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Research article

The highly pathogenic avian influenza H5N8 sublineage 2.3.4.4b virus: A critical analysis of genetic steadiness based on full HA gene sequencing in Egypt

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Abstract

The highly pathogenic avian influenza virus (HPAIV) continues to be a significant threat to the poultry industry despite the implementation of vaccination programs. The main goal of this study was to explore the incidence of avian influenza viruses (AIVs) and the stability of the hemagglutinating (HA) gene sequence. For this drive, oropharyngeal swabs (n=425) were collected from various wild and domesticated bird species from 13 provinces in Egypt from 2019 to 2021. Viral isolation in specific pathogen-free chicken embryos revealed positive HA activity in 9.88% (n=32) of the wild and 36.37% (n=64) of domesticated species. Only eight H5N8 avian influenza viruses were delivered (4/each category) in mixed infection with Newcastle disease virus (NDV). Clinically, domesticated examined birds exhibited clinical findings suggesting avian influenza, but AIVs were delivered from both apparently healthy and sick wild birds. A/chicken-cobb/Egypt/55/2019(H5N8) sequence generated in this study has been submitted to GenBank under the accession number ON724339, and its *in vivo* pathogenicity was evaluated in four-week-old chickens, which revealed 90% mortality and typical clinical findings of HPAIV. Based on the full HA gene sequence, the strain has a Furin-sensitive cleavage site in the HA protein, which is an HPAIV. It has been clustered in clade 2.3.4.4b within AIVs from 2016 to 2023 and coexistence of genetically stable viruses 2016-2019 with 99.8-100% similarity. The existing strain is fully identical to three strains: A/green-winged teal/Egypt/877/2016(H5N8), A/northern shoveler/Egypt/MB-D-816OP/2016(H5N8), and A/duck/Egypt/N13736E/2017 (H5N8), with the only point of mutation at position 284 (G284E). In our analysis, the majority of H5NX AIV strains detected in Egypt since 2016 possessed 156T. As a result, there is evidence suggesting that H5N8 has been introduced sequentially through the migration of European wild birds along the Black Sea–Mediterranean and East Asia–West Africa flyways. This raises the possibility of future adaptation to mammals due to spillover from avian-origin strains, especially in native aquatic birds such as geese and ducks.

Keywords: Full HA gene sequence, HPAIV-H5N8, Wild birds, Domesticated birds, Pathogenicity

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Introduction

Since the first invasion of highly pathogenic avian influenza virus (HPAIV) subtype H5N8 of clade 2.3.4.4b into Egypt in 2016 (Kandeil et al., 2017; Selim et al., 2017), the virus evidenced its incrimination in ongoing breaks in poultry till now (Tourky et al., 2024). The HPAIV remains a noteworthy hazard to the poultry industry despite the application of vaccination programs (Hegazy et al.,2022; Ali et al.,2024). Avian influenza is an infectious disease that affects domestic and wild birds. It is caused by avian influenza A viruses ofthe family *Orthomyxoviridae*. The viruses are

non-enveloped with a negative-sense single-stranded and segmented RNA genome. Avian influenza viruses are generally categorized depending on their surface proteins, hemagglutinin (HA), and neuraminidase (NA) into highly pathogenic and low pathogenic AIVs (Tonegawa et al., 2003). HA is the humoral immune response's primary target and is responsible for the attachment of the virus to the host cell. Immunological escape of viral subtypes and continuous antigenic alterations are linked to the host population's HA-specific immunological pressure against influenza A (Smith et al., 2004). Throughout history, the influenza A virus has attention due to its role in several epidemics and pandemics (Zoric et al., 2020), along with its significant financial losses to the poultry industry (Kupferschmidt, 2023).

In 1997, HPAI H5N1 (Gs/GD/96) goose/Guangdong lineage emerged in China (Wan, 2012). In 2000, H5N1 banquets among poultry in many countries, such as Indonesia, Thailand, Netherlands, and Vietnam (WHO, 2005). After 2005, H5N1 continued to blow out to poultry across Europe and Africa (Jonassen and Handeland, 2007; Ducatez et al., 2007) and was first introduced in Egypt in 2006 (Aly et al., 2008). The emergences of non-N1 recombinant AIV strains have been witnessed in several countries including H5N2, H5N6, and H5N8. The outbreak of reassortant HPAI H5N8 clade 2.3.4.4 was first reported in live bird markets in China in 2010 (Lee et al., 2014). At the end of 2014, waterfowl migratory birds played a critical role in the spread of HPAIV H5N8 throughout Europe, North America, and East Asia (Bouwstra et al., 2015; Hanna et al., 2015; Lee et al., 2015; Pasick et al., 2015). From 2016 to 2017, there was the beginning of a new wave of a reassortant of H5N8 and H5N5 within clade 2.3.4.4b in migrating birds that resulted in millions of bird deaths across many countries globally (Antigua et al., 2019). Since then, the reassortment of the virus with other influenza A subtypes has caused ongoing evolutionary divergence, leading to the formation of different genotypes and additional transmission to domestic birds (Lycett et al., 2020). In February 2021, the first known occurrence of the virus HPAI H5N8 infection in humans was reported in two farmers who had previously come into contact with infected birds in Russia (WHO, 2021). In Egypt, H5N8 was introduced in late 2016 via the common coot (Fulica atra) (Selim et al., 2017). Phylogenetically, the original virus was related to H5N8 viruses of clade 2.3.4.4b in Russia (Yehia et al., 2018). After that, the HPAI H5N8 virus became endemic and has since been found in vaccinated poultry across several Egyptian provinces (Shehata et al., 2019; Abdien et al., 2023).

All indications have proven that the movement of migratory birds across the world was the main reason for the spread of the bird flu virus (Lee et al., 2015 and 2017; Verhagen et al., 2015), including Egypt. Egypt is considered a habitat for a staggering variety of bird species, particularly the north Mediterranean coast and Nile Delta. Throughout the annual migration, Egypt is an essential stopover for millions of migratory birds (Ibrahim, 2011). Abdien et al. (2023) concluded that two Anseriformes-migratory birds, including a northern shoveler and a green-winged teal, harbored more than 50% of the isolated AIVs in Egypt. Moreover, it has been demonstrated that domestic ducks act as a reservoir for numerous avian influenza virus subtypes and permit their reassortment, contributing to the ecology, spread, and creation of novel AIV genotypes (Barber et al., 2010; Parvin et al., 2020). In 2017, HPAIV H5N8 was recorded in domestic ducks and geese in Egypt (Anis et al., 2018). Based on the whole genome sequence, six genotypes of the HPAI H5N8 virus were recognized in both domestic and migratory birds in Egypt (Yehia et al., 2018; Salaheldin et al., 2018; Moatasim et al., 2019; Hassan et al., 2020; Yehia et al., 2020). After that, in a very short time, the virus spread among the domestic poultry sector in different provinces of Egypt, presenting a serious risk to the poultry industry (Hassan et al., 2020; Yehia et al., 2020). Lately, in 2020, The HPAI H5N8 viruses detected in Europe were phylogenetically related to those viruses isolated in Egypt in 2019, depending on the sequencing of the HA gene (Lewis et al., 2021; Beerens et al., 2021).

