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Influenza A viruses in birds and humans: Prevalence, molecular characterization, zoonotic significance and risk factors' assessment in poultry farms

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ABSTRACT

This study aimed to investigate the prevalence of influenza A viruses in birds and humans residing in the same localities of Sharkia Province, Egypt and the risk factors' assessment in poultry farms. A total of 100 birds comprised of 50 chickens, 25 ducks and 25 wild egrets were sampled. Swab samples were collected from 65 people (50 poultry farm workers and 15 hospitalized patients). All samples were screened for the presence of influenza A viruses using isolation and molecular assays. Avian influenza viruses were only detected in chicken samples (18%) and molecularly confirmed as subtype H5. The infection rate was higher in broilers (40%) than layers (8.6%). Influenza A (H1) pdm09 virus was detected in a single human case (1.54%). All the isolated AI H5 viruses were clustered into clade (2.2.1.2) and shared a high similarity rate at nucleotides and amino acid levels. In addition, they had a multi-basic amino acid motif (__PQGEKRRKKR/GLF__) at the H5 gene cleavage site that exhibited point mutations. Chicken breed, movement of workers from one flock to another, lack of utensils' disinfection and the introduction of new birds to the farm were significant risk factors associated with highly pathogenic AI H5 virus infection in poultry farms ($p \leq 0.05$). Other factors showed no significant association. The HPAI H5 viruses are still endemic in Egypt with continuous mutation. Co-circulation of these viruses in birds and pdm09 viruses in humans raises alarm for the emergence of reassortant viruses that are capable of potentiating pandemics.

1. Introduction

Influenza A viruses (IAVs) are important zoonotic pathogens that infect wide range of hosts including; aquatic birds, humans and swine. Avian influenza viruses (AIVs) and swine influenza viruses (SIVs) which are members of genus IAVs, have sparked concern due to their ability to cause devastating losses in poultry and animals worldwide as well as zoonotic importance. Wild aquatic birds are serving as an important natural reservoir and a risk for virus transmission to domestic poultry [1,2]. Avian influenza (AI) virus infection in poultry constitutes a major threat to the poultry industry worldwide and comprises two forms; highly pathogenic and low pathogenic viruses. Highly pathogenic AI (HPAI) viruses are associated with some strains of H5 or H7 haemagglutinin subtypes that can cause severe systemic disease with 100% mortality [3]. Low pathogenic AI (LPAI) viruses induce manifestations that range from asymptomatic infection to mild respiratory diseases and drop in egg production. However, some LPAI strains can mutate into virulent HPAI that cause high mortalities in poultry population and pandemics in humans [4]. In Egypt, 363 confirmed AI H5N1 human cases were reported during the period from March 2006 to April 2015 [5]. Avian influenza virus infection can be transmitted to humans through inhalation of droplet aerosols from infected birds, direct handling of infected poultry and consumption of uncooked poultry products [6]. Consequently, Egypt became an epicenter for H5N1 and one of the endemic countries with the virus despite vaccination [7]. Pigs are susceptible to avian and human influenza viruses as they possess ∝ 2-3, ∝ 2-6 sialic acid receptors for both strains which in turn make them an efficient mixing vessel for the occurrence of genetic reassortment (antigenic shift). Thus, can result in the emergence and dissemination of hybrid strains capable of infecting humans and causing pandemics [8]. Additionally, pigs played an important role in the rapid generation of the 2009 pandemic influenza A (H1N1) pdm09 virus (reassortant swine influenza virus) that emerged among humans [9]. The current study aimed to investigate the prevalence of IA viruses

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in birds and humans as well as the risk factors' assessment in poultry farms.

2. Materials and methods

2.1. Ethical statement

This study was approved by The Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt. Informed consent was obtained from the persons participated in this study.

