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H5N8 AVIAN INFLUENZA VIRUS IN ASWAN GOVERNORATE

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Received: 26 February 2019; **Accepted:** 11 March 2019

ABSTRACT

Egypt has experienced outbreaks of avian influenza (AI) since 2006. Tracheal and cloacal swabs were collected from three hundred and thirty four domestic poultry (one hundred and sixty eight ducks, one hundred and fifty six chickens, ten geese) from 50 houses from different parts in Aswan governorate. Tracheal and cloacal swabs were examined for the presence of avian influenza virus by real-time RT-PCR. H5N8 genome was detected in 26 out of 334 examined birds, giving a ratio of 7.7%. The species wise distribution of RT-PCR results was 8.3%, 7.6%, 0.0% for ducks, chickens and geese respectively. Clinical signs of avian influenza were appeared on most of positive cases which have been examined for AI in the form of: significant mortality may be as high as 100% within 3-4 days after infection, respiratory signs such as (cough, sneezing and respiratory discharge), swelling and cyanosis in comb and wattles and some birds that survive longer exhibit nervous signs. The main clinical feature in ducks was nervous signs (torticollis, other unusual positions of the head and complete reluctance to move) with high mortality rate from 50% up to 100%.

In conclusion, the predominance of H5 infection indicates a need for continuous monitoring of AIV among avian species and the awareness against public health risk.

Key words: Avian influenza virus, H5N8, backyard ducks, chicken, geese.

INTRODUCTION

Avian influenza (AI) is a highly contagious disease and the global spread in bird populations represents a major problem. Both, highly pathogenic avian influenza (HPAI) H5N1 and low pathogenic avian influenza (LPAI) H9N2 viruses have been endemic in Egyptian poultry flocks since 2006 and 2011, respectively (Saad *et al.,* 2007; Arafa *et al.,* 2012). Recently, H5N8 HPAI virus of clade 2.3.4.4 has been introduced to Egypt through migratory birds in 2016 (Kandeil *et al.,* 2017; Selim *et al.,* 2017). Later, the same lineage of virus was isolated from domestic ducks in 2017 (Yehia *et al.,* 2018).

Aquatic birds, including ducks, are generally considered to be the natural reservoir of LPAI viruses. HPAI viruses of certain H5 and H7 strains are thought to be derived from LPAI viruses of wild bird origin (Webster *et al.,* 1992; Olsen *et al.,* 2006).

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The geographical location of Egypt makes it an important migration spot for migratory birds crossing Europe, Asia and Africa. Spread of AI virus from wild to domestic populations occurred both within and between regions, while viral flow from domestic to wild birds was restricted within a geographic region (Bahl *et al.,* 2016). The transmission of AI virus from migratory birds to domestic poultry can represent an additional public health threat in Egypt.

H5N8 subtype clade 2.3.4.4 was first detected in domestic poultry in China in 2010. By 2014, H5N8 HPAI viruses had caused a series of outbreaks among domestic ducks, chickens, geese and wild birds in South Korea, and outbreaks followed in Japan, China, Europe and North America (Lee *et al.,* 2015, 2016). As of March 2017, the virus had spread across most European countries, the Middle East and Africa (World Organisation for Animal Health [OIE], 2018).

The cross-reactivity between the classical 2.2.1 and the variant 2.2.1.1 H5N1 viruses was low and both clusters showed significant antigenic differences by using cross haemagglutination inhibition (HI) assay

(Ibrahim *et al.,* 2013). Importantly, the protective efficacy of the commercial inactivated vaccines used in Egypt against the circulating variant 2.2.1.1 viruses was impaired (Abdelwhab *et al.,* 2011). This can be explained by the mutations in major antigenic sites as previously demonstrated (Ibrahim *et al.,* 2015). No cross-reactivity was observed between HPAI H5N1 strains from different clades and two HPAI H5N8 viruses isolated in 2014 from the Netherlands and Korea, although H5N1 vaccines tested in these studies conferred significant cross-protection against HPAI H5N8 virus challenge (De Vries *et al.,* 2015; Park *et al.,* 2016).

