High prevalence of co-infections with bovine herpesvirus 1 and 5 found in cattle in southern Brazil

F.S. Campos a,b,*, A.C. Franco a,b, S.O. Hübner c, M.T. Oliveira a,b, A.D. Silva d, P.A. Esteves d, P.M. Roehe a,b, F.A.M. Rijsewijk a,b

a Virology Laboratory, Microbiology Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul (UFRGS), Av. Sarmento Leite 500, Porto Alegre, CEP 90050-170, Rio Grande do Sul (RS), Brazil
b FEPAGRO – Animal Health – Institute for Veterinary Research “Desidério Finamor” (IPVDF), Estrada do Conde 6000, Eldorado do Sul, CEP 92990-000, Rio Grande do Sul (RS), Brazil
c Virology Laboratory, Department of Preventive Veterinary Medicine, Faculty of Veterinary Medicine, Federal University of Pelotas (UFPel), Pelotas, University Campus, Post Box 354, CEP 96010-900, Rio Grande do Sul (RS), Brazil
d Embrapa CNPSA, BR 153, Km 110, Concórdia, Post Box 21, CEP 89700-000, Santa Catarina (SC), Brazil

1. Introduction

Bovine herpesvirus 1 (BoHV-1) is a member of the order of Herpesvirales, the family Herpesviridae, the subfamily Alphaherpesvirinae and the genus Varicellovirus (Davison et al., 2009). BoHV-1 is an important pathogen of cattle causing economic losses worldwide. BoHV-1 is mainly implicated in respiratory infection (infectious bovine rhinotracheitis, IBR), genital disease (vulvovaginitis/pustular balanoposthitis, IPV/IBP) and abortion (Kahrs, 2001). Sporadically, BoHV-1 is associated with encephalitis (Roels et al., 2000; Silva et al., 2007a).

BoHV-1 infections go through three phases: a primary infection that lasts about 2 weeks, followed by a lifelong latent infection, occasionally followed by reactivation of latent virus that causes renewed virus shedding for several days (for a recent review on BoHV-1, see Muylkens et al., 2009).
2007). Reactivation and re-excretion of BoHV-1 is mainly depending on the health conditions of the host. Among the tissues where BoHV-1 virus remains latent are the sensory ganglia of the nerves that innervate the sites of the primary infection. These tissues are, for example, the lumbo-sacral ganglia after a genital infection (Van Engelenburg et al., 1995a) and the trigeminal ganglia after a respiratory infection (Van Engelenburg et al., 1995b). In those tissues viral DNA remains in the nuclei of neuronal cells (Muylkens et al., 2007). In practice, animals that have a genital BoHV-1 infection also develop a BoHV-1 respiratory infection due to animal-to-animal transmission (Mollema et al., 2006). This makes trigeminal ganglia a preferred site to detect genomic DNA of latent BoHV-1.

On the other hand, the closely related bovine herpesvirus 5 (BoHV-5), which has about 70% sequence identity with BoHV-1 (Delhon et al., 2003) is implicated in bovine encephalitis or meningoencephalitis, although sometimes the virus has also been recovered from both genital and respiratory tracts of cattle (Bratanich et al., 1991). Unlike BoHV-1, which is usually associated with low mortality rates, BoHV-5 meningoencephalitis is highly fatal, with mortality rates approaching 100% if it concerns bovines <1 year of age (Salvador et al., 1998). After an intranasal infection of BoHV-5, latent BoHV-5 DNA can be found in several areas of the central nervous system, in lymphoid tissues and tracheal and nasal mucosae, but BoHV-5 DNA is also consistently found in trigeminal ganglia (Meyer et al., 2001; Vogel et al., 2003). BoHV-5 does not have a worldwide distribution, but is predominantly found in Australia and South America.

The state Rio Grande do Sul has a cattle population of 14 million (IBGE, 2007) that for the biggest part is roaming free in the fields and a large part of this population is found in the south of this state. Based on several small scaled studies (Silva et al., 2007b; Rissi et al., 2008) and serological studies, which have little sensitivity and cannot discriminate between BoHV-1 and BoHV-5 antibodies (Roehe et al., 1997; Holz et al., in press), it can be inferred that part of this cattle population is infected with BoHV-1 or BoHV-5.

