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The Occurrence of Low Pathogenic Avian Influenza H9 Viruses in Broiler Farms Within Ismailia Province, Egypt

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Abstract

The Egyptian broiler farms are easy prey for the low pathogenic avian influenza (LPAI) H9 viruses that cause great economic losses despite their weak nature. This study focused on the occurrence and molecular characterization of the LPAI H9 viruses circulating in commercial broiler farms in different villages of Ismailia Province during 2020-2021. Tracheal, cloacal swabs, and tissue pools were collected from 34 broiler farms suffering from respiratory signs. In this study, LPAI H9 was detected in 41.17% of farms (14/34) using real-time PCR. The positive samples were propagated in 11-day-old embryonated chicken eggs (ECEs) and three isolates were selected for partial hemagglutinin (HA) gene sequencing. Sequence analysis results showed an obvious genetic evolution in comparison with the original virus (A/quail /Egypt/113413v /2011) that shared 94-94.3% and 96.7-97.1% homology at the level of nucleotides and amino acids respectively. The identity percentage of the isolates with five selected commercial vaccine seed viruses revealed a higher identity with the local vaccines than the imported ones. The molecular analysis revealed 14-15 mutations in the amino acid residues with genetic stability in the main sites in comparison with the reference Egyptian strain. Phylogenetically, the H9 isolates were grouped in the G1 lineage similar to the Middle East circulating viruses with close phylogeny to Israeli viruses. The continuous genetic evolution of H9 viruses detected in this study necessitates regular virus monitoring for better control.

KEYWORDS
Broiler farms, Hemagglutinin gene, Ismailia, LPAI H9, PCR

INTRODUCTION

According to the genetic behaviors and disease severity in poultry, avian influenza viruses (AIVs) are classified into highly pathogenic and devastating avian influenza viruses that can cause 100% mortalities in various bird species and the milder low pathogenic avian influenza viruses (LPAI) that frequently goes unreported in domestic poultry (Cui *et al*., 2017). However, infection with the LPAI H9N2 viruses frequently results in immune suppression and co-infection with other pathogens, both of which can increase morbidity and mortality rates in chickens (Kim *et al*., 2006; Bonfante *et al*., 2017).

The LPAI H9N2 outbreaks were widespread in chickens all around the world, notably between 1994 and 1999 (Alexander, 2000; Matrosovich *et al*., 2001). In Egypt, the LPAI H9N2 of G1 lineage was reported for the first time in 2011, it was isolated from commercial bobwhite quail that suffered from neither clinical signs nor mortalities (El-Zoghby *et al*., 2012).

Since 2011, Egypt has been suffering from the endemicity of the LPAI of the H9 subtype. The genome of the LPAI H9N2 viruses is unstable and mutates continuously with H9N2 variants arising from the natural phenomenon of antigenic drift in the HA gene (Peacock *et al*., 2018). The H9N2 viruses are the main participants in the antigenic shift phenomena because they frequently donate their internal genes to other AIVs during the co-infection (Kandeil *et al*., 2017; Hagag *et al*., 2019; Peacock *et al*., 2019).

To achieve effective control of LPAI H9N2 in the field, vaccination must be a part of an overall integrated disease control program including ongoing national monitoring, biosecurity, and differentiating infection in vaccinated animals (DIVA) strategies (Lee and Song, 2013). There are several inactivated vaccines against the LPAI H9 viruses in the Egyptian field, however, there are many factors affecting their efficacy as the degree of HA homology between the vaccine seed virus and the challenge virus as well as the antigen content in the vaccine (Talat *et al*., 2020).

This study targeted molecular detection, virus isolation, and HA gene sequencing of the LPAI H9 viruses among broiler farms as this sector represents the largest poultry production sector. Furthermore, the dense broiler populations and the co-existence of other pathogens with weak biosecurity measures in most farms complicate the situation. Therefore, the surveillance of the LPAI H9 viruses in Ismailia broiler farms is essential for a better understanding of the ecology and occurrence of the virus for better prevention and control strategies.

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MATERIALS AND METHODS

Sampling and samples processing

One hundred fifty-two broiler chickens of different ages representing 34 flocks (3-5 birds/ flock) with a history of respiratory signs were collected from Ismailia province, Egypt during the period from 2020 to 2021 and submitted for clinical and post-mortem examination. Two hundred ninety-four tracheal and cloacal swabs, besides 11 tissue pools (trachea, lungs, liver, spleen, duodenum, pancreas, cecal tonsils, heart, and kidneys) were collected and stored at -20 ºC until used. Samples were prepared and processed according to Numan *et al.* (2008).

