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Genetic Evolution Correlated with Pathogenicity of H9N2 Circulated in Lower Egypt during 2022

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Abstract | However, the low pathogenicity of avian influenza (H9N2), can have significant economic losses in poultry farms associated with other viral and bacterial infections. The purpose of this research is to study the genetic evolution and pathogenicity of the H9N2 virus during 2022. The forty tracheal samples were gathered from broiler chicken farms from 5 governorates (El-Dakahlia, El-Sharqia, Alexandria, Al-Qalyubia, and El-Gharbia) and tested using Real-Time Reverse Transcription polymerase chain reaction (RT-PCR) by specific primers detect avian influenza type A and H9 typing. The nine samples selected represent different governorates for sequencing the HA gene. Also, the pathogenicity of the virus was positive experimentally in specific pathogen-free (SPF) chicks. In this research, the prevalence was 45% (18 out of 40 farms) with mild respiratory signs and 15-20% mortality. According to HA gene's phylogenetic study of nine selected strains, they belong to G1/97-B like Eurasian sub-lineages (EGY-2 group) that had circulated in Egypt since 2014 and they were clustered with the 2021-2022 strains in the Egy-2b subgroup. Compared with Quail/Hong Kong/G1/97, the strains in this research had a specific characteristic mutation at L87I in A-chicken-Egypt-RN1-2022 and A-chicken- Egypt-RN9-2022, D405N in A-chicken- Egypt-RN5-2022, A-chicken-Egypt-RN6-2022, A-chicken-Egypt-RN9-2022. Also, new characteristic mutations in antigenic site II at L234Q and N201D in A-chicken-Egypt-RN1-2022 and A-chicken-Egypt-RN2-2022 were detected which may be due to vaccination pressures that may be affecting vaccine efficacy. The pathogenicity of the virus has been examined, and it has been found to prompt mostly mild respiratory signs with diarrhea in some birds without any mortality, as well as high and persistent viral shedding from 2 dpi to 10 dpi and mean antibody titer at 7 and 14 dpi (6±0.52 and 6.8±0.61 log2) respectively. The histopathological alterations do not only affect the respiratory and digestive systems; they also affect the heart, liver pancreas, and proventriculus. Also, the spleen showed severe lymphoid depletion and necrosis causing immunosuppression. Regular virus monitoring and study of the pathogenicity is necessary for better control of virus infection.

Keywords: H9N2, Genetic evolution, Pathogenicity, Histopathology, Avian influenza

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INTRODUCTION

Avian influenza (AIV) viruses are a member of the family Orthomyxoviridae. The avian influenza had eleven and eighteen subtypes according to Neuraminidase (NA) and hemagglutinin (HA), respectively (Webster *et al*., 2005; Tong *et al*., 2012, Wu *et al*., 2014). In 1966, the H9N2 virus was first found in American turkeys (Homme *et al*., 1970). From this date, it is classified into American and Eurasian lineages based on genetic differences of the hemagglutinin (HA) gene (Banks *et al*., 2000). The American viruses were detected in wild birds, and the Eurasian viruses had three lineages, each having a unique prototype: the G1 lineage (A/quail/Hong Kong/G1/1997), Y280 lineage (Beijing/1/94 and A/duck/HK-Y280-1997), and the Korean lineage (A/chicken/Hong Kong/Y439/1997) (Guo *et al*., 2000). The G1 lineage has been divided phylogenetically into 4 groups (A, B, C, and D) (Fusaro *et al*., 2011). Then, Avian influenza (H9N2) cases have been reported worldwide. It was initially noted in Asia prior (Cameron et al., 2000) to being documented in the Middle East and Africa (Roussan *et al*., 2009). The H9N2 virus was initially isolated in Egypt in May 2011 (El-Zoghby *et al*., 2012) belonging to the Qa/HK/G1/97 lineage closely linked to Israeli viruses, then it spread throughout Egyptian governorates (ELbayoumi *et al*., 2013; Abdel-Moneim *et al*., 2012). They are additionally categorized as cluster B upon the sequencing of the HA gene and clustering into the Egy-1 group (Kandeil *et al*., 2014).

Parallel to the spread of H9N2, Egypt developed as a reservoir for several AIV subtypes, including H5N8 and H5N1 (Zanaty *et al*., 2019; Selim *et al*., 2017; Naguib and Harder, 2018). Re-assortment is highly likely to occur as long as H9N2 is co-circulating with distinct subtypes of AIVs with significant consequences for public health (Naguib and Harder, 2018; Lee *et al*., 2016). For instance, a distinct H5N2 reassortant has been produced by combining the NA gene from the Egyptian H9N2 with the HA gene and additional internal genes from the Egyptian HPAI H5N8 (Hagag *et al*., 2019). In 2014, pigeons revealed numerous cases of reassortant H9N2 viruses, which have internal genes from various subtypes of avian influenza observed in wild birds with the HA gene categorized into the Egy-2 group (Kandeil *et al*., 2017) and still found till now (Hassan *et al*., 2020; Adel *et al*., 2022; El Sayes *et al*., 2022; Bedair *et al*., 2024). Moreover, because of alterations in the HA gene, the LPAI H9N2 acquires various antigenic variations through genetic drift mutations (Peacock *et al*., 2016; Adel *et al*., 2019).

In chicken farms, the low pathogenic avian influenza (LPAIV) H9N2 virus produces mild respiratory illnesses and a considerable mortality rate, particularly when associated with secondary viral and bacterial diseases (Alexander, 2000). H9N2 virus infection in chickens is generally more common than in ducks (Kayali *et al*., 2014). Furthermore, several H9N2 infections in people have been documented due to acquiring some mutation in receptor binding sites adapted the virus to infect the human. Most infections were probably caused by direct contact with H9N2 virus-infected poultry. According to the Egyptian Ministry of Health, three H9N2 infections in humans were verified in laboratories (Peacock *et al*., 2019). This study aims to monitor the genetic evolution of the HA gene of the H9N2 virus in Egypt and study the pathogenicity of the current circulating strain in Lower Egypt during 2022.

