

# Molecular identification of bovine gammaherpesvirus 6 in water buffaloes (*Bubalus bubalis*) with histological evidence of pulmonary and intestinal lesions

## Identificação molecular do gammaherpesvirus bovino 6 em búfalos (*Bubalus bubalis*) com evidência histológica de alterações pulmonares e intestinais

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### Highlights

First association of BoGHV6 with pulmonary and intestinal lesions in buffaloes.  
BoGHV6 may be a possible etiologic agent of respiratory disease in buffaloes.  
Infections by BoGHV6 in ruminants may be widespread in Brazil.

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## Abstract

Bovine gammaherpesvirus 6 (BoGHV6), formerly known as bovine lymphotropic virus, belongs to the genus *Macavirus*, which includes the porcine lymphotropic herpesviruses (PLHV) and members collectively referred to as malignant catarrhal fever virus (MCFV) group. This study investigated the possible participation of BoGHV6 in the development of intestinal and pulmonary diseases of buffaloes. Intestinal and pulmonary fragments were obtained from 19 buffaloes from Goiás, Central-western Brazil. Histopathology revealed interstitial pneumonia in all pulmonary fragments evaluated, while two of these had suppurative bronchopneumonia. Furthermore, all intestinal fragments demonstrated atrophic enteritis. BoGHV6 DNA was amplified from 31.6% (6/19) of the buffaloes with interstitial pneumonia and in 26.3% (5/19) with atrophic enteritis. The phylogenetic evaluation revealed that the strain of BoGHV6 amplified from these buffaloes had 100% nucleotide (nt) sequence identity with the reference strains of BoGHV6, but only 69.5 to 73.7% and 65.8 – 69.5% nt sequence identity with members of the MCFV and the PLHV, respectively. Additionally, molecular assays to detect important pathogens of ruminants did not amplify the respective nucleic acids in the tissues evaluated. These results demonstrated that BoGHV6 was amplified from tissues of buffaloes with histopathologic diagnoses of interstitial pneumonia and atrophic enteritis, suggesting the possible participation of this virus in the development of the intestinal and pulmonary lesions herein identified.

**Key words:** BoGHV6. Bovine lymphotropic virus. *Macavirus*. Enteritis. Histological patterns of disease. Interstitial pneumonia.

## Resumo

Gamaherpesvírus bovino 6 (BoGHV6), anteriormente conhecido como vírus linfotrópico bovino, pertence ao gênero *Macavirus*, que inclui o herpesvírus linfotrópico dos suínos (PLHV) e membros coletivamente referidos como vírus da febre catarral maligna (VMVF). Este estudo investigou a possível participação do BoGHV6 no desenvolvimento de doenças intestinais e pulmonares em búfalos. Fragmentos intestinais e pulmonares foram obtidos de 19 búfalos de Goiás, Centro-Oeste do Brasil. A histopatologia revelou pneumonia intersticial em todos os fragmentos pulmonares avaliados, sendo que dois deles apresentavam broncopneumonia supurativa. Além disso, todos os fragmentos intestinais demonstraram enterite atrófica. O DNA do BoGHV6 foi amplificado em 31,6% (6/19) dos búfalos com pneumonia intersticial e em 26,3% (5/19) com enterite atrófica. A avaliação filogenética revelou que a cepa de BoGHV6 amplificada desses búfalos tinha 100% de identidade de sequência de nucleotídeos (nt) com as cepas de referência de BoGHV6, mas apenas 69,5 a 73,7% e 65,8 – 69,5% de identidade de sequência nt com membros do VMCF e o PVHS, respectivamente. Adicionalmente, ensaios moleculares para detectar importantes patógenos de ruminantes não amplificaram os respectivos ácidos nucléicos nos tecidos avaliados. Esses resultados demonstraram que BoGHV6 foi amplificado a partir de tecidos de búfalos com diagnóstico histopatológico de pneumonia intersticial e enterite atrófica, sugerindo a possível participação desse vírus no desenvolvimento das lesões intestinais e pulmonares aqui identificadas.

**Palavras-chave:** Vírus linfotrópico bovino. *Macavirus*. Enterite. Padrões histológicos de doenças. Pneumonia intersticial.