2.2. Study area and sampling

Ten commercial chicken farms (seven layers and three broilers) and five duck flocks (three commercials and two backyards) located in five different cities at Sharkia Province, Egypt, were included in this study upon the convenience of sampling and agreement of the owners. These farms were suspected to be infected with avian influenza viruses and showed high mortality rates. Twenty-five wild egrets (*Bubulcus ibis*) were hunted by a professional sniper from the same localities. Internal organs (intestine, liver, brain, trachea and lung) were collected from 50 commercial chickens, 15 commercial ducks and 25 wild egrets. Moreover, oropharyngeal and cloacal swabs (10, each) were collected from 10 backyard ducks. In addition, oropharyngeal and nasal swabs were collected from 50 asymptomatic poultry farm workers in the enrolled farms and 15 patients admitted to five general hospitals located in the same cities from which the bird samples were collected. These patients exhibited at least one of the following respiratory symptoms (cough, sputum production, dyspnea, pleuritic pain, shivering, body aches and fever $\geq 38\,^{\circ}C$). A structured questionnaire was filled with information relevant to the investigated birds and persons during each visit. All the collected samples were inserted into viral transporting media, labeled, ice packed and transported to Virology Laboratory, Faculty of Veterinary Medicine, Zagazig University for further processing and examination.

2.3. Isolation and propagation of AIVs from bird samples

All procedures were carried out in a biosafety level 3 laboratory (BSL-3) under strict precautions. Avian influenza viruses were isolated from the processed tissue homogenate and the swab supernatant of bird samples by inoculation of specific pathogen free embryonated chicken eggs (SPF-ECEs) as previously described [10].

2.4. Slide Haemagglutination (HA) test

The harvested allantoic fluids were tested for the presence of haemagglutinating AI viruses using slide HA test.

2.5. Molecular identification and subtyping of AI viruses

2.5.1. Real-time reverse transcriptase PCR (rRT-PCR)

The allantoic fluids of HA-positive bird samples were pooled into four pools (one pool from each positive farm) then subjected to RNA extraction using QIAamp Viral RNA Mini Kit (QIAGEN, catalogue No. 52904) according to the manufacturer's instructions. The one-step rRT-PCR was performed to identify AI viruses subtypes H5, H7 and H9 targeting haemagglutinin (H5, H7 and H9) genes. The sequences of the primers, TaqMan Probe sets (Metabion, Germany) were listed in Table 1. The reaction was conducted using QuantiTect Probe RT-PCR kit (Qiagen) in Applied Biosystem Step One Real-Time PCR System machine (Stratagene MX3005 P). Each PCR cycle was followed by plate read for fluorescence acquisition and the cycle threshold (CT) was detected by The MX3005 P QPCR system.

2.5.2. Conventional reverse transcriptase PCR (RT-PCR)

The rRT-PCR positive bird samples were confirmed by one-step RT-PCR using primer pairs supplied from Metabion (Germany) to amplify H5 gene cleavage site (311bp) (Table 1).The reaction was carried out in a T3 Thermal cycler (Biometra). PCR products, negative, positive controls and ladder (cat. no. 239035, QIAGEN, USA) were loaded onto the gel and visualized under ultraviolet trans-illuminator (gel documentation system, Consort, Belgium). The data were analyzed using computer software.

2.6. Molecular detection and subtyping of IAVs in human samples

2.6.1. Real-time RT-PCR

The viral RNA was directly extracted from the swab samples using QIA amp Viral RNA Mini Kit. The extracted RNA was subjected to one-step rRT-PCR for the identification of H5 gene (specific for AIVs subtype H5) and H1 gene (specific for influenza A (H1) pdm09). The sequences of the primers and TaqMan probe sets (Metabion, Germany) and the reaction conditions were illustrated in Table 1.

2.6.2. Conventional RT –PCR

The rRT-PCR positive human sample was confirmed by one-step RT-PCR using primer pairs supplied from Metabion (Germany) to amplify H1 gene (115bp) specific for influenza A (H1) pdm09). The reaction conditions were illustrated in Table 1.

