Research Article

Prevalence of Two Distinct Genotypes of Highly Pathogenic Avian Influenza A/H5N8 Viruses in Backyard Waterfowls in Upper Egypt During 2018

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Abstract | Highly pathogenic avian influenza (HPAI) H5N8 virus of clade 2.3.4.4 was first emerged in China. The virus disseminated throughout migrating birds into different countries in Asia, America, Europe and Africa. HPAI A/H5N8 viruses were initially reported in Egypt in winter of 2016. However, backyard poultry production sector is commonly found in rural areas in Egypt with biosecurity deficiencies and could play a key role in epidemiology of AIVs in Egypt. Thus, the aim of this study was to determine the prevalence of AIVs in backyard ducks and geese in Assuit governorate, Egypt during 2018, and to study the genetic features of detected AIVs. A total of 245 oropharyngeal and cloacal swabs were collected from backyard waterfowls (ducks ≈ 92 and geese ≈ 32) either apparent healthy or demonstrated severe clinical signs of AIV infection from 5 different sites. AIVs were successfully isolated in SPF-ECE from 50 swabs collected from both waterfowls. All isolates were subtyped as H5N8 viruses. H5N1 and H9N2 subtypes were not identified among collected samples. Two representative H5N8 isolates from duck and goose were subjected to full genome sequencing and genetic analysis. Although both of H5N8 viruses were closely related to clade 2.3.4.4b, they were genetically distinct and had genetically different constellation forms. This study highlights the need of active surveillance of AIVs in backyard production sector for assessing the epidemiology, evolution, and diversity of currently circulating viruses in Egypt.

Keywords | Highly Pathogenic Avian Influenza, H5N8, Backyard, Waterfowls, Egypt.

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INTRODUCTION

HPAI viruses is a source of major concern for poultry and human health. The hemagglutinin (HA) genes of subtype H5 evolved through reassortment with various neuraminidase subtypes (1–9) to comprise AI H5NX viruses. The co-circulation of group A viruses with low pathogenic avian influenza (LPAI) viruses resulted in emergence of novel reassortants; H5N1 and H5N8 (El-Shesheny et al., 2017). H5N8 viruses has been emerged in Asia since 2010 where the first outbreaks in breeding ducks occurred in China then South Korea, Japan and Taiwan 2014. then quickly spread to other continents as Europe, North America and Africa (Lee et al., 2014).

In Egypt during December 2016, influenza A/H5N8 viruses were detected and isolated from common coot and the green-winged teal at a fish market in Damietta, and a live-bird market in Port Said City; respectively (Kandeil et al., 2017; Selim et al., 2017). Because of the venerable location of Egypt which represents a bridge between Africa, Asia and Europe, millions of migrating birds go over during their flights annually especially in winter. On the other hand, there is a magnificent number of bird species inhabitants in Egypt either in intensive farms or farmer rearing. Reassortment with other influenza A subtypes caused evolutionary divergence, resulting in the emergence of various genotypes and further spread to domestic birds (Lycett et al., 2020). After the first report of H5N8 in Egypt in 2016, six distinct genotypes of H5N8 were found to have genetically different reassortant combinations (Kandeil et al., 2022b; Moatasim et al., 2019; Yehia et al., 2018) . Based on their whole genome sequence, these six genotypes of the HPAI H5N8 viruses were identified in both migratory and domestic birds in Egypt (Kandeil et al., 2022b; Moatasim et al., 2019; Yehia et al., 2018). The virus spread rapidly among Egyptian domestic poultry populations in various governorates, posing a significant threat to the poultry industry. (Kandeil et al., 2022b). Reassortment events between LPAI H9N2 and HPAI H5N8 was the cause of emergence of novel HPAI H5N2 virus reported in Egypt by 2018/2019 (Hagag et al., 2019; Hassan et al., 2020). HPAI H5N8 viruses that detected in Europe in 2020 were found to be phylogenetically related, according to sequence of HA (haemagglutinin) gene, to HPAI H5N8 viruses that isolated from domestic poultry in Egypt in 2019 (Beerens et al., 2021; Kandeil et al., 2022a; Lewis et al., 2021). Epidemiological data suggested that HPAI H5N8 virus (clade 2.3.4.4b) has substituted the Egyptian H5N1 virus (clade 2.2.1.2) becoming the most common subtype of H5 in Egypt (Amer et al., 2021). It is current evident that HPAI H5N8 viruses circulate in endemic manner in domestic poultry in Egypt (Hassan et al., 2021; Kandeil et al., 2019). Indeed, the genetic evolution and epidemiology of Egyptian HPAI H5N8 viruses among backyard waterfowls remain unknown.

