Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 3, 356-361

Molecular characterization of avian influenza viruses (H5N2, H5N8, H5Nx and H9N2) isolated from chickens and ducks in the South of Egypt 2020 – 2021

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ARTICLE INFO

Recieved: 14 September 2023

Accepted: 25 October 2023

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Keywords:

AIV
H5 and H9 subtypes
Ducks and chickens
Phylogenetic analysis
Egypt

ABSTRACT

This study was conducted to investigate the epidemiological situation of avian influenza viruses (AIV) and the molecular identification of the different AIV subtypes circulating among chickens and duck farms in South Egypt. A total of 143 samples were collected from chicken and duck farms in Qena (n = 105) and Luxor (n=38) governorates during 2020. The organs and swabs were collected from diseased chickens and healthy ducks. The viruses were isolated in embryonated chicken eggs (ECEs) and their propagation was confirmed by hemagglutination test (AHT) and molecular detection of matrix gene by reverse transcription polymerase chain reaction (RT-PCR). AIV subtypes were identified by RT-PCR and specific primers. Phylogenetic analysis of sequenced partial H5, N2, and N8 genes was performed. The results revealed that 15 AIVs were subtyped to 2 H5N2, 2 H5N8, 8 H5Nx, and 2 H9N2. While an isolate could not be subtyped by used primers. The H5-based evolutionary tree of 4 isolates revealed their categorization with the 2.3.4.4b clade with close relation to H5N8 isolates from Egypt in 2021 and Kazakhstan in 2020. In conclusion, the occurrence of H5 and H9 viruses pays attention to a public health concern. Also, non-identified HxNx reveals a new AIV HA and NA subtype may be present among chickens.

Introduction

Influenza viruses belong to the family *Orthomyxoviridae* which is composed of Influenzavirus A, Influenzavirus B, Influenzavirus C, and Influenzavirus D genera (Nuñez and Ross, 2019). Its genome comprises eight segments, single-stranded, negative-sense RNA encoding over 11 proteins. These proteins are hemagglutinin (HA) and neuraminidase (NA), which formed surface glycoproteins, transcriptase proteins (PB2, PB1, and PA), matrix proteins (M1 and M2), nucleoprotein (NP), and nonstructural (NS1 and NS2) proteins (Wright, 2007).

Influenza A viruses are classified according to the antigenic properties of the surface glycoproteins HA and NA into 16 HA and 9 NA subtypes identified in poultry and wild birds (Wang *et al*., 2022). All avian influenza virus subtypes have been reported in wild birds, the natural reservoir, which usually shows asymptomatic infections, with few reported cases suffering from clinical signs (Alexander, 2007).

Avian influenza (AI) is a significant risk factor for poultry investment in Egypt because many AI virus subtypes are circulated among poultry on the same farms. High-pathogenic (HP) AI viruses of subtype H5N1 (clade 2.2) and low-pathogenic (LP) AI H9N2 have been circulating in poultry farms since their detection in 2006 and 2010, respectively (El-Zoghby *et al*., 2012). The emergent chicken H9N2 reassortant containing viral genes from quail H9N2 (HA, NP, and NA segments) and pigeon H9N2 (PB2, PB1, PA, and M segments) isolated in 2014 has been reported as the parental donor of seven gene segments for the novel HPAIVH5N2 reassortant from broiler chicken flocks in Beheira and Fayoum in 2019 (Hagag *et al*., 2019; Hassan *et al*., 2020). Continuous evolution of H5N2 clades 2.3.2.1a and 2.3.4.4c through genetic reassortment with other AIV HA subtypes has been recorded in many Asian countries such as Chian and Bangladesh (Cui *et al*., 2020; El-Shesheny *et al*., 2020; Li *et al*., 2020). Since 2016, HPAIVH5N8 has become endemic with repeated virus detection among poultry flocks in Egypt (Selim *et al*., 2017; Yehia *et al*., 2018; Sultan *et al*., 2022) and several genotypes, about 7 genotypes, have been demonstrated for clade 2.3.4.4b H5Nx among birds in Egypt since 2016. Also, wild birds have contributed to internal genes of H5Nx and H9N2 circulating in Egypt (Hassan *et al*., 2020). In addition to that, LPAIVH7N3 has been detected in wild birds donating gene segments to HPAIVH5N8 circulating among chicken farms (Naguib *et al*., 2019). Because of HPAIVH5N8, 2,782 outbreaks resulted in the death of approximately 38 million birds in over 25 countries (Cui *et al*., 2022). Since 2014 AI H5N8 viruses have been reported as the prevalent H5 subtype among avian species worldwide (Lycett *et al*.,2020). Although HPAI H5N8 viruses were repeatedly detected in wild and domesticated birds during 2020 – 2021 all over the world (Lewis, *et al*.,2020), they were isolated from workers in an infected poultry farm in Russia (WHO,2021). Some LPAIV subtypes, particularly H5 and H7, can evolve into HPAIVs by recombination and mutation (Alexander, 2000; Tang *et al*., 2021).

The current study was designed to investigate the AIV subtypes' epidemiological status among chickens and ducks in the south of Egypt during 2020-2021 through isolation, molecular characterization, subtyping and genetic analysis of circulating AIV isolates.

Materials and methods

Collection of samples

A total of 143 samples were collected from broiler chicken (n=99)

ISSN: 2090-6277/2090-6269/

and duck (n=44) flocks at different localities in Qena (n=105) and Luxor (n=38) governorates during 2020. The tracheal swabs were collected from alive broiler chickens of 7 - 35 days old showing respiratory and nervous manifestations, while organs (proventriculus, liver, trachea, spleen, lung, intestine, and cecal tonsils) were collected from diseased freshly dead birds showing cyanosis in comb and wattles with facial edema, and hemorrhage in the hock and shank (Fig. 1). Intestinal congestion, congestion in the bursa and petechial hemorrhage in proventriculus were observed during the postmortem (PM) examination (Fig. 1). Cloacal swab samples were collected from apparently healthy ducks (Muscovy and French) of 2 - 3 months old. The swabs were mixed with phosphate buffer saline (PBS) containing antibiotics and antifungals. The collected samples were transferred on ice to the laboratory of Virology, Microbiology department, Faculty of Veterinary Medicine, South Valley University, and kept at -08°C until processing.

