

EPIZOOTIOLOGICAL AND MOLECULAR CHARACTERIZATION OF EQUINE INFLUENZA VIRUS 2008 OUTBREAK IN EGYPT

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SUMMARY

Equine influenza symptoms were detected in population of equines in different governorates in Egypt (Cairo, Giza, Helwan, Alexandria, Minoufia, Behaira,, Assiut and Aswan) during July - August 2008. High temperature, inappetence, conjunctivitis, redness of nasal mucosa, serous to mucopurulent nasal discharge and a harsh dry cough were the most common clinical manifestations. Horses of all ages and both sexes were affected. Free movement of the infected animals and direct contact at markets and races facilitated the rapid spread of the disease. Nine suspected cases represented eight governorates were examined for equine influenza virus (EIV) where 107 nasal swabs and 107 serum samples were used for diagnosis. Real-time reverse transcription-polymerase chain reactions (rRT-PCR) assay was applied to

detect the matrix (M) gene of influenza type A viruses in nasal swabs and 6 out of the 8 cases were positive. Three cases were positive by virus isolation on embryonated chicken egg inoculation and the hemagglutination test. The hemagglutination inhibition (HI) was performed to identify the isolated influenza virus using reference antisera against A/Equi-1 (H7N7) and A/Equi-2 (H3N8). In this study, full characterization of the isolated virus was carried out through molecular techniques for typing of hemagglutinin (HA) and neuraminidase (NA) genes by RT-PCR and partial sequencing of the HA gene of one isolate (A/Equine/Egypt/21AHRI/2008(H3N8)) and the results confirmed that H3N8 virus was the causative agent of this outbreak

KEYWORDS:

Equine Influenza virus - Hemagglutination inhibition test – Real time PCR. HA gene sequencing- subtypes H3N8-Egypt.

INTRODUCTION:

Equine influenza is a highly contagious viral disease that affects equine population (Burrows et al., 1982). It is an acute, contagious respiratory disease caused by two distinct subtypes (subtype 1: H7N7 and subtype 2: H3N8) of influenza A viruses within the genus *Influenza virus A*, of the family *Orthomyxoviridae* (OIE .2008).

The first equine influenza virus was isolated in the Czech Republic in 1956. Later, it became a reference strain for subtype 1 and was designated A/Eq 1/56 (H7N7) (Pospisil et al., 2002). In 1963, major epizootics of respiratory disease, affecting 50%-90% of horses, occurred in the United Kingdom (UK) and the United States of America (USA) (Radostits et al., 2000). One of the outbreaks in the USA that year occurred in Miami, where a new subtype of equine influenza virus emerged and was designated A/Eq2/Miami/63 (subtype 2) (H3N8).

It was noted that antigenic and genetic variants of equine H3N8 viruses co-circulated, and that variants circulating in Europe and the USA were distinguishable from one another in terms of both the antigenic reactivity and the genetic structure of the HA1 portion of the haemagglutinin (HA) molecule (Daly et al, 1996).

Clinically, equine influenza is characterized by pyrexia, mucopurulent nasal discharge and

severe persistent cough (OIE, 2008 and Radostits et al., 2000).

Outbreaks of equine influenza occurred among vaccinated race horses with Japanese commercial equine influenza vaccine at Kanazawa race horses in Ishikawa prefecture in Japan in 2007 (Ito et al., 2008).

Equine influenza (EI) virus (H3N8) was identified in the Australian horse population for the first time in August 2007. The principal molecular diagnostic tool used for detection was a TaqMan real-time reverse transcription-polymerase chain reactions (rRT-PCR) assay specific for the matrix (MA) gene of equine influenza virus (EIV). This assay showed sensitive results that able to detect EIV from day 1 or 2 post-challenge, as early as virus isolation, and before clinical signs of disease were observed (Spackman et al., 2002 and Foord et al., 2008).

During local respiratory disease outbreaks, occurring in 2003 and 2004 in horse training stables within race-tracks in Rome, and on a stud horse farm in Bari in 2005, four strains of equine influenza (EI) virus were isolated. All outbreaks occurred in flu-vaccinated horses. The Rome and Bari isolates were identified as members of the American lineage (Damiani et al., 2008).

