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 in 2011, the virus has distributed rapidly and widely in different poultry sectors in Egypt causing severe economic losses and the problematic situation in poultry production especially with a co-infection with other circulating pathogens. In this study, 1026 confirmed positive H9N2 cases by RT-PCR out of 23182 different examined samples of different species and sectors in Egypt, with a prevalence rate 4.4% by the year 2015/2016. However, the LPAI H9N2 showed a wide range distribution with high geo-prevalence rate in 2015/2016 (96.3%) as positive cases were recorded in 26 governorates., the positive samples were distributed in 783 farms with the highest prevalence rate (76.5%), Also, the most of positive cases were detected in chicken as the highest prevalence (90%) among all the examined species, Genetically, the genetic sequence for the Hemagglutinin (HA) and Neuraminidase (NA) of circulating Egyptian viruses are belonging to the Middle East G1- like viruses in group B that were closely related to each other and scattered phylogenetically in different subgroups with the presence of variant viruses in the quail during 2015.

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Introduction

The infection with H9N2 in poultry population resulted in great economic losses in the poultry industry when it is complicated with other pathogens (Naeem et al., 1999). H9N2 is a subtype of 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 in the world countries (Homme and Easterday, 1970).

In Egypt, the first detection of H9N2 virus was reported in May 2011 has been isolated from bobwhite quail (El-Zoghby et al., 2012), consequently, Egypt has been endemic with H9N2 avian influenza virus (Kim, 2018). 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 depending on HA gene sequence (Kandeil et al., 2017). The 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 (Soliman et al., 2014), Except for chicken, the prevalence of infection in quails was relatively high in comparison with other species (Soliman et al., 2014). While, the prevalence of virus infection in duck was lower than in chicken (Kayali et al., 2014). The presence of LPAI H9N2 may add risk factor to the poultry industry in Egypt, especially with the endemic situation of HPAI H5N1 and presence of other pathogens with low biosecurity level in some commercial sectors that permit easy virus transmission and add more stress to the condensed poultry populations. Besides the newly introduction of the HPAI H5N8 in 2016 (Selim et al., 2017; Yehia et al., 2018).

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 circulating H9N2 viruses in Egypt.

Materials and Methods

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 cases of Cloacal and oropharyngeal swab samples were collected from different poultry species including chicken, quail, turkey, and ducks then were characterized at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP, Egypt).

Extraction of Viral RNA using a QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, RNA extracts have been tested by real-time RT-PCR to determine the positivity of samples for H9 subtype by specific primers as shown in Table 1 (Ben Shabat et al., 2010), with One-Step RRT-PCR Kit (QIAGEN, Hilden, Germany). Thermal profile for amplification of HA gene of H5, H7and H9 subtypes: 50 °C for 30 min, 95 °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 (according the manufacturer manual of QIAGEN, Hilden, Germany). The real-time PCR reactions have run on Stratagene MX3005P real-time PCR machine (Stratagene, Amsterdam, and the Netherlands).

Virus isolation and propagation

Thirty- eight positive influenza A samples with RT-PCR were selected depending on the geographical distribution from backyard poultry holdings and commercial farms representing Upper and Lower Egypt including different poultry species. They 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 have been confirmed for H9N2 by real-time PCR, then stored at -80 C until use.

The study was done for samples represent the H9N2 virus circulating in Egypt during 2015-2016.

Amplification and sequencing of the full NA gene and the HA gene

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 primers 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 directed for sequencing reactions using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's, the reaction product was purified by exclusion chromatography in CentriSep columns (Princeton Separations, Adelphia, NJ). The recovered materials sequenced using a 3500 x l DNA Analyzer (Applied Biosystems).

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

BioEdit 7.0 was used for multiple sequence alignment by ClustalW method and percent identity matrices

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 and NA genes.*

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 as recorded in table 1. 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.*

comparing the genes under study to each other. We generated neighbor-joining Phylogenetic trees for HA and NA genes segments using the distance-based method in MEGA version6. We calculated bootstrap values based on 1,000 replicates (Tamura et al., 2013). The trees included the most available Egyptian H9N2 virus sequences available in the GenBank database, closely related H9N2 viruses from other Middle Eastern countries, representative viruses from the groups A-D (Monne et al., 2013), Major ancestral H9N2 strains as shown by BLAST search (Basic Local Alignment Search Tool). Selection pressure was estimated by Data monkey Adaptive evolution server when the p-value is less than 0.05, it is considered significant at a 5% level (Suzuki and Gojobori, 1999)

Epidemiological analysis

The epidemiological data were analyzed by Excel 2013.

