

## Bovine Herpesviruses Do Not Play a Major Role in the Differential Diagnosis of Rabies in Cattle in Southern Brazil

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### ABSTRACT

**Background:** Rabies has long been recognized as the major cause of encephalitis in cattle in Latin American countries. It has been estimated that nearly 50.000 cattle heads per year are lost due to encephalitis in that subcontinent, with a significant economic impact on cattle productive chains. In Brazil only, 2.500 to 3.000 cattle heads are estimated to be lost every year due to rabies. However, it is believed that rabies incidence in cattle is much larger, since usually only a few samples from affected animals in disease outbreaks are submitted to diagnostic laboratories. Rabies encephalitis is promptly and accurately diagnosed; however, particularly when rabies is excluded as *causa mortis*, the agent responsible for neurological disease of infectious origin often remains undetermined. Two bovine herpesviruses (BoHVs), bovine herpesvirus type 1 (BoHV-1) and bovine herpesvirus type 5 (BoHV-5) are major pathogens of cattle which are widely disseminated in Brazil. As usual in herpesvirus' biology, these tend to infect a large number of hosts and establish lifelong latent infections which may occasionally be reactivated. Both viruses, particularly BoHV-5, are often recovered from cases of neurological disease in cattle. The participation of BoHVs in the differential diagnosis of rabies must be evaluated. Besides, there might be associations between the occurrence of rabies and BoHV infections that deserve investigation. The aim of this study was to investigate whether bovine herpesvirus 1 and 5 would play a significant role in cases of neurological disease where rabies was the presumptive clinical diagnosis. In addition, associations between the occurrence of rabies and BoHV infections were searched for. The approach adopted for conducting such investigations was based on the search for viral nucleic acids as well as classical virus isolation on tissues of cattle submitted to rabies diagnosis over a two-year period, including rabies-positive and rabies-negative specimens.

**Materials, Methods & Results:** Brain tissue samples of 101 cattle originally submitted to rabies diagnosis were collected over a two year period (2009-2010) from various municipalities within the state of Rio Grande do Sul, Brazil. Thirty nine of these samples had the diagnosis of rabies confirmed by standard laboratory diagnostic methods. Aliquots of tissues were submitted to DNA extraction and examined in search for genomes of bovine herpesviruses (BoHV) types 1 (BoHV-1) and 5 (BoHV-5) by as well as for infectious virus. Bovine herpesvirus genomes were detected in 78/101 (77.2%) samples, in which BoHV-1 genomes were detected in 26/78 (25.7%), BoHV-5 genomes in 22/78 (21.8%) and mixed BoHV infections (BoHV-1 and BoHV-5 genomes) were detected in 30/101 (29.7%) samples. In the 39 samples with confirmed rabies diagnosis, BoHV-1 DNA was detected in 9/39 (23%), BoHV-5 DNA in 6/39 (15.4%) and mixed infections with both BoHV types in 16/39 (41%) samples. However, no infectious herpesvirus was recovered from any of the specimens examined.

**Discussion:** The high prevalence of BoHV1 and BoHV-5 infections was evidenced in the sampled population, but the absence of infectious BoHVs indicate that these were not associated to the occurrence of the cases of encephalitis where rabies was the primary suspicion. In addition, no association was detected between occurrence of rabies and detection of BoHVs, since the frequency of detection of herpesvirus genomes did not significantly differ between rabies-positive and rabies-negative samples. The detection of BoHV DNA in scattered areas of the brain with no infectious virus suggests that latency may take place in different regions of the brain.

**Keywords:** encephalitis, bovine herpesvirus, BoHV-1, BoHV-5, rabies, nested PCR.

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## INTRODUCTION

Rabies is the major cause of infectious encephalitis in cattle in South America. In Brazil, 2.500 to 3.000 cattle deaths caused by rabies are reported every year. It has been estimated that nearly 50.000 cattle heads per year are lost due to encephalitides in South American countries with a significant economic impact on the cattle productive chains [4].

However, although rabies diagnosis is performed with adequate accuracy, particularly when rabies is excluded as *causa mortis*, the actual cause of neurological disease of infectious origin often remains undetermined. Two bovine herpesviruses (BoHVs), bovine herpesvirus type 1 (BoHV-1) and bovine herpesvirus type 5 (BoHV-5) are major pathogens of cattle which are widely disseminated in Brazil [2,4]. As usual in herpesvirus' biology, these tend to infect a large number of hosts and establish lifelong latent infections which may occasionally be reactivated [17]. Both viruses, particularly BoHV-5, are often recovered from cases of neurological disease in cattle [2,4,19,20]. In view of these, the participation of BoHVs in the differential diagnosis of rabies must be evaluated. Besides, there might be associations between the occurrence of rabies and BoHV infections that deserve investigation.

