Mutation signature in neuraminidase gene of avian influenza H9N2/G1 in Egypt

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Received: 8 November 2016 / Accepted: 18 February 2017 / Published online: 23 May 2017
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Abstract The low pathogenic avian influenza (LPAI) H9N2 subtype has become the most prevalent and widespread in many Asian and Middle Eastern countries. It causes an enzootic situation in commercial poultry and known as a potential facilitator virus that can be transmitted to human from birds. The neuraminidase (NA) gene plays an important role the release and spread of the virus from infected cells and throughout the bird. The complete nucleotide sequences of the NA gene of seven H9N2 viruses collected from apparent healthy chicken and quail flocks in Egypt during 2014–2015, were amplified and sequenced. The phylogenetic relationships were investigated and all viruses were belonging to the A/Q/HK/G1/97 strain (G1-like). There were no insertions or deletions or shortening in NA stalk regions when compared to Y280-lineage and the human H9N2 isolates. No obvious changes NA interactions with antiviral drugs. We found that the Egyptian H9N2 viruses have seven glycosylation sites like the most recorded H9N2 viruses in the country, except A/Q/Egypt/14864V/2014 virus which has only six. The NA has four amino acid substitutions distributed in different parts of the hemadsorbing site. The most characteristic substitutions in this site were S372A and W403R these

substitutions were a distinctive feature resembling to human H9N2, H2N2 and H3N2 viruses but differs from the other avian influenza viruses. These Special features of surface glycoproteins of LPAI-H9N2 viruses refer to the tendency for enhanced introductions into humans and ensuring the importance of poultry in the transfer influenza viruses.

Keywords Avian influenza viruses · (LPAI) H9N2 · H3N2 · Hemadsorbing (HB) sites · Phylogenetic analysis · Neuraminidase gene

Introduction

The low pathogenic avian influenza LPAI-H9N2 subtype was occurring frequently in several countries from 1966 till now [22]. The H9N2 virus persists in chicks and spreads to non-affected flocks without displaying much of severe signs [7]. When other pathogens accompany it, it causes high economic losses in poultry [19]. The H9N2 virus has been detected in bird populations in Asia, Europe, the Middle East and Africa [39]. Recently, H9N2 virus acquired a considerable importance because of constant and rapid spread of the virus in domestic fowl. More importantly, H9N2 can cross the species barrier and have the ability to be transmitted to humans in Hong Kong and Guangzhou, China [30, 40].

The most of human cases of avian influenza infection (H5, H7, and H9) have been reported mainly as a result of poultry-to-human transmit [53]. H9N2 virus does not seem to transmit easily between humans and tends to result in mild clinical disease [33], the Egyptian H9N2 virus showing a lot of markers that correlated with increased affinity of the virus to the human host [27]. Recently four

Electronic supplementary material The online version of this article (doi:10.1007/s13337-017-0367-7) contains supplementary material, which is available to authorized users.

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human cases of H9N2 avian influenza from Egypt have been reported from March 2015 until May 2016. The infections were through exposure to infected poultry or contaminated environment with poultry waste [10, 14].

The H9N2-LPAI is belonging to influenza A of family *Orthomyxoviridae* and possesses eight negative-sense single-stranded RNA segments [3]. H9N2 virions display two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), both recognizing sialic acid-containing receptors on the host cell surface [52]. The level of NA activity should compensate the HA binding activity to ensure the release of progeny virus [15]. NA resembles a homotetramer of mushroom shape comprises 470 amino acid residues. The NA three-dimensional structure consists of four domains: a cytoplasmic tail, a transmembrane domain, a stalk region and a globular head [48]. The enzyme active site and calcium binding domain situated in the head of NA which stabilizes the enzyme structure at low pH values [23], the hem adsorbing binding site function has yet to be clarified. It has been suggested that it plays a role as an alternative binding site [33]. The main function of NA is the promotion of virus release by removing sialic acid from newly synthesized HAs and NAs, which are sialylates by cellular enzymes, and believed to prevent the aggregation of newly synthesized virions. So that it facilitates the spreading of virus progeny [1, 2, 40] NA has also been considered a suitable target for antiviral drugs since it possesses an active site whose amino acid sequence is conserved among all influenza virus types and subtypes [9].