In April 2004 an outbreak of equine influenza has been occurred at the Zagreb, Croatia, the genetic characterization of the HA1 portion of

the haemagglutinin (HA) gene of virus isolated from the outbreak indicated that the isolate (A/equine/Zagreb/04) was an H3N8 strain (Barbic et al., 2009).

The broad distribution and prevalence of H3 subtype influenza viruses in avian and mammalian hosts constitute a global threat to both human and veterinary health (Pu et al., 2009).

The hemagglutination inhibition (HI) assay is a widely used serological method to measure the levels of protective antibody responses against influenza viruses (Jia et al., 2008).

One-step RT-PCR assay followed by

sequencing is a rapid, accurate, and specific method for detection and sub typing of different neuraminidase subtypes of influenza A virus strains from a range of host species in different geographical locations (Alvarez et al., 2008).

The purpose of the present study is to document the recent outbreak in 2008 and describe the clinical, virological and molecular methods used for diagnosis of equine influenza virus in Egypt. As well as to match the genotype (s) of field isolates from national outbreak by the sequencing with other worldwide circulated strains.

MATERIAL AND METHODS:

Field investigation

Clinical examinations were performed during the outbreak, including mucous membrane examination and percussion and auscultation of the lungs as well as the rectal temperature of the diseased equines.

Virological diagnosis

1- Serum samples

Whole blood from clinically-ill animals was collected in sterile test tubes without anticoagulant. A total of 107 blood samples were collected (Table 1). These samples were centrifuged at 1,000 g for 10 min., and the sera were collected and frozen. All collected sera

were heated first at 56°C for 30 minutes to inhibit the non specific reactors.

2- Nasal swabs

One hundred and seven nasal swabs were collected from affected animals and placed in sterile Phosphate Buffer Saline (PBS). The specimens were transported to the laboratory in an ice container. The swab contents were clarified at low speed centrifugation; 1,000 g for 10 minutes. Antibiotics (100 IU of penicillin and 100 µg of streptomycin per ml) were added to the supernatant, which was left at 4°C for one hour then kept at -20°C until used.

Table (1) The number of samples collected from different governorates in Egypt at summer 2008:

Site	Governorate	Housing	Serum	Swabs
Proke hospital	Alexandria	Hospital	5	5
Zahraa Ein Shams	Cairo	Equestrian club	10	10
EL Mansoria	Giza	Stables	7	7
Edfo	Aswan	Sporadic	31	31
VACSERA	Helwan	Stables	31	31
Shebene-Elkoom	Menofia	Stables	5	5
Abbasia	Cairo	Stables	11	11
EL Mahmudia	Assiut	Stables	2	2
Dammhour	Behira	Stables	5	5
Total			107	107

The ages of the diseased equines were ranged from 6 months up to 8 years

Real time RT-PCR:

The received 107 swab samples from suspected outbreaks were tested by Real time RT-PCR. Briefly, RNA was extracted from nasal swabs suspensions from each case by using QIAamp Viral RNA Mini Kit (Qiagen, Germany). Samples were amplified using One-Step Real-time reverse transcription-PCR (RRT-PCR) kit for detection of Matrix (M) gene of influenza A virus (PG-Biotech; Qiagen, Germany) according to the manufacturer instructions and the primers & PCR condition as described by (Spackman et al., 2002).

Virus isolation and subtyping:

As method described in OIE manual (OIE, 2008), 0.2 ml of prepared nasal swab suspensions were inoculated into the allantoic cavity of eight-to-nine-day specific pathogen free (SPF) embryonated hen eggs, they were

further incubated for five days at 35°C. The allantoic fluids were then harvested and tested for haemagglutinating activity. Negative fluids were further passage twice.

The positive HA cases were titrated by HA test and 4 HA units were used for subtype identification by HI test with both H3 and H7 reference antiserum against A/Equi-1 (H7N7) and A/Equi-2 (H3N8).

The haemagglutination inhibition (HI) test was also used to detect equine influenza subtype in the collected serum samples against reference antigen of A/Equi-1 (H7N7) and A/Equi-2 (H3N8).