## Introduction

Bovine Gammaherpesvirus 6 (BoGHV6), formerly known as bovine lymphotropic virus, belongs to the genus *Macavirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae* (ICTV, 2022). The genus *Macavirus* contains viruses that can infect a wide range of domestic and wild ruminants and swine (Russell et al., 2009). Additional members of this genus include ovine gammaherpesvirus 2 (OvGHV2), alcelaphine gammaherpesvirus 1 and 2 (AIGHV1 and 2), caprine gammaherpesvirus 2 (CpGHV2), porcine lymphotropic herpesviruses (PLHVs; SuGHV1-3), and hippotragine gammaherpesvirus 1 (ICTV, 2022).

Several members of the *Macavirus* genus (e.g., OvGHV2, AIGHV1 and 2, and CpGHV2) share the 15A epitope (Li et al., 1994), are known to produce malignant catarrhal fever (MCF) in mammals worldwide, and are referred to as the MCF virus (MCFV) group (Li et al., 2000; Crawford et al., 2002). Additionally, all members of the *Macavirus* genus are phylogenetically related but distinct (Li et al., 2003; Li et al., 2014). However, while the diseases associated with MCFV members are well known and documented, the exact participation of BoGHV6 in disease and/or clinical manifestations in domestic animals is currently unknown (Headley et al., 2022b).

Since the initial identification of BoGHV6 in cattle infected with the bovine leukemia virus in the USA (Van der Maaten & Boothe, 1972; Rovnak et al., 1998), this virus was detected in several countries, including within vaginal secretions of cows with chronic metritis from the UK (Cobb et al., 2006; Banks et al., 2008) and Belgium (Garigliany et al., 2013). Additionally, BoGHV6 was identified in

an aborted bovine fetus from Canada (Gagnon et al., 2010), and in several fetuses from Brazil that were concomitantly infected by pathogens of bovine reproductive diseases (Headley et al., 2022b). Furthermore, BoGHV6 was associated with lymphoproliferative diseases in buffaloes (Oliveira et al., 2015) and in a cow with pulmonary disease (Headley et al., 2023b) from Brazil. Alternatively, there was no association of BoGHV6 with any disease process in cows from the USA (Collins et al., 2000), in cattle from several European countries (Rosato et al., 2021), as well as from the state of Minas Gerais, Brazil (Oliveira et al., 2015).

Water buffaloes (*Bubalus bubalis*) are considered as being more susceptible to infections by *Macavirus* relative to cattle (Stahel et al., 2013), while the occurrence of MCFV in the African buffalo (*Syncerus caffer*) is an emerging infectious disease (Pfitzer et al., 2015). Furthermore, most of the studies done with *Macavirus* in buffaloes have been based on the diagnosis of MCF due to OvGHV2 (Tham, 1997; Costa et al., 2009; Stahel et al., 2013; Pfitzer et al., 2015), CpGHV2 (Dettwiler et al., 2011; Stahel et al., 2013), and AIGHV1 (Tham, 1997; Pfitzer et al., 2015). Alternatively, there is only one study that investigated the possible role of BoGHV6 in buffaloes (Oliveira et al., 2015). In addition, there are only two studies that have investigated the occurrence of *Macavirus* in buffaloes from Brazil: the first described an outbreak of MCF in Murrah buffaloes (Costa et al., 2009), while the second associated BoGHV6 with lymphoproliferative diseases in water buffaloes (Oliveira et al., 2015). Accordingly, this study provides additional information as to the occurrence of this *Macavirus* in buffaloes.

## Materials and Methods

### *Study location, animals, organ collection, and histopathologic evaluation*

The buffaloes used in this study originated from one farm located in the State of Goiás, Central western, Brazil. These buffaloes were approximately 2 years of age, were raised for beef production, and maintained on grass pastures. Sheep are not reared at this farm but were reared by other farmers within proximity. Animals at this farm are only routinely immunized against foot and mouth disease, clostridiosis, and brucellosis. During the period of collection, there was no report of buffalo morbidity or mortality at this establishment.

Buffaloes from this farm were slaughtered at an abattoir, located within the State of Goiás, during which fragments of the small intestines and lungs were randomly collected. All tissues were collected at the same moment during slaughter, and then routinely processed for histopathological evaluation with the Hematoxylin and eosin stain to determine possible patterns of disease. Additionally, fragments of these organs, collected in duplicate, were maintained at -80 °C until used in molecular assays designed to amplify the nucleic acids of specific disease agents of ruminants based on the histological pattern of disease observed.

