Molecular confirmation of ovine herpesvirus 2-induced malignant catarrhal fever lesions in cattle from Rio Grande do Norte, Brazil[1]

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ABSTRACT.- Headley S.A., Sousa I.K.F., Minervino A.H.H., Barros I.O., Barrêto Júnior R.A., Alfieri A.F., Ortolani E.L. & Alfieri A.A. 2012. **Molecular confirmation of ovine herpesvirus 2-induced malignant catarrhal fever lesions in cattle from Rio Grande do Norte, Brazil**. *Pesquisa Veterinária Brasileira 32(12):1213-1218.* Laboratório de Patologia Veterinária, Faculdade de Medicina Veterinária, Universidade Norte do Paraná, PR 218 Km 1, Cx. Postal 560, Arapongas, PR 86702-670, Brazil. E-mail: headleysa@gmail.com

Molecular findings that confirmed the participation of ovine herpesvirus 2 (OVH-2) in the lesions that were consistent with those observed in malignant catarrhal fever of cattle are described. Three mixed-breed cattle from Rio Grande do Norte state demonstrated clinical manifestations that included mucopurulent nasal discharge, corneal opacity and motor incoordination. Routine necropsy examination demonstrated ulcerations and hemorrhage of the oral cavity, corneal opacity, and lymph node enlargement. Significant histopathological findings included widespread necrotizing vasculitis, non-suppurative meningoencephalitis, lymphocytic interstitial nephritis and hepatitis, and thrombosis. PCR assay performed on DNA extracted from kidney and mesenteric lymph node of one animal amplified a product of 423 base pairs corresponding to a target sequence within the ovine herpesvirus 2 (OVH-2) tegument protein gene. Direct sequencing of the PCR products, from extracted DNA of the kidney and mesenteric lymph node of one cow, amplified the partial nucleotide sequences (423 base pairs) of OVH-2 tegument protein gene. Blast analysis confirmed that these sequences have 98-100% identity with similar OVH-2 sequences deposited in GenBank. Phylogenetic analyses, based on the deduced amino acid sequences, demonstrated that the strain of OVH-2 circulating in ruminants from the Brazilian states of Rio Grande do Norte and Minas Gerais are similar to that identified in other geographical locations. These findings confirmed the active participation of OVH-2 in the classical manifestations of sheep associated malignant catarrhal fever.

INDEX TERMS: Cattle disease, ovine herpesvirus 2, malignant catarrhal fever, necrotizing vasculitis, gammaherpesvirus.

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RESUMO.- [**Diagnóstico molecular de herpesvírus ovino tipo 2 em surto de febre catarral malígna em bovinos do Rio Grande do Norte.**] Os achados moleculares confirmaram a participação do herpesvírus ovino tipo 2 (OVH-2) nas lesões observadas em um surto de febre catarral malígna em bovinos. Três bovinos oriundos de propriedade rural de Mossoró, Rio Grande do Norte apresentaram manifestações clínicas, que incluíram secreção nasal mucopurulenta, opacidade da córnea e incoordenação motora. A necropsia revelou ulcerações e hemorragias da cavidade oral, opacidade da córnea e linfonodomegalia. Os achados histopatológicos significativos incluíam vasculite necrosante generalizada, meningoencefalite não supurativa, nefrite intersticial linfocítica, hepatite linfocítica e trombose. A PCR, realizada a partir

de DNA extraído do rim e do linfonodo mesentérico de um dos animais, amplificou um produto com 423 pares de base do gene da proteína do tegumento do herpesvírus ovino 2 (OVH-2). O sequenciamento direto dos produtos da PCR e a análise pelo Blast demonstraram que o produto amplificado apresentava 98-100% de identidade com sequências do OVH-2 depositadas no GenBank. As análises filogenéticas, baseadas nas sequências deduzidas de aminoácidos demonstraram que a cepa de OVH-2 circulando em ruminantes nos estados de Rio Grande do Norte e Minas Gerais são semelhantes àquelas identificadas em outras regiões geográficas. Esses achados confirmam a participação ativa de OVH-2 nas manifestações clássicas de febre catarral maligna em ovinos.

TERMOS DE INDEXAÇÃO: Doenças de bovinos, herpes vírus ovino tipo 2, febre catarral malígna, vasculite necrosante, gammaherpesvírus.