Genotyping by RT-PCR:

The positive cases by real-time RT-PCR were further tested by conventional RT-PCR for subtyping of both HA and NA genes. Briefly, the extracted RNAs were amplified using one step RT-PCR kit (Reddy Mix PCR master

mix, Thermo, UK). PCR assay was done as described by the manufacturer instructions using the primer pairs: eq/H3/9/ [CAG GGG ATA TTT CTG TCA ATC ATG] and eq/H3/1741/ [AGT AGA AAC AAG GGT GTT TTT AAC] for the HA gene (Ilobi et al., 1998) and eq/N8/2/+ [GCA AAA GCA GGA GTT TAA AAT G] and eq/N8/1460/- [GTA GAA ACA AGG AGT TTT TTT CG] for the NA gene (Manuguerra et al., 2000) and run on thermal cycler (ABI-2720). The samples were incubated at 94 °C for 5 min. Then, 30 cycles of amplification were carried out (45 s at 94°C, 45 s at 45°C, 1.5 min at 72°C) followed by a 5 min incubation at 72°C. The size of the PCR products, specific for the H3 are 1730 and for N8 genes are 1460 bp. The products were run on electrophoresis gel with GelPilot 200 bp ladder (Cat no.239055, Qiagen, Germany).

HA gene sequencing:

Nucleotide sequence analysis of the HA gene was conducted to identify the subtype of the

RESULTS

Epizootiological investigations:

Clinical investigations among diseased animals revealed symptoms ranged from mild to moderate severity usually with rising in rectal temperature (39°C-41°C), redness of the nasal mucosa, and in most of the affected cases there were conjunctivitis, serous to mucopurulent nasal discharge and a harsh dry cough, increase in the intensity of the normal breath sounds. Depression, anorexia and reluctance to move were common signs.

isolated virus. The PCR products of the HA gene fragments (1730 bp) were directly sequenced. Partial nucleotide sequencing of the HA gene was carried out. The amplified HA products were purified using a PCR purification kit (Qiagen, Hilden, Germany) and sequenced by using the forward and reverse primers: eq/H3/9 and eq/H3/1741 by using a BigDye Terminator v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI-3130; Applied Biosystems). The sequenced fragments of HA gene of the Egyptian isolate were compared to other strains isolated worldwide by using BLAST tool of NCBI.

Phylogenetic analysis was carried out on HA gene. Multiple and pairwise sequence alignments were constructed using the ClustalV algorithm and a phylogenetic tree were constructed using the neighbour-joining of MegAlign program from LaserGene Biocomputing Software Package (DNASTAR, Madison, WI).

Morbidity was about 95% and was no mortality. The affected race horses were under stress, as a result of heavy and continued work and were suffered from more severe clinical symptoms and low performance.

Results of Real time RT-PCR (rRT-PCR):

Real-time RT PCR was used to detect the M gene of Equine influenza virus in nasal swab samples and the results were positive for 6 affected equine populations in 5 governorates

(Cairo, Giza, Aswan, Helwan and Menofia) with a total of 83 out of 107 swab samples

(77.6 %) as shown in Tables (2).

Table (2): The Results of rRT-PCR for EIV:

Site	Swabs	positive	Negative
Proke hospital	5	-	5
Zahraa Ein Shams	10	7	3
EL Mansoria	7	4	3
Edfo	31	31	-
VACSERA farm	31	31	-
Shebene-Elkoom	5	4	1
Abbasia	11	6	5
EL Mahmodia	2	-	2
Dammhour	5	-	5
Total	107	83 (77.6%)	24 (22.4%)

Virus isolation, identification and subtyping:

Positive RRT-PCR samples were subjected for viral isolation in SPF chicken embryos, 3 cases out of the 6 were positive rRT-PCR cases (Zahraa Ein Shams, Edfo and VACSERA farm) had HA activity detected in the allantoic fluid after the first passage. The HA titer of the isolated viruses ranged from 5-7 log₂.

The 3 positive HA cases were identified by HI test with both H3 and H7 antisera and were positive only with H3 antisera.

The serum samples of the 6 positive cases by rRT-PCR were subtyped by HI test with both H3 and H7 antigens and were positive only with H3.