2.7. Sequencing and phylogenetic analysis of AI H5 gene

The RT-PCR amplicons (311bp) of H5 gene cleavage site were purified using QIAquick PCR Product Extraction kit (Qiagen, Valencia CA) and sequenced by Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA Cat. number 4336817). The nucleotide sequences were analyzed and compared with other H5 gene sequences available in the GenBank using NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment was performed by the Clustal W method using MEGA 6 program software and the phylogenetic tree was generated using the neighbor-joining method with 1000 bootstrap. Consequently, the nucleotide, amino acid identities and the deduced amino acid sequences of the obtained H5 gene and other relevant H5 genes retrieved from the GenBank were determined using DNAStar software (Lasergene version 7.1.0 (44); DNASTAR, Madison, WI, USA).

2.8. Statistical analysis of risk factors

The data in the completed questionnaires relevant to the investigated chicken farms were analyzed by the univariate logistic regression model using the computer program SPSS, Inc. version 22 (IBM Corp. 2013, Armonk, NY). These data included the flock type, the age of birds, previous vaccination and medication, number of birds per flock, clinical signs, post-mortem findings, mortality and morbidity rate, movement of workers, utensils' disinfection and the introduction of new birds to flocks. Odds ratios (OR) and their 95% confidence interval [95% CI] were noted. P-values ≤ 0.05 were considered statistically significant in the analysis.

3. Results

In this study 9 (18%) of the examined domestic chickens showed an evidence of AI virus infection through lesions and mortality in chicken embryo and agglutination of chicken RBCs using HA test. The HA-positive pooled samples (n = 4) were molecularly confirmed as AI viruses subtype H5 and none of the samples were positive for subtypes H7 and H9. The higher prevalence of AI H5 viruses was observed in commercial broiler chickens (40%) compared with layers (8.6%). AI viruses couldn't

Table 1
Sequences of the primers, probes and reaction conditions used in this study.

Target gene	Primers' sequences 5\–3\	Reverse Transcription	1st denaturation	Amplification cycles			Final extension	Reference
				2nd denaturation	Annealing	Extension		
H5	F:H5LH1: ACATATGACTAC CCACARTATTCA G R: H5RH: AGACCAGCT AYC ATGATTGC Probe: H5PRO: TCWACA GTGGCGAGT TCCCTAGCA	50 °C/30 min	94 °C/15 min	94 °C/15 s	54 °C/30 s	72 °C/10 s	72 °C/ 10 min.	[11]
H7	F: LH6H7: GGC CAG TAT TAG AAA CAA CAC CTA TGA R: RH4H7:GCC CCG AAG CTA AAC CAA AGT AT Probe: H7pro11: CCG CTG CTT AGT TTG ACT GGG TCA ATC T							[12]
H9	H9 F: GGAAGAATTAATTATTATTGGTCGGTAC H9 R: GCCACCTTTTTCAGTCTGACATT H9 Probe: AACCAGGCCA GACATTGCGAGTAAGATCC							[13]
H1	SW1 H1 F: GTG CTA TAA ACA CCA GCC TYC CA SW1 H1: R: CGG GAT ATT CCT TAA TCC TGT RGC SW1 H1 Probe: CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A		94 °C/15 min	94 °C/15 s	55 °C/30 s	55 °C/30 s	72 °C/ 10 min.	[14]
H5311bp	F: H5-kha-1: CCT CCA GAR TAT GCM TAY AAA ATT GTC R : H5-kha-3: TAC CAA CCG TCT ACC ATK CCY TG		94 °C/15min	94 °C/30 s	56 °C/45 s	72 °C/45 s	72 °C/ 10 min	[15]
H1 115bp	F: SW1 H1: GTG CTA TAA ACA CCA GCC TYC CA R :SW1 H1: CGG GAT ATT CCT TAA TCC TGT RGC		94 °C/15 min	94 °C /15 s	55 °C/30 s	55 °C/30 s	72 °C/ 10 min.	[14]

Table 2
Prevalence of AIVs in the examined bird samples.

