

Detection of Local Isolates of *Equine Herpes virus Type 1* Associated with Abortion and Respiratory Manifestation in Horses in Giza

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ABSTRACT

Equine herpesvirus-1 (EHV-1) is one of the infectious viruses affecting *family equidae* causing epidemic abortion, neonatal mortality, respiratory diseases and neurological disorders. Due to the economic significant of the disease it is essential to update the circulating types, so this study aimed to isolate the *EHV-1* with antigenic and molecular identification of circulating isolates in Giza. A total of 30 tissue samples (aborted fetal tissues, fetal fluids and placenta) were collected from aborted mares of native breeds and different ages during 2018-2020) and 42 vaginal and nasal swabs were collected from aborted mares and adult horses of different ages with a history of respiratory manifestations. The samples were prepared and a trial for virus isolation was done on Vero cell culture. Positive samples showing a characteristic cytopathic effect for EHV-1. Identification of positive EHV-1 isolates was done by fluorescence antibody technique (FAT) and polymerase chain reaction (PCR) targeting the glycoprotein B (gpB). The study revealed that 12.5% of the samples were positive for *EHV-1*. In conclusion, *EHV-1* infection monitoring was used to investigate and to gain knowledge about natural infection as well as to implement and regulate existing directives concerning clinical diseases. This study recommended that; further molecular characterization will help a better understanding of the virus epidemiology and effective control measures.

Keywords: EHV-1, Giza, Isolation, Local isolates, Molecular identification.

INTRODUCTION

Equine herpes virus-1 (EHV-1) is a viral pathogen with a high prevalence rate in horse populations throughout the world (Patel and heldens, 2005). It has a major economic and welfare impact on all sectors of the horse industry worldwide, due to its serious clinical forms in horses, including abortion in pregnant mares, respiratory and neurological disorders and paralysis (Ata et al., 2018).

Equine herpes virus 1 (EHV-1) is linear double-stranded DNA viruses that belong to the family Herpesviridae, subfamily *Alpha-herpesvirinae*, and genus *Varicello virus* (Lunn et al., 2009).

The genome is approximately 150 kbp long which exists in two isomeric forms since the short region can invert relative to the fixed orientation of the long region. The EHV-1 genome was expected to include 80 open reading frames (ORFs) encoding 76 different

genes, with four duplicated ORFs in the terminal repeat sequence (TRS), about 30 viral proteins including at least 13 glycoproteins have been identified (Crabb and Studdert, 1996).

EHV-1, like other herpes viruses, can establish a lifelong latency status in lymphocytes and neural tissues months or years after the primary infection (Edington et al., 1994; Baxi et al., 1995 and Chesters et al., 1997) making it difficult to be eradicated. Intermittent viral shedding from asymptomatic carriers, which occurs when the virus is re-activated by stress-induced circumstances and shed into the environment, is considered as an essential factor in the transmission of infection among susceptible animals (Patel and Heldens, 2005). And likely the occurrence of unexpected outbreaks in closed populations (Welch et al., 1992). This situation represents a challenge to control the transmission of the virus because clinical signs are usually hard to detect during the reactivation process (Slater, 2014).

Different *EHV-1* strains are circulating in the field have been classified as abortigenic or neurovirulent phenotype (Nugent et al., 2006; Goodman et al., 2007 and Lunn et al., 2009).

Abortion is one of the most serious consequences of *EHV-1* infection in mares, which might be a major cause of losses for the pure breed and industry. In affected mares abortion can occur at any time within 1-14 days after the onset of the disease. The majority of mares abort during the last half of gestation (5-10 months) (Schulman et al., 2015).

EHV-1 infection is not limited to a specific age but other factors such as sex, breed, stress (during transportation, weaning, pregnancy, racing), immune status, primary infection, and reinfection all play a role in the infection (Allen, 2008).

EHV-1 has a high rate of morbidity as long as it can be transmitted by direct contact with the aborted fetus, fetal membranes and aerosols after shedding from the nasal cavity, or by

large virus loads that are present in aborted fetuses and the placenta (OIE, 2021).

Wherever, virus isolation constitutes an important role in the diagnosis of *EHV-1* either on embryonated chicken eggs (ECE) or cell culture (Warda, 2003). Virus neutralization test and ELISA using specific poly or monoclonal antibodies (Gilkerson et al., 1998) and restriction endonuclease (Rola, 1997) were performed for virus identification. Conventional PCR (Seham et al., 2004), real-time PCR (Abd El-Hafeiz et al., 2010), or multiplex nested PCR (Soha et al., 2017 and Rashwan et al., 2019) was carried out as a recent sensitive and rapid diagnosis of *EHV-1*. However, there is a significant defect in information regarding the most prevalent and circulating EHV-1 in Egypt. This work aimed to isolate EHV-1 with antigenic and molecular identification to be a preliminary step for tissue culture vaccine preparation from the locally identified isolated strain to induce a specific good immune response.

