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Avian Disease

Molecular Characterization of Highly Pathogenic Avian Influenza H5Nx in Upper Egypt during 2023

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ABSTRACT

The highly pathogenic avian influenza (HPAI) H5Nx viruses circulate worldwide causing high economic losses and infecting some species of mammals including humans. Epidemiological investigations for respiratory viruses of poultry in Upper Egypt are limited so, this study investigated them in 20 chicken farms in Assiut, Sohag, and Qena Governorates during 2023. Two farms from Assiut were positive for H5N1 (10%) while the other two farms from Sohag were positive for H5N8 (10%), while 4 farms were tested positive for H9N2 AIV (20%). Two HPAI H5N8 and H5N1 have been isolated, and HA gene was sequenced with PLREKRRKRGLF sequence in cleavage site indicating a virus of high pathogenicity. Three amino acids (aa) conserved mutations observed between H5N8 and H5N1 strains; T140A/S in antigenic site A, N189K in the antigenic site B, and N236D close to the receptor binding domain (RBD) compared to A/North shoveler/Egypt/MB-D-819OP/2016 H5N8 strain. Antigenically all strains tested from clade 2.3.4.4b including H5N1 or H5N8 subtypes were similar but antigenically different from H5N1 clade 2.2.1 virus. Therefore, using vaccines with seed strains from clade 2.3.4.4b is recommended to give a specific immune response and efficient protection and this would help in disease control and reduce the evolution of H5Nx viruses in Egypt.

Keywords: HPAI, LPAI, Egypt, H5N8, H5N1.

INTRODUCTION

The H5Nx AI viruses have been circulating in migratory birds for the last 20 years and they become endemic in poultry in some countries around the world (Claes et al., 2016; Lee et al., 2017). The HPAI H5N8 virus was detected for the first time in China in 2010. The virus emerged through genetic reassortment of various avian influenza subtypes, (Zhao et al., 2013), then in 2014, a new reassortant H5N8 virus was discovered in South Korea (Lee et al., 2014). Later on, it travelled to Europe and North America via migratory birds, resulting in a number of outbreaks. Phylogenetically H5N8 virus clustered into clade 2.3.4.4a (Bevins et al., 2016; Lewis et al., 2021). New reassortants of this virus were discovered in migratory birds across a number of nations during 2016–2017, exhibiting a distinct gene cluster

designated as clade 2.3.4.4b (Lycett et al., 2020; Fusaro et al., 2019). Since then, this virus has continued to diverge evolutionary by reassortment with other influenza A subtypes, giving rise to different genotypes and expanding to domestic birds (Lycett et al., 2020).

In Egypt, the HPAI H5N1 virus (clade 2.2.1) was reported early in 2006 and became endemic in different poultry sectors for 10 years (Abdelwhab et al., 2011). Moreover, the LPAI H9N2 virus was detected in quails and chickens in 2011, and also it became endemic in Egyptian poultry species till now (El-Zoghby et al., 2012). Interestingly in 2016, a new HPAIV of H5N8 subtype belonging to clade 2.3.4.4b was detected in Egypt. Firstly, it was detected in migratory birds (Selim et al., 2017; Kandeil et al., 2017), then in domestic poultry populations across the country (Tarek et al., 2021; Kandeil et al., 2022). Surveillance studies during 2017 indicated that this new virus has displaced the H5N1 virus of the old clade 2.2.1.2 (Amer et al., 2021).

Recently, Subclade 2.3.4.4b H5N1 viruses have been extensively circulating in wild birds and detected in different continents of the world since October 2020. This virus was reported in poultry from live bird markets in Egypt for the first time in 2021 (El-Shesheny et al., 2023). The cocirculation of LPAI H9N2 plus the HPAI H5N1 and H5N8 viruses which have a high ability for reassortment in Egyptian poultry sectors represents a high risk to the poultry industry and human.

Studies on the epidemiology of cocirculating AIVs in the Upper Egypt region are very few, so in this study we targeted to survey the circulation of AIVs in the Upper Egypt region during the 2023 season.

MATERIALS AND METHODS

Ethical approval

This study was performed under the ethical approval given by the Institutional Animal Care and Use Committee (University of Sadat City No VUSC-056-1-22).

