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CLINICO-PATHOLOGICAL DIAGNOSIS OF EQUINE HERPES VIRUS-1(EHV-1) INFECTION IN EGYPT

By

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ABSTRACT

One hundred eighty two equines of different ages, sexes, localities and breeds were examined for EHV-1 infections by clinical examinations, pathological examinations and virus isolation and identification. The EHV-1 was isolated from aborted foeti, nasal swab, vaginal swabs and preputeal swabs on both embryonated chicken eggs and baby hamster kidney (BHK) cell culture. The EHV-1 was identified by serum neutralization test (SNT) using reference antisera specific to EHV-1. The aborted fetuses and their internal organs were grossly and histopathologically examined for the detection of alterations caused by the EHV-1 infection and its inclusion bodies. The results showed that, the clinical signs in EHV-1 infected equines were fever, respiratory manifestations, abortions, ataxia, and hind limb paralysis and limb edema. The lung of aborted foeti appeared edematous with areas of rubbery texture and the lung tissues showed degenerated bronchiolar epithelium and the alveoli were lined by degenerated epithelial cells. The liver of aborted foeti appeared enlarged, friable, dark and congested. The liver of aborted fetuses showed necrosis of all hepatic tissue and activation of Kupffer cells with hemosiderin. The eosinophilic intranuclear inclusion bodies of EHV-1 were detected in the examined tissues of the aborted foeti. The EHV-1 had pock lesions on the chorio-allantoic membranes of embryonated chicken eggs. Also EHV-1 had the cytopathic effects in tissue cultures. It was concluded that EHV-1 infections could be diagnosed clinically and confirmed by pathological changes of aborted foeti organs with virus isolation and identification.

Keywords:

Equine herpesvirus-1,diagnosis, clinical, pathological, virus isolation.

INTRODUCTION

Equine herpesvirus-1 (EHV-1) is one of family herpesviridae and is prevalent in most horse populations all over the world, it causes illness in horses with extensive economic losses through its variable outbreaks of respiratory disease, abortion, neonatal foal death, and myeloencephalopathy **(Kydd *et al.*, 2006; Slater *et al.*,2004; Allen *et al.*,2004; Slater,2007**). Infections caused by EHV-1 are more common in young performance horses, and it causes latent infection within the first weeks of life **(Foote *et al.*, 2004**) with subsequent viral reactivation causing clinical disease and viral shedding during periods of stress. The clinical forms of EHV-1 in the equine population are three which include: (i) sporadic occurrence of mild respiratory disease associated with pyrexia, principally affecting horses under two years of age. (ii) Abortion which usually occurring during the 3rd trimester of pregnancy. (iii) Outbreaks of neurological disease (equine herpes myeloencephalopathy) cause economical loss and loss of life **(USDA and Ceah, 2007)**. Diagnosis of EHV-1 depends upon virus isolation and identification, detection of intranuclear inclusion bodies, polymerase chain reactions and serologic tests. Aim of this work is the studying of EHV-1 infection by clinical and pathological examinations and virus isolation and identification in equine populations.

MATERIAL AND METHODS

Animals:

One hundred and eighty two horses from different localities from different governorates were examined for EHV-1 infections. They included different breeds (Arabian and native breeds), Sexes (male and female) and ages (different ages) and during different seasons of year (all over year). The clinical signs appeared on these animals and their contacts were recorded.

Samples:

Different samples were collected from the examined animals for the diagnosis of viral causes of the reproductive problems as follows.

Aborted foeti:

Five aborted foeti from aborted mares were collected. Parts of lungs and livers of these foeti were preserved in 10 % formalin solution and examined by histopathology to detect pathological changes and inclusion bodies specific to EHV-1, EHV- 4 and EVA. Other parts of foetal tissues were preserved at -2 ^{o}C for viral isolation.

Nasal swab:

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.

Vaginal swab:

Twelve vaginal swabs were collected from equines showed nervous signs and abortion and also the contact animals, then kept in the transfer media for viral isolation.

Preputeal swab:

Ten Preputeal swabs were collected from stallions in the groups with mares showed nervous signs and abortion and their contact animals, then kept in the transfer media for viral isolation

Clinical examination:

Equines showing abortions and infertility and their contacts were generally clinically examined by measuring body temperature, respiratory rate, heart rate and mucous membranes according to **Radostitis *et al.* (2008)**. Results of clinical examinations and any clinical signs were recorded.

Virus isolation and adaptation:

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 37^{o}C for 5 days with periodic candling. Deaths within the first 24 hours were discarded as non-specific. After chilling at 4^{o}C for one hour, the CAMs were harvested and examined for the presence of pock lesions. Further serial passages (4 - 6) were made on CAM.

