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Original Research Article

Using indirect ELISA and PCR for the diagnosis of equine herpes virus-1 (EHV-1) infection in Egypt

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ABSTRACT

The present study was carried out for studying the prevalence of equine herpes virus-1 infection among equines in different Governorates of Egypt. One hundred eighty two equines of different ages, sexes, localities and breeds showing signs of fever, respiratory manifestations, abortions, ataxia, dog sitting position, limb edema, foal depression and death were examined for the isolation of EHV-1 on both embryonated chicken eggs and baby hamster kidney (BHK) cell culture, the aborted fetuses were histologically examined for the detection of inclusion bodies. Serum samples were collected to detect immunoglobulin-G specific to EHV-1 by using ELISA. Nasopharyngeal swabs isolated virus and the aborted fetal tissues were tested by PCR using specific primers to prove the infection with EHV-1. The prevalence of EHV-1 infection in the examined animals was recorded as 4.94%. It was prevalent in Cairo, Giza, Kafr Elsheikh, Monofeia, El Sharkia Governorates. The EHV-1 was isolated on the embryo chicken eggs and the pock lesion was appeared on the chorio-allantoic membranes. The cytopathic effects were also observed in tissue cultures. The Liver of aborted fetuses showed necrosis of all hepatic tissue and activation of Kupffer cells with hemosiderin and the detection of eosinophilic intranuclear inclusion bodies. Indirect ELISA could detect IgG in all infected equines (*n*=9) from which EHV-1 isolated. PCR proved the infection with EHV-1 in the aborted fetal (*n*=3) tissues and gave similar results by using the horses nasopharyngeal swabs isolated virus where 489 bp PCR products were detected in both. In conclusion, EHV-1 is prevalent in different Governorates in Egypt. EHV-1 infection could be diagnosed by intranuclear eosinophilic inclusion bodies in the aborted fetal tissues. The indirect ELISA could diagnose EHV-1 infection in all ages and sexes groups. PCR applied on aborted fetal tissues is better for the diagnosis of EHV-1 infection than that based on the horse nasopharyngeal swabs isolated virus due to saving time and money.

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1. Introduction

There is a great interest with equine industry all over the world, especially in Egypt and Arabian countries which plays an important role in the national income. Abortion in horses may result from a variety of infectious agents, such as bacteria, viruses or fungi that attack the fetus or its membranes, resulting in fetal death and expulsion.

EHV-l is one of group of alpha-herpes viruses. Both EHV-l and EHV-4 have extensive antigenic cross-reactivity and were previously considered subtypes of the same virus (EHV-l). DNA fingerprinting has demonstrated both the genetic divergence of the two virus species (Radostitis et al., 2010). EHV-1 is an imported pathogen and has been responsible for causing four recognized clinical syndromes in horses throughout the world: upper respiratory disease in young horses, abortions in late pregnant mares, perinatal foal mortality and occasionally neurological disorders (Reed and Toribio, 2004).

Economic losses caused by equine herpes virus -1 is summarized in abortion and loss of fetus, death of horse due to encephalomyeilitis, loss of semen of infected stallion, loss of infected newborn foals and loss of training time and opportunities to perform during convalescence and quarantine.

Equine herpesvirus (EHV-1 abortion virus) is most often associated with abortions in mares, while herpesvirus EHV-4 (rhinopneumonitis virus) is usually associated with respiratory disease in young horses. Both subtypes have the potential to cause respiratory disease and abortion. Vaccines are available as aids to prevent abortion due to EHV-1 infections. The herpes family of viruses has the capacity to persist in the body of its host in a dormant state as an inapparent carrier after the primary infection. Months or years after the primary infection, the latent herpesvirus may again become manifest with renewed replication and with the potential for initiating new outbreaks of disease in its host as well as susceptible stable mates. Therefore, it is the existence of these latently infected carrier horses, from which the virus is re-activated by stress-induced circumstances and shed into the environment to infect other individuals, that initiates a new outbreak of the disease.

Abortion in affected mares can occur at any time within 1-14 days after the onset of the disease. Most mares abort during the last half of gestation (5-10 months). Incidence of abortion is up to 50 percent. The disease can cause death in horses with a course of disease of 2-15 days.