Species	Origin or production sector	No. of examined birds	Positive no. (%) using HA test	Identified strains using RT-PCR
Domestic Chickens	Commercial Layers	35	3 (8.6)	AIVs H5
	Commercial broilers	15	6 (40)	
	Total	50	9 (18)	
Domestic ducks	Commercial flocks	15	0	–
	Backyard flocks	10	0	
	Total	25	0	
Wild egrets (*Bubulcus ibis*)		25	0	
Total		100	9 (9)[a]	

Neither AIVs H7 nor H9 was identified.

[a] These positive samples were from four chicken farms (3 broilers and 1 layer). HA positive allantoic fluids of the 9 chicken samples were pooled into four pools (one pool from each positive farm) then molecularly identified using rRT-PCR and confirmed by conventional RT-PCR.

Table 3
Prevalence of IAVs in the examined human samples.

Examined samples	Poultry farm workers (n = 50)			Hospitalized patients (n = 15)			Total (n = 65)	
	No.	H5 Positive no. (%)	pdm09 (H1) Positive no. (%)	No.	H5 Positive no. (%)	pdm09 (H1) Positive no. (%)	H5 Positive no. (%)	pdm09 (H1) Positive no. (%)
Nasal swabs	25	0	0	7	0	0	0	0
Oropharngeal swabs	25	0	0	8	0	1(12.5)	0	1 (3)
Total	50	0	0	15	0	1(6.67)	0	1(1.54)

be isolated from duck flocks and wild egrets (Table 2). None of the examined human samples were positive for AI H5 viruses (Table 3). Moreover, influenza A (H1) pdm09 virus was molecularly identified and confirmed in a single human case with an overall percentage of 1.54% (6.67% in hospitalized patients versus 0 in poultry farm workers) (Tables 3 & 4).

Only three AI H5 viruses produced good quality sequences of H5 gene cleavage site and these sequences were submitted to the GenBank (Table 5). The studied AI H5 viruses were clustered into clade (2.2.1.2) which is circulating in Egypt recently and shared 98.6–98.7% and 99–100% at the nucleotides (nt) and the amino acids (aa) levels, respectively (Fig. 1). Moreover, these isolates had respective strongest identities of 98.8–99.3% nt versus 99–100% aa and 97.9–98.3% nt versus 99–100% aa with the latest H5N1 human isolate N0005-2015 in Egypt (GenBank: KP864435.1) and the latest H5N1 duck isolate 1435 CAS-2014 at Sharkia Governorate (GenBank: KP209291.1). Remarkably, the nucleotides versus the amino acids sequences of the H5 gene of the studied viruses shared a high level of similarity (98.5–98.7% nt vs. 98.1–99% aa) and (97.6–98% nt vs. 97.1–98.1% aa) with the H5 gene sequences of the Egyptian H5N1 human isolate N0004-2015 (GenBank: KP864434.1) and the latest H5N1 chicken isolate F611-2015 at Sharkia Governorate (GenBank: KT359061.1), respectively. The H5 gene sequences of the studied isolates displayed 95.9–96.7% nt and 97.1%–98.1% aa homology with the H5 gene sequences of the Egyptian chicken parent 2006 H5N1 (GenBank: EU372943.1), chicken classic 2009 H5N1 (GenBank: JF746741.1), chicken variant 2008 H5N1 (GenBank: GQ184238.1). In addition, the H5 gene sequences of the studied isolates shared 94.8–95% nt, 96.2–97.1 % aa versus 92.4–93.2% nt, 92.4–94.1% aa identities with H5 gene sequences of the Egyptian 2010 H5N1 vaccine strain

Table 4
Prevalence of IAVs in the examined persons with respect to their clinical characteristics.

