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Highly Pathogenic Avian Influenza H5N1 in Chickens in Upper Egypt

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Abstract

One-year surveillance of HPAI (H5N1) virus infection in different chicken flocks (141 flock) from different Governorates in Upper Egypt was carried out, during the period from January 2015 to January 2016. The detection rate of highly pathogenic avian influenza (HPAI) H5N1 virus was 21.3% (30/141) which was highest during the winter season compared with the other seasons. The investigated flocks suffered from respiratory and nervous signs and cyanosis of the comb and wattles was observed in some birds as well as subcutaneous hemorrhages in shanks. The thirty positive H5N1 samples were tested for the existence of other respiratory viruses. The results were as follows: single H5N1 virus infection was only detected in 5 flocks (16.6%). The mixed H5N1 with H9N2 in 2 flocks (6.6%); H5N1with NDV in 12 farms (40%); H5N1 with IBV in 7 farms (23.3%) while H5N1, H9N2 and NDV were detected together in 4 flocks (13.3 %). The sequence analysis of the HA gene from five selected H5N1 isolates revealed that these strains were clustered with Egyptian classical H5N1 viruses (sub-clade 2.2.1.2) of the Eurasian origin. It is concluded that, the five H5N1 subtype isolates of HPAI are clustered with classical H5N1 viruses to subclade 2.2.1.2 of Eurasian origin. Therefore, vaccination of the backyard chicken as well as chicken farms with suitable homologues vaccine to eliminate HPAI virus from Egypt is recommended.

Keywords: Highly Pathogenic Avian Influenza H5N1, Chickens, Upper Egypt.

Introduction

Avian Influenza (AI) viruses are members in the family Orthomyxoviridae, of the genus influenza virus type A. The genome of AI viruses is composed of single stranded RNA with eight gene segments encoding at least ten viral proteins [1]. Based on the haemagglutinin (HA) and neuraminidase (NA), the two-main surface transmembrane glycoproteins, there are at least 18 HA and probably 10 NA subtypes [1]. According to their pathogenicity in chickens, AIV_s which cause asymptomatic infection are recognized as low pathogenic AIV (LPAIV). Meanwhile those AIV_s causing high mortalities are recognized as highly pathogenic AIV (HPAIV).

In Egypt, in February 2006, severe outbreaks of HPAIV H5N1 emerged in several Governorates and were associated with drastic mortality up to 100% in infected chickens [2]. The Egyptian strains belonged to subclade 2.2 of the H5N1 virus of Eurasian origin, which is also circulating in the Middle East region. Genetic and antigenic analysis of the circulating field viruses in vaccinated flocks revealed subtype antigenic variation and antigenic drift of the virus in comparison to vaccine strains [3-9].

These mutations allowed the virus to evade the receptor of host immune responses after vaccination [10]. Studies have reported that stable lineages of H5N1 viruses are found in vaccinated chickens and humans in Egypt [11]. Two different H5N1 groups of viruses are currently co- circulating in Egypt referred to as classic strains 2.2.1 which is usually isolated from backyard birds (rarely from vaccinated small commercial farms) and humans and variant strains 2.2.1.1 and 2.2.1.2 subclades, isolated from vaccinated and backyard birds, respectively [12,13]

In order to recognize the spread of newly emerging influenza variants, continuous surveillance and phylogenetic analysis of the circulating HPAIV H5N1 are necessary [14]. This study aimed to investigate the occurrence of HPAIV H5N1 in commercial chicken flocks in Upper Egypt and determination of genotypic characters of H5 gene.

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Materials and Methods

Samples

Tracheal, cloacal swabs (n=282) and specimens from internal organs (n=141) including (liver, spleen, lung and trachea) prepared as pooled samples, were collected from diseased and freshly dead birds. These specimens represented 141 chicken flocks located in 6 different Governorates in Upper Egypt including Assiut (n=87), El-Minia (n=32), Sohag (n=14), Quena (n=3), Aswan (n=3) and Elwady Elgadid (n=2).

Virus isolation

The tissue samples were homogenized and centrifuged at 3000 rpm for 15 min and the supernatant was collected. The collected swabs and homogenized fluid were mixed with antibiotic solution and inoculated aseptically into 9-11 day old embryonated chicken eggs (free from antibodies against H5N1) via allantoic sac (AS) route using 0.1 mL/egg. The inoculated eggs were incubated at 37^{o}C and candled daily for 7 days and the allantoic fluids from dead embryos were collected.