MATERIAL AND METHODS

Samples (3-5 birds/flock) were taken from forty infected broiler chicken farms gathered in 2022 from the five Egyptian governorates of 10 El-Dakahlia, 10 El-Sharqia, 5 Alexandria, 5 Al-Qalyubia, and 10 El-Gharbia (Table 1) and transported to the lab. In an ice box for clinical and P/M examination, the 10 tracheal and cloacal swabs/flock were pooled in sterile phosphate-buffered saline (PBS) containing amphotericin B, streptomycin, and penicillin (OIE, 2014) and kept at -20 ˚C until used. Ten farms were vaccinated with killed H9 and H5 2.2.1, and others were not vaccinated with killed H9, vaccinated only with H5 (2.2.1) (Table 1). The chicken showed a mortality rate of 15-20% with mild respiratory symptoms. The samples also showed ruffled feathers, depression, coughing, sneezing, nasal and ocular discharge, and diarrhea in some cases.

H9N2 Virus Detection

Real-time PCR analysis of the extracted samples was performed using Real-Time Reverse Transcription polymerase chain reaction (RT-PCR). The Qiamp viral RNA kit (Qiagen, Germany) was utilized for the extraction in compliance with the manufacturer's guidelines, the samples were lysed and then buffered to optimize the binding condition to Qiamp membranes then loaded to the membrane with two washing step to remove any impurities then eluted in RNase free buffer. The extracted RNA was examined for AIV type A and H9 typing by real-time RT-PCR using the Qiagen one-step kit (Qiagen, Germany) (Spackman *et al*., 2002; Ben Shabat *et al*., 2010).

H9N2 Virus Isolation

By standard methodology, 0.2 mL of sample were injected into specific pathogen-free (SPF) embryonated chicken eggs (ECE) at the age of 9 to 11 days (3 egg/sample) and incubated at 37^{0}c incubator and observed daily (OIE, 2015) for 2-3 days and chilled at 4^{0}c for 4hr. Then the allantoic fluid was harvested and examined using the haemagglutination (HA) test (OIE, 2015) and confirmed by real-time RT-PCR.

Table 1: Epidemiological data of suspected broiler farms and result of RRT-PCR.

No.	Date of collection	Species	Age/ days	Governorate	Vaccination regime	Mortality percent	Result of RRT-PCR
1	1-2022	Chicken	30	El-Dakahlia	H5(2.2.1)	20%	+ve
2	2-2022	Chicken	25	Al-Qalyubia	H5(2.2.1)	18%	-ve
3	4-2022	Chicken	30	El-Sharqia	H5(2.2.1)	15%	+ve
4	5-2022	Chicken	40	El-Dakahlia	H5(2.2.1)	18%	+ve
5	7-2022	Chicken	42	Alexandria	H5(2.2.1)	20%	-ve
6	3-2022	Chicken	35	Al-Qalyubia	H5(2.3.2)	15%	-ve
7	2-2022	Chicken	20	El-Gharbia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	20%	-ve
8	11-2022	Chicken	23	Alexandria	H5(2.2.1)	19%	+ve
9	8-2022	Chicken	35	El-Dakahlia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	20%	+ve
10	9-2022	Chicken	35	Alexandria	H5(2.2.1)	17%	+ve
11	12-2022	Chicken	25	Alexandria	H5(2.2.1)	15%	+ve
12	11-2022	Chicken	20	El-Dakahlia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	15%	+ve
13	3-2022	Chicken	25	El-Sharqia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	17%	+ve
14	3-2022	Chicken	30	El-Dakahlia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	20%	-ve
15	1-2022	Chicken	35	El-Dakahlia	H5(2.2.1)	18%	-ve
16	4-2022	Chicken	40	El-Sharqia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	16%	+ve
17	11-2022	Chicken	22	Alexandria	H5(2.2.1)	17%	-ve
18	5-2022	Chicken	24	El-Dakahlia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	15%	-ve
19	7-2022	Chicken	30	El-Dakahlia	H5(2.2.1)	18%	+ve
20	8-2022	Chicken	33	El-Dakahlia	H5(2.2.1)	16%	+ve
21	9-2022	Chicken	40	El-Sharqia	H5(2.2.1)	20%	-ve
22	1-2022	Chicken	35	El-Dakahlia	H5(2.2.1)	17%	+ve
23	4-2022	Chicken	40	El-Sharqia	H5(2.2.1)	18%	+ve
24	8-2022	Chicken	20	El-Gharbia	H5(2.2.1)	16%	-ve
25	5-2022	Chicken	22	El-Gharbia	H5(2.2.1)	15%	+ve
26	4-2022	Chicken	25	El-Gharbia	H5(2.2.1)	20%	-ve
27	3-2022	Chicken	30	El-Gharbia	H5(2.2.1)	17%	+ve
28	4-2022	Chicken	35	El-Sharqia	H5(2.2.1)	15%	-ve
29	5-2022	Chicken	22	Al-Qalyubia	H5(2.2.1)	18%	-ve
30	1-2022	Chicken	25	Al-Qalyubia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	20%	+ve
31	1-2022	Chicken	30	Al-Qalyubia	H5(2.2.1)	18%	+ve
32	2-2022	Chicken	32	El-Sharqia	H5(2.2.1)	16%	-ve
33	4-2022	Chicken	25	El-Gharbia	H5(2.2.1)	15%	-ve
34	2-2022	Chicken	20	El-Sharqia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	15%	-ve
35	3-2022	Chicken	22	El-Gharbia	H5(2.2.1)	20%	-ve
36	5-2022	Chicken	25	El-Gharbia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	16%	-ve
37	3-2022	Chicken	30	El-Gharbia	Killed H9 at 3 days of age then H5 (2.2.1) at 8 day	16%	-ve
38	6-2022	Chicken	35	El-Gharbia	H5(2.2.1)	18%	-ve
39	5-2022	Chicken	40	El-Sharqia	H5(2.2.1)	19%	-ve
40	3-2022	Chicken	22	El-Sharqia	H5(2.2.1)	19%	-ve

The post-mortem lesions included fibrinopurulent and catarrhal inflammations with lung and tracheal congestion. Also, enteritis, pancreatitis, and congestion of the spleen and liver were observed.

