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Surveillance on Avian Influenza H5N1 and H9N2 Subtypes In Egypt 2012-2013

Shakal MA[1]*, Youssef YI[1], El Zeedy SA[2], Ibrahim SM[2] and Al Baroudi BM[1]

[1]Department of Poultry and Rabbit Diseases, Faculty of Veterinary Medicine, Cairo University, Egypt
[2]Department of Genetic Engineering, Veterinary Serum & Vaccine Research Institute (VSVRI), Agricultural Research Center (ARC), Cairo, Egypt

Abstract

Surveillance on Avian Influenza Virus in Egypt during 2012 - 2013 was undertaken to update the epidemiology of avian influenza virus infections among poultry flocks in Egypt. A total of 148 commercial chicken farms were tested by single-plex RT-PCR for direct detection of avian influenza viruses using type A specific nucleoprotein (NP) gene primer sets. The results revealed 5 positive chicken flocks infected with type-A AIV. These viruses were successfully isolated and propagated in SPF eggs. H5N1/HI test was positive only for three chicken flocks. Subtype specific (RT-PCR) revealed 3 positive H5 chicken flocks (2%) and 2 positive H9 chicken flocks (1.35%). All examined chicken flocks were negative for H7 subtype. Multiplex RT-PCR using H5, H7 and H9 subtypes specific primers confirmed these results. This shows the continuous co-circulation of the AIV H5 and H9 in commercial chicken flocks in Egypt complicating the respiratory problems in affected flocks.

Keywords: Avian influenza virus, H5N1, H9N2, Single-plex RT-PCR, Egypt

Introduction

Devastating highly pathogenic avian influenza (HPAI) H5N1 virus infection was first recorded in Egypt in mid-February 2006 in infected commercial poultry production sectors and backyards causing great socioeconomic losses in poultry industry [1-6].

Meliegy [7] stated that poultry industry suffered losses of billions due to avian influenza affecting 1.5 million people working on poultry in Egypt. In Egypt, influenza viruses other than H5N1 subtypes were isolated as H3N1, H4N1 [8], H7N1 [9], H7N7 [10] and recently H9N2 [11-13].

Egypt declared endemic for H5N1 to the OIE and new cases in birds and humans are notified [14]. This endemic situation of H5N1 in Egypt may be associated to wild and migratory birds, the intensive poultry production, backyard poultry raising and the wide geographical distribution of live poultry market in Egypt. Hassan [15] found that backyard birds play an important role for endemic avian influenza infections in Egypt.

Therefore the incidence of avian influenza subtypes infections in chicken flocks needed to be newly investigated during 2012-2013 in different governorates in Egypt. Single-plex RT-PCR was used for direct detection of avian influenza viruses in tracheal swabs using type specific nucleoprotein (NP) gene primer set. Positive RT-PCR samples were subjected to virus isolation using SPF ECE and hem-agglutination test followed by hem-agglutination inhibition (HI) test. Thereafter, multiplex RT-PCR using H5, H7 and H9 subtypes specific primers was performed for subtyping of the isolated virus.

Material and Methods

Cloacal and tracheal swabs

Twenty tracheal and cloacal swabs were taken once per chicken flock not less than three weeks. They were taken randomly from 12, 14, 11, 11, 9, 13, 17, 11, 8, 12, 10, 10, 10 farms from Qalyubia, Dakahlia, Al Sharqia, Gharbia, Fayum, Giza, Damietta, Minya, Asyut, Alexandria, Beheira, Beni Suef and Kafr el-Sheik governorates consequently in Egypt between 2012 - 2013. Swabs were placed in 1-2 ml isotonic phosphate buffered saline (PBS) PH 7.0-7.4, chilled immediately on frozen gel packs and submitted to the laboratory as quickly as possible.

Tracheal and cloacal swabs collected from each commercial poultry flock were pooled separately. Tracheal swabs were screened with RT-PCR for AIV detection, but cloacal swabs in PBS with antibiotics were subjected for virus isolation.

