Animal Health Research Journal Vol. 7, No. 2, June 2019 pp. 74-82

ISSN: 2356-7767

Some studies on *Equine Influenza virus* in Menofiya Governorate

Khamees, A.K.S.*; Wafaa, A. Hosny and Suzan, S.M.*****

Animal Health Research Institute, *Shebeen El-Kome Branch; **Elisa and Virus Strains Bank Unit, *** Shebeen El-Kome Branch

Received in 9/6/2019
Accepted in 14/7/2019

Abstract

Background: Equine Influenza virus (EIV) is a major pathogen of respiratory diseases in horses, donkeys and mules. It is characterized by a very rapid spread and remains a disease with high economic stakes for the equine industry. The disease is OIE listed A&B disease of equines, ponies, mules and donkeys. The rapid diagnosis of EIV is critical to the implementation of control measures.

Objectives: Studies on equine influenza are rare in Menofiya Governorate. Therefore, this work aimed to virological and molecular study on equine influenza virus since 2017 in Menofiya Governorate.

Methods: Serum samples from a total 50 horses, donkeys and mules showing acute clinical signs were tested using the ID Screen Influenza A Antibody Competition ELISA which detects antibodies against the EI viral nucleoprotein (NP). A total of 50 nasopharyngeal swabs were used for virus isolation in allantoic cavities of specific-pathogen free (SPF) embryonated chicken eggs, 9- 11 day old. Isolated virus was identified by Haemagglutination assay. Further identification was carried out by Real-time RT-PCR and haemagglutination inhibition assay.

Results: On testing of collected serum samples from 50 horses, donkies and mules by using ID Screen Influenza A Antibody Competition ELISA, the number of total positive samples was 20 samples from 50 tested samples. Out of 50 samples were inoculated for four passages on embryonated chicken eggs, 5 isolates were positive and showed haemagglutinating activity. By using Real-time RT-PCR on five samples, there are two samples are positive. The two positive RT-PCR samples were positively identified as equine H3N8 influenza viruses by haemagglutination inhibition assay.

Conclusion: Rapid and specific etiological diagnosis is important for early detection and control of influenza in horses. The sensitive rapid ELISA may be useful screening test in the exposure and management of EI. Like other influenza A viruses, equine influenza virus undergoes antigenic drift, therefore isolation of the virus and molecular identification are important.

Keywords: *Isolation; Molecular detection; ID Screen Influenza A Antibody Competition ELISA; Equine Influenza virus and El Menofiya, Egypt.*

Introduction

Equine influenza is a highly contagious, infectious upper respiratory disease of horses and other equidae characterized by pyrexia, anorexia, dry cough, dyspnea, and nasal discharges **(van Maanen and Cullinane, 2002)**.

Equine influenza is caused by the equine influenza virus (EIV), an RNA virus, which belonging to the family Orthomyxoviridae, genus Influenza A **(Virmani, *et al.,* 2010)**. on the basis of antigenic characteristics of haemagglutinin and neuraminidase, there are two EIV subtypes H7N7 (A/Equi 1/H7N7) and H3N8 (A/Equi 2/ H3N8) are recognized to have been established in horses, co-circulating for nearly two decades, the H7N7 subtype which was first isolated in horses in 1956 has not been in circulation

since 1978, however, serological evidence of this virus subtype has been reported in India in the recent past. The H3N8 (avian-origin influenza) which was first isolated in 1963 has continued to spread panzootically among horses **(Newton *et al.,* 2006 ; Gildea *et al.,* 2012)**. To date, H3N8 subtype have been the cause of all outbreaks of Equine influenza since 1979 **(Gildea *et al.,* 2013)**.

Equine influenza A (H3N8) viruses, initially, evolved as a single phylogenetic lineage (**Kawaoka *et al.,* 1989**). Two phylogenetic lineages, the American and Eurasian lineages, named according to the geographic origin of the isolates, emerged in the late 1980s **(Daly *et al.* 2011)**. Using viruses isolated in North America, the American lineage divergence into three sublineages: a South American lineage, a Kentucky lineage and a Florida lineage **(Lai *et al.* 2004)**. Further evolution of the Florida lineage (or sublineage, as it is now known) has resulted in the emergence of two groups of viruses with divergent HA sequences: Florida sublineage clade 1, containing influenza A/Equine/ Wisconsin/03 (A/eq/Wisconsin/03)-like viruses, and Florida sublineage clade 2, containing A/eq/ Newmarket /5/ 03-like viruses (**Lewis *et al.,* 2011**).