In Egypt, backyard poultry production is a major source of meat and eggs, providing over 53% of daily protein requirements and comprises around 10% and 30% of the market meat and egg production sectors; respectively. Otherwise, backyard poultry rearing constitute a risk as a constant reservoir for AIV; often composed of mixed bird species, easy wild bird contact, inferior biosecurity and shortage of veterinary notice or vaccination (Zanaty et al., 2019). While most of domestic backyard geese and ducks in Egypt are not vaccinated, Wild and domestic waterfowls form an additional pressure as it could be infected with highly pathogenic viruses with showing no or mild symptoms (Calindo et al., 2022; van den Brand et al., 2018). The current study was carried out to determine the prevalence of AIVs in backyard ducks and geese in South Egypt and study the genetic features of detected AIVs and their correlation with other contemporary H5N8 strains.

MATERIAL AND METHODS

Ethics and biosafety

Animal experiments were approved by the Medical Research Ethics Committee of the National Research Centre (Ethical permission code: 18040 in April 2018). All animal experiments were conducted in accordance with the guidelines of the Egyptian Animal Welfare Legislation. Viruses were handled and cultured under biosafety level 3 (BSL-3) conditions.

Sampling

During winter 2018, a total of 245 different samples (121 cloacal and 124 oropharyngeal swabs) were collected from 124 vaccinated and non-vaccinated backyard waterfowls (92 ducks and 32 geese) in five different sites including Dairut (n=40) Assuit (n=35) El Ghanayem(n=64), Abnub (n=58), and Manfalut (n=48) at Assuit governorate in Upper Egypt (Figure 1). Samples were collected from healthy and sick waterfowls exhibiting influenza like symptoms. Each swab sample was collected in 1ml viral transport medium (PBS supplemented with 10% penicillin-streptomycin mix (WHO, 2002; Tolba et al., 2018), and stored under cooling conditions at -80 °C.

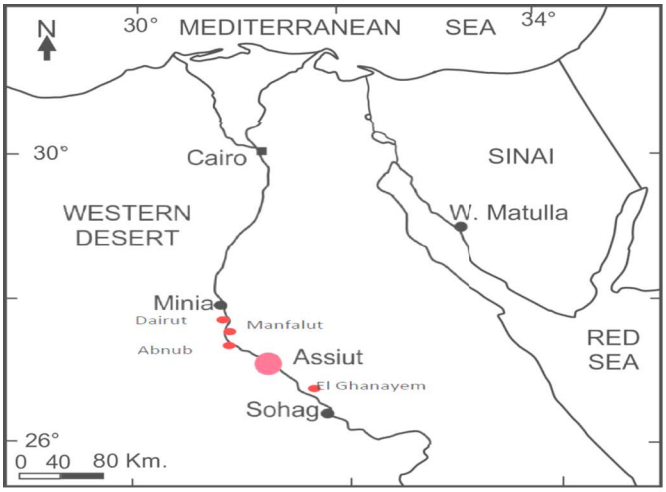

Figure 1: The geographic sites which the samples were collected in Assiut governorate in Egypt, Red dots represent the geographic sites which the samples were collected in Assiut governorate in Egypt.

Virus isolation and HA test

All collected samples were individually prepared and inoculated into allantoic cavities of 9-11 days old specific pathogen free embryonated chicken' eggs (SPF ECE) and in

Table 1: The significance of AIV H5 detection ratio as estimated for sample type, site, host species and bird vaccination status

Variable	No. Collected Samples (%)	No. of AIV -Positive Samples (%)	*p*-Value
Sample Type			
Oropharyngeal	124(50.61)	30(12.24)	
Cloacal	121(49.39)	20(8.16)	NS
Species			
Duck	183(74.69)	41(16.73)	
Goose	62(25.31)	9(3.67)	$p < 0.05$
Site			
Dairut	40(16.33)	12(4.90)	
Asyut	35(14.29)	6(2.45)	
El-Ghanayem	64(26.12)	4(1.63)	
Abnub	58(23.67)	28(11.43)	
Manfalut	48(19.59)	0(0)	$p < 0.05$
Vaccination			
Vaccinated	40(16.33)	13(5.31)	
Non vaccinated	205(83.67)	37(15.10)	$p < 0.05$

Table 2: Percent of positive cases examined from different regions from backyard waterfowls during winter 2018 in Upper Egypt.

Site	Examined (%)	AIV-subtyping			Positive cases (%)
		H5N1	H5N8	H9N2	
Dairut	40(16.33)	0	12	0	12(4.9)
Asyut	35(14.29)	0	6	0	6(2.45)
El-Ghanayem	64(26.12)	0	4	0	4(1.63)
Abnub	58(23.67)	0	28	0	28(11.43)
Manfalut	48(19.59)	0	0	0	0(0)
Total count	245(100)	0	50	0	50(20.41)

cubated for up to two days as described by (WHO, 2002). Inoculated eggs were then checked daily for embryo death by candling. The allantoic fluid was harvested and subjected to HA test using 0.5 % chicken erythrocytes according to WHO protocol (WHO, 2002).

