



Epidemiological Disclosing and Molecular Subtyping for the Highly Pathogenic Avian Influenza Viruses H5N8 in Commercial Broilers and Layer Chickens in some Egyptian Governorates



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HIGHLY pathogenic avian influenza virus (HPAIV) H5N8 is a serious and fatal respiratory viral disease with an increase in the series of outbreaks in poultry field since its first emergence in Egypt from 2016 till now. Oral and cloacal swabs pool were collected from Sixty domestic poultry flocks of commercial broilers and layer chickens in four Egyptian governorates during 2023 that suffered from classic clinical disease of HPAIV with various mortalities reached 70% during the surveillance period. Molecular analysis was employed to analyze HPAIV subtypes H5 and NA (N1 and N8) by reverse transcriptase polymerase chain reaction (RT-PCR) and further partial hemagglutinin (HA) gene sequencing was carried out also to four positive representative isolates. The results initially revealed the negativity of all tested samples to H5N1 subtype, while H5N8 genome was predominantly positive in 17 out of the 60 examined flocks with prevalence rate 28.3%. Furthermore, the sequencing and phylogenetic tools indicated that our H5N8 isolates were in a close proximity to the clade 2.3.4.4b with the characteristic amino acid motif (PLREKRRKR/GLF) in the HA gene cleavage site which is representative for pathogenic AIV strains. This study concluded the prevalence and predominance of HPAIV H5N8 in the domestic poultry farms in Egypt even in immunized flocks with the strict need of regular monitoring and active surveillances to those circulated strains as well as re-evaluation of the prevention and control protocols and also the vaccination efficacy assessment to overcome such endemic disease.

Keywords: Highly pathogenic avian influenza virus; H5N8 subtype; surveillance; Domestic poultry; clade 2.3.4.4b.

Introduction

Highly pathogenic avian influenza virus (HPAIV) belongs to influenza A viruses are a single stranded, negative sense RNA viruses related to family Orthomyxoviridae [1]. They are classified according to their antigenic properties of the surface glycoproteins of 16 Hemagglutinin (HA) and 9 Neuraminidase (NA) subtypes [2].

Basically, HPAIV H5N8 of clade 2.3.4.4 were classified into two groups A and B which are highly pathogenic to both domestic poultry and wild birds without any reports about human cases hazard [3, 4]. The HPAIV A/ H5N8 was firstly reported and isolated in 2010 in china

from live bird markets [5], thereafter, continuous outbreaks of H5N8 clade 2.3.4.4 occurred in domestic and wild birds in the early 2014 in South Korea [6]. By the late 2014, the virus further spread to other localities in Asia, Europe and North America through migratory birds causing frequent outbreaks with the clade 2.3.4.4a [7, 8]. Later on and recently in 2017, a new variant H5N8 virus was isolated and arised in Russia, Middle East and many localities of Africa [9, 10].

In Egypt during the late 2016, the H5N8 virus was firstly recognized and isolated from green-winged teals and common-coots [10, 11]. Thereafter,

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a definitely six genotypes of H5N8 with various reassorted combinations were reported in both domestic and migratory birds in Egypt in which the virus spreads vigorously in Egyptian poultry farms causing significant hazard to the poultry sectors [2, 12, 13].

HPAIV H5N8 that was identified in Europe and Asia during 2019 and 2020 were found in a close proximity and phylogenetically related to those isolated in Egypt at the same time from the clade 2.3.4.4 based on HA gene sequencing suggesting that most of H5N8 isolates may be originated from the same ancestral descent [8, 12, 14].

Based on the clinical picture, the HPAIV H5N8 infection is recognized by rapid onset of sudden deaths with increased mortalities and birds have multiple signs of lethargy, off-food, eye edema, cyanosis in combs and shank and severe respiratory illness along with obvious nervous manifestations [15].

