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SOME EPIDEMIOLOGICAL STUDIES ON THE MOST PREVALENT AVIAN INFLUENZA VIRUS SUBTYPE AFFECTING SOME BROILER FLOCKS IN EGYPT USING REAL TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RRT-PCR)

By

Yasmin, A. Sh.[1], Khelfa, D. G. A. [2], Shaban, Kh. S. [2], Salman, O. G. A.[1]

[1] Newcastle Disease department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

[2]Department of poultry diseases, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

ABSTRACT

In this study, thirty-one broiler chicken flocks were monitored for determining the most prevalent subtype of Avian Influenza Virus (AIV) circulating in the field. It was found that, the monitored broiler flocks suffered from clinical manifestation suspected to be infected with Highly Pathogenic Avian Influenza Virus (HPAIV). Three hundred and ten tracheal swabs were collected from moribund and freshly dead birds for isolation and detection of AIV using Specific Pathogen Free Embryonated Chicken Eggs (SPF-ECEs) and through RRT-PCR. Twenty-seven out of thirty-one samples (27/31) gave haemagglutination activity after isolation in SPF-ECEs. By using Real time Reverse Transcriptase Polymerase Chain Reaction (RRT-PCR), Ten out of thirty-one samples (10/31) were found to be positive for HPAIV (H5) and there was no detectable AIV (H7). Mixed infection of more than one virus was common in the monitored farms where, (AIV H5 and Newcastle disease virus (NDV)) were found to be positive in 3/31, (AIV H9 and NDV) were positive in 11/31, (AIV H5, H9 and NDV) were found in 7/31. Mono infection was found to be rare where 3/31 were positive for AIV (H9) only, 3/31 were positive for NDV only and 4/31 were found to be negative for the previously tested viruses. From the afforded mention results, it could be concluded that subtype H9 of AIV was the most prevalent AIV subtype in Egypt.

Keywords:

Avian Influenza disease -Avian Influenza Virus (AIV) -HPAIV-AIV (H5N1) - AIV H7- Real time Reverse Transcriptase Polymerase Chain Reaction (RRT-PCR).

INTRODUCTION

Avian Influenza is a viral disease of many kinds of poultry, wild and cage birds characterized by marked variation in morbidity, mortality, signs and lesions. In addition, the infection causes periodically epidemics in human, horses, pigs, seals, whales and variety of birds **(Swayne and Halvorson, 2003).** Based on the severity of clinical signs seen in birds, AIVs are classified into two patho-types, HPAI and low pathogenic avian influenza (LPAI) **(Alexander, . and Subbarao and Joseph, 2007)**. In Egypt, HPAI subtype H5N1 virus was first reported in poultry in 2006 and was declared to be enzootic in 2008 **(Aly *et al.,* 2006**) while in 2007, the Egyptian Ministry of Environment, in cooperation with US Naval Medical Unit No. 3, reported isolating the low-pathogenic avian influenza H7 subtype from wild migratory ducks from El-Abassa Lake - El-Sharkia **(EL-Zoghby *et al.,* 2011)**. There was serological evidence for the presence of AIV H9N2 infection in chicken population during 2001 in Egypt (**Hussien and El-Azab, 2002)**. In addition, H9N2 has already detected and sequenced from samples obtained from live birds in 2003 and submitted through NAMMRU3 to SEPRL, USA to complete characterization **(Arafa *et al.,* 2012a)**. In Egypt, Mixed infection in poultry flocks with AIV H5N1 and H9N2 plus subclinical infection of pigs and human by H1N1 and H5N1 may lead to Reassortment of these viruses, so situation of influenza virus must be understood to control the disease **(Abdelwhab and Abdel-Moneim, 2015).** The objective of the present study is to determine the most prevalent AIV subtype in poultry flocks in Egypt during 2016 - 2017.

MATERIAL AND METHODS

Samples:

Three hundred and ten tracheal swabs were collected from moribund and freshly dead birds from 31different broiler chicken flocks suffered from clinical manifestation suspected to be infected with HPAIV and located in seven governorates (Sharkia, Elfayoum, Monofia, Kaluobia, Giza, Ismailia, and Menia) in Egypt.

SPF-ECEs:

They were purchased from the specific pathogen free egg project, Kom Oshim, Fayoum Governorate. The eggs were incubated at 37°C and 60% humidity until inoculated at 9-11 days of age via allantoic sac route. They were used for isolation of AIV from suspected samples.

