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Epidemiological studies and Genetic characterization of highly pathogenic avian influenza A/H5N1 isolated from Egyptian field strains during 2015-2016

Mohamed Elhusseiny[*], Nahed Yehia[*], Osama Mahana[*], Motaz Elsayed[*], Marwa Ali[*], Mohamed Samy[*], Abdel Sattar Arafa[*], Naglaa Hagag[*], Ahlam Yonis[*], and Wafaa Mohamed[1].

*Reference lab. For Veterinary Quality Control On Poultry Production (RLQP)-
Animal Health Research Institute (AHRI),
Agriculture Research Central (ARC)

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Abstract

Introduction:

Avian influenza is a highly contiguous disease in poultry production causing severe economic losses and poses a serious risk for humans.

Method: The surveillance of AI (A/H5N1) occur in this study in late 2015-2016 by collect 14130 tracheal and cloacal swabs from suspected cases in different sectors (10952 farms – 2765 household – 413 live bird market) represent the most government of Egypt. Viral RNA was extracted and examined for Matrix, H5 and N1 genes by real-time reverse-transcription polymerase chain reaction (RT-q PCR). The positive samples were isolated in SPF egg and representative samples were selected for the sequence of the cleavage site.

Result: In this surveillance, the avian influenza subtype H5N1 was detected in 190 cases (25 farms – 100 household – 65 LBM) with prevalence rate (0.23% - 3.6% - 15.7%) respectively with a high prevalence rate in live bird market. The prevalence of the disease was high in winter in Upper Egypt. It occurred mainly in commercial & household chicken except in the live bird market it occurred in duck. Most infected farms were vaccinated. The cleavage site of the selected strains was PQGEKRRKKRGLF belongs to the endemic clade 2.2.1.2 that found in Egypt from 2011.

Conclusion: in this surveillance during 2015-2016, we reported a circulation of AIV (A/H5N1) especially in Upper Egypt in all sectors mainly in a live bird market in winter. The molecular characterization of the cleavage site was similar to clade 2.2.1.2. Interestingly, The infected farms in this study were vaccinated, so surveillance and updating vaccinal seeds should be continued to minimize the spread of the virus in Egypt.

Keywords: *avian influenza, (A/H5N1), cleavage site, genetic characterization*

Introduction

Avian influenza (AI) is a segmented RNA virus that belongs to the orthomyxovir dea family. **(Swyane, 2008).** It was classified as highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) according to pathogenicity and 16 different HA subtype (H1- H16) and 9 different NA subtypes (N1-N9) according to the antigenicity of the Hemaglutinin (HA) and Neuraminidase (NA) glycoproteins **(Fouchier, *et al.,* 2005, OIE, 2006).**

In 1996, The A/goose/Guangdong/1/1996 (H5N1) virus was detected firstly in southern China then spread across Asia, Europe and Africa **(Wan, 2012).** It was recorded in Egypt in 2006 and lead to heavy economic losses for the poultry industry and unemployment of about 1.5 million workers in the poultry industry in Egypt **(Meleigy, 2007).** Afterward, the vaccination strategy was applied using different vaccinal seeds such as low pathogenic (A/

H5N2) and Asian H5N1 strains **(Abdel-Moneim, 2009, Arafa, *et al.,* 2010)**

Dif-
eent factors have contributed to an increase in t he incidence of (AH5N1) infections in birds an d humans, such asintensive poultry production, backyard poultry growth and wide geographic distribution of the live bird market (LBM), which facilitated the disease to be endemic in Egypt due to lack of biosecurity measures and closely contact with wild and migratory birds (**Abdelwhab, *et al.,* 2010 and 2011, Buscaglia, *et al*., 2007, Cristalli and Capua, 2007 and Kayali *et al.,* 2014).**

The highly pathogenic avian influenza virus found in Egypt belongs to clade 2.2.1 **(WHO/ OIE/FAO H5N1 Evolution Working Group. 2008).** After the application of vaccines in 2007-2011, the virus has evolved with high mutations in the HA gene into variant strains belong to clade 2.2.1.1 (**Arafa, *et al.,* 2010**). The endemic cluster 2.2.1.2 appeared in 2008 to 2014 (**Arafa, *et al*., 2015)** and it was responsible for most human infections **(Dudley, 2009).**

The H5N1 viruses acquire many genetic changes that increase its virulence. **(Jiao *et al.,* 2008 and Fan *et al.,* 2009).** The Identification of these changes will understand the pathogenicity and virulence of the virus.

