BIOLOGICAL SCIENCES

Fetal lesions of EHV-1 in equine

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Abstract: EHV-1 infection is responsible for huge economic losses in equines due to abortion and neonatal mortality. In this study, we describe 4 cases of abortion and neonatal deaths from pregnant mares and a she-donkey from different localities in Egypt during the period from May 2015 to October 2017. Attempts were made to isolate and identify EHV-1, in addition to compare the different pathological lesions in various tissues of the necropsied cases. EHV-1 was successfully isolated from two aborted fetuses and one dead neonatal foal from mares, beside one aborted fetus from a she-donkey. The positive cases showed cytopathic effect on embryonated chicken eggs scattered on chorioallantoic membrane. Moreover, PCR was applied for the pock lesions and revealed positive results for EHV-1. Interstitial pneumonia, bronchopneumonia and necrosis of hepatic, myocardial, microcotyledonary tissues besides disseminated thrombi were the main encountered lesions. Intranuclear inclusion bodies were demonstrated in brain, liver, placenta and pulmonary tissues. Here, we describe EHV-1 induced brain lesions represented by degenerated neurons, vascular endotheliosis with intranuclear inclusion bodies in the aborted she-donkey fetus. Lesions were more severe in the aborted fetuses from mares than the one from the she-donkey. EHV-1 antigen was detected by immunohistochemistry staining.

Key words: abortion, EHV-1, fetus, neonatal, mares, she-donkey.

INTRODUCTION

Equine herpes virus 1 (EHV-1) and 4 (EHV-4) are linear double-stranded DNA viruses belong to the family *Herpesviridae*, genus *Varicellovirus* and subfamily *Alphaherpesvirinae* (Davison 2009). These viruses are ubiquitous, endemic in horse populations and considered as the most important infection in equine industry all over the world. Moreover, they are responsible for huge economic losses in equine industry through different tissue tropism. Clinical signs began by sporadic occurrence of mild respiratory disease, principally affecting horses under 2 years of age, represented by fever with depression, anorexia, and oculonasal discharges. Later, infected mares abort during the 3rd trimester of

pregnancy, resulting in further dissemination of the virus. Eventually, outbreaks of equine herpes myeloencephalopathy (EHM) which causes huge economic losses resulted from the fatal neurological disease, the extensive movement restrictions and the difficulties in management at training centers and horse events (Pusterla et al. 2005, Lunn et al. 2009, Azab & Osterrieder 2013). EHV-1 is considered as the most important equine viral disease causing equine abortion (Smith et al. 2003). EHV-4 induces only 4% of the EHV-1 abortions (Van Maanen et al. 2000). Only few researchers studied the EHV infections in Egypt for many years until EHV-1 was isolated on chorioallantoic membrane (CAM) of embryonated chicken eggs from internal

organs of aborted equine fetus (Hassanien et al. 2002). Amer et al. (2011) used ELISA, semi-nested polymerase chain reaction (PCR) assays for the diagnosis of EHV-1. In a surveillance study, the seroprevalence of EHV-1 in Egyptian horses was 62.5% (El-sayyad et al. 2015). EHV-1 latent infection was recorded in 60-80% of randomly selected equine populations (Smith 1997). Establishment of latent infection by EHV-1 within the 1st week of life in young performance horses is common (Foote et al. 2004). Horses with latent infection act as carriers of infections and have the ability to disseminate viruses when stressed (Sharma et al. 2012). In this study, we described the EHV-1 induced pathological lesions in both aborted fetuses and placentae from mares and one she-donkey that were positive for EHV-1 by virus isolation, IHC and PCR.