Antiserum

Specific polyclonal antiserum against avian influenza subtype H5N1 was obtained from X-OVO (flock screen, Batch: 1/09) produced by OIE/FAO and National Reference Laboratory for Newcastle disease and Avian Influenza Virology Department. Istituto Zooprofilattico delle Venezie, Italy

Nucleic acid extraction

Ribonucleic acid was extracted from tracheal swabs of each flock separately, using Gene JET™ RNA Purification Kit (Fermentas International Inc., Canada, Thermo Fisher Scientific) and GF -1 Viral Nucleic Acid Extraction Kit. (Vivantis, Malaysia) commercial.

Single-Plex and multi-Plex PCRs

Amplification of cDNA was performed with primer set type-specific (NP) gene fragment in singleplex PCR for detection of type-A avian influenza virus. Subtype-specific multiplex reverse transcription-polymerase chain reaction (RT-PCR) was applied according to [16]

***Corresponding author:** Shakal MA, Department of Poultry and Rabbit Diseases, Faculty of Veterinary Medicine, Cairo University, Egypt, Tel: 00201003939101; E-mail: shakal2000@gmail.com

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to simultaneously detect three subtypes (H5, H7 and H9) of avian influenza virus (AIV). PCR was performed in 50 µl volume in thin-walled 0.2 ml PCR tubes and the reaction mixtures were transferred to the thermal cycler for amplification of the target (NP) gene sequences (Figure 1) in the single-plex PCR and (H5, H7 and H9) gene sequences in the multiplex PCR (Figure 3). cDNA synthesis was carried out using the commercial RT-PCR kits Viva 2- steps RT- PCR Kit (Vivantis, Malaysia) and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Canada) using NP reverse specific primer in RT for single-plex PCR and the hexamer random primer for cDNA synthesis in RT for multiplex PCR. First strand cDNA synthesis from the total RNA extract of the tested samples was accomplished [16].

Oligonucleotide primers

Four different sets of primers (forward and reverse) specific for NP, H5, H7 and H9 of influenza type-A according to Chaharaein et al. [16] were used in this study (Table 1).

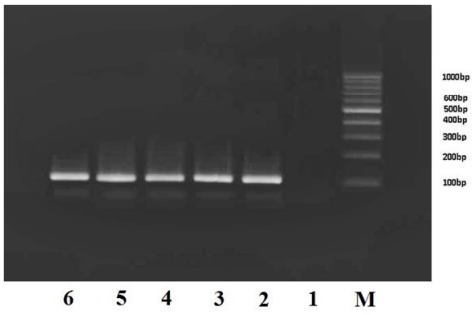

Figure 1: Agarose gel electrophoresis of RT-PCR of NP gene amplified from total RNA extracted from avian influenza infected farm samples compared to 100 bp molecular weight marker (Lane M). Negative control (Lane 1), amplification of 106 bp fragments using type specific NP gene primer set (Lanes 2, 3, 4, 5, 6) from Al Sharqia, Dakahlia, Gharbia, Kafr el-Sheikh and Qalyubia governorates respectively.

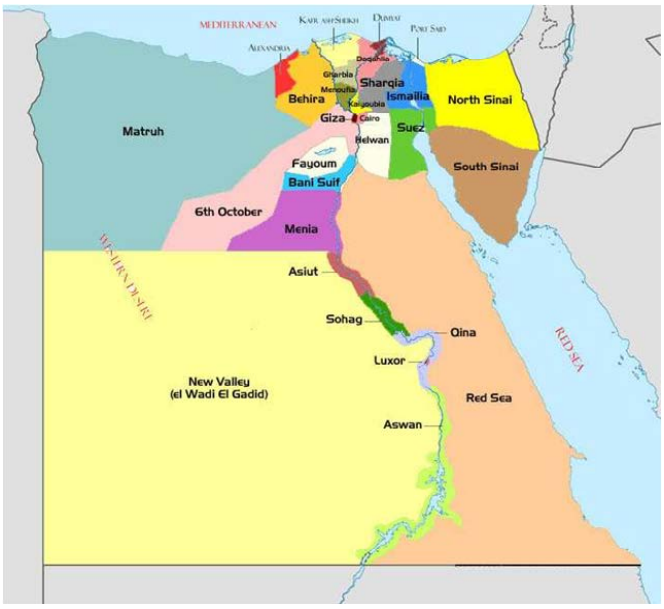

Figure 2: Egypt Governorates.