In this study, we follow up the situation, epidemiological mapping and genetic characterization for the cleavage site of HPAI (A/H5N1) in the backyard, farm and live bird market based on the surveillance conducted by reference laboratory for veterinary quality control on poultry production (RLQP) in Egypt during 2015 – 2016.

Materials and Methods:-

Sampling:-

Surveillance was conducted from different geographical sectors in Egypt. Cloacal and oropharyngeal swabs were collected from 14130 suspected cases for the examination of HPAI (H5N1) virus (10952 farms – 2765 household – 413 live bird market) during the period from 2015 to 2016.

Detection of AIV H5 subtype by real-time RT-PCR

Following the manufacturer's instructions, the viral RNA was extracted from samples using a QiaAmp ® Viral RNA Mini Kit (QIAGEN, Hilden, Germany), then the RNA was tested for influenza type A by One Step Real-Time RT-PCR Kit (QIAGEN, Hilden, Germany) using specific sets of M-gene primers then tested for sub typing by specific sets of primers and probe specific to H5 & N1 **(Montserrat *et al.,* 2007 and Spackman *et al.,* 2002)**

Virus isolation

Virus isolation was performed by intra-allantoic inoculation of positive sample in SPF ECE aged 9–11 days, then incubated for 3–5 days at 37 ° C. The isolates were tested by Hemagglutination (HA) and Hem-agglutination inhibition (HI) tests using (A/H5N1) specific antiserum for the existence of avian influenza virus (A/H5N1) **(OIE, 2012, WHO. 2002).**

Amplification of HA gene cleavage site of H5N1viruses

We selected 35 positive cases for HPAI (A/ H5N1) from different sectors (19 backyards – 4 farms – 12 LBM) which represent governorates, species and production sectors as shown in table.1. The RNA was extracted from the positive isolates using QiaAmp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and the area flanking HA cleavage site was amplified by using specific primers KH1 5-CCTCCAGARTATGCMTAYAAAATTGTC-3, KH3 5TACCAA CCGTCTACCATKCCY-TG-3 using one-step RT-PCR with Qiagen®kit (QIAGEN, Hilden, Germany) **(Njouom, *et. al.,* 2008)** according to manual instruction. Then theQi-
aquick gel extraction kit (QIAGEN, Hilden, Ge rmany) was used to purify the positive amplico ns.

Sequencing of HA gene cleavage site:-

The sequencing of the HA gene was carried out using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA) according to manual instruction. Then, the sequencing reactions were purified using a spin column Centrisep® kit (Applied Biosystems,

USA) then loaded into the sequencer machine (Applied Biosystems 3130 genetic analyzers, USA).

Genetic analysis of HA gene cleavage site:-

DNAStar software's MegAlign module (Laserg ene version 7.2; DNASTAR, Madison, WI, US) has been used to align nucleotide sequences o f current study isolates and other HA gene sequ ences available in GenBank using the Clustal W.

Result:-

The result of RRT-PCR of all 14130 suspected cases was positive for HPAI (A/H5N1) virus in 190 cases (25 farm – 100 household – 65 LBM) with prevalence rate (0.23% - 3.6% - 15.7%) respectively with a high prevalence rate in live bird market.

Despite the low prevalence rate of HPAI (A/ H5N1), the virus showed a high geo- prevalence rate (84.6%) as the positive cases were recorded in 22 governorates during 2015 and 2016 with high incidence in Upper Egypt as shown in fig.1-2.