INTRODUCTION

Malignant catarrhal fever (MCF) is usually a fatal disease of domestic cattle, wild ruminants, and occasionally pigs that occurs worldwide (Brown et al. 2007, MacLachlan & Dubovi 2011). This disease is induced by cross species infection of members of the genus *Rhadinovirus*, subfamily *Gammaherpesvirinae* (MacLachlan & Dubovi 2011). There are two distinct classical epidemiologically recognized manifestations of MCF: 1) occurring within Africa and occasionally in wildlife outside of this continent that is caused by *Alcelaphine herpesvirus* 1 (AlHV-1), which uses wildebeest (*Connochaetes gnu* and *C. taurinus*) as a carrier; 2) a disease occurring outside Africa, which affects cattle, bison, and deer that is caused by ovine herpesvirus 2 (OVH-2), in which sheep are recognized as carriers (Russell et al. 2009, MacLachlan & Dubovi 2011, Zachary 2012b). These manifestations are frequently referred to as wildebeest associated (WA-MCF) and sheep-associated (SA-MCF) malignant catarrhal fever, respectively (Brown et al. 2007, Russell et al. 2009). Although SA-MCF is predominantly diagnosed in ruminants (Brown et al. 2007, MacLachlan & Dubovi 2011, Zachary 2012a,b), the disease was recently identified in a foal (Costa et al. 2009b).

The gross lesions associated with MCF, irrespective of the type of manifestation, include multisystemic ulcerations and erosions associated with hemorrhages and hypertrophy of lymphoid organs (Brown et al. 2007, MacLachlan & Dubovi 2011, Zachary 2012a,b). Histological alterations are characterized by multisystemic lymphoproliferative and necrotizing vasculitis with fibrinoid necrosis (degeneration) to the wall of affected vessels (MacLachlan & Dubovi 2011, Zachary 2012b); but thrombi are not easily demonstrated histologically (Brown et al. 2007). Although characteristic histopathology findings are recognized by the World Organization for Animal Health as being diagnostic for FCM, sequencing of PCR products of the agent is ideal for phylogenetic and epidemiological studies (Russell et al. 2009). Several studies have described the phylogenetic relationship based on OVH-2 (Kleiboeker et al. 2002, Li et al. 2003, Jacobsen et al. 2007).

MCF has been described in all five macroregions of Brazil, with disease being characterized predominantly by characteristic histopathological changes (Lemos et al. 2005, Rech et al. 2005, Macêdo et al. 2007) and a combination of histopathology with PCR without complete molecular diagnosis through sequencing of the amplified product (Garmatz et al. 2004, Mendonça et al. 2008). However, characterization of MCF based on the sequencing of the OVH-2 tegument protein gene has been done in an outbreak occurring in buffaloes (Costa et al. 2009a) and in a unique case in a foal (Costa et al. 2009b) from Minas Gerais, using previously described specific primers (Baxter et al. 1993, Li et al. 1995).

This paper describes the molecular findings (PCR, sequencing, and phylogenetic analyses) that confirmed the participation of OVH-2-induced lesions in three cases of MCF occurring in cattle.

MATERIALS AND METHODS

Animals, clinical history, and necropsy

Three mixed-breed cattle that were maintained on a farm located within the municipality of Mossoró/RN, Brazil, were admitted at the Veterinary Teaching Hospital (Hovet-UFRSA), Universidade Federal Rural do Semi-Árido, RN, between November 2008 to April 2009; the biological data of the animals are given in Table 1. The small subsistence farm contained 22 cattle that were raised semi-extensively in pastures that contained horses and sheep (n=70). The owner reported that several (41%; 9/22) of these died suddenly after demonstrating clinical manifestations that included fever, mucopurulent nasal discharge, motor incoordination, muscular spasms, and respiratory difficulties. Three of these were seen individually at the Hovet-UFRSA beginning in October and December 2008 and later in November 2009. All animals were clinically evaluated daily until the final outcome. The animals were treated with combinations of antibiotic therapy (Terramicina LA; Pfizer 5mg/kg), antipyretic medications (DipironaVetnil -Algivet; 25mg/kg), and vitamin complex. All animals that died were necropsied soon after; selected tissue fragments (brain, liver, lungs, kidneys, spleen, lymph nodes, and heart) were fixed by immersion in 10% buffered formalin solution and routinely processed for histopathological evaluation. For comparative analysis, the intensity of characteristic histological alterations and the degree of vascular involvement were subjectively graded using a previously established system as absent, 0; discrete, +; moderate ++, and severe +++ (Liggitt & DeMartini 1980, Headley et al. 2012). Duplicates of the tissues mentioned above were aseptically collected from one animal and maintained frozen at -20°C until used for molecular analyses.