AIV detection and subtyping

The viral RNA was extracted from the positive HA samples using a QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's protocol, then they were classified as negative or positive for influenza virus using a taqMan real time RT-PCR test for the M gene (Kamel et al., 2022; WHO, 2017) . The positive M gene samples were further subtyped for HA (H5 and H9) and NA (N1-N2 and N8) genes (Fereidouni et al., 2009; Lee et al., 2001). The significance of AIV H5 detection ratio was estimated for each of the following characteristics: sample type, site, host species and bird vaccination status using SPSS (Tables 1 and 2) (Liu et al., 2018).

Sequence analysis and phylogenetic tree construction

Full genome of two AI virus isolates (one from geese and one from duck) was amplified using universal primers and SuperScript IV One-Step RT-PCR System (Hoffmann et al., 2001). The purified PCR Amplicons were subjected to DNA Sanger sequencing at Macrogen sequencing facility (Macrogen, South Korea). Sequences were assembled using SeqMan DNA Lasergene 7 software (DNASTAR, Madison, WI) then were deposited in GenBank under the accession numbers listed in Table 6.

Publicly available sequences of the AIV A/H5N8 viruses from Egypt and reference strains were obtained from the Global Initiative on Sharing All Influenza Data (GISAID) (http://platform.gisaid.org/epi3/). Nucleotide sequences for each segment were subjected to multiple alignments using the ClustalW tool in BioEdit software version 7.2.5. The aligned sequences were trimmed then eight neighbor-joining phylogenetic trees were constructed using the

MEGA X software with Kimura's two-parameter distance model and 1000 replicates in bootstrap.

Translated sequences for each segment of the viruses in this study and reference viruses were obtained using the annotation tool available on the NCBI influenza virus resources, aligned and analyzed using BioEdit software version 7.2.5.

RESULTS

Virus isolation, HA test and real time RT-PCR

Out of 245 collected swab samples, 50 samples (30 Oropharyngeal (12.24%) and 20 Cloacal (8.16%) were positive for influenza virus (Table 1), in a total percentage of 20.4 % (Table 2). This percentage was differed significantly according to sites, host species and vaccination (Table 1). The detection rate in tested swabs from ducks was significantly higher than geese samples test result ($P \leq 0.05$) (Table 1). The detection percentage by site ranged from 1.63% in El-Ghanayem to 11.43% in Abnub, and influenza A viruses were not detected in Manfalut ($P < 0.05$). Influenza A viruses were detected among vaccinated and non-vaccinated birds. The detection rate of influenza A in vaccinated birds was statistically significant comparing with non-vaccinated birds ($P \leq 0.05$). All influenza A positive samples were subtyped as A/H5N8 viruses. Influenza A/H5N1 and A/H9N2 viruses were not detected (Table 2).

Full genome sequencing and Phylogenetic analysis

Two representative A/H5N8 isolates from duck and goose were subjected to full genome sequencing and genetic analysis. Phylogenetic analysis of the two selected A/H5N8 isolates from duck and goose were applied on the 8 segments nucleotide sequences. Highly similar sequences were retrieved from GenBank of each obtained sequence, and reference strains from the GISAID and GenBank. Analysis of generated HA sequences indicated that both the detected H5N8 viruses belongs to 2.3.4.4b clade and diversified into subgroup I for one A/goose/Egypt/BA71C/2018(H5N8) virus, and subgroup II for the second A/duck/Egypt/BA3C/2018(H5N8) virus (Figure 2). The HA gene of both strains are grouped with the Egyptian strains of 2016-2018 with identity 98.9 -99.2%.

No reassortment was detected through our analysis of internal segments of both HPAI H5N8 viruses with endemic H9N2 viruses in Egypt. the two H5N8 viruses in this study had different origin of genome (Figure 3). Goose/BA71C/2018 genetic constellation showed that the 8 segments belong to the Eurasian and the Russian and Asian linages, while the A/duck/Egypt/BA3C/2018 virus was closely related to the Russian and European linage (Figure 4).