Characteristic		Poultry farm workers (n = 50)		Hospitalized patients (n = 15)	
		No. examined	Positive no.(%)	No. examined	Positive no. (%)
Gender (sex)	Male	50	0	6	1(16.7)
	Female	–	–	9	0
Age group/ year	≤ 18 years	8	0	5	0
	> 18 years	42	0	10	1(10)
Direct or indirect exposure to	Avian	50	0	6	0
	Swine	–	–	4	1(25)
	None	–	–	5	0
Locality	Zagazig	–	0	8	0
	Abu-Hammad	10	0	4	1(25)
	Hehia	5	0	2	0
	Diarb Negm	5	0	–	–
	Belbis	10	0	1	0
	El-Qurain	20	0	–	–
Influenza like symptoms		0	0	15	1(6.7)
Vaccination	Vaccinated	20	0	0	0
	None	30	0	15	1(6.7)

(GenBank: CY099579.1) and the ancestral H5N1strain (A/Goose/Guangdong/1/96) (GenBank: NC_007362.1), respectively. The H5 gene cleavage site of all studied AI H5 viruses had a multi-basic amino acid motif (__PQGEKRRKKR/GLF__) that exhibited point mutation (Fig. 2). Thus, resulted in an amino substitution at position 325 (arginine→lysine) compared with the reference Egyptian AI H5N1 viruses (chicken parent, classic and variant). Additionally, the cleavage site of our AI H5 viruses exhibited point mutation that resulted in an amino acid substitution at position 325 (glycine→lysine) compared with the Egyptian 2010 H5N1 vaccine strain. Chicken breed (P = 0.014), movement of workers from one flock to another (P = 0.02), disinfection of utensils (P = 0.003) and the introduction of new birds to the farm (P = 0.05) were significant risk factors strongly associated with HPAI H5 virus infection in chicken farms (Table 6).

4. Discussion

In the current study, the isolation rate of AI H5 viruses from chickens (18%) coincided with previous reports in Egypt **[16]**. On the contrary, lower rates [7,17,18] and higher rates [16,19,20] were also recorded in Egypt. The variation in the isolation rates of AI viruses could be attributed to the methods used for virus detection and vaccination. In fact, about 90% of the examined chickens were previously vaccinated by the H5N1 vaccine which provide a partial protection against infection. The lack of detection of AI H7 and H9 viruses in the examined chicken samples coincided with previous studies in India [21] and contradicted other reports from Egypt [22]. Such results could be explained by the predominance of subtype H5 in commercial chicken farms at Sharkia Governorate. The lack of virus detection in domestic ducks could be related to the establishment and adaptation of the virus to the environmental and climatic conditions. Thus, the lowest prevalence rate of the disease is suspected during hot months [23]. AI H5N1 viruses were previously detected with low percentages in ducks in Egypt [7,17] and Vietnam [24]. The lack of virus detection in wild egrets in this study coincided with previous studies in Eastern Europe, Middle East and Africa [25], Egypt [26] and Australia [27]. However, other studies in Egypt [28] reported a low prevalence of AI viruses in wild birds despite the large sample size and direct virus detection using RT-PCR.

A notable feature is that all the isolated AI H5 viruses were clustered into clade (2.2.1.2) which is recently circulating and emerged from the continuous mutation of the virus since its incursion in Egypt in 2006. The close genetic relatedness between these viruses indicated that they shared an immediate ancestor. The strongest identity between the isolated AI H5 viruses and other Egyptian AI H5N1 viruses of bird origin in the GenBank indicated the presence of a common source of infection and executed the newly introduced sources of infection till that time. Furthermore, the strongest homology between the studied AI H5 viruses and other Egyptian AI H5N1 viruses of human origin in the GenBank highlighted an evidence of zoonotic transmission. AI H5N1 viruses can cross the species barrier and transmit to humans through occupational exposure to infected poultry. Previous studies in Egypt have reported an association between human infection with AI H5N1 viruses and poultry exposure [29]. The presence of multi-basic amino acid motif (__PQGERRRKKR/GLF__) at the H5 gene cleavage site indicated that the isolated viruses were highly pathogenic, and this coincided with the acute clinical picture and the observed high mortalities in the investigated chickens. This motif is considered a pathogenicity marker for the HPAI virus. It enables the dissemination and replication of the virus in extra pulmonary organs resulting in an increased infection and mortality of birds [30]. The point mutation (R325 K substitution) at the multi-basic cleavage site motif was previously reported in the AI H5N1 viruses isolated from ducks and human cases in Egypt [31], broiler and layer chickens in Egypt [19] and ducks at Sharkia Governorate [29]. In accordance with previous studies, these data suggested that the isolated AI H5 viruses were direct drift progeny of the initially introduced virus to Egypt in 2006. The presence of such mutations in the isolated viruses may result in the emergence of new variants that circumnavigate vaccine-induced immunity. This explained the reason for the occurrence of high mortalities among the investigated chickens despite their vaccination.