DNA extraction, PCR assay, and sequencing

DNA was extracted from tissue fragments of the mesenteric lymph node and kidney of animal 1 by using the DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), and maintained at -20 °C until usage. The PCR protocol consisted of the primer pairs 566 (AGTCTGGGGTATATGAATCCAGATGGCTCTC) and 755 (AAGATAAGCACCAGTTATGCATCTGATAAA) to amplify the desired 422 base pairs (bp) of the OVH-2 tegument protein gene as previously described (Baxter et al. 1993, Li et al. 1995), but without utilizing the second round of PCR.

The obtained PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and examined under UV light. The products were then purified (illustra GFX PCR DNA and Gel Band Purification Kit; GE Healthcare, Buckinghamshire, UK) and submitted for direct sequencing with sense and anti-sense primers. The partial nucleotide sequences were

initially compared by the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) program with similar sequences deposited in GenBank. Phylogenetic trees and sequence alignments were then created by using MEGA 5 (Tamura et al. 2011), constructed by the Neighbor--Joining method, based on 1,000 bootstrapped data sets. The generated tree was constructed based on the deduced amino acid sequences, using the nucleotide sequences as data base inputs. Distances values were calculated by using the Kimura 2 parameter model. The nucleotide sequences used for phylogenetic analyses during this study are given in Figure 1; murine herpesvirus from the same gene was used as the outgroup.

RESULTS

Clinical findings

The principal clinical manifestations observed are summarized in Table 1; corneal opacity and bilateral mucopurulent nasal discharge (Fig.2) were observed in all cases (100%; 3/3) while motor incoordination occurred in 2 (66%) animals. However, animal 1 was more severely affected, with manifestations of fever, bloody diarrhea, motor incoordination, and dehydration with spontaneous death occurring three days after the onset of clinical signs. Clinical manifestations of discomfort were less severe in animal #2 and # 3; however, the clinical status of the second animal deteriorated rapidly despite therapy and the owner solicited euthanasia seven days after initial manifestations of clinical signs. Animal # 3 died spontaneously five days after the onset of clinical manifestations.

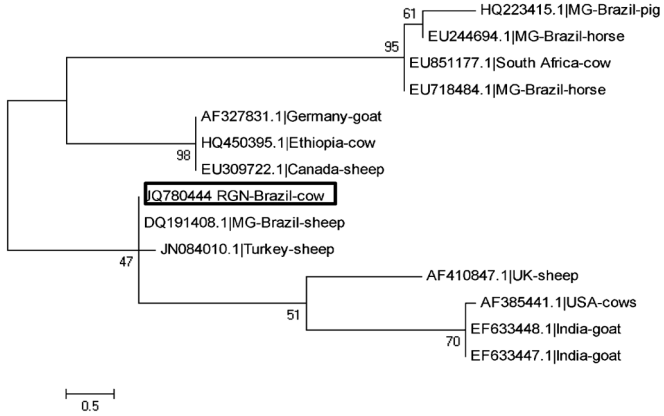

Fig.1. Phylogenetic relationship constructed from the translatedprotein sequences of the tegument protein gene of ovine herpesvirus-2. The sequences drived from this study are highlighted (box).

Fig.2. There is mucopurulent nasal discharge (Cow 1); malignant catarrhal fever.

Gross and histological findings

The significant gross findings observed in all animals (100%; 3/3) was corneal opacity; but most (66%; 2/3)

Table 1. Principal clinical and gross findings associated with ovine herpesvirus 2 infections in mixed-breed cows during an outbreak in Mossoró, RN

Case	Signalment	Principal clinical manifestations	Main gross findings	Outcome
1	Male, 7 months	Apathy	Congestion of meningeal vessels (cerebrum and cerebellum)	Spontaneous death
		Abdominal discomfort		
		Bloody diarrhea	Corneal opacity	
		Corneal opacity	Edema of the glottis	
		Dehydration	Erosions of the hard palate	
		Fever	Lymph node enlargement	
		Sternal decumbency	Tracheal hyperemia	
		Motor incoordination	Ulcerative glossitis, esophagitis and stomatitis	
		Mucopurulent nasal discharge		
		Muscular spasms		
		Respiratory distress		
		Excessive salivation		
2	Male, 18 months	Corneal opacity	Corneal opacity	Euthanasia
		Congestion of ocular vessels	Hemorrhagic glossitis and stomatitis	
		Dehydration		
		Mucopurulent nasal discharge		
		Sternal decumbency		
3	Female, adult	Anorexia	Corneal opacity	Spontaneous death
		Apathy	Ulcerative glossitis and stomatitis	
		Cornel opacity	Tracheal and esophageal hyperemia	
		Fever		
		Motor incoordination		
		Mucopurulent nasal discharge		
		Photophobia		
		Increased pulmonary and cardiac rate		