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 37^{o}C then maintenance medium was added and incubated at 37^{o}C 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)**.

Viral identification by using serum neutralization test (SNT)

SNT was carried out according to **Doll *et al.* (1956)** for identification and typing of the viral isolate using reference antisera against EHV-I as follows. Reference antisera (Freeze-dried rabbit anti-EHV-I serum produced kindly by Dr. Jannet Wellington, Research Fellow, Department of Biological Science, Macquarie Univ., NSW, Australia. It was used in serum neutralization test.) were inactivated at 56ºC for 30 minutes and 1/4 dilution was prepared maintenance.100 ID^{50}/0.2 ml of previous titrated infected CAMs the isolated virus was mixed with equal volume (1:1) of diluted reference antisera. The serum-virus mixture was incubated at 37ºC for one hour to allow antigen antibody reaction. Inoculation of 0.4 ml of virus- serum mixture on chorio-allantoic membrane (CAM) of embryonated chicken egg, incubation at 37ºC for 5 days with daily observation then examined for the development of pock lesion. Back titration of the virus was made. Four embryonated chicken eggs were left un-inoculated as a control. Calculation of 50% end point of neutralization was done according to **Reed and Muench (1938)**.

Pathology:

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 haematoxylin and eosin (H and E) then examined by light microscope to detect EHV-1 associated tissue changes and intranuclear inclusion bodies.

Experiment and results

I - Clinical Examination:

The total of one hundred and eighty two horses of both sexes, different age groups and different governorates were clinically examined by measuring body temperature, pulse and respiration rates. Inspection of mucous membranes and lymph nodes was also done. Clinical signs suggestive for EHV-1 infection were recorded.

The results are shown in (Tables 1 and 2)

II - Pathological Examination:

Different samples were collected from equines suffering from abortion and nervous signs, including serum, aborted fetus, fetal membranes and examined for gross pathological changes related to EHV-1.Histopathological examination was also applied.

The results are shown in photo (1-10).

III - Virological Examination:

Fetal fluids, vaginal swabs, Preputeal swabs and nasal swabs. Samples were tested for equine herpes virus -1 isolation by inoculation in ECE and cultivation in tissue culture.

The results are shown in photo (11).

Results of clinical examinations

Table (1): The recorded Clinical signs suggestive for EHV-1 infection

Number of equines infected with EHV-1	Abortion	Fever	Ataxia	Hind limb paralysis	Limb edema	Respiratory signs	Age (years)	Sex
1	+	+	-	-	-	+	5	Female
2	+	+	+	-	+	-	6	Female
3	+	+	-	-	-	+	12	Female
4	+	+	-	-	-	-	15	Female
5	+	+	-	-	-	-	14	Female
6	+	-	-	-	-	-	18	Female
7	+	+	+	-	+	-	7	Female
8	+	+	+	-	-	-	10	Female
9	+	-	-	-	-	-	15	Female
10	+	+	+	-	-	-	12	Female
11	+	-	-	-	-	-	13	Female
12	+	+	+	-	-	-	14	Female
13	-	+	+	+	-	-	12	Female
14	-	+	+	-	+	-	8	Male

Table (2): Epidemiological data of the examined equines.

Number of equines infected with EHV-1	Geographical distribution	Months of year	Age (years)	Sex
1	Giza	9	5	Female
2	Giza	10	6	Female
3	Cairo	11	12	Female
4	Monofiya	11	15	Female
5	Al sharkia	9	14	Female
6	Beni-sueif	10	18	Female
7	Kafr alsheikh	10	7	Female
8	Alexandria	3	10	Female
9	Behira	4	15	Female
10	Cairo	5	12	Female
11	Cairo	6	13	Female
12	Giza	8	14	Female
13	Giza	9	12	Female
14	Giza	12	8	Male

RESULTS OF PATHOLOGY

A-Gross pathological changes

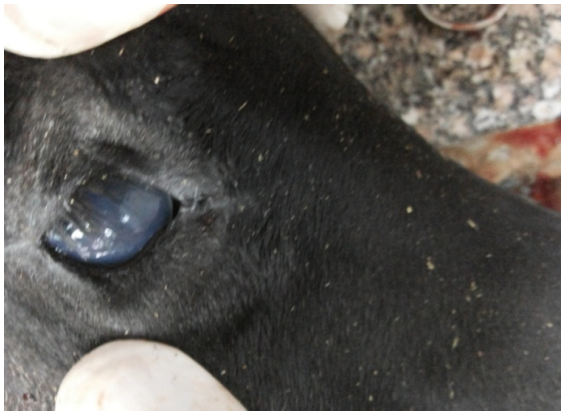


Photo (1): Abnormal discoloration of the eye of the aborted foetus with EHV-1.

