International Journal of Microbiological Research 1 (1): 26-32, 2010
ISSN 2079-2093
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Isolation of Equine Herpesvirus-1 (EHV-1) as a Cause of Reproductive Disorders with Emphasis on Antigenic and Genetic Identifications

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Abstract: Equine herpesvirus (EHV-1), is an important pathogen of horses which causes serious economic losses in the horse industry worldwide. The goal of this study was to isolate the EHV-1 as a cause of reproductive disorders with emphasis on antigenic and genetic identifications. Samples of fetal fluid (FF; n= 8) from aborted, foreign breed mares at 5-7th months of gestation and semen (n=4) from native breed stallions, in a horses farm having history suggestive of EHV-1 infection, were collected. Virus was isolated on Vero cell culture and identified with fluorescence antibody (FA), polymerase chain reaction (PCR) and Real-time PCR (Rt-PCR). The main cytopathic effects (CPEs) in inoculated Vero cells were observed after 2-3 days post inoculation (PI) at the 5th passage of FF-samples (7 out of 8) while no CPEs were observed in semen-inoculated Vero cells. Intranuclear fluorescence granules were observed only in 6 out of 8 FF-inoculated Vero cells. The expected PCR fragment (99 base pair) was successfully amplified in 7 out of 8 FF-inoculated Vero cells. Simultaneously 6 of these samples were also positive FA, while the seventh one was negative FA despite it showed CPEs. On the other hand, no amplified products were developed for semen-inoculated Vero cells. Real-time PCR detected the EHV-1 DNA only in 5 out of 8 FF-inoculated Vero cells. In conclusion, EHV-1 cause abortion that diagnosis of the virus must be rapid and sensitive. Further studies on the molecular characterization of these isolates, molecular epidemiology and pathogenesis of EHV-1 will help a better understanding of factors necessary for the effective control.

Key words: EHV-1 · Isolation · FA · PCR · Rt-PCR

INTRODUCTION

The *alphaherpesvirus*, equine herpesvirus-1 (EHV-1), is the causative agent for major economic losses and welfare problems in horses; it is among etiological agents causing respiratory and neurological diseases as well as abortion of pregnant mares [1-4]. EHV-1 was first described in 1932 [5] and became latent in around 80% of horses [1, 6, 7], involving lymphocytes and the trigeminal ganglion [8]. The inclusion of lymphocytes leads to the development of a cell associated viraemia [9] and this is responsible for the rapid dissemination of virus to sites of secondary replication, including the spinal cord, gravid uterus, testes and accessory sex glands without noticeable effects on fertility of stallions [2, 10-12]. Abortion can occur as early as 5th month of gestation, but is more usual around the 8th month [13].

The viral genome is 155 kilo base pair (kbp) in length, encodes 76 genes of which 63 are located in the unique long (UL), 9 in the unique short (US) and 6 in the internal repeat (IRs) regions [3]. The glycoprotein B (gB) is the most conserved glycoprotein in the family *Herpesviridae*, suggesting that this glycoprotein plays a critical role (s) in propagation of the virus [14].

Diagnosis of EHV-1 must be rapid and sensitive so early intervention policies, aimed at reducing the effect of virus spread, can be take place [15-17]. Routine diagnosis of EHV-1 infection in live animals is usually achieved by virus isolation in cultured cells from nasopharyngeal secretions and blood, or from the tissues of aborted fetuses [18, 19]. Several rapid and innovative diagnostic techniques based on enzyme linked immunosorbent assay (ELISA) [19-21], immunohistochemical staining with peroxidase [13, 22], polymerase chain reaction (PCR)

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[23, 24], competitive PCR-ELISA [25], multiplex nested-PCR [19], Real-time PCR (Rt-PCR) [18, 26, 27] or nucleic acid hybridization probes [28], have been described. Although, virus isolation is a time consuming and not very sensitive, it still the gold standard technique [4, 9].

