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Molecular Characterization of Avian Influenza Virus Subtype H9N2 in Poultry in Egypt During 2011-2013 with Emerging of a New Variant in Quails

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Abstract: Avian influenza virus subtype H9N2 has become established in poultry in Egypt and together with highly pathogenic H5N1 causing serious threats in the field of poultry production. Since the first isolation of H9N2 AIV in May 2011, continuous monitoring of this subtype in poultry population has occurred. The epidemiological situation of H9N2 virus during the period from August 2011 to January 2013 was studied. The results of H9N2 virus detection indicated that these viruses were still circulating in different species of domestic poultry like chickens, ducks, turkeys and quails during that period. The H9N2 viruses were detected in 343 commercial flocks and 17cases from house hold birds in 20 different Governorates. According to the phylogenetic analysis, the Egyptian H9N2 viruses can be subdivided into two subgroups based on HA gene variation. Both two subgroups were circulating during 2011to 2013 in different locations. The genetic analysis indicated 5-20 amino acid substitutions in HA gene in comparison to the original A/Quail/Egypt/113413v/2011 virus. The highest variation was recorded in quail isolate (A/Quail/ Egypt/122313V/2012) which has been isolated during 2012 and had 20 A.A. substitutions distributed along HA protein including the receptor binding domain and antigenic epitopes proposed to be a new variant of H9N2 virus in quail. The results from this work indicate the importance of periodical monitoring of circulating viruses especially in endemic countries like Egypt to update the epidemiological situation and that will help the efforts to control.

Key words: H9N2 subtype · Genetic characterization · HA gene sequencing · Phylogenetic analysis

INTRODUCTION

Influenza A viruses are represented in many anti-genic subtypes; 18 haemagglutinin (HA) and 11 neuraminidase (NA) subtypes; the recent subtypes H17N10 and H18N11were detected in bats [1, 2]. Since the 1966s, the first detection of H9N2 AIV [3], the poultry populations in many Countries became susceptible to infection with this subtype as Since the 1990s, great economic losses in poultry industry occurred in many countries in Asia, Europe and the Middle East due to many outbreaks of H9N2 AIV which might be as a result of co- infection [4,5].Although the low pathogenicity of H9N2 AIV subtype proved by absence of multiple basic amino acids at the cleavage sites, it is remarkable between other subtypes as it infect many species including chickens [6], pigeons [7], turkeys, ducks [8], geese, pigs [9] in addition to its ability to infect human [5]. Also, infection with mild or no clinical signs facilitated the viruses spread rapidly with frequent antigenic variation due to the difficulty of true diagnosis [10]. Among many poultry species quail are highly susceptible to infection with H9N2 AIV, as the first detection of H9N2 virus infection in land-based poultry in Asia was in 1988 then the virus already acquired some of the molecular marker facilitated its adaptation in terrestrial poultry [11]. In Egypt, a previous report was described the isolation and identification of H9N2 virus from a commercial quail flock in May, 2011 from Giza Province as well as the preliminary genetic characterization of partial HA and NA sequences

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has also been made [12], Subsequent isolation and characterization of H9N2 AIV from chicken in Egypt were reported [13,14]. Relative host restriction is present for H9N2 viruses as G1-like viruses were commonly detected in quail, less frequently detected in other minor poultry species and not detected in chickens, However G1like viruses caused outbreaks in chickens in Middle Eastern and European countries [15]. In this study, the epidemiological situation of H9N2 virus in Egypt during August 2011 to January 2013 was investigated. In addition to the isolation and genetic characterization of H9N2 viruses from chicken, turkey and quail in Egypt was indicated.

MATERIALS AND METHODS

Sampling: In this study a total of 12497 samples from 20 different provinces throughout Egypt were collected and examined. Most of these cases were apparently healthy, although, there were cases of respiratory distress and mortalities from chicken, all these cases could be associated with other pathogens which aggravated the case.

The samples were collected during the period of August 2011 to January 2013from chicken, duck, turkey and quail (Table 1, 2) Swab sampling of birds was conducted according to published guidelines of the OIE [16]. The samples were submitted to National Laboratory for Quality Control on Poultry Production (NLQP), Animal Health Research Institute, through farm and house-hold birds surveillances.

Table 1: Total number of samples that were collected from different poultry species during the study:

Species	Samples numbers
Chicken	11545
Duck	593
Turkey	345
Quail	14
Total numbers	12497

Virus Isolation: Fertile Specific pathogen Free 9 - 11 day-old Embryonated chicken Eggs (SPF-ECE) were inoculated with 0.1 ml of pooled swab samples through the allantoic route and the inoculated eggs were incubated at 37°C [16]. Inoculated eggs were examined daily for embryo mortality. Amnio-allantoic fluid was harvested after five days and tested for hemagglutination activity according to the recommended protocol [16]. The test was carried out using 1% chicken erythrocytes in 96-well V-shape plates (Nunc, Wiesbaden, Germany) [16].

PCR-Examination: RNA was extracted from a pool of five cloacal and five tracheal swabs by using a QiaAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The extracted RNA was used for RRT-PCR and regular PCR using primers and probe listed on (Table 3).