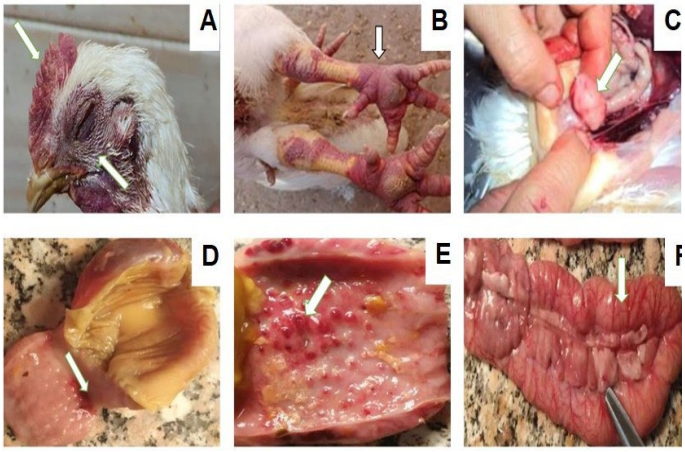

Fig.1. Showing the characteristic clinical signs and PM lesions (white arrow) of AIV observed on alive or dead birds during sample collection. A) Cyanosis in the comb and wattles, and periorbital edema. B) Ecchymotic hemorrhage on the shank. C) Congestion in bursa. D) Hemorrhages at a tip of the proventriculus. E) Petechial hemorrhage in all proventriculus, and F) Congestion in blood vessels of duodenum.

Preparation of sample and isolation of AIV into embryonated chicken eggs (ECEs)

To isolate the virus from organs, 1 g of the target organ was cut into small pieces, grinded in a porcelain mortar, and suspended in PBS containing an antibiotic and antifungal mixture in a 1:9 ratio. After incubation overnight in the refrigerator the mixture was centrifuged at 3000 rpm/10 min. the isolation of the virus from tracheal and cloacal swabs was prepared by dissolving the swab content in 1 ml PBS containing an antibiotic and antifungal mixture and debris was precipitated by centrifugation at 3000 rpm / 10 min. A total of 0.2 ml of the prepared sample supernatant was inoculated in 10-day-old embryonated chicken eggs via the allantoic cavity. The inoculated ECEs were incubated at 37°C for 4 days with daily observing of the embryo viability. The allantoic fluids (AFs) were collected from dead or alive ECEs after overnight chilling and used for virus confirmation by hemagglutination test. The dead embryos before 24 hr. were considered non-specific death.

Detection of AIV propagated in embryonated chicken eggs using HA test

The collected allantoic fluids were subjected to an HA test according to WOAH (2021). The HA-negative samples were re-inoculated in ECEs to confirm the presence or absence of the virus. The HA-positive samples were stored at -80°C for molecular study.

Molecular detection of AIV using of RT-PCR

Extraction of Nucleic acid: The RNA was extracted from the allantoic fluids showing HA activity using EasyPure Viral DNA/RNA kit (TransGen biotech, China) according to manufacturer instructions. The obtained RNA was used as a template for reverse transcription polymerase chain reaction (RT-PCR). To detect the presence of AIV one one-step RT-PCR was conducted using a OneStep RT-PCR kit (GeneDirex Inc., Korea) and specific primers for the M gene of AIV (Table 1). The reaction mixture was adjusted for 25ul containing; 12.5ul 2x reaction mix, 1ul of each primer (10uM), 1ul RT/HotStar Taq mix, 5ul from extracted RNA, and 4.5ul RNase-free water. The thermal profile was carried out according to Starick *et al.* (2000).

Molecular, genetic identification, subtyping of AIV M gene of positive samples

The positive AIV isolates were subtyped by RT-PCR using specific primers (Table 1) detecting N2, H5, H9, and N8 subtypes following the same RT-PCR reaction master mix and thermal profiles described by Wright *et al.* (1995); Starick *et al.*, (2000); Chaharaein *et al.* (2006) and Qiu *et al.* (2009).

Molecular identification, sequencing and phylogenetic analysis of AIV

QIAquick Gel Extraction Kit (Qiagen, USA) was used to purify positive amplicons of H5, N2, and N8 from the gel as per the manufacturer manual. BigDye Terminators v3.1Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used to directly sequence the purified PCR products using ABI PRISM3500 genetic analyzer (Applied Biosystems, USA) used to sequence the purified products directly. To identify the sequence identity BioEdit software and Clustal W version 7.2 were used, and connected sequence data were aligned with reference strains obtained from GenBank. The phylogenetic tree was constructed using the maximum likelihood (ML) method, the general likelihood reversible substitution model, and performed with bootstrap test (1000 replication)

Table 1. Oligonucleotide primer sequences are used for the detection, subtyping, and sequencing of isolated AI viruses.

Target gene	Primer name	Primer Sequence (5′- 3′)	RT-PCRproduct (bp)	References
M	AIV-M1-F	AGCGTTAGACGCTTTGTC	601	Starick *et al.* (2000)
	AIV-M1-R	GACGATCAATCCAC		
H5	H5-F	GGAACATCAACACTRAAYCAAGAG	456	
	H5-R	TACACATTGGGTGATTG		
H9	H9-F	ATCGGCTGTTAATGGAATGTGTT	221	Chaharaein *et al.* (2006)
	H9-R	TGGGCGTCTTGAATAGGGTAA		
N2	N2-F	ATGGTCCAGCTCAAGTTGTCA	434	Wright *et al.* (1995)
	N2-R	TCCAGTTATGTGTTGCTCAGG		
N8	N8-F	ACAGTCRTTAGGGAATAC	362	Qiu *et al.* (2009)
	N8-R	TACACATTGGGTGATTG		

using MEGA 6 software (Tamura *et al*., 2013).

Accession numbers of AIV

The sequences of the obtained isolates in this study have been deposited in GenBank under the following accession numbers: OR262330 for A/chicken/Egypt-Qena/47/2020 (H5), OR262331 for A/chicken/Egypt-Luxor/15/2020 (H5), OR262332 for A/chicken/Egypt-Luxor/18/2020 (H5), OR262333 for A/chicken/Egypt-Qena/32/2020 (H5), OR262338 for A/chicken/Egypt-Qena/47/2020 (N2), OR262339 for A/chicken/Egypt-Qena/32/2020 (N2), OR263457 for A/Muscovy-duck/Egypt-Qena/13D/2020 (N8) and OR263458 for A/chicken/Egypt-Luxor/18/2020 (N8).

