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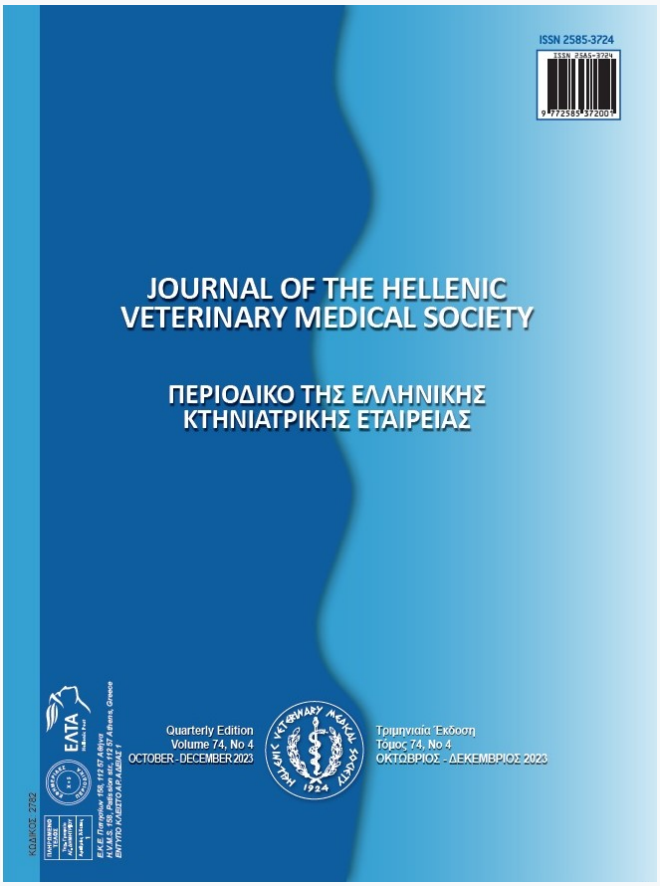

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Research article
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Investigation of the presence of equine herpesvirus-1 (EHV-1) in tissue samples of the aborted foals using histopathological, immunohistochemical, and Real-Time PCR techniques

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Abstract: Equine herpesvirus-1 (EHV-1) causes some manifestations in horses including; pneumonia, encephalitis, and abortion of live or dead feti in the last trimester of pregnancy. The aim of this study is to investigate the incidence of EHV-1 infection in the tissue samples collected from the aborted stillborn and live-born foals and also the foals which died soon after birth, as well as to compare and correlate the conventional histopathologic diagnosis with IHC and Real-Time PCR. For this purpose, tissue samples (lung, liver, spleen, kidney, placenta, and brain) of 38 foals from Istanbul, Kocaeli, Bursa, and Eskişehir provinces of Turkey between August 2009 and March 2011 were examined using IHC and Real-Time PCR. The obtained results showed 52.6 % positive Real-Time PCR rests (20/38 of examined samples) and 39.4 % positive immunohistopathological (IHC) tests (15/38 of examined samples), which indicated a correlation between these two diagnostic methods. In histopathological evaluation, the inclusion body was found in at least one of the tissue sections belonging to the lung, spleen, and mostly liver of 20 foals (52.6%). Tissue samples of the foals found to be positive by IHC and PCR revealed histopathologic findings compatible with the characteristics of the disease.

Keywords: Horse; abortion EHV-1; immunohistochemistry; Real-Time PCR

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INTRODUCTION

Equine herpesvirus-1 (EHV-1) is a highly contagious virus that causes pneumonia, encephalitis, and abortion in horses (Rimstad and Evensen,1993; Hazıroğlu and Milli 2001). Transmission easily occurs through contact with infectious materials such as aerosols, fomites, acutely infected virus-shedding individuals, aborted fetuses or placenta, and the use of common nursing stables (Oladunni et al., 2019). Clinical symptoms include upper respiratory tract infection in foals, abortion, and delivery of stillborn or weak foals in the last trimester of pregnant mares, and sometimes, as neurological signs in adult horses (Rimstad and Evensen,1993; Hazıroğlu and Milli 2001). Edema in the lungs, hemorrhage in the pericardium, and the presence of liver lesions are common characteristic histopathological lesions in the aborted feti (Yeşildere et al., 1994). The presence of intranuclear inclusion bodies in the hepatocytes is a pathognomonic feature for the disease. Inclusion bodies can also be present in the pulmonary alveolar epithelium, bile duct epithelial cells, lymph nodes, lymphocytes in the white pulp of the thymus and spleen, and vascular endothelial cells. Although the inclusion bodies are pathognomonic for the diagnosis of the disease, but they can not be detected in about 30-40% of the infested samples, which necessitate development of other diagnostic methods (Rimstad and Evensen,1993; Yeşildere et al., 1994; Pamukçu, 1975; Szeredi et al., 2003).

For this purpose, polymerase chain reaction (PCR), *in situ* hybridization, and immunohistochemical (IHC) staining are frequently used in diagnosis (Rimstad and Evensen,1993; Galosi et al. 2001; Diallo et al., 2006). The present study aimed to investigate the presence of EHV-1 using histopathological, immunohistochemical (IHC), and Real-Time PCR methods from tissue samples of aborted foals and to evaluate the compatibility of diagnostic methods.