Equine influenza is endemic in horse populations in many countries worldwide and which occurs sporadically in epidemic form time to time. Countries free from equine influenza include Iceland, Australia and New Zealand. Outbreaks are possible and occur in endemic countries (**Tu *et al.,* 2009 and Gildea *et al.,* 2013).** The risk associated with the increase in the international movement of horses by air transport for racing and breeding purposes means that it is imperative that there are sensitive virus detection systems available for the rapid diagnosis of equine influenza (**Timoney, 1996**).

Equine influenza diagnosis is based on the clinical signs (fever, dry deep cough, nasal discharge) and the infection is confirmed by virological examination **(Rozek *et al.,* 2011)**. Equine influenza virus isolation is done by inoculation of embryonated chicken eggs into the allantoic or amniotic cavity (**Yondon *et al.,* 2013**) or by inoculation of the Madin–Darby canine kidney (MDCK) cell line (**Liu *et al.,* 2009**). The detection of virus directly in secretions may be done by reverse-transcription polymerase chain reaction (RT-PCR) assays or by commercial diagnostic kits (**Donofrio *et al.,* 1994**). Serological diagnosis of equine influenza relies on detection of an increase in the titre of specific antibodies in paired serum samples (the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2 weeks later). The haemagglutination inhibition test and the single radial haemolysis test are most frequently used in serological diagnosis of equine influenza.

Nashwa, *et al.,* (2004) isolated EIV which was designated as A/equi-2/Cairo-2/2000. **Abd El-Rahim and Hussein (2004)** described an epizootic of respiratory tract disease caused by ifluenza virus in a large populationin Luxor and Aswan governorates. **Ahmed, *et al.,* (2011)** made sequence analysis of 373 bp fragment of the haemagglutinin gene of the isolated Egyptian EIV A/Equine/Egypt/ URLCU/2008(H3N8). **Hussein, *et al.,* (2011)** isolated EIV which designated as A/Equine/ Egypt/ URLCU/2008(H3N8).

The present study was carried in El Menofiya governorate due to appearance of respiratory symptoms between horses, donkeys and mules so rapid diagnostic assay for EIV using different type of clinical samples was applied for disease management. In this study trial to isolate and identify Equine influenza virus in Menofiya governorate, Egypt during 2017-2018.

Materials and Methods.

Collection of samples:-

A total of 50 horses, donkies and mules of unvaccinated, all ages, different breeds and of both sexes showing acute clinical signs were

sampled under proper restraint during 2017 and 2018 from different localities in Menofiya governorate.

Nasopharyngeal swabs were collected in medium containing phosphate buffered saline (PBS) containing either 40% glycerol or 2% tryptose phosphate broth with 2% antibiotic solution (penicillin [10,000 units], streptomycin [10,000 units] in sterile distilled water [100 ml]), and 2% fungizone (250 mg/ml stock)), **(Quinlivan *et al.,* 2004).** These samples were immedietely submitted to Animal Health Research Institute where they were centrifuged at 3000 r.p.m for 5 minutes and the supernatant were preserved in -80°C till used for virus isolation and identification **(Kitching and Donaldson, 1987).**

Blood samples were collected and prepared by centrifugation at 1500 r.p.m for 10 minutes for serum separation. The serum samples were stored at -20°C until they were tested by ID Screen Influenza A Antibody Competition ELISA, (**Coles, 1986).**

Influenza A Antibody Competition ELISA:-

It was applied in Virology department, Animal Health Research Institute, Egypt.

