A RETROSPECTIVE SEARCH FOR BOVINE HERPESVIRUS 5 (BOHV-5) IN THE BRAIN OF CATTLE AFFECTED BY NEUROLOGICAL DISEASE IN RIO DE JANEIRO STATE

Pinto, A.M.V.1,2; Leite, J.P.G.2; Romijn, P.C.3; Silva, R.C.F.3; Flores, E.F.4; Weiblen, R.4; Paixão, I.C.N.P.5

1. Instituto Biomédico, Universidade Federal Fluminense, Rua Professor Hernani Melo, 101, São Domingos, CEP: 24210-130, Niterói-RJ, Brazil.
2. Laboratório de Virologia Comparada e Ambiental, Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Av. Brasil, 4365, Manguinhos CEP: 21040-360 Rio de Janeiro-RJ, Brazil.
4. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Santa Maria, RS.
5. Instituto de Biologia, Universidade Federal Fluminense, Campus do Valonguinho, Centro, CEP: 24020-141, Niterói-RJ, Brazil

ABSTRACT

Bovine herpesvirus 5 (BoHV-5) is an important agent of meningoencephalitis in cattle and has been identified in outbreaks of neurological disease in several Brazilian states. However, no report of BoHV-5-associated neurological disease in Rio Janeiro state (RJ) has been published to date. This article reports a retrospective investigation for BoHV-5 performed in 146 brain specimens of cattle affected by neurological disease, submitted to the virology laboratory of the Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (PESAGRO-RJ) between 1998 and 2001. Most specimens came from herds in the RJ state (130 or 89%) and a few from Minas Gerais counties (16 or 11%), close to the RJ border. All brain samples examined in this study were previously diagnosed negative for rabies virus. The specimens were submitted to PCR/nested-PCR for BoHV-5 sequences and virus isolation. From 146 analysed brains samples 48 (33%) were positive in at least one of the tests (PCR or virus isolation), 20 samples (14%) were positive in both assays, 19 samples (13%) were positive in Nested-PCR (n-PCR) and nine samples (6%) were characterized as BoHV-5 after virus isolation. The northeast region of the RJ contributed with most clinical specimens (92/146 or 63%) and presented the highest positivity (28/92 or 30%). These results demonstrated BoHV-5 association with neurological disease in bovine herds of RJ and MG and should therefore be included in differential diagnosis of neurological disease in cattle. The limitations of each of the diagnostic assays used here in led us to recommend the concomitant use of PCR and virus isolation to improve the sensitivity of the diagnosis of BoHV-5 infection.

Keywords: Bovine herpesvirus 5, differential diagnostic, nested-PCR

INTRODUCTION

Bovine herpesviruses 1 (BoHV-1) and 5 (BoHV-5) are large, enveloped double-stranded DNA viruses, belonging to the genus Varicellovirus, subfamily Alphaherpesvirinae, family Herpesviridae (http://www.ictvonline.org/virusTaxonomy.asp). The main biological property of alphaherpesviruses is the ability to establish lifelong latent infections in their hosts after primary infection. The latent infection by alphaherpesviruses is established mainly in sensory and autonomic nerve ganglia and can be periodically reactivated by natural or dexamethasone induced stress. Virus reactivation and shedding provide adequate means for these viruses to be maintained in nature (Rock 1994). Virus reactivation is only occasionally accompanied by clinical signs yet frequently courses with virus shedding and transmission to other animals (Ackermann et al. 1982, Meyer et al. 2001, Vogel et al. 2003). Thus, latent infection plays a pivotal role in the epidemiology and pathogenesis of human and animal alphaherpesviruses (Rock 1994, Jones 1998, Jones et al. 2011).

Bovine herpesvirus 1 is an important pathogen of cattle. BoHV-1 is associated with severe respiratory infection, conjunctivitis, abortions, vulvovaginitis and balanoposthitis and fatal multisystemic infection in neonate calves (Wyler et al. 1989, Kahrs, 2001). BoHV-1 infection is distributed worldwide with the exception of some European countries (Austria, Denmark, Finland, Norway, Sweden, Switzerland and several Italian provinces,) that have eradicated the BoHV-1 infection.
in herds (Ackermann and Engels 2006). In Brazil, seroepidemiological, clinical and virological studies have demonstrated the presence of BoHV-1 in cattle herds (Wizigmann et al. 1972, Ravazollo et al. 1989, Lovato et al. 1998, Roehe et al. 1998).

