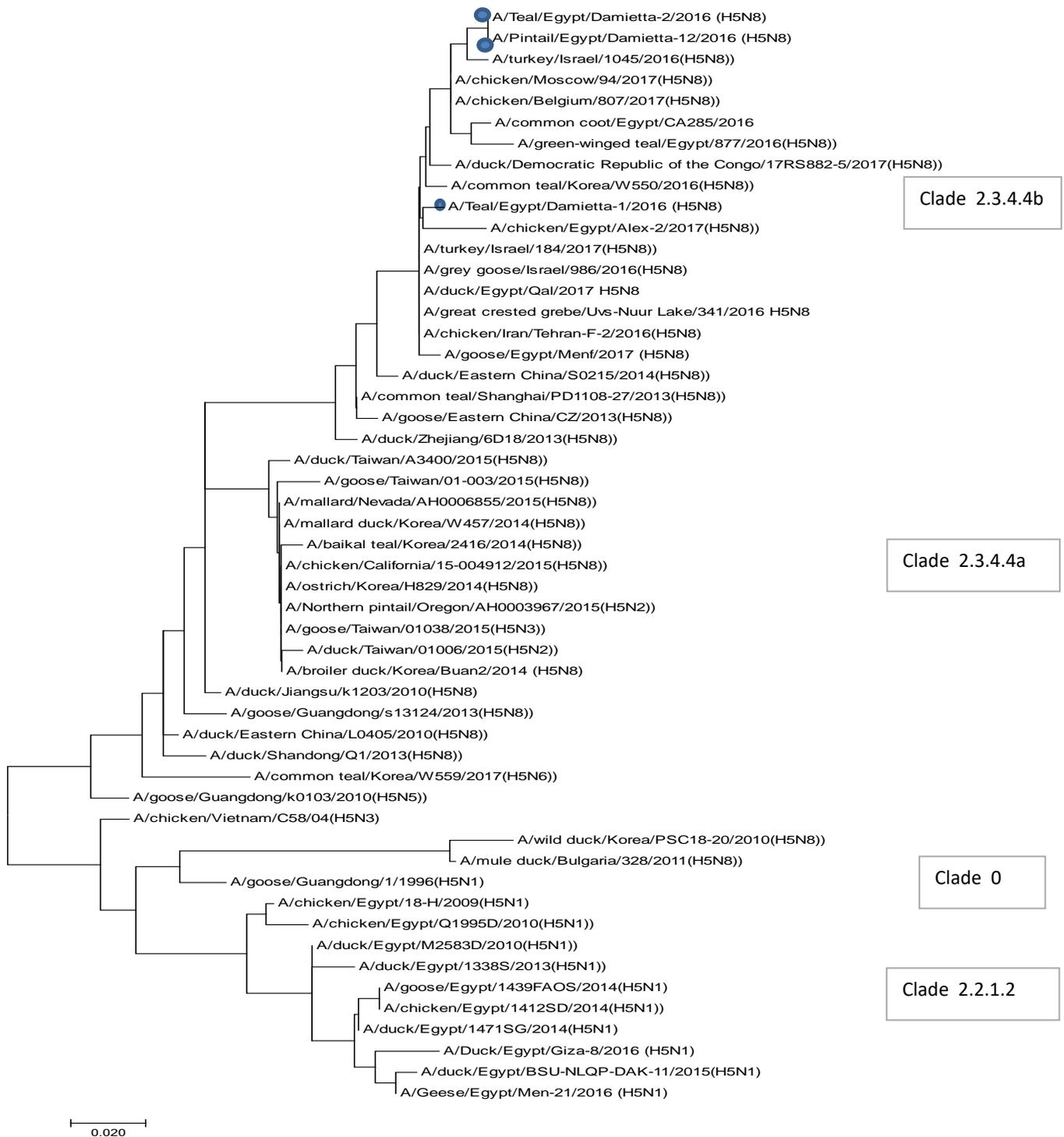


Fig. (1): Phylogenetic relationship of three H5N8 and two H5N1 AIV local field isolates /2016 to other selected AIV isolates based on nucleotide sequence of the (HA) gene. Tree was generated using Mega 7 program using Maximum likelihood method. The five isolates are labeled with round marks while, the vaccinal strains are labeled with triangular marks