Table 2: Total number of samples that were collected during the present study *(8/2011 to 1/2013):

		Surveillance		Date of collection (M/Y)		
Serial	Provinces	Farms	Backyards	8-12/2011	1-12/ 2012	1/ 2013
1	Giza	779	265	549	453	42
2	Qalubia	1027	9	521	511	4
3	Cairo	15	2	5	12	0
4	Minufiyah	3267	245	1060	2296	155
5	Dakahlia	853	879	1083	584	65
6	Gharbia	209	106	88	204	23
7	Sharkia	915	364	794	390	95
8	Behaira	2312	78	724	1552	114
9	Alexandria	36	0	5	29	2
10	Domiatt	37	51	38	47	3
11	Suez	36	2	19	19	1
12	Ismailia	205	9	62	142	10
13	Kafr El-Sheikh	88	24	50	60	2
14	Qena	70	2	19	51	2
15	Fayoum	97	39	36	97	3
16	BeniSweef	73	91	121	41	2
17	El-menia	111	102	105	106	2
18	Aswan	23	0	6	17	0
19	Souhaj	33	3	11	24	1
20	Luxor	24	16	18	10	12
Total numbers		10210	2287	5314	6645	538

*(M/Y): (month/ year)

Table 3: The Sequence of primers and probe used in amplification of HA gene of H9N2:

Primer	Sequence	References
H9F:primer	GCC ACC TTT TTC AGT CTG ACA TT	Ben Shabat *et al*., 2010 [17]
H9R: primer	GGA AGA ATT AAT TAT TAT TGG TCG GTA C	
H9:Probe	Joe- AAC CAG GCC AGA CAT TGC GAG TAA GAT CC- Tamra	
F1-6 (forward)	TAG CAA AAG CAG GGG AAT TTC TT	Designed by NLQP-Manufactured by
R-1320 (reverse)	ATC TTG TAT TTG GTC ATC AAT C	(Metabion-Germany)
F 950 (forward)	AAG GTG GCT TAA TCA GTA CAT TG	
R 1740 (reverse)	TAG AAA CAA GGG TGT TTT TGC TA	

Sequencing 0nd Phylogenetic Analysis: Determination of nucleotide sequences for HA gene of the selected isolates were carried out for each amplified gene using a Big Dye Terminator Kit (Version 3.1; Applied Biosystems, Foster City, CA) on a 3130 Genetic Analyzer (Applied Biosystems, Foster City,CA), using specific primers for HA gene sequencing. Primer sequences for the HA segment were described in (Table 3). The nucleotide and amino acid sequence analysis were performed for HA genes of the isolated Egyptian viruses in comparison with other related strains, including the coding regions HA, 1-1683. Genotyping and phylogenetic analysis of the isolates sequences used for comparison in this study were obtained from GenBank and were available from the National Center for Biotechnology Information (NCBI), Influenza Viruses Resource (http:// www. ncbi. nlm.Nib.gov/genomes/FLU/Database). The pair-wise nucleotide percent identity matrix for H9N2 isolates from Egypt and other countries was calculated using the CLUSTAL-V algorithm in the MegAlign program of the Lasergene software suite (DNASTAR, Madison, Wisconsin). Sequence alignments of nucleotide and deduced amino acid sequences were generated using the CLUSTAL-W program [16]. Phylogenetic and molecular evolutionary analyses had been conducted using MEGA version 5 [18], with neighbor-joining method. The robustness of the groupings in the neighbor-joining analysis was assessed with 1000 bootstrap replicates. The sequences of the HA genes of the Egyptian viruses were sent to Genbank under accession numbers KJ781207- KJ781216.

RESULTS

Results of H9N2 Virus Detection: Comparison between number and percentage of H9N2 positive cases between farm and backyard raring systems in each governorate was illustrated in (Table 4).The incidence of H9N2 AIV among different species chicken, duck, turkey and quail was investigated (Table 5).Comparison between incidence of infection during summer and winter seasons was indicated (Table 6).

Results of Genetic Sequencing: In the present investigation, the positive H9N2 cases by Real time-PCR were isolated from swab samples in embryonated chicken eggs after one passage. The isolated viruses were found to be haemagglutinating viruses using an HA test. The sequenced part of the HA gene contains 1683 nucleotides, encoding 561 amino acids.

Amino acid substitutions M7V, V13I, N17S, M87I, F264Y, V370I and M539T were characteristic for A/Chicken/Egypt/1339V/2013; while amino acid substitutions V287I, V411I, K501N were characteristic for A/Chicken/Egypt/13 139V/2013 within subgroup 1.

Amino acid substitutions P333H, T526A were typical for the following tested viruses within subgroup 2 [A/Chicken/Egypt/1256S/2012, A/Chicken/ Egypt /123140V/2012, A/Chicken/ Egypt/123243V/2012, A/Chicken /Egypt/ 123245V /2012 and A/Turkey/ Egypt/1341V/2013]. While amino acid substitutions L6M and E93K were characteristic for (A/Chicken/ Egypt/ 123243V/2012, A/Chicken /Egypt/ 123245V/2012 and A/Turkey/Egypt/1341V/2013) and amino acid substitution L535I was characteristic for (A/Chicken/ Egypt/123245V/ 2012 and A/Turkey/Egypt /13 41V/2013).