Bovine herpesvirus type 5 (BoHV-5) formerly classified as a subtype of BoHV-1 (BoHV-1.3) has been associated with fatal meningoencephalitis in cattle (Carrillo et al. 1983, Schudel et al. 1986), infertility and venereal disease in bovine (Kirkland et al. 2009). BoHV-5 was detected as etiologic agent of apoptotic effect on in vitro produced bovine embryos in Brazil (Silva et al. 2010a; Silva et al. 2010b) and semen of healthy bull from Australia and Brazil (Diello et al. 2010, Oliveira et al. 2011). Neurological disease by BoHV-5 has been sporadically reported in Austrália (French 1962), United States (d’Offay et al. 1993) and Europe (Bartha et al. 1969).

BoHV-5 infection and neurological disease in bovine have been frequently observed in Brazil and Argentina, affecting mainly - but not exclusively - young animals (Carrillo et al. 1983, Schudel et al. 1986, Salvador et al. 1998). BoHV-5 has been identified in outbreaks of neurological disease in cattle in several Brazilian states, including Rio Grande do Sul (Riet-Correa et al. 1989, Weiben et al. 1989, Roehe et al.1998), Mato Grosso do Sul (Salvador et al. 1998) and Minas Gerais (Gomes et al. 2002). The seroprevalence of BoHV-5 infection among Brazilian cattle is difficult to ascertain with the routine serological tests due to the high percentage of cross-reactivity with BoHV-1 what prevents the distinction between the antibody response to BoHV-1 and BoHV-5 (Bratanich et al. 1991, Vogel et al. 2002).

Antibodies reacting with BoHV-1 (and likely reacting also with BoHV-5) have been detected among cattle population from Rio de Janeiro state (Roehe et al.1998). However, to date there has been no report on the direct identification of either BoHV-1 or BoHV-5 associated with clinical disease in this state. The present article presents a retrospective investigation for BoHV-1 and BoHV-5 in brain specimens of cattle suffering from neurological disease and previously diagnosed negative for rabies in the state of Rio de Janeiro between 1998-2001. Additionally, some specimens from MG counties near the state boundary were also examined.

**MATERIAL AND METHODS**

**Clinical specimens**

From January 1998 through December 2001, brains (whole brain of partial sections) collected from animals which died after developing neurological disease were submitted to the virology laboratory of the Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (PESAGRO-RJ). Rabies was the initial clinical suspect in all cases. Accordingly, these specimens were submitted to rabies diagnosis (direct immunofluorescence, histopathology and mouse inoculation) following the WHO (World Health Organization) recommendations. Only the 146 specimens that were negative for rabies were included in the present study. These specimens have been originated from RJ counties (130 specimens) and from MG counties (16 samples) near the RJ boundary (Figure 1). The brain samples were stored at -20°C after the respective rabies diagnosis and until tested for BoHV-1/BoHV-5.

**Cells, viruses and monoclonal antibodies (Mabs)**

The standard virus strains (BoHV-1 Cooper; BoHV-5 A-663) and pestivirus-free bovine kidney cells (Madin Darby bovine kidney, MDBK) used for virus multiplication and isolation were kindly provided by the virology section of the Federal University of Santa Maria, RS (SV/UFSM). MDBK cells were cultured in Eagle's minimal essential medium (E-MEM) supplemented with 10% equine serum, containing 0.075% sodium bicarbonate; antibiotics and antifungics (penicillin 200 UI/ml, streptomycin 200 µg/ml and 0.50 µg/ml amphotericin. Monoclonal antibody 11H6 that recognize only BoHV-1 and 2G5 antibody that recognize both BoHV-1 and BoHV-5 (Souza et al. 2002) used in fluorescent antibody assays (FA) were provided by Dr. Paulo Michel Roehe (Centro de Pesquisas Desidério Finamor/FEPAgro and Departamento de Microbiologia of the Federal University of Rio Grande do Sul, UFRGS). The anti-mouse IgG FITC-conjugated secondary antibody used in FA tests was purchased from Sigma (Sigma Inc., St.Louis, MO).