MATERIALS AND METHODS

Samples

A total of seventy-two samples were collected, from which 30 tissue samples and placentae (placenta: n = 7; tissues from aborted fetuses: n = 19 and fetal fluids: n=4) were collected from aborted fetuses and mares immediately after abortion from 2018 to 2020. The aborted mares were of various ages, breeds. Samples were collected and processed according to Mahy and Kangro (1996). In 5 ml phosphate-buffered saline (PBS), 1 gm of the specimen was homogenized and centrifuged at 3000xg/5 minutes in tissues homogenizer. The cellular precipitate was discarded and the cellular suspension was used to inoculate the cell culture. And 42 swabs (nasal swabs: n= 37 and vaginal swabs: n=5) were collected from aborted mares and adult horses of different ages with a history of respiratory manifestations using sterile swabs. The swabs were placed directly in the virus transport medium (serum-free MEM with 1% penicillin

streptomycin, 1% gentamicin, and 0.1% fungizone). After collection, all samples were packed in coolers with ice packs and transported immediately to the laboratory for analysis

Cell culture

Green Monkey Kidney cells (Vero) were tested against abortive infection with bovine viral diarrhoea virus (BVDV) and *Mycoplasma*, and cultured in Eagle's minimal essential medium (EMEM; Biowest, France) supplemented with fetal bovine serum (FBS; Biowest, France) at 2 and 7% of maintenance M-EMEM and growth G-EMEM respectively (Mahy and Kangro, 1996). The medium was supplied with antibiotic/antimycotic (100X; Biowest, France) at the recommended concentration of 1X.

Virus isolation

According to guidelines of (OIE Terrestrial manual 2013), A confluent sheet of Vero cell line (70-80%) was inoculated with a cellular suspension of samples previously prepared supplemented with antibiotics after discarding the growth medium. It was left for one hour for adsorption at 37°C then maintenance medium was added and incubated at specific conditions (37°C, 5% CO₂, and 85% humidity) with daily examination for the development of cytopathic effects (CPEs) of the virus. After the 1st passage of the specimen on cellular culture, the inoculated cells were scratched and part of them was used for the fluorescence antibody assay (FA) and the others were used for further two successive passages.

Fluorescence antibody (FA) detection

The immunofluorescence antibody (FA) assay was carried out as described by (Bolin et al., 1991). Detection of *EHV-1* antigens in inoculated Vero monolayers on glass cover slips after 24 hours post inoculation using fluorescence isothiocyanate (FITC) as described in the manufacture instruction (VMRD, INC. Pullman, WA. USA) and examined using an inverted epifluorescence phase-contrast trinuclear microscope.

Molecular investigation

Extraction of nucleic acid: from specimens inoculated cell culture was carried out using ID Gene Spin Universal Extraction Kit, on silica spin-columns (ID.vet, France). Killed virus vaccine strain (Pneumab01t4&+ 1 b; Fort Dodge Animal Health, Iowa 50501, USA) was used as a positive control in the genetic identification of the isolates.

Primer design: As cited by (Elia et al., 2006) and published by (Abd El-Hafeiz et al., 2010) a primer set was selected to amplify a region of 99 bp within a highly conserved glycoprotein B (gpB). The primer sequence is *EHV-1* F 5—GCT CTC AGG TTT TAC GAC ATC—3 (2360-2381) for the forward primer and *EHV-1* R 5—TTT CAA GGG CCT GGG TAA AG—3 (2440-2460) for the reverse primer.

Polymerase Chain Reaction: As a standard protocol and in a final volume of 25 µl, PCR was carried out using 5 µl of DNA as a template, 25 pmol of each

Primer and PCR master mix (Taq master/high yield, Jena Bioscience). The amplification cycles were carried in a PT-100 thermal cycler (MJ Research, USA). Reaction conditions were optimized to be 95° C/3 min as 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 water was used as a negative control to confirm the absence of contaminant while the positive control is DNA of *EHV-1* vaccinal strain (Abd El-Hafeiz et al., 2010).

The amplification products were electrophoresed in 1 % agarose gel. The size of the amplified fragments was determined using a 50 bp DNA ladder (Aβ gene, UK).