The goal of this study was isolation of the EHV-1 as a cause of reproductive disorders with emphasis on antigenic and genetic identification.

MATERIALS AND METHODS

Animals and Samples: Foreign breed mares were imported to a horse's farm during July, 2006. After the adaptation of these mares to the Egyptian environment and at September, 2007, mares came in estrus and bred three times, each other day, starting from diagnosis of mature ovulating follicles of > 3.5 cm in Ø using ultrasound scanning (Novek ultrasound scanner, Canada). Later on, many mares get aborted at the 5th to 7th month of gestation. The stallions in this farm were Egyptian native breed.

Fetal fluid (FF; n= 8) from aborted mares and semen (n=4) from the stallions, were collected and diluted in phosphate buffered saline (PBS) with addition of antibiotic/antimycotic preparation (Sigma-Aldrich, Ames Iowa, USA) at the recommended concentration and as described briefly by Hierholzer and Killington [29].

Cell Culture and Virus Strain: Green monkey kidney cells (Vero cells), tested against latent infection with mycoplasma and bovine viral diarrhea virus (BVDV), were cultured in Eagle's minimal essential medium (EMEM; Gibco, Life Technologies, Scotland, UK) supplemented with 2 or 10 % irradiated, heat inactivated fetal bovine serum (FBS; Biowest, Franc), as maintenance (M-EMEM) and growth (G-EMEM) media, respectively.

Killed virus vaccine strain (Pneumabort-K®+ 1b; Fort Dodge Animal Health, lowa 50501, USA) was used as a positive control in genetic identification of the isolates.

Virus Isolation: As described by Ataseven *et al.* [19] but with Vero cells and in 24 well tissue culture (TC) plate (NUNC, Denmark), the cells were grown under the standard culture conditions (37°C, 5% Co_2 and 85% humidity) using an initial concentration 1.5×10^5 cells/ml in G-EMEM. At 85% of cell confluence monolayers, 20 µl of each sample was inoculated in its appropriate well and incubated for 1 hour under the standard culture conditions. Subsequently, the inoculum's was replaced with M-EMEM and the plate was incubated under the standard culture conditions. The cells were monitored daily for the development of viral cytopathic effects (CPEs) using an inverted epifluorescence phase-contrast trinuclear microscope (Nikon ECLIPSE-TS100, Japan) with 20X plan a chromatic lens and a digital camera DS-U2 with its software, NIS elements. Cells and supernatants were subjected for freezing/thawing several times and re-inoculated on cellular monolayers for further passages. The cells and supernatants were harvested and stored at -70°C in small aliquots. Non inoculated monolayers were used as cellular control.

Fluorescence Antibody (FA) Technique: Fluorescence antibody (FA) detection of EHV-1 antigens in inoculated Vero monolayers after 24 hours post inoculation (PI) from the 3rd passage of each appropriate sample using fluorescence isothiocyanate (FITC) conjugated anti-EHV-1(VMRD, INC. Pullman, WA, USA) was carried out on coverslips as outlined by Bolin *et al.* [30] and examined using an inverted epifluorescence phase-contrast trinuclear microscope.

Extraction of DNA: From the 3rd passage of inoculated samples in Vero cells, the DNA was extracted using proteinase K, sodium deodecyl sulfate (SDS) and phenol-chloroform isoamyl alcohol method as described by Sambrook and Russell [31].

Design of Primers: According to the genomic sequence of EHV-1 as published by Telford *et al.* [32] and as cited by Elia *et al.* [18], region of gB was chosen to design a primer set for specific detection of the virus. The forward primer (fP) 5'---GCT CTC AGG TTT TAC GAC ATC---3' and the reverse primer (rP) 5'---TTT CAA GGG CCT GGG TAA AG---3' were synthesized in Biobasic Company, Canada and used to amplify a specific band of 99 bp (base pair) of EHV-1 genome.