In order to address such questions, the present study was set up to examine the participation of bovine herpesviruses in the differential diagnosis of rabies encephalitis, as well as in cases with a confirmed rabies diagnosis, in search for possible correlations between such infections.

## MATERIALS AND METHODS

### *Brain tissue samples*

Cattle brain tissues (fragments of cortex, spinal cord, cerebellum, thalamus, midbrain, pons, medulla oblongata, cervical medulla) submitted to the official laboratory for rabies diagnosis in the State of Rio Grande do Sul, Brazil. These comprised the totality of cattle brain samples submitted to rabies diagnosis in the period 2009-2010. Rabies laboratory diagnosis was performed by usual methods [27]. Samples were then stored in sealed containers and frozen at -70°C until processed. During that period, 101 cattle brain tissue samples were received from different regions within the State. Out of the 101 specimens, 39 had the diagnosis of rabies confirmed.

### *DNA extraction*

About 100 µg of tissue were collected from different brain fragments, pooled and mixed with 1 mL of lysis buffer (20 mM Tris-HCl, pH 7.4; 10 mM EDTA; 200 mM NaCl<sub>2</sub>; 100 µg proteinase K; 10 % SDS; pH 8.0) and incubated for 1 h at 56°C. This mixture was then centrifuged at 12.000 x g for 10 min and 400 µL of the supernatant were collected. After a standard phenol extraction [21] the purified DNA was precipitated in ethanol, washed and resuspended in 100 µL TE (10 mM Tris, pH 7.4; 1 mM EDTA, pH 8.0). The quantity and quality of the DNA was estimated by agarose gel electrophoresis by comparison with known amounts of phage lambda DNA.

### *Nested PCR*

For BoHV detection and type differentiation, a type-specific nested PCR (nPCR) was performed as described [6]. Briefly, for the first round of amplification, primers were prepared with basis on the sequence of the glycoprotein C (gC) gene of both BoHV-1 and BoHV-5 (nucleotides 873-1447 and 813-1384 from Genbank sequence accession numbers Z49223 and Z49224, respectively). The primers<sup>1</sup> (PF2: 5' CGGC-CACGACGCTGACGA 3' and PR1: 5' CGCCGCC-GAGTACTACCC 3') are expected to anneal to a gC region shared by both BoHV-1 and BoHV-5, giving rise to fragments whose expected sizes are 575 bp (BoHV-1) and 572 bp (BoHV-5), respectively [12]. The PCR was set up in a total volume of 25 µL, with 1 mM MgCl<sub>2</sub>, 0.3 µM of each primer, 10% DMSO, 1 U Taq DNA polymerase<sup>2</sup>, 10% PCR buffer and 0.6 mM DNTPs per reaction. Twenty five molecules of an internal control template (control product length: 440 bp; [5]) were added to each reaction. Amplifications were performed in a thermal reactor<sup>3</sup> under the following cycling conditions: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C, followed by 5 min at 72°C.

The second round of amplification was carried to allow type-specific differentiation using the products of the first PCR as templates. To detect BoHV-1 DNA, primers PF: 5'-CTAACATGGAGCGCCGCTT-3' and PR: 5'-CGGGGCGATGCCGTC-3' were used. These are expected to give rise to a product of 161 bp. To detect BoHV-5 DNA, primers PF: 5'-GTGGAGC-GCCGCTTCGC-3' and PR: 5'-TATCGCGGAGAG-CAGGCG-3' were employed to amplify an expected product of 236 bp [6]. Each PCR was carried out in a

total volume of 25 µL containing 0.2 µM of each the primer pairs above plus the same components used for the first PCR. The cycling conditions were: 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C and a final extension step of 5 min at 72°C. To avoid contamination, separate rooms were designated to prepare the PCR buffers, to perform DNA extractions from tissues and to carry out analyses of PCR products. Filter tips were used throughout. Work benches were decontaminated with ethanol 70% and UV. Negative controls (water) were included in every three PCR reactions [6].

*Cells*

Madin-Darby bovine kidney (MDBK) cells<sup>4</sup> (CCL-22), were used for BoHV-1 and BoHV-5 isolation. Cells were kept in Eagle’s Minimal Essential Medium (EMEM)<sup>5</sup> supplemented with 10% goat serum (previously tested to ensure the absence of BoHV or BoHV-inhibiting antibodies) and antibiotics at usual concentrations [100 µL/L enrofloxacin<sup>6</sup>; 400 µL/L amphotericin B<sup>7</sup>].