demonstrated ulcerative rhinitis, stomatitis, and glossitis (Fig.3-4) with hyperemia of the tracheal and esophagus (Table 1). Petechial hemorrhages of the oral mucosa and the tongue were observed in animal # 2. Additionally, there was widespread enlargement of lymph nodes and ulcerations of the tongue and erosions of the hard palate of the first animal and severe congestion of ocular mucosa of the second.

The intensity of the principal significant histological alterations of these cases is summarized in Table 2. All animals demonstrated discrete to moderate non-suppurative meningoencephalitis, characterized by necrotizing vasculitis at the meninges (Fig.5) and the cerebral and cerebellar white matter. Multifocal non-suppurative interstitial nephritis was more severe in the first animal relative to the other two, being characterized by the severe accumulations of lymphocytic inflammatory cells. Lymphocytic portal hepatitis, with bridging extensions, was more intense in the first animal (Fig.6). The affected tissues of all animals (brain, kidneys, liver, lungs, lymph nodes, and spleen) demonstrated lymphocytic vasculitis and perivasculitis with fibrinoid necrosis, which affected primarily the intima and media of arteries. However, associated vascular thrombi were observed within the lymph nodes (Fig. 7), lungs, and spleen of the second animal. Discrete non-suppurative myocarditis with fibrinoid degeneration was observed only in the third case. Additionally,

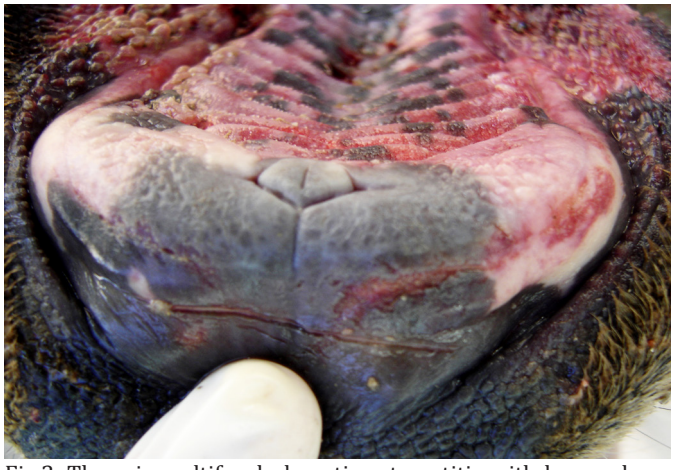

Fig.3. There is multifocal ulcerative stomatitis with hemorrhage and necrosis of the tip of the buccal papillae (Cow 2); malignant catarrhal fever.

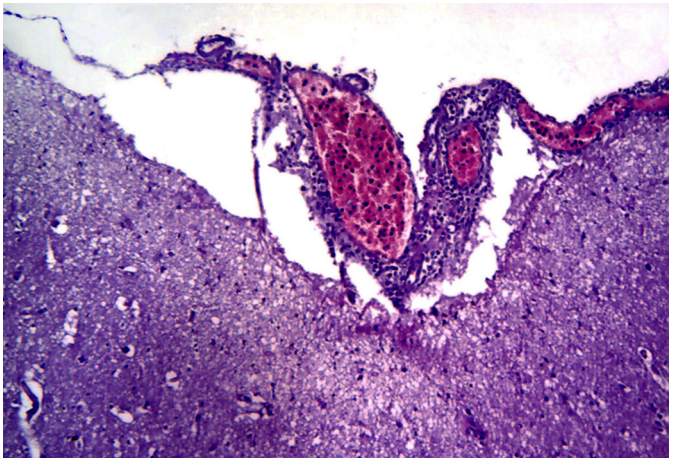

Fig.5. Necrotizing vasculitis of the leptomeningesof the brain; histopathology. Hematoxylin and eosin stain, 100x)