Table (4): Amino acid sequences identity of obtained H5N8 and H5N1 HPAI isolates with HPAI strains circulating in Egypt and other countries and Avian Influenza vaccines showing identity and divergence percent based on A.A sequence comparison, black squares indicate identical sequence:

Percent Identity																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1	■	87.0	86.6	84.4	87.4	97.4	96.9	97.8	86.1	97.4	96.9	96.5	87.4	96.9	97.8	97.4	90.9	97.8	84.8	96.9	1
2	87.0	■	96.5	95.8	97.2	89.2	87.8	88.9	96.2	88.9	88.2	89.9	98.6	88.2	88.9	88.5	95.3	88.9	95.1	88.9	2
3	86.6	96.5	■	99.0	99.3	89.2	87.8	88.9	99.7	88.2	87.8	89.9	97.9	88.2	88.9	88.5	95.2	88.9	98.6	88.9	3
4	84.4	95.8	99.0	■	97.9	85.4	83.9	84.9	99.5	84.4	84.9	85.9	96.9	83.9	84.9	84.9	95.2	84.9	97.9	84.9	4
5	87.4	97.2	99.3	97.9	■	89.9	88.5	89.6	99.0	88.9	88.6	90.6	98.6	88.9	89.6	89.2	95.3	89.6	97.9	89.6	5
6	97.4	89.2	89.2	85.4	89.9	■	97.9	99.0	88.9	99.0	98.4	97.2	89.9	98.3	99.0	99.3	92.9	99.0	87.8	98.3	6
7	96.9	87.8	87.8	83.9	88.5	97.9	■	99.0	87.5	98.3	97.6	97.2	88.5	99.0	99.0	97.9	92.9	99.0	86.5	98.3	7
8	97.8	88.9	88.9	84.9	89.6	99.0	99.0	■	88.5	99.3	98.4	98.3	89.6	99.3	100.0	99.0	92.9	100.0	87.5	99.3	8
9	86.1	96.2	99.7	99.5	99.0	88.9	87.5	88.5	■	87.8	87.5	89.6	97.6	87.8	88.5	88.2	95.1	88.5	99.0	88.5	9
10	97.4	88.9	88.2	84.4	88.9	99.0	98.3	99.3	87.8	■	98.0	97.6	89.6	98.6	99.3	99.0	94.1	99.3	86.8	98.6	10
11	96.9	88.2	87.8	84.9	88.6	98.4	97.6	98.4	87.5	98.0	■	96.4	88.6	98.4	98.4	98.4	92.9	98.4	86.3	99.2	11
12	96.5	89.9	89.9	85.9	90.6	97.2	97.2	98.3	89.6	97.6	96.4	■	90.6	97.6	98.3	97.2	92.9	98.3	88.5	97.6	12
13	87.4	98.6	97.9	96.9	98.6	89.9	88.5	89.6	97.6	89.6	88.6	90.6	■	88.9	89.6	89.2	95.3	89.6	96.5	89.6	13
14	96.9	88.2	88.2	83.9	88.9	98.3	99.0	99.3	87.8	98.6	98.4	97.6	88.9	■	99.3	98.3	92.9	99.3	86.8	99.3	14
15	97.8	88.9	88.9	84.9	89.6	99.0	99.0	100.0	88.5	99.3	98.4	98.3	89.6	99.3	■	99.0	92.9	100.0	87.5	99.3	15
16	97.4	88.5	88.5	84.9	89.2	99.3	97.9	99.0	88.2	99.0	98.4	97.2	89.2	98.3	99.0	■	92.9	99.0	87.2	98.3	16
17	90.0	95.3	95.2	95.2	95.3	92.9	92.9	92.9	95.1	94.1	92.9	92.9	95.3	92.9	92.9	92.9	■	92.9	95.1	92.9	17
18	97.8	88.9	88.9	84.9	89.6	99.0	99.0	100.0	88.5	99.3	98.4	98.3	89.6	99.3	100.0	99.0	92.9	■	87.5	99.3	18
19	84.8	95.1	98.6	97.9	97.9	87.8	86.5	87.5	99.0	86.8	86.3	88.5	96.5	86.8	87.5	87.2	95.1	87.5	■	87.5	19
20	96.9	88.9	88.9	84.9	89.6	98.3	98.3	99.3	88.5	98.6	99.2	97.6	89.6	99.3	99.3	98.3	92.9	99.3	87.5	■	20
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	

