

## Neurological disorder in cattle associated with bovine herpesvirus 4

[Desordem neurológica em bovinos associada ao herpesvírus bovino 4]

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### ABSTRACT

A nested PCR assay was used to diagnose bovine encephalitis through herpesviruses including bovine herpesvirus 5 (BHV-5), bovine herpesvirus 1 (BHV-1), Aujeszky's disease virus (SHV-1), and ovine herpesvirus 2 (OHV-2) in 14 fragments of central nervous system (CNS) from cattle that died with neurological signs. In addition, as some samples of bovine herpesvirus type 4 (BHV-4) have been isolated from neural tissue, it was also tested by nested PCR. The cases of encephalitis occurred in isolation at different times of the year and did not present any seasonality. The duration of the clinical course ranged between 1 to 15 days, and in 64.3% of the cases it manifested between 1 to 2 days. The most frequently observed neurological signs were ataxia, recumbency, unsteadiness and inability to stand, opisthotonus, paddling movements, nystagmus and ptialism. In the nested assay, there was no evidence of: BHV-1, SHV-1 or OHV-2 in the DNA obtained from the CNS in any of the samples. But the presence of BHV-4 was found in all fragments of the CNS in cattle which died presenting neurological signs. Moreover, BHV-5 was found in association with BHV-4 in two of these samples.

Keywords: cattle, bovine herpesvirus 4, central nervous system, encephalitis

### RESUMO

*Nested PCR foi utilizada para o diagnóstico de encefalite bovina por herpesvírus incluindo o herpesvírus bovino 5 (BHV-5), o herpesvírus bovino 1 (BHV-1), o vírus da doença de Aujeszky (SHV-1) e o herpesvírus ovino 2 (OHV-2) em 14 fragmentos do sistema nervoso central (SNC) de bovinos que morreram com sinais neurológicos. Embora o BHV-4 não seja reconhecido como vírus neurotrófico, foi detectado nos casos de encefalite que ocorreram isoladamente em diferentes épocas do ano e não apresentaram nenhuma sazonalidade. A duração do curso clínico variou entre 1 e 15 dias, e em 64,3% dos casos manifestou-se entre 1 e 2 dias. Os sinais neurológicos mais freqüentemente observados foram ataxia, apatia, instabilidade, opistótono, movimentos de pedalagem, nistagmo e sialorréia. Nos ensaios de PCR nested realizados a partir do DNA obtido do SNC, não foi encontrado evidência de: BHV-1, SHV-1 ou OHV-2 em nenhuma das amostras. Mas, a presença de BHV-4 foi encontrada em todos os fragmentos do SNC de bovinos que morreram com sinais neurológicos. Além disso, o BHV-5 foi encontrado em associação com o BHV-4 em duas dessas amostras.*

*Palavras-chave: bovinos, herpesvírus bovino 4, sistema nervoso central, encefalite*

### INTRODUCTION

Out of all bovine herpesviruses, the BHV-4 is the most intriguing because it is isolated in apparently healthy bovines as well as in a wide range of clinical conditions such as abortions,

skin lesions, metritis, mastitis, ulcerative mammillitis, bladder and rumen tumors, cases of malignant catarrhal fever and ocular, respiratory, genital, enteric and neurological infections (Asano *et al.*, 2003; Dewals *et al.*, 2006).

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Due to the small number of carriers, the slow reproductive cycle with low viral titer/load and slow cytopathic effect, common properties in the cytomegaloviruses (CMV), the BHV-4 was firstly grouped in the betaherpesvirus subfamily. However, because of its preference for lymphoid organs and based on its genomic structure, the BHV-4 was included in the lymphotropic herpesviruses group of the gammaherpesviruses family, Rhadinoviruses subfamily (Goyal and Naeem, 1992; Zimmermann *et al.*, 2001).

