Research Article

Molecular and Epidemiological Overview on Low Pathogenic Avian Influenza H9N2 in Egypt between 2015 and 2016

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Abstract | Since the first isolation of the H9N2 low pathogenic avian influenza virus (LPAI) in 2011, the virus has been distributed rapidly and widely in different Egyptian poultry sectors causing severe economic losses and the problematic situation in poultry production especially with a co-infection with other circulating pathogens. In this study, a total of 23182 cloacal and tracheal samples were collected from suspected cases between 2015 and 2016 and submitted to the Reference laboratory of veterinary quality control on poultry production (RLQP) from different poultry sectors and bird species which are distributing all over the Egyptian governorates for examination of LPAI (H9N2) virus by real time RT-PCR. The results confirmed positive samples from 1026 for H9N2 with prevalence rate 4.4%. However, the LPAI H9N2 showed a wide range distribution with high geo-prevalence rate in 2015/2016 (96.3%) as positive samples were recorded from 26 governorates. Totally, the positive samples were distributed in 783 farms with the highest prevalence rate (76.5%), then 167 live bird markets (LBM) (16.5%) and 76 households (7%), respectively. Also, the highest number of positive samples were detected in chicken with highest prevalence (90%) for H9N2 infection among all the examined species, followed by Turkey (4%), duck (2.6%) and quail (2.4%), respectively. Genetically, the genetic sequence for the Hemagglutinin (HA) of 38 viruses circulating Egyptian viruses during 2015 and 2016 were belonging to the Middle East G1- like viruses and scattered phylogenetically in different subgroups with the presence of variant viruses in the quail during 2015.

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Introduction

Infection with H9N2 in poultry population resulted in great economic losses in the poultry industry especially when being complicated with other pathogens (Naeem et al., 1999). H9N2 of subtype avian influenza viruses had been classified as influenza type A of the family *Orthomyxoviridae*. The first isolation of H9N2 was from turkeys in Wisconsin in 1966 (Tu/WS/66), then it became widely distributed to other countries (Homme and Easterday, 1970).

In Egypt, the first detection of H9N2 virus was reported in May 2011 that has been isolated from bobwhite quail (El-Zoghby et al., 2012), consequently, Egypt has been reported with endemicity of H9N2 avian influenza virus. The circulating Egyptian viruses are closely related to H9N2 viruses from Israel and

belonged to Asian G1-like (Monne et al., 2013) and classified as cluster B based on HA gene sequence (Kandeil et al., 2017). Infections with H9N2 viruses in Egypt are higher in chicken more than other species, mostly in apparently healthy broilers and recorded in layers and breeders, also, infection in other species was recorded but with low incidence. Except for chicken, the prevalence of infection in quails is relatively high in comparison with other species (Soliman et al., 2014). While, the prevalence of virus infection in duck is lower than in chicken (Kayali et al., 2014). The circulation of LPAI H9N2 may add a risk factor to the Egyptian poultry industry, especially with the endemic situation of HPAI H5N1 and presence of other pathogens with low biosecurity level in some commercial sectors which permit rapid virus transmission and add more stress to the condensed poultry populations. In this study, we tried to follow up the epidemiological status of LPAI H9N2 in Egypt and the genetic evolution of both Hemagglutinin (HA) and Neuraminidase (NA) genes of the currently circulating viruses in Egypt.

Materials and Methods

Molecular detection of H9N2 virus by real time RT-PCR

As a part of routine avian influenza surveillance program conducted in Egypt; a total 23182 suspected cloacal and oropharyngeal samples were collected from different poultry species including chicken, quail, turkey and ducks then were characterized at the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP, Egypt).

Extraction of viral RNA using a QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) was done according to the manufacturer's protocol, extracted RNA has been tested for influenza type A by real-time RT-PCR to the positivity of samples for H9 subtype by specific primers as shown in Table 1 (Shabat et al., 2010), with One-Step RT-qPCR Kit (QIAGEN, Hilden, Germany). Thermal profile for amplification of HA gene ofH9 subtype was as follow; 50 °C for 30 min, 94 °C for 15 min, cycling steps 94°C for 10 sec, 54°C for 30 sec and 72°C for 10 sec repeated for 40 cycles. The real-time RT-PCR have been run on Stratagen MX3005P real-time PCR machine (Stratagene, Amsterdam, Netherlands).

Virus isolation and propagation

Thirty-eight positive influenza A samples with RT-PCR were initially isolated in 10-day-old specific pathogen free embryonated chicken eggs (SPF Eggs Production Farm, Kom Oshim, Egypt) according to standard protocols (OIE, 2015). The allantoic fluid was harvested, tested for Hemagglutination, and then stored at -80 C until use.

The 38 selected samples represent the H9N2 virus circulating in Egypt during 2015-2016 from backyard poultry holdings and commercial farms representing Upper and Lower Egypt including chicken and quails.

Amplification and sequencing of the full HA and NA genes

The first-strand cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and Uni-12 primer as per the manufacturer's protocol. The full length of each gene was amplified using gene specific forward and reverse primer (Table 2). Using a platinum® Taq DNA polymerase high fidelity (Invitrogen, Carlsbad, CA). HA and NA genes were amplified using gene specific primer and the appropriate expected size of each gene was confirmed by gel electrophoresis. The electrophoresis of PCR products was done on ethidium bromide-stained agarose gel and the amplified products of expected correct size visualized by gel documentation system BDA digital – Image capture (Biometra, Germany).

Amplicons of the appropriate sizes were subsequently gel purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The purified PCR products were used directly for sequencing using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instruction, the reaction products were purified by exclusion chromatography in CentriSep columns (Princeton Separations, Adelphia, NJ). The recovered materials sequenced using a 3500xl DNA Analyzer (Applied Biosystems).