The competition ID Screen Influenza A Antibody Competition Multispecies ELISA for the detection of antibodies to the internal nucleocapsid of the Influenza A virus in bird, pig and horse sera was carried out in accordance with the manufacturer's instructions (ID Vet Innovative Diagnostics, Montpellier, France). The wells of the test plate were already coated with Antigen A. **(Kittelberger *et al.,* 2010).**

The competition percentage was calculated for each sample tested.

$$\text{Competition \%} = \frac{\text{OD sample}}{\text{OD NC}} \times 100$$

The manufacturer's suggested interpretation of the results is as follows: the samples of a Competition % ≤45% considered Positive, samples of Competition % > 45% and < 50% are doubtful but samples of a Competition % ≥ 50% considered Negative. A sample that produced a doubtful result was classified as negative. A seroconversion was defined as a decrease in competition percentage of 45% or more, or a change from seronegative to seropositive between acute and convalescent samples.

Virus isolation and haemagglutinating (HA) activity:-

It was applied in ELISA unit and viral strains bank, A. H.R. I., Dokki, Giza, Egypt.

Nasopharyngeal swabs were inoculated into allantoic cavities of 10- day- old specific-pathogen free (SPF) embryonated chicken eggs for four passages. The allanoic fluids were harvested and tested for haemagglutination activity according to **(OIE, 2012)**. The hemagglutination positive samples were confirmed by real -time RT–PCR.

Real-time RT–PCR (Quinlivan *et al.*, 2005):-

RNA extraction. RNA extraction from samples was done using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH). Briefly, 140 µl of the sample suspension was incubated with 5.6 µl of carrier RNA and 560 µl of AVL lysis buffer at room temperature for 10 min. After incubation, 560 µl of absolute ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 60 µl of AE elution buffer provided in the kit.

Oligonucleotides. Primers and probes used to amplify **AI (M) gene** were synthesized by Metabion (Germany) according to **Slomka *et al.*, 2007**.

Sep1: AGATGAGTCTTCTAA CCGAGGTCG
Sep 2: TGCAAAAACATCTTC AAGTCTCTG
SEPRO: [FAM] TCAGGCCCC CTCAAAGCCGA [TAMRA]

DNA amplification. PCR amplifications were performed in a final volume of 25 µl containing 3 µl of RNA template, 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 8.625 µl

PCR grade water, 0.25 µl of each primer (50 pmol conc.) and 0.125 µl of each probe (30 pmol conc.) and 0.25 µl of QuantiTect RT Mix. Reverse transcription was done at 50oC for 30 min. followed by primary denaturation at 94 oC for 15 min, followed by 40 cycles of denaturation at 94 oC for 15 s, annealing and extension at 60 oC for 20 s. The reaction was done in stratagene MX3005P real time PCR machine.

Haemagglutination inhibition assay (HI):-

It was applied in ELISA unit and viral strains bank, A. H.R. I., Dokki, Giza, Egypt.

It was performed according to the standard OIE procedures **(OIE, 2000).**

Results

ID Screen Influenza A Antibody Competition ELISA:-

On screening of serum samples from 50 horses, donkies and mules for antibodies against the internal nucleocapsid of the Influenza A virus by ID Screen Influenza A Antibody Competition ELISA, a number of 20 samples out of 50samples were positive **(Table 1).**

Virus isolation and Haemagglutinating activity:-

The prepared nasopharyngeal samples collected from clinically suspected horses, donkies and mules at Menofiya governorate during 2017 and 2018 were inoculated in embryonated chicken eggs. It was found that out of 50 nasopharyngeal samples, 5 samples showed haemagglutinating activity after isolation on embryonated chicken eggs for four passages in embryonated chicken eggs **(Table 1).**

Real-time RT–PCR:-

It was found that out of tested samples there are two samples positive to EI virus with CT (cycle threshold) value ranged from 30-35 cycles as shown in **Table (1) and Figure (1).**

Haemagglutination inhibition assay:-

It was found that out of tested samples there are two samples positive to equine H3N8 influenza viruses as shown in **Table (1).**

Table (1). Results of ELISA test, haemagglutination, Haemagglutination inhibition and Real time PCR:-

No. of samples	Type of test	Result		
		+ve	-ve	Positive %
50 Serum samples	ELISA test	20	30	40%
50 Nasopharyngeal swabs	HA	5	45	10%
	R.T. PCR	2	48	4%
	HI	2	48	4%