MATERIALS AND METHODS

Tissue samples (lung, heart, liver, spleen, kidney, brain, thymus, lymph node, intestine and placenta) were collected from 38 aborted foals (23 stillborn and 15 died shortly after birth). Between August 2009 and March 2011, the samples were delivered from the Istanbul, Izmit, and Karacabey stud farms of the Jockey Club of Turkey (TJK) to Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine. Tissues were examined for EHV-1 by histopathology, immunohistochemistry and Real-Time PCR. Information regarding anamnesis, tissues taken, clinical and macroscopic findings of the mare and aborted foals used in this study are given in Table 1.

Table 1. Information regarding anamnesis, clinical findings and macroscopic findings of the mare and aborted foals used in the study

Case No	Age of the foal	Age of the mare	Vaccination Information of Mares	Clinical signs and Anamnesis	Examined Tissues	Macroscopy
1	Abortion at the 5th month *(necropsy)*	3	Vaccinated	Chronic weight loss	Lung, liver, spleen, kidney, intestine, heart, thymus, brain	A lentil-sized white focal lesion under the capsule in the spleen, hyperemia in the lung
2	Abortion at the 5th month *(necropsy)*	N/A	Vaccinated	N/A	Lung, liver, spleen, kidney, heart, thymus, brain	Organs were autolytic
3	Abortion at the 10th month	13	Vaccinated	N/A	Lung, liver, spleen, kidney,	No macroscopic findings were observed
4	Abortion at the 10th month	6	Vaccinated	N/A	Lung, liver, spleen, kidney, placenta	No macroscopic findings were observed
5	Unknown	N/A	N/A	N/A	Lung, liver, kidney, placenta	No macroscopic findings were observed
6	Unknown	N/A	N/A	N/A	Lung, liver, spleen, kidney, intestine, heart	No macroscopic findings were observed
7	1 week old *(necropsy)*	N/A	Vaccinated	Rapid breathing, insufficient milk sucking, stagnation, dropping of the 3rd eyelid in foal.	Lung, liver, spleen, kidney, intestine	Hemorrhagic areas in the lungs. Generalized icterus.
8	Unknown	N/A	N/A	Unknown	Lung, liver, spleen, kidney, placenta	No macroscopic findings were observed
9	Abortion at the 9th month *(necropsy)*	N/A	N/A	8th birth of the mare. Caesarean birth.	Lung, liver, spleen, kidney, intestine, thymus, brain	Renal hemorrhage with muddy brown appearance.
10	Abortion at the 10th month	N/A	Vaccinated	7th birth of the mare.	Lung, liver, spleen, kidney, intestine, placenta	No macroscopic findings were observed

Case No	Age of the foal	Age of the mare	Vaccination Information of Mares	Clinical signs and Anamnesis	Examined Tissues	Macroscopy
11	Abortion at the 5th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, brain	Hemorrhagic ascites, diaphragm rupture, subcutaneous ecchymoses in thoracal area.
12	Died shortly after birth	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart	No macroscopic findings were observed
13	Abortion at the 9th month	N/A	N/A	N/A	Liver, spleen, kidney, placenta	No macroscopic findings were observed
14	Abortion at the 9th month	N/A	N/A	N/A	Liver, spleen, kidney, placenta	No macroscopic findings were observed
15	Abortion at the 8th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart	No macroscopic findings were observed
16	Abortion at the 7th month	N/A	Vaccinated	2nd abortion of the mare.	Lung, liver, spleen, intestine, heart, brain	No macroscopic findings were observed
17	4 days old	11	Vaccinated	7th birth of the mare, three of her foals died. Foal has abdominal breathing.	Lung, liver, spleen, kidney, heart	No macroscopic findings were observed
18	Abortion at the 9th month	6	Vaccinated	2nd birth of the mare.	Lung, liver, spleen, kidney, heart, thymus	No macroscopic findings were observed
19	Abortion at the 9th month	N/A	Vaccinated	3rd birth of the mare.	Lung, liver, spleen, kidney, thymus, placenta	No macroscopic findings were observed
20	Abortion at the 10th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, thymus, placenta	Generalized icterus. The liver was orange in color.
21	Stillbirth at the 9th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart	No macroscopic findings were observed.
22	Abortion at the 9th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart	No macroscopic findings were observed.
23	Died 2,5 hours after birth	N/A	N/A	Lethargy	Lung, liver, spleen, kidney, heart, brain, placenta	No macroscopic findings were observed.
24	Died 20 hours after birth	N/A	N/A	Wheezing. Antibiotics were used for treatment.	Lung, liver, spleen, kidney, heart, brain	Hemorrhagic intestines.
25	Unknown	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart, brain	No macroscopic findings were observed.
26	Died 12 hours after birth	N/A	N/A	Abdominal breathing and wheezing, lethargy	Lung, liver, spleen, kidney, intestine, heart, thymus, brain	Fibrin deposition on the splenic capsule, hemorrhagic intestines.
27	2 weeks old	N/A	N/A	Cough, dyspnea. Antibiotics were used for treatment.	Lung, liver, spleen, kidney, intestine	No macroscopic findings were observed.
28	Abortion at the 10th month	N/A	N/A	Unknown	Lung, liver, spleen, kidney, heart, thymus, brain	The liver is in autolytic appearance.
29	Died 4 hours after birth	N/A	N/A	The foal dies in agony.	Lung, liver, spleen, kidney, thymus, brain	Hemorrhage in the heart muscle.
30	Stillbirth at the 11th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart, thymus, brain	Icteric appearance in internal organs, white necrotic areas 2 cm in diameter in the lung.
31	3 days old	N/A	N/A	Abdominal breathing, melena in the stool, leukopenia in the foal. The mare has not previously aborted	Lung, liver, spleen, kidney, thymus, brain	No macroscopic findings were observed.
32	Unknown	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart, placenta	No macroscopic findings were observed.
33	Died the day it was born	N/A	N/A	Respiratory failure	Lung, liver, spleen, kidney, heart, thymus, placenta	No macroscopic findings were observed.
34	Stillbirth at the 11th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart, thymus, brain	No macroscopic findings were observed.