### *Molecular investigations of specific agents of ruminants*

Nucleic acid extraction was performed from 500 µl proteinase K pre-treated aliquots of tissue suspensions of the lung and intestine

as described (Boom et al., 1990; Alfieri et al., 2006), after which the extracted nucleic acids were eluted in UltraPure™ DEPC-treated water (Invitrogen™ Life Technologies, Carlsbad, CA, USA) and maintained at -80 °C until used in molecular assays.

Molecular assays were performed to amplify the nucleic acids of specific infectious disease pathogens of ruminants. These included BoGHV6 (Oliveira et al., 2015), OvGHV2 (Baxter et al., 1993), bovine viral diarrhea virus, (BVDV) (Vilcek et al., 1994b), bovine respiratory syncytial virus, (BRSV) (Vilcek et al., 1994a), bovine alphaherpesvirus 1, (BoAHV1) (Claus et al., 2005), bovine coronavirus, (BCoV) (Takiuchi et al., 2006), and bovine parainfluenza virus 3, (BPIV3) (Zhu et al., 2011). All agents listed were used in molecular assays to amplify the respective nucleic acids from the pulmonary fragments; while molecular assays were used to detect BVDV, BCoV, and OvGHV2 from the intestine.

Positive controls consisted of the utilization of nucleic acids of these infectious agents derived from previous studies: OvGHV2, BoGHV6 (Headley et al., 2015; Headley et al., 2022b), BPIV3, BVDV, BoAHV1 (Headley et al., 2014; Headley et al., 2018; Oliveira et al., 2020). The negative control consisted of sterile ultrapure water; positive and negative controls were included in each molecular assay.

### *Sequencing and phylogenetic analysis of BoHV-6 polymerase gene*

The products of the BoGHV6 polymerase gene amplified from the lung and intestine obtained from the specific BoGHV6 nested-PCR assay were purified

with a commercial Gel Extraction and PCR Purification Combo Kit (Invitrogen® Life Technologies, Carlsbad, CA, USA), quantified with a Qubit® Fluorometer (Invitrogen® Life Technologies, Eugene, OR, USA), and submitted to sequencing in both directions with the forward and reverse primers used in the respective molecular assays in an ABI3500 Genetic Analyzer sequencer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA).

Sequence quality analyses and consensus sequences were obtained using PHRED and CAP3 software (<http://asparagin.cenargen.embrapa.br/phph/>), respectively. Similarity searches of the BoHV-6 polymerase gene were performed with nucleotide (nt) sequences deposited in GenBank using the BLAST software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The identity of the nt sequences was confirmed by comparison with reference sequences of BoGHV6 available in GenBank. Sequence alignment and identity matrix were performed by using the BioEdit software (Hall, 1999). The phylogenetic analysis of the BoHV-6 polymerase gene was then obtained using the Maximum Likelihood method with the Kimura 2-parameter model (Kimura,

1980) with the MEGA software, version 7.0.26 (Kumar et al., 2016), during which the fragment derived from this study was compared with reference strains of *Macavirus* including BoGHV6, OvGHV2, AIGHV1, and 2, and the particle fragments of CpGHV2 and PLHV1, 2 and 3.