Results of RT-PCR for H3 and N8 genes:

The 6 positive cases by real-time RT-PCR were further tested by conventional RT-PCR for subtyping of both HA and NA genes. All the 6 cases tested were positive for both H3 and N8 gene specific PCR primers. The positive result of H3 gene detection were indicated at 1730 bp, while the N8 gene indicated at 1460 bp. Only one isolate (A/Equine/Egypt/21AHRI/2008(H3N8)) was selected for sequencing of H3 gene.

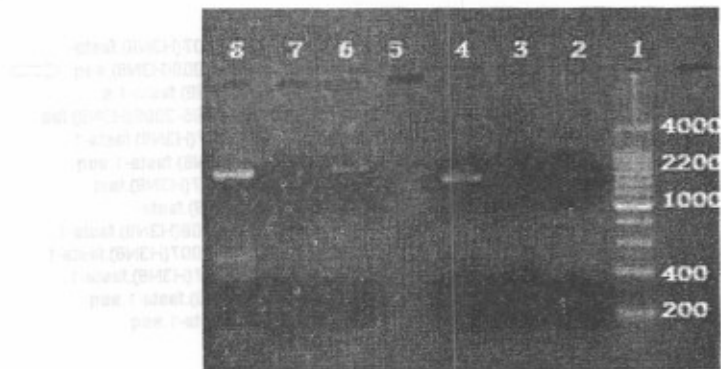


Photo1. Results of RT-PCR for H3 and N8 genes; lane 1 DNA ladder, lanes 2, 3 and 4 represent N8 gene detection where the positive result indicated at 1460 bp, lanes 6, 7 and 8 represent H3 gene detection where the positive result indicated at 1730 bp. Lanes 2 and 6 are positive controls of N8 and H3 respectively. Lanes 3 and 7 are negative controls of N8 and H3 respectively. Lanes 4 and 8 the positive isolate(A/Equine/Egypt/21AHRI/2008(H3N8)). Lane 5 no template controls.

HA gene sequencing and phylogenetic analysis:

The results of partial HA gene sequencing for the PCR products of the HA gene fragments (1730 bp) were directly sequenced by using the forward primers (eq/H3/9) produced 600 nucleotides sequences for the first part of the

H3 gene and by using the reverse primer (eq/H3/1741) produced 800 nucleotides sequences from the end of the gene. After comparison of the obtained sequences by the available international strains from the genbank by using BLAST tool of NCBI revealed that the HA gene of the Egyptian strain (A/Equine/Egypt/21AHRI/2008(H3N8)) of the equine influenza virus was very closely related to H3N8 subtype of American strains from California, Florida and Pennsylvania in 2006 and 2007 by identity percent reached to 98% and other strains circulated in Asia in last 2 years like in Japan and China and also European strains of H3N8 from UK and Italy isolated from 2003 till 2007 by identity percent ranged from 95.6 to 98%.

(Figures 1 and 2).