So as to, in the present study, oral and cloacal swabs were collected from commercial broilers and layer chickens in 4 Egyptian governorates with a history of suspected clinical disease to HPAIV for isolation and identification of AIV HA and NA subtypes and further partial HA gene sequencing in order to determine the prevalence rate and the phylogenetic homology of the currently circulated strains from HPAIV in chicken flocks in Egypt during 2023.

Material and Methods

Ethical approval and Biosafety

Birds swabbing and sampling were approved by the Medical Research Ethics Committee (MREC) of the National Research Centre, Dokki, Cairo, Egypt (Ethical approval number: 0620223-1 in October 2023) and compatible with the strict protocol for well handling and care of birds.

Birds sampling and surveillance study

A total of sixty commercial poultry flocks (35 Broilers and 25 Layers) were swabbed by oropharyngeal and cloacal swabs in which samples were collected from February till August 2023 from clinically manifested birds exhibiting flu like symptoms or freshly died ones in 4 different Egyptian governorates including Alexandria, El Behera, El Fayoum and El Gharbia. Oropharyngeal and cloacal swabs from each flock were pooled together and suspended in 2 ml phosphate buffer saline supplemented with 1% antibiotic-antimycotic solution and clarified by centrifugation at 5000 rpm for 10 min following the recommendations of OIE

[16] and then stored in -80°C for further isolation and subtyping identification for AIV.

Virus isolation and Haemagglutination test (HA)

All collected samples were inoculated singly in the allantoic sac of 11 days-old specific pathogen free embryonated chicken eggs (ECE) and monitored daily for 5 days for any embryonic deaths, and then the allantoic fluids were harvested and tested for HA test via 0.5 % chicken red blood cells following the standards of OIE [17].

AIV one step RT-PCR and subtyping

All positive HA samples were subjected to viral RNA extraction using QIA amp viral RNA mini kits (Qiagen, Germany) in accordance with manufacturer procedures. All samples were initially tested for AIV by one step RT-PCR targeting the HA gene (H5) of type A influenza virus with specific primers according to Lee *et al.* [18] as shown in table.1 with thermal cycling condition indicated as; reverse transcription (42°C for 45 min), initial denaturation (95°C for 3 min), 40 cycles of 95°C for 30 s as denaturation, annealing (50°C for 40 s) and extension (72°C for 40 s), followed by 72°C for 10 min for final extension. The positive H5 AIV samples were further subtyped for NA gene (N1 and N8) as previously described by Fereidouni *et al* [19] with primers specific to NA genes as presented in table.1 and thermal cycle condition of 30 min at 50 °C as a reverse transcription cycle and then an initial denaturation of 95 °C for 2 min followed by five touch-down PCR cycles starting with 94 °C for 15 s, 60 °C (decrement of 1 °C per cycle) for 30 s, 68 °C for 1 min and followed by 30 cycles of 94 °C for 15 s, 54 °C for 15 s, 68 °C for 1 min and a final extension at 68 °C for 5 min.

Sequence analysis and phylogenesis

Partial HA gene sequencing of 4 selected H5-positive isolates representative to the four Egyptian governorates with obvious viral load was carried out using Sanger dideoxy sequencing after purification of those samples from gel using QIA quick gel extraction kits (Qiagen). Multiple available sequences of HPAI H5N8 viruses from Egypt and other global or neighbouring countries were obtained from influenza data base in Genbank. Thereafter, multiple alignments and blasting were achieved using Clustal W tool in Lasergene software for the obtained sequences. Also amino acid identity matrix was analyzed for the selected isolates with other reference strains using DNA star Lasergene software. Finally sequences of the selected positive AIV isolates were submitted to Genbank data base and phylogenetic evaluation was conducted through 1000 trials boost trap using maximum like hood software analysis.

TABLE 1. Oligonucleotide primers targeting AIV HA and NA genes for one step RT-PCR.