The BHV-4 pathogenicity has not been well defined as of yet (Izumi *et al.*, 2006). It seems that the BHV-4 infection has increased and there is a strong association between BHV-4 high antibodies loads and reproductive system infections in bovines (Goyal and Naeem, 1992). Nonetheless, experimental inoculations do not replicate the clinical disease, suggesting that the BHV-4 reactivation may be related to additional factors or conditions in order to become pathogenic (Goyal and Naeem, 1992).

It is likely that the route of the BHV-4 infection is typical of herpesviruses, which primarily replicates in the epithelial cells of the respiratory tract and presumably also in the intestinal mucous membranes (Egyed *et al.*, 1996). After replication in epithelial cells of the respiratory tract the virus spreads to the intramucous lymph nodes and is transported to various organs of the body by infected leukocytes (Egyed *et al.*, 1996; Zimmermann *et al.*, 2001). High amounts of viral DNA were detected in immune system organs (spleen, tonsils, and thymus) and organs which are densely infiltrated with immune cells (kidney and intestines) suggesting that the immune cells are the main sites of replication and latency (Goyal and Naeem, 1992; Egyed *et al.*, 1996).

Although the BHV-4 is not considered a neurotropic virus, some BHV-4 samples have been isolated in neural tissues in cases of persistent infections (Bona *et al.*, 2005; Donofrio *et al.*, 2005; Izumi *et al.*, 2006) and in animals which presented neurological signs such as ataxia and encephalitis typical histopathological lesions (Fridgut and Stram., 2006; Zimmermann *et al.*, 2001). However, it is still not very clear the direct interaction between the BHV-4 and the neurons. It is suggested, in these cases, that the nervous system is also included as a latency site,

though its function in clinical and latent infections is questionable (Izumi *et al.*, 2006).

Besides, *in vitro* studies showed that bovine endothelial cells were highly susceptible to BHV-4 infection (Egyed and Baska, 2003). Experimental transmission in rabbits showed that the BHV-4 is able to replicate in the cardiovascular system tissues and is able to initiate histopathological lesions in these cells (Donofrio *et al.*, 2005; Egyed and Baska, 2003). These lesions are only found in BHV-4 chronic infections, characterized by a local acute or chronic vasculitis, chronic endocarditic signs with accumulation of mononuclear cells (histiocytes and lymphocytes) and thrombus (Egyed and Baska, 2003).

There are BHV-4 accounts in several countries, such as the United States of America, Sweden, Germany, Belgium, South Africa, Spain, Japan and Israel. In Brazil, the studies on the presence of the BHV-4 are still scarce.

This paper reports the presence of the BHV-4 DNA in the central nervous system of cattle which died with neurological disorders in Minas Gerais State, Brazil. Furthermore, the association between the histopathological and clinical profile of these encephalitis and the BHV-4 has been described.

## MATERIALS AND METHODS

A total of 14 fragments of central nervous system (CNS) derived from cattle that died with neurological signs were sent to the Laboratory of Compared Virology for encephalic herpesvirus diagnosis. The survey for encephalic herpesvirus included bovine herpesvirus 5 (BHV-5), bovine herpesvirus 1 (BHV-1), Aujeszky's disease virus (SHV-1), and ovine herpesvirus 2 (OHV-2) using PCR assays. In addition, the bovine herpesvirus 4 (BHV-4) DNA was also tested, although is not considered a neurotropic virus.

These samples had been previously submitted to direct immunofluorescence (IF) and mice inoculation (MI) tests and all samples tested were negative for rabies. These cases occurred in 2005. Age, gender, breed, kind of production (dairy, beef breeds or crossbred), clinical history, duration and extension of clinical signs of each animal were recorded in a questionnaire by

attending veterinarians and owners. Fragments of the CNS were usually sent frozen and were stored at -20°C. Repeated freezing and thawing of samples was avoided.