Results

Detection of AIV propagated in embryonated chicken eggs using HA test

The prevalence of AIV in broiler chickens and duck samples was reported. Out of 143 samples collected from broiler chickens and ducks in Qena and Luxor governorates 15 samples could be propagated in ECEs and were positive by HA test. These samples were confirmed as AIV using RT-PCR and specific primers targeting the M gene with a PCR product size of 601 bp (Table 2 and Fig. 2). The AIV was detected in 12/99 (12.1%) samples from broiler chickens and 3/44 (6.8%) samples from ducks. Interestingly, HA titers were 25 – 210 for isolates obtained from chickens showing clinical signs characteristic of AIV while these titers were 210 for isolates from ducks with no clinical signs (Table 3). The AIV isolates were detected at a higher rate in samples collected from birds in Luxor 15.8% (6/38) than these samples collected from birds in Qena 8.6% (9/105).

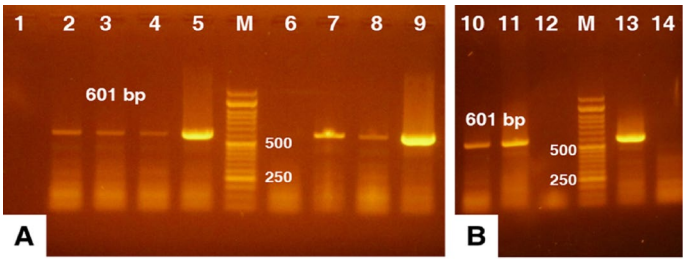

Fig. 2. RT-PCR results of extracted nucleic acid from HA-positive isolates using M gene primers for AIV showing amplicon size of 601 bp. (A) Lanes 1 and 6: negative samples, lanes 2 – 5 and 7 - 9: positive AIV isolates (12, 39D, 28, 22, 45, 48, and 15, respectively). B) Lanes 10 - 11, and 13: positive AIV isolates (13D, 32, and 79), Lanes 12 and 14: negative samples; Lane M: 50 bp DNA Ladder.

Molecular detection, genetic identification, subtyping of AIV M gene, Sequencing and Phylogenetic Analysis of AIV subtypes in chicken and duck samples

The AIV M gene-positive isolates were identified for HA and NA gene subtypes using H5, H9, N2, and N8 specific primers to detect the most common subtypes circulating among chicks and ducks. Out of 15 AIV isolates assessed with H5 primers, 12 isolates were positive for H5 with RT-PCR product size of 456 bp (Table 3 and Fig. 3). The H9 subtypes were detected in 2 isolates and the other isolate was non-H5 or H9 (Table 3). The co-infection of both subtypes (H5 and H9) could not be detected (Table 3). Further subtyping of the NA gene revealed that 4 isolates were N2 with PCR products of 434 bp (Table 3 and Fig. 4) and 2 isolates were N8 subtypes producing PCR products of 362 bp as shown in Fig. 5 and Table 3.

The subtyping results of HA and NA revealed that 2 H5N8 isolates

Table 2. The prevalence of AIV isolates obtained from collected samples.

No. of samples	Type of sample	Avian species	Vaccination status for AIV	No. of AIV positive sample
10	Tracheal swab	Broiler chickens	Non vaccinated	2
44	Cloacal swab	Ducks	Non vaccinated	3
25	Liver	Broiler chickens	Non vaccinated	4
14	Lung	Broiler chickens	Non vaccinated	2
15	proventriculus	Broiler chickens	Non vaccinated	2
25	Intestine	Broiler chickens	Non vaccinated	1
10	Duodenum	Broiler chickens	Non vaccinated	1

Table 3. Serological and molecular identification of the isolated AI viruses from broiler chickens and ducks.

Sample ID	Species	Governorate	HA	RT-PCR AIV-M gene	H5	H9	N2	N8
18	Chicken broilers	Luxor	27	601bp	+ ve	*NS	NS	+ ve
32	Chicken broilers	Qena	29	601bp	+ ve	NS	+ ve	NS
47	Chicken broilers	Qena	27	601bp	+ ve	NS	+ ve	NS
12	Chicken broilers	Qena	25	601bp	+ ve	NS	NS	NS
15	Chicken broilers	Luxor	26	601bp	+ ve	NS	NS	NS
22	Chicken broilers	Luxor	26	601bp	+ ve	NS	NS	NS
23	Chicken broilers	Luxor	26	601bp	+ ve	NS	NS	NS
28	Chicken broilers	Luxor	29	601bp	+ ve	NS	NS	NS
39	Chicken broilers	Qena	210	601bp	+ ve	NS	NS	NS
45	Chicken broilers	Qena	210	601bp	+ ve	NS	NS	NS
48	Chicken broilers	Qena	210	601bp	+ ve	NS	NS	NS
79	Chicken broilers	Qena	26	601bp	NS	NS	NS	NS
12D	Ducks	Qena	210	601bp	NS	+ ve	+ ve	NS
39D	Ducks	Luxor	210	601bp	NS	+ ve	+ ve	NS
13D	Ducks	Qena	210	601bp	+ ve	NS	NS	+ ve

*NS means not subtyped using current primers of H5, H9, N2 and/or N8

were identified from a chicken and a duck origin while 2 H5N2 subtype isolates were detected from chickens only and 2 H9N2 isolates were obtained from ducks only. H5Nx (non-N2 or N8) subtypes were identified from 8 chicken samples (Table 3). One isolate from chicken could not be subtyped for HA and NA subtype by these primers (Table 3).

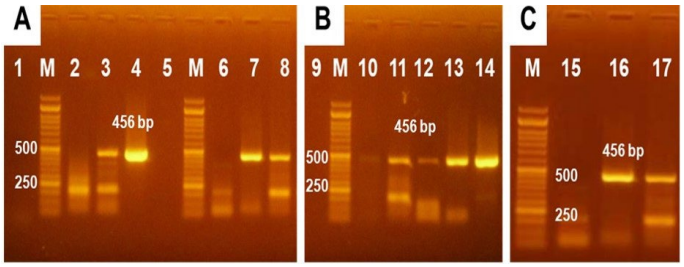

Fig. 3. Results of H5 subtyping by RT-PCR for AIV positive isolates using H5 primers, 456 bp amplicon indicating H5 subtype. Lane M: 50 bp DNA Ladder. A) Lanes 3 - 4, and 7 -8: H5 positive isolates, lanes 1, 5, and 6: non-H5 subtype AIV isolates. B) Lanes 10 - 14: H5 positive isolates, lane 9: non-H5 subtype AIV isolates. C) Lanes 16- 17: H5 positive isolates, lane 15: non-H5 subtype AIV isolates.

