Research Article

Genetic Characterization Of Avian Influenza Virus (H9N2) Hemagglutinin Genes In Broiler Chickens Of Luxor Governorate, Egypt

Mohamed. E. Taha[1], Mohamed S. Ahmed[2], Ahmed I. Ahmed[2], Amany Adel[3], Salama Rehab[4,5*], Nabila Osman[2]**

[1]Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Institute, Agriculture Research Center, Luxor, Egypt; [2]Department of Poultry Diseases, Faculty of Veterinary Medicine, South Valley University, Qena,Egypt;[3]Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Institute, Agriculture Research Center, Cairo, Egypt; [4]Department of Poultry Diseases, Faculty of Veterinary Medicine, Aswan University, Aswan, Egypt; [5]Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, , Obihiro, Hokkaido, Japan.

Abstract | Avian influenza virus (AIV) subtype H9 is a genuine threat to the poultry industry and human. However, it causes mild to moderate respiratory signs. The infection may be more serious when complicated with other respiratory pathogens. In the current study AIV H9 was identified among broiler chickens in Luxor governorate in 2016. Five hundred (n= 500) samples were collected from fifty farms (5 tracheal and 5 cloacal pools from each farm) have been examined by real time RT-PCR for the prevalence of AIV H9. The results revealed that ten farms (20%) were positive for AIV (H9N2). Three isolates with high CT values have been selected for complete sequencing of hemagglutinin gene. Amino acid sequences showed that these isolates were identical to the Asian strain "A Quail-Hong-Kong-G1-97" (91-92%), as well as the other Egyptian strains "A/chicken/Egypt/ME543V/2016" (99-100%), and "A/quail/Egypt/113413v/2011" (98-99%). Also, six of the N-glycosylation sites were identical to the Hong Kong isolate "HK/G1/97", while the other two glycosylation sites at amino acid residues "188–191" and "200–203" were mutated. The phylogenetic analysis clearly highlights the aggregation of the sequenced AIV H9 isolates with the Egyptian H9N2 viruses G1 lineage in the group B and the Israeli strains. Although the strains are low pathogenic, the presence of mammalian binding amino acid markers indicated their public health concern. Overall conclusion indicated that AIV (H9N2) still circulates among poultry farms in the Southern Egypt. Furthermore, isolated strains were closely related to strains of Israel, Middle East and Asia.

Keywords | Avian Influenza, H9N2, Broiler chickens, Real Time RT-PCR, Sequencing.

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***Correspondence** | Rehab Youssef Salama, Nabila Osman, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-11 Inada-cho, Obihiro, Hokkaido 080-8555, Japan; Department of Poultry Diseases, Faculty of Veterinary Medicine, South Valley University, 83523, Qena, Egypt; **Email:** rehabyousif@vet.aswu.edu.eg; nabila.osman@vet.svu.edu.eg
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INTRODUCTION

Avian influenza virus (AIV) subtype H9 became widely distributed in Middle Eastern countries resulted in great economic losses for the agricultural and health sectors particularly if mixed with other pathogens (Adel et al., 2017). Despite being low pathogenic, infected birds are more susceptible to secondary infections (Umar et al., 2017). Several evidences showed that H9 AIV is potentially increasing the flock mortality up to 65% (Nili and Asai, 2002), as well as decreasing the body weight of broiler chickens and the egg production and quality in layers and breeder chickens (Qi et al., 2016).

Since the 1990s, H9N2 subtype has globally circulated in wild and domestic birds, pigs, and human (Alexander, 2000). It has been also transmitted to the Middle Eastern and African countries (Tombari et al., 2011). North American and Eurasian are the two major lineages of influenza A (H9N2) viruses prevailing in domestic and wild birds (Guo et al., 2000).

From 1998 till 2010, the full genome sequencing of H9N2 viruses from nine countries in central Asia and the Middle East indicated the presence of four different circulating groups "A, B, C, and D". Each group has been extensively subjected into inter-and intra- subtypes assortments with the emerging of viruses with different biological properties (Fusaro et al., 2011). Groups A and B were widely distributed in the Middle East since their detection in 1999 till present. The evolution of H9N2 in the Middle East has played an important role in shaping the viral genetic diversity (Fusaro et al., 2011; Bashashati et al., 2013).