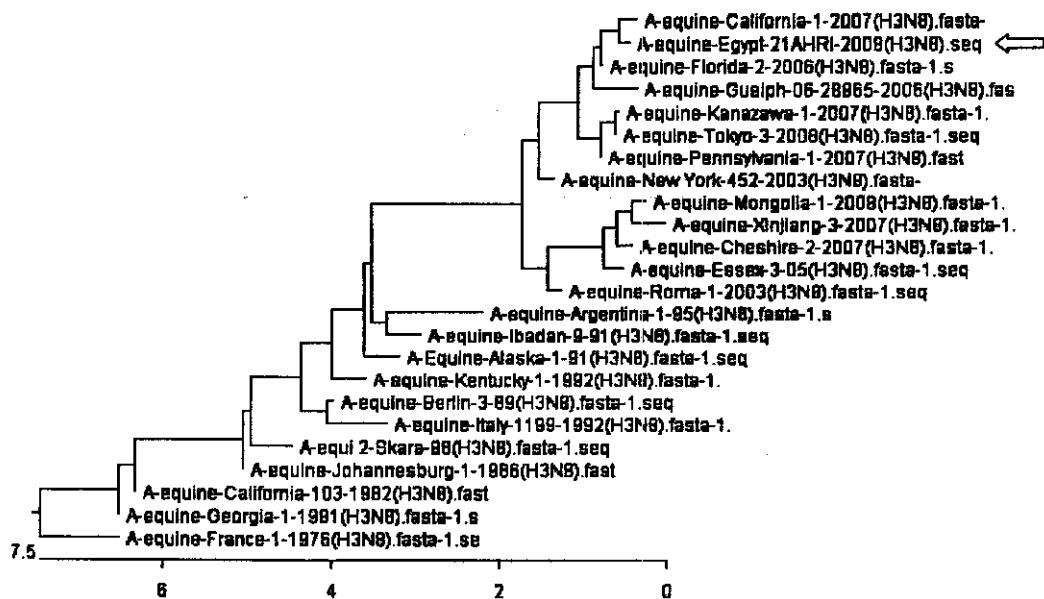


Fig.1. Phylogenetic analysis of Egyptian strain (A/Equine/Egypt/21AHRI/2008(H3N8)) compared to other strains from America, Europe and Asia. The analysis was done by using Clustal V method of DNA star software.

		Percent Identity																									
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
Divergence	1	100	97.7	98.2	97.0	95.0	96.8	95.3	93.7	95.3	95.7	96.8	92.0	88.9	96.2	87.2	93.8	95.8	93.8	93.8	95.5	95.2	95.4	92.5	92.8	1	
	2	2.3	100	98.3	94.7	96.4	96.7	96.4	94.9	96.8	94.4	96.8	95.0	99.9	97.5	87.3	96.6	87.9	84.7	96.5	87.0	97.2	97.0	96.1	94.6	2	
	3	2.8	2.2	100	93.0	95.8	94.3	95.6	94.2	95.0	94.4	94.4	82.3	87.5	95.0	94.7	94.0	95.4	93.9	93.8	96.1	98.8	98.0	92.7	93.3	3	
	4	1.4	1.4	2.5	100	93.8	97.7	93.7	92.3	94.1	95.7	97.6	95.7	98.2	99.2	98.8	96.1	98.7	95.6	98.6	94.2	92.9	93.1	95.3	98.8	4	
	5	5.0	3.5	4.3	4.2	100	94.0	87.5	87.7	90.5	81.8	83.8	98.7	96.3	95.0	95.3	98.8	97.3	98.8	98.0	98.3	98.0	97.8	98.4	98.4	5	
	6	1.7	3.1	3.8	2.4	6.1	100	94.3	92.6	94.3	98.1	99.8	94.4	97.3	97.4	98.9	94.5	97.8	81.2	95.5	94.4	93.1	94.2	94.8	91.6	6	
	7	4.8	3.7	4.4	4.4	2.5	5.8	100	98.4	97.8	92.0	94.2	97.4	98.3	94.8	95.8	97.8	98.1	96.5	98.1	97.7	98.8	98.8	98.2	98.4	7	
	8	4.7	3.8	4.2	4.2	2.3	5.8	0.6	100	90.4	92.5	95.7	94.9	93.3	93.8	98.2	94.4	98.3	96.4	97.9	97.3	97.8	97.1	98.8	98.8	8	
	9	4.8	3.0	4.0	3.9	0.5	5.7	2.2	2.0	100	92.1	94.2	99.2	96.8	95.3	95.6	98.4	98.2	97.8	98.1	98.5	98.8	98.5	97.3	98.8	98.8	9
	10	3.9	6.2	5.8	4.3	8.3	1.9	8.1	8.0	7.9	100	98.3	92.8	94.9	95.8	97.1	92.8	95.7	98.8	93.7	92.2	92.8	92.0	92.8	98.8	98.8	10
	11	1.8	3.2	3.9	2.5	6.2	0.2	6.0	5.9	5.8	1.8	100	94.3	97.2	97.3	98.8	94.4	97.4	91.1	95.4	94.3	93.2	94.1	94.5	91.8	91.1	11
	12	5.8	3.8	4.8	4.2	1.2	5.5	2.5	3.0	0.7	7.2	5.8	100	95.3	95.8	98.8	95.8	94.1	98.5	98.1	95.0	98.8	98.8	98.0	98.0	98.0	12
	13	2.5	1.0	1.5	1.8	3.7	2.8	3.8	3.8	3.2	4.8	2.9	3.8	100	98.4	98.2	98.5	95.3	97.2	98.7	98.2	98.8	98.3	94.4	94.4	94.4	13
	14	2.3	2.3	3.2	0.8	5.1	2.8	5.3	5.1	4.8	4.5	2.7	4.6	2.1	100	98.5	95.4	98.3	92.0	98.5	95.4	93.8	95.3	95.4	93.1	93.1	14
	15	0.5	1.8	2.8	1.1	4.6	1.1	4.4	4.4	4.3	2.8	1.2	4.3	1.7	1.5	100	95.7	98.7	92.3	98.7	95.8	93.7	95.7	95.8	92.8	95.8	15
	16	4.8	3.0	4.0	3.8	1.1	5.2	2.4	2.4	0.8	7.0	5.4	1.2	3.5	4.4	4.1	100	95.9	94.6	98.8	98.8	98.4	100.0	97.2	97.5	96.8	16
	17	1.8	1.2	2.0	1.3	4.8	2.5	3.8	3.7	3.7	4.3	2.8	4.1	1.5	1.9	1.4	3.8	100	96.9	96.4	94.2	98.3	98.1	93.8	93.8	17	
	18	4.8	3.8	4.5	4.5	2.8	5.8	0.5	0.7	2.3	8.1	8.0	3.2	3.8	5.2	4.8	2.5	3.8	100	94.9	97.5	98.8	97.2	98.4	98.2	18	
	19	4.1	2.5	3.4	3.4	1.4	4.5	1.8	1.8	0.8	8.3	4.8	1.3	2.8	3.8	3.3	1.1	3.1	2.8	100	98.4	98.9	97.8	97.5	97.5	97.5	19
	20	4.5	2.8	3.9	3.8	1.0	5.6	2.3	2.1	0.5	7.8	5.7	0.8	3.3	4.7	4.2	0.1	3.8	2.4	1.0	100	98.7	98.0	97.2	97.2	97.2	20
	21	3.5	3.4	3.2	3.0	1.7	5.0	1.2	1.1	1.2	7.1	5.1	2.1	2.8	4.3	3.7	1.5	3.2	1.4	0.9	1.3	100	98.8	95.8	98.7	98.7	21
	22	4.7	3.0	4.1	4.0	1.1	5.8	2.5	2.4	0.8	8.0	5.9	1.0	3.4	4.8	4.4	0.0	3.7	2.7	1.1	0.1	1.4	100	97.8	97.5	22	
	23	5.3	4.1	4.7	4.8	3.0	5.4	0.8	1.1	2.6	7.2	5.6	3.0	3.8	4.7	4.2	2.8	4.0	0.8	2.0	2.7	1.5	2.9	100	95.6	23	
	24	8.1	4.1	5.3	5.8	0.2	7.0	2.4	2.2	0.3	8.7	7.0	0.7	4.3	5.7	5.7	1.2	4.8	2.5	1.2	1.8	2.0	1.2	3.2	100	24	