Amino acid substitutions I9V, H25Y, S102T, I121M and S388A were characteristic for A/Chicken/ Egypt/ 1373Vd/ 2013 within subgroup 2.

Amino acid substitutions L88F, S127N, D153E, N168T, F186Y, A198V, N201G, T206I, I235A, N239D, N256D, V390M and N392S were characteristic for A/Quail/Egypt/122313V/2012 (H9N2) that appeared to have special divergence from other H9N2 AIVs within subgroup 2 (Table 7).

Derived amino acid sequence showed that the motif of HA cleavage site of the tested isolates is ^{335}RSSR↓GLF^{341} (H9numbering). HA receptor-binding pocket analysis showed that Egyptian H9N2 isolates at the position 234(H9 numbering) had Leucine (L) amino acid instead of Glutamine (Q) which preferred human receptor 2,6-linked sialic acid and at the position 236 (H9 numbering) had Glycine (G).

Table 4: Results of positive cases tested by rRT-PCR for AI H9N2 of both farms and backyard from different provinces:

		Total Farm surveillances				Backyard surveillance				
				Positive cases			Positive cases		Total Positive cases	
Serial	Governorate	Total tested cases	No. of tested cases	No.	%	No. of tested cases	No.	%	No.	%
1	Giza	1044	779	27	3.4	265	5	1.88	32	3
2	Qalubia	1036	1027	42	4.0	9	1	11.1	43	4.1
3	Cairo	17	15	1	6.6	2	0	0	1	5.8
4	Minufiyah	3512	3267	88	2.6	245	1	0.4	89	2.5
5	Dakahlia	1732	853	19	2.2	879	2	0.2	21	1.2
6	Gharbia	315	209	16	7.6	106	5	4.7	21	6.6
7	Sharkia	1279	915	20	2.1	364	1	0.3	21	1.6
8	Behaira	2390	2312	78	3.37	78	0	0	78	3.3
9	Alexandria	36	36	5	13.8	0	0	0	5	14
10	Demiatta	88	37	1	2.7	51	0	0	1	1.1
11	Suez	38	36	1	2.7	2	0	0	1	2.6
12	Ismailia	214	205	2	1.0	9	0	0	2	0.9
13	KafrEl-Sheikh	112	88	2	2.27	24	1	4.16	3	2.6
14	Qena	72	70	5	7.1	2	0	0	5	7
15	Fayoum	136	97	23	23.7	39	0	0	23	17
16	BeniSweef	164	73	3	4.1	91	0	0	3	1.8
17	El-menia	213	111	2	1.8	102	0	0	2	0.9
18	Aswan	23	23	1	4.3	0	0	0	1	4.3
19	Souhaj	36	33	2	6.0	3	1	33.3	3	8.3
20	Luxor	40	24	5	20.8	16	0	0	5	12.5
Total		12497	10210	343	3.35	2287	17	0.74	360	2.9

Table 5: Results of total positive cases tested by rRT-PCR for AI H9N2 in different species:

Species	Total cases tested	No. of positive cases	Percentage of positive samples (+/T)
Chicken	11545	343	3%
Duck	593	8	1.4%
Turkey	345	6	1.7%
Quail	14	3	21.4%
Total numbers	12497	360	2.8%

Table 6: Comparison of the incidence of H9N2 infection between the summer and winter seasons during study period:

	Summer		Winter	
Season				
Results	*+ve/T	%	*+ve/T	%
2011	9/109	8.2	100/109	91.8
2012	56/198	28.2	142/198	71.7
8/2011-1/2013	295/360	82%	65/360	18%

*(+ve/T): Positive samples/ total positive samples

Table 7: Variability of HA sequences among the H9N2 isolates analyzed in this study

Isolate	Amino acids																																			
A/Chicken/Egypt/115512V/2011	V	N	N	M	L	E	S	I	S	D	N	F	A	N	T	I	N	H	N	F	V	V	P	A	Q	V	S	V	N	V	N	K	G	T	L	M
A/Chicken/Egypt/1256S/2012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	A	-	-
A/Chicken/Egypt/1225VL/2012	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	D	-	E	-	-	-
A/Quail/Egypt/122313V/2012	-	-	-	-	F	-	-	-	N	E	T	Y	V	G	I	A	D	-	D	-	I	-	-	-	-	-	-	M	S	-	-	-	-	-	-	-
A/Chicken/Egypt/123140V/2012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	K	-	-	-	-	-	-	-	-	A	-	-
A/Chicken/Egypt/123243V/2012	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	A	-	-
A/Chicken/Egypt/123245V/2012	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	A	I	-
A/Chicken/Egypt/1339V/2013	I	S	S	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	S	-	I	-	-	-	-	D	-	E	-	-	T
A/Turkey/Egypt/1341V/2013	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	A	I	-
A/Chicken/Egypt/13139V/2013	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	S	-	-	-	-	-	I	D	N	E	-	-	-
A/Chicken/Egypt/1373VD/2013	-	S	-	-	-	-	T	M	-	-	-	-	-	-	-	-	-	Y	-	-	I	-	-	-	-	-	A	-	-	-	-	-	E	-	-	-

Analysis of HA protein sequences showed that H9N2 viruses had seven sites of glycosylation with N-X-T/S motif in which X may be any amino acid other than proline. Five of these sites were located in HA1 and 2 of them were located in HA2 (Table 8).

