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Pathological and virological studies on herpes virus in horses

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Abstract

The study performed in a horse breeding farm which showed signs of respiratory manifestation and abortion suspected equine herpes virus infections (EHVs) during the winter and spring of 2019. Tissue specimens (lung, liver, spleen and kidney) from 6 aborted fetuses and 4 neonatal foals were collected along with 10 blood samples from mares and 5 from the asymptomatically contact ones. The diagnosis was performed by inoculation of embryonated chicken eggs (free from pathogen, 11day old), through chorio-allantoic membrane (CAM), then identified by antigen detection enzyme linked immune-sorbent assays (ELISA) and immune-fluorescent technique (IF) utilizing a polyclonal antibody against EHV-1 and EHV-4 strains. The results revealed the presence of EHV-1 in 5 cases and EHV-4 in 3 cases and in 2 mixed infections of both strains. Histopathological examination confirms EHVs infection by detection of intra-cytoplasmic and intra-nuclear inclusion bodies in liver and lung.

Keywords: *Herpes Virus, Virology, Hematology, Histopathology, Arabian horses*

Introduction

The equine herpes viruses (EHVs) are endemic worldwide due to high incidence of infection early in life. Equine herpes virus EHV-1 & EHV-4 are members of the Herpesviridae family and the subfamily Alphaherpesvirinae and Genus Varicello-virus. In horse population the most important are the alpha herpes viruses EHV-1 and EHV-4. These viruses are the main respiratory tract pathogens. However, EHV-1 may induce abortion, still birth and neurological disorders. It can act as immunosuppressive agents or may take long -live latent infection in recovered horses **(Allen *et al.,* 2004; Brown *et al,* 2007; Gerst *et al,* 2003; Landolt & Lunn, 2009; Schlipf & Smith, 2009).** Currently, it is estimated that as many as 80% of all horses are latently infected with EHV-1, and seroprevalence for EHV-4 is even higher **(Pusterla & Hussey, 2014).**

Equine herpesvirus-1 has been prevalent in Egypt as a major cause of epidemic respiratory disease and sporadic abortion in Arabian and Thoroughbreds horse, resulting in serious economic losses among the horse industry **(Meselhy *et al,* 2019; Alkhalefa *et al,* 2018, Salib *et al,* 2016); Ahmed *et al.,* 2015; Amer *et al.,* 2011; Warda *et al.,* 2003).** The first EHV-1 isolation in Egypt was recorded by **Hassanain *et al.* (2002).** However in the last two decades, EHV-4 had been isolated from aborted fetuses or neonatal foals among horse breeding farms in several governorates in Egypt **(Khattab *et al,* 2022, Azab *et al.,* 2019; Al-Shammari *et al;* Afify *et al.,* 2017; Ahmed *et al.,* 2015; Amer *et al* 2015)**

Different methods have been used for EHV infections diagnosis in clinical samples **(OIE, 2008).** The virus isolation has been the standard diagnostic approach, then identification by immunofluorescence, ELISA or immunoperoxidase using type-specific monoclonal antibodies, in addition to immunohistochemistry and electron microscopical investigations **(Elia *et al.,* 2006; Landolt & Lunn, 2009; Larson, 2011; Lunn *et al,* 2009; Ataseven *et al.,* 2009 Salib *et al,* 2018).** Rapid molecular diagnostic techniques based on PCR were applied for the diagnosis of EHvs infection **(Amer *et al.* 2015; Ataseven *et al.,* 2009, Al-Shammari *et al,* 2016; Khattab *et al,* 2022).**

The present study aims to point out the various types of EHVs infection as single or mixed infection through virus isolation, ELISA, IF and could be diagnosed by the presence of eosinophilic inclusion bodies in the prenatal and aborted fetal tissues.

Materials and Methods

Case History: Signs of respiratory manifestations (tachypnea and dyspnea), marked depression, pyrexia, weakness and symmetrical hind limb ataxia were manifested in a horse breeding farm during the winter and spring of 2019. Abortion at the trisemister period of gestation occurred for several mares and neonatal foals died few days after birth.

