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Avian Influenza Virus Screening in Some Egyptian Provinces

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ABSTRACT

Avian influenza virus (AIV) is one of the most important viral diseases that widely affect the Egyptian commercial poultry industry, with varying mortality rates and clinical symptoms. AIV is a catastrophic disease of poultry belonging to the family *Orthomyxoviridae,* genus Influenza A virus. This study aims to identify, analyze, and resolve issues related to the epidemiology, and diagnosis of currently prevalent AIV subtypes in Egyptian field. Molecular evidence of AIV subtypes was investigated in domesticated commercial poultry in three Egyptian provinces (Ismailia, Sharqia, and Dakahlia) between 2022 and 2023. 50 tissue samples were examined by Real-time reverse transcription polymerase chain reaction (RT-qPCR) followed by isolation trial and serological identification through hemagglutination (HA) and hemagglutination inhibition test (HI) of the 9 positive samples (8 H9, and 1H5). The isolated samples were negative for AIV by HI test; this result may be due to several reasons as the presence of degraded nucleic acid, but not active virus or the samples collected during the convalescent stage. From this result, it can be concluded that the continuous circulating of H9 and H5 viruses detected in this study necessitates regular virus monitoring for better control. The objective of this study was molecular investigation of AIV in domestic in Egypt, focusing on H5, H9 and H7 subtypes.

INTRODUCTION

Influenza, known also as "Flu", is an acute highly contagious respiratory disease of humans and bird caused by influenza viruses. AI is either mild or highly fatal disease of poultry; the latter is also known as "bird flu" mainly when the virus transmits from birds to humans. The word influenza was first described in the fifteenth century in Italy from the Latin word "influential", as it means a disease caused by unfavorable astrological circumstances **(El-Zoghby et al. 2012)**. Avian influenza viruses (AIVs) are members of the family *Orthomyxoviridae* and genus influenza A virus. Influenza

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viruses are divided into four categories, A, B, C, and D, based on the nucleoprotein (NP) and matrix (M) proteins **(Asha and Kumar, 2019)**.

Two AI pathotypes are recognized based on their virulence: High Pathogenic AIV (HPAIV) and Low Pathogenic AIV (LPAIV). The arrangement of the amino acids at an endoproteolytic cleavage site in the HA protein reflects, the variation in these virus's pathogenicity to poultry **(Alexander, 2000)**. It is important to note that currently AIVs have 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes, giving 198 possible subtypes. All AIV subtypes are known to infect birds **(Dey et al. 2023; Hutchinson et al. 2014)**, except H17N10 and H18N11 viruses, which have recently been found in bats **(Tong et al. 2012)**.

In birds, there are a lot of potential HxNx combinations of AIV (e.g., H1N1, H2N3, H5N1, H9N2, etc.), each of which must have one HA and one NA subtype **(Fouchier et al. 2005)**. The genome of AIV is single-stranded RNA with eight segments that encode at least 11 viral proteins **(Pinto et al. 2021)**. Virus isolation is the gold standard for AIV diagnosis, which is typically done by inoculating swabs or tissues in specific pathogen free embryonated chicken eggs (SPF-ECEs). AIV can be diagnosed molecularly using methods for RNA detection, viral genome sequencing and phylogenetic analysis. Reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (RT-qPCR) are the most frequently used methods for detecting RNA of influenza virus. Despite the Egyptian authorities' best efforts, including trade restrictions and mass vaccination campaigns, an enzootic of the virus in local poultry populations could not be avoided. Poor vaccination and antigenically unrelated vaccines generated suboptimal immunological responses in vaccinated birds, resulting in virus variants escaping from the vaccine-induced immunity **(Abdel-Moneim et al. 2009)**.

MATERIALS and METHODS

Sampling and Sample Processing

One hundred and fifty-eight samples including tissues and swaps from domestic birds (50 flocks) of commercial farms and backyard flocks at different ages showed respiratory signs with moderate to high mortality in some Egyptian provinces (Ismailia, Dakahlia, and Sharqia) during the period from 2022-2023 were collected. Collected samples from each flock were pooled, then labeled and transported immediately in the icebox to the laboratory for storage at -80c^0 till processing.