## Results and Discussion

### *Pathological evaluations*

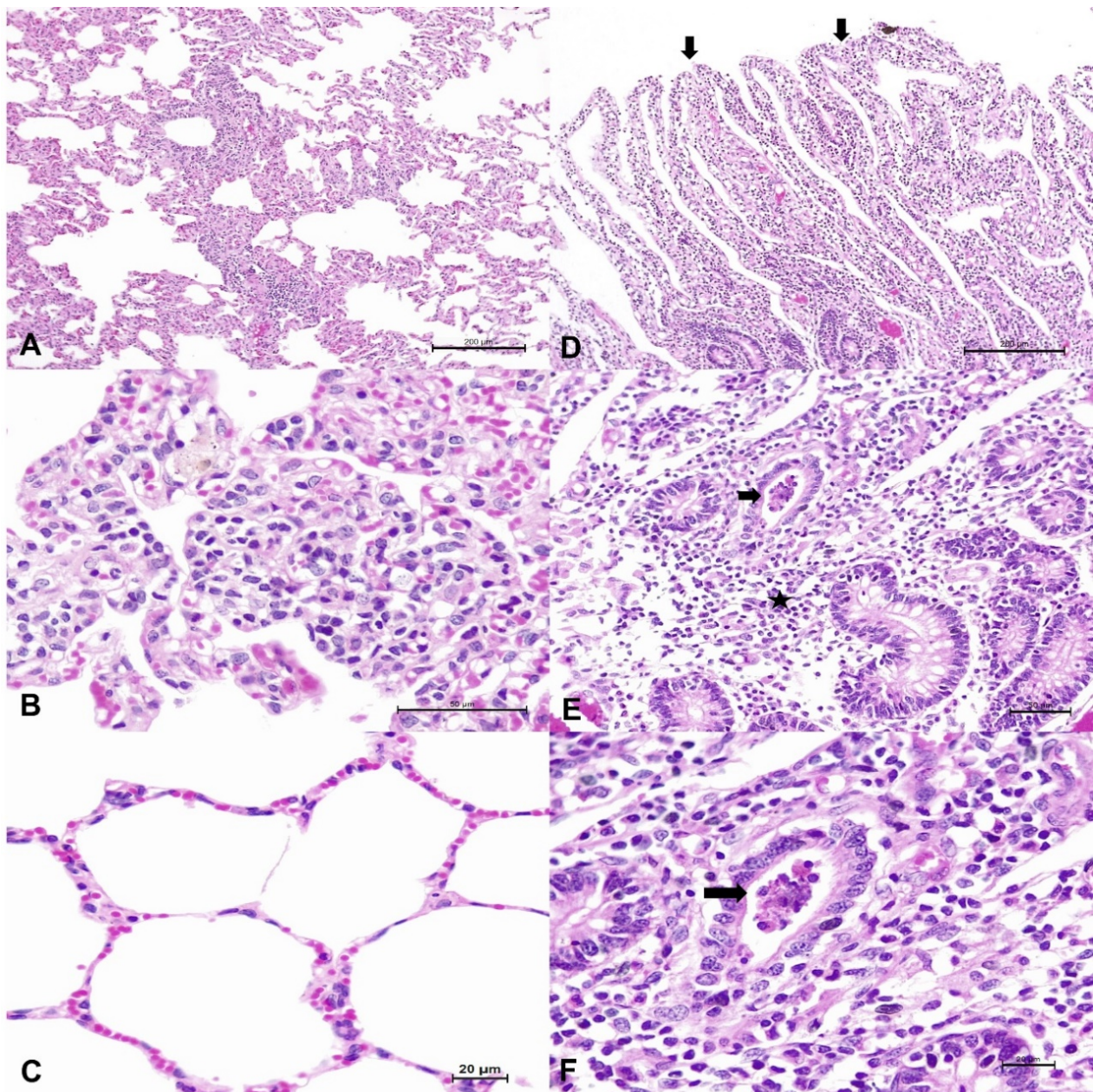
During this study, intestinal and pulmonary fragments from 19 buffaloes were collected. Gross alterations were not observed in any of the organs evaluated. All pulmonary fragments showed foci of moderate interstitial pneumonia, characterized by the proliferation of type II pneumocytes with mild accumulations of lymphocytes (Figure 1A-C). Additionally, mild areas of suppurative bronchopneumonia were identified in two buffaloes (10.5%; 2/19) with interstitial pneumonia, resulting in the concomitant identification of these two patterns of pneumonia in these buffaloes (Table 1).

**Table 1**

**Relationship between the principal histopathological diagnoses and the molecular detection of gammaherpesvirus 6 in the lungs and intestines of buffaloes**

Histopathologic diagnosis observed in buffaloes	BoGHV6 nested-PCR
Atrophic enteritis ( $n=19$ )	5 (26.3%)
Interstitial pneumonia ( $n=17$ )	5 (26.3%)
Suppurative bronchopneumonia and interstitial pneumonia ( $n=2$ )	1 (5.3%)
Simultaneous pulmonary and intestinal lesions ( $n=19$ )	0
Total	11 (57.9%)





**Figure 1.** Histopathologic findings observed in buffaloes infected with bovine gammaherpesvirus 6. There is interstitial pneumonia (A), which is better appreciated at a closer view (B), as compared to the normal-looking lung (C). There is mild blunting (arrow) of the intestinal villi (D) with accumulations of necrotic debris (arrow) within intestinal crypts and lymphocytes (\*) at the intestinal mucosa (E-F). Hematoxylin and eosin stain; Bar, A and D, 200 µm; B, 50 µm; C, E, and F 20µm.

