Original Research

Journal of Advanced Veterinary Research
(2025) Volume 15, Issue 2, 248-254

Analysis of the hemagglutinin polypeptide of highly pathogenic avian influenza H5 clade 2.3.4.4b viruses isolated from backyard ducks and commercial chickens in Egypt

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ARTICLE INFO

Recieved: 19 January 2025

Accepted: 24 March 2025

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Keywords:

Antigenic sites, Backyard ducks, Dual receptors avidity, HPAI H5 clade 2.3.4.4b

ABSTRACT

Highly pathogenic avian influenza (HPAI) viruses of the H5 subtype garner global attention due to their severe impact on the poultry industry and the risk of human infections with high fatality rates. This study aimed to identify and characterize HPAI H5 viruses circulating in two major poultry production sectors: commercial farms and backyard flocks. Tracheal and cloacal swabs were collected from 75 flocks (57 farms and 18 backyard flocks) representing various domestic species and age groups, all exhibiting respiratory and/or nervous symptoms with high mortality rates. Real-time PCR results identified 24 positive samples for avian influenza virus (AIV) (24/75, 32%), which were further subtyped into 8 HPAI H5 (8/75, 10.66%) and 16 low pathogenic avian influenza (LPAI) H9 (16/75, 21.33%) cases. Among HPAI H5 infections, a higher detection rate was observed in backyard ducks (5/8, 62.5%) compared to chickens and turkeys on commercial farms. Three H5 viruses were isolated, and their hemagglutinin (HA) genes were sequenced using primers designed for this study. Sequence analysis revealed 96.5–97.1% and 97.8–98.6% identity with A/Common-coot/Egypt/CA285/2016 (the original H5N8 Egyptian virus), and 97.4–98.2% and 97.7–99% identity with A/Astrakhan/3212/2020 (the first H5N8 human isolate) at the nucleotide and amino acid levels, respectively. Several unique mutations were identified in the HA polypeptide of the duck isolates. Analysis of receptor binding sites (RBS) indicated a probable dual receptor-binding affinity in the H5 isolates studied. Phylogenetic analysis grouped the H5 isolates within clade 2.3.4.4b, showing close relatedness to Russian viruses. The mutational findings suggest the potential emergence of H5N8 escape mutants from backyard ducks.

Introduction

Egypt's intercontinental location is considered a cornerstone for wild bird migrations as two main migration lines cross there (El-Zoghby *et al*., 2013). This geographic location makes Egypt more vulnerable to Eurasian avian influenza viruses (AIVs) and those originating from Africa because migratory birds are the main reservoir of AIVs (Hagemeijer and Mundkur, 2006).

Based on their pathogenicity and virulence in avian hosts, AIVs are classified into two main categories: highly pathogenic avian influenza (HPAI) viruses, which can cause up to 100% mortality in various bird species, and low pathogenic avian influenza (LPAI) viruses, which typically result in mild or asymptomatic infections in poultry (Cui *et al*., 2017).

Since 2006, Egypt has suffered from HPAI H5N1 virus of Asian origin (Saad *et al*., 2007). This introduction was accompanied by huge losses in the Egyptian poultry sector estimated at 1 billion dollars, with 30 million birds culled. It also severely affects the income of 1.5 million people working in this field (Meleigy, 2007). In 2016, a second wave of HPAI viruses was reported but of the H5N8 subtype (Kandeil *et al*., 2017; Selim *et al*., 2017). Since 2017, several HPAI H5N8 outbreaks have been recorded (Salaheldin *et al*., 2018; Hassan *et al*., 2021).

Despite co-infection of H5N1 and H9N2 AIVs in the same avian host for several years, no HPAI reassortant virus has occurred (Kayali *et al*., 2014; Hassan *et al*., 2016), whereas dual infection with HPAI H5N8 and LPAI H9N2 led to the emergence of HPAI H5N2 that has been isolated from ducks and chickens in Fayoum, Dakahlia, and Beheira governorates (Hagag *et al*., 2019; Hassan *et al*., 2020a; Hassan *et al*., 2021). Seven different genotypes of H5 clade 2.3.4.4b were reported in Egypt, five of them are H5N8 while only two of them are H5N2 (Hassan *et al*., 2020b).

The endemicity of AIVs in Egypt provides a favorable environment for viral genetic evolution, potentially leading to the emergence of novel subclades with enhanced zoonotic potential. Furthermore, the first human case of HPAI H5N8 clade 2.3.4.4b, reported in Russia in December 2020, has heightened concerns about the risk of human infections (Pyankova *et al*., 2021).

This study aimed to detect, isolate, and sequence the HA gene of HPAI H5N8 viruses circulating in commercial and backyard flocks across various domestic species in select Egyptian governorates. A key research objective was to compare the HA gene of the study isolates with that of the first Russian H5N8 virus isolated from humans, to assess the potential risk of human cases of this subtype emerging in Egypt.

Materials and methods

Ethics approval and consent to participate

The ethical policies of the journal were fulfilled. This study was approved by the Scientific Research and Bioethics committee of the faculty of veterinary medicine, Suez Canal University with ethical approval number (2019025). The study's methodology, including sampling and examination, was performed according to the ethical guidelines of Suez Canal University, Egypt. Swabs taken from live birds were collected with minimum pain by specialized veterinarians, while the post-mortem examination was done on already-dead birds following strict hygienic measures.

Sampling and clinical examination

A total of 238 birds representing 75 flocks (1-5 birds per flock) of various ages and species, including 58 chicken, 8 duck, 6 turkey,1 quail and 2 pigeon flocks, were sampled from seven Egyptian governorates during 2020–2021. These birds had a history of respiratory and/or nervous signs accompanied by high daily mortality rates. The governorates included Ismailia (53 flocks), Port Said (7 flocks), Sharkia (8 flocks), Cairo (4 flocks), Assiut (1 flock), Beheira (1 flock), and Suez (1 flock). A total of 371 tracheal and cloacal swabs (2–10 swabs per flock) were collected and stored at -20°C until they were prepared and processed, as described by

ISSN: 2090-6277/2090-6269/

Numan *et al*. (2008).

Real-time RT-PCR (rRT-PCR)

The Biospin Virus RNA Extraction Kit (BioFlux, China; Cat. No. BSC62M1) was used for viral RNA extraction. Primers and probes specific for M1 (Spackman *et al*., 2002), H9 (Shabat *et al*., 2010), H5 and N8 genes (ABT Q PCR KITs, Applied Biotechnology) were used. AgPath-ID™ One-Step RT-PCR KIT (Thermo Fisher Scientific, Cat# AM1005) was used, with a reaction volume of 25 µL (Table 1). The 7500 Real-Time PCR system (AppliedBiosystems) was used following the company's instructions.

