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Rapid Detection Of Equine Influenza Virus By Using Polymerase Chain Reaction

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ABSTRACT

Equine influenza virus is a cause of epizootic respiratory disease of equine. Thirty nasal swabs from horses suffering from respiratory manifestation were tested by reverse transcriptase (RT) PCR using primers specific for HA gene. Primer specificity was tested by using RNA extracted from amnioallantoic fluid infected with local isolate of A/equi-2/Cairo-2/2000 (H3N8). Also virus isolation from previous nasal swabs was performed in specific pathogen free embryonated chicken egg (SPF-ECE) and Madin Darby Canine Kidney (MDCK) cells. The overall viral detection with these methods were 43%, 33% and 26% respectively. RT-PCR proved to be the most sensitive and rapid method for viral detection followed by isolation in ECE and MDCK cells.

INTRODUCTION

Influenza virus is one of the main biological groups of viruses known to cause upper respiratory disease in equidae. However, it remains the most important one in countries with substantial breeding and racing industries because of its unparalleled morbidity which can result in epizootic outbreaks of the disease, particularly in the young foals *(1)*.

Equine influenza (EI) caused by virus belong to family Orthomyxoviridae, type A which comprises two antigenically distinct serotypes represented by reference strains. A/Equi-1/Praque-1/56 (H7N7) and A/equi-2/Miami-2/63 (H3N8) *(2-4)*.

EI virus has (13.6 kb) single stranded, segmented RNA genome. It has eight segments that code for eight structural protein and two non-structural protein, segments 1 to 6 code for polymerase protein (PB1, PB2, PA), hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) respectively. Segment 7 code for Matrix protein (M1, M2). Segment 8 code for two non-structural proteins (NS1-NS2). These genome in close association with nucleoprotein and surrounded by the matrix protein followed by lipid envelope containing surface glycoprotein spikes (haemagglutinin and neuraminidase) *(5)*.

In Egypt, the first outbreak of EI disease was recorded on October 1989 where EI virus subtypes 1 and 2 were isolated *(6-7)*. The second outbreak was recorded in Winter 1999-2000 where subtype 2 was isolated *(8,9)*.

As the risk associated with the increase in the international movement of horses for racing and breeding purposes, it is imperative to have specific and sensitive virus detection method available for rapid diagnosis of equine influenza virus.

Traditionally, equine influenza virus is diagnosed by viral isolation from naso-pharyngeal swabs in embryonated chicken eggs (ECE) *(4)* and Madin Dabry canine kidney cells *(10)*, or measurement of a rise in antibody titre in paired sera sample by haemagglutination inhibition assay *(11)*.

These established techniques are time consuming and laborious, further more the sensitivity of virus isolation is dependent on the presence of infectious particles and some virus strains are difficult to isolate *(12)*.

So, the main objective of this study is based on application and standardization of RT-PCR as reliable diagnostic tool for rapid amplification and detection of nucleic acid of EI virus (Subtype-2) (H3N8) and compare its sensitivity with viral isolation in ECE and TC.

MATERIAL AND METHODS

Virus

A locally identified isolate of EI virus A/equi-2/Cairo-2/2000. Ep 10 with HA unit 2^{10} and infectivity titre $10^{9.5}$ EID_{50}/0.1 ml was

obtained from Equine Disease Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and used as positive control ***(9)***.

Samples

Thirty nasal swabs placed in virus transport medium (Tryptose phosphate broth) containing 0.1 % gelatin with a concentration of 800 units of penicillin and 40 ug of streptomycin/ml were collected from suspected cases of viral respiratory disease from different places. They stored at -70°C until used for virus isolation and RT-PCR application ***(4,13,14)***.

Virus isolation

In Embryonated Chicken Eggs (ECE)

Samples were agitated and centrifuged at 1500 g for 15 minutes to remove bacteria and debris. 0.2 of ach sample was inoculated into the amnio-allantoic cavities of 9-11 day old specific pathogen free (SPF) ECE. Amnioallantoic fluid was harvested after 2 to 3 days of incubation at 35°C. The harvested fluid was clarified by centrifugation at 1500 rpm for 15 minutes and tested for haemagglutinating activity (HA) ***(4)*** and infectivity titre egg infective dose 50 (EID_{50}) ***(15,16)***. Negative fluids and those with titres less than 1/16 were repassaged. Egg fluids with haemagglutination (HA) unit more than 1/16 were identified by haemagglutination inhibition test (HI) using type specific antisera supplied by Department of Virology, Faculty of Veterinary Medicine, Cairo University.

In Tissue Culture

Madin Darby Canine Kidney (MDCK) cell sheets in screw capped test tubes were propagated at 37°C in Earl's minimum essential medium (EMEM) with 10% new born calf serum. Growth media was removed and cells were washed with phosphate buffer saline (PBS) solution to remove serum remains. For virus isolation, the cells were maintained in serum free medium containing 0.5–2.0 µg/ml trypsin TPCK treated (L-1-tosylamide-2 phenyl ethyl chloromethyl ketone). Cells and supernatant medium were examined daily for evidence of cytopathic effect (CPE) and HA activity. Negative fluids and those with titres less than 1/16 repassaged. $TCID_{50}$ were carried out on samples that were frozen within 24 hours of collection and had subsequently been thawed once ***(17-19)***.

