JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Clinical, epidemiological, and pathological findings of ovine gammaherpesvirus 2 infections in cattle from Southern Brazil

Selwyn Arlington Headley[1,2], Juliana Torres Tomazi Fritzen[3], Érika Fernandes Lopes Maturana[1], Natali Araujo Correia Alves de Alvarenga[4], Rafael Vince Rodrigues[1], Ana Laura Paulino Leite Gomes[1], Maria Constanza Rodriguez[5], Maria Izabel Pedra Sogari[1], Ana Aparecida Correa Xavier[1], Flávia Helena Pereira Silva[1], Priscilla Fajardo Valente Pereira[4], Vitor Bruno Bianconi Rosa[6], Karina Keller Marques da Costa Flaiban[7], Giovana Wingeter Di Santis[1], Thais Helena Constantino Patelli[6], Júlio Augusto Naylor Lisbôa[4], Amauri Alcindo Alfieri[2,3]

[1] Laboratory of Animal Pathology, Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[2] Multi-User Animal Health Laboratory (LAMSA), Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[3] Laboratory of Animal Virology, Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[4] Large Animal Internal Medicine, Department of Veterinary Clinics, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[5] Marcos Enrietti Diagnostic Center, Agência de Defesa Agropecuária do Paraná (ADAPAR), Curitiba, Paraná, Brazil
[6] Department of Veterinary Medicine and Animal Production, Universidade Estadual do Norte do Paraná, Bandeirantes, Brazil
[7] Laboratory of Clinical Pathology, Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

Abstract

Introduction: Sheep associated-malignant catarrhal fever is a frequently fatal, lymphoproliferative, and vascular disease caused by ovine gammaherpesvirus 2 (OvGHV2), a member of the malignant catarrhal fever virus (MCFV) complex. OvGHV2-related epidemics normally have reduced morbidity with elevated lethality.

Methodology: This study investigated the causes of elevated morbidity, mortality, and lethality in cattle maintained on *Brachiaria* grass pastures and with neurological and enteric disease syndromes from nine farms in Paraná, Southern Brazil.

Results: The principal histopathological findings included necrotizing lymphocytic vasculitis, proliferative vascular lesions, and toxic cholangiohepatitis. An immunohistochemical (IHC) assay utilizing the 15A monoclonal antibody (15A-MAb), which is specific for MCFV, revealed positive intracytoplasmic immunoreactivity within the epithelial cells of the lungs, intestine, liver, and kidneys in most animals, confirming infections by MCFV. PCR detected singular infections by OvGHV2 (n = 3) and bovine gammaherpesvirus 6 BoGHV6 (n = 3) in cattle with positive intracytoplasmic immunoreactivity by the 15A-MAb IHC assay. In one animal with positive immunoreactivity to the 15A-MAb IHC assay, neither *Macavirus* was identified through molecular testing.

Conclusions: These findings suggested that these two *Macavirus* were associated with the positive IHC findings. Additionally, the non-detection of OvGHV2 and BoGHV6 in the organs of one animal, despite typical vascular lesions and with positive IHC results, suggests that another *Macavirus* may be involved. Moreover, the identification of BoGHV6 DNA in cattle not infected by OvGHV2 but containing MCFV antigens, indicates cross-reactivity of BoGHV6 with the 15A-MAb assay. The possibe role of *Bracharia* on the occurrence of these infections is discussd.

Key words: Bovine gammaherpesvirus 6; *Brachiaria* spp. poisoning; diagnostic immunohistochemistry; *Macavirus*; malignant catarrhal fever; 15A epitope.

J Infect Dev Ctries 2025; 19(1):124-139. doi:10.3855/jidc.19951

(Received 01 February 2024 – Accepted 11 May 2024)

Introduction

Malignant catarrhal fever (MCF) is a frequently fatal, lymphoproliferative disease of artiodactyls (cloven-hoofed animals) that is caused by specific members of the genus *Macavirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae* [1]. The 15A-monoclonal antibody (15A-MAb) is one of several major MAb of members of the *Macavirus* genus [2]. This MAb is considered restricted to *Macavirus* that cause MCF since they all share the 15A antigenic epitope [3], are well-conserved within the DNA polymerase gene [4], and are collectively referred to as the malignant catarrhal fever virus (MCFV) complex [4,5]. The 15A antigenic epitope, located on the viral

glycoprotein complex [2,6], was the base for the development of competitive inhibition enzyme-linked immunosorbent assays [6,7]. Furthermore, the 15A-MAb was standardized to be used in immunohistochemical (IHC) assays [8] for the identification of MCFV tissue antigens, considering that this epitope is restricted to the MCFV complex [4,5]. This IHC assay was then used for the detection of intralesional tissue antigens of MCFV in cattle with renal [9], and pulmonary [10] alterations and in ruminants [11,12] infected by OvGHV2.

Members of the *Macavirus* genus known to be associated with the development of MCF in susceptible mammalian hosts include ovine gammaherpesvirus 2 (OvGHV2), alcelaphine gammaherpesvirus 1 and -2 (AlGHV1 -2), and caprine gammaherpesvirus 2 [13-15]. Alternatively, the participation of bovine gammaherpesvirus 6 (BoGHV6), another *Macavirus*, in the pathogenesis of disease processes in ruminants remains controversial [16] and was never associated with MCF.

Epidemiologically, two widely accepted forms of MCF are recognized and intensively studied: sheep associated-malignant catarrhal fever (SA-MCF) and wildebeest associated-MCF (WA-MCF), in which sheep and wildebeest, respectively, are the reservoir hosts for the associated virus [13-15]. WA-MCF is caused by AlGHV1 and -2 and occurs predominantly in artiodactyls from Africa and in wildlife maintained in some zoological parks in the USA [17,18]. Alternatively, SA-MCF occurs worldwide in numerous ungulates and is caused by OvGHV2, during which the reservoir host is asymptomatic, while the dead-end hosts develop several typical clinical manifestations of MCF [13,14,18]. Although continental Brazil may harbor several members of the *Macavirus* genus, only OvGHV2 has been associated with the development of MCF in mammalian hosts [15].

The characteristic clinical manifestations of SA-MCF observed in the dead-end hosts are the head-and-eye [14], alimentary [17,19], neurological [17], and cutaneous [3,19,20] forms as well as peracute/acute and chronic manifestations [19,21,22]. The course of the alimentary form of MCF is frequently acute [19,21] and is characterized by profuse diarrhea and dysentery [21], without typical ocular lesions, and occurs predominantly in cattle without direct contact with sheep [19], with death occurring 12-72 hours after the onset of diarrhea or dysentery [23]. Furthermore, in the alimentary form of MCF, diarrhea may be the only noticeable clinical manifestation which frequently results in misdiagnosis with other common enteric diseases of cattle, including bovine viral diarrhea [21,23]. Additionally, the cutaneous manifestations of SA-MCF can be clinically confused with hypersensitivity and/or photosensitivity reactions [20].

This study presents the findings observed in outbreaks associated with affections due to OvGHV2 in cattle from Southern Brazil.

Methodology

Animals, geographical locations of study, and epidemiological data

From early April to the end of October 2022, farmers and veterinarians from diverse geographical regions of Paraná (PR) State, Southern Brazil, reportedly informed that there was elevated cattle morbidity and mortality associated with a mysterious disease. The Veterinary Teaching Hospital, Universidade Estadual de Londrina (VTH-UEL), received animals and/or tissues (n = 16) submitted from farmers and veterinarians to investigate and provide a diagnosis of the ongoing cattle mortality. However, not all farmers with similar problems sought to investigate the cause of cattle mortality. The geographical locations of the related cattle mortality with the principal ongoing clinical syndromes reported at each farm are provided (Table 1). Furthermore, these farms are located in some mesoregions of Paraná state (Figure 1), where elevated

Table 1. Epidemiological data associated with clinical manifestations of SA- MCF in cattle farms from Paraná, Southern Brazil, 2022.

Farm #	Geographical location	Period of occurrence	Principal clinical syndrome	Presence of sheep	Animals					
					Total	Sick	Dead	Morbidity (%)	Mortality (%)	Lethality (%)
A	Ribeirão Claro	April	Neurological	No	300	1	1	0.3	0.3	100
B	Pitanga	June	Neurological	Commingling with cattle	300	5	5	1.7	1.7	100
C	Bandeirantes	August - October	Alimentary	Yes	450	200	200	44.4	44.4	100
D	Ibaiti	August - September	Alimentary	No	1800	80	46	4.4	2.6	57.5
E	Jandaia do Sul	September	Alimentary	No	1000	10	10	1.0	1.0	100
F	Santo Antônio de Platina	August - September	Alimentary	No	340	32	28	9.4	8.2	87.5
G	Abatiá	August - September	Alimentary	No	790	150	60	19.0	7.6	40
H	Apucarana	August - September	Alimentary	Sheep within proximity	70	26	21	37.1	30.0	80.8
I	Londrina	August	Alimentary	Yes	14	2	2	14.3	14.3	100
Total					5064	506	373	10.0	7.4	73.7
Average								14.6	12.2	85.1
Median								**9.4**	**7.6**	**100**
1 Quartile								1.7	1.7	72.5
3 Quartile								19.0	14.3	100

prevalence levels of MCFV tissue antigens were previously identified in cattle with renal lesions [9].

Cattle at most of these farms were routinely immunized against bovine rabies, bovine viral diarrhea virus (BVDV), bovine alphaherpesvirus 1 (BoAHV1), and *Clostridium* spp. Furthermore, there were reported frustrating attempts at treating cattle with severe alimentary syndrome since some animals reportedly responded to diverse antibiotic and maintenance therapies, but most succumbed to intestinal disease.

Clinical and laboratory evaluations

Cattle (n = 7) *in extremis* from three farms (C, D, and E) were evaluated clinically; blood and/or serum samples from four of these were submitted for hematological and/or biochemical analyses. However, not all animals evaluated clinically were submitted for routine *post-mortem* evaluations.

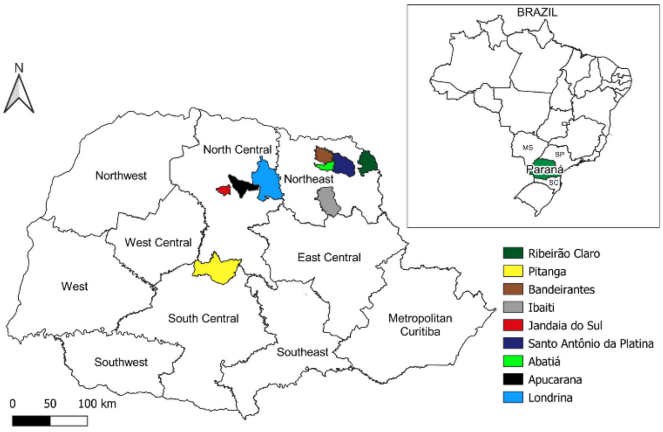
Post-mortem evaluations, histopathology, and histochemistry

Tissue sections obtained during *post-mortem* evaluations (n = 4) and received for histopathological analyses (n = 12) were collected in duplicates for routine pathological and molecular diagnostics. Tissues for histopathological evaluation were routinely processed with the Hematoxylin and eosin stain. Selected sections of some of these were evaluated with the Verhoeff-Van Gieson (VVG) histochemical method to identify the integrity of elastin within arteries. Selected tissue sections were used in an immunohistochemical (IHC) assay to detect intralesional tissue antigens of MCFV. Tissue fragments of freshly collected and/or received organs were used in molecular assays for the detection of common disease agents of cattle were maintained at -80 ºC until used in molecular assays.