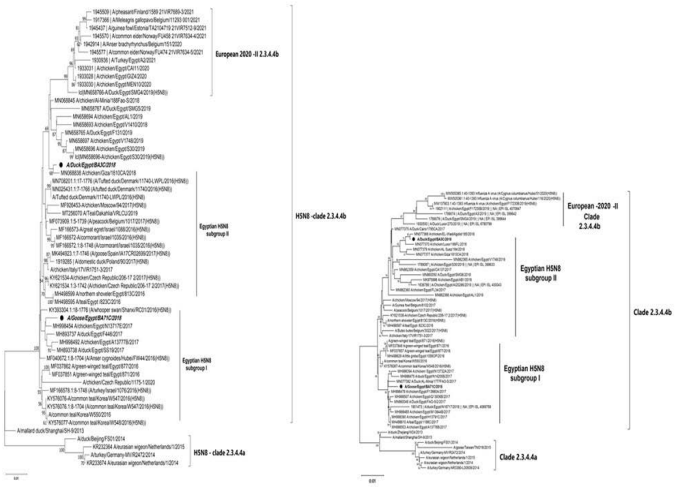

Figure 2: Phylogenetic analysis of HA and NA segments of the Egyptian H5N8 viruses, both the two isolates of this study are related to Egyptian H5N8 strains of clade 2.3.4.4.b which indicated with a black dot. Duck/BA3C/2018 is related to the Egyptian subgroup II and the Goose/BA71C/2018 is related to Egyptian subgroup I

Genetic characterization

Two Egyptian HPAI H5N8 viruses displayed PLREKRRKR/GLF motif at the HA cleavage site. There are no alterations in amino acid sequences of receptor binding sites, glycosylation sites and antigenic sites in comparison with other Egyptian strains as shown in Table 3, except for M140 in antigenic site A of Goose/BA71C/2018 virus and V140 of Duck/BA3C/2018. E79D, P88T and Q342L mutations in HA of goose sample are characteristic to clade 2.3.4.4b.

The NA gene of both H5N8 isolates in this study are related to Egyptian H5N8 strains of clade 2.3.4.4.b which belong to the Russian reassortant H5N8 circulated in 2018 with identity percent ranged from 98.5 to 99.2%. There are no changes in the hemadsorption sites and glycosylation sites of NA gene in both H5N8 samples compared other Egyptian strains, while Goose/BA71C/2018 has acquired new glycosylation site at 42NGTC. Also, there is no change in the oseltamivir resistance marker I312V.

The PB2 proteins of both H5N8 viruses did not have the E627K substitution mutation, which has been linked to mammalian host adaptation and virulence (Tables 4 & 5), but acquired the PB2 (504V and 701 N) linked to mammalian virulence (Table 4). Both of Egyptian H5N8 isolates encoded an N-terminally truncated PB1-F2 protein (52 amino acids in length). The current Egyptian H5N8 viruses have many molecular markers associated with virulence in mammals and adaptation to mammalian hosts