The first official report of human influenza A (H9N2) was in 1998, after that, 59 additional cases have been reported from Bangladesh, China, Egypt, Pakistan, and Oman (Peacock et al., 2019). All human influenza A (H9N2) viruses were genetically similar to poultry viruses isolated at that time (Peacock et al., 2019).

In May 2011, the first case of AIV (H9N2) from apparently health commercial bobwhite quail as "A/quail/Egypt/113413v/2011/H9N2" has been reported in Egypt (EL Zoghby et al., 2013). Later, it has been isolated from broiler breeder, commercial broiler, and layer chicken flocks (Shakal et al., 2013). The phylogenetic analysis of hemagglutinin (HA) genes showed that the Egyptian H9 isolates have been grouped together with the quail/Hong Kong/G1/97-like lineage similarly to viruses distributed throughout the Middle East, and closely related to the Israeli strains (Arafa et al., 2012).

Based on HA gene sequences, the Egyptian AIV(H9N2) has been identified as group B (Abdel-Moneim et al., 2012), which is able to obtain essential amino acids in the HA binding peptide, thus becoming highly pathogenic. The cocirculation of H5N1 and H9N2 subtypes may affect the reduction of spread and the epizootiologic pattern of infection for both subtypes particularly in combination with different vaccine applications (Arafa et al., 2012).

Accordingly, this study was conducted to determine the genetic characterization of low pathogenic (LP) AIV (H9N2) to provide an update about the situation of H9N2 circulating among commercial broiler chicken flocks in Luxor governorate in 2016.

MATERIALS AND METHODS

Sampling

Five hundred pooled cloacal and tracheal swabs (n=500) were collected from fifty commercial broiler flocks in Luxor governorate from three locations (Esna, Armant, and Luxor) in 2016 (Table 1). Each pool represented as five cloacal swabs and five tracheal swabs. All farms had a history of mild respiratory signs with low mortality. The samples were collected in phosphate buffered saline (PBS) (pH 7.2) with antibiotics according to (WHO 2002), then transferred to the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute, Luxor, Egypt.

Detection of AIV H9 subtype by real-time RT-PCR

For detecting AIV H9, specific primers and probes were used as described in table 2. The RNA was extracted via QiaAmp® Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions, followed by One-step real-time RT-PCR using (Quantitect probe RT-PCR master mix Kit (Qiagen, Germany). The real time RT-PCR was conducted at Stratagene MX3005P (Stratagene, USA). Thermal profile was adjusted as following, reverse transcription at 50°C for 30 min then polymerase heat activation at 95°C for 15 min, followed by 40 cycles of initial denaturation at 94°C for 10 s, annealing at 54°C for 30 s and final extension at 72°C for 10 s.

Virus isolation

AIV (H9) positive samples were isolated in specific pathogen free embryonated chicken eggs (ECEs). Briefly, original pooled samples were diluted with a penicillin-streptomycin (Oxoid, UK) and PBS solution (1:1), filtered (0.22 µm), and inoculated into the allantoic cavity of 9–11day old ECEs. The allantoic fluid was harvested at 3-5 days post-inoculation. The presence of AIV was verified by hemagglutination assay (OIE, 2018) followed by real time RT-PCR for the AIV H9 using specific primers and