The continual circulation of HPAIV highlights the necessity for ongoing genomic investigation to better understand the virus's evolution and impact on poultry, mammalian adaptation, and human health hazards. Therefore, the current study goal was to investigate the occurrence of AIVs among wild and domesticated birds in Egypt from 2019 to 2021, in addition to a thoughtful molecular analysis of the full HA gene sequence.

Materials and methods

Institutional review-board statement

We followed relevant institutional, national, and international guidelines for the care and use of animals for research. The guidelines provided in the guide for the care and use of birds in scientific research are followed by the Ministry of Environment when capturing wild birds. Holders of a hunting identity card, as well as adhering to the technical and environmental guidelines set forth by the Ministry of Environment under Law No. 4 of 1994 and Ministerial Resolutions No. 1270 of 8/9/2018 and No. 209 of 6/8/2019, are required to capture wild birds. Besides, the Institutional Animal Care and Use Committee at Suez Canal University in Egypt's guidelines for using animals in experiments (approval No.: 8112018).

Birds and samples collection

Between 2019 and 2021, 425 oropharyngeal swabs were collected from various wild and domesticated bird species across 13 provinces in Egypt. In wild birds, about 324 swabs were collected from 22 different species from six provinces in Egypt (Table 1). Wild birds were either captured using traps (baited nets) set up at the entrances of the Al Manzala, Bardawil, and Bitter Lakes in Port-Said, North Sinai, and near Ismailia provinces, respectively, or gathered from live bird markets (LBMs) that are present in the provinces of Sharkia, Dakahlia, and Damietta. Among 324 wild birds, 44 showed clinical signs, and the remaining birds were apparently healthy. The 44 unhealthy birds were captured in nests for observation, and during this time, the birds that died were anatomized and examined. In domesticated birds, 101 pooled swabs were collected from 101 flocks (3 swabs/flock), including 60 chicken flocks, 38 duck flocks, 2 pelican flocks, and one turkey flock. The domestic bird flocks were located in 13 Egyptian provinces representing different species (Table 2). Swabs were collected from both healthy and diseased birds exhibiting typical AI signs, then underwent clinical and post-mortem examinations.

Sterile cotton swabs were utilized to collect samples. The samples were placed in a maintenance essential medium (MEM) at pH 7.2, containing 5% glycerol, and supplemented with antibiotics (Penstrept, Lonza Walkersville, Inc., Walkersville, USA). They were preserved at 4°C during transportation to the laboratories of the Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig and Suez Canal Universities, Egypt, within 24 h, and then stored at -80°C until further processing.

Virus isolation

The collected swabs were clarified by centrifugation at 3,000 rpm for 10 minutes; then supernatants were inoculated into 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) according to the recommendations of the World Organisation for Animal Health (WOAH, 2021). Three fertile SPF-ECEs were used per swab, and at least three consecutive embryo passages were applied for each sample to be negative. The collected allantoic fluids (AFs) were screened using slide HA assay.

Viral RNA extraction and RT-PCR amplification of matrix and hemagglutinin genes

RNA was extracted from positive HA allantoic fluids using the QIAamp MinElute Virus Spin kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted RNAs were reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Loughborough, UK). The reaction mixture and the thermal profile used for reverse transcription were adjusted following the manufacturer's instructions. The PCR was conducted using a set of primers specific for the fusion (F) gene segment of NDV, following the cycling conditions detailed in Eid et al. (2022). Table 3 describes the matrix (M) and hemagglutinin (H5) gene segments of the AIV.

The PCR was conducted using 2× Dream Taq Green PCR Master Mix (ThermoFisher Scientific, Loughborough, UK), in which the PCR was first performed to screen the presence of AIV and NDV genomes using the conserved M and F genes, respectively. Next, the PCR confirmed that AIVs were discriminated for the H5 subtype using its specific primer. Cycling conditions were performed as an initial denaturation step at 95°C for 3 min, followed by 40 amplification cycles (denaturation at 95°C for 30 s, annealing at 50°C (M) or 55°C (F and H5) for 30 s, and extension at 72°C for 45 s). The reaction was finalized with a final extension step at 72°C for 5 min. Finally, the PCR products were visualized using ethidium bromide-stained DNA gel electrophoresis 120V/30 min. The confirmed positive products were subjected to next-generation sequencing.

Next-generation sequencing of the selected AIV subtype H5

For comprehensive characterization of the isolated AIV H5, after analyzing the HA gene by PCR using H5 gene-specific primers, the PCR products of selected samples (sharp band) were shipped to the Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey, GU24 0NF in the UK for sequencing. The full H5 gene sequence data was obtained.

Data availability statement

The new sequence generated in this study has been submitted to GenBank under the accession number ON724339. The strain was named A/chicken-Cobb/Egypt/55/2019(H5N8).

Alignment and phylogenetic analysis

Initially, 165 AIV HA sequences were retrieved for the NCBI GenBank using a nucleotide blast research tool. Similar sequences were eliminated to facilitate the analysis, and thirty-three aa sequences originating from wild migratory and domesticated birds were aligned. MEGA 4.1 software (Tamura et al., 2007) was started with a set of aligned sequences of gene segments using Clustal W (Thompson et al., 1994). The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length=0.07771280 is exposed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 33 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 567 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 1: Species and number of wild birds captured and examined by provinces during 2019-2021, Egypt.