For the DNA recovery, 1.0g was used from fragments of CNS homogenized in a 500µl sodium iodide (6M NaI) solution. One aliquot of 100µl was used for DNA extraction through the silica procedure (Boom *et al.*, 1990). One microliter (200ng) of DNA was used as a template for each PCR. An internal control of the amplification efficiency was used in a separate PCR assay with primers for the highly conserved

mammalian insulin-like growth factor 1 (IGF-1) gene (Mikawa *et al.*, 1995). In samples which failed to amplify this gene, the DNA extraction and PCR were repeated. Positive control for each PCR was included in every test, which consisted of purified DNA as follows: BHV-5, strain EVI-88 (Spilki *et al.*, 2002); SHV-1, strain A031 (Flatschart and Resende, 2001); OHV-2, strain F1 (Andrade *et al.*, 2006). In all field samples internal control (IGF-1) gene was used. In addition, as negative control, water was used for each PCR assay. The PCR assay for each virus was performed as described in Table 1.

Table 1. Polymerase chain reaction primers used in virus surveillance

Virus*	Sequences	PCR type
BHV-1 and BHV-5 (Ros <i>et al.</i> , 1999)	5'-gccgagagmccgccrgmccgaga-3'	F flank
	5'-ggccacgccgcagtgacgtacag-3'	R flank
	5'-gcctcgcgctcgcagagcatccac-3'	R semi-nested
BHV-4 (Egyed <i>et al.</i> , 1996)	5'-gttgggcgtcctgtatggtagc-3'	F flank
	5'-atgtatgcccaaaactataatgaccag-3'	R flank
	5'-ttgatagtgcgttgttgggatgtgg-3'	F nested
	5'-cactgcccggtgggaatagca-3'	R nested
OHV-2 (Baxter <i>et al.</i> , 1993)	5'-agtctgggtatgaatccagatggctctc-3'	F flank
	5'-aagataagcaccagttatgcatctgataaa-3'	R flank
	5'-ttctgggtagtggcgagcgaaggcttc-3'	R semi-nested
SHV-1 (Scherba <i>et al.</i> , 1992)	5'-tccacagctcaacaatgaagtggg-3'	F flank
	5'-tccgaggagcgggacgatacgt-3'	R flank

\*BHV-5 = bovine herpesvirus 5; BHV-4 = bovine herpesvirus 4; OHV-2 = ovine herpesvirus 2; SHV-1 = suine herpesvirus 1 (also known Aujeszky's Disease Virus)

The amplified products were analyzed by electrophoresis in 2% agarose gels by using 0.5 X Tris-borate-EDTA as the running buffer. The ethidium bromide-stained bands were visualized with UV light. The molecular size of the fragments was compared with those of a 100-bp DNA ladder molecular marker (Invitrogen Corp., Carlsbad, CA).

The identity of the PCR product was confirmed by the sequencing of four amplicons randomly chosen from the sampling total. PCR products were purified using the PureLink PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and submitted to nucleotide sequencing in a MEGABACE sequencer (Amersham Biosciences). The nested PCR products were sequenced three times in both orientations by the dideoxy method using ET Dynamic Terminator for MEGABACE (GE

Healthcare, Fairfield, CT, USA). The nucleotide (nt) sequences of BHV-4 thymidine kinase partial gene were assembled using the CAP3 Sequence..., (CAP3..., 2006) (<http://pbil.univ-lyon1.fr/cap3.php>) and deposited in GenBank under accession numbers EU244697 (MGArom), EU244698 (MGA696), EU244699 (MGA514), EU244700 (MGA1075). These nucleotide sequences and inferred amino acid sequences were aligned and compared with BHV-4 sequences in GenBank using the ClustalW 1.6 program (Thompson *et al.*, 1994).

Fragments of CNS fixed in 10% neutral formalin were processed routinely and embedded in paraffin, and 5µm thick sections were cut and stained with hematoxylin and eosin for histological examination (Luna, 1968).