Sequence analysis and Phylogeny of the full-length HA and NA genes

Bio Edit 7.0 software was used for multiple sequence alignment and percent identity matrices comparing the genes under study to each other. We generated neighbor-joining phylogenetic trees for HA and NA genes using the distance-based method in MEGA version6. We calculated bootstrap values based on 1,000 replicates (Tamura et al., 2013) including most of the Egyptian H9N2 virus sequences available on GenBank database, closely related H9N2 viruses from

Table 1: *Primers and probes used for Real tine RT-PCR identification of H9N2 viruses in this study.*

Prime ID	Primer sequences	Ref.
H9 subtype	**For:** GGA AGA ATT AAT TAT TAT TGG TCG GTA C **Rev:** GCC ACC TTT TTC AGT CTG ACA TT **H9probe:** [FAM] AAC CAG GCC AGA CAT TGC GAG TAA GAT CC [TAMRA]	(Ben Shabat et al., 2010)

Table 2: *Primers used in Reverse Transcriptase-Polymerase Chain Reaction (onestep RT-PCR) and Sequance reaction of HA gene.*

Prime ID	Primer Sequence for HA gene amplification	Reference
F1-6	5'TAG CAA AAG CAG GGG AAT TTC TT 3'	RLQP
H9- Rev	5' GCC ACC TTT TTC AGT CTG ACA TT 3'	Ben Shabat etal., 2010
H9-For	5'GGA AGA ATT AAT TAT TAT TGG TCG GTA C 3'	Ben Shabat etal., 2010
HT7R	5'TAA TAC GAC TCA CTA TAA GTA CAA ACA AGG GTG 3'	SEPRL
	Primer Sequence for NA gene amplification	
Forward primer	5' GGC ACA ACA CAT GAT AGA AGT CCC 3'	SEPRL
Reverse primer	5' CGC CAA CAA GTA CTA AGC ACA CAT 3'	SEPRL

Table 3: *Suspected and positive cases examined for H9N2 in different sectors in Egypt during 2015-2016.*

Year/ sector	Farm		Backyard		LBM		total	
	+ve cases/total	Prevalence rate	+ve cases/ total	Prevalence rate	+ve cases/ total	Prevalence rate	+ve cases/total	Prevalence rate
2015	353/7835	4.5%	44/2869	1.5%	55/218	25.2%	452/10922	4%
2016	430/9538	4.5%	32/2358	1.4%	112/364	31%	574/12260	4.7%
Total	783/17373	4.5%	76/5227	1.5%	167/582	28.7%	1026/23182	4.4%

The suspected cases were examined by real time PCR which resulted in 1026 positive samples. In comparison to the total number of the examined suspected cases in each sector, the highest prevalence rate of H9 was recorded in LBM (28.7%) followed by farms (4.5%) while the lowest rate was recorded in household.

other Middle Eastern countries, representative viruses from groups A-D of G1 lineage (Monne et al., 2013). Major ancestral H9N2 strains as shown by BLAST (Basic Local Alignment Search Tool).

Selection pressure was estimated by Data monkey Adaptive evolution server (Weaver et al., 2018), which was measured by ω (dN/dS), as dN=non-synonymous substitution rate (non-synonymous changes per non-synonymous site) and dS=synonymous substitution rate (synonymous changes per synonymous site). Maximum Likelihood analysis of natural selection codon-by-codon method was conducted using HyPhy software package, the test statistic dN - dS was used for detecting codons that had undergone positive selection, if dN>dS and ω>1, it means that amino acid changes provide better fitness. The p-value is calculated, when p-value is less than 0.05, it is considered significant at a 5% level (Suzuki and Gojobori, 1999).

Epidemiological data analysis

The epidemiological data were prepared and analyzed in Excel spreading sheet.

Results and Discussion

Epidemiological analysis

During 2015 and 2016 RLQP received 23182 suspected samples for examination of LPAI H9N2 virus from different sectors (17373 farms – 5227 household – 582 live bird market), the examination of the collected samples by real time RT-PCR resulted in 1026 positive samples for H9N2 (783 farm – 76 household – 167 LBM) with prevalence rate (76.5% - 7% - 16.5%) respectively with highest prevalence rate in the farm as shown in Table 3. Time distribution of the positive samples during 2015 and 2016 reported in Figure 1 which revealed higher incidence of virus distribution during 2016 than 2015 which reached to the peak in the 2nd quarter of 2016 from March to June followed by a dramatic decline in the last bisection of 2016. The distribution of the positive cases in chicken from the farm sector is the highest in all the governorates except in Fayoum

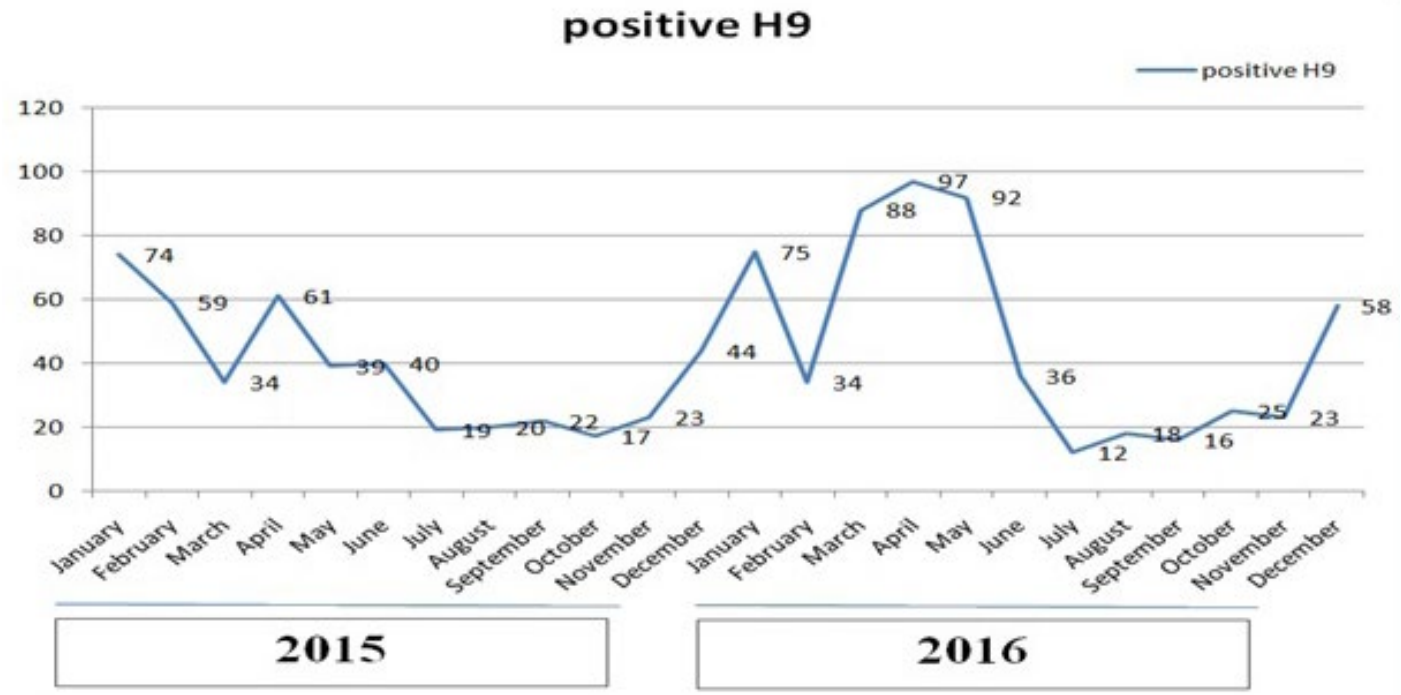

Figure 1: *Time distribution of the positive cases during 2015 and 2016; the incidence of virus distribution is higher during 2016 than 2015.*