Bird species (Common name)	Sharkia	Dakahlia	Damietta	Ismailia	Port-said	North Sinai	Total
Corvus cornix (Hooded crow)	27	11	1	1	10	-	50
Larus canus (Common gull)	-	2	-	-	4	3	9
Numenius minutus (Little curlew)	5	-	-	-	3	2	10
Coturnix ypsilophora (Brown quail)	-	-	-	-	-	2	2
Streptopelia turtur (European turtle dove)	12	6	1	-	7	-	26
Nycticorax nycticorax (Black-crowned night heron)	3	1	1	1	4	-	10
Gallinula chloropus (Common moorhen)	16	8	1	-	18	1	44
Bubulcus ibis (Cattle egret/ Little egret)	25	13	-	3	23	-	64
Anas platyrhynchos (Mallard duck)	-	-	-	-	1	2	3
Ardenna pacifica (Wedge-tailed shearwater)	-	-	-	-	1	-	1
Spatula clypeata (Northern Shoveler)	-	-	-	-	3	-	3
Ardea cinerea (Grey heron)	-	-	-	-	3	5	8
Porphyrio madagascariensis (Purple gallinule (African swamphen)	-	-	-	-	4	6	10
Anthus rubescens (American pipit)	-	-	-	-	1	-	1
Actitis hypoleucos (Sandpiper)	6	2	-	1	6	-	15
Vanellus spinosus (Spurwinged lapwing)	7	1	-	1	6	1	16
Halcyon smyrnensis (White throat kingfisher)	2	2	-	1	7	-	12
Ceryle rudis (Pied kingfisher)	6	1	-	-	4	-	11
Scolopax rusticola (Wood cock)	8	2	1	-	3	-	14
Plegadis falcinellus (Glossry ibis)	1	1	-	-	1	1	4
Nyctyornis amictus (Red-bearded bee-eater)	3	2	-	-	1	-	6
Acridotheres tristis (Common myna)	1	-	-	-	4	-	5
Total	122	52	5	8	114	23	324

Table 2: Species and number of domestic bird flocks examined by provinces during 2019–2020, Egypt.

Bird species/ Breed	Sharkia	Dakahlia	Gharbia	Alexandria	Damietta	Suez-Canal	Cairo	Sohag	Assuit	Port-said	North Sinai	South Sinai	Total
Chickens													
Cobb	9	1	-	5	-	-	-	3	4	-	1	2	25
Sasso	-	1	-	-	1	-	-	1	-	-	-	-	3
Avian 48	-	-	-	-	-	2	-	-	-	-	-	1	3
Hay-line	-	2	-	-	-	-	-	-	-	-	-	-	2
Ross	-	-	-	3	-	-	-	-	-	-	1	-	4
Indian river	-	-	-	-	-	2	-	-	-	-	1	-	3
Bovanes	2	-	-	3	-	-	-	-	-	-	-	-	5
Hubbard	-	-	-	-	1	-	-	-	-	1	-	-	2
Lehman	-	-	-	1	-	-	-	-	-	-	-	1	2
Isapapcock	2	-	-	-	-	-	-	-	-	-	-	-	2
Baladi	7	-	-	-	-	-	-	1	1	-	-	-	9
Duck													
Muscovy		9	8	-	-	-	-	-	-	-	-	-	17
Mallard	-	2	3	-	-	-	-	-	-	-	-	-	5
Pekin	1	6	9	-	-	-	-	-	-	-	-	-	16
Turkeys													
Converter	1	-	-	-	-	-	-	-	-	-	-	-	1
Pelicans													
Pelecanus crispus	-	-	-	-	-	-	2	-	-	-	-	-	2
Total	22	21	20	12	2	4	2	5	5	1	3	4	101

Table 3: Oligonucleotide primers used for the amplification of fusion (F), matrix (M), and hemagglutinin (H5) genes.

Gene		Primers (5`---- 3`)	Size	References
F	F	CACAGCAGGTCGGTGTAGAA	306 bp	Eid et al., 2022
	R	TCTCCAAATAGGT-GGCACGC		
M	F	ATG AGY CTT YTA ACC GAG GTC GAA ACG	244 bp	WHO, 2007
	R	TGG ACA AAN CGT CTA CGC TGC AG		
H5	F	GAT GGT TGG TAT GGG TAC C	511 bp	This study
	R	AGT ATT TGG TAA GTT CCT ATT G		

Pathogenic characterization of AIV-H5N8 in chickens

To assess the pathogenicity of the A/chicken-cobb/Egypt/55/2019(H5N8) strain, firstly, the allantoic fluid was purified from NDV-mixed infection using hyperimmune serum and reinoculated in SPF-ECEs, then submitted to RT-PCR assays for both AIV and NDV for purity assurance. Further, it was titrated in SPF-ECEs for the median embryo infective doses (EID_{50}) calculation. Twenty newly hatched commercial broiler chicks were obtained from a commercial hatchery. These chicks have no antibodies against NDV and AIV using the hemagglutination inhibition (HI) test. They were reared in the experiment units under strict hygienic conditions and kept under observation until the time of the experiment. At 4 weeks of age, the birds were divided into two groups (GA and GB). GA was inoculated intraocularly with 0.1 mL virus fluid containing 10^7 EID_{50} after titration in 10-days-old of SPF ECEs (Kandeil et al., 2023). GB was inoculated with phosphate-buffered saline (PBS) as a sham-inoculated control and housed separately from the other birds as a negative control group. Over 10 days, all birds were monitored two or three times daily, during which clinical signs and mortality were recorded, the dead birds during observation were anatomized and examined for lesions.

Results

Virus isolation

The inoculated swab samples of the wild birds (n=324) and domesticated birds (n=101) in SPF-ECEs showed that 32 (9.88%) and 64 (63.37%) samples produced hemorrhagic and congested embryos with positive HA activity, respectively, suggesting possible hemagglutinating virus presence, either NDV and/or AIV. The positive HA samples were submitted for confirmation by RT-PCR using specific F, M, and H5 genes primers.

Molecular identification

Out of 32 positive samples for HA in wild birds, eight (25%) were positive for AIV detection using the conversed M gene primer. Further analysis using a specific primer targeting the H5 gene showed that four samples among eight were positive. However, 30 swab samples (93.75%) exhibited the presence of NDV. It was noted that all AIVs were co-infected with NDV (Figures 1a and b). AIVs were detected in 4 provinces (Port-Said, Sharkia, Dakahlia, and Ismailia), including the four isolates of AIV-H5, one isolate per province. Eight wild bird species had AIV, of which the AIV-H5 was identified in four species, including the hooded crow, cattle egret, European turtle dove, and sandpiper; those were captured or collected from Dakahlia, Ismailia, Port-Said, and Sharkia, respectively(Figures 1c and d). Regarding the domesticated birds, 13 of the 64 positive HA swabs (20.31%) had AIV; four were specific to AIV-H5, following the examination with a particular H5 gene primer. Furthermore, NDV was confirmed in 58 flocks. Notably, 7 out of 13 AIVs were found combined with NDV (Figures 2a and b). AIVs in domesticated flocks were distinguished in Sharkia, Dakahlia, Gharbia, Alexandria, Damietta, and North Sinai, where the four AIV-H5 were recognized in Sharkia (n=1), Dakahlia (n=2), and North Sinai (n=1). The Cobb and Ross chickens and Muscovy ducks were the breeds that were affected by the AIV-H5 (Figures 2c and d).