Polymerase Chain Reaction (PCR): As a standard protocol and in a final volume 25 µl, PCR was carried out using 5 µl of DNA as template, 25 pmol of each primer and 1X PCR master mix (Taq master/high yield, Jena Bioscience). The amplification cycles were carried in a PT-100 thermocycler (MJ Research, USA). Reaction conditions were optimized to be 95°C/3 min as an initial

denaturation, followed by 40 cycles of 94°C/45 seconds, 60°C/60 seconds and 72°C/60 seconds. A final extension step at 72°C/10 min was done. Sterile molecular biology grade was used as a negative control to confirm absence of contaminant while the positive control was DNA of EHV-1 vaccine strain. All testes were repeated twice to ensure reproducibility of the PCR assay.

The amplification products were electrophorezed in 1.5% agarose gel. The size of the amplified fragments was determined using 100 bp DNA ladder (Aβ gene, UK).

Real-Time PCR (Rt- PCR): Real-time amplification of the EHV-1 DNA was performed in a total volume 20 µl using the same primer set as in PCR. The amplification reaction consisted of 1X ABsolut™ QPCR SYBR Green mix (Thermoscientific, Aβgene, USA), 900 nM of each primer and 3 µl of DNA template. The thermal cycle protocol was performed on a rotor gene 6000 real-time detection system (Corbett Research, Australia) using the following program: An initial activation step for DNA polymerase at 95°C/15 min, followed by 45 cycles of denaturation at 94°C/15 sec, annealing at 60°C/30 sec and extension at 72°C/30 sec. Melt curve analysis was performed by heating the PCR products from 60°C to 95°C. The fluorescence data was collected at the end of each extension step and continuously during the melt curve program by SYBR Green channel. Each run included both positive (DNA template of EHV-1 vaccine strain) and negative (no DNA template) controls. Samples were considered positive when its fluorescence exceeds the threshold and its melt curve analysis was specific.

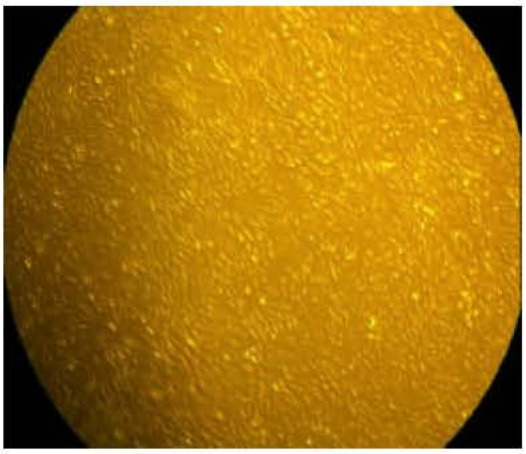

Fig. 1: Non inoculated Vero monolayers cell culture as cellular control

RESULTS

Virus Isolation: The main CPEs in inoculated Vero cells were observed after 2-3 days PI at the 5th passage for FF-samples (7 out of 8) while no CPEs were observed in semen-inoculated Vero cells. The infected cells became rounded, dispersed with grapes-like appearance in the fluid phase and intranuclear inclusion bodies were observed (Figure 2) as compared with the cellular control (Vero cells without inoculation; Figure 1).

Virus Identification

Fluorescence Antibody (FA): The viral antigens were detected in 6 out of 8 FF-inoculated Vero cells whereas fluorescence granules were observed intranuclear in the inoculated cells (Figure 3). This test was negative for the semen-inoculated Vero cells.

Polymerase Chain Reaction (PCR): The expected PCR fragment (99 bp) was successfully amplified in 7 out of 8 FF-inoculated Vero cells. Simultaneously 6 of these samples were also positive FA, while the seventh sample was negative FA despite it showed CPEs. On the other hand, no amplified products were developed for semen-inoculated Vero cells (Figure 4).

Real-Time PCR: Real-time PCR detected the EHV-1 DNA in 5 out of 8 FF-inoculated Vero cells, while it was not detected in semen-inoculated Vero cells. Figure 5 showed the amplification curve of representative positive and negative samples, while figure 6 showed their melt curve analysis.