Table 1. Reagent quantities and thermal profile for rRT-PCR reactions

	Regents	Amounts per reaction		
Reagents used	2x AgPath buffer	10 µL		
	Forward primer	15 Pmol (1.5 µL)		
	Reverse primer	15 Pmol (1.5 µL)		
	Probe	5 Pmol (0.5 µL)		
	Enzyme mix	0.8 µL		
	PCR grade water	5.7 µL		
	Template RNA	5 µL		
Thermal profile	Stage	Temp	Time	Cycle
	Reverse transcription	45˚C	10 min	1
	Primary denaturation	95˚C	10 min	1
	Second denaturation	95˚C	15 sec	
	Annealing	57˚C	30 sec	45
	Extension	72˚C	30 sec	

Virus isolation and propagation

rRT-PCR positive samples for H5 were subjected to virus isolation using 11-day-old embryonated chicken eggs (ECEs) from native Baladi breeds that had no prior vaccination or infection with AIVs (Woolcock, 2008). Up to three passages were performed per sample to confirm isolation failure. Following isolation, a hemagglutination (HA) assay was performed on harvested allantoic fluids using washed chicken red blood cells (RBCs) as described (Killian, 2008). The presence of HPAI H5 isolates was subsequently reconfirmed by rRT-PCR.

Hemagglutinin (HA) gene partial sequencing

Two sets of primers to the H5 were designed using Geneious software (Table 2) and used for gene amplification by the overlapping technique in separate PCR tubes alongside the WHO-recommended primers (WHO, 2002). Two-step RT-PCR was applied, the reagents and the thermal profile used were according to Shelkamy *et al*. (2022). The expected correct size of each segment was visualized by the agar gel electrophoresis of PCR products on a 1% ethidium bromide-stained agarose gel. The PCR products were sent to SolGent Co., Ltd. (South Korea) for purification and sequencing using direct Sanger sequencing based on forward and reverse primers.

Table 2. Primers used for amplifying HA gene in H5 study isolates by RT-PCR.

Primer sequence	Product size	Reference
H5F1: CATGCAAACAAYTCGACAGAGCA H5R1: TGGGGCATTCCCCRATGGTG	889	This study
H5F2: TGGAATATGGCCACTGYAAYACCAA H5R2: TGCAAATTCTGCACTGTAAHGACCCA	836	This study
HA-1144: GGAATGATAGATGGNTGGTAYGG	591	WHO (2002)
H5-1735R: GTGTTTTTAAYTAMAATCTGRACTMA		

Sequence and phylogenetic analysis for the HA gene

Chromas software (version 2.6.6) was used to assemble sequences. Related reference viruses were identified using BLAST analysis. Sequence of reference isolates were obtained from both the NCBI and GISAID databases, and mature H5 numbering was followed (Burke and Smith, 2014). Multiple sequence alignment (Clustal W) and homology percent determination were achieved using BioEdit version 7.2 (Hall, 1999). The glycosylation sites were identified using the NetNGlyc1.0 server (Gupta *et al*., 2004). Amino acid substitutions analysis and potential antigenic sites prediction were performed using the FluSurver (Flusurver, 2020).

The phylogenetic tree was constructed using MEGA 11 software (Tamura *et al*., 2021) based on the HA nucleotide sequences of the isolates, employing the Neighbor-Joining method with the Kimura 2-parameter model. The tree's robustness was evaluated by calculating bootstrap values based on 500 replicates.

Results

AIVs detection using rRT-PCR

Out of the 75 flocks tested, 24 were positive for AIV (24/75, 32%). Among these, eight flocks tested positive for H5 (8/75, 10.66%), which were subsequently subtyped as N8 (except for one broiler chicken flock "F2" that was not tested for N8). The remaining 16 flocks were subtyped as H9 (16/75, 21.33%). The highest occurrence of H5 infection was found in backyard duck flocks (5/8), followed by commercial broiler flocks (2/8) and turkey flocks (1/8). In terms of poultry production sectors, the detection of HPAI H5 virus was higher in backyard flocks compared to commercial farms, with infection rates of 27.77% (5/18) and 5.26% (3/57), respectively. Notably, all commercial farms infected with H5 had a history of H5 vaccination and experienced mortality rates ranging from 1.4% to 2.5%. In contrast, all infected backyard flocks were unvaccinated and had mortality rates as high as 36.36%

Clinical and post-mortem findings of the H5 infected birds

Clinically, infected broiler chickens and turkeys primarily exhibited respiratory signs, including sneezing, nasal discharge, conjunctivitis, and facial edema. Additionally, some nervous manifestations were observed in the infected turkey flock. Infected broilers frequently showed cyanosis of the comb and wattles, along with subcutaneous hemorrhages and edema of the shanks and feet. In contrast, nervous manifestations (such as imbalance, head tremors, and convulsions) were the predominant signs in affected ducks, which also displayed severe beak congestion (Figure 1). On necropsy, affected broilers and turkeys exhibited hemorrhagic tracheitis, pinpoint hemorrhages on the pancreas, coronary fat, muscles, and carcass serosa. Lymphatic organs, including the cecal tonsils, Peyer's patches, and spleen, were congested, edematous, and hemorrhagic. Post-mortem examination was available for only one duck flock, which showed hemorrhages in the cecal tonsils.

Virus isolation and HA test

Based on rRT-PCR results, all field samples positive for H5 were selected for virus isolation in ECEs. Three viruses were successfully isolated, with HA titers ranging from 2log8 to 2log10. Early embryonic deaths occurred within 24 to 48 hours post-inoculation, accompanied by diffuse hemorrhagic lesions and stunted embryos, compared to the negative control (Figure 2).

H5 gene amplification using RT-PCR for partial sequencing

The primers designed for the study successfully amplified the HA

gene of the three H5 isolates, while the WHO-recommended primers failed. Specific bands were visualized on the agarose gel electrophoresis at 889 bp and 836 bp for each gene (Figure 3).

The obtained sequences of the three H5 isolates: A/Duck/Egypt/F6Ismailia/2021 (F6), A/Duck/Egypt/F16Ismailia/2021 (F16), and A/Chicken/Egypt/F2Asyut/2021 (F2) were assembled and submitted to the GenBank with accession numbers OM912781, OM912782, and OM912783, respectively.

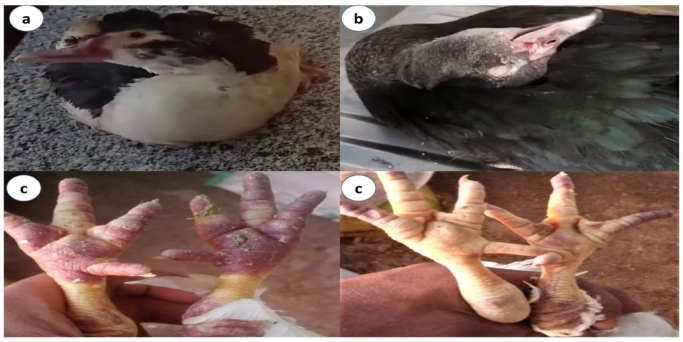

Fig. 1. The clinical findings in HPAI H5 clade 2.3.4.4b infected birds, a) Backyard Muscovy duck exhibiting head tilt and tremors with beak congestion, b) Baladi duck displaying nervous signs including opisthotonus, c) Broilers with subcutaneous hemorrhages and edema of the hocks and feet.