The avian influenza H9N2 subtype is classified into two major genetic lineages in both poultry and wild birds into North American and the Eurasian H9N2 lineages [18, 51]. The Eurasian lineages consist of two sublineages represented by their prototype strains: A/Q/HK/G1/97 (G1-like), A/D/HK/Y280/97 (Y280-like) [54]. There were four distinct groups (A, B, C and D) co-circulating in The Middle Eastern and Central-Asian countries. Groups A and B are circulating extensively in The Middle Eastern countries from 1999 [13, 27] to date.

Depending on the NA gene sequence, the Egyptian H9N2 viruses are classified within cluster B of the G1 sublineage (A/Q/HK/G1/97). They showed significant similarity with G1-like viruses and other H9N2 influenza viruses isolated from humans in Hong Kong and those from Israel 2008 strains [32]. The A/QL/HK/G1/97 thought to be the donor of the six internal genes to the poultry and the human H5N1viruses [49].

Because of different roles of the NA gene in virus pathogenicity, host range, and antiviral properties, mutations in NA gene can affect the virus biological properties. This study was carried out to determine in-depth genetic analysis of the neuraminidase NA gene and to study its

molecular markers that can promote the NA activity in H9N2 viruses isolated from Egypt during 2014–2015. The phylogenetic relationship to the other Asian H9N2 lineages and the mutation analysis of NA gene including the HB sites were carried out in comparison with the H9N2 viruses from human to study the tendency of these viruses for human infection. From a public health point of view, since both H9 and H5 subtypes have the capability to infect human so this study makes highlighting on preparation for more understand the mutation disposition to inter-species transmission of H9N2 viruses.

Materials and methods

Virus isolation and propagation

Cloacal and oropharyngeal swabs were collected as part of ongoing long-term avian influenza surveillance; the samples were characterized at the National Laboratory for Veterinary Quality Control on Poultry Production, Egypt (NLQP, Ministry of Agriculture). 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 for influenza type A by real-time RT-PCR to amplify 244 bp of the M segment [45], then Sub-typing of the samples (H5, H9, H7) using specific sets of primers and probes to determine the HA and NA subtypes [11, 35] With One-Step RRT-PCR Kit (QIAGEN, Hilden, Germany). Thermal profile for amplification of HA gene of H5, H7 and H9 subtypes: 50 °C for 30 min, 95 °C for 15 min, cycling steps 94 °C for 10 s, 54 °C for 30 s and 72 °C for 10 s repeat 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).

The extracted RNA was tested further for IBV and NDV by real-time RT-PCR to make sure that the isolates are free from other important pathogens affecting respiratory system [25, 42]. Each positive sample for influenza A virus by 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 [44]. The allantoic fluid was harvested, tested for Hemagglutination, and then stored at −80 °C until use.

According to HA phylogenetic grouping, seven viruses were selected representing H9N2 virus groups circulating in Egypt. Isolates were collected from January 2014 to July 2015 from backyard poultry holdings and commercial farms representing Upper and Lower Egypt and including chicken and quails. The epidemiological data of the selected samples is shown in (Table 1).

Table 1 Epidemiological data of selected Egyptian H9N2 isolates used in this study

No.	H9N2 isolate	Collection date	Collection site	Governorate	CT ^a	HA log activity (log ²)	Gene Bank no.
1	A/chicken/Egypt/1455V/2014	January	Farm	Giza	18	9	KY078294
2	A/quail/Egypt/14864V/2014	May	Farm	Monofia	12	7	KY078295
3	A/chicken/Egypt/141794V/2014	August	Farm	Monofia	17	8	KY078296
4	A/chicken/Egypt/14153VS/2014	September	Farm	Giza	13	9	KY078297
5	A/chicken/Egypt/158SG/2015	January	backyard	Al Daqahlia	14	7	KY078298
6	A/chicken/Egypt/1560VG/2015	February	Farm	Al Daqahlia	15	5	KY078299
7	A/chicken/Egypt/152537V/2015	July	Farm	Asyut	15	9	KY078300