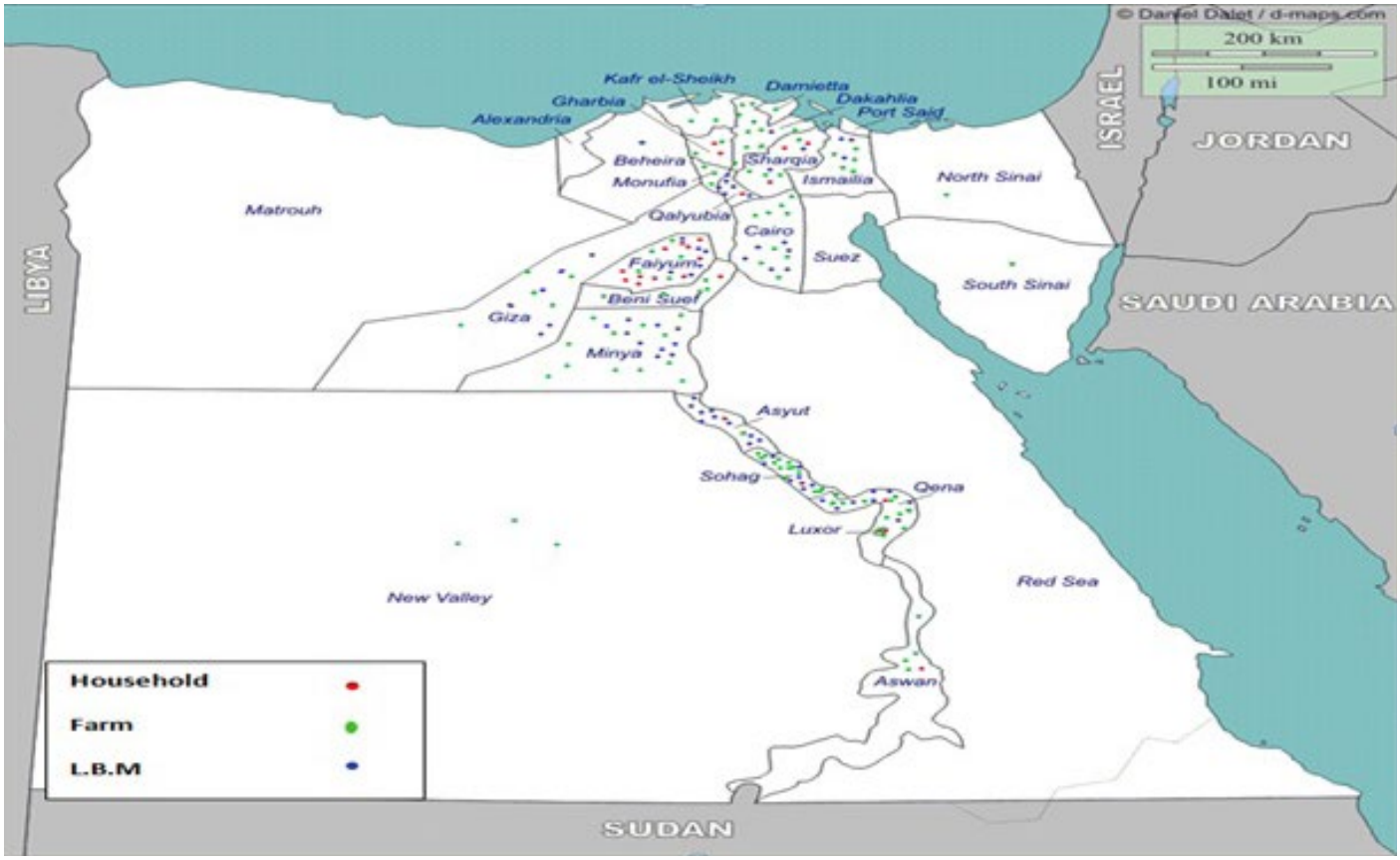

Figure 2: *Geographical distribution of H9N2 in Egypt.*

which had a high incidence among the household population as shown in Figure 2. Totally, the Lower Egypt governorates recorded a higher infection rate than the Upper Egypt ones especially Dakahlia that showed the highest incidence during 2016 as shown in Figure 3. According to the distribution of virus among the species as shown in Figure 4, chickens have the highest percent in all the breeding sectors, followed by quails.