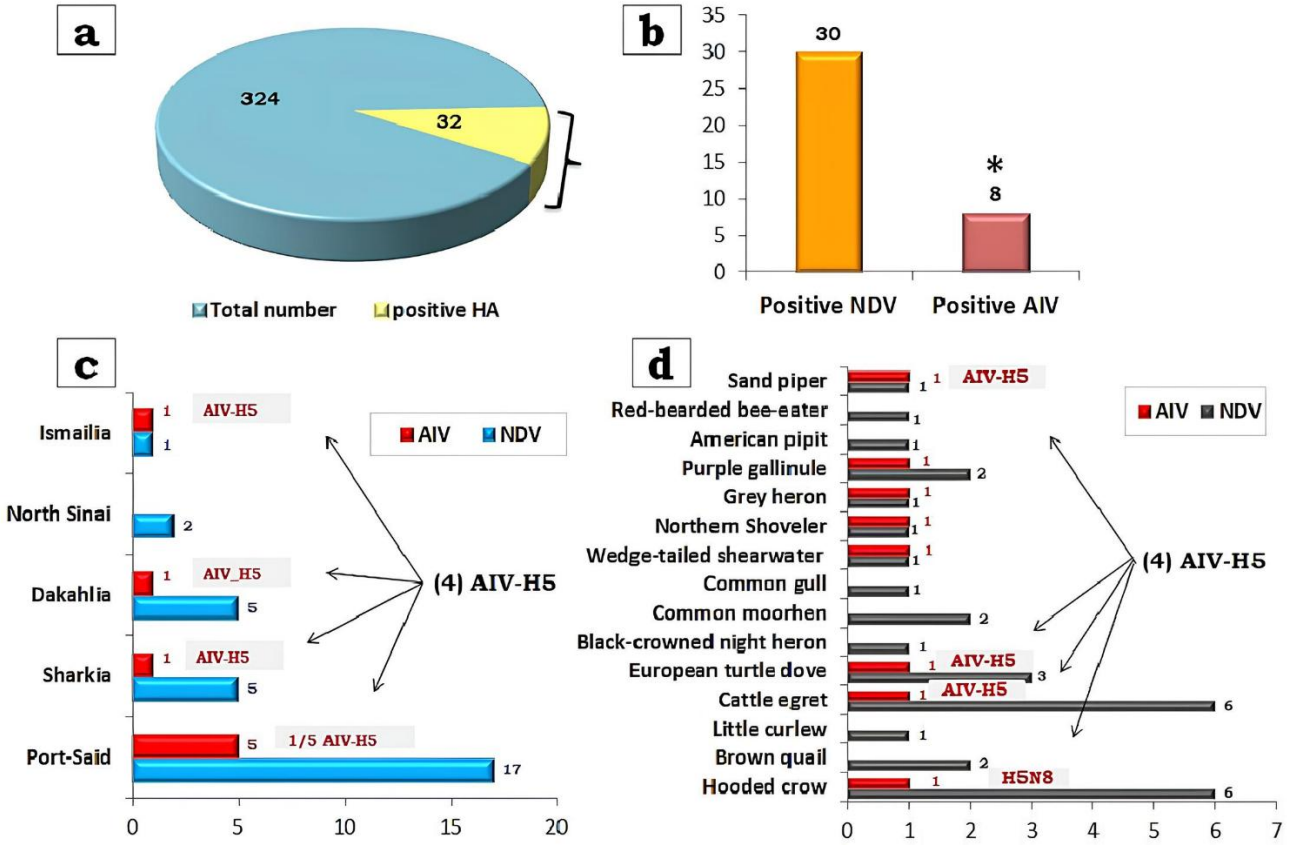

Figure 1: Screening results for AIVs and NDVs in wild bird swabs utilizing HA and RT-PCR assays. (a) total HA positive samples, (b) positive samples for AIVs and NDVs, the geographical distribution of detected AIVs and NDVs, (c) positive samples for AIVs and NDVs among Egyptian provinces, (d) positive samples for AIVs and NDVs among wild bird species; (*) indicates that all AIVs were co-infected with NDV.

