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Detection of bovine herpesvirus 2 and bovine herpesvirus 4 DNA in trigeminal ganglia of naturally infected cattle by polymerase chain reaction

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Abstract

Establishment of latent infection within specific tissues in the host is a common biological feature of the herpesviruses. In the case of bovine herpesvirus 2 (BoHV-2), latency is established in neuronal tissues, while bovine herpesvirus 4 (BoHV-4) and ovine herpesvirus 2 (OvHV-2) latent virus targets on cells of the monocytic lineage. This study was conducted in quest of BoHV-2, BoHV-4 and OvHV-2 DNA in two hundred trigeminal ganglia (TG) specimens, derived from one hundred clinically healthy cattle, majority of them naturally infected with bovine herpesvirus 1 (BoHV-1) and bovine herpesvirus 5 (BoHV-5). Total DNA extracted from ganglia was analyzed by polymerase chain reactions (PCR) designed to amplify part of the genes coding for BoHV-2, and BoHV-4 glycoprotein B and, for OvHV-2, the gene coding for phosphoribosylformylglycinamidine synthase-like protein. BoHV-2 DNA was detected in TG samples of two (2%) and BoHV-4 DNA in nine (9%) of the animals, whereas OvHV-2 DNA could not be detected in any of the TG DNA. The two animals in which BoHV-2 DNA was identified were also co-infected with BoHV-1 and BoHV-5. Within the nine animals in which BoHV-4 DNA was detected, six were also co-infected with BoHV-1 and BoHV-5. This report provides for the first time evidence that viral DNA from BoHV-2 and BoHV-4 can be occasionally detected in TG of naturally infected cattle. Likewise, in this report we provided for the first time evidence that the co-infection of cattle with three distinct bovine herpesviruses might be a naturally occurring phenomenon.

Keywords: bovine herpesvirus; ovine herpesvirus; PCR; latency.

1. Introduction

Bovine herpesvirus type 2 is member of the subfamily *Alphaherpesvirinae*, while BoHV-4 and OvHV-2 are members of the subfamily *Gammaherpesvirinae* (Davison et al., 2009). The latent infection is a hallmark of herpesvirus' biology. In the case of alphaherpesviruses, latency is known to be established in neuronal tissues. BoHV-2 would also be expected to induce latency in nerve ganglia, although based on the usual primary target of the infection, which is the mammary gland, the virus would be expected to remain latent in inguinal nerves. Torres et al. (2009a) showed that lambs intranasally inoculated with BoHV-2 harbored latent viral DNA in trigeminal ganglia, tonsils and regional lymph nodes. So far, BoHV-4 infection has not yet been clearly associated with disease (Donofrio et al., 2007). Like other gammaherpesviruses, BoHV-4 latency is expected to be established in cells of the monocytic lineage (Osorio and Reed, 1983; Dubuisson et al., 1989), although it has also been detected in peripheral and/or central nervous system tissues (Egyed et al., 1996). OvHV-2 is the causative agent of sheep-associated malignant catarrhal fever (MCF). The virus is transmitted mainly by the respiratory route and may be shed intermittently in nasal secretions. Similarly to BoHV-4, monocytes are also presumed to be the site for latency of OvHV-2 in sheep (Li et al., 2004).

To date, serological tests for detection of BoHV-2- and OvHV-2-antibodies are not commercially available (Li et al., 2013). In addition, BoHV-2 virus isolation from infected animals is easily accomplished but essentially requires sampling of clinically apparent lesions. BoHV-4 virus isolation is common, but not routinely successful. Attempts on OvHV-2 recovery from clinical MCF cases have constantly failed. Thus, a more reliable method for identification of above mentioned viruses, would have to be based on genome detection by PCR (Egyed and Bartha, 1998; Torres et al., 2009a; Li et al., 2011).