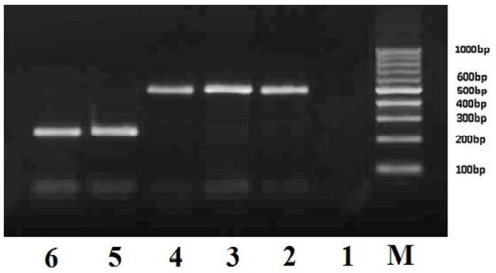

Figure 3: Agarose gel electrophoresis of multiplex RT-PCR product resulting from the amplification of total RNA extracted from avian influenza infected farm samples in comparison to 100 bp molecular weight marker (Lane M). Negative control (Lane 1), amplification of 499 bp fragments (Lanes 2, 3, 4) from Al Sharqia, Dakahlia and Qalyubia respectively, amplification of 221 bp fragments (Lanes 5, 6) from Al Gharbia and Kafr el-Sheikh respectively. The multiplex RT-PCR was done using subtype specific H5, H7 and H9 primer set. No amplification of 409 bp fragments (H7) has happened with any tested sample.

Virus isolation

Isolation of avian influenza virus was performed from cloacal swabs of NP RT-PCR positive cases. Antibiotic treated cloacal swabs were centrifuged and inoculated through allantoic sac route according to the OIE and WHO manuals on animal influenza diagnosis and surveillance [17,18]. Positive embryo showed lesions, as well as the presence of hem-agglutinin in the allantoic fluid (>3HA). Allantoic fluids for each isolate was labeled and stored at 70°C.

HA and HI

Hemagglutination (HA) and hem-agglutination inhibition (HI) test were performed for AIV H5 subtype identification following (OIE terrestrial manual) [17,18] using H5N1 specific antiserum.

Results and Discussion

One hundred forty eight poultry flocks in 13 Egyptian governorates were randomly sampled and investigated for presence of avian influenza type-A subypes between 2012 and 2013.

Twelve, 14, 11, 11, 9, 13, 17, 11, 8, 12, 10, 10, 10 farms from Qalyubia, Dakahlia, Al Sharqia, Gharbia, Fayum, Giza, Damietta, Minya, Asyut, Alexandria, Beheira, Beni Suef and Kafr el-Sheik governorates consequently (Table 2) were sampled and tested for presence of avian influenza type-A subypes. Forty-five of them 30.4% showed respiratory manifestation.

Five out of 148 farms (3.37%) showed positive results in RT –PCR for NP gene as determined by the amplification of target sequence in NP gene with the expected molecular weight (106 bp) for type-A avian influenza virus infection. Two out of 76 broiler farms (2.63%) were positive for AIV infection from Gharbia and Kafr el-Sheikh governorates. Three out of 66 layer farms (4.45%) were positive for AIV infection from Al Sharqia, Dakahlia and Qalyubia governorates (Table 2) indicating higher incidence in layer type chickens between the investigated flocks. Supporting our notion, previous reports described the AIV isolation from layer farms in Qalyubia, Al Sharqia, Alexandria, Bohera, Cairo, ELfayom, Gharbia, Giza and Dakahlia, governorates [15,19]. Positive single-plex RT-PCR samples (Figure 1) were subjected to virus isolation in SPF-ECE. Only three isolates inoculated eggs showed embryonic death after 48–72 hr post inoculation. The allantoic fluid collected from all infected SPF ECE were HA positive. Those three isolates were H5N1/HI positive using specific polyclonal H5N1

antiserum representing 2.02% of the examined flocks in Egypt between 2012 and 2013, where they represent 6.6% (3/45) of flocks showing respiratory manifestation between the other respiratory pathogens.