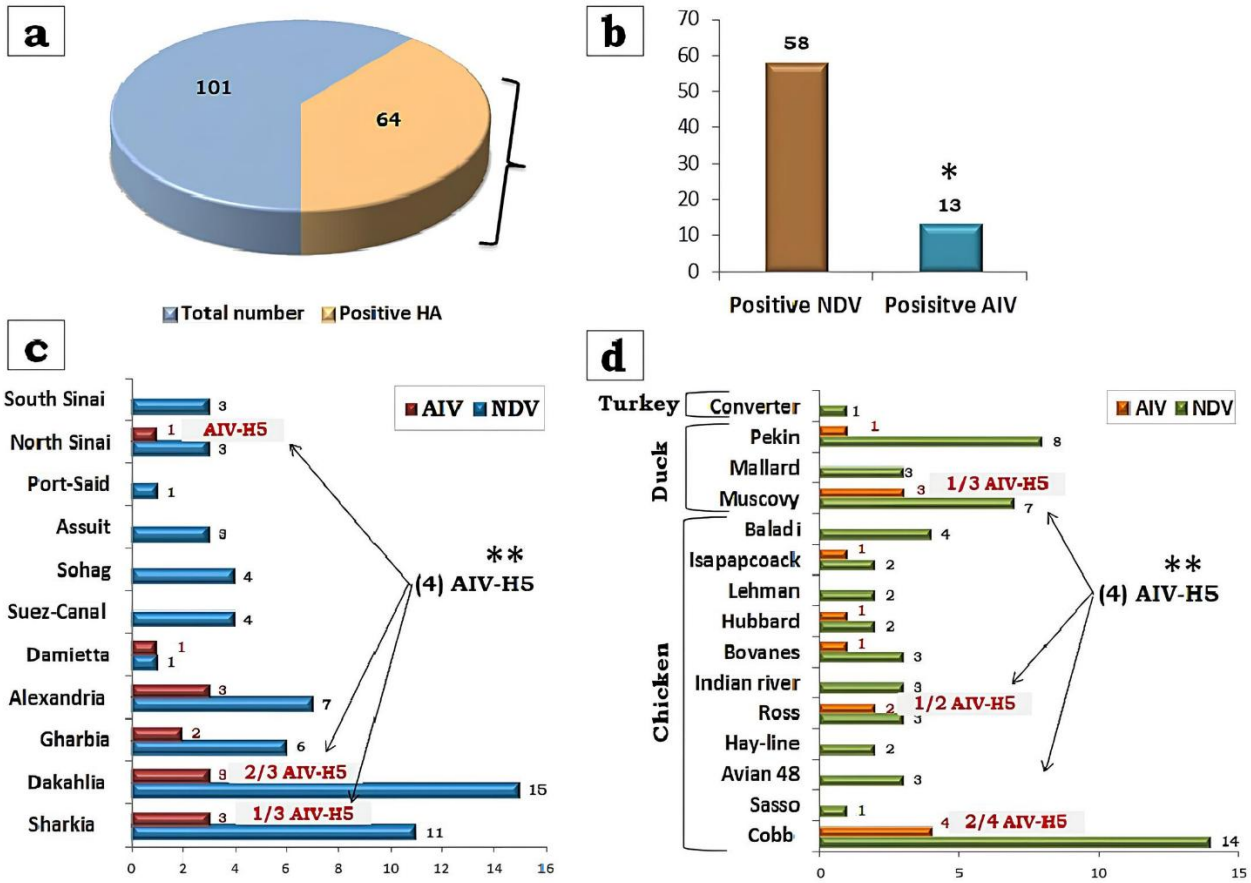

Figure 2: Screening results for AIVs and NDVs in domestic bird swabs utilizing HA and RT-PCR assays. (a) the HA positive samples among the total number examined, (b) positive samples for AIVs and NDVs, the geographical distribution of detected AIVs and NDVs, (c) positive samples for AIVs and NDVs among Egyptian provinces, (d) positive samples for AIVs and NDVs among bird species; (*) means that 7 out of 13 AIVs were co-infected with NDV, (**) means that all AIV-H5 were co-infected with NDV.

Clinical findings of naturally infected birds

Most wild birds that underwent clinical examination were healthy and showed no symptoms. On the other hand, forty-four birds showed clinical signs such as respiratory symptoms, diarrhea with depression, and ruffled feathers. Additionally, edema and cyanosed heads were observed in certain birds. Almost all clinically sick birds died in the caged nest throughout the monitoring period. The post-mortem examination showed that the most common lesions were respiratory tract inflammation, including tracheitis, air saculitis, and congested lungs, as well as the septicemic picture in all essential organs. A handful of the birds under examination also showed pancreatitis, liver necrosis, pericarditis, hemorrhages on coronary fat, and cecal tonsillitis (Table 4).

The domestic birds exhibited several clinical signs, including ruffled feathers, greenish diarrhea, and respiratory troubles (sneezing, swollen eyes, and gasping), which were the predominant signs in the chickens. Some of them also displayed cyanosis of the head, comb, wattles, and nervous distress. In ducks, the main clinical signs were greenish diarrhea and nervous signs. During the post-mortem examination, septicemic internal organs and greenish intestinal content with inflammation were observed in all examined birds. Hemorrhages in the cecal tonsils, coronary fate, and the mucosa and glands of the proventriculus were shown, particularly in chickens (Table 5).

Alignment phylogenetic and analysis based on the full genome of HA protein

Thirty samples were submitted for next-generation sequencing. However, due to the shutdown and extensive troubleshooting and the successive lockdown in the UK institute during the COVID-19 epidemic, only one strain was recovered with a positive full-length hemagglutinin gene.

Table 4: Data and clinical findings in detail for the wild birds that tested positive for AIV subtype H5.

Bird species	Common name	Date of collection	Province	Clinical findings
Corvus Cornix	Hooded Crow	August 2019	Dakahlia	Apparent health
Bubulcus ibis	Cattle egret	August 2019	Ismailia	Frothy exudate from the beak, watery diarrhea, congested head, air saculitis, enteritis, congested heart, enlarged liver and kidney
Streptopelia turtur	European Turtle Dove	October 2019	Port-Said	Apparent health
Actitis hypoleucos	Sandpiper	October 2020	Sharkia	Diarrhea, ocular nasal discharges, enlarged liver with necrosis, pericarditis

Table 5: Data and clinical findings in detail for the domestic birds that tested positive for AIV subtype H5.

Breed	Bird species	Aged /day	Date of collection	Province	Flock density	Mortality rates/ 3 days	Clinical findings
Chickens	Cobb	30	February 2019	Sharkia	8,500	270	Cyanosed head, comb, and wattles, greenish diarrhea, respiratory signs, hemorrhages in the cecal tonsils, and coronary fat.
	Ross	33	March 2019	North-Sinai	14,000	600	Respiratory signs, greenish diarrhea, septicemia
	Cobb*	34	March 2019	Dakahlia	4,000	120	Nervous, greenish diarrhea, septicemia
Ducks	Muscovy	60	August 2019	Dakahlia	4,000	113	Greenish diarrhea, septicemia

* A/chicken-cobb/Egypt/55/2019(H5N8)

The strain, called Influenza A virus (A/chicken-Cobb/Egypt/55/2019(H5N8), was submitted to Genbank under accession number ON724339.1. The Influenza A virus (A/chicken-Cobb/Egypt/55/2019(H5N8) was employed as a model to research the virus's behaviors throughout the last six years.

The alignment of the complete aa sequences (567 aa) of the HA protein of 33 AIV strains revealed that all the analyzed strains displayed the H5N8 cleavage motif PLREKRRKR except the A/common buzzard/Denmark/4079-1p1/2017(H5N8) displayed PLRERRRK. The alignment analysis revealed that two AIV subtypes were introduced in 2016 due to migratory bird spillover. The first is the AIV strain A/green-winged teal/Egypt/877/2016 (H5N8) and A/northern shoveler/Egypt/MB-D-816OP/2016(H5N8).

This specific sequence remained stable up to 2019 and had 100% aa identity with the many other strains detected in 2016 and up to 2019 in domesticated duck and chicken flocks such as (A/duck/Egypt/N13736E/2017(H5N8), (A/duck/Egypt/N14200B/2017(H5N8) and our studied model (A/chicken-Cobb/Egypt/55/2019(H5N8).

