

Characterization of Avian Influenza H9N2 Virus in Broiler Chicken Backyard Flocks in Three Egyptian Governorates during Late Autumn 2023

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ABSTRACT

Background: Low pathogenic avian influenza viruses (LPAIV) H9N2 considered as one of the most prevalent infectious agents in Egyptian poultry farms since its first introduction in 2011 and one of the most important potential pandemic threats.

Methods: Thirty samples were collected from backyard broiler chicken flocks from three Egyptian governorates (Menoufia, Gharbia and Buhayrah) during late autumn of 2023. All of these flocks were not vaccinated against LPAIV H9N2. All collected samples were screened by Real time reverse transcriptase polymerase chain reaction (rRT-PCR) for detection of Avian influenza viruses (H9, H5 and H7), Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) then virus isolation of H9 positive samples followed by molecular and phylogenetic analysis of two isolates from Menoufia and Gharbia governorates through full Haemagglutinin (HA) gene sequencing.

Results: The results of rRT-PCR showed that the prevalence of LPAIV (H9N2) was 20 % while the prevalence of NDV and IBV was 63 % and 56% respectively. Molecular and phylogenetic analysis reveals that our isolates were clustered with the recent Egyptian strains from 2020 till 2023. our isolates harbor the molecular markers 191H, 234L and 198A at the HA gene which indicate their potential zoonotic significance. Additionally, the potential glycosylation sites in our isolates are similar to all compared Egyptian strains since 2011. Analysis of the amino acid (aa) identity % shows the highest identity % between the two isolates of the current study (98.8), however the identity percent with the Egyptian isolate in 2011 was about 95%.

Conclusions: This study highlights the continued prevalence and evolution of H9N2 LPAIV in poultry flocks in Egypt with co-circulation of other respiratory viruses (NDV and IBV). Moreover, the role of vaccination besides biosecurity can't be neglected as effective control measures.

Keywords: H9N2, LPAIV, Unvaccinated, Egyptian.

INTRODUCTION

In 1966, in the USA was the first isolation of the low pathogenic avian influenza virus (LPAIV) H9N2 from turkeys (Homme and Easterday, 1970). Thus, LPAIV H9N2 was frequently found in both domestic and wild birds worldwide (Alexander, 1997). Infections with the LPAI H9N2 virus in poultry became common in Asia and the Middle East (Guo et al., 2000 and Peacock et al., 2019). The host range of AIVs is greatly expanded by terrestrial poultry, particularly quails and chickens (Xu et al., 2007, Gambaryan et al., 2008). Recently, LPAIV H9N2 is one of the most significant possible pandemic risks and has been identified as a zoonotic agent (Deng et al., 2010, Ma et al., 2019).

Influenza A virus is considered as a member of the Orthomyxoviridae family. At least ten viral proteins (hemagglutinin (HA), neuraminidase (NA), polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), nucleoprotein (NP), matrix proteins (M1 and M2), and non-structural proteins (NS1 and NS2 or the nuclear export protein [NEP]) are encoded by its segmented RNA genome, which consists of eight negative sense ssRNA segments (Mostafa et al., 2018).

Most often, the LPAI H9N2 causes moderate, asymptomatic infections that go unnoticed. This enables the virus to evolve by inducing a widespread

enzootic infection and adaptive mutations. Genetic reassortments through the interchange of its genomic segments with other avian influenza virus subtypes, such as the H5N1, H7N7, and H1N1 subtypes, are another way for virus evolution. This can therefore result in the appearance of new reassortant viruses or variant strains, which may alter the biological characteristics of the newly emerged viruses (Iqbal et al., 2009; Ashraf et al., 2017).

The influenza virus H9N2 has been divided into two distinct lineages: the Eurasian and North American lineages. The Eurasian lineage generally comprises three main sub-lineages: the Korean lineage, the G1 lineage and the Y280 lineage (Chen et al., 2001, Aamir et al., 2007).

In 2024, a recent classification suggestion for AIV H9 divided it into three primary lineages (B, G, and Y). The G lineage, formerly recognized as G1 or h9.4.1, the B lineage, formerly recognized as BJ/94, Y280, G9, or h9.4.2, and the Y lineage, which includes the formerly recognized American (or h9.1–h9.2) and Y439 (or Korean, h9.3) lineages (Fusaro et al., 2024).

In Egypt, AIV H9N2 has been endemic since 2011 with difficult control. In this study we monitored H9N2 in unvaccinated poultry flocks.

MATERIAL AND METHODS

Ethical approval:

Ethical approval for this study was given by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, University of Sadat City, Egypt under the No. VUSC – 058-1-22.