Fig. 2: EHV-1 inoculated Vero cells became rounded, dispersed with grapes-like appearance in the fluid phase and intranuclear inclusion bodies were observed.

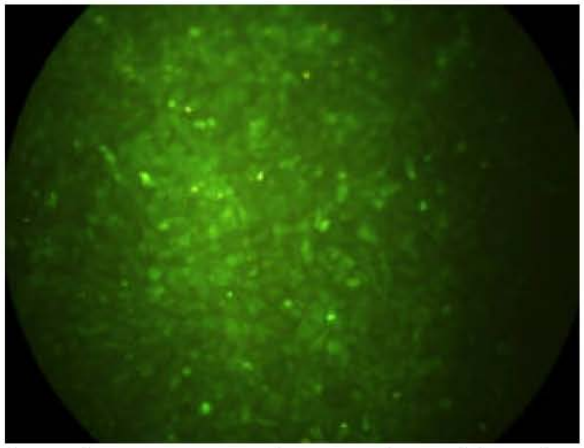

Fig. 3: Fluorescence granules were observed in 24 hours post inoculated Vero cells at the 3rd passage of inoculated samples using FITC conjugated anti-EHV-1.

Fig. 4: Positive 99 bp PCR products of EHV-1 amplified using EHV-1 forward and reverse primers. Lane 1 was 100 bp ladder DNA marker, lane 2 was EHV-1 vaccine strain as positive DNA control, lanes 3-6 were positive samples, lane 7 was a negative sample and lane 8 was a negative control.

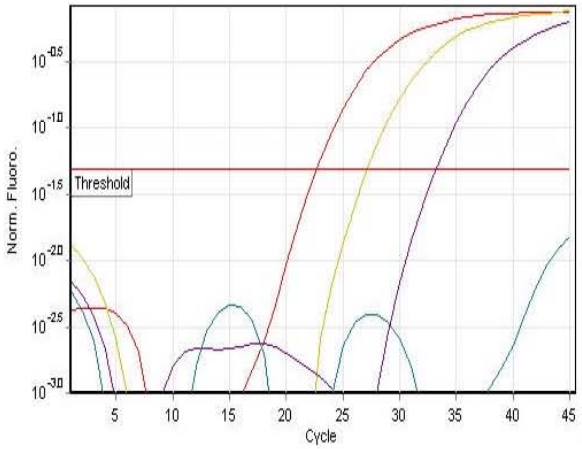

Fig. 5: Rotor gene results showing amplification curves of representative positive and negative samples and no template negative control.

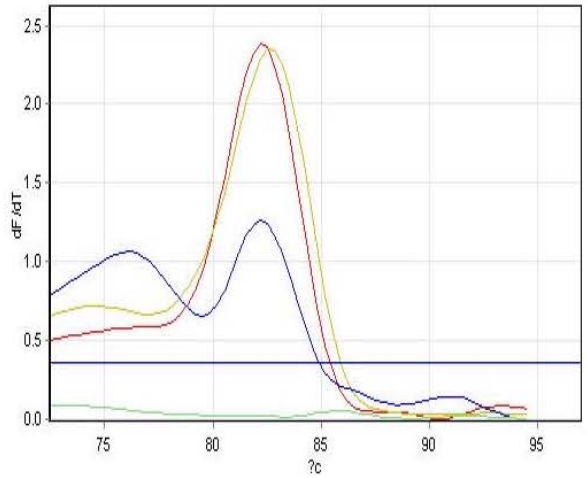

Fig. 6: Rotor gene results showing melt curves of representative positive, negative samples and no template negative control. Samples having melting temperature area of 80-85°C represent the positive samples.