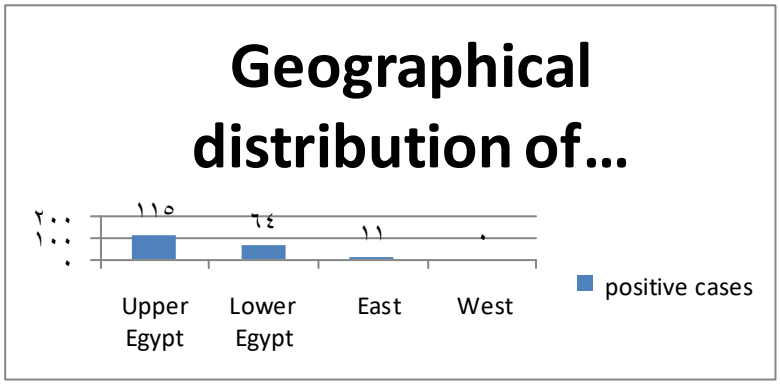

Fig.(1). Geographical distribution map of positive cases in Egypt between 2015 and 2016

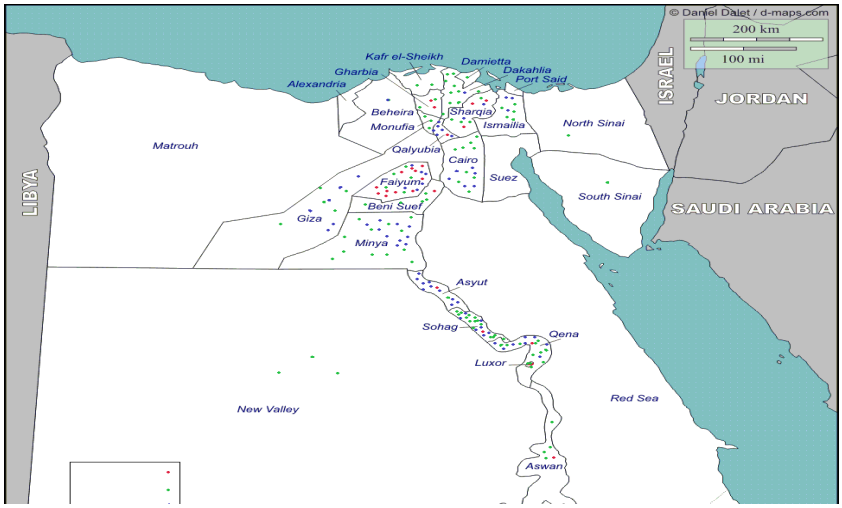

Fig.(2). Geographical distribution in Egypt between 2015 and 2016 of positive cases

The positive cases mostly recorded in chicken either in farm or in household sectors while ducks repre- sent the highest records in case of the live bird market and the positive cases were highly recorded in win- ter and spring as shown in fig.3-4

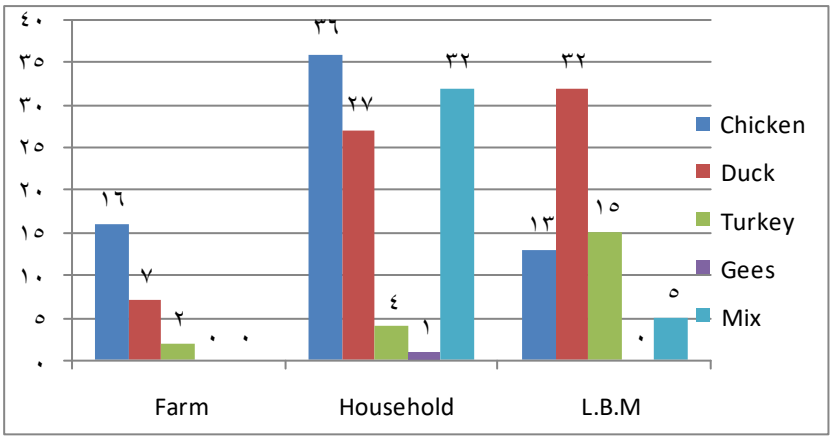

Fig (3). chart for species and production sector of positive cases in Egypt during late 2015 to 2016:-