Table 1: Showing the case history of collected samples

H9 vaccination	Age of flock (days)	Capacity	Location	Bird type	Farm. No.
Vaccinated	35	10000	Esna city	Broiler	1
Unvaccinated	28	6000	Esna city	Broiler	2
Unvaccinated	35	1000	Esna city	Broiler	3
Vaccinated	29	2800	Esna city	Broiler	4
Unvaccinated	25	4500	Esna city	Broiler	5
Unvaccinated	29	4000	Esna city	Broiler	6
Unvaccinated	28	3000	Esna city	Broiler	7
Unvaccinated	30	5000	Esna city	Broiler	8
Vaccinated	35	10000	Esna city	Broiler	9
Unvaccinated	33	5000	Esna city	Broiler	10
Unvaccinated	25	6000	Esna city	Broiler	11
Vaccinated	27	10000	Esna city	Broiler	12
Unvaccinated	29	7000	Esna city	Broiler	13
Unvaccinated	32	3000	Esna city	Broiler	14
Unvaccinated	30	2500	Esna city	Broiler	15
Unvaccinated	30	5000	Esna city	Broiler	16
Unvaccinated	33	2000	Esna city	Broiler	17
Unvaccinated	24	4000	Esna city	Broiler	18
Vaccinated	38	7000	Esna city	Broiler	19
Unvaccinated	25	3500	Esna city	Broiler	20
Unvaccinated	30	3000	Esna city	Broiler	21
Unvaccinated	30	5000	Esna city	Broiler	22
Unvaccinated	29	10000	Luxor city	Broiler	23
Unvaccinated	28	5000	Esna city	Broiler	24
Unvaccinated	35	4500	Esna city	Broiler	25
Unvaccinated	24	5000	Esna city	Broiler	26
Unvaccinated	28	6000	Esna city	Broiler	27
Unvaccinated	29	8000	Esna city	Broiler	28
Unvaccinated	33	5000	Esna city	Broiler	29
Unvaccinated	25	10000	Esna city	Broiler	30
Unvaccinated	24	6000	Esna city	Broiler	31
Unvaccinated	23	10000	Luxor city	Broiler	32
Vaccinated	37	5000	Luxor city	Broiler	33
Vaccinated	30	10000	Luxor city	Broiler	34
Vaccinated	33	10000	Luxor city	Broiler	35
Unvaccinated	34	9000	Luxor city	Broiler	36
Unvaccinated	33	7500	Luxor city	Broiler	37
Unvaccinated	35	6000	Luxor city	Broiler	38
Unvaccinated	29	5000	Luxor city	Broiler	39
Unvaccinated	30	6000	Luxor city	Broiler	40
Unvaccinated	32	4000	Armant city	Broiler	41
Unvaccinated	31	7000	Armant city	Broiler	42
Unvaccinated	36	10000	Armant city	Broiler	43
Vaccinated	34	10000	Armant city	Broiler	44
Vaccinated	33	9000	Armant city	Broiler	45

Unvaccinated	32	5000	Armant city	Broiler	46
Unvaccinated	35	6000	Armant city	Broiler	47
Unvaccinated	36	4000	Armant city	Broiler	48
Unvaccinated	28	5000	Armant city	Broiler	49
Vaccinated	33	7000	Armant city	Broiler	50

Table 2: Primers and probe sequences used for identification of avian influenza virus subtypes by Real tine RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction (one step RT-PCR) and Sequence reaction of full HA

Prime ID	Primer Sequence	Reference
H9 subtype (RT-PCR)	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) (Ben Shabat et al., 2010) (Ben Shabat et al., 2010) (Ben Shabat et al., 2010)
H9-For	**5'**GGA AGA ATT AAT TAT TAT TGG TCG GTA C **3'**	(Ben Shabat et al., 2010)
H9- Rev (Conventional PCR)	**5'** GCC ACC TTT TTC AGT CTG ACA TT **3'**	(Ben Shabat et al., 2010)
F1-6	**5'**TAG CAA AAG CAG GGG AAT TTC TT **3'**	(Adel et al., 2017)
HT7R (Sequencing)	**5'**TAA TAC GAC TCA CTA TAA GTA CAA ACA AGG GTG **3'**	(Adel et al., 2017)

probes (Table 2). Negative samples were passaged three times in ECEs after which viral isolation was deemed unsuccessful.

Full H9 genes amplification

The previously extracted RNA of 3 selected isolates was used as a template for full H9 gene amplification by conventional RT-PCR using Qiagen One Step RT-PCR Kit (Qiagen- USA) and thermocycler 2720 ABI (Applied Biosystems, USA) with specific primers both for conventional RT-PCR and sequencing (Table 2). The thermal profile was conducted as following, reverse transcription at 50°C for 30 min, then initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 45s, annealing at 56°C for 45s and extension at 72°C for 2 min, then final extension at 72°C for 10 min. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide 0.5ug/ml, and viewed under UV trans-illuminator.