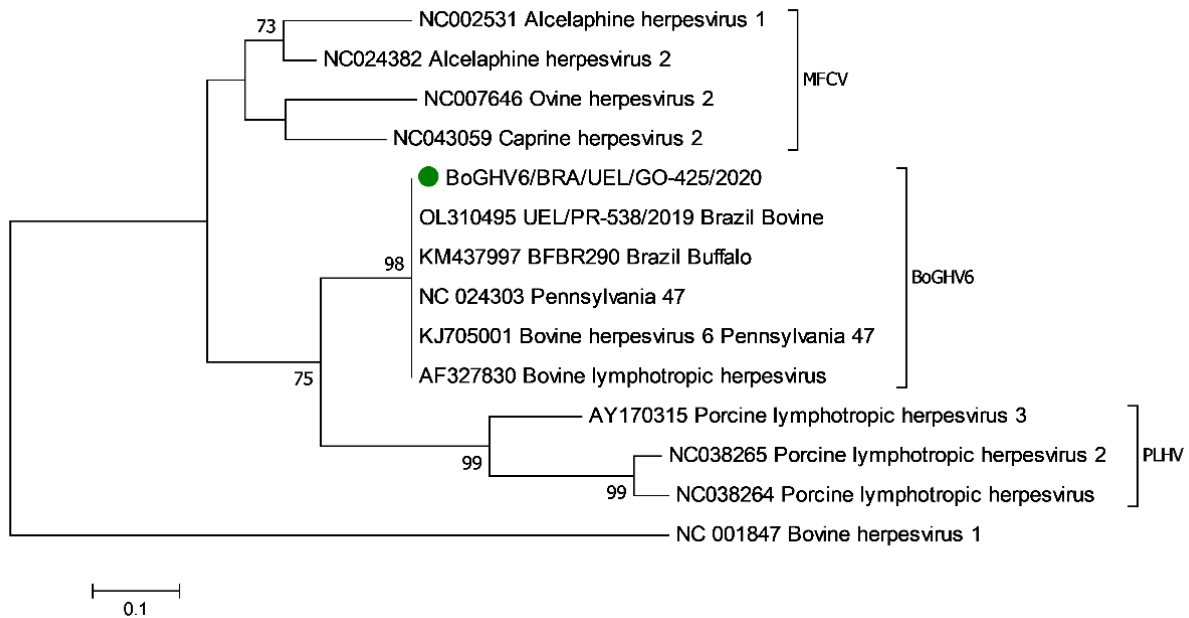
Histopathological evaluation of the intestinal fragments revealed mild to moderate atrophic enteritis characterized by varying degrees of fusion and blunting of the intestinal villi, accumulations of necrotic debris within the intestinal crypts (Figure 1 D-E) and intestinal mucosa with an accumulation of lymphoplasmacytic infiltrate at the mucosa of all fragments evaluated.

#### *Relationship between histological changes and molecular identification of BoGHV6*

BoGHV6 nucleic acids were amplified from 26.3% (5/19) of the pulmonary fragments containing only interstitial pneumonia. Additionally, BoGHV6 was also amplified from the lungs of one buffalo (1/19; 5.3%) with simultaneous interstitial pneumonia and suppurative bronchopneumonia. Alternatively, BoGHV6 DNA was amplified in 26.3% (5/19) of intestinal fragments with atrophic enteritis. Furthermore, there was no simultaneous amplification of BoGHV6 from the intestinal and pulmonary fragments of the same buffalo. Collectively, 57.9% (11/19) of the buffaloes investigated contained BoGHV6 DNA. Additionally, the nucleic acids of BVDV, OvGHV2, BRSV, BPIV3, BCoV, and BoAHV1 were not amplified from any of the tissues evaluated.