It has been previously described that BoHV-2 can establish latent infections in sensory ganglia (Letchworth and Carmichael, 1982). BoHV-4 DNA can be found in bone marrow cells at 62 d.p.i., suggesting that this virus may persist in this tissue (Egyed and Bartha, 1998). With respect to OvHV-2, this virus has been found in nasal secretions of sheep, but not in bovines (Li et al., 2004). Recently, we have investigated the presence of BoHV-1 and BoHV-5 in TG samples from cattle (Campos et al., 2009). The present study was extended in quest for BoHV-2, BoHV-4 and OvHV-2 DNA in the same TG samples in order to find out whether or not, cell types other than lymphocytes can be latently infected by BoHV-4 and OvHV-2, as well as to clearly define whether or not BoHV-2 can establish latency in TG from cattle.

2. Materials and methods

2.1. Cells and virus

The CRIB cell line, a bovine viral diarrhea virus-resistant clone derived from Madin-Darby bovine kidney cells (MDBK) (Flores and Donis, 1995), was used for virus propagation of BoHV-4. The cells were maintained in Eagle's minimal essential medium (E-MEM, Gibco) supplemented with 10 % fetal bovine serum (Gibco), 10 µg/mL streptomycin (Vitalfarma), 100 µg/mL gentamicin (Gentamax®, Marcolab), and 2 µg/mL amphotericin B (Cristália). The BoHV-4 strain Movar was isolated in Europe by Bartha et al (1966).

2.2. Collection of trigeminal ganglia

Two hundred TG specimens, derived from 100 animals of mixed breeds of both genders, with a mean age of about 4 years were collected in a slaughterhouse in the city of Pelotas, southern Rio Grande do Sul, Brazil. Details on the sampling procedure and identification of samples were provided elsewhere (Campos et al., 2009).

2.3. Extraction of total ganglion DNA

Total DNA extraction method from ganglia was described previously (Campos et al., 2009). In summary, fragments of TG of approximately 50 mg were lysed with TEN buffer [20 mM Tris-HCl (Affymetrix, USB), pH 7.4; 10 mM EDTA (Invitrogen), pH 8.0 and 200 mM $NaCl_2$ (J.T.Baker®)], 100 µg proteinase K (BioAmerica Inc.) and 1 % SDS (Serva). A standard phenol (Invitrogen) extraction was performed. To check the quantity and quality of the DNA, 10 µl were loaded on agarose (Agargen) gels and compared with known quantities of lambda phage DNA (New England Biolabs). One hundred nanograms of sample DNA were added to each tube as templates for the first round PCRs that preceded nested and semi-nested reactions.

2.4. BoHV-2, BoHV-4 and OvHV-2 PCR assays

For detection of BoHV-2, BoHV-4 and OvHV-2 DNA by PCR assay, the amplification conditions of previously published PCR systems were optimized (Table 1). Total DNA extracted from ganglia was subjected to PCR assay designed to amplify part of the genes coding for BoHV-2 and BoHV-4 glycoprotein B (gB) and, for OvHV-2, the gene coding for phosphoribosylformylglycinamidine synthase-like protein. The

assays were carried out in two steps: in a first round of reactions, 100 ng of total DNA and fixed amounts of an internal control (IC; see below for details) were added; then, in a second round of reactions, 1 µl of the first reaction was used as template. All reactions of the first PCR assay, including apparently negative results, were subjected to nested polymerase chain reaction (nPCR) (for BoHV-4 detection) and to semi-nested polymerase chain reaction (snPCR) (for BoHV-2 and OvHV-2 detection). Table 1 shows the targeted genes, primers and sizes of the expected products. All amplification assays were performed in a Mastercycler apparatus (Eppendorf), in a final volume of 25 µl. Each reaction tube contained 1 mM $MgCl_2$ (Invitrogen), 0.2 µM of each primer (IDT), 10 % dimethylsulfoxide (DMSO; Nuclear), 1 U Taq DNA polymerase (Invitrogen), 10 % PCR buffer (Invitrogen) and 0.4 mM deoxynucleoside triphosphates (GE Healthcare). Amplification reactions were performed under the following cycling conditions: initial denaturation of 94 ºC for 3 min; 35 cycles of 94 ºC denaturation for 50 sec, 51-56 ºC annealing for 30-50 sec (see Table 1), and 72ºC extension for 50 sec; a final elongation step at 72 ºC for 3 min. The snPCR for OvHV-2 and the nPCR for BoHV-4 were carried out in 32 cycles. In the nPCR for BoHV-4 thirty sec of denaturation and extension times were applied.