Photo (2): the aborted foetus with EHV-1 showed congested viscera.

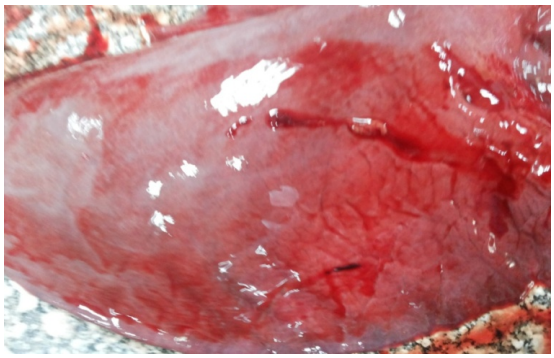


Photo (3): Lung of aborted foetus with EHV-1 showed edema and areas that is rubbery in texture with whitish coloration on the surface.

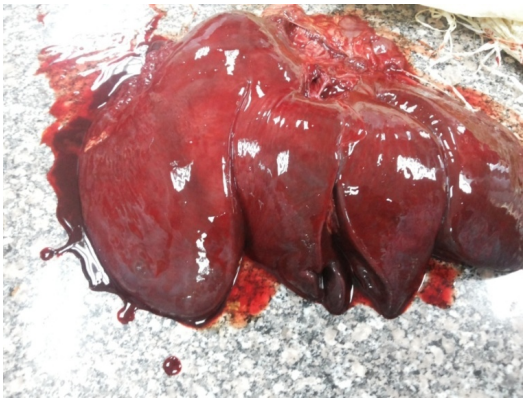


Photo (4): Liver of the aborted foetus with EHV-1 showed enlarged, friable, dark and congested areas at the pripheryof the lobules with whitish colouration onthe surface.

B-*Histopathological changes*:

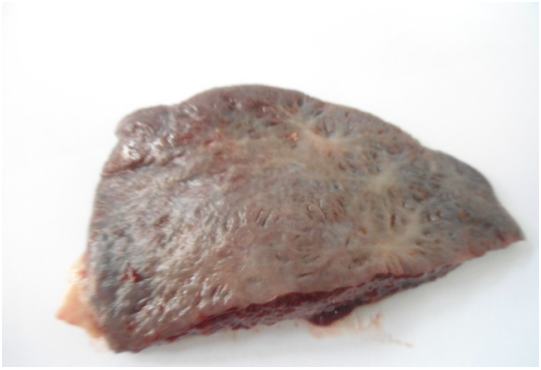


Photo (5): Spleen of aborted foetus with EHV-1 showing congestion and edema.

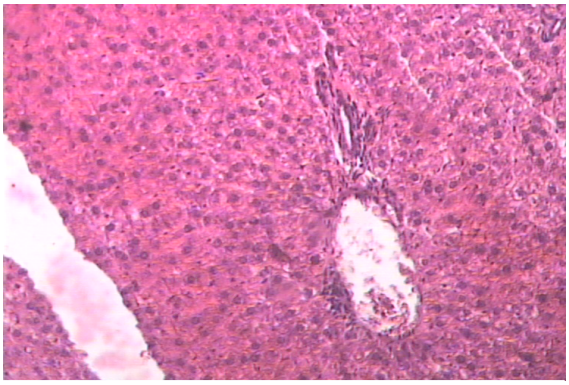


Photo (6): Liver of aborted foetus with EHV-1 showed degenerative changes of hepatocytes with focal area of inflammatory cell aggregations at the portal area. (H&E.X100).

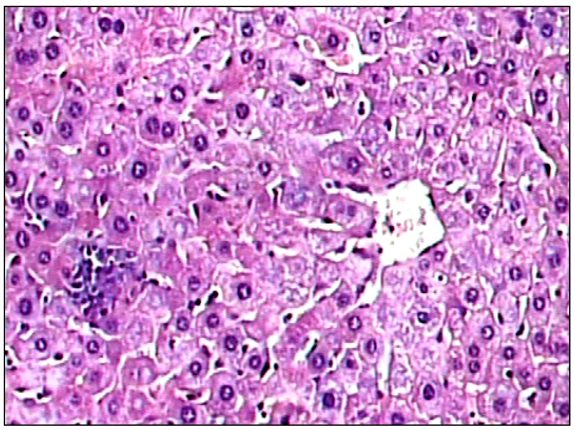


Photo (7): liver of aborted foetus with EHV-1 showedareasofhydropic degeneration of hepatocytes with focal areas of hepatic necrosis. Focal inflammatory cell aggregation. (H&E.X400).