H5	H5-155f	5-ACACATGCYCARGACATACT	545 bp
	H5-699r	5-CTYTGRTTYAGTGTTGATGT	
N1	N1-f	AGR ^a CCTGYTCTGGGTTGA	126 bp
	N1-R	ACCGTCTGGCCAAGACCA	
N8	N8-F	GGTCAGGATAYAGYGGTTCYTTCAC	145 bp
	N8-R	CCACACATCACAATGGAGCT	

^a Codes for ambiguous bases position and NTP analogues: R = A/G, Y = C/T, I = inosine. bp: base pair

Results

Features of outbreak during sampling

During our surveillance the suspected AIV infected flocks showed a comprehensive signs of weakness, lack of food, ruffled feather and eventually sudden death along with respiratory signs of sneezing, cough, rales and nasal discharges. Moreover, most of investigated chicken flocks exhibit marked septicaemia with cyanosis in comb and wattles as well as shank subcutaneous haemorrhages. In addition to, birds with long coarse disease exhibited nervous signs of tremors and torticollis with elevated mortalities and also sever decline in egg productivity in layer chicken flocks. In the base of post-mortem gross picture the necropsy of examined dead birds detected presence of muscle and subcutaneous tissue haemorrhages, enlarged and necrotic spleen, hemorrhagic tracheitis, fibrinous pneumonia, nephritis and haemorrhages on heart fats. Most of positive AIV suspected flocks expressed mortality rates reached 75% even those flocks have been vaccinated against HPAI either with classic inactivated H5 vaccines or viral-vectored H5 vaccines.

Virus isolation, propagation and HA test

Out of 60 farms in 4 different Egyptian governorates were surveyed in this study, most of inoculated samples in ECE revealed the characteristic lesions of hemorrhagic embryos accompanied with deaths of some embryos during the first 48 hours post-inoculation. In addition to, a total of 25 samples were found positive for HA test (n=25/60) with a percentage of 41.6% of tested samples as described in supplementary table 2.

RT-PCR detection of HPAIV

A total of sixty samples from tracheal and cloacal swabs pool were collected and positive HA samples were screened by one step RT-PCR for presence of AIV H5, N1 and N8 subtypes. Initially, all tested samples were found negative to H5N1 subtype, while H5N8 genome was discovered in 17 out of the 60 examined chicken flocks (Fig 1& 2) with detection rate 28.3% and species distribution of 3 and 6 positive H5N8 cases from 35 broiler flocks in Alexandria and El Behera respectively, moreover 8 H5N8 positive cases in 25 commercial layers flocks in both El Gharbia and El Fayoum governorates as mentioned in table 2.

TABLE 2. Percent of HA and RT-PCR positive cases for examined chicken flocks in different Egyptian governorates

Governorate	Flock type	No of collected samples	HA positive samples	HPAIV subtype by RT-PCR	
				H5N1	H5N8
Alexandria	Broilers chickens	10 flocks	5	0	3
El Behera	Broilers chickens	25 flocks	10	0	6
El Fayoum	Layers chickens	10 flocks	5	0	4
El Gharbia	Layers chickens	15 flocks	5	0	4

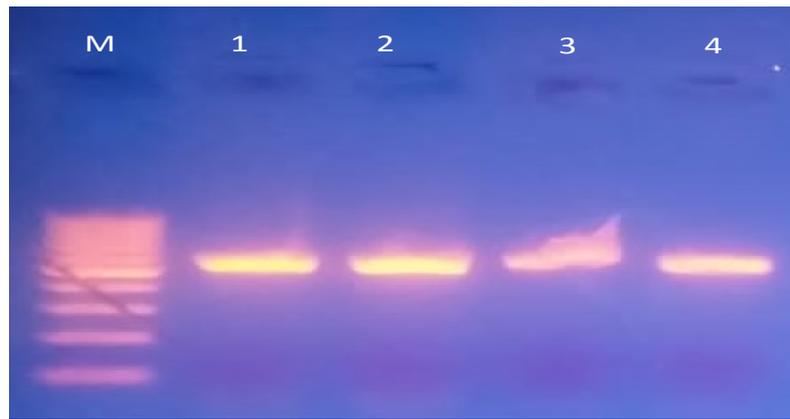


Fig. 1. Agarose gel electrophoresis pattern of the amplified products (545 bp) by RT-PCR for H5 subtype. M: 100 bp DNA ladder; Lane 1: Control positive for H5; Lane 2, 3 and 4: Positive H5 subtype specific bands of 545 bp amplicon.