2.5. Construction of internal controls

Internal controls (ICs) used in the first round of PCRs were constructed by amplification of DNA extracted from TG with the primer pairs indicated in Table 1. Amplification reactions were run under low stringency conditions (melting temperature: 50 – 55 ^{o}C) and high $MgCl_2$ concentration (2 mM). After the agarose gel electrophoresis, amplicons that differed in size when compared with the expected products (Table 1) were chosen as templates for ICs. These amplicons (753 bp for BoHV-2, 322 bp for BoHV-4 and 515 bp for OvHV-2) were purified, cloned into pCR™ 2.1 vector (TOPO® TA Cloning® kit, Invitrogen) and sequenced (Supplementary data 1). The resulting plasmids were used as ICs in the first round PCRs and were used to determine the sensitivity of the PCR assays.

2.6. Sensitivity of the PCRs

The sensitivity of the PCRs was determined by amplifying 10-fold dilution series of known quantities of IC (2.5×10^6 to 2.5×10^1 molecules). The conditions of the reactions (reagent concentrations and set of cycling parameters) were optimized to allow amplification of low copy numbers of IC molecules. Amplification of at

least 25 molecules of any of the IC PCR templates were sufficient to obtain a visible PCR product on 1.5 % ethidium bromide-stained agarose gel (Supplementary data 2). This implies that the lower detection limit of these PCRs was about 25 IC molecules. Consequently, in all first round reactions, 25 molecules of the IC template were added to each reaction.

2.7. Sequencing

All products of the BoHV-2 snPCR and of the BoHV-4 nPCR were cloned into pCR™ 2.1 vector (Invitrogen) and subjected to nucleotide sequence analysis. Sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, UK) in an ABI-PRISM 3100 Genetic Analyzer (ABI, Foster City, CA), according to the manufacturer's instructions.

2.8. Phylogenetic analysis

GenBank (http://www.ncbi.nlm.nih.gov/genbank/) deposited sequences of BoHV-2, BoHV-4 and other phylogenetically related herpesviruses sequences were used for comparisons with partial sequences of gB gene of the BoHV-2 and of the BoHV-4 detected in TG. The analysis involved nine nucleotide sequences related to BoHV-2 and 19 nucleotide sequences related to BoHV-4. Alignment of the nucleotide sequences was performed using the ClustalW application, version 2.0. The tree was calculated with the MEGA 5 software. The evolutionary history was inferred by using the Maximum Likelihood method to BoHV-2 and the Neighbor-Joining method to BoHV-4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

3. Results and discussion

3.1. Detection of BoHV-2 and BoHV-4 DNA in TG by PCR

The analyses of total DNA isolated from 200 TG derived from 100 cattle by herpesvirus specific PCR assays identified the presence of BoHV-2 DNA in 2 % of analyzed animals, while BoHV-4 DNA was detected in 9 %. For OvHV-2, the PCR specific assay was negative for all analyzed samples and no evidence for the presence of viral DNA was observed. Hitherto, the detection of these viruses had been performed only in experimentally inoculated animals (Torres et al., 2009a; Egyed and Bartha, 1998). Here, a larger number of samples from naturally exposed animals were analyzed.

One of the problems of using a PCR assay as a diagnostic test is the generation of false-negative results that may be caused by random or non-random failures of amplification. In order to minimize such problem, ICs were constructed and used in all PCRs. ICs were also used to determine the sensitivity of PCR, which could be fixed at about 25 molecules for all assays. A highly sensitive method is crucial for the detection of viral genomes in tissues that contain low copy numbers of viral DNA, such as the TG (Wang et al., 2005).