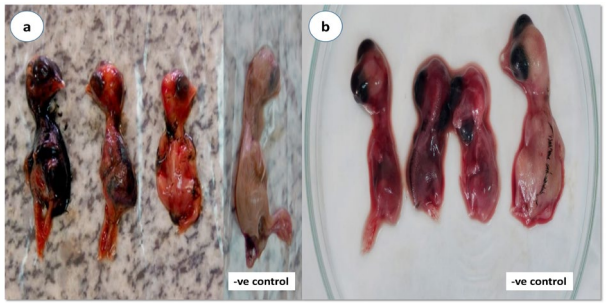

Fig. 2. Embryonic lesions due to H5 virus isolation in ECEs, a) Embryos inoculated with the F2 chicken isolate, which died within 48 hours post-inoculation, showing diffuse hemorrhagic lesions b) Embryos inoculated with the F16 duck isolate, which died within 24 hours post-inoculation, exhibiting body congestion, hemorrhages, and stunted growth.

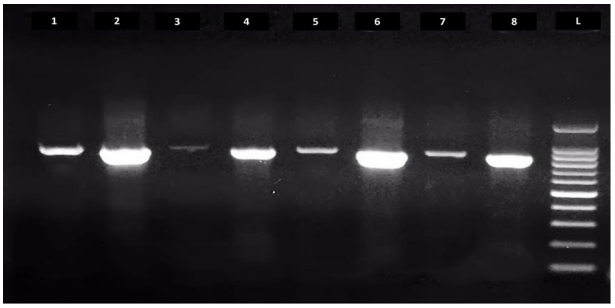

Fig. 3. Electrophoresis of amplified segments of the H5 gene using the designed primers. Specific bands were visualized in ethidium bromide-stained agarose gel at 889 bp and 836 bp. Lane L: DM10-100bp DNA Ladder Marker (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500 bp), lanes 1, 2 & lanes 3, 4 for isolate F2 (Two different ECEs), lanes 5, 6 for isolate F6, and lanes 7, 8 for isolate F16.

Sequence and phylogenetic analysis

A complete analysis was performed, and the H5 numbering of the mature HA polypeptide was followed. The sequences of the isolates were compared with the first reported H5N8 strain in Egypt (A/Common-coot/Egypt/CA285/2016), eight commercially available vaccine strains in the Egyptian market, and the first H5N8 case reported in humans. The H5 isolates shared 97.6-99.5% and 97.6-99.8% homology with each other as well as 96.5-97.1% and 97.8-98.6% homology with the original Egyptian virus (A/Common-coot/Egypt/CA285/ 2016) at the level of nucleotides and amino acids respectively.

Molecular analysis of the F6, F16, and F2 isolates revealed 14, 11, and 9 amino acid substitutions, respectively, of which four substitutions (T140A, Y487D, V522A, and V532M) were common compared to the original Egyptian virus (Table 3). Additionally, the isolates exhibited multiple basic amino acids in the HA cleavage site (321PLREKRRKR/GLF332).

This study focused on three major antigenic sites: A (133,140 and 141), B (154, 156, and 184), and E (71, 83, and 86) as well as ten other sites at positions: 94, 120, 162, 227, 252, 263, 282, 11, 151, and 168. The study isolates had a common mutation (T140A) at the antigenic site A. The chicken isolate (F2) had a mutation at A184V (antigenic site B). The HA polypeptide of the duck isolates (F6 and F16) showed several unique amino acids substitutions at I151V, I162M, and N168Y. In addition, isolate F6 had other unique mutations at N11K (Glycosylation site) and Q15L (binding small ligand as predicted and defined by the FluSurver). Mutations in the antigenic sites of the study isolates relative to the Egyptian reference strain as well as the vaccine seed viruses used in the analysis are shown in Table 4.

Twelve receptor-binding sites (RBS) were investigated to assess the probable avidity of the study isolates. The study isolates contained the following amino acids at these sites: S94, P123, A133, N154, A156, N189, K192, N220, Q222, R223, G224, and T315. Regarding N-linked glycosylation sites, the sequenced fragment revealed five sites at positions 10, 23, 165, 286, and 483. Notably, the F6 and F2 isolates had 10NKST instead of 10NNST, compared to the original virus. Phylogenetically, the study isolates clustered with group B H5N8 viruses from clade 2.3.4.4, showing close phylogeny to Russian viruses (Figure 4).

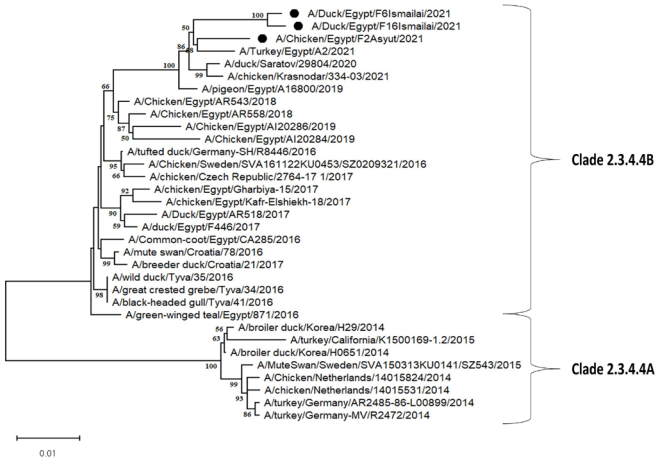

Fig. 4. Phylogenetic analysis of H5 gene sequences (1569 bp) using the neighbor-joining method-Kimura 2-parameter model, tested by 500 bootstrap values. Solid circles refer to the study isolates.

Eight commercially available H5 vaccine seeds were used in the analysis to determine their identity percentage with the H5 study isolates at the level of deduced amino acids. The vaccine strains included: (RG A/green-winged teal/Egypt/877/2016 (H5N8), A/chicken/Egypt/ME-

Table 3. Amino acid substitutions in the mature HA polypeptide of H5 study isolates compared to the original Egyptian virus

The reference isolate	Isolate F6 (duck-H5N8)	Isolate F16 (duck-H5N8)	Isolate F2 (chicken-H5)
A/Common- coot/Egypt/CA285/2016	N11K, Q15L, T140A, I151V, I162M, N168Y, R169Q, I198V, L430F, E479A, Y487D, T512I, V522A, V532M	T140A, I151V, I162M, N168Y, R169Q, I198V, E479A, Y487D, T512I, V522A, V532M	N11K, T140A, A184V, N236D, G268E, Y487D, S503G, V522A, V532M

Table 4. Amino acid substitutions at antigenic sites in H5 study isolates and selected H5 vaccine virus seeds, with identity percentages of the HA polypeptide compared to the original Egyptian virus