Sampling was conducted according to the ethical guidelines of Suez Canal University, Egypt.

Molecular detection of LPAI H9 using Real-time RT-PCR (rRT-PCR)

Viral RNA was extracted using Biospin Virus RNA Extraction Kit (BioFlux) Cat. No. BSC62M1 following the manufacturer's directions. Specific primers and probes (Shabat *et al*., 2010, Metabion) were used for H9 virus detection. A SensiFAST Probe No-ROX One-Step master mix Kit (Meridian Bioscience) was used, with a total volume of 20 µl containing 2x SensiFast probe one-step mix (10 µl), forward primer 50 Pmol (0.5 µl), reverse primer 50 Pmol (0.5 µl), probe (0.125 µl), reverse transcriptase (0.2 µl), RNase inhibitor (0.4 µl), PCR grade water (3.275 µl), and template RNA (5 µl). The rRT-PCR thermal conditions for the H9 gene were 50 °C for 30 min, then 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 sec, 54 °C for 30 sec and 72 °C for 10 sec. The MX3005P machine (Stratagene) was used for PCR reactions following the manufacturer's guide.

Virus isolation in embryonated chicken eggs (ECEs)

The H9-positive samples using rRT-PCR were selected for virus isolation in 11-day-old ECEs via the allantoic cavity route (Woolcock, 2008). The ECEs were obtained from native balady hens that had no history of avian influenza vaccination nor infection. Suspected samples were passaged blindly three times in ECEs. The collected allantoic fluids were tested for hemagglutination (HA) activity (Killian, 2008), and the LPAI H9 isolation was reconfirmed by rRT-PCR. Furthermore, the hemagglutination inhibition (HI) test was done for detection of Newcastle disease virus (NDV) in the HA-positive allantoic fluids using NDV antiserum for LaSota strain (GD lab.) Cat. No. VLDIA053 (Alexander, 1988).

Partial sequencing of hemagglutinin (HA) gene

The predicted RNA of the three selected H9 isolates was extracted using the same extraction kit. Two sets of H9-specific forward and reverse primers were designed in this study using Geneious software (Table 1) and used for HA gene amplification. Two-step RT-PCR took place using ABT H-minus cDNA synthesis kit (Applied Biotechnology) Cat. No. ABT009 was used for cDNA synthesis from the virus RNA template (first step) and WizPure PCR 2X Master Mix (wizbiosolutions) Cat. No. W1401 contains a loading dye and is used for PCR reactions (second step), the reaction mix volumes and thermal conditions are shown in Tables 2 and 3. The thermal cycler (Techne) was used.

The expected molecular weight of each HA gene segment was visualized via agar gel electrophoresis using 1% ethidium bromide and a gel documentation system. The amplified PCR products were shipped for purification and Sanger sequencing in SolGent Co., Ltd. (South Korea).

Sequence analysis and phylogeny of HA gene

Chromas software (version 2.6.6) was used for assembling and editing the sequences. A BLAST analysis was performed on each sequence to determine the related viruses. The reference sequences used in the analysis were downloaded from NCBI and GISAID databases. The H9 numbering of the mature peptide was used during this study.

Clustal W method for multiple sequence alignment, as well as nucleotide and amino acids identities determination, were

Table 1. The designed forward and reverse primers for H9 hemagglutinin gene amplification using RT-PCR.

Primer sequence	Product size	References
H9F1: TGCCAAAGAATTRCTCCACACAGAGC	900 bp	This study
H9R1: TCCAGCTATRGCTCCAAATAGCCCT		This study
H9F2: GTCAGACTGAAAAAGGTGGCCTAAA	688 bp	This study
H9R2: CCCCTCAGATTCCAGYTTAACTCCCT		This study

Table 2. The reaction mix volumes per reaction of H9 gene amplification using 2 step RT-PCR

	Reagent	Volume/ reaction
cDNA synthesis (first step) using ABT H-minus cDNA synthesis kit	5×First-strand Buffer	4µl
	H minus MMLV (RT Enzyme) (200 unit/ ul)	0.5µl
	dNTPs mixture (10mM)	2µl
	Random Hexamer Primer	1µl
	PCR grade Water	7.5µl
	RNA template	5µl
	Total volume	20µl
	Reagent	Volume/reaction
PCR (2nd step) using WizPure™ PCR 2X Master	Wizpure master	20 µl
	Forward primer	0.2 µl (20 Pmol)
	Reverse primer	0.2 µl (20 Pmol)
	PCR grade water	14.6 µl

Table 3. Thermal profile and cycling conditions of H9 gene amplification using two step RT-PCR reaction.