Case No	Age of the foal	Age of the mare	Vaccination Information of Mares	Clinical signs and Anamnesis	Examined Tissues	Macroscopy
35	Stillbirth at the 10th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart, brain	No macroscopic findings were observed.
36	Abortion at the 9th month	N/A	N/A	N/A	Lung, liver, spleen, kidney, heart, thymus, brain	Icteric placenta, hemorrhagic ascites, fibrin deposition on the splenic capsule, expanded white pulp structures, hepatized areas in the lungs, and the cross-sectional surface of the kidneys were observed with cyanotic appearance.
37	Abortion at the 11th month *(necropsy)*	N/A	N/A	Abdominal breathing.	Lung, liver, spleen, kidney, heart, thymus, brain	Generalized icterus, foci of miliary necrosis in the liver, fibrin deposition on the splenic capsule, expanded white pulp structures, pale and edematous heart muscle, and cross-sectional surface of the kidneys were observed with cyanotic appearance.
38	Unknown	N/A	N/A	N/A	Liver, spleen, brain	No macroscopic findings were observed.

Histopathology

Tissue samples taken for histopathological and immunohistochemical (IHC) examinations were fixed in 10% formaline for 24 hours. The tissues were subjected to routine laboratory procedures, to be embedded in paraffin wax, sectioned in 4-5 μm thickness using rotary microtome (Leica RM2245, Germany), stained with hematoxylin eosin (H&E), and finally examined under a light microscope.

Immunohistochemistry

The paraffin sections were mounted on positively charged slides and treated with polyclonal ERV/EHV-1 antiserum (VMRD, catalog no: 210-70-ERV) using the streptavidin-biotin peroxidase technique. These sections were dewaxed and rehydrated before being subject to antigen retrieval by incubation with heated citrate buffer solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) using a microwave oven (~70°C, 4x5 minutes, and 20 minutes cooling inside the same solution). To quench the endogenous peroxidase sections, they were incubated for 10 minutes in 0.3% H_2O_2 in acetone-free methanol. To block the endogenous biotin sections were incubated in reconstituted powdered dried milk (2% dried milk solution). Afterward, sections were washed two times with phosphate-buffered saline (PBS; pH 7.4, 0.1M) and then incubated with a commercial blocking solution (ABCAM, catalog no: 93677) for 5 min. The slides were incubated with a polyclonal antibody at a dilution of 1 in 20, 000 (37°C for 1.5 h). After washing the slides two times with PBS, SPlink HRP Detection kit (Golden Bridge International, catalog no: D01-110/60) was applied according to the manufacturer's instructions. Finally, the sections were treated at room temperature for 30 min with 3-amino-9-ethyl carbazole (AEC) (UltraVision, catalog no: TA-060-HA), washed two times with distilled water, counterstained with Mayer's hematoxylin (LabVision, catalog no: TA-125-NM) and cover slipped under an aqueous mounting medium (LabVision, catalog no: TA-060-UG). As chromogen 3, 3'-diaminobenzidin (DAB) gives a brown reaction similar to the exogenous pigments (Taylor et al., 2006) which can be encountered in the respiratory system, AEC was preferred. Positive control sections were from a foal that had focal coagulation necrosis, intra-nuclear inclusions, and hepatitis in histopathological examination and positive results with Real-time PCR. For negative control, the primary antibody was substituted with PBS. After examining the staining of positive and negative controls; slides were evaluated as negative or positive according to the presence or absence of immune reaction by scanning different areas under a light microscope.

REAL-TIME PCR ASSAY FOR DETECTION OF EHV-1

Samples were homogenized and DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, catalog no: 69506,) as recommended by the manufacturer. The presence of EHV-1 was investigated in DNA samples using specific primers in real-time PCR by SYBR Green. Primers were used where the nucleotide positions for the glycoprotein B gene were selected from the range 297-314 for the Forward primer (EHV-1 MGB F1: 5-CAT GTC AAC GCA CTC

CCA-3) and 343-359 for the Reverse primer (EHV-1 MGB R1: 5-GGG TCG GGC GTT TCT GT-3) as previously studied by Diallo et al. (2006). Positive controls were the samples that were previously tested and confirmed by sequence analysis in the Virology Department of Istanbul University while negative controls were DNA extracted from a repeatedly EHV-1 negative sample and nuclease-free water.