Sampling

In this study 30 backyard broiler chicken flocks suspected to be infected with H9N2 AIV were sampled from three Egyptian governorates (Menoufia, Gharbia & Buhayrah). The number of samples collected from every governorate was (13-11-6 respectively). Ten tracheal tissue samples were collected from every flock as a pool. The age of the collected samples ranged from

17 to 35 days during late autumn of 2023 with mortality rates ranging between 6 to 13%. All the tested flocks were unvaccinated against H9N2 AIV while almost all of them were vaccinated by live vaccines against NDV and IBV at the first week of age then another live vaccine against NDV by the end of the second week. The history of these sampled farms as shown in Table 1.

The samples were kept at -75°C until further use in the central laboratory of diagnosis of viruses, Faculty of Veterinary Medicine, University of Sadat City.

Table 1. History of collected samples of the current study.

No	Collection date	Age (days)	Breed	Governorate	Mortality %
1	11-2023	31	Broiler	Menoufia	7.5%
2	11-2023	29	Broiler	Buhayrah	10%
3	11-2023	32	Broiler	Buhayrah	8%
4	11-2023	21	Broiler	Gharbia	10%
5	11-2023	32	Broiler	Gharbia	9%
6	11-2023	29	Broiler	Gharbia	11%
7	11-2023	30	Broiler	Gharbia	7%
8	11-2023	30	Broiler	Menoufia	13%
9	11-2023	28	Baladi	Buhayrah	6%
10	11-2023	24	Broiler	Menoufia	11%
11	11-2023	26	Broiler	Buhayrah	7.5%
12	11-2023	23	Broiler	Menoufia	10%
13	11-2023	30	Broiler	Menoufia	8%
14	11-2023	35	Broiler	Menoufia	7%
15	11-2023	17	Broiler	Menoufia	10%
16	11-2023	33	Broiler	Gharbia	10%
17	11-2023	24	Broiler	Gharbia	11%

18	12-2023	21	Broiler	Gharbia	7%
19	12-2023	26	Broiler	Menoufia	9.5%
20	12-2023	28	Broiler	Menoufia	7%
21	12-2023	27	Broiler	Menoufia	11%
22	12-2023	35	Broiler	Gharbia	9%
23	12-2023	28	Broiler	Gharbia	8%
24	12-2023	28	Broiler	Gharbia	12%
25	12-2023	29	Broiler	Menoufia	8%
26	12-2023	31	Broiler	Menoufia	11%
27	12-2023	27	Broiler	Menoufia	10%
28	12-2023	27	Broiler	Gharbia	8%
29	12-2023	18	Broiler	Buhayrah	10%
30	12-2023	19	Broiler	Buhayrah	10%

Molecular detection of the causative agent(s) by real time RT-PCR

Total genomic viral RNA was extracted from tracheal tissue homogenate fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real time reverse transcriptase polymerase chain reaction (rRT-PCR) was

performed using Thermo Scientific superscript III platinum One-Step qRT-PCR kit (Invitrogen, USA) with gene specific primers for detection of influenza A virus, AI H9 virus, AI H5 Virus, AI H7 virus, Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) as shown in the following table:

Table (2): Primers for detection of influenza A virus, AI H9 virus, AI H5 Virus, AI H7 virus, Newcastle disease virus (NDV) and infectious bronchitis virus (IBV):

Virus	Target gene	Forward primer	Reverse primer	Probe	References
AIV (A)	Matrix (M)	AGATGAGTCTTCTAA CCG AGG TCG	TGCAAAAACATC TTCAAG TYT CTG	FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1	Spackman et al., 2002
AIV (H9)	HA	GGAAGAATTAATTAT TATTGGTCGGTAC	GCCACCTTTTTCAG TCTGACATT	[FAM]AACCAGGCCAGA CATTGCGAGTAAGATC C[TAMRA]	Ben Shabat et al., 2010
AIV (H5)	HA	ACATATGACTAC CCACAR TATTCA G	AGA CCA GCT AYC ATG ATT GC	FAM-TCWACA GTGGCGAGTTCC CTAGCA - BHQ1	Löndt et al., 2008
AIV (H7)	HA	TTTGGTTTAGCTTCG GG	ACATGATGCCCCGA AGCTAAAC	FAM- CATCATGTTTCATACTT CTGGCCAT-BHQ	Monne et al., 2008

NDV	Matrix (M)	AGTGATGTGCTCGGA CCTTC	CCTGAGGAGAGGC ATTTGCTA	[FAM]TTCTCTAGCAGTG GGACAGCCTGC[TAMR A]	Wise et al. 2004
vvNDV	Fusion (F)	TCCGGAGGATACAA GGGTCT	AGCTGTTGCAACCC CAAG	[FAM]AAGCGTTTCTGTC TCCTTCCTCCA[TAMRA]	Wise et al. 2004
IBV	Untranslated region UTR	ATGCTCAACCTTGTC CCTAGCA	TCAAAGTGGGATC ATCACGT	FAM TTGGAAGTAGAGTGAC GCCCAAACCTTCA-BHQ	Callison et al., 2001

Table (3): The thermal profile used in rRT-PCR using one step qRT-PCR kit shown in the following table.