Virus	Antigenic site A			Antigenic site B			Antigenic site E			New antigenic sites							In-silico predicted by FluSurver			Identity percentage		
	133	140	141	154	156	184	71	83	86	94	120	162	227	252	263	282	11	151	168	F6	F16	F2
A/Common-coot/CA285/2016	A	T	P	N	A	A	I	A	A	S	S	I	D	Y	T	V	N	I	N	97.8	98	98.6
A/Duck/Egypt/F6Ismailia/2021(H5N8)	A	A	P	N	A	A	I	A	A	S	S	M	D	Y	T	V	K	V	Y	100	99.8	97.6
A/Duck/Egypt/F16Ismailia/2021(H5N8)	A	A	P	N	A	A	I	A	A	S	S	M	D	Y	T	V	*	V	Y	99.8	100	97.8
A/Chicken/Egypt/F2Asyut/2021(H5)	A	A	P	N	A	V	I	A	A	S	S	I	D	Y	T	V	K	I	N	97.6	97.8	100
RG A/green-winged teal/Egypt/877/2016	A	T	P	N	A	A	I	A	A	S	S	I	D	Y	T	V	N	I	N	97.3	97.5	98.6
A/chicken/Egypt/ME-2018/2018(H5N8)	A	T	P	N	A	A	I	A	A	S	S	I	D	Y	T	V	N	I	N	96.9	97.1	98.2
A/duck/Potsdam/1402-6/1986(H5N2)	S	R	S	N	A	A	L	D	I	D	S	R	E	Y	A	M	N	I	N	87.9	88.1	87.9
RG A/chicken/Egypt/18-H/2009 (H5N1)	S	R	T	N	T	E	P	I	A	N	S	E	E	N	T	I	N	I	N	88.5	88.6	89.4
A/duck/Egypt/M2583D/2010(H5N1)	S	R	S	N	A	A	L	I	A	N	D	K	E	N	T	I	N	T	N	90.4	90.6	90.9
A/duck/ China/E319-2/03 (H5N1)	S	S	S	N	A	A	I	A	A	N	S	R	D	Y	A	M	N	I	N	92.3	92.5	93.1
A/turkey/England/N28/73(H5N2)	S	R	S	N	A	A	L	D	T	D	S	R	E	Y	A	P	N	I	N	87.7	87.9	87.5
A/Swan/Hungary/4999/ 2006 (H5N1)	S	R	S	D	A	A	L	I	A	N	S	R	E	N	T	I	N	I	N	90.4	90.6	90.9

NB/Underlined letters indicate amino acid substitutions in antigenic sites, * indicates partial sequence

2018/2018 (H5N8), A/duck/Potsdam/1402-6/1986 (H5N2), RG A/chicken/Egypt/18-H/2009 (H5N1), A/duck/Egypt/M2583D/2010 (H5N1), A/duck/China/E319-2/03 (H5N1), A/turkey/England/N28/73 (H5N2) and A/Swan Hungary/4999/2006 (H5N1)). The results revealed identity percentages of 97.3-98.6%, 96.9-98.2%, 87.9-88.1%, 88.5-89.4%, 90.4-90.9%, 92.3-93.1%, 87.5-87.9% and 90.4-90.9% respectively (Table 4).

Compared to A/Astrakhan/3212/2020 (the first H5N8 human isolate), the study isolates showed 97.4-98.2% and 97.7-99% homology at the nucleotide and amino acid levels, respectively. The HA polypeptide of A/Astrakhan/3212/2020 exhibited five mutations (T140A, N236D, Y487D, V522A, and V532M) compared to A/Common-coot/ Egypt /CA285 /2016. All five mutations were found in the chicken isolate, while only four were observed in the duck isolates.

Discussion

Our study revealed positive cases of HPAI H5N8 virus in both major sectors, backyard flocks, and commercial farms using real-time PCR. The occurrence was higher in backyard ducks than in commercial gallinaceous farms, similar findings reported by Kandeil *et al.* (2019). These findings could be attributed to a lack of sanitation, and vaccination strategies, coupled with direct contact with wild birds or their droppings in the backyard sector (Iqbal, 2009; Sheta *et al.*, 2014). On the other hand, the ducks' feeding style seems to be positively associated with AIV infection (Hill *et al.*, 2010).

Although the first detection of H5N8 in Egypt was from apparently healthy migratory birds (Kandeil *et al.*, 2017; Selim *et al.*, 2017), our investigations revealed that the HPAI H5N8 clade 2.3.4.4b infected various breeds of ducks (Pekin, Muscovy, Mallard, and Baladi), all exhibiting similar clinical symptoms, primarily neurological signs such as head tremors and convulsions. These symptoms have been reported in several studies (Bányai *et al.*, 2016; Sun *et al.*, 2016; Gaide *et al.*, 2021; Saad *et al.*, 2024)

This neurological presentation is a significant characteristic of the genetic evolution of AIV H5N8, potentially enabling the virus to cross the blood-brain barriers of infected ducks. Notably, the first HPAI H5N8 virus isolated in Ireland in 1983 was unable to breach the blood-brain barrier in ducks and was not associated with neurological manifestations (Kawaoka *et al.*, 1987). Moreover, several HPAI H5N8 outbreaks, specifically of clade 2.3.4.4a, did not induce any signs in ducks despite efficient transmission (Kim *et al.*, 2014). Similarly, several European outbreaks during 2014-2015 lacked these neurological signs (Bouwstra *et al.*, 2015; Hanna *et al.*, 2015; Harder *et al.*, 2015; Verhagen *et al.*, 2015).

Dual mutations: T204I and R496G (mature H5 numbering) in the HA polypeptide of H5N8 AIV were recorded (Bányai *et al.*, 2016) , and it was noted that these mutations may have a role in the neuroinvasiveness and neurovirulence of H5N8 AIVs in ducks. However, our study tracked the original T204 and R496 residues in all H5N8 AIVs isolated from ducks that experienced neurological signs. Our findings suggest that some mutations and other factors may have a role in this neurological form and further studies are needed.

Infected chicken and turkey flocks showed classical respiratory signs, in addition to cyanosis of the comb and wattles, subcutaneous hemorrhages with edema of shanks and feet in chickens. The post-mortem examination revealed systematic carcass congestion, pinpoint haemorrhages on multiple organs, and enlarged lymphatics explaining the high pathogenicity of the viral strain causing infection (Capua and Alexander, 2002; Swayne and Pantin-Jackwood, 2008; Bae *et al.*, 2015; Stoute *et al.*, 2016; Sun *et al.*, 2016; Ameji *et al.*, 2021; Ali *et al.*, 2024)

Variable mortalities were observed among infected flocks, ranging from 1.4% to 2.5% in commercial farms and up to 36.36% in backyard flocks. This study identified a possible correlation between higher mortality rates and the absence of vaccination. Notably, all commercial farms affected by AIV H5 clade 2.3.4.4b had a history of H5 vaccination, whereas none of the infected backyard flocks was vaccinated. These findings underscore the crucial role of H5 vaccination in mitigating mortality rates in infected flocks, irrespective of vaccine clades. Similar results were reported by Tarek *et al.* (2021), where unvaccinated farms experienced a mortality rate of 43.1%, compared to only 5% in vaccinated ones. However, Ali *et al.* (2024) reported up to 100% mortalities in vaccinated layer chicken flocks.