1- **Samples for virology, hematology and pathology:**

Blood and Tissue Samples: Tissue specimens (lung, liver, spleen and kidney) from 6 aborted fetuses and 4 neonatal foals were collected along with 10 blood samples from mares and 5 from the asymptomatically contact ones. Two blood samples were collected from each, (one on EDTA as anticoagulant for study complete blood picture and the other one without anticoagulant for separation of serum for detecting antibodies against EHVs.

The virology examination; isolation and identification:

Pathogen free, fertilized chicken eggs of eleven day old embryo were incubated according to biosecurity roles and used for Virus isolation by inoculating homogenized tissues into Chorioallantoic membrane (CAM) of Embryos. Candle the embryos for viability. Disinfect with Bioguard and punch a hole directly in the top of the air cell. Use a 26 or 28-gauge, 1/2 in. needle. Insert the needle straight down the top of the egg the full length of the needle. Pull the needle back out about 1/4 in. and release the inoculum. Tissues samples has prepared for egg inoculation according to Payment and Trudal methods **(Payment, & Trudel, 1995).**

Virus identification & Immunofluorescent technique:

The inoculated embryonated chicken eggs that shows easily visible foci or "pocks.", were subjected to further examination. CAM were sectioning and fixed in acetone and examined by immunofluorescent techniques. The test performed on CAM by using the indirect method according to Payment and Trudel methods **(Payment, & Trudel, 1995).**

Hematological studies:

Complete blood picture was demonstrated for the blood samples on EDETA using Exigo Veterinary Haematological Analyzer (Manufacturer is Boule Medical AB, Sweden). Differential leukocytic count using Giemsa stained blood film **(Kerr, 2002).**

Histopathology:

Specimens from different organs were preserved on 10 % neutral formalin, routinely processed by standard paraffin embedding technique, processed and 5 micron histology sections were prepared. Slides were stained with hematoxylin and eosin stains (H&E) and phloxine & tartrazine **(Suvarna *et al,* 2012).**

Statistical Analysis:

Data obtained were statistically analysed using t- student test at significant level of $P \leq 0.05$ **(SPSS 14, 2006).**

Results

Virology results:

Virus growth and replication in the CAM is indicated by visible lesions (pocks); grey white area in transparent CAM. Each pock is derived from a single virion. CAM were sectioning and fixed in acetone and examined by immunofluorescent techniques which revealed the presence

of EHV-1 in 5 cases and EHV-4 in 3 cases and in 2 mixed infections (EHV-1 and EHV-4). ELISA utilizing a polyclonal antibody against EHV-1 and EHV-4 strains showed similar positive results as immunofluorescent study.

Hematological results:

Tables (1) demonstrated changes in hemogram of Herpes virus infected horses. The erythrogram showed slightly significant increase of RBCs count with increased significantly in Hb content and PCV value (dehydration status), as compared to control values. The leucogram demonstrated slightly significant leucocytosis and significantly lymphopenia, as compared to control values.

Table (1). Values of heamogram (mean ± SE) of horses infected with herpes virus.

Groups	Control	Positive herpes virus	P <
Total RBCs X10^6 / µl	7.02 ± 0.158	8.53 ± 0.474*	3.022
Hb g/dl	12.13 ± 0.233	13.71 ± 0.324**	4.085
PCV %	30.00 ± 0.598	35.78 ± 0.740**	4.849
MCV fl	42.93 ± 0.642	42.19 ± 0.280	-1.050
MCH pg	17.33 ± 0.224	16.80 ± 0.112	-2.095
MCHC g/dl	40.40 ± 0.127	39.86 ± 0.157	-2.681
Total WBCs X10^3 / µl	7.80 ± 0.164	8.51 ± 0.148*	3.214
Neutrophils X10^3 / µl	3.66 ± 0.364	2.81 ± 0.440	-1.478
Lymphocytes X10^3 / µl	3.76 ± 0.186	2.05 ± 0.203 **	-4.800
Monocytes X10^3 / µl	0.22 ± 0.325	0.17 ± 0.035	-1.063
Eosinophil X10^3 / µl	0.16 ± 0.0058	0.26 ± 0.055	1.845
Basophils X10^3 / µl	0 ±0	0 ±0	_