MATERIALS AND METHODS

Studied cases

The current study was conducted on 4 cases of abortion and neonatal deaths in mares and a jenny. The animals originated from different localities in Egypt and were recruited between May 2015 until October 2017. Clinical signs and postmortem lesions on the fetuses, neonatal foal and placentae were recorded. Data about history of aborted animals was recorded (case no., submission date, age, breed, reproductive status, vaccination status, previous medication, gestation period, history of abortion and time of abortion) (Table I). The tissue specimens were obtained from the aborted fetuses included liver, lungs, kidneys, spleen, heart, brain and placenta of aborted fetuses. They were subjected for viral isolation and histopathological examination. Specimens from fetal organs and placenta were immediately kept in 10% neutral buffer formalin for 48 hours for histopathological and immunohistochemical examination

(Suvarana et al. 2013). The histopathological lesion grading was calculated by description of histomorphological changes in 5 fields per section for each examined organ according to (Katherine et al. 2013). Samples for virological examination were transported in proper containers with minimal essential medium (MEM) supplemented with 500 IU/ml penicillin and 500 µg/ml streptomycin. These samples were sent to the Department of Virology, Animal Health Research Institute (AHRI), El-Dokki, Giza Egypt, for preparation and stored at -80°C for viral isolation, PCR and sequencing.

IHC technique

Immunohistochemical investigation for detection of gB antigen of EHV-1 was done using gB monoclonal antibody (Allen & Yeargan 1987) at dilutions of 1:1000 (Abcam®), Cat. #: ab80436 according to (Suvarana et al 2013). Briefly, 5 µm thick paraffin sections of fetal tissues and placentae were prepared and bring to water then antigen retrieval was performed. Blocking of endogenous peroxidase with hydrogen peroxide (H₂O₂) was done. The primary antibody was added on the slides and incubated overnight at 4 °C then complement was added for 10 minutes at room temperature to facilitate binding primary antibody with secondary one. Addition of horse reddish peroxidase (HRP) conjugate (Secondary antibody) (Expose mouse and rabbit specific HRP/DAB detection kit, abcam; Ready-to-use; Cat.#: ab80436) for 15 minutes at room temperature was performed. Addition of the substrate (one drop 3'-Diaminobenzidine (DAB) chromogen to 50 drops of DAB substrate) for detection of immunoreactivity. Finally, Mayer's Haematoxylin stain was used as a counter stain .

Virus isolation on CAM

Virus isolation was performed using specific pathogen free (SPF) embryonated chicken

Table I. History of aborted cases and neonatal deaths.

Case number	1	2	3	4
Site of collection	El-Zahraa	El-Tahawiya	El-Zahraa	Zoo animal
Species	Arabian mare	Arabian mare	Arabian mare	She-donkey
Submission date	17-4 - 2016	30 -8-2016	13-1-2017	22-4-2017
Age of dam	7 Y	9 Y	8 Y	7 Y
Time of abortion	10.5 months of gestation	9 months of pregnancy	Neonatal foal death after 2 weeks	10 months of gestation
Reproductive status	2 nd foaling	multiparous	Multiparous	Multiparous
History of abortion	first abortion	abortion in the previous year	non	First abortion
Vaccination with equine herpes	Vaccinated (Inactivated vaccine)	Non vaccinated	Vaccinated (Inactivated vaccine)	Non vaccinated

eggs 11-13 day old according to (Hassanien et al. 2002). Tissue specimens from different organs of aborted fetuses and placentae were homogenized to give approximately 10% (w/v) suspension in PBS pH 7.2 containing 100IU/ml penicillin and 100µg/ml streptomycin. The homogenized samples were centrifuged at 1000 rpm for 15min at 4°C. The supernatant was filtered through a 0.45µm membrane filter. Amount of 150µl of each filtrated sample was inoculated in CAM of embryonated chicken eggs (11-13 days) and incubated at 37°C for 5days and examined twice daily. Those that died within 24h after inoculation were discarded. Mortality after 24 hours' post inoculation (PI) was considered to be non-specific death. After 3 passages, the CAM and fluid was harvested aseptically and positive results appear as cytopathic effect

(CPE) on CAM in form of pock lesion after 5-6 days of incubation at 37°C. The pock lesion was increased by progressive virus passages till the 3rd passage.