Immunohistochemical identification of MCFV

IHC assay was done on selected formalin-fixed paraffin-embedded (FFPE) tissue sections derived from animals (n = 16) submitted to *post-mortem* evaluations and/or received for histopathological analyses. Whenever possible, specific FFPE sections of the lungs, kidney, intestine, and liver were used in an IHC assay designed to identify intralesional antigens of MCFV using the 15A-MAb as previously described [8]. Positive controls consisted of FFPE tissue sections known to contain antigens of OvGHV2 from previous studies [8,11]. Two negative controls were used in all IHC assays: the first consisted of replacing the 15A-MAb with its diluent, while in the second, the 15A-MAb was placed on FFPE tissues known to demonstrate negative immunoreactivity to OvGHV2. Negative and positive controls were included in all IHC assays.

Figure 1. Map demonstrating the geographical locations of each farm within the mesoregions of Paraná state, Southern Brazil.



Molecular detection of infectious disease agents associated with respiratory, enteric, and neurological diseases of cattle

Nucleic acid extractions using the phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate method as described in Alfieri *et al.* and Boom *et al.* [24,25] were done only from tissue suspensions of the freshly collected organs; molecular analyses were not done on tissues fixed in formalin solution or derived from FFPE tissue blocks. The extracted nucleic acid was eluted in 50 µL of 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 DNA and/or RNA of the common infectious disease agents associated with respiratory, enteric, and neurological diseases of cattle. These molecular assays were done based on previously described protocols for OvGHV2, BoGHV6, BVDV, bovine respiratory syncytial virus (BRSV), bovine alphaherpesvirus 1 and 5 (BoAHV1- and 5), bovine coronavirus (BCoV), bovine rotavirus A (BRV), bovine parainfluenza virus 3 (BPIV-3), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasmopsis bovis* (formerly *Mycoplasma bovis*), and mollicutes. A list of the specific genes targeted and the required amplicon to be amplified by these molecular assays is provided (Supplementary Table 1). Some of these agents are specific to a particular organ/system, but most are associated with infections in multiple organs/systems of cattle. Sterile, ultrapure water was used as the negative

control in all nucleic acid extractions and molecular procedures.

The products of the OvGHV2 and BoGHV6 PCR purified using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen® Life Technologies, Carlsbad, CA, USA), quantified by using 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 homepage (http://asparagin.cenargen.embrapa.br/phph/), respectively. Similarity searches of the OvGHV2 and BoGHV6 gene were performed with nucleotide (nt) sequences deposited in GenBank using the Basic Local Alignment Search Tool homepage (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Furthermore, specific segments of the brains of the two animals with neurological syndromes were submitted to the Official Regional Diagnostic Veterinary Laboratory (Centro de Diagnóstico Marcos Enrietti), Curitiba, Paraná, to detect the presence of bovine lyssavirus.

Determination of infections by OvGHV2, BoGHV6, and MCFV in the establishment of singular and concomitant infections

The characterization of singular and/or multiple infections observed in each animal was determined due to the combination of the IHC detection of tissue antigens of MCFV and the molecular amplification of nucleic acids of the specific infectious disease pathogen in one and/or several organs of the same animal. Consequently, a diagnosis of OvGHV2-related infections was established due to the detection of MCFV tissue antigens by IHC with the concomitant amplification of OvGHV2 DNA by PCR. Similarly, BoGHV6-associated diseases were confirmed due to the detection of MCFV tissue antigens with the simultaneous molecular detection of BoGHV6 DNA by PCR. An infection was termed MCFV-associated in animals that did not contain either OvGHV2 or BoGHV6 DNA by PCR but demonstrated positive immunoreactivity with the 15A-MAb IHC assay.

Results

Epidemiological findings and clinical syndromes

The first reported mortality received at the Laboratory of Animal Pathology, UEL, occurred in mid-April from a farm located in Ribeirão Claro, PR, where one cow suddenly died of neurological manifestations (Table 1). This was then followed by another report of neurological disease affecting cattle from the city of Pitanga, PR, in June. It must be highlighted that both of these regions had previous episodes of confirmed bovine and equine rabies; therefore, rabies was suspected by the farmers and consulting veterinarians.

Seven reports of elevated cattle mortality in several regions of Paraná associated with diarrhea between August and October were examined (Table 1). At all these farms the main reported clinical manifestation was profuse diarrhea followed by emaciation resulting in death within a few days. Cattle at these farms with enteric disease were reared on pastures containing *Brachiaria* spp., supplemented predominantly by mineral salt; water was provided *ad libitum* from wells or streams within these farms.

The overall data associated with cattle morbidity and mortality from nine farms located within distinct geographical locations of the State of Paraná, Southern Brazil are presented in Table 1: a total of 5,064 heads of cattle were at risk, 10% (506/5,064) were sick, and 7.4% (373/5,064) of these died during these outbreaks. The median morbidity rate (Table 1) was calculated as 9.4% (Q1: 1.7%; Q3: 19%); the median mortality rate was determined as 7.6% (Q1: 1.7%; Q3: 14.3%), while the median lethality rate was estimated as 100% (Q1: 72.5%; Q3: 100%).

Two principal clinical manifestations were observed at these farms in association with affections due to OvGHV2, resulting in alimentary (n = 7) and neurological (n = 2) syndromes. When the rates of morbidity, mortality, and lethality were compared with the type of clinical disease manifestations, the morbidity rate associated with cattle that died of neurological syndromes was very low (0.3 – 1.7%), while cattle with alimentary manifestations had morbidity rates ranging from 1-44.4% (Table 1). Additionally, all cattle with neurological manifestations died (100% lethality); while the lethality rate associated with intestinal disease fluctuated between 40 - 100%. Comparatively, more cattle demonstrated the alimentary syndrome relative to the neurological manifestations, while some cattle with the alimentary form recovered from these affections.

Sheep were not reared within the proximity and/or on most (55.5%; 5/9) of these farms (Table 1). However, commingling between sheep and cattle occurred in animals that died of neurological disease (Farm B), while sheep were reared (Farms C and I) or within 50 km of cattle (Farm H) with alimentary syndrome. Furthermore, the most elevated indices of morbidity and mortality (44.4%) with elevated lethality (100%) were identified at Farm C, where cattle developed alimentary disease and were reared concomitantly with sheep. The farms from this study are located in three mesoregions (Northeast, n = 5; North central, n = 3; South central, n = 1) of Paraná state and correspond to regions with comparatively elevated positive immunoreactivity to MCFV antigens in cattle with renal lesions [9].

Clinical and laboratory information

Seven animals with severe intestinal disease were evaluated clinically at the VTH-UEL and/or Universidade Estadual do Norte do Paraná, Paraná, Southern Brazil; however, *postmortem* evaluations and/or tissues were not received from all animals that were clinically evaluated by large animal clinicians at these institutions. The principal clinical manifestations, duration of disease progression, and the clinical outcome reported at each farm are provided in Table 2. The age of the affected cattle ranged from 1.5 to 7 years, with most animals being more than 2 years of age.

Cattle from farms with reported neurological syndromes died within 24-72 hours after the onset of clinical manifestations; one was prostrated with ataxia and lateral recumbency while the other was found dead after a brief episode of muscular tremors. The alimentary manifestations reported from animals of the seven farms were quite similar with the affected cattle demonstrating some form of diarrhea (liquid, fetid, or bloody), with progressive emaciation and death occurring at most farms within 24-78 hours after the onset of clinical manifestations. However, the enteric syndrome at Farm F reportedly occurred for 5-7 days. Additionally, cutaneous photosensitivity was only observed in cattle from Farms D and E. Most cattle (56.3%; 9/16) evaluated from Farms with the neurological (A and B) and enteric (D, F-I) syndromes died spontaneously after episodes of clinical manifestations, while the animals from Farms C (n = 6) and E (n = 1) with enteric disease were euthanized *in extremis* (Table 2).

The results of the hematological and biochemical analyses obtained from blood and/or serum collected from animals at three farms are resumed in Table 3. Laboratory evaluation of the red blood cells did not reveal anemia in any of the animals evaluated; however, three animals were moderately dehydrated. Leukocytosis with neutrophilia was the most significant hematological alteration observed. A similar pattern of alterations to the white blood cells was observed for the animals from Farms C (Animal #3), D (Animal #9), and E (Animal #11), where leukocytosis with neutrophilia was very severe in animals from Farms D and E; these alterations were accompanied by marked hyperfibrinogenemia (animals from all Farms). These results are indicators of an ongoing inflammatory process.

Table 2. Biological data, principal clinical manifestations, disease progression, and clinical outcome observed in cattle infected with immunohistochemical and molecular detection of *Macavirus*.

Farm #	# animal	Biological data	Principal clinical manifestations reported at each farm	Disease progression (hours)	Clinical outcome
A	1	3.5- year- old, mixed breed male	Muscular tremors	24 hrs	Spontaneous death
B	2	5- year- old, Nellore, cow	Prostration, ataxia, lateral decumbency	48 - 72 hrs	Spontaneous death
C*	3 4 5 6 7 8	2- year- old, Nellore, cow	Fetid, liquid, brown- colored diarrhea, dehydration, permanent lateral decumbency, ruminal hypomotility, flaccid tetraparesis	24 - 48 hrs	Euthanized *in-extremis*
D*	9 10	1.5- year- old, Nellore, cow	Liquid diarrhea, progressive emaciation, external decumbency, ruffled hair- coat, cutaneous photosensitivity lesions	72 - 96 hrs	Spontaneous death
E	11	1.5- year- old, Nellore, cow	Fetid, liquid, brown- colored diarrhea, chest edema, cutaneous photosensitivity lesions,	48 - 72 hrs	Euthanized *in-extremis*
F	12	7- year- old, Nellore, cow	Fetid, profuse, bloody diarrhea, progressive emaciation, aggressive behavior	120 - 168 hrs	Spontaneous death
G	13	5- year- old, Nellore, cow	Diarrhea	24 - 72 hrs	Spontaneous death
H	14	4- year- old, Nellore cow	Diarrhea and respiratory difficulties	24 - 48 hrs	Spontaneous death
I	15	6- year- old, Nellore, cow			Spontaneous death
	16	12- year- old, Holstein cow	Bloody diarrhea	24 - 48 hrs	Spontaneous death

*: biological data is only available for one animal from these farms.