Chicken red blood cells (RBCs):

RBCs from healthy chicken were collected on 4% sodium citrate as anticoagulant. The red cells were washed three times with physiological saline and used in the rapid slide haemagglutination test (HA) as 1:10 suspension in saline (V/V).

QIAamp Viral RNA MINI kit:

It was supplied from (Qiagen, Valencia, Calif**.,** USA), commercial licensed kit cat.no.52906. It was used for RNA extraction.

Quantitative RT-PCR superscript® lll platinum® one-step:

It was supplied from Invitrogen, Cat. No 11732-088. It was used for RRT-PCR.

Sample preparation:

The collected swab samples were placed in 2 ml isotonic phosphate buffered saline (PBS) containing antibiotics as transport media. The swabs were collected during monitoring activities for virus identification according to the standard protocol **(OIE, 2015)**. The collection vial vigorously agitated with vortex then centrifuged at 10000 rpm/15minute. The supernatant passed through 22µm filter (to get rid of bacterial contamination) then transferred to a new vial for preservation at - 80°C until further virological examination. After preparation of the samples, every flock was considered as one sample after pooling.

Isolation of suspected viruses on SPF - ECE 9-11 days old:

A volume of 0.2 ml of the prepared sample was inoculated into the allantoic cavity of 5 SPF -ECEs /sample. The eggs were incubated at 37°C and 60% humidity for 5 days. Deaths on the first 24 hours post inoculation were considered nonspecific while recorded deaths after that were took in consideration as specific results. All eggs at the end of incubation period were collected and chilled at 4°C for 4 hrs or overnight before harvesting. Allantoic fluid was collected with a sterile syringe and presence of virus was confirmed by rapid HA test.

Extraction of viral RNA from suspected samples:

RNA extraction was applied according to instructions of QIAamp Viral RNA MINI kit that supplied from (Qiagen, Valencia, Calif**.,** USA), commercial licensed kit cat.no.52906.

RRT-PCR of suspected HPAIV:

One step RRT- PCR method used according to Invitrogen superscript® lll platinum® one-step Quantitative RT-PCR Cat. No 11732-088.

Table (1): Primers and dual labeled TaqMan® probe used in RRT-PCR for detection of HA gene H5 subtype **(Speckman *et al.*, 2002).**

Primer	Sequence (5´-3´)
Forward H5+1456	**ACG TAT GAC TAT CCA CAA TAC TCA G**
Revere H5- 1685	**AGA CCA GCT ACC ATG ATT GC**
Probe H5+ 1637	**FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA**

RRT-PCR of AIV (H7):

One-step RRT- PCR method used according to Invitrogen superscript® lll platinum® one-step Quantitative RT-PCR Cat. No 11732-088.

Table (2): Primers and dual labeled TaqMan® probe used in RRT-PCR for detection of HA gene H7 subtype **(Speckman *et al.*, 2002).**

Primer	Sequence (5´-3´)
Forward H7+1244	**ATT GGA CAC GAG ACG CAA TG**
Revere H7-1342	**TTC TGA GTC CGC AAG ATC TAT TG**
Probe H7+1281	**FAM-TAA TGC TGA GCT GTT GGT GGC A-TAMRA**

Detection of mixed infection with other respiratory viruses (NDV and AIV H9) in field samples:

RRT-PCR for detection of NDV and H9 from field samples:

One-step RRT PCR method used according to Invitrogen superscript® lll platinum® one-step Quantitative RT-PCR Cat. No 11732-088

Table (3): Primers and dual labeled TaqMan® probe used in RRT-PCR for detection of ND **(Wise *et al.*, 2004).**

Primer	Sequence (5´-3´)
F+4839	**TCCGGAGGATACAAGGGTCT**
f-4939	**AGCTGTTGCAACCCCAA/GT**
F+4894 probe	**FAM-AAGCGTTTCTGTCTCCTTCCTCCA-TAMRA**

Table (4): Primers and dual labeled TaqMan® probe used in RRT-PCR for detection of H9 **(Shabat *et al.*, 2010).**

Primer	Sequence (5´-3´)
H9F	GGAAGAATTAATTATTATTGGTCGGTAC
H9R	GCCACCTTTTTCAGTCTGACATT
H9 probe	FAM-AACCAGGCCAGACATTGCGAGTAAGATCC-TAMRA

RESULTS

Field investigation to monitor different poultry outbreaks suspected to be infected with HPAI.