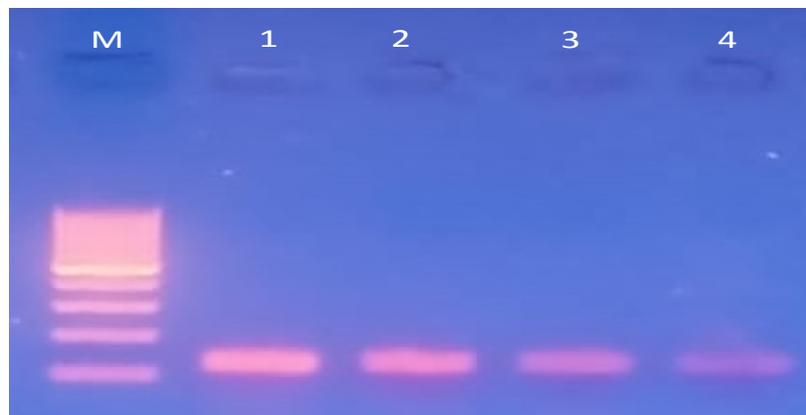


Fig. 2. Agarose gel electrophoresis pattern of the amplified products (145 bp) by RT-PCR for N8 subtype. M: 100 bp DNA ladder; Lane 1: Control positive for N8; Lane 2, 3 and 4: Positive N8 subtype specific bands of 145 bp amplicon.

Genetic evaluation and phylogenetic analysis of HPAI H5N8

Four representative molecularly identified influenza A H5N8 isolates were subjected to partial HA gene sequencing and submitted to Genbank which designated as; A/Broiler/EGY/Alex/NRC2023/H5N8, A/broiler/EGY/BH/NRC2023/H5N8, A/Layer/EGY/FAY/NRC2023/H5N8 and A/Layer/EGY/GH/NRC2023/H5N8 with accession numbers; OR636449, OR636453, OR636485 and OR636486, respectively as shown in table 3.

The phylogenetic analysis of HA gene of the 4 selected isolates in compared to other Egyptian H5N8 isolates and strains from related and neighbouring countries and global ones revealed that our isolates are clustered in the definite 2.3.4.4b clade with closely relation to other H5N8 viruses from Egypt, China, Iran, Iraq, Israel, Saudi Arabia and France currently circulated in wild birds, aquatic birds and domestic chicken flocks (Fig.3). Moreover, the HA gene multibasic

cleavage site characteristic for HPAIV was detected in our isolates with amino acid (AA) motif PLREKRRKR/GLF as well as in other reference Egyptian and global reference strains supplemented in this study and mentioned in Fig.4. Furthermore, the genetic analysis of AA identity percent showed that our study isolates have identity percent among each others varied from 99.0 to 99.7%. While our isolates identity with other Egyptian strains like A/Chicken/Egypt/134FAO-s/2022, A/chicken/Layer/Egypt/MR18/2018 and A/common teal/Egypt/MB-D-8290P/2016 was found ranged from 95.6 to 99.4%. In addition to, AA identity percent of our isolates with other global reference H5N8 strains from China, Iran, Iraq, Israel, Saudi Arabia and France were inside the range of 95.7 to 99.8% suggesting that most of H5N8 isolates of the clade 2.3.4.4 may be descendant from similar ancestor isolates (Table 4).