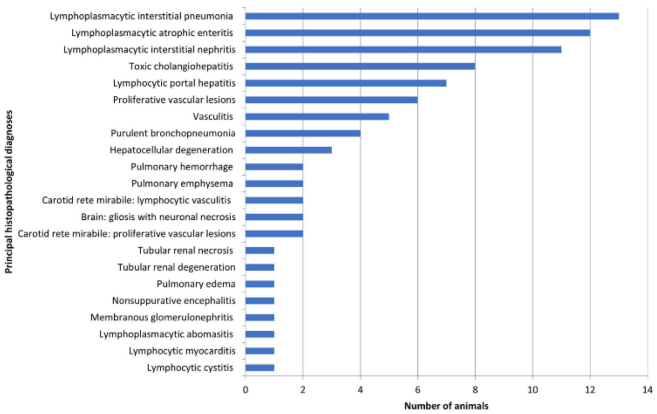
Table 3. Hematological and biochemical findings observed at cattle farms during outbreaks of neurological and alimentary infections associated with ovine gammaherpesvirus 2.

Laboratory parameters	Animals from Farms				Physiological
Hemogram	**C1**	**C2[a]**	**D**	**E**	**Interval[1]**
Red blood cells (× 10⁶/mm³)	7.85	ND	8.39	8.78	5 – 10
Hemoglobin (g/dL)	11.1	ND	13.3	10.7	8 – 15
PCV (%)	38	ND	40	34.6	24 – 46
MCV (fl)	48.4	ND	47.6	39.4	40 – 60
MCH (pg)	14.1	ND	15.8	12.2	11 – 17
MCHC (%)	29.2	ND	33.3	30.9	30 – 36
White blood cells (/mm³)	17,000	ND	66,400	41,400	4,000 – 12,000
Metamyelocytes (/mm³)	170	ND	0	0	0
Band neutrophils (/mm³)	170	ND	0	0	0 – 120
Mature neutrophils (/mm³)	12,410	ND	55,776	33,534	600 – 4,000
Lymphocytes (/mm³)	4,250	ND	10,624	7,038	2,000 – 7,500
Monocytes (/mm³)	0	ND	0	828	25 – 800
Fibrinogen (mg/dL)	1,200	ND	1,000	1,200	200 – 700
Serum biochemistry					
Total Protein (g/dL)	7.2	ND	6	8.4	6.7 – 7.4
Albumin (g/dL)	1.2	1.3	2.2	ND	3.0 – 3.5
Creatinine (mg/dL)	1.5	1.2	2.6	2,1	1.0 – 2.0
Urea (mg/dL)	84	ND	99	ND	20 – 30
ALP (U/L)	ND	ND	ND	270	0 – 488
GGT (U/L)[b]	111	175	38	305	6.1 – 17.4
AST (U/L)[b]	538	1,190	856	709	78 – 132
CK (U/L)	39,280	78,375	68,950	3,813	35 – 280
Total Bilirubin (mg/dL)	2.9	4.3	ND	5.2	0.01 – 0.50
Conjugated bilirubin (mg/dL)	1	2	ND	2.2	0.04 – 0.44
Unconjugated bilirubin (mg/dL)	1.9	2.3	ND	3	0.03
Observation	Icteric serum+ + +	Icteric serum+ +	Icteric serum + +	Icteric serum+ +	

[a]: only serum was collected from this animal; [b]: hepatic activities (GGT and AST) were extremely elevated in cattle with toxic cholangiohepatitis. PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; AST: aspartate aminotransferase; CK: creatine kinase; ND: not done. + : mild; + + : moderate; + + + : severe.
[1]: Radostits OM, Gay CC, Hinchcliff KW, Constable PD (2007) Veterinary Medicine: a textbook of the disease of cattle, horses, sheep, pigs, and goats. 10th ed. Saunders Elsevier, Edinburgh. 2156p.

Analysis of the biochemical alterations indicated liver dysfunction as a common pattern in all animals at these three Farms, with the renal system not being affected since the urea and creatinine values were within physiological limits. The elevated enzymatic activities of serum gamma-glutamyl transferase and aspartate aminotransferase indicated that all animals evaluated had cholangitis and hepatocytic lesions, respectively. Furthermore, elevations of total, direct, and indirect bilirubin concentrations were detected in most cattle, indicating cholestasis and decreased ability of the liver to conjugate bilirubin. Additionally, the hypoalbuminemia observed in all animals and the icteric serum are further evidence of impaired liver function and the loss of enteric protein. The elevation of serum creatine kinase activity indicated that all animals suffered from muscular lesions, which could have been related to permanent recumbency or primary muscular damage.

Figure 2. Principal histopathological findings in cattle infected with *Macavirus*.

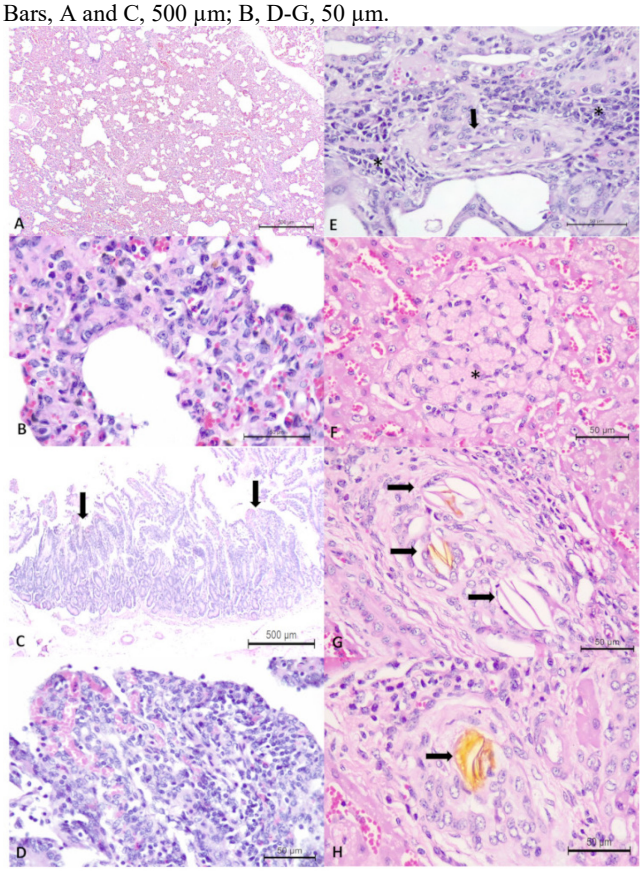


Pathological and histochemical findings

Four of the animals (#4, 10, 11, and 15) evaluated were submitted to complete *post-mortem* evaluations at the VTH-UEL. Tissues were received for histopathological diagnosis for the other 12 animals. Tissues from animal #3 were not submitted for pathological diagnosis, while significant histopathological alterations were not observed in the organs of animal #16. The principal histopathological patterns diagnosed during this study are summarized in Figure 2, with interstitial pneumonia (n = 13), atrophic enteritis (n = 12), interstitial nephritis (n = 11), and toxic cholangiohepatitis (n = 8) being the most frequent lesions identified.

The principal histopathological alterations observed in each animal evaluated are summarized in Table 4. Brain fragments were only received from one of the animals (#2) with clinical manifestations of a neurological syndrome. In this animal, histological findings indicative of brain disease were restricted to neuronal necrosis with lymphocytic vasculitis and proliferative vascular lesions (PVL), also referred to as arteriopathy, at the arteries of the carotid rete mirabile (CRM); these lesions at the CRM are diagnostic for SA-MCF [26]. Most animals that died of the alimentary syndrome had histopathological evidence of simultaneous pulmonary, enteric, and renal disease. Furthermore, most (61.5%; 8/13) of these cattle evaluated by histopathology, had evidence of toxic cholangiohepatitis.

Figure 3. Histopathological findings observed in cattle infected with OvGHV2. There is widespread lymphoplasmacytic interstitial pneumonia (A), which is better appreciated with a closer view (B). Observe areas of blunting (arrows) of the intestine (C) with severe accumulations of lymphoplasmacytic infiltrates at the mucosa (D), and foci (*) of lymphoplasmacytic interstitial nephritis (E) surrounding a degenerated artery (arrow) within the kidney. There is an accumulation of foamy macrophages (*) within the liver (F), negative images of several crystals (arrows) within bile ducts (G), and ductal (arrow) biliary stasis (H). Hematoxylin and eosin stain. Bars, A and C, 500 µm; B, D-G, 50 µm.



Most animals (81.2%; 13/16) developed lymphoplasmacytic interstitial pneumonia (LIP) with simultaneous foci of purulent bronchopneumonia (n = 4) and extensive areas of pulmonary hemorrhage (n = 2). LIP was characterized by marked thickening of pulmonary alveoli due to severe proliferation of Type II pneumocytes with accumulations of lymphoplasmacytic inflammatory infiltrates and erythrocytes within pulmonary interstitium but without any neutrophilic exudate within pulmonary airways (Figure 3A-B). Most animals (85.7%; 12/14) that died of the alimentary syndrome had histological evidence of enteric disease, being characterized as lymphoplasmacytic atrophic enteritis due to blunting and/or shorting of intestinal villi with varying degrees of accumulations of lymphoplasmacytic infiltrates within the intestinal mucosa (Figure 3C-D). Histological evidence of renal alterations was observed in cattle with clinical manifestations of both alimentary and neurological manifestations, with lymphoplasmacytic interstitial nephritis (Figure 3E) being the most predominant lesion identified in the kidney. Two distinct patterns of hepatic lesions were observed: lymphocytic portal hepatitis (n = 7), occurring in cattle with both clinical syndromes and

Figure 4. Vascular lesions identified in cattle infected with ovine gammaherpesvirus 2. There is a proliferative vascular lesion (arteriopathy) in the lungs (A) and the muscular layer of the small intestine (B); compared with varying degrees of fibrinoid degeneration (change) at the muscular layer of the same animal (C-E). Observe arteriopathy at the carotid rete mirabile (F), which is easily identified with the Verhoeff-Van Gieson histochemical stain for elastin (G). Hematoxylin and eosin stain, A-F. Bars, A, 100 µm; B, C, E, and F, 50 µm; D, 20 µm.

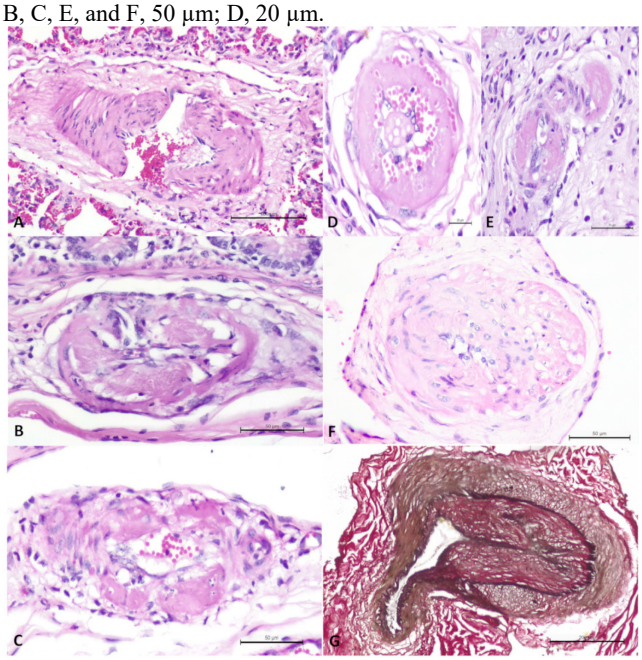


Table 4. Principal histopathological diagnoses observed in cattle infected with ovine gammaherpesvirus 2.