Viral identification by PCR (extraction of DNA)

DNA extraction from different tissue samples was done using QIAamp DNA Mini Kit (Metabion, Germany) according to the manufacturer instructions (Hafshejani et al. 2015). PCR amplification using a set of specific primers targeting partial 342bp of EHV-1 glycoprotein B (gB) was performed (Table II).

Cycling conditions of the primers during PCR

Temperature and cycling conditions of the two primers were performed according to the Emerald Amp GT PCR master mix (Takara®)

kit. Amplification was performed for 35 cycles in a Thermal Cyclic Reactor (Hoei Science Co. Ltd. Japan). Each cycle consisted of primary denaturation at 94 °C for 5 min, secondary denaturation for 94 °C for 30 seconds, primer annealing at 55°C for 40 seconds and extension at 72°C for 45 seconds with an additional final 10 min incubation at 72°C to complete all extensions.

DNA sequencing

The conventional PCR products of selected EHV-1 positive samples were excised from the agarose gel and purified by DNA gel extraction kit (Qiagen inc. Valencia CA). The nucleotide sequence of the purified fragments was determined using an automated DNA sequencer (ABI, 3130, USA). Sequence result analysis was conducted using BLAST web interface (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and phylogenetic analyses were performed using neighbour joining and maximum parsimony in MEGA6.06 (Tamura et al. 2013).

RESULTS AND DISCUSSION

Four cases were positive for EHV-1 by forming CPE on embryonated chicken eggs in the form of focal thickening with presence of yellowish or grayish areas scattered on CAM (pock lesion) after 5-6 days of incubation at 37°C in the 3rd

passage (Fig. 1). The forementioned cytopathic effect (CPE) was described by (Hassanien et al. 2002) who isolated EHV-1 from aborted fetal organs on CAM of embryonated chicken eggs. Moreover, PCR was applied on pock lesions for EHV-1 and EHV-4 and revealed positive results for EHV-1 (Fig.2a) and negative results for EHV-4 (Fig. 2b). Our results were in harmony with (Elia et al. 2006, Abdel-Hafez et al. 2010 & Amer et al. 2011) who detected EHV-1 by PCR from several infected equine fetal tissues. Our nucleotide partial sequence of 342 bp of gB gene which was selected from one of the PCR positive products (aborted mare fetus sample) and was deposited in the Genbank with an accession number of (MG593231). Our EHV-1- Sharkia-1.sqn 1 strain is closely related to the different EHV-1 sequences that were isolated from different localities as Australia, Turkey, Germany, Japan, USA and United Kingdom. The sequence of this study had 100% identity to TR01 strain (JN705794.1) from Turkey/2012. Furthermore, it was highly similar to other EHV-1 strain with a similarity ranged from (99- 95%) available on Genbank (Fig. 3). The recorded clinical signs in one of two sporadic cases of EHV1 aborted mares included anorexia with mild respiratory manifestation followed by the abortion, while the other mare aborted without any detectable clinical signs. Retention of placentae was not recorded in both cases. Our results were

Table II. primers sequences Metabion® Germany.

Virus	Gene	Sequence 5→ 3`	Amplified product	Reference
<i>EHV1</i>	<i>Glycoprotein B</i>	GCAAACAACAGAGGGTCGATAGAAG	342 bp	Hafshejani et al (2015)
		GTCGATGTCGTA AACCTGAGAG		
<i>EHV4</i>		TATTGTTCCGCCACTCTTGACG	508 bp	
		GTAGAATCGGAGGGCGTGAAGC

Figure 1. CAM of embryonated chicken egg (3rd passage) showing pock lesion which represented by grayish or yellowish necrotic areas (arrow).

