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Molecular Survey and Characterization of H5N8 Isolates during 2016-2017 on Egypt

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ABSTRACT

Avian influenza (AI) disease still threat poultry industry in Egypt causing great economic losses. In order to identify and characterize the agent of suggestive clinical cases of AI disease, 28 flocks showing clinical signs suspected to be due to AI infections have been investigated. By slide Haemagglutination (HA), the positive samples were 14/28 and concerning the results of real time- reverse transcriptase polymerase chain reaction (RRT-PCR), 2/14 samples were positive to AI H5, 7/14 to New castle disease virus (NDV), 1/14 to H9 and 4/14 co-infected (2 samples had NDV + AI H5 and others had NDV + AI H9). These positive PCR samples were subjected to further characterization by genotyping and sequencing analysis. The two isolated of H5 AI strain were classified to H5N8 which, related to Russian strains (clade 2.3.4.4) and the genetic analysis approved little relationship between these two H5N8 strain and the commercial AI vaccines with percent (80- 91.7%). So, the researchers should have more monitoring for these viral diseases with effective biosecurity and quarantine measures to minimize the disease occurrence.

Key words: Avian influenza, flocks, molecular, survey

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) of the subtype H5N1 is a contagious pathogen that causes severe respiratory disease with high mortality in poultry (Ali et al., 2015). Type A influenza belongs to the orthomyxoviridae virus family, is enveloped, and is pleomorphic with a size ranging from 80-120 nm (Spackman, 2008). According to Hemagglutinin (HA) and Neuraminidase (NA), there are 18 HA subtypes and 11 NA subtypes. Many different combinations of HA and NA proteins are possible (Tong et al., 2013). AI viruses are categorized as either Low Pathogenic (LP) or High Pathogenic (HP). HPAI viruses produce severe, systemic disease with high mortality in chickens and other gallinaceous poultry birds, but usually don't produce infection or possible mild disease in ducks. The newer H5 and the H7 HPAI viruses have shifted to increased virulence in chickens (Pantin-Jackwood and Swayne, 2009). HPAI H5N1 virus has been endemic in Egypt since 2006. The main symptoms were ecchymosis on the shanks and feet, cyanosis of the comb and wattles, subcutaneous edema of the head and neck for chickens, and nervous signs (torticollis) for ducks. Within 48-72 hours of the onset of illness, the average mortality rates were 22.8-30 % and 28.5-40 % in vaccinated chickens and non-vaccinated ducks respectively (Hagag et al., 2015). A newly emerged H5N8 influenza virus was isolated from green-winged teal in Egypt during December 2016. Multiple peculiar mutations were characterized in the Egyptian H5N8 viruses (Kandiel et al., 2017). Although some of the commercial vaccines protected chickens from mortality by H5N8, they failed to prevent chickens from shedding of virus. Accordingly, so the updating and reinforcing of H5N8 prevention and control strategies in Egypt is very important (Kandiel et al., 2018). Influenza viruses are inherently unstable, as these viruses lack a genetic proof-reading mechanism, small errors that occur when the virus copies itself go undetected and uncorrected. Specific mutations and evolution in influenza viruses cannot be predicted (WHO, 2006). Viruses in vaccinated poultry populations displayed higher mutation rates at the immunogenic epitopes, promoting viral escape and reducing vaccine efficiency (Abdelwhab et al., 2016). The aim of this study is epidemiological survey of AI in commercial

chicken flocks during 2016-2017 on El-Behera, El-Gharbia, Dimiatta governorates through Real Time Polymerase Chain Reaction (RT-PCR) and sequencing analysis to follow recent and current changes that occur on AI viruses.

MATERIAL AND METHODS

Chicken flocks

Twenty-eight broiler chicken flocks of different breeds and age ranged from (25-35 days) at El-Behera, El- Gharbia and Damietta governorates, Egypt during period from June 2016 to May 2017 showing high mortality rate with respiratory manifestations and cyanosis of comb and wattles and diarrhea, the course of disease ranged between 3-5 days. All flocks had history of non-vaccination against AI disease. Samples were taken from trachea, lung and liver of freshly slaughtered birds in each flock separately and transported to laboratory of poultry and fish disease department, faculty of veterinary medicine, Alexandria University, Egypt on ice box.