The incidence of H5N1 in this study is showing a different pattern of the disease incidence from the period of 2006 to 2009 as reported before [1,2,4,5,6,20,21]. AI surveillance program in commercial poultry flocks carried out in 2007 – 2008 showed that AIV infections were detected in 35/3,610 (0.97%), 27/8,682 (0.31%) of examined commercial poultry farms in these years respectively [22]. Also Hassan [15] isolated two AIV isolates from 49 examined chicken farms (4%) in 2012 -2103.

While El-Zoghby et al. [23] recorded only 23 positive farms out of 22024 totals examined farm giving 0.1% in 2009 (Figure 2). Surprisingly, in late 2009-2010, seventy different chicken flocks showed high mortality rates with respiratory manifestation examined for avian influenza infection and 31 farms (40%) were positive for avian influenza virus infection [19,12] indicating high rate of infection in the studied farms.

According to clinical signs and P.M lesion, 45 out of 148 farms were suffering from respiratory manifestation (30.4 %). Out of farms with respiratory manifestation (11.11%) were positive to AIV infection. Thirty three broiler farms with respiratory manifestation out of 76 and (6%) of these farms were positive to AIV infection. Twelve layer examined farms with respiratory manifestation out of 66 and (25%) of these farms were positive to AIV infection. Geographically AIV infected farms were at Qalyubia, Dakahlia, Al Sharqia , Gharbia and Kafr el-Sheikh and the percentage of infections were (8.33 %, 7.14 %, 9%, 9 % and 10 %) respectively out of all examined farms (Tables 2,4).

The obtained results indicate that the incidence of AIV infection in Egypt greatly varied between Lower and Upper Egypt. The increased incidence in Lower Egypt is explained by the ecology of poultry

Table 1: Primer sets to amplify type-specific (NP) and subtype-specific (H5, H7 and H9) genes of influenza type A.

Primer	Target gene	Type	Sequence (5′ - 3′)	Product size
NPF	NP gene	Forward	5' TGTACGGACTTGCTGTGGCC 3'	106 bp
NPR	NP gene	Reverse	5' GAGACTGAAGACCTGGCTGTT 3'	
H5F	H5 gene	Forward	5' ACAAAGCTCTATCAAAACCCAAC 3'	499 bp
H5R	H5 gene	Reverse	5' TACCCATACCAACCATCTACCAT 3'	
H7F	H7 gene	Forward	5' CAGGCGGAATTGATAAGGAG 3'	409 bp
H7R	H7 gene	Reverse	5' TGCCCCATTGAAACTGAAAG 3'	
H9F	H9 gene	Forward	5' ATCGGCTGTTAATGGAATGTGTT 3'	221 bp
H9R	H9 gene	Reverse	5' TGGGCGTCTTGAATAGGGTAA 3'	

Table 2: Incidence of avian influenza using RT-PCR type NP and subtype specific H5, H7 and H9 genes in different governorates.