interpreted by (Sellon & Long 2007) who mentioned that these respiratory signs could be seen due to epitheliotropism of EHV-1 either from reactivation of the virus from the latent sites to the respiratory epithelium or due to primary infection. The previous results were also in partial agreement with (Van Maanen 2002) who recorded that the infection of pregnant mares with EHV1 passed unnoticed with appearance of few clinical signs such as anorexia and edema of the lower limbs. Our results disagreed with (Lunn et al. 2009) who reported that EHV1 infection in pregnant mare was generally inapparent and abortion occurred without any previous clinical signs. Gross lesions in the aborted fetuses from mares were mainly pulmonary edema with exudate in bronchial tree and necrotic hepatic areas together with edematous and congested placentae. Placenta of she-donkey fetus showed dark red patches (Fig.4a). Such lesions were in partial accordance with (Mumford 1992) who reported multifocal necrotic areas in the lungs, liver and lymphoid tissues of aborted foals infected with EHV-1. Our gross findings in the lungs were in agreement with (Hamir et al 1994)

who recorded thoracic lesions related to foal infected by EHV-1 such as excessive serosanguineous fluid in the thoracic cavity with extensive pulmonary edema and multifocal areas of necrosis and hemorrhages. The recorded gross lesions may be attributed to vasculitis. The aforementioned lesion was in accordance with (Zachary 2017) who mentioned that EHV-1 placental lesions could be due to endometrial microvasculature vasculitis and edema. The latter lesions led to separation of the maternal and fetal layers of placenta resulting in abortion. One neonatal foal (2 weeks old) died from EHV-1 developed some signs as depression, weakness, pyrexia and respiratory distress prior to death. Similar findings were described by (Sellon & Long 2007, Dunowska 2014). Mortality of the foal may be attributed to intense bronchointerstitial pneumonia and/or immunodeficiency (lymphoid depletion) that followed by secondary bacterial invasion (Murray et al. 1998). Regarding to the histopathological changes, the liver revealed various types of cell injuries mainly microsteatosis and necrosis. Moreover, disseminated thrombi within the hepatic

Figure 2. (a) PCR (4 pock lesion positive samples) analyses for EHV-1 using specific primers for partial sequences of gB gene. The amplification products for gB appeared at the expected molecular weights for EHV-1 at 342 bp. Lane M represents 100 bp DNA molecular weight ladder (ABgene). (b) PCR (4 pock lesion positive samples) analyses for EHV-4 using specific primers for partial sequences of gB gene showing negative results for EHV-4 at 508 bp. Lane M represents 100 bp DNA molecular weight ladder (ABgene)

vasculature besides eosinophilic intranuclear inclusions bodies (EINIB) inside the degenerated hepatic cells were noticed. The achieved results were in concurrent with (Bosschere et al. 2005, Easton et al. 2009, Van Der Kolk & Veldhuis 2013). Lung tissues in our investigation showed edema in some alveolar spaces and others contained extravasated erythrocytes beside aspirated eosinophilic keratin squames within some bronchi. Some pulmonary blood vessels exhibited vasculitis, perivascular lymphocytic aggregation and organized thrombi inside its lumina (Fig. 4b). EINIB were observed in some degenerated bronchial epithelium. The forementioned results were in parallel with (Jubb et al. 2007) who mentioned that respiratory tract lesions related to EHV-1 infection in aborted fetuses included extensive pulmonary and tracheal edema, fibrin casts in the bronchial lumina, bronchiointerstitial pneumonia and

multifocal necrosis of lung parenchyma with EINIB in bronchial epithelium. The previous results may be attributed to the replication of EHV-1 in upper respiratory air way epithelium which induces erosion of the respiratory mucosa followed by dissemination of the virus to the internal organs including pulmonary vascular endothelium resulting in severe necrotizing vasculitis followed by thrombosis, edema, hemorrhage and focal necrotic areas of the lung tissues (Sellon & Long 2007). Moreover, the detected eosinophilic keratin squames within bronchial lumina may be due to aspirated amniotic fluid according to (Van Der Kolk & Veldhuis 2013). The kidneys exhibited focal degenerative or necrotic changes in some renal tubules and distorted glomeruli. Some necrotic tubules had evidence of focal calcification. The previous results were in partial concurrence with (Van Der Kolk & Veldhuis 2013) who found