Results and Discussion

Epidemiological analysis

During 2015 and 2016 RLQP received 23182 suspected cases for examination of avian influenza viruses of LPAI H9N2 from different sectors (17373 farms – 5227 household – 582 live bird market) the examination revealed 1026 positive cases for H9N2 by RT- PCR (783 farm – 76 household – 167 LBM) with prevalence rate (76.5% - 7% - 16.5%) respectively. the Farm samples showed highest prevalence rate with highest prevalence rate in the farm as shown in Table 3. Time distribution of the positive cases during 2015 and 2016 reported in Figure 1 which revealed a 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 was the highest in all the governorates except in Fayoum which has 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 in Figure 4, chickens have the highest percentage in all the breeding sectors, followed by quails.

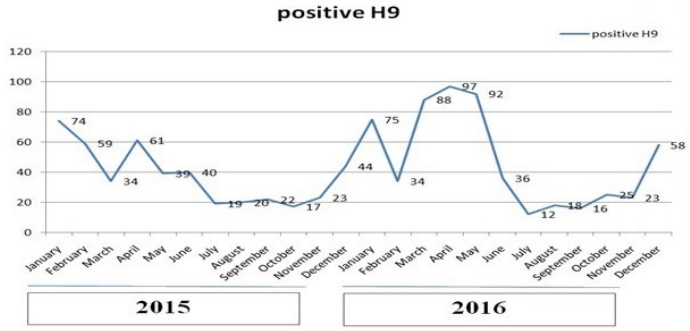

Figure 1: *Time distribution of the positive cases during 2015-2016.*

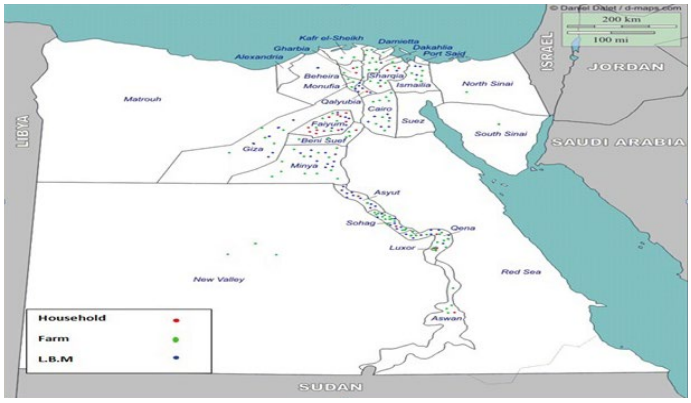

Figure 2: *The prevelence of H9N2 occurrence in Egypt.*

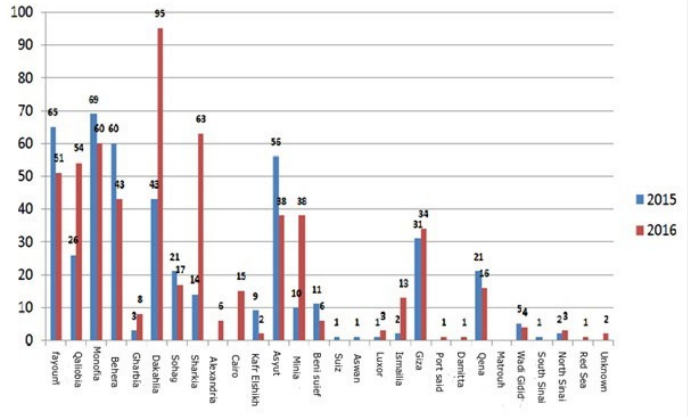

Figure 3: *Geographical distribution of H9N2 in Egypt.*

Virus isolation and confirmation

The selected 38 Egyptian H9N2 viruses were isolated from apparently healthy broiler chickens and quails in Egypt that were positive for H9N2 by real-time RT-PCR in the routine surveillance along 2015-2016. Viruses are randomly selected; representing five Governorates from Upper and Lower Egypt. According to the type of breeding the selected viruses represent backyard poultry holdings, live bird markets, and commercial farms. The HA activity ranged from 7 to 9log2 and the CT values for H9 subtype ranged from 12 to 18.