Another reassortant virus was detected in purple heron (A/purple heron/Egypt/MB 933C/2016(H5N5) with 99.5% aa identity compared with the green-winged teal-originated strain. However, the virus mentioned above has not been detected in any domesticated duck or chicken flocks in Egypt. It possessed distinct features such as (R185L), (E492K), (S525L) and (V538A). After that, AIV strains introduced in 2017, rather than the originally introduced virus in 2016, had nearly identical sequences to the 2016 strains except for consistent mutation (L191M). This aa mutation was detected in strains isolated from wild migratory and domesticated birds such as A/common teal/Egypt/MB-F-1204C/2017(H5N8), A/ commonteal/Egypt/MB-F-1199OP/ 2017 (H5N8), A/duck/Egypt/F13666A /2017(H5N8), A/chicken/Egypt/Q13804A /2017(H5N8) and A/chicken/Egypt/H13795A/2017 (H5N8). Alignment comparison with reference strains isolated from the wild migratory birds in Denmark (2016-2017), A/great black-backed gull/Denmark/19069-1/2016 (H5N8), (A/tufted duck/Denmark/11740/2016 (H5N8) and A/duck/Denmark/19062-5Sp1c/2017 (H5N8), revealed high identity percent (99.5%) with consistent mutations (D13N) compared with the A/green-winged teal/Egypt/877/2016(H5N8) and our studied model A/chicken-Cobb/Egypt/55/2019(H5N8). Interestingly, AIV

strains, 2017-2018, had the same point of mutation at position 284 (G 284 E), with the Denmark-originated strains, for examples, A/duck/Egypt/N13736E/2017 (H5N8), A/chicken/Egypt/Q13804A/2017 (H5N8), A/chicken/Egypt/A15037/2018 (H5N8) and A/chicken/Egypt/N15168C/2018 (H5N8) viruses. Two novel mutations were nearly consistent in 2018 strains (R88S) and (T156A) in chicken-originated isolates or M in goose-originated strains. In 2021, a new virus with a distinct mutation profile at the amino acid level was introduced via the spillover of wild birds such as A/common pochard/Egypt/DT19799C /2021 (H5N8) and A/duck/Egypt/BA19903OP /2021 (H5N8) viruses. This strain had (A99N), (T156A), (R185Q), (N199S), (N252D), (V538A), (M547I) and (V 548 M). A new subtype (H5N1) emerged in (2021-2023) with novel aa mutations such as (K388R) and (V548I) (Supplementary Table 1).

The phylogenetic study of 33 amino acid sequences of the full-length HA protein represented AIV strains of the clade 2.3.4.4b subtype isolated throughout 2016 and 2023 was performed. The UPGAMA method was used for the evolutionary analysis, rooted with Influenza A virus (A/duck/Jiangsu/k1203/2010(H5N8), which revealed that the 33 strains under consideration were classified into three groups (1-3). The strains in phylogroup 1 were first found in migratory wild birds in Egypt in 2016 and were detectable until 2019 with 99.8-100% aa identity. Strains found in 2017-2019 are associated with European strains detected in migratory birds in 2016-2017, such as A/common buzzard/Denmark/4079-1p1/2017(H5N8), which had 99.3-99.6% aa identity, compared with group 1. Phylogroup 3 was separated into two sub-phylogroups: 3a, which included H5N8 strains isolated in 2021, and 3b, which included H5N1 strains isolated in birds between 2021 and 2023, with aa identities ranging from 98.2 to 98.8%, compared with phylogroup1 (Figure 3). The phylogenetic study of 33 amino acid sequences of the full-length HA protein represented AIV strains of the clade 2.3.4.4b subtype isolated throughout 2016 and 2023. The UPGAMA method was used for the evolutionary analysis, rooted with Influenza A virus (A/duck/Jiangsu/k1203/2010(H5N8), which revealed that the 33 strains under consideration were classified into three groups (1-3). The strains in phylogroup 1 were first found in migratory wild birds in Egypt in 2016 and were detectable until 2019 with 99.8-100% aa identity. Strains found in 2017-2019 are associated with European strains detected in migratory birds in 2016-2017, such as A/common buzzard/Denmark/4079-1p1/2017 (H5N8), which had 99.3-99.6% aa identity. Phylogroup 3 was separated into two sub-phylogroups: 3a, which included H5N8 strains isolated in 2021, and 3b, which included H5N1 strains isolated in birds between 2021 and 2023, with aa identities ranging from 98.2 to 98.8% (Figure 3).

Clinical manifestation and gross lesions observed in chickens infected experimentally with HPAIV-H5N8

The next experiment aimed to determine the clinical outcome after HPAIV-H5N8 infection in broiler chickens. In this study, group A of ten 4-week-old chickens was inoculated intraocularly with a dose of 0.1 mL containing 10^7 EID_{50} of infectious virus, and during the observation period of 10 days post-infection (dpi), specific clinical signs of HPAI were noted on the birds as early as 3 dpi. 70% of birds suffered from conjunctivitis with closed eyes, and 80% exhibited cyanosis in comb and wattle. Neurological symptoms, including torticollis and opisthotonus, along with greenish diarrhea, were observed later in 40% and 100% of the infected chickens, respectively, by 7 dpi. The mortality rate reached 90% within 10 days, which started as early as 24 hours post–infection.

All birds that were infected and either died during observation or were euthanized at the end of the experiment underwent necropsy and examination. The gross lesions were recorded as submandibular edema with gelatinous fluid in 80% of birds, septicemic internal organs in 100%, and hemorrhages on the shank, proventriculus, and pancreas in 100%, 80%, and 70%, respectively. Additionally, mottled spleen was noted in 80% of the birds, while nephritis was observed in 50%. On the other hand, Sham-inoculated chickens (control group) did not exhibit any clinical signs all over the observation period.