**Virus isolation and identification**

Approximately 1g of each brain specimen was homogenized with sterile sand, resuspended in culture medium (10% weight/volume), centrifuged at 1500 x g for 10 min, aliquoted and stored at -70°C until processing (virus isolation or DNA extraction). For virus isolation, 100 µl of each tissue homogenate were inoculated onto MDBK cells, allowed to adsorption for one hour at 37ºC and then discarded. Maintenance medium (with 2% equine serum) was added and the cultures were incubated at 37ºC in a CO₂ incubator. Monitoring for appearance of cytopathic effect (CPE) was performed for four days, after which the supernatant was harvested and inoculated onto a freshly cell monolayer and observed for additional four days. All homogenates were submitted to five passages of four days each before considering them negative for virus. Positive (cell monolayers inoculated with a BoHV-1/5 positive material) and negative (cells inoculated with MEM) were included in all experiments. Cell monolayers showing characteristic CPE and those not showing CPE at the end of the fifth passage were submitted to indirect fluorescence antibody assay (IFA), using the above described Mabs as primary antibody, according to protocols described elsewhere.
were recorded. Sixteen specimens were submitted, with a few specimens; no submissions from region III (northwest RJ), each of the other six regions contributing roughly the same 33% versus 33% (Table 1). It should be emphasized that we refer to submission to all clinical specimens analysed in this study were administrative regions of the state of RJ from which positive specimens, per year and region. Sixty three positive BoHV-5 specimens by region in brackets during period studied.

Table 1. Distribution of BoHV-5 positive specimens relative to total submissions among Rio de Janeiro and Minas Gerais regions

<table>
<thead>
<tr>
<th>Region</th>
<th>1998</th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Metropolitan</td>
<td>0/1</td>
<td>1/1</td>
<td>0</td>
<td>0</td>
<td>1/2</td>
</tr>
<tr>
<td>II - Northwest</td>
<td>8/39</td>
<td>10/25</td>
<td>1/2</td>
<td>9/16</td>
<td>29/92</td>
</tr>
<tr>
<td>IV - Serrana region.</td>
<td>1/4</td>
<td>1/5</td>
<td>0/3</td>
<td>0/1</td>
<td>2/13</td>
</tr>
<tr>
<td>V - Baixada Litorânea</td>
<td>1/2</td>
<td>1/8</td>
<td>0/2</td>
<td>5/6</td>
<td>7/18</td>
</tr>
<tr>
<td>VI - Médio Paraíba</td>
<td>0</td>
<td>0</td>
<td>1/1</td>
<td>2/2</td>
<td>2/3</td>
</tr>
<tr>
<td>VII - Southcentral</td>
<td>0</td>
<td>1/1</td>
<td>0</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td>VIII - Costa Verde</td>
<td>0</td>
<td>1/1</td>
<td>0</td>
<td>0</td>
<td>1/1</td>
</tr>
</tbody>
</table>

| Positive from RJ (%) | 10/46 | 22% | 15/41 | 37% | 2/18 | 11% | 16/25 | 64% | 43/130 | 33% |
| Positive from MG | 1/4 | 3/6 | 1/3 | 0/3 | 5/16 |
| Total Positive Submitted (%) | 11/50 | 22% | 18/47 | 38% | 3/21 | 14% | 16/28 | 57% | 48/146 | 33% |

* Political-administrative RJ regions, according to the map (Figure 1). No specimens were received from herds in Region III.

Within the studied period (1998-2001), the number of submissions remained between 18 (2000) and 46 (1998) (Table 1). Minas Gerais herds contributed to 16 submissions (11%) within the period. The overall BoHV-5 positivity for RJ and MG submissions were roughly the same 33% versus 33% (Table 1). It should be emphasized that we refer to submission to all clinical specimens submitted to diagnosis and found negative for rabies. Only those specimens were included in our study.

Figure 1. Adapted from the map of the administrative regions of Rio de Janeiro State (http://www.mapas-rio.com) shows regions from which were collected the brain samples of cattle with neurological disease diagnosed negative for rabies. I: RJ Metropolitan region; II: Northwest region; III: Northern fluminense region; IV: Serrana region; V: Baixada Litorânea region; VI: Médio Paraíba region; VII: Southcentral fluminense region; VIII: Costa Verde region; Minas Gerais (MG); 1-3 MG counties. Number of positive BoHV-5 specimens by region in