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).

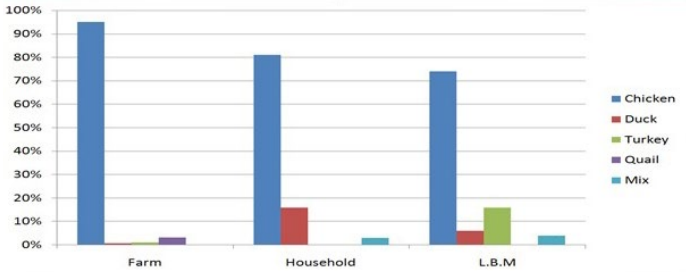

Figure 4: *Positive % of H9N2 cases in different breeding sectors and poultry species during 2015-2016.*

Phylogenetic analysis of the full-length HA gene

The thirty – eight isolates were sequenced for the hemagglutinin gene and analyzed in comparison with each other and the previous sequences of the Egyptian viruses the accession numbers were -listed in supplementary Table 1. All isolates in this study were closely similar to each other with high identity 98-100%, except two isolates designated as QU/Egy/2890V/2015 and QU/Egy/2792V/2015 were genetically related to the Egyptian variant viruses have been arised since 2012 (Adel et al., 2017). Both 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 still belonging to the Egyptian viruses which circulating since 2011 and 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 three major subgroups. One group represents circulating viruses since the first introduction in 2011 and persisted till 2013, the second group represents the viruses which were predominant from 2013 till 2016