Samples

Tracheal swabs and/or tracheas were collected from 20 commercial chicken flocks showing respiratory manifestations from 3 Governorates in Upper Egypt between the period of January to June 2023. Flocks history is shown in Table 1. The samples were transferred in ice box to the Reference Laboratory for Quality Control on Poultry Production (RLQP, Egypt), Animal Health Research Institute (AHRI), Dokki, Giza, Egypt for testing according to (OIE, 2008).

Table 1: History of suspected examined flocks for AIV, recorded from January to June 2023.

Sample code	Area	Collection date	Flock type	Flock size	Age/ Days	Vaccination with H5 vaccine	Mortality* (%)
1	Assiut	2023-01-28	Broiler	7200	29	One dose	19.4%
2	Qena	2023-02-05	Broiler	10000	32	One dose	7.5%
3	Assiut	2023-03-04	Broiler	3000	35	One dose	11.7%
4	Sohag	2023-03-13	Broiler	20000	33	One dose	3.6%
5	Assiut	2023-03-25	Layer	7000	143	Four doses	40%
6	Sohag	2023-04-11	Broiler	1500	31	One dose	32%
7	Sohag	2023-04-13	Broiler	2500	37	One dose	64%
8	Assiut	2023-04-20	Broiler	18000	24	One dose	22.8%
9	Assiut	2023-04-21	Broiler	9500	36	One dose	3.4%
10	Qena	2023-04-26	Broiler	3000	23	One dose	18.3%
11	Sohag	2023-05-01	Broiler	5000	27	One dose	6.2%
12	Assiut	2023-05-08	Broiler	5600	35	One dose	79%
13	Assiut	2023-05-10	Broiler	9500	32	One dose	9.7%
14	Assiut	2023-05-15	Broiler	7000	30	One dose	4.5%
15	Assiut	2023-05-15	Broiler	20000	31	One dose	6.6%
16	Sohag	2023-05-21	Broiler	13000	30	One dose	8.5%
17	Assiut	2023-05-28	Layer	5300	115	3 doses	6.2%
18	Sohag	2023-06-04	Layer	9000	138	3 doses	36%
19	Qena	2023-06-11	Broiler	11500	25	One dose	7%
20	Sohag	2023-06-14	Broiler	7500	28	One dose	5.3%

*Mortality percent calculated within 1 week after respiratory manifestation

Molecular detection of the causative agent by real time RT-PCR

Extraction of RNA from tracheal swabs/tissues suspensions was done using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real time RT-PCR was performed using Quantitect probe RTPCR kit (Qiagen, Inc. Valencia CA) with specific primers and probes supplied from Metabion® (Germany) and used for detection of AIV subtypes (H5, H9 & N1, N8) and NDV-F and IBV-N genes (Table 2).

Table 2: Primers and probes used for real-time PCR detection of AIV subtypes (H5, H9 & N1, N8) and NDV-F and IBV-N genes.

ID	Oligo name	Sequence (5' to 3')	Reference
AI-H5 subtype	H5LH1 H5RH1 H5PRO	ACATATGACTAC CCACARTATTCA G AGACCAGCT AYC ATGATTGC [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	Löndt et al., 2008
AI-H9 subtype	H9F H9R H9 Probe	GGAAGAATTAATTATTATTGGTCGGTAC GCCACCTTTTTCAGTCTGACATT [FAM]AACCAGGCCAGACATTGCGAGTAA GATCC[TAMRA]	Ben Shabat et al., 2010
NDV-F-gene	F+4839 F-4939 F+4894	TCCGGAGGATACAAGGGTCT AGCTGTTGCAACCCCAAG [FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]	Wise et al., 2004
IB-N-gene	AIBV-fr AIBV-as AIBV-TM	ATGCTCAACCTTGTCCCTAGCA TCAAACTGCGGATCATCACGT (FAM-TTGGAAGTAGAGTGACGCCCAAACTTCA-TAMRA)	Meir et al. (2010)
AI-N1 subtype	N1 forward N1 reverse N1 Probe	TAYAACTCAAGGTTTGAGTCTGTYGCTTG ATGTTRTTCCTCCAACTCTTGATRGTGTC FAM-TCAGCRAGTGCYTGCCATGATGGCA-Tamra	Li et al., 2013
AI-N8 subtype	N8-1296F N8-1423R N8-1354	TCC ATG YTT TTG GGT TGA RAT GAT GCT CCA TCR TGC CAY GAC CA FAM- TCH AGY AGC TCC ATT GTR ATG TGT GGA GT-Tamra	Hoffmann et al., 2016

Virus isolation:

The clarified tracheal suspensions from rRT-PCR positive samples were filtered by 0.2 µm filter then the allantoic cavity route was used in 10-day-old SPF embryonated chicken eggs (ECE) (for each sample five eggs were inoculated). Eggs were incubated at 37 ◦C and candling was performed for three successive days. At the end of the third day, eggs were chilled to 4 ◦C for 2 hours then allantoic fluids were harvested and examined for hemagglutination.