Diagnosis of EHV-1 must be rapid and sensitive so early intervention policies aimed to reduce the virus spread. Routine diagnosis of EHV-1 infection in live animals is usually achieved by virus isolation in SPF embryonated chicken egg, cultured cells from nasopharyngeal secretions or from the tissues of aborted fetus (Elia et al., 2006). Several rapid and innovative diagnostic techniques based on PCR and enzyme linked immunosorbent assay (ELISA) were applied for the diagnosis of EHV-1 infection (Ataseven et al., 2009).

The goals of this study are to investigate the prevalence of equine herpes virus-1 infection and comparing indirect ELISA and PCR with virus isolation as diagnostic tools.

2. Materials and methods

Animals

One hundred and eighty two horses from different localities in different governorates were examined for investigation of viral causes of abortions including different breeds (Arabian and native breeds), sexes, ages and seasons. These groups of horses were suffering from problem of abortion and nervous signs and their contacts were also examined.

Samples

One hundred and eighty two serum samples were collected from the examined horses and frozen at -20ºC till use. Serum samples were screened by indirect ELISA for detection of antibodies specific to equine herpes virus-1. Five aborted fetuses were collected and parts of lungs and livers were preserved in 10% formalin solution and examined by histopathology to detect the pathological changes and inclusion bodies specific to equine herpes virus 1. Other parts of fetal tissues were preserved at -20ºC for viral isolation and PCR. Twenty two nasal swabs were collected from equines showed nervous signs and abortion and also their contact animals, then kept in the transfer media for viral isolation.

ELISA kits were used to detect IgG antibodies specific to equine herpes virus-1 (Glory Science Co., Ltd, Add: 2400 Veterans Blvd. Suite 16-101, Del Rio, TX 78840, USA). Two primers were used to amplify specific sequence of EHV-1: (5' -ACA CCA ACT CAC ACA ACT CCG AAT C- 3') reverse: (5'

-GGC ATA CAA GGA CCA CAC GTA AAT G-3') (Fermentas) using PCR to detect EHV-1 in aborted fetuses and the cells containing the isolated EHV-1.

Clinical examination

Equines showing abortions and nervous signs and their contacts were clinically examined by measuring body temperature, respiratory rate, heart rate and mucous membranes according to Radostits et al. (2007). All revealed clinical signs were recorded.

Histopathology

Aborted fetuses were examined to record gross and histopathological changes (OIE, 2015). Tissue samples including lung, liver and spleen were fixed in formalin 10% then washed, dehydrated by alcohol, cleared in xylene, embedded in paraffin, sectioned at 5 µm and stained with H&E for light microscopy to detect tissue changes and EHV-1 intranuclear inclusion bodies.

Virus isolation and adaptation

a- In embryonated chicken eggs

It was made according to Warda et al. (2013). Attempts were made to isolate the virus from the nasopharyngeal swabs and lung tissue of aborted fetus of equine. The swabs and 10% of lung tissue suspension were inoculated into chorio-allantoic membrane (CAM) of embryonated chicken egg (11-13 days) and incubated at 37ºC for 5 days with periodic candling. Deaths within the first 24 hours were discarded as non-specific. After chilling at 4ºC for one hour, the CAMs were harvested and examined for the presence of pock lesions. Further serial passages (4-6) were made on CAM.

b. In tissue culture

A confluent sheet of BHK-21 cell line (70-80%) was inoculated with 10% suspension of previously prepared infected CAMs supplemented with antibiotics after discarding the growth medium. It was left for one hour as adsorption time at 37ºC then maintenance medium was added and incubated at 37ºC with daily examination for the development of cytopathic effect of the virus. The collected virus fluid was tested for its sterility and infectivity titration was undertaken. Further passages were done till obtaining high virus titer (Warda et al., 2013).

c. Alternative passage of EHV-I in tissue culture and embryonated chicken egg

The fourth chorio-allantoic passage of the isolated virus was inoculated in primary chicken embryo fibroblast cells (CER). Then alternative passage was done on tissue culture. The collected chorio-allantoic passage was again inoculated in CER to confirm the presence of the virus (Warda et al., 2013).