## RESULTS

The BHV-4 sequences were identified by PCR in all samples of the CNS analyzed. There was no detection of BHV-1, SHV-1 e AHV-1 in the DNA extracted from the SNC in any of the analyzed animals. Out of the samples positive for BHV-4, two were positive for BHV-5. The internal control was amplified by PCR in all fourteen clinical samples included in this study. The use of primers for mammalian IGF-1 gene was important in the detection of false negatives or invalid results.

The translation of the sequences obtained by PCR assay is part of the open reading frame 2 (ORF 2) of the thymidine kinase gene. This ORF 2 is homologous to the gammaherpesvirus Epstein-Barr virus (EBV) BXRFl gene (Lomonte *et al.*, 1992). All four BHV-4 thymidine kinase partial sequences were closely related to each other. The identity ranged between 94 to 100% at the deduced amino acid sequence level and between 96 to 99% at the nucleotide sequence level. By comparison with homologous sequences of BHV-4 isolates available in the GenBank, the highest identity degree at nucleotide and deduced amino acid sequence levels was observed in sequences S49773, AB035515, AB035516 and AB035517. The variation identity among homologous sequences ranged between 91 to 98% and 95 to 99% at the deduced amino acid and nucleotide sequence levels, respectively.

Additionally, the Brazilian strain MGA696 (EU244698) presented 3-nt deletion corresponding to nt 529-531 (based on GenBank PubMed Protein Query accession number AAB24378), causing the loss of 1 lysine (K). This characteristic was not observed in any other BHV-4 homologous sequence. The MGArom (EU244697), MGA696 (EU244698) and MGA1075 (EU244700) sequence strains exhibited 1 amino acid substitution (threonine to proline) when compared to the S49773 sequence (position 388 to 390). Moreover, the MGArom strain sequence (EU244697) also exhibited 1 amino acid substitution (proline to serine) when compared to the same sequence (position 397 to 399).

Due to the lack of material preserved in formalin, the encephalon histological evaluation was performed in six out of the fourteen BHV-4 cases. In all cases a non suppurative encephalitis was

observed, affecting the cerebellum and the brain, also extending to the meninges, with intense hyperemia (Fig. 1A) and mononuclear infiltrates (lymphocytes and monocytes, predominantly) within the meninges and filling the Virchow-Robin perivascular spaces (perivascular cuffing) (Fig. 1B). In 5 of these samples a degeneration of the nervous tissue such as gliosis, neuronophagia, and darkened shrunken neurons was observed (Fig. 1C), sometimes with vascular lesions such as congestion, focal hemorrhage (Fig. 1D) and thrombosis.

The neurological signs reported by veterinarians and owners, in decreasing order of frequency, were ataxia (12/14 - 85.7%), recumbency (8/12 - 57.1%), inability to stand (7/14 - 50.0%), restlessness (6/14 - 42.9%), opisthotonus (5/14 - 35.7%), paddling movements (4/14 - 28.6%), nystagmus (4/14 - 28.6%), ptialism (4/14 - 28.6%) and unsteadiness on standing position (4/14 - 28.6%). The development of the neurological signs in the affected bovines ranged between 1 to 15 days and in 64.3% (9/14) of the cases it was manifested between 1 to 4 days. Bovines of both genders were affected and females comprised 85.7% (12/14) of the cases. The age of the affected bovines ranged from 3 months to 7 years, and 50% (7/14) of the animals were 2-year-olds.

In most properties (10/14 - 71.4%) the neurological disease was sporadic, affecting one to four bovines per herd, and in four properties (28.6%) 6 to 30 bovines were affected. The BHV-4 cases did not present a seasonal character. However, as in these cases only one animal was sent to diagnosis, it was not possible to ascertain that there was an association between the neurological disease and the BHV-4 in all bovines from these herds.

In two properties, besides the neurological signs, the animals presented respiratory symptoms such as coughing, breathlessness and dyspnea. According to information given by the owners and responsible veterinarians, during the necropsy it was found that the bovine's lungs were 60% congested. In these two properties, a serological examination for the bovine respiratory syncytial virus (BRSV) showed evidence of seropositive animals. In addition, in all properties affected by the BHV-4, mastitis cases and reproductive disorders were frequently observed.