RESULTS

Virus isolation

The characteristic CPEs for the herpes viruses were observed at the 3rd passages of inoculation, that the cells became rounded, dispersed in the medium as grapes like (Fig.1). The non-inoculated cells as control (Fig.2) did not show any CPEs. Out of 72

tested samples, 9 samples were positive for *EHV-1*. 2/37 of nasal swabs, 3/19 of tissue samples, 2/7 of the placenta, 1/4 of fetal fluids and 1/5 of vaginal swabs were positive for *EHV-1* (table 1).

Fluorescence antibody (FA) test

Positive isolates of *EHV-1* was identified by IFAT; the result showed that apple green fluorescent granules were detected in

inoculated Verocells (Fig.3). 9 samples were positive by IFAT.

Polymerase chain reaction

Conventional PCR using type-specific primer for *EHV1* within a highly conserved glycoprotein B (gpB) was applied. 9 samples had successfully amplified the expected 99 bp bands (Fig 4).

Table (1): Results of clinical samples by virus isolation, IFAT, and PCR:

Sample type	Number of samples	Positive for virus isolation	Positive for IFAT	Positive for PCR
Aborted fetal tissue	19	3	3	3
Placenta.	7	2	2	2
Fetal fluids	4	1	1	1
Vaginal swabs	5	1	1	1
Nasal swabs	37	2	2	2
Total	72	9	9	9

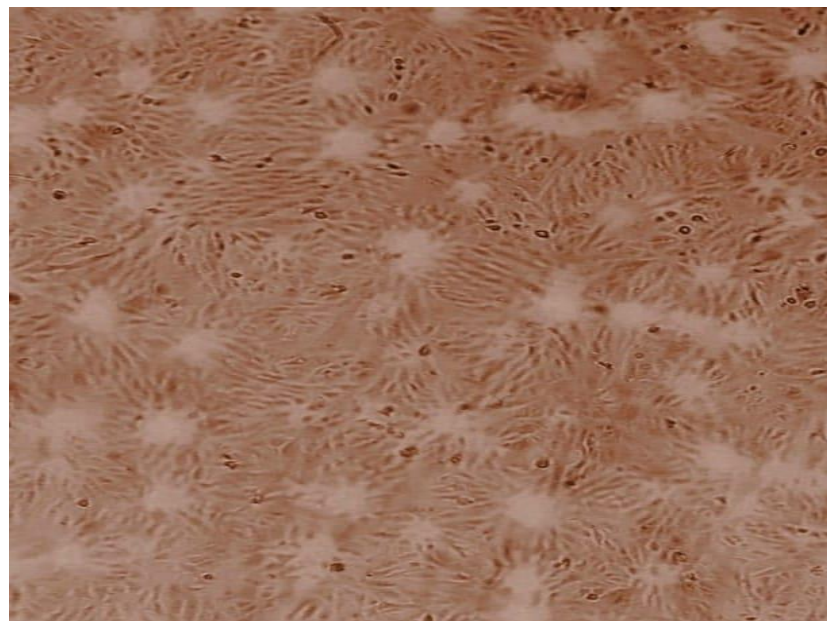


Figure (1): Cytopathic effect of *EHV-1* isolates on Vero cells showed rounding and cell detachment.

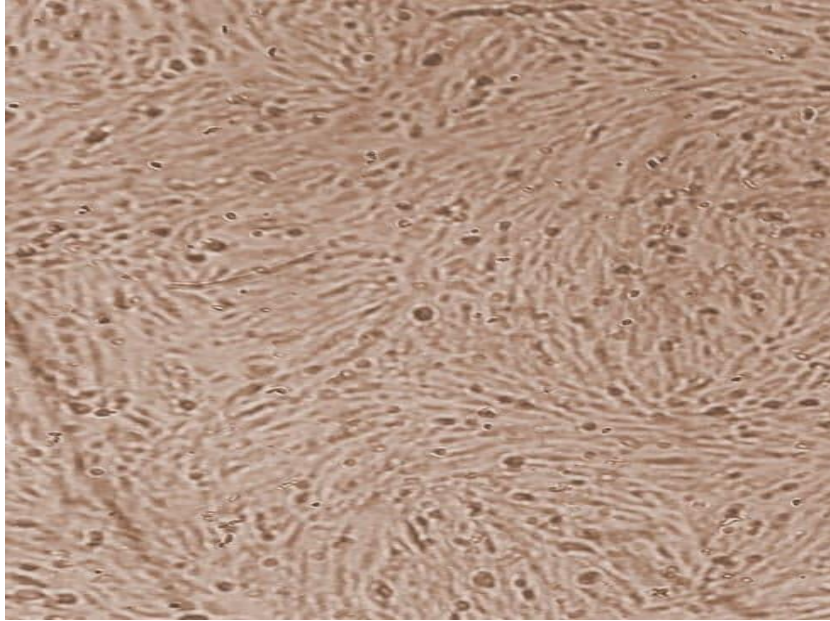


Figure (2): Control non-infected complete sheet of Vero cells.