*Significant at P < 0.05 **Significant at P <0.01

Histopathology results:

Lung: pulmonary tissues showed focal interstitial and fibrinous pneumonia with bronchiolitis. Where, inflammatory cells infiltration (specially lymphocytes and neutrophils) was observed. Eosinophilic exudate scattered filling the alveolar lumens with early proliferation of fibrinous threads were detected. Also, thickening of bronchiolar wall was noticed. Some alveoli were collapse, meanwhile others were emphysematous. Intracytoplasmic inclusion bodies were seen within the epithelial cells lining the bronchioles (figure 1).

Thrombosis in peri - alveolar blood capillaries with sometimes dilated blood vessels were detected, in addition to some areas of hemorrhagic pneumonia. Peri -bronchiolar inflammatory cells infiltration and fibrosis were seen with desquamation of lining epithelium of some bronchioles. Intracytoplasmic inclusion bodies within the bronchial epithelial cells were noticed (figure 2). Inflammatory cells revealed distorted nuclei and eosinophilic intracytoplasmic inclusion bodtes (figure 3). Some parts showed loss of architecture and cellular details. positive fluorescent of lung tissue as antigen of EHV-1 detected inside cytoplasm of infected cells, positive reaction also seen inside cellular exudates (Fig.7).

Liver: Hepatic tissue revealed hepatocytes with nucleoli enlarged, nucleoli displaced toward the nuclear membrane, the nuclear membranes appears distorted and duplicated, some nuclei showed disintegration (Figure 4). Eosinophilic intra-nuclear inclusion bodies within hepatocytes (Fig, 5 a) were very obvious. Degeneration was detected as vacculated hepatocytes, focal necrosis (Fig. 5 b) , severe inflammatory reaction accompanied by inflammatory cells infiltrations and hemorrhages.

Spleen: Hyperplasia of megakaryocytes within the red pulp were observed (Fig. 6).

Kidney: Renal tissues showed shrinkage of glomerular tuft with widen of peri - glomerular space. Some renal tubules showed different stages of degeneration with obliteration of luminal space, while others showed coagulative necrosis.

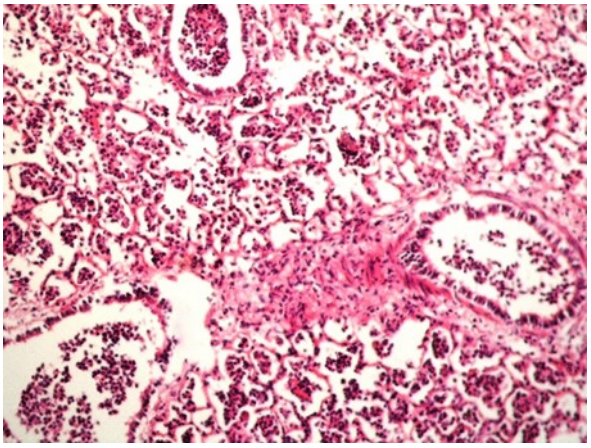

Fig. (1): Lung of aborted fetus showing interstitial and fibrinous pneumonia; with predominant inflammatory cells type of neutrophil and lymphocyte. Intracytoplasmic inclusion bodies (black arrow) within the epithelial cells lining the bronchiole (H&E, X 200).

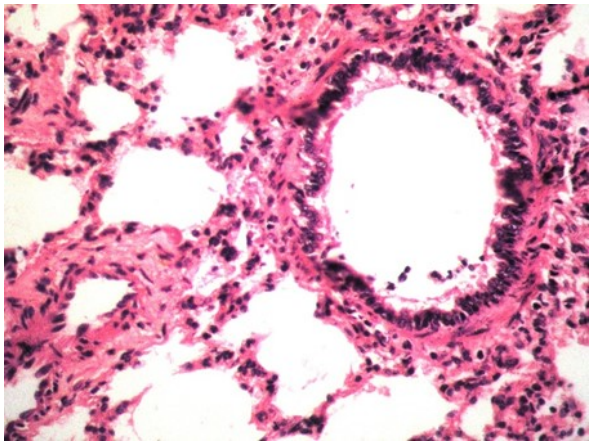

Fig. (2): Lung of aborted fetus showing fibrinous pneumonia; with predominant inflammatory cells type of neutrophil and lymphocyte. Intracytoplasmic inclusion bodies (black arrow) within the bronchial epithelial cells (H&E, X400).