Molecular Detection of AIVs Using Multiplex RT-qPCR

Viral RNA extractions were done using Intron veterinary DNA/RNA extraction Kit Cat. No.17159. All steps were performed according to the manufacturer's instructions using reagents provided in these kits. The multiplex RT-qPCR was conducted using one-step RT-qPCR (*HERA* RT-qPCR Kit Cat. No. WF10301005) to detect several subtypes of AIVs (H9, H5, and H7) in suspected samples using sets of primers and probes as shown in Table 1. The reactions were performed using the Stratagene MX3005P system **(Stratagene, USA)** following the manufacturer's guide. The thermocycler conditions of primers and probes used for the detection of AIV subtypes (H5, H7, H9) targeting the HA are shown in Table no 2.

Table 1. Primers and probes used for avian viruses subtype identification by RT-qPCR

Subtype	Primers and probes	Primer sequence	Reference
H5	H5 LH1 -Eng14 (Forward)	5'-ACGTATGACTACCCTAAGTATTCAG3'	(Slomka et al., 2007)
	H5 RH1 -Eng14 (Reverse)	5'- AGACCAGCCACTATGATTGC-3'	
	H5 PRO	5'[FAM]TCWACA GTGGCGAGT TCCCTAGCA [TAMRA]-3'	
H9	H9F (Forward)	5'-GGAAGAATTAATTATTATTGGTCGGTAC-3'	(Shabat et al., 2010)
	H9R (Reverse)	5'-GCCACCTTTTTCAGTCTGACATT-3'	
	H9PRO	5'[FAM] AACCAGGCCAGACATTGCGAGTAAGATCC[BHQ] 3'	
H7	LH6H7	5'-GGC CAG TAT TAG AAA CAA CAC CTA TGA-3'	(Slomka et al., 2009)
	RH4H7	5'-GCC CCG AAG CTA AAC CAA AGT AT-3'	
	H7pro11	5'[HEX] CCG CTG CTT AGT TTG ACT GGG TCA ATCT [TAMRA]3'	

Table 2. The thermocycler conditions of RT-qPCR

Item	Cycles					
	Reverse transcription	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
Temperatures	45°C	94°C	94°C	52°C	72°C	72°C
Duration	30 min.	5 min.	40 sec.	40 sec.	60 sec.	10 min
No. Cycles	-	-	35 cycles	35 cycles	35 cycles	-

Virus Isolation

Prepared AIV samples were inoculated and propagated via an allantoic sac of SPF-ECEs according to **(OIE, 2018)**.

HA and HI Test

HA and HI tests were performed for the collected allantoic fluid for confirmation of the virus subtypes using reference antisera against H5N8, H5N1, and H9N2 subtypes with cat. No (VLDIA331),(VLDIA257), and (VLDIA150) respectively according to **(OIE, 2018)**.

RESULTS

Detection AIV Subtypes of in Examined Domestic Flocks Using Multiplex RT-qPCR

There were 9 positive samples for AIVs by Multiplex RT-qPCR out of 50 samples which were collected from both commercial farms and backyard sectors. Samples were gathered from some Egyptian provinces as Ismailia, Dakahlia (2/9), and Sharqia (1/9). Nine positive samples were 6 from Ismailia provinces (6/9), 2 from Dakahlia provinces (2/9), and 1 from Sharqia provinces (1/9). The incidence of positive flocks for AIVs from total collected flocks tested against AIV subtypes was 18% (9/50), with the highest incidence rate for the H9 subtype by a percentage of 16% (8/50),and 2% only for H5(1/50), with no detection of H7 subtype as shown in table 3 and figures 1, 2, and 3.

Table 3.The incidence of AIV subtypes using Multiplex RT-qPCR

AIV subtypes	Infected flocks	
	Number	Percent % (Positive/ Total tested)
H9	8	16% (8/50)
H5	1	2% (1/50)
H7	0	-
Total	9	18% (9/50)

Virus isolation followed by HA and HI test

All positive samples with multiplex RT-qPCR were subjected to virus isolation then HA and HI tests. However, all these samples did not show any lesions in the embryo during virus isolation and were negative in HA and HI tests.