In this study, influenza A (H1) pdm09 virus was detected in a single human case exhibited influenza-like symptoms with a history of direct contact with swine and not vaccinated. The pdm09 virus was previously detected in patients from Kenya using the same primers and probe used in the current study [32]. Several studies have documented the incidence of reassortant swine influenza virus H1N1 among swine farm workers [33,34]. The inability to conduct sequencing for pdm09 influenza virus H1 gene is one of our study limitations and could be attributed to the low quantity of the virus (CT value 28). The fact that AI H5 viruses couldn't be detected in humans in this study could be assumed to that 31% (20/65) of the examined participants received influenza vaccine which provided a partial protection against the infection. In addition, the small sample size obtained from the persons due to difficulties in collecting the samples.

Remarkably, this study revealed a significant association between HPAI H5 virus infection in chicken farms and the breed of the examined chickens. Broilers were 7.11 times more likely to be infected with HPAI H5 virus than layers. Such finding was similar to that reported by Desvaux et al. [35]. The potential risk proposed by broilers could be

Table 5
The isolated AIVs H5 in this study and their accession numbers.

No.	Virus	Abbreviation	HA accession number	Origin/Production sector	Province	Date of isolation
1	H5	A/Chicken/Egypt/AM-14/2015	KX230059	Broiler chickens /commercial farm	Sharkia	15-February-2015
2	H5	A/Chicken/Egypt/AM-15/2015	KX230060			05-March-2015
3	H5	A/Chicken/Egypt/AM-16/2015	KX230061			20-March-2015