Virus isolation

The tissue was homogenized with three successive freezing and thawing forming tissue homogenate 10% with Phosphate Buffer Saline (PBS) and centrifuged at 3000 rpm for 15 min. Each sample was inoculated in five eggs with 0.1 ml of tissue homogenate per egg via allantoic sac route, two eggs inoculated with 0.1 ml PBS used as negative control. The fertile specific pathogen free Eggs (SPF ECEs) obtained from kom oshem, fayom, Egypt is used in present study. Inoculated eggs were incubated at 37°c for five days with daily candling for mortalities with discarding non- specific death at first 24 hours of inoculation (OIE, 2008). Dead embryos were examined for gross lesions and their allantoic fluids were harvested and tested by slide (HA) test (Charles, 1989) and the negative allantoic fluids were inoculated on SPF ECEs for two successive passages.

Real time-reverse transcription polymerase chain reaction (RRT-PCR)

Fourteen positive HA allantoic fluid samples were extracted by QIAamp viral RNA mini kits {cat.no .52904, (QIAGEN), Germany} as described by manufacturer manual of Qiagen RNA extraction kit. RRT-PCR was performed using primers of H5 (Lòndt et al., 2008), H9 (Ben Shabat et al., 2010), IB (Meir et al., 2010) and ND (Wise et al., 2004) as shown in table 1.

Sequencing and phylogenetic analysis

The positive samples for AI H5 by RT-PCR were subjected to nucleotide sequencing using primer according to Slomka et al. (2007) (Table 2) using 301bp on an Applied Bio systems 3130 automated DNA sequencer *(*ABI, 3130, USA*)* using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA) (cat-number 4336817) for performing gene sequencing using an Applied Biosystems 3130 genetic analyzer (HITACHI, Japan). QIA quick PCR product extraction kit (Qiagen Inc. Valencia CA), was used for purification of the PCR product on (1.5%) agarose gel directly. Using Centrisep (spin column, Cat number: CS-901) of 100 reactions according to the instruction of the manufacture for Purification of the sequence reaction. A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of Meg Align module of laser gene DNA star software Pairwise, which was designed by Thompson et al. (1994) to determine nucleotide and amino acid sequence similarities and relationships. Phylogenetic analysis was done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

Ethical approval

Direct collection of tissues and organs from freshly slaughtered birds was carried out in strict accordance with the recommendations of Alexandria university, Egypt for the care and use of laboratory animals. Also, slaughtered chickens were humanly handled.

RESULTS

Investigated chicken flocks

Twenty-eight chicken farms suffered from clinical signs and post mortem lesions suspected to be of AI disease were investigated in this study. The diagnosis of AI depends on observation of clinical signs like cyanosis at comb, wattles and legs and sever sinusitis and severe respiratory signs and swelling in the head and post mortem pathological lesions like, tracheitis, petechial hemorrhage on proventriculus, congestion of pectoral and thigh muscles and all internal organs as liver, kidney, spleen, heart, brain and thymus in addition to sever pancreatitis with necrosis.

Virus isolation

All samples were isolated in fertilized SPF ECEs at age 10 days and the embryos mortality began after 48

hours post incubation. The dead embryos characterized by sever hemorrhage in all the body. By slide (HA), the positive samples were (3/8) from Dimiatta (4/9) from Gharbia and (7/11) from El-Behera (Table 4).

Real time-reverse transcription polymerase chain reaction

Concerning the results of RRT-PCR, (2/14) samples were positive to AI H5 (7/14) to NDV, (1/14) to H9 and (4/14) co-infected (2 samples had ND +AI H5 and others had (ND +AI H9) (Table 4).