Haemagglutination (HA) test was performed and if there was no HA activity, another second passage was done before identifying the specimen as negative [15].

Detection of viral RNA using rRT-PCR

All HA positive isolates were further identified using Real Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) with specific primers and probes for AI subtype H5 [16]. The sequences of the primers and probe are listed in Table (1). Extraction of Viral RNA was carried by Thermo Scientific GeneJET Viral RNA Purification Kit according to the manufacturers' instructions.

Table 1: Sequences of primers and probes for detection of AI, ND and IB viruses in chickens in Upper Egypt

Target gene	Primer	Sequence (5'-3')
H5 (rRT-PCR)	H5LH1	5'-ACA TAT GAC TAC CCA CAR TAT TCA G-3'
	H5RH1	5' AGA CCA GCT AYC ATG ATT GC 3'
	H5PRO (Probe)	5' FAM-TCW ACA GTG GCG AGT TCC CTA GCA TAMRA 3'
H9	H9-f	5'-CTY CAC ACA GAR CAC AAT GG-3'
	H9-R	5'-GTC ACA CP GTT GTTGTR TC-3'
NDV	ND-f	5'-TGG AGC CAA ACC GCG CAC CTG CGG-3'
	ND-R	5'-GAG GAT GTT GGC AGC AT-3'
IB	XCE3	5'-CAGATTGCTTACAACCACC -3'
	BCEI	5'-AGTAGTTTTGTGTATAAACCA-3'
	DCEI	5'- TTCCAATTATATCAAACCAGC-3'
	MCEI	5'- AATACTACTTTTACGTTACAC-3'
H5 (RT-PCR)	H5- kho-l	5'-CCT CCA GAR TAT GCM TAY AAAATT GTC-3'
	H5- kho-3	5'-TAC CAA CCG TCT ACC ATK CCYTG-3'

The thermo scientific verso 1- step qRT-PCR kit plus Rox vial was used. A total volume of 25 µL containing nuclease free water (3.75 µL), 2X 1- step PCR ready mix (12.5 µL), RT- enhancer (1.25 µL), forward primer (1 µL), reverse primer (1 µL), Verso enzyme mix (0.25 µL), probe (0.25 µL), extracted RNA (5 µL). Thermal cycling consisted of 30 min at 50°C, 15 min at 95°C; then 40 cycles of 10 sec at 95°C, 30 sec at 54°C, and 10 sec at 72°C.

Detection of coinfection using RT-PCR

All positive samples with H5 subtype using rRT-PCR were re-tested for the existence of co-infection with H9 subtype [17], NDV [18] and IBV [19] using Reverse Transcription (RT- PCR) (Table 1).

Viral RNA was extracted from all allantoic fluids using Gene JET Viral DNA and RNA purification kit (Thermo scientific, USA) Cat. No. Ko821. The procedure was performed according to the manufacturer's instruction. Amplification reaction was carried out using Verso one step RT-PCR ready-Mix (Cat. No

AB- l454/LD/A) (Thermoscientific, Germany). Reaction mix of RT- PCR: A total volume of 25 µL containing 12.5 µL RT-PCR Master mix, 1 µL of forward primer and 1 µL of the reverse primer, 0.5 µL of RT enzyme mix, 1 µL of RT enhancer, 5 µL of template RNA with 4 µL of RNase-free water. Thermal cycling consisted of 30 min at 50°C, 15 min at 95°C; then 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C. The reaction was performed in a Stratagene MX3005P RT-PCR machine. For visualization of the amplified products, 2% agarose solution was prepared in Tris Acetate EDTA (TAE) and 5 µL of Ethidium Bromide was added after heating. Ladder DNA (5 µL) was added in the 1st lane then 5 µL of each sample were added in each lane and the gel was visualized after 40 min of electrophoresis by UV trans-illuminator.