	Regents		Temperature	Time	Cycle
cDNA synthesis (first step) using ABT H-minus cDNA synthesis kit	PCR grade water Random Hexamer Primer RNA		65˚C	5 min	1
	5×First-strand Buffer dNTPs mixture (10mM) H minus MMLV (RT Enzyme) (200 unit/ ul)		42˚C	1 hr	1
	Reaction termination		75˚C	5 min	
PCR (2nd step) using WizPure™ PCR 2X Master	Stage		Temp	Time	Cycle
	Primary denaturation		95˚C	5 min	1
	Amplification	Second denaturation	95˚C	30 sec	35
		Annealing	55˚C	30 sec	
		Extension	72˚C	1 min	
		2nd Extension and elongation	72˚C	5 min	1

performed using BioEdit version 7.2 (Hall, 1999). The glycosylation sites were determined by NetNGlyc 1.0 server (Gupta *et al.*, 2004). The phylogenetic trees were constructed for isolates' HA nucleotide sequences by the Neighbor-joining method- Kimura 2-parameter model based on 500 bootstrap values using MEGA 11 software (Tamura *et al.*, 2021).

RESULTS

Clinical signs and post-mortem lesions

The examined flocks had signs of depression, reduced feed consumption, ruffled feathers, poor growth in some flocks, and white or greenish diarrhea. The main clinical signs were respiratory signs such as cough, sneezing, nasal and ocular discharge, conjunctivitis, respiratory noises, and facial edema with mortality rates ranging from 1.1% to 2.8%.

The post-mortem examination revealed sticky mucous on the oropharynx, sinusitis, hemorrhagic tracheitis, caseous plug at the tracheal bifurcation (Figure 1), lung congestion, airsaculitis with congested blood vessels, congested liver and spleen with pinpoint hemorrhages on the pancreas, fibrinous exudate on the visceral organs and mucoid enteritis. Thickening of the proventriculus and intestinal wall with atrophy of the pancreas and enlarged kidneys with nephritis were found in some cases.

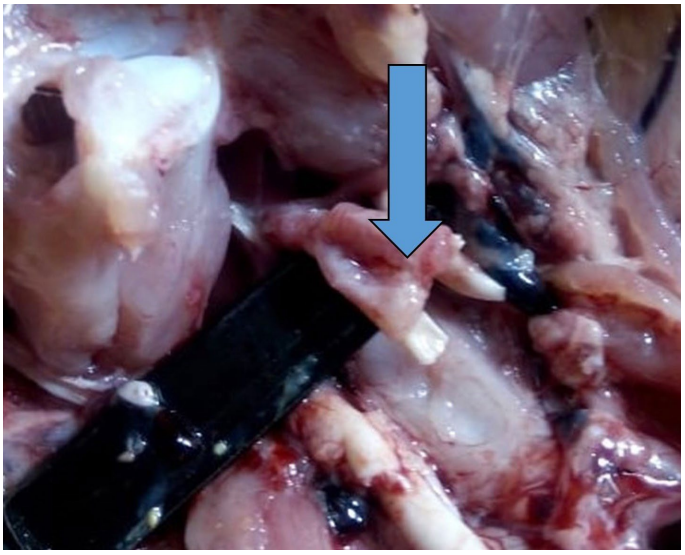

Fig. 1. Broiler chicks aged 35 days showing caseous plug at tracheal bifurcation.

Real-time PCR results

The examined field samples representing 34 broiler flocks revealed 14 H9- positive flocks with an occurrence of 41.17% (14/34). The cycle threshold (CT) value ranged from 16 to 30.

Results of virus isolation, HA, and HI tests

Eight hemagglutinating agents were isolated in ECEs out of the 14 H9 positive samples. Four of them were confirmed by rRT-PCR as H9 isolates, while the four remaining isolates were Newcastle viruses using the HI test. Depending on the rRT-PCR, virus isolation, HA, and HI results, mixed infection of the LPAI H9 and ND in four broiler farms was confirmed (4/34, 11.76%).