Virus isolation and screening

The selected 38 Egyptian H9N2 viruses were isolated

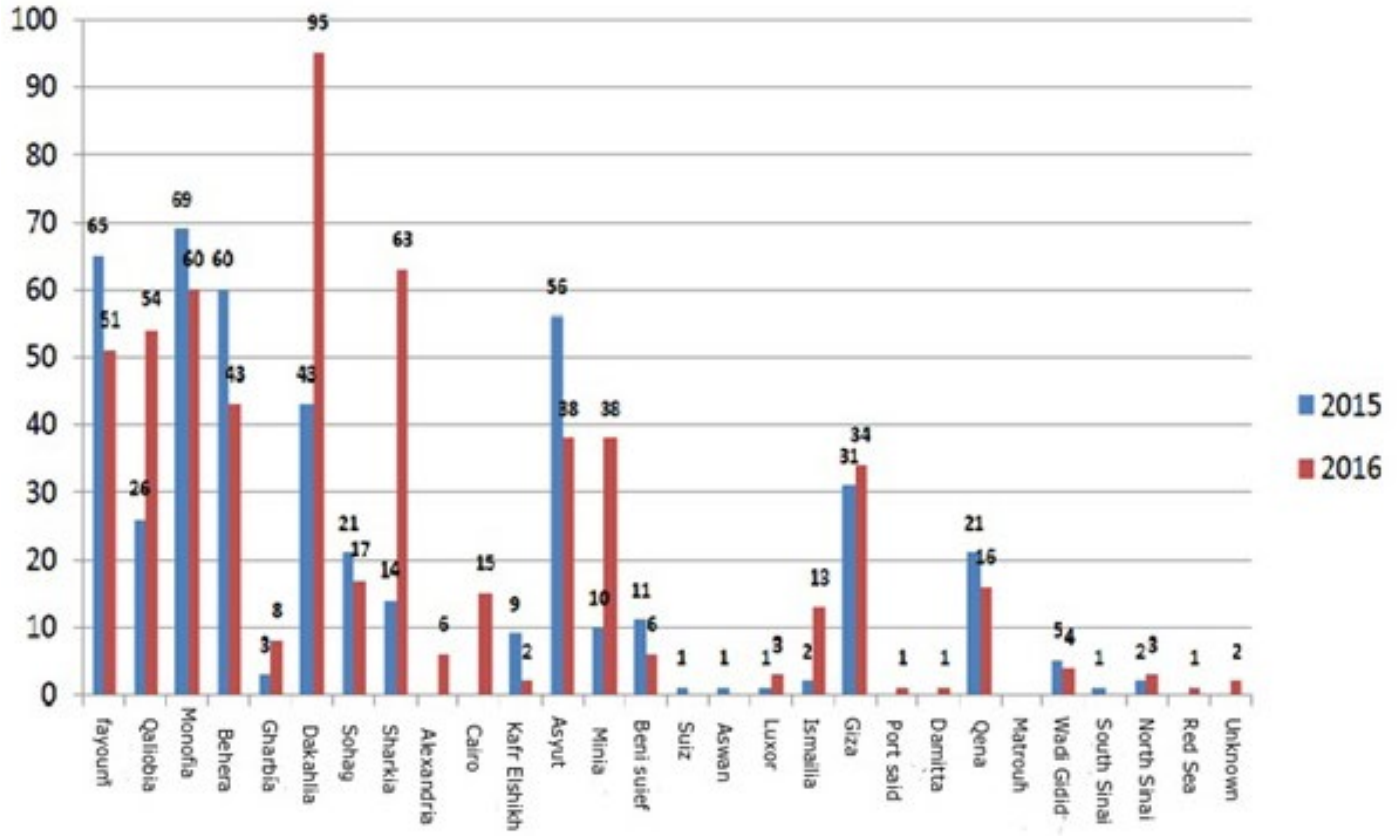

Figure 3: *Incidence % of H9N2 occurrence in different Egyptian governorates reveals high record in Dakahlia governorate during 2016.*

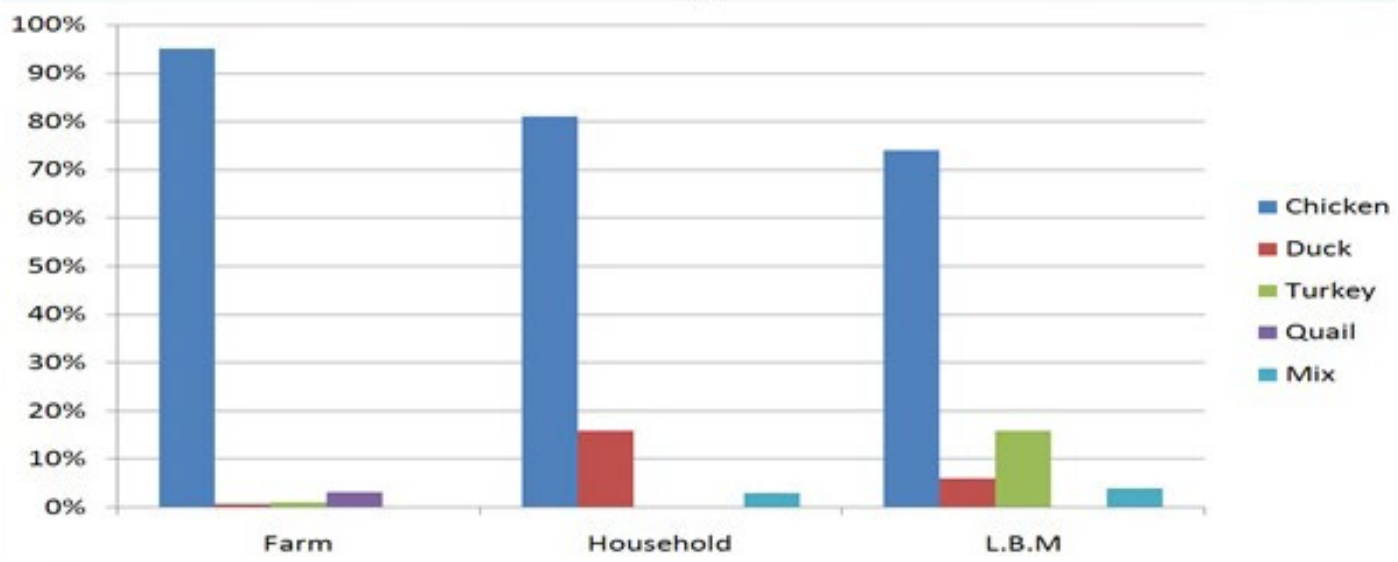

Figure 4: *Positive % of H9N2 cases in different breeding sectors and poultry species during 2015 and 2016.*

from apparently healthy broiler chickens and quails in Egypt that were positive for H9N2 by real time RT-PCR during routine surveillance along 2015and 2016. Viruses are representing five Governorates from Upper and Lower Egypt. According to the type of breeding the selected viruses represent backyard poultry holdings, live bird markets (LBM), and commercial farms. The HA titers were ranged from 7 to 9 log2 and the CT values for H9 subtype ranged from 12 to 18.

Phylogenetic analysis of the full-length HA gene

The selected isolates were sequenced for hemagglutinin gene and the obtained sequences were analyzed in comparison to each other and the previous sequences of the Egyptian viruses that were submitted to GenBank., the accession no. recorded in supplementary Table 1. All isolates in this study are closely similar with high identity % ranged between 98-100%, except 2 isolates designated as QU/Egy/2890V/2015 and QU/Egy/2792V/2015 which

were genetically related to the Egyptian variant viruses which arise since 2012 (Adel et al., 2017). Both quail isolates were highly similar to each other with identity % 98-99%, but less similar to the common circulating Egyptian H9N2 strains with identity % 93-95%.