Figure 3. Phylogenetic tree construction of EHV-1_Sharkia-1.sqn 1 (marked with black circle) using MEGA 6.06 based on partial nucleotide analysis of 342 bp of the amplified viral specific glycoprotein B gene compared to other Gen Bank- derived reference EHV-1 strains.

focal renal necrosis, hemorrhages between the necrotic tubules and EINIB inside the tubular epithelium of the aborted fetuses infected with EHV-1. The hemorrhages and necrotic changes in the renal tubules may be attributed to the replication of the EHV-1 in the endothelial cells and pericytes of the small arterioles of the kidneys followed by vasculitis and thrombosis (Zachary 2017). The spleen showed intense lymphoid depletion of the white pulps, focal sub capsular hemorrhages and intense hemolysis. Some central arterioles had endothelial destructions and thickened media. These lesions were similar to those obtained by (Mumford 1992) who reported multifocal necrosis in the spleen and the lymph nodes of the infected aborted fetuses with EHV-1. The lymphoid depletion of the white pulps may be attributed to cytoskeletal damage of lymphocytes during EHV-1 intracellular replication cycle (Lyman & Enquist 2009) or due to lymphocytolysis (Zachary 2017). Some myocardial muscle fibers of the aborted mare fetuses revealed myolysis (Fig. 4c). Our results

were in partial agreement with (Machida et al. 1997) who found vascular lesions of coronary arteries together with degeneration and necrosis of cardiac myocytes and edematous fluid containing inflammatory cells separating cardiac muscle fibers. Our results were in contrast to (Kennedy & Miller 1993) who noticed no significant morphologic lesions in the heart of the infected aborted fetus with EHV-1 despite the detection of the EHV-1 from the heart specimens by PCR. This apparent conflict in results can be explained by the fact that EHV-1 in horses is transported via leukocytes in the blood stream (leukocytic trafficking) and therefore, the most likely source of the virus dissemination to the cardiac blood. Examination of the placentae showed microcotyledonary infarction. The latter was represented by partially or completely necrotic tissues containing numerous inflammatory cells with evidence of calcification (Fig. 4d). A few trophoblasts showed syncytia formations with EINIB. The majority of the chorionic blood vessels and capillaries had disseminated recent

Figure 4. (a) Placenta of full term aborted a she-donkey fetus showing dark red patches on fetal membrane (arrow). (b) Lung of aborted shedonkey fetus showing vasculitis and organized thrombus within pulmonary blood vessel (star) H&E Bar 50µm. (c) Heart of aborted mare fetus showing myolysis (star) or degeneration of some cardiac muscle fibers (arrow heads) and intermuscular edema (arrows) H&E bar 50 µm. (d) Placenta of aborted mare fetus showing necrosis (stars) and calcification (arrows) of the chorionic villi. H&E bar 100 µm.

thrombi. Similar lesions were described by (Walker et al. 1999) who showed necrosis of the chorionic villi with congestion of the chorionic capillaries in the mare placenta infected with EHV1. Our results were supported by (Gerst et al. 2003) who demonstrated EHV-1 EINH in trophoblast epithelium and endothelium of chorionic capillaries. The highly disseminated recent thrombi in fetal placentae were illustrated by (Gentry et al. 1992) who found gradual increase in coagulation factors in blood as (plasma fibrinogen, factor VIII and Von willebrand factor) from mid gestation until parturition and these factors increased the risk of thrombosis to be developed in virus –infected endothelial cells of maternal and fetal placenta which enhance abortion in the last 3rd of gestation. Moreover, (Robinson & Maxie 1993) reported that thrombosis and abortion occurred at late pregnancy had relation with local release of high level of vasoactive substances such as serotonin, thromboxanes and prostaglandins from aggregated platelets. Our data declared that EHV-1 in she-donkey fetus induced brain lesions following viremia and vascular damage which one of the sequel of EHV-1 endothelial