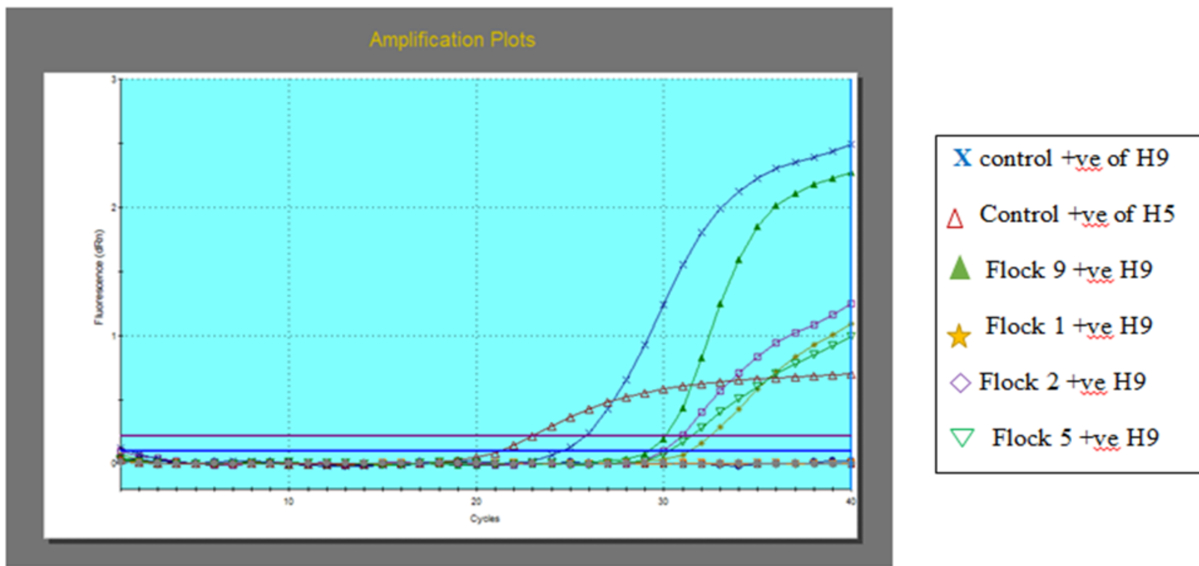

Figure 1: Amplification plot of H9 positive flocks (1, 2, 5, and 9) with positive a negative control generated by the Stratagene MX3005P machine.

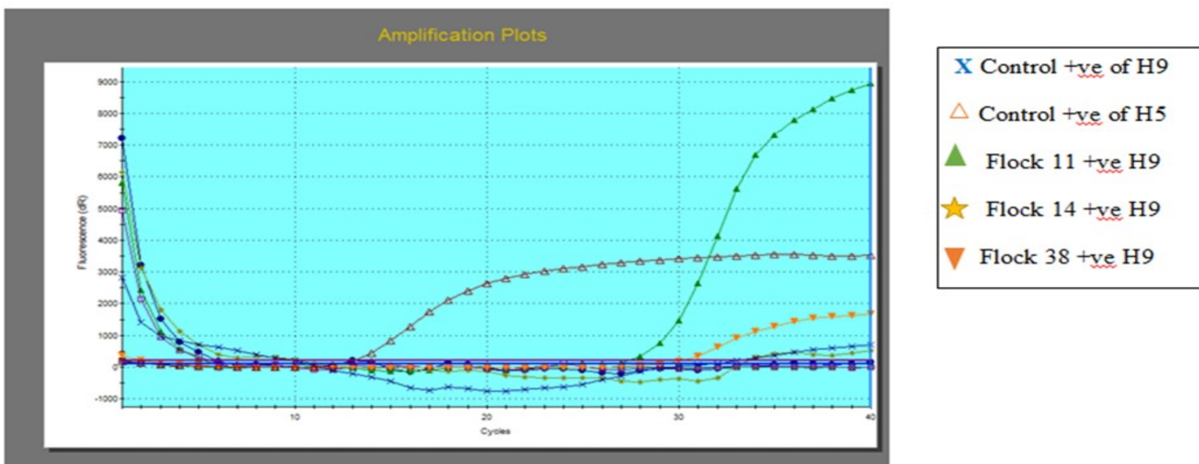

Figure 2. Amplification plots of AIV H9 and H5 positive flocks (flocks 11, 14, and 38) with positive and negative control generated by Stratagene MX3005P machine.