TABLE 3. Characteristic features of the obtained Egyptian H5N8 isolates of the current study

Isolate name	Accession number	Locus	Cleavage site	Host	Isolation year	Province
A/Broiler/EGY/Alex/NRC2023/H5N8	OR636449	HA gene	PLREKRRKR/GLF	Broilers	2023	Alexandria
A/Broiler/EGY/BH/NRC2023/H5N8	OR636453	HA gene	PLREKRRKR/GLF	Broilers	2023	El Behera
A/Layer/EGY/FAY/NRC2023/H5N8	OR636485	HA gene	PLREKRRKR/GLF	Layers	2023	El Fayoum
A/Layer/EGY/GH/NRC2023/H5N8	OR636486	HA gene	LREKRRKR/GLF	Layers	2023	El Gharbia

TABLE 4. Amino acid sequence identity of the obtained H5N8 isolates with other strains circulating in Egypt and other countries showing identity and divergence percent, black squares indicate identical sequence.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Divergenc	1	99.0	99.3	99.7	98.4	97.0	97.4	97.2	97.9	97.7	97.6	95.0	96.9	97.4	97.6	1
	2	0.4	99.2	99.0	98.7	96.3	95.7	95.6	98.0	97.0	97.9	96.3	97.2	95.7	98.9	2
	3	0.4	0.4	99.1	98.6	96.4	99.6	99.4	98.4	98.9	99.8	97.2	99.1	99.6	99.8	3
	4	0.8	0.8	0.8	98.2	96.1	97.2	97.0	97.9	96.5	97.4	96.2	96.7	97.2	97.4	4
	5	0.4	0.4	0.4	1.1	97.8	99.6	99.4	95.7	99.3	99.8	97.6	99.4	100.0	99.8	5
	6	3.4	3.8	3.8	3.8	3.4	92.4	92.2	98.0	92.0	92.6	97.0	97.2	98.8	98.6	6
	7	0.4	0.4	0.4	1.1	0.4	3.8	99.4	97.4	98.9	99.8	97.2	99.1	99.6	99.8	7
	8	0.6	0.6	0.6	1.3	0.6	4.0	0.6	97.6	99.1	99.6	97.0	99.3	99.4	99.6	8
	9	4.1	4.4	4.8	5.1	4.4	6.7	4.8	4.6	96.3	95.6	96.1	95.6	95.7	96.6	9
	10	1.1	1.2	1.1	1.9	0.7	4.2	1.1	0.9	3.8	99.1	96.9	99.1	99.3	99.1	10
	11	0.2	0.2	0.2	0.9	0.2	3.6	0.2	0.4	4.6	0.9	97.4	99.3	99.8	100.0	11
	12	2.9	3.0	2.8	3.3	2.5	3.2	2.8	3.0	6.2	3.2	2.6	97.0	97.6	97.4	12
	13	1.0	1.0	0.9	1.7	0.6	4.0	0.9	0.7	4.6	0.9	0.7	3.0	99.4	99.3	13
	14	0.4	0.4	0.4	1.1	0.0	3.4	0.4	0.6	4.4	0.7	0.2	2.5	0.6	99.8	14
	15	0.2	0.2	0.2	0.9	0.2	3.6	0.2	0.4	4.6	0.9	0.0	2.6	0.7	0.2	15
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	

- ▶ A-Broiler-EGY-Alex-NRC2023-H5N8
- ▶ A-broiler-EGY-BH-NRC2023-H5N8
- ▶ A-Layer-EGY-FAY-NRC2023-H5N8
- ▶ A-Layer-EGY-GH-NRC2023-H5N8
- A-goose-Iran-180-2016
- A-chicken-Egypt-134FAO-S-2022
- A-chicken-layer-Egypt-MR18-2018
- A-commonteal-Egypt-MB-D-829OP-2016
- A-commonteal-Shanghai-PD1108-13-2013
- A-duck-EasternChina-S1109-2014
- A-greagret-Israel-1084-2016
- A-Layinghen-Iraq-H5N8-2021
- A-ostrich-SaudiArabia-KFU-HKU3594-2017
- A-swan-France-171377-2017
- A-turkey-Israel-184-2017