tropism. The brain of she-donkey fetus exhibited mild vascular lesions characterized by endotheliosis of the blood vessels and capillaries with EINH (Fig. 5a). Moreover, few pyramidal cells of the cerebral cortex showed degenerative and pyknotic changes, satellitosis and neuronophagia (Fig. 5b). Minute scattered hemorrhagic areas in the cerebellum together with a few submeningeal lymphocytic infiltrations were encountered. Our results declared that lesions were more severe in the aborted mare fetus than that of the she-donkey aborted fetus. In addition to, pronounced bronchointerstitial pneumonia in neonatal foal (Fig. 5c). Moreover, EHV1 antigen was detected by immunohistochemistry, which represented by golden yellow or dark brown granules within the degenerated bronchial epithelial cells of the lungs (Fig. 5d) and many degenerated trophoblast cells of the chorioallantoic membrane. The above mentioned results were in partial agreement with (Szeredi et al. 2003). Comparative studies of our summarized lesion scores among examined equine fetal tissues were listed in Table III.

Figure 5. (a) Brain tissue of aborted shedonkey showing Eosinophilic intranuclear inclusion bodies within proliferative vascular endothelial lining (arrow head) H&E Bar 20µm. (b) Brain tissue of aborted shedonkey fetus showing degenerated neurons (arrow head), satellitosis (curved arrow) and neuronophagia (open arrow) H&E Bar 50µm. (c) Lung of postnatal dead foal showing suppurative bronchiolitis (stars) and necrotic alveolitis in the adjacent pulmonary tissue (arrows) H&E bar 50µm. (d) Mare aborted fetal lung infected with EHV-1 showing weak reaction of EHV-1 antigen in bronchial epithelial cells as a golden yellow granules (arrow). IHC counterstaining with Mayer's haematoxylin. Bar 50µm.

Table III. Summarized the main histopathological lesions scores among examined organs of fetal and neonatal tissues beside placentae.

Organ	Main lesions	Aborted fetus from mare	Neonatal foal from mare	Aborted fetus from She-donkey
Liver	Focal Hepatic necrosis	++	+++	+
	Lymphocytic hepatitis	+++	+	++
	Focal Hemorrhage	+++	+	+
	EINIB	+	++	-
Lung	Interstitial pneumonia	+	+++	+
	Bronchopneumonia	-	++	-
	EINIB	+	+	-
Kidney	Tubular necrosis	++	++	+
	Hemorrhage	++	+	+++
Spleen	Lymphoid depletion and necrosis	+	++	+++
Heart	Degenerative changes and necrosis	++	++	+
	Lymphocytic myocarditis	+	++	+
Placenta	Microcotyledonary infarction	+++	Not examined	++
	Disseminated thrombus	+++	Not examined	++
	EINIB	+	Not examined	-
Brain	Satellitosis and neuronophagia	Not examined	Not examined	++
	Endotheliosis with EINIB	Not examined	Not examined	++
	Viral encephalitis	Not examined	Not examined	+

The represented scores in the table are the mean lesion score. Histologic lesions were scored for severity (- absent, + mild, ++ moderate, +++ severe).

Finally, it could be concluded that the lesions in the aborted fetus of the she-donkey and neonatal foal were similar to those observed in the aborted fetuses of the mares beside pronounced bronchointerstitial pneumonia in the neonatal foal. Also our results declared that presence of brain lesions in she donkey fetus as vascular endotheliosis with EINIB.

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AAA, NAR designed the study and critically revised the manuscript. MSS did the main experiment. NAA read and approved the final manuscript.