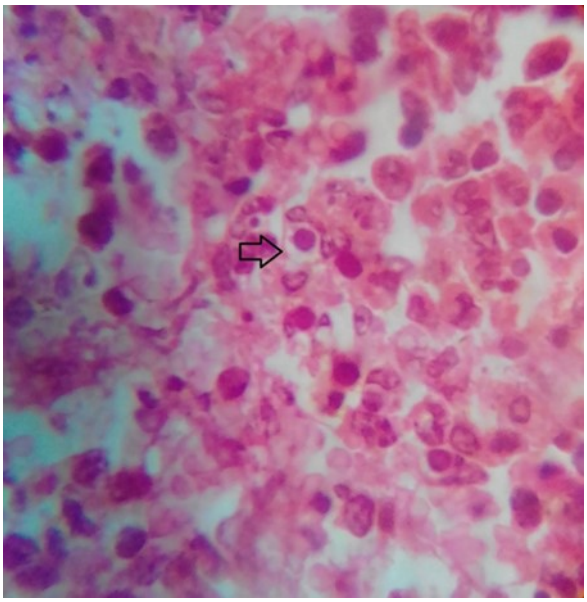

Fig. (3): Lung of neonatal foal showing inflammatory exudate and inflammatory cells with distorted nuclei and eosinophilic intracytoplasmic inclusion body. (arrow) (phloxine & tartrazine, X 1000).

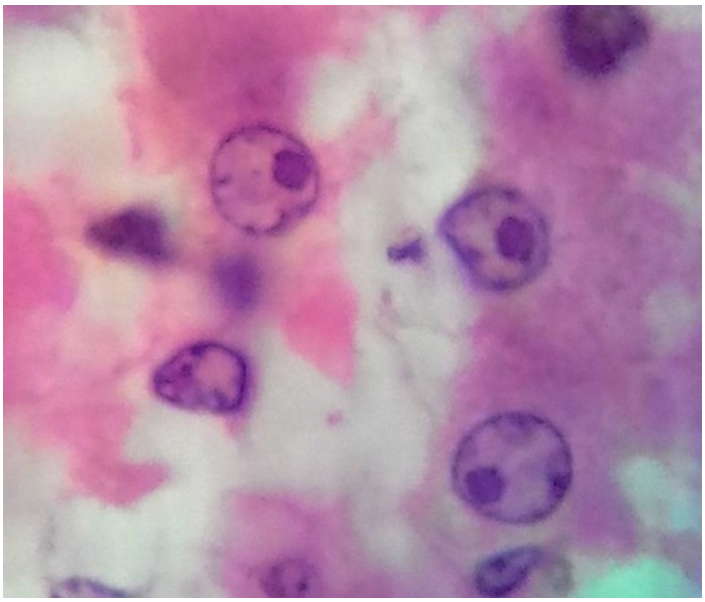

Fig. (4): Liver of aborted fetus showing hepatocytes with enlarged nucleoli, displaced toward the nuclear membrane and the nuclear membranes appears distorted and duplicated, some nuclei were disintegration. (H&E, X 1000)

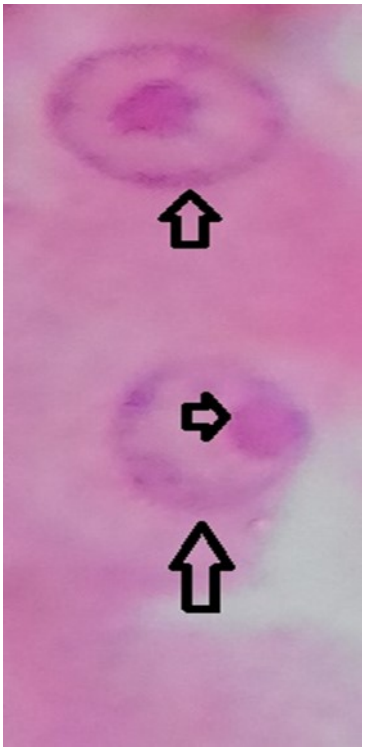

Fig. (5 a) : Liver of aborted fetus showing intra-nuclear inclusion bodies within hepatocytes (arrows) (H&E, X 1000).