RT-PCR for HA gene amplification

RT-PCR was done with HA gene-specific primers amplifying HA1 region and it was carried out using RT-PCR Master Mix from Willowfort, Birmingham, UK. The amplified RT-PCR products were separated through agarose gel electrophoresis, and then bands purified using the QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany).

Sequencing of the amplified HA gene segment

Sequencing of the purified bands was done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). The amplified sequences were then cleaned and processed through an ABI PRISM 3100 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The sequence was assembled using

Bioedit clustal W software (Hall, 1999).

Phylogenetic Analysis

Sequence analysis was conducted with the BioEdit program using the Clustal W alignment algorithm then the phylogenetic tree was constructed using neighbor-joining method (Mega 11 program).

Polyclonal antibodies preparation

Vaccine formulations were prepared by mixing the formalin-inactivated allantoic fluids of H5N1 and H5N8 viruses with Montanide ISA 71 VG oil adjuvant (Seppic, France) in a ratio of 30/70. The inactivated allantoic fluids of RG-A/Chicken/Egypt/D10552B/2015, RG-A/Chicken/Egypt/RG-F192-VV/2020, and RG-A/chicken/Egypt/A19673/2021 obtained from the repository of the Egyptian Company for Biological & Pharmaceutical Industries (Vaccine Valley), 6th October City, 12511, Giza, Egypt. The inactivated allantoic fluids of two H5N1 and H5N8 strains were isolated in this study prepared in AHRI. Chicks (SPF) of 3 weeks age were inoculated with the formulated vaccines by 0.5 ml subcutaneously and booster inoculated after weeks. Chicks were bled after three weeks for serum separation.

Hemagglutination inhibition (HI)

Sera were inactivated (56°C/30 min) and then tested by HI assay. HI was carried out according to the guidelines of OIE manual (OIE, 2008).

RESULTS

Epidemiology of avian influenza in examined farms:

In this study, we examined seventeen broiler farms with ages ranging from 23 to 37 days old from Assiut, Sohag, and Qena governorates with mortalities ranging from 3.4% to 79%, in addition to two-layer farms from Assiut plus another layer farm from Sohag with mortalities ranged from 6.2% to 40%.

Results of real time RT-PCR:

Samples were examined first by using primers specific for H5 and/or H9 then positive samples for either H5 or H9 were further tested for the presence of IBV and NDV. Also, positive samples for H5 were NA subtyped by using primers specific for N1 and N8. Two farms from Assiut were positive for H5N1 (10%) while the other two farms from Sohag were positive for H5N8 (10%), 4 farms were examined positive for H9N2 AIV (20%), results shown in Table 3.

Table 3: CT values of Real time RT-PCR

Sample code	CT*			
	H5	H9	ND	IB
1	-VE	-VE		
2	-VE	-VE		
3	-VE	-VE		
4	-VE	-VE		
5	26 (H5N1)	-VE	-VE	-VE
6	23 (H5N8)	-VE	-VE	-VE
7	-VE	32	-VE	29
8	-VE	-VE		
9	-VE	-VE		
10	-VE	-VE		
11	-VE	-VE		
12	20 (H5N1)	-VE	28	-VE
13	-VE	30	-VE	27
14	-VE	-VE		
15	-VE	-VE		
16	-VE	26	29	-VE
17	-VE	-VE		
18	27 (H5N8)	-VE	30	-VE
19	-VE	31	27	-VE
20	-VE	-VE		

*CT: Threshold value.

Virus isolation

The tracheal swab/tissue suspensions from two selected flocks for isolation in SPF-ECE; layer flock number 5 (Layer5) that tested positive for H5N1 only and broiler flock number 6 (Br6) that tested positive for H5N8 only. The allantoic fluids showed HA positivity by HA assay with 6 $\log_2$ titers obtained in the second passage.