The clinical signs observed in ducks infected with HPAI H5N8 virus include lethargy, nasal discharge and neuronal signs such as torticollis and other unusual positions of the head with a remarkable increase in mortality rate from 50% up to 100%.

The aim of this study was to study the prevalence of HPAI (H5N8) in poultry among different locations in Aswan governorate.

MATERIALS AND METHOD

Tracheal swabs were collected from domestic birds from backyards in Aswan Governorate. The swabs were pooled and the maximum size of pool consist of five samples, while suspected samples were tested without pooling.

For taking a swab sample: Insert the swab into the cloaca or trachea, swabbing the area thoroughly. Place the swab into 1-2 mL of viral transport media and swirl vigorously to dispel the contents of the swab into the media. Lift the swab out of the media; press the swab firmly against the side of the tube to remove any remaining liquid from the swab. Discard the swab into a disinfectant solution. Transport samples immediately to the laboratory on wet ice for RT-PCR examination.

Firstly, the viral RNA was extracted from the collected swab samples by using QIAamp Viral RNA Mini Kit (QIAGEN) catalogue No. 52904, and the protocol was conducted according to the kit instructions. Then, one step RT-PCR was carried out using QuantiTect® probe RT-PCR kit catalogue No. 204443.

Oligonucleotide Primers and probes. Primers and probes used were supplied from **Metabion® (Germany)** are listed in table (1).

Virus	Gene	Primer/ probe sequence 5'-3'	Ref
AI	H5	**H5LH1** ACATATGACTAC CCACARTATTCA G	**Lòndt *et al.*, 2008**
		H5RH1 AGACCAGCT AYC ATGATTGC	
		H5PRO [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	
	H9	**H9F** GGAAGAATTAATTATTATTGGTCGGTAC	**Ben Shabat *et al.*, 2010**
		H9R GCCACCTTTTTCAGTCTGACATT	
		H9 Probe [FAM]AACCAGGCCAGACATTGCGAGTAA GATCC[TAMRA]	
	N1	**N1 forward** TAYAACTCAAGGTTTGAGTCTGTYGCTTG	**Li *et al.*, 2013**
		N1 reverse ATGTTRTTCCTCCAACTCTTGATRGTGTC	
		N1 Probe FAM-TCAGCRAGTGCYTGCCATGATGGCA-Tamra	
	N8	**N8-1296F** TCC ATG YTT TTG GGT TGA RAT GAT	**Hoffmann *et al.*, 2016**
		N8-1423R GCT CCA TCR TGC CAY GAC CA	
		N8-1354 FAM- TCH AGY AGC TCC ATT GTR ATG TGT GGA GT-Tamra	

Cycling conditions of Primers and probes used are listed in table (2):

Virus	Reverse Transcription	Primary Denaturation	Secondary Denaturation	Annealing	Extension	No. of cycles
H5	50˚C 30 min.	95˚C 15 min.	94˚C 15 sec.	54˚C 30 sec.	72˚C 10 sec.	40
H9	50˚C 30 min.	95˚C 15 min.	94˚C 15 sec.	60˚C 45 sec.		40
N1	50˚C 30 min.	95˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 10 sec.	
N8	50˚C 30 min.	95˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 10 sec.	

RESULTS

Avian influenza virus (H5N8) subtype was detected by using real time PCR (RT-PCR)

A total 668 cloacal and tracheal swabs were collected from different localities in Aswan Governorate; samples were collected from household chicken, duck and geese.

These samples were examined for the presence of avian influenza virus (H5,H9 and N1,N8) subtypes by RT-PCR in Reference Laboratory for Veterinary Quality Control on Poultry Production in Giza.