Sequencing and phylogenetic analysis of H5N8 HPAI isolates results

The phylogenetic tree of HA gene was revealed that the two analyzed isolates (H5) were H5N8 HPAI Influenza Avirus{A/chicken/Egypt/Alex-1/2017(H5N8)} {A/chicken/Egypt/Alex-2/2017(H5N8)} and clustered together and belonged to clade 2.3.4.4 viruses which circulating in Russia and the two isolates were closely related to the first Egyptian isolates (EPIISL224580) which was named A/common coot/Egypt/CA285/2016 (EG-CA285) in Egypt which was isolated from El-fayoum (Figure 1). In addition, amino acid sequencing revealed that the arrangement (PLREKRRKR/GLF) was found in the two isolates and were compared with other H5N8 isolates and commercial AI vaccines on the gene bank and found that the two isolates located at one group with Russian strains (Table 3). These two isolates have sequence identity equal to 99%. But the similarities between two isolated samples and other H5N8 sample which recorded on gene bank ranged between (94.1-99.3%). The similarity between the two isolates and other isolate which isolated in Egypt (EPIISL) is 97.4 – 97.7 %, also percent of similarity between the two isolated samples and other vaccines for AI virus ranged from 80–91.7 % (Figure 2). These two isolates of H5N8 were submitted on gene bank under accession numbers MF182406 and MF182407.

Table 1. Oligonucleotide primers which used in RRT/PCR for identifying the samples oligonucleotide primers and probes used were supplied from Metabion (Germany) these analyses were done by national laboratory for veterinary quality control on poultry production, animal health research institute, Giza, Egypt at 2017

Primer/ probe sequence 5'-3'		References
Avian influenza (H5)	H5LH1(F)ACATATGACTAC CCACARTATTCAG H5RH1(R)AGACCAGCTAYCATGATTGC H5(PRO)[FAM]TCWACAGTGGCGAGT TCCCTAGCA[TAMRA]	Löndt et al., 2008
Avian influenza (H9)	H9(F)GGAAGAATTAATTATTATTGGTCGGTAC H9(R)GCCACCTTTTCAGTCTGACATT H9 (PRO)[FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[BHQ]	BenShabat et al., 2010
Infectious Bronchitis (IB)	AIBV-(F)ATGCTCAACCTTGTCCCTAGCA AIBV-(R)TCAAACTGCGGATCATCACGT AIBV-(PRO)[FAM]TTGGAAGTAGAGTGACGCCCAAACTTCA [TAMRA]	Meir et al., 2010
New castle Disease Virus (NDV)	M+4100 (F)AGTGATGTGCTCGGACCTTC M-4220 (R)CCTGAGGAGAGGCATTTGCTA M+4169(PRO)[FAM]TTCTCTAGCAGTGGGACAGCCTGC [TAMRA]-3’	Wise et al., 2004

F: forward of the primer, R: reverse of the primer, PRO: probe of the primer

Table 2. Primers (for sequencing) of H5 isolates, primers were supplied from Metabion (Germany), these analyses were done by national laboratory for veterinary quality control on poultry production, animal health research institute, Giza, Egypt at 2017

Virus	Primer/ probe	Amplified product	Reference
H5	sequence5'-3'H5-kha-1(F)CCTCCAGARTATGCM TAY AAA ATT GTC H5-kha-3(R)TACCAACCGTCTACC ATKCCYTG	311 bp	Slomka *etal*., 2007

F: forward, R: reverse

Table 3. Amino acid sequence of H5N8 avian influenza virus subtype isolates these analyses were done by national laboratory for veterinary quality control on poultry production, animal health research institute, Giza, Egypt at 2017

Avianinfluenza virus	Amino acid sequence	Accession number
InfluenzaAvirus{A/chicken/Egypt/Alex1/2017(H5N8)}	PEYAYKIVKKGDSTIMKSEVEYGHCNTKCQTPVGAINSSMPFHNIHPLTIGECPKYVKSNKLVLATGLRNS***PLREKRRKRGLF***GAIAGFIEGGWQGMVDGW	MF182406
InfluenzaAvirus{A/chickenEgypt/Alex-2/2017(H5N8	PEYAYKIVKKGDSTIMKSEVEYGHCNTKCQTPVGAINSSMPFHNIHPLTIGECPKYVKSNKLVLATGLRNC***PLREKRRKRGLF***GAIAGFIEGGWQGMVDGWY	MF18240

