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Isolation and Identification of Equine Influenza H3N8 Virus from Clinical Cases in Khartoum State, Sudan

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Abstract

In June 2013, a severe respiratory disease with high morbidity 75% (75/100) and low mortality (2%) occurred at the Khartoum Horse Racing Stable (KHRS); nasal discharge "serous to mucopurulent", dry hacking cough and pyrexia were the main clinical signs observed. The one animal subjected to Post mortem examination showed, congestion in lung and liver and myocarditis. During the course of the disease which lasted about one week. 20 out of 23 horses (87%) at Halfaya Stable (Khartoum north), showed clinical signs similar to that of Khartoum Stable, but none of the horses died. Equine influenza H3N8 virus was isolated from lung and nasal swab samples inoculated into allantoic cavity of 10 day embryonating chicken eggs. The virus was confirmed serologically by HA & HI tests and by one step RT-PCR. The results of the study confirmed the presence of influenza a virus in horses in Khartoum state as well as the isolation of the virus for the first time in the country from clinical cases.

Keywords

Equine Influenza, Clinical Sign, One - Step RT - PCR

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1. Introduction

Equine influenza (EI) virus is a highly infectious disease affecting equines and it is endemic in the horse populations in countries other than Iceland and Newzealand (Cowled *et al*., 2009). The disease is caused by the genus *Influenzavirus* A of the family *Orthomyxoviridae*. Two subtypes H3N8 and H7N7 have been isolated from horses. The H7N7 subtype was first identified in horses in Czehoslovakia in 1956 (Sovinova *et al.,* 1958), However, this subtype appears to be distinct and the last published isolation of an H7N7 virus was in Egypt in 1989 (Ismail *et al*., 1990). The subtype H3N8 is the most common influenza subtype that caused EI in horses. This subtype was first isolated in USA, Miami in 1963 (Wadell *et al*., 1963).

The disease has an incubation period between one and seven days (Paillot *et al*., 2006); and the first symptom appeared is pyrexia followed by a deep dry hacking cough, inappetance and a clear nasal discharge (Barbic *et al*., 2009). The nasal discharge may become mucopurulent, due to secondary bacterial involvement (Happold and Rubira., 2011). In horses used in transportation or exposed to unsuitable climate conditions, the disease characterized by severe cough leading to bronchitis, pneumonia (Glass *et al*., 2002).Transmission of infection occurs principally by droplets from the virus laden cough (Miller, 1965) and possibly by contaminated subjects (Gupta *et al*., 1993). Equine influenza has high morbidity, in many outbreaks infection rates up to 100% have been

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reported, but few animals (5%) die from the disease (Happold and Rubira, 2011; Gue *et al*., 1995).

The virus may be isolated in embryonated hens' eggs or less frequently, in Madin–Darby canine kidney cells (OIE, 2015). In Sudan, EI virus antibodies were serologically detected in serum samples collected from horses in South Darfur (Algezoli and Kheir, 2014). The present study, describes the laboratory diagnosis for the causative agent of severe respiratory signs occurred among horses of Khartoum and Halfaya Stables, Khartoum State.

2. Materials and Methods

2.1. Case History

In June 2013, severe respiratory signs were observed among horses at Khartoum Horse Racing Stable. Affected horses were adult and of different breed (cross and exotic) and sex (male and female). The main clinical signs were dry cough, pyrexia and serous to mucopurulent nasal discharge. 75% (75/100) of the horses of the Stable showed the signs, and 2 of them died. Lesions observed at post mortem were congestion and oedema of the lung, congestion of the liver with fatty changes, splenomegaly and icterus. During the course of the disease which lasted about one week, clinical signs similar to that of Khartoum Stable appeared in horses of Halfaya Stable at Khartoum north. Few days later, 20 out of 23 horses (87%) showed severe respiratory signs, but none died. The following specimens were collected under aseptic condition, and were kept cool till transported to laboratory and used for virus isolation:

Twenty nasal swabs from affected animals of KHRS and Halfaya Stable (ten from each stable), were collected in tubes containing transport medium (consists of PBS containing 2% tryptose phosphate broth with 2% antibiotic solution "penicillin, streptomycin and fungizone" (OIE, 2015). Lung sample from necropsied horse of Khartoum Stable.