- 1-(A/chicken/Egypt/Alex-2/2017/H5N8)
- 2-(A/chicken/Egypt/Q1995D/2010(H5N1))
- 3-(A/duck/Egypt/1471SG/2014(H5N1))
- 4-(A/duck/Egypt/BSU-NLQP-DAK- 11/2015(H5N1))
- 5-(A/duck/Egypt/M2583D/2010(H5N1))
- 6-(A/goose/Egypt/Menf/2017_(H5N8))
- 7-(A/green-winged teal/Egypt/877/2016 (H5N8))
- 8-(A/grey goose/Israel/986/2016(H5N8))
- 9-A/Geese/Egypt/Men-21/2016 (H5N1)
- 10- A/Teal/Egypt/Damietta-1/2016 (H5N8)
- 11- A/Teal/Egypt/Damietta-2/2016 (H5N8)

- 12-A /breeder_duck/Korea/Gochang1/2014 H5N8
- 13-A/chicken/Egypt/18-H/2009(H5N1)
- 14-A/common_coot/Egypt/CA285/2016
- 15-A/duck/Eastern_China/S1109/2014_H5N8
- 16- A/duck/Egypt/Qal/2017_H5N8
- 17- A/chicken/Vietnam/C58/04
- 18- A/great_crested_grebe/Uvs-uur_Lake/341/ 2016(H5N8)
- 19- A/Duck/Egypt/Giza-8/2016 (H5N1)
- 20- A/Pintail/Egypt/Damietta-12/2016 (H5N8)

Table (5): Cross haemagglutination inhibition (HI) test between two HPAI (H5N8 & H5N1) field isolates/2016 and between three commercial H5 vaccines:

Antigen	Antisera				
	HI* titer means Log ₋₂				
	S1	S2	S3	H5N1 ^{S*}	H5N8 ^{S*}
V1	9.5	6.5	2.5	4.0	0.8
V2	7.5	9.0	4.2	4.5	1.8
V3	7.2	7.4	8.5	1.4	0.8
H5N1 ^{4*}	6.0	8.5	8.2	9.0	1.8
H5N8 ^{5*}	3.3	3.8	3.5	2.0	6.5

V1: A/chicken/Egypt/18-H/2009/H5N1

V2: A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1

V3: A/chicken/Vietnam/C58/04/H5N3

H5N1^{4*}: A/Geese/Egypt/Men-21/2016/H5N1 local isolate

H5N8^{5*}: A/Teal/Egypt/Damietta-1/2016/H5N8 local isolate

S1: antiserum against A/chicken/Egypt/18-H/2009/H5N1

S2: antiserum against A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1

S3: antiserum against A/chicken/Vietnam/C58/04/H5N3

H5N1^{S*}: antiserum against A/Geese/Egypt/Men-21/2016/H5N1 local isolate

H5N8^{S*}: antiserum against A/Teal/Egypt/Damietta-1/2016/H5N8 local isolate

HI*: Haemagglutination inhibition

-The homologous titers are shown in bold.