Sequencing of HA Gene of H9N2 Viruses

In this study we selected 9 samples for sequencing of full HA gene representing the five governorates (Table 2), the QIAmp viral RNA mini kit, which served as an extraction

method of the RNA virus from the isolates compliance following manufacturer's guidelines, Super Script TMIII reverse transcriptase (Thermo Fisher Scientific, MA, USA) was utilized to synthesize the cDNA by adding 1µl of oligo-(dt), 5µl of RNA and 1 µl 10 mM dNTP then put at 65°c for 5 minutes and incubate on ice for one minute then add 4 µl 5X First-Strand Buffer, 1 µl 0.1 M DTT, 1 µl RNase OUT and 1 µl of SuperScript™ III-RT and incubate in 25°C for 5 minutes, 50°C for 45 minutes then 70°C for 15 minutes. The PCR was carried out by using Phusion® high fidelity DNA polymerase (Thermo Fisher Scientific, MA, USA) and gene-specific primers as directed by the kit manufacturer (Naguib *et al*., 2015), To identify the PCR results, agarose gel electrophoresis was used. The QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was utilized for PCR product purification. The sequencing was done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA), and An ABI 3500 Genetic Analyzer was utilized to ascertain the nucleotide sequence (USA: California; Life Technologies).

Table 2: Accession number of sequenced strains.

Number of strain	Name	Accession number
1	A-chicken-Egypt-RN1-2022	PQ187665
6	A-chicken-Egypt-RN2-2022	PQ187666
11	A-chicken-Egypt-RN3-2022	PQ187667
12	A-chicken-Egypt-RN4-2022	PQ187668
13	A-chicken-Egypt-RN5-2022	PQ187669
15	A-chicken-Egypt-RN6-2022	PQ187670
17	A-chicken-Egypt-RN7-2022	PQ187671
21	A-chicken-Egypt-RN8-2022	PQ187672
27	A-chicken-Egypt-RN9-2022	PQ187673

Mutation and Phylogenetic Analysis

Using Bio-Edit software version 7.2 using cluster W parameter (Hall, 1999) to align the full HA gene DNA sequence of the H9N2 virus with other strains from the National Center for Biotechnology Information (NCBI). We choice using this program due to it is easy to analyze, align, and edit the sequence data. Using 1,000 bootstrap replications and the Neighbour-Joining approach in MEGA 6, a phylogenetic tree was created (Tamura *et al*., 2013). The DNASTAR Lasergene 9 (Madison, WI, USA) was used to measure the genetic identity between several viruses. PyMOL software was used to predict 3D structures (Arnold *et al*., 2006), and Net N-Glyc 1.0 Server was used to identify possible glycosylation sites (Gupta *et al*., 2004).

Pathogenicity of H9N2 Avian Influenza Virus

Ethical approval: *The animal experiment was ethically approved by* Veterinary Medicine, Suez Canal University with code SCU-VET-REC-2024036.

Virus:

The A-chicken-Egypt-RN1-2022 represents to recently circulated Egyptian strain that was titrated in SPF at 9-11 days in accordance with (OIE, 2015). Using a conventional methodology, EID_{50} was measured (Reed and Muench, 1938, OIE, 2014). The extracted allantoic fluid was analyzed using real-time RT-PCR for the H9N2 virus, AIV H5N1 virus, infectious bronchitis virus, and Newcastle disease virus, to confirm that it was positive for H9N2 virus and negative for other viruses.

Experimental design: There were two equal groups of forty SPF chicks (20 chicks each). 0.1ml of LPAI (H9N2) (10^6 EID_{50}/ml) dropped in the nostrils and eyes at age 21 days (G1), and G2 serves as a negative control. After the infection, the clinical symptoms and death were tracked for 14 days.

Virus shedding: On 2, 4, 7, 10, and 14-day post-infection (dpi), oropharyngeal swab samples from all birds were collected using a 2% antibiotic solution of neomycin, streptomycin, and penicillin in sterile phosphate-buffered saline (PBS) and quantify the copy number of virus using Real-Time Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR) by a standard curve was created using a tenfold serial dilution of the challenge virus in order to link the qRT-PCR cycle titrated (CT) and virus titer (expressed in EID50/ml). Between positive and negative shedding, a CT value of 35 was employed as the cutoff. The data is expressed as a mean viral titer ± SD.

Hemagglutination inhibition (HI): 1.5 cm blood samples from the wing veins of each group of birds were taken at seven and fourteen days. The serum was separated, and the haemagglutinin inhibition (HI) test was carried out in accordance with standard methodology, as outlined in the OIE manual (OIE, 2014) using 4HAU of H9N2 antigen prepared from infected virus and 1% (v/v) of chicken red blood cells. The controls consisted of known positive and negative sera. The titers were converted to log2 and represented as the reciprocal of the positive samples' serum dilution. The data is expressed as a mean HI titer ±SD.

Histopathological: Three chicks from both infected and non-infected groups were ethically sacrificed and subjected to necropsy. All tissues of the two groups trachea, lung, heart, liver, spleen, proventriculus, intestine, and pancreas were removed at the 7th dpi, preserved in 10% formalin, dehydrated in various alcohol grades, and embedded according to Bancroft *et al*. (2013), in paraffin, sectioned at 4 u thicknesses, and stained with H&E stain. The histopathological lesions were scored for both groups as previously described (Rohaim *et al*., 2021). Briefly, each aforementioned lesion as mentioned in Table 4, was

evaluated in each bird at the 7th dpi based following scoring system: (–) the lesion was absent, (+) the lesion was mild, (++) the lesions were moderate and (+++); the lesion observed were severe. The severity of the histopathological lesions compared to those observed in the non-infected group.

RESULTS AND DISCUSSION

Clinical Signs

The chicken showed a mortality rate of 15-20% with mild respiratory symptoms. The samples also showed ruffled feathers, depression, coughing, sneezing, nasal and ocular discharge and diarrhea in some cases. The postmortem lesions included fibrinopurulent and catarrhal inflammations along with minor lung and tracheal congestion. Also enteritis, pancreatitis and congestion of spleen and liver, were observed.

H9N2 Virus Detection and Isolation

18 out of 40 tested samples (7/10 El-Dakahlia, 4/10 El-Sharqia, 3/5 Alexandria, 2/5 Al-Qalyubia, and 2/10El-Gharbia) were positive for H9N2 virus in vaccinated and non vaccinated farms by real-time RT-PCR(Table 1). The positive samples isolated in ECE with HA titer ranging from 7-8 log2 HAU and confirmed positive by real-time RT-PCR.