^a Cycle threshers

Amplification, sequencing and phylogenetic analysis of the full NA gene

The first-strand cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and Uni-12 primer 5'AGCRAAAGCAGG3' as per the manufacturer's protocol. The full NA gene of the isolates was amplified using gene specific forward primer 5'GGCACAACACATGATAGAAGTCCC3' and reverse primer 5'CGCCAACAAGTACTAAGCACACAT3' Using a platinum[®] taq DNA polymerase high fidelity (Invitrogen, Carlsbad, CA). The entire NA gene for each AI isolate was amplified. The expected size of the PCR product was confirmed by gel electrophoresis. 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 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 3130xl DNA Analyzer (Applied Biosystems). Sequences were assembled using seqscape, SeqMan DNALasergene 6 software (DNASTAR, Madison, WI, USA).

MegAlign (DNASTAR) and BioEdit 7.0 were used for multiple sequence alignment [20]; Percent identity matrices comparing the genes under study to each other were obtained. We generated neighbor-joining Phylogenetic trees for all gene segments using the distance-based method in MEGA6. We calculated bootstrap values based on 1000 replicates [47]. The phylogenetic tree was generated from the deduced amino acid sequences of the NA gene using the neighbor-joining method. The tree included other NA genes of Egyptian H9N2 sequences available in the GenBank database and closely related H9N2 viruses from other Middle Eastern countries [36], major ancestral H9N2 strains, and other influenza virus subtypes with closely

related H9N2 genes. The tree was constructed rooted with A/Turkey/Wisconsin/1/1966.

The NA gene selection pressure

Selection can be measured by ω (dN/dS), in which 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 [41] and Hasegawa–Kishino–Yano model of nucleotide substitution and the selection at the codon level was created by MEGA6 software [47].

The test statistic dN–dS is used for detecting codons that have undergone positive selection, If dN > dS and $\omega > 1$, it means that amino acid changes provide better fitness. The *p* value is calculated, when *p* value is <0.05, it is considered significant at a 5% level [41].

Results

Virus isolation and confirmation

HA activity and real-time RT-PCR results for seven isolates showed in (Table 1). HA activity ranged from 7 to 9 log2 and the cycle threshold (Ct) gene values for H9 subtype ranged from 12 to 18.

Results of Conventional RT-PCR

The PCR for NA gene using forward and reverse primers were visualized by gel documentation system BDA digital—image captures (Biometra, Germany). The PCR products electrophoresis was done on an ethidium bromide stained agarose gel and the amplified gene of expected correct size at 1410 bp

Genetic and phylogenetic analysis of NA

In this study, 1410 base pairs of the NA genes (ORF region) were sequenced and 469 amino acid sequences of the NA of the seven isolates were deduced from the nucleotide sequence as shown in (Fig. 1). The H9N2 of the seven tested viruses in this study belonged to group B H9N2 and cluster tightly with those of Israeli, Iran, United Arabia Emirates and Pakistan specially closed to A/turkey/Israel/311/2009 of the HK-G1 strain and away from strains which clustered in A, C and D groups.

The Egyptian isolates clustered into multiple minor subgroups. The A/Quail/Egypt/14864V/2014 virus from quail origin were not clustered with other published viruses isolated from quail origin in the same year.

The NA genes showed the nucleotide and deduced amino acid similarities ranged from (92 to 99%) and (84 to 99%), respectively between strains under study and all Egyptian isolate. the strain A/Quail/Egypt/14864V/2014 showing the lower nucleotide and deduced amino acid similarities with all Egyptian isolates (92 and 84) respectively. The seven tested viruses shared nucleotide and deduced amino acid homologies with each other ranged from (96 to 99%) and (95 to 100%) and with G1-leinage 91 and 89–91%, respectively, the amino acid identities of commercial H9N2 vaccine strains used in Egypt (A/chicken/Egypt/114940v//2011, A/chicken/Saudi-Arabia/CP7/1998 and A/chicken/Iran/av1221/1998) were ranged from 96 to 98, 91 to 92 and 90 to 92% respectively. NA genes of Egyptian isolates showed the higher similarity to those of A/turkey/Israel/311/2009 (95–97%) (Supplementary Table 1).