Amino acids associated with the receptor binding site were highly conservative among the analyzed Egyptian isolates. Only three residues in the positions 198from RBS and 235,239 from left-edge of binding pocket showed remarkable variations: 198A, 235I and 239N, respectively were found in most of the Egyptian viruses isolated from chickens and turkey, Whereas 198V, 235A and 239D, respectively were found in A/Quail/Egypt/12313V/2012 (Table 9).

Five antigenic epitopes have been identified in HA protein of AIV. All isolates in this study had conservative composition with two amino acids variations were found in A/Quail/Egypt/12313V/2012 (Table 9).

Phylogenetic analysis: The phylogenetic relationships between the HA genes of the tested isolates and those of selected H9N2 viruses isolated in several other countries were analyzed. All the Egyptian isolates were closer to the viruses isolated in the Middle East with more close relationship to the Israeli strains that together formed a characteristic group amongst G1 like viruses. The Egyptian strains could be divided into two subgroups according to HA gene (Fig. 1).

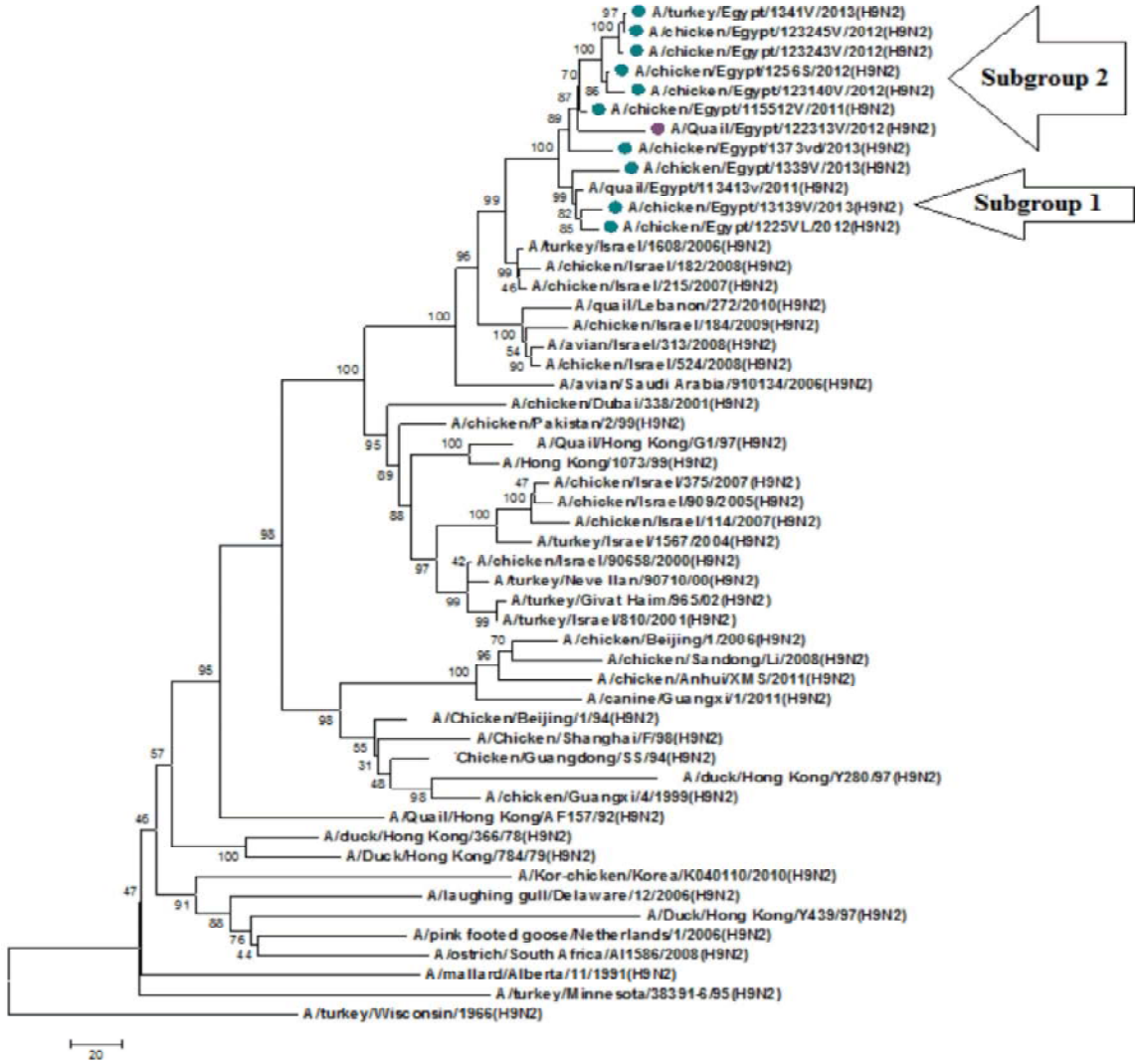

Fig. 1: Amino acids phylogenetic tree of H9 protein of the eleven H9N2 AI isolates analyzed in this study (Marked) and other H9N2 strains published in Gen Bank.