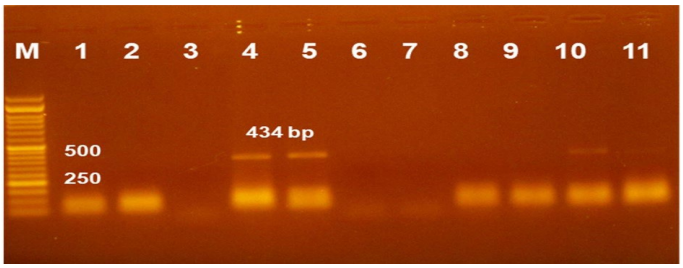

Fig. 4. RT-PCR subtyping of N2 for AIV positive isolates using N2-specific primers where positive N2 subtype isolates show 434 bp product size. Lane M: 50 bp DNA Ladder. Lanes 4 – 5, and 10 -11: indicating N2 subtype AIV isolates. Lanes 1 -3, and 6 – 9: indicating non-N2 subtype AIV isolates.

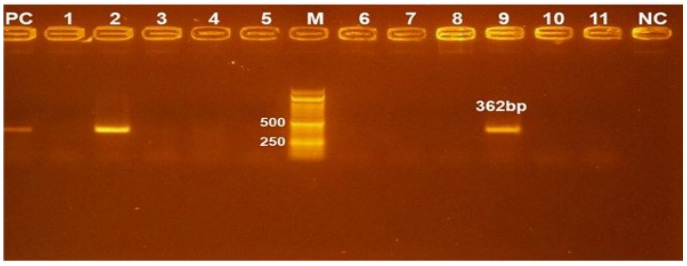

Fig. 5. RT-PCR results of N8 subtyping for AIV positive samples showing positive isolates with an amplicon size of 362 bp. PC: positive control (H5N8 lab. isolate). Lane M: 50 pb DNA Ladder. Lanes 2 and 9: indicating subtype N8 AIV isolates (13D and 18). Lanes 1, 3 - 8, and 10 – 11: non-N8 AIV subtype isolates. NC: negative control (master mix without template RNA).

Phylogenetic Analysis

The nucleotide sequences of the 4 H5 (456 nt), 2 N2 (434 nt), and 2 N8 (362 nt) viruses from this investigation were aligned with other isolates from Egypt and worldwide and deposited in GenBank. The evolutionary phylogenetic trees were constructed based on these sequences.

H5 tree

Fig. 6 showed that the phylogenetic tree based on sequence analysis of nucleotide sequences of 4 H5 isolates; A/chicken/Egypt-Luxor/15/2020(H5), A/chicken/Egypt-Luxor/18/2020(H5), A/chicken/Egypt-Qena/32/2020(H5) and A/chicken/Egypt-Qena/47/2020(H5) in comparison with the represented sequences obtained from the GenBank. They found nearby these isolates from duck, chicken, turkey, and pigeon that isolated in Egypt (2017, 2019, 2020, and 2021 as well as Spain (2022) and Kazakhstan (2020) isolates (Fig. 6). The isolates were clustered into two groups; one group contains A/chicken/Egypt-Qena/47/2020(H5), and the second group contains the other three isolates located nearby the A/chicken/Kazakhstan/23/2020/H5N8 isolate. The isolates A/chicken/Egypt-Luxor/15/2020 (H5), A/chicken/Egypt-Qena/32/2020(H5), and A/chicken/Egypt-Qena/47/2020(H5) are closely related to the isolate A/chicken/Kazakhstan/23/2020(H5N8) with nucleotides identities of 99.56%, 98.9% and 99.24%, respectively, (Table 4). In the other hand, the isolate A/chicken/Egypt-Luxor/18/2020 (H5) was nearby the isolate A/Duck/Egypt/BEH2/2020(H5N8) and showed a nucleotide identity of 98.69 %.

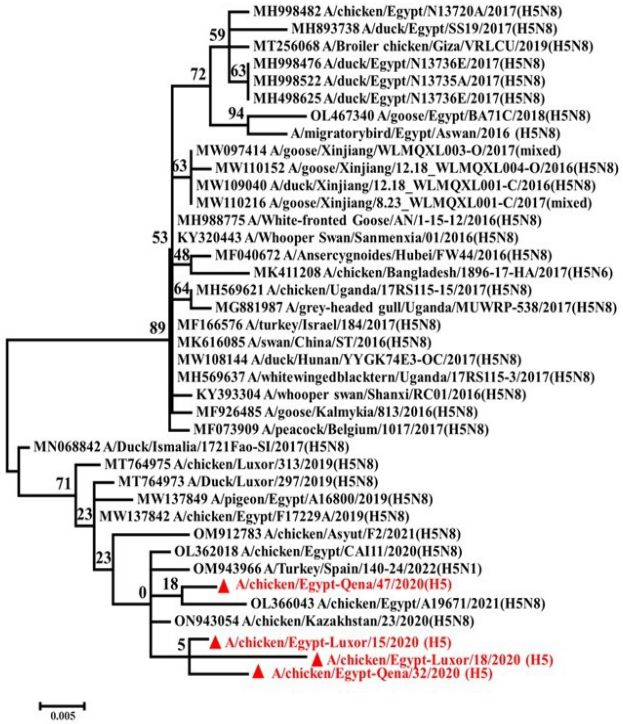

Fig. 6. Maximum-likelihood phylogenetic tree based on H5 nucleotide sequences of avian influenza isolates from Qena and Luxor governorates indicated by red color and triangle and reference strains of H5 subtypes available in the GenBank database. Numbers at each node indicated bootstrap values. Phylogenetic analysis was conducted using MEGA 6 software, the general likelihood reversible substitution model, and a bootstrap value of 1000 repeats. The scale bar indicates 0.005 nucleotide substitutions per site.

Table 4. The nucleotide identities between current isolated avian influenza viruses and closely related strains obtained from GenBank.

Current isolate ID	Closely Related Strain from GenBank	Identity (%)	Accession no.
A/chicken/Egypt-Luxor/15/2020 (H5)	A/chicken/Kazakhstan/23/2020(H5N8)	99.56	ON943054
A/chicken/Egypt-Luxor/18/2020 (H5)	A/Duck/Egypt/BEH2/2020(H5N8)	98.69	OL362014
A/chicken/Egypt-Qena/32/2020 (H5)	A/chicken/Kazakhstan/23/2020(H5N8)	98.9	ON943054
A/chicken/Egypt-Qena/47/2020(H5)	A/chicken/Kazakhstan/23/2020(H5N8)	99.24	ON943054
A/Muscovy duck/Egypt-Qena/13D/2020 (N8)	A/reassort ant/IDCDC-RG71A (Astrakhan/3212/2020 Puerto Rico/8/1934) (N8)	98.87	OM403994
A/chicken/Egypt-Luxor/18/2020 (N8)	A/reassort ant/IDCDC-RG71A (Astrakhan/3212/2020 Puerto Rico/8/1934) (N8)	98.87	OM403994
A/chicken/Egypt-Qena/47/2020 (N2)	A/Arizona/4/2006(N2)	100	EU100495
A/chicken/Egypt-Qena/32/2020 (N2)	A/Arizona/4/2006(N2)	100	EU100495