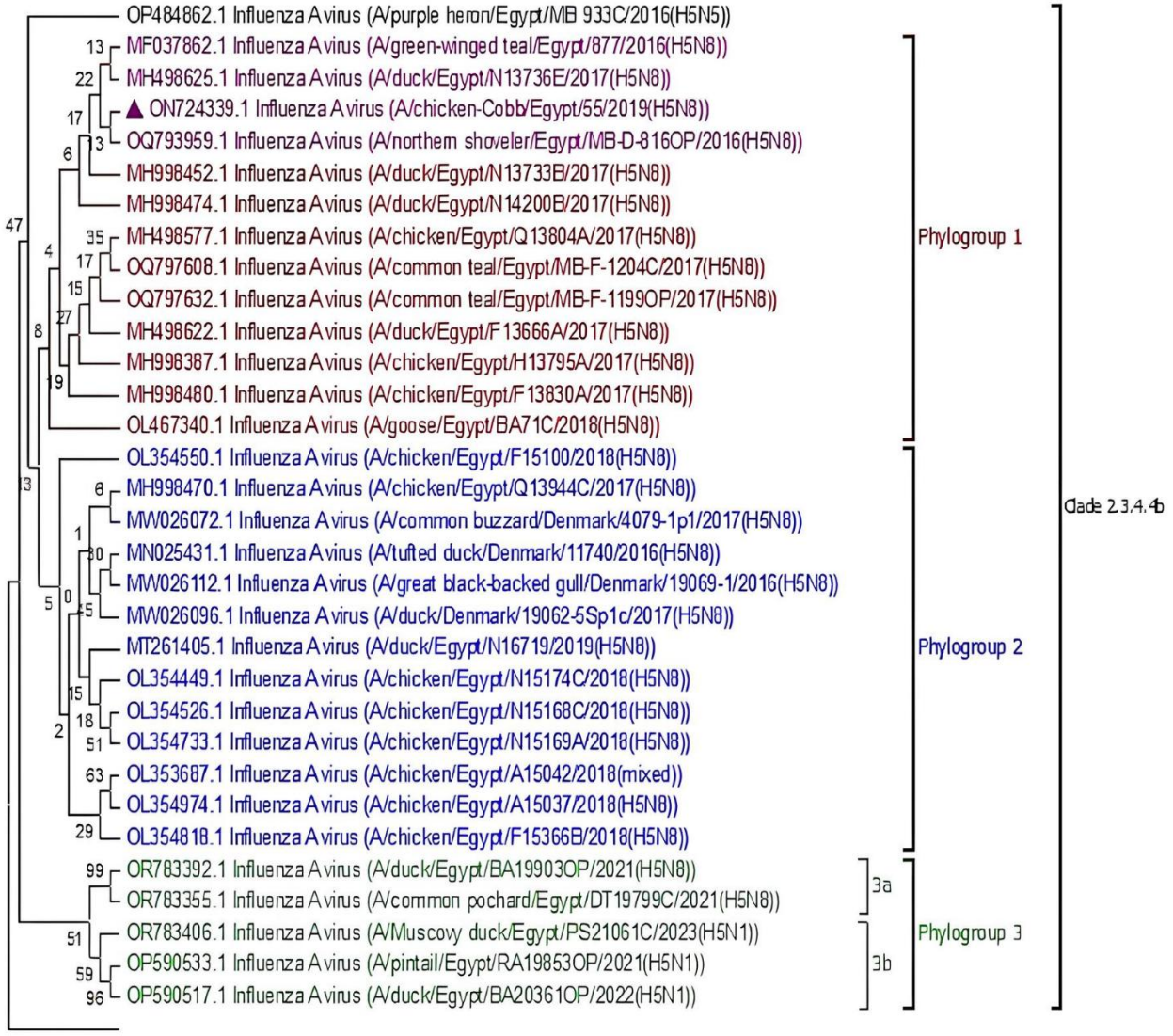

Figure 3: The evolutionary analysis of the hemagglutinin gene of H5NX (Clade 2.3.4.4b). Phylogenetic study of the total 567 amino acids from the HA protein of 33 AIV strains H5NX (Clade 2.3.4.4b). The taxa include 33 strains isolated from migratory and domesticated birds since 2016, as well as some European models. The tree is divided into three phylogroups: one named red and purple, two labeled in blue, and three labeled in green. Our examined model is represented by the purple triangle. The clade history was inferred using the UPGMA method (Sneath and Sokal, 1973). The bootstrap consensus tree reliant on 1000 replicates (Felsenstein, 1985) is taken to denote the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Discussion

Up till now over, H5N8 subtype clade 2.3.4.4b HPAIV has been responsible for destructive outbreaks in Egypt in domestic poultry since its first introduction in 2016through migratory birds (Kandeil et al., 2017; Selim et al., 2017; Yehia et al., 2018; Shehata et al., 2019). Wild birds are highly influential in the evolution and dissemination of AIVs during their seasonal migration, especially wild aquatic birds (Kandeil et al., 2017), and have a principal role in introducing different AIV subtypes into Egypt (Naguib et al., 2019). In addition, domestic waterfowl can be suggested to act as an intervening factor in avian influenza spreading and evolution among birds (Li et al., 2004), as in H5N8 HPAIV (Hill et al., 2015). In this study, the HPAI H5N8 subtype of clade 2.3.4.4b from Egypt in 2019–2021 was isolated and genetically characterized.

The epidemic source of HPAI resurgences, the underlying genome evolution in domestic and wild populations, and its role in the maintenance and transmission of the virus need further investigation. To address these issues, 425 oropharyngeal swabs from wild birds and domestic flocks were collected from 13 provinces in Egypt from 2019 to 2021 to explore the incidence of AIV likewise, the analysis of full HA gene sequence stability among 33 HPAI H5N8 viruses since 2016 that is available on the GenBank comparable with our isolate (A/chicken-cobb/Egypt/55/2019(H5N8).

It was noticed that 9.88% (n=32/324) and 36.37% (n=64/101) of the collected swabs from wild birds and domesticated species, respectively, contained hemagglutinating viruses. More investigation into the hemagglutinating samples using a molecular technique with the M gene primer revealed that 25% (8/32) were AIV positive in wild birds with a prevalence of 2.47% (8/324) compared to 20.31% (13/64) in domestic birds with a prevalence 12.87% (13/101), and using a specific H5 primer indicated that four samples from each wild and domestic species were specific to AIV-H5. Nearly similar prevalence of AIV in wild birds was recorded by Kandeil et al. (2017), in which only two samples from green-winged teals were positive for M gene using real-time PCR among 128 samples collected from 64 birds of six species and identified as H5N8. Furthermore, Nabil et al. (2020) isolated ten AIV from 148 wild birds (6.76%), representing 14 species, and identified them as H5N8 in 4 samples using PCR subtyping and pan-HA/NA sequencing assays. Meanwhile, Salaheldin et al. (2022) indicated a 45.1% prevalence rate of AIV in domestic birds such as chickens, turkeys, and ducks in various areas in Egypt from 2019 to 2021, and all of them were H5N8. This was higher than that mentioned in the current study, which may be attributed to the different numbers and types of the collected samples.

Interestingly, all AIVs detected in the wild bird samples in this study were co-infected with NDV, where NDV was identified in 30 out of 32 swab samples. On the other hand,7 out of 13 AIVs in domestic birds were co-infected with NDV, whereas all AIV-H5 were co-infected with NDV. This incidence of co-infection has been previously reported (Abd El-Hamid et al., 2020; Musa et al., 2020; Eid et al., 2022) and indicated that wild birds act as carriers for many pathogens and transmit them across countries and different bird species (Musa et al., 2020; Blagodatski et al., 2021; Eid et al., 2022; Martelli et al., 2023).