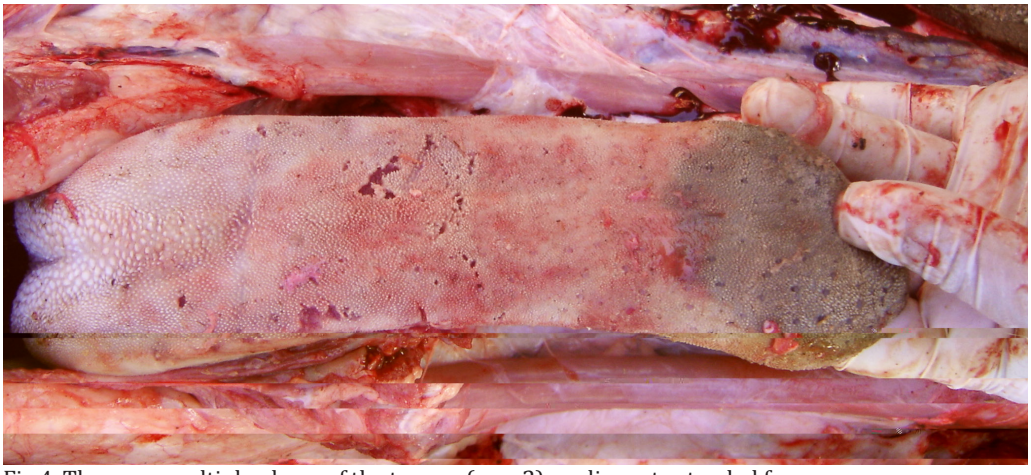

Fig.4. There are multiple ulcers of the tongue (cow 2); malignant catarrhal fever.

Table 2. Main histopathological findings associated with ovine herpesvirus-2 in cattle

Animal#	Histopathological findings					
	Nonsuppurative meningoencephalitis	Interstitial nephritis	Portal hepatitis	Interstitial pneumonia	Fibrinoid degeneration[a]	Thrombus[a]
1	++	+++	+++	+	+++	0
2	+	+	+	+	+++	+++
3	+	+	+	++	+++	+

0 Absent, + discrete, ++ moderate, +++ severe.[a], based on the presence of these lesions observed in several organs/tissues from the same animal.

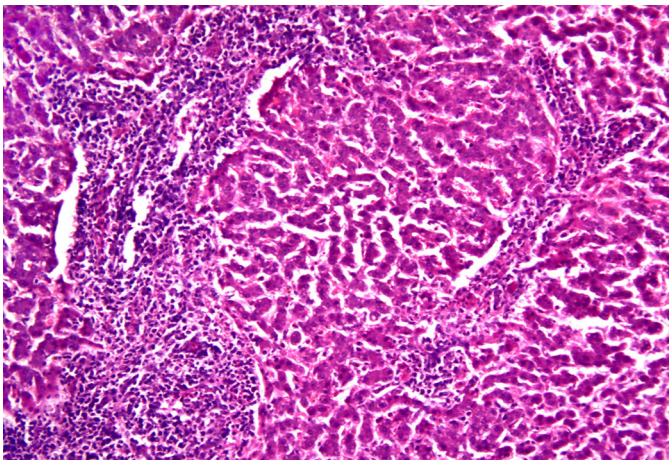
Fig.6. Bridging lymphocytic hepatitis with vasculitis; histopathology. Hematoxylin and eosin stain, 200x.

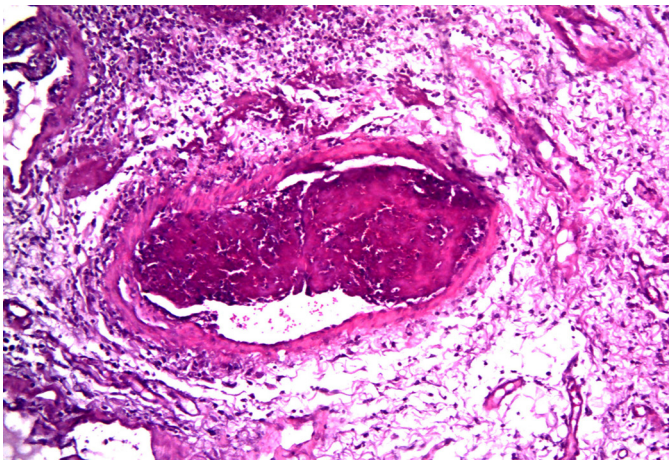
Fig.7. There is a mural thrombus within the lymph node; histopathology. Hematoxylin and eosin stain, 200x)

all animals demonstrated discrete to moderate interstitial pneumonia.