N8 tree

The phylogenetic tree based on sequence analysis of 2 N8 nucleotide sequences for A/Muscovy-duck/Egypt-Qena/13D/2020(N8) and A/chicken/Egypt-Luxor/18/2020(N8) in comparison with the represented sequences obtained from the GenBank (Fig. 7). The two isolates were nearby A/spot-billed-duck/shanghai/JDS20867/2020(H5N8), A/Cygnus Columbians/Hubei/116/2020(H5N8), A/reassortant/ID-CDC-RG71 (Astrakhan/3212/2020 XPuertoRico/8/1934(H5N8)), A/Mule_duck/France/20338/2020 (H5N8), A/Chicken/Nigeria/NRD21 _21VIR2288-5/2021 (H5N8), and A/chicken/Nigeria/VRD21 _100 _21VIR2370-423/2021(H5N8). These isolates are in clade 3.3.4 group B. The isolates A/Muscovy-duck/Egypt-Qena/13D/2020(N8), and A/chicken/Egypt-Luxor/18/2020(N8) showed nucleotides identity of 98.87% to A/reassortant/IDCDC-RG71 (Astrakhan/3212/2020 XPuertoRico/8/1934 (H5N8)) as shown in Table 4.

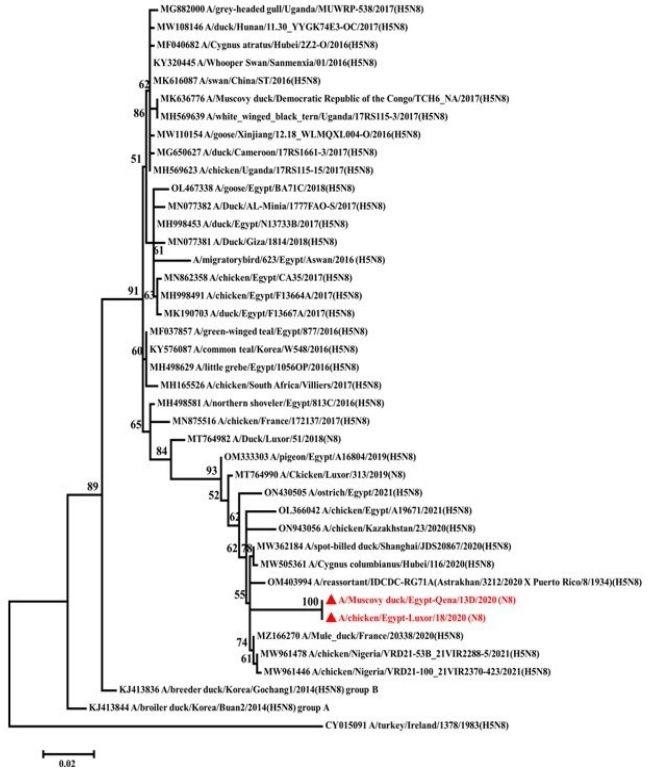

Fig. 7. Maximum-likelihood phylogenetic trees of the N8 nucleotide sequences of avian influenza isolates from Qena and Luxor governorates (red color and triangle) and reference strains of N8 subtypes retrieved from the GenBank database. Numbers at each node indicate bootstrap values. Phylogenetic analysis was conducted using MEGA 6 software, the general likelihood reversible substitution model, and a bootstrap value of 1000 repeats. The scale bar indicates 0.02 nucleotide substitutions per site.

N2 tree

The phylogenetic tree based on nucleotide sequence analysis of A/chicken/Egypt- Qena/47/2020(H5N2), and A/chicken/Egypt-Qena/32/2020(H5N2) isolates in the current study in comparison with the represented sequences retrieved from the GenBank (Fig. 8) showed their close relation to A/Homosapiens/India/149/2017 (N2), A/Chicken/Belgium/150/1999 (H5N2), A/Swine/Kansas/14-032899/2014 (H1N2), A/Arizona/4/2006 (N2) and A/Michigan/16/2003 (N2). The two isolates A/chicken/Egypt-Qena/47/2020 (N2) and A/chicken/Egypt-Qena/32/2020 (N2) have nucleotides identity of 100% to A/Arizona/4/2006(N2) as shown in Table 4.

Discussion

Egypt has been considered an AIVs endemic country since February 2006 (Mohamed *et al*., 2019). This study was conducted to evaluate the

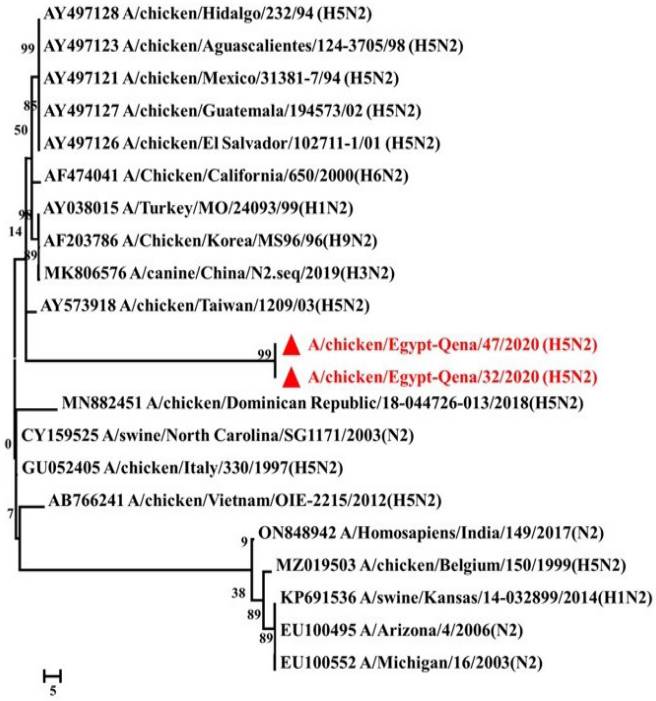

Fig. 8. Maximum-likelihood phylogenetic trees of the N2 gene of avian influenza isolates in (Qena and Luxor) with reference strains of N2 subtypes available in the GenBank database. The H5N2 strains isolated in 2020 are indicated by red color and triangle. Numbers at each node indicate bootstrap values. Phylogenetic analysis was conducted using MEGA 6 software, the general likelihood reversible substitution model, and a bootstrap value of 1000 repeats. The scale bar indicates 0.02 nucleotide substitutions per site.

epidemiological status and elucidate the prevalence of different subtypes of AIV among chickens and ducks that have been reared in Qena and Luxor governorates in South Egypt during 2020. The RT-PCR targeting the M segment is widely used to estimate the prevalence of AIV and is sometimes used to screen samples for virus isolation and characterization (Ferro *et al*., 2008; 2010).