BoHV-2 DNA was found in two percent of the target population, indicating that the improved PCR setup used in this study is reliable for the detection of BoHV-2 gB specific DNA in bovine TG. Kálmán and Egyed (2005) reported about detection of BoHV-2 DNA in non-bovine ruminants (roe deer, red deer, fallow deer, mouflon and domestic sheep). The prevalence of the viral DNA in these natural wild life reservoir species ranged from 3 % to 50 %. The differences found, when these results are compared, could be due to the latency site studied. While we analyzed TG, the previous study used lymphoreticular tissues to detected viral DNA (lymph nodes or spleen). Interestingly, Torres et al. (2009a) examined the distribution of latent BoHV-2 DNA in different tissues of sheep [dorsal root (lumbar) ganglia, trigeminal ganglia and lymph nodes] and they observed, in some cases, BoHV-2 positive lymph nodes and negative TG and the opposite situation was also found. Our findings suggest that TG of bovines can be also the sites in which BoHV-2 may remain latent. BoHV-2 infections in bovines have been detected in cattle from several countries by the detection of anti-viral antibodies (Dardiri and Stone, 1972; Martin and Gwynne, 1968; Imai et al., 2005). A serologic survey for BoHV-2 antibodies in cows revealed a prevalence of 24.5% (543/2.213) in southern Brazil (Torres et al., 2009b). In addition to the differences between these studies such as type of sample and methodology, the

highest rate of prevalence found by Torres et al (2009b), in comparison to our results, could be due to the gender of the animals studied, since mammillitis outbreaks are described in females. In addition, the virus may establish latency in other ganglia as well as in the lymph nodes.

BoHV-4 DNA was detected in nine out of the 100 animals analyzed in this study. The available literature data on the detection of BoHV-4 DNA in the neural tissues is scarce and conflicting. Egyed et al. (1996) have studied *in vivo* distribution of BoHV-4 in the natural host and they found that the nervous system remains free of viral DNA after experimental infection. However, two years later, Egyed and Bartha (1998) examined various tissues of experimentally infected calves for the presence of BoHV-4 DNA using a sensitive nPCR and these authors detected BoHV-4 in neural tissues and other organs that had never been associated with virus persistence. These findings highlight the importance of using a highly sensitive PCR for detection of virus DNA in tissue samples. Although BoHV-4 has been detected in cells of the monocytic lineage of latently infected cattle (Dubuisson et al., 1989), we have shown here, for the first time, that virus DNA can be present in TG of naturally infected cattle. However, we cannot rule out the possibility that these findings could result from the detection of viral DNA present in blood mononuclear cells irrigating the ganglia, due to the high sensitivity of the PCR used here. On the other hand, previous studies have also shown the presence of gammaherpesviruses in the central nervous system of theirs hosts. Thiry et al. (1990) reviewed the biology of BoHV-4 and reported that after primary infection BoHV-4 persists in a latent state in the nervous ganglia and mononuclear blood cells, thus, corroborating with ours results. In Brazil, BoHV-4 DNA was also detected by PCR, using thymidine kinase specific primers, in all fragments (n=14) of central nervous system (CNS). Interestingly, two of the BoHV-4 positive animals were concurrently positive for BoHV-5 DNA, indicating the coexistence of both DNA viruses in fragments of CNS (Costa et al., 2011). In the United States, a seroprevalence of 36% (107/296) was detected for BoHV-4 infection in one dairy herd (Frazier et al., 2002), while in Canada, the prevalence of anti-BoHV-4 antibodies in milk samples of 176 tested cows was 98.2%, but PCR positive results were only 1.3% (Ali et al., 2011). Factors such as the sanitary status of the herd, type of sample and methodology used can help to explain the variation in the results shown in different studies, including this study.