Sequencing of 5 selected H5 isolates

The HA gene of H5 subtypes was amplified using conventional RT-PCR [20] with specific primers (Table 1). The reaction conditions were performed with a first step of reverse transcription at 50°C for 30 min (one cycle) followed by one cycle of initial denaturation at 94°C for 5 min. The amplification cycles consisted of 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec and extension at 72°C for 45 sec followed by 1 cycle of final extension at 72°C for 10 min. The amplified products were visualized as previously described. Extraction and purification of the amplicons was performed using QIAquick PCR Product Extraction kit (Qiagen, Valencia CA). The purified products were sequenced with Big dye terminator v 3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA Cat. number 4336817).

Phylogenetic analysis

The truncated nucleotide sequences obtained from PCR products were aligned with other HA gene sequences available in GenBank by the ClustalW method, using the MegAlign module of DNAStar software (Lasergene version 7.2; DNASTAR, Madison, WI, USA). The phylogenetic tree was generated using the neighbor-joining method in MEGA version 5 (www.megasoftware.net). The tree topology was evaluated by 1000 bootstrap analyses.

Results

Specimens from 141 commercial chicken flocks were collected from diseased and freshly dead birds suffering from high mortality, respiratory and nervous signs, cyanosis of comb and wattles as well as subcutaneous hemorrhages in the shank (Figures 1.1 and 1.2) from January 2015 till January 2016.

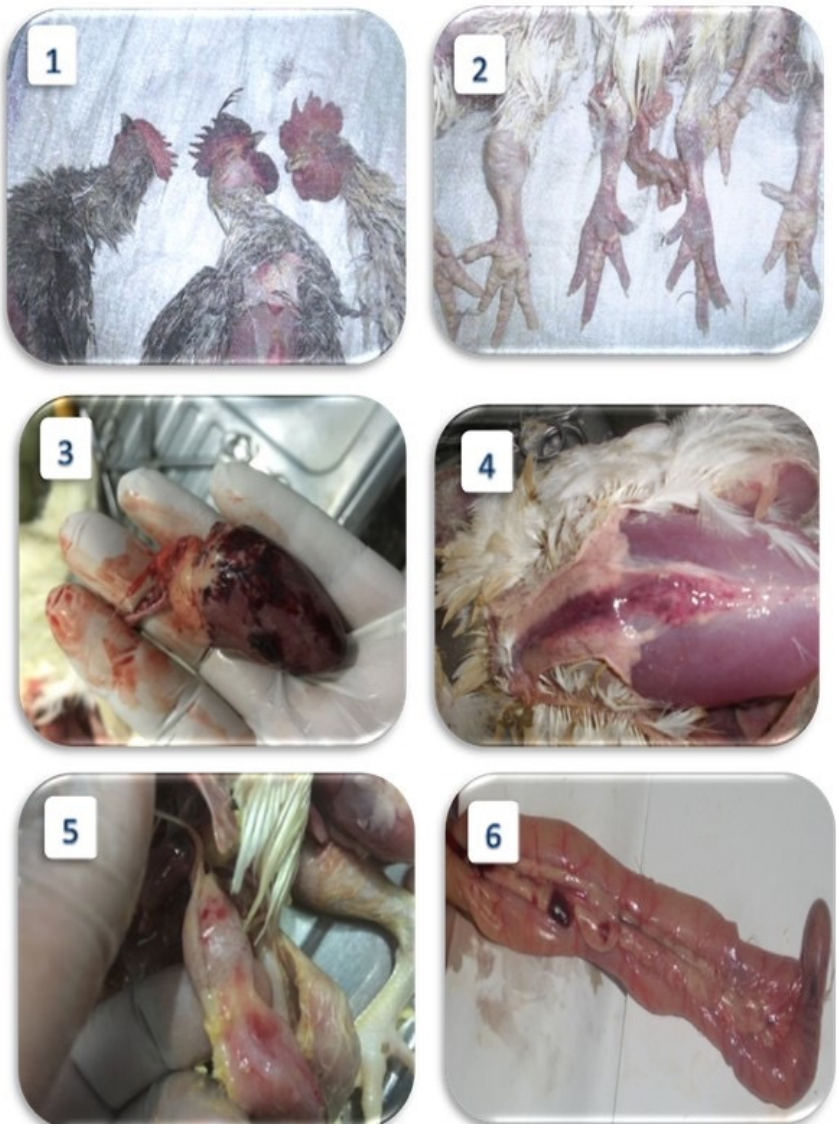

Figure 1: Clinical findings of naturally infected Chicken with HPAI of the H5N1 subtype: 1: Native breed breeders showing cyanosis of comb and wattles; 2: Broiler chicken showing hemorrhages of the shank; 3: Chicken displaying petechial hemorrhages in the myocardium; 4: Chicken, Presenting with abundant serofibrinous subcutaneous edema and hemorrhages in sternal region.;5: Chicken showing petechial haemorrhages on the serosal surface of the proventriculus and 6: Chicken exhibiting hemorrhagic pancreatitis.