Sequence analysis of the obtained HA gene partial sequence:

The first segment of the HA gene from two isolated strains Layer5/23 H5N1 and Br6/23 H5N8 was successfully amplified and sequenced. The sequence of the HA cleavage site in both H5N1 and H5N8 strains from clade 2.3.4.4b was PLREKRRKRGLF indicating high pathogenicity virus which contains 5 basic amino acids. Three amino acids (aa) conserved mutations observed between H5N8 and H5N1 strains; T140A/S in antigenic site A, N189K in the antigenic site B, and N236D close to the receptor binding sites (RBD) compared to A/North shoveler/Egypt/MB-D-819OP/2016 H5N8 strain. In addition, sporadic aa mutations were recorded in some selected H5Nx strains used in this analysis which are more prevalent in H5N8 strains than H5N1 strains. Broiler6/23 H5N8 strain has R72K mutation in antigenic site E, N109K, P123T in antigenic site A, R169Q in antigenic site Ca, A184V in antigenic site B, and G268E in antigenic site C, while Layer5/23 H5N1 has only I114T in antigenic site A (Figure 1).

Phylogenetically both Layer5/23 H5N1 and Broiler6/23 H5N8 strains were clustered within clade 2.3.4.4b with strains from different countries from Africa, Asia, Europe and Americas (Figure 2).