Phylogenetically, all the viruses in this study belong to the Egyptian viruses which circulating since 2011 which are genetically related to group B of G1 lineage of H9N2 that predominant in the Middle East countries and other neighboring countries including Israel, Iran, KSA, and UAE. The Egyptian viruses were classified in the phylogenetic tree at least into 3 major subgroups. One group represents circulating viruses since the first introduction in 2011 and persisted till 2013, another group represents the viruses which were predominant from 2013 till 2016 without obvious amino acids alterations between the 2 groups. The third group represents the variant group (Egy/G1var) that was detected previously in quail from 2012 and was recorded up to 2015 as shown in Figure 5.

Analysis of the deduced amino acid sequence of HA protein

This study depends on the comparison between the Egyptian viruses at the level of the pathogenic determinants of HA protein, the receptor binding site (RBS), antibody binding epitopes, and the proteolytic cleavage site (PCS). The cleavage site in all the Egyptian viruses showed typical motif of the low pathogenic avian influenza H9N2 (^{315}PARSSRGLF^{323}). The amino acid mutations along the HA molecule shown in Table 4.

There are six distinct glycosylation sites in all Egyptian viruses definitely five sites on HA1 at ^{11}NSTE^{14}, ^{87}NGTC^{90}, ^{123}NVTY^{126}, ^{280}NSTL^{283} and ^{287}NISK^{290}, in addition to one site on HA2 at ^{474}NGTY^{477}. In this study, all sequences of HA protein have the previous glycosylation sites with acquisition of new additional sites in QU/2792V/2015 and QU/2890V/2015 at amino acid residues ^{127}NGTS^{130}, ^{148}NGSY^{151} and ^{189}NTTT^{192} which are characteristic for the viruses isolated from quails as mentioned in previous study (Adel et al., 2017). Also, there is a new additional site on the HA2 of ch/860VG/2015 at amino acid residue ^{398}NMTI^{401}.

The selection pressure of the HA gene of 70 sequences from 2011 – 2016 reveals positive selection at amino acid residue 216 (p-value <0.05 and ω≥1).

Phylogenetic analysis of the NA gene

In this study, five selected viruses were sequenced for NA gene which were selected according to the phylogenetic tree of HA gene of viruses in this study. The NA genes showed the nucleotide and deduced amino acid similarities ranged between 96 99% and 85 to 99%, respectively between the isolated viruses in this study in correlation with most of previously reported Egyptian viruses. The five tested isolates shared nucleotide and deduced amino acid homologies with each other ranged from (96 to 99 %) and (95-100%) and with G1-linage 90% and 88-90 %, respectively, while NA genes of Egyptian isolates showed the highest similarity to those of A/turkey/Israel/311/2009 (95-97%).

Phylogenetically, The Egyptian isolates clustered into multiple minor subgroups. The phylogenetic clustering among the Egyptian viruses was not related to species, time or geographical distribution from Upper or Lower Egypt during 2015 and2016 which displayed continuous virus diversity. Those viruses belonged to group B which includes the Middle East viruses from Israeli, Iran, United Arab Emirates and Pakistan, as shown in Figure 6.

Analysis of the deduced amino acid sequence of NA protein

The stalk length, hemadsorbing site, enzyme active site, and the number of glycosylation sites have a potential role in neuraminidase activity. Analysis of stalk length revealed that no stalk deletions at sites 38-39 or 46-50. But, there are different mutations at sialic acid binding pocket of the hemadsorbing sites in comparison to G1 lineage, all the five viruses of this study have a substitution S372A in locus 1 of the HB sites except the virus of Qu/Egy/V2792/2015 which has the same motif of HK/G1/97, also the locus 2 shows two substitutions in all the target viruses of this study as shown in Table 5, while all the target viruses have identically the same motif of the locus 3 of HB sites of HK/G1/97 virus.

However, sequence analysis of binding-pocket residues involved in interactions with antiviral drugs revealed that no mutations were present, all Egyptian viruses showing 119E, 198D, 222I, 274H and 292R substitutions.

There are eight glycosylation sites located on the NA protein at positions (44, 61, 69/70, 86, 146, 200, 234 and 402) of the HK/G1/97 virus, while the NA genes