The AIV H9 isolation revealed embryonic deaths within 24 to 120 h. with hemorrhagic lesions and congestion of the head and legs, however, AIV H9 isolation in some cases caused no deaths nor lesions. The AIV H9 isolates had HA titers ranging from 2 $\log^4$ to 2 $\log^{11}$. It was noticed that H9 isolates of HA titer of 2 $\log^5$ or more were accompanied by embryonic deaths, unlike the lower titer.

Partial sequencing of HA gene of H9 AIV isolates

H9 gene amplification using RT-PCR

Three H9 isolates were selected for partial HA gene amplification using our designed primers. The electrophoresis run applied to the PCR products showed the presence of specific DNA bands detectable by ethidium bromide in agarose gel at the expected molecular weight (900 bp and 688 bp/each gene) (Figure 2).

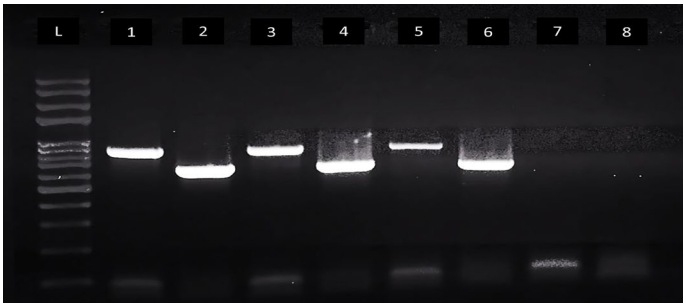

Fig. 2. Agarose gel electrophoresis of RT-PCR products of HA gene of the H9 isolates showing specific sharp bands at 900 bp and 688 bp for each isolate. Lane L: DM11-100bp Plus DNA Ladder Marker (100, 200, 300,400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 3000, 5000 bp). Lanes 1, 2 for H9 isolate 92, lanes 3, 4 for isolate 88, lanes 5, 6 for isolate 82 and lanes 7, 8 for negative control.

The assembled nucleotide sequences of the 3 H9 isolates: A/ Chicken /Egypt/ 92 Ismailia/2021(92), A/Chicken/Egypt/88Ismailia/2021(88), and A/ Chicken /Egypt /82 Ismailia/ 2021(82) were submitted to the Genbank with accession numbers OM912777, OM912778, and OM912779 respectively.

HA gene sequence analysis of the three isolates

BLAST analysis of the studied H9 isolates showed high homology with the Egyptian viruses since 2011. The three isolates shared 98.7-99.1% and 99.5-100% homology at the level of nucleotides and deduced amino acids respectively. In comparison with the original Egyptian virus (A/quail /Egypt/ 113413v /2011), they shared 94-94.3% and 96.7-97.1% identity at the level of nucleotides and amino acids respectively. While concerning Egyptian viruses circulating from 2011 until 2021, the study isolates shared identities of 94-99.2% and 95.6-100% at the level of nucleotides and amino acids respectively.

The studied H9 isolates shared a homology of 97.5-98.4%, 91.7-92.3%, 92-92.3%, 91.9-92.5% and 95.1-95.8% with five H9 commercially available vaccine seeds: A/Chicken/Egypt/ ME543V/2016, A/Chicken/Iran/AV1221/1998, A/Chicken /UAE/ AG537/ 1999, A/ Chicken /Saudi Arabia/CP7/98, and A/Chicken/ Egypt/ S10490 /2015 respectively (Table 4). The molecular analysis of isolates (sample code numbers) 92, 88, and 82 revealed 14, 14, and 15 amino acid mutations respectively (M40K, I57V, M69L, D165N, A191T, V194I, N198S, K204T D295N, S339A, T395N, I404V, V411I, A445, K483M, and E489G) in comparison with the original Egyptian virus.

The HA cleavage motif sequence of the H9 isolates was 315PARSSRGLF323. Regarding the glycosylation sites in the sequenced portion of the HA gene: 87NGTC90, 123NVTY126, 280NSTL283, 287NISK290, and 474NGTY477 were recorded. The potential seven receptor binding sites (RBS) in the HA1 were: Y91, W143, T145, H173, A180, L184, and Y185, while the left edge and right edge of the receptor-binding pocket were (214NGLIGR219) and (128GTSKS132) respectively. We focused on two overlapped antigenic sites: Epitope I (129, 147, and 152), overlapping area (127, 179, and 188), and Epitope II (135, 183, and 216) in addition to 12 antigenic sites at positions: 146, 149, 150, 178, 182, 189, 163, 262, 264, 267, 278, and 316. Our H9 isolates showed no mutations in these sites in comparison to the original virus.