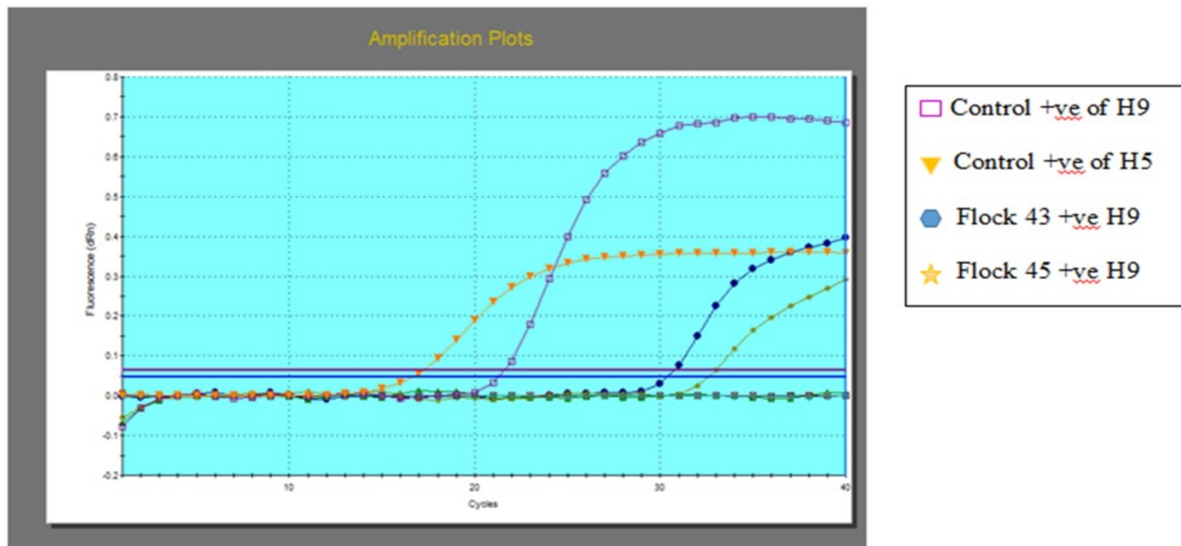

Figure 3. Amplification plots of AIV H9 positive flocks (43, and 45) with positive and negative control generated by Stratagene MX3005P machine.

DISCUSSION

The introduction and spread of different viral diseases have been linked to the expanding poultry industry in Egypt, global trade and the movement of live birds. The ecology and epidemiology of several poultry diseases are now a global concern **(Radwan et al. 2013)**. Over the past few years, outbreaks of respiratory diseases that may be caused by AIV, virulent NDV, or IBV have increased in Egyptian commercial poultry flocks with varying mortality rates and clinical symptoms. Because of their capacity to cause diseases either alone or in conjunction with one another, these pathogens have a major significant economic impact **(Hassan et al. 2021)**.

Our surveillance showed the occurrence of H9, and H5 in 8, and 1flock respectively, from 50 flocks collected from some Egyptian provinces, similar findings were also reported by **Shehata et al. (2019)** that detected H9, and H5 in 8, and 1flocks respectively out of 39 commercial farms in some Egyptian provinces during the period from January 2016 to December 2017. The previous researchers interpreted the persistence of AIV infection in Egypt due to the uneffectiveness of the vaccines to stop the virus circulation especially when the backyard birds play an essential role in its circulation **(Mohamed et al. 2019)**.

The higher incidence of H9 than the H5 subtype was in consistent with **Helal et al. (2017)**, who reported a higher incidence of occurrence of H9 than H5 in positively infected samples in live bird markets in the Suez Canal region. Among AIV subtypes, the H9 subtype spreads quickly and becomes one of the most common LPAI viruses in domestic poultry, resulting in significant economic losses due to respiratory symptoms and moderate to high mortalities, particularly when accompanied by concurrent infection with another respiratory disease **(Lee and Song, 2013)**.

Although virus isolation is typically expensive, labor-intensive, and sluggish, it is considered the standard diagnostic for the diagnosis of avian respiratory viruses **(Suarez et al. 2007)**. RT-PCR and RT-qPCR are examples of quick diagnostic techniques that have been developed for the detection of viral nucleic acids or viral antigens **(Bashashati et al. 2013)**.
The isolated samples were negative for AIVs by HI test; this result may be due to several reasons as the presence of degraded nucleic acid but not an active virus, or the samples col-

lected during the convalescent stage. Also, the same finding was detected and explained by **Zowalaty et al. (2011)**, who reported the inability of AIVs isolation from positively infected flocks using RT-qPCR. This lack of AIV recovery could be due to the presence of inactivated virus. It is also possible that such samples need additional blind passages in SPF-ECEs or permissive cell cultures to support the AIV recovery. Additionally, it should be mentioned that certain AIV strains might not be able to adjust to growth in measurable titers in SPF-ECEs. Further studies are recommended to determine whether samples that were negative by virus isolation SPF-ECEs could yield virus on inoculation in cell cultures.

CONCLISION

In conclusion, Egyptian LPAI H9, and HPAI H5 viruses are still circulating endemically in some studies poultry sectors which were commercial farms or backyard sectors so continuous monitoring is very important.

Conflict of interests

The authors have not declared any conflict of interest.

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