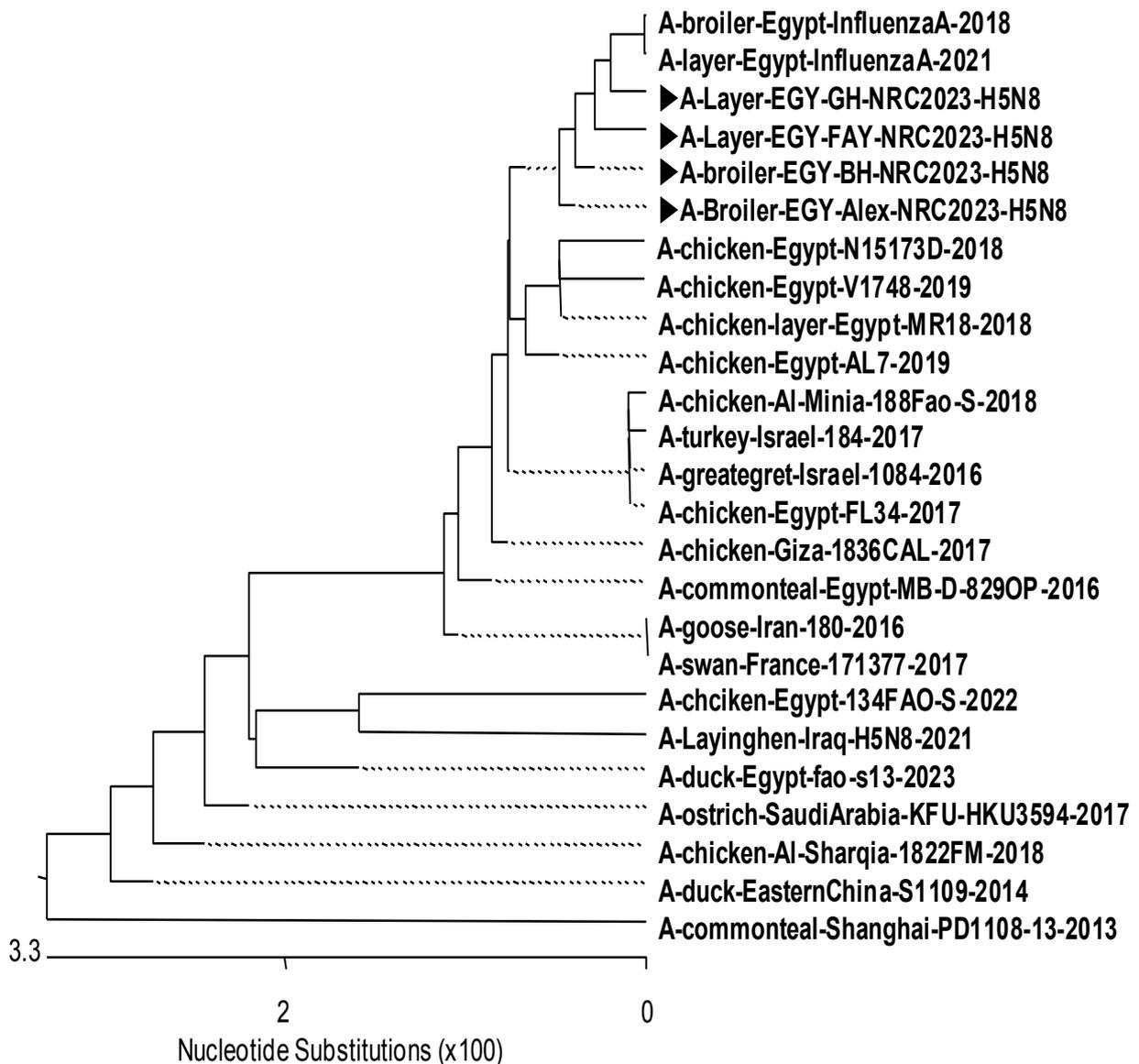


Fig. 3. Phylogenetic relationship between the H5N8 isolates clade 2.3.4.4b obtained in this study and other previously isolated in Egypt with some reference strains retrieved from the Genbank by Maximum likelihood method. ► Isolates of the study.