Phylogenetic Analysis of H9N2 Virus

For sequencing, we have chosen nine isolates to represent different governorates and submit them into NCBI under accession number (Table 2). Using the phylogenetic study of LPAIV's (H9N2) HA gene, the Egyptian viruses in this investigation, belonged to the G1/97-B like Eurasian sub-lineages (A/quail/Hong Kong/G1/97-like) with 89.6-90.3% A.A. identity percent and closely with Israeli strains (A/Chicken/Israel/5-2/2013) with A.A. identity percent 93.8-94.5%. The Egyptian strains were clustered into two groups (Egy-1 (2011-like) and Egy-2 (pigeon-like). Most prevalent strains are found in the Egy-2 from 2015 till now. The strains in this study were clustered into EGY-2 with A.A. identity percent 97-97.7% with A-pigeon-Egypt-D14794-2017.The Egy-2 was divided into two subgroups (Egy-2a, Egy-2b). The subgroup Egy-2a contains strains from 2018-2019. Subgroup Egy-2b containing strains from 2021-2022.The strains in this study cluster into EGY-2b with A.A. identity percent 99.1-100% with A-chicken-Egypt-DT20674OP-2022 (Figure 1 and 2).

Mutation Analysis

The mutation analysis of strains in this study comparing with A/quail/Hong Kong/G1/97, reveals that A126S, I134M, T145S, S158N, N179T, N218D, V224L as Egyptian strains and, V429I and N41G as strains belong to EGY-2 (pigeon-like) and T313N, T413N, I422V as strains belong to subgroup Egy2b isolated 2021-2022 and they had specific A.A. changes in L87I in A-chicken- Egypt-RN1-2022 and A-chicken- Egypt-RN9-2022 and D405N in A-chicken-Egypt-RN5-2022, A-chicken- Egypt-RN6-2022 and A-chicken-Egypt-RN9-2022.

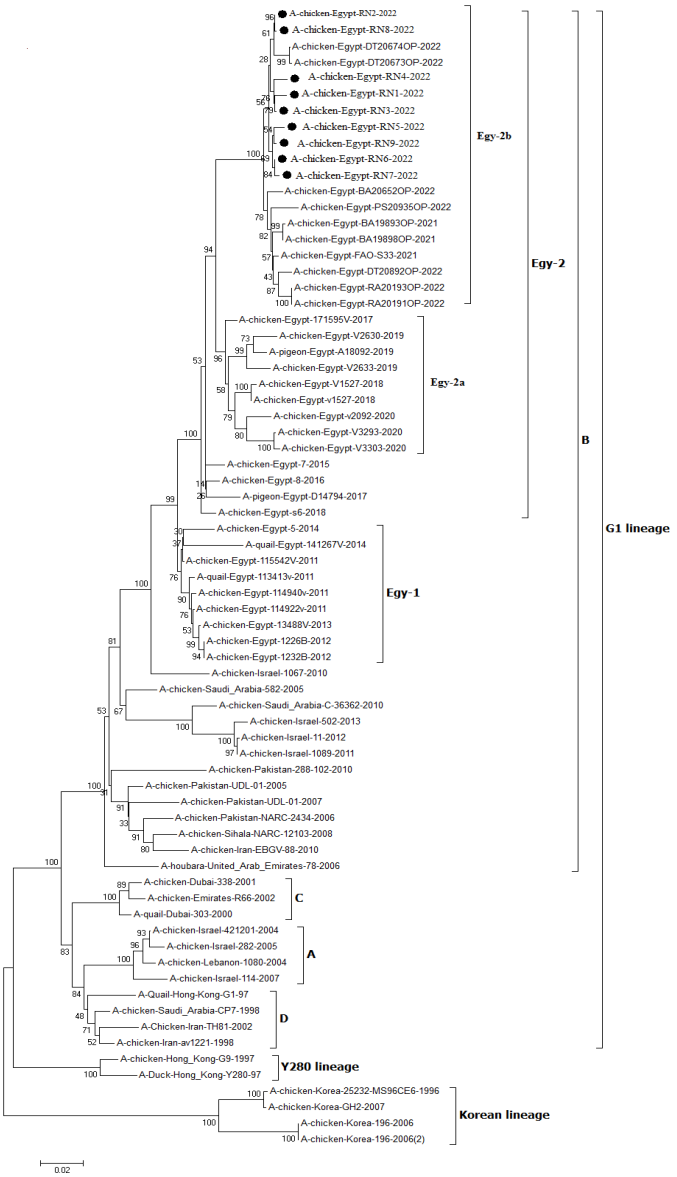

Figure 1: The H9N2 virus phylogenetic tree. The H9N2 viruses included in this study are marked by a black dot.

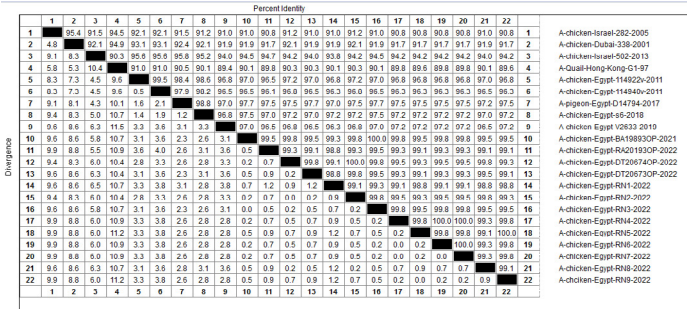

Figure 2: The amino acid identities of the sequenced virus were compared with strains from Hong Kong, Egypt, Israel, Saudi Arabia Dubai and Iran.

The cleavage site of our strains was PARSSRGLF. It indicates low pathogenic as other Egyptian strains. The RBS preferred to human-like α 2,6 sialic acid was detected in all strains in 191H, 232N, 234L, 235I, and 236G except A-chicken- Egypt-RN1-2022 had 234Q.

All strains had a mutation in the antigenic site at S166N in the antigenic site I and G153D in antigenic site II as other Egyptian strains with specific mutations in N201D and L234Q mutations in antigenic site II found only in A-chicken- Egypt-RN1-2022 and A-chicken-Egypt-RN2-2022 (Table 3). Also, our strains had mutations in other different antigenic sites at T121I as other Egyptian strains, D216S, M58K, I75V and V212I as EGY-2 (pigeon-like) strains and S183N found only in strains related to EGY-2b. The strains in this study had 7 glycosylation sites at 29 (NSTE), 82 (NPSC), 105 (NGTC), 141 (NVTY), 298(NSTL), 305(NISK), and 492 (NGTY).