To amplify the HA gene via conventional RT-PCR, we first increased the virus titer through isolation in ECEs. Isolation of HPAI H5 resulted in early embryonic deaths within 24 to 48 hours post-inoculation, accompanied by diffuse hemorrhagic lesions and dwarfed embryos, indicating the high virulence of the strain, comparable findings were reported (El-Araby *et al.*, 2018). The study's RT-PCR primers outperformed the WHO-recommended primers, which failed to amplify the targeted sequence due to mutations in the annealing sites. These mutations likely interfered with primer binding, leading to amplification failure (Dauber *et al.*, 2011; Yang *et al.*, 2016; Graaf *et al.*, 2017). This underscores the need for continuous development of RT-PCR primers used in AIV diagnosis to align with prevalent AIV strains in the Egyptian field, thereby preventing missed diagnoses and virus characterization.

Sequence analysis of the H5 study isolates, compared to the H5N8

reference strain, showed 96.5-97.1% and 97.8-98.6% identity at the nucleotide and deduced amino acid levels, respectively. This is lower than the >99% identity reported by Yehia *et al.* (2018), indicating that H5N8 AIVs are continually evolving in the Egyptian field.

The cleavage site is the major pathogenic determinant of AIVs. The sequence of the examined isolates revealed 321PLREKRRKR/GLF332, confirming their high pathogenic nature due to the presence of many basic amino acids, as observed in the original viruses (Kandeil *et al*., 2017; Selim *et al*., 2017) and other Egyptian H5N8 isolates (Salaheldin *et al*., 2018; Hassan *et al*., 2020b; Moatasim *et al*., 2024; Saad *et al*., 2024)

When comparing our H5 isolates to the reference strain (A/Common-coot/CA285/2016) and the vaccines commercially available in Egypt, focusing on the three major sites (A, B, and E) and the seven new sites (Kandeil *et al*., 2017; Flusurver, 2020), the study isolates exhibited the presence of T140A at the A antigenic site. Similar results were reported in H5N8 isolates in 2018 (Yehia *et al*., 2020), and in 2021-2022 (Moatasim *et al*., 2024). Furthermore, some unique mutations (I151V, I162M, and N168Y) were detected in the antigenic sites of the backyard duck isolates. These mutations may prevent previously secreted antibodies from neutralizing these epitopes, creating the possibility of escape mutant viruses and leading to vaccine failure (Escorcia *et al*., 2008; Ibrahim *et al*., 2015).

A mutation in the N-linked glycosylation site at position 10 was recorded in F6 and F2 isolates (10NKST instead of 10NNST). Since changes in glycosylation site patterns can influence the host range and pathogenicity of AIV viruses (Kawaoka and Webster, 1988; Wang *et al*., 2010), further investigation is needed to determine the impact of this mutation on the virulence of the viruses and their host range.

The possibility of AIV transmission to humans is a global concern, particularly regarding HPAI H5. With this in mind, we investigated the HA of H5 isolates compared to the first H5N8 human isolate (Pyankova *et al*., 2021). Interestingly, five mutations (T140A, N236D, Y487D, V522A, and V532M) observed in the human isolate A/Astrakhan/3212/2020 were also present in the chicken isolate (F2), while only four of them (T140A, Y487D, V522A, and V532M) were found in the duck isolates (F6 and F16). This suggests a potential for zoonotic transmission, as discussed by Ali and his colleges (Ali *et al*., 2021).

Additionally, the investigated receptor binding sites based on (Yamaji *et al*., 2020), revealed that the study isolates exhibit four RBS (P123, A133, A156, and R223) with avidity to human like receptors along with deglycosylation at site 154-156 (Gao *et al*., 2018) strengthens our hypothesis of possessing dual affinity to the avian and human receptors. Although our conclusions are based solely on in-silico analysis of the HA polypeptide, which is insufficient for confirming zoonosis, they serve as a cautionary indication for implementing appropriate virus control measures in the Egyptian field to mitigate this potential risk. Whole-genome sequencing will provide deeper insights into the eight segments of the study isolates, offering a more comprehensive understanding of their zoonotic potential.

The common belief is that vaccination efficacy increases with the tighter antigenic matching between vaccine seed virus and field strain (Swayne and Kapczynski, 2008; Beato *et al*., 2010; Fouchier and Smith, 2010). Local RG vaccine seeds (A/green-winged teal/Egypt/877/2016 (H5N8) and A/chicken/Egypt/ME-2018/2018 (H5N8) showed higher identity percentage to study isolates. In contrast, other vaccine seeds from H5N1 and H5N2 subtypes shared much lower identity with the study isolates. Kandeil *et al.* (2017) supported our findings using the HI assay that revealed impaired reactivity of elevated serum against H5N8 AIVs clade 2.3.4.4 to clades 2.2.1, 2.2.1.1, 2.2.1.2 and 2.3.2.1c. Conversely, other studies have shown high efficacy of H5N1 and H5N2 vaccine seeds in protecting against HPAI H5N8 (Gamoh *et al*., 2016). Although A/duck/China/E319-2/03 (H5N1) and A/ Swan /Hungary /4999 /2006 (H5N1) vaccine seeds, used in commercially available vectored vaccines, showed identity percentages of 92.3-93.1 and 90.4-90.9, respectively, with the study isolates, they provided high protection against HPAI H5N8 under experimental conditions (Steensels *et al*., 2016; Sultan *et al*., 2019). The reason may be due to the high efficacy of recombinant technology in inducing both cell-mediated and humoral immunity (Palya *et al*., 2018).

Phylogenetically, H5 study isolates clustered together in group B of H5N8 viruses of clade 2.3.4.4, with a tight lineage to Russian viruses, similar findings were reported (Mady *et al*., 2019; Ali *et al*., 2024; Moatasim *et al*., 2024). Recently, El-Shesheny *et al.* (2023) isolated HPAI H5N1 virus, clade 2.3.4.4.b, from wild Pintail and domestic Pekin ducks in Egypt, which poses a potential zoonotic risk based on whole genome sequences analysis.

Conclusion

The backyard flocks are central to HPAI virus endemicity in Egypt, primarily due to poor biosecurity, inadequate vaccination, and inconsistent AIV monitoring. The identification of dual receptor avidity and mutations in antigenic and glycosylation sites highlights the need for stronger control measures by Egyptian authorities. These mutations suggest a potential for H5N8 strains to evade immunity, especially those from backyard ducks. Additionally, genetic analyses of Clade 2.3.4.4b (H5Nx) viruses, spread mainly by migratory ducks, emphasize the ongoing evolution and transmission of these viruses across poultry species, underscoring the need for enhanced surveillance and containment efforts.