Histopathological diagnoses	Animals[1]													
	1	2	4	5	6	7	8	9	10	11	12	13	14	15
Brain: gliosis with neuronal necrosis		x	x											
Carotid rete mirabile: lymphocytic vasculitis		x								x				
Carotid rete mirabile: proliferative vascular lesions										x				x
Hepatocellular degeneration										x	x			x
Lymphocytic cystitis			x											
Lymphocytic myocarditis											x			
Lymphocytic portal hepatitis	x	x	x		x	x		x					x	
Lymphoplasmacytic abomasitis	x													
Lymphoplasmacytic atrophic enteritis			x	x	x	x	x	x	x	x	x	x	x	x
Lymphoplasmacytic interstitial nephritis	x	x	x		x	x	x		x	x	x	x		x
Lymphoplasmacytic interstitial pneumonia	x	x	x	x	x	x	x	x	x	x	x	x	x	
Membranous glomerulonephritis														x
Nonsuppurative encephalitis									x					
Proliferative vascular lesions	x	x	x								x	x	x	
Pulmonary edema				x										
Pulmonary emphysema										x				x
Pulmonary hemorrhage	x		x											
Purulent bronchopneumonia		x						x	x	x				
Tubular renal degeneration				x										
Tubular renal necrosis													x	
Toxic cholangiohepatitis				x				x	x	x	x	x	x	x
Vasculitis	x	x	x								x	x		

[1]Tissues were not received for histopathological evaluations from animal #3; significant pathological alterations were not observed in animal #16.

toxic cholangiohepatitis (TCH, n = 8), diagnosed only in cattle with the alimentary syndrome. TCH was characterized due to the accumulations of foamy macrophages, negative images of crystals, proliferation of bile duct epithelial cells, biliary and canicular stasis, and bridging fibrosis (Figure 3F-H); these lesions are characteristic of the intoxication of saponins due to the ingestion of *Brachiaria* spp. [27-29].

Two types of vascular lesions (Table 4) were identified by histopathology in multiple tissues of cattle that died of both clinical syndromes: vasculitis and PVLs (also referred to as angiopathy). PVLs were characterized due to proliferation and/or degeneration of the intima and media of arteries with consequent luminal obstruction. In animals with vasculitis, there was severe fibrinoid degeneration (or change) and/or lymphocytic vasculitis at the arteries within the muscular layer of the small intestine, CRM, as well as the lungs (Figure 4A), and kidneys. In the muscular layer of the small intestine of animal #11, there was a distinct transition from necrotizing vasculitis with fibrinoid change to PVL (Figure 4B-E). Additionally, the PLV identified by routine histopathology at the CRM was easily visualized with the VVG histochemical method to identify elastin within arteries (Figure 4F-G).

Table 5. Immunohistochemical and molecular findings observed in cattle from farms infected by ovine gammaherpesvirus 2 (OvGHV2).

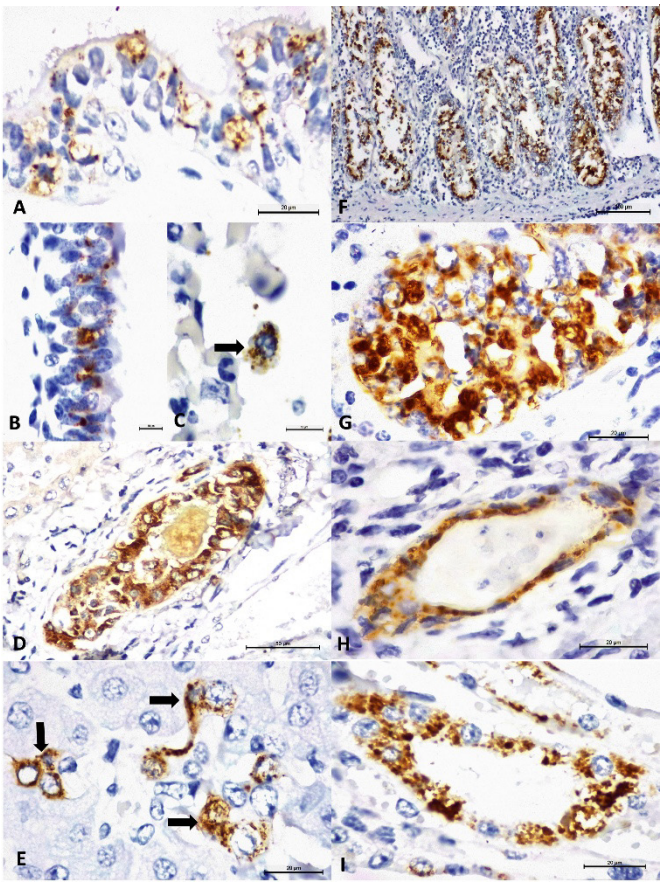
Farms #	Animal#	MCFV IHC				OvGHV2 tegument protein nPCR				Additional pulmonary infections by PCR			Type of Infection[1]	Results
		Lung	Liver	Kidney	Sm. Int	Lung	Liver	Kidney	Sm. Int.	BoGHV6	BCoV	*HS*		
A	1	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	- ve	+ ve	- ve	+ ve	Triple	OvGHV2 + BoGHV6 + *H. somni*
B	2	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	NC	+ ve	- ve	- ve	Dual	OvGHV2 + BoGHV6
C	3	NC	NC	NC	NC	+ ve	- ve	- ve	- ve	+ ve	- ve	- ve	Dual	OvGHV2 + BoGHV6
	4	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	Singular	BoGHV6
	5	+ ve	+ ve	+ ve	+ ve	NC	NC	NC	NC	NC	NC	NC	Singular	MCFV
	6	+ ve	+ ve	+ ve	+ ve	NC	NC	NC	NC	NC	NC	NC	Singular	MCFV
	7	+ ve	+ ve	- ve	+ ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	Singular	BoGHV6
	8	+ ve	NC	+ ve	+ ve	+ ve	NC	- ve	NC	- ve	- ve	- ve	Singular	OvGHV2
D	9	- ve	- ve	NC	- ve	- ve	- ve	NC	NC	- ve	+ ve	- ve	Singular	BCoV
	10	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	- ve	- ve	- ve	+ ve	Dual	*H. somni* + MCFV
E	11	- ve	- ve	- ve	- ve	- ve	- ve	+ ve	+ ve	- ve	- ve	+ ve	Dual	OvGHV2 + *H. somni*
F	12	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	Singular	BoGHV6
	13	- ve	- ve	- ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	- ve	Singular	OvGHV2
G	14	+ ve	+ ve	- ve	+ ve	+ ve	- ve	+ ve	- ve	- ve	- ve	- ve	Singular	OvGHV2
H	15	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	+ ve	- ve	- ve	Dual	OvGHV2 + BoGHV6
I	16 [a]	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	- ve	+ ve	+ ve	- ve	Quadruple	OvHGV2 + BoHV6 + BCoV + BoAHV1
n		9	9	8	10	7	1	2	2	8	2	3		

- ve: negative: + ve: positive; NC: not collecetd; MCFV IHC: malignant catarrhal fever virus immunohistochemistry; snPCR: seminested PCR; OvGHV2: ovine gammaherpesvirus 2; BoGHV6: bovine gammaherpesvirus 6; MCFV: malignant catarrhal fever virus; BCoV: bovine coronavirus; *H. somni: Histophilus somni*. [1]: based on the molecular and/or immunohistochemical detection of infectious disease agent(s) within one or more organs of the same animal. [a]: lung infected with bovine alphaherpesvirus 1 by PCR.

Immunohistochemical identification of MCFV tissue antigens

Intralesional tissue antigens of MCFV were detected in multiple organs (lungs, liver, kidney, and small intestine) from most of the cattle (68.7%; 11/16) evaluated, with the exception being in FFPE sections derived from cattle maintained at Farms A, E, and I, as well as one animal from Farms D and F (Table 5). Additionally, from the animals with positive immunoreactivity by the 15A-MAb IHC assay, most (54.5%; 6/11) of these contained intralesional tissue antigens of MCFV in all four organs evaluated. Furthermore, positive intralesional intracytoplasmic immunoreactivity for MCFV antigens was detected in cattle with histopathologic confirmation of enteric (n = 10), pulmonary, hepatic (n = 9), and renal (n = 8) diseases.

Figure 5. Immunohistochemical detection of intralesional tissue antigens of malignant catarrhal fever virus (MCFV) in the organs of cattle intoxicated by the ingestion of *Brachiaria* spp. Observe positive intracytoplasmic immunoreactivity to MCFV antigens within the bronchial epithelium (A, B) and pulmonary macrophage (C, arrow) of the lung. There is intracytoplasmic detection of MCFV antigens within epithelial cells of bile ducts (D) and hepatocytes and Kupffer cells (E, arrows) of the liver, epithelial cells of intestinal crypts (F-G), and renal tubular epithelium (H-I). Immunoperoxidase counterstained with Hematoxylin. Bars, A, E, G, H, and I, 20 µm; B, C, 10 µm; D, 50 µm; F, 100 µm.



Positive, patchy, intralesional, intracytoplasmic immunoreactivity to MCFV antigens were identified principally within bronchiolar and bronchial epithelium as well as the epithelium of peribronchial glands of the lungs and rarely within pulmonary macrophages (Figure 5A-C) in the lungs of cattle with interstitial pneumonia. Similarly, positive intracytoplasmic immunoreactivity was detected predominantly within bile duct epithelium and within several, randomly located, hepatocytes of the liver (Figure 5D-E) of animals with lymphocytic portal hepatitis. Additionally, positive intracytoplasmic immunoreactivity to MCFV antigens was intense and widespread within the epithelial cells of the intestinal crypts with lymphoplasmacytic atrophic enteritis (Figure 4F-G) and within epithelial cells of the renal tubules of cattle with lymphoplasmacytic interstitial nephritis (Figure 5H-I).