Statistical analysis

The Medical Tests Assessment Method (Özdamar, 1999) was used to evaluate the sensitivity, specificity, positive identification, negative identification, and accuracy rates of the IHK method according to the Real-Time PCR method.

Sensitivity (Se)% = 100xGP / (GP + YN); Specificity (Sp)% = 100xGN / (YP + GN); Positive identification rate (PPV)% = 100xGP / (GP + YP); Negative identification rate (NPV)% = 100xYN / (YN + GN); Accuracy rate (DA)% = 100x (GP + GN) / n.

RESULTS

The histopathological, immunohistochemical, and PCR results of the tissues from the aborted foals examined in the current study were shown in Table 2 as a collective comparison.

Table 2. Comparison of histopathology, IHC, and PCR results

Case no.	Histopathology						IHC			PCR
	Liver		Lung		Others		Liver	Lung	Others	All organs
	Necrosis	inclusion body	Necrosis	inclusion body	Necrosis	inclusion body				
1	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	-	-	-	-	-
5	-	-	-	+	+	-	-	-	-	+
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	+	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	--	-	-
11	-	-	-	-	-	-	-	-	-	-
12	+	-	-	-	-	-	-	-	+	-
13	+	+	No tissue	No tissue	-	+	-	No tissue	+	+
14	+	+	-	-	-	-	+	-	+	+
15	+	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	+	+	-	+	-	-	-	-	-	+
18	+	+	-	-	-	-	-	-	-	-
19	+	+	-	+	-	-	-	-	+	+
20	-	-	-	-	-	-	-	-	-	-
21	+	+	-	-	-	+	+	-	+	+
22	+	+	-	-	-	+	+	-	-	+
23	+	+	-	-	-	-	+	-	+	+
24	+	-	-	-	-	-	-	-	+	+
25	+	-	-	+	-	-	-	-	-	+
26	-	-	-	-	-	-	+	-	+	+
27	-	-	-	-	-	-	-	-	-	-
28	+	+	-	+	+	-	-	-	+	+
29	+	+	-	+	-	-	-	-	+	+
30	+	+	-	+	+	+	-	-	+	+
31	+	+	-	+	+	+	-	-	-	+
32	-	-	+	-	-	-	-	-	-	-
33	+	+	+	+	-	-	+	-	+	+
34	+	-	-	-	-	-	-	-	-	-
35	+	+	-	+	-	-	-	-	-	+
36	+	+	+	+	+	-	+	-	+	+
37	+	+	+	+	+	+	+	-	+	+
38	+	+	No tissue	No tissue	-	-	-	-	+	+

Histopathological findings

Lung tissues from 36 of 38 foals were available. In most of those tissues, respiratory capillary vessel distention and edema in the alveoli were present (26/36) (Figure 1a). Fibrine formations varying from mild to moderate levels were observed in the alveoli. In addition, there were mild leukocyte infiltrations in the inter and intra alveolar areas, and mild to moderate leukocyte infiltration (21/36). Coagulative necrosis of the alveolar walls was observed in a small number of animals (6/36). Intranuclear eosinophilic inclusion bodies were detected in bronchial epithelial cells and a small number of alveolar epithelial cells (12/36) (Figure 1b).

In most of the liver samples, hepatic cord structures were disrupted, sinusoids appeared to be congested, and parenchymal cells were swollen and degenerated. Mild to moderate neutrophil leukocyte infiltrates and to a lesser extent, lymphocyte infiltrates were observed in sinusoids. Areas of focal coagulative necrosis were observed in hepatocytes (23/38) (Figure 1c). Moreover, intranuclear inclusion bodies were found in the parenchymal cells around the necrotic areas (Figure 1d) in animals with coagulative necrosis (18/23).

However, capillary distention, hyperemia, and focal glial cell proliferation in the neuropil were observed in the brain parenchyma (Figure 1 e).