All chicken flocks (141) had mortality rate of 10-25% at last 3 days before sampling, the dead birds showed gross lesions of myocardium hemorrhages (Figure 1.3), sternal edema (Figure 1.4), proventriculus outer serosal surface hemorrhages (Figure 1.5), and pancreatitis (Figure 1.6).

Detection of H5N1 virus in chicken flocks in Upper Egypt

Seventy-one samples exerted positive HA activity of the inoculated allantoic fluid. Thirty samples were positive for H5 subtype of AIV using rRT-PCR, the detailed data are presented in Table (2).

The thirty positive samples (30) for subtype H5 were selected for further tested via using RT-PCR against AI H9, NDV and IBV to detect the co-infection in positive samples. The results revealed the existence of H5 and NDV in 12 samples, H5with H9 in 2 samples, H5 and IBV in 7 samples meanwhile H5, H9 and NDV were detected in 4 samples and only

5 samples were positive for H5 single infection.

There was a high incidence of HPAIV subtype H_5N_1 during winter season (76.7%) then gradually decreased during the rest of the year.

Sequencing for HA gene for the selected isolates

Partial hemagglutinin (HA) gene sequencing (segment 4) was done for five selected isolates which have single infection with HPAI (H5N1) representing Assiut (3), Elmenia (1) and Elwady Elgaded (1) Governorates was conducted in order to perform genetic characterization of the investigated isolates. The isolates were taken from non-vaccinated flocks, single infection was confirmed by RT-PCR to exclude mixed infection.

Table 2: PCR results of H5N1 virus infection in chicken flocks of upper Egypt Governorates

Governorate	No. of tested flock	No. of + ve HA	No. of + ve H_5	% of +ve 30 flocks
Assiut	87	40	17	56.6%
Elmenia	32	18	7	23.4%
Sohage	14	9	2	6.6%
Quena	3	1	1	3.4%
Aswan	3	1	1	3.4%
Elwady Elgaded	2	2	2	6.6%
The Total	141	71	30	21.3% of +ve (H_5)

Phylogenetic analysis

The obtained partial sequences of HA gene of H5 subtype-AI viruses were compared with other strains from Asia and Europe available from Gene Bank. The data revealed that all the examined strains belonged to subclade 2.2.1.2 of the H5N1 virus of Eurasian origin, which is also circulating in the Middle East region and was introduced into Africa since early 200