As shown in (Table 5), the highest numbers of the poultry outbreaks suspected to be infected with HPAI during this study were located in Sharkia (38.7 %) followed by Monofia (16.1%) then Kaluobia and Fayoum (12.9 %). All suspected flocks suffered from clinical signs which varied from dullness, depression, off food, off water and ruffled feather to cyanosis of comb and wattles Fig.(1), facial edema, ecchymosis on the shanks and feet, respiratory and nervous signs. Post mortem lesions appeared as hemorrhagic lesion in internal organ and the skin, congestion of the visceral organ, sinusitis, air saculitis, severe hemorrhagic tracheitis, subcutaneous edema and petechial hemorrhages on internal membrane surfaces of visceral organ and coronary fat of the heart Fig.(2). Flocks that were not vaccinated showed high mortality rate reach to 100 % and rapid course with clear clinical signs while in vaccinated flocks, it was found that, the clinical signs were more or less severe. This was correlated to co-infection when it was present; the clinical signs were severe with acute course. Vaccination history of broilers for suspected outbreaks was present in 29 out of 31. Vaccination against AIV (H5) was started at day 4 to 18 of age and the most common age was at 10 days. Average age of birds at time of clinical disease was 32 days. The interval between time of vaccination and that of infection ranged from 2: 4 weeks. Mortality rate ranged from as low as 8.6% to as high as 100 %. The highest mortality percent was mostly observed with AIV (H5) infection that was mixed with NDV and/or AIV (H9) (30:100 %), while the lowest one was observed with AIV (H9) infection alone (9.8:11.25%). The breeds in the monitored flocks in the different investigated farms were different. They were Cubb, Hubbard, Sasso, Avian 48, H&N and

native (Balady) breeds. There was no difference observed in susceptibility of these breeds to infection with suspected HPAIV (H5).

At time of clinical disease, the age of birds ranged between 18: 42 days. It was found that all birds were equally susceptible to AIV (H5 or H9) and NDV at these ages.

Table (5): History of monitored poultry flocks suspected to be infected with HPAIV through the clinical disease.

Flock No.	Govern.	breed	Age at time of clinical disease	Total no. of chicks	Mortalities at exam. day	Total dead birds	Mortality %	History of AI Vaccination	Most prevalent C.S	Most prevalent P.M
1	Sharkia	Cubb	29 d	11000	350	2200	20%	At 4 d	High mortalities Depression, Dullness, Ruffled feather, Respiratory and Nervous signs	Septicemia All viscera congested Air Sacculitis Cyanosis of comb and wattles Hem. On the Internal organs
2	Monofia	Hubbard	29 d	2000	1500	2000	100%	Non vaccinated		
3	Monofia	Hubbard	30 d	4000	200	660	16.5%	At 12 d		
4	Monofia	Cubb	28 d	8500	300	3620	42.6%	Non vaccinated		
5	Monofia	Sasso	40 d	6000	220	950	16%	At 10 d		
6	Ismailia	Cubb	27 d	8500	450	1950	22.9 %	At 14 d		
7	Sharkia	Hubbard	31 d	10000	470	3680	36.8 %	At 4 d		
8	Giza	Sasso	42 d	3000	300	1500	50%	At 10 d		
9	Sharkia	H&N	32 d	5000	370	1150	23%	At 10 d		
10	Sharkia	Sasso	28 d	10000	500	1800	18%	At 7d		
11	Fayoum	Sasso	27 d	3000	90	260	8.6%	At 18 d		
12	Kaluobia	H&N	30 d	8000	200	700	8.75%	At 10 d		
13	Sharkia	Avian 48	36 d	4000	120	450	11.25%	At 7 d		
14	Menia	Sasso	35 d	5000	100	490	9.8%	At 18 d		
15	Sharkia	Sasso	18 d	6500	220	1600	24.6%	At 14 d		
16	Menia	Sasso	27 d	6000	550	1800	30%	At 10 d		
17	Kaluobia	Hubbard	30 d	5000	620	2300	46%	At 10 d		
18	Monofia	Avian 48	23 d	5000	125	500	10%	At 17 d		
19	SharkiA	Cubb	35 d	3000	450	1200	40%	At 13 d		
20	Fayoum	Baladi	28 d	4500	270	2000	44.4%	At 7 d		
21	Kaluobia	Cubb	30 d	11000	180	1050	9.5%	At 10 d		
22	Sharkia	Cubb	34 d	5000	170	800	16%	At 12 d		
23	Sharkia	Cubb	32 d	1000	150	380	38%	At 10 d		
24	Fayoume	Hubbard	35 d	4000	340	1550	38.75%	At 13d		
25	Giza	Cubb	32 d	6000	100	600	10%	At 7d		
26	Sharkia	Hubbard	26 d	5000	230	2050	41%	At 10 d		
27	Ismailia	Cubb	33 d	9000	400	4000	44.4%	At 11 d		
28	Kaluobia	Baladi	30 d	7000	350	2500	35.7%	At 9 d		
29	Sharkia	Sasso	18 d	20000	550	11000	55%	At 7 d		
30	Fayoume	Sasso	25 d	7500	2000	6000	80%	At 18 d		
31	Sharkia	Cubb	38 d	4000	500	2300	57.5%	At 10 d		