Deduced amino acid sequence analysis

The stalk length, hem adsorbing site, enzyme active site, and the number of glycosylation sites have a potential role in neuraminidase activity [34]. Analysis of stalk length revealed that no stalk deletions at sites 38–39 or 46–50 (Table 2). Amino acids of HB site in the NA stalk are three locations which include first locus (366–373), second locus (399–404) and third locus (431–433) (Fig. 2). The sialic acid binding pocket of the hemadsorbing site of all Egyptian viruses shows motif (³⁶⁶IKKDSRAG^{373/399}-DSDSW^{404/431}PQE⁴³³) (Table 2). Four substitutions were observed in hemadsorbing site: (K367N, S372A and I402G/N) Different from HB of G1-linage virus precursor. K367N was observed in A/chicken/Egypt/141794V/2014, A372S in A/Quail/Egypt/14864V/2014, and I402G/N observed in A/chicken/Egypt/1560VG/2015, A/chicken/Egypt/1455V/2014 and A/chicken/Egypt/141794V/2014. The W403R substitution observed in A/chicken/Egypt/1560VG/2015 but it is similar to G1-linage virus precursor

and different from other Egyptian viruses (R403W) (Table 2). 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.

NA glycosylation sites with the N-X-T/S motif (in which X may be any amino acid except proline). Seven of these sites were located in the NA protein positions (44, 61, 69/70, 86, 146, 200, and 234). 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, The Egyptian virus A/Quail/Egypt/14864V/2014 contain only six sites as it lost glycosylation site at ^{SGT}69. The glycosylation site in 402 was not found in the Egyptian isolates (Table 3).

NA gene selection pressure

To make sure whether mutations in NA are under certain selective pressure or not, we calculated ratios of substitutions per nonsynonymous and synonymous sites (dn/ds). The finding indicates p-value of the seven tested viruses was ranged from 0.4 to 1.0 (more than 0.05). These results indicate that the NA genes of our viruses have not been under positive selection pressure.

Discussion

since mid-1990s the H9N2 Avian influenza viruses have seriously affected the poultry production of the Far and the Middle East and are conceived one of the most probable candidates causes a new influenza humans pandemic [13]. Subsequently, the virus revealed in North African countries: Egypt, Libya and Tunisia [26, 35]. H9N2 was detected in Egyptian farms from bobwhite quail in May 2011 [4]. Also, H9N2 was isolated in mammalian species such as cats and dogs [56]. World Health Organization continuous monitors Human case of H9N2 influenza infection [24]. In Countries affected, H9N2 virus high seroprevalences were detected in poultry farms workers and poultry processing plants [28].

In this study, the NA genes of seven H9N2 influenza viruses were isolated and genetically characterized from poultry farms and backyards in Egypt 2014–2015. The Phylogenetic analysis revealed that all tested isolates belonging to group B and clustered with those of Iran, Pakistan, United Arab Emirates and Israeli and very close to A/turkey/Israel/311/2009. All H9N2 in our analysis are within a single lineage originated from A/Q/HK/G1/97 (G1 lineage) [27]. This finding indicates a common origin of those viruses from a single progenitor of the Asian origin (Fig. 1). The Egyptian isolates clustered into multiple

Fig. 1 Phylogenetic tree of the NA genes of avian influenza H9N2 viruses isolated in Egypt during 2014–2015 and reference isolate from Gen Bank. The Phylogenetic analysis was conducted by using the neighbor-joining algorithm with the Kimura 2-parameter model. Strain A/Turkey/Wisconsin/1/1966 was used as the root of the tree and the reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1000 replications. Evolutionary analysis was conducted by using MEGA6 (<http://www.megasoftware.net>). A *black rhomboid* indicates isolates sequenced specifically for this study *boldface* indicates avian viruses