RESULTS
Figure 1 depicts the geographic, politico-administrative regions of the state of RJ from which the clinical specimens analysed in this study were originated. Table 1 presents the number of submissions and positive specimens, per year and region. Sixty three percent (92/146) of the submissions came from region II (northwest RJ), each of the other six regions contributing with a few specimens; no submissions from region III were recorded. Sixteen specimens were submitted from MG counties, close to the RJ border line (Figure 1, table 1). The northwest region of RJ (II) also showed the highest positivity (28/48 = 58%) of all positive submissions for BoHV-5 (Table 1).
Table 2 summarizes the results of examination of bovine brains for BoHV-1/BoHV-5 by nested-PCR and virus isolation within the period. From a total of 146 specimens submitted between 1998 and 2001 and found negative for rabies, 48 (33%) were positive for BoHV-5 in at least one assay (PCR or virus isolation). Twenty specimens (14%) were positive in both tests. Thirty nine (81% of the positives samples or 27% out of the total) were positive by PCR performed in total DNA extracted directly from the clinical specimens. Twenty nine specimens (60% of the positive; 20% of the total) were positive in virus isolation, twenty (42% of the positive; 14% of the total) were positive also in PCR and nested PCR direct assay and nine specimens (19% of the positive; 6% of the total) were identified as BoHV-5 by PCR and nested PCR after virus isolation.

All cell cultures inoculated with tissue homogenates and presenting CPE, and the cell cultures not presenting CPE at the end of the fifth passage as well, were submitted to IFA. Although the Mabs used (2G5 and 11H6) present different specificities (Souza et al. 2002), they were used as a mixture to widen the spectrum of reactivity. In other words, these Mabs were used to detect BoHV-1/BoHV-5 antigens rather than to distinguish between these viruses.

In all specimens positive in PCR and nested-PCR the amplicons size (589 pb and 166 bp) were respectively compatible with that of BoHV-5 standard A-663 (Figure 2). No amplicon corresponding to the BoHV-1 expected size (274 bp), as that observed in BoHV-1 strain Cooper was observed in any test sample. Therefore, all positive results in PCR were thereafter referred as positive for BoHV-5. No amplification was ever observed in the negative controls.

The positive animals (PCR, virus isolation or both) had ages ranging from one month to three years (23 animals), nineteen were adult ones (age > 3 years) and nine positive submissions were not accompanied by data concerning animal age. Three specimens that resulted BoHV-5 positive in virus isolation had been previously been diagnosed positive for Babesia sp.

Table 2. Results of a retrospective search for BoHV-5 in freezing brain specimens of cattle with neurological disease in Rio de Janeiro and Minas Gerais regions

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Specimens</th>
<th>Nested-PCR positive</th>
<th>Nested-PCR negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>Virus Isolation</td>
<td>Virus Isolation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive*</td>
<td>Negative*</td>
</tr>
<tr>
<td>1998</td>
<td>50</td>
<td>6 (12%)</td>
<td>4/6</td>
</tr>
<tr>
<td>1999</td>
<td>47</td>
<td>18 (40%)</td>
<td>11/18</td>
</tr>
<tr>
<td>2000</td>
<td>21</td>
<td>3 (14%)</td>
<td>0/3</td>
</tr>
<tr>
<td>2001</td>
<td>28</td>
<td>12 (43%)</td>
<td>5/12</td>
</tr>
<tr>
<td>Total</td>
<td>146</td>
<td>39/146 (27%)</td>
<td>20/146 (14%)</td>
</tr>
</tbody>
</table>

* Positive samples for cytopathic effect and/or immunofluorescence of cell monolayers inoculated with homogenates of the clinical material.  
* Negative samples for cytopathic effect and/or immunofluorescence after five passages in MDBC cells.
standard BoHV-5 strain (A-663); lane 4: negative control.
B) Second round (nested) of PCR amplification for BoHV-5 sequences. M: Molecular weight marker (Invitrogen); lane 1: (negative control, MDBK cells); lane 2: standard BoHV-5 strain (A-663); lanes 3 and 4: negative control (no template) for PCR and nested PCR; lane 5: specimen 16/2000; lane 6: specimen 42/2001

**DISCUSSION**

Several serological studies have demonstrated the wide distribution of bovine herpesvirus infection among Brazilian cattle (Wizigmann et al.1972, Ravazollo et al. 1989, Roehe et al. 1998). BoHV-1 and BoHV-5 are antigenically closely related and display an extensive serological cross-reactivity (Bratanich et al. 1991). The antigenic similarity between BoHV-1 and BoHV-5 is such that most serological tests are unable to distinguish between the antibody responses to each of these viruses. As a consequence, the real seroprevalence of BoHV-1 and BoHV-5 infections is rather difficult to predict in regions where the two viruses co-circulate (d’Offay et al. 1994, Vogel et al. 2002). Nevertheless, in addition to the vast serological data, many clinic-pathological and virological reports have demonstrated the occurrence of both BoHV-1 and BoHV-5 infection and associated clinical diseases in our country (Riet-Correa et al. 1989, Weiblen et al. 1989, Flores et al. 1998, Roehe et al. 1998, Salvador et al. 1998, Gomes et al. 2002). In addition to the published reports, outbreaks of respiratory and/or genital disease and neurological disease with laboratory confirmation of the etiological agent (BoHV-1 and BoHV-5) have been frequently registered by regional diagnostic services in several states (Flores 2005, Silva et al. 2010a, Silva et al. 2010b, Oliveira et al. 2011). Taken together, these data support that both BoHV-1 and BoHV-5 infections and their respective associated diseases as well, are endemic among the cattle population in most Brazilian regions and other regions of the world (Diafio et al. 2010, Rodriguez et al. 2012).