Sequencing and phylogenetic analysis

The PCR product was purified using PCR purification kits (Qiagen, Valencia, CA), and direct sequencing was done using specific primers as listed in Table 2. The sequencing reaction was performed using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Perkin- Elmer, Foster, CA). To identify the identity of our isolates to the reference sequences from the GenBank, sequence were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/) and the sequence alignment was carried out using CLUSTAL W (Thompson et al., 1994).

The phylogenetic relatedness of A1 (H9N2) was detected to other HA subtypes by retrieving relevant gene sequences from the GenBank database. The analyses were performed by the neighbor-joining method with Kimura 2-parameter. The consensus tree was obtained after bootstrap analysis, with 1,000 replications using the MEGA X software program (Tamura et al., 2018).

Three reference isolates were used for comparison to sequenced isolates in this study, with the following accession number: (AF156378) for A-Quail-HongKong-G1-97, (JN828570) for A/quail/Egypt/113413v/2011, and (MF434468) for A/chicken/Egypt/ME543V/2016.

RESULTS

Detection, isolation and identification of H9 subtype.

In the current study, screening of fifty farms (500 samples) revealed that ten farms (20%) were positive for AIV (H9N2) by real-time RT-PCR, with different values of cycle threshold (*Ct*). Additionally, all samples were negative for other HA subtypes such as H5 and H7, as well as other hemagglutinating viruses including Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV). The harvested allantoic fluid of the tested isolates for HA activity showed HA titers 6-9 Log 2.

Sequence analysis of HA gene

Three samples with high Ct Values were selected from positive isolates for sequencing (F18, F23 and F26). Conventional RT-PCR was conducted for amplification of the HA gene (H9N2). The result showed amplified products of expected size at 963 bp for HA1 and 1028 bp for HA2

Table 3: Shows comparison of amino acids according to different sides of RBS and HA areas (HA1 and HA 2) in comparison *. A-Quail-HongKong-G1-97 , (-) no changes in comparison to A-Quail-Hong Kong-G1-97, and the variation in the RBS are identified by bold underline under the letter with H9 numbering for mature protein , our isolates were identified by *.

A/chicken/Egypt/1626F/2016*	A/chicken/Egypt/1623F/2016*	A/chicken/Egypt/1618F/2016*	A/chicken/Egypt//ME543V/2016	A/quail/Egypt/113413v/2011	A-Quail-Hong-Kong-G1-97	Isolate ID		A/chicken/Egypt/1626F/2016*	A/chicken/Egypt/1623F/2016*	A/chicken/Egypt/1618F/2016*	A/chicken/Egypt//ME543V/2016	A/quail/Egypt/113413v/2011	A-Quail-HongKong-G1-97	Isolate ID	
A	A	A	A	A	E	180	Mutation HA1 in amino acids	GTSKS	GTSKS	GTSKS	GTSKS	GTSKS	GISRA	Right side edge of RBS (128-132)	
T	T	T	T	T	I	186									
T	T	T	T	T	N	188		NGLI-GR	NGLI-GR	NGLIGR	NGLIGR	NGLIGR	NDLQGR	Left side edge of RBS (214-219)	
I	I	I	I	_	V	194									
N	N	N	N	N	D	198									
D	D	D	D	D	N	200		K	K	K	K	_	M	40	Mutation HA1 in amino acids
L	L	L	L	L	V	206		N	N	N	N	N	S	45	
_	A	_	_	_	V	213		N	N	N	N	N	H	48	
N	N	N	N	N	D	221		I	I	I	I	I	V	61	
F	F	F	F	F	Y	246		M	M	M	M	M	L	69	
E	E	E	E	E	G	253		G	G	G	G	G	E	72	
N	N	N	N	N	K	264		P	p	P	P	P	S	83	
_	_	R	_	_	S	265		I	I	I	I	I	T	103	
D	D	D	D	D	T	295		S	S	S	S	S	A	108	
I	I	I	I	I	V	300		M	M	M	M	M	I	116	
G	G	G	G	G	R	301		S	S	S	S	S	T	127	
I	I	I	I	I	V	309		N	N	N	N	N	S	140	
V	V	V	V	V	I	365	Mutation HA2 in amino acids	N	N	N	N	N	S	148	
I	I	I	I	_	V	411		N	N	N	N	N	F	150	
M	M	M	M	K	R	483		T	T	T	T	T	N	161	
G	G	G	G	_	E	489		D	D	D	D	D	S	165	
_	_	F	_	_	V	496		D	D	D	D	D	Y	178	