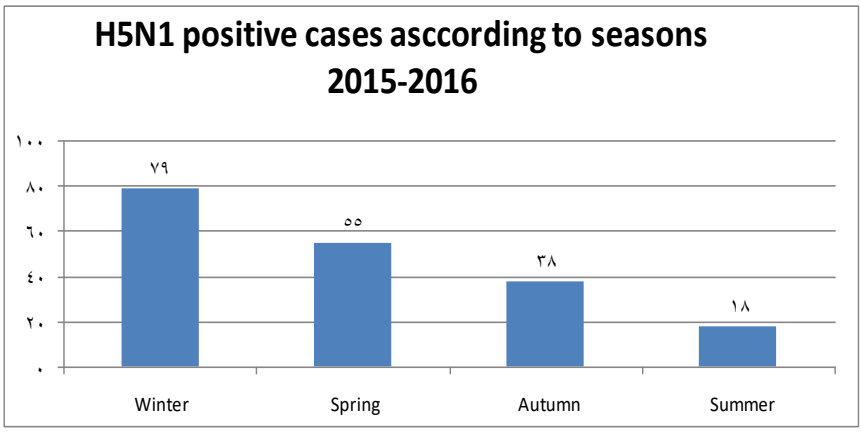

Fig (4). chart for positive cases in Egypt from 2015 to 2016 according to seasons:-

The cleavage site of 35 selected isolates were sequenced. The cleavage site of all Egyptian strains contained multiple basic amino acids (PQGEKRRKKRGLF) belong to clade 2.2.1.2.

Discussion:-

HPAIVs (A/H5N1) was recorded in 2006 in 21 out of 26 Governorates of Egypt then become endemic till now. It has caused high economic losses in the poultry industry and human infection **(Aly, *et al.*, 2006).** Despite the implementation of all control measures, the virus still circulated in all governments of Egypt and highly evolved to overcome the immunity of birds after vaccine application in 2007 **(Hussein, 2009).** It is important to know the current situation of the circulating H5N1 viruses.

To study the current situation of the HPAI (A/H5N1) in Egypt. The surveillance was applied in 14130 suspected cases represent to all governments of Egypt (upper, lower) and represent to all sectors (10952 farms – 2765 household – 413 live bird market), the results revealed a high incidence of positive cases in the upper Egypt which indicate the improper application of biosecurity measures.

On the farm, the positive cases were 25/10952 with a prevalence rate of 0.23% mainly in chicken. It is the lowest prevalence rate in three sectors of production due to a lack of reporting of suspected farm cases.

The positive farms in this study were expected to be vaccinated with (A/H5N1) or (A/H5N2) vaccines. Interestingly, recording positive cases for HPAI (A/H5N1) in pre vaccinated farms was previously recorded **(Arafa, *et al.*, 2012, El-Zoghby., *et. al*, 2012, Shakal, *et al.*, 2013).**

This indicates that AIV viruses continue to circ ulate in the vaccinated farm and the available vaccine may fail to protect the farms from infections or defects in the vaccination process and poor biosecurity measures. So it is important to annually update the used vaccinal seeds and its efficiency with the application of restricted bio-security measures.

