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Communication

[*Comunicação*]

Identification of equine herpesvirus type 1 as cause of abortion in mares in Southern Brazil

[*Identificação do herpesvirus equino tipo 1 como causa de abortos em éguas no sul do Brasil*]

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Equine herpesvirus type-1 (EHV-1) is classified into the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, gênero *Varicellovirus* (International…, 2018). This virus is a ubiquitous pathogen that affects the horse population worldwide causing epidemic abortion, neonatal death, respiratory disorders and myeloencephalopathy (Schulmann *et al*., 2015). Infection with this virus may cause a serious economic impact in the horse industry, especially for breeding farms (Gilkerson *et al*., 1999). In Brazil, serological studies have demonstrated the circulation of EHV-1 in horses (Lara *et al*., 2010; Diaz *et al*., 2015). Latency has been demonstrated in both lymphoid and neural tissues and allows the virus to circulate silently in the horse populations (Patel *et al*., 2005).

Equine herpesvirus-1 abortion is diagnosed by detailed examination of the aborted fetus using a combination of gross findings, histopathology, virus isolation, polymerase chain reaction (PCR), and immunostaining (Reed *et al*., 2004). Conventionally, these tests have been applied to selected target organs, particularly the liver, lung, thymus and spleen (Easton *et al*., 2009). The main purpose of this study was to describe and confirm EHV-1 as cause of abortion in southern Brazil.

The epidemiological data of each case were obtained from the necropsy submission forms of the Laboratório Regional de Diagnóstico of the Universidade Federal de Pelotas, RS, Brazil (LRD/UFPel). Sections of paraffin embedded tissues were subjected to hematoxylin and eosin staining and samples of the liver, spleen and lungs were processed for immunohistochemical (IHC) analysis using a polyclonal antibody specific to EHV-1 (PAB-ERV – VMRD) and analyzed using a commercial streptavidin-alkaline phosphatase kit (LSAB+System-AP, Dako Cytomation, K0689 [Dako Cytomation, 6392 Via Real, Carpinteria, CA, USA]) according to a previous study (Easton *et al*., 2009). Positive (a previously confirmed EHV-1 case) and negative controls (liver, spleen and lungs from a healthy, EHV-1-free horse) were included. A specific EHV-1 and EHV-4 multiplex nested PCR was performed on DNA extracted from sections of paraffin-embedded tissues by using *Quick-DNA FFPE MiniPrep (Zymo Research)* according to the protocol provided by the manufacturer (GE Healthcare). PCR conditions and primers were previously described (Ataseven *et al*., 2009). An aliquot of the product from the first round of amplification was used as template for the second round of PCR. Amplicons specific for EHV-1 (188bp) were visualized after electrophoresis in agarose gel (1.0%) stained with *Gel Red.* After, the PCR

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products were excised from the gel, purified using the *Gene JET Gel Extraction Kit* (*Thermo Scientific)* and, subjected to DNA sequencing using both reverse and forward primers (ABI 3700 DNA analyzer). The sequences were trimmed and the homology searches performed with BLAST (Basic Local Alignment Search Tool). DNA extracted from a reference strain of EHV-1 propagated in RK13 cells was used as a positive control.

Four EHV-1 infection outbreaks in thoroughbred mares were identified in the necropsy protocols of the LRD/UFPel. These cases occurred between 1994 and 2012. In the first outbreak, eight out of 16 pregnant mares aborted. Seven abortions occurred between 10 and 11 months of gestation, and one abortion occurred in a 9-month-pregnant mare. The mares of this stud farm were not vaccinated against equine rhinopneumonitis. In another outbreak, there were two abortions and one stillbirth out of 35 pregnant mares. In the third outbreak, one abortion of a 9-month old fetus occurred in one mare out of 50 pregnant mares. In the fourth outbreak, one abortion of a 10 months old fetus was recorded in a herd of 50 pregnant mares, which have been vaccinated with a commercial vaccine against rhinopneumonitis annually. No serologic status of the vaccinated animal was available.

In all outbreaks, only formalin-fixed organ samples were received for evaluation at the laboratory. The clinicians described the findings grossly as a diffuse yellow tinge to all tissues and abundant amber fluid in body cavities.

Microscopic evaluations in all cases showed multiple random foci of degeneration and necrosis (Figure 1A) with moderate infiltration by lymphocytes and macrophages in the liver. Several acidophilic nuclear inclusion bodies were also observed in the hepatocytes and Kupffer cells (Figure 1A). Multifocal necrosis was also evident in the spleen along with multiple acidophilic intranuclear inclusion bodies. The lungs exhibited multifocal necrotic foci randomly distributed in the parenchyma and acidophilic intranuclear inclusion bodies were also observed in the epithelia of the bronchioles. Extramedullary hematopoiesis was observed was evident in some less affected areas of the liver.

EHV-1 antigen was detected using IHC staining within the nucleus and cytoplasm of several hepatic cells and leukocytes of the tissue samples from all the cases. Positive IHC staining was associated with necrotic foci in the liver (Figure 1B), lung and in the spleen in all analyzed samples.