fragments. Sequencing of the PCR product was conducted in both directions and a sequence of 1509 nucleotides were used for nucleotide analysis and deduced amino acid analysis. The sequenced isolates were submitted to GenBank and assigned as A/chicken/Egypt/1618F/2016, A/chicken/Egypt/1623F/2016, and A/chicken/Egypt/1626F/2016 and their accession numbers were MH734794, MH734795, and MH734796, respectively.

To elucidate how the recent H9N2 strains have been evolved in the south of Egypt (Luxor governorate), the deduced amino acid sequences (503 amino acids) of each isolate were determined and aligned with the reference "A-Quail-HongKong-G1-97" (Fig 1).

Analysis of the proteolytic cleavage site "PCS" revealed that the isolates have the original cleavage site "PARSS-RGLF" motif from amino acid 315 to 323, which indicated their low pathogenicity (Fig 1). In comparison to "A-Quail-HongKong-G1-97", the current isolates showed an assortment on the right edge of "RBS" at three positions of the amino acid residues, including 129 (Isoleucine was changed to Threonine), 131 (Arginine was shifted to Lysine), and 132 (Alanine was altered by Serine). Additionally, they had an alteration on the left edge of "RBS" at position 215 (Aspartate is substituted by Glycine), and 217 (Glutamine shifted to Isoleucine). Within "RBS", all three Egyptian isolates were H173, T179 and

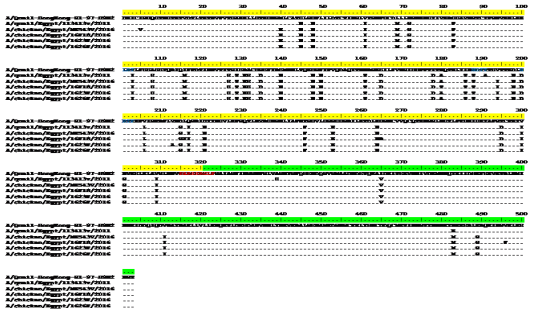

Figure 1: Alignment of HA amino acid sequences for the isolated strains in comparison to the reference one in GenBank using "A-Quail-HongKong-G1-97", A/quail/Egypt/113413v/2011 and A/chicken/Egypt/ME543v/2016. HA1 region of H9 (AIV) was highlighted as yellow color, encoding amino acids (1-320). While HA2 region of H9 (AIV) was highlighted as green color, encoding amino acids (321-503). Cleavage site were marked as red color, including amino acids (315 to 323). The two mutated glycosylation sites were marked as blue color, including sites (188-191), and (200-203).

L216, which are associated with the preferential binding of cell receptors existing in various epithelial cells of the human's respiratory tract. While A180 and G 218 are avian receptors (Fig 1). Other alterations have been observed in the HA1 and HA2, were listed in Table 3. Investigation of

Table 4: The glycosylation sites of our isolates on HA genes compared to A-Quail HongKong G1-97, A/quail/Egypt/113413v/2011, and A/chicken/Egypt/ME543V/2016 the glycosylation sites variation appears bold with underlined letter, amino acid sites with H9 numbering.