Virus	RT (reverse transcription)	Initial denaturation	PCR *		
			Denaturation	Primer annealing	Extension
AIV (A)	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	52.5 °C / 30 sec.	72 °C / 30 sec.
AIV (H9)	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	48 °C / 30 sec.	72 °C / 30 sec.
AIV (H5)	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	52 °C / 30 sec.	72 °C / 30 sec.
AIV (H7)	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	52 °C / 30 sec.	72 °C / 30 sec.
NDV (M)	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	54 °C / 30 sec.	72 °C / 30 sec.
vv NDV	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	54 °C / 30 sec.	72 °C / 30 sec.
IBV	50 °C / 15 min.	95 °C / 2 min.	95 °C / 30 sec.	46 °C / 30 sec.	72 °C / 30 sec.

*The number of PCR cycles was 45 cycles for all viruses.

Isolation of avian influenza virus in specific pathogen free (SPF) embryonated chicken eggs (ECE) and hemagglutination (HA) test:

In this study 15 samples were inoculated in 9-11 days old SPF-ECE via the allantoic sac route in the first passage. These samples were selected according to the result of rRT-PCR and also according to the severity of clinical signs and postmortem lesions. Five of these were passed for additional passage. The procedures of virus isolation and HA test were performed according to OIE terrestrial manual (2021).

HA gene sequence

The extracted RNA for 2 selected samples from Menoufia and Gharbia governorates were sent to Macrogen laboratory (South Korea) for full HA gene sequencing by Sanger technology.

HA gene sequence analysis

Sequence assembly and editing were performed using Bioedit® software package version 7.7.1.0 (Hall, 1999). Confirmation of homology and identity were done by BLAST <http://www.ncbi.nlm.nih.gov>. To detect the molecular and epidemiological

relationships of our AIV H9N2 isolates, some AIV H9N2 reference strains isolated from Egypt and Middle East at different periods were downloaded from GenBank. These reference strains were representative of all the different AIV H9N2 strains. Molecular and Phylogenetic analysis were performed using MEGA version 10 (Kumar et al. 2018). Phylogenetic tree of full HA gene (Amino acid sequences) was constructed by the Neighbor-Joining method with the Jones-Taylor-Thornton model at 1000 bootstrap replicates.

RESULTS

Molecular identification of different respiratory agents by rRT-PCR

rRT-PCR test was performed on all samples using primers specific for the M

gene for detection of AIV type A then other primers specific for the HA gene for detection of H9, H5 and H7 AIVs.

For detection of NDV, specific primer for M gene (common gene) followed by typing of positive samples using specific primers for F gene, while for detection of IBV specific primer for the UTR was used.

The results showed that six samples were positive by PCR for H9 AIV, all samples were AIV H5 & H7 Negative. For IBV, 17 out of 30 samples showed positive results by rRT-PCR.

For NDV, 19 out of 30 samples showed positive results by rRT-PCR, none of them was classified as Velogenic Viscerotropic NDV. The results of rRT-PCR are shown in Table 2.

Table 4. Results of rRT-PCR (Ct values) of the current study.

Sample No	rRT-PCR results (Ct values)						
	AIV (A)	H9	H5	H7	IBV	ND Common	VVNDV
1	Negative				27	25	Negative
2	Negative				34	28	Negative
3	Negative				29	23	Negative
4	Negative				26	Negative	
5	Negative				33	34	Negative
6	Negative				28	24	Negative
7	Negative				Negative	30	Negative
8	Negative				29	27	Negative
9	Negative				30	Negative	
10	Negative				22	21	Negative
11	Negative				Negative	Negative	
12	Negative				20	25	Negative
13	Negative				24	30	Negative
14	Negative				Negative	Negative	
15	Negative				30	29	Negative
16	22	20	Negative	Negative	Negative	Negative	
17	Negative				Negative	29	Negative
18	Negative				Negative	28	Negative
19	22	20	Negative	Negative	Negative	Negative	
20	19	18	Negative	Negative	Negative	Negative	
21	Negative				24	28	Negative
22	21	20	Negative	Negative	Negative	Negative	
23	20	18	Negative	Negative	Negative	Negative	
24	Negative				20	23	Negative
25	Negative				Negative	30	Negative

26	Negative				23	33	Negative
27	21	22	Negative	Negative	Negative	Negative	
28	Negative				24	Negative	
29	Negative				Negative	33	Negative
30	Negative				22	29	Negative

Results of virus isolation and HA test:

In this study 15 samples were inoculated on ECE - via the allantoic sac route- in the first passage. These samples were selected according to the result of rRT-PCR for AIV H9 and also according to

the severity of clinical signs and postmortem lesions. The results showed that 8 samples were positive for the HA test. Five of these were passed for additional passage. The HA titers shown in Table 5.