F: forward, R: reverse

Table 4. History of investigated flocks, results of slide heamagglutination test and real time reverse transcriptase polymerase chain reaction, this investigation was done at 2016-2017

Number of flock	Age of birds /days	Breed of birds	Total number of flocks	Mortality %	Location	HA Slide test	RRT-PCR
1	26	Cobb	7000	8	El-Behera	+	NDV
2	29	Cobb	10000	9	EL-Behera	+	NDV
3	28	Avian48	15000	12	El-Behera	+	H9+NDV
4	33	Ross	5000	10	El-Behera	-	-
5	25	Avian48	6000	9	El-Behera	-	-
6	31	Ross	5000	9	El-Behera	-	-
7	35	Cobb	7000	8	El-Behera	-	-
8	34	Cobb	15000	20	Damietta	+	H5
9	33	Cobb	15000	8	Damietta	+	NDV
10	27	Cobb	20000	8	Damietta	+	NDV
11	29	Cobb	15000	9	Damietta	-	-
12	34	Avian48	5000	10	Damietta	-	-
13	26	Avian48	5000	8	Damietta	-	-
14	28	Ross	7000	8	Damietta	-	-
15	25	Cobb	5000	10	Damietta	-	-
16	33	Ross	8000	8	El-Gharbia	+	H9
17	31	Ross	20000	20	El-Gharbia	+	H5+NDV
18	34	Avian48	15000	20	El- Gharbia	+	H5+NDV
19	25	Cobb	10000	10	El- Gharbia	+	NDV
20	27	Cobb	6000	8	El-Gharbia	-	-
21	35	Avian48	10000	10	El-Gharbia	-	-
22	28	Ross	5000	19	El-Gharbia	-	-
23	28	Avian48	8000	10	El-Gharbia	-	-
24	35	Cobb	6000	8	El-Gharbia	-	-
25	27	Cobb	20000	15	El-Behera	+	H5
26	31	Avian48	5000	10	El-Behera	+	NDV
27	33	Cobb	6000	9	El-Behera	+	NDV
28	28	Cobb	10000	13	El-Behera	+	H9+ND

HA: hemagglutination test, RRT-PCR: real time reverse transcriptase polymerase chain reaction, H5: avian influenza virus H5, NDV: Newcastle Disease Virus, H9: avian influenza virus H9, +: positive, -: negative