Table 8: Glycosylation sites at HA1, HA2 of the Egyptian isolates:

*Glycosylation sites at HA1	Amino acid motif
29	NST
105	NGT
141	NVT
298	NST
305	NIS
Glycosylation sites at HA2	Amino acid motif
492	NGT
551	NGS

*Glycosylation sites are H9-numbering

Table 9: Receptor-binding pocket, cleavage site and Antigenic sites of H9N2 Egyptian isolates:

				Antigenic epitope sites				
Virus	Receptor binding site	Left-edge of binding pocket	Right-edge of binding pocket	143	153	165	170	201
A/Chicken/Egypt/115512V/2011	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/1256S/2012	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/1225VL/2012	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Quail/Egypt/122313V/2012	PWTHVLY	NGLAGRID	GTSKS	T	E	K	P	G
A/Chicken/Egypt/123140V/2012	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/123243V/2012	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/123245V/2012	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/1339V/2013	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Turkey/Egypt/1341V/2013	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/13139V/2013	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N
A/Chicken/Egypt/1373VD/2013	PWTHALY	NGLIGRIN	GTSKS	T	D	K	P	N

Receptor-binding site (RBS)—amino acid residues at position 110,161, 163, 191, 198, 202 and 203; Left-edge of binding pocket—amino acid residues at position 232-239 ; Right-edge of binding pocket—amino acid residues at position 146-150 ; numbering according to H9 HA.

DISCUSSION

Although H9N2 viruses did not satisfy the criteria for highly pathogenic AI, they were unique among this category, infecting a wide variety of species [6]. The emergence of H9N2 infection in Egypt was described in quail in May 2011 [12]. Here, we have studied the epidemiological situation of H9N2 virus in Egypt during Aug 2011 to Jan 2013, in addition to the investigation of the molecular characterization of eleven isolates from different species and regions. Geographical distribution of H9 cases revealed that the infection was recorded in 20 governorates throughout Egypt without geographical selection (Table 4), this mean that H9N2 AIV became persisted in Egypt. The total percentage of positive cases was 2.9% indicated that the incidence of infection with H9N2 increased gradually from the beginning of its emergence in May 2011 but it was still in low rate among the poultry population.

The density of poultry population among different governorates in Egypt was not equal. The governorates that had the highest poultry density as Menofia, Behaira, Sharkia, Dakahlia, Qalubia, Giza showed variable low rates which were (2.5%,3.3%, 1.6%, 1.2%, 4.1%, 3 %) respectively. While the highest rates of H9N2 infection were recorded in Fayoum, Luxor and Souhaj in the Upper Egypt as well as Alexandria (17%, 12.5%, 8.3%, 14%) respectively. The prevalence of positive H9N2 cases in different governorates in poultry farms (343/10210 with percentage 3.35%) was higher than that in backyards (17/2287 with percentage 0.74%) (Table 4). The presence of H9N2in commercial farms may indicate some defect in applying the biosecurity measures and that will threaten the poultry industry especially with the frequent presence of mortalities associated with other pathogen infections. The pattern of H9N2 incidence was 295/360 cases recorded during winter months with percentage of 82%, while it was 65/360 cases during summer months with percentage of 18%, that indicates the prevalence of the virus infection in the winter was more than that in the summer (Table 6), that supports the theory of increasing the activity of H9N2 AIVs by low temperature. The results mentioned here were agreed with previous studies that found the AIV H9N2 causing lesions and mortalities during winter in northern Pakistan higher than that occurred in the summer season in southern Pakistan [19, 20].

The phylogenetic relationships between the HA genes of eleven Egyptian isolates and those of selected H9N2 viruses isolated in other countries were analyzed. All the Egyptian isolates were closer to viruses isolated in the Middle East which belonging to viruses from G1 lineage with more close relationship to the Israeli strains [15].

According to the phylogenetic analysis, the Egyptian strains could be distributed among two subgroups according to HA protein that was independent in chronological or in geographical location (Fig. 1). Amino acid residues S16, S357, D428, E507 were typical for subgroup1, while N16, A357, N428, G507 were typical for subgroup 2. These changes were occurred as a result of multiple drift mutations in the HA gene. From the phylogenetic tree we found that group1 was closer to A/Quail/Egypt/113413v-2011 [12].The second subgroup included the new proposed genetically variant A/Quail/ Egypt/122313V/2012 that had characteristic divergence among the other H9N2 AIVs within the same subgroup.

The cleavage site of different H9N2 viruses showed 15 amino acid motifs which none of them had multi alkaline amino acid that is related to HPAI isolates [15].While other study reported 19amino acid motifs [21]. In our study, all the tested viruses had cleavage site motif RSSR/GLF suggested that they were of low pathogenic nature [10]. However Further pathogenicity study is needed to confirm the low pathogenicity of the Egyptian isolates as there was varying in pathogenicity of H9N2 viruses with a stable-conserved RSSR cleavage site motif as it caused different outbreaks in Asian and Middle eastern countries [22] especially when poultry were co-infected with other respiratory pathogens, such as IBV [4].