Table (6): Antigenic relatedness^a values (%) of two HPAI (H5N8 & H5N1) field isolates/2016 and three vaccinal strains and their interpretation^b:

Item	V1		V2		V3		H5N1 ⁴		H5N8 ⁵	
	R value %	Intpr.	R value %	Intpr.	R value %	Intpr.	R value %	Intpr.	R value %	Intpr.
V1	-	-	75.5	LND	47.2	MSD	53.0	MSD	20.7	MjSD
V2	-	-	-	-	63.7	MSD	68.7	MSD	31.0	MjSD
V3	-	-	-	-	-	-	38.7	MSD	22.5	MjSD
H5N1 ^{4*}	-	-	-	-	-	-	-	-	24.8	MjSD
H5N8 ^{5*}	-	-	-	-	-	-	-	-	-	-

V1: A/chicken/Egypt/18-H/2009/H5N1

V2: A/chicken/Egypt/Q1995D/2010 - A/duck/Egypt/M2583D/2010/H5N1

V3: A/chicken/Vietnam/C58/04/H5N3

4*: A/Geese/Egypt/Men-21/2016/H5N1 local isolate

5*: A/Teal/Egypt/Damietta-1/2016/H5N8 local isolate

^a: antigenic relatedness was calculated using the **Archetti an Horsfall formula (1950)**, to calculate R-values based on the cross HI titers.

^b: The interpretation of the results was done according to **Brooksby (1967)**.

R value: Relatedness value.

Intpr.: Interpretation

LND: Little or no subtype differences.

MSD: Minor subtype difference.

MjSD: Major subtype difference

DISCUSSION

Highly pathogenic avian influenza viruses of the H5 subtype remain a serious concern for poultry and human health. Since the emergence of HPAIV H5N1 (A/goose/Guangdong/1/1996) in China in 1996, descendants of this strain continue to spread among avian species and their HA has evolved into multiple distinct phylogenetic clades and subclades (Smith and Donis, 2015). Many studies implicated the involvement of wild migratory birds in HPAIV spread and endemic outbreaks in poultry (Dalby and Iqbal, 2015; Lee *et al.*, 2015; Ozawa *et al.*, 2015; Hill *et al.*, 2015; Pantin-Jackwood *et al.*, 2016 & Lee *et al.*, 2017). Domestic waterfowls that are in contact with wild birds and also other poultry species can act as intermediate key in the transmission of avian influenza among birds (Li *et al.*, 2004) and also have been implicated in the dissemination and evolution of H5N1 HPAIV (Wasilenko *et al.*, 2011) as well as H5N8 (Hill *et al.*, 2015). In 2010, HPAI H5N8 of clade 2.3.4.4 was detected in domestic ducks in eastern China. By 2014, this virus had caused multiple outbreaks among domestic ducks, chickens, geese, and wild birds in South Korea and subsequent outbreaks in Japan, China, Europe, and North America (Lee *et al.*, 2014). Later on, the virus has been detected during the 2016 spread wave of HPAI H5N8 viruses in Russia (Lee *et al.*, 2017), Germany (Pohlmann *et al.*, 2017), India (Nagarajan *et al.*, 2017), Italy (Fusaro *et al.*, 2017), and Iran (Ghafouri *et al.*, 2017). The spread of HPAIV (H5N8) strains has been linked to the overlapping flyways of migratory wild birds (Lee *et al.*, 2015). Previous study reported that clade 2.3.4.4 HPAI viruses are more adapted to wild aquatic bird species than H5N1 viruses (Kwon *et al.*, 2018). In 2016, the HPAI H5N8 clade 2.3.4.4 was reported in wild birds (common coots and green-winged teal) documenting its introduction to Egypt through migratory birds (Selim *et al.*, 2017; Kandeil *et al.*, 2017). Egypt also is endemic with HPAI H5N1 since 2008 and the distinct cluster of HPAI H5N1 clade 2.2.1.2 has emerged since 2012 (El-Shesheny *et al.*, 2014). In the current study, we investigated thirty-two tracheal samples that were collected from waterfowls (wild and domestic) during the