Phylogenetic analysis of the HA gene

The phylogenetic analysis of our LPAI H9 isolates' HA gene revealed that they were grouped in the G1 lineage, which is similar to the viruses circulating in the Middle East with close phylogeny to 2020 Egyptian and Israeli viruses (Figure 3).

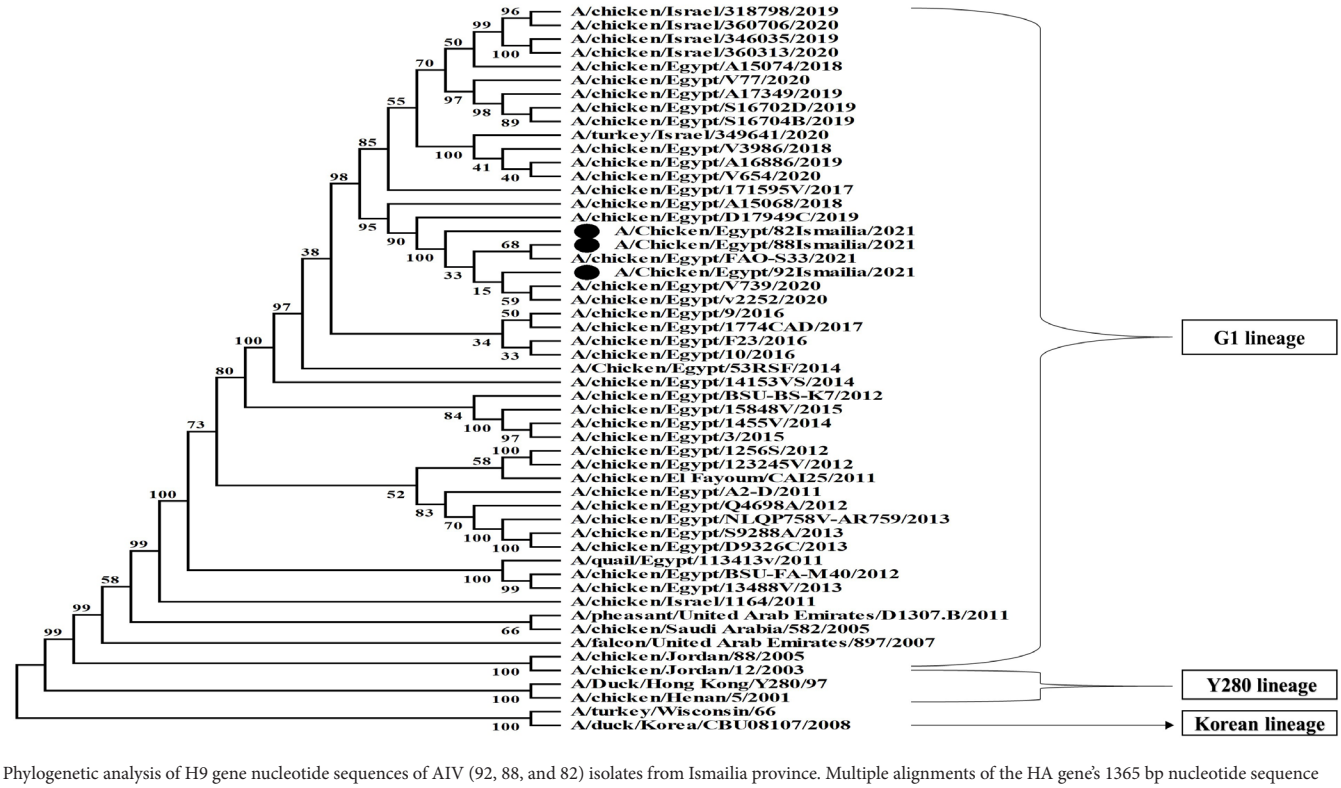

Fig. 3. Phylogenetic analysis of H9 gene nucleotide sequences of AIV (92, 88, and 82) isolates from Ismailia province. Multiple alignments of the HA gene's 1365 bp nucleotide sequence were used to construct the tree in MEGA 11 using the neighbor-joining method -Kimura 2-parameter model based on 500 bootstrap values. Isolates in this study are marked with solid circles.