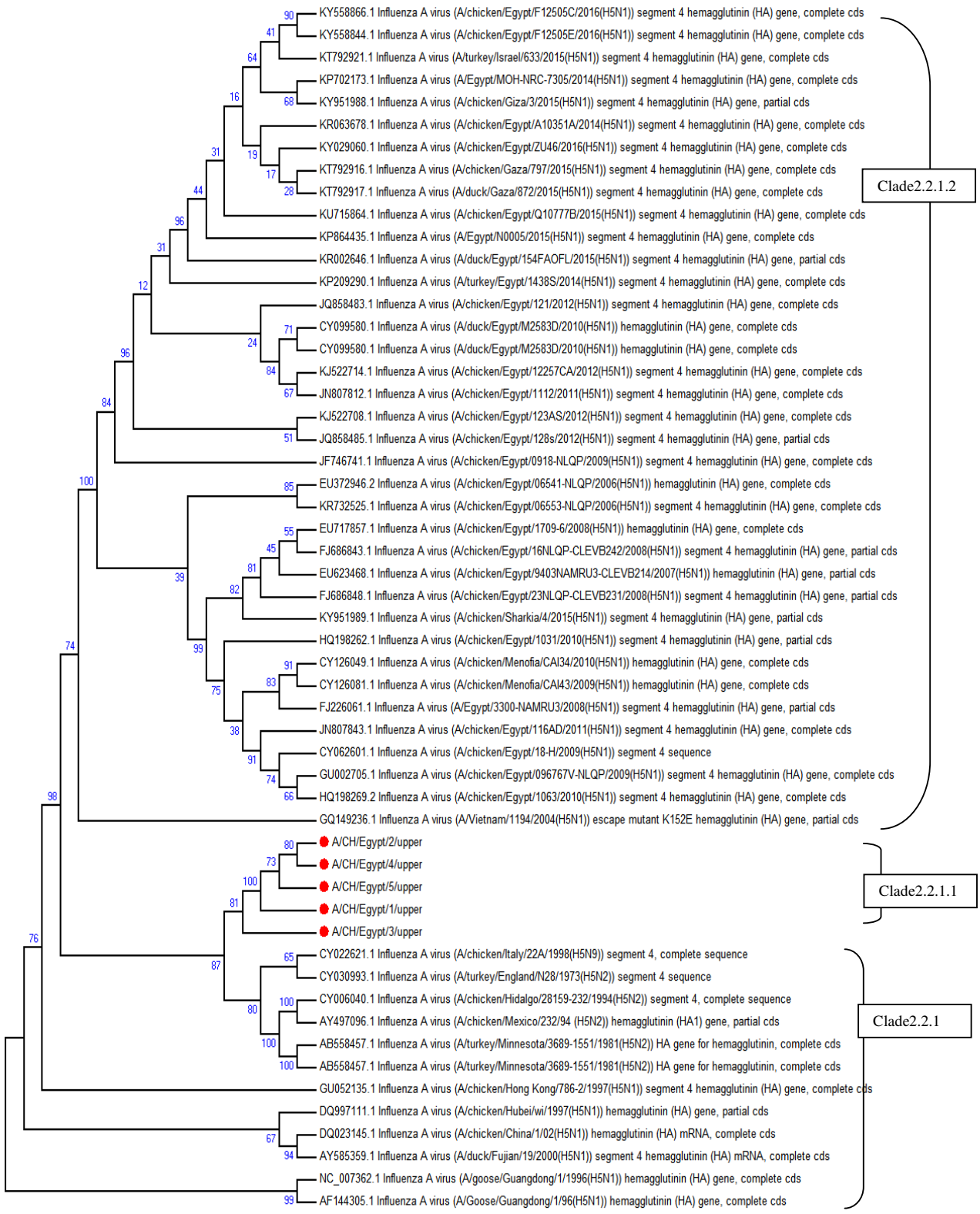

Figure 2: Phylogenetic analysis of HA gene truncated nucleotide sequences of avian influenza virus (AIV) isolated from chicken flocks, upper Egypt Governorates, showing the genetic relationship between representative HPAI (H5N1) virus in the phylogenetic tree and the five studied isolates in the period of 2016 for HA gene segment (4) the five isolates is marked with red circle. The phylogenetic tree was generated using the neighbor-joining method in MEGA version 5 (www.megasoftware.net).

Discussion

Poultry infection with type A influenza virus has been recognized since the late 19th century causing mainly sporadic but serious outbreaks of the disease. In 1997, HPAIV H5Nl containing a group of genes originated from wild aquatic birds was able to replicate causing severe mortalities in gallinaceous birds in Hong Kong [21]. Following culling of all live poultry across Hong Kong, the virus was successfully eradicated; however, in 2003, re-emergence of A/ H5Nl in domestic and wild birds occurred in Qinghai Lake, China [22-24].

Insufficient economic conditions and poultry infrastructure in Egypt facilitated the entrance and spread of the first of A/ H5Nl across poultry production sectors and marketing chains resulting in the endemicity of the disease [11]. More than 30 million birds were culled and over one billion US $ losses in poultry industry have been estimated following the first introduction of infection in 2006 [25].

This study aimed to assess the detection rate of HPAIV/H5N1 in Upper Egypt through detection of AIV in chicken flocks of H5N1 viruses isolated from commercial poultry farms. The data revealed that 71 flocks out of 141 tested chicken flocks in Upper Egypt Governorates were HA positive (71%). All these samples were tested by rRT-PCR against subtype H_5, and 30 flocks were positive with the percentage of 21.3% and this percentage was higher than that recorded by Awad *et al,* [26] who reported H5N1 in 12% of chicken flocks during late 2013 and 2015 in Alexandria.