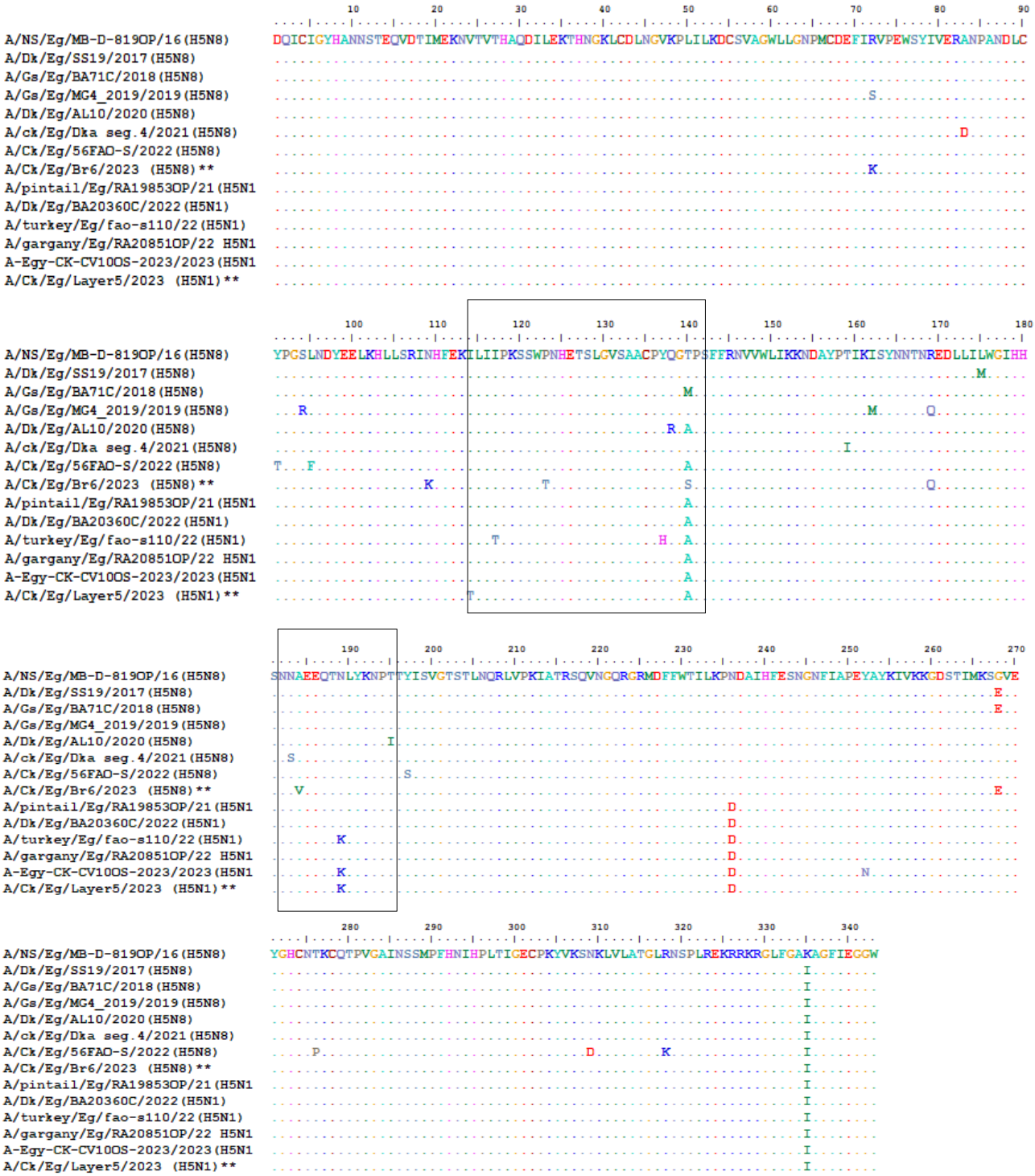

Fig (1): Amino acids sequence alignment for the HA gene of the H5N1 and H5N8 HPAIVs clade 2.3.4.4b isolated in this study and selected reference strains from Egypt.