H5N8 genome was detected in 26 out of 334 examined birds, giving a ratio of 7.7%. The species wise distribution of RT-PCR results was 8.3%, 7.6%, 0.0% for ducks, chickens and geese respectively (Table 3). These results appear within one hour and 49 min after starting the run. The samples are negative when they have no CT value, no crossing point and no amplification curve and the samples are positive when they have crossing point cycles Fig. (2), Fig. (3)

Clinical signs of avian influenza were appeared on most of positive cases which have been examined for AI in the form of: significant mortality may be as high as 100% within 3-4 days after infection, respiratory signs such as (cough and sneezing), swelling and cyanosis in comb and wattles, some birds that survive longer exhibit nervous signs characterized by prostration, complete reluctance to move, paralysis of wings, torticollis, opisthotonus and abnormal gait (Fig. 1).

Table 3: Prevalence of avian influenza among the examined chicken, duck and geese

Type of birds	No of samples	Total No of samples	Total No of +ve samples	No of +ve samples	% of +ve samples	Total % of +ve samples
Chicken	156			12	7.6%	
Duck	168	334	26	14	8.3%	7.7%
Geese	1o			0	0.0%	

Figure (1): Clinical signs appear in chickens and ducks due to AIV infection Nervous sigs, ruffled feather & Swelling and cyanosis of comb and wattles in chickens

Nervous signs in duck and chicken (unusual position of the head)

Fig. (2): PCR profile showing positive and negative H5 samples (Positive samples have different crossing points but negative samples have no crossing points).

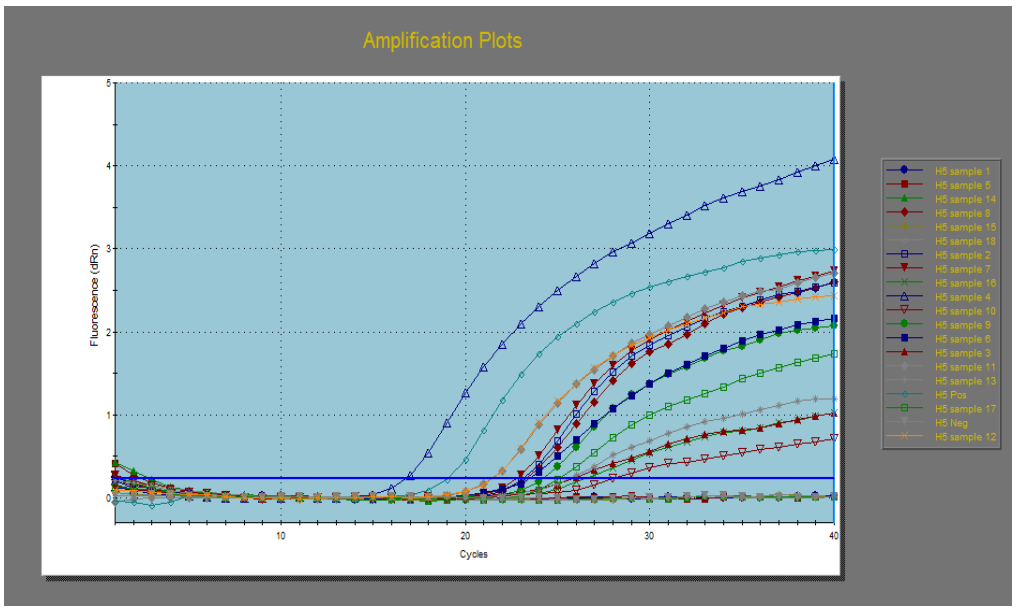
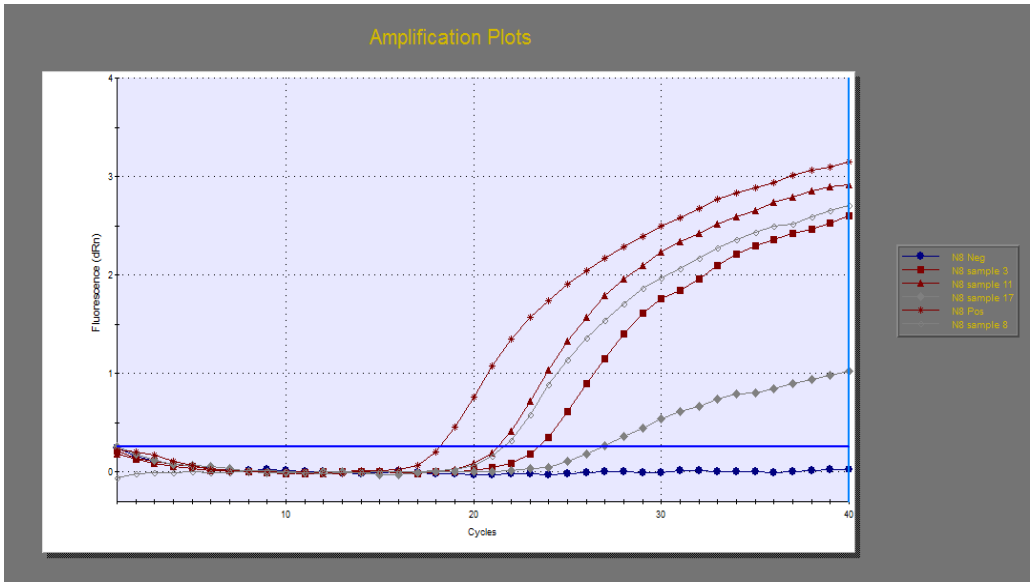