RNA extraction

The viral RNA was extracted from nasal swabs and infected allantoic fluid using RNA Qia Amp viral RNA kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. RNA was eluted in 60µl DEPC treated water and kept at – 80°C after the addition of RNase inhibitor until using.

Oligonucleotide primers

The primers used in this study were designed by ***(20)*** from highly conserved region of the HA gene. These primers synthesized by DNA Denmark and expected to amplify 707 pb amplicons.

Table (1): Forward and reverse primers for EIV-subtype-2 HA gene

Forward primer Lp/1/93-931/F	5` GAA GCA TCC CCA ACG ACA AACC
Reverse primer Lp/1/93-1637R	5` GGC GAA TGA AAT CCA CAG TAT CC

RT-PCR

Revert Aid first strand DNA synthesis kit (Fermentas) was used for 1st strand synthesis. A mix containing 10 µl of viral RNA, 1 µl random hexamer primer (0.2 µg). 1 ml DEPC-treated water were mixed and incubated at 70°C for 5 minutes, then rapidly cooled on ice, mixture containing 5 µl 5x buffer reaction, 1 µl ribonuclease inhibitor (20 u), 2 µl of 10 mM DNTP mix were added to the tube, mixed and incubated at 25°C for 5 minutes. After adding 1 µl of (200 u) M-Mulv reverse transcriptase, the reaction mixture was

incubated at 25°C for 10 minutes at 42°C for 60 minutes and then 70°C for 10 minutes. For PCR step, the PCR amplification was carried out with PCR reaction mix by using 2 µl of cDNA, 1 µl of 20 uM of each forward and reverse primers (final conc 0.2 uM) 25 µl of 2x (Promega) mix buffer and 2 µl MgCl2 (final conc. 2.5 uM) and nuclease free H2O to 50 µl volume reaction. PCR cycle program were as follows: denature step at 95°C for 2 minutes, then 40 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute with final extension at 72°C for 10 minutes.

Agarose gel electrophoresis for analysis of amplified products

10 µl of each final amplified products were analyzed by electrophoresis on a 1.2 % Agarose gel using DNA molecular size standards (100 bp ladder, Invitrogen). The gel is stained with ethidium bromide 0.5 µg/ml and visualized by UV transilluminator.

RESULTS AND DISCUSSION

Thirty nasal swabs from horses suffering from respiratory manifestation were tested for the presence of equine influenza virus by using RT/PCR technique, in addition to virus isolation in ECE as well as tissue culture (TC).

Ten haemagglutinating agents were secured from 10 nasal swabs out of the thirty where the isolation was carried out in SPF-ECE by intra amnioallantoic sac routes. Seven isolates were detected from the first passage and three from the second passage. With HA titre expressed in log2 ranged from 2 to 8 and EID_{50} expressed in log10 from 6 to 8. These results agreed with ***(21)***.

On the other hand, cytopathic changes were detected on MDCK cell lines from the third blind passage (8 nasal swabs out of thirty) with incubation period ranged from 5 to 9 days and HA titre expressed in log2 ranged from 2 to 5 and $TCID_{50}$ expressed in log_{10} ranged from 6 to 7.2 ***(18)***.

Identification of the previous isolates were carried out with haemagglutination inhibition test using standard antisera to H7N7 and H3N8 strains of equine influenza virus ***(4)***.

All virus isolates showed positive results with HI test against EI-subtype (H3N8) antisera giving significant titre ranged from 1/40 to 1/160.

Before the application of PCR on suspected nasal swabs, the primers were tested in the PCR reaction containing the RNA extracted from infected amnioalantoic fluid with local isolate of equine influenza virus subtype-2. The reaction yields a PCR product of characteristic size (707 bp) as shown in Fig. 1.

Specific PCR primers of EI virus subtype-2 were specifically amplified the 707 pb of the HA gene of EI virus subtype-2 from 13 nasal swabs. Among the 13 RT-PCR positive nasal swabs, 8 were positive by isolation in ECE and MDCK (cells), 2 were positive by isolation in ECE only and 3 were positive only by RT/PCR as shown in table (2).

The data presented here demonstrated that PCR can be valuable tool in the surveillance of equine influenza virus for several aspects:

This method is allowing amplification of the most conserved region of HA gene i.e. common sequence of all equine influenza virus subtype-2 which give successful recognize and identity to the virus ***(22,23)***.

It is proved to be the most sensitive assay used, as the viral nucleic acid was detected in 13 nasal swabs with overall detection rate 43% compared to 33% and 26% for virus isolation in ECE and TC respectively.

It is also rapid, less labor intensive, could be performed within one day of receiving clinical materials and it does not depend on the presence of viable virus. So, we can diagnose the samples which may have been inactivated during transportation or storage for long time.