The alteration in the glycosylation pattern influenced the adaptation of AI viruses to poultry by altering their pathogenicity and antigenicity and it was considered the characteristic of the pathogen to escape from the host defense [23]. H9N2 viruses of this study had seven glycosylation sites, five of these sites were located in HA1 and two of them were located in HA2 (Table 8), unlike Indian H9N2 isolates which had nine glycosylation sites, seven of these sites were located in the HA1 region and two in HA2 [7].

Five antigenic epitopes have been identified in HA protein of AIVs, substitution of amino acids in these epitopes usually resulted in antigenic drift of HA protein [30]. Amino acid changes at any 9 sites on the HA protein of H9 subtype AIV led to the emergence of a variant [31]. A recent report suggested that there should be additional amino acid residues located at the antigenic epitopes on HA protein, based on co-crystal structures between HA and antibody protein [32]. Changes in the antigenic epitope sites between H9N2 AIVs were detected in previous study [33]. In the present study, we found that A/Quail/Egypt/12313V/2012 was different from other viruses in 9 amino acid residues (S127N, D153E, N168T, F186Y, N201G, I235A, N239D, N256D and V390M). In addition, 2 of these 9 sites were located at the previously reported antigenic sites (Table 9), so we suspect that A/Quail/Egypt/ 12313V/2012 was an emergence of a new H9N2 genetically variant but further antigenicity and pathogenicity studies are needed.

The amino acid residues of HA1 involved in the receptor binding site (RBS) comprised seven residues [24], the presence of PWTQALY and PWTHALY amino acid residues in the RBS among the Israeli isolates were detected [8].Ten isolates from chicken and turkey in this study had conserved amino acid residues in the RBS which were PWTHALY, but it was variable in A/Quail/Egypt/12313V/2012 which was PWTHVLY (Table 9) (the variation between quail isolate and the other isolates was in the amino acid no. 198).The mutation at position 198 (H9 numbering) displayed variations among H9N2 viruses as it influence the affinity of virus binding to SA receptor; high affinity to the human like receptor being with V, intermediate with T and low with A Matrosovich *et al.* [25]. H9N2 viruses were isolated in this study from chicken and turkey were highly conserved in the amino acid 198, having alanine (A) the same as most of Egyptian isolates published in GenBank, in contrast A/Quail /Egypt /12313V/2012 had valine (V), showed a characteristic variability. So from this study, we can predict that the H9N2 viruses isolated from chicken and turkeys had a moderate or low affinity binding to human like receptor, but a higher affinity can be predicted for the virus isolated from quail in this study. This finding needs to be further confirmed by experimental studies.

The Right-edge of binding pocket were highly conserved among our isolates (GTSKS), while The Left-edge of binding pocket showed variability as it contained eight amino acids (NGLIGRIN) in all our isolate with exception A/Quail/Egypt/ 12313V/2012 had (NGLAG RID) (Table 9), finding of variations between our isolates matched with Perk *et al.* [21] that detected variability among H9N2 AIVs. The receptor specificity of influenza viruses was one factor that allowed the virus to cross the species barrier. The RBD involved in the RBS of the avian-like motif ‘‘GQQG’’ was found in the prototype chicken H9N2 strains ‘‘BJ/9400 and Tu/WS/66 [26]. In this

study the H9N2 isolate from quail carry ''GLAG'', while other viruses isolated from chicken and turkey showed ''GLIG'' (233-236-H9 numbering) the same as the most of the viruses were isolated in Egypt.

Amino acid differences in the RBS of HA were associated with differences in receptor binding specificity possession of leucine at 234 (H9 numbering) was indicative of human influenza virus-like receptor specificity [25]. All H9N2 isolates in this study carried a substitution Q234L, this mutation correlated with a shift in affinity of the HA from the ''avian'' type sialic receptors to the human type and reflected the virus change ending up to human risk [27]. In contrast with the prototype strain Tu/WS/66 that had glutamine (Q) indicated low binding affinity to the 2, 6-linked SA found in human [26].

The H9N2 virus transmission to human was enhanced by the presence of a combination of four key amino acid residues at the RBS of the HA molecule (H191, A197, E198 and L234-H9 numbering) which appeared to enhance the respiratory droplet transmission of a reassortant virus carrying the surface proteins of an avian H9N2 virus in a human H3N2 backbone [28]. Here, lacking of A197 was observed in our isolates, where it had amino acid T197, Interestingly, an alanine at position 197 was rarely observed among H9N2 viruses (Only 6 of 847 Eurasian H9 sequences available in GenBank showed A197 and the Saudi Arabian isolate was the only virus from the G1 lineage that carried this substitution [28]. The deduced amino acid sequence at the RBS showed the presence of H191which was similar to Qa/HK/G1/97 [29]. So in this study, all of 11 isolates carried amino acid residues H191 and L234 which showed to enhance the virus transmission to human, in addition to V198 in quail isolate, all those results suggested that the suspected new variant from quail may have higher ability to infect humans based on the structure of RBS of the HA molecule.