Table 5. Results of the HA test of the current study.

Sample No.	HA titers (log2)	
	1st passage	2nd passage
8	0	Not done
13	0	Not done
14	0	Not done
16	8	8
17	5	Not done
18	8	8
19	8	Not done
20	8	8
21	0	Not done
22	5	6
23	6	8
25	0	Not done
27	7	Not done
28	0	Not done
30	0	Not done

Genetic analysis of HA gene of H9N2 AIV strains:

Complete HA gene sequence for 2 H9N2 isolates detected in this study was successfully done by Sanger technology. The cleavage motif sequences of the HA of the current study Egyptian isolates in 2023 were PARSSRGLF which have been associated with low pathogenicity as shown in Table 4. Analysis of the aa

residues within the antigenic sites as shown in Table 5 reveals the presence of five aa substitutions when compared with the G1/97 ancestral strain. The four substitutions (G153D, N206T, S158N and E198A) which were firstly reported in the Egyptian strain in 2011 and remained established till 2023 according to our isolates in addition to the substitution M58K which was reported

in Egypt since 2014 and remained till 2023 in our isolates.

Analysis of the aa residues at the receptor binding sites reveals presence of some substitutions within the binding sites, the right edge and the left edge as shown in Table 6. Within the binding sites only one substitution E198A which was firstly reported in the Egyptian strain at 2011 and remained till 2023 in our isolates, within the right edge three aa substitutions (I147T, R149K and A150S) which were reported in Egyptian strain since 2011 and remained till 2023 in our isolates, While two substitutions were detected within the left edge, D233G and Q235I which were reported in Egyptian strain since 2011 and

remained till 2023 in our isolates. The two isolates of the current study shared the same seven PGS (potential glycosylation sites) at the same positions of the HA gene. Interestingly, all these sites are identical to the PGS of all compared Egyptian H9N2 strains from 2011 till 2023 as shown in Table 8. Analysis of the identity % of the full HA gene based on aa sequences as in Table 7, shows the highest identity % between the two isolates of the current study (99.8 %) while the identity percent between the current study isolates and the Egyptian isolate in 2011 was about 95%, however it was about 90% when compared with the ancestral G1/97 strain.

Table 6. Amino acid substitutions detected within cleavage sites compared to the ancestral G1/97 H9N2 strain.

H9N2 isolates	Cleavage sites								
	333	334	335	336	337	338	339	340	341
A/Quail/Hong Kong/G1/97	P	A	R	S	S	R	G	L	F
A/chicken/Egypt/114940v/2011	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/1225VL/2012	-	V	-	-	-	-	-	-	-
A/chicken/Egypt/CL42/2013	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/S9668D/2014	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/1433RSF/2014	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/ABD7/2015	-	-	-	-	G	-	-	-	-
A/chicken/Egypt/15226VD/2015	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/F12054D/2016	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/A-chicken-1/2017	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/A15068/2018	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/Q17897C/2019	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/S19326C/2020	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/S19712/2021	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/BA20656OP/2022	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/CV16/2023	-	-	-	-	-	-	-	-	-
A/Chicken/Egypt/GH/22/2023	-	-	-	-	-	-	-	-	-
A/Chicken/Egypt/MF/27/2023	-	-	-	-	-	-	-	-	-
A/Duck/Hong Kong/Y280/97	-	-	-	-	-	-	-	-	-
A/Duck/Hong Kong/Y439/97	-	-	A	-	N	-	-	-	-

A/Chicken/Hong Kong/G9/97	-	-	-	-	-	-	-	-	-
A/turkey/Wisconsin/66	-	-	V	-	-	-	-	-	-
A/Chicken/Shanghai/F/98	-	-	-	-	-	-	-	-	-
A/chicken/Iran/av1221/1998	-	-	-	-	-	-	-	-	-
A/Duck/Hong Kong/168/77	-	-	A	-	G	-	-	-	-
A/chicken/Korea/01310_CE20/2001	-	-	T	-	G	-	-	-	-

Table 7. Amino acid substitutions detected within known antigenic sites compared to the ancestral G1/97 H9N2 strain.