Acknowledgments

We acknowledge the EpiFlu and NCBI databases for supplying us with AIV sequences.

Conflict of interest

The authors declare that they have no conflict of interest

References

Ali, A.A., Kotb, G., Soliman, A., Swede, K.S., 2024. Isolation and Identification of The Highly Pathogenic Avian Influenza H5N8 Virus Isolated From Commercial Layer Chickens in Al-Sharkia Province in Egypt During 2019-2021. Zagazig Veterinary Journal 52, 117-129.

Ali, M., Yaqub, T., Shahid, M.F., Wong, F.Y., Mukhtar, N., Naeem, M., Lam, P., Jayakumar, J., Smith, G.J., Su, Y.C., 2021. Genetic characterization of highly pathogenic avian influenza A (H5N8) virus in Pakistani live bird markets reveals rapid diversification of clade 2.3. 4.4 b viruses. Viruses 13, 1633.

Ameji, N.O., Oladele, O.O., Jambalang, A.R., Adanu, A.W., Chinyere, C.N., Meseko, C.A., Lombin, L.H., 2021. Multiple Outbreaks and Clinico-pathological Features of Highly Pathogenic Avian Influenza H5N1 and H5N8 in Poultry Farms in Jos Metropolis, Plateau State, Nigeria. Journal of World's Poultry Research 11, 376-386.

Bae, Y.J., Lee, S.B., Min, K.C., Mo, J.S., Jeon, E.O., Koo, B.S., al., H.I.K., 2015. Pathological evaluation of natural cases of a highly pathogenic avian influenza virus, subtype H5N8, in broiler breeders and commercial layers in South Korea. Avian Diseases 59, 175-182.

Bányai, K., Bistyák, A.T., Thuma, Á., Gyuris, É., Ursu, K., Marton, S., Farkas, S.L., Hortobágyi, E., Bacsadi, Á., Dán, Á., 2016. Neuroinvasive influenza virus A (H5N8) in fattening ducks, Hungary, 2015. Infection, Genetics and Evolution 43, 418-423.

Beato, M.S., Monne, I., Mancin, M., Bertoli, E., Capua, I., 2010. A proof-of-principle study to identify suitable vaccine seed candidates to combat introductions of Eurasian lineage H5 and H7 subtype avian influenza viruses. Avian Pathology 39, 375-382.

Bouwstra, R., Heutink, R., Bossers, A., Harders, F., Koch, G., Elbers, A., 2015. Full-genome sequence of influenza A (H5N8) virus in poultry linked to sequences of strains from Asia, the Netherlands, 2014. Emerging Infectious Diseases 21, 872-4.

Burke, D.F., Smith, D.J., 2014. A recommended numbering scheme for influenza A HA subtypes. PloS One 9, e112302.

Capua, I., Alexander, D.J., 2002. Avian influenza and human health. Acta Tropica 83, 1-6.

Cui, J., Qu, N., Guo, Y., Cao, L., Wu, S., Mei, K., Sun, H., Lu, Y., Qin, Z., Jiao, P., 2017. Phylogeny, pathogenicity, and transmission of H5N1 avian influenza viruses in chickens. Frontiers in Cellular and Infection Microbiology 7, 328.

Dauber, E., Glock, B., Mayr, W., 2011. A primer binding site mutation at the D2S1338 locus resulting in a loss of amplification. Forensic Science International: Genetics Supplement Series 3, e87-e88.

El-Araby, I.E., ElBakrey, R.M., Ali, H., Mohamed, M.H., Mansour, S.M., Eid, A.A., 2018. Detection of DNA alterations in muscovy ducks (cairina moschata) naturally infected with highly pathogenic avian influenza virus subtype H5N1. Slovenian