The HA glycoprotein is the primary determinant of Influenza A viruses virulence with receptor binding properties [30]. The HA cleavage site of the most previously isolated H5N8 strains encodes the motif PLREKRRKR/GLF which is characteristic for pathogenicity of those isolates, our results concluded that and were similar to other recent investigations by many studies [6, 13, 21, 23, 31] who concluded that their selected H5N8 isolates have the same motif in HA cleavage site.

Furthermore, the genetic analysis of AA identity percent showed that our study isolates have identity percent among each others varied from 99.0 to 99.7% as in previously reported by Setta *et al.* [23] and similar to our results who detected a close proximity to their H5N8 isolates to each others, while found them spaced out in AA identity percent of their isolates with H5 vaccinal strains. In addition to, AA identity percent of our isolates with other global strains from China, Iran, Iraq, Israel, Saudi Arabia and France were inside the range of 95.7 to 99.8% suggesting that most of H5N8 isolates of the clade 2.3.4.4 are coming down from the same ancestor origin [12].

Conclusion

During our active surveillance, we recorded the prevalence of HPAIV H5N8 in poultry flocks in four different Egyptian governorates with high mortality rate in infected flocks raising the importance to the need of periodical screening to those circulated strains and updating the prevention and control measures as well as the awareness with usage of matched vaccination regimes to the currently endemic clade 2.3.4.4b of HPAIV H5N8 in poultry field in Egypt.

Author's Contribution

All authors equally participated in design, experimental procedure, writing, revised, and reviewing the manuscript.

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Conflict of interest

The authors have declared no conflict of interest.

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التقصي الوبائي والتصنيف الفرعي الجزيئي لفيروسات أنفلونزا الطيور H5N8 شديدة الضراوة في دجاج التسمين التجاري والدجاج البياض في بعض المحافظات المصرية

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فيروس أنفلونزا الطيور شديد الضراوة H5N8 هو مرض فيروسي تنفسي خطير ومميت مع التزايد في معدل تفشي المرض في حقول الدواجن منذ ظهوره لأول مرة في مصر من عام 2016 حتى الآن. تم جمع مسحات الفم وفتحة المجمع من ستين قطيعاً من الدواجن من دجاج اللحم التجاري والدجاج البياض في أربع محافظات مصرية خلال عام 2023 والتي عانت من المرض السريري الكلاسيكي لفيروس أنفلونزا الطيور مع معدلات نفوق مختلفة وصلت إلى 70% خلال فترة المراقبة. تم استخدام التحليل الجزيئي لتحليل الأنواع الفرعية لفيروس (H5, NA (N1 and N8 عن طريق تفاعل البوليميراز المتسلسل العكسي (RT-PCR) وتم إجراء تسلسل جيني جزئي للهيماجلوتينين (HA) أيضاً على أربع عزلات تمثيلية إيجابية. وكشفت النتائج مبدئياً سلبية جميع العينات التي تم اختبارها للنوع الفرعي H5N1، في حين كان جينوم H5N8 إيجابياً وهو الغالب في 17 من أصل 60 قطعاً تم فحصها بمعدل انتشار 28.3%. علاوة على ذلك، أشارت أدوات التسلسل والتطور إلى أن عزلات H5N8 الخاصة بنا كانت على مقربة من الفرع b2.3.4.4 مع نموذج الحمض الأميني المميز (PLREKRRKR/GLF) في ممثل موقع إنشطار الجينات HA لسلاسل فيروس أنفلونزا الطيور المسببة للأمراض. خلصت هذه الدراسة إلى مدى انتشار وهيمنة فيروس H5N8 في مزارع الدواجن المحلية في مصر مع الحاجة الصارمة للمراقبة المنتظمة والمراقبة النشطة لتلك السلالات المنتشرة وكذلك إعادة تقييم بروتوكولات الوقاية والسيطرة وكذلك تقييم فعالية التحصينات للتغلب على مثل هذا المرض المستوطن.