To study the prevalence of BoHV-1 and BoHV-5 in bovines in the south of Rio Grande do Sul in a more resolute way, it was decided to try to detect latent viral DNA in their trigeminal ganglia. To this end a quantitative PCR was developed that allowed the amplification of both BoHV-1 and BoHV-5 genomic DNA. The quantitative PCR was sensitive enough to detect most BoHV DNA infected trigeminal ganglia and showed that at least 87% of the studied bovines were positive for BoHV. To discriminate between BoHV-1 and BoHV-5 infected animals, two type-specific PCRs were developed – one for each virus – that used the products of the first PCR as a template. It was found that the majority of the BoHV infected animals are infected with both BoHV-1 and BoHV-5.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby bovine kidney (MDBK) cells, subcultured from ATCC CCL-22, were used to multiply BoHV-1 and BoHV-5 viruses. The cells were kept in Eagle’s minimal essential medium (EMEM, Gibco) with 10% fetal bovine serum (FBS, Cultilab) supplemented with a cocktail of antibiotics [10 IU/ml penicillin (Cultilab), 10 μg/ml streptomycin (Cultilab) and 2 μg/ml amphotericin B (Cristália)]. The BoHV-5 strain used in this study is EVI 88/95 and the BoHV-1 strain is EVI 123/98. Both strains were isolated in Rio Grande do Sul in, respectively, 1995 and 1998 (Roehe et al., 1997; D’Arce et al., 2002).

2.2. Collection of trigeminal ganglia and sera

Both the left and the right trigeminal ganglion of 200 cattle of mixed breeds, both male and female, with a mean age of about 4 years and originating from 40 districts spread over the south of Rio Grande do Sul, were collected in a slaughterhouse in Pelotas in the far south of Rio Grande do Sul. In short, the heads of the animals just slaughtered were randomly chosen and opened with an electric saw to take out the brains. Subsequently, the trigeminal ganglia were collected in pairs using sterilized pincers, scissors and chirurgical knives. The ganglia were stored individually in 6-well plates, placed on dry ice and transported. In the laboratory the ganglia were cut into several pieces and each piece was placed in a separate well of a 24-well plate and frozen at −70 °C until processed. All samples were carefully processed to avoid cross-contamination.

From all 200 heads 10 ml blood samples were collected and after coagulation the blood samples were centrifuged for 10 min at 3000 rpm (Damon/IEC Division) to obtain the serum fraction of each sample. These samples were stored in 1.5 ml microtubes at −20 °C until further use.

2.3. Virus neutralization tests (VNTs)

The 24 h VNTs were performed according to the protocols of the OIE (OIE, 2008). First, the sera have been inactivated by a 30 min incubation at 56 °C. After that, two fold serial dilutions were made in tissue culture medium using sterile 96-wells plates. The VNTs were done in duplicate using 50 μl per well of each serum. Then, 50 μl of each serum were mixed with 50 μl of sample containing 100–200 TCID50 (50% tissue culture infectious dose) of either BoHV-1 (EVI 123/98) or BoHV-5 (EVI 88/95). Strong positive control sera, weak positive control sera, and negative sera were included in each test. After a 24 h pre-incubation at 37 °C in a 5% CO2 incubator (Forma Scientific), 100 μl of a cell suspension were added to the pre-incubation mixture at a concentration of 3 × 104 cells per well and the plates were further incubated at 37 °C in a 5% CO2 incubator. Four days after the start of the VNTs the cells were examined for the presence of the characteristic cytopathic effect of BoHV-1/BoHV-5. The titers were calculated using the method of Reed and Muench (1938).

2.4. Extraction of total ganglion DNA

Fragments of trigeminal ganglia of about 50 μg were placed in 1 ml lysis buffer [20 mM Tris–HCl (Invitrogen), pH 7.4; 10 mM EDTA (Invitrogen), pH 8.0 and 200 mM NaCl
(Nuclear); 100 μg proteinase K (USB Corporation) and 1% SDS (Promega) and incubated overnight at 37 °C. After this, the mixture was centrifuged at 12,000 × g for 10 min to precipitate the non-digested material and 400 μl supernatant was collected. After a standard phenol (Invitrogen) extraction and ethanol (Pro Analysis) precipitation (Sambrook and Russell, 2001) the purified DNA was dissolved in 200 μl TE (10 mM Tris pH 7.4; 1 mM EDTA pH 8.0). To check for the quantity and quality of the obtained DNA, 10 μl were analyzed on an agarose (Invitrogen) gel and compared with known quantities of phage lambda DNA (Fermentas). It has been established using predetermined amounts of internal control templates that the amount of total ganglion DNA that still allowed an efficient PCR amplification was 200 ng.