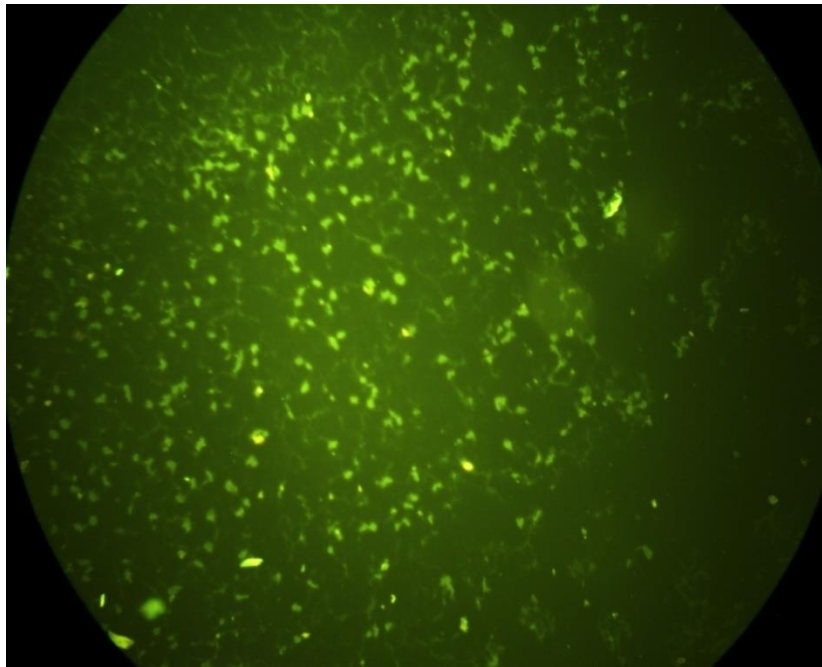


Figure (3): Vero cells inoculated with positive *EHV-1* sample, and stained by FTIC conjugated anti-EHV-1 showed apple green fluorescent granules.

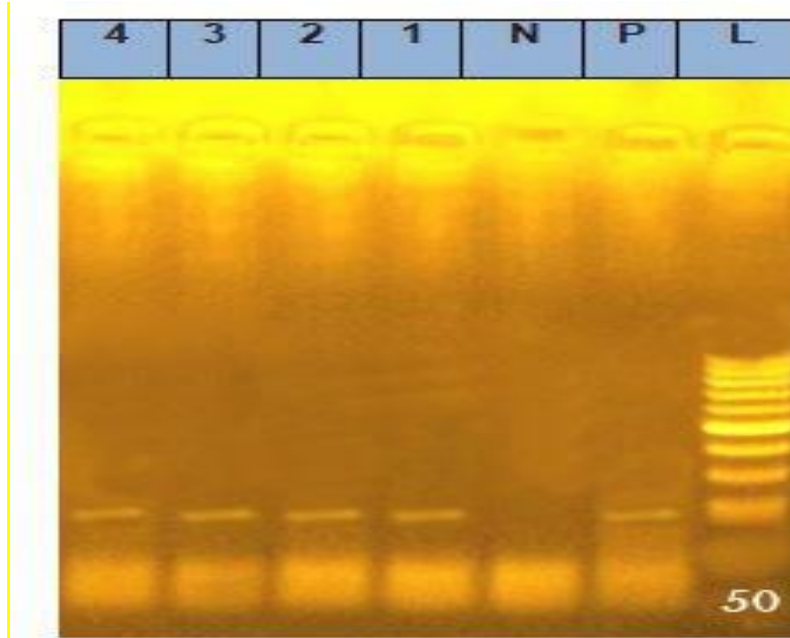


Figure (4): Positive 99 bp PCR products of *EHV-1* amplified using *EHV-1* forward and reverse primers. Lane L was 50 bp ladder, lane P was *EHV-1* vaccine strain as positive DNA control, lane N was a negative control and lanes 1-4 were positive samples.

DISCUSSION

EHV-1 possesses an emerging threat to equine health of all ages and economic prosperity of horses-related businesses wherever it affected three organ systems during clinical infection including respiratory system, reproductive system and central nervous system. Usually this disease is beginning with vascular endothelial damage of the infected tissues followed by ischemia, thrombosis and necrosis. Death customarily resulted from myeloencephalopathy (Gilkerson et al., 1998; Allen et al., 1999 and Radostitis et al., 2007). Successful identification of *EHV-1* infection in horses depends on a variety of factors such as suitable case selection with emphasis on timing of sample collection, selection of appropriate sample(s) based on the clinical manifestations, application of relevant diagnostic technique(s) and/or test(s), and careful evaluation and interpretation of laboratory results (Balasuriya et al., 2015).