period from March 2016 till March 2017. Seven samples were collected from domestic waterfowls and twenty-five samples were collected from four different species of wild ducks (teal, pintail, mallard, and coot). Most of the tested wild ducks were apparently healthy and showed no clinical signs in agreement with previous studies (Kandeil *et al.*, 2017 & van den Brand, *et al.* 2018). Sun *et al.*, (2016) concluded that the recent H5 reassortants belonging to clade 2.3.4.4 have reduced virulence compared to the parental H5N1 virus as evident by a decrease in the intravenous pathogenicity index and increased mean death time.

A reverse transcription polymerase chain reaction (RT-PCR) is a confirmed molecular technique which is used worldwide as a diagnostic tool of most poultry viral diseases including H5 Avian influenza virus (Slomka *et al.*, 2007). RT-PCR was carried out for partial amplification of HA gene including the HA cleavage site. Fourteen samples from wild ducks (14/25) and two samples from domestic waterfowls (2/7) were H5 positive. All H5Nx strains detected in migratory ducks were characterized as HPAIV H5N8, clade 2.3.4.4. The other two H5Nx strains detected in domestic waterfowls were characterized as HPAIV H5N1, clade 2.2.1.2.

Partial HA gene sequencing of five selected samples was performed, the amino acid sequences of HA cleavage site revealed polybasic amino acid which is characteristic of HPAIV. The amino acid sequences of HA cleavage of H5N8 isolates was (PLREKRRKR/GLF) as found previously (Selim *et al.*, 2017; Kandeil *et al.*, 2017; Anis *et al.*, 2018; Salaheldin *et al.*, 2018 & Shehata *et al.*, 2019). H5N1 isolates cleavage site revealed PQGEKRRKRR/GLF as found previously by Arafa *et al.*, (2016).

Since 2017, multiple cases of H5N8 infection have been reported in poultry in the Nile Delta and some have extended to Upper Egypt (OIE, 2017 & Yehia *et al.*, 2018).

In order to examine the correlation between the genetic and antigenic distances of currently circulating HPAI H5Nx of different clades, we first performed a phylogenetic relationship between some H5N8 and H5N1 local field

isolates\2016 and other AIV isolates based on the HA gene nucleotide sequence (Fig. 5). The three H5N8 isolates clustered in clade 2.3.4.4b. The three H5N8 isolates shared 99.3 similarity with the Russian HPAI H5N8 virus A/great_crested_grebe/Uvs-Nuur_Lake/341/2016, which strengthen the suggestion of Yehia *et al.*, (2018) that the Egyptian H5N8 viruses have emerged from a common ancestor virus (A/great_crested_grebe/Uvs-Nuur_Lak/341/2016).

This cluster includes other H5N8 strains recently isolated From Asia (Iran – Korea – Russia – Israel) with amino acid identity percent with each other ranging from (96.4% - 99.3%), Africa (Congo), Europe (Belgium) and Eastern China with amino acid identity percent with each other ranging from (98.4% - 99.3%). This result agreed with recent studies (Kandeil *et al.*, 2017; Salaheldin *et al.*, 2018 & Shehata *et al.*, 2019). The two H5N1 isolates clustered in clade 2.2.1.2 with other recent Egyptian isolates. The three vaccine strains used in this study were genetically distinct from the H5N8 viruses currently circulating in Egypt in agreement with (Kandeil *et al.*, 2018 & Shehata *et al.*, 2019).

Vaccination has been used in Egypt since 2006 to control HPAI H5N1 epidemics. Different viruses were used as vaccine seed strains, including classical H5 lineage viruses and reverse genetics–engineered reassortant viruses containing H5N1 virus HA and NA genes and the remaining genes from A/Puerto Rico/8/1934(H1N1) (Kayali *et al.*, 2016). Multiple factors influence the efficacy of poultry vaccine. One of these critical factors is the genetic and antigenic matching between the circulating viruses and commercial vaccine strains (Wong and Webby, 2013).