2.5. The first PCR reaction to detect BoHV-1/BoHV-5 simultaneously

The primers used in the first PCR assay were based on the glycoprotein C (gC) coding sequences of both BoHV-1 and BoHV-5, which were available in the database of the National Center for Biotechnology Information (NCBI, 2007). The primers (PF2: 5’-CGGCAAGCCGCTGAGCA-3’ and PR1: 5’-CGGCGCCGATCTATACCC-3’) anneal to a region shared by both BoHV-1 and BoHV-5 and amplify a region of 575 and 572 bp, respectively (Esteves et al., 2008). To be able to detect the low number of BoHV genomes present in ganglia, it was needed to optimize the PCR developed by Esteves et al. (2008). The improved PCR reaction (25 μl) contained 1 mM MgCl₂ (Invitrogen) 0.3 μM of each primer (IDT), 10% dimethylsulfoxide (DMSO; Acros Organics), 1 U Taq DNA polymerase (Invitrogen), 10% of PCR buffer (Invitrogen) and 0.6 mM deoxynucleoside triphosphates (Abgene) per reaction. To each reaction, 25 molecules of the internal control template (product length 440 bp) were added to detect false negative reactions. Reactions were performed in the Eppendorf Mastercycler under the following cycle conditions: 5 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 1 min at 62 °C, 1 min at 72 °C; followed by 5 min at 72 °C.

To avoid contaminations with PCR products, separate spaces were used to prepare the PCR reaction buffer, the extraction of total ganglion DNA, and the analysis of the PCR products. Moreover, filter tips were used, work benches were decontaminated with UV light, and negative controls were included in every five PCR reactions.

2.6. The second and third PCR reactions to detect BoHV-1 and BoHV-5 separately

To differentiate between BoHV-1 and BoHV-5, two subtype-specific PCRs were performed using the product of the first PCR as a template. To detect BoHV-1 DNA the primer pair (PF 5’-CTAACATGGAGCGCCGCTTT-3’ and PR 5’-CGGGCGATGCCGTC-3’) was used, which gives a product of 161 bp. To detect BoHV-5 the primer pair (PF 5’-CGGAGCCGCGTTCCG-3’ and PR 5’-TATCGCCGGAGGCAGGGCC-3’) was used, which gives a product of 236 bp. The reaction conditions of the second and third PCR were the same as for the first PCR with exception of the primers concentration, which was 0.2 μM. The cycling conditions were: 94 °C for 5 min, followed by 35 cycles of 1 min 94 °C, 1 min 61 °C and 1 min 72 °C and a final extension time of 5 min 72 °C. Some products were sequenced to confirm their identity (data not shown).

3. Results

3.1. BoHV virus neutralization tests (VNTs)

To know what the prevalence of BoHV-1/BoHV-5 infected bovines would have been when standard VNTs were used, the serum samples of all the 200 bovines used in this study were tested in a 24 h VNT. The results revealed an overall neutralizing antibody prevalence of 49.5% (99/200). Of these sera, 44.0% (88/200) neutralized BoHV-1 and 41.5% (83/200) neutralized BoHV-5.

3.2. Construction of an internal control molecule for the BoHV PCR

To be confident that genomic BoHV DNA could be amplified in the presence of isolated ganglion DNA, an internal control molecule was constructed that could be amplified by the same primers as the BoHV viral DNA and was added to each PCR reaction. To differentiate between the viral product and the internal control product the amplified region of the internal control construct was made 135 bp shorter than the viral product (575/572 bp). To this end a plasmid containing the viral PCR product of BoHV-1 (EVI 123/98) was digested with BsmBI, separated from its 135 bp BsmBI insert and recircularized (Fig. 1a). The resulting construct, named internal control (IC) was added to the PCR reactions and gave rise to a 440 bp fragment. The presence of the IC product in the absence of a viral product implied a negative result, at least a result below the detection limit of the method. On the other hand, the absence of both a viral product and an IC product implied an amplification failure and could be a false negative result. Ganglion DNA samples that even after several attempts failed to allow the amplification of the IC were considered non-interpretable, which corresponded to about 2.5% of the samples.