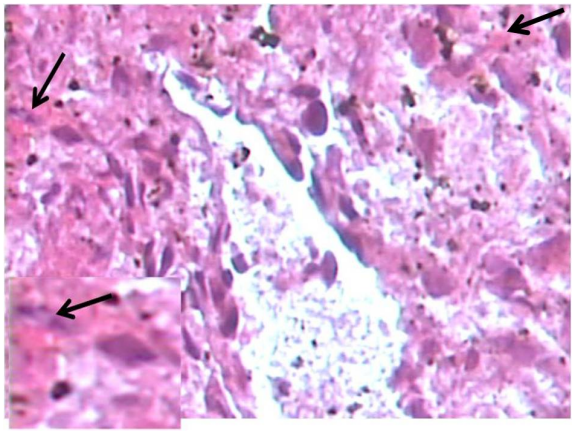
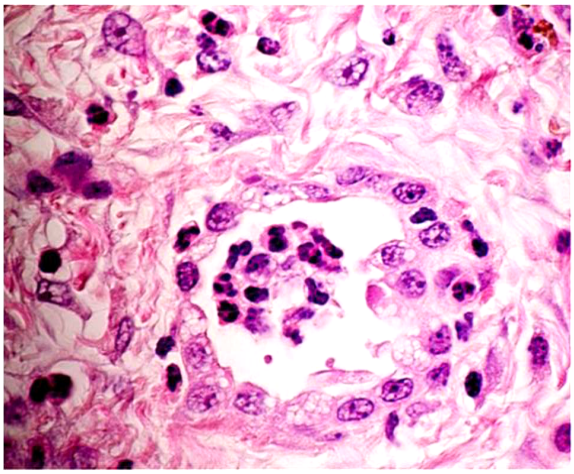


Photo (8): Liver of aborted foetus with EHV-1 showed necrosis of all hepatic tissue and activation of kuffer cells with heamosidren. Esinophilic Intranuclear inclusion bodies (black arrow). (H&E.X400).



Photo(9): Lung of aborted foetus with EHV-1 showed 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 eutrophils. H&E.X400.

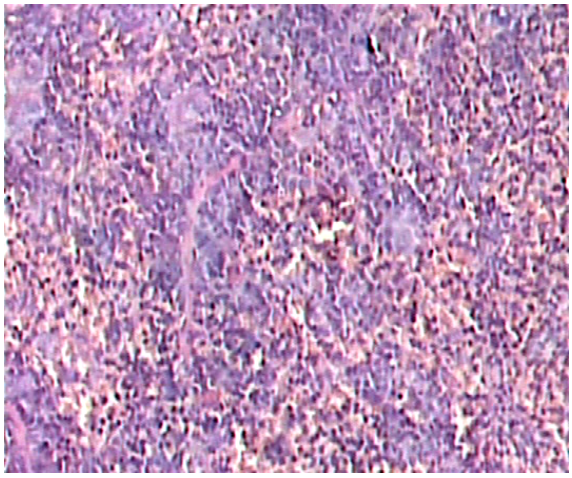


Photo (10): Spleen of aborted foetus with EHV-1 showed congestion with depletion of spleen follicles and necrosis of others. H&E. X100.

Results of virus isolation and identification:

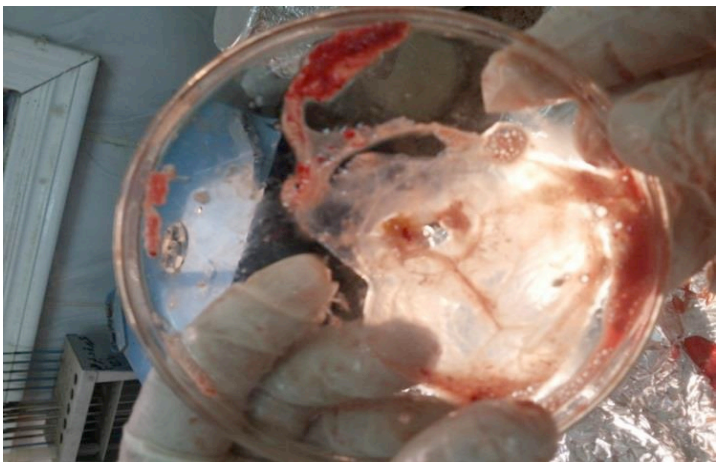


Photo (11): Pock lesions of EHV-1 on CAM of embryonated chicken egg.