Necrotic areas which characterized by diffuse

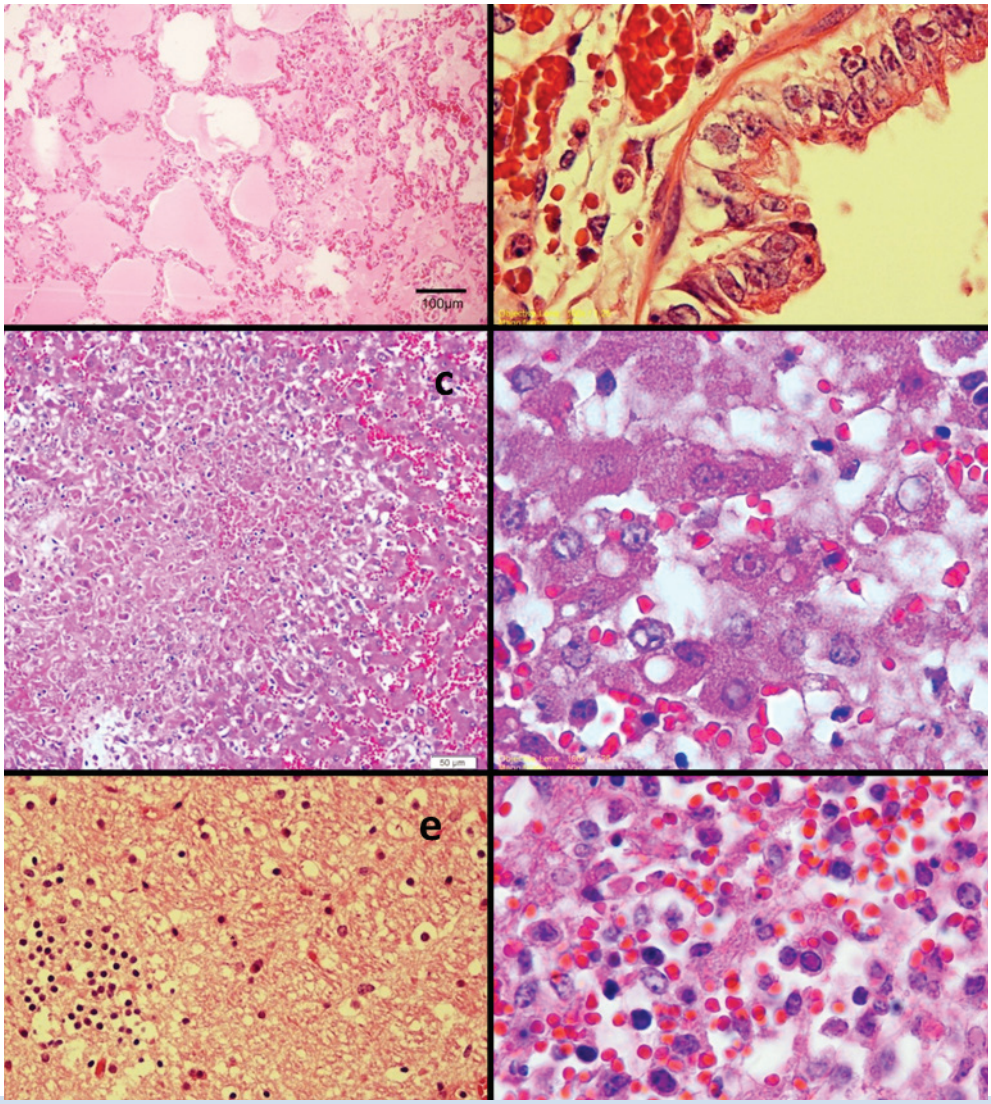

Figure 1. (a) Edema of alveolar lumens in the lung (H&E, Bar=100µm), (b) Intranuclear inclusion body in bronchial epithelium (H&E, Bar=10µm), (c) Focal coagulative necrosis in the liver (H&E, Bar=50µm), (d) Intranuclear eosinophilic inclusion body in hepatocyte in liver (H&E, Bar=10µm), (e) Focal gliosis in neuropil in the brain (H&E, Bar=20µm), (f): Intranuclear eosinophilic inclusion body in the spleen (H&E, Bar=10µm)

karyorrhexis and karyolysis were observed in lymphocytes in the white pulp of the spleen (7/38). Also, intranuclear eosinophilic inclusion bodies were found in some intact lymphocytes around these necrosis areas (3/7) (Figure 1f). The kidneys were generally congested, and the parenchymal degeneration was detected in the tubulus epithelium (9/36). In some cases, there were hemorrhages in the intertubular areas (11/36). In addition, the thickening of glomerular capillary membranes and proteinaceous fluid in Bowman's space were observed in many sections (23/36).

In the thymus, there were vessel distention, neutrophil leukocyte and plasma cell infiltrates, and edema in the interfollicular areas under the capsule and in the cortex. Occasional karyopyknosis and karyorrhexis were observed in lymphoid cells in the cortex.

Intestinal tissues from 5 of the 38 foals were available. Enteritis characterized by intestinal villi exfoliation, necrosis, and degeneration in the glands were present in some cases (3/5).

The examined placentas (13) showed desquamation with vacuolar and degenerative changes in trophoblastic epithelial cells, which usually lined the villi in placentas (7/13). The heart samples of 26 foals were available. Moreover, the cardiac muscle fibers of the examined heart samples were separated from each other, which might be due to distention and edema in the vessels (22/26). Rarely, mononuclear cell infiltrates were observed between the cardiac muscle fibers.

Immunohistochemical findings

Lung, heart, spleen, thymus, lymph node, and intestinal tissues which were stained immunohistochemically with EHV-1 antiserum showed no positive staining. However, intracytoplasmic staining was observed in hepatocytes in the liver tissues (8/38), as widespread areas in some (5/8) and limited areas in others (3/8) (Figure 2a, b). Necrosis and intranuclear eosinophilic inclusion bodies were observed in seven of the 8 liver tissues with positive staining. In the brain tissues, nine (9/18) of the patients had poor staining in the neuropil (Figure 2c). In addition, intracytoplasmic staining was detected in the trophoblastic epithelium in four (4/13) placentas (Figure 2 d). Cytoplasmic positive staining was observed in tubule epithelium in only two kidneys (2/36) (Figure 2e). There was no staining in the negative control tissues.