3.3. Sensitivity of the BoHV PCR

The sensitivity of the BoHV PCR was determined using 10-fold serial dilutions of known quantities of IC molecules. Moreover, the co-amplification of both viral DNA and IC DNA was established to check for any major difference in DNA amplification efficiency. The products of 25 molecules of IC and viral template were still easily visualized on an ethidium bromide-stained agarose gel (Fig. 1b). This implies that the lower detection limit of this PCR experiment is positioned between 25 and 2.5 molecules. Because in practice the PCR is performed using 200 ng of total ganglion DNA, the sensitivity test was also done in the presence of 200 ng total ganglion DNA from a BoHV positive cow (Fig. 1c). 200 ng genomic bovine DNA is the amount found in 5 × 10⁴ bovine cells. This experiment showed that also in the presence of 200 ng total ganglion...
Submitted to the BoHV PCR. 5 ng of total DNA was mixed with tenfold dilutions of IC molecules and in the presence of total DNA extracted from a bovine trigeminal ganglion. (BioLabs). (C) Co-amplification of target viral DNA with the IC molecules and 2.5 (lane 5). Lane 1: negative control; lane 6: 100 bp DNA Ladder analyzed by electrophoresis in a 1.5% agarose gel. The total number of IC molecules were 2500 (lane 1), 250 (lane 3), 25 (lane 4) and 2.5 (lane 5). Negative controls (lanes 2 and 6) and Lambda HindIII marker (lane 7).

DNA, 25 molecules of IC gave an easily detectable band on an agarose gel (Fig. 1c, lane 4). The absence of a visible viral band in the presence of 2500 IC molecules (Fig. 1c, lane 1) is due to competition between both templates.

3.4. Quantification of the mean number of BoHV genome copies in trigeminal ganglia

The presence of a known number of internal control molecules in the PCR reaction made it possible to estimate the mean number of BoHV genomes present in a portion of a latently infected ganglion. The IC fragment is amplified with the same efficiency as the viral DNA fragment (see Fig. 1b and c, lane 4). Therefore, when the intensities of the bands of the PCR products of both the IC fragment and the viral fragment are the same, the amount of viral genomes found in 200 ng of total DNA is about 25 molecules. Using these co-amplification results it was estimated that in 5 × 10⁴ trigeminal ganglion cells up to some 200 BoHV genomes could be detected, like in Fig. 2a, lane 7. On the other hand, often only a few BoHV genome copies are detected in 5 × 10⁴ trigeminal ganglion cells (Fig. 2a, lane 2 and Fig. 2b). This implied that some (parts of) ganglia may harbor even less BoHV genome copies than the detection limit of our test and will not score positive.

3.5. Percentage of bovines that scored positive for the presence of BoHV genomes in their trigeminal ganglia

The results of the first BoHV PCR test used in this study showed that of the 400 ganglia tested, 74.8% (299/400) scored positive, 20.5% (82/400) scored negative and 4.7% (19/400) gave a non-interpretable result (Table 1). In some cases only one of both ganglia scored positive, under-scoring the non-homogeneous distribution of trigeminal ganglia cells positive for BoHV genomes. As a consequence, the percentage of animals that scored positive for the presence of BoHV genomes in (only one of) their trigeminal ganglia was higher: 87.0% (174/200) scored positive, 10.5% (21/200) scored negative and 2.5% (5/200) were non-interpretable (Table 1).

3.6. Type-specific PCRs for BoHV-1 and BoHV-5 that used the products of the BoHV PCR as a template

Within the amplified region of the BoHV PCR, two pairs of primers were selected that were specific for BoHV-1 and for BoHV-5, respectively. Using the products of the first PCR as templates the primers pairs gave products of 161 bp for BoHV-1 and 236 bp for BoHV-5 (Fig. 3). All 299 BoHV templates gave rise to a product in at least one of the type-specific PCRs. In many cases the products of the first PCR gave a positive result in both type-specific PCRs (Fig. 3).