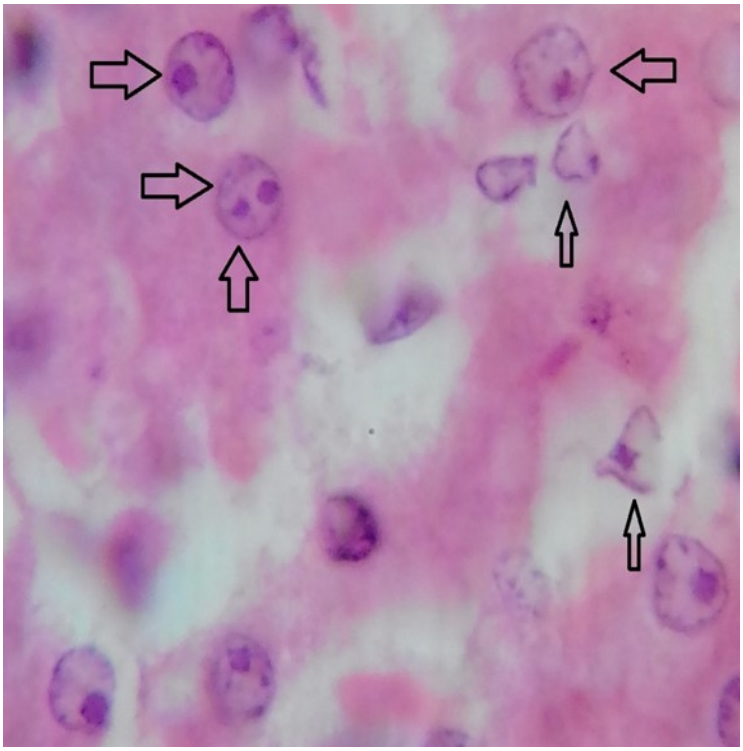

Fig. (5 b): Liver of aborted fetus showing vacuolation and necrosis of hepatic cells, hepatocytes showed nucleoli enlarged and nucleoli displaced toward the nuclear membrane and the nuclear membranes appears distorted and duplicated, some nuclei showed fragmentations and others showed disintegration (arrows) (H&E, X 1000).

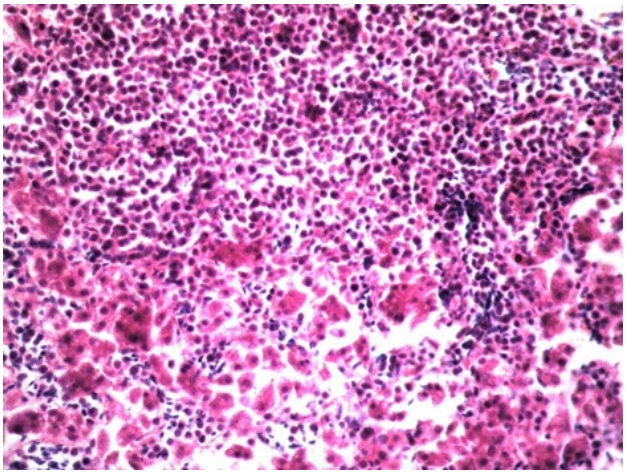

Fig. (6): Spleen of aborted fetus showing numerous number of megakaryocytes within the red pulp (H&E, X400).