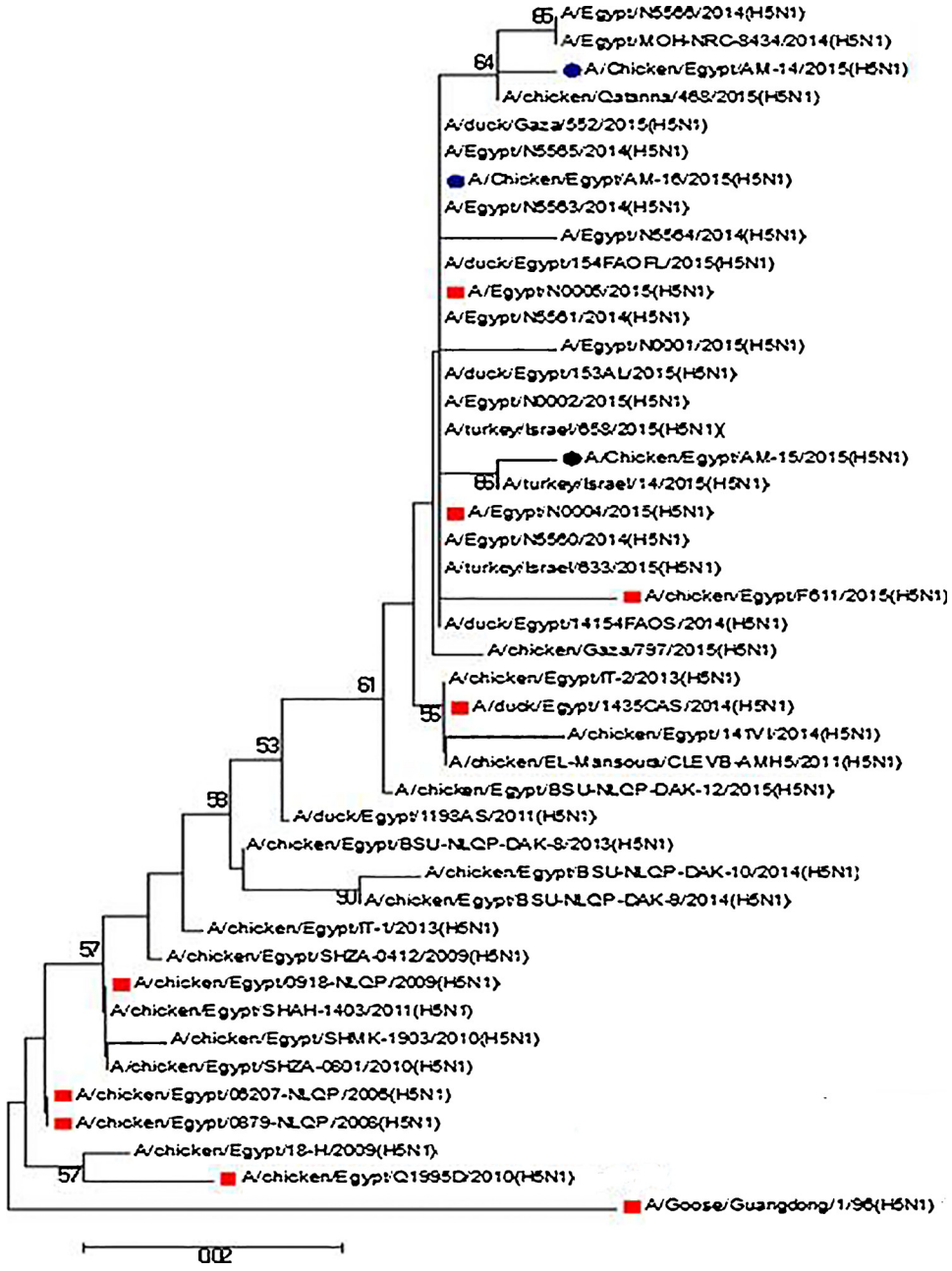

Fig. 1. Phylogenetic analysis of H5 gene nucleotide sequences of AI viruses subtype H5 isolated from chickens at Sharkia Governorate, Egypt and other H5 gene sequences in the GenBank. The isolated viruses were marked with a blue solid circle, while the aligned sequences were marked with a red solid quadrilateral.

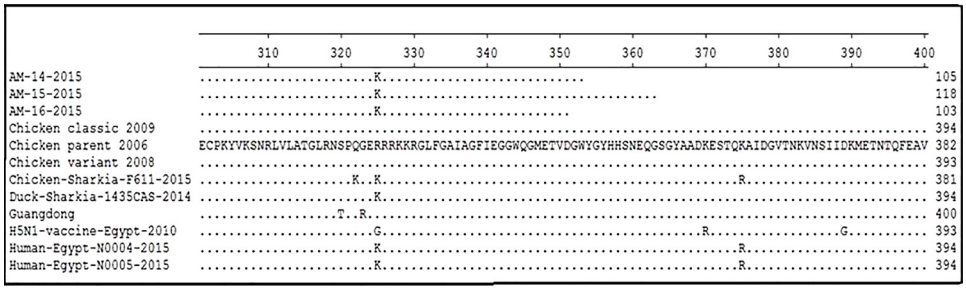

Fig. 2. The deduced amino acid sequences of the H5 gene cleavage site of the isolated AI H5 viruses in comparison with nine representative reference AI H5N1 viruses of bird and human origin retrieved from the GenBank. Dots indicated amino acids that were identical to the corresponding H5 gene cleavage site sequence of the reference strains. Amino acid position in which mutations occurred was indicated by solid bars. K: lysine, R: arginine, G: glycine, Q: glutamine, T: threonine, S: serine. The H5 influenza numbering was based on the alignment with A/Goose/Guangdong/1/96(H5N1) minus the 16 amino acids known as HA signal peptide.

Table 6
Univariate analysis of risk factors associated with HPAI H5 virus infection in chickens.