Fig. (3): RT-PCR profile showing positive N8 samples which have different crossing points as well as positive control.



DISCUSSION

Detection of the H5N8 virus in Egypt is of concern since it is very likely this will further complicate control of H5N1 HPAI there. H5N1HPAI virus of clade 2.2.1.2 and its earlier relatives have been entrenched in Egypt since the first intercontinental wave in 2005–2006. The H5N8 strain is antigenically distant from vaccine strains currently used to assist in the control of this disease in Egypt's commercial sector and this may result in an increase in outbreaks and accelerated virus spread if it becomes established in poultry. In addition, the presence of two distinct strains will make laboratory detection more complex although options using clade-specific rapid diagnostic tools exist and have been used (Naguib *et al.,* 2017). As of June 2017 multiple cases have been reported in poultry in the Nile Delta (OIE, 2017e) and some have extended to Upper Egypt, suggesting the virus is likely to become widespread in poultry in this area with a potential spread to other parts of Egypt. Reassortment of genes between the H5N8 virus and the pre-existing H5N1 viruses and/or H9N2 viruses is possible.

In this study a total 334 cloacal and tracheal swabs were collected from different localities in Aswan Governorate; samples were collected from backyard chicken, duck and geese. These samples were examined for the presence of avian influenza virus (H5, H9 and N1, N8) subtypes by RT-PCR. All positive samples were for H5N8 avian influenza virus. HPAI H5N8 virus was detected in domestic ducks and geese in Egypt in 2017 by (Anis *et al.,* 2018) and was detected in 4 poultry flocks not

vaccinated for H5 in the Nile Delta by (Salaheldin *et al.,* 2018).

The transmission of HPAI H5N8 virus to domestic poultry represents a problem in the control and prevention of AI, particularly in Egypt where HPAI H5N1 and LPAI H9N2 viruses are endemic (Monne *et al.,* 2013). Interestingly, the emergence of novel reassortants of HPAI H5N8 virus through acquiring new genes from the endemic AI viruses is expected and this could be a potential threat to the public health. Therefore, strict control measures should be implemented and followed by all partners in poultry industry in Egypt. Movement control, systematic surveillance for wild and domestic birds, biosecurity measures and vaccination should be applied to minimize the losses from AI epizootic.

H5N8 genome was detected in 26 out of 334 examined birds, giving a ratio of 7.7%. The species wise distribution of RT-PCR results was 8.3%, 7.6%, 0.0% for ducks, chickens and geese respectively. This result was lower than (El-Zoghby *et al.,* 2013) who detect AIV in backyard birds in Egypt and the positive percentage was 10.5% and this may be due to that the surveillance was performed in 24 provinces (larger area and higher bird population). And higher than (Osman *et al.,* 2015) who detect AI virus (AIV) in Qena and Luxor provinces and positive samples were (5.64%) and this may be due to that Aswan is a large province and contain high population of birds.