H9N2 isolates	Antigenic sites													
	143 ^a	165 ^b	170 ^b	153 ^a	201 ^b	234 ^d	14 ^a	197 ^b	206 ^b	58 ^c	93 ^c	158 ^a	182 ^b	198 ^b
A/Quail/Hong Kong/G1/97	T	K	P	G	N	L	T	T	N	M	E	S	K	E
A/chicken/Egypt/114940v/2011	-	-	-	D	-	Q	-	-	T	-	-	N	-	A
A/chicken/Egypt/1225VL/2012	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Egypt/CL42/2013	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Egypt/S9668D/2014	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Egypt/1433RSF/2014	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/ABD7/2015	-	-	-	D	-	-	-	-	T	K	-	N	-	T
A/chicken/Egypt/15226VD/2015	-	-	-	D	-	-	-	-	T	K	K	N	-	A
A/chicken/Egypt/F12054D/2016	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/A-chicken-1/2017	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Egypt/A15068/2018	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/Q17897C/2019	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/S19326C/2020	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/S19712/2021	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/BA20656OP/2022	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/chicken/Egypt/CV16/2023	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/Chicken/Egypt/GH/22/2023	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/Chicken/Egypt/MF/27/2023	-	-	-	D	-	-	-	-	T	K	-	N	-	A
A/Duck/Hong Kong/Y280/97	S	-	-	D	-	-	-	-	T	-	-	-	-	T
A/Duck/Hong Kong/Y439/97	-	-	-	N	-	Q	-	-	A	-	K	-	-	-
A/Chicken/Hong Kong/G9/97	S	-	-	D	-	-	-	-	T	-	-	-	-	A
A/turkey/Wisconsin/66	-	-	-	N	D	Q	-	-	A	V	-	-	E	-
A/Chicken/Shanghai/F/98	S	-	-	D	-	Q	-	-	T	-	K	-	-	A
A/chicken/Iran/av1221/1998	-	-	-	-	-	Q	-	-	-	-	-	-	-	A
A/Duck/Hong Kong/168/77	-	-	-	N	-	Q	-	-	A	-	-	-	-	-
A/chicken/Korea/01310_CE20/2001	-	-	-	-	-	Q	G	-	A	-	-	-	-	-

Table 8. Amino acid substitutions detected within known receptor binding sites compared to the ancestral G1/97 H9N2 strain.

H9N2 isolates	receptor binding sites																	
	Binding sites							Right edge				Left edge						
	110	161	163	191	198	202	203	146-150				232-237						
A/Quail/Hong Kong/G1/97	P	W	T	H	E	L	Y	G	I	S	R	A	N	D	L	Q	G	R
A/chicken/Egypt/114940v/2011	-	-	-	-	A	-	-	-	T	-	K	S	-	G	Q	I	-	-
A/chicken/Egypt/1225VL/2012	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/CL42/2013	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/S9668D/2014	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/1433RSF/2014	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/ABD7/2015	-	-	-	-	T	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/15226VD/2015	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/F12054D/2016	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/A-chicken-1/2017	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/A15068/2018	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/Q17897C/2019	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/S19326C/2020	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/S19712/2021	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/BA20656OP/2022	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Egypt/CV16/2023	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/Chicken/Egypt/GH/22/2023	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/Chicken/Egypt/MF/27/2023	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/Duck/Hong Kong/Y280/97	-	-	-	N	T	-	-	-	T	-	K	-	-	G	-	-	-	-
A/Duck/Hong Kong/Y439/97	-	-	-	-	-	-	-	-	T	-	-	-	-	-	Q	-	-	-
A/Chicken/Hong Kong/G9/97	-	-	-	N	A	-	-	-	T	-	K	-	-	G	-	-	-	-
A/turkey/Wisconsin/66	-	-	-	-	-	-	-	-	T	-	-	-	-	G	Q	-	-	-
A/Chicken/Shanghai/F/98	-	-	-	N	A	-	-	-	T	-	K	-	-	G	Q	-	-	-
A/chicken/Iran/av1221/1998	-	-	-	-	A	-	-	-	T	-	K	-	-	G	Q	-	-	-
A/Duck/Hong Kong/168/77	-	-	-	-	-	-	-	-	T	-	-	-	-	G	Q	-	-	-
A/chicken/Korea/01310_CE20/2001	-	-	-	-	-	-	-	-	T	-	K	-	-	G	Q	-	-	-

Table 9. Identity percent between H9N2 (H9 gene) strains based on amino acid sequences.