AIV isolates among non-vaccinated chickens were detected by RT-PCR where 12/15 (80%) isolates were H5 (9 from chickens and 3 from ducks). H5 AI viruses are not only causing severe mortality among various avian species worldwide, but they can also spill over to infect mammals and humans (Kuiken, 2023).

Subtyping revealed that 2 isolates were H5N2, 2 isolates were H5N8, and 8 isolates H5Nx. The isolation of H5N2 from chickens in the current study is congruent with Hagag *et al*., (2019) and Hassan *et al*., (2020) who stated the presence of novel H5N2 reassortant among chickens in lower Egypt.

HPAI H5N8 viruses exhibit asymptomatic disease in geese and ducks with prolonged virus shedding (Son *et al*., 2018) while adaptation of the virus in chickens may be the main cause of its high pathogenicity (Pantin-Jackwood *et al*., 2017; Rohaim *et al*., 2021). It agrees with the current results of H5N8 isolation from a duck cloacal swab showing no clinical signs and chicken organs showed AIV clinical signs. Several outbreaks have been reported in backyards and commercial farms in various Egyptian governorates attributed to H5N8 clade 2.3.4.4b which was closely related to Russian H5N8 (OIE, 2017; Hassan *et al*., 2020; Yehia *et al*., 2018; Yehia *et al*., 2020). This detection of H5N8 among chick flocks poses a public health concern where this virus could be transmitted to workers (Pyankova *et al*., 2021; WHO, 2021).

The high prevalence of H5Nx viruses significantly harms poultry production (Bertran *et al*., 2019) due to its ability to evolve the antigenic sites of the surface glycoprotein antigens HA and NA (Sautto *et al*., 2018). In the last decade, influenza A viruses of subtype (H5Nx) have continued to circulate in migratory birds and spread to infect domestic poultry in many countries worldwide (Pohlmann *et al*., 2022). AIV H5Nx viruses are a severe threat to poultry production (Bertran *et al*., 2019), as they can introduce mutations in the antigenic sites of the surface glycoprotein antigens HA and NA (Sautto *et al*., 2018). According to a previous study, broilers were in high danger from AIVs by industry stakeholders. Their results revealed that although various AIV subtypes such as H5Nx and H9N2 were generating rare outbreaks, they are enabling AIV mutations (Shi *et al*., 2018). The high prevalence of H5 AIV may be attributed to the following; the lack of bio-security measures in the area of sample collection, the low percentage of vaccinated flocks, vaccination inefficient, collection of samples either from outbreaks or individual cases and bird

species (Suarez and Perdue, 1998; Hassan *et al*., 2020).

Also, 2/15 samples were H9N2, and one sample was Hx (non-H5 or H9) Nx (non-N2 or N8) this result indicated that there may be another subtype circulating in the field other than H5 and H9 subtypes. The low prevalence of H9N2 in comparison with H5 subtypes agrees with the results reported by Osman *et al*. (2015) as H5 viruses were highly prevalent among avian species in Qena and Luxor governorates and H9 could be detected in chickens only in 2011. Interestingly, in this study, the H9N2 isolates were detected in duck flocks. These results (HxNx) indicated the presence of new HA and NA subtypes of AIV circulated among chickens in South Egypt.

In this study, phylogenetic analysis of the partial H5 and N8 gene of the H5N8 viruses from chicken and duck indicated that they clustered together in group B of H5N8 viruses of clade 2.3.4.4 with close relation to these viruses from Egyptian governorates, Russia, and Nigeria.

The H5N2 HPAIVs with clades 2.3.2.1a and 2.3.4.4c HA genes have still evolved via genetic reassortment with different AIV subtypes, especially in some Asian regions including China and Bangladeshi (Cui *et al*., 2020; El-Shesheny *et al*., 2020; Li *et al*., 2020). The H5N2 evolutionary tree revealed that 2 isolates segregated in a distinct clade than other H5N2 isolates which may indicate that further genetic evolution occurs and a novel reassortant may present.

Conclusion

The isolation and molecular characterization of AIV among chickens and ducks in South Egypt during 2020-2021 revealed that AI-H5Nx (non H5N8, H5N2, or H9N2) viruses subtype are predominantly circulating among chickens. Also, AIV HxNx (non-H5 nor H9) was reported indicating dissemination of another HA subtype among chickens. The occurrence of the H5 subtype in high prevalence raises a public health concern and enhances the molecular investigation for AIV HA and NA subtyping other than H5N8, H5N2, and H9N2 subtypes.

Conflict of interest

The authors declare that they have no conflcit of interest.

References

Alexander, D.J., 2000. A review of avian influenza in different bird species. Veterinary Microbiology 74, 3–13. https://doi.org/10.1016/S0378-1135(00)00160-7.

Alexander, D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine, 25, 5637–5644. https://doi.org/10.1016/j.vaccine.2006.10.051.

Bertran, K., Pantin-Jackwood, M.J., Criado, M.F., Lee, D.H., Balzli, C.L., Spackman E. Suarez, D.L., Swayne D.E., 2019. Pathobiology and innate immune responses of gallinaceous poultry to clade 2.3.4.4A H5Nx highly pathogenic avian influenza virus infection. Vet Res. 50, 89. https://doi.org/10.1186/s13567-019-0704-5.

Chaharaein, B., Omar, A., Aini, I., Yusoff, K., Hassan, S.S., 2006. Reverse transcriptase-polymerase chain reaction-enzyme linked immunosorbent assay for rapid detection of avian influenza virus subtype H9N2. Arch. Virol. 151, 2447–2459. https://doi.org/10.1007/s00705-006-0809-9.

Cui, Y., Li, Y., Li, M., Zhao, L., Wang, D., Tian J., Bai, X., Ci, Y., Wu, S., Wang, F., Chen, X., Ma, S., Qu, Z., Yang, C., Liu, L., Shi, J., Guan, Y., Zeng, X., Tian, G., Cui, P., Deng, G., Jiang, Y., Chen, P., Liu, J., Wang, X., Bao, H., Jiang, L., Suzuki, Y., Li, C., Li, Y., Chen, H., 2020. Evolution and extensive reassortment of H5 influenza viruses isolated from wild birds in China over the past decade. Emerg. Microbes Infect. 9, 1793–1803. https://doi.org/10.1080/22221751.2020.1797542.