MCF-susceptible species (including cattle, deer, bison, water buffalo and pigs) generally are thought to be dead-end hosts that do not transmit virus to other animals. The possible reason for the lack of virus spread

between MCF-susceptible animals is likely to be that the virus replicates in a cell-associated manner in these species and cell-free virus is not produced. When the virus infection occurs in cattle, OvHV-2-associated lesions are more apparent in mesenteric lymph nodes, with the presence the lymphoid cell infiltrations. In an *in vitro* study, a cell line of bovine large granular lymphocyte (LGL) infected with OvHV-2 expressed most of the unique genes. However, viral genomes were mainly circular, suggestive for latency; yet, no evidence of transcription of the latency associated gene (ORF73) could be found in these cells. Thus, it appears that LGL cells may have features that support both latent and productive life cycles, suggesting that the normal programme of viral gene expression is defective in these hosts (Russell et al., 2009). MCF caused by OvHV-2 has occasionally been reported in Brazil, indicating that this virus circulates among Brazilian cattle (Garmatz et al., 2004; Headley et al., 2013). In our study, the OvHV-2 specific PCR previously described by Baxter et al. (1993) was used. This technique is recommended by the OIE (2013) for detection of OvHV-2. However, the PCR conditions were slightly modified to optimize the annealing temperature of the primers, and to increase the sensitivity of the test. Thus, we believe that the lack of detection of viral DNA in our TG samples may indicate a restricted distribution of this virus in Brazil – at least when compared with BoHV-1 and BoHV-5 (Campos et al., 2009) – or, alternatively, that TG are not the preferential latency sites of this virus.

3.2. Sequences deposited in GenBank and phylogenetic analysis

The two partial sequences amplified from BoHV-2 gB gene present in TG were deposited in GenBank (accession numbers JQ958306 and JQ958307). These sequences were 99 - 100 % identical to BoHV-2 sequences available at GenBank and displayed 0.8 % (JQ958306) and 0.6 % (JQ958307) nucleotide differences (data not shown) in relation to the sequences deposited in GenBank. These differences lead to amino acid exchanges in relation to the corresponding sequences (JQ958306: threonine to isoleucine – T174I and glutamine to leucine – Z206L; JQ958307: threonine to isoleucine – T174I) (Supplementary data 3). This amino acid exchange (T174I), is also observed in macacine herpesvirus 1 (accession number U14664), at the same position, indicating that this mutation also occurs in related species. In addition, these results show that there are gB gene sequence variations among BoHV-2 isolates and they may be related to the geographical localization of viruses, however this should be further investigated when more sequences become available in GenBank (currently, only four sequences of BoHV-2 gB are available in GenBank). Phylogenetic

reconstructions based on the alignment of the gB gene nucleotide sequences of BoHV-2 are shown on Figure 1a. The Brazilian isolates form a subcluster within the BoHV-2 group. BoHV-2 sequence analysis obtained here suggests that they are genetically related to the samples described by Hammerschmidt et al. (1988), who observed that the BoHV-2 gB gene is highly conserved compared to the gB gene of human herpesvirus 1.

From the nine BoHV-4 gB gene PCR products, four were sequenced and deposited in GenBank (accession numbers KC540702, KC540703, KC895399 and KC895400). From the sequenced ones, only one displayed 0.3 % (KC895400) nucleotide difference (data not shown) in comparison with others published BoHV-4 gB genes in GenBank. This nucleotide difference results in the exchange of alanine to valine at amino acid position 86 (KC895400) (Supplementary data 3). To date, this amino acid exchange was not observed in any other sequences available in GenBank. Phylogenetic reconstructions based on the alignment nucleotide sequences of the BoHV-4 gB gene are shown on Figure 1b. Of note, the Brazilian isolates of BoHV-4 grouped with other GenBank BoHV-4 sequences from Belgium, Hungary, Ireland, Italy and Turkey, emphasizing that this region of gB is indeed conserved among the BoHV-4 strains.

In conclusion, the detection of BoHV-2 and BoHV-4 DNA in TG tissues from cattle shown in this report confirms that bovines can be co-infected with more than one herpesvirus. To our knowledge, this is the first time that a co-infection with three different bovine herpesviruses is described in cattle. On the other hand, co-infections with several herpesviruses have been commonly described in humans, and apparently can also be detected in cattle. Likewise, this report shows for the first time the genetic relatedness, based on gB gene sequences, between BoHV-2 and BoHV-4 from Brazil and other countries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:….