3.7. High prevalence of co-infections with bovine herpesvirus 1 and 5

Application of both type-specific PCRs to all the first PCR products of the 174 BoHV positive animals showed...
that 82.8% (144/174) of them was positive for BoHV-1, 75.9% were found positive for both viruses. The percentage of BoHV-1 and BoHV-5 co-infections is 66%.

<table>
<thead>
<tr>
<th>Results per ganglion</th>
<th>Number</th>
<th>BoHV positives</th>
<th>BoHV negatives</th>
<th>Inhibited</th>
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<tr>
<td>Percentage</td>
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<td>299&lt;sup&gt;b&lt;/sup&gt;</td>
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<table>
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<th>Results per animal</th>
<th>Number</th>
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<th>BoHV negatives</th>
<th>Inhibited</th>
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<tbody>
<tr>
<td>Percentage</td>
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<td>174&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>87.0</td>
<td>10.5</td>
<td>2.5</td>
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<sup>a</sup> In the samples that were inhibited neither the viral product nor the product of the 25 molecules of the internal control could be detected.

<sup>b</sup> The subtype-specific PCRs were accomplished using in the products of the BoHV positive samples.

<sup>c</sup> The percentage of BoHV-1 and BoHV-5 positive animals is based on the number of BoHV positives animals. In the total population of 200 animals the percentage of BoHV-1 and BoHV-5 co-infections is 66%.

4. Discussion

The identification of herpesvirus infected animals based on the presence of genomic viral DNA has, to our knowledge, never been performed on such a large scale as in this study. Of the 200 animals used here 49.5% (99/200) were found positive for either BoHV-1 and/or BoHV-5 in a standard 24 h VNT, while 87% of them scored positive in the PCR assay. This study also shows for the first time the identification of herpesvirus infected animals based on the presence of genomic viral DNA has, to our knowledge, never been performed on such a large scale as in this study.

Other research groups also developed BoHV-1/BoHV-5 specific PCR assays (Ros et al., 1999; Abril et al., 2004). Unfortunately, these PCRs were never used to detect BoHV-1 and BoHV-5 in samples that harbor both viruses at the same time. The sensitivities of the PCR assays of Ros et al. (1999) and Abril et al. (2004) [6–60 molecules and 1–10 molecules, respectively] appear to be comparable to the sensitivity of the PCR assay in the study presented here. However, the sensitivities of these PCRs were determined using either purified viral genomic DNA (Ros et al., 1999) or purified plasmid DNA (Abril et al., 2004). In practice, PCR amplification of viral sequences present in total DNA from a tissue sample has a reduced sensitivity due to the inhibiting effects of DNA and other components in the sample. Furthermore, “the sensitivity of a PCR” is not a stable number of molecules. This is due to tube to tube variations. This subject is important for the study presented here for two reasons. (1) To obtain a clear-cut estimation of the (often low) number of BoHV-1 or BoHV-5 genome copies in bovine trigeminal ganglion samples and (2) to reduce the number of false negative results.

For both reasons, adding a small amount of an internal control (IC) molecule in the same tube is essential. The tube to tube variation is immediately perceptible. As long as the IC is giving a signal, an accurate estimation of the number of viral genome copies in the sample is possible. Alternatively, if the signal of the IC is not detectable the PCR reaction has to be repeated to reduce the number of false negative results.

Abril et al. (2004) used an internal control kit for their real-time PCR (RT-PCR). Unfortunately, this kit uses a standard template and a primer pair that have optimal amplification conditions distinct from the optimal conditions for the amplification of the target DNA, making the internal control procedure less precise. Moreover, in RT-PCR relative differences in the amounts of product between samples can be accurate, but the absolute values calculated for the experimental samples are without meaning. This makes the RT-PCR an unsuitable tool to determine very low numbers of viral genome copies per tissue sample.