Egypt's household poultry plays an important r ole in the environmental spread of the HPAI (A/H5N1) virus **(Aly, *et al.,* 2008).** The exact number of household poultry in Egypt is unknown. It was estimated 4 to 9.5 million **(Fasina *et al*., 2015).** The prevalence of AI (A/H5N1) in the backyard in Egypt in late 2015-2016 (100/2765) was 3.6%. It is higher than the prevalence of the disease in the farm due to lack of biosecurity measures and the absence of veterinarian observation, application of immunization strategy and monitoring. The same situation was previously recorded in 2009 to 2014 surveillance (**Arafa, *et al.,* 2016, El-Zoghby, *et al.,* 2013)**

LBMs are known to play a key role in AI (A/H5N1) transmission and distribution. Due to various sources, species and age of poultry from small farms, household poultry producer without any biosecurity measures **(Ibrahim *et al.,* 2007).**

Due to illegal trading of poultry from farms and household sector without any veterinarian inspection into LBMs, The prevalence of the AI (A/H5N1) was high from 2009-2014 especially in the duck and geese and in the winter season **(ElMasry, *et al.,* 2015)**. In this study during 2015-2016, the prevalence ratio reaches 15.7% more than farm and household poultry production.

The number of infected ducks in the live bird market sector was higher than in farms and in the backyard. The inherent risk of the duck infection is silent infection without any clinical signs that allow easy transmission of the virus to other species, the environment and humans **(Kim *et al.,* 2009).**

Despite the low prevalence rate of HPAI (A/H5N1), the virus showed a high geo-prevalence rate (84.6%) as positive cases were recorded in 22 governorates which indicate the continuous spread of HPAI (A/H5N1) virus in Egypt as shown in fig.1.

The molecular characterization of the cleavage site of AI (A/H5N1) is important to identify the pathogenicity of the virus. There were 13 groups of viruses identified according to the sequence of the cleavage site (**OFFLUE, 2014**).

The previous study showed PQGERRRKKQG cleavage motif dominant in clade 2.2.1 and 2.2.1.1 then the cleavage site began to mutate in 2011 to PQGEKRRKKRGLF in 10% of Egyptian strains **(El-Shesheny, *et al.,* 2014)** then this mutation became dominant in 2013 in clade 2.2.1.2 **(Arafa *et al.,* 2016).** In this study, the cleavage site of all isolated strains was PQGEKRRKKRGLF closely related to the dominant cluster 2.2.1.2.

The Changes of the cleavage site has important role in the pathogenicity of the virus as recorded by **Zhang *et al.,* 2012**, change of cleavage site motif from PQGEGRRKKRQG to PQGERRRKKRQG increase the pathogenicity of the virus but the change from PQGERRRKKRQG to PQGEGRRKKRQG motif reduce the pathogenicity of the virus in mice (**Yoon *et al.,* 2013)**. Further investigations to study the effect of the dominant motif PQGEKRRKKRGLF on the pathogenicity of the virus are recommended.

Conclusion:-

Our study showed wide circulation of endemic A / H5N1 among poultry in Egypt, particularly in Upper Egypt, during 2015-2016. The virus was detected in all sectors (farms, backyards and live bird markets) but it is markedly detected in live bird markets than other sectors because the live bird market links all species from all age and different sectors due to low biosecurity measures. In addition, the prevalence of A/H5N1 mainly in winter in chicken except in the live bird market it occurred in ducks. The infected farms were vaccinated so we need to evaluate the commercial vaccines and continuous updating of the used vaccinal seeds. The cleavage site of all selected isolates was PQGEKRRKKRGLF belong to endemic clade 2.2.1.2 that found in Egypt from 2011.

Continuous surveillance, tracing the source of infection and strict biosecurity measures are good poultry rearing practices are needed to minimize the spread of the virus in Egypt.