This high incidence in Upper Egypt occurred in winter season (16.1) and decreased during the rest of the year, because low temperature and winds in winter increase the viability of the virus. This result coincides with WHO/OIE/FAO H5N1 Evolution Working Group [27] who declared that H5N1 became endemic in Egypt resulting in severe losses in poultry industry mainly in winter season. The percentage of positive H5N1 subtype HPAI samples from chicken farms from Upper Egypt during 2016 is 21.3% is higher than 12% recorded in the same year in chicken farms in Alexandria [27].

Partial hemagglutinin (HA) gene (segment 4) sequencing for 5 isolates of AI H5N1 were selected to identify the genetic characterization of these isolates. The obtained data revealed that the 5 HPAIV H5N1 isolates were located at one group of Egyptian viruses and their sequences belong to highly diverse clade 2.2.1.2 viruses and this indicated the predominance of this clade. From 2006 till 2014 the subclade 2.2.1.1 of H5N1 subtype of HPAI was dominant in the previously recorded isolates, but in the last two it disappeared due to the vaccination pressure in the chicken farms, however the subclade 2.2.1.2 is the mostly viruses appear in isolation which originated from backyards [27]. In Egypt, continuous vaccination and/or continuous transmission of the virus among the same species and other species explain the recent increased antigenic variation among H5 viruses [28].

Conclusion

It is concluded that, the five H5N1 subtype isolates of HPAI are clustered with classical H5N1 viruses to subclade 2.2.1.2 of Eurasian origin. Vaccination of the backyard chicken as well as chicken farm with suitable homologues vaccine to eliminate HPAI virus from Egypt is recommended.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

انفلونزا الطيور شديدة الضراوة H_5N_1فى الدجاج فى صعيد □صر

حاتم صلاح الدين عبد الحميد ، هانى فوزى اللقانى، احمد رجب البستاوى وعبدالقادر محمد عبد القادر
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أجريت مراقبة لمدة سنة واحدة للعدوى□فيروس (H_5N_1) في قطعان الدجاج المختلفة (١٤١ قطيع) من محافظات مختلفة في صعيد مصر، خلال الفترة من يناير ٢٠١٥ إلى يناير ٢٠١٦. كشف معدل انفلونزا الطيور □ديدة الإمراضية وكان فيروس H_5N_1 (٢١.٣٪)(١٤١/٣٠) الذي كان أعلى خلال فصل الشتاء مقارنة مع مواسم أخرى. وقد عانت القطعان المستكشفة من علامات تنفسية وعصبية، وأظهرت□عض الطيور زراق فى العر□ والدلايات ، فضلا عن نز□ تحت الجلد على الساق. تم اختبار ثلاثين عينة إيجا□ية ١N٥H لوجود فيروسات الجهاز التنفسي الأخرى. وكانت النتائج على النحو التالي: وجود فيروس الانفلونزا من النوع (H_5N_1) منفردا في (٥)عينات فقط (١٦.٦٪) من مجموع العينات التي تم اختبارها،حيث وجد فيروسى H_5N_1مع H_9N_2 في قطيعين (٦.٦)، (H_5N_1 و NDV)في(١٢)مزرعة (٤٠٪)، (H_5N_1 و IBV) فى (٧) مزارع (٢٣.٣٪) و (H_5N_1و H_9N_2 و NDV)في (٤) قطعان (١٣.٣٪). كما كشف تحليل التتا□ع الجينى لخمس معزولات مختارة من H_5N_1 أن هذه السلالات كانت متجمعة مع الفيروسات الكلاسيكية المصرية (H_5N_1) (الكليد الفرعي ٢.٢.١.٢) من أصل أوراسيا. وخلصت الدراسة إلى أن خمسة معزولات فرعية من النمط H_5N_1 من أنفلونزا الطيور □ديدة الإمراضية H_5N_1 تتجمع في□جرة النشوء والتطور مع فيروسات H_5N_1 الكلاسيكية إلى الفئة الفرعية ٢.٢.١.٢ من أصل أوراسيا ولذلك، فإننا نوصي□زيادة التركيز في تحصين الدجاج سواء□المزارع او التر□ية المنزلية□لقاح مماثل للعترة السارية وذلك للتخلص على فيروس (H_5N_1) من مصر.