*BoHV isolation*

Each brain tissue sample was processed individually in laminar flow cabinets to prevent cross contamination. Fragments were collected from different regions of the brain, weighed, pooled and approximately one gram of the tissue pool was macerated with sterile sand in 10 mL of EMEM and centrifuged at 2.500 x g for 10 min. After centrifugation, 1 mL of supernatant was collected and added to overnight grown MDBK cell monolayers cultured in 25 cm<sup>2</sup> flasks. After adsorption for one hour at 37°C, the inoculi were removed, the monolayers washed three times with PBS (phosphate buffered saline, pH 7.2) and 10 mL of EMEM (supplemented as above) added. Inoculated cells and mock infected controls were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and

examined daily in search of cytopathic effect (CPE). After 7 days, if no CPE was evident, the monolayers were frozen at -80°C, thawed, clarified by low speed centrifugation and the supernatants passaged again. Samples which did not give rise to CPE following three subsequent passages in cell cultures were considered negative for the presence of infectious herpesvirus.

*Statistical analysis*

The results were analyzed using the Pearson Chi-Square in the program SPSS 17.0. The comparisons between rabies positive, BoHV-infected, and rabies negative, BoHV-non-infected samples were considered significant when *P* < 0.05%.

**RESULTS**

The results of the search for bovine herpesvirus genomes are summarized on Table 1. Among the 101 samples examined, BoHV-1 and/or BoHV-5 genomes were detected in 78 (77.2%), of which 25.7% (26/101) contained BoHV-1 DNA, 21.8% (22/101) contained BoHV-5 DNA and 17.8% (18/101) contained both BoHV-1 and BoHV-5 DNA (Table 1). In samples where rabies diagnosis was confirmed, samples with BoHV genomes were identified in 79.5% of the samples, whereas in rabies-negative samples were identified in 75.8% of the samples. These differences were not statistically significant when comparing rabies-positive and rabies-negative cases; therefore, no correlation - either positive or negative - could be identified between the occurrence of rabies and detection of BoHVs (*P* < 0.05).

Virus isolation attempts revealed that no infectious virus could be recovered from the tissues examined.

**Table 1.** Nested polymerase chain reaction (nPCR) for bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) DNA on brain tissue samples of cattle submitted to rabies diagnosis (n = 101).

Detection of BoHV-1 and/or BoHV-5 DNA					
BoHV-1	BoHV-5	BoHV-1 plus BoHV-5	Total BoHV positive*	Negative	Total of samples examined
26 (25.7%)	22 (21.8%)	30 (29.7%)	78 (77.2%)	23 (22.8%)	101 (100%)

\*Total n° of samples containing BoHV-1 and/or BoHV-5 DNA.

When examining BoHV genome recovery in relation to the positive or negative rabies diagnosis, on the 39 samples where rabies diagnosis was confirmed, BoHV-1 DNA was present in 23%, BoHV-5 DNA in 15.4% and mixed BoHV-1 and BoHV-5 infections were detected in 41% samples. However, on the 62 samples with a negative rabies diagnosis BoHV-1 genomes were found in 27.4% of the samples, BoHV-5

genomes in 25.8% and mixed BoHV-1/BoHV-5 infections in (22.6%) of the samples (Table 2). Statistical analysis demonstrated that the frequencies of detection of BoHV genomes were not significant between rabies-positive and rabies-negative samples ( $P < 0.05$ ). Therefore, the presence of BoHV genomes showed no relationship with the incidence of rabies on the examined population.

**Table 2.** Nested polymerase chain reaction (nPCR) analysis for BoHV-1 and BoHV-5 DNA in brain tissue samples of cattle with a confirmed rabies diagnosis positives samples (n = 39) and rabies negative samples (n = 62).

Detection of BoHV-1 and/or BoHV-5 DNA						
Rabies diagnosis	BoHV-1	BoHV-5	BoHV-1 plus BoHV-5	Total BoHV positive**	Negative	Total of samples examined
Positive*	9 (23%)	6 (15.5%)	16 (41%)	31 (79.5%)	8 (20.5%)	39 (100%)
Negative*	17 (27.4%)	16 (25.8%)	14 (22.6%)	47 (75.8%)	15 (24.2%)	62 (100%)

\*Refers to results at rabies diagnosis. \*\*Total n° of samples containing BoHV-1 and/or BoHV-5 DNA.