Table 3: Antigenic sites of HA gene of H9N2 virus in this study comparing with A/quail/HongKong/G1/97 reference strains.

	Antigenic site						Overlapping site		
	Antigenic site I			Antigenic site II					
	143	166	170	153	201	234	141	197	206
A/quail/Hong Kong/G1/97	T	S	P	G	N	L	N	T	N
A-chicken-Egypt-RN1-2022	T	N	P	D	D	Q	N	T	T
A-chicken-Egypt-RN2-2022	T	N	P	D	D	Q	N	T	T
A-chicken-Egypt-RN3-2022	T	N	P	D	N	L	N	T	T
A-chicken-Egypt-RN4-2022	T	N	P	D	N	L	N	T	T
A-chicken-Egypt-RN5-2022	T	N	P	D	N	L	N	T	T
A-chicken-Egypt-RN6-2022	T	N	P	D	N	L	N	T	T
A-chicken-Egypt-RN7-2022	T	N	P	D	N	L	N	T	T
A-chicken-Egypt-RN8-2022	T	N	P	D	N	L	N	T	T
A-chicken-Egypt-RN9-2022	T	N	p	D	N	L		T	T

A 3D model of the HA gene represents the mutation detected in antigenic sites and RBS of strains in this study (Figure 3).

Virus Pathogenicity

9–11 day SPF chicks were used to titrate the A-chicken-Egypt-RN1-2022. It was verified as being H9 positive and infectious bronchitis virus, AIV H5N1 virus, and Newcastle disease virus were negative by real-time RT-PCR. It had a $10^{8.5}$ EID_{50} titration and then adjusted to 10^{6} EID_{50}.

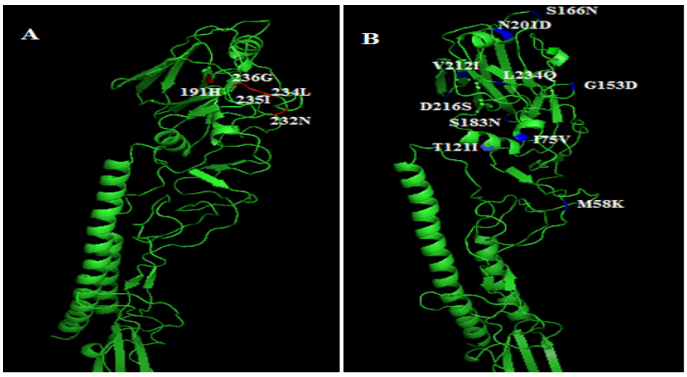

Figure 3: 3D structure of HA protein of H9N2 virus A:RBSa propensity for binding to human-like α2,6 sialic observed in H9N2 virus in this study indicated as Red colour. B: Antigenic site mutation observed in all H9N2 viruses in this study (all antigenic mutations found in all strains except L234Q and N201D found only in A-chicken- Egypt-RN1-2022 and A-chicken- Egypt-RN2-2022) indicated as blue colour.

Clinical Signs and Gross Lesion

Following infection, about 85% of birds suffered mild depression and anorexia, ruffled feathers, cough, mild sneezing and gasping, and tracheal rales in 2 dpi to 14 dpi of the H9N2 virus and reaching to maximum at 7 dpi. Diarrhea began from the 5th dpi till the end of the experiment reaching to maximum of 7 dpi in about 30% of chicken. There is no mortality was detected. The post-mortem lesion showed tracheal mucosa swollen and congested accompanied by sticked tracheal exudates (Figure 4A). Air sacculities and bronchopneumonia was seen. Other lesions were found, such as congestion of visceral organs, mild to moderate enteritis (Figure 4B), and mild pancreatitis (Figure 4C) were noticed. No lesions were observed in the bursa of Fabricius and thymus whereas the spleen was usually congested and atrophied. Conversely, there were no obvious symptoms or gross lesions in the negative control group.

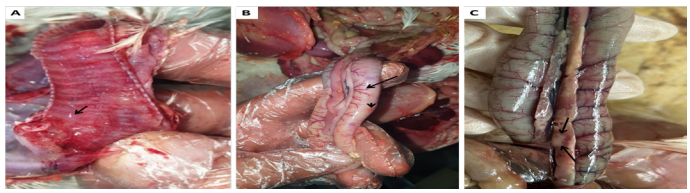

Figure 4: gross lesion of SPF chicks after infection of H9N2 virus. **A:** Trachea is swollen with congested tracheal mucosa and mild sticked exudat. **B:** Severe hyperemia of mesenteric blood vessels with presence of mild scattered serrosal ulcers. **4C:** Pancreas showing pale scattered areas of the surface of pancreas.

Virus Shedding

The mean virus shedding of infected chicken was 2±0.18, 2.5±0.21, 3±0.11 and 2.3±0.08 log10 EID_{50}/ml at 2, 4, 7 dpi in 100% of infected chicken and 10 dpi in 60% of infected chicken respectively and then disappears at 14 dpi. There is no viral shedding in negative control.

Hemagglutination Inhibition (HI)

The sera of birds from negative control (G1) were negative in the hemagglutination inhibition test, while those of the infected bird had mean HI titer (6±0.52 and 6.8±0.61 log2) at 7 and 14dpi respectively.

Histopathological Examination

The trachea of the infected group characterized by severe tracheitis with luminal exudate consists of mucus, erythrocytes, and inflammatory cells, besides desquamated sheets (Figure 5A) and thickness in mucosa with edema, hyperemic blood vessels, and leukocytes mainly heterophils and lymphocytes infiltration with desquamated lining epithelia (Figure 5B). The bronchi of lung showed moderate perivascular edema, partial destruction of their lining epithelia and disseminated hemorrhage with leukocytes infiltration (Figure 5C, D and E). Some air vesicles contained extravasated erythrocytes or serofibrinous exudate. Air sacs showed focal edema and inflammatory cells mainly heterophils and lymphocyte infiltration (Figure 2F).