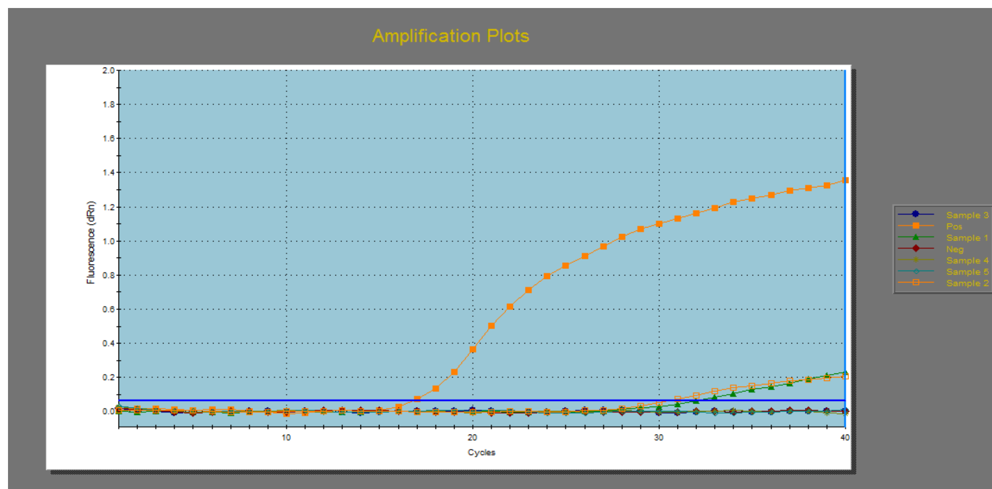

Figure 1. Amplification curves of sensitivity test of EI virus by using real-time PCR that showing positive results for only two. Positive CT value ranged from 30-35cycle and negative CT value considered 40 cycles.

Discussion

Equine influenza is an important equine respiratory pathogen and a high priority disease for the equine industry globally **(van Maanen and Cullinane, 2002 and Meseko *et al.,* 2016)**.

The causative agent, equine influenza virus, has a global distribution, belonging to the family of the Orthomyxoviridae, genus Influenza virus, type A and is a major cause of respiratory diseases in horses. Only two antigenic subtypes of EIV (H7N7 and H3N8) have been isolated from horses, although highly pathogenic avian influenza virus H5N1 was isolated from donkeys in Egypt **(Abdelmoneim *et al.,* 2010 and OIE, 2016).** Viruses of the H7N7 subtype have not been isolated since the late 1970s.

Since an outbreak of EI can cause severe economic losses to the horse industry due to the cancellation of equestrian events and the implementation of movement restrictions, rapid diagnostic tests were required to facilitate the implementation of effective control measures (**Gildea *et al.,* 2013; Meseko *et al.,* 2016).**

A clinical diagnosis of equine influenza can be confirmed in the laboratory using one of several different techniques. Diagnosis may be based on virus isolation, serological response to the infection as demonstrated by a significant rise in antibody levels in paired serum samples collected approximately two weeks apart, or antigen or genome detection **(OIE, 2012).**

The success of virus diagnosis largely depends on the quality of the specimen and the conditions for transport and storage of the specimen before it is processed in the laboratory **(Watson *et al.,* 2007)**. Specimens for isolation of respiratory viruses in cell cultures or embryonated chicken eggs and for the direct detection of viral antigen or nucleic acids should generally be taken during the first 3 days after onset of clinical symptoms for influenza (**Lennette, 1995.)**Samples required for the diagnosis of equine influenza include nasopharyngeal swabs for virus detection or clotted bloods for the detection of antibodies directed against the virus **(OIE, 2012)**.

Nasopharyngeal swabs should be collected as early as possible after the onset of fever to isolate or detect the virus **(Timoney, 1996)**. A delay in veterinary intervention means that a low rate of isolation may result as peak shedding may be missed **(Gildea *et al.,* 2011).** Native horses may shed the EI virus for 7-10 days after infection. Partially immune horses only shed the virus transiently **(van Maanen and Cullinane, 2002)**. On collection the nasopharyngeal swab must then be immersed in cool viral transport medium (VTM) and transported as quickly as possible to the laboratory.