Table (1). Data of selected isolate for sequence analysis:-

	Code	Breeding	Governorate	The sequence of the cleavage site
1	A/CK/Egypt/1611CA/2016(H5N1)	Backyard	Asyut	PQGEKRRKKRGLF
2	A/CK/Egypt/1612CA/2016(H5N1)	Backyard	alqahera	PQGEKRRKKRGLF
3	A/CK/Egypt/15139CAG/2015(H5N1)	Backyard	Al Daqahlia	PQGEKRRKKRGLF
4	A/CK/Egypt/161CAL/2016(H5N1)	Backyard	Qena	PQGEKRRKKRGLF
5	A/CK/Egypt/164CAL/2016(H5N1)	Backyard	Aswan	PQGEKRRKKRGL
6	A/CK/Egypt/156FAO-FL/2015 (H5N1)	Farm	Sohag	PQGEKRRKKRGLF
7	A/DK/Egypt/162FAO-SL/2016 (H5N1)	Market	Sohag	PQGEKRRKKRGLF
8	A/CK/Egypt/1652S/2016(H5N1)	Backyard	Minea	PQGEKRRKKRGLF
9	A/CK/Egypt/166SL/2016(H5N1)	Backyard	Sohag	PQGEKRRKKRGLF
10	A/TK/Egypt/1610FAO-SL/2016 (H5N1)	Market	QENA	PQGEKRRKKRGLF
11	A/CK/Egypt/169CAL/2016(H5N1)	Farm	ASWAN	PQGEKRRKKRGLF
12	A/CK/Egypt/1658CA/2016(H5N1)	Backyard	Cairo	PQGEKRRKKRGLF
13	A/DK/Egypt/1610A/2016(H5N1)	Backyard	Minea	PQGEKRRKKRGLF
14	A/DK/Egypt/1662CA/2016(H5N1)	Backyard	South Sina	PQGEKRRKKRGLF
15	A/CK/Egypt/164AL/2016(H5N1)	Backyard	Luxor	PQGEKRRKKRGLF
16	A/CK/Egypt/1611AL/2016(H5N1)	Backyard	Qena	PQGEKRRKKRGLF
17	A/CK/Egypt/1661CA/2016(H5N1)	Backyard	Qena	PQGEKRRKKRGLF
18	A/DK/Egypt/16149CA/2016(H5N1)	Backyard	Beni-sueif	PQGEKRRKKRGLF
19	A/CK/Egypt/1674FAO-S/2016 (H5N1)	Market	Cairo	PQGEKRRKKRGLF
20	A/DK/Egypt/1687FAO-S/2016 (H5N1)	Market	Qaliobia	PQGEKRRKKRGLF
21	A/TK/Egypt/1689FAO-S/2016 (H5N1)	Market	Asute	PQGEKRRKKRGLF
22	A/DK/Egypt/1697FAO-S/2016 (H5N1)	Market	Qaliopia	PQGEKRRKKRGLF
23	A/DK/Egypt/16100FAO-S/2016 (H5N1)	Market	Asute	PQGEKRRKKRGLF
24	A/TK/Egypt/16156FAO-S/2016 (H5N1)	Market	Giza	PQGEKRRKKRGLF
25	A/CK/Egypt/16212S/2016 (H5N1)	Backyard	wadigidid	PQGEKRRKKRGLF
26	A/DK/Egypt/16127FAO-S/2016 (H5N1)	Market	Al Qaliobia	PQGEKRRKKRGLF
27	A/CK/Egypt/1674FAO-S/2016 (H5N1)	Market	Al-minia	PQGEKRRKKRGLF
28	A/CK/Egypt/16186S/2016 (H5N1)	Backyard	Luxor	PQGEKRRKKRGLF
29	A/DK/Egypt/1633VS/2016 (H5N1)	Farm	Al Sharqiya	PQGEKRRKKRGLF
30	A/CK/Egypt/1641CAI/2016 (H5N1)	Backyard	Ismailia	PQGEKRRKKRGLF
31	A/TK/Egypt/1669CA/2016 (H5N1)	Backyard	Cairo	PQGEKRRKKRGLF
32	A/DK/Egypt/16241FAO-S/2016 (H5N1)	Market	Asyut	PQGEKRRKKRGLF
33	A/DK/Egypt/167120FAO-SI/2016 (H5N1)	Market	Ismailia	PQGEKRRKKRGLF
34	A/DK/Egypt/1651CAI/2016 (H5N1)	Backyard	Ismailia	PQGEKRRKKRGLF
35	A/CK/Egypt/16180S/2016 (H5N1)	FARM	Al Fayioum	PQGEKRRKKRGLF

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