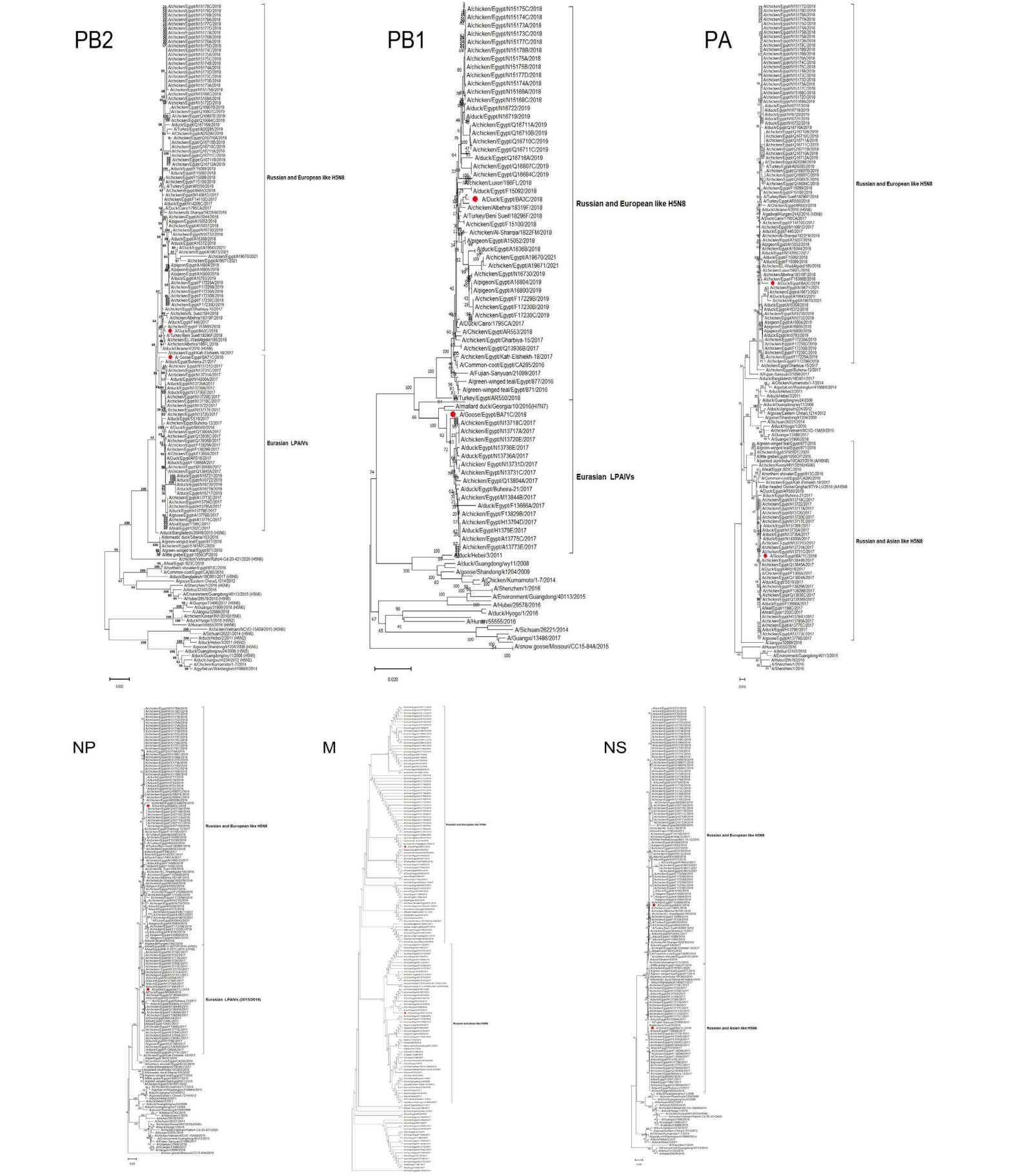

Figure 3: Neighbor joining phylogenetic trees of PB2, PB1, PA, NP, M, and NS genes of the detected H5N8 viruses in backyard in upper of Egypt comparing with published H5N8 sequences.

Table 3: Mutations in HA gene Receptor binding sites and Antigenic sites of duck and goose isolates in comparison with clade 2.3.4.4.a and clade 2.3.4.4.b

	Cleavage sites	Receptor binding sites						Antigenic sites								
								A			B			E		
		103	129	186	221	222	224	133	140	141	154	156	184	71	83	86
2.3.4.4a	RERRRKR-GLF	H	L	E	G	Q	G	A	A	S	N	A	A	I	A	A
2.3.4.4b	REKRRKR-GLF	H	L	E	G	Q	G	A	T	P	N	A	A	I	A/T	A/V
Goose/ BA71C/2018	REKRRKR-GLF	H	L	E	G	Q	G	A	M	P	N	A	A	I	A	A
Duck/ BA3C/2018	REKRRKR-GLF	H	L	E	G	Q	G	A	V	P	N	A	A	I	A	A

Table 4: Analysis of mammalian virulence determinants in the viral PB2, PB1, PA, NP, M2, NS1, and NS2 proteins of H5N8 ducks and geese isolates.

Protein	aa site	Virulent	A virulent	Duck/BA3C	Goose/BA71C	
PB2	627	K	E	E	E	(Wang, Sun et al. 2012)
	147	L	M	I	I	(Wang, Sun et al. 2012)
	250	G	V	V	V	(Wang, Sun et al. 2012)
	504	V	I	V	V	(Rolling, Koerner et al. 2009)
	701	N	D	N	N	(Teng, Zhang et al. 2013)
	591	K	Q	Q	Q	(Mok, Lee et al. 2014)
PB1	317	I	M/V	M	M	(Chen, Chang et al. 2006, Lee, Deng et al. 2007)
PA	127	V	I	-	V	(Lycett, Ward et al. 2009)
	672	L	F	L	L	(Li, Chen et al. 2005)
	100	R	V	-	V	(Otte, Sauter et al. 2015)
	550	L	I	L	L	(Rolling, Koerner et al. 2009)
NP	470	R	K	-	K	(Chen, Wang et al. 2017)
M2	64	S/A/F	P	S	S	(Lycett, Ward et al. 2009)
	69	P	L	P	P	(Lycett, Ward et al. 2009)
NS1	42	S	A/P	S	S	(Jiao, Tian et al. 2008)
	92	E	D	D	D	(Lee, Deng et al. 2007)
	103	L	F	F	F	(Dankar, Wang et al. 2011)
	106	I	M	M	M	(Dankar, Wang et al. 2011)
	189	N	D/G	D	D	(Subbarao and Shaw 2000)
	PDZ motif (227-230)	presence	deletion	deletion	presence	
NS2	31	I	M	M	M	(Subbarao and Shaw 2000)
	56	Y	H/L	H	H	(Subbarao and Shaw 2000)

including PB2 (504V), PA (672L), M2 (64S, and 69P), in addition to mammalian marker (398Q) in NP genes of both isolates (Tables 4 & 5).