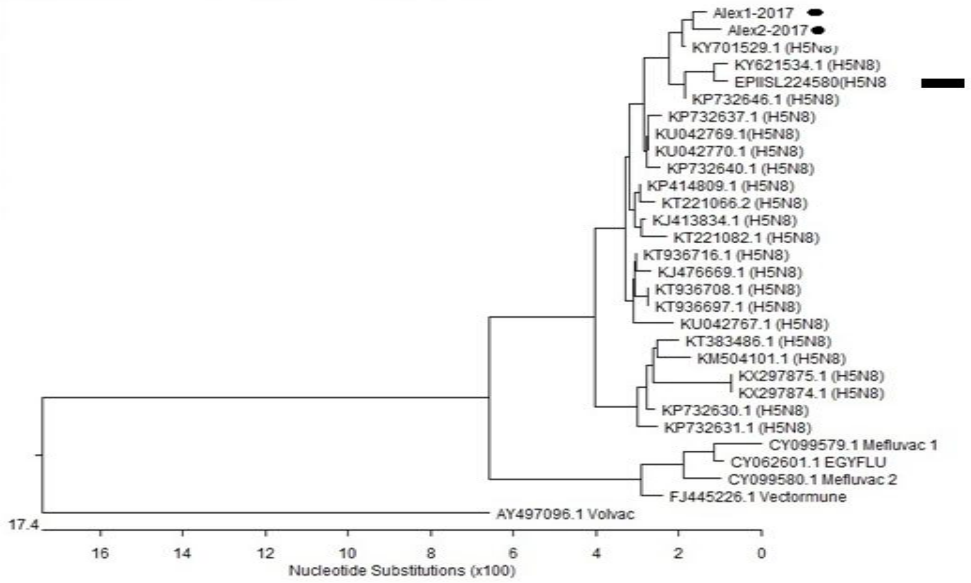

Figure 1. Phylogenetic tree for the two field isolates highly pathogenic H5N8 (●) and the first Egyptian isolate (■) (EPiiSL) with other H5N8 strain and commercial vaccines against avian influenza

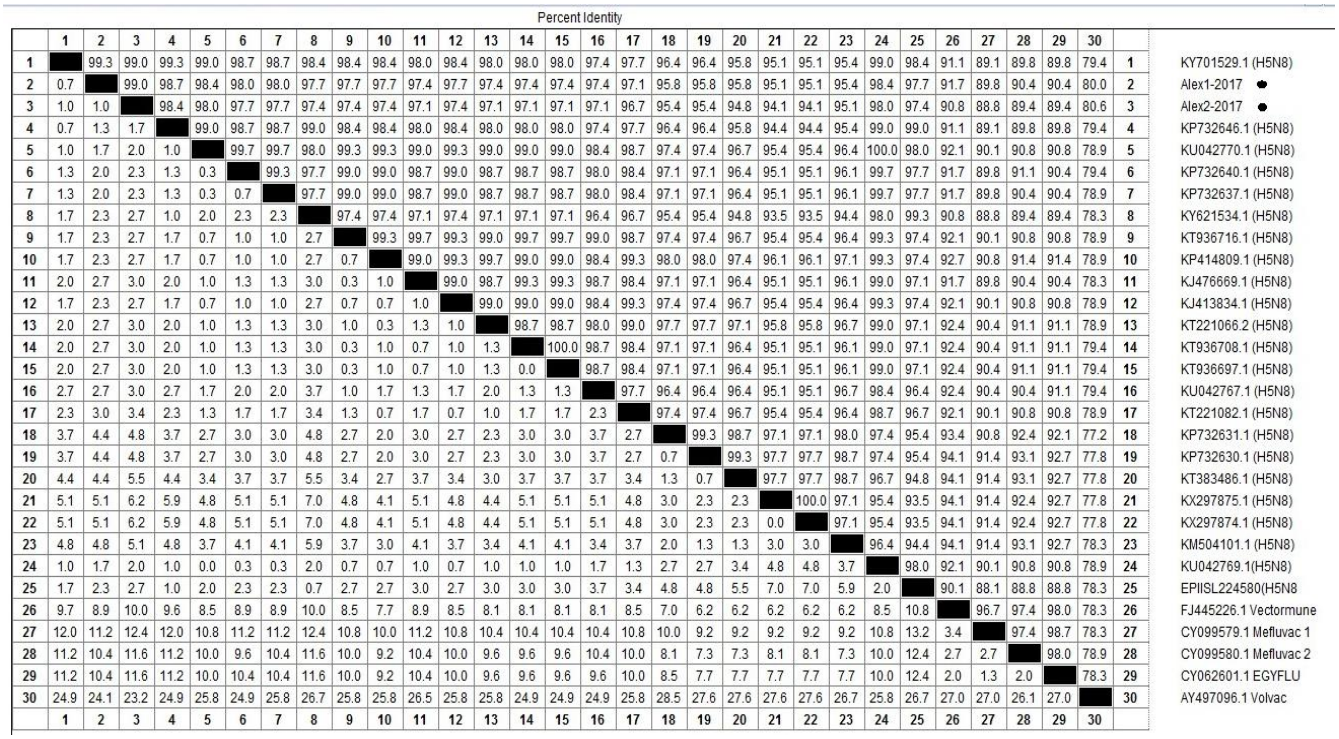

Figure 2. Identity and diversity of amino acid sequence of isolated tow field H5N8 (●) high pathogenic avian influenza compared with other H5N8 viruses and some commercial avian influenza vaccine