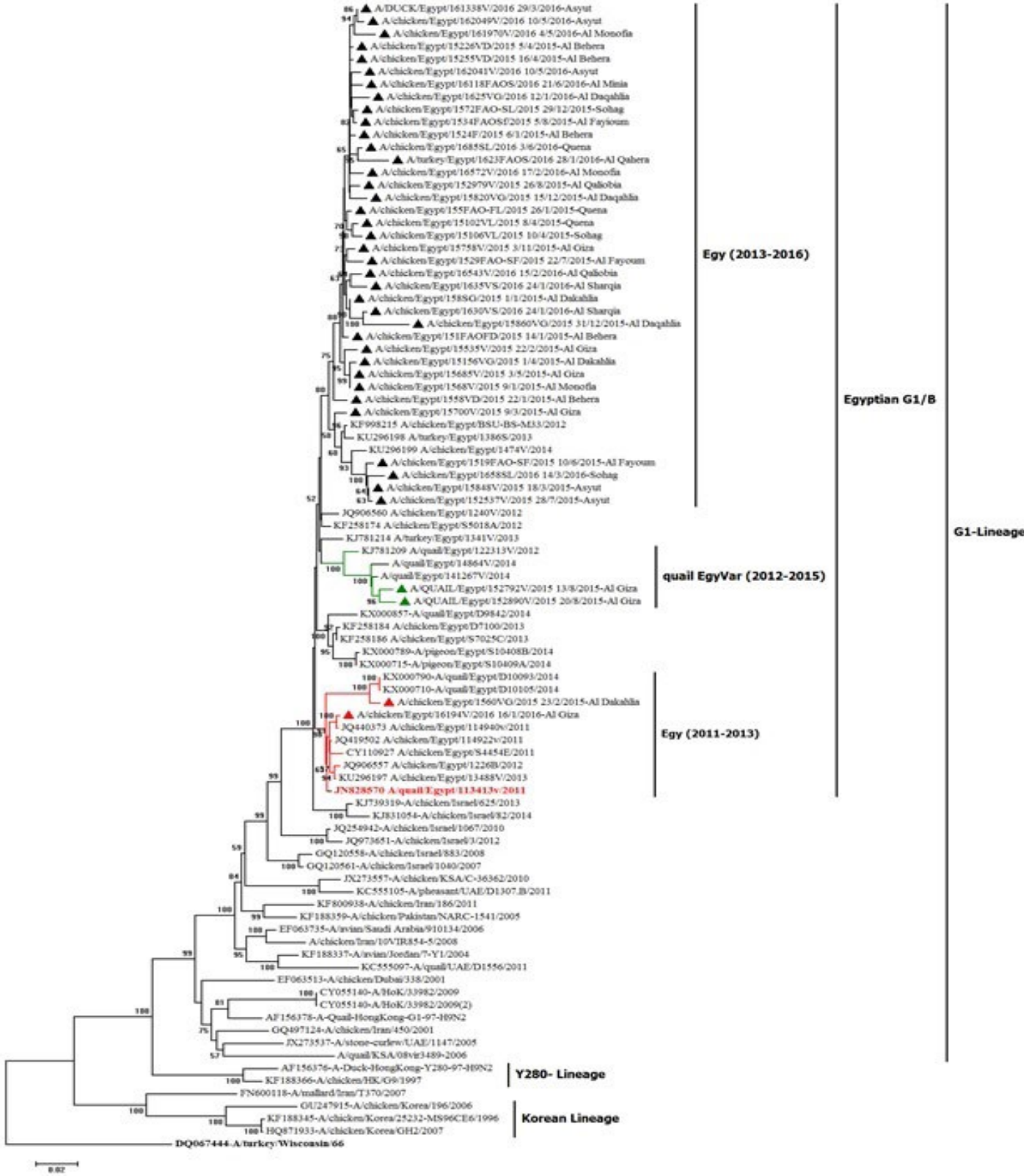

Figure 5: *Phylogenetic tree of HA gene of LPAI H9N2, Egyptian viruses were labeled with the black dots as the target sequences of this study. The phylogenetic analysis was applied by MEGA 6 software, using Neighbor Joining method with bootstrap of 1000 replicates.*

without obvious amino acids alterations between both groups. The last one 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 Hemagglutinin

This study aims to compare the Egyptian H9N2 viruses at the level of the pathogenic determinants of HA protein, the receptor binding site (RBS),