serial	Governorate	Type of flock	Number of flocks	Flocks with respiratory manifestations		RT-PCR positive rate (NP gene) to total No. of flocks		RT-PCR positive rate (NP gene) to respiratory affected flocks		Results of multiplex RT-PCR positivity rate (H5, H7 and H9) gene primer sets					
										H5 gene		H7 gene		H9 gene	
				Rate	%	Rate	%	Rate	%	Rate	%	Rate	%	Rate	%
	Qalyubia	Broiler	7	2/7	28.57	0/7	0			0/7	0	0/7	0	0/7	0
		Layer	5	1/5	20	1/5	20			1/5	20	0/5	0	0/5	0
		Total	12	3/12	25	1/12	8.33	1/3	33.33	1/12	8.33	0/12	0	0/12	0
	Dakahlia	Broiler	5	3/5	60	0/5	0			0/5	0	0/5	0	0/5	0
		Layer	9	1/9	11.11	1/9	11.11			1/9	11.11	0/9	0	0/9	0
		Total	14	4/14	28.57	1/14	7.14	1/4	25	1/14	7.14	0/14	0	0/14	0
	Al Sharqia	Broiler	2	1/2	50	0/2	0			0/2	0	0/2	0	0/2	0
		Layer	9	2/9	22.22	1/9	11.11			1/9	11.11	0/9	0	0/9	0
		Total	11	3/11	27.27	1/11	9	1/3	33.33	1/11	9	0/11	0	0/11	0
	Gharbia	Broiler	6	3/6	50	1/6	16.6			0/6	0	0/6	0	1/6	16.6
		Layer	4	0/4	0	0/4	0			0/4	0	0/4	0	0/4	0
		Broiler breeders	1	0/1	0	0/1	0			0/1	0	0/1	0	0/1	0
		Total	11	3/11	27.27	1/11	9	1/3	33.33	0/11	0	0/11	0	1/11	9
	Faiyum	Broiler	5	2/5	40	0/5	0			0/5	0	0/5	0	0/5	0
		Layer	4	0/4	0	0/4	0			0/4	0	0/4	0	0/4	0
		Total	9	2/9	22.22	0/9	0	0/2	0	0/9	0	0/9	0	0/9	0
	Giza	Broiler	7	3/7	42.85	0/7	0			0/7	0	0/7	0	0/7	0
		Layer	6	2/6	33.33	0/6	0			0/6	0	0/6	0	0/6	0
		Total	13	5/13	38.46	0/13	0	0/5	0	0/13	0	0/13	0	0/13	0
	Damietta	Broiler	6	3/6	50	0/6	0			0/6	0	0/6	0	0/6	0
		Layer	10	2/10	20	0/10	0			0/10	0	0/10	0	0/10	0
		Broiler breeders	1	0/1	0	0/1	0			0/1	0	0/1	0	0/1	0
		Total	17	5/17	29.41	0/17	0	0/5	0	0/17	0	0/17	0	0/17	0
	Minya	Broiler	7	3/7	42.85	0/7	0			0/7	0	0/7	0	0/7	0
		Layer	4	2/4	50	0/4	0			0/4	0	0/4	0	0/4	0
		Total	11	5/11	45.45	0/11	0	0/5	0	0/11	0	0/11	0	0/11	0

	Asyut	Broiler	5	2/5	40	0/5	0			0/5	0	0/5	0	0/5	0
		Layer	3	0/3	0	0/3	0			0/3	0	0/3	0	0/3	0
		Total	8	2/8	25	0/8	0	0/2	0	0/8	0	0/8	0	0/8	0
	Alexandria	Broiler	9	3/9	33.3	0/9	0			0/9	0	0/9	0	0/9	0
		Layer	3	1/3	33.3	0/3	0			0/3	0	0/3	0	0/3	0
		Total	12	4/12	33.3	0/12	0	0/4	0	0/12	0	0/12	0	0/12	0
	Beheira	Broiler	4	3/4	75	0/4	0			0/4	0	0/4	0	0/4	0
		Layer	2	0/2	0	0/2	0			0/2	0	0/2	0	0/2	0
		Broiler breeders	4	0/4	0	0/4	0			0/4	0	0/4	0	0/4	0
		Total	10	3/10	30	0/10	0	0/3	0	0/10	0	0/10	0	0/10	0
	Beni Suef	Broiler	6	2/6	33.3	0/6	0			0/6	0	0/6	0	0/6	0
		Layer	4	0/4	0	0/4	0			0/4	0	0/4	0	0/4	0
		Total	10	2/10	20	0/10	0	0/2	0	0/10	0	0/10	0	0/10	0
	Kafrel-Sheikh	Broiler	7	3/7	42.85	1/7	14.2			0/7	0	0/7	0	1/7	14.2
		Layer	3	1/3	33.3	0/3	0			0/3	0	0/3	0	0/3	0
		Total	10	4/10	40	1/10	10	1/4	25	0/10	0	0/10	0	1/10	10
Total		148		45/148	30.4	5/148	3.37	5/45	11.11	3/148	2.02	0/148	0	2/148	1.35

Table 3: Clinical history and postmortem lesions of Avian influenza type-A isolates.