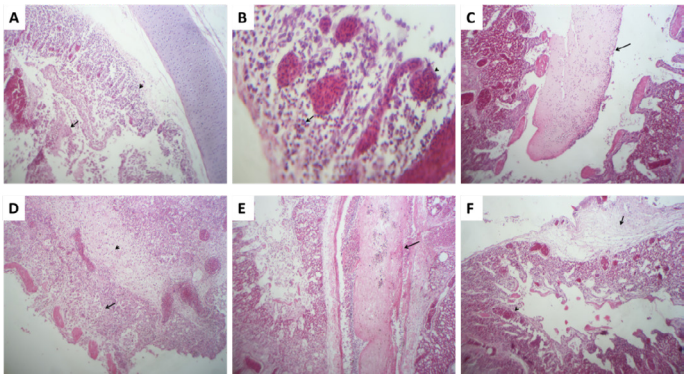

Figure 5: Histopathological lesion of trachea and lung of SPF chicks after infection of H9N2 virus; **A:** Tracheitis represented luminal exudates (arrow) and mucosal leukocytic infiltrates (arrow head); **B:** Trachea containheterophilic infiltrates (arrow) and hyperemic mucosal blood vessels (arrow head); **C:** the lung had Mucus casts (arrow) within the lumen of bronchi; **D:** bronchitis represented by leukocytic infiltration (arrow) and peribronchial inflammatory edema (arrow head); **E:** Thrombus within pulmonary blood vessels (arrow); **F:** thickened air sacs characterized by edema and little leukocytic infiltrates (arrow) beside disseminated hemorrhage in lungs (arrow head).

The myocardial muscles of the heart showed moderate intermuscular edema, heterophilic aggregates, and partial hyalinization besides mild pericarditis (Figure 6A). The hepatic parenchyma of the liver suffered from moderate microsteatosis of hepatic cells, dilated sinusoids with portal leukocytic infiltrates mainly heterophils and lymphocytes, beside congested blood vessels (Figure 6B). The spleen, showed severe lymphoid depletion with or without extravasated erythrocytes (Figure 6C). The intestine showed moderate enteritis represented by the destruction of villous mucosa with leukocytic infiltrates in addition to luminal exudate from mucus epithelia, epithelial sheets and little inflammatory cells (Figure 6D). Proventriculus shown mild changes represented by luminal mucus and a few mucosal inflammatory cells infiltrates (Figure 6E), and Pancreas shown moderate pancreatitis represented by focal destruction of the exocrine pancreas and replaced by inflammatory cells mainly lymphocytes and edema with degenerated and necrotic pancreatic acini (Figure 6F).

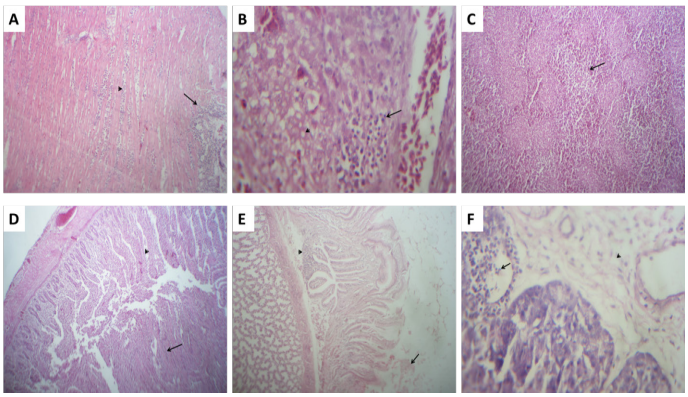

Figure 6: Histopathological lesion of different organs of SPF chicks after infection of H9N2 virus; **A:** the heart shown focal pericarditis (arrow) and intramuscular heterophilic aggregates (arrow head); **B:** Liver showed portal lymphocytic infiltrates (arrow) and microsteatosis of hepatic cells (arrow head); **C:** Spleen shown lymphoid depletion of white pulps (arrow); **D:** Intestine shown enteritis characterized by luminal exudates (arrow) and mucosal inflammatory cells (arrow head); **E:** Proventriculus had mucus in lumen (arrow) and a few mucosal leukocytes (arrow head); **F:** Pancreas had focal inflammatory cells replacing pancreatic acini (arrow) and interstitial edema (arrow head).

The histopathological lesion score of different organs in the infected group were severe in the trachea and spleen, followed by the lung, intestine, liver, heart, pancreas, and proventriculus but there is no lesion in the examined organs of the noninfected group (Table 4).

The H9N2 subtype of AIVs is one of the most common LPAI viruses in domestic chickens. It spreads quickly and causes mild to high mortality as well as respiratory symptoms that cause significant economic loss, particularly when combined with the co-occurrence of another respiratory pathogen (Lee and Song, 2013). The H9N2 virus was

initially found in Egypt in 2011, Since then, it has dispersed widely since it coexists with the endemic clade 2.2.1 H5N1 viruses (El Zoghby *et al*., 2012, Kayali *et al*., 2014) and the virus affected vaccinated and non vaccinated flocks with continues evolution from 2011 till now (El-Zoghby *et al*., 2012; Adel *et al*., 2022). Due to its detrimental effects on the poultry sector as well as the pattern of its genetic alterations, it has grown to be one of the most significant illnesses in Egypt. Therefore, there is a strong need to regularly monitor the circulating strain. In this study, we monitor the genetic evolution of the full HA gene to study the phylogenetic analysis, genetic marker related to virulence, antigenic site and preference for mammalian host. Also, studying the pathogenicity of the circulated strains in Lower Egypt during 2022.

Table 4: Histological lesion score of different organs in infected and non-infected groups at 7 dpi with LPVI (H9N2) virus.

Organ	Lesion	Intensity in infected group (G1)	Non-infected Group (G2)
Trachea	Tracheal exudates	+++	-
	Mucosal leukocytic infiltrates	+++	-
	Mucosal hyperemic blood vessels	+++	-
Lung	Bronchial casts	+++	-
	Bronchial mucosal leukocytic infiltrates	++	-
	Thrombosis of blood vessels	++	-
	Air sacculitis	++	-
Heart	Pericarditis	+	-
	Heterophilic myocarditis	++	-
Liver	Microsteatosis	++	-
	portal heterophilic infiltrates	++	-
Spleen	Lymphoid depletion	+++	-
Intestine	Luminal catarrhal exudates	++	-
	leukocytic infiltrates in mucosa	+	-
	Destructed villous enterocytes	++	-
Proventriculus	Mucosal infiltrates	+	-
Pancreas	Necrotic pancreatitis	++	-
	leukocytic infiltrates	++	-

(–) lesion was absent; (+) the lesion was mild; (++) the lesions were moderate and (+++); the lesion observed were severe.