Table 2 Amino acid substitutions in the hemadsorbing sites and stalk region

H9N2 virus	Deletion In stalk region	HB-Site																
		366–373					399–404					431–433						
A/Quail/Hong Kong/G1/97 ^{4a}	38–39	I	K	K	D	S	R	S	G	D	S	D	I	R	S	P	Q	E
A/Hong Kong/1073/99 (Human-H9N2) ¹	38–39	–	–	–	–	–	–	–	–	–	–	–	N	W	–	–	–	–
A/Beijing/39/1975 (H3N2) ³	NO	–	S	E	–	–	–	–	–	–	–	–	N	–	–	E	–	–
A/Lebanon/11L046/2012 (H3N2) ³	NO	–	N	E	T	–	–	L	–	–	R	G	D	–	–	K	E	–
A/Egypt/84/2001(H1N2) ³	NO	–	S	E	K	L	–	–	–	–	R	G	N	–	–	N	–	–
A/Chicken/Hong Kong/G9/97 ¹	NO	–	–	–	–	–	–	–	–	–	–	–	N	W	–	–	–	–
A/Duck/Hong Kong/Y280/97 ²	63–65	–	–	E	–	–	–	–	–	–	–	–	N	W	–	–	–	–
A/Turkey/Wisconsin/1/1966	NO	–	S	–	–	–	–	–	–	–	–	S	N	W	–	–	–	–
A/quail/UAE/D1556/2011	NO	–	N	E	–	L	–	K	–	–	–	–	N	W	–	–	–	–
A/Quail/Lebanon/272/2010	NO	–	–	–	–	–	–	A	–	–	–	–	N	W	–	–	–	–
A/chicken/Israel/375/2007	NO	–	N	–	–	–	–	A	–	–	–	–	N	L	–	–	–	–
A/Chicken/Egypt/D4692A/2012	NO	–	–	–	–	–	–	A	–	–	–	–	G	W	G	–	R	G
A/Chicken/Egypt/D7108E/2013	NO	–	–	–	–	–	–	A	–	–	–	–	N	W	–	–	–	–
A/Chicken/Egypt/SCU9/2014	NO	–	–	–	–	–	–	A	–	–	–	–	N	W	–	–	–	–
A/chicken/Egypt/114940V/201 ^{4b}	NO	–	–	–	–	–	–	A	–	–	–	–	G	W	–	–	–	–
A/chicken/Egypt/Q10660B/2015	NO	–	–	–	–	–	–	A	–	–	–	–	G	–	–	–	–	–
<i>A/chicken/Egypt/1455V/2014</i>	NO	–	–	–	–	–	–	A	–	–	–	–	G	W	–	–	–	–
<i>A/quail/Egypt/14864V/2014</i>	NO	–	–	–	–	–	–	–	–	–	–	–	N	W	–	–	–	–
<i>A/chicken/Egypt/141794V/2014</i>	NO	–	N	–	–	–	–	A	–	–	–	–	G	W	–	–	–	–
<i>A/chicken/Egypt/14153VS/2014</i>	NO	–	–	–	–	–	–	A	–	–	–	–	N	W	–	–	–	–
<i>A/chicken/Egypt/158SG/2015</i>	NO	–	–	–	–	–	–	A	–	–	–	–	N	W	–	–	–	–
<i>A/chicken/Egypt/1560VG/2015</i>	NO	–	–	–	–	–	–	A	–	–	–	–	G	–	–	–	–	–
<i>A/chicken/Egypt/152537V/2015</i>	NO	–	–	–	–	–	–	A	–	–	–	–	N	W	–	–	–	–

Amino acid residues were compared in the neuraminidase stalk region, HB site of influenza A H9N2 viruses. Residues differences and similarities are compared with reference strains of lineages

1—Human isolate (H3N2), 2—G1-like, 3—Y280-like, 4—vaccine strains, 4a—A H9N2 Available vaccine virus (G1-LIKE; WHO report, 2010), 4b—A H9N2 commercial Egyptian vaccine virus

The viruses under study in italic

Dashes identically identical conserved residues

minor subgroups. The phylogenetic clustering among the Egyptian viruses from different governorates clustered together without being restricted by species or geographical distribution. Whereas, A/Quail/Egypt/14864V/2014 virus from quail origin was not clustered with other published viruses isolated from quail origin in the same year. And that displayed continuous virus diversity. This is means that this is the dominant subgroup that may be circulating in the country from 2011 till now.