Fig.2. Identity percent of Egyptian strain (A/Equine/Egypt/21AHRI/2008(H3N8)) compared to other strains from America, Europe and Asia. The analysis was done by using Clustal V method of DNA star software.

DISCUSSION

Although influenza viruses are endemic in many countries and circulate continuously in the equine population, explosive outbreaks occurred at intervals of several years when the immunity of the equine population wanes, and sufficient antigenic drift in the virus has occurred, allowing the virus to evade vaccinal immunity (OIE, 2008).

Historically, equine influenza was recorded and notified in Egypt to the world organization for animal health (OIE) in 1989 and 1999/2000 and finally in summer 2008.

Equine influenza is highly contagious and the virus spreads rapidly through groups of horses in aerosolized droplets dispersed by coughing. The severity of clinical signs depends on the degree of existing immunity, among other factors. Horses that are partially immune can become sub clinically infected and shed virus. (Lai et al., 2001)

From the epizootological point of view, Equine influenza outbreak in Egypt started in July 2008 in Alexandria governorate "Proke Hospital" as many affected cases specially drought horses the signs in the form of high temperature and nasal discharge. The spread of the disease from Alexandria to Cairo, Giza, Assiut, Helwan, Aswan, Menofia, Cairo and finally in Behira governorate in August 2008 was rapid.