Table 4. The identity percentage of the study H9 isolates' HA peptide with the 5 commercially available H9 vaccines used in the analysis.

Vaccine seed	Company	Accession number	Identity percentage		
			92%	88%	82%
A/Chicken/Egypt/ME543V/2016	MEVAC	MF434468.1	98.4	98.2	97.5
A/Chicken/Iran/AV1221/1998	Boehringer Ingelheim	KF800947.1	92.3	92.1	91.7
A/Chicken/UAE/AG537/1999	Intervet (MSD)	AJ781824.1	92.3	92.3	92
A/Chicken/Saudi Arabia/CP7/98	-	CY081264	92.5	92.3	91.9
A/Chicken/Egypt/S10490/2015	Vaccine Valley	KT216664.1	95.8	95.6	95.1

N.B: (-) indicates unavailable data

DISCUSSION

Among AIVs subtypes, the H9N2 subtype spreads rapidly and becomes one of the main dominant LPAI viruses in domestic poultry resulting in a significant economic loss due to respiratory signs and moderate to high mortalities especially when combined with concurrent infection with other respiratory pathogens (Lee and Song, 2013).

Clinically, AIV H9 infected broiler flocks showed mainly respiratory signs with general signs of illness (cough, sneezing, nasal and ocular discharge, conjunctivitis, respiratory noises, and facial edema), similar clinical signs were reported (Capua and Terregino, 2009; Abdel-Moneim *et al*., 2012; Gado *et al*., 2017; Samir *et al*., 2019). On necropsy, the post-mortem examination revealed mainly severe tracheitis, caseous plug at the tracheal bifurcation, pneumonia, and nephritis, similar findings were reported (Swayne and Pantin-Jackwood, 2008; Gado *et al*., 2017; Elfeil *et al*., 2018). In some H9 infected flocks, there was atrophy of the pancreas accompanied by poor growth. This may be attributed to the effect of AIV H9 on the pancreatic tissues resulting in flaws in its enzymatic functions (Subtain *et al*., 2011).

In this study, LPAI H9 viruses were detected in 41.17% (14/34) of Ismailia broiler farms using real-time PCR, which is higher than detection rates in Alexandria, Gharbia, Kafer El-Sheikh, and Sharkia governorates but lower than Beheira governorate according to (Awad *et al*., 2013; Gado *et al*., 2017). This may be due to the lower density of poultry farms, lack of biosecurity, and improper vaccination programs in Ismailia province.

Concurrent infections of the LPAI H9 with ND in four broiler flocks (4/34, 11.76%) were recorded based on real-time PCR, HA, and HI tests. These findings were parallel to the immunosuppressive activity of H9 AIVs that cause epithelial damage to the respiratory tract and other lymphatic tissues which facilitate the entrance of NDV (Bonfante *et al*., 2017). Several studies based on AIV H9 experimental infection in broilers revealed several pathological lesions in the thymus, bursa of Fabricius, and cecal tonsils of the infected birds (Hadipour *et al*., 2011; Qiang and Youxiang, 2011; Abdel Hamid *et al*., 2016; Gado *et al*., 2017).

To amplify the HA gene of the detected H9 viruses via conventional PCR for gene sequencing, the virus titer must be increased through its propagations in ECEs. The H9 AIV isolation revealed embryonic deaths within 24-120 h., with hemorrhages concentrated on the head and legs, comparable findings were reported by Lee *et al*. (2000) and Moatasim *et al*. (2017). One of the main noticed features during H9 AIVs isolation was the ability of these viruses to cause embryonic deaths with obvious lesions despite their low pathogenic nature, although Haghighat-Jahromi *et al*. (2008) reported their inability. The explanation of this feature was titer dependent because H9 isolates with HA titer below 2 $\log^5$ did not able to kill embryos while the HA titer more than 2 $\log^5$ of the same H9 isolates, as well as other H9 isolates, were able to cause embryonic deaths.

The molecular characterization was carried out for the HA gene of H9 isolates focusing on the cleavage site, receptor binding sites (RBS), antibody binding epitopes, and the glycosylation sites in comparison to the original reference viruses as well as other Egyptian viruses. The H9 numbering of the mature peptide was used during this study.