Governorate	Type of birds	Flock size	Flock age	AI Vac. history	Clinical signs	Timeline	Deaths	P.M findings	Results of AI examination		
									RT-PCR (NP)	Virus Isolation	HI against H5
Al Sharkia	Layer	10000	11 W	H5 N1	Depression, nervous manifestation, paralysis, cyanosis of comb and wattles and diarrhea.	November 2012	980	Hemorrhages and necrosis of internal organs, subcutaneous hemorrhage and hemorrhages on the pericardium, and in pectoral muscles.	Positive	Positive	Positive
Dakahlia	Layer	9000	17 W	H5N2	nervous manifestation, paralysis, cyanosis of un feathered area of skin	December 2012	364	Hemorrhages and necrosis of internal organs, subcutaneous hemorrhage and hemorrhages on the pericardium and in pectoral muscles	Positive	Positive	Positive
Gharbia	Broiler	5000	41 D	H5N2	Coughing, sneezing, rales and diarrhea.	October 2012	63	Sinusitis, tracheal exudates, airsaculitis, pneumonia, nephrosis and nephritis.	Positive	Positive	Negative
Qalyubia	Layer	10000	10 W	H5N2	Depression, Coughing, rales, diarrhea and drop of food and water consumption.	September 2013	220	Necrosis in liver, spleen and kidney, hemorrhages in heart and lung, hemorrhagic enteritis.	Positive	Positive	Positive
Kafr El Sheikh	Broiler	5000	32 D	H5N2	Coughing, sneezing, depression and drop in food and water consumption.	September 2013	82	Sinusitis, tracheal exudates, airsaculitis, enlarged kidney, perihepatitis.	Positive	Positive	Negative

production in Egypt [24,25]. The majority of poultry production is concentrated in Lower Egypt (Delta) than Upper Egypt where climatic and environmental circumstances encourage intensive poultry production in parallel with high density of human population as well.

As shown in Table 3, the deaths recorded were higher in flocks infected with H5N1 rather than those with other AIV (H9 by RT-PCR subtyping), which is a criteria of the HP H5N1 infections. Interestingly, all AIV infected flocks were vaccinated either with H5N1 or H5N2 vaccines (Table 3). This indicates that the AIV viruses are still circulating and the available vaccines did not completely protect chicken flocks in Egypt against AIV infections.

Supporting our notion, the obtained data come in accordance with the findings of El-Zoghby et al. [12], who isolated H5N1 from vaccinated commercial layer flock in Egypt. Therefore causes of vaccine failure against AIV should be extensively studied and strict bio-security measures should be applied.

The AIV positive cases in our study were found during fall 2012 and fall 2013. This explains the role of weather change as a load on the immune system of birds and provides favorable environmental conditions for AIV transmission. These result in adverse effect on the host pathogen relationship for AIV infection. These observations of the seasonal effect and environmental changes on avian influenza infections in chicken flocks were also explained by Douglas and Edurards [26].

Simultaneous detection of H5, H7 and H9 subtypes of avian

Table 4: Incidence of avian influenza in different governorates using RT-PCR type specific NP* gene.

Type of flocks	Number of flocks	Flocks with respiratory manifestations		RT-PCR positive rate (NP gene) to total No. of flocks		RT-PCR positive rate (NP gene) to respiratory affected flocks	
		Rate	%	Rate	%	Rate	%
Broiler	76	33 / 76	43.42 %	2 / 76	2.63%	2 / 33	6 %
Layer	66	12 / 66	18.18 %	3 / 66	4.45%	3/ 12	25 %
Broiler breeders	6	0 / 6	0 %	0 /6	0 %	0 / 6	0 %
Total	148	45 / 148	30.4 %	5 /148	3.37 %	5 /45	11.11 %

NP: Avian influenza type A specific nucleoprotein gene primer sets

Table 5: Incidence of avian influenza subtypes (H5, H7 and H9) in different flocks.