2.2. Virus Isolation and Identification

Ten percent homogenate (W/V) of the lung in antibiotic solution was prepared (OIE, 2015). The homogenate suspension and the nasal swabs fluid were centrifuged at 800 g for 5 minutes. The clarified fluid was collected, and used for virus isolation into allantoic cavity of 10 day old embryonating chicken eggs (0.2 ml/egg). Inoculated eggs were incubated at 35°C for 3 days and candled daily. Embryos died within 24hrs were discarded, and eggs containing embryos died more than 24hrs or live embryos were chilled, and their allantoic fluid was harvested and tested for HA activity. Aliquots of each harvest were pooled, and passage into further eggs (up to 4 passages). The presence of EI H3N8 virus in the clarified allantoic fluid of the inoculated eggs was confirmed serologically by HA and HI tests using reference EI virus antigen and antisera (OIE, 2015) and through molecular technique (one-step RT-PCR).

2.2.1. Haemagglutination Test

The haemagglutination test was used to detect equine influenza virus and was carried out according to the method described by OIE (2015). All titrations were carried out in U-shaped microtitre plates. Twenty five microliters of PBS were dispensed into the individual wells of each plate. Twenty five microlitres of the allantoic fluid were added to the wells of the first row and thoroughly mixed. Twenty five microlitres were transferred from row 1 to row 2 and so on to make a two-fold dilution. An extra 25 µl of PBS was added to all wells. Fifty microlitres of 1% chicken red blood cells (RBCs) suspended in PBS were added to all wells including control wells. The microplate was incubated at room temperature for 30 minutes until the cell control wells showed a button of deposited RBCs.

2.2.2. Haemagglutination Imhibition (HI)

The samples showing agglutination of chicken erythrocytes were identified and typed using the HI assay with standard antisera for EIV H3N8 (equine influenza H3N8 American like / Newmarket) Obtained from European Pharmacopeia Reference Standard.

2.3. Molecular Identifications

2.3.1. RNA Extraction

RNA was extracted from the clarified allantoic fluid using QIAamp® Viral RNA Mini kit (Qiagen) following the manufacturer's instructions. The extracted RNA of each sample was divided into 10 µl aliquots and frozen at-80°C till used.

2.3.2. One-Step RT-PCR

One-step RT-PCR was performed using Qiagen one-step RT. PCR kit.

Reverse transcription reactions were carried out under standard conditions using primers: M52C (5′-CTTCTAACCGAGGTCGAAACG-3′) and M 253R (5′-AGGGCATTTTGGA/TCGTCTA-3′), designed (by Invitrogen, Carlsbad, CA, USA,) from highly conserved regions of the matrix gene. The reverse transcription and PCR were performed as described by Fouchier *et al*. (2000), with some modification in the cycling conditions as follows, 30 min at 42°C and 2 min at 94°C once, and then 40 cycles of 15 sec at 94°C, 30 sec at 45°C, and 1 min at 72°C, with a final extention of 3 min at 72°C. PCR products of 244 bp

(matrix primers) were visualized on 1% agarose gels stained with ethidium bromide.

3. Results

Equine influenza (H3N8) virus was isolated from lung and nasal swab samples inoculated into allantoic cavity of 10 day embryonating chicken eggs. The clarified harvested allantoic fluid of the inoculated eggs agglutinated 1% chicken RBCs (HA test). Agglutination was inhibited when reference EI virus antiserum (was used (HI test). Presence of EI virus (5 isolates) in the allantoic fluid was also confirmed by PCR (Fig. 1) and the results showed the same designated molecular size 244 bp (matrix primers).