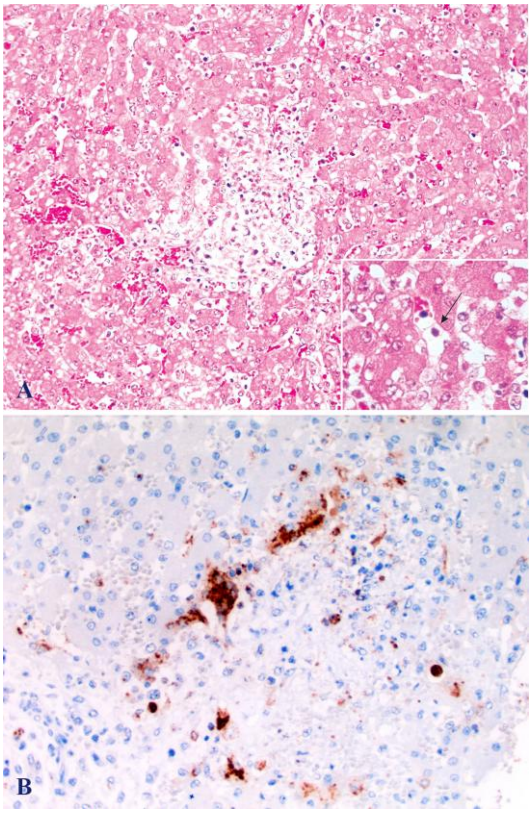

Figure 1. Equine abortion by EHV-1. A. There is a necrotic focus in the hepatic parenchyma and congestion of sinusoids. H.E. Obj. 20x. Inset. Acidophilic nuclear inclusion body is observed in degenerated hepatocyte (arrow). H.E. Obj. 40x. B. Labeling of EHV-1 viral antigen in the hepatocytes of the aborted foal.
90x137mm (300 x 300 DPI).

The amplification of a specific 188bp amplicon from DNA extracted from two paraffin-embedded tissues confirmed the presence of EHV-1 in tissue samples from two aborted fetuses. Additionally, the sequencing of the PCR products and BLAST results obtained with the consensus sequence (100% identity with multiple gB sequences available in the GenBank) confirmed the presence of EHV-1 DNA in the tissues analyzed.

EHV-1 abortion was first diagnosed in Southern Brazil in 1994 by isolation of virus associated with the high prevalence of EHV-1 antibodies in the equine population (Weiblen *et al*., 1994). These authors suggested that abortions due to this virus are more frequent than reported as a consequence of absence of laboratory diagnosis. Several other reports have demonstrated the presence of specific antibodies to EHV-1 in Brazil (Vargas e Weiblen, 1991). However, it was demonstrated in the present study that the prevalence of viral abortion was less frequent in Southern Brazil compared with other causes of equine abortion in the same region. EHV-1 abortion accounted for 3.8% out of 104 abortion outbreaks diagnosed in this region between 1978 and 2016 (Schild *et al*., 2017). The low prevalence observed in these cases could be explained, at least partially, by the systematic vaccination of the animals in stud farms.

Abortion outbreaks are described in vaccinated mares from a reproductive center that specializes in the impregnation of mares using artificial insemination and embryo transfer. The authors suggest that broodmare management and improvement of the vaccine efficacy could prevent abortion outbreaks (Barrandeguy *et al*., 2002). Foote *et al*. (2006) demonstrate that EHV-1 and EHV-4 circulate in vaccinated populations of mares and their unweaned foals. This could explain the occurrence of sporadic abortion in vaccinated animals, which was observed in the present report. The annual vaccination without serological monitoring of mares may also have contributed to the occurrence of the abortion.

Histological lesions observed in the present study were characteristic of abortions by EHV (Schlafer e Miller, 2007). IHC analysis showed the presence of EHV-1 antigen in tissues of aborted fetus. IHC analysis is an important diagnostic tool especially in cases suspected of EHV infection in which specific histopathological changes are not found (Szeredi *et al*., 2003). In the present study the viral etiology of the abortions outbreaks was confirmed based on the histological lesions along with the detection of antigens of EHV-1 by immunohistochemical analysis. Furthermore, specific EHV-1 DNA was detected (by the multiplex nested-PCR) and confirmed (by nucleotide sequencing and BLAST analysis) on tissue samples of two cases, corroborating the findings of IHC and histological analysis. Taken together, these findings indicated that EHV-1 should be considered in the differential diagnosis of equine abortion in southern Brazil.

Keywords: abortion, equine herpesvirus-1, immunohistochemistry, PCR

RESUMO

Foi realizado um estudo retrospectivo dos casos suspeitos de aborto por herpesvírus equino observados na região sul do Rio Grande do Sul entre 1978 e 2016. Foram revisados os protocolos de necropsia do Laboratório Regional de Diagnóstico da Faculdade de Veterinária da Universidade Federal de Pelotas resgatando-se os dados epidemiológicos, lesões macroscópicas e histológicas de cada caso. Foram observados dois surtos da enfermidade com prevalência entre 5,7% e 50% nos diferentes estabelecimentos, e dois casos individuais. Em todos os casos foram enviados fragmentos de órgãos fetais formolizados. Histologicamente, em todos os casos foram observados focos de necrose no fígado, pulmão e baço e presença de corpúsculos de inclusão acidofílico em hepatócitos, células epiteliais pulmonares e leucócitos. A imuno-histoquímica utilizando anticorpo policlonal comercial para herpesvirus equino-1 (EHV-1) revelou marcação positiva em todos os casos. Além disso, foi extraído DNA dos tecidos emblocados em parafina dos casos e submetidos à técnica de nested-PCR seguida de sequenciamento genômico dos amplicons em duas amostras. Estes achados indicam, que EHV-1 deve ser considerado como diagnóstico diferencial em casos de aborto em equinos no sul do Rio Grande do Sul.

Palavras-chave: aborto, herpesvirus equino-1, imuno-histoquímica, PCR

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