The highest positive result by rRT-PCR was recorded in ducks. This may reveal that the avian influenza become more endemic in ducks in Egypt, as domestic ducks play an important role in the epidemiology of highly pathogenic H5N8 avian influenza. Free-ranging ducks are implicated in the transmission of virus to the environment and subsequently to other ducks or other species, since water in which ducks swim, drink, and eat presents a high exposure risk to humans and other birds. Therefore the risk is greatest in rural areas of affected countries, where domestic ducks and chickens often mingle, frequently sharing the same water supply where the viruses transmitted to chickens under these conditions (Gilbert and Slingenbergh, 2004).

During this study we noticed that the main source of infection was introduction of new birds from market, these new birds transmitted the infection from market to household poultry and this mean that the live bird market has important role in spreading AIV and this comes agree with (Abdelwahab *et al.,* 2010) who said that the LBMs have an important role in the spread of HPAI in Egypt through three main aspects. First, LBMs collect birds from different sources and multiple species of birds as chickens, ducks, geese, turkey, pigeons and rabbits. Second, maintenance of birds all together provides suitable condition for inter- and intra-species transmission. Third, LBMs spread the virus or newly emerged variants to poultry and human in a large extended area.

Highly pathogenic avian influenza (HPAI) H5N8 virus was recently detected in wild birds and domestic poultry in Egypt in the 2016/2017 winter season. Vaccination based on commercial H5 vaccines is used as an essential control strategy in Egyptian poultry. Most of the commercial poultry H5 vaccines used in Egyptian poultry are ineffective because the seed viruses in these vaccines are genetically distinct from the H5N8 viruses currently circulating in Egypt, although some of the commercial vaccines protected chickens from mortality, they failed to prevent chickens from shedding the virus (Kandeil *et al.,* 2018).

CONCLUSION

The predominance of H5 infection indicates a need for continuous monitoring of AIV among avian species and the awareness against public health risk. Also, We are in need of updating and reinforcing the H5N8 prevention and control strategies in Egypt. The vaccination strategy should be reconsidered based on currently circulating viruses.

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انفلونزا الطيور (H5N8) فى محافظة أسوان

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هذه الرسالة تهدف الى الدراسات الإبديميولوجية لمرض أنفلونزا الطيور(شديدة الدراوة) فى الدواجن فى محافظة اسوان حيث شهدت مصر تفشي لمرض أنفلونزا الطيور منذ عام ٢٠٠٦ حيث تم جمع (٣٣٤) مسحة من القصبة الهوائية والمجمع من الدواجن منزلية (٥٠ منزل) وكانت (١٦٨ من البط و١٥٦ من الدجاج و١٠ من الأوز) من شتى أنحاء المحافظة. تم اختبار العينات التى تم جمعها باستخدام تفاعل إنزيم البلمرة التسلسلى العكسى وقد وجدت العترة إتش ٥ إن ٨ (H5N8) فى ٢٦ من إجمالى ٣٣٤ طائر والذى أعطى نسبة دراوة (٧.٧%) وكانت دراوة المرض فى كل من البط والدجاج والاوز هى ٨.٣% و ٧.٦% وصفر على التوالى وكانت الاعراض الظاهرية لمعظم الطيور تتمثل فى نسبة نفوق تصل إلى مائة بالمائة خلال ٣-٤ يوم وأعراض تنفسية مثل الرشح والسعال وسيلان من الأنف بالإضافة إلى انتفاخ وإزرقاق فى العرف كما أن الطيور التى قاومت المرض لفترة أطول ظهرت عليها أعراض عصبية. أما البط فكانت الأعراض الرئيسة هى أعراض عصبية مثل مواضع غير عادية للرأس والرفض التام للحركة مع نسبة نفوق تبدأ من ٥٠ بالمائة إلى مائة بالمائة.

فى الختام: الهيمنة للعترة إتش ٥ (H5) كمسبب لمرض أنفلونزا الطيور تحتاج منا إلى ملاحظة ورصد للمرض بين أنواع الطيور والوعى ضد المخاطر الصحية العامة