Govern: Governorate **hem: hemorrhage** **C.S: Clinical Sign**

P.M: Post Mortem **exam: examination** **d: days**

Fig .(1) : Chicken suspected to be infected with HPAI showing cyanosis of comb and wattles.

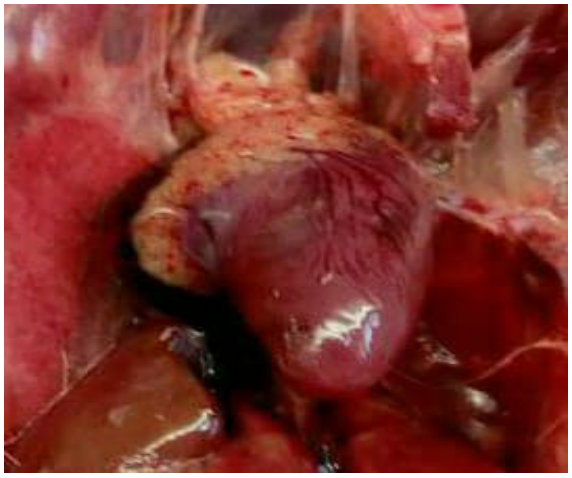

Fig. (2) : Heart of chicken suspected to be infected with HPAI showing petechial hemorrhage on the coronary fat.

Detection of HPAIV from a field samples by using RRT-PCR:

As shown in (Table 6).

Twenty-seven samples out of thirty-one were positive for rapid HA test and four samples were negative.

Ten samples out of thirty-one collected pooled samples were positive for HPAI using H5 gene primer (32.2%).

All samples were negative for AIV (H7).

When all samples were examined for other respiratory viruses (NDV and AIV (H9)), the following results were obtained:

Seven samples out of ten positive HPAIV (H5) were mixed with (ND and H9) and the other three positive HPAIV (H5) samples were mixed with (NDV) alone.

Eleven samples were positive for both NDV and AIV H9 (35.48 %).

Three samples were positive for NDV only (9.67 %).

Three samples were positive for AIV (H9) only (9.67 %).

Four samples were negative for all viruses (12.9 %).

Table (6): Result of slide HA test and RRT-PCR of field samples.

No.	Target (CT)				HA test	Comment
	H5	H9	H7	NDV		
1	-ve	+ve	-ve	+ve	+ve	H9 / NDV
2	+ve	+ve	-ve	+ve	+ve	H5 / H9 /NDV
3	-ve	+ve	-ve	+ve	+ ve	H9 / NDV
4	-ve	+ve	-ve	+ve	+ ve	H9 / NDV
5	-ve	+ve	-ve	+ve	+ve	H9 / NDV
6	-ve	+ve	-ve	+ve	+ve	H9 / NDV
7	-ve	+ve	-ve	+ve	+ve	H9 / NDV
8	-ve	+ve	-ve	+ve	+ve	H9 / NDV
9	-ve	-ve	-ve	+ve	+ve	NDV
10	-ve	-ve	-ve	+ve	+ve	NDV
11	-ve	-ve	-ve	-ve	-ve	-ve
12	-ve	-ve	-ve	-ve	-ve	-ve
13	-ve	+ve	-ve	-ve	+ve	H9
14	-ve	+ve	-ve	-ve	+ve	H9
15	-ve	+ve	-ve	+ve	+ve	H9 / NDV
16	+ve	-ve	-ve	+ve	+ve	H5 / NDV
17	+ve	-ve	-ve	+ve	+ve	H5 / NDV
18	-ve	+ve	-ve	-ve	+ve	H9
19	+ve	-ve	-ve	+ve	+ve	H5 / NDV
20	-ve	+ve	-ve	+ve	+ve	H9 / NDV
21	-ve	-ve	-ve	-ve	-ve	-ve
22	-ve	-ve	-ve	+ve	+ve	NDV
23	-ve	+ve	-ve	+ve	+ve	H9 / NDV
24	-ve	+ve	-ve	+ve	+ve	H9 / NDV
25	-ve	-ve	-ve	-ve	-ve	-ve
26	+ve	+ve	-ve	+ve	+ve	H5 / H9 / NDV
27	+ve	+ve	-ve	+ve	+ve	H5 / H9 / NDV
28	+ve	+ve	-ve	+ve	+ve	H5 / H9 / NDV
29	+ve	+ve	-ve	+ve	+ve	H5 / H9 / NDV
30	+ve	+ve	-ve	+ve	+ve	H5 / H9 / NDV
31	+ve	+ve	-ve	+ve	+ve	H5 / H9 / NDV