Isolate I D	Glycosylation sites							
A-Quail-Hong-Kong-G1-97	^{11}NSTE^{14}	^{87}NGTC^{90}	^{123}NVTY^{126}	^{188}NDTT^{191}	^{200}NRTF^{203}	^{280}NSTL^{283}	^{287}NISK^{290}	^{474}NGTY^{477}
A/quail/Egypt/113413v/2011	NSTE	NGTC	NVTY	**T**DT**A**	**D**RTF	NSTL	NISK	NGTY
A/chicken/Egypt/ME543V/2016	NSTE	NGTC	NVTY	**T**DTT	**D**RTF	NSTL	NISK	NGTY
A/chicken/Egypt/1618F/2016	NSTE	NGTC	NVTY	**T**DTT	**D**RTF	NSTL	NISK	NGTY
A/chicken/Egypt/1623F/2016	NSTE	NGTC	NVTY	**T**DTT	**D**RTF	NSTL	NISK	NGTY
A/chicken/Egypt/1626F/2016	NSTE	NGTC	NVTY	**T**DTT	**D**RTF	NSTL	NISK	NGTY

Table 5: Percent of identity among isolated strains in compare to A-Quail-HongKong-G1-97, A/quail/Egypt/113413v/2011, and A/chicken/Egypt/ME543V/2016

	Seq ID	1	2	3	4	5	6
1	A-Quail-HongKong-G1-97	ID	92%	91%	91%	91%	92%
2	A/quail/Egypt/113413v/2011	92%	ID	98%	98%	98%	99%
3	A/chicken/Egypt/ME543V/2016	91%	98%	ID	99%	100%	100%
4	A/chicken/Egypt/1618F/2016	91%	98%	99%	ID	99%	99%
5	A/chicken/Egypt/1623F/2016	91%	98%	100%	99%	ID	100%
6	A/chicken/Egypt/1626F/2016	92%	99%	100%	99%	100%	ID

the glycosylation sites demonstrated that the current isolates had the same six sites of the "HK/G1/97" virus except for those two sites at amino acids 188–191 and 200–203, which were distinct as shown in Table 4. This distinguished difference attributed to the substitution of Asparagine to Threonine at position 188, while the other lost was due to the substitution of Asparagine to Aspartate at position 200.

Percent of identity among selected strains

As shown in Table 5, amino acid sequences revealed that the three local isolates (A/chicken/Egypt/1618F/2016, A/chicken/Egypt/1623F/2016, and A/chicken/Egypt/1626F/2016) were 91%, 91% and 92% identity, respectively, to Asian strain "A Quail-HongKong-G1-97". However, two isolates "A/chicken/Egypt/1623F/2016 and A/chicken/Egypt/1626F/2016" showed 100% identity to the Egyptian strain "A/chicken/Egypt/ME543V/2016", while A/chicken/Egypt/1618F/2016 had 99% identity with the same strain. Additionally, all three isolates were similar to other Egyptian strain "A/quail/Egypt/113413v/2011" with identity percentage ranged from 98% to 99%.

Phylogenetic analysis of HA gene

As shown in the current study, all three isolates were classified as EGY/G1. As well, they were very close to the Egyptian strains isolated during 2011-2016, and Israeli strains (2008, 2010, and 2012) (Fig 2).

DISCUSSION

Since 1990, LPAI H9N2 has been constantly circulated in poultry farms from the Far East to the Middle East (Fusaro et al., 2011), and associated with great zoonotic consequences (Butt et al., 2003).

In December 2010, active monitoring studies have been conducted to isolate AIV or influenza A virus H9N2from broiler chickens in Egypt (Monne et al., 2013), and LPAIV H9N2 has been identified for the first time in Egypt in November 2011 from bobwhite quails. The isolated strains were closely related to the G1 strain which isolated from neighboring countries, thus increases the possibility of epidemiological pervasion (El-Zoghby et al., 2012). In 2016, we conducted another active survey in the poultry farms of Luxor governorate, in the southern of Egypt. About five hundred samples (cloacal and tracheal swabs) were collected from fifty farms of three local centers (Esna, Armant, and Luxor) within the governorate. All farms were suffering from mild respiratory signs with moderate to high

mortality. The current screening revealed that ten farms (20%) were positive for LPAI H9N2 by real-time