DISCUSSION

Highly pathogenic avian influenza A (H5N8) viruses of clade 2.3.4.4 spread into West Africa in late 2016 during the autumn bird migration. Genetic characterization of the complete genome of these viruses detected in wild and domestic birds in Cameroon on January 2017 demonstrated the occurrence of multiple virus introductions **(**Wade et al., 2018). The first report of highly pathogenic H5N8 avian influenza detection in Egypt, made it the third country in the Middle East that report the new H5 clade, which has also spread to several European countries after it was first identified in Russian migratory birds (Cidrap, 2016). The clinical signs and post mortem lesions recorded in this study supported by Abou Rawash et al. (2012); Horimoto and kawaoka (2001); Wasito et al. (2016); ElBakrey et al. (2013); Twabela et al. (2018). All investigated flocks were not vaccinated against avian influenza virus, and that may contribute to high mortality percent in the flocks. All samples were inoculated in SPF-ECEs as AI was completely adapted in (ECEs) via allantoic sac inoculation route , and lead to death of the embryo with hemorrhagic lesions (Brauer and chen, 2015; Salaheldin et al., 2017). Molecular identification of AI was carried out by using real time RT /PCR as it consider a sensitive method for accurate AI detection (Hanna et al., 2015; Bouwstra et al., 2015) and in present study, 4/28 samples were positive to H5 from which two samples were positive pure. Performing real time RT/PCR followed by sequencing and phylogenetic analysis is very important and valuable technique to know strains of AI (Salaheldin et al., 2018; Li et al., 2017; Bouwstra et al., 2015) Two PCR positive samples (pureH5)were sequenced. The partition of amino acids (PLREKRRKR/GLF) means that the two isolates are high pathogenic AI virus (Kandeil et al., 2017; Selim et al., 2017). EG-CA285 contain three amino acid assignment differences in the HA protein, namely R22K , E268G and D487Y which distinguished EG-CA285 from the recent HPAIV (H5N8) clade 2.3.4.4b strain isolated in Russia (A/great-crested-grebe/Uvs-Nuur-Lake/341/2016) and in the NA protein, they observed four substitution mutations (V8A, V31L, G126E,I407T) that distinguished the EG-CA285 from the subtype found in Russia (Selim et al., 2017). And Phylogenetic analysis explained that relation-ship between the first Egyptian H5N8 isolate (EPI ISL 224580), two H5N8isolates and other H5N8 isolates (Figure 1) so, the two isolates (Alex1, Alex2) are related to clade 2.3.4.4b.

A total 301 deduced amino acids sequences from a.a position 1 to 301 were analyzed**.** The two isolates had

some changes of amino acids like S71C, A176G, A211T and G225A in Alex1–Alex 2 respectively but this difference has very little effect on the percent of identity between the two isolates equal to 99%. Also identity percent between the two isolates and other commercial AI vaccines ranged from 80–91.7% which approve the little genetically relation-ship between the two isolates and commercial vaccines so chicken sera will be of little or no titer and this approved also by Kandeil et al. (2017) who stated that, chicken sera induced by commercial inactivated H5- vaccines showed no or very low reactivity with H5N8 viruses so, it should be depend on biosecurity in prevention programs against AI H5N8 and put all possible rules for protecting the poultry industry in Egypt. Egypt is consider the bridge between Europe, Asia and Africa where millions of migrating birds pass during their flights annually particularly in winter. Thus, Egypt is under huge pressure from migratory birds from the entire world and researchers must improve the criteria of deal with any spot of infection.

CONCLUSION

We concluded from present study many respiratory viral diseases threaten poultry industry in Egypt and new avian influenza H5N8 strain isolated from different area in Egypt in addition to high distribution of avian influenza H5N1, so this industry needs to more efforts by veterinary authorities to reduce these spreading in Egypt and protect the industry to keep away human from the zoonotic infections.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Author`s contributions.

All authors participated in making the design, performing the experiment, analyses of the data, and writing the paper.

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