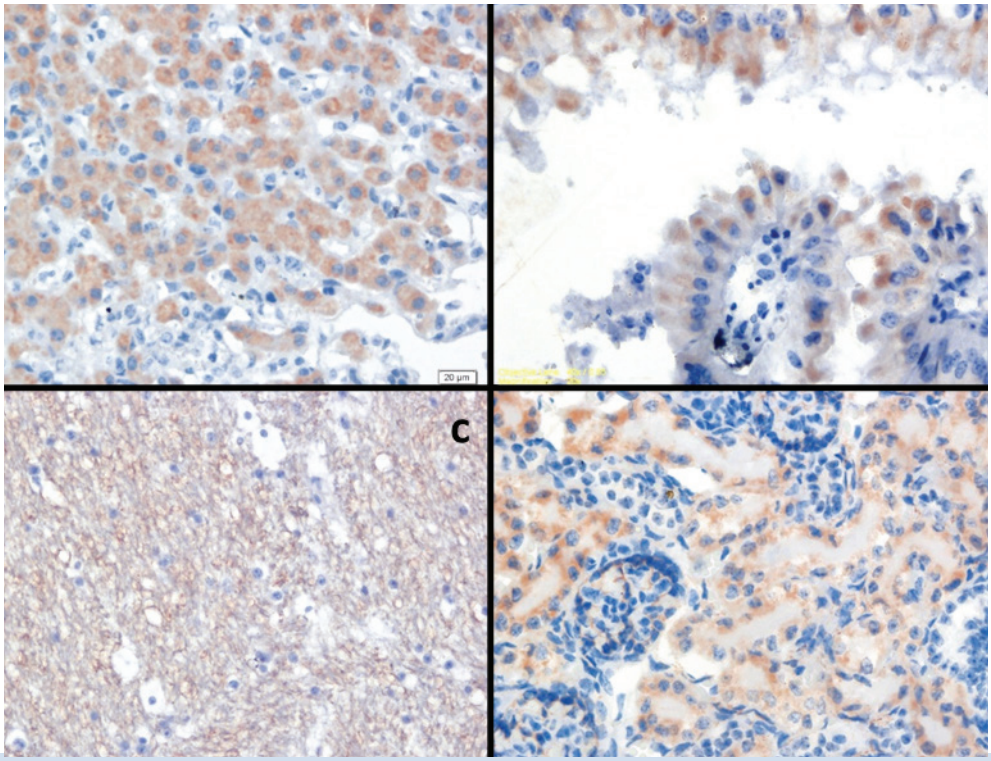

Figure 2. (a) Cytoplasmic positive staining in hepatocytes in the liver (Streptavidin biotin staining, bar = 20µm), (b) cytoplasmic positive staining in trophoblastic epithelial cells in placenta (Streptavidin biotin staining, bar=20µm), (c) poor staining in neuropil in the brain (Streptavidin-biotin staining, bar = 20µm), (d) cytoplasmic positive staining in renal tubular epithelium (Streptavidin-biotin staining, bar = 20µm)

Real-Time PCR Findings

Viral DNA was detected in 52% (20/38) of the foals using Real-Time PCR. Out of 228 tissue samples from 38 foals, 119 were positive and 109 were negative for EHV-1. The positive samples consisted of 20 liver, 19 spleen, 19 kidney, 18 lung, 16 heart, 12 brain, 10 thymus and 5 placenta samples.

Primers used in the Real-Time PCR analysis were synthesized to detect the glycoprotein B gene. In this direction, the amplification image of the glycoprotein B gene of the EHV-1 virus in the examined tissues was seen in Figure 3. C_T values of positive tissues were between 12 and 20 for lungs, 26 and 37 for livers, 24 and 30 for brains, 30 and 34 for spleens, 32 and 34 for hearts, and 14 and 31 for thymuses.

Statistical Findings

About 52% of the cases (20/38) were positive with Real-Time PCR, while about 39% of the cases (15/38) gave positive results in IHC methods. However, 39% of the cases (15/38) tested positive with Real-Time PCR and IHC methods. Meanwhile, there were 13% of the positive PCR cases (5/38) showed no positive staining in IHC.

Based on the Real-Time PCR method, the statistical analysis showed that the sensitivity of IHC was 75%, the specificity value was 100%, the positive identification rate was 100%, the negative identification rate was 21%, and the accuracy rate was 86%.

DISCUSSION

Equine herpesvirus-1 infection is an important sporadic and epidemic disease which characterized by respiratory and neurological disorders and abortions in horses that causes economic losses (Sugahara et al., 1997; Zhang et al., 1998; Gilkerson et al., 1999; Siedek et al., 1999; Taouji et al., 2002). In this study, histopathology, IHC and PCR were used together to investigate the presence of EHV-1 and compatibility of diagnostic methods.