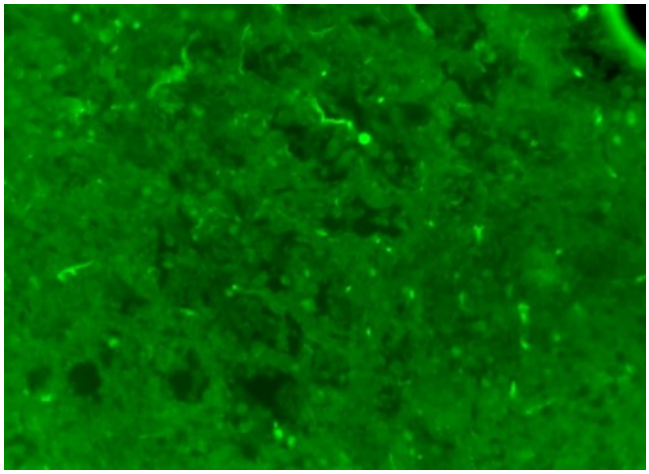

Fig. (7): Lung of neonatal foal showing positive fluorescent as antigen of EHV-1 detected inside cytoplasm of infected cells, positive reaction also seen inside cellular exudates (IF X 60).

Discussion

Equine herpes viruses (EHVs) are most frequently causes equine abortion, fetal neonatal illness, or neurologic disease. The EHVs infection occurs within groups of horses, usually in the winter and spring and most commonly causes respiratory disease, abortion, still births and may develop neurological disorder **(Slater, 2007; Coetzer & Tustin, 2004).**

Diagnosis of EHV-1 must be rapid and sensitive so early intervention policies aimed to reduce the virus spread.

The present results of virus isolation on the CAM revealed the presence of EHV-1 in 5 cases and EHV-4 in 3 cases and in 2 mixed infections (EHV-1 & EHV-4). ELISA assays using polyclonal antibody against EHV-1 and EHV-4 strains showed similar positive results as immunofluorescent study. Mixed infection of both EHV-1 & EHV-4 has been reported by **Amer *et al,* (2015)** in one case during screening of clinical samples from Arabian horses in Cairo breeding farm.

Although, the isolation of EHV-4 in cases of abortion is rare since it is predominately restricted to the upper airways and is not generally associated with abortion or neurologic disease, However in our study 4 positive cases for EHV-4 were documented to cause abortion and neonatal death. Our finding are in agreement with many authors who reported that EHV-4 were endemic in Egypt and can induce abortion and neurological disease. **(Khattab *et al,* 2022, Azab *et al.,* 2019; Al-Shammari *et al;* Afify *et al.,* 2017; Ahmed *et al.,* 2015; Amer *et al.,* 2015).**

Regards to hematological study, there were no significant changes correlated to the EHVs infection although due to the clinical signs of pyrexia and dehydration due to the viral infection and the tissue damage, leucogram demonstrated slightly significant leucocytosis with significantly lymphopenia, that explain tissue demond. Moreover, erythrogram showed slightly significant increase of RBCs count with increased significantly in Hb content and PCV value (dehydration status). **Coles (1986)** recorded that, in dehydrated animal, the packed cell volume, hemoglobin and total erythrocyte count are increased. The author added that parameters are of more value for following the treatment of dehydration.

In this study, microscopical lesions of lung showed Thrombosis in peri - alveolar blood capillaries.

EHV-1 is endotheliotropic virus. That infects and replicates in mucosal epithelial and endothelial cells of respiratory system **(Zachary, 2017).** In addition to dehydrated state of animal which theoretically, is related to a hyper-

coagulable state because it can decrease regional blood flow and increase blood viscosity. These risk factors induce thrombus formation.
Lopez & Martinson (2017) recorded that dehydration increase the viscosity of mucus, reducing or stopping mucociliary movement.