With respect to the difference between the serological results (49.5%) and the PCR results (87%) several explanations can be put forward. One would be that false positive results added to the score of the PCR test. It is true that gel analysis of the amplified PCR products can be a source of contamination. To avoid such contaminations a strict separation was applied consequently between the site of gel analysis and the site where the PCR reactions were mounted. Moreover, negative controls were included in all series of samples. When one of the negative controls scored positive, the whole series was discarded and the PCR analysis was repeated. If a contamination with BoHV-1 or BoHV-5 would have occurred during the period that this PCR analysis was performed one would expect that later series would have a higher BoHV-1 or BoHV-5 score. This is, however, not the case (see graph of the test results in supplementary data).
Alternatively, it can be argued that a 24 h VNT to detect anti-BoHV-1 or BoHV-5 antibodies may be not sensitive enough. Indeed, Kramps et al. (1996) showed that some ‘home-made’ blocking ELISAs scored positive early after infection (7 days), while 24 h VNTs were not able to detect these low anti-BoHV-1 titers. In experimentally infected cattle anti-BoHV-1 antibodies persist at least 3 years at easily detectable levels (Kaasheok et al., 1996). However, data about antibody responses against BoHV-1 or BoHV-5 under field conditions are not available. The ‘persistence’ of anti-BoHV-1 antibodies in calves held in captivity may be the result of regular reactivation and this may be an essential difference with a field situation (Van Reenen et al., 2000).

It is also unclear how cattle under field conditions are infected with BoHV-1 and/or BoHV-5. A possible additional explanation for the difference between the serological and the PCR results are seronegative carriers. Especially when the animals are infected when maternal antibodies against BoHV-1 are still present, seronegative BoHV-1 carriers may arise (Lemaire et al., 2000). So, seronegative carriers and the limited sensitivity of the 24 h VNT test are the most likely explanations for the difference between the serological results and the PCR results.

Meyer et al. (2001) developed a cross-neutralization assay for BoHV-1 and BoHV-5. Their method is based on serum samples of calves experimentally infected with a known virus: either BoHV-1 or BoHV-5. The serum samples of the presented study here are from cattle with an unknown infection history and are often infected with both BoHV-1 and BoHV-5. Therefore, cross-neutralization assays would give only un-interpretable results.

Hence, two subtype-specific PCRs (one for BoHV-1 and one for BoHV-5) were developed and applied to the DNA products obtained with the first PCR. The high percentage of doubly infected animals is unprecedented. Again it could be argued that contaminations are responsible for such a result. However, again the precautions to avoid contaminations have been taken and observed strictly.

The biological consequences of the observed co-infection of BoHV-1 and BoHV-5 in the same ganglion are a matter of further investigation. Co-infection of BHV-1 variants in ganglia has been observed before (Thiry et al., 1985; Whetstone & Miller, 1989). The first consideration is that the presence of both viruses in the same ganglion may give rise to recombinants. Meurens et al. (2004) co-infected MDBK cells with an MOI of 10 and obtained two BoHV-1/BoHV-5 recombinants. BoHV-1 variants that reactivated from latency can form recombinants (Schyns et al., 2003; Muylkens et al., 2006), but whether BoHV-1 and BoHV-5 also form recombinants after reactivation is not known. Until now no BoHV-5 viruses with substantial regions of BoHV-1 (or vice versa) have been isolated from the field. Hence such recombinations are either very rare or the recombination products cannot compete with the wild type versions of BoHV-1 or BoHV-5.

BoHV-1 and/or BoHV-5 infected cattle and semen are confronted more and more with international trade limitations. The approach to control BoHV-1 and BoHV-5 infections in a cattle population is much dependent on the prevalence of the viral infection. With a low number of infected animals the “stamping out” method is preferred, while with a high number of infected animals vaccination with a marker vaccine followed by selective slaughter is favored (Ackermann and Engels, 2006). The data presented here will help to decide how to face the BoHV-1 and BoHV-5 infections of cattle in Rio Grande do Sul.

5. Conclusions

A quantitative PCR has been developed to detect both BoHV-1 and BoHV-5 DNA in trigeminal ganglia of cattle. The PCR method had a detection limit between 2.5 and 25 molecules of BoHV-1 or BoHV-5 DNA. Using this PCR it was found that the number of BoHV-1 or BoHV-5 DNA molecules present in 5 × 10⁴ ganglion cells ranged from several hundreds to only a few. Within the detection limits of the newly developed PCR, BoHV-1 or BoHV-5 DNA could be detected in 87% of the bovines in south of Rio Grande do Sul, Brazil. To differentiate between BoHV-1 and BoHV-5 infections two subtype-specific PCRs were developed using the amplified region of the first PCR as a template. The results of the subtype-specific PCRs implied that of the 87% of BoHV-1 or BoHV-5 positive trigeminal ganglia 75.9% were found positive for both BoHV-1 and BoHV-5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.05.015.

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