In this study, using real-time PCR, LPAI H9 viruses were found in 45% (18/40) of the 7/10 El-Dakahlia, 4/10 El-Sharqia, 3/5 Alexandria, 2/5 Al-Qalyubia and 2/10 El-Gharbia broiler farms. It was recorded in vaccinated and non-vaccinated farms as previously recorded (Abdel-Moneim *et al*., 2012; Yehia *et al*., 2021; El Sayes *et al*., 2022; Bedair *et al*., 2024). The majority of the clinical symptoms displayed by AIV H9-infected chicken were mild respiratory, comprising cough, nasal and ocular discharge, sneezing, respiratory noises, and ruffled feathers and diarrhea in some cases with 15-20% mortality rate as previously described (Capua and Terregino, 2009, Abdel-Moneim *et al*., 2012, Gado *et al*., 2017, Samir *et al*., 2019, Abdel-Latif *et al*., 2020; Yehia *et al*., 2021), Upon necropsy, the post-mortem finding showed fibrinopurulent and catarrhal inflammations along with lung and tracheal congestion. Also, enteritis and congestion of liver and spleen as similar findings were reported (Samir *et al*., 2019; Abdel-Latif *et al*., 2020; Yehia *et al*., 2021; Mostafa *et al*., 2022).

Following its initial introduction to domestic poultry in Egypt in 2011, the AI H9N2 virus spread throughout Egypt, eventually becoming endemic and the HA gene was categorized in EGY-1 with minor subgroups (El-Zoghby *et al*., 2012; Adel *et al*., 2022). In 2014, the reasserted H9N2 virus were detected in pigeons in Egypt that had five genes (PB2, PB1, PA, NP, and NS) from Eurasian AIVs in wild birds and three genes (HA, NA, and M) from Egyptian H9N2 viruses, with HA gene being clustered to EGY-2 (Kandeil *et al*., 2017). In Late 2014, there was a spontaneous reassortment with H9N2 virus in Egypt, sharing just PB2, PB1, PA, and NS with HA gene being clustered to EGY-2 (Hassan *et al*., 2020; Adel *et al*., 2022; El Sayes *et al*., 2022; Bedair *et al*., 2024).

The phylogenetic analysis of HA gene in this study belong to G1/97- B like Eurasian sub-lineages (A/quail/Hong Kong/G1/97-like) with 89.6-90.3% tightly with Israeli strains (A/Chicken/Israel/5-2/2013) with identity percent 93.8-94.5% and cluster as EGY-2 group (Pigeon like) with 97-97.7% with A-pigeon-Egypt-D14794-2017 as previously described (Adel *et al*., 2022; El Sayes *et al*., 2022; Bedair *et al*., 2024). The EgY-2 group was divided into two subgroups (Egy-2a, Egy2-b) due to the acquiring of some specific mutations. Egy-2a contains strains from 2017 to 2020 similar to the finding in (Adel *et al*., 2022), and strains in this study grouped with 2021 and 2022 strains in the Egy-2b subgroup.

According to a genetic study, the HA cleavage motif contained RSSR*GLF as other Egyptian strains (El Sayes *et al*., 2022; Bedair *et al*., 2024), which indicates that H9N2 viruses that have been found in chickens in the Middle East and Asia are low pathogenic (Aamir *et al*., 2007; Golender *et al*., 2008; Tosh *et al*., 2008).

By genetic analysis strains in this study had specific mutation as other Egyptian strains Egy-2 group (Mohamed *et al*., 2021; Mostafa *et al*., 2022; El says *et al*., 2022) with

specific mutations (T313N, T413N, I422V and S183N) specify to group Egy-2b in 2021 and 2022 as previously described (Mostafa *et al*., 2022). In addition to specific characteristic mutations in A-chicken- Egypt-RN1-2022 and A-chicken- Egypt-RN9-2022 in L87I and A-chicken-Egypt-RN5-2022, A-chicken-Egypt-RN6-2022, A-chicken- Egypt-RN9-2022 in D405N. We need more investigation to determine how these mutations affect on the virus. According to LPAI H9N2 virus antigenic mapping (Kaverin *et al*., 2007; Smith *et al*., 2004; Okamatsu *et al*., 2008), The strains in this study had multiple alterations in the antigenic sites on the HA molecule as previously detected in Egyptian strains (Adel et al., 2022; Mohamed *et al*., 2021; El says *et al*., 2022; Mostafa *et al*., 2022). In addition, novel antigenic changes in the N201D and L234Q in antigenic site II in A-chicken-Egypt-RN1-2022 and A-chicken-- Egypt-RN2-2022 that may be due to vaccination pressures that lead to changes in the antigenicity of the virus and vaccination failure. We need more research to assess the effectiveness of vaccines and this highlights the necessity of regularly updating commercially available vaccines to match the strains that are currently in circulation.

Worldwide, AI H9N2 viruses sporadically spread to humans and pigs (Carnaccini and Perez, 2020). The first human instance of infection of the H9N2 virus was documented in China in 1998 (Peiris *et al*., 1999). The AI H9N2 virus has infected 95 people in laboratories; these cases have been confirmed in humans and mostly occurred in China, followed by Egypt where verified human infection in three people in the laboratory (Peacock *et al*., 2019). The majority of the strains in our study possessed 191H, 232N, 234L, 235I, and 236G in their RBS, which enhances their binding affinity to the human α 2, 6 SA receptor as recorded in (El says *et al*., 2022; Bedair *et al*., 2024).

A genetic study of the HA sequence revealed seven probable N-linked glycosylation sites in the Egyptian H9N2 strain: positions 29 (NSTE), 82 (NPSC), 105 (NGTC), 141 (NVTY), 298 (NSTL), 305 (NISK), and 492 (NGTY). These areas are critical for infectivity, pH stability, protein folding, and host immune responses. The position 82 N-linked glycosylation site identified in our strains was characteristic of Egyptian strains in subgroup EGY-2b isolated in 2021-2022 (Bedair *et al*., 2024). The changes in the glycosylation pattern can affect viral pathogenicity (Kaverin *et al*., 2007; Iqbal *et al*., 2009; El says *et al*., 2022).