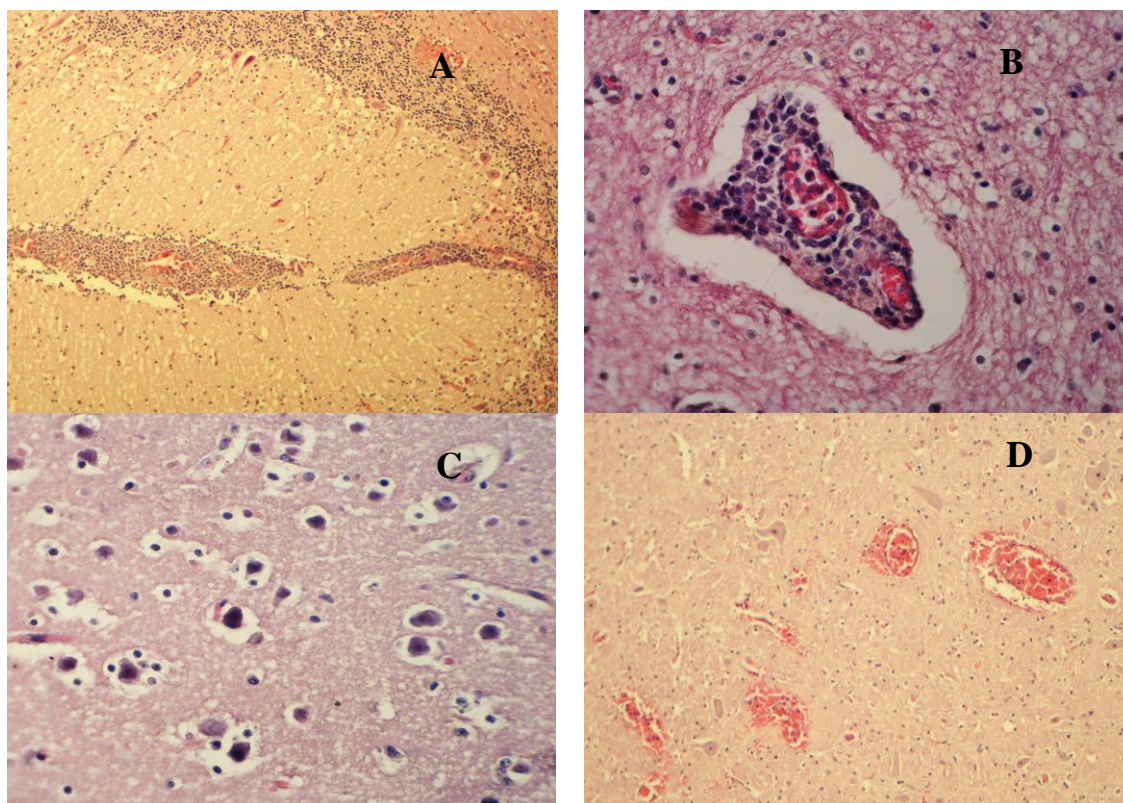


Figure 1. Encephalon histopathological examination of cattle that died presenting neurological signs and a BHV-4 positive diagnosis. **A.** Cut of cerebellum showing the congestion of molecular layer, Purkinje cell layer, and granular layer. Meninges thickened by intense hyperemia and mononuclear infiltrate (lymphocytes and monocytes, predominantly), HE. Obj. 10X. **B.** Fraction of the brain white matter showing retraction and thickening of the vessels, perivascular mononuclear cuffing (lymphocytes and monocytes, predominantly) and focal hemorrhages, HE, obj. 40X. **C.** Fragment of the brainstem (obex) showing dark and shrunken neurons surrounded by glial cells, HE, obj. 40X. **D.** Cut of the brainstem. Non suppurative encephalitis with inflammatory thickening and congestion of the vessels and hemorrhage in the neutropil, HE, obj. 20X.