Partial HA gene sequencing revealed that the obtained isolates shared 99.5-100% similarity at the level of deduced amino acids among each other. In comparison with A/quail/ Egypt/ 113413v/ 2011, they shared 96.7-97.1% similarity. Comparable findings were recorded by Gado *et al*. (2017) and Adel *et al*. (2021) whose H9 isolates shared 93.3-99.1% and 94.7-96.2% similarity with the original Egyptian strain respectively.

The cleavage site is an essential determinant of influenza viruses' pathogenicity. The study isolates had 315PARSSR/GLF323 cleavage motif sequence, revealing the dibasic amino acid R-S-S-R motif that confirmed the low pathogenic nature of AIV H9 as mentioned by Steinhauer (1999). However, this motif makes isolates from this study have the potential to be highly pathogenic by acquiring nucleotide substitutions forming multiple basic amino acids in the HA-connecting peptide as reported by Aamir *et al*. (2007) and Parvin *et al*. (2020).

The receptor binding sites (RBS) are essential for identifying the susceptible host range (Gambaryan *et al*., 2002). Liu *et al*. (2003) mentioned that there are seven amino acid residues in HA1 involved in the receptor-binding domain, and six of them are highly conserved according to (Huang *et al*., 2010). Isolates from the present study have the six conserved residues (Y91, W143, T145, H173, L184, and Y185), similar findings were recorded by Arafa *et al*. (2012) and Samir *et al*. (2019). The amino acid residue at position 216 has a critical role in H9 avidity, the study isolates have L216 instead of Q216 which means that they have avidity to mammalian cell receptors with possible human infection (Wan and Perez, 2007). Furthermore, the study H9 isolates had H173 and T179 residues that confirm their affinity to the mammalian sialic acid receptors with a replication ability in the respiratory epithelium of humans (Wan *et al*., 2008).

Previous investigations mapped the antigenic epitopes on the HA of H9N2 viruses, revealing two overlapping antigenic sites I, and II (Kaverin *et al*., 2004) and additional antigenic sites also predicted (Wu *et al*., 2010; Wan *et al*., 2014). There were no mutations tracked in our H9 isolates' antigenic epitopes, indicating their stability in Ismailia province at the level of specific antibodies neutralization.

The alterations in the glycosylation site pattern influence the host range as well as the virulence of influenza viruses (Kawaoka and Webster, 1988; Wang *et al*., 2010). The study H9 isolates have no changes in the glycosylation sites in comparison with A/ quail /Egypt /113413v /2011 and other Egyptian isolates since 2011.

Despite the genetic stability in the main sites of the study H9 isolates, an obvious antigenic drift was reported. There were several tracked mutations: M40K, I57V, M69L, D165N, A191T, V194I, N198S, K204T D295N, S339A, T395N, I404V, V411I, A445, K483M, and E489G that require further investigations to determine their role. There were five common mutations reported by Adel *et al*. (2021): M40K/R, I57V, V194I, N198S, and T395N.

The consensus is that the more antigenic relatedness between the vaccine and the field viruses, the higher the vaccination effectiveness. Five H9 vaccine seeds were used to identify their homology percentage with H9 isolates from this study, A/ Chicken/Egypt/ME543V/2016 seed showed the best identity percentage among its competitors by 97.5-98.4% followed by A/ Chicken /Egypt /S10490 /2015 that showed 95.1-95.8 homology %. These findings proved that vaccines prepared from recent local H9 isolates are better than the imported ones, similar findings were reported under experimental conditions (Ebrahim and Seioudy, 2020; Talat *et al*., 2020).

Phylogenetically, the obtained isolates are placed in the G1-lineage like the Middle East circulating viruses with close phylogeny to 2020 Egyptian and Israeli viruses. Comparable results were recorded (Gado *et al*., 2017; Adel *et al*., 2021).

CONCLUSION

The LPAI H9 viruses are real threats facing broiler farms despite their low pathogenic nature. Ismailia governorate is suffering from a high occurrence percentages of AIVs of the H9 subtype this may be due to a lack of biosecurity measures and/or futile vaccination programs. H9 viruses are in continuous genetic evolution with potential zoonotic risks. Continuous updates in vaccine seed virus isolated from recent and local isolates are required to avoid escape mutants formation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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