Outbreaks or single cases of neurological disease associated with BoHV-5 have been described in several states, including Rio Grande do Sul (RS), São Paulo (SP) and Mato Grosso do Sul (MS) (Riet-Correa et al.1989, Weiblen et al.1989, Salvador et al. 1997). Gomes et al. (2002) reported a retrospective investigation for BoHV-5 (by PCR and histopathology) in the brain of cattle affected by neurological disease in MG, detecting BoHV-5 sequences and/or histological changes indicative of BoHV-5 infection in 5 out of 22 specimens. Nonetheless, no report of the occurrence of BoHV-5 neurological disease (or respiratory disease associated with BoHV-1) in RJ state has been published to date. The only reference to these infections in the state refers to a serological study demonstrating the presence of BoHV-1 antibodies in RJ cattle (Roehe et al. 1998).

Two main reasons may explain the little emphasis historically given to BoHV-5 as a possible cause of neurological disease in RJ (and in other states as well): 1. the high prevalence of bovine rabies in the state directs most of the clinical and laboratory investigations on cases of neurological disease in cattle. 2. The lack of an effective diagnostic laboratory network with adequate equipments, methodology and reagents to perform differential diagnosis for BoHV-5 on those negative cases for rabies.

The present study was performed to collect evidence concerning the occurrence of BoHV-1 and BoHV-5-associated diseases in RJ. The large number of brain submissions (neurological disease) with the diagnostic negative for rabies first suggested the occurrence of BoHV-5 infection in the state. Taken together with studies from other states, these data were indicative of the occurrence of other neurological pathology than rabies, probably BoHV-5-infection. In fact, the results presented herein demonstrated that BoHV-5 infection is present and seems to be quite frequent among the cattle herd in the RJ state, mainly in the northeast region, characterized by intensive dairy farming. Positive results were detected in cattle belonging to different ages, corroborating previous observations (Flores et al.1998, Salvador et al. 1998, Gomes et al. 2002). Although adult cattle may also be affected, most outbreaks of BoHV-5 neurological disease involve mainly young cattle (Carrillo et al. 1983, Schudel et al. 1986, Salvador et al. 1998). Post-weaning and transportation stress, associated with crowding and concomitant diseases (parasitic, bacterial) are likely responsible for the higher occurrence of BHV-5 neurological among young cattle (Flores 2005). Pre-existing immunity to BoHV-1, due to natural infection and/or vaccination may also explain the lower incidence of neurological disease among adult animals comparing to young ones (d’Offay et al. 1993).

Outbreaks of neurological disease affecting several young cattle are likely associated with introduction of the virus into the herd and/or reactivation infection from latently infected adult cattle. Single, isolated cases of neurological disease by BoHV-5 in adult cattle are more likely to be due to reactivating virus rather than due to introduction of new virus into the herd (Vogel et al. 2003, Flores 2005). In the present study, the lack of additional clinic-epidemiological information accompanying many submissions did not allow a more comprehensive and detailed analysis of the epidemiology of the infection within the studied area.

Although most cases of neurological disease by bovine herpesviruses have been now attributed to BoHV-5, BoHV-1 has been occasionally associated with bovine meningoencephalitis (Furuoka et al. 1995, Flores et al. 1998, Varela et al. 2010). Thus, we used a PCR strategy that would detect both BoHV-1 and BoHV-5 genomes while distinguishing between them based on the size of the amplicon. PCR amplification of BoHV-1 genome would result in a 274 bp product, whereas the BoHV-5
genome would yield a 166 bp amplified product. Using this strategy, all positive samples originated amplicons of the expected size for BoHV-5 (Figure 2). Likewise, the identification of the virus producing CPE after cell culture inoculation (as BoHV-1 and BoHV-5 produce undistinguishable CPE) was performed with two Mabs: antibody 11H6 recognizes only BoHV-1 and antibody 2G5 that reacts with BoHV-1 and BoHV-5 (Souza et al. 2002). However, although Mab 11H6 would be able to distinguish between BoHV-1 and BoHV-5, this differentiation was not performed as the PCR results pointed out for BoHV-5 amplicons.