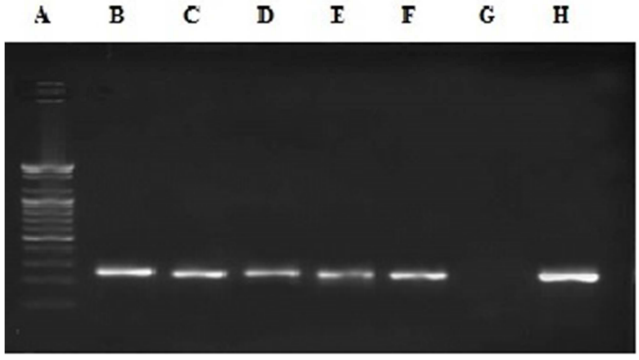

Fig. 1. PCR products of 5 isolates of equine influenza a virus where.

Lane A: DNA marker Ladder
Lane B: allantoic fluid (Lung tissue) Khartoum stable
Lane C and D: allantoic fluid (nasal swab) Khartoum stable
Lane E and F: allantoic fluid (nasal swab) Halfaya stable
Lane G: negative control
Lane H: EI virus positive control

4. Discussion

In this study, a severe respiratory disease with high morbidity (75%) and low mortality (2%) occurred at the KHRS and Halfaya Stable at Khartoum north, with high morbidity (87%), but no mortality was investigated. Observed clinical signs of the disease in this study resembled the previous reports (Gildea *et al*., 2013). EI (H3N8) virus was isolated in allantoic cavity of embryonating chicken eggs and confirmed serologically by HA and HI tests and molecularly by one-step RT-PCR in lung and nasal swab samples collected from infected animals.

Equine influenza has been found to be transmitted more easily within, rather than, between racing stables (Satou and Nishiura, 2006). This type of infection is suggestive of both droplet and contact transmission being the dominant routes of transmission (Rosanowski, 2012). In the present study, contact transmission between infected horses of KRHS and susceptible horses of Halfaya during racing might be the possible route of transmission. Movement and the mixing of infected and susceptible animals is the highest risk activity for the spread of disease (Bates *et al*., 2001). Internationally, the movement of horses and horse products have been identified as key for spreading disease between countries (Sluyter, 2001), and many outbreaks in susceptible populations have occurred through the importation of infected horses (Guthrie *et al*., 1999; Callinan, 2008). The movement of horses between properties, sale yards, race meetings and competitions has been implicated in the spread of the disease (Guthrie *et al*., 1999).

At pasture, the disease in horses has been reported to be less severe than in horses stabled in a dusty environment (Dalglish, 1992). Similar observation was observed in an EI outbreak occurred in Australia in 2007. This observation may partly reflect the closer inspection and monitoring associated with horses that are stabled (EI Epidemiology Support Group, 2009).

During the EI outbreak in the Australian horse population in 2007, some deaths of young foals and mature horses occurred (Begg *et al*., 2001; Patterson-Kane *et al*., 2008). These fatally affected animals had extensive pulmonary lesions (Begg *et al*., 2001; Patterson-Kane *et al*., 2008). However, the pathological lesions in these cases consisted mainly of suppurative bronchopneumonia resulting from secondary bacterial infection, which is considered to be only part of the pathological process of EI (Van Maanen and Cullinane, 2002). In this study, the main pathological lesions observed in 77% of the infected animals were serous to mucopurulent nasal discharge.

In the present study, EI virus was isolated and confirmed by HA, HI and one-step RT-PCR assays. There have been several reports of the use of RT-PCR assays for the detection of influenza virus in clinical specimens; however, such assays were not widely used for the routine diagnosis (Donofrio *et al*., 1994, Oxburgh, and Hagstrom, 1999). This have changed; however, following the introduction of EI virus into Australia in 2007, when an rRT-PCR developed to detect the avian influenza virus matrix gene then, used as the molecular diagnostic method of choice for EI virus (Foord *et al*., 2009; Spackman *et al*., 2002).

5. Conclusion

The present study confirmed the presence of influenza a virus in horses in Sudan and the isolation of the virus from clinical cases for the first time in the country.

Further studies are needed to identify the subtype(s) circulating in Sudan, and the economic impact of the disease on the Sudanese equine breeding.

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