PCR, sequencing, and phylogenetic analysis

The PCR assay amplified the desired band from fragments of the lymph node and kidney of animal #1, which on direct sequencing revealed 423 bp of OVH-2 tegument protein gene. The partial sequences obtained during this study have been named RGN-PR/UEL 1 and 2 and have been deposited in GenBank (Accession numbers JQ780444 and JQ780445). Initial BLAST analyses demonstrated that these partial nucleotide sequences demonstrated 98-100% similarity with other sequences of OVH-2 deposited in GenBank. Phylogenetic analyses based on the deduced amino acid sequences revealed that the OVH-2 DNA derived from ruminants of Brazil and other geographical locations were clustered into one group, while those obtained from horses (isolated in Minas Gerais) formed a distinct cluster that was derived from the larger clade (Fig.1).

DISCUSSION

The clinical manifestations and the pathological findings observed in these animals are consistent with those described in SA-MCF (Russell et al. 2009, MacLachlan & Dubovi 2011, Zachary 2012b). Moreover, molecular data obtained from genomic DNA extracted from the kidney and lymph node of cow #1 revealed the desired 423 bp amplicon of the OVH-2 tegument protein gene as previously described (Baxter et al. 1993, Li et al. 1995). Similar molecular approaches that have used the same primers but with nested--PCR also amplified OVH-2 DNA from buffalos (Costa et al. 2009a) and horses (Costa et al. 2009b) from the state of Minas Gerais, and in an experimental cattle study done in Rio Grande do Sul (Garmatz et al. 2004). Therefore, these findings confirmed that the lesions were induced by OVH-2 and add to the few cases (Costa et al. 2009a,b) that have fully characterized this disease by molecular techniques in Brazil. Additionally, this study represents the first characterization of MCF in the state of Rio Grande do Norte by the combination of pathological findings and molecular biology; an older study previously diagnosed this disease in cattle in this state by characteristic histopathological alterations (Tokarnia et al. 1959).

The results of these initial phylogenetic analyses have suggested that the strain of OVH-2 circulating in ruminants within the states of Minas Gerais and Rio Grande do Norte is similar, while that of horses from the state of Minas Gerais might be different to that observed in ruminants. Nevertheless, these Brazilian OVH-2 DNA sequences derived from ruminants are similar to those identified in ruminants from other geographical regions. This would then imply that one strain of OVH-2 might be associated with SA-MCF in ruminants worldwide. However, a more detailed phylogenetic epidemiological survey is needed to fully understand the distribution of this pathogen within Brazil, but this is currently impossible due to the few sequences of the Brazilian OVH-2 strains that are available in GenBank. Alternatively, the clinicopathological aspects of MCF are well described in most geographical regions of Brazil (Garmatz et al. 2004, Lemos et al. 2005, Rech et al. 2005, Macêdo et al. 2007, Mendonça et al. 2008), but most studies have not fully characterized OVH-2 by sequence analysis. Nevertheless, studies performed in different Brazilian geographical regions, that have used molecular techniques, have confirmed that FCM is associated with OVH-2 (Garmatz et al. 2004, Mendonça et al. 2008, Costa et al. 2009a,b). This was further confirmed when experimentally induced manifestations of the disease were described in cattle (Garmatz et al. 2004).

Unique to this study was the finding of vascular thrombosis in two of the animals evaluated. Thrombosis is not frequently observed in cases of MCF (Brown et al. 2007), and has only been related in few studies (Mendonça et al. 2008) described in Brazil. Surprisingly, thrombosis was more severe in the second case that presented discrete clinical manifestations of disease. The interstitial pneumonia observed in these cases have been previously described in MCF affecting a horse (Costa et al. 2009a), cattle (Macêdo et al. 2007), and buffalos (Costa et al. 2009a) and might be due to vasculitis of the alveolar capillaries and arteries. The acute onset of the disease cumulating in death of all affected animals is a salient feature of MCF and has been descri-

bed in outbreaks occurring in buffaloes (Costa et al 2009a) and cattle (Rech et al. 2005) from different geographical regions of Brazil and also in cattle experimentally infected by OVH-2 (Garmatz et al. 2004). During this outbreak, the affected animals in addition to horses and sheep were grazing within the same pastures; we believe that asymptomatic sheep would have been the most likely source of contamination. Unfortunately, samples (blood or tissue) were not collected from other species of domestic animals, hence it is unknown if the horses were also affected, since OVH-2 was recently identified in a foal from Minas Gerais with histopathological features consistent with MCF (Costa et al. 2009b).

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