Although the reported lesions in the examined aborted feti (38) differed depending on the organs, however, the pathological changes were found mostly in the lungs (26/38) and then in the livers (23/38). In accordance with Alibaşoğlu (1969), Correa (1970), Eaglesome and Garcia (1979) and Yeşildere et al. (1994) these changes were observed as areas of focal coagulative necrosis in the lung, liver, and spleen,as well as presence of intranuclear eosinophilic inclusion

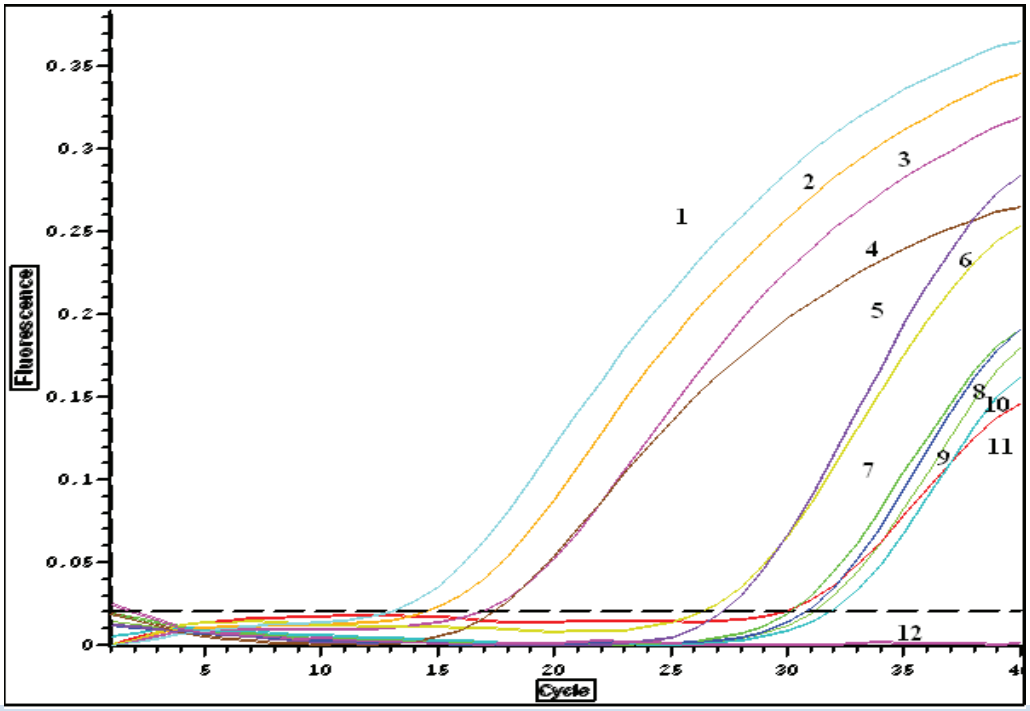

Figure 3. Amplification image of glycoprotein B gene of the EHV-1 virus

Line 1: Lung (Foal no.33), Line 2: Thymus (Foal no.31), Line 3: Lung (Foal no.31), Line 4: Positive Control, Line 5: Placenta (Foal no.33), Line 6: Liver (Foal no.31), Line 7: Spleen (Foal no.31), Line 8: Kidney (Foal no.31), Line 9: Thymus (Foal no.33), Line 10: Heart (Foal no.31), Line 11: Brain (Foal no.31), Line 12: Negative Control.

bodies around the necrotic areas or in other locations.

Regarding the pathogenesis of the EHV-1 infection, it has been reported that the virus causes vasculitis and thrombosis in the endometrium and placenta, nasal mucosa as well as the pulmonary veins of the fetus. (Edington et al., 1991; Smith et al., 1996; Smith et al., 2001; Szeredi et al., 2003). In the current study, hyperemia was common in the tissues, while vasculitis and thrombosis could not be observed in the vessel walls. Although vascular lesions could not be detected in 13 placentas, 4 of them showed positive staining in IHC, and 5 of them were Real-Time PCR positive. Similarly, other studies indicate that vascular lesions in the placenta are rarely detected, while antigen presence can be detected with other diagnostic methods such as IHC, PCR and in situ hybridization (Edington et al., 1991; Mukaiya et al.,2000; Szeredi et al., 2003; Gerst et al., 2010).

Today, EHV-1 is diagnosed using a florescent antibody (FA), in situ hybridization (IHS) immunoperoxidase (IP), virus isolation (VI), IHC, and PCR techniques together or separately (Rimstad and Evensen,1993; Schultheiss et al., 1993; Mukaiya et al., 2000; Smith et al., 2001; Szeredi et al., 2003; Gerst et al., 2010; Léon et al., 2008).The aborted feti under investigaion (38) showed positives staining with IHC in 15 feti (39%) and viral DNA by Real-Time PCR in 20 feti (52%). About 15 cases were positive for EHV-1 in both Real-Time PCR and IHC staining. Although positivity was detected by Real-Time PCR (13%), no staining was detected with IHC in five cases. According to our results, the positivity was mostly obtained by Real-Time PCR. However, when it was evaluated on a tissue basis, much greater differences were detected between Real-Time PCR and IHC results. For example, 20 liver and 19 lung tissues were positive in PCR, while only 8 of the liver tissues showed positive staining in IHC. Based on these findings, there was no correlation between organ-based PCR and IHC. Rimstad and Evensen (1993) recorded 21 of 31 EHV-1 suspect samples (32%) to be positive with PCR, IHC, and virus isolation, however, these results were given per animal and tissue-based individual evaluation results were not given.