-ve: negative+ve :positive NDV: Newcastle Disease Virus

C.T: Cycle Threshold

DISCUSSION

HPAI outbreaks due to clade 2.2 in poultry were reported from February 2006 in Egypt **(Abdelwhab and Hafez, 2011)**. Despite the wide vaccination strategy of poultry in Egypt to combat H5N1, AIV is continuously circulating in vaccinated commercial and backyard poultry **(Hafez *et al.,* 2010)**. In this study data were collected from 31 broilers flocks of different ages suspected to be infected with HPAIV and located in seven governorates in Egypt. Swab samples were collected from moribund and freshly dead birds then transmitted in clean sterile plastic bags and transported in ice tank to the laboratory; the samples were preceded according to the standard protocol **(OIE, 2015)**. The prepared samples were subjected for RNA extraction and examined using RRT-PCR to investigate the presence or absence of HPAIV (H5). Also testing all field samples for other respiratory viruses (NDV & H9) by using RRT-PCR was done according to **Speckman *et al.,* (2002); Wise *et al.,* (2004) and Shabat *et al.,* (2010).** In this study, 90.3 % of suspected flocks were vaccinated against AI and 9.67 % were unvaccinated. Severe symptoms appeared in the unvaccinated flocks comparing to the vaccinated ones, which agree with those reported by **Hafez *et al.,* (2010) and Abdelwhab *et al.,* (2011).** Those authors stated that vaccination of some broiler flocks with inactivated H5N1 vaccine gave partial protection that decrease mortalities but does not prevent infection that might explain the low mortality rate and mild infection commonly recorded in countries, which make a vaccination as a control strategy. The examined flocks showed variation in clinical signs and lesions, such result might be related to the presence of mixed infection. Also, the immunological status of the infected flocks at time of clinical disease and this result are in agreement with **(Arafa *et al.,* 2012 b)** who examined 220 flocks during 2011 and 2012 and detected HPAI, LPAI and mixed infection of both and this explain the clinical signs and lesion of HPAI (H5N1) in that time.In addition, our results are similar to **Swayne, (2008)** who mentioned that co-infection played an important role in severity and course of the disease and mortalities. The average age of birds of the examined flocks at time of notification and appearance of clinical signs and start of deaths was 32 days. The interval between time of vaccination and that of infection ranged from 2:4 weeks and this is the time for good to maximum protection that means there was a failure in vaccination. HPAI samples had been examined for presence of other respiratory viral pathogen specially ND and AI (H9) viruses by RRT-PCR, to know if high mortality rate was due to HPAIV

alone or due to mixed infection. Our results revealed that, All HPAIV positive samples were accompanied with other viral infection and the high mortalities in the field caused by circulation of mixed respiratory viral infection. These results are compatible with other investigators **Abo-Elkhair *et al.*, (2012); EL-bayoumi *et al*., (2013) and Hussein *et al.*, (2013)** who stated that in the last two years, presence of mixed infection especially infection with H5N1, H9N2, NDV and Infectious Bronchitis (IB) viruses may play an effective role in the increased mortalities in poultry industry. In conclusion, LPAIV H9 is the most prevalent AIV subtype either alone or as co-infection with other viruses resulting in aggravation of clinical disease affecting broiler flocks. In addition, it could be concluded that, HPAIVs compromise the poultry industry and cause a serious public health threat in Egypt and in spite of vaccination; there are infections in commercial poultry flocks.

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