### DISCUSSION

Bovine herpesvirus 4 (BHV-4) is one of the four bovine herpesviruses that occur worldwide. BHV-1, BHV-2 and BHV-5 are responsible for infectious bovine rhinotracheitis-pustular vulvovaginitis, bovine herpesvirus mammilitis and bovine encephalitis herpesvirus, respectively (Lomonte *et al.*, 1992). Even though the BHV-5 is the bovine herpesvirus causative of encephalitis, cases of neurological disorders and encephalitis in bovines infected with the BHV-1 and BHV-4, despite being less frequent, have been reported (Yamamoto *et al.*, 2000; Spilki *et al.*, 2002; Fridgut and Stram, 2006).

In all BHV-4 cases detected in this study, derived from CNS samples of bovines which died after presenting neurological signs, non-suppurative encephalitis was observed in the histopathologically analyzed encephala. However, the BHV-4 function in the nervous system is still obscure. Conversely to the other herpesviruses, it was not possible to prove that the BHV-4 is the causative agent of any diagnosed disease (Fridgut and Stram, 2006), especially neurological disorders.

The genomic structure, the gene arrangement and the biological properties confirm that BHV-4 belongs to the genus *Rhadinovirus* (Zimmermann *et al.*, 2001). According to the analysis of the LUR regions outside the

conserved gene blocks from many BHV-4 strains, the existence of DNA insertions or deletions in homologous sequences give evidence of significant strain differences with consequences on the ORFs (Zimmermann *et al.*, 2001). As the BHV-4 strains represent a heterologous group of viruses, it is possible that different strains vary in their biological properties, including cellular tropism and latency sites leading to a variety of clinical disorders, likely, in their capacity, to invade the nervous system (Asano *et al.*, 2003; Egyed and Baskas, 2003).

The majority of Alphaherpesviruses avoid their elimination by the host immune system through latency within the nervous system cells, such as the ganglia, between the disease episodes. On the other hand, the Beta and Gammaherpesviruses avoid their elimination through latency within the lymphocytes. Blood samples in EDTA tubes for the BHV-4 detection in the leukocytes (PBL), as well as the bovines' encephala, were collected in only property in this study. In this case, the BHV-4 was detected in both specimens, proving that the virus was associated to the CNS and the leukocytes. Besides, the BHV-4 presence in the leukocytes of the dead calves' mothers suggests that they are the virus transmission source, since the virus can be found and can multiply in the mammary epithelium and in leukocytes present in the milk (Wellenberg *et al.*, 2002).

In the other cases, only the association to the CNS was confirmed. When the BHV-4 DNA is found in the nervous system and in lymphocytes, it is suggested that the BHV-4 is latent in both tissues (Yamamoto *et al.*, 2000). However, there are reports of BHV-4 latent in only one of these sites (Izumi *et al.*, 2006).

Several gammaherpesviruses are associated to lymphoproliferative diseases and tumor development. These diseases seem to be associated to immunosuppression or a late-in-life infection of the natural host as well as with the infection of a related non-natural host. There is no established link between the BHV-4 and such diseases (Zimmermann *et al.*, 2001). Gammaherpesvirus genes homologous to cellular genes have been demonstrated to be often involved in cell growth or cell survival, in the nucleotide metabolism and in immune escape. The set of such genes in the BHV-4 is reduced

when compared to other gammaherpesviruses with known transforming capacity. In conclusion, the possibility that BHV-4 has a lymphoproliferative or transforming capacity under certain conditions cannot be excluded, but there is no evidence that this virus has the genes required to cause such diseases (Egyed *et al.*, 1996; Zimmermann *et al.*, 2001). Meanwhile, for the occurrence of any clinical manifestation of the BHV-4, an association with bacteria, fungi or another virus may be necessary (Goyal and Naeem, 1992; Fridgut and Stram, 2006). Studies reveal that in approximately 75% of the BHV-4 infection cases where there was a clinical manifestation of any disease, the animals were co-infected by other pathological agents (Goyal and Naeem, 1992).