Veterinary Research 55, 323-332.
El-Shesheny, R., Moatasim, Y., Mahmoud, S.H., Song, Y., El Taweel, A., Gomaa, M., Kamel, M.N., Sayes, M.E., Kandeil, A., Lam, T.T., 2023. Highly pathogenic avian influenza A (H5N1) virus clade 2.3. 4.4 b in wild birds and live bird markets, Egypt. Pathogens 12, 36.
El-Zoghby, E.F., Aly, M.M., Nasef, S.A., Hassan, M.K., Arafa, A.S., Selim, A.A., Kholousy, S.G., Kilany, W.H., Safwat, M., Abdelwhab, E., 2013. Surveillance on A/H5N1 virus in domestic poultry and wild birds in Egypt. Virology Journal 10, 1-10.
Escorcia, M., Vázquez, L., Méndez, S.T., Rodríguez-Ropón, A., Lucio, E., Nava, G.M., 2008. Avian influenza: genetic evolution under vaccination pressure. Virology Journal 5, 1-5.
Flusurver, 2020. BII Flusurver–Prepared for the next wave. Available at. (https://flusurver.bii.a-star.edu.sg/) (Accessed 12 November 2020.)
Fouchier, R.A., Smith, D.J., 2010. Use of antigenic cartography in vaccine seed strain selection. Avian Diseases 54, 220-223.
Gaide, N., Foret-Lucas, C., Figueroa, T., Vergne, T., Lucas, M. N., Robertet, L., Souvestre, M., Croville, G., Loc'h, L., Delverdier, M., 2021. Viral tropism and detection of clade 2.3. 4.4 b H5N8 highly pathogenic avian influenza viruses in feathers of ducks and geese. Scientific Reports 11, 1-9.
Gamoh, K., Nakamizo, M., Okamatsu, M., Sakoda, Y., Kida, H., Suzuki, S., 2016. Protective efficacy of stockpiled vaccine against H5N8 highly pathogenic avian influenza virus isolated from a chicken in Kumamoto prefecture, Japan, in 2014. Journal of Veterinary Medical Science 78, 139-142.
Gao, R., Gu, M., Liu, K., Li, Q., Li, J., Shi, L., Li, X., Wang, X., Hu, J., Liu, X., 2018. T160A mutation-induced deglycosylation at site 158 in hemagglutinin is a critical determinant of the dual receptor binding properties of clade 2.3. 4.4 H5NX subtype avian influenza viruses. Veterinary Microbiology 217, 158-166.
Graaf, A., Beer, M., Harder, T., 2017. Real-time reverse transcription PCR-based sequencing-independent pathotyping of Eurasian avian influenza A viruses of subtype H7. Virology Journal 14, 1-10.
Gupta, R., Jung, E., Brunak, S., 2004. Prediction of N-glycosylation sites in human proteins.http://www.cbs.dtu.dk/services/NetNGlyc
Hagag, N.M., Erfan, A.M., El-Husseiny, M., Shalaby, A.G., Saif, M.A., Tawakol, M.M., Nour, A.A., Selim, A.A., Arafa, A.-S., Hassan, M.K., 2019. Isolation of a novel reassortant highly pathogenic avian influenza (H5N2) virus in Egypt. Viruses 11, 565.
Hagemeijer, W., Mundkur, T., 2006. Migratory flyways in Europe, Africa, and Asia and the spread of HPAI H5N1. In: Proceeding of International Scientific Conference On Avian Influenza and Wild Birds, Rome, Italy.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Proceeding of Nucleic Acids Symposium series No. 41, pp. 95-98.
Hanna, A., Banks, J., Marston, D.A., Ellis, R.J., Brookes, S.M., Brown, I.H., 2015. Genetic characterization of highly pathogenic avian influenza (H5N8) virus from domestic ducks, England, November 2014. Emerging Infectious Diseases 21, 879-82.
Harder, T., Maurer-Stroh, S., Pohlmann, A., Starick, E., Höreth-Böntgen, D., Albrecht, K., Pannwitz, G., Teifke, J., Gunalan, V., Lee, R.T., 2015. Influenza A (H5N8) virus similar to strain in Korea causing highly pathogenic avian influenza in Germany. Emerging Infectious Diseases 21, 860-3.
Hassan, K.E., El-Kady, M.F., EL-Sawah, A.A., Luttermann, C., Parvin, R., Shany, S., Beer, M., Harder, T., 2021. Respiratory disease due to mixed viral infections in poultry flocks in Egypt between 2017 and 2018: Upsurge of highly pathogenic avian influenza virus subtype H5N8 since 2018. Transboundary and Emerging Diseases 68, 21-36.
Hassan, K. E., King, J., El-Kady, M., Afifi, M., Abozeid, H. H., Pohlmann, A., Beer, M., Harder, T., 2020a. Novel reassortant highly pathogenic avian influenza A (H5N2) virus in broiler chickens, Egypt. Emerging Infectious Diseases 26, 129-133.
Hassan, K.E., Saad, N., Abozeid, H.H., Shany, S., El-Kady, M.F., Arafa, A., Azza, A.E., Pfaff, F., Hafez, H.M., Beer, M., 2020b. Genotyping and reassortment analysis of highly pathogenic avian influenza viruses H5N8 and H5N2 from Egypt reveals successive annual replacement of genotypes. Infection, Genetics and Evolution 84, 104375.
Hassan, K.E., Shany, S.A., Ali, A., Dahshan, A.H.M., Azza, A., El-Kady, M.F., 2016. Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poultry Science 95, 1271-1280.
Hill, N.J., Takekawa, J.Y., Cardona, C.J., Ackerman, J.T., Schultz, A. K., Spragens, K.A., Boyce, W. M., 2010. Waterfowl ecology and avian influenza in California: do host traits inform us about viral occurrence? Avian Diseases 54, 426-432.
Ibrahim, M., Sultan, H.A., Razik, A. G.A., Kang, K.-i., Arafa, A. S., Shehata, A.A., Saif, Y. M., Lee, C.-W., 2015. Development of broadly reactive H5N1 vaccine against different Egyptian H5N1 viruses. Vaccine 33, 2670-2677.
Iqbal, M., 2009. Controlling avian influenza infections: The challenge of the backyard poultry. Journal of Molecular and Genetic Medicine, 3, 119-120.
Kandeil, A., Hicks, J.T., Young, S.G., El Taweel, A.N., Kayed, A.S., Moatasim, Y., Kutkat, O., Bagato, O., McKenzie, P.P., Cai, Z., 2019. Active surveillance and genetic evolution of avian influenza viruses in Egypt, 2016–2018. Emerging Microbes & Infections 8, 1370-1382.
Kandeil, A., Kayed, A., Moatasim, Y., Webby, R.J., McKenzie, P.P., Kayali, G., Ali, M.A., 2017. Genetic characterization of highly pathogenic avian influenza A H5N8 viruses isolated from wild birds in Egypt. The Journal of General Virology 98, 1573-1586.
Kawaoka, Y., Nestorowicz, A., Alexander, D.J., Webster, R.G., 1987. Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. Virology 158, 218-227.
Kawaoka, Y., Webster, R.G., 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proceedings of the National Academy of Sciences 85, 324-328.
Kayali, G., Kandeil, A., El-Shesheny, R., Kayed, A.S., Gomaa, M.M., Maatouq, A.M., Shehata, M. M., Moatasim, Y., Bagato, O., Cai, Z., 2014. Active surveillance for avian influenza virus, Egypt, 2010–2012. Emerging Infectious Diseases 20, 542-51.
Killian, M.L., 2008. Hemagglutination assay for the avian influenza virus. In: Avian influenza virus, Humana press, Totowa, NJ, USA, pp. 47-52.
Kim, Y.I., Pascua, P.N.Q., Kwon, H.I., Lim, G.J., Kim, E.H., Yoon, S.W., Park, S.J., Kim, S. M., Choi, E. J., Si, Y. J., 2014. Pathobiological features of a novel, highly pathogenic avian influenza A (H5N8) virus. Emerging Microbes & Infections 3, 1-13.
Mady, W., Yehia, N., Elhussieny, M.H., Shalaby, A.G., El Sayed, M.M., Bakry, N.R., Shaaban, A., Arafa, A.S., Mahmoud, A.A.B., Hassan, W.M., 2019. Genetic characterization of highly pathogenic avian influenza virus (H5N8) in backyard poultry production sector during Mid-2017 to autumn 2018. Hosts and Viruses 6, 75-84.
Meleigy, M., 2007. Egypt battles with avian influenza. The Lancet, 370, 553-554.
Moatasim, Y., Aboulhoda, B.E., Gomaa, M., El Taweel, A., Kutkat, O., Kamel, M.N., El Sayes, M., GabAllah, M., Elkhrsawy, A., AbdAllah, H., 2024. Genetic and pathogenic potential of highly pathogenic avian influenza H5N8 viruses from live bird markets in Egypt in avian and mammalian models. PloS One 19, e0312134.
Numan, M., Siddique, M., Shahid, M., Yousaf, M., 2008. Characterization of isolated avian influenza virus. J. Vet. Anim. Sci. 1, 24-30.
Palya, V., Tatár-Kis, T., Walkóné Kovács, E., Kiss, I., Homonnay, Z., Gardin, Y., Kertész, K., Dán, Á., 2018. Efficacy of a recombinant turkey herpesvirus AI (H5) vaccine in preventing transmission of heterologous highly pathogenic H5N8 clade 2.3. 4.4 b challenge virus in commercial broilers and layer pullets. Journal of Immunology Research 1, 3143189.
Pyankova, O.G., Susloparov, I.M., Moiseeva, A.A., Kolosova, N.P., Onkhonova, G.S., Danilenko, A. V., Vakalova, E. V., Shendo, G. L., Nekeshina, N. N., Noskova, L. N., 2021. Isolation of clade 2.3. 4.4 b A (H5N8), a highly pathogenic avian influenza virus, from a worker during an outbreak on a poultry farm, Russia, December 2020. Eurosurveillance 26, 2100439.
Saad, M.D., Lu'ay, S.A., Gamal-Eldein, M.A., Fouda, M.K., Khalil, F.M., Yingst, S.L., Parker, M. A., Montevillel, M. R., 2007. Possible avian influenza (H5N1) from migratory bird, Egypt. Emerging Infectious Diseases 13, 1120-1.
Saad, N., Esaki, M., Kojima, I., Khalil, A.M., Osuga, S., Shahein, M.A., Okuya, K., Ozawa, M., Alhatlani, B.Y., 2024. Phylogenetic Characterization of Novel Reassortant 2.3. 4.4 b H5N8 Highly Pathogenic Avian Influenza Viruses Isolated from Domestic Ducks in Egypt During the Winter Season 2021–2022. Viruses 16, 1655.
Salaheldin, A.H., Abd El-Hamid, H.S., Elbestawy, A.R., Veits, J., Hafez, H.M., Mettenleiter, T.C., Abdelwhab, E.M., 2018. Multiple introductions of influenza A (H5N8) virus into poultry, Egypt, 2017. Emerging Infectious Diseases 24, 943-6.
Selim, A.A., Erfan, A.M., Hagag, N., Zanaty, A., Samir, A.H., Samy, M., Abdelhalim, A., Arafa, A.-S. A., Soliman, M. A., Shaheen, M., 2017. Highly pathogenic avian influenza virus (H5N8) clade 2.3. 4.4 infection in migratory birds, Egypt. Emerging infectious Diseases 23, 1048-1051.
Shabat, M. B., Meir, R., Haddas, R., Lapin, E., Shkoda, I., Raibstein, I., Perk, S., Davidson, I., 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. Journal of Virological Methods 168, 72-77.
Shelkamy, M.M., Abdien, H.M., Hamed, D.M., Soltan, M.A., Abdallah, M.S., 2022. The Occurrence of Low Pathogenic Avian Influenza H9 Viruses in Broiler Farms Within Ismailia Province, Egypt. Journal of Advanced Veterinary Research 12, 346-352.
Sheta, B.M., Fuller, T.L., Larison, B., Njabo, K.Y., Ahmed, A.S., Harrigan, R., Chasar, A., Aziz, S.A., Khidr, A.-A.A., Elbokl, M.M., 2014. Putative human and avian risk factors for avian influenza virus infections in backyard poultry in Egypt. Veterinary Microbiology 168, 208-213.
Spackman, E., Senne, D.A., Myers, T., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., Suarez, D.L., 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 40, 3256-3260.
Steensels, M., Rauw, F., van den Berg, T., Marché, S., Gardin, Y., Palya, V., Lambrecht, B., 2016. Protection afforded by a recombinant Turkey herpesvirus-H5 vaccine against the 2014 European highly pathogenic H5N8 avian influenza strain. Avian Diseases 60, 202-209.
Stoute, S., Chin, R., Crossley, B., Gabriel Senties-Cue, C., Bickford, A., Pantin-Jackwood, M., Breitmeyer, R., Jones, A., Carnaccini, S., Shivaprasad, H., 2016. Highly pathogenic Eurasian H5N8 avian influenza outbreaks in two commercial poultry flocks in California. Avian Diseases 60, 688-693.
Sultan, H.A., Arafa, A.E., Talaat, S., Gaballa, A.A., Kilany, W.H., Elfeil, W.K., Shehata, A.A., Amarin, N., 2019. Efficacy of clade 2.3. 2 H5-recombinant baculovirus vaccine in protecting muscovy and pekin ducks from clade 2.3. 4.4 H5N8 highly pathogenic avian influenza infection. Avian Diseases 63, 219-229.
Sun, H., Pu, J., Hu, J., Liu, L., Xu, G., Gao, G. F., Liu, X., Liu, J., 2016. Characterization of clade 2.3. 4.4 highly pathogenic H5 avian influenza viruses in ducks and chickens. Veterinary microbiology, 182, 116-122.
Swayne, D. E., Kapczynski, D., 2008. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunological reviews, 225, 314-331.
Swayne, D. E., Pantin-Jackwood, M., 2008. Pathobiology of avian influenza virus infections in birds and mammals. In: Avian influenza, Blackwell, Ames, Iowa, USA, pp. 87-122.
Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Molecular biology and evolution, 38, 3022-3027.
Tarek, M., Naguib, M.M., Arafa, A.S., Tantawy, L.A., Selim, K.M., Talaat, S., Sultan, H.A., 2021. Epidemiology, genetic characterization, and pathogenesis of avian influenza H5N8 viruses circulating in Northern and Southern Parts of Egypt, 2017–2019. Animals 11, 2208.
Verhagen, J.H., Van der Jeugd, H., Nolet, B.A., Slaterus, R., Kharitonov, S., De Vries, P., Vuong, O., Majoor, F., Kuiken, T., Fouchier, R., 2015. Wild bird surveillance around outbreaks of highly pathogenic avian influenza A (H5N8) virus in the Netherlands, 2014, within the context of global flyways. Eurosurveillance 20, 21069.
Wang, W., Castelán-Vega, J.A., Jiménez-Alberto, A., Vassell, R., Ye, Z., Weiss, C.D., 2010. A mutation in the receptor binding site enhances infectivity of 2009 H1N1 influenza hemagglutinin pseudotypes without changing antigenicity. Virology 407, 374-380.
WHO (World Health Organization), 2002. WHO manual on animal influenza diagnosis and surveillance. World Health Organization. https://iris.who.int/han-