Molecular detection of OvGHV2 and other viral disease pathogens of cattle

The summary of the molecular detection of viral and bacterial disease pathogens evaluated during this study is presented in Table 5. OvGHV2 was the pathogen most frequently (64.3%; 9/14) amplified from at least one tissue of most animals evaluated by molecular diagnostics since fresh samples from two animals (#5 and 6) were not submitted for molecular identification. Consequently, OvGHV2 was amplified in cattle originating from all Farms, except Farm D, where the two animals were infected by a combination of BCoV, MCFV, and *H. somni*. Furthermore, in five animals (#1, 2, 3, 15, and 16) there was simultaneous amplification of OvGHV2 and BoGHV6 DNA, with positive immunoreactivity for MCFV tissue antigens being detected in two (# 2 and 15) of these. Interestingly, only BoGHV6 DNA was amplified from the tissues of three animals (#4, 7, and 12) that contained intralesional tissue antigens of MCFV. Furthermore, animal #10 was only infected by *H. somni;* the organs (liver, kidney, lungs, and intestine) of this animal demonstrated positive immunoreactivity by the MCFV IHC assay but without the molecular detection of either OvGHV2 or BoGHV6.

At these Farms singular (n = 7), dual (n = 5), triple (n = 1; OvGHV2, BoGHV6, *H. somni*), and quadruple (n = 1; OvGHV2, BoGHV6, BCoV, BoAHV1) infections were identified by molecular characterization, with MCFV being confirmed in the other two animals by IHC. Additionally, the nucleic

acids of BVDV, BRSV, bovine rotavirus A, BoAHV5, *M. haemolytica*, *P. multocida*, *M. bovis*, and mollicutes were not amplified from the tissues evaluated during this study. Furthermore, bovine lyssavirus was not confirmed from the brain tissues of the two cows with neurological syndrome that were submitted to the Official Regional Diagnostic Veterinary Laboratory

Relationship between vascular lesions and the detection of Macavirus

All vascular lesions identified in multiple organs of cattle from this study occurred in animals with a diagnosis of *Macavirus* (OvGHV2 and BoGHV6) by PCR assays are provided in Table 6. PVLs were more frequently identified (53.3%; 8/15) relative to vasculitis (40%; 6/15), considering that the organs of one animal were not received for histopathological diagnosis. OvGHV2 was detected in all animals with vasculitis, except for animals # 4 and 12 which had PVLs but were only infected by BoGHV6. Furthermore, at least one of the *Macavirus* investigated was detected by PCR in three animals (#7, 8, and 16) without histopathological evidence of vasculitis in any of the organs evaluated. Additionally, in animal #10 without histological evidence of vascular disease, MCFV was identified by IHC without the concomitant molecular detection of OvGHV2 and BoGHV6.

Discussion

The lesions identified during the histopathological and histochemical investigations of this study are consistent with those described in cattle [15,30] and bison [31] infected with OvGHV2. The positive immunoreactivity to the 15A MAb IHC assay in most tissues of the cattle investigated indicated that these animals were infected by a MCFV [8], considering that all *Macavirus* known to induce MCF share the 15A epitope [6,32], which cannot be differentiated within members of MCFV complex [3]. The molecular identification of OvGHV2 with the Baxter PCR assay [33], in most animals with positive immunoreactivity by the 15A-MAb IHC assay, confirmed that these were infected by OvGHV2, since differentiation of infections induced by MCFV requires the utilization of specific primers [32]. However, in three animals with positive immunoreactivity for the 15A-MAb by IHC in multiple tissues, only BoGHV6 was detected by PCR, suggesting that this *Macavirus* was associated with the intralesional tissue antigens identified by IHC in these cases. Additionally, in one cow with positive immunoreactivity to the 15A-MAb IHC assay, neither OvGHV2 nor BoGHV6, was identified by molecular assays. These findings may suggest the possible participation of a different *Macavirus* in the development of the lesions identified in this animal. Furthermore, the non-amplification of the DNA/RNA of BVDV, BRSV, BRV, BoAHV5, *M. haemolytica*, *P. multocida*, *M. bovis*, and mollicutes suggest that these were not associated with the disease processes identified during these outbreaks.

Macavirus-associated lesions in cattle

The histopathological findings observed during this investigation were previously described in ruminants infected with OvGHV2, but with [12,22] and without [11,22,34] the typical clinical manifestations of SA-MCF. During this investigation, infected cattle had comparatively more PVLs (also referred to as

Table 6. Relationship between the histopathological identification of vascular lesions and the diagnosis of *Macavirus* by PCR or immunohistochemistry[1].

Animal #	Types of vascular lesion		Diagnosis of *Macavirus*		
	Proliferative Vascular Lesions	Vasculitis	OvGHV2 PCR	BoGHV6 PCR	MCFV IHC
1	+ ve	+ ve	+ ve	+ ve	
2	+ ve	+ ve	+ ve	+ ve	
3[a]			+ ve	+ ve	
4	+ ve	+ ve		+ ve	
5[b]					+ ve
6[b]					+ ve
7				+ ve	
8			+ ve		
9					
10					+ ve
11	+ ve	+ ve	+ ve		
12	+ ve	+ ve		+ ve	
13	+ ve	+ ve	+ ve		
14	+ ve		+ ve		
15	+ ve		+ ve	+ ve	
16			+ ve	+ ve	

[1]: only positive results are shown; *Macavirus* was not diagnosed in the other animals. [a]: tissues not received for histopathological evaluations; [b]: organs not collected for molecular characterization. + ve: positive. MCFV IHC: malignant catarrhal fever virus immunohistochemistry; OvGHV2: ovine gammaherpesvirus 2; BoGHV6: bovine gammaherpesvirus 6.

angiopathy) relative to vasculitis, while all animals with PVLs were infected by at least one of these *Macavirus*. These findings demonstrated that although the salient histopathological alteration in OvGHV2-associated infections is vasculitis with fibrinoid degeneration [14,15,23], a histopathological diagnosis of OvGHV2-2-associated infections should not be based exclusively on vasculitis [11]. This is because the vascular lesions associated with OvGHV2-related infections are progressive, from acute arteritis to chronic hyperplastic arteriopathy with occlusion of vascular lumen and disruption of vascular elastin [31]. These progressive vascular alterations were observed in the intestine of one cow with lymphoplasmacytic atrophic enteritis during this study. Recent investigations have demonstrated that the vascular alterations in SA-MCF are most likely associated with a currently unknown trigger mechanism that results in the infection of T cells, monocytes, proliferating macrophages, and the activation of vascular endothelial cells [26].

An interesting finding during this investigation was the occurrence of interstitial pneumonia in cattle (n = 13) with both clinical syndromes. Furthermore, three of these had positive immunoreactivity to the 15A-MAb IHC assay, with the amplification of only OvGHV2 (n = 2) and BoGHV6 (n = 1) DNA from the pulmonary samples by PCR. Additionally, three animals with interstitial pneumonia were simultaneously infected by only OvGHV2 and BoGHV6. These are additional findings to support the participation of OvGHV2 as a possible cause of pulmonary disease in cattle [15] since viral replication occurs initially within pulmonary epithelial cells [35,36]. Moreover, these results are in accord with a recent study that demonstrated the role of OvGHV2 in the development of bovine respiratory disease in dairy calves with clinical manifestations of respiratory impairment [12]. Furthermore, interstitial pneumonia is frequently diagnosed in cattle with SA-MCF [15], while BoGHV6 was previously associated with the development of pneumonia in a cow [37]. Consequently, these findings are additional evidence to support the role of, at least OvGHV2 in the development of pulmonary disease in ruminants. It must be highlighted that most animals infected by these *Macavirus* were poisoned due to the ingestion of *Brachiaria* spp. (see below). However, there is no association between *Brachiaria* spp. intoxication and the development of pulmonary disease in cattle [27,28], suggesting that the interstitial pneumonia identified during this study was of viral origin.

The 15A-MAb was initially used in serological evaluations to detect the presence of MCFV antibodies in a wide range of mammalian species from the USA [5,6] and Europe [38-40]. We later designed an IHC to detect intralesional antigens of MCFV in tissues of cattle where confirmation of infection by OvGHV2 was obtained by PCR [8] and used this assay to detect MCFV [9,10] and/or OvGHV2 [11,12,41] in ruminants. Collectively, these studies demonstrated that the 15A-MAb can be used effectively to identify intralesional tissue antigens of MCFV and OvGHV2 (when used in conjunction with specific PCR assays). In this study, only BoGHV6 was detected in three animals (#4, 7, and 12) with positive immunoreactivity by the 15A-MAb IHC assay for MCFV and with vascular lesions typical of MCF in two of these (#4 and 12); OvGHV2 was not detected in any of the tissues analyzed from these animals by PCR. Consequently, these findings demonstrated that the 15A-MAb IHC assay can also be used to detect intralesional tissue antigens of BoGHV6, with conformation of BoGHV6 being obtained with specific molecular assays.

These findings then raise the question as to whether BoGHV6 is an infectious disease pathogen or a commensal organism of ruminants and, consequently, its importance in veterinary medicine. Several studies have identified BoGHV6 DNA in tissues of ruminants and have suggested the possible participation of this *Macavirus* in the development of disease processes of ruminants with reproductive [42-46], lymphoproliferative [47], fetal [16], and pulmonary [37] lesions. Alternatively, no association was determined between the occurrence of BoGHV6 in ruminants with disease syndromes in studies done in Europe and the USA, while additional studies were suggested as necessary to confirm the participation of this virus in disease processes [45,46]. The possible role of BoGHV6 in the development of pulmonary disease was described above. Additionally, during this study two animals with clinical manifestations of the enteric syndrome with typical MCF-related vascular lesions were infected by only BoGHV6, suggesting that this agent could have contributed to the development of the enteric disease with the histopathological vascular alterations observed in these cases. Since these findings are interesting and suggest that BoGHV6 may be directly associated with the development of these vascular lesions, studies are being initiated to determine the viral load of this agent in these animals in an attempt to correlate with possible infectability and/or pathogenicity.

Epidemiological data of Macavirus-related infections

Cattle infected during this study did not present the classical "head and eye" form of SA-MCF, so infections by OvGHV2 were only suspected during histopathological evaluations and then confirmed by molecular detection. Similar reports were described in cattle [8,11,48-50] and bison [7,31,51], which may suggest that these animals were subclinically infected. Additionally, both animals from this investigation with the neurological syndromes died acutely without clinical manifestations of typical SA-MCF; this seems to be the pattern for cattle with the neurological manifestations of OvGHV2, as was observed in other cases [8,48,49]. Consequently, it is recommended that OvGHV2-induced infection could be used as a differential diagnosis in areas where bovine rabies is endemic, since during this investigation, as well as in other cases of acute neurological infections due to OvGHV2 [8,48,49], the initial clinical diagnosis of cattle was bovine rabies, considering that bovine rabies is probably the most frequent cause of neurological disease in cattle from Brazil [52-54].

The animals from this study were maintained on farms located within geographical regions of Paraná state where elevated prevalence (41-69.2%) of MCFV tissue antigens were identified in cattle with renal lesions [9], suggesting that a MCFV, most likely OvGHV2, may be endemic within this state. Consequently, seroepidemiological surveys are being implemented to estimate the number of cattle exposed to OvGHV2 in Paraná, and more extensively Brazil, since MCF is endemic in most geographical regions of this continental nation and results in an annual estimated economic loss of 3.2 - 4.8 billion USD [15]. Additionally, MCF is one of the most frequently diagnosed inflammatory neurological diseases of cattle from the southern states of Paraná [53] and Rio Grande do Sul [52], as well as the semiarid Northeastern region of Brazil [54].