Two co-infection cases of the BHV-4 with the BHV-5 were found in this study and in two properties the bovines were seropositive for the bovine respiratory syncytial virus (BRSV). It is worth pointing out that the BHV-5 is a causative agent of encephalitis in bovines. In the BRSV co-infection cases the calves not only presented neurological signs, but also respiratory symptoms similar to the ones occurred in BRSV infections. Additional diagnostics for bacteria or fungi were not performed in any BHV-4 cases.

The BHV-4 is present in the bovine population at rates which vary according to the region and it can be activated when the animals are exposed to some kind of stress, such as long distance transportation, parturition, sudden climate changes and dexamethasone treatments (Izumi *et al.*, 2006). In the case of samples where the BHV-4 is latent in the nervous system, the virus activation by stress induced immunosuppression may result in a replication in the latency site and it might lead to a neurological disease. Studies reveal that though the blood lymphocytes are one of the BHV-4 latency sites, the virus replication occurs in them during the first seven weeks of the infection, and the number of infected cells is much larger from 2 to 5 weeks (Egyed *et al.*, 1996). However, a neurological disorder in bovines infected with BHV-4 may not be related to its presence in the nervous system (Yamamoto *et al.*, 2000).

Studies have shown that the BHV-4 is able to replicate and initiate histopathological lesions in the vascular system cells of experimentally

infected rabbits and that endothelial cells cultures are a 100 to a 1000 times more sensitive to the BHV-4 than the other cells normally used for viral isolation such as the *Madin Darby Bovine Kidney* (MDBK) continuous lineage cells (Goyal and Naeem, 2002; Egyed and Baska, 2003). On account of the tropism for endothelial cells and because it causes vascular lesions in mammals, another hypothesis for the encephalitis in some bovines infected by the BHV-4 would be the virus replication in the nervous system endothelial cells, causing an inflammatory reaction through the vascular damages. That would explain the fact that in this study some of the BHV-4 infected bovines presented encephalitis with notable vascular lesions such as hemorrhages, thrombus and lymphocytic vasculitis. It could be that the tropism for lymphoid and epithelial cells of some of the BHV-4 samples, results in histopathological signs similar to the malignant catarrhal fever, in which a generalized lymphoid vasculitis is always observed (Goyal and Naeem, 1992; Asano et al., 2003).

The BHV-4 is disseminated worldwide among bovine populations, especially in North America, Japan, Hungary, Thailand, Israel and some other European countries (Egyed and Baska, 2003; Fridgut and Stram, 2006; Izumi et al., 2006). In the present study, the BHV-4 cases were reported in seven of the ten administrative regions of Minas Gerais, therefore spread throughout the whole State. In Minas Gerais the majority of properties have animals showing problems with mastitis, reproductive disorders and abortions, including the properties where the BHV-4 was detected. Because these diseases involve several causative agents, it is very difficult to establish the diagnosis. As the BHV-4 is frequently associated to these diseases, it is possible to include it as a probable aggravating factor, together with other causative agents, since the reports indicate that outbreaks of diseases involving BHV-4 result from multifactorial processes (Fridgut and Stram, 2006). Nonetheless, a serological research in the bovine population should be carried out to verify the prevalence of the BHV-4 in Minas Gerais. However, the BHV-4 associated with encephalitis appears to be a sporadic event and it does not mean that it is the cause of neurological diseases or other diseases such as abortions and mastitis outbreaks (Fridgut and Stram, 2006).

## CONCLUSIONS

In all cases of non-suppurated BHV-4, DNA was detected in 14 samples. In two samples BHV-5 DNA was also detected. These samples were negative for rabies virus, BHV-1, OHV-2 and SHV-1 genome. In 12 cases of encephalitis, only BHV-4 was detected.

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