Titration of the isolated virus

The infectivity titer of the isolated virus was determined by titration of the fifth passage on CAM of embryonated chicken egg and BHK tissue culture (Warda et al., 2013). The virus titer was calculated according to Reed and Muench (1938).

Viral identification of EHV-1 by serum neutralization test (SNT)

It was carried out according to Doll et al. (1956) for identification and typing of the viral isolate using reference antisera against EHV-1. Calculation of 50% end point of neutralization was carried out according to Reed and Muench (1938).

Indirect ELISA

ELISA was carried out on 182 serum samples according to Warda et al. (2013) to detect IgG antibodies specific to equine herpes virus-1 (Glory Science Co., Ltd, Add: 2400 Veterans Blvd. Suite 16-101, Del Rio, TX 78840, USA)

The reading of the test validity was recorded as follows:

The average of positive control well ≥ 1.00,

The average of negative control well ≥ 0.10.

The calculated critical (CUT OFF) equals the average of negative control well + 0.15. Negative sample for EHV-1 was reported when its OD< calculated Critical (CUT OFF). Positive sample for EHV-1 was reported when its OD $\geq$ calculated Critical (CUT OFF.

DNA extraction from tissues of aborted fetuses

DNA of EHV-1 was extracted from tissues of aborted fetuses as described by Galosi et al. (2001). Fifty mg of sample were digested for 5 h at 60°C in 3 ml of TEN buffer (1 M Tris, 0.05 M EDTA and 0.3 M NaCl) containing 10 % SDS and 0.1 mg of proteinase K/ml. One ml of 6 M NaCl was added, and after shaking ethanol precipitation was carried out. Total DNA was suspended in 40 µl of TE buffer and 10 µl of pure supernatant, and 10 µl of a 1: 10 dilution in TE buffer of the preparations was used for PCR.

Extraction of DNA from tissue culture containing EHV-1

DNA of EHV-1 was extracted from tissue culture cells containing virus by treatment with 100µg/100 ml of proteinase K and 0.1% sodium dodecyl sulfate and purified by phenol/chloroform and then precipitated with ethanol (Sambrook and Russell, 2001)

PCR amplification

PCR assay was performed according to Galosi et al. (2001) in total volume of 25 µl reaction mix contain 5 µl of template DNA, 20 p mol of each primer targeting glycoprotein C, forward: 5' -ACA CCA ACT CAC ACA ACT CCG AAT C- 3' reverse: 5'-GGC ATA CAA GGA CCA CAC GTA AAT G- 3' and 1X of PCR mix (Fermentas). The conditions for PCR amplification were initial heating to 94°C for 5 min; 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 90 s. The PCR products were run in a horizontal 1.5 % agarose gel in TBE buffer (50 mM Tris pH 8.0, 50 mM boric acid and 1 mM EDTA) at 7 V/cm for one hour and then visualized using a UV transilluminator after staining with 0.5 g of ethidium bromide.

3. Results

Clinical signs

Signs of EHV-l infection included several clinical forms: nervous system involvement (myeloencephalopathy) (Fig. 1), abortion (Fig. 2), respiratory diseases, fever and limb edema.

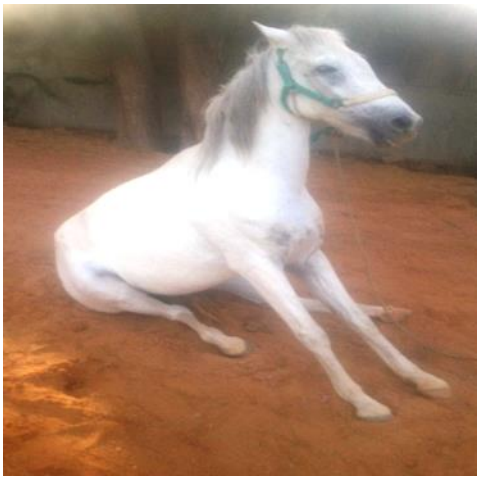

Fig. 1. Dog sitting position in EHV-1 infected stallion.