CONCLUSION

The H9N2 AIVs have circulated in domestic poultry in Egypt during the recent years and were detected from chicken, ducks, turkeys and quails from commercial flocks and house hold birds in 20 governorates. Continuous evolution of H9N2 AIV was recorded and that classifying the Egyptian viruses into two subgroups that were not dependent on time or geographical location. Multiple antigenic drifts were detected, the highest variation was recorded in quail isolate (A/Quail/Egypt/ 122313V/2012) that had 20 A.A. substitutions distributed along HA protein including the receptor binding domain and antigenic epitopes proposed to be a new variant of H9N2 virus in quail. Continuous monitoring of the H9N2 viruses evolution among different poultry species in Egypt is important.

REFERENCES

1. Tong, S., Y. Li, P. Rivailler, C. Conrardy, D.A. Castillo, L.M. Chen, S. Recuenco, J.A. Ellison, C.T. Davis, I.A. York, A.S. Turmelle, D. Moran, S. Rogers, M. Shi, Y. Tao, M.R. Weil, K. Tang, L.A. Rowe, S. Sammons, X. Xu, M. Frace, K.A. Lindblade, N.J. Cox, L.J. Anderson, C.E. Rupprecht and R.O. Donis, 2012. A distinct lineage of influenza A virus from bats. Proc. Natl. Acad. Sci. USA, 109(11): 4269-4274.
2. Tong, S., X. Zhu, Y. Li, M. Shi, J. Zhang, M. Bourgeois, H. Yang, X. Chen, S. Recuenco, J. Gomez, L.M. Chen, A. Jackson, Y. Tao, C. Dreyfus, W. Yu, R. McBride, P.J. Carney, A.T. Gilbert, J. Chang, Z. Guo, C.T. Davis, J.C. Paulson, J. Stevens, C.E. Rupprecht, E.C. Holmes, I.A. Wilson and R.O. Donis, 2013. New World bats harbor diverse influenza A viruses. PLoSPathog. 9:e1003657. doi:10. 1371/ journal.ppat.1003657.
3. Homme, P.J. and B.C. Easterday, 1970. Avian influenza virus infections. I. Characteristics of influenzaA-turkey-Wisconsin-1966 virus. In: Avian Dis. 14 (1), S 66.
4. Nili, H. and Asasi K. Natural, 2002.Cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol, 31: 247-252.
5. Li, K.S., K.M. Xu, J.S.M. Peiris, L.L.M. Poon, K.Z. Yu, K.Y. Yuen, K.F. Shortridge, R.G. Webster and Y. Guan, 2003. J. Virol. 77: 6988-6994.
6. Wu, R., Z.W. Sui, H.B. Zhang, Q.J. Chen, W.W. Liang, K.L. Yang, Z. Xiong, Z.W. Liu, Z. Chen and D.P. Xu., 2008. Characterization of a pathogenic H9N2 influenza A virus isolated from central China in 2007.Arch Virol.; 153:1549-1555. doi:10.1007/s00705 -008-0139-1.
7. Nagarajan, S., K. Rajukumar, C. Tosh, V. Ramaswamy, K. Purohit, G. Saxena, P. Behera, B. Pattnaik, H.K. Pradhan and S.C. Dubey, 2009. Isolation and Pathotyping of H9N2 Avian Influenza viruses in Indian poultry. Vet. Microbiol., 133: 154-163.