	Strain	1	2	3	4	5	6	7	8	9	10	11
1	A/Quail/Hong Kong/G1/97		91.8%	91.0%	91.0%	92.1%	89.5%	90.5%	90.7%	90.1%	90.1%	90.3%
2	A/chicken/Egypt/114940v/2011	91.8%		97.5%	97.2%	98.6%	95.7%	95.9%	95.7%	95.5%	95.5%	95.7%
3	A/chicken/Egypt/CL42/2013	91.0%	97.5%		97.2%	98.5%	95.9%	95.9%	95.7%	95.5%	95.5%	95.7%
4	A/chicken/Egypt/D10945E/2015	91.0%	97.2%	97.2%		98.5%	97.5%	97.9%	97.7%	97.2%	97.5%	97.7%
5	A/chicken/Egypt/A-chicken-1/2017	92.1%	98.6%	98.5%	98.5%		97.0%	97.2%	97.0%	96.6%	96.8%	97.0%
6	A/chicken/Egypt/Q17897C/2019	89.5%	95.7%	95.9%	97.5%	97.0%		97.7%	97.5%	97.2%	97.0%	97.2%
7	A/chicken/Egypt/S19712/2021	90.5%	95.9%	95.9%	97.9%	97.2%	97.7%		99.4%	99.2%	99.2%	99.4%
8	A/chicken/Egypt/BA20656OP/2022	90.7%	95.7%	95.7%	97.7%	97.0%	97.5%	99.4%		98.6%	98.6%	98.8%
9	A/chicken/Egypt/CV16/2023	90.1%	95.5%	95.5%	97.2%	96.6%	97.2%	99.2%	98.6%		98.8%	99.0%
10	A/Chicken/Egypt/GH/22/2023	90.1%	95.5%	95.5%	97.5%	96.8%	97.0%	99.2%	98.6%	98.8%		99.8%
11	A/Chicken/Egypt/MF/27/2023	90.3%	95.7%	95.7%	97.7%	97.0%	97.2%	99.4%	98.8%	99.0%	99.8%	

Table 10. Potential glycosylation sites detected within the studied H9N2 isolates in comparison with Egyptian H9N2 strains.

Strain No	Potential glycosylation sites									Total No
	29	105	141	206	218	298	305	492	551	
A/chicken/Egypt/114940v/2011	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/1225VL/2012	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/CL42/2013	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/S9668D/2014	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/F12054D/2016	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/A-chicken-1/2017	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/A15068/2018	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/Q17897C/2019	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/S19326C/2020	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7

A/chicken/Egypt/S19712/2021	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/BA20656OP/202 2	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/chicken/Egypt/CV16/2023	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/Chicken/Egypt/GH/22/202 3	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7
A/Chicken/Egypt/MF/27/202 3	NS T	NG T	NV T			NS T	NI S	NG T	NG S	7

Phylogenetic analysis

The phylogenetic tree based on full HA gene aa sequences showed that H9N2 isolates sequenced in this study were clustered with recent H9N2 strains from

Egypt in 2020 till 2023 and all Egyptian isolates are clustered with other Middle east and African strains which were related to the G1 Clade (Fig. 1).