Routine diagnosis of *EHV-1* infection in live animals is commonly from nasopharyngeal secretions and blood as well as from the tissues

of aborted fetuses (Rashwan et al., 2019). Furthermore early intervention policies aimed to reduce the virus spread. So the diagnosis of *EHV-1* must be rapid and sensitive.

Depending on pathogenicity of *EHV-1*, aborted foeti were considered to be the ideal samples for virus detection and isolation due to the ability of virus to reach the fetus via blood circulation (Harless and Pusterla, 2006) so the collected samples in this study include aborted foeti and its fluids in almost half of collected samples.

A variety of non–equine-derived cell types can be used for *EHV-1* isolation as Madin–Darby bovine kidney [MDBK], baby hamster kidney-21 [BHK-21], pig kidney- 15 [PK-15], (rabbit kidney-13 [RK-13], and green monkey kidney cell culture [Vero] which used as a model to study the growth kinetic of *EHV-1* due to its high titer at the same passage compared with the other used cell lines that agreed with Warda (2007).

CPE appeared as cell rounding with the appearance of cells dispersed in the medium as grapes like progressing to its lyses. These

results agreed with (Plummer and Waterston., 1963) and (Warda, 2007) who said that cell rounding, granulation of the cytoplasm and cell degeneration that end with detachment of cells from the culture surface leaving empty spaces is characteristic CPE for *EHV-1*.

Detection of *EHV-1* from three of the aborted fetuses, two placentae, one fetal fluids and one vaginal swab was anticipated as the role of *EHV-1* in equine abortion is well documented through the world (van Maanen, 2002 and Léon et al., 2008). Likewise, In Egypt (Abd El Hafeiz et al., 2010; Amer et al., 2011; EL-Sayyad et al., 2015 and Azab et al., 2019) which indicated that *EHV-1* is the most serious viral cause of abortion in mares and results in major economic loss to the horse-breeding industry.

Two nasal swabs out of 37 were positive for virus isolation of *EHV-1* in this study. This result give agreement with many studies which had been indicated that *EHV-1* virus could be isolated during acute infection from nasal swabs (Matsumura et al., 1992 and Ghoniem et al., 2017) so it is probable that a primary infection at the time of sampling.

Samples of post-mortem tissues collected from aborted equine fetuses or fetal fluid as well as placentae provides imperative samples for diagnosis of herpes virus than nasopharyngeal swabs due to saving time and money (Slater et al., 2006; Salib et al., 2017 and Warda et al., 2021).

On the other hand, the virus isolation was confirmed by immunofluorescent detection of viral antigen in the inoculated cell culture using type specific monoclonal antibodies, a yellowish green fluorescence was observed in positive samples inoculated Vero cells. This result was similar to that obtained by (Warda et al., 2013) who stated that virus isolation and confirmation using indirect florescent antibody technique (IFAT) of *EHV-1* is strongly supportive of a diagnosis of disease in a horse with clinical signs.

However, virus isolation is considered the "gold standard" of virological methods, it is time consuming and not viable in some laboratories, led to using molecular methods in the diagnosis of this infection (Ohta et al., 2011 and Nisavic et al., 2016). Several PCRs with primers specific for *EHV-1* were developed for the detection of virus in clinical samples, tissues, or cell cultures inoculated with the virus (Wagner et al., 1992; Lawrence et al., 1994 and Dynon et al., 2001).

PCR was carried on DNA extracted from Vero cells inoculated with samples which showed the positive result of a band of 99-bp which agree with (Yamada et al., 2008; Kasem et al., 2008 and Abd El-Hafeiz et al., 2010). From the previous data, we found that there is a high correlation between PCR techniques and viral isolation in the diagnosis of *EHV-1* from aborted foals which was agreed with (Dynon et al., 2001; Hornyak et al., 2006 and Warda et al., 2013). Elsewhere, PCR was proved to be sensitive and suitable tool for screening of latent *EHV-1* infection in horses (Borchers et al., 1997, Allen, 2006 and Pusterla et al., 2012). It could be concluded that samples collected from aborted foeti provide indispensable samples for diagnosis of *EHV-1* than nasopharyngeal swabs. As well as diagnostic laboratories are being pushed to exchange their classical procedures for new analytical methodologies more suited to fulfill new requirements.

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