DISCUSSION

A fortuitous combination of several unique biological properties possessed by this member of the equine herpesvirus accounts for its capacity for systemic spread beyond the local environment of the horse's respiratory tract to cause abortion and/or paralytic disease [4, 33]. The end pathogenetic result of these specialized properties for EHV-1 is infection of endothelial cells by virus-infected leukocytes as they circulate through the vasculature of the gravid mare's endometrium, or the central nervous system (CNS) [9, 10]. The proportion of at risk mares that abort cannot be predicted and may be as low as a single animal or as high as 70% of those exposed to the virus [34, 35].

Because EHV-1 is a highly contagious disease with the potential for occurring as explosive outbreaks with high mortality from abortigenic or neurological sequelae [36, 37], rapid diagnostic methods are important.

A number of cell types may be used for isolation of EHV-1 (e.g. rabbit kidney [RK-13], baby hamster kidney [BHK-21], Madin-Darby bovine kidney [MDBK], Vero, etc.), but equine-derived cell cultures are most sensitive [9]. Samples of post-mortem tissues collected

from aborted equine fetuses or fetal fluid provides indispensable samples to the veterinary diagnostic laboratory for diagnosis of herpesvirus [3]. In this study, the main CPEs in FF-inoculated Vero cells were observed after 2-3 days PI at the 5th passage (7 out of 8) while no CPEs were observed in semen-inoculated Vero cells. The infected cells became rounded, dispersed with grapes-like appearance in the fluid phase and intranuclear inclusion bodies were observed.

The basis for identification of any herpesvirus isolates recovered from the specimens submitted from suspected cases is its immune reactivity with specific anti-sera. Specific identification of an isolate can be quickly and simply accomplished by immunofluorescent detection of viral antigen in the inoculated cell culture using type-specific poly or monoclonal antibodies [38]. In the present study, the viral antigens were only detected in 6 out of 8 FF-inoculated Vero cells (but not in semen) that fluorescence granules were observed intranuclear of inoculated Vero cells at the 3rd passage.

The PCR can be used for rapid detection of EHV-1 nucleic acids in clinical specimens, or inoculated cell cultures [23, 24]. Although a variety of type-specific PCR primers have been designed, the lack of laboratory protocol standardization and quality assurance procedures remain ongoing challenges and need a consensus on the interpretation of the results [4, 27]. However, in the present study, a preliminary trail was used to detect EHV-1 DNA in samples-inoculated Vero cells using both conventional and Rt-PCR assays. The primer set was designed previously by Elia *et al.* [18], to target a 99 bp specific region of the gB. The expected PCR fragment was successfully amplified in 7 out of 8 FF-inoculated Vero cells. Simultaneously 6 of these samples were also positive FA, while the seventh sample was negative FA despite it showed CPEs. On the other hand, no amplified products were developed in semen-inoculated Vero cells which confirmed both results of virus isolation and FA tests. All the results were reproducible when performed twice.

The increasing application of PCR in EHV-1 detection in practice settings has presented new dilemmas in test interpretation, since such assays are unable to differentiate between replicating (lytic), non-replicating or latent virus. Advances in technology and the use of novel PCR platforms, such as Rt-PCR, enable the quantification of viral loads for equine herpesviruses [18, 26, 39-41]. In the present study, the same primer pair was used in preliminary trial for detecting EHV-1 using Rt-PCR assay. Real-time PCR detected EHV-1 DNA in 5 out of the 8 FF-inoculated Vero cells while all the semen-inoculated Vero cells were negative. Differences between both PCR and Rt-PCR assays results could be attributed to lack of reaction optimization, less optimum reaction condition or to less sufficient DNA extraction method. As Rt-PCR assay offers several advantages over the conventional PCR, more optimization trails are required to increase the sensitivity of the assay. Also, utilization of specific TaqMan probe instead of the non-specific SYBR Green intercalating dye will increase both sensitivity and specificity of the assay.

In conclusion, EHV-1 cause abortion that diagnosis of the virus must be rapid and sensitive. Further studies on the molecular characterization of these isolates, molecular epidemiology and pathogenesis of EHV-1 will help a better understanding of factors necessary for the effective control.

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