Comparison of the nucleotide sequences identity of NA genes of the seven H9N2 viruses isolated in this study from different types of birds with those of H9N2 viruses isolated from human A/HK/G1/1073/99 was 90%. The lower nucleotide and deduced amino acids identity among the seven isolates was from A/Quail/Egypt/14864V (Supplementary Table 2). The local commercial H9N2 vaccine

strains (A/chicken/Egypt/114940v//2011) in Egypt showing the highest similarity in comparison to other commercial vaccine used, some recent studies detect H9N2 viruses in vaccinated chicken flocks [5], so that some vaccines did not make full protection against H9N2 infection, and vaccine candidates from local circulating strains should be used and continuously updated. The comparison with the earliest Egyptian virus A/avian/Egypt/920431/2006 indicates that nucleotide and amino acid similarities between our isolates are the lowest (93 and 83% respectively). That means the H9N2 viruses are under continuous evolution with frequent mutations from 2006 till now. When comparing the similarity among our isolates and others from the neighbor countries, we found that the NA gene of Egyptian viruses showed the higher similarity with A/turkey/Israel/311/2009 (95–97%) (Supplementary Table 2),

Fig. 2 The three-dimensional structure of NA gene. Showing **1** crystal structure of the neuraminidase tetramer of the H9N2 influenza virus (overview) homotetramer chain A, B, C, and D. **2** Showing location of the hemadsorbing site of NA protein of Egyptian H9N2 on chain D, **3** showing that HB compose of 3 overlapped chains, I402G (yellow) and R403W (blue) amino acid substitutions at the HB binding site distinguish A/chicken/Egypt/60VG/2015 virus from other H9N2 viruses circulating in Egypt using PyMol viewer (color figure online)

Table 3 Sequence of amino acids in glycosylation sites between the N2 neuraminidase proteins

H9N2 virus	Amino acid site (s)							
	44	61	69, 70	86	146	200	234	402
A/Hong Kong/1073/99	NTS	NIT	NNT	NWS	NGT	NAT	NGT	NWS
A/Beijing/39/1975/(H3N2)	SPA	NIT	NTT	NWS	NDT	NAT	NGT	NRS
A/quail/Hong Kong/G1/97	NPS	NIT	NNT	NWS	NGT	NAT	NGT	IRS
A/chicken/Hong Kong/G9/97	NSS	NIT	NST	NWS	NGT	NAT	NGT	NSS
A/duck/Hong Kong/Y280/97	NPS	–	NST	NWS	NGT	NAT	NGT	NWS
A/turkey/Wisconsin/1/1966	PPS	NIT	NIT	NWS	NGT	NAT	NGT	NWS
A/white bellied bustard/UAE/D1520/2011	NLS	NTT	NIT	IWS	NGT	NAT	NGT	NWS
A/quail/Lebanon/272/2010	NSS	NIT	NGT	NWS	NGT	NAT	NGT	NWS
A/turkey/Israel/1567/2004	NPS	NIT	NNT	NWS	NGT	NAT	NGT	NLS
A/chicken/Egypt/D4692A/2012	NTS	NIT	NGT	NWS	NGT	NAS	NGT	GWG
A/chicken/Egypt/D7108E/2013	NTS	NLT	DGP ^a	NWS	NGT	NAT	NGT	SWS ^a
A/chicken/Egypt/SCU9/2014	NTS	NIT	NGT	NWS	NGT	NAT	NGT	SWS ^a
A/chicken/El Fayoum/CAI25/2011	NTS	NIT	NGT	NWS	NGT	NAT	NGT	SWS ^a
A/chicken/Egypt/1455V/2014	NTS	NIT	NGT	NWS	NGT	NAT	NGT	GWS ^a
A/quail/Egypt/14864V/2014	NTS	NIT	SGT ^a	NWS	NGT	NAT	NGT	SWS ^a
A/chicken/Egypt/141794V/2014	NTS	NIT	NGT	NWS	NGT	NAT	NGT	GWS ^a
A/chicken/Egypt/14153VS/2014	NTS	NIT	NGT	NWS	NGT	NAT	NGT	SWS ^a
A/chicken/Egypt/158SG/2015	NTS	NIT	NGT	NWS	NGT	NAT	NGT	SWS ^a
A/chicken/Egypt/1560VG/2015	NTS	NIT	NGT	NWS	NGT	NAT	NGT	GRS ^a
A/chicken/Egypt/52537V/2015	NTS	NIT	NGT	NWS	NGT	NAT	NGT	SWS ^a

Dashes indicate losing the glycosylation in this site by deletion

The viruses under study in italic

^a Star indicates losing the glycosylation in this sites by change the Asparagine to any another amino acid

that may indicate the possible origin of the recent Egyptian viruses.