The genetic analysis of amino acid residues associated with amantadine resistance in the M2 protein revealed that both isolates had L26, V27, A30, S31, and G34, indicating amantadine sensitivity.

The PDZ motif (X-S/ T-X-V) (227 to 230) was found at the C-terminus of the NS1 protein in the form of GSEV in Goose/BA71C H5N8 isolate (Tables 4 & 5). S42 virulence determinants were found in the NS1 of Egyptian H5N8 viruses. Mammalian-specific residues in the viral proteins NS1 and NS2 were not found in either Egyptian H5N8 isolate. The sequences of both viruses exhibited no substitutions of NS2 virulence determinant residues at po

Table 5: Analysis of genetic determinants of host range in the PB2, PB1, PA, NP, M1, M2, NS1, and NS2 proteins in H5N8 viruses. The host preference markers are shown.

Viral protein	aa site	Avian preference	Mammalian preference	Duck/BA3C	Goose/BA71C	
PB2	44	A	S	A	A	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	64	M	T	M	M	(Guilligay, Tarendeau et al. 2008)
	81	T	M	T	T	(Shaw, Cooper et al. 2002)
	199	A	S	A	A	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	591	Q	K	Q	Q	(Mok, Lee et al. 2014)
	627	E	K	E	E	(Wang, Sun et al. 2012)
	661	A	T	A	A	(Kuzuhara, Kise et al. 2009)
	701	D	N	D	D	(Teng, Zhang et al. 2013)
	702	K	R	K	K	(Kuzuhara, Kise et al. 2009)
PB1	336	V	I	V	V	(Chen, Chang et al. 2006)
	375	N	S	N	N	(Jeffery K. Taubenberger 2005)
PA	28	P	L	-	P	(Wanitchang, Jengarn et al. 2011)
	55	D	N	-	D	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	57	R	Q	-	R	(Chen, Chang et al. 2006)
	100	V	A	-	V	(Wang, Tang et al. 2015)
	133	E	G	-	E	(Brown, Liu et al. 2001)
	225	S	C	-	S	(Finkelstein, Mukatira et al. 2007)
	241	C	Y	-	C	(Yamaji, Yamada et al. 2015)
	268	L	I	L	L	(Finkelstein, Mukatira et al. 2007)
	356	K	R	K	K	(Chen, Chang et al. 2006)
	382	E	D	E	E	(Shaw, Cooper et al. 2002, Taubenberger, Reid et al. 2005)
	404	A	S	A	A	(Chen, Chang et al. 2006)
	409	S	N	S	S	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	552	T	S	T	T	(Finkelstein, Mukatira et al. 2007)
	615	K	L	R	K	(Gabriel, Dauber et al. 2005)
NP	33	V	I	V	V	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	61	I	L	I	I	(Shaw, Cooper et al. 2002, Finkelstein, Mukatira et al. 2007)
	109	I	V	I	I	(Chen, Chang et al. 2006)
	136	L	M	L	L	(Shaw, Cooper et al. 2002)
	214	R	K	R	R	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)

	313	F	Y	F	F	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	357	Q	K	-	Q	(Chen, Chang et al. 2006)
	372	E	D	-	E	(Chen, Chang et al. 2006)
	398	K	Q	-	Q	(Chen, Chang et al. 2006)
	455	D	E	-	D	(Chen, Chang et al. 2006)
M1	15	V	I	V	V	(Katz, Lu et al. 2000)
	115	V	I	V	V	(Finkelstein, Mukatira et al. 2007)
	121	T	A	T	T	(Finkelstein, Mukatira et al. 2007)
	137	T	A	T	T	(Shaw, Cooper et al. 2002, Finkelstein, Mukatira et al. 2007)
M2	11	T	I	T	T	(Chen, Chang et al. 2006)
	16	E	G/D	E	E	(Shaw, Cooper et al. 2002)
	20	S	N	S	S	(Shaw, Cooper et al. 2002, Chen, Chang et al. 2006)
	28	I	I/V	I	I	(Shaw, Cooper et al. 2002)
	57	Y	H	Y	Y	(Chen, Chang et al. 2006)
	55	L	F	L	L	(Pan and Jiang 2009)
	86	V	A	V	V	(Chen, Chang et al. 2006)
NS1	227	E	K/R	deletion	G	(Soubies, Volmer et al. 2010)
	full length	217	230	217	230	

Table 6: Accession Numbers of two representative HPAI/H5N8 isolates of backyard duck and goose collected during winter 2018 in Upper Egypt.