It has been demonstrated previously that matching antigenic similarity of the HA between the vaccine and challenge virus provides the best protection against mortality and virus shedding (Lee *et al.*, 2004; Swayne and Kapczynski, 2008; Romer-Oberdorfer *et al.*, 2008 & Pica and Palse, 2013). Other studies showed that when the vaccine and the challenge virus belong to same H5N1 subtype with expected high HA homology, vaccinated ducks were completely protected against challenge virus (Tian *et al.*,

2005; Webster *et al.*, 2006 & Kim *et al.*, 2008). However, other studies revealed that genetically more distinct vaccines can also protect ducks against infection with H5N1 viruses (Beato *et al.*, 2007; Pfeiffer *et al.*, 2010 & Van der Goot *et al.*, 2008).

Several experimental studies were conducted in chickens to evaluate the efficacy of different types of commercial vaccines against challenges with different Egyptian H5N1 viruses of clades 2.2.1, 2.2.1.1, 2.2.1.1a, and 2.2.1.2. The serological results showed that different types of commercial AI vaccines provided variable reactivity against the previously described antigens of Egyptian H5N1 viruses (isolates from 2006 to 2009, clade 2.2.1), but that reactivity declined with recent circulating viruses of clade 2.2.1.2 (Kayali *et al.*, 2016).

In our study, two field isolates were selected (A/Teal/Egypt/Damietta-1/2016/H5N8 clade 2.3.4.4 and A/Geese/Egypt/Men-21/2016/H5N1 clade 2.2.1.2) and used for preparation of two experimental vaccines to be used in the antigenic analysis study using Cross haemagglutination inhibition (HI) test. HI titers are considered to be of predictive value concerning protective efficacy if suitable matching pairs of HI antigen and challenge virus are used (Swayne, 2009). Serological monitoring of H5 vaccinated flocks by the HI test using the homologous vaccine antigen is a routine laboratory procedure to evaluate vaccination efficacy of poultry in Egypt and elsewhere (Hafez *et al.*, 2010). Titres greater than 4-log₂ have been claimed to be an indicator for clinical protection and titres greater than 6-log₂ indicator for prevention of viral shedding as previously stated (Tian *et al.*, 2005, and Kumar *et al.*, 2007).

In this study, the H5N8 isolate A/Teal/Egypt/Damietta-1/2016/H5N8 clade 2.2.3.4 showed very low reactivity with H5 antisera raised against H5N1 vaccinal strains of clades 2.2.1.1, 2.2.1.2 and clade 1. Similarly, duck hyperimmune sera raised against the experimental inactivated H5N8 vaccine (homologous titer 6.5-Log₂) showed very low reactivity with heterologous H5 antigens of clades 2.2.1.1, 2.2.1.2 and clade 1 in agreement with (Kandeil *et al.*, 2017 & Shehata *et al.*, 2019). These results also agreed with (Hiono *et*

al., 2015) and (Ohkawara *et al.*, 2017) who demonstrated that antigenicity of the viruses of clade 2.3.4.4 differed significantly from that of the viruses of other clades.

The H5N1 isolate A/Geese/Egypt/ Men-21/2016/H5N1 clade 2.2.1.2 showed reactivity with H5 antisera raised against H5N1 commercial vaccinal strains while, it showed very low reactivity with the experimental H5N8 vaccine. The very low antigenic reactivity between H5N8 and H5N1 subtypes correlated with the genetic dissimilarity.