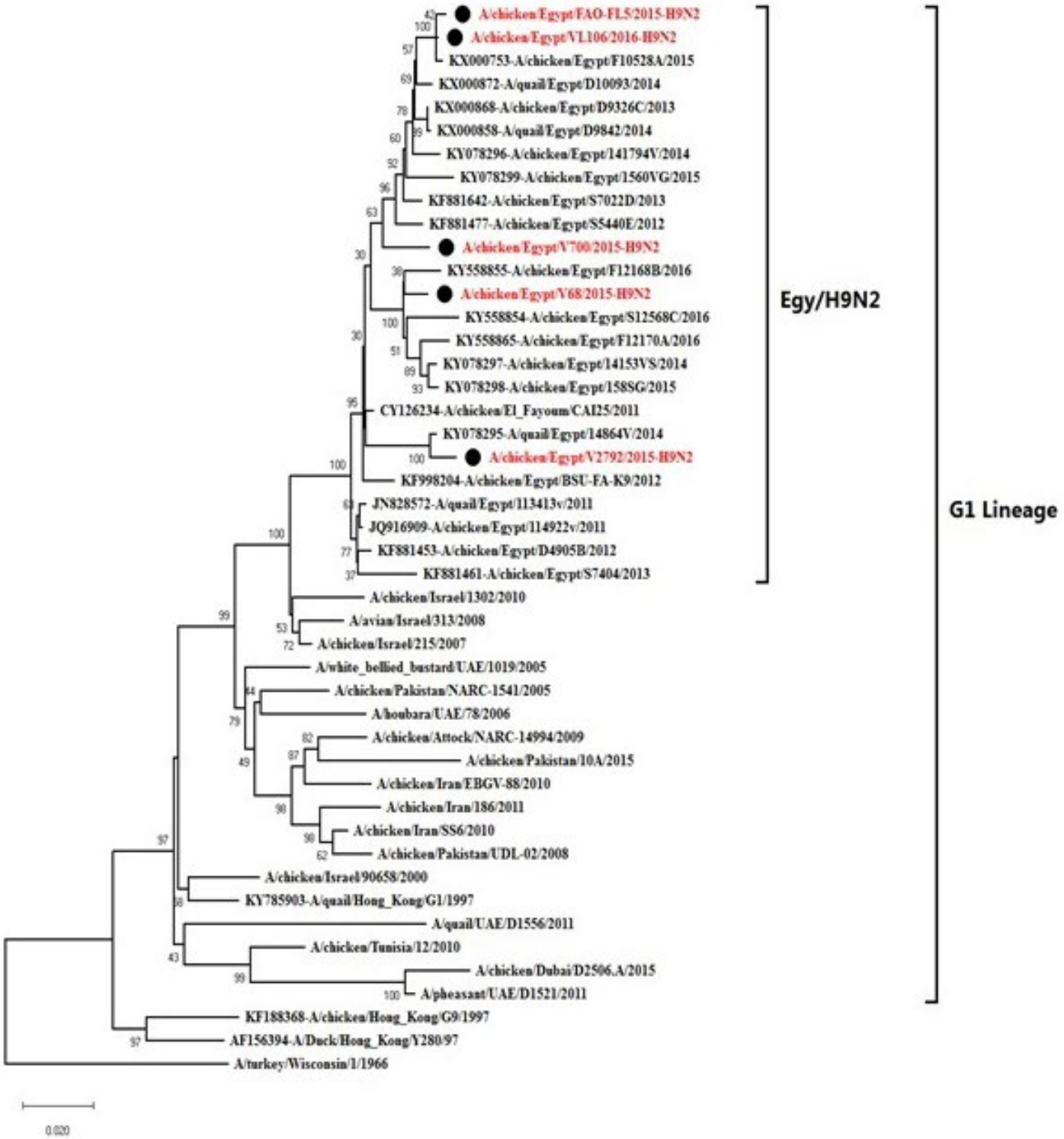

Figure 6: *Phylogenetic tree of NA gene of LPAI H9N2, 5 Egyptian viruses were labeled with the colored circles as the target sequences of this study. The phylogenetic analysis was applied by MEGA 6 software, using Neighbor Joining method with bootstrap of 1000 replicates.*

antibody binding epitopes, and the proteolytic cleavage site (PCS). The cleavage site in all the Egyptian viruses showed a typical motif of the low pathogenic avian influenza H9N2 (^{315}PARSSRGLF^{323}). The amino acid mutations along the HA molecule were shown in Table 4. There are six distinct glycosylation sites in all Egyptian H9N2 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 the 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} - Also, there was 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, revealed positive selection at amino acid residue 216 (p-value <0.05 and ω≥1).

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

Phylogenetic analysis of the NA gene

Five randomly selected viruses according to the phylogenetic tree of the HA gene were sequenced for the NA gene. The NA genes sequences of selected viruses showed the nucleotide and deduced amino acid similarities in correlation with the most previously published Egyptian viruses and the percentage of identities were ranged between 96 to 99% and 85 to 99% respectively. But the nucleotide and deduced amino acid homologies between the

five strains showed an identity between each other (96 to 99 %) and (95-100%) and with G1-lineage 90% and 88-90%, respectively, in addition to the NA genes of Egyptian isolates showed the highest similarity to those of A/turkey/Israel/311/2009 (95-97%). Phylogenetically, the entire 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 and 2016 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 Neuraminidase

Stalk length of the studied viruses were analyzed and exposed that no stalk deletions at sites 38-39 or 46-50. But, there were different mutations at sialic acid binding pocket of the hemadsorbing sites in comparison to G1 lineage, all the five viruses of this study had a substitution S372A in locus 1 of the HB sites except the virus of Qu/Egy/V2792/2015 which had the same motif of HK/G1/97, also the locus 2 showed two substitutions in all the target viruses of this study as shown in Table 5, while all the target viruses had identically the same motif of the locus 3 of HB sites of HK/G1/97 virus. However, t no mutations were present in the pocket residuesall Egyptian viruses showing 119E, 198D, 222I, 274H, and 292R substitutions. There were 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 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 was no positive selection pressure for the NA gene of the Egyptian viruses at p-value < 0.05 and ω ≥1.