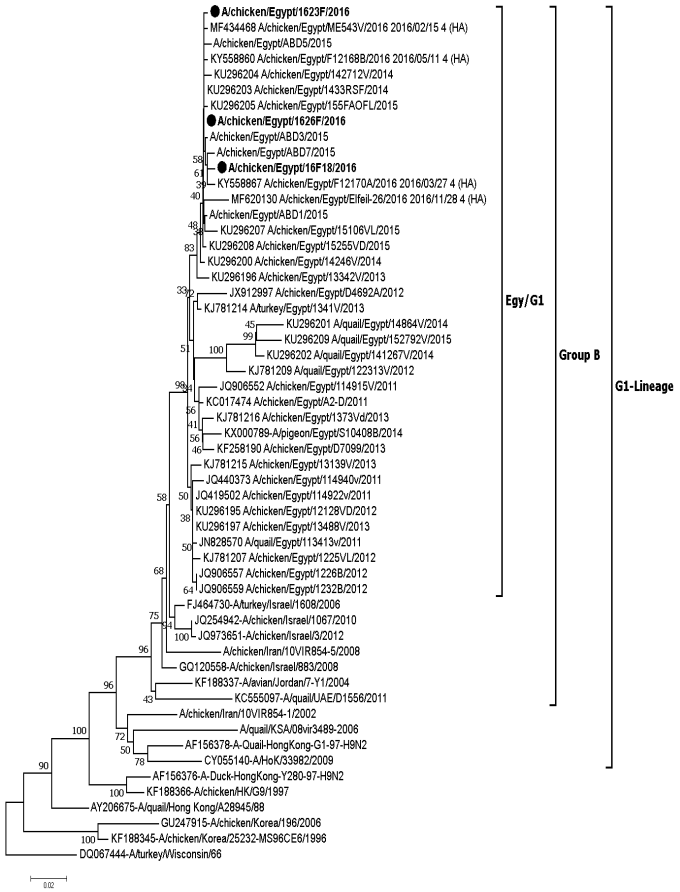

Figure 2: The evolutionary relationship among HA genes of A(H9N2) retrieved from GenBank database and sequences data in this study (MH734794 to MH734796) was inferred by using Neighbor Joining Estimation method based on the Kimura 2- parameter model derived from full gene sequence data. Values on branches are the percent of bootstrapping using 1,000 replicates. All positions containing gaps and missing data were trimmed. Evolutionary analyses were conducted in MEGA X software. The viruses of this study are indicated in black bold dot (●).

RT-PCR, which was lower than Abdel Hamid et al. (2016) who reported 32% (32 /100) incidence of AIV (H9N2). Kayali et al. (2014) performed an active surveillance for AIV from 2010-2012 and reported 18.7% positive cases for AIV (H9N2) and 21.7% co-infected cases of AIV H9N2 and H5N1, which was somewhat in the same line as our result. Other studies were conducted in Egypt, showed that the incidence of AIV (H9N2) among broilers was ranged from 10% to approximately 55% (Shalaby et al., 2014; Dabbour, 2015).

Genetic evolution of the avian influenza viruses depending mainly on the alterations and mutations of different sites on HA genes, which are important for pathogenicity determinant and host range. Proteolytic cleavage site, the receptor-binding site (RBS), and the presence or absence of glycosylation sites near the RBS in the HA gene are identified as fundamental keys for the molecular determinants of pathogenicity and viral transmission (Baigent and McCauley, 2003). Based on proteolytic cleavage site, the amino acid sequences of the 3 isolates have "PARSSRGLF" motif, thus can be identified as LPAIV (Steinhauer, 1999).

RBS of the HA gene influences the generation of human viruses from avian precursors and also it is essential for host cellular receptor specificity. Analysis of the RBS in the current study showed that all 3 isolates had variations in both right side edge and left side edge. In case of the right side the changes were determined at three positions such as "129" where Isoleucine has been shifted to Threonine, at "131" in which Arginine was changed to Lysine, and at "132" Alanine was substituted by Serine. Also, the left edge of the RBS showed mutations at positions " 215" Aspartate was altered to Glycine, and "217" Glutamine was replaced by Isoleucine. Furthermore, analysis of the RBS showed that all 3 isolates had H173, and L216 which had been previously identified as human markers, these results came parallel with those of Neumann and Kawaoka, (2006). Thus, explain the zoonotic transmission of AIV (H9N2). According to Wan et al. (2008), AIV (H9N2) has acquired a typical receptor binding resembling that of human strains. These findings might increase the probability of assortment in both human and pig respiratory tracts. It was noticed that the combination of H173, E180, and L216 was typical of the early human isolates H3N2 (Sorrell et al., 2009). However, the presence of human-like receptor was determined at positions T179 and G218 which was identified before in the avian virus of G9 lineage (A/Ch/HK/G9/97). The presence of H173, T179, and L216 residues in HA1 is supposed to sustain the binding to mammalian α2,6-sialic acid-linked receptors and the replication in the human respiratory epithelial cells (Sorrell et al., 2009; Wan et al., 2008).