Cui, P., Zeng, X., Li, X., Li, Y., Shi, J., Zhao, C., Qu, Z., Wang, Y., Guo, J.,Gu, W.,Ma, Q., Zhang, Y., Lin, W., Li, M., Tian, J., Wang, D., Xing, X., Liu, Y., Pan, S., Zhang, Y., Bao, H., Liu, L., Tian, G., Li, C., Deng, G., Chen, H., 2022. Genetic and biological characteristics of the globally circulating H5N8 avian influenza viruses and the protective efficacy offered by the poultry vaccine currently used in China. Sci. China Life Sci. 65, 795–808. https://doi.org/10.1007/s11427-021-2025-y.

El-Shesheny, R., Franks, J., Turner, J., Seiler, P., Walker, D., Friedman, K., Mukherjee, N., Kercher, L., Hasan, M.K., Feeroz, M.M., Krauss, S., Vogel, P., McKenzie, P., Barman, S., Webby, R.J., Webster, R.G., 2020. Continued evolution of H5Nx avian influenza viruses in Bangladeshi live poultry markets: pathogenic potential in poultry and mammalian models. J. Viro. 94, e01141- 20. https://doi.org/10.1128/jvi.01141-20.

El-Zoghby, E.F., Arafa, A.S., Kilany, W.H., Aly, M.M., Abdelwhab, E.M., Hafez, H.M., 2012. Isolation of avian influenza H5N1 virus from vaccinated commercial layer flock in Egypt. J. Viro. 9, 1–8. https://virologyj.biomedcentral.com/articles/10.1186/1743-422X-9-294.

Ferro P.J., El-Attrache J., Fang X., Rollo S.N., Jester A., Merendino T., Peterson M.J., Lupiani B., 2008. Avian influenza surveillance in hunter-harvested waterfowl from the Gulf Coast of Texas (November 2005-January 2006. J. Wildl. Dis. 44, 434–439. https://doi.org/10.7589/0090-3558-44.2.434.

Ferro P.J., Peterson M.J., Merendino T., Nelson M., Lupiani B., 2010. Comparison of real-time reverse transcription-PCR and virus isolation for estimating the prevalence of avian influenza virus in hunter-harvested wild birds at waterfowl wintering grounds along the Texas mid-Gulf Coast (2005- 2006 through 2008-2009. Avian Dis. 54, 655–659. https://doi.org/10.1637/8810-040109-ResNote.1.

Hagag, N.M., Erfan, A.M., ElHusseiny, M., Shalaby, A.G., Saif, M.A., Tawakol, M.M., Nour, A.A., Selim, A.A., Arafa, A.S., Hassan, M.K., Hassan, W.M.M., Fahmy, H.A., Ibraheem, E., Attia, M., Abdelhakim, A.M.M., Shahein, M.A., Naguib, M.M., 2019. Isolation of a novel reassortant highly pathogenic avian influenza (H5N2) virus in Egypt. Viruses. 11, 565. https://doi.org/10.3390/v11060565.

Hassan, K.E., King, J., El-Kady, M., Afifi, M., Abozeid, H.H., Pohlmann, A., Beer, M., Harder, T., 2020. Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt. Emerg Infect Dis. 26, 129-133. https://doi.org/10.3201/eid2601.190570.

Lewis, N.S., Banyard, A.C., Whittard, E., Karibayev, T., AlKafagi, T., Chvala, I., Byrne, A., Li Y.T., Chen C.C., Chang A.M., Chao D.Y., Smith G.J.D., 2020. Cocirculation of both low and highly pathogenic avian influenza H5 viruses in current poultry epidemics in Taiwan. Virus Evol. 6, veaa037. https://doi.org/10.1093/ve/veaa037.

Lycett, S.J., Pohlmann, A., Staubach, C., Caliendo, V., Woolhouse, M., Beer, M., Kuiken, T., 2020. Genesis and spread of multiple reassortants during the 2016/2017 H5 avian influenza epidemic in Eurasia. Proc. Natl. Acad. Sci. USA 117, 20814–20825. https://doi.org/10.1073/pnas.2001813117.

Mohamed, M.E.M., Ahmed, H.A., Erfan, A.M., Abdelkarim, L., Awadallah, M.A.I., 2019. Endemic Status and Zoonotic Potential of Avian Influenza Viruses in Egypt, 2006-2019. Advances in Animal and Veterinary Sciences 7, 154–162. http://dx.doi.org/10.17582/journal.aavs/2019/7.s2.154.162.

Naguib, M.M., Verhagen, J.H., Samy, A., Eriksson, P., Fife, M., Lundkvist, Å., Ellström, P., Järhult, J.D., 2019. Avian influenza viruses at the wild domestic bird interface in Egypt. Infect. Ecol. Epidemiol. 9, 1575687. https://doi.org/10.1080/20008686.2019.1575687.

Nuñez, I.A., Ross, T.M.A., 2019. Review of H5Nx Avian Influenza Viruses. Therapeutic Advances in Vaccines and Immunotherapy, 7. https://doi.org/10.1177/2515135518821625.

OIE, (2017. Update on highly pathogenic avian influenza in animals (type H5 and H7. Office International Des Epizooties, Paris, France.

Osman, N., Sultan, S., Ahmed, A.I., Ibrahim, R.S., Abd El-Wanes, S.A., Ibrahim, E.M., 2015. Molecular epidemiology of avian influenza virus and incidence of H5 and H9 virus subtypes among poultry in Egypt in 2009-2011. Acta Virologica 59, 27–32. https://doi.org/10.4149/av_2015_01_27.

Pantin-Jackwood, M.J., Costa-Hurtado, M., Bertran, K., DeJesus, E., Smith, D., Swayne, D.E., 2017. Infectivity, transmission, and pathogenicity of H5 highly pathogenic avian influenza clade 2.3. 4.4 (H5N8 and H5N2) United States index viruses in Pekin ducks and Chinese geese. Vet. Res. 48, 1-14. https://doi.org/10.1186/s13567-017-0435-4.

Pohlmann A., King J., Fusaro A. Zecchin B., Banyard AC., Brown I.H., Byrne A.M. P., Beerens N., Liang Y., Heutink R., 2022. Has Epizootic Become Enzootic? Evidence for a Fundamental Change in the Infection Dynamics of Highly Pathogenic Avian Influenza in Europe, 2021. mBio. 13, 00609 –22. https://doi.org/10.1128/mbio.00609-22.