Anis *et al.*, (2018) previously reported that the sera from H5N8- infected duckling did not show cross reactivity with an H5N1 Egyptian strain. Previous study also reported that serum from infected ferret with H5N1 A/Vietnam/1194/2004 (homologous titre 80) completely failed to react in HI assay with H5N8 A/chicken/ Netherlands/EMC-3/2014 virus. Moreover, H5N8 specific ferret serum (homologous titre 160) completely failed to cross-react with A/Vietnam/1194/2004 H5N1 virus (De Vries *et al.*, 2015).

The genetic dissimilarity and poor reactivity between the H5 commercial vaccines used in Egypt and the currently circulating H5N8 viruses proves that the vaccines might not be effective in the field or may provide only partial protection and thus could lead to vaccine-induced escape mutant strains (Kandeil *et al.*, 2018).

The antigenic relatedness between local antigens A/Geese/Egypt/Men-21/2016/H5N1 clade 2.2.1.2 and A/Teal/Egypt/Damietta-1/2016/H5N8 clade 2.3.4.4b was low with R value 24.8% indicating major subtype difference between the two strains as detected by (Shehata *et al.*, 2019). These results agree with the genetic dissimilarity between the two subtypes. The R value was ranging from 38.7% to 68.7% between the A/Geese/Egypt/Men-21/2016/H5N1 clade 2.2.1.2 and the three vaccinal strains which indicate a minor subtype difference between them in agreement with the genetic similarity.

Kandeil *et al.*, (2018) tested the efficacy of eight commercial vaccines against challenge with the HPAI A/duck/Egypt/F13666A/2017 (H5N8)

virus clade 2.3.4.4b in chickens. Most of the commercial vaccines protected chickens from mortality but did not reduce or prevent virus shedding. The experimental homologous H5N8 vaccine provided the best protection against a challenge with the clade 2.3.4.4 virus.

Contrary to these results, a previous study revealed good protective efficacy of a stockpiled vaccine based on A/duck/Hokkaido/Vac-1/2004/ H5N1 against HPAIV H5N8 challenge (Gamoh *et al.*, 2016). Also, Steensels *et al.*, (2016) concluded that despite the high antigenic divergence of the challenge H5N8 strain, a single administration of rHVT-H5 vaccine expressing the H5 gene of a clade 2.2 H5N1 strain at 1 day old resulted in a full clinical protection against challenge and a significant reduction of viral shedding in the vaccinated birds.

Sultan *et al.*, (2019) evaluated a recombinant subunit commercial H5-vaccine prepared from clade 2.3.2 H5-segment on baculovirus in Pekin and Muscovy ducks after experimental infection with the Egyptian HPAI H5N8 isolate clade 2.3.4.4bA/commoncoot/Egypt/CA285/2016/H5 N8. It was concluded that the H5-baculovirus-based vaccine can be used in ducks with better vaccination regime based on double-dose vaccination at 10 and 28 days of age.

Our results indicated that, these vaccines are genetically and antigenically distinct from the HPAI H5N8 virus currently circulating in Egypt. Since vaccination has been used as an essential control strategy in Egypt, updating HA of H5 Vaccine to be antigenically closer to the field viruses is necessary for optimal protection as previously suggested (Kandeil *et al.*, 2017; Kandeil *et al.*, 2018; Yehia *et al.*, 2018; Sultan *et al.*, 2019 & Shehata *et al.*, 2019). Finally, regular monitoring of wild birds should be adopted to predict and prevent possible AIV outbreaks.

CONCLUSION

In the present study, two subtypes of HPAI viruses of different clades currently circulating in Egypt, H5N8 clade 2.2.3.4b and H5N1 clade 2.2.1.2 were characterized. HPAI H5N8 virus is genetically and antigenically distinct from HPAI

H5N1 virus. In addition, some commercial vaccines which are used to control avian influenza in Egypt are genetically and antigenically distinct from HPAI H5N8 virus currently circulating in Egypt. Updating the HA of H5 Vaccine to be antigenically closer to the field viruses is recommended together with regular monitoring of wild birds to predict and prevent possible AIV outbreaks.

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