Glycosylation sites of the HA gene are significant for detection of the virus virulence, shielding antigenic epitopes, as well as host cell receptors (Wanzeck et al., 2011). Two potential glycosylation sites were lost from the current isolates. Previous studies showed a relationship between the addition of glycosylation sites to the HA and the loss of H5N1 virulence, as well as the reduction in the specificity of H2 receptor binding (Kaverin et al., 2000). Additionally, others indicated that change in the glycosylation pattern may result in adaptation of AIV (H9N2) in poultry host (Baigent and McCauley, 2003).

The identity percent of the current study exhibited that all three isolates were closely related to each other at a

range from 99% to 100%, as well as their relatedness to the Egyptian and Middle East strains. Furthermore, one isolate "A/chicken/Egypt/1626F/2016" possessed 92% identity to the Asian strain. While, other 2 isolates "A/chicken /Egypt / 1618F/ 2016" and "A/ chicken/ Egypt/ 1623F/ 2016" had 91% identity. Similarly, "A/chicken/Egypt/1626F/2016" showed 99% identity to the Egyptian strain of 2011. While both isolates "A/ chicken/ Egypt/ 1618F/ 2016" and "A/chicken/Egypt/1623F/2016" showed 98%. All of these findings were quite similar to that have been identified by Abd ElHamid et al., (2018) who showed that AIV H9N2 isolates had 92.3-97.1% identity with the Egyptian strain A/Quail/Egypt/113413v/2011.

The phylogenetic evolution of the current isolates demonstrated a very close relatedness to "G1/97", which was firstly recorded in china during 1997, then disseminated through the Eurasian countries such as, Iran, Pakistan, and Italy with a permanent evolution. Afterward they distributed to North Africa and Middle East including, Tunisia, Egypt, Saudi Arabia, UAE and Israel (Alexander, 2000, EL Zoghby et al., 2012; Perk et al., 2006; Tombari et al., 2011). Egyptian H9 viruses were classified previously into 2 sublineages of group B according to Fusaro et al. (2011) and Kandeil et al. (2014). These findings were in the same as Banks et al. (2000), who identified the close relation of all Egyptian strains to those isolated from the Middle East as G1 lineage with close relation to the Israeli strains.

CONCLUSION

Based on current findings, AIV (H9N2) still circulating among poultry farms in the Southern of Egypt (Luxor governorate). Furthermore, isolated strains were closely related to strains from Egypt, the Middle East, and Asia. Although, they are still low pathogenic,the risk of zoonotic transmission still exists. So that, regular monitoring of the further changes is demanded to understand the periodical evolution of the virus, which provides a standard map for which strains can be used for vaccination. Finally, adequate health measures in the poultry farms are highly recommended to control infection and decline the chance of zoonotic transmission.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

NOVELTY STATEMENT

This study illustrated the current situation of AI (H9N2) in luxor governorate in Egypt, and confirmed that AI H9N2 isolates were low in pathogenicity, as well as the isolated strains were closely related to strains from Egypt, the Middle East, and Asia.

AUTHORS CONTRIBUTION

Conceptualization, A.I., N.O., R.S.; Data curation, M.E.T., N.O., M.S., R.S., A.A; Investigation, M.E.T., N.O., M.S.; Supervision, A.I., N.O., M.S.; Visualization, A.I., N.O., M.S.; Writing—original draft, M.E.T., N.O., and M.S.; Writing—review and editing, M.E.T., N.O., M.S., R.S.

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