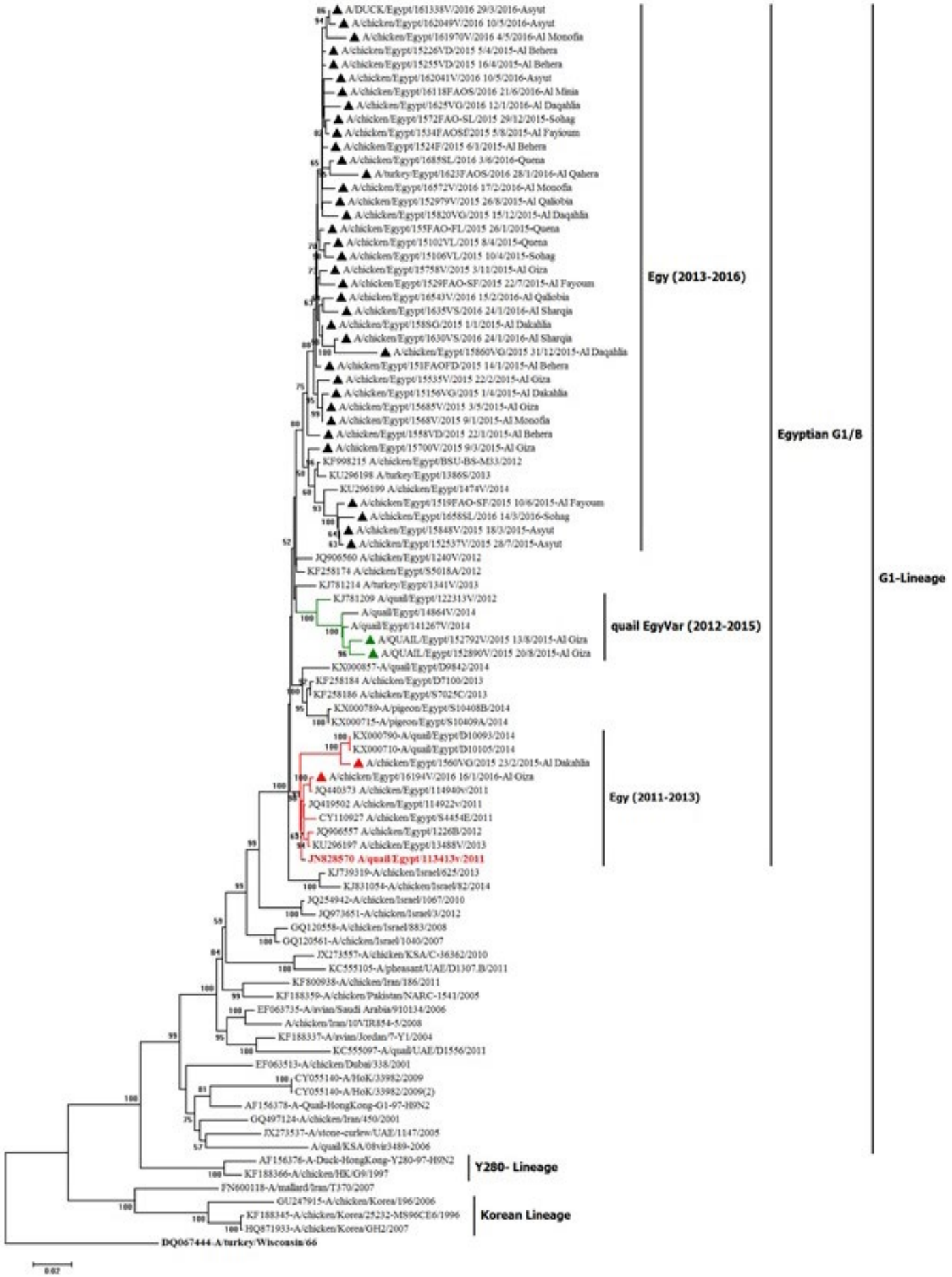

Figure 5: *Phylogenetic tree of HA gene of LPAI H9N2, Egyptian viruses were labeled with the colored triangles as the target sequences of this study. The phylogenetic analysis was constructed by MEGA 6 software, using Neighbor Joining method with bootstrap of 1000 replicates.*

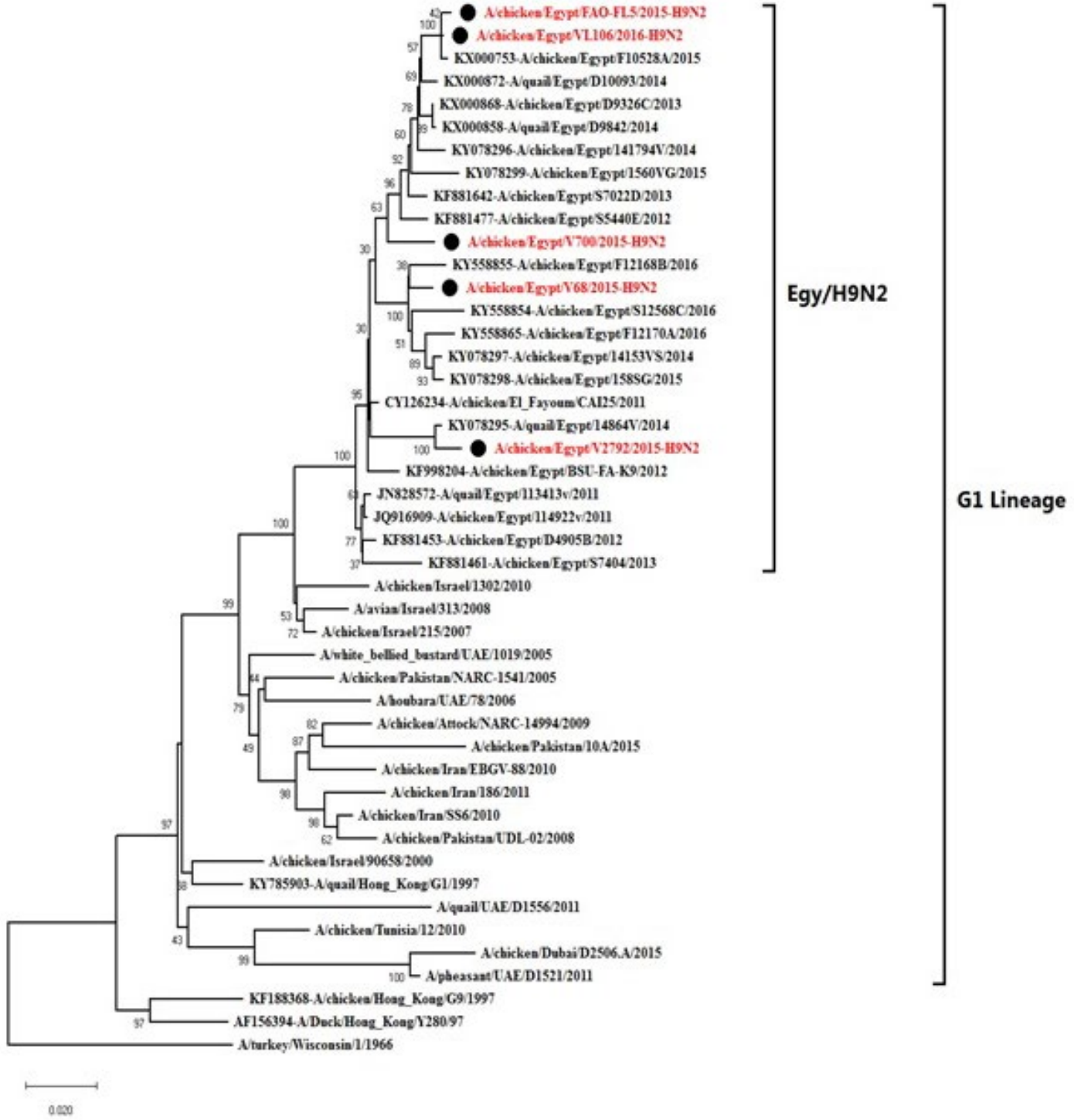

Figure 6: *Phylogenetic tree of NA gene of LPAI H9N2, five Egyptian viruses were labeled with the colored circles as the target sequences of this study. The phylogenetic analysis was constructed by MEGA 6 software, using Neighbor Joining method with bootstrap of 1000 replicates.*

of the Egyptian viruses contained seven glycosylation sites, at positions ^{NTS}44, ^{NIT}61, ^{NGT}69, ^{NWS}86, ^{NGT}146, ^{NAT}200 and ^{NGT}234, with loss of the glycosylation site 402. There is no positive selection pressure for the NA gene of the Egyptian viruses at p-value < 0.05 and ω ≥1.