dle/10665/68026
Woolcock, P.R., 2008. Avian influenza virus isolation and propagation in chicken eggs. In: Avian influenza virus, Humana press, Totowa, NJ, USA, pp. 35-46.
Yamaji, R., Saad, M.D., Davis, C.T., Swayne, D.E., Wang, D., Wong, F.Y., McCauley, J.W., Peiris, J.M., Webby, R.J., Fouchier, R.A., 2020. Pandemic potential of highly pathogenic avian influenza clade 2.3. 4.4 A (H5) viruses. Reviews in Medical Virology 30, e2099.
Yang, G., Jones, J., Jang, Y., Davis, C.T., 2016. Multiplex assay for subtyping avian influenza A viruses by cDNA hybridization and adapter-mediated amplification. Applied Microbiology and Biotechnology 100, 8809-8818.
Yehia, N., Hassan, W.M., Sedeek, A., Elhusseiny, M.H., 2020. Genetic variability of avian influenza virus subtype H5N8 in Egypt in 2017 and 2018. Archives of Virology 165, 1357-1366.
Yehia, N., Naguib, M.M., Li, R., Hagag, N., El-Husseiny, M., Mosaad, Z., Nour, A., Rabea, N., Hasan, W.M., Hassan, M.K., 2018. Multiple introductions of reassorted highly pathogenic avian influenza viruses (H5N8) clade 2.3. 4.4 b causing outbreaks in wild birds and poultry in Egypt. Infection, Genetics and Evolution 58, 56-65.