A comparison of histopathological findings with IHC and PCR results revealed more significant differences. Despite histopathological examination revealed necrosis in 23 cases (60%) and inclusion bodies in 18 cases (47%) in the liver samples, only 8 of the liver samples showed positive staining with IHC (21.05%) and 12 of the liver samples were positive with PCR (31.57%). However, edema (26/36), fibrin (21/36), coagulation necrosis (6/36), and intranuclear inclusion bodies (12/36) were detected in the lungs in the histopathological examination, there was no staining in IHC. Nevertheless, EHV-1 positivity was detected in 18 samples by PCR in the lungs. These results show that there was no direct correlation between histopathology, IHC, and PCR, and the most sensitive diagnostic method is the Real-Time PCR. Schultesis et al. (1993), also pointed out that there is no direct correlation between IP, FA, VI, and histology in the examination of the liver and lungs.

The examined liver tissues (18) showed inclusion bodies in with PCR positive reaction in 12 samples, while the lung tissues, inclusion bodies appeared in 12 cases and PCR positivity was detected in 18 cases. Detection of the inclusion body is thought to be a viral particle during viral infections (Erer et al., 2000), which help in detection of DNA using PCR. So the failure to detect viral DNA by PCR in 6 samples with inclusion bodies in our study might be due to mixture occurred between the inclusion bodies with the intra-nuclear structures (nucleoli) which could be sained eosinophilic with H&E.

The EHV-1 infection positivity is usually detected in the liver using IHC (Rimstad and Evensen,1993; Schultheiss et al., 1993; Mukaiya et al., 2000;). In our study, the majority of positive IHC staining was seen in the liver. Similar to previous studies (Rimstad and Evensen,1993; Schultheiss et al., 1993) positive staining with IHC in the liver especially in the cytoplasm of intact hepatocytes around necrotic areas. While Kupffer cells mostly stained positively, intranuclear inclusion bodies in hepatocytes were not stained. In parallel with Schulthesis et al. (1993), staining was observed in the tubular epithelial cells of the kidney in the areas extending from the cortex to the medulla in two foals (Foal no.14 and 21). Several studies also reported positive IHC staining in the lung, lymph nodes, spleen, intestine, and thymus (Rimstad and Evensen,1993; Schultheiss et al., 1993, Murray et al., 1998). Contrarily, there was no positive staining in any of the organs examined in this study.

In the current study, the histopathological changes could not be observed in the placental samples belonging to about 13 cases of the aborted feti. However, cytoplasmic staining was detected by IHC in the trophoblastic epithelial cells of four of these placentas, and viral DNA belonging to EHV-1 was detected in

five of them by Real-Time PCR. Szeredi et al. (2003) found nonspecific changes in the placenta due to normal birth, such as edema, hyperemia, congestion, and mild vacuolar degeneration in the chorion epithelium, and they also observed mild lymphohistiocytic vasculitis in allantoic vessels in only one placenta. The latter authors observed a positive immunohistochemical staining in 11 of 76 of placental samples, especially in the cytoplasm of trophoblastic epithelial cells. However, Mukaiya et al. (2000) recorded no changes in the histopathological examination of 7 placental samples, and none of them stained with IHC, but viral DNA belonging to EHV-1 is detected in all of them by Real-Time PCR, these findings came in one line with our results.

In the neurological syndrome caused by EHV-1, it has been reported that the viral antigen does not actually settle in neurons but is in the endothelial cells of the brain and spinal cord (Patel et al., 1982; Kydd et al., 1994a; Kydd et al., 1994b). In our study, the brain tissues of 12 foals were positive by the Real-Time PCR method (12/18), and in nine of these 12 tissues, IHC staining was poor in the neuropil.

Our study revealed that the most positivity is obtained with Real-Time PCR, therefore, we attributed that the Real-Time PCR technique gave more accurate results in the diagnosis of EHV-1, which supported by the findings of Mackie et al. (1996), who stated that positive results were obtained by PCR in cases where virus isolation was negative. For this reason, Real-Time PCR results were used as a reference in the statistical comparison applied in this study. In addition, Real-time PCR results were verified by performing sequence analysis of positive samples. Rimstad and Evensen (1993) also used PCR results as a reference in their study using virus isolation, PCR and IHC. According to the calculations made when Real-Time PCR method was taken as a reference; the sensitivity of IHC was 75%, the specificity value was 100%, the positive identification rate was 100%, the negative identification rate was 21%, and the accuracy rate was 86%.

CONCLUSIONS

The current study revealed that Real-Time PCR and IHC were highly effective methods in the diagnosis of EHV-1 infection. Real-Time PCR was the most reliable method which might be due to its definite results. Our result reported that the confirmation of the findings in histopathological examination using IHC and Real-Time PCR could be more useful for the diagnosis of the disease.

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