References

Ali, H., Keefe, G.P., Cepica, A., 2011. Bovine herpesvirus-4, a potential cause of mastitis in Canadian dairy cows. Br. J. Dairy Sci. 2, 31-34.

Bartha, A., Juhasz, M., Liebermann, H., 1966. Isolation of a bovine herpesvirus from calves with respiratory disease and keratoconjuntivitis. Acta. Vet. Acad. Sci. Hung. 16, 357-358.

Baxter, S.I.F., Pow, I., Bridgen, A., Reid, H.W., 1993. PCR detection of the sheep-associated agent of malignant catarrhal fever. Arch. Virol. 132, 145-159.

Campos, F.S., Franco, A.C., Hübner, S.O., Oliveira, M.T., Silva, A.D., Esteves, P.A., Roehe, P.M., Rijsewijk, F.A., 2009. High prevalence of coinfections with bovine herpesvirus 1 and 5 found in cattle in southern Brazil. Vet. Microbiol. 139, 67-73.

Costa, G.A., Vasconcelos, A.C., Bomfim, M.R.A., Amorim, H.B., Lima, G.B.L., Coelho, F.M., Resende, M., 2011. Neurological disorder in cattle associated with bovine herpesvirus-4. Arq. Bras. Med. Vet. Zootec. 63, 828-835.

Dardiri, A.H., Stone, S.S., 1972. Serologic evidence of dermophatic bovine herpesvirus infection of cattle in the United States of America. Proc. Annu. Meet. U. S. Anim. Health. Assoc. 76, 156-171.

Davison, J.A., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order *Herpesvirales*. Arch. Virol. 154, 171-177.

Donofrio, G., Herath, S., Sartori, C., Cavirani, S., Flammini, C.F., Sheldon, I.M., 2007. Bovine herpesvirus 4 is tropic for bovine endometrial cells and modulates endocrine function. Reproduction. 134, 183-197.

Dubuisson, J., Thiry, E., Bublot, M., Thomas, I., Van Bressem, M.F., Coignoul, F., Pastoret, P.P., 1989. Experimental infection of bulls with a genital isolate of bovine herpesvirus-4 and reactivation of latent virus with dexamethasone. Vet. Microbiol. 21, 97-114.

Egyed, L., Ballagi-Pordány, A., Bartha, A., Belák, S., 1996. Studies of in vivo distribution of bovine herpesvirus type 4 in the natural host. J. Clin. Microbiol. 34, 1091-1095.

Egyed, L. and Bartha, A., 1998. PCR studies on the potential sites for latency of BHV-4 in calves. Vet. Res. Commun. 22, 209-216.

Flores, E.F., Donis, R., 1995. Isolation of a mutant MDBK cell line resistant to bovine virus diarrhoea virus (BVDV) due to a block in viral entry. Virology 208, 565–575.