By experimental infection of the recently circulated strains. In this study, the virus had the ability to affect the respiratory and digestive systems (Kye, *et al*., 2021; Abdel Hamid *et al*, 2016; Subtain *et al*., 2011) with seroconverted at 7 and 14dpi that indicate the presence of the infection as previously described by (Yehia *et al* 2024; Song *et al*., 2019; Abdel Hamid et al., 2016; kye *et al*., 2021). The chicken had mild signs mainly respiratory signs as coughing, and sneezing in 85% of chickens from 2 days till the end of the experiment and reached maximum in 7day with mild stick exudate in the trachea with occasional occlusion of airways resulting in abnormalities in respiration and bronchopneumonia without mortality similar to finding (Mohamed *et al*., 2021; Abdel Hamid *et al*., 2016; Yehia *et al*., 2024; Bóna *et al*., 2023; Slemons *et al*., 1990; Slemons *et al*.,1991) and some birds had diarrhea with enteritis associated with congestion of visceral organs accepted with (Abdel Hamid *et al*., 2016; Mostafa *et al*., 2022; subtain *et al*., 2022; Kim *et al*., 2006) contrary to others the H9N2 virus was infected chicken without any clinical signs and mortality and this increase in case of co-infection only (Nili and Asasi, 2002; Kishida *et al*., 2004; Adel *et al*., 2022) or other infect the respiratory tract only (Hassan *et al*., 2017) it may be due to presence of trypsin-like proteases. That facilitate the virus's replication in the respiratory and intestinal epithelium (Shaib *et al*., 2011). Also, the chicken had an atrophied and congested spleen that decreased the immunity of the chicken and increased the incidence of co-infection which led to an increase in the mortality rate in chicken (Kim *et al*., 2006; Bonfante *et al*., 2017). It has been accepted by many other published papers (Adel *et al*., 2022; Mostafa *et al*., 2022; Song *et al*., 2019; Bano *et al*., 2003).

Curiously, it was observed that the degree of clinical signs that the infected birds displayed Lesions in the upper respiratory tract, mainly in the nasal cavity (rhinitis) and tracheal sections (tracheitis) began from 2nd day till the end of the experiment and reached maximum in 7day. It showed a substantial correlation with the tracheal shedding of the H9N2 virus (Morales *et al*., 2009). The tracheal viral shedding begins on 2nd day after infection and reaches a maximum at 7 days in 100% of chickens and continues till 10-day post infection accepted with (Song *et al*., 2019; Abdel Hamid et al., 2016) this contrary recorded by (El says *et al*., 2022; Mohamed *et al*., 2021; Adel *et al*., 2022; kye *et al*., 2021) that H9N2 viruses isolated at 2019 were shedding begin at 2, 3 days and then decrease at 7-day post-infection and then disappear at 10 days. This indicates the prolonged viral shedding.

Histopathological examination revealed mainly pathological alteration in respiratory and gastrointestinal tissue (tissue expressing trypsin) with an increase in severity in the trachea then lung and intestine as observed by (Swayne, 2007; Adel *et al*., 2022). It appeared as severe trachitis, with hemorrhage and inflammatory cells mainly lymphocytic infiltration, and moderate bronchitis with edema, hemorrhage, and leukocytic infiltration. Additionally, the pathological lesions in the intestine associated with H9N2 infection were moderate enteritis with leukocytic infiltrates in the mucosa accompanied by destructed villous enterocytes due to replication of the virus and induce inflammatory

cytokine (IL6 and IFN-ɣ) that induce inflammation and tissue damage as previously mentioned by (Kye, *et al*., 2021; Hassan *et al*., 2017; Subtain *et al*., 2011).

Furthermore, the H9N2 virus spreads systematically inducing damage in visceral organs resulting in moderate pancreatitis due to the damage of pancreatic acinar epithelium in addition to moderate hepatitis and mild pathological change in proventriculus. mild pericarditis with moderate intermuscular edema besides heterophilic aggregates appeared in the myocardial muscle of the heart indicating extrapulmonary pathogenesis of the H9N2 virus as recorded in (Kye *et al*., 2021; Abdel Hamid et al., 2016).

In our study, the spleen showed severe lymphoid depletion and necrosis or apoptosis of lymphocytes following H9N2 infection. This lesion may be attributed to the dissemination of H9N2-infected lymphocytes through the body via blood or lymph vessels (Kwon *et al*. 2008). This damage of lymphocytes may lead to immunosuppression and subsequent coinfection by other pathogens (Hadipour *et al*., 2011) which explains severe clinical signs and lesions related to H9N2. Our results were in agreement with previous studies by (Doustar *et al*. 2012; Mohamed *et al*. 2021; Adel *et al*., 2022).

CONCLUSIONS AND RECOMMENDATIONS

Despite their low pathogenicity, the LPAI H9 viruses pose a serious threat to broiler farms. The high occurrence percentage of AIVs of the H9 subtype in the El-Dakahlia,El-Sharqia, Alexandria, Al-Qalyubia, and El-Gharbia governorates with possible zoonotic dangers, H9 viruses are constantly evolving genetically. They acquired multiple mutations in the antigenic site that may be affect the effectiveness of vaccines. By studying the pathogenicity of the virus, the virus in this study affects both the respiratory and digestive tract. It had mainly mild respiratory signs and diarrhea in some birds without mortality with persistent high viral shedding from 2 dpi to 10 dpi. The histopathological changes aren't restricted to the respiratory and digestive but extended to the heart, liver, pancreas, and proventriculus. Also, the spleen showed severe lymphoid depletion causing immunosuppression. It is necessary to continuously molecular monitoring of the H9N2 virus all over Egypt and study the vaccine efficacy of commercially used vaccines.

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NOVELTY STATEMENT

H9 viruses are always changing genetically, as evidenced by the high prevalence of AIVs of the H9 subtype in five governorates in southern Egypt, which may pose a zoonotic risk. They developed several alterations in the antigenic sites, which could have an impact on vaccination efficacy. Through research on the pathogenicity of the virus, which in this study affects not only the digestive and respiratory tracts but also the heart, liver, pancreas, and spleen.

AUTHOR'S CONTRIBUTIONS

Nahed Yehia contributed in genetic evolution of avian influenza H9N2 and data analysis and write the manuscript. RaNia i. MohaMed contributed in samples collection and study the pathogencity of the virus with patholoogical changes.

Conflict of Interest

The authors have no conflict of interest to declare.

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