Discussion

LPAI H9N2 viruses are endemic in poultry in the Middle East region from the mid-1990s. However, in Egypt virus appeared from December 2010 (Abdel-Moneim et al., 2012; Monne et al., 2013) to May 2011 when the first record was done in quail (El-Zoghby et al., 2012), then this virus distributed in all the domestic poultry species around Egypt (Arafa et al., 2012). Previously, there was evidence for the presence of H9N2 viruses in LBM but the virus was not isolated (Abdelwhab and Abdel-Moneim, 2015), Also, the serological investigation revealed that the H9N2 virus

Table 4: *Amino acids variation of the HA of Egyptian H9N2 viruses.*

	code	Right edge of RBS (128–132)	Left edge of RBS (214–219)	Cleavage site (315–323)
		GISRA	NDLQGR	PARSSRGLF
1	A/chicken/Egypt/1558VD/2015	GTSKS	NGLIGR	PARSSRGLF
2	A/chicken/Egypt/151FAOFD/2015	GTSKS	NGLIGR	PARSSRGLF
3	A/chicken/Egypt/155FAO FL/2015	GTSKS	NGLIGR	PARSSRGLF
4	A/chicken/Egypt/15535V/2015	GTSKS	NGLIGR	PARSSRGLF
5	A/chicken/Egypt/1560VG/2015	GTSKS	NDLTGR	HARSSRGLF
6	A/chicken/Egypt/15700V/2015	GTSKS	NGLIGR	PARSSRGLF
7	A/chicken/Egypt/15848V/2015	GTSKS	NGLIGR	PARSSRGLF
8	A/chicken/Egypt/15156VG/2015	GTSKS	NGLIGR	PARSSRGLF
9	A/chicken/Egypt/15226VD/2015	GTSKS	NGLIGR	PARSSRGLF
10	A/chicken/Egypt/15102VL/2015	GTSKS	NGLIGR	PARSSRGLF
11	A/chicken/Egypt/15106VL/2015	GTSKS	NGLIGR	PARSNRGLF
12	A/chicken/Egypt/15255VD/2015	GTSKS	NGLIGR	PARSSRGLF
13	A/chicken/Egypt/15685V/2015	GTSKS	NGLIGR	PARSSRGLF
14	A/chicken/Egypt/1519FAO SF/2015	GTSKS	NGLIGR	PARSSRGLF
15	A/chicken/Egypt/1529FAO SF/2015	GTSKS	NGLIGR	PARSSRGLF
16	A/chicken/Egypt/152537V/2015	GTSKS	NGLIGR	PARSSRGLF
17	A/chicken/Egypt/1534FAOSf/2015	GTSKS	NGLIGR	PARSSRGLF
18	A/QUAIL/Egypt/152792V/2015	GTSKA	NGQAGR	PARSSRGLF
19	A/QUAIL/Egypt/152890V/2015	GTSRA	NGQAGR	PARSSRGLF
20	A/chicken/Egypt/152979V/2015	GTSKS	NGLIGR	PARSSRGLF
21	A/chicken/Egypt/15758V/2015	GTSKS	NGLIGR	PARSSRGLF
22	A/chicken/Egypt/15820VG/2015	GTSKS	NGLIGR	PARSSRGLF
23	A/chicken/Egypt/1572FAO SL/2015	GTSKS	NGLIGR	PARSSRGLF
24	A/chicken/Egypt/15860VG/2015	GTSKS	NGLIGR	PARSSRGLF
25	A/chicken/Egypt/1625VG/2016	GTSKS	NGLIGR	PARSSRGLF
26	A/chicken/Egypt/16194V/2016	GTSKS	NGQIGR	PARSSRGLF
27	A/chicken/Egypt/1630VS/2016	GTSKS	NGLIGR	PARSSRGLF
28	A/chicken/Egypt/1635VS/2016	GTSKS	NGLIGR	PARSSRGLF
29	A/turkey/Egypt/1623FAOS/2016	GTSKS	NGLIGR	PARSSRGLF
30	A/chicken/Egypt/16543V/2016	GTSKS	NGLIGR	PARSSRGLF
31	A/chicken/Egypt/16572V/2016	GTSKS	NGLIGR	PARSSRGLF
32	A/chicken/Egypt/1658SL/2016	GTSKS	NGLIGR	PARSSRGLF
33	A/DUCK/Egypt/161338V/2016	GTSKS	NGLIGR	PARSSRGLF
34	A/chicken/Egypt/161970V/2016	GTSNP	NGLIGR	PARSSRGLF
35	A/chicken/Egypt/162041V/2016	GTSKS	NGLIGR	PARSSRGLF
36	A/chicken/Egypt/162049V/2016	GTSKS	NGLIGR	PARSGRGLF
37	A/chicken/Egypt/1685SL/2016	GTSKS	NGLIGR	PARSSRGLF
38	A/chicken/Egypt/16118FAOS/2016	GTSKS	NGLIGR	PARSSRGLF

was wide-spread in the commercial sector between February 2009 and April 2012 (Afifi et al., 2013). Since the introduction of the LPAI H9N2 in Egypt, it becomes endemic in parallel co-circulation with H5N1 (Arafa et al., 2012) and recently with newly introduced HPAI H5N8 (Selim et al., 2017; Yehia et al., 2018). According to this situation of the multiple introductions of the different influenza subtypes, the viruses become at the high risk for reassortment in spite of there is no record for that till now (Naguib et

al., 2017).

In this study, the epidemiological data reveal that the LPAI H9N2 virus still circulating with a higher incidence in chicken of commercial sectors more than other species and other sectors. These findings are compatible with the previous studies that were accomplished on H9N2 in Egypt (Abdelwhab and Abdel-Moneim, 2015; Adel et al., 2017; Kandeil et al., 2017).

Table 5: *Mutation in hem adsorbing site of NA in comparison to the qu/HK/G1/97.*

Isolate ID	HB-Site		
	366–373	399–404	431–433
A/Quail/Hong Kong/G1/97	IKKDSRSG	DSDNRS	PQE
A/chicken/Egypt/FAO-FL5/2015	IKKDSRAG	DSDGWS	PQE
A/Quail/Egypt/V2792/2015	IKKDSRSG	DSDSWS	PQE
A/chicken/Egypt/V68/2015	IKKDSRAG	DSDSWS	PQE
A/chicken/Egypt/V700/2015	IKKDSRAG	DSDSWS	PQE
A/chicken/Egypt/VL106/2015	IKKDSRAG	DSDGWS	PQE

As known, the incidence of avian influenza increases in cold weather during winter (Park and Glass, 2007), however, the last studies recorded many outbreaks all over the year regardless the weather (Abdelwhab and Abdel-Moneim, 2015; Arafa et al., 2012). In this study, the high positive cases for H9N2 were recorded during the spring and early summer of 2016, while during winter of 2015-2016 the positive cases were at the lowest level. Depending on these finding, we could approve the previous studies that reports there is no obvious impact of the weather on the distribution of the virus (Gilbert et al., 2008).