#### *Characterization, sequencing, and phylogenetic analysis of the BoGHV6 polymerase gene*

The BoGHV6 nested PCR amplified the desired partial fragment of the polymerase gene, which was confirmed by direct sequencing. The partial nt sequence derived from this study was named BoGHV6/BRA/UEL/GO-425/2020 and is deposited in GenBank (accession #ON184015). Phylogenetic analysis revealed three distinct clusters formed by members of the BoGHV6, MCFV, and PLHVs (Figure 2). Furthermore, the strain herein identified clustered with the reference strain of BoGHV6 and other strains circulating in Brazil and was quite distant from members of the MCFV and PLHVs. Additionally, the strain herein identified has 100% nt identity with the reference strains of BoGHV6 (NC02430; KJ705001) as well as strains of BoGHV6 circulating in ruminants (OL310495, cattle; KM437997, buffalo) from Brazil. In addition, the strain identified in the buffaloes from this study has 69.5 to 73.7% nt sequence identity with members of the MCFV group and was 65.8 – 69.3% different from members of the PLHV.



**Figure 2.** Phylogenetic analysis of members of the Macavirus genus by the Maximum Likelihood method using the polymerase gene. Evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved six strains of bovine gammaherpesvirus 6 and representative strains of each species of the Macavirus genus; bovine alphaherpesvirus 1 was used as the out-group. The gammaherpesvirus 6 strain identified in this study is highlighted (●).

Histological evaluation revealed interstitial pneumonia and atrophic enteritis in all pulmonary and intestinal fragments evaluated. Additionally, BoGHV6 was detected in 31.6% of the buffaloes with pneumonia and 26.3% of the intestinal fragments of buffaloes with enteritis, suggesting that this virus was probably involved in the development of the pulmonary and intestinal lesions herein described. Moreover, gross lesions were not observed, while the histological alterations identified in all buffaloes were not severe. Interestingly, buffaloes were considered as the intermediate hosts for *Macavirus* (Stahel et al., 2013), and may explain the absence of severe lesions in these animals during this study.

Furthermore, the nonamplification of nucleic acids of BVDV, OvGHV2, BRSV, BPIV3, BCoV, and BoAHV1 suggests that these viruses did not participate in the etiopathogenesis of the pulmonary and enteric lesions herein described in these buffaloes during the time of collection. However, caution must be exercised with the interpretation of these results since the simple amplification of BoGHV6 DNA does not necessarily confirm ongoing intestinal and pulmonary disease processes in these buffaloes. Nevertheless, these findings represent the second study to identify BoGHV6 in buffaloes worldwide; in a previous study, BoGHV6 was identified in 2.2% (7/314) of buffaloes but only one had



an associated lymphoproliferative disease (Oliveira et al., 2015). Alternatively, in this study BoGHV6 was identified in 57.9% of the buffaloes investigated. Although the reason for these differences in the occurrence of BoGHV6 in these two studies are unknown, the first study (Oliveira et al., 2015) investigated buffaloes from eight farms located in the States of Pará (n=7) and São Paulo (n=1) and used blood samples for molecular evaluation, while in this study all buffaloes were within the same establishment and molecular detection was done on tissues. Therefore, the number of animals investigated, and the type of samples used in each study could have contributed towards these differences. Notwithstanding the above, these findings suggest that BoGHV6 may be an infectious disease pathogen of not only cattle, but also buffaloes.

The identification of BoGHV6 in pulmonary tissues of buffaloes with histologic diagnosis of interstitial pneumonia is an interesting finding. BoGHV6 was previously identified in the fetal lungs of cattle with histological evidence of pulmonary disease (Oliveira et al., 2022) and in a cow with interstitial pneumonia (Headley et al., 2023b). Although the role of *Macavirus* in the development of pulmonary diseases in ruminants remains unclear (Headley et al., 2023b), we have identified a MCFV, more likely OvGHV2, acting singularly or in association with other pulmonary pathogens in the etiopathogenesis of pulmonary diseases of cattle (Headley et al., 2020a; Oliveira et al., 2021; Headley et al., 2022a) and sheep (Headley et al., 2021). Furthermore, OvGHV2 was associated with the development of pulmonary disease in cattle with clinical manifestations of respiratory impairment

(Headley et al., 2023a). Accordingly, it was proposed that OvGHV2 may be a potential inductor of bovine respiratory disease (BRD) or an innocent bystander (Oliveira et al., 2021) and should be considered a possible infectious agent of BRD (Headley et al., 2020b). Therefore, one wonders if BoGHV6, another member of the *Macavirus* genus, plays a similar role in the development of pulmonary diseases in ruminants. To this end we are perusing the development of in situ diagnostic assays as well as the development of infectious studies to effectively determine the role of BoGHV6 in the pathogenesis of diseases in ruminants. Alternatively, in most studies done with buffaloes infected by MCFV, there was clear presentation of clinical manifestations of disease with typical histopathological alterations of MCF (Costa et al., 2009; Dettwiler et al., 2011; Stahel et al., 2013; Pfitzer et al., 2015), while in one study no clinical manifestation of MCF was reported (Tham, 1997). These findings contrast the differences associated with the detection of these *Macavirus* in buffaloes, where MCFV produces the classical disease syndromes, while the association between BoGHV6 and disease still has to be fully investigated.