## DISCUSSION

Accurate determination of the causative agent of encephalitis of infectious origin is essential to define adequate preventive and control measures. The similarity in clinical signs makes presumptive diagnosis insufficient to define the microorganism involved. As rabies and BoHV infections are two major causes of infectious encephalitis in Brazil, it became of interest to examine whether BoHV-1 and BoHV-5 might be involved in cases where rabies was the main suspicion. Since herpesviruses can establish latent, lifelong infections [17], it would be important to determine whether these, if present, would be in a stage of productive infection, with the generation of infectious virus. As the biology of herpesviruses involves the establishment of latent infections with only occasional production of infectious virus, it would not be unexpected to find BoHV infections in cattle, as revealed by the presence of viral genomes, though with no involvement with disease.

The results reported here indicate that BoHVs do not play a major role in the differential diagnosis of rabies in cattle. In a previous study, we searched for infectious BoHV-1 in 70 bovine brain samples submitted to rabies diagnosis [5]. However, on that occasion, no searches for herpesvirus genomes were

performed. Moreover, on that study, infectious BoHV-1 was recovered from only two samples, while no BoHV-5 was identified. Those observations are now complemented by the present work, showing that, despite the absence of virus in infectious form, BoHV infections are widespread in the population examined. Nevertheless, these do not play a significant role in the differential diagnosis of rabies. Other authors detected infectious BoHV in outbreaks of meningoencephalitis in about 38% of 47 samples examined [10]. However, those did not correlate rabies with BoHV infections and examined only rabies-negative samples.

As a complement to this study, we searched for associations between the presence of BoHVs and the occurrence of rabies, since herpesviruses might have some effect on rabies incidence. No such association could be found, since there was no statistically significant difference between the frequencies of detection of BoHV DNA in rabies-positive or rabies-negative animals. Therefore, BoHV infections were shown not to have any effect on the occurrence of rabies in the sampled population.

There may also be important differences in the geographic region from which samples were collected, as the incidence of rabies, as well as the incidence of herpesvirus infections, may vary in geographically distinct regions. As such, it is likely that the degree of



endemicity of herpesvirus infections may also vary. Thus, the present report refers to samples from the region examined and not necessarily reflect similar situations in other geographic areas, although it seems very likely that BoHV infections are widespread in the cattle population in most cattle farming areas of Brazil. Moreover, it seems quite likely that the absence of association between rabies and BoHV infections is universal.

Differences in sensitivity of the methodology employed may also have played some part in the outcome of these studies. The PCRs for BoHV detection in different studies show different specificities; the nested PCR employed here was able to detect 2.5 to 25 copies of viral DNA [6]. Other authors have calculated PCR sensitivity by different methods, what renders comparisons difficult [1,9,10]. Nevertheless, such differences do not alter significantly the main findings reported here in that BoHVs are widely disseminated in the cattle population, though not participating significantly in the differential diagnosis of rabies.

Detection of herpesvirus DNA in the absence of infectious virus indicates that such viruses were present in a latent state. Although we have not performed tests to confirm latency, such as the detection of mRNA of latency related transcripts [23], BoHV DNA was detected in different regions of the brains examined without detection of infectious virus. Accumulated evidence suggests that alphaherpesvirus latency can occur in a number of extra-ganglionic sites and not only in the trigeminal ganglia, as usually investigated. Others authors have already pointed out the presence of latent BoHV-1 DNA in neural as well as in lymphoid

tissues (trigeminal ganglia, CNS, tonsils, regional lymph nodes), in peripheral blood mononuclear cells and in several other ganglia, lymphnodes and semen [13,18,22,24,25]. In swine, latent pseudorabies virus (PRV) DNA was detected in tonsils and in some brain regions [8,25]. In humans, HSV-1 DNA has also been detected in different regions of the central nervous system of a significant percentage of humans, as well as in the vagus nerve and bone marrow [3,7,11,14-16]. Thus, the finding of viral genomes in the absence of infectious virus cannot be considered unusual.

In conclusion, the present study revealed that bovine herpesvirus infections do not play a significant role in the differential diagnosis of rabies in the sampled population. Moreover, the frequency of detection of BoHV DNA bears no correlation with the occurrence of rabies.

#### SOURCES AND MANUFACTURERS

<sup>1</sup>IDT - Integrated DNA Technologies Inc., Coralville, Iowa, IA, USA.

<sup>2</sup>Invitrogen Life Technologies, São Paulo, SP, Brazil.

<sup>3</sup>Eppendorf Mastercycler, Hauppauge, NY, USA.

<sup>4</sup>American Type Culture Collection (ATCC), Manassas, VA, USA.

<sup>5</sup>Gibco® Life Technologies, New York, NY, USA.

<sup>6</sup>Bayer®, São Paulo, SP, Brazil.

<sup>7</sup>Cristália Produtos químicos farmacêuticos Ltda., São Paulo, SP, Brazil.

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