Of epidemiological importance during this investigation, was the absence of sheep on most farms where OvGHV2-related infections were diagnosed; similar findings were described in an outbreak of SA-MCF in cattle with 100% lethality from Paraná state [53] and other geographical regions of Brazil [15]. Sheep are the asymptomatic hosts and are responsible for the dissemination of OvGHV2 to susceptible animals [13,14,17]. Accordingly, the absence of sheep at Farm D, where neither OvGHV2 nor BoGHV6 was not detected in cattle with typical histopathological lesions of OvGHV2, is another indication of the possible circulation of an undiagnosed *Macavirus* in cattle herds from Brazil where sheep may not be the asymptomatic host. Additionally, most animals were maintained on farms that presented the alimentary manifestations associated with OvGHV2 infections with variable lethality rates (40-100%); elevated lethality was related to enteric disease induced by OvGHV2 in cattle [19] and bison [31], with the clinical course being between 1 to 3 days [19]. Most cattle that succumbed to the alimentary syndrome during this study died within three days of the onset of clinical manifestations. Although there seems to be no seasonal occurrence of OvGHV2-induced infections, during this study most outbreaks occurred during August to September 2022, which corresponds to the end of winter and the beginning of spring in Brazil.

Possible effects of Brachiaria spp. poisoning on viral infections

During this study, eight animals from farms with enteric disease demonstrated histopathological evidence of TCH, characteristic of poisoning due to the ingestion of *Brachiaria* spp. [28,29,55]. Additionally, cutaneous photosensitization was observed in two of these, while there was significant elevation in the serum activities of hepatic enzymes (GGT and AST) of three animals; these are demonstrations of the typical clinical manifestation and biochemical alterations characteristics of TCH due to *Brachiaria* spp. poisoning [27-29]. Furthermore, MCF was previously diagnosed in cattle maintained on *Brachiaria brizantha* pastures [56] and in cattle with suspected ingestion of bracken fern [57], while OvGHV2 DNA was amplified from nasal secretions (61.1%; 88/144) of sheep reared predominantly on *Brachiaria* spp. [58]. However, a possible association between plant poisoning and infection due to OvGHV2 was only suggested for cattle that ingested bracken fern [57]. Additionally, cattle maintained on *Brachiaria brizantha* that ingested sprouts of *Senecio brasiliensis* were simultaneously infected by BVDV [59]. These findings may suggest that under special conditions plant intoxications may have a synergic effect on the development of viral infections in cattle.

In 2022, there was an extremely prolonged and abnormal dry period during most of the winter and the beginning of spring within most mesoregions of Paraná state, with early rainfall beginning during the transition from winter to spring. Coincidentally, the transition between winter and spring in Southern Brazil is associated with the early regrowth of *Brachiaria* spp. grass due to ideal relative humidity, temperature, and insolation [29]. Furthermore, there is an excessive

increase in the saponin concentration of the newly reborn grass [27,29]. Consequently, the elevated concentration of the toxins contained in *Brachiaria* spp. during this transitional period probably favored plant poisoning, resulting in severe TCH at several farms, which may have partially been associated with the elevated cattle mortality with infections by OvGHV2 being stimulated by the toxic components of *Brachiaria* spp. or simply a simultaneous occurrence.

The toxic compounds identified in *Brachiaria* spp. are several steroidal saponins, including dichotomin, dioscin [27], and protodioscin [27,29,55]. Experimental studies have demonstrated that the concentration of protodioscin increases progressively during plant growth, flowering, seeding, and regrowth [29,55]. The effects of saponins on animal production, health, and performance have been extensively reviewed [60,61], with a wide range of positive relative to negative functions being identified, including the stimulation of the cell-mediated immune system due to the production of cytokines [60] and the increase of microbial protein synthesis [61]. It must be highlighted that SA-MCF affects immune cell subtypes, particularly CD8+ lymphocytes [17,26], and increases in cellular activation may exacerbate the clinical signs and pathological manifestations associated with this disease in affected cattle. However, the direct influences of the toxic compounds contained in *Brachiaria* spp. on possible synergic effects on OvGHV2 infections in ruminants have not been investigated and are currently unknown, so the occurrence during this investigation may be simply coincidental and without any effect on the development of viral diseases. Nevertheless, studies are being implemented to investigate a possible association between the concentrations of the plant toxin and the viral load in affected ruminants maintained on *Brachiaria* spp. pastures.

Conclusions

Cattle maintained on *Brachiaria* spp. pastures developed neurological and enteric clinical syndromes with histopathological evidence of infections predominantly by OvGHV2. Immunohistochemistry detected the antigens of MCFV in multiple organs of all animals with the 15A-MAb IHC assay. Molecular investigations confirmed singular infections of OvGHV2 and BoGHV6 in cattle with positive intracytoplasmic immunoreactivity with the 15A-MAb assay. These findings demonstrated that this MAb can also be used to detect tissue antigens of BoGHV6. The detection of intralesional MCFV tissue antigens with the simultaneous amplification of OvGHV2 from the lungs of cattle with interstitial pneumonia in singular infectious due to this pathogen provides additional evidence of the association of OvGHV2 with the development of pulmonary disease in cattle. Furthermore, most animals infected during this investigation had histopathological evidence of toxic cholangiopathies characteristics of poisoning by *Brachiaria* spp. Therefore, it is rather likely that the elevated cattle mortality observed during these outbreaks was partially associated with intoxication by *Brachiaria* spp., with infections by OvGHV2 being a concomitant occurrence or possibly exacerbated due to stimulation of immune cells by the toxic components of *Brachiaria* spp.

Acknowledgements

The authors are extremely grateful to Dr. George Cameron Russell, Moredun Research Institute, Scotland, UK, for the critical review of this manuscript. The authors thank all consulting veterinarians and farmers who submitted animals and/or organs for pathological and molecular analyses that were used during this study.

Ethics approval

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 Estadual de Londrina (CEUA/UEL; protocol, 835.2019.45).

Data Availability

The nucleotide sequence of the OvGHV2 and BoGHV6 strains identified during this study is deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank).

Funding

Headley, S.A., and Alfieri, A.A. are recipients of the Brazilian National Council of Scientific and Technological Development (CNPq; Brazil) fellowships. Xavier, A.A.C. and Silva, F.H.P. are recipients of graduate fellowships from the Coordination for the Improvement of Higher Education Personnel (CAPES; Brazil). We are grateful for all financial resources received from CNPq, CAPES, Araucária Foundation (FAP/PR), and the National Institute of Science and Technology for Dairy Production Chain (INCT – LEITE) that were used during the development of this investigation.

Authors' Contributions

Conceptualization, S.A.H.; molecular investigations, J.T.T.F., M.C.R., and A.A.A.; pathological investigations: S.A.H., E.F.L.M, R.V.R., A.L.P.L.G., M.I.P.S., A.A.C.X,

F.H.P.S., and G.W.D.S.; immunohistochemical and histochemical analyses: S.A.H., A.A.C.X, and F.H.P.S.; clinical evaluations, N.A.C.A.A., P.F.V.P., V.B.B.R, T.H.C.P., and J.A.N.L.; clinical pathology, K.K.M.C.F. and J.A.N.L.; writing—original draft preparation, S.A.H.; writing—review and editing, S.A.H, J.T.T.F., M.C.R, K.K.M.C.F., G.W.D.S, T.H.C.P., J.A.N.L., and A.A.A.; supervision, S.A.H. and A.A.A.; project administration, S.A.H.; funding acquisition, S.A.H. and A.A.A. All authors have read and agreed to the published version of the manuscript.

Corresponding author

Prof. Selwyn Arlington Headley, DVM, MSc, PhD
Laboratory of Animal Pathology,
Department of Preventive Veterinary Medicine,
Universidade Estadual de Londrina,
Rodovia Celso Garcia Cid, PR 445 Km 380,
Campus Universitário, Londrina, Paraná, Brazil. 86057-970.
Tel: + 55 43 3371-4766.
E-mail: selwyn.headley@uel.br

Conflict of interests

No conflict of interests is declared.