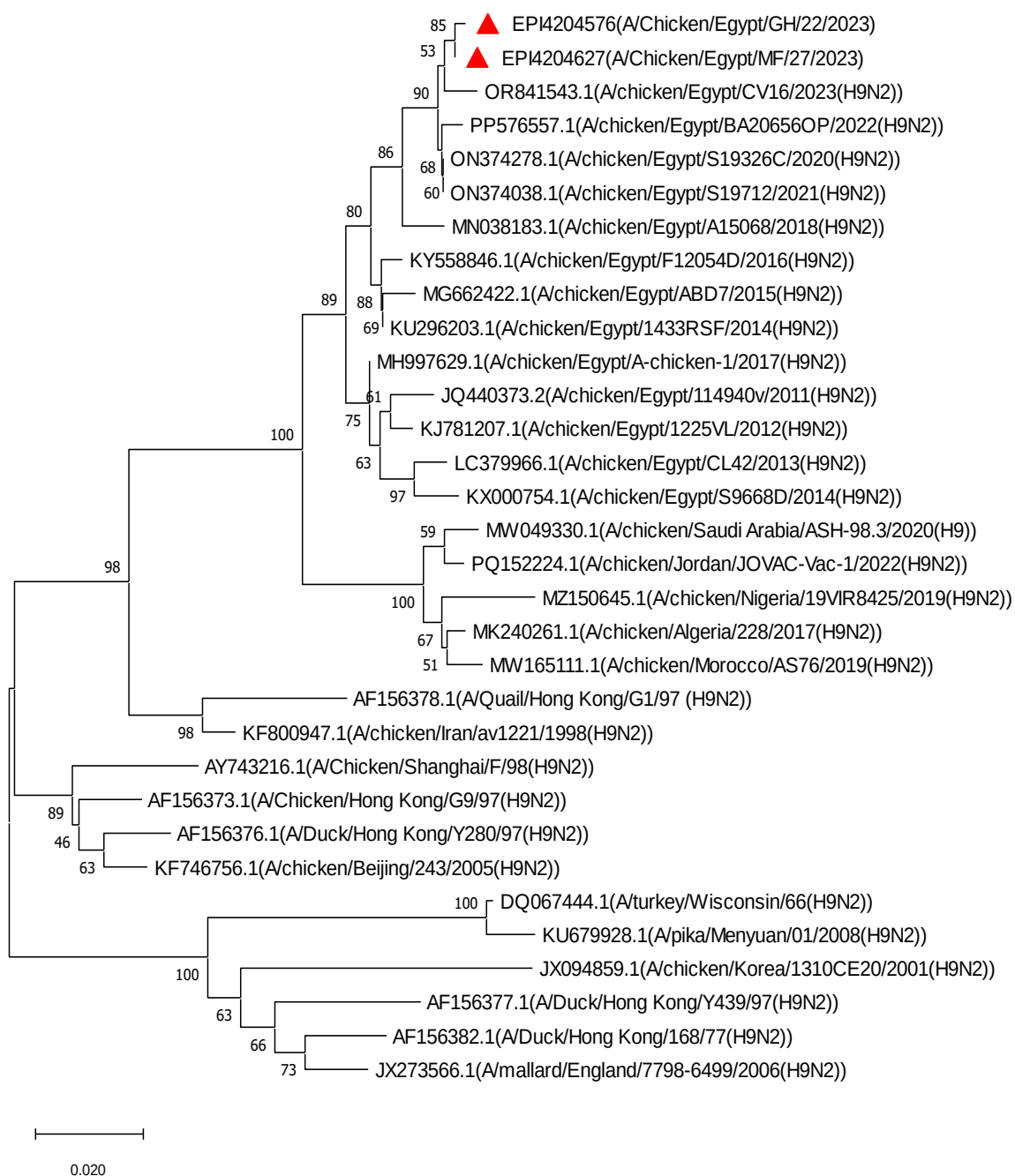


Figure 1. Phylogenetic tree based on aa sequence of full HA genes from G1 lineage. The tree was generated by the Neighbor-Joining method with the Jones-Taylor-Thornton model at 1000 bootstrap replicates with the MEGA 10 program.

DISCUSSION

The first reports of detection of G1-like lineages of H9N2 AIV viruses in Egypt were in 2010 (Arafa et al., 2012; Kandeil et al., 2014). Later, the virus spread widely among domestic poultry. Egyptian H9N2 viruses were divided into two genotypes based on phylogenetic analysis: genotype I, that was detected in Egyptian poultry between 2010 and 2013, in addition to genotype II, that appeared as a result of reassortment between H9N2 AIV G1 and Eurasian strains of H9N2 AIV isolated from wild birds in 2014 (Kandeil et al., 2017; El Sayes et al., 2022).

Four different groups (A, B, C, and D) emerged from the LPAIV H9N2 isolates from the Central Asia and Middle East between 1998 and 2010 (Fusaro et al., 2011).

In the current study 30 samples from 3 different governorates were screened for detecting H9 AIV by rRT-PCR and then by virus isolation, the results showed that the prevalence of H9 AIV virus was 20 % by rRT-PCR test which is matched with the results obtained by Bedair et al., 2024 as the prevalence rate was 23% in samples collected from the same and some neighboring governorates.

The data in our study indicated also the high prevalence of NDV in this area (63%), none of them was classified as vvNDV. Additionally, we found that about 56% of samples were positive for IBV. Interestingly, the H9 AIV positive samples of the current study were negative for both NDV and IBV. These results can explain the reason for variable mortality rates between farms (6 to 13%) mortality.

LPAI H9N2 was classified phylogenetically into two lineages: North American and Eurasian. The majority of the strains that were reported were categorized into two main clades (G1 and Y280) within the Eurasian lineage, while the Eurasian lineage includes other clades. The prototype virus A/Quail/Hong Kong/G1/1997, which mostly present in Central Asia, Southern China, in addition to the Middle East, is the primary representative of the G1 clade, whereas the A/Duck/Hong Kong/Y280/1997 prototype virus primarily represents the Y280 clade viruses, which primarily circulate in China (Group et al., 2013). Based on the obtained HA gene sequences, the detected H9 AIV isolates in the current study are phylogenetically clustered with the recent Egyptian H9N2 strains at (2023,2022 ,2021 and 2020) within a separate cluster within the G1 clade.