The distribution of H9N2 in Lower Egypt is more spreading than in Upper Egypt (Abdelwhab and Abdel-Moneim, 2015; Arafa et al., 2012) and this situation persists till 2016, as the highest record of positive cases were recorded in Dakahlia governorate in Lower Egypt followed by Monofia, Behera, and Sharkia. On other hand, El Fayioum and Asyut recorded a higher incidence in Upper Egypt.

The LPAI H9N2 virus shows continuous evolution since its first introduction in Egypt. Previous studies classified the Egyptian viruses into at least clusters (Adel et al., 2017; Kandeil et al., 2017; Naguib et al., 2017), that were belonged to the Middle East viruses of group B - G1 lineage (Fusaro et al., 2011). Through the evolution of the virus, a group of genetic and antigenic variant viruses adapted in quail was raised from 2012 (Adel et al., 2017; Kandeil et al., 2017) and persist till our recent study in 2015. Hemagglutinin (HA) protein is the most important surface antigen of Influenza virus that plays a crucial role in virus attachment and evasion from the host humeral immunity (Laursen and Wilson, 2013; Wilson et al., 1981).

Thirty eight H9N2 positive samples represent the different sectors and the geographic distribution, were selected for virus isolation and propagation, then sequenced for Hemagglutinin (HA) gene and phylogenetic analysis which revealed that most of the selected isolates were located within the circulating Egyptian viruses isolated since 2013, except two isolates (Qu/152792V and Qu/152890V) related to the most recent circulating Egyptian variant viruses which arised since 2012 (Adel et al., 2017) and characterized genetically with acquisition of additional glycosylation sites at amino acid residues 127, 148 and 189 around the right edge of the receptor binding sites, also these isolates have avian specific marker Q216 on the left edge of the receptor binding sites. Epidemiologically, these variant viruses restricted to Giza governorate and isolated from quail species in farm sector (Adel et al., 2017).

The viruses of this study have the typical proteolytic cleavage site (PARSSR/GLF) on HA protein of low pathogenic avian influenza virus (Steinhauer, 1999). Like all previous Egyptian H9N2 viruses, the viruses of interest possess conserved receptor binding domains at residues Y91, W143, T145, L184 and Y185 (Arafa et al., 2012). There are no changes at the antigenic sites on HA protein of the viruses of this study in comparison to the other previously isolated Egyptian LPAI H9N2 viruses, due to the viruses of interest are genetically closed to the circulating viruses in Egypt since 2013 with no impacted alterations (Adel et al., 2017).

However, the two reported viruses in our study (Qu/152792V and Qu/152890V) are related genetically to the variant Egyptian viruses and possess the same substitutions that are characteristic

to this group including; S127N and D189N at the overlapping antigenic site of H9N2 which resulted in acquisition of two new additional glycosylation sites in comparison to the other Egyptian viruses. Both of these substitutions were proved that they have a robust impact on the antigenicity of these viruses against the other circulating Egyptian viruses (Adel et al., 2017). In addition to substitutions in the antigenic site II at residues D135G, N183D and L216Q.

Neuraminidase is one of the surface antigen of influenza virus which has the main function in the release of virus by removing sialic acid from newly synthesized HAs and NAs and prevent the aggregation of newly synthesized virus particles (Peiris et al., 1999). Also, it possesses an active conserved motif that has antiviral drugs property (Colman et al., 1989). In this study, the NA genes of five H9N2 influenza viruses represent the viruses of our study, were sequenced. The Phylogenetic analysis revealed that sequenced NA genes are belonging to LPAI H9N2 lineage G1 that originated from A/QU/HK/G1/97. However, the Middle east viruses classified according to NA sequences into four groups (Fusaro et al., 2011), the Egyptian viruses including the viruses of our study clustered with the group B that includes the viruses from Iran, United Arab Emirates and Israeli (Mosaad et al., 2017).

The stalk length, hemadsorbing site, enzyme active sites and the number of glycosylation sites had a potential role in neuraminidase activity (Gubareva et al., 2000). In the stalk region of NAs of our Egyptian viruses, there are no deletion at the amino acid residues 38 to 39 that similar to the other Egyptian viruses and the viruses from the Middle Eastern origin (Israel, Lebanon, and United Arab Emirates) (Kandeil et al., 2017). The hemadsorption site is a conserved area located on the NA surface plays a specific role in virus replication (Aamir et al., 2007). There is a substitution in the HB site 1(366–373) at residue S372A in the viruses of our study like all the Egyptian viruses (Kandeil et al., 2017), except the Qu/Egy/V2792/2015 virus which have the same residue (S372) like the QU/HK/G1/97 virus and other human viruses as A/Beijing/39/1975 (H3N2) and A/Egypt/84/2001 (H1N2) (Mosaad et al., 2017), in addition to substitution at the aa residues N402G/S and R403W in the HB site 2 (399-404).

All the Egyptian viruses including viruses of this study showing 119E, 198D, 222I, 274H and 292R substitutions indicate no resistance to the sialidase inhibitors oseltamivir and zanamivir antiviral (Gubareva et al., 1997). The viruses of this study possess seven glycosylation sites in comparison to the prototype of G1 lineage (QU/HK/G1/97) due to substitutions of the amino acid residues N400S/G and R401W in the locus 2 of the HB site. In addition to the loss of another glycosylation site ^{NGT}69 on the NA molecule of Qu/Egy/V2792/2015 virus which related to the Egy/var group (Mosaad et al., 2017).

In conclusion, Egyptian LPAI H9N2 viruses are still circulating endemically in all poultry sectors especially in the commercial broiler chicken. In spite of the climatic conditions, the prevalence of the virus circulation reached the peak in the summer season of 2016. Overall, LPAI H9N2 Egyptian viruses reveal continuous evolution according to the genetic sequence of both surface genes HA and NA, particularly in quails in which the virus became antigenically and genetically more variant than other Egyptian circulating viruses.

Authors Contribution

Amany Adel , Wesam Mady and Zienab performed the gene sequenes, Amany Adel analyzed the data, Asmaa shabaan prepared the received samples, Fatma Amer applied the real time RT-PCR, Dalia Said isolated and propagated the viruses on ECE, Marwa Ali prepared the epidemiologial data, Abdelsatar Arafa, Mohamed K. Morsi and Mohamed K. Hassan revised the results and writing. All author read and approved the final version of the manuscript for submission.

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