References

1. ICTV (n.d.) International committee on taxonomy of viruses. Subfamily: Gammaherpesvirinae, Genus: Macavirus. Available: https://ictv.global/report/chapter/herpesviridae/herpesviridae/macavirus. Accessed: 13 October 2022.
2. Li H, Shen DT, Davis WC, Knowles DP, Gorham JR, Crawford TB (1995) Identification and characterization of the major proteins of malignant catarrhal fever virus. J Gen Virol 76: 123-129. doi: 10.1099/0022-1317-76-1-123.
3. Crawford TB, Li H, Rosenburg SR, Norhausen RW, Garner MM (2002) Mural folliculitis and alopecia caused by infection with goat-associated malignant catarrhal fever virus in two sika deer. J Am Vet Med Assoc 221: 843-847. doi: 10.2460/javma.2002.221.843.
4. Li H, Keller J, Knowles DP, Crawford TB (2001) Recognition of another member of the malignant catarrhal fever virus group: an endemic gammaherpesvirus in domestic goats. J Gen Virol 82: 227-232. doi: 10.1099/0022-1317-82-1-227.
5. Li H, Gailbreath K, Bender LC, West K, Keller J, Crawford TB (2003) Evidence of three new members of malignant catarrhal fever virus group in muskox (*Ovibos moschatus*), Nubian ibex (*Capra nubiana*), and gemsbok (*Oryx gazella*). J Wildl Dis 39: 875-880. doi: 10.7589/0090-3558-39.4.875.
6. Li H, Shen DT, Knowles DP, Gorham JR, Crawford TB (1994) Competitive inhibition enzyme-linked immunosorbent assay for antibody in sheep and other ruminants to a conserved epitope of malignant catarrhal fever virus. J Clin Microbiol 32: 1674-1679. doi: 10.1128/jcm.32.7.1674-1679.1994.
7. Li H, McGuire TC, Muller-Doblies UU, Crawford TB (2001) A simpler, more sensitive competitive inhibition enzyme-linked immunosorbent assay for detection of antibody to malignant catarrhal fever viruses. J Vet Diagn Invest 13: 361-364. doi: 10.1177/104063870101300417.
8. Headley SA, Oliveira TES, Li H, Lisbôa JAN, Queiroz GR, Fritzen JTT, Flores EF, Alfieri AA, Cunha CW (2020) Immunohistochemical detection of intralesional antigens of Ovine Gammaherpesvirus-2 in cattle with sheep-associated malignant catarrhal fever. J Comp Path 174: 86-98. doi: 10.1016/j.jcpa.2019.11.002.
9. Xavier AAC, Queiroz GR, Lisbôa JAN, Cunha CW, Headley SA (2023) Immunohistochemical identification of a malignant catarrhal fever virus in cattle with renal diseases from Paraná state, Southern Brazil: a retrospective epidemiological study. Trop Anim Health Prod 55: 344. doi: 10.1007/s11250-023-03740-y.
10. Oliveira TES, Scuisato GS, Pelaquim IF, Cunha CW, Cunha LS, Flores EF, Pretto-Giordano LG, Lisbôa JAN, Alfieri AA, Saut JPE, Cunha PHJ, Headley SA (2021) The participation of a virus within the malignant catarrhal fever virus group and *Mycoplasma bovis* in the development of single and mixed infections in beef and dairy cattle with bovine respiratory disease. Front Vet Sci 8. doi: 10.3389/fvets.2021.691448.
11. Headley SA, de Lemos GAA, Dall Agnol AM, Xavier AAC, Depes VCA, Yasumitsu CY, Oliveira TES, Silva LE, Faccin TC, Alfieri AA, Lisboa JAN (2022) Ovine gammaherpesvirus 2 infections in cattle without typical manifestations of sheep-associated malignant catarrhal fever and concomitantly infected with bovine coronavirus. Braz J Microbiol 53: 433-446. doi: 10.1007/s42770-021-00653-6.
12. Headley SA, Dall Agnol AM, Bessegato JA, Frucchi APS, Maturana É FL, Rodrigues RV, Xavier AAC, Alfieri AF, Alfieri AA (2023) Association of ovine gammaherpesvirus 2 with an outbreak of acute respiratory disease in dairy cattle. Sci Rep 13: 5623. doi: 10.1038/s41598-023-30133-w.
13. Li H, Cunha CW, Taus NS, Knowles DP (2014) Malignant catarrhal fever: inching toward understanding. Annul Rev Anim Biosci 2: 209-233. doi: 10.1146/annurev-animal-022513-114156.
14. O'Toole D, Li H (2014) The pathology of malignant catarrhal fever, with an emphasis on ovine herpesvirus 2. Vet Pathol 51: 437-452. doi: 10.1177/0300985813520435.
15. Headley SA, Oliveira TES, Cunha CW (2020) A review of the epidemiological, clinical, and pathological aspects of sheep-associated malignant catarrhal fever with emphasis on Brazil. Braz J Microbiol 51: 1405–1432. doi: 10.1007/s42770-020-00273-6.
16. Headley SA, Fritzen JTT, Bon VR, Xavier AAC, Dall Agnol AM, Zucoloto NZ, Silva FHP, Figueiredo JRX, Alfieri AF, Okano W, Alfieri AA (2022) Detection of bovine gammaherpesvirus 6 in tissues of aborted fetuses from dairy cows concomitantly infected by *Histophilus somni*. Microb Pathog 169: 105621. doi: 10.1016/j.micpath.2022.105621.
17. Russell GC, Stewart JP, Haig DM (2009) Malignant catarrhal fever: a review. Vet J 179: 324-335, doi: 10.1016/j.tvjl.2007.11.007.
18. Li H, Cunha CW, Taus NS (2011) Malignant catarrhal fever: understanding molecular diagnostics in context of epidemiology. Int J Mol Sci 12: 6881-6893. doi: 10.3390/ijms12106881.
19. Constable P, Hinchcliff KW, Done S, Gruenberg W (2017) Malignant catarrhal fever. In Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs, and goats, 11 ed.; Elsevier: St. Louis, Missouri. pp. 2076-2080.
20. David D, Perl S, Brenner J, Dagoni I, Garazi S (2005) Two cases of the cutaneous form of sheep-associated malignant catarrhal fever in cattle. Vet Rec 156: 118-120. doi: 10.1136/vr.156.4.118.

21. Van Metre D, Tennant BC, Whitlock RH (2008) Malignant catarrhal fever. In Diseases of dairy cattle, 2nd ed.; Divers TJ, Peck SF, Eds.; Saunders Elsevier: St Louis, 2008; pp. 276–279.
22. O'Toole D, Li H, Miller D, Williams WR, Crawford TB (1997) Chronic and recovered cases of sheep-associated malignant catarrhal fever in cattle. Vet Rec 140: 519-524. doi: 10.1136/vr.140.20.519.
23. Uzal FA, Plattner BL, Hostetter JM (2016) Malignant catarrhal fever. In Jubb, Kennedy & Palmer's Pathology of Domestic Animals, 5th ed.; Maxie MG, Saunders WB Volume 2, pp. 131-136.
24. Alfieri AA, Parazzi ME, Takiuchi E, Médici KC, Alfieri AF (2006) Frequency of group A rotavirus in diarrhoeic calves in Brazilian cattle herds, 1998–2002. Trop Anim Health Prod 38: 521–526. doi: 10.1007/s11250-006-4349-9.
25. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J (1990) Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28: 495–503. doi: 10.1128/jcm.28.3.495-503.1990.
26. Saura-Martinez H, Al-Saadi M, Stewart JP, Kipar A (2021) Sheep-associated malignant catarrhal fever: role of latent virus and macrophages in vasculitis. Vet Pathol 58: 332-345. doi: 10.1177/0300985820978310.
27. Low S (2015) Signal grass (*Brachiaria decumbens*) toxicity in grazing ruminants. Agriculture 5: 971-990. doi: 10.3390/agriculture5040971.
28. Riet-Correa B, Castro MB, Lemos RA, Riet-Correa G, Mustafa V, Riet-Correa F (2011) *Brachiaria spp.* poisoning of ruminants in Brazil. Pesq Vet Bras 31: 183-192. doi: 10.1590/S0100-736X2011000300001.
29. Ferreira MB, Brum KB, Fernandes C, Martins C, Pinto GS, Castro VS, Rezende KG, Correa Peñuela J, Haraguchi M, Wysocki HL, Lemos R (2011) Variation in saponin concentration in *Brachiaria brizantha* leaves as a function of maturation: preliminary data. In Poisoning by plants, mycotoxins, and related toxins, Riet-Correa F, Pfister J, Schild AL, Wierenga TL, Eds.; CAB International, pp. 118-123.
30. O'Toole D, Li H, Roberts S, Rovnak J, DeMartini J, Cavender J, Williams B, Crawford T (1995) Chronic generalized obliterative arteriopathy in cattle: a sequel to sheep-associated malignant catarrhal fever. J Vet Diagn Invest 7: 108-121. doi: 10.1177/104063879500700118.
31. O'Toole D, Li H, Sourk C, Montgomery DL, Crawford TB (2002) Malignant catarrhal fever in a bison (Bison bison) feedlot, 1993-2000. J Vet Diagn Invest 14: 183-193. doi: 10.1177/104063870201400301.
32. Li H, Keller J, Knowles DP, Crawford TB (2001) Recognition of another member of the malignant catarrhal fever virus group: an endemic gammaherpesvirus in domestic goats. J Gen Virol 82: 227-232. doi: 10.1099/0022-1317-82-1-227.
33. Baxter SI, Pow I, Bridgen A, Reid HW (1993) PCR detection of the sheep-associated agent of malignant catarrhal fever. Arch Virol 132: 145-159. doi: 10.1007/BF01309849.
34. Powers JG, VanMetre DC, Collins JK, Dinsmore RP, Carman J, Patterson G, Brahmbhatt D, Callan RJ (2005) Evaluation of ovine herpesvirus type 2 infections, as detected by competitive inhibition ELISA and polymerase chain reaction assay, in dairy cattle without clinical signs of malignant catarrhal fever. J Am Vet Med Assoc 227: 606-611. doi: 10.2460/javma.2005.227.606.
35. Li H, Cunha CW, Davies CJ, Gailbreath KL, Knowles DP, Oaks JL, Taus NS (2008) Ovine herpesvirus 2 replicates initially in the lung of experimentally infected sheep. J Gen Virol 89: 1699-1708. doi: 10.1099/vir.0.2008/000554-0.
36. Taus NS, Schneider DA, Oaks JL, Yan H, Gailbreath KL, Knowles DP, Li H (2010) Sheep (*Ovis aries*) airway epithelial cells support ovine herpesvirus 2 lytic replication in vivo. Vet Microbiol 145: 47-53. doi: 10.1016/j.vetmic.2010.03.013.
37. Headley SA, Dall Agnol AM, Oliveira TES, Bon VR, Scuisato GS, Xavier AAC, Yasumitsu CY, Alfieri AF, Alfieri AA (2023) Possible association of bovine gammaherpesvirus 6 with pulmonary disease in a cow. Animals 13. doi: 10.3390/ani13030417.
38. Frölich K, Li H, Müller-Doblies U (1998) Serosurvey for antibodies to malignant catarrhal fever-associated viruses in free-living and captive cervids in Germany. J Wildl Dis 34: 777-782. doi: 10.7589/0090-3558-34.4.777.
39. Probst C, Speck S, Hofer H (2011) Serosurvey of zoo ungulates in central Europe. Int Zoo Yb 45: 168-182. doi: 10.1111/j.1748-1090.2010.00117.x.
40. Müller-Doblies UU, Li H, Hauser B, Adler H, Ackermann M (1998) Field validation of laboratory tests for clinical diagnosis of sheep-associated malignant catarrhal fever. J Clin Microbiol 36: 2970-2972. doi: 10.1128/jcm.36.10.2970-2972.1998.
41. Headley SA, Fritzen JTT, Silva DC, Xavier AAC, Yasumitsu CY, Silva FHP, Alfieri AF, Soethe AM, Alfieri AA (2023) *Histophilus somni* disease conditions with simultaneous infections by ovine gammaherpesvirus 2 in cattle herds from Southern Brazil. Braz J Microbiol 54: 1169-1179. doi: 10.1007/s42770-023-00915-5.
42. Banks M, Ibata G, Murphy AM, Frossard JP, Crawshaw TR, Twomey DF (2008) Bovine lymphotropic herpesvirus and non-responsive post-partum metritis in dairy herds in the UK. Vet J 176: 248-250. doi: 10.1016/j.tvjl.2007.02.005.
43. Cobb SP, Banks M, Russell C, Thorne M (2006) Bovine lymphotrophic herpesvirus in a UK dairy herd. Vet Rec 158: 807-808. doi: 10.1136/vr.158.23.807-a.
44. de Boer MW, Zheng T, Buddle BM, McDougall S (2014) Detection of bovine herpesvirus type 4 antibodies and bovine lymphotropic herpesvirus in New Zealand dairy cows. N Z Vet J 62: 351-355. doi: 10.1080/00480169.2014.933683.
45. Gagnon CA, Allam O, Drolet R, Tremblay D (2010) Quebec: Detection of bovine lymphotropic herpesvirus DNA in tissues of a bovine aborted fetus. Can Vet J 51: 1021-1022.
46. Garigliany MM, Bayrou C, Cassart D, Jolly S, Desmecht D (2013) Bovine lymphotropic herpesvirus detected in Belgium. Vet Rec 172: 535-536. doi: 10.1136/vr.f3127.
47. Oliveira CH, Oliveira FG, Gasparini MR, Galinari GC, Lima GK, Fonseca AA, Jr., Barbosa JD, Barbosa-Stancioli EF, Leite RC, Dos Reis JK (2015) Bovine herpesvirus 6 in buffaloes (*Bubalus bulalis*) from the Amazon region, Brazil. Trop Anim Health Prod 47: 465-468. doi: 10.1007/s11250-014-0733-z.
48. Luvizotto M, Ferrari H, Cardoso T (2010) Malignant catarrhal fever-like lesions associated with ovine herpesvirus-2 infection in young calves (*Bos indicus*): a case report. J Venom Anim Toxins incl Trop Dis 16: 178-185. doi: 10.1590/S1678-91992010005000012.
49. Headley SA, Lisbôa JAN, Fritzen JTT, Queiroz GR, Alfieri AF, Oliveira RAM, Bracarense APFRL, Flaiban KKMC, Alfieri AA (2013) Ovine herpesvirus type 2-induced malignant catarrhal fever in a heifer. Semin: Cenc-Agrar 34: 3903-3908. doi: 10.5433/1679-0359.2013v34n6Supl2p3903.
50. Martins MSN, Castro AMMG, Lima MS, Pinto VSC, Silva TG, Fava CD, Depes CR, Okuda LH, Pituco EM (2017) Malignant catarrhal fever in Brazilian cattle presenting with