In the present study, a nasopharyngeal swabs, from 50 clinically suspected horses, donkies and mules, showing acute clinical signs, of all ages, different breeds and of both sexes at dif-

ferent localities of Menofiya Governorate, were collected and transferred to a tube containing transport medium immediately and were preserved at -70°C at Animal Health Research Institute till used**.**

The ELISA is a useful supplementary test for the diagnosis of EI **(Kirkland and Delbridge, 2011)**. The ID Screen Influenza A Antibody Competition ELISA can detect the rise in antibodies earlier than the single radial haemolysis test suggesting that in native populations this ELISA could provide a faster diagnosis than traditional serology methods **(Galvin *et al.,* 2013)**.

In this study, examination of serum samples from 50 horses, donkies and mules indicated that 20 samples out of 50 samples were positive as shown in **table (1).**

Virus isolation is a highly sensitive and very useful technique for the diagnosis of viral infection when used with clinical specimens of good quality. In fact, isolation of a virus in eggs or cell culture along with subsequent identification by immunologic or genetic techniques (or by electron microscopy) are standard methods for virus diagnosis. One important advantage of virus isolation is that the virus is available for further antigenic and genetic characterization, and also for vaccine preparation or drug-susceptibility testing if required **(Kirkland *et al.,* 2011 and OIE 2012).**

In the present study, 50 nasopharyngeal samples were passaged for four passages in the allantoic cavities of 10- day old specific-pathogen free (SPF) chicken eggs. The allantoic fluids were harvested and tested by hemagglutination using using 1% sheep red blood cells (**OIE 2012**). The hemagglutinin (HA) protein agglutinates erythrocytes; hence, the derivation of its name. The traditional method for identifying influenza field isolates takes advantage of this property (**Shortridge and Lansdell 1972).**

Out of 50 passaged samples 5 samples were positive by haemagglutination test as shown in **table (1).** These results come in agree with the study applied by **Watson *et al.,* (2011)**

Polymerase Chain Reaction (PCR) is a powerful technique for the identification of influenza virus genomes even when they are present at very low levels.

RT-PCR is the most sensitive technique available for the detection of EI. This was consistent with previous studies (**Foord *et al.,* 2009; Read *et al.,* 2012).** This test also appears to be one of the most specific, based on the low proportion of false positives.

Real time RT-PCR was used for identification of five samples using Oligonucleotide specific primers and probe to amplify specific sequence related to EI virus. It was observed that two samples from the five tested samples were positive with CT (cycle threshold) value ranged from 30-35 cycles as shown in **table (1)** and **Figure (1)**.

The HA subtype of new isolates of equine influenza viruses may be determined by haemagglutination inhibition using H7N7- and H3N8-specific antisera. Isolates may first be treated with Tween 80/ether, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity. Standard antigens must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/ Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A /eq/ Newmarket/2/93, and A/eq/ South Africa/4/03 and A/eq/Richmond/1/07). Virus strains may be obtained from OIE Reference Laboratories. The standard antigens should be treated with Tween 80/ether to avoid cross-contamination (**OIE 2016**).

Using haemagglutination inhibition assay on the two positive RT-PCR samples, the two samples were positively identified as equine H3N8 influenza viruses as shown in **table (1).** These results come in agree with **Hannant and Mumford (1996)** who said that the identity of the haemagglutinating agent is confirmed in haemagglutination inhibition (HI) tests using specific antisera to H7N7 and H3N8 viruses.

An improved understanding of the distribution of the horse population, horse movements and other contacts between horse premises in El Menofiya governorate are required to better

inform risk prediction of the course of a future outbreak of equine influenza (or another EAD affecting horses such as West Nile /Kunjin virus or African horse sickness). A consistent national approach to the collection of data on the distribution and demographics of the Egyptian horse population is required to enable rapid identification of the population at risk in any future outbreaks of emergency animal diseases affecting horses.

Conclusion

Equine influenza A H3N8 viruses continue to cause serious diseases in horses despite control measures, including quarantine and vaccination, and the international spread of the virus occurs during exchanges and participation horses in competitions. Moreover, monitoring antigenic drift and emergence of new strains that allow the production of effective vaccines is critical. Finally, the vaccination of horses by modern and effective vaccines will considered to be a new weapon to control this disease.

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