DISCUSSION

Equine herpes viruses are double-stranded DNA viruses that have spherical to pleomorphic and icosahedral symmetry, their diameter ranged from 150 to 200 nm. There are 12 serotypes of herpes virus only three serotypes (1, 3 and 4) are pathogenic and could produce diseases in

equines.EHV-1 multiply rapidly in nasopharyngeal mucosa and subsequently detected in the upper respiratory tract, the soft palate and main stem bronchus within 12 hours then invade whole respiratory system and local blood vessels. The viruses circulate in blood and invade blood lymphocytes from which it could be isolated. When EHV-1 invades primary the respiratory tract associated with a febrile reaction and followed by abortion and viraemia then lymphocyte-associated transport of the virus to the vasculature of the CNS **(Borchers *et al.,* 2006)**. There are two theories of the vascular damage effect of EHV-1 infection: EHV-1 invasion and multiplication inside vascular endothelium lead to death of endothelial cells, inflammation, activation of clotting factors and formation of blood clots in small vessels that obstruct blood vessels and decrease blood flow to adjacent tissues resulted in necrosis **(Borchers *et al.,* 2006)**. (I) the deposition of antigen-antibody complexes in small vessels results in an Arthus reaction with subsequent ischemia. But this theory is weak because it was noticed that mares with no antibody titer to EHV-1 were at increased risk of developing myeloencephalopathy that does not support a role for type III hypersensitivity **(Smith *et a1,* 2001)**. The clinical signs and pathological changes are interpreted by infection of vascular endothelial cells with EHV-1 in small blood vessels of the brain and spinal cord is followed by vasculitis and thrombosis that result in ischaemia, myelomalacia and haemorrhage that explain the clinical signs of hind limb paralysis and ataxia as recorded in (Table 1) and (photos 1 and 2). Similar vascular lesions happened in the pregnant uterus resulting in abortion (Table1) and pathological changes in aborted foeti tissues. The pulmonary vasculitis explains the appearance of respiratory signs and lung congestion that is illustrated in (photos 3 and 9). The immune status of the infected animal play a role in determination of the severity of the infection where immuno-pathological mechanisms such as immune complexes and cytokines derived from cytotoxic T-cells (CTL) influenced the propagation and severity of lesions of EHV-1-induced vasculitis **(Borchers *et al.,*2006; Radostitis *et al*., 2008).** Intrauterine infection with EHV-1 may cause foals survive to full term of gestation and become stillborn or weak and die soon after birth with pulmonary (photos 3 and 9), hepatic (photos 4, 6, 7 and 8), and cardiac lesions. On other hand, infection of foals with EHV-1 at birth is usually (I) a self-limiting, (ii) mild infection of the upper respiratory tract and associated with leukopenia,(iii) a transitory immune suppression and (iv) low incidence of uveitis and corneal opacity (photo 1) and (v) death **(Smith *et a1*.2004; Smith *et a1*.1992)**. The spleen is also infected with EHV-1 and it was congested and swollen with the depletion

and necrosis of splenic follicles as in (photos 5 and 10). The EHV-1 infection caused abortion in pregnant mares by damage to the placenta, endometrium or fetus **(Studdert *et al.,* 2003)**. Virus isolation is considered the gold standard test for a laboratory diagnosis of EHV-1 infection and should be attempted, especially during outbreaks of EHV-1. Isolation and identification of EHV-1 from nasal swabs or aborted foeti or buffy coat samples is strongly supportive of a diagnosis of EHV-1 infection in a horse with compatible clinical signs. **(Mumford, 1984)**. The drawbacks of virus isolation is false negative cases that is because (i) passing the peak of virus shedding by the time of neurological signs appeared, (ii) intermittent shedding of EHV-1 and (iii) interference of local antibodies with virus recovery. So to reduce false negative cases of EHV-1 isolation, the sampling of EHV-1 should be carried out by monitoring in-contact horses and collecting nasal swab and buffy coat samples from these animals during the prodromal febrile phase before appearance of neurological signs. It should be taken in consideration that, the interpretation of positive viral cultures is confusing researchers because EHV-1 has been isolated from the respiratory tract of healthy horses **(Mumford, 1984)**. It was concluded that EHV-1 infection could be diagnosed by combination of clinical signs, pathological changes, intranuclear inclusion bodies and virus isolation and identification. It is better to evaluating new diagnostic test such as PCR and indirect ELISA for the diagnosis of EHV-1 to avoid disadvantages of virus isolation and save the time and effort.

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