neurological syndrome. Braz J Microbiol 48: 366-372. doi: 10.1016/j.bjm.2016.10.021.
51. Sausker EA, Dyer NW (2002) Polymerase chain reaction and DNA sequencing for detection of Ovine Herpesvirus 2 in American Bison (*Bison bison*). J Vet Diagn Invest 14: 40-46. doi: 10.1177/104063870201400108.
52. Sanches AWD, Langohr IM, Stigger AL, Barros CSL (2000) Diseases of the central nervous system in cattle of southern Brazil. Pesq Vet Bras 20: 113-118. doi: 10.1590/S0100-736X2000000300005. [Article in Portuguese]
53. Queiroz GR, Oliveira RAM, Flaiban KKMC, Santis GWD, Bracarense APFRL, Headley SA, Alfieri AA, Lisbôa JAN (2018) Differential diagnosis of neurologic diseases of cattle in the state of Paraná. Pesq Vet Bras 38: 1264-1277. doi: 10.1590/1678-5150-PVB-5429. [Article in Portuguese]
54. Galiza GJN, Silva MLCR, Dantas AFM, Simões SVD, Riet-Correa F (2010) Diseases of the nervous system of cattle in the semiarid of Northeastern Brazil. Pesq Vet Bras 30: 267-276. doi: 10.1590/S0100-736X2010000300014. [Article in Portuguese]
55. Gaspar AO, Guizelini CC, Roberto FC, Difante GS, Brumatti RC, Ítavo CCBF, Lemos RAA, Lee ST (2021) Protodioscin levels in *Brachiaria* spp. in a sheep production system and a brief review of the literature of Brachiaria spp. poisoning in ruminants. Pesq Vet Bras 41: e06921, doi: 10.1590/1678-5150-PVB-6921.
56. Headley SA, Pimentel LA, Oliveira VH, Toma HS, Alfieri AF, Carvalho AM, dos Santos MD, Alfieri AA (2015) Transplacental transmission of ovine herpesvirus 2 in cattle with sheep-associated malignant catarrhal fever. J Comp Path 153: 206-211. doi: 10.1016/j.jcpa.2015.10.175.
57. Twomey DF, Holt GJ, Reid HW (2002) Malignant catarrhal fever in cattle with suspected bracken poisoning. Vet Rec 151: 486-487.
58. Eloi RSA, Marçola TG, Paludo GR, Araújo RR, Colodel EM, Lima EMM, Castro MB (2017) Ovine herpesvirus type 2 (OvHV-2) infection rate in sheep herds of the Federal District, Brazil. Pesq Vet Bras 37: 657-661. doi: 10.1590/S0100-736X2017000700001. [Article in Portuguese]
59. Headley SA, Alfieri AA, Fritzen JTT, Queiroz GR, Lisbôa JAN, Pontes Netto D, Okano W, Flaiban KKMC, Alfieri AF (2014) Concomitant bovine viral diarrhea, mycotoxicosis, and seneciosis in cattle from northern Paraná, Brazil. Semin: Cenc-Agrar 35: 2563-2576. doi: 10.5433/1679-0359.2014v35n5p2563.
60. Francis G, Kerem Z, Makkar HP, Becker K (2002) The biological action of saponins in animal systems: a review. Br J Nutr 88: 587-605. doi: 10.1079/bjn2002725.
61. Kholif AE (2023) A review of effect of saponins on ruminal fermentation, health and performance of ruminants. Vet Sci 10: 450. doi: 10.3390/vetsci10070450.

Annex – Supplementary Items

Supplementary Table 1. Targets genes, primers, and amplicon size of the molecular assays used to identify infectious pathogens of respiratory, neurological, and enteric diseases of cattle.

Pathogens	Target genes	Primer sequences (5' – 3')	Amplicons size (bp)	Reference
Viral				
OvGHV2	Tegument protein	Fw – AGTCTGGGTATATGAATCCAGATGGCTCTC Rv - AAGATAAGCACCAGTTATGCATCTGATAAA Rv – TTCTGGGGTAGTGGCGAGCGAAGGCTTC	422 238	1
BoGHV6	polymerase gene external region	Fw- ACAGACGGGCAGCAGATAAG Rv- ATGGTTCGCCCCTGTAGAGT Rv- AGTCTACCACGAGCACAGGA	551 166	2
BCoV	N gene	Fw - CGATGAGGCTATTCCGAC- Rv – TGTGGGTGCGAGTTCTGC Fw - TTGCTAGTCTTGTTCTGGC	454 251	3
BVDV	5'UTR	Fw – ATGCCCWTAGTAGGACTAGCA Rv – TCAACTCCATGTGCCATGTAC	288	4
BRSV	Glycoprotein G	Fw - CCACCCTAGCAATGATAACCTTGAC Rv - AAGAGAGGATGCYTTGCTGTGG Fw - CATCAATCCAAAGCACCACACTGTC Rv - GCTAGTTCTGTGGTGGATTGTTGTC	603 371	5
BoAHV1	Glycoprotein C	Fw - CAACCGAGACGGAAAGCTCC Rv- AGTGCACGTACAGCGGCTCG	354	6
BoAHV5	Glycoprotein C	Fw - CGGACGAGACGCCCTTGG Rv- AGTGCACGTACAGCGGCTCG	159	6
BPI3	HN gene	Fw - GAATGACTCATGATAGAGGTAT Rv - AGGACAACCAGTTGTATTACAT	647	7
BRV	VP7 gene	Fw- GGCTTTAAAAGAGAGAATTTCCGTCTGG Rv - GGTCACATCATACAATTCTAATCTAAG	1062	12
Bacterial				
Mannheimia haemolytica	lktA-artJ intergenic region	Fw - GTCCCTGTGTTTTCATTATAAG Rv - CACTCGATAATTATTCTAAATTAG	385	8
Histophilus somni	16S rDNA	Fw - GAAGGCGATTAGTTTAAGAG Rv - TTCGGGCACCAAGTRTTCA	400	9
Pasteurella multocida	ORF KMT1 clone	Fw – GCTGTAAACGAACTCGCCAC Rv - ATCCGCTATTTACCCAGTGG	460	10
Mycoplasma bovis	ITS region	Fw - CCGTCAAACYATGGGAGC Rv – GTGYCCCGCCMTACTCAGG Fw - GTACACTTGTCTTTTATCACTATA Rv - AAGGTATCTCGCTTTATGTCCT	864 488	11

1. Baxter SI, Pow I, Bridgen A, Reid HW (1993) PCR detection of the sheep- associated agent of malignant catarrhal fever. Arch Virol 132: 145- 159. doi: 10.1007/bf01309849.
2. Oliveira CH, Oliveira FG, Gasparini MR, Galinari GC, Lima GK, Fonseca AA, Jr., Barbosa JD, Barbosa- Stancioli EF, Leite RC, Dos Reis JK (2015) Bovine herpesvirus 6 in buffaloes (*Bubalus bulalis*) from the Amazon region, Brazil. Trop Anim Health Prod 47: 465- 468. doi: 10.1007/s11250- 014- 0733- z.
3. Takiuchi E, Stipp DT, Alfieri AF, Alfieri AA (2006) Improved detection of bovine coronavirus N gene in faeces of calves infected naturally by a semi-nested PCR assay and an internal control. J Virol Methods 131: 148- 154. doi: 10.1016/j.jviromet.2005.08.005.
4. Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ (1994) Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch Virol 136: 309- 323. doi: 10.1007/bf01321060.
5. Vilcek S, Elvander M, Ballagi- Pordany A, Belak S (1994) Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. J Clin Microbiol 32: 2225-2231. doi: 10.1128/jcm.32.9.2225- 2231.1994.
6. Claus MP, Alfieri AF, Folgueras- Flatschart AV, Wosiacki SR, Médici KC, Alfieri AA (2005) Rapid detection and differentiation of bovine herpesvirus 1 and 5 glycoprotein C gene in clinical specimens by multiplex- PCR. J Virol Methods 128: 183- 188. doi: 10.1016/j.jviromet.2005.05.001.
7. Zhu YM, Shi HF, Gao YR, Xin JQ, Liu NH, Xiang WH, Ren XG, Feng JK, Zhao LP, Xue F (2011) Isolation and genetic characterization of bovine parainfluenza virus type 3 from cattle in China. Vet Microbiol 149: 446- 451. doi: 10.1016/j.vetmic.2010.11.011.
8. Angen O, Thomsen J, Larsen LE, Larsen J, Kokotovic B, Heegaard PM, Enemark JM (2009) Respiratory disease in calves: microbiological investigations on trans- tracheally aspirated bronchoalveolar fluid and acute phase protein response. Vet Microbiol 137: 165- 171. doi: 10.1016/j.vetmic.2008.12.024.
9. Angen O, Ahrens P, Tegtmeier C (1998) Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. Vet Microbiol 63: 39- 48. doi: 10.1016/s0378- 1135(98)00222- 3.
10. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ (1998) Development of PCR assays for species- and type- specific identification of *Pasteurella multocida* isolates. J Clin Microbiol 36: 1096-1100. doi: 10.1128/jcm.36.4.1096-1100.1998.
11. Voltarelli DC, de Alcântara BK, Lunardi M, Alfieri AF, de Arruda Leme R, Alfieri AA. (2018) A nested- PCR strategy for molecular diagnosis of mollicutes in uncultured biological samples from cows with vulvovaginitis. Anim Reprod Sci 188:137- 143, doi: 10.1016/j.anireprosci.2017.11.018.
12. Gouvea V, Glass R I, Woods P, Taniguchi K, Clark HF, Forrester B, Fang Z Y. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J Clin Microbiol. 1990 Feb;28(2):276-82. doi: 10.1128/jcm.28.2.276-282.1990