No.	Accession No.	Name of The Gene
1	OL467344.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 1 polymerase PB2 (PB2) gene, partial cds
2	OL467343.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 2 polymerase PB1 (PB1) gene, partial cds
3	OL467342.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 3 polymerase PA (PA) and PA-X protein (PA-X) genes, complete cds
4	OL467341.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 8 nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds
5	OL467340.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 4 hemagglutinin (HA) gene, complete cds
6	OL467339.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 7 matrix protein 2 (M2) gene, partial cds; and matrix protein 1 (M1) gene, complete cds
7	OL467338.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 6 neuraminidase (NA) gene, partial cds
8	OL467337.1	Influenza A virus (A/goose/Egypt/BA71C/2018(H5N8)) segment 5 nucleocapsid protein (NP) gene, complete cds
9	OL467331.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 1 polymerase PB2 (PB2) gene, complete cds
10	OL467330.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 2 polymerase PB1 (PB1) gene, partial cds
11	OL467329.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 3 polymerase PA (PA) gene, partial cds

12	OL467328.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 8 nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds
13	OL467327.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 4 hemagglutinin (HA) gene, partial cds
14	OL467326.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 7 matrix protein 2 (M2) gene, partial cds; and matrix protein 1 (M1) gene, complete cds
15	OL467325.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 6 neuraminidase (NA) gene, partial cds
16	OL467324.1	Influenza A virus (A/Duck/Egypt/BA3C/2018(H5N8)) segment 5 nucleocapsid protein (NP) gene, partial cds

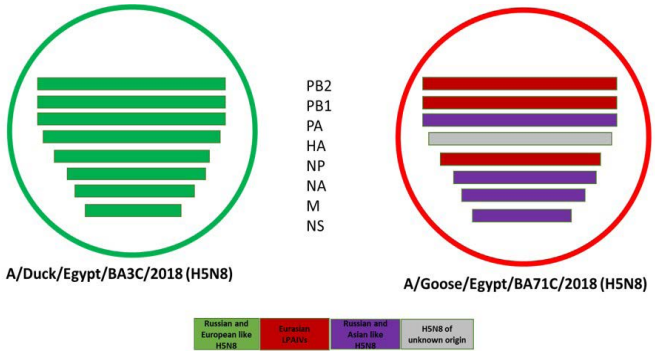

Figure 4: Genetic constellation of two Egyptian HPAI H5N8 viruses that identified in backyard in upper of Egypt in 2018.

sition 31 and 56 (Table 4).

The full genome sequences from the 2 isolated strains were deposited in the Genbank database under the accession numbers listed in Table 6.

DISCUSSION

The HPAI H5N8 clade 2.3.4.4b has been considered a reason of panzootic waves spanning Asia, Europe, the Middle East, and Africa since 2016 (Kleyheeg et al., 2017; WHO, 2017). In Egypt during December 2016, influenza A/H5N8 influenza viruses were detected and isolated from wild bird in the late of 2016 (Kandeil et al., 2017; Selim et al., 2017). After that the virus spread all over Egypt and has been recovered from numerous various poultry production sectors causing vast economic losses (Kandeil et al., 2019; Kandeil et al., 2022b; Yehia et al., 2020). Shortly after evolution of HPAI H5N8 virus in Egypt, genotyping research demonstrated multiple incursion in Egypt (Kandeil et al., 2022b; Salaheldin et al., 2018; Yehia et al., 2018).

In the current study, we surveyed live backyard ducks and geese either apparent healthy or showed severe AIV suspected clinical signs reared in different regions in Assiut governorate. The prevalence rate of AIVs (20.4%) was detected among collected samples , which is higher than previously reported by (Ahmed et al., 2019; Hagag et al., 2022; Mady et al., 2019).

Full genome sequence analysis of representative the two H5N8 isolates showed complete of genetic diversity of these viruses. As previously mentioned, multiple introductions of H5N8 were detected in Egyptian poultry, and our data also support that. one obtained from a duck is related to the Egyptian subgroup II and the other one from goose is related to Egyptian subgroup I. The Phylogenetic analysis of HA gene of the Egyptian H5N8 viruses indicated that they clustered together in group B of clade 2.3.4.4. The genetic constellation of the 2 sequenced viruses belong to different genetic linages. Based on our analysis and Kandeil et al. (2022), Goose/BA71C/2018 was classified as Genotype 1 (G1) similar to those isolated from the migratory and domestic poultry at the first wave of introduction of H5N8 in Egypt 2016-2017. while A/duck/Egypt/BA3C/2018 virus was closely related to G3, similar to those isolate from domestic poultry in 2017-2019 (Kandeil et al., 2022b).