Type of flocks	Number of flocks	Results of RT-PCR positive rate (H5, H7 and H9) gene primer sets					
		H5		H7		H9	
		Rate	%	Rate	%	Rate	%
Broiler	76	0 / 76	0 %	0 / 76	0 %	2 / 76	2.63 %
Layer	66	3 / 66	4.54 %	0 / 66	0%	0/ 12	0 %
Broiler breeders	6	0 / 6	0 %	0 /6	0 %	0 / 6	0 %
Total	148	3 / 148	2.02 %	0 / 148	0 %	2 / 148	1.35 %

influenza viruses using multiplex reverse transcription-polymerase chain reaction revealed that three out of 148 examined farms (2.02 %) were positive in multiplex RT –PCR for H5 gene with the presence of specific DNA bands at the expected molecular size (499 bp) confirming the results obtained for virus isolation and identification using HI test against H5 (Tables 2,5) (Figure 3). These positive results were obtained from layer farms in Qalyubia, Dakahlia and Al Sharqia and the percent in layer farms was 4.54 % out of examined layer farms, but 2% of the total examined farms (Tables 2,5).

Two farms out of 148 total examined farms (1.35%) showed positive result in multiplex RT –PCR for H9 gene with presence of specific DNA bands at the expected molecular size (221 bp) (Table 5) (Figure 3). Positive results were obtained from broiler farms in Gharbia and Kafr el-Sheikh and the percent in broiler farms was (2.63%) out of total broiler farms (Table 5). These results come in accordance with previous reports stating circulation of H9N2 subtype in Egypt [11-13,15].

Sequencing of our isolates and phylogenetic analysis results of our isolates (H5N1 and H9N2) in this study are available and to be published in a separate article.

The co-circulation of these avian influenza subtypes in the same susceptible chicken population may result in the emergence of novel viruses as a consequence of natural reassortment. Monne et al. [13] warned off the circulation of H9 subtype in a highly pathogenic H5N1 influenza virus endemic population provides an opportunity for genetic reassortment and emergence of novel viruses and raises concerns on its control and on the public health implications of such co-circulation. Thus, continuous monitoring of genetic and antigenic changes in HPAI H5N1, development of vaccines using local field isolates, and standard challenge studies for correct evaluation of the efficacy of vaccines are recommended. All examined farms were negative when tested by RT-PCR using H7 gene primer set.

This excludes the transmission of AIV H7 subtype infection in chicken flocks in the tested Egyptian flocks (Table 5), while the virus infection is wide spread in Mexico [27,28]. Aly et al. [10] found H7 in backyard chicken around Abasa lake north of Egypt during 2007.

In this study the highest incidence was recorded in layer followed by broiler this goes along with the previously mentioned data [19,24]. Here it's important to highlight that all the isolated H5N1 were from layer farms vaccinated either by homologous or heterogonous AI vaccine. This indicates that outbreaks are still occurring despite of the vaccination of AIV H5 subtypes since 2006, pointing out the occurrence of mutations in the virus genome and providing a room for virus escape from vaccination. At the same time, the percentage of Avian Influenza cases in our study shows that the control measure is paying off in Egyptian poultry flocks. On the contrary, poultry industry still is in strong need of more aggressive bio-security measures, monitoring of the susceptible and already vaccinated poultry flocks. These speculations go along with El-Zoghby et al. [25] and Eladl et al. [29].

The cocirculation of the two subtypes H5N1 and H9N2 avian influenza may affect the epizootiologic pattern of AIV infections for both subtypes especially when different vaccination and biosecurity approaches are applied [11].

Therefore, the permanent and updated surveillance programs as early warning measure should be taken for prompt diagnosis of avian influenza infections and to allow the implementation of restriction, eradication and vaccination policies and to help in developing new control strategies [23,30-34].

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