The main function of NA is to release the virus from the cell and to help its spread through its action on the neuraminic acid in the receptors [12]. The stalk length, hemadsorbing site, enzyme active sites and the number of

glycosylation sites had a potential role in neuraminidase activity [17, 23].

The sequence and length of the stalk region known to vary among NA subtypes [17], however, the shortening of the NA stalk by deletion of the amino acid is discriminatory feature of the highly pathogenic H5 and H7 influenza

[38]. The Longer stalk length of viral NA enhances replication of influenza virus [34]. The amino acid deletion from 38 to 39 in the NA was found to decrease the virus ability to release from the cell [31]. Analysis of NA stalk region of the Egyptian H9N2 viruses revealed that no stalk deletion at (38–39) [27] and this Similar to our findings in this study from seven Egyptian viruses as well as other viruses from the Middle Eastern origin (Israel, Lebanon and United Arab Emirates) with their sequence available in the GenBank (Table 2). This deletion found to be characteristic to G1 strain includes the H9N2 viruses isolated from humans in Hong Kong (A/HK/1073/99), it is not known whether this deletion in the NA of H9N2 viruses is correlated with pathogenicity in chickens or not.

The avian H9N2 viruses neuraminidase (NA) has a separate sialic acid binding site (hemadsorption site HB) and the NA hemadsorbing site is a second sialic acid-binding pocket on the NA protein surface, in addition to the catalytic site that is not present in human viruses. The biological significance of the hemadsorption activity of NA in avian influenza H9N2 viruses remained elusive and its biological function is not completely understood [49]. The hemadsorption site is located on the NA surface away from the enzymatic site and its conservation suggests that it is maintained for a specific role in virus replication [1]. The HB site in the NA of Asian H9N2 viruses was found to be under positive selection pressure and reassortment resulting compatible combinations from HA and NA [15]. The mutations in the HB site were previously seen in viruses that bind to α -2,6-linked receptors [43]. the NA hemadsorption site has a role in virus replication in birds but is either not essential or disadvantageous for the virus replication in humans [29].

In our findings, substitution recorded in different positions of hemadsorption site (Fig. 2) as a substitution at site K367N which firstly observed in H9N2, this substitution observed in most of the human H3N2 virus as A/Lebanon/11L046/2012 (Table 2). This situation may lead to the generation of viruses with unknown biological properties.

Nevertheless, several single or multiple amino acid substitutions in N2 NAs may abolish the hemadsorption activity without apparent effects on enzymatic activity [21]. There were 2 distinct mutations in hemadsorption site in our seven viruses, S372A and I402G/N. The substitution S372A was observed in all Egyptian viruses except for one virus (A/Quail/Egypt/V864/2014) which lack this mutation similar to the original G1/97 strain and the human G1-like virus precursor (A/HK/1073/99) and other human viruses ((A/Beijing/39/1975(H3N2) and A/Egypt/84/2001(H1N2)). The substitution I402N was observed in 4 out of 7 viruses in our study, while I402G was observed in 3 viruses (Table 2). The substitution W403R, which was detected before in most of human virus A/Beijing/39/1975 (H3N2)

as A/Egypt/84/2001(H1N2), detected in recent Egyptian H9N2 virus A/chicken/Egypt/60VG/2015. This substitution also observed in A/Q/HK/G1/97(H9N2), Pakistan strains isolated in 2008 and A/chicken/Iran/186/2011. This site is located on the surface of NA protein far from the enzymatic site (Fig. 2).