Characteristics	No. of examined birds	Positive no. (%)	Unadjusted OR	95% CI	P-value
Breed					
Layer	35	3 (8.6%)	1	1.48–34.21	0.014*
Broiler	15	6 (40%)	7.11		
Vaccination					
Yes	45	7 (15.6%)	1	0.509–25.755	0.199
No	5	2 (40%)	3.619		
Movement of workers					
Yes	20	7 (35%)	7.538	1.372–41.413	0.02*
No	30	2 (6.7%)	1		
Disinfection of utensils					
Yes	35	2 (5.7%)	1	2.506–83.168	0.003*
No	15	7 (46.7%)	14.437		
New introduced birds					
Yes	10	4 (40%)	4.667	0.967–22.529	0.05*
No	40	5 (12.5%)	1		
Disposal of dead birds and wastes					
improper	20	6 (30%)	3.857	0.836–17.793	0.084
proper	30	3 (10%)	1		

* Represent statistically significant difference ($P \leq 0.05$).

attributed to that broiler production is characterized by a high conversion rate of feed within a short period. This affects the immune system of the birds, exposes them to stress and become susceptible to the infection. Moreover, the movement of workers from one flock to another was a significant risk factor associated with 7.538 higher odds of HPAI H5 virus infection in persons who moved from one farm to another compared with the persons who didn't. Similarly, a significant association between HPAI H5 virus infection and movement of workers from one farm to another in Nigeria was reported [36]. The presence of this association could be explained by the fact that workers don't follow the guidelines and the biosecurity principles in the farms. For instance; they rarely wash their hands or change their clothes and shoes when they move from one flock to another. The lack of utensils' disinfection was 14.437 times more likely to be associated with HPAI H5N1 virus infection in the farm than the application of disinfection. Such findings commensurated with previous studies in China [37,38] and could be related to that some disinfectants (e.g. chlorine compounds, aldehydes and quaternary ammonium) can work effectively against AI viruses, thereby decreasing the incidence of the infection [39]. In this study, the newly introduced birds to the farm were 4.667 times more likely to be infected with HPAI H5 virus than the none introduced ones. Similarly, the introduction of new birds to the farm was significantly associated with HPAI H5 virus infection in Nigeria [36] and China [37]. The introduction of new birds is a major threat because birds may carry the virus and transport it to the farms [37]. In addition, the farmers may sell sick birds during the outbreak to minimize the financial losses [40]. Surprisingly, the disposal of dead birds and wastes from chicken farms wasn't significantly associated with HPAI H5 virus infection, although the risk of the infection was higher in the practice of improper disposal of dead birds and wastes compared with the other practice. This could be attributed to the small sample size in this investigation. Conversely, previous studies reported a significant association between the improper disposal of dead birds and HPAI H5 virus infection in poultry farms [36,38]. The absence of significant association between HPAI H5 virus infection and the vaccination in the current study could be related to the mutation of the virus which may result in incomplete protection provided by the used vaccine. In addition, the technical errors during the administration of the vaccine by the farm owner. However, the non-vaccinated birds were 3.619 times more likely to be infected with HPAI H5 viruses than the vaccinated ones. The risk of HPAI H5N1 virus outbreaks was significantly increased in non-vaccinated flocks [41].

5. Conclusions

This study concluded that the highly pathogenic AI H5N1 viruses are still endemic and circulating at Sharkia Governorate, Egypt especially in broiler chickens than in layers despite large-scale vaccination campaigns. Therefore, there is no exotic introduction of the virus. Mutations are still undergoing in the circulating viruses, resulting in the emergence of new variants that escape the vaccine-induced immunity and lead to more difficulties in controlling the disease. The isolation of AI H5 viruses from birds and influenza A (H1) pdm09 virus from humans at the same location provided a major chance for the emergence of reassortant viruses with novel characteristics that threaten not only avian health but also human. Farm management and biosecurity measures have a vital role in combating avian influenza viral infection in commercial farm sectors. This study recommended the annual updating of the used vaccine and regular sequencing for the circulating IAVs to understand the genetic and phenotypic characteristics of the newly diverged strains and reduce the risk for zoonotic infections.

Conflicts of interest

None.

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