While, virus isolation in ECE or tissue culture takes several days. Infection usually

identified by HA, and virus identity has to be confirmed by HI test with specific antisera *(13)*.

As equine influenza virus undergo antigenic drifts *(24, 25)*. It is important to monitor this antigen variation. This is done by HI and monoclonal antibodies after the virus had been isolated. This is often unsuccessful. Evaluating the antigenicity could be made by sequencing the amplified segment from RT/PCR assay.

In conclusion, RT-PCR protocol described here for detection of equine influenza virus in circumstance in which virus was not isolated from nasal swabs by using conventional ECE and tissue culture techniques and it will be useful in elucidating the epidemiology of the virus.

Table (2): Detection of equine influenza by RT-PCR and virus isolation

No. of nasal swabs	Number of positive samples detected by :		
	RT-PCR	ECE	MDCK cells
30	13	10	8

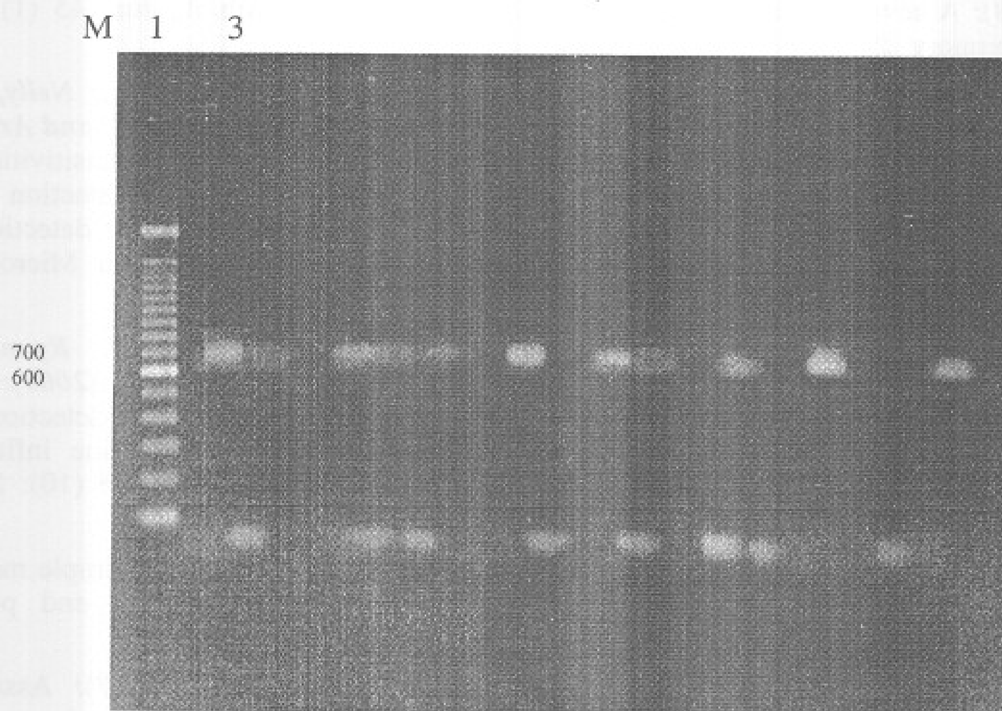

Fig. (1): Agarose gel electrophoresis showing the amplified PCR product from infected allantoic fluid with local isolate of EI-2 and nasal swab samples, HA gene (707 bp)

Lane M: 100 bp DNA marker (Invitrogen)
Lane 1: Positive control A/equi-2, H3N8 infected amnioallantoic fluid.
Lane 3: Negative control
Lane 2, 4, 5, 6, 8, 10, 11, 13, 15, 18: Positive samples

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المخلص العربى

الكشف السريع لفيروس أنفلونزا الخيول باستخدام اختبار البلمرة المتسلسل

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تم استخدام الناسخ العكسى لتفاعل البلمرة المتسلسل (RT-PCR) للكشف عن فيروس انفلونزا الخيول فى عدد ثلاثون مسحة أنفية من الخيول تعانى من أعراض تنفسية باستخدام بادئات متخصصة لجين التلازن الدموى (HA).

كما تم اختبار كفاءة البادئات على الحمض النووى الريبوزى المستخلص للعترة المعزولة محلياً (A/equi-2/Cairo-2/2000) والممرة فى السائل اللقانقى لأجنة بيض خالى من المسببات المرضية.

كما تم ايضاً محاولة لعزل الفيروس من المسحات الأنفية السابقة على أجنة البيض المخصب الخالى من المسببات المرضية وعلى خلايا MDCK.

لوحظ أن نسبة الكشف عن الفيروس باستخدام RT-PCR والعزل على كل من البيض والخلايا هى كالأتى: ٤٣%، ٣٣%، ٢٦% بالترتيب.

بناءاً على هذه النتائج أثبت تفاعل البلمرة المتسلسل أنه الأكثر سرعة وحساسية فى الكشف عن الفيروس ثم يليه العزل على البيض والخلايا.