The results of all previous studies of the receptor specificity of Egyptian H9N2-HA encoded amino acid H183 and L226 (H3 numbering) which were reported to Correlate with a shift the affinity of the HA from the avian towards the human-like sialic acid linkage [50] [15]. The HB site of the NA is conserved in viruses with a high affinity for avian 2, 3-linked receptors and human 2, 6-linked receptors [57]. Species of aquatic birds act as a natural reservoir for influenza viruses and could serve as an intermediate host in the zoonotic transmission into humans. The substitutions W403R and S372A were detected in Hong Kong H9N2 viruses from poultry and both H2N2 and H3N2 subtypes that causing human pandemics, in the HB-site sequence [33]. The features of surface glycoproteins of H9N2 viruses suggested a tendency for favorable introduction into humans and asserted the significance of poultry infection for influenza virus zoonoses.

The antiviral drug targeting Neuraminidase protein because it plays a major role in influenza virus propagation [37], NA, as well as HA, under continues evaluation, which resulting in resistance to neuraminidase inhibitors. Any mutation may affect on NA enzyme sensitivity to antiviral drugs, The most common neuraminidase mutation which arises under pressure of antiviral drug is the conserved Glu 119(119E), cause 100-fold reduction in the sensitivity of the enzyme to zanamivir [46]. Substitution on the position R292K is manifested as resistance to Oseltamivir and Zanamivir [55]. Another mutation, which brings resistance to Oseltamivir, is H274Y [8]. Sequence analysis of binding-pocket residues involved in interactions with antiviral drugs show that there is no mutations were present. All Egyptian viruses including viruses under study showing 119E, 198D, 222I, 274H, and 292R substitutions And lacking 119I/V, 198N, 222R, 274Y, and 292K substitutions, which are associated with resistance to the sialidase inhibitors oseltamivir and zanamivir, Antiviral [16].

NA glycosylation sites were similar in most H9N2 viruses with seven glycosylation sites [22]. The NA genes of the Egyptian viruses contained also seven glycosylation sites 44, 61, 69/70, 86, 146, 200 and 234. In addition, the Egyptian viruses showed conserved sequences in all tested viruses, the mutation observed in most H9N2 viruses was found at position 69 (Table 3). Only one isolate A/quail/Egypt/14864V/2014 from quail origin missed N-link 69 due to amino acid substitution at this position, so it contains only six glycosylation sites, similar to A/chicken/Egypt/D7108E/2013 virus which recently recorded to miss

this site. The glycosylation site at 146 is conservative for all NAs [4]. Asparagine (Asn) in position 146 site seems to have a regulatory function. It has been shown that the NA enzymatic activity affected by carbohydrate chain at Asn146, causing a 20-fold decrease in activity [18]. The mechanism by which an additional glycosylation site in NA may affect pathogenicity is obscure, but it is supposed to be linked to the HA cleavage in cells. For example, the highly glycosylated NA of the highly pathogenic virus may facilitate the increasing activation of the host proteases required to cleave the HA of the virus [9]. The glycosylation site 402, which was described previously as a characteristic of H9N2 viruses [6], was not observed in Egyptian viruses or in G1 avian origin virus (A/quail/HK/G1/97) and in contrast, site 402 present in (A/HK/1073/99) G1 human origin. Addition or loss of potential glycosylation may contribute toward increased virulence due to altered sialidase activity and antigenicity [9].

In conclusion the Genetic characterization of LPAI-H9N2 viruses has remarkable important to grasp the key features for the emergence of H9N2 among poultry and as a potential pandemic virus. Phylogenetically the recent Egyptian H9N2 viruses have no significant changes versus previous years; the virus is spreading in the country without being restricted to geographical distribution. Numbers of new substituting mutations were detected in NA proteins may be of possible public health concern to word the human transmission. Our results showed that the genetic changes in the NA represent another indicator of continued acclimation of avian influenza to humans and the gradual acquisition and accumulation of virus mutation. Further molecular studies on H9N2 internal genes are needed to compare with the activity of surface proteins (HA and NA).

Acknowledgements Thanks to the entire gene analysis unit staff, National Laboratory for Quality Control on Poultry Production (NLQP), Animal Health Research Institute, Cairo, Egypt for their great help during the study. Special thanks to Drs. Abdelsatar Arafa, Amany Adel and Naglaa Hagag.

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