Like most previously detected H5N8 viruses in Egypt, the current HAs of the Egyptian H5N8 viruses had PLREKRRKR/GLF motif at the HA cleavage site. The viral surface HA glycoprotein is a major determinant of influenza virus and contributed in the virulence , host range and is greatly influenced by HA receptor-binding properties (Matrosovich et al., 2000; Neumann and Kawaoka, 2006). Our results were similar to the previous study in HA glycoprotein of H5N1 in which four mutations (Q222L, G224S, T156A, and H103Y) known to facilitate the transmission of the virus in ferrets via respiratory droplets were reported (Herfst et al., 2012). N-linked glycosylation sites of the HA of influenza A viruses affect receptor-binding specificity and/or masking antigenic sites on the globular head. The receptor-binding site (RBS) is a critical viral factor for receptor specificity and influences the generation of human viruses from avian sources (Kandeil et al., 2017).

There is no change in the oseltamivir resistance marker I312V (Kandeil et al., 2017) who found that the NA genes of the Egyptian viruses contained five glycosylation sites at positions 54, 67, 84, 144 and 293, N-linked glycosylation sites on NA gene (54NETV, 67NTSV, 84NNTE, 144NGTV, 293NWTG and 398NWSG. We report a new

glycosylation site at 42NGTC in goose originated isolate besides those previously reported in clade 2.3.4.4b,(Kandeil et al., 2017). The NA of the Egyptian H5N8 viruses had the I314/312V (N2/N8 numbering) substitution, which is a molecular marker for oseltamivir resistance (Orozovic et al., 2011).

The receptor binding pocket of the HA protein of the tw Egyptian isolates revealed amino acids H103, N182, G221, Q222, and G224 (H5 numbering), suggesting an avian-like α2,3-sialic acid receptor binding preference (Yehia et al., 2018). Mutation by substitutions were detected in E79D, P88T and Q342L in goose isolate which are characteristic for the clade 2.3.4.4b that has been reported in early introduction of the H5N8 in Egypt in 2016.

The I504V substitution which is associated with increased polymerase complex activity was reported (Rolling et al., 2009). The PB2 protein did not have the E627K substitution mutations, which have been linked to mammalian host adaptation and virulence. Also, the studied isolates are encoded an N-terminally truncated PB1-F2 protein (52 amino acids in length).

Analysis of drug resistance and mutations associated with amantadine resistance showed that the N31S signature motif, associated with resistance to amantadine, was observed in the M2 protein of the Egyptian H5N8 viruses as well as viruses within the Russian-like reassortant cluster. The genetic analysis of amino acid residues associated with amantadine resistance in the M2 protein revealed that both duck and goose isolates had L26, V27, A30, S31, and G34, indicating amantadine sensitivity, in addition to the current mammalian adaptation and virulence markers as PA (550L and 672L) and NP (398Q) (Kandeil et al., 2017).

The C terminal sequences of NS1 viral protein of certain influenza viruses contain PDZ motif (Obenauer et al., 2006). Previous research has shown that this motif is one of the important motifs associated with virulence in influenza viruses (Soubies et al., 2010; Zielecki et al., 2010). Egyptian H5N1 exhibited four PDZ motif forms (ESKV, EPKV, ESEV, and ESEI) (Kandeil et al., 2017). The PDZ motif (X-S/ T-X-V) was found at the C-terminus of the NS1 protein in the form of GSEV. S42 virulence determinants were also found in the NS1 of Egyptian H5N8 viruses. Mammalian-specific residues in the viral proteins NS1 and NS2 were not found in both duck and goose H5N8 isolate. In addition, no substitutions of NS2 virulence determinant residues at position 31 and 56 were reported.

This study also highlights the need for active surveillance and monitoring of AIVs among mixing species of poultry in backyard production sector in Egypt. Detection of H5N8 viruses among vaccinated flocks insights the requirement to evaluate commercially used vaccines against circulating AIVs in Egypt. Continuous surveillance of backyard and other production sectors to follow up the molecular epidemiology and diversity of HPAI in Egypt is needed. Data revealed that viruses had undergone antigenic drift due to the accumulation of mutations in antigenic sites A.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

NOVELTY STATEMENT

In Egypt in 2018, there was a high prevalence rate and co-circulation of highly pathogenic avian influenza A/H5N8 viruses in backyard waterfowl.

AUTHORS' CONTRIBUTION

AA.M., A.K., HA.H., A.H., A.N.E, NS.A., A.A., and M.A.A. designed the study; AA.M., Y.M., HA.H., M.A.A., A.H., A.N.E., and M.A.A performed the experiments; AA.M., Y.M., A.K., A.N.E., A.A., HA.H. and M.A.A. analyzed the data; A.K, Y.M., A.N.E , A.A ,M.A.A., and HA.H. drafted the manuscript; all authors approved the manuscript.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript. All sequences presented in this

study are openly available in GenBank under the accession numbers listed in the manuscript.

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