8. Perk, S., N. Golender, C. Banet-Noach, E. Shihmanter, S. Pokamunsky, M. Pirak, Y. Tendler, M. Lipkind and A.A. Panshin, 2009. Phylogenetic analysis of hemagglutinin, neuraminidase and nucleoprotein genes of H9N2 avian influenza viruses isolated in Israel during the 2000-2005 epizootic. Comp Immunol. Microbiol Infect Dis.m 32:221-238. doi:10. 1016/j.cimid.2007.06.008.
9. Yu, H., R.H. Hua, T.C. Wei, Y.J. Zhou, Z.J. Tian, G.X. Li, T.Q. Liu and G.Z. Tong, 2008. Isolation and genetic characterization of avian origin H9N2 influenza viruses from pigs in China. Vet. Microbiol., 131: 82-92.
10. Steinhauer, D.A., 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology, 258: 1-20.
11. Perez, D., R. Lim, Wilina, Seiler, P. Jon, Yi, Guan, Peiris, Malik, Shortridge, F. Kennedy,Webster and G. Robert, 2003. Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. In: J. Virol., 77(5): S. 3148-3156.
12. Elham F. El-Zoghby, Abdel-Satar Arafa, Mohamed K. Hassan, Mona M. Aly, Abdullah Selim, Walid H. Kilany, Usama Selim, Soad Nasef, Mohamed G. Aggor, E.M. Abdelwhab and Hafez M. Hafez, 2012. Isolation of H9N2 avian influenza virus from bobwhite quail(Colinusvirginianus) in Egypt. Arch Virol., 157: 1167 -1172.
13. Ahmed, S. Abdel-Moneim, Manal A. Afifi and Magdy F. El-Kady, 2012. Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. Virology Journal, 9: 173.
14. Abdelsatar A. Arafaa, Naglaa M. Hagag, Nahed Yehia, Aly M. Zanaty, Mahmoud M. Naguib and Soad A. Nasef, 2012. Effect of co-circulation of highly pathogenic avian influenza H5N1 subtype with low pathogenic H9N2 subtype on the epizootiological nature of outbreaks. Avian Dis., 56(4): 849-57.
15. Banks, J., E.C. Speidel, P.A. Harris and D.J. Alexander, 2000. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathol., 29: 353-60.
16. Alexander, D.J., 2009. Avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals 2010, chap 2.3.4. World Organisation for Animal Health, Paris. http://www. oie.int/ fileadmin /Home/eng/Health_standards/tahm/2.03.04_AI.pdf. Accessed 27 May 2011.
17. Ben Shabat, M., R. Meir, R. Haddas, E. Lapin, I. Shkoda, I. Raibstein, S. Perk and I. Davidson, 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses Journal of Virological Methods, Volume 168, Issues 1-2, September 2010, pp: 72-77.
18. Tamura Koichiro, Daniel Peterson, Nicholas Peterson, Glen Stecher, Masatoshi Nei and Sudhir Kumar, 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Method. Molecular Biology and Evolution, 28: 2731-2739.
19. Naeem, K., A. Ullah, R.J. Manvell and D.J. Alexander, 1999. Avian influenza A subtype H9N2 in poultry in Pakistan. Vet. Rec., 145, 560.
20. Naeem, K., M. Naurin, S. Rashid and S. Bano, 2003. Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. Avian Pathology, 32(3): 285-289.
21. Perk, S. Caroline Banet-Noacha, Ester Shihmantera, Shimon Pokamunskib, Michael Pirakb, Michael Lipkinda, Alexander Panshina and Comp, 2006. Genetic characterization of the H9N2 influenza viruses circulated in the poultry population in Israel , Immun. Microbiol. Infect. Dis., 29: 207-223.
22. Shaib, H.A., N. Cochet, T. Ribeiro, N.A.M. Abdel, G. Nemer and E.K. Barbour, 2010. Impact of embryonic passaging of H9N2 virus on pathogenicity and stability of HA1-amino acid sequence cleavage site. Medical Sci-ence Monitor, 16: 333-337.
23. Escorcia, M., K. Carrillo-Sánchez, S. March-Mifsut, J. Chapa, E. Lucio and G. Nava, 2010. Impact of antigenic and genetic drift on the serologic surveillance of H5N2 avian influenza viruses. Vet. Res., pp: 1746-61.
24. Liu, H., X. Liu, J. Cheng, D. Peng, L. Jia and Y.Huang, 2003. Phylogenetic analysis of the hemagglutinin genes of twenty-six avian influenza viruses of subtype H9N2 isolated from chickens in China during 1996-2001. Avian Dis., 47: 116- 127.
25. Matrosovich, M., S. Krauss and R.G. Webster, 2001. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology, 281: 156-162.
26. Huang, Y., B. Hu, X. Wen, S. Cao, B.K. Gavrilov, Q. Du and M.I. Khan and X. Zhang, 2010. Diversified reassortant H9N2 avian influenza viruses in chicken flocks in northern and eastern China. Virus Res., 151: 26-32.

27. Wan, H., E.M. Sorrell, H. Song, M.J. Hossain and G. Ramirez-Nieto, 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS ONE 3: e2923.
28. Sorrell, E.M., H. Wan, Y. Araya, H. Song and D.R. Perez., 2009. Minimal molecular constraints for respiratory droplet transmission of avian-human H9N2 influenza A virus. Proc. Natl. Acad. Sci. U. S. A. 106: 7565-7570.
29. Guan, Y., K.F. Shortridge, S. Krauss, P.S. Chin, K.C. Dyrting, T.M. Ellis., 2000. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. In: J. Virol., 74(20): S. 9372-9380.
30. Caton, A.J., G.G. Brownlee, J.W. Yewdell and W. Gerhard, 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31: 417-427.
31. Kaverin, N.V., I.A. Rudneva, N.A. Ilyushina, A.S. Lipatov, S. Krauss and R.G. Webster, 2004. Structural differences among hemagglutinins of influenza A virus subtypes are reflected in their antigenic architecture: analysis of H9 escape mutants. J. Virol., 78: 240-249.
32. Ekiert, D.C., G. Bhabha, M.A. Elsliger, R.H.E. Friesen, M. Jongeneelen, M. Throsby, J. Goudsmit and I.A. Wilson, 2009. Antibody recognition of a highly conserved influenza virus epitope. Science, 324: 246-251.
33. Zhang Yi, Yanbo Yin, Yuhai Bi, Shouchun Wanga, Shouzhen Xu, Jianlin Wang, Shun Zhou, Tingting Sun and Kyoung-Jin Yoon, 2012. Molecular and antigenic characterization of H9N2 avian influenza virus isolates from chicken flocks between 1998 and 2007 in ChinaVeterinary Microbiology, 156: 285-293.