Most of the positive specimens were originated from herds lacking basic biosafety and/or preventive sanitary measures such as herd screening, serologic tests for new additions and/or vaccination. The lack of such preventive measures likely favors the introduction and maintenance of latently infected animals in the herds. Latently infected animals represent the main source of alphaherpesvirus infection due to periodic episodes of naturally occurring virus reactivation and transmission (Rock 1994).

The nested-PCR test used herein showed a higher sensitivity compared to virus isolation. From 39 specimens detected as positive by PCR nineteen (49%) virus isolation was not detected – a possible explanation for this discrepancy is that the long storage of brain specimens at -20 °C for years would have resulted in loss of infectivity of the virions in the sample. In this sense, the PCR has a major advantage when used for diagnostic purposes. Molecular methods as PCR and nested PCR detected small amounts of viral DNA in samples even if the particles are inactivated. Inactivation of virus infectivity due to improper collection and/or conservation and/or submission occurs very commonly among field clinical specimens submitted to diagnostic laboratories and thus would result negative in tests assaying for viable virus, i.e. virus isolation.

An additional explanation for the higher number of positive specimens by PCR is that some of the animals found positive in this technique (and not by virus isolation) had possibly latent BoHV-5 infection. In this sense, in spite of its high sensitivity, PCR should be used with caution in the diagnosis of neurological diseases in cattle since BoHV-5 DNA may be present in several areas of the brain of latently infected cattle (Vogel et al. 2003). Thus, if BoHV-5 latently infected animals develop a neurological disease of any other etiology and their brains are submitted to PCR for BoHV-5 sequences, they may be erroneously diagnosed as positive. In other words, PCR would detect the BoHV-5 DNA in the brain of cattle with neurological disease regardless whether the disease was indeed caused by the virus or by another agent. These results evidence the need for associating molecular methods (i.e. PCR) with other diagnostic tests (e.g. virus isolation) as to achieve a highly sensitive, specific and precise diagnostic of BoHV-5-associated neurological disease.

On the other hand, infectious virus was detected in nine specimens (9/107) that were negative by PCR. This finding argues against a general higher sensitivity of PCR over virus isolation. The presence of PCR inhibitory substances in the original material or during the DNA extraction procedure, or the presence of a number of genome copies below the threshold of detection would be possible explanations for the failure of PCR to detect BoHV-5 in these specimens. The sensitivity (number of genome copies) of our PCR has not been determined yet it is plausible that lack of sensitivity was responsible for some of these false-negative results comparing to virus isolation.

In the present study, three specimens wherein Babesia sp has been previously detected gave positive results for BoHV-5 in virus isolation. The clinic-epidemiological relevance of the association of BoHV-5 with Babesia sp and other agents well) in neurological disease in cattle deserves further investigation. For that purpose, a differential diagnosis for agents usually involved in neurological manifestations would be necessary. Concomitant BoHV-5 and rabies infection in the brain of cattle suffering from neurological disease has already been described (Batista et al. 2004). A possible explanation for these cases (Babesia sp or rabies concomitant with BoHV-5) is that animals with latent BoHV-5 infection were subsequently infected by other infectious agents (with possible contribution of BoHV-5 infection as well). Thus, reactivation of BoHV-5 (and its detection in clinical material) would have been triggered by the other pathologies.

In summary, the results presented herein corroborate data from previously published reports regarding the importance of BoHV-5 infection and disease in cattle. BoHV-5 associated neurological disease - confirmed either by PCR or by virus isolation, or both – was confirmed in an expressive number of brain specimens of animal suffering from neurological disease in RJ and MG. Thus, differential BoHV-5 diagnosis (and other agents involved in neurological disease as well) in those cases negative for rabies should thereafter be included and adopted by diagnostic laboratories. Likewise, the concomitant use of PCR and virus isolation would be advisable as to achieve a higher sensitivity of BoHV-5 diagnostic.

Conflict of interest statement
None.

ACKNOWLEDGEMENTS
The authors thank the Brazilian agencies CNPq, CAPES, FAPERJ FOPESQ-UFF, PESAGRO-RJ and FIOCRUZ for financial support.
REFERENCES


Flores EF 2005. Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900. e-Address: flores@ccr.ufsm.br (personal communication).


Salvador SS, Lemos RAA, Riet-Correa F, Roehe PM,