Histopathology

In aborted fetuses, EHV-1 eosinophilic intranuclear inclusion bodies (Fig. 4) were found in hepatic cells at the periphery of areas of hepatic necrosis. Lung of aborted tissues of aborted fetuses showed degenerated bronchiolar epithelium (Fig. 5) and the alveolus were lined by degenerated epithelial cells (vacuolar) and surrounded by a fibrous tissue proliferation. The alveolar spaces were filled by numerous inflammatory cells. The characteristic microscopic lesion associated with EHV-1 neuropathy was a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

Virus isolation

The pock lesions of EHV-1 on the CAM of embryonated chicken egg were recorded. After first passage, they appeared as pin headed necrotic foci and opacity (Fig. 3) and the density increased from passage to passage. CPE was characterized by cell rounding, granulation of the cytoplasm and cell degeneration that end with detachment of cells from the culture surface leaving empty spaces.

Molecular investigation

It has been found that PCR products revealed EHV-1 specific band at 489 bp. The reaction based on aborted fetus tissues obtained from Cairo (one animal) and Giza (2 animals) (Table 3 and Fig. 6).

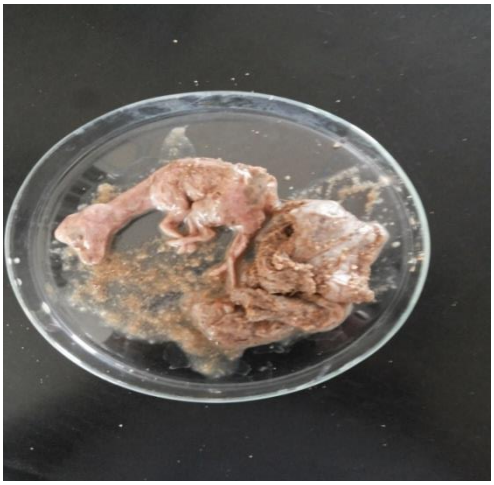

Fig. 2. Aborted equine fetuses (different ages)

Table 1. Results of indirect ELISA and PCR for diagnosis of EHV-1 in equines.

Governorate	No. of horses	Sex		Indirect ELISA (positive EHV-1)	PCR (positive EHV-1)
		Male	Female		
Cairo	26	10	16	+ (2)	+ (1)
Giza	39	12	27	+ (4)	+ (2)
Al sharkia	18	9	9	+ (1)	-
Beni-Suef	10	4	6	-	-
Monofia	23	8	15	+(1)	-
Kafr alsheikh	20	6	14	+ (1)	-
Behira	22	9	13	-	-
Alexandria	24	11	13	-	-
Total	**182**	**69**	**113**	**9 ***	**3**

*The prevalence of EHV-1 in the examined animals was recorded as 4.94% by using indirect ELISA.

Table 2. Comparing indirect ELISA based on sera of aborted mares and PCR based on the aborted fetuses.

Sequence of aborted mare	Governorate	Sex of aborted fetuses	Abortion (months of gestation)	Indirect ELISA (EHV-1) on sera of aborted mares	PCR (EHV-1) on aborted fetuses
1	Giza	Female	+ (7)	+	+
2	Giza	Male	+(8)	+	+
3	Cairo	Female	+(9)	+	+
4	Kafr alsheikh	Female	+(6)	+	-(macerated fetus)
5	Cairo	Male	+(10)	+	-(macerated fetus)
6	Monofia	-	-	+	-
7	Al sharkia	-	-	+	-

Table 3. Comparing indirect ELISA, PCR and EHV-1 isolation from nasopharyngeal swabs.