Pyankova, O.G., Susloparov, I.M., Moiseeva, A.A., Kolosova, N.P., Onkhonova, G.S., Danilenko, A.V., 2021. Isolation of clade 2.3.4.4b a(H5N8), a highly pathogenic avian influenza virus, from a worker during an outbreak on a poultry farm, Russia, December 2020. Euro. Surveill. 26, 2100439. https://doi.org/ 10.2807/1560-7917.ES.2021.26.24.2100439.

Qiu, B.-F., Liu, W.-J., Peng, D.-X., Hu, S.-L., Tang, Y.-H., Liu, X.-F., 2009. A reverse transcription-PCR for subtyping of the neuraminidase of avian influenza viruses. J. Virol. Meth. 155, 193–198. https://doi.org/10.1016/j.jviromet.2008.10.001.

Rohaim, M.A., El Naggar, R.F., Madbouly, Y., AbdelSabour, M.A., Ahmed K.A., Munir, M., 2021. Comparative infectivity and transmissibility studies of wild-bird and chicken-origin highly pathogenic avian influenza viruses H5N8 in chickens. Comp. Immunol. Microbiol. Infect Dis. 74, 101594. https://doi.org/10.1016/j.cimid.2020.101594.

Sautto, G.A., Kirchenbaum, G.A., Ross, T.M., 2018. Towards a universal influenza vaccine: different approaches for one goal. Virol J. 15, 17. https://doi.org/10.1186/s12985-017-0918-y.

Selim, A.A., Erfan, A.M., Hagag, N., Zanaty, A., Samir, A.H, Samy, M., Abdelhalim, A., Arafa, A.A., Soliman, M.A., Shaheen, M., Ibraheem, E.M., Mahrous. I., Hassan, M.K., Naguib, M.M., 2017. Highly Pathogenic Avian Influenza Virus (H5N8) Clade 2.3.4.4 Infection in Migratory Birds, Egypt. Emerg Infect Dis. 23, 1048-1051. https://doi.org/10.3201/eid2306.162056.

Shi, J., Deng, G., Ma, S., Zeng, X., Yin, X., Li, M., Zhang, B., Cui, P., Chen, Y., Yang, H., Wan, X., Liu, L., Chen, P., Jiang, Y., Guan, Y., Liu, J., Gu, W., Han, S., Song, Y., Liang, L., Qu, Z., Hou, Y., Wang, X., Bao, H., Tian, G., Li, Y., Jiang, L., Li, C., Chen, H., 2018. Rapid evolution of H7N9 highly pathogenic viruses that emerged in China in 2017. Cell Host Microbe. 24, 558–568.e7. https://doi.org/10.1016/j.chom.2018.08.006.

Son, K., Kim, Y.K., Oem, J.K., Jheong, W.H., Sleeman, J.M., Jeong, J., 2018. Experimental infection of highly pathogenic avian influenza viruses, clade 2.3. 4.4 H5N6 and H5N8, in mandarin ducks from South Korea. Transbound. Emerg. Dis., 65(3): 899-903. https://doi.org/10.1111/tbed.12790.

Starick, E., Römer-Oberdörfer, A., Werner, O., 2000. Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV. J. Vet. Med., Series B 47, 295–301. https://doi.org/10.1046/j.1439-0450.2000.00386.x.

Suarez, D.L., Perdue, M.L., 1998. Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Research 54, 59-69 https://doi.org/10.1016/S0168-1702(98)00011-2.

Sultan, S., Eldamarany, N.M.I., Abdelazeem, M.W., Fahmy, H.A., 2022. Active Surveillance and Genetic Characterization of Prevalent Velogenic Newcastle Disease and Highly Pathogenic Avian Influenza H5N8 Viruses Among Migratory Wild Birds in Southern Egypt During 2015-2018. Food Environ Virol. 14, 280-294. .https://doi.org/10.1007/s12560-022-09532-1.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725– 2729. https://doi.org/10.1093/molbev/mst197.

Tang, W., Li, X., Tang, L., Wang, T., He, G., 2021. Characterization of the low-pathogenic H7N7 avian influenza virus in Shanghai, China. Poultry Science 100, 565–574. https://doi.org/10.1016/j.psj.2020.11.018

Wang, Y., Wang, M., Zhang, H., Zhao, C., Zhang, Y., He, G., Deng, G., Cu, P., Li, Y., Liu, W., Shen, J., Sun, X., Wang, W., Zeng, X., Li, Y., Chu, D., Peng, P., Guo, J., Chen, H., Li, X., 2020. Emergence, Evolution, and Biological Characteristics of H10N4 and H10N8 Avian Influenza Viruses in Migratory Wild Birds Detected in Eastern China in 2020. Microbiol Spectr. 10, e0080722. https://doi.org/10.1128/spectrum.00807-22.

WHO, 2021. Human Infection with Avian Influenza A (H5N8)-the Russian Federation; World Health Organisation: Geneva, Switzerland, 2021. Available online: https://www.who.int/csr/don/26-feb-2021-influenza-a-russian-federation/en/ (accessed on 2 March 2021)

WOAH, 2021. Highly Pathogenic Avian Influenza IN EGYPT Summary experiences, challenges faced and socio-economic impact on the livelihood (2006-2021. https://rrmiddleeast.woah.org/wpcontent/uploads/2021/11/4-highlypathogenic-avian-influenza-2021-egypt.pdf.

Wright, K.E., Wilson, G.A.R., Novosad, D., Dimock, C., Tan, D., Weber, J.M., 1995. Typing and subtyping of influenza viruses in clinical samples by PCR. Journal of Clinical Microbiology 33, 1180–1184. https://doi.org/10.1128/jcm.33.5.1180-1184.1995.

Wright, P.F., 2007. Orthomyxoviruses. Fields Virology, 1691–1740.

Yehia, N., Hassan, W.M.M., Sedeek, A., Elhusseiny, M.H., 2020. Genetic variability of avian influenza virus subtype H5N8 in Egypt in 2017 and 2018. Arch. Virol. 165, 1357–1366. https://doi.org/10.1007/s00705-020-04621-7

Yehia, N., Naguib, M.M., Li, R., Hagag, N., El-Husseiny, M., Mosaad, Z., Nour, A., Rabea, N., Hasan, W.M., Hassan, M.K. Harder, T., Arafa, A.A., 2018. Multiple introductions of reassorted highly pathogenic avian influenza viruses (H5N8) clade 2.3.4.4b causing outbreaks in wild birds and poultry in Egypt. Infect. Genet. Evol. J. 58, 56–65. https://doi.org/10.1016/j.meegid.2017.12.011.