The wild bird species positive for AIV-H5 were a hooded crow, cattle egret, European turtle dove, and sandpiper, and were captured or collected from Dakahlia, Ismailia, Port-Said, and Sharkia. The positive AIV-H5 in domestic birds was in the breeds of Cobb and Ross in chicken species and Muscovy duck, which are the highly reared breeds in Egyptian farms and were collected from North Sinai, Sharkia, and Dakahlia. Dakahlia and Sharkia provinces have live bird markets (LBM), where impressive migratory, free-living, and domestic birds of different species are mixed, and this gives a chance for dissemination of several infectious agents as different influenza subtypes, and this may be mixing vessels for possible mutations (Nabil et al., 2020). In the intervening time, Ismailia, North Sinai, and Port-Said are provinces that have Al Manzala, Bardawil, and Bitter Lakes, as they are one of the migratory bird paths in Egypt (Abdien et al., 2023).

This is the same consequence that occurred during the H5N1 pandemic in Egypt in late 2005, which suggested the introduction of this virus through migratory birds. Given that AIV is a highly contagious disease that affects birds of all ages (Spackman, 2008), the clinical findings observed in the domestic birds that were infected with AIV-H5, including chickens and ducks, were

cyanosed heads, greenish diarrhea, respiratory and nervous signs, general septicemia in internal organs, and hemorrhages. These findings are consistent with those recorded in the domestic birds naturally infected with AIV-H5 (Anis et al., 2018; Hashim et al., 2022; Djurdjevic et al., 2023).

In this study, most of the captured or collected wild birds were healthy without any clinical signs. However, some birds, such as Cattle egret and Sandpipers infected with AIV-H5, showed mild to moderate symptoms, including respiratory signs, watery diarrhea, congested head, air sacculitis, enteritis, and congested internal organs. This is comparable to Kandeil et al. (2017), who recorded that the different wild bird species showed no clinical signs of HPAI infection during the sampling. As well, in the majority, avian influenza A viruses cause asymptomatic or mild clinical signs in wild bird hosts (Shriner and Root, 2020). Concerning, those wild birds that exhibited clinical disease were likewise reported in preceding studies. Krone et al. (2018) corroborated the findings, revealing that the highly pathogenic influenza virus H5 clade 2.3.4.4b resulted in a significant number of fatal infections and deaths among white-tailed sea eagles in Germany, which were linked to severe neurological symptoms confirmed through immunohistochemical analysis. In 2017, Pohlmann et al. (2017) identified a novel HPAI H5N8 classified within group b, subtype 2.3.4.4, which was responsible for lethal infections in numerous wild bird species, including northern pintails, mallards, European gulls, carrion crows, great-crested grebes, swans, geese, and domestic birds involved ducks, chickens, and turkeys in Germany.

For genetic characterization, this study used the fully deduced amino acid sequences of the hemagglutinin derived from 144 HPAI A (H5Nx) viruses, later coalesced to 33, to inspect the virus evolution throughout this varying epidemiological outline. The HA-deduced amino acid sequence similarities among Egyptian strains ranged from 98.1% to 100% (Kandeil et al., 2022). The HAs were clustered in clade 2.3.4.4b with the reference strain A/duck/Jiangsu/k1203/2010(H5N8).

We investigated the evolutionary changes of the virus from 2016-2023, providing evidence of ongoing modification in transmission dynamics and disease epidemiology. We demonstrated the coexistence of genetically stable viruses between 2016 and 2019 with 99.8-100% identity and genetically diverse viruses with novel points of mutations. Those mentioned above could be attributed to the multiple introductions of H5N8 via the migration events of the European wild bird population (through the Black Sea–Mediterranean and East Asia–West Africa flyways). The evolutionary analysis clustered the investigated viruses into three phylogroups. The clustering was mainly associated with the existence of new viruses in the migratory wild birds. The year of isolation, the geographical location, and even the host of origin did not correlate with the virus clustering. Phylogroup 1 included viruses from 2016-2019, phylogroup 2 included viruses from 2017-2018, and other H5N8 viruses from different European countries. Phylogroup 3 included virus strains from 2019 to 2023.

Alteration at the amino acids could be responsible for the affinity of the virus to certain species receptors, especially at the N-glycosylation site. It has been documented earlier that the existence of 156 Alanine in the H5 could be responsible for the receptor-binding specificity of the influenza virus to human receptors. Shifting to threonine could be accountable for decreasing HA binding to mammalian sialylated glycans and limiting virus transmission in guinea pigs (Gao et al., 2009). Most avian species had threonine mutation at this position. Accordingly, in our analysis, the majority of H5NX AIV strains detected in Egypt since 2016 possess 156T, except certain strains detected in domesticated birds in 2018.

Furthermore, H5Nx AIV strains were detected in wild migratory birds such as common pochard and pintail and domesticated birds, which had 156T. The previous raised alarm about the possibility of future mammalian adaptation from the spillover of avian-originated strains. Accordingly, Egyptian H5 subtypes of 2021 displayed two mammalian virulence mutations that were not present in published Egyptian H5N8 sequences: NS1 189N in four isolates and NS2 31I in two isolates (Kandeil et al., 2022).

In goose species, T 156 M was detected in virus isolated from Assuit province (A/goose/Egypt/BA71C/2018(H5N8)), confirming the responsibility of this position to the affinity of the virus to a certain host range (Abdel-Ghany et al., 2023). In accordance, the later study supports the multiple introductions

of H5N8 to Egypt, as it reported two viruses in 2018 belonging to different genetic lineages (Kandeil et al., 2022). One virus obtained from a duck is correlated to the Egyptian subgroup II and the other goose one is related to Egyptian subgroup I. Another point of mutation was abundant in phylogroup 2 strains (G284E), however, the recent isolates 2019-2023 have 284 G residues similar to 2016 viruses.

After studying the genetic analysis of the influenza strain isolated in the present study (A/chicken-cobb/Egypt/55/2019(H5N8), it was subjected to study its pathogenic characterization in broiler chickens at 4 weeks of age via inoculation intraocularly. The exhibited clinical signs and postmortem lesions in the infected birds with a record high mortality rate reached 90% within 10 dpi. These results are consistent with former pathogenicity studies of clade 2.3.4.4 H5N8 viruses (Moatasim et al., 2019; Foret-Lucas et al., 2023).

Conclusions

The results of the current criticizing investigation on full HA gene sequence revealed the persistence of H5N8 sublineage 2.3.4.4b succeeding its first introduction in Egypt during 2016 via migratory aquatic birds till now among both domesticated and wild birds. The existence of genetically stable viruses 2016-2019 with 99.8-100% similarity with the complete identity of our current strain with three Egyptian strains form green-winged teal/ 2016(H5N8), northern Shoveler /2016(H5N8), and duck/2017(H5N8). There is an indication of sequential introductions of H5N8 via the migration of European wild birds to African flyways, which suggests the possibility of future mammalian adaptation from the AIV spillover, principally in native aquatic birds.

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