During this study BoGHV6 was identified from pulmonary and intestinal tissues with histologic evidence of disease; this contrast with the previous investigation done in buffaloes where BoGHV6 was associated with lymphoproliferative disease due to the serological identification of bovine leukemia virus without any histological demonstration of disease (Oliveira et al., 2015). Furthermore, most previous studies that have detected BoGHV6 in cattle only examined the possible role of infection by molecular identification of this virus without any associated histologic

evidence of tissue injury (Rovnak et al., 1998; Cobb et al., 2006; Banks et al., 2008; Gagnon et al., 2010; Garigliany et al., 2013; de Boer et al., 2014). Alternatively, our group has demonstrated a plausible association between BoGHV6 and fetal alterations (Headley et al., 2022b; Oliveira et al., 2022) and pulmonary lesions in cattle (Headley et al., 2023b). Accordingly, *in situ* evaluations, such as immunohistochemistry and/or *in situ* hybridization, must be done to investigate the possible association of BoGHV6 with the development of disease processes.

The phylogenetic analysis showed that the strains of BoGHV6 circulating in Brazil are similar to the reference strains of BoGHV6 deposited in GenBank; similar results were described (Oliveira et al., 2015; Headley et al., 2023b). Furthermore, all strains of BoGHV6 identified formed a distinct cluster relative to other members of the *Macavirus* genus, demonstrating that all members of this genus are phylogenetically related (Li et al., 2003; Li et al., 2014). Moreover, the detection of BoGHV6 in buffaloes (Oliveira et al., 2015) and cattle (Oliveira et al., 2022; Headley et al., 2023b) from different geographical regions of Brazil may suggest that this virus may be more prevalent in ruminants from this country than previously considered. This is because 57.9% of the buffaloes from the state of Goiás, Central-western Brazil herein investigated were infected by BoGHV6, while 7.2% of buffaloes from the states of Pará and Minas Gerais, Northern and Southeastern Brazil, respectively, and 66.7% of cattle from these regions were infected by BoGHV6 (Oliveira et al., 2015). Furthermore, BoGHV6 was associated with the development of pulmonary disease in a cow from Southern Brazil (Headley et al., 2023b). Collectively,

these studies demonstrate that BoGHV6 may be widespread in Brazil than previously suspected. In addition, the prevalence of BoGHV6 in ruminants from Brazil is less than that described in the USA where 91% of cattle were infected (Rovnak et al., 1998), and more than the average (32%) of infections detected in several European countries (Rosato et al., 2021). Consequently, these are accumulating evidence that BoGHV6 was associated with infections in ruminants from several countries, but there is the need to investigate the direct association of this virus with the development of disease processes and/or syndromes in ruminants.

## Conclusions

BoGHV6 was detected in the lungs and intestines of buffaloes with histologic evidence of disease. Phylogenetic analyses revealed that the strain of BoGHV6 herein detected is similar to the reference strain of BoGHV6 as well as to strains circulating in cattle and buffaloes from different geographical regions of Brazil. The detection of BoGHV6 in ruminants from diverse geographical regions of Brazil may suggest that this virus is more widespread than previously considered. However, additional studies are required to associate BoGHV6 with the development of disease syndromes in ruminants.

## Data Availability

The nucleotide sequence of the BoGHV6 strain identified during this study is deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

## Statement of Animal Rights

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Additionally, permission was obtained from the owner of this animal relative to the utilization in scientific studies. Moreover, permission to realize studies in ruminants was obtained from the National Council for the Control of Animals in Experiments (CONCEA; Brazil) and approved by the Animal Ethics Committees for Animal Usage of the Universidade Norte do Paraná (CEUA, UNOPAR; protocol #008/20; 02/22) and Universidade Estadual de Londrina (CEUA/UEL; protocol, 835.2019.45).

## Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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