292 Frazier, K., Baldwin, C.A., Pence, M., West, J., Bernard, J., Ligget, A., Miller, D., Hines, M.E., 2002.
293 Seroprevalence and comparison of isolates of endometriotropic bovine herpesvirus 4. J. Vet. Diagn. Invest.
294 14, 457-462.
295 Garmatz, S.L., Irigoyen, L.F., Rech, R.R., Brown, C.C., Zhang, J., Barros, C.S.L., 2004. Malignant catarrhal
296 fever in cattle in Rio Grande do Sul, Brazil: experimental transmission to cattle and characterization of the
297 etiological agent. Pesq. Vet. Bras. 24, 93-103.
298 Hammerschmidt, W., Conraths, F., Mankertz, J., Pauli, G., Ludwig, H., Buhk, H.J., 1988. Conservation of a
299 gene cluster including glycoprotein B in bovine herpesvirus type 2 (BHV-2) and herpes simplex virus type 1
300 (HSV-1). Virology 165, 388-405.
301 Headley, S.A., Lisboa, J.A.N., Fritzen, J.T.T., Queiroz, G.R., Alfieri, A.F., Oliveira, R.A.M., Bracarense,
302 A.P.F.R.L., Flaiban, K.K.M.C., Alfieri, A.A., 2013. Ovine herpesvirus type 2-induced malignant catarrhal
303 fever in a heifer. Sem. Ciên. Agr. 34, 3895-3900.
304 Imai, K., Ishihara, R., Nishimori, T., 2005. First demonstration of bovine herpesvirus 2 infection among cattle
305 by neutralization test in Japan. J. Vet. Med. Sci. 67, 317-320.
306 Kálmán, D. and Egyed, L., 2005. PCR detection of bovine herpesviruses from nonbovine ruminants in
307 Hungary. J. Wild. Dis. 41, 482-488.
308 Letchworth, G.J. and Carmichael, L.E., 1982. Bovid herpesvirus 2 Latency: failure to recover virus from
309 central sensory nerve. Can. J. Comp. Med. 46, 76-79.
310 Li, H., Cunha, C.W., O'Toole, D., Nicola, A.V., Knowles, D.P., Taus, N.S., 2013. Development of an in vivo
311 system to measure antibody-blocking of ovine herpesvirus 2 entry. J. Virol. Meth. 188, 104-107.
312 Li, H., Cunha, C.W., Taus, N.S., 2011. Malignant catarrhal fever: understanding molecular diagnostics in
313 context of epidemiology. Int. J. Mol. Sci. 12, 6881-6893.
314 Li, H., Taus, N.S., Lewis, G.S., Kim, O., Traul, D.L., Crawford, T.B., 2004. Shedding of ovine herpesvirus 2
315 in sheep nasal secretions: the predominant mode for transmission. J. Clin. Microbiol. 42, 5558-5564.
316 Martin, W.B., Gwynne, M., 1968. Antibodies to the group II lumpy skin disease virus in the sera of cattle in
317 Kenya. Bull Epizoot. Dis. Afr. 16, 217-222.
318 OIE, World Organization for Animal Health, 2013. Manual of diagnostic tests and vaccines for terrestrial
319 animals. Chapter 2.4.15: Malignant catarrhal fever. OIE terrestrial manual, 1-12.

320 Osorio, F.A. and Reed, D.E., 1983. Experimental inoculation of cattle with bovine herpesvirus-4: evidence for
321 a lymphoid-associated persistent infection. Am. J. Vet. Res. 44, 975-980.
322 Russell, G.C., Stewart, J.P., Haig, D.M., 2009. Malignant catarrhal fever: A review. Vet. J. 179, 324-335.
323 Thiry, E., Dubuisson, J., Bublot, M., Van Bressem, M.F., Pastoret, P.P., 1990. The biology of bovine
324 herpesvirus-4 infection of cattle. Dtsch. Tierarztl. Wochenschr. 97, 72-77.
325 Torres, F.D., Almeida, S.R., Silva, M.S., Weiblen, R., Flores, E.F., 2009a. Distribution of latent bovine
326 herpesvirus 2 DNA in tissues of experimentally infected sheep. Res. Vet. Sci. 87, 161-166.
327 Torres, F.D., Bernardes, L.M., Weiblen, R., Flores, E.F., 2009b. Prevalência de anticorpos contra o vírus da
328 mamilite herpética em bovinos do Rio Grande do Sul, Brasil. Cienc. Rural 39, 1901-1904.
329 Wang, K., Lau, T. Y., Morales, M., Mont, E.K., Straus, S.E., 2005. Laser-capture microdissection: refining
330 estimates of the quantity and distribution of latent herpes simplex virus 1 and varicella-zoster virus DNA in
331 human trigeminal ganglia at the single-cell level. J. Virol. 79, 14079-14087.
332 Wellenberg, G.J., Verstraten, E.R.A.M., Belak, S., Verschuren, S.B.E., Rijsewijk, F.A.M., Peshev, R., Van
333 Oirschot, J.T., 2001. Detection of bovine herpesvirus 4 glycoprotein B and thymidine kinase DNA by PCR
334 assays in bovine milk. J. Virol. Methods 97, 101-112.