LPAI H9N2 virus is endemic in poultry specially in the Middle East region, from the mid-1990s (Kim, 2018). However, in Egypt the 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 spread in all the domestic poultry species all over Egypt (Arafa et al., 2012). Previously, there was a shared evidence for the presence of H9N2 viruses in LBM but the virus wasn't isolated (Abdelwhab and Abdel-Moneim, 2015), Also, a serological investigation revealed that theH9N2 virus 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 revealed that the LPAI H9N2 virus still circulating with a higher incidence in the chicken of commercial sectors more than other species and other sectors as mentioned before in Table 3 and Figure 5. that finding was compatible with the previously studied in Egypt (Abdelwhab and Abdel-Moneim, 2015; Adel et al., 2017; Kandeil et al., 2017).

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 in spite of the weather (Abdelwhab and Abdel-Moneim, 2015; Arafa et al., 2012). The highest positivity for H9N2 were recorded during the spring and early summer of 2016 (88-97%), while during winter of 2015-2016 the positive cases were at the lowest level (20-23%)as shown in Figure 1. Accordingly, we settle the finding of (Gilbert et al., 2008) that there was no obvious impact of the weather on the distribution of the H9N2 virus.

The distribution of H9N2 in lower Egypt (Delta) was 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 the other hand, El Fayioum and Asyut recorded a higher incidence in Upper Egypt as shown in Figure 2.

The LPAI H9N2 virus show continues evolution since its first introduction in Egypt, Different previous studies classified the Egyptian viruses into at least three clusters (Adel et al., 2017; Kandeil et al., 2017; Naguib et al., 2017), that were belonging to the middle east viruses of group B - G1 lineage (Fusaro et al., 2011).

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

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 persists in 2015.

Hemagglutinin is the most important surface antigen of Influenza virus that plays a crucial role in virus attachment and evasion from the host 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 isolation and propagation, then sequenced for Hemagglutinin gene and the phylogenetic analysis as shown in Figure 5 were the most of the selected isolates were located within the circulating Egyptian viruses isolated since 2013, except 2 isolates (Qu/152792V and Qu/152890V) related to the most recent circulating Egyptian variant viruses which raised 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 in the left edge of the receptor binding sites. Epidemiologically, these two variant viruses restricted in Giza governorate, isolated from quail species in the same farm at different dates (Adel et al., 2017).

The viruses of this study have the typical proteolytic cleavage site (PARSSR/GLF) on HA of low pathogenic avian influenza virus (Steinhauer, 1999). Like all the 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 were no changes in the antigenic sites on the HA of the viruses in comparison with other published Egyptian LPAI H9N2 viruses, as the viruses of interest were genetically closed to the circulating viruses in Egypt since 2013 with no impacted alterations (Adel et al., 2017).

However, both strains (Qu/152792V and Qu/152890V) were related genetically to the variant Egyptian viruses (Egy/Var) possessed the same substitutions that characterized the Egy/var viruses including S127N and D189N at the overlapping antigenic site of H9N2; and in the acquisition of both new additional glycosylation sites in comparison to the other Egyptian viruses. Both substitutions were proved that they have a robust impact on the antigenicity of these viruses against the other circulating Egyptian viruses, that was studied previously by cross antigenicity (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 antigens 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).

The Phylogenetic analysis of the NA genes of five H9N2 influenza viruses and Figure 6, revealed that sequenced NA genes were belonging to LPAI H9N2 lineage G1 that originated from A/QU/HK/G1/97 of Hongkong. However, the middle east viruses classified according to NA sequences into 4 groups (Fusaro et al., 2011), the Egyptian viruses including the viruses of our study clustered to the group B that includes the viruses of 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 the examined Egyptian viruses, there was 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 was a substitution in the HB site 1 (366–373) at residue S372A in the studied viruses as 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 a.a 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 possessed seven glycosylation sites in comparison to the prototype of G1 lineage (QU/HK/G1/97) due to substitutions of the amino acid residues N400 S/G and R401W in the locus 2 of the HB site. In addition to loss of another glycosylation site ^{NGT}69 on the NA molecule of Qu/Egy/V2792/2015 virus that related to the Egy/var virus (Mosaad et al., 2017).

In conclusion, the Egyptian LPAI H9N2 viruses 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 at the summer season of 2016. Genetically, the LPAI H9N2 Egyptian viruses reveal a 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 the other Egyptian circulating viruses.

Continuous further study is recommended to follow up with the continuous evolution of the virus.

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