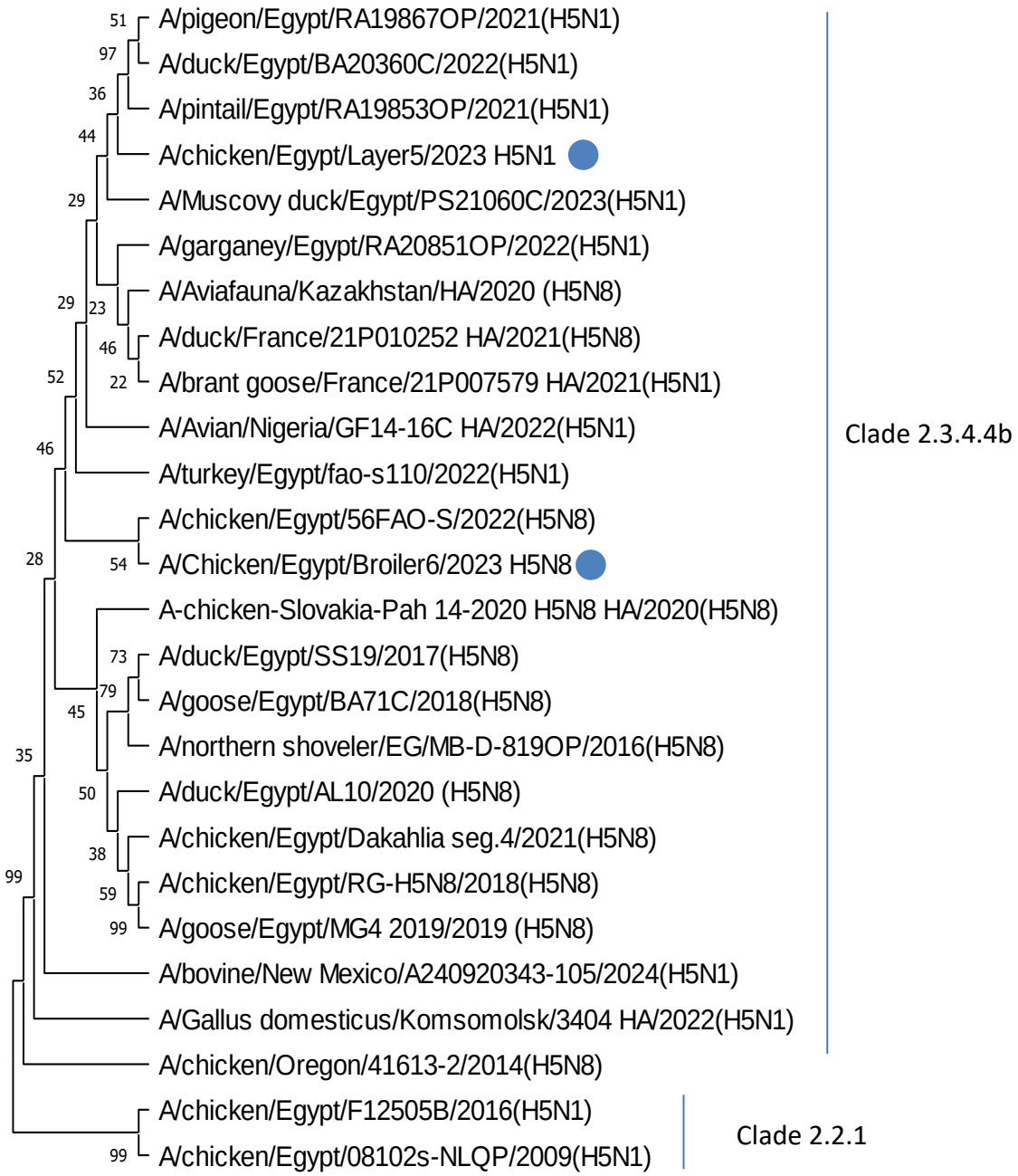

Fig (2): Phylogenetic tree based on nucleotide sequences of the HA gene. The circles indicate the strains isolated in this study. The tree was generated using the neighbor-joining method and phylogenetic relationships were estimated through a bootstrap trial of 1000.

Antigenic analysis of H5N1 and H5N8 co-circulating strains:

The H5N1 strain clade 2.2.1 showed reduced cross reactivity with up to 6 log_2 (64-fold) reduction compared to the 4 strains from clade 2.3.4.4b. Also, this was observed for H5N8 and H5N1 strains clade 2.3.4.4b which showed reduced HI titers with the heterologous H5N1 clade 2.2.1 antisera, but they give high HI titers with the homologous antisera from the same clade indicating that strains from clade 2.3.4.4b either H5N1 or H5N8 were antigenically similar (Table 4).

Table 4: Cross HI titers for the prepared antisera:

Antigen		antisera				
Strain name	clade	D10552B	RG-F192-VV	A19673	Layer 5	Broiler 6
A/Ck/Egypt/D10552B/2015	**2.2.1**	**10***	5	5	4.5	4
A/Ck/Egypt/RG-F192-VV/2020		5	**11**	10	10	10
A/Ck/Egypt/A19673/2021	**2.3.4.4**	4.5	10	**10**	10	10
A/Ck/Egypt/Layer5/2023	**b**	4	11	10	**10**	10
A/Ck/Egypt/Broiler6/2023		5	10	9	10	**9**

*HI titters represented as log2

DISCUSSION

The emergence of the clade 2.3.4.4 of H5 AI viruses represents a high risk for global poultry production due to its potential to reassort and generate multiple genotypes of H5NX viruses. H5N8 virus clade 2.3.4.4b was detected in Egypt during 2016 in wild birds (Selim et al., 2017; Kandiel et al., 2017). Subsequent to multiple introductions, H5N8 viruses were detected in Egyptian domestic poultry from different geographical regions (Yehia et al., 2018; Kandiel et al., 2019). The newly introduced H5N8 viruses predominate over the previously existing H5N1 clade 2.2.1 viruses and take their place within a short period. Moreover, in a few separated occurrences, H5N2 reassortants between the co-circulating H5N8 and H9N2 subtypes have been found in domesticated birds (Hassan et al., 2020).

In the current study, molecular detection of the circulating AI viruses in three Governorates from Upper Egypt followed by genetic and antigenic analysis of two isolated HPAI viruses. H9N2 was detected in 20% of the examined chicken farms with 10% coinfection with IBV and 10% coinfection with NDV. In a recent study in the Governorates of Delta region from January 2022 to October 2022, the detection rate for coinfection of H9N2 and either IBV or NDV was as follows; 7.52% for H9 + IB, and 3.90% for ND + H9 while single H9N2 infection ranged from 8.7% to 22.2% in different Governorates (El-Shemy et al., 2024). The H5Nx AI viruses were detected in 20% of the examined chicken farms as H9N2 with the H5N1 and H5N8 each detected in 10% of examined farms. The single infection with H5N1 and H5N8 was 5%. Also, the coinfection for both viruses with NDV was 5%. El-Shemy et al. (2024) detected the AI-H5 viruses in 3.57%–

11.11% in different Governorates with a mean of 5.85% as single infection without detection of coinfection with any other virus-like NDV, H9N2, and IBV. In another study, 329 samples were tested for the presence of AIVs from 2016 to 2018; 48% were H9N2, 37.1% were H5N8, and 7.3% were coinfections with 2 of the 3 subtypes (Kandeil et al., 2019). In Assiut, detection rate for H9N2 was 3.2% and H5N8 was 1.3% while in Sohag only H9N2 was detected in 1% of examined samples (Kandeil et al., 2019). Detection rate in Upper Egypt is lower than Delta region may be due to differences in density of poultry production and the hot weather helps in low spread of AIVs.