The cleavage site sequences of the current study strains were 335RSSR/GLF341, which indicates their low pathogenicity (Okazaki et al., 2004). This motif has been reported to be typical of Asian LPAIV H9N2 viruses. and in the Middle East (Chrzastek et al., 2018) and perfectly adapted to poultry (Aamir et al., 2007). Interestingly this motif was reported also in Egyptian poultry farms at 2023 (Bedair et al., 2024).

Mutations in the aa residues in the AIV H9N2, HA protein's antigenic sites are crucial because they may alter the virus's antigenicity, which could impact the effectiveness of vaccinations or the virus's ability to bind to cell receptors (Matrosovich et al., 2001; Meng et al., 2016).

Any alteration to the nine amino acids in the antigenic sites (143, 166, 170, 153, 201, 234, 141, 197, and 206) of the HA glycoprotein of H9 AIV resulted in the emergence of new variants, according to study in 2004 (Kaverin et al., 2004). In the current study four substitutions (G153D, N206T, S158N and E198A) which were firstly reported in the Egyptian strain in 2011 and remained till 2023 in our isolates. In addition to the substitution M58K which was reported since 2014 and continued till 2023 in our isolates.

For host receptor preference, the HA's receptor binding sites are very important. For AI H9N2 viruses to spread in ferrets, amino acid substitutions within the receptor binding sites Q191H, T197A, A198E, Q234L, and G236S (H9 numbering) are crucial (Wan et al., 2008).

Other important determinants include the aa residues 191, 198, 234, 235, and 236 (H9 numbering) at the HA protein's RBS (Iqbal et al., 2009; Ghorbani et al., 2016). Residue to the position 234 is very important for the host specificity. Thus, Avian influenza virus having the aa Q at 234 positively bind effectively to avian host receptor via α [2, 3] Gal-type binding (Weis et al., 1988). However, the affinity for human-like receptors is increased by substitution Q234L (Weis et al., 1988; Naguib et al., 2017). Furthermore, it was recommended that residue 234 L was typical for the human pandemic caused by H2 and H3 AIV (Matrosovich et al., 2008). Additionally, the 191H residue was also linked to a predilection for binding effectively to receptors on respiratory epithelial cells in human (Kandeil et al., 2014) and H9N2 AIV transmission through direct

contact between ferrets (Wan et al., 2008).

However, the α [2–6] receptor binding (human-like receptor) and subsequent infections of mammals can be affected by residue 198 (H9 numbering) (Matrosovich et al., 2001). Numerous substitutions, including 198 T/V/A, were previously reported at the same position. (Zhu et al., 2018). The H9 AIV strains may therefore be able to bind to α [2–6] human-like receptors if they have 198 T, but its affinity was lower than that of residues A and V at the same site (Matrosovich et al., 2001).

In the current study only one substitution E198A within the binding sites which was firstly reported in the Egyptian strain in 2011 and remained till 2023 isolates. However, within the right edge three aa substitutions (I147T, R149K and A150S) which were reported in Egyptian strains since 2011 and remained till 2023 in our isolates. Additionally, within the left edge: two substitutions D233G and Q235I which were reported in Egyptian strains since 2011 and remained till 2023 in our isolates. Interestingly, these substitutions were reported in Algerian poultry at 2017 (Barberis et al., 2020). Consequently, according to all detected substitutions within in the receptor binding sites, the current study isolates may harbor higher affinity for human-like receptors.

The two isolates of this study have the same seven potential glycosylation sites (PGS) at the same positions of the HA gene which are identical to the PGS of all compared Egyptian H9N2 strains from 2011 till 2023 as shown in Table 8. According to earlier research, differences in influenza virus PGS patterns may affect the pathogenicity,

receptor binding affinity, and specificity of AIVs (Kaverin et al., 2004; Iqbal et al., 2009) and most likely infection of a new host. In addition, it had been described that any loss of PGS is linked with higher affinity of AIV to human like receptors and also increased virulence (Reading et al., 2009; Sun et al., 2013). Nevertheless, it has been found that more PGS on HA protein can reduce pathogenicity (Matsouka et al., 2009).

CONCLUSION

This study demonstrates the continuous circulation of old and recent strains of LPAIV (H9N2) in Egyptian poultry farms with continuous evolution. So Effective control measures for AIV should be considered due to economic losses in poultry with public health concerns through continuous surveillance, biosecurity and vaccination.

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