		Indirect ELISA (EHV-1)		PCR (EHV-1)		Total
		Positive	Negative	Positive	Negative	
EHV-1 isolated from Nasopharyngeal swabs	Positive	9	0	9	0	9
	Negative	2	171	0	173	173
Total		11	171	9	173	182

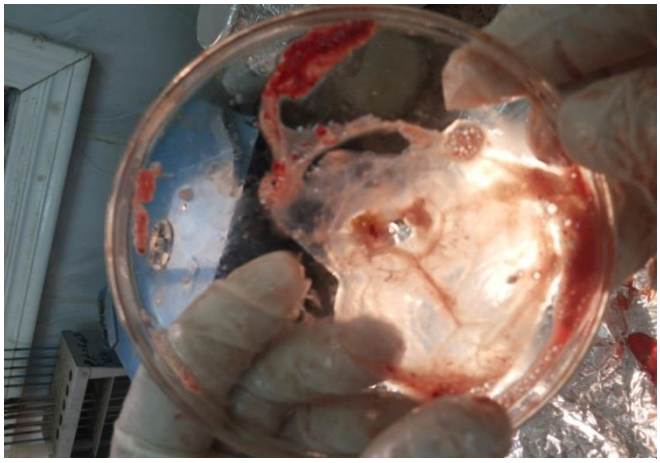

Fig. 3. Pock lesions of EHV-1 on CAM of embryonated chicken egg.

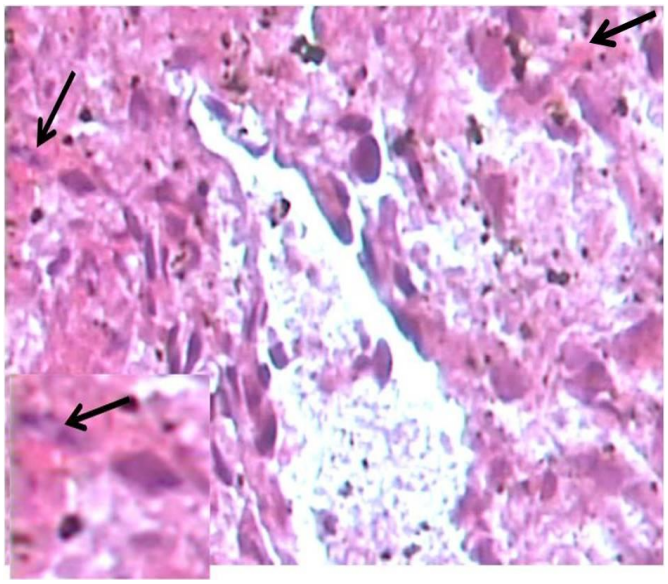

Fig. 4. Liver of aborted foal showing necrosis of hepatic tissue and activation of kuffer cells with hemosiderin. Eosinophilic Intranuclear inclusion bodies (black arrow) (HE x400).

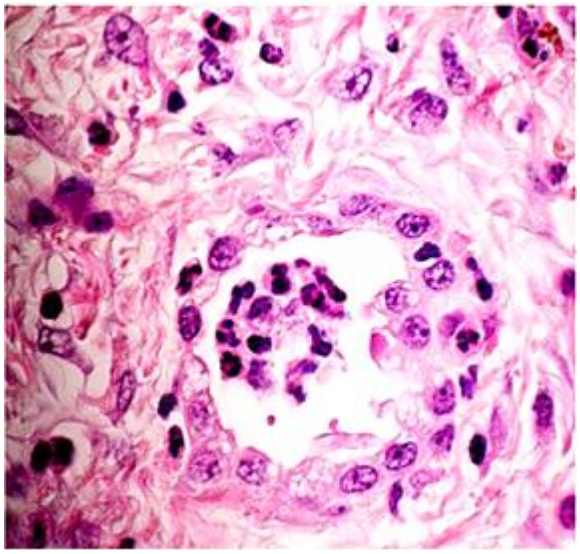

Fig. 5. Lung of aborted foal showing degenerated bronchiolar epithelium and the alveolus lined by degenerated epithelial cells (vacuolar) and surrounded by a fibrous tissue proliferation. The alveolar space filled by numerous inflammatory cells, primarily neutrophils (HE x400).