335 **Table 1.** Descriptions of the primers*, sizes of products and target genes used in the PCR, nPCR and snPCR.

Virus	Assay	Primer (annealing)	Amplicon size	Target gene	GenBank Accession number	Reference
BoHV-2	PCR[a]	Forward[d] 5'-TATGGAGAAGGAAGAGCCCG-3' Reverse1[d] 5'-TTTTTAGCCGCGTGGTGTGC-3' (62 ºC for 50 sec)	608 bp	gB[e]	M21628 region: 6363..6970	Torres et al., 2009a
	snPCR[b]	Forward[d] 5'-TATGGAGAAGGAAGAGCCCG-3' Reverse2 5'-CGGTGGTCTCAAGGTTGTTC-3' (65 ºC for 50 sec)	512 bp		M21628 region: 6363..6874	Torres et al., 2009a
BoHV-4	PCR[a]	gB1 5'-CCCTTCTTTACCACCACCTACA-3' gB2 5'-TGCCATAGCAGAGAAACAATGA-3' (59 ºC for 50 sec)	615 bp	gB[f]	AF318573 region: 11381..11995[h]	Wellenberg et al., 2001
	nPCR[c]	PFnested 5'-CAACAACATCAACAAGCAAGC-3' PRnested 5'-GACCACCCTCTGTAAACTG-3' (58 ºC for 30 sec)	364 bp		AF318573 region: 11532..11895	This study
OvHV-2	PCR[a]	P556modF[d] 5'-GGTATATGAATCCAGATGGC-3' P755modR[d] 5'-AAGCACCAGTTATGCATCTG-3' (51 ºC for 50 sec)	411 bp	FGAM-synthase[g]	DQ198083 region: 121120..121530	Baxter et al., 1993
	snPCR[b]	P556modF[d] 5'-GGTATATGAATCCAGATGGC-3' P555modR2[d] 5'-GTTTCTGGGGTAGTGGCG-3' (58 ºC for 50 sec)	197 bp		DQ198083 region: 121120..121316	Baxter et al., 1993

336 a Polymerase chain reaction (PCR) – first round reaction.

337 b Semi-nested polymerase chain reaction (snPCR).

338 c Nested polymerase chain reaction (nPCR).

339 d Primers designed with highlighted modifications.

340 e Glycoprotein B (gB), UL 27.

341 f ORF 8 (glycoprotein B).

342 g Phosphoribosylformylglycinamidine (FGAM) synthase-like protein, ORF 75.

343 h Primers amplify a region comprising a small portion (50 nt) of ORF 7 (transport protein) and a larger portion (575 nt) of ORF 8.

344 *Primers specificity was checked by blast and by the alignment of BoHV-1, BoHV-2, BoHV-4, BoHV-5 and OvHV-2 gB gene (data not

345 shown).

346 **Figure caption**

347 **Figure 1**. Phylogenetic trees constructed with BoHV-2 and BoHV-4 sequences. The tree is drawn to scale,
348 with branch lengths measured in the number of substitutions per site. The percentage of trees in which the
349 associated taxa clustered together is shown next to the branches. Fig 1a shows the phylogenetic relatedness
350 between Brazilian BoHV-2 samples, previously reported BoHV-2 sequences and other alphaherpesviruses.
351 The analysis comprised 9 nucleotide sequences. Fig 1b shows the phylogenetic relatedness between Brazilian
352 BoHV-4 samples (KC540702, KC540703, KC895399 and KC895400, filled black squares) and previously
353 reported BoHV-4 sequences from Belgium (JN133502), Hungary (AF318573 and Z15044), Ireland
354 (AJ609274), Italy (AJ617687 and AJ617688) and Turkey (EU055543, GQ246863, GQ246865, GQ246866,
355 GQ246867, GQ375280, JX644988 and JX644989). The analysis was composed of 19 BoHV-4 nucleotide
356 sequences and Macaca mulatta rhadinovirus (from US, GU233160) was used as an outgroup.

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