The mortality rates showed great differences between examined flocks. Generally, H5Nx infected flocks showed high mortality rates (32%-79%), while H9N2 infected flocks showed lower mortality rates. Interestingly, flock no.7 showed high mortality (64%) in the presence of H9N2 and IBV infection compared to low mortality 9.7% in flock no.13 also compared to flocks no.16 and no.19 showed low mortality 8.5% and 7%, respectively in the presence of H9N2 and NDV coinfection. This can be due to the presence of early infection of other viruses, especially immunosuppressive ones and/or bad management. Recently, one broiler flock in Menoufia Governorate in November 2024 experienced about 60% mortality after infection by H9N2 and IBV at 32 days old but by reviewing the history of this flock it was previously infected by IBDV at 24 days old.

The H5N8 and H5N1 strains isolated in this study had PLREKRRKRGLF sequence in the cleavage site with multiple basic amino acids which are specific for high pathogenicity as previously recorded (Kandiel et al., 2022; Yehia et al., 2023). They had 5 amino acids in the HA gene H103, N182, G221, Q222, and G224 specific for avian-like α2,3-sialic acid receptor binding preference (Cai et al., 2012; Mair et al., 2014).

Phylogenetic analysis revealed clustering of the H5N8 and H5N1 isolates from this study in clade 2.3.4.4b, with other Egyptian H5N8 isolates from 2016 till now and H5N1 isolates from 2021 till now. The amino acid identity percentage ranging from (96.9% - 99.3%), while the amino acid identity percentage between H5N1 clade 2.2.1 vaccinal strains and H5N8/H5N1 clade 2.3.4.4b field isolates range from (86.3% - 89.6%), as previously reported (Hamouda et al., 2019).

The HA had conserved aa mutation in the antigenic site A; T140S or T140A in the H5N8 Broiler6/23 and H5N1 Layer5/23 strains, and N189K in the antigenic site B. Also, broiler6/23 H5N8 strains have R72K in antigenic site E, N109K, P123T in antigenic site A, R169Q in antigenic site Ca, A184V in antigenic site B, and G268E in antigenic site C, while, Layer5/23 H5N1 has only I114T in antigenic site A compared to A/North shoveler/Egypt/MB-D-819OP/2016 H5N8 strain (Figure 1) as previously recorded (kandiel et al., 2020; Yehia et al., 2022). It may be due to the immune pressure on circulating viruses due to the massive use of several H5 vaccines from different clades that lead to point mutations in antigenic sites that might alter the antigenicity (Lee et al., 2004). Also, virus circulation in different poultry species that reared together in backyard flocks or in close contact with different farms in Egypt might be the cause of the continuous evolution of H5Nx viruses.

Antigenic analysis by cross HI assay revealed that currently circulating H5Nx viruses were antigenically similar to each other while, they were antigenically different with clade 2.2.1 H5N1 viruses (Table 4). Our observation agreed with earlier study using cross HI to detect the antigenic relatedness between H5N8/2016 isolates and H5N1 clade 2.2.1 vaccinal strains from 2009-2016, significant antigenic difference observed between isolates of clade 2.3.4.4b and 2.2.1 (Hamouda et al., 2019). Also, a previous study showed that Egyptian H5N8 HPAIVs were antigenically distant from used vaccine strains and using such mismatched vaccines led to drifted strains identified from vaccinated chicken flocks (Kandiel et al., 2018). Interestingly, a recent study compared H5Nx strains antigenically from different clades (2.3.4.4b, 2.3.4.4h, and 2.3.2.1d), cross HI titters showed 64-fold difference between clades 2.3.4.4b and 2.3.2.1d strains while 32-fold difference between strains from clades 2.3.4.4b and 2.3.4.4h (Cui et al., 2022). Based on these results, usage of H5 vaccines with seed strains from clade 2.3.4.4b is highly recommended to give specific antibodies with high affinity to circulating viruses and this will result in efficient protection and reduction in environment contamination.

Epidemiological studies for respiratory viruses in Upper Egypt are very limited so continuous surveillance and monitoring of circulating viruses in these Governorates is important. Cocirculation of H5N1, H5N8, and H9N2 AIVs in combination with NDV and IBV is recorded in Assiut, Sohag and Qena Governorates which is similar to most areas in Egypt. H5N8 and H5N1 viruses of clade 2.3.4.4b were antigenically similar and using strains from this clade in used vaccines will help in control the disease.

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