Loss of body water when exceeds intake, dehydration occur. This disturbance of water results from excessive water loss without compensatory increased intake. Consumption of water in amounts necessary to replace the water lost is the principal compensatory mechanism. Also, the kidney is very sensitive, through the action of antidiuretic hormone (ADH) which control any change in water balance and immediately acts to conserve water when plasma osmolarity is increased **(Coles, 1986).**
In the present investigation, renal tissues were suffered from different stages of degeneration in some renal tubules with obliteration of luminal space while others showed coagulative necrosis. Dilatation of peri-glomerular space with collapsed of glomerular tuft were also noticed. So kidney conservation of water may be disturbed, in addition to, the infected horses suffered from fever and some cases were recumbent. So water is inadequately compensated for greater water loss. Dehydration become a major problem. **Coles (1986)** recorded that excessive loss of body fluid may occur in prolonged fever, sweating and an uncontrolled polyurea if there is in adequate water intake to compensate for the loss.
Microscopical examination of spleen revealed hyperplasia of megakaryocytes in red pulp. That indication to hemorrhage, where pulmonary tissue seen hemorrhagic pneumonia.
Histopathological findings were in the form of focal liver necrosis, the bronchial and alveolar epithelium and intra-nuclear inclusion bodies in hepatocytes. Changes in host chromatin in Equine that observed in the nucleolus; it becomes enlarged, displaced toward the nuclear membrane, and ultimately disaggregates or fragments. Concurrently, host chromosomes become marginated, and later in infection the nucleus becomes distorted. The numerous protrusions and distortions have in the past been mistaken for amitotic division. Margination of the chromosomes may or may not be linked with the chromosome breakage reported by numerous investigators. Hepatocytes showed nucleoli enlarged and nucleoli displaced toward the nuclear membrane and the nuclear membranes appears distorted and duplicated, some nuclei showed disintegration and eosinophilic intra-nuclear inclusion bodies **(Caswell & Williams ,2007: Jones & Hunt, 1983).**

The presence of the intra cytoplasmic and intranuclear inclusion bodies is a pathognomonic findings in Herpes infection but it does not differentiate between the various types of herpes infection. However Immune-fluorescent methods using specific monoclonal antibodies can identify the various types. Immune -fluorescent staining technique showed the EHVs antigen inside walls of blood vessels, and inside the cytoplasm and nuclei on the infected cells. The viral antigen was also seen inside various types of inflammatory cells and the cellular exudates. Cells productively infected with herpes viruses do not survive. Almost from the beginning of reproductive cycle, the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction. **(Allen, 2002; Gerst *et al,* 2003; Landolt & Lunn, 2009).**
Virus infects and replicates in mucosal epithelial cells of respiratory system, next in contiguous mucosal and submucosal lymphocytes, macrophages, monocytes and cells, then spreads via leucocytes trafficking in afferent lymphatic vessels to regional lymph node. Viral envelop glycoproteins likely attach to glycosaminoglycan receptors of target cell membranes. Virus uses this binding to enter the cells listed earlier **(Zachary, 2017).** Equine herpes viruses are major horse pathogens. They cause a variety of clinical forms, respiratory diseases, neurological diseases, abortion and neonatal death.
In the current study, pulmonary tissue revealed focal interstitial, fibrinous and hemorrhagic pneumonia with broncheolitis. Inflammatory cells infiltration and thickening of bronchiolar wall were observed. Eosinophilic exudate scattered filling the alveolar lumens, in addition to some alveoli were collapse, while others were compensatory emphysematous. Intracytoplasmic or intranuclear inclusion bodies were seen

within the epithelial cells lining the bronchioles or hepatocytes. These findings agreed with the study of several authors **(John & Hunts, 1983; Slater, 2007; Salib *et al,* 2016).**

In conclusion, EHV 1 and EHV-4 are prevalent in Egypt, the diagnosis of the infection must be rapid and sensitive so early intervention policies aimed to reduce the virus spread. PCR are the most sensitive methods for rapid diagnosis of EHVs if you have the facilities to perform it. Viral isolation and ELISA is also sensitive methods as well as Immunofluorescent and Immune-histochemistry techniques. However, histopathology can also be used in routine pathology laboratories for diagnosis of EHVs by detection the pathognomonic eosinophilic inclusion bodies within the infected cells.Equine herpes pathogens are endemic and continuous threat for horses in absence of vaccination programs. Equide herpes viruses affect equine health and induce major economic loss. They have a severe impact on the equine industry. Genetically, Arabian horses are the most valuable worldwide. Recommendation for control, must be given more care by using prophylactic program, healthy feeds and clean environment.

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