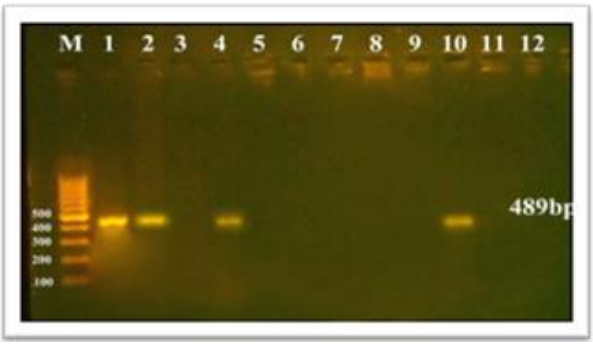

Fig. 6. PCR products showed that EHV-1 specific band at 489 bp. M (DNA marker), lane-1(control positive), lane-2 (positive), lane-3 (negative), lane-4(positive), lane-5(negative), lane-6(negative), lane-7(negative), lane-8 (negative), lane-9(negative), lane-10 (positive), lane-11(negative), lane-12(control negative). The previous PCR reaction was also obtained by using DNA extracted from the cultured cells containing the isolated EHV-1

4. Discussion

During the clinical EHV-1 infection, three organ systems are affected including the respiratory tract, uterus and placenta, and central nervous system. The fate of this disease is usually beginning with damage to vascular endothelium in the infected body tissues followed with ischemia, thrombosis and necrosis. Death usually resulted from myeloencephalopathy (Radostitis et al., 2007).

The prevalence of EHV-1 in the examined animals was recorded as 4.94% (9/182) (Table 1) by using indirect ELISA that showed that EHV-1 infection has low morbidity but causes high economic losses due to abortion, deaths due to myeloencephalopathy and losses of infected foals (Patel and Heldens, 2005). This disagrees with (Crabb and Studdert, 1995; Gilkerson et al., 2000) who recorded the seroprevalence of EHV- 1 as 9-28% horses in Australia. Sixty-one percent of eighty two normal horses and horses with upper respiratory tract disease had antibodies to EHV-1 in New Zealand (Dunowska et al., 2002). The first EHV-1 isolation in Egypt was recorded by Hassanain et al. (2002).

The obtained results revealed that EHV-1 was able to induce its pock lesions on CAM of embryonated chicken egg and specific cytopathic effect (CPE) on the tissue culture used. CPE was appeared as cell rounding, granulation of the cytoplasm and cell degeneration that end with detachment of cells from the culture surface leaving empty spaces that agreed with Warda (2007).

Indirect ELISA could detect antibodies specific to EHV-1 in sera of 11 horses (9 of them were infected with EHV-1 and the virus was isolated and identified) (Table 3). Two serum samples were positive but not suffered from EHV-1 infection and the virus was not isolated, that could be explained by that the two horses were infected and cured from EHV-1 infection but the immunoglobulin specific to the virus persist for long time.

Identification of EHV-1 in the isolated virus tissue culture and different tissues from aborted equine fetuses were carried out by using PCR (Table 2). DNA of the EHV-1 was extracted and used in PCR technique to confirm EHV-1 infection in the aborted fetuses and equines suffered from respiratory and nervous signs and their contacts. The results obtained by PCR were compared with those of standard virus isolation from both nasopharyngeal swabs and aborted fetuses. The PCR products and DNA marker were run in agarose gel and the reaction was recorded. The results showed that, in all cases, the amplified DNA segments were specific for EHV-1.

The results obtained by PCR have been compared with those of standard virus isolation from all samples that were positive by virus isolation and PCR. No negative cultured tissue samples were found positive by PCR. These results might have been due to the good preservation which keep EHV-1 intact and active, which will give a positive result by cultivation but which is crucial for the identification of DNA fragments by PCR. The present study indicates that PCR technique on the tissues of aborted fetuses has more excellent applicability to diagnose EHV-1 abortion in the aborted mares than EHV-1 isolation from fetal tissues using traditional cell culture techniques (Galosi et al., 2001).

In conclusion, EHV-1 is prevalent in Egypt in different governorates. EHV-1 infection could be diagnosed by intranuclear eosinophilic inclusion bodies in the aborted fetal tissues. The indirect ELISA could diagnose EHV-1 infection in all ages and sexes groups. PCR on aborted fetal tissues is better for the diagnosis of EHV-1 infection than that on the horse virus culture positive nasopharyngeal swabs.

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