No vaccination policy against EIV in Egypt. The highly spreading of the infection due to many factors as highly susceptible none

vaccinated horses, aerosol infection and importation of equine from endemic countries. The main clinical signs in most affected horses in different governorates were pyrexia, rising of temperature, low performance, and nasal discharge as the same signs were recorded by (Radostits et al., 2000).

Equine influenza is spread via aerosolized respiratory secretions and fomites, including contaminated inanimate objects and people moving between infected and uninfected horses. The most common source of infection and outbreak is the introduction of a new animal into the herd. The incubation period is usually one to three days. Morbidity associated with EI in naïve populations is estimated at 60 to 90%; (OIE, 2008).

In the present study, the successful detection of Equine influenza virus was achieved by real time PCR and confirmed by other serological and virological diagnosis. The isolated virus of Egyptian strain (A/Equine/Egypt/21 AHRI/2008 (H3N8)) of Equine Influenza virus was successfully sub typed by HI test and confirmed by HA and NA subtype specific RT- PCR and also by sequencing of the HA gene and found that it was very closely related by identity percent reached to 98% to H3N8 subtype of American and some other Eurasian strains circulated in last 2 years 2006-2007 that may give a speculation that the transportation of live animals may play an important role in spreading the infection from

region to another and the disease was wide spread during short period and recorded in different governorates in Upper and lower Egypt in short time. As well as cross species transmission was also reported from infected dogs that may help in the transmission of the disease. (Crawford et al., 2005).

In conclusion, this paper documents the most characteristic features of equine influenza virus outbreak in Egypt in summer 2008 and describes the accurate and rapid diagnosis of the field cases by real-time RT-PCR. Virus isolation and full characterization of the isolated virus from clinically infected cases through the conventional tests and finally by molecular techniques for typing of hemagglutinin (HA) and neuraminidase (NA) genes and proteins by RT-PCR and HI tests as well as partial sequencing of the HA gene of

one isolate (A/ Equine /Egypt / 21AHRI /2008 (H3N8)), for the first time in EIV outbreak in Egypt. The results confirmed that H3N8 virus was the causative agent of this outbreak. The HA gene sequencing results and phylogenic analysis help to understand the genetic characteristics of the causative agent and to study the epizootiological features of the recorded 2008 outbreak in Egypt.

Our recommendation that the exhibition of our *Equidae* to infection from time to time with EIV stimulate us for production of national vaccine from the field isolates to protect these wealth from the threat of the EIV, in addition to the application of restriction regulation for the importation of equines.

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التوصيف الوبائي و الجزيئي لوباء فيروس انفلونزا الخيول في مصر لعام ٢٠٠٨

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الملخص العربي

تم رصد عراض انفلونزا الخيول في التجمعات الخيلية في المحافظات الاتيه بجمهورية مصر العربية(القاهرة-الجيزة-حلوان-اسكندرية-البحيره-المنيا-اسيوط-واسوان) خلال شهرين يوليو و اغسطس عام ٢٠٠٨ وهي ارتفاع درجة الحرارة-فقد الشهية-التهاب القرانية-احمرار مع وجود افرازات انفيه تصل الي المخاطية الصديدية وكحة جافة.الخيول المصابة كانت من جميع الاعمار وامن الجنسين. نتيجة حركة الحيوانات المصابة والاتصال المباشر في الاسواق ساعد علي الانتشار السريع للمرض. تم فحص ٩ حالات من ٨ محافظات لفيروس انفلونزا الخيول حيث استخدمت ١٠٧ مسحة انفية و ١٠٧ عينة سيرم من الحيوانات المصابة للتشخيص. تم عمل اختبار انزيم البلمرة العكسي باستخدام جين A للمسحات وكانت هناك ٦ حالات ايجابية من ٨. وقد تم عزل الفيروس علي البيض من ٣ حالات و اجراء الاختبار التلزن الدموي وعمل اختبار التلزن الدموي المثبط للتعرف علي الفيروس الانفلونزا باستخدام اجسام مضادة للانتجين. في هذه الدراسة تم توصيف الفيروس من خلال الاختبارات البيولوجيه الجزيئية كما تم